SKELETAL MUSCLE AS A STRESS SENSOR: FOCUS ON MUSCLE-DERIVED INTERLEUKIN-6 PRODUCTION DURING HEAT STRESS

By

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ACTB	beta actin
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AP-1	activator protein 1
APR	acute phase response
ATF	activating transcription factor
ATP	adenosine tri-phosphate
c.a.	constitutively active
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CCAAT	cytidine-cytidine-adenosine-adenosine-thymidine
C/EBP	CCAAT enhancer binding protein
cGMP	cyclic guanosine monophosphate
CREB	cAMP response element-binding protein
d.n.	dominant negative
DAMPS	damage associated molecular patterns
DMEM	dulbecco’s modification of eagle’s medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
eHSP	extracellular heat shock protein
eIF	eukaryotic initiation factor
ELISA	enzyme-linked immunosorbent assay

EPI	epinephrine
ER	endoplasmic reticulum
ERK	extracellular signal-related kinases
EV	empty vector
FBS	fetal bovine serum
FDR	false discovery rate
Fig.	figure
FNDC5	fibronectin type III domain containing 5
GAPDH	glyceraldehyde-3-phosphate
GRE	glucocorticoid responsive elements
h	hours
HMGB1	high-mobility group protein 1
HRP	horse radish peroxidase
HPRT	hypoxanthine guanine phosphoribosyl transferase
HSE	heat shock element
HSF	heat shock factor
HSPs	heat shock proteins
HSP72	heat shock protein 72
IgG	bovine gamma globulin
IL-1B	interleukin 1 beta
IL-1RA	interleukin 1 receptor antagonist
IL-6	interleukin 6
IL-8	interleukin 8

IL-10	interleukin 10
IL-15	interleukin 15
IL-18	interleukin 18
IP	intraperitoneal
IP-10	interferon gamma-induced protein 10
IRAK	interleukin 1 receptor associated kinase
IRE	insulin response element
IRF	interferon regulatory factor
JNK	c-Jun-NH_2 terminal kinase
KNK437	3,4-methylenedioxy-benzylidine-γ-butyrolactam
LDH	lactate dehydrogenase
L_o	optimal length
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein 1
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation primary response gene 88
NF-	nuclear factor
NF-κB	nuclear factor κB
NO	nitric oxide
nNOS	neuronal nitric oxide synthase
NOS	nitric oxide synthase

P1	purinergic receptor 1
P2	purinergic receptor 2
PAMPS	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PKC-δ	protein kinase C δ
PLB	passive lysis buffer
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SOCS	suppressor of cytokine signaling
SNARE	specific soluble NSF attachment protein receptor
sTNFR	soluble tumor necrosis factor receptor
TE	tris-EDTA
T_{ENV}	environmental temperature
TLRs	toll-like receptors
TMB	tetramethylbenzadine
TNF-α	tumor necrosis factor alpha
TRIF	tri-domain-containing adapter molecule interferon-β
UPR	unfolded protein response
UTR	untranslated region
WT	wild-type
XBP	x-box-binding protein

ΔΔCT	delta delta cycle threshold

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SKELETAL MUSCLE AS A STRESS SENSOR: FOCUS ON MUSCLE-DERIVED INTERLEUKIN-6 PRODUCTION DURING HEAT STRESS

By

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Skeletal muscle has been identified as an endocrine organ due to its capacity to produce and secrete a variety of proteins with autocrine/paracrine/endocrine function (myokines). To date, myokines have primarily been studied in response to exercise or metabolic challenges. However, numerous observations suggest that muscle releases myokines in response to internal or external stress. In this study, we focus on the regulation of IL-6 as a prototypical stress response myokine and demonstrate that IL-6 gene regulation in muscle is organized to respond to a variety of internal and external stressors. Systemic IL-6 during exercise is attenuated when core temperature is reduced. Therefore, we hypothesized that muscle may contribute to circulating IL-6 and that hyperthermia is an important component for muscle-derived IL-6. In C2C12 myotubes, hyperthermia increased IL-6 mRNA and secreted IL-6 protein. Ex-vivo soleus muscles from mice, exposed to heat (41°C) yielded similar IL-6 gene responses. When whole animals were exposed to passive hyperthermia, such that core temperature increased to 42.4°C, IL-6 mRNA in soleus increased compared to time-matched controls. Muscle HSP72 mRNA also increased as a function of the intensity of heat and IL-6 mRNA respond proportionally. Pharmacological inhibition of heat shock factor-1

(HSF-1) attenuated heat-induced HSP72 and IL-6 mRNA. Follow-up experiments show the importance of heat as a co-stimulus for stress-induced IL-6 production. Heat combined with epinephrine or lipopolysaccharide (LPS) treatment potentiated IL-6 mRNA formation and differentially regulated IL-6 protein in myotubes. Heat exposure during epinephrine treatment, but not during LPS treatment also potentiated IL-6 protein. To verify that the signaling pathway by which heat activates IL-6 is via HSF-1, myotubes were transfected with either a dominant negative HSF-1 or a constitutively active HSF-1. The results demonstrate an important role for HSF-1 in regulation of IL-6 in hyperthermia that is likely to transcend many other conditions of stress. Since IL-6 can initiate protective, anti-inflammatory or restorative processes throughout the organism during life-threatening conditions, we present the argument that skeletal muscle has a physiological function as a sensor and responder to stress. Furthermore, we suggest that it may comprise a fundamental component of the organism's acute stress response.

CHAPTER 1
INTRODUCTION

Skeletal muscle research has often exclusively involved contractile and locomotive functions. However, skeletal muscle is a multifunctional organ. The recent discovery that skeletal muscle can produce and release cytokines (myokines) has identified a novel role for skeletal muscle in health and disease beyond that of contraction and locomotion (144, 145). The discovery of new paracrine and endocrine roles for muscle may lead to novel approaches to disease prevention and treatment. In Chapter 2, the groundwork is developed for the underlying molecular mechanisms used by skeletal muscle to detect internal and external stress that apply to both exercise and conditions of organismal stress. We then focus specifically on how these stress signals link directly to the regulation of IL-6. Establishing the background and defining the underlying hypotheses we wish to test. In this study we aim to identify a unique stimulus for skeletal muscle derived interleukin (IL)-6, elucidate the molecular mechanisms for its regulation, and to test the functionality when hyperthermia is coupled with unrelated stress stimuli.

Specific Aim 1: To Test the Hypothesis that Hyperthermia is a Unique Stimulus for IL-6 Formation in Skeletal Muscle.

Skeletal muscle operates in a dynamic temperature range during physiological conditions (18, 59). Muscle can be exposed to high body temperature due to a breakdown in the body's ability to thermoregulate, but it is also capable of generating its own heat. The exothermic metabolic reaction driving muscle contraction yields muscle temperatures often about 1°C above core temperature and as high as 41°C in human (157) or 44°C in rats (18). Myokine research has largely been viewed in the context of exercise and metabolism (144). Little consideration has been given to the importance

of hyperthermia to myokine production, although evidence suggests that temperature is an important co-factor for circulating cytokine production (153, 172). In this aim we will focus on the role of skeletal muscle as an IL-6 generator in hyperthermia models of acute stress.

Specific Aim 2: To Determine the Molecular Mechanisms by which Hyperthermia Stimulates IL-6 Formation.

The physiological role of IL-6 during acute stress is poorly understood. It is a multifunctional cytokine that plays a central role in inflammation and tissue injury, but has correlatively been linked to positive and negative pathological outcomes (45). Regardless of beneficial or deleterious outcomes, the determination of the molecular mechanisms regulating the IL-6 gene is important for future therapeutic strategies. In this aim, we will test candidate regulatory mechanisms of hyperthermia-induced skeletal muscle-derived IL-6.

Specific Aim 3: To Test the Role Of Hyperthermia and/or Heat Shock Factor-1 Activation as a Co-Stimulus or Potentiator of IL-6 Gene Expression in Response to Distinct Stress Stimulants: Epinephrine and Lipopolysaccharide.

There is accumulating evidence that heat shock is an important regulator of IL-6 (81, 181, 192). The activation of heat shock transcription factors (HSF-1) mediates the heat shock response. In this aim, we test HSF-1 as a master regulatory sentinel that could account for the regulation of stimulated IL-6. We chose epinephrine (EPI) and lipopolysaccharide (LPS) as known general stimulators of IL-6 (47, 48) that could be present during acute stress events in which heat is also present (1, 67). Using a series of experiments we will test the regulatory role of HSF-1 during EPI- and LPS-induced IL-6. HSF-1 will be activated using a series of different methodologies to account for associated signals that accompany heat stress.

Significance:

Skeletal muscle is increasingly being recognized as an endocrine organ because of its ability to generate cytokines (144, 145, 175). In this study, we set out test if hyperthermia alone is a novel stimulus of skeletal muscle IL-6, to determine the molecular mechanisms of hyperthermia-induced IL-6 regulation, and to test the regulatory role of heat/HSF-1 on EPI or LPS stimulated IL-6. The results will provide a basic understanding of the mechanisms for the regulation of IL-6 during hyperthermia and may provide the groundwork for the development of future therapeutic approaches.

CHAPTER 2
THE REGULATION OF IL-6 IMPLICATES SKELETAL MUSCLE AS AN INTEGRATIVE STRESS SENSOR AND ENDOCRINE ORGAN [1]

Background

In this review, we will discuss emerging evidence in support of the hypothesis that skeletal muscle plays an important physiological role as a whole organism "stress sensor" and that muscle endocrine responses to stress can contribute, and perhaps be essential to, the ability of an organism to survive life-threatening conditions. The endocrine response of skeletal muscle is still poorly understood. Much of what we do know has centered on the ability of muscles to generate and secrete cytokines in response to exercise or metabolic challenges (144, 145). These cytokines have more recently been termed "myokines," defined as "cytokines and other peptides that are produced, expressed, and released by muscle fibers and exert paracrine, autocrine, or endocrine effects" (144). The idea that stress may also be an important stimulus for myokine production was inspired by a recent observation that heat stress stimulates interleukin-6 (IL-6) production in skeletal muscle and that IL-6 transcription can be blocked by application of heat shock factor (HSF) inhibitors (192). The heat shock response, mediated by HSFs, is the quintessential evolutionarily conserved stress response used by cells to cope with environmental and physiological stresses.

This review will focus on IL-6 as a model signal that represents a potential efferent arm of skeletal muscle's ability to contribute to the stress response of the organism. We will explore experimental evidence of how various stimuli known to accompany acute

[1] Reprinted with permission from *Experimental Physiology:*
Welc SS, Clanton TL. The regulation of IL-6 implicates skeletal muscle as an integrative stress sensor and endocrine organ. *Exp. Physiol.* (August 31, 2012). doi: 10.1113/expphysiol.2012.068189.

internal and external stress, injury, infection or trauma can be linked to IL-6 production in muscle and in other cell types. During the discussion we will evaluate the promoter region of the IL-6 gene to outline the relationships between known signaling pathways and their links to stress signals that can be detected by muscle. Finally, we will briefly review the potential physiological significance of IL-6 in stressed tissue and in the whole organisms.

An Overview of Cytokine Response to Stress: Comparison to Exercise

In many forms of stress there are striking changes in circulating cytokines which have historically been attributed to the response of immune cells to endotoxin. Endotoxin is thought to arise from the breakdown of the intestinal barrier and leakage of bacterial wall fragments into the circulation. Stressors such as hemorrhagic shock (43), hyperthermia (144), burns (30), and trauma (35) have all been shown to induce intestinal barrier dysfunction and to produce cytokine responses. However, it is unknown if endotoxin is the primary stimulus and whether immune cells are the chief effector cells.

Similar issues have historically surrounded the cytokine responses to endurance exercise. In prolonged exercise there are often elevations of circulating endotoxin, also presumably arising from intestinal barrier dysfunction (13). One of the arguments against endotoxin as the stimulus for exercise-induced cytokine expression is that the timeline of the release pattern and the relative concentrations of specific cytokines are distinctly different in exercise compared to endotoxin exposure (144). For example, the endotoxin response is characterized by an early rise in TNF-α and IL-1β and a later elevation of IL-6, IL-10 and IL-1RA; whereas in exercise, pro-inflammatory cytokines

(TNF-α and IL-1β) are blunted or absent, but there are marked elevations in circulating IL-6, IL-1RA and IL-10 (144).

IL-6 has been one of the more predictable and dominant cytokines expressed following endurance exercise (36). The cellular source of circulating IL-6 accompanying exercise was not known for many years. However, experiments sampling across the vasculature of the human leg during exercise demonstrated a net production of IL-6 secreted into the circulation. These findings were the first to show that muscle can be a significant source of circulating IL-6 (175). Additional experiments demonstrating elevations in IL-6 protein within muscle cells following exercise and IL-6 protein released in high concentrations into the muscle interstitium during exercise provided important supporting data, as reviewed in Pedersen et al. (144). These experiments resulted in a paradigm shift in muscle biology, beyond our basic understanding of muscle as a motor, to a realization that it may have unrecognized functions as an endocrine and immune organ. Besides IL-6, many other myokines have since been identified in various muscle preparations, these include: TNF-α, IL-1β, IL-8, IL-10, IL-15, IL-18, HMGB1, FNDC5-Irisin among many others, as recently reviewed (145). Based on these observations, the source of the circulating cytokine profile seen in intense exercise cannot be attributed solely to a response to endotoxin alone. Though endotoxin cannot be excluded as a co-stimulus, in many conditions of exercise, it is clear that it is not a necessary pre-requisite for a cytokine response to intense exercise.

Like exercise, the cytokine response to some stress exposure differs from endotoxin exposure. For example, in hyperthermia, the circulating TNF-α and IL-1β responses are also either blunted or are absent, but very strong IL-6, IL-1RA, and IL-10

signals occur, either during or shortly after heat exposure (154). In hemorrhagic shock, IL-6 and TNF-α elevations tend to coincide, but with a predominant IL-6 response (127). These patently different cytokine profiles following stress more closely resemble those seen following endurance exercise than those seen after endotoxin exposure.

IL-6 is a dominant signal seen in the circulation during and following stress conditions like hyperthermia (192). For example, in a mouse model of heat stroke, plasma IL-6 can exceed 1000 pg/ml, measured 2 hours (h) after heat exposure (192). Equally high levels of IL-6 are seen 2 h following hemorrhagic shock in mice (83) However, though there is ample evidence that skeletal muscles upregulate IL-6 and other myokines in stress, as we shall discuss, the link between circulating levels observed and muscle production has not yet been established in stress, as it has in exercising muscle.

The Capacity for Skeletal Muscles to Respond to Stress Signals

Skeletal muscles are exposed to a variety of “internal” and “external” stress stimuli, as illustrated in Figure (Fig.) 2-1. Muscles appear to be able to withstand internal stress intensities that would severely damage most other tissues. Some of these internal stresses include: hypoxia (28), mechanical stress, thermal stress, osmotic stress, oxidative stress (as reviewed in (198)), as well as nitrosative stress (188) and energy imbalance (57). However, muscle is also exposed and is sensitive to blood-borne mediators of stress, via membrane receptor systems, such as catecholamines, bacterial wall products, ATP and its metabolites, oxidized lipids, alarmins and pro-inflammatory cytokines commonly produced in many life-threatening conditions. These might be considered “external stressors” that indicate that the entire organism or surrounding tissues are being threatened (Fig. 2-1). This review will consider the evidence that

skeletal muscle is equipped to function as an acute sensor of both external and internal stress signals and how these signals are linked to IL-6 expression at the genomic level.

"External" Stress Signals and IL-6 Regulation

Toll-like receptors: PAMPS and DAMPS

The early response of the innate immune system to endotoxin or other foreign pathogens is through the initiation of an inflammatory cascade by way of a family of toll-like receptors (TLRs) (180). TLRs are evolutionarily conserved receptors that were originally identified to function in recognizing microbial structures like endotoxin , now referred in general as pathogen-associated molecular patterns (PAMPs) (180). The innate immune system's ability to mount an immune response to a pathogen, without prior exposure to an antigen, is mediated largely through TLRs. TLRs are often associated with immune cells, but can also be highly expressed on other cell phenotypes such as skeletal muscle. Skeletal muscles express multiple TLRs, this repertoire of TLRs makes it responsive to pathogens via a wide range of different microbial components (106). Frost, Lang and colleagues have characterized the interactions between bacterial endotoxin, lipopolysaccharide (LPS), TLR-4 and cytokine production in skeletal muscle (46). They found muscle to be highly responsive to LPS and that the response is independent of immune cell activation. Injection of a bolus of LPS into the gastrocnemius strongly induced IL-6 mRNA in the injected leg, but not in the contralateral leg, suggesting that LPS acts directly on the muscle and not via a secondary systemic immune response (49).

Skeletal muscle is potentially exposed to multiple types of PAMPs. As mentioned, in addition to infection, many acute stress events and even strenuous exercise can result in endotoxin or inflammatory mediator release from the intestine (13). Since

muscle represents a high percentage of body mass, approximately 40% in man, it has an enormous potential sensing capacity for blood-borne changes, particularly in high metabolic states such as in exercise, where both capillary cross-sectional area and turnover of interstitial fluid is elevated many fold. In a classic experiment by Mathison et al. the accumulation of LPS in tissues of rabbits and primates at rest was characterized following an LPS injection. They found that the liver accounts for the most tissue-bound LPS (≈40%) and muscle accounts for the second highest tissue-bound LPS (≈5%), largely because of the large relative mass of muscle (121).

In addition to detecting the presence of invading microorganisms, TLRs function in "sterile inflammation" by detecting ligands derived from damaged cells in the extracellular environment. These are sometimes referred to as "damage associated molecular patterns (DAMPs), "alarmins" or "danger signals" and include: extracellular heat shock proteins (eHSPs), β-defensins, high mobility group box 1 protein (HMGB1), free nucleic acids from released DNA or RNA, mitochondrial membranes (which resemble bacterial membranes), and oxidized lipids (187). They all initiate signaling events via subsets of TLRs. For example, though HSPs are well known as molecular chaperones inside the cell, some HSPs such as HSP72 are released into the extracellular space, particularly during times of stress, and can exhibit potent innate and acquired immune responses (89), most likely via TLR activation (38). β-defensins are another type of TLR-ligand (51) that consists of anti-microbial peptides produced by skeletal muscle and other tissues (52). The HMGB1 protein was one of the first identified DAMPs, and though normally a constitutive nuclear protein, it is considered a cytokine, released only from damaged or dying cells (20). Finally, lipids are oxidized in

the presence of reactive oxygen species (ROS) or reactive nitrogen species (RNS), that are often elevated in stress, producing a variety of oxidized membrane-like products, some of which can signal via TLRs (209).

PAMPs and DAMPs bind to TLRs and signal through a number of proteins such as myeloid differentiation primary response gene 88 (MyD88), TRI-domain-containing adapter molecule interferon-β (TRIF), and interleukin-1 receptor associated kinase (IRAK), ultimately leading to the activation of downstream transcription factors. Fig. 2-2 shows the primary regulatory sites in the promoter regions for IL-6 gene transcription that communicate with TLRs and other stress signals (the basic elements reviewed in, (17)). Though TLRs can signal through a variety of pathways, all TLRs signal via mobilization of the P60/P65 elements of the NF-κB protein, which translocate into the nucleus and activate the NF-κB response element on the promoter region of IL-6 (142). Another pathway that appears to be critical and perhaps obligatory for IL-6 signaling from some TLRs such as TLR3 and TLR4 is via “interferon regulatory factor” activation (IRF), particularly IRF-3,-5 or -7 (142). Finally, nearly all TLRs can activate the IL-6 promoter region via mitogen activated protein kinases (MAPK), specifically c–Jun terminal kinase (JNK) and P38 MAPK (142).

The p38 and JNK pathways are often grouped together and are referred to as “stress-activated” protein kinases. They induce downstream phosphorylation of c-fos/c-jun and the activation of AP-1. AP-1 can then bind to its transcription site within the promoter region to stimulate IL-6 transcription (122) (Fig. 2-2). This pathway is probably the most common stress-activated transcriptional pathway for IL-6. Besides TLRs, it is activated by oxidative stress, toxins, hyper-osmolarity, ischemia-reperfusion,

ceramide, TLR-activation, catecholamine-activation and endoplasmic reticular (ER) stress (i.e. the unfolded protein response, UPR) (135). Importantly, our most recent understanding of contraction-induced IL-6 regulation has been shown to be highly dependent on the activation of the AP-1 response element via JNK phosphorylation and AP-1 (193).

Catecholamines

Stressful conditions initiate a well-coordinated physiological response marked by increased secretion of catecholamines, epinephrine and norepinephrine, from the sympathetic nervous system and adrenal medulla. In classic work, Walter Cannon coined the term “fight or flight” to describe this response to physical or psychological stress. Later, Hans Selye suggested a consistent integrative multi-organ response to stress across a wide variety of threatening stimuli. Epinephrine acts through α- and β-receptors to elicit mainly cardiovascular and metabolic effects, but less well known is the fact that it can activate components of the innate immune system. For example, it promotes an anti-inflammatory cytokine milieu by stimulating IL-1RA, IL-10 (148), and pleiotropic IL-6 (177). Additionally, catecholamines inhibit pro-inflammatory cytokines such as TNF-α and IL-1β (148).

Both α- and β-adrenergic receptors are expressed on skeletal muscle cells (3). Epinephrine predominantly binds β1/2-adrenergic receptors and is a strong stimulant for IL-6 synthesis in skeletal muscle (48). Though less studied, norepinephrine, which binds α-receptors, also stimulates IL-6 formation in C2C12 muscle cells, in a similar dosage range as epinephrine (48). The β-receptor density on muscle is proportional to the percentage of slow-twitch fibers, and the receptors’ affinities for their antagonists is higher in fast- than slow- or mixed-fibers (120). In rat, infusion of epinephrine, at a

dosage sufficient to achieve plasma concentrations comparable to sepsis, yielded a 40-fold and 15-fold increase of IL-6 mRNA and protein in skeletal muscle, respectively (48). Others have found that only supra-physiological doses of epinephrine (>100 nM) stimulate IL-6 gene expression in skeletal muscle (77). However, the latter study exposed intact soleus muscles for just 1 h of epinephrine and did not test norepinephrine. Considering the time required for diffusion, transcriptional and translational responses to occur, this may not be sufficient to see protein production in an isolated intact muscle setting. Another problem with isolated muscle experiments that we have observed is that muscle isolation is its own strong stimulus for IL-6 transcription (192), which tends to minimize the true physiological responses when treated samples are compared against sham controls.

Since the most common second messenger system for catecholamine stimulation in most cell systems is cyclic AMP (cAMP), upstream of CRE signaling on the IL-6 promoter region (Fig. 2-2), one would think that this would comprise the most likely signaling pathway for catecholamines. However, there is no evidence for this at this time. The primary pathway appears to be through the MAP Kinase signaling described above for TLR activation, i.e. JNK/AP-1 (48), Fig. 2-2.

ATP and adenosine

Several lines of evidence have shown that extracellular ATP, ADP or adenosine, cause stimulation of IL-6 formation and/or release in skeletal muscle (21) or in other cell phenotypes. ATP and its metabolites are released into the extracellular medium after pathophysiological events including hypoxia, cell swelling, shear stress, or inflammation (104, 162). The fact that cellular damage is a source of extracellular ATP (156) has resulted in ATP being sometimes included in the list of “alarmins,” as discussed above,

though purinergic receptors for ATP or adenosine do not signal via the same mechanisms as TLRs (152).

Muscle also releases considerable quantities of ATP, AMP and adenosine during contraction (21). Most of the extracellular adenosine arises from the degradation of AMP or other adenine nucleotides within the extracellular space via an extracellular ecto-5'nucleotidase. The adenosine is then resorbed for preservation of adenonucleotide concentrations in the muscle (116). P1 receptors are stimulated by adenosine, whereas P2 receptors are stimulated by ATP and/or ADP (151) and skeletal muscle sarcolemma is generally equipped with both categories of receptor (115).

The roles of P1-adenosine receptors in IL-6 regulation in adult skeletal muscle are not well understood. However, in human and rat primary cultured muscle cells, adenosine effects on IL-6 can be inhibited by a general MAPK inhibitor, but which MAPK pathway is involved is not clear (76). In cardiac fibroblasts adenosine exposure stimulates IL-6 mRNA via the p38/AP-1 pathway (144) and via PKC-δ, which is upstream of p38, Fig. 2-2 (42). In cultured myotubes and in rat muscle cells extracellular adenosine stimulates G-coupled cAMP activity, but the complete link with IL-6 regulation and P1 receptor activity has not been clearly established (71).

The P2 receptors can activate a number of physiological responses in muscle cells. P2X mediate rapid and selective permeability to several cations (151), P2Y reduce Cl- conductance (189) and stimulate G-protein-coupled activation of phospholipase C and PKC (151). Via these multiple pathways, P2 stimulation mobilizes "slow release" intracellular Ca^{2+} stores, which are then linked in several ways to IL-6 transcription (21). In several non-skeletal muscle phenotypes there are also links

between P2-ATP receptor stimulation through activation of the cAMP-PKA-CRE pathway that is synergistic with elevations in cytosolic Ca^{2+} (Fig. 2-2) (167).

Inflammatory cytokines: TNF-α and IL-1β

The innate immune system responds to acute invasion of micro-organisms, as in sepsis, with a rapid increase in circulating plasma TNF-α and somewhat later with elevations in IL-1β, see review (144). Skeletal muscle contains both IL-1β and TNF-α receptors, which are present in normal conditions, but are generally upregulated only in diseased or damaged muscle (207). The response of cultured muscle cells and intact skeletal muscle (47) to inflammatory cytokines emulates the response seen in the circulation, in that marked increases in IL-6 are produced.

As shown in Fig. 2-2, the skeletal muscle IL-6 response to TNF-α and IL-1β is mediated in part through a the JNK/AP-1 signaling network discussed previously (47). However, in addition, CCAAT enhancer binding protein C/EBP binding site (80) and NF-κB signaling (109) have been implicated as important transcriptional mediators in inflammatory cytokine-induced IL-6 formation.

Of some interest in the context of an integrated response to inflammation is the influence of anti-inflammatory signaling via glucocorticoids. As shown in Fig. 2-2, glucocorticoids inhibit IL-6 via the intracellular glucocorticoid receptor and glucocorticoid responsive elements (GRE) on the promoter region (7). Therefore, IL-6 works in a global feedback loop where stress/inflammatory signals stimulate IL-6 production and anti-inflammatory signals from steroid release feedback to suppress IL-6 release.

“Internal” Stress Signals and IL-6 Regulation

Reactive oxygen species

IL-6 is elevated in cultured myotubes but not myoblasts in response to exposure to reactive oxygen species (ROS) (100), and in humans IL-6 release from contracting skeletal muscle is inhibited by supplementation with the antioxidants, vitamins C and E (44). In vivo, short-term vitamin E supplementation reduces IL-6 mRNA and protein production in skeletal muscle and attenuates LPS-induced increases in NF-κB activation (79). Also, reduced oxidative stress with N-acetylcysteine decreases ERK1/2, p38, and NF-κB signaling proteins and also reduces IL-6 formation (171). These data suggest that ROS are an active component of internal stress-induced IL-6 signaling.

ROS are generated in a variety of different stress-like conditions in skeletal muscle (as reviewed in (198)). For example, increased ROS are produced during both exercise and periods of inactivity; as well as during exposure to hypoxia, hyperthermia and sepsis, among many other examples. ROS do not have to be formed endogenously within the cell; they can also come from external sources, but most ROS are short-lived, local signals, ideal for early signaling of local, intracellular stress. One potential stress signaling pathway that can be activated by ROS is the stress MAPKs-pathway discussed above. In skeletal muscle cell culture IL-6 has been shown to be upregulated via ROS exposure through AP-1 JNK/p38 signaling (100) (Fig. 2-2). Recent studies of in intact muscle have also suggested that the primary redox sensitive pathway is via p38 MAPK (171).

Another redox regulated signaling pathway potentially involved in IL-6 transcriptional regulation is the NF-κB family of proteins. There is considerable support

for the concept that one of the chief ways ROS influences most transcriptional targets in oxidative stress is via NF-κB signaling (87). In addition, a number of cell culture studies using different phenotypes have shown that NF-κB activation can stimulate IL-6 transcription (26). However, most recent studies have ruled out NF-κB as the predominant signaling link between ROS and IL-6 in both intact skeletal muscles (171) and in cardiac fibroblasts, in favor of a p38/AP-1 mechanism (158).

Reactive nitrogen species

Nitric oxide (NO) and other reactive nitrogen species (RNS) may also play roles in muscle's ability to respond to acute stress. All 3 NO synthases (NOS) have been detected in skeletal muscle in various conditions (19), but the neuronal isoform (nNOS) is abundantly expressed in in fast fibers and is most prevalent along the sarcolemma of fast twitch fibers (98). NO production is significantly elevated in muscle during contraction (98, 150), hypoxia (86), endotoxin exposure (48), ischemia-reperfusion (183), crush injury (155) and by exposure to inflammatory cytokines (195). NO may mediate redox-sensitive transcriptional control via several pathways discussed above (176), as well as by modification of proteins within the cytoplasm or nucleus. Pharmacological inhibition of NO during exercise attenuates increases in IL-6 mRNA (176).

The gene regulatory link between NO and IL-6 transcription has not been entirely determined in skeletal muscle, but results from other phenotypes have pointed to multiple pathways. In mononuclear cells, NO stimulates guanylyl cyclase to form cGMP, which can then activate NF-κB (170) (Fig. 2-2). Interestingly, the influence of NO is not linear. High concentrations of NO, in the pathophysiological or

pharmacological range, inhibit IL-6 formation; whereas, low concentrations (<10 μM) common to normal physiological signaling, stimulate IL-6 (170). Recent reports in osteoblasts have also demonstrated the importance of CREB/CRE signaling via cGMP for NO-stimulated IL-6 formation (17) (Fig. 2-2).

Another possible link for both RNS and ROS with IL-6 is through their influence on Ca^{2+} signaling and cytosolic Ca^{2+} concentrations. There are many well-known effects of ROS and RNS on Ca^{2+} regulation that are dose dependent. In general, both reactants, in low concentration, stimulate Ca^{2+} release from the SR or activate pathways responsible for elevating cytosolic Ca^{2+} via intracellular stores (39). A demonstration of the influence of cellular Ca^{2+} on IL-6 release is shown in the application of Ca^{2+} ionophores, which elevate intracellular Ca^{2+} and stimulate IL-6 production in skeletal muscle (77).

Hyperthermia

During intense exercise in hot environments, muscle operates about 1°C above core temperature, often reaching as high as 41°C in humans (157) and can reach ≈44°C in rats in hyperthermic exercise (18). Therefore muscle has excellent potential as a heat sensor because of the wider dynamic range of exposure during physiological conditions. We have recently identified hyperthermia to be an independent stimulus of IL-6 in skeletal muscle (192). IL-6 mRNA was upregulated at temperatures ≥41°C in a variety of experimental settings. Interestingly, the response is proportional to the intensity of heat exposure and parallels the expression of HSP72 mRNA.

Hasselgren and colleagues have shown a link between hyperthermia, heat shock proteins (HSPs) and IL-6 formation in intestinal epithelial cells (140). Through the use

of indirect activators of the heat shock transcription factors, HSF-1 and HSF-2, the IL-6 mRNA and protein expression were increased in the absence of hyperthermia (149). Therefore, the stimulus is likely via HSF interaction with the IL-6 gene. We have also provided supportive data showing that HSF inhibitor (KNK437) blocks IL-6 transcriptional responses to heat in skeletal muscle cells (192).

As a transcriptional regulator, HSF-1 is an important cofactor that functions to partially open the chromatin structure of the IL-6 promoter region, allowing for other activators and repressors to bind to it more efficiently (81). Additionally, HSF-1 affects the gene regulation of other cytokines such as TNF-α and IL-1β. HSF-1 also has an apparent direct interaction with the C/EBP (sometimes called “nuclear factor (NF) of IL-6”), an essential regulator of IL-1β transcription (199) and of IL-6 transcription (Fig. 2-2). However, in contrast to the observed physiological effects of heat on IL-6, HSF inhibits C/EBP binding to its promoter region and therefore would potentially suppress IL-6 (Fig. 2-2).

HSFs primarily bind to heat shock elements (HSE) on the DNA. HSF monomers normally present in the cytosol form trimers in protein-stress conditions that comprise the necessary configuration for nuclear translocation and DNA interaction. The HSEs on the DNA are identified as a canonical repeat of GAA-TAA nucleotide sequences with preferably two but sometimes seven intermediate nucleotides between the repeats, i.e GAAnnTTCnnGAA on the 5’-3’ strand (64, 201) (Fig. 2-3). The effectiveness of these HSE sites for binding HSF trimers has been studied under in vivo conditions (64). In both humans and mice, putative HSE sequences have been identified on the IL-6 gene. For example, Pritts et al. identified three potential HSEs within the IL-6 promoter (149),

though these sequences are still unproven and are atypical of most HSEs (201). More classical sequences are present on the human promoter at about -660 nucleotides from the origin (Fig. 2-3) (81). This HSE binding site on IL-6 has been confirmed for the human gene (81). We have identified a similar potential site in the mouse gene at -964 nucleotides from the start site, (Fig. 2-3). Note that few effective HSEs exactly fit the canonical sequence (Fig. 2-3), and relaxation of nucleotides at several locations are most often seen for actual in vivo binding sites (64). Interestingly, we have found sequences outside the 5' UTR promoter region in the human and mouse genomes that fit closely to the canonical HSE sequence (Fig. 2-3) and lie within the 2nd intron (Figs. 2-2 and 2-3), about 200 nucleotides from the end of the second exon. Though these sites have not yet been verified with respect to the physiological function, there are several examples of effective gene regulation by HSFs on intronic promoter regions (25, 103).

Unfolded protein response

A newly described stress signal that is emerging as a regulator of IL-6 and other cytokines such as IL-8 and MCP-1 is the "unfolded protein response" (UPR) (53). The UPR is initiated when the endoplasmic reticulum (ER), where most proteins are synthesized, is placed under stress such that proteins being produced remain incomplete or unfolded. This sets up a series of signaling pathways through activation of three primary signaling proteins, i.e. PERK, IRE1α and ATF6. These proteins release or activate second messengers such ATF4 and the X-box-binding protein-1 (XBP1), among others (196). UPR pathways are upregulated in a variety of muscle disorders and challenges, and following chronic loading and unloading of living muscle and therefore are very relevant to muscle pathophysiology (31).

Cytokine regulation in response to UPR is not well studied yet in skeletal muscle, but in endothelial cells and in purified B cells, IL-6 transcription is greatly stimulated by UPR via ATF4 and XBP1 (84). The signaling systems for how UPR elevates IL-6 transcription appear to be via JNK/AP-1, NF-κB, and/or C/EBP (31). However, the predominate linkage between UPR and IL-6 transcription in skeletal muscle remains unexplored.

Energy imbalance

Pedersen and Febbraio (2008) suggest that muscle's cytokine response to contraction is not inflammatory, but centered on the ongoing metabolic needs of the muscle, initiating shifts in metabolic pathways in target organs that favor energy availability. Muscle IL-6 gene expression (95) and protein release (173) are intensified during times of low intramuscular glycogen. Additionally, glucose ingestion during exercise attenuates IL-6 protein release from leg muscle (40).

Low glycogen depots are detrimental to moderate and intense exercise performance (69). The drop in glycogen storage during conditions of continued energy demands could be perceived as an "internal stress" where it is being asked to function in a condition that is incompatible with normal energy homeostasis. These conditions result in IL-6 upregulation and release. We speculate that when glycogen stores are compromised in conditions of stress or extreme exercise, the functional role of muscle may be switched to include a role as a more sensitive stress sensor/effector for the whole organism. For example, low intramuscular glycogen availability is necessary for contraction-induced HSP72 gene and protein expression, (41), suggesting the involvement of HSFs. In addition, low glycogen during contraction leads to the phosphorylation and transfer of p38 MAPK to the nucleus (23), which can upregulate IL-

6 via the AP-1 site (Fig. 2-2). Therefore, several signaling pathways are likely involved with IL-6 responses to energy imbalance.

Significance of Skeletal Muscle as a Stress Sensor

This proposed function of skeletal muscle as a stress sensor would presumably provide the whole organism with an “early warning” or “preconditioning” message. What evolutionary advantage would this provide? First, muscle is distributed throughout the body and can sample regionally or globally. Since it makes up to 40% of the body weight in man, it has an enormous capacity to sample and respond to stress signals coming from anywhere in the circulation. If the circulation is cut off to one organ system in trauma, muscles in other regions are available. Second, as discussed previously, muscle is incredibly resistant to stress, making it an ideal candidate as a resilient tissue capable of sensing stress in harsh environments. Third, muscle has abundant stores of protein as substrate for signaling molecules that can be used without greatly compromising the metabolism of contracting muscle cells.

Though it is likely that an array of signaling proteins could be produced by skeletal muscle in response to stress (144, 145), IL-6 has many important potential functions that may make it a unique prototype. Despite its reputation as a pro inflammatory cytokine, it inhibits the production of TNF-α, IL-1β, and other pro-inflammatory cytokines from inflammatory cells (200). It also promotes anti-inflammatory cytokine formation, such as IL-1RA, IL-10, as well as corticosteroid production (40). As the primary mediator initiating the acute phase response arising from the liver and other tissues, it has a critical role in responding to infection, hemorrhage, heat stroke, malignancy, or severe trauma (70).

IL-6 also has many protective and restorative roles throughout the body, particularly in acute stress settings. For example, in hemorrhagic shock, IL-6 supplementation protects the lung from injury, it protects the intestine from ischemia, and prevents circulatory collapse and cell apoptosis (4, 125, 129). In heart muscle IL-6 and associated STAT3 signaling pathways are critical for pre-conditioning and post-conditioning stimuli that protect the ischemic or damaged heart (29). In heat stroke, IL-6 deficient mice have much higher mortality (108). IL-6 has neuroprotective functions in the brain during hypoxia (11) and it promotes wound healing in a variety of tissues (124). Its strong effects on mobilization of energy stores in the liver, muscle and other organs during intense exercise (145), are also likely to play a significant role in orchestrating energy storage and release in life-threatening conditions when energy supply and delivery are compromised. Like other more classic stress hormones such as epinephrine and norepinephrine, chronic or excessive exposure to IL-6 during inappropriate times can promote and even cause serious illnesses. Therefore, as a stress hormone, it has its place and time, and functions in a delicate balance between its life-saving and life-threatening impacts on whole body homeostasis.

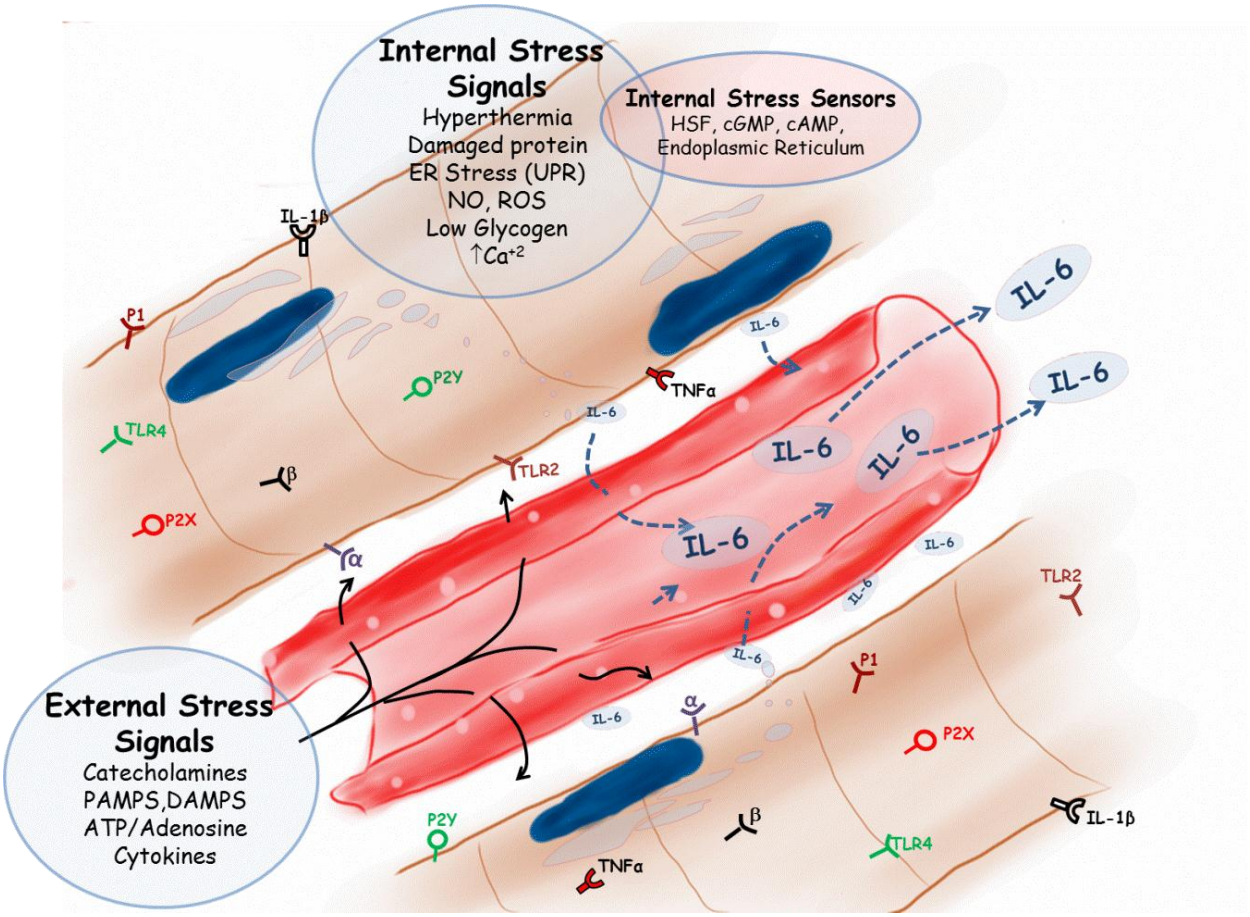


Figure 2-1. The internal and external stress-related signals that skeletal muscle is equipped to detect via receptors or 2nd messengers and that are genomically linked to regulation of the IL-6 gene.

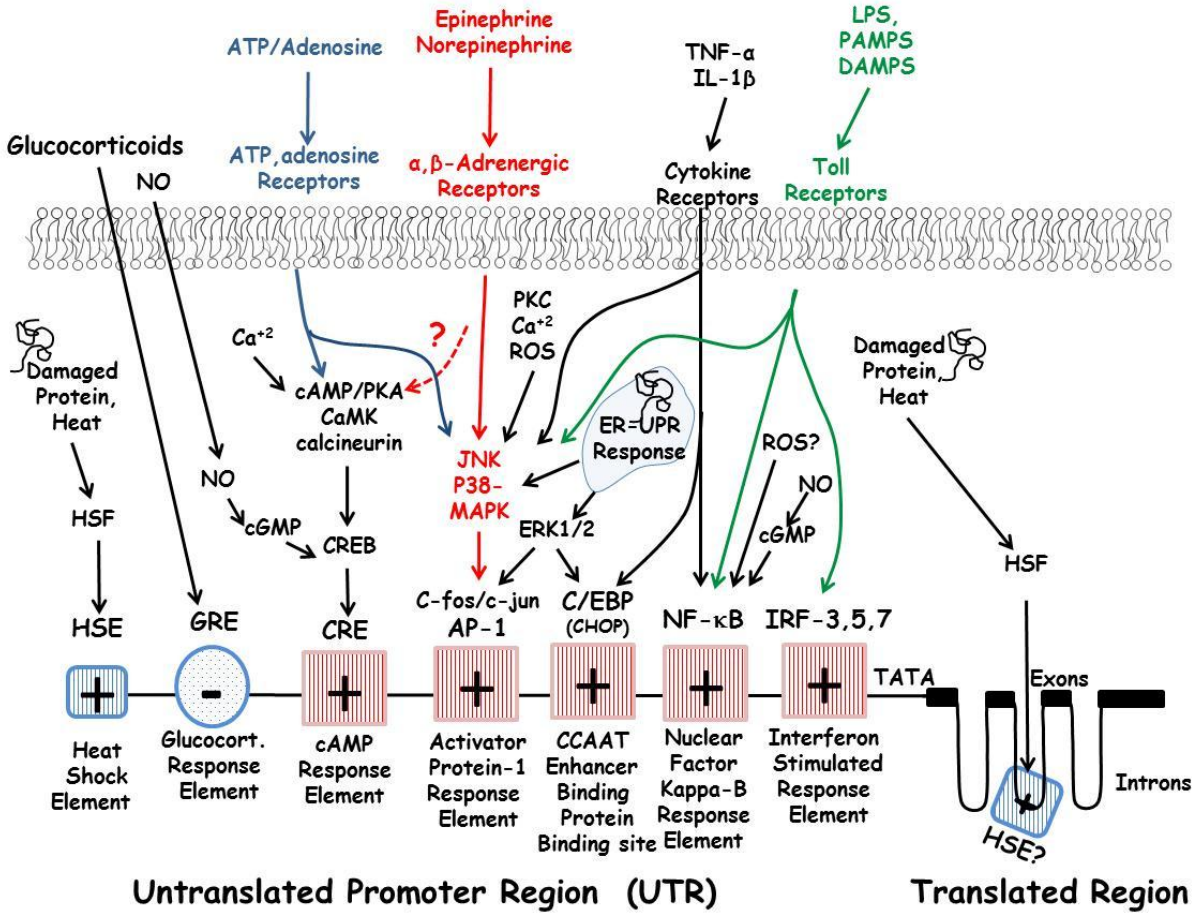


Figure 2-2. Acute stress stimuli and their relationship to the IL-6 promoter region. Note that these regulatory sites are not accurately placed in their respective location along the gene. For abbreviations, see text.

CANONICAL HSE SEQUENCE		nTTCnnGAAnATTCn	
Within the promoter			
HUMAN	-660 FROM ORIGIN	AGTTCACCCGACTTCGT	REVERSE 3′ to 5′ *confirmed*
MOUSE	-964 FROM ORIGIN	TGTTGTTGAAGTTTCAT	REVERSE 3′ to 5′
Intronic Sites			
MOUSE	+218 from exon 2	ATTTCTAGAAAATTCAC	Forward 5′ to 3′
HUMAN	+192 from exon 2	CTTTCTGGAAAATGCAG	Forward 5′ to 3′

Figure 2-3. Potential matches of the canonical HSE sequence for HSF binding in the human and mouse IL-6 gene.

CHAPTER 3
HYPERTHERMIA INCREASES IL-6 IN MOUSE SKELETAL MUSCLE [1]

Background

Muscle cytokine production has largely been viewed in the context of exercise. Early observations linked the elevations of circulating cytokines (22) with inflammatory cell activation due to muscle injury or less well defined inflammatory mediators such as endotoxin in the blood (36). However, in recent years, a paradigm shift has occurred with the observation that during exercise muscle can behave as an endocrine organ, producing significant amounts of cytokines such as interleukin-6 (IL-6) and others (144, 175). Muscle is not just a downstream effector of exercise-related circulating factors, it can also be locally stimulated to produce cytokines with contraction (75, 134, 146, 175). In addition, skeletal muscle tissue and cells have been shown to be responsive to stimuli that are not necessarily exercise related, including: endotoxin (46), inflammatory cytokines (47, 114, 186), catecholamines (48, 77, 101), low glycogen (95, 173), ATP (21), intracellular Ca^{2+} (2, 90), surgical manipulation (161), reactive oxygen species (ROS) (100), and nitric oxide (NO) (117, 176). Most of these stimuli occur during conditions of "stress" at either the tissue or the whole organism level.

Previous studies have demonstrated some interactions between core body temperature and IL-6 production during exercise. For example, when core temperature is decreased during endurance exercise in humans, the IL-6 response is attenuated (153) and when endurance exercise is performed in a heated environment, the circulating IL-6 response is augmented (172) suggesting that the skeletal muscle IL-6

[1] Reprinted with permission from: *American Journal of Physiology - Cell Physiology:* **Welc SS, Phillips NA, Oca-Cossio J, Wallet SM, Chen DL, Clanton TL**. Hyperthermia increases interleukin-6 in mouse skeletal muscle. *Am. J. Physiol., Cell Physiol.* 303: C455–466, 2012.

response is a temperature – related phenomenon. In addition, IL-6 is one of the most consistent and highly expressed circulating cytokines following heat exposure and heat stroke (15, 16, 107, 154). For example, in humans, therapeutic hyperthermia, which is used for cancer treatment (41.8°C core temperature for 30 min), results in striking elevations of IL-6, in the absence of heat illness (154). The role of IL-6 in this context is not well understood. However, IL-6 knockout animals have a greater morbidity to acute hyperthermia exposure (108) and IL-6 has the potential to suppress local and systemic inflammatory responses by reducing expression of pro-inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (160, 200). It is also the principal mediator of the acute phase inflammatory response, an important component of the hyperthermia response resulting in production of protective acute phase proteins (54). In this context it is often considered a pro-inflammatory mediator. Its actions are truly complex.

The cellular source of the IL-6 and other circulating cytokines seen during and after hyperthermia is also unknown. However, since skeletal muscle is capable of producing significant levels of cytokines in exercise, and previous studies have shown that core temperature during exercise influences circulating IL-6 (153, 172) we hypothesized that hyperthermia may be an additional and unrecognized stimulus for muscle IL-6 production. Through the use of in-vivo, ex-vivo, and cell culture models, we provide evidence to support this hypothesis, establishing that physiologically relevant acute heat exposure, within the typical range of muscle temperatures during exertional hyperthermia, high fever, or severe heat illness (41-42°C), is a novel stimulus for

muscle IL-6. In addition, we explore the influence of hyperthermia on muscle TNF-α and other cytokines and chemokines produced by skeletal muscle cells.

Materials and Methods

Anesthetized Heat Stroke Model

Male C57BL6 mice, 3-4 months old, were purchased from Jackson Laboratories (Bar Harbor, ME) and housed at the University of Florida. All animal housing and procedures were performed at The University of Florida and were approved by The University of Florida's Institutional Animal Care and Use Committee. A total of 18 adult mice were given ad libitum access to food and water prior to experimentation. Animals were anesthetized with ≈0.3 ml of a 10% pentobarbital solution (Neumbutal) IP; 0.05 mL supplements were given IP, as needed. A 0.5 ml supplement of sterile saline was given before commencing temperature-specific treatment in order to prevent dehydration and circulatory collapse during the extended experiments. No further hydration supplements were provided. Core temperature was monitored using a YSI 400 rectal thermistor connected to a temperature monitor/PID servo-control unit (Digi-sense). Data were recorded in 15 sec intervals with Digi-sense software (Eutech). Core temperature was either normothermic, maintained at 37°C, or was incrementally elevated over ~3.5 h to a peak of 42.4°C. This peak corresponds to the core temperature required to induce mild heat stroke in unanesthetized mice, as described by Leon et al. (2005). The method of temperature elevation was achieved using a protocol described in detail previously (136). Briefly, temperature was initially elevated to 39.5°C for 30 min using a long wavelength infrared heat lamp (4-14 μm λ) and then was incrementally increased 0.5°C every 30 min until the target peak was achieved for one, 20 sec sample period. This was followed by a 30 min recovery period, during which core temperature was allowed

to return to 37°C. Some additional experiments were performed with a recovery period of 2 h to follow plasma cytokine production. At the conclusion of either protocol a cardiac puncture was performed to collect blood, and the soleus was harvested and stored at -80°C for later analysis.

Ex Vivo Muscle Preparation Experiments

Soleus muscles from three groups of mice were studied in oxygenated muscle baths to understand the influence of heat exposure on muscle mRNA and protein release. In Group 1 (n = 8), mice were asphyxiated with CO2 and the soleus muscles quickly removed and placed in oxygenated Krebs Ringer solution at 22°C, containing (in mM) 121 NaCl, 5 KCl, 1.8 CaCl2, 0.5 MgCl2, 0.4 NaH2PO4, 24 NaHCO3, 5.5 glucose, 0.1 EGTA, 10 uM D-Tubocurarine. The buffer was further dosed with 10 μg/mL Polymyxin B Sulfate (Calbiochem) to eliminate the effects of potential contamination with endotoxin in the open baths. This dose has been shown to block LPS-induced activation of toll like receptor (TLR)-4 (60). Muscles were continuously bubbled with 95% O2/5% CO2, mounted and placed in custom-made 1.5 ml, pre-set to 37˚C, then maintained at 37°C (sham controls) or heated to 41˚C. The temperature was elevated in the heated baths by switching the inflow of the chamber jacket to a preheated water bath at a temperature sufficient to maintain a steady state bath temperature at 41°C. Elevating bath temperature required approximately 5 min. Two platinum wire electrodes lined the interior of each chamber to deliver current. Both muscles were stimulated to twitch with a custom-made constant current generator, driven by Grass Instruments Model S88 Stimulator. Chamber temperature was monitored using a YSI thermistor. Max stimulation current was adjusted to exceed by 10% the current required to

generate maximal twitch force and intermittent twitches were used to set optimal length (L_O). Group 1 muscles were subjected to twitches at 1/min throughout the remaining 2 h protocol. This low level stimulation has previously been shown to not fatigue isolated muscles and was used to continually monitor contractile function during the heat stimulus, as described in previous studies from our laboratory (137, 197). Following completion of the protocol the muscles were quickly blotted on tissue paper, weighed, flash-frozen in liquid nitrogen, and stored at -80˚C for subsequent analysis.

In response to results in Group 1, we hypothesized that the muscle stimulation and asphyxia protocol could have influenced the outcome. Therefore, a second group (Group 2) was studied in which animals were anesthetized with pentobarbital, prior to muscle isolation, to avoid any possible influences of CO2 asphyxiation (n=8). The soleus muscles were then prepared at room temperature in oxygenated buffer identical to that described for Group 1. However, a 4 ml standard muscle bath (Radnoti, USA) was used, and Lo was adjusted to a standard preload of 1 g, without stimulation. The muscles then underwent an identical protocol as in Group 1, but without any electrical stimulation at any time. An additional sub-group of animals (n = 6) were studied at this time, in which soleus muscles were rapidly removed from newly anesthetized animals and flash-frozen for later mRNA analysis. These tissues were used to estimate the relative changes in IL-6 and TNF-α mRNA message, relative to housekeeping genes, in both Groups 1 and 2.

To determine the influence of heat exposure on contractile function, a third group of isolated soleus muscles was studied (Group 3, n = 4). These were put through a nearly identical protocol as Groups 2, but force frequency curves were generated at

baseline and after the total two hour experimental period. As before, one soleus was exposed to continuous 37°C for 2 h and the matched soleus from the same animal was exposed to 41°C for 1 h, followed by recovery at 37°C for 1 h.

Cell Culture

The C2C12 mouse myoblast cell line was purchased from the American Type Culture Collection (Mannassas, VA). Laboratory cell stocks were routinely tested for mycoplasma infection using the method described by Zakharova et al (205). Cells were grown in 6-well cell culture plates (Corning Inc, Corning, NY), 2 ml media volume, and cultured in Dulbecco's Modification of Eagle's Medium (Mediatech Inc, Mannassas, VA) containing 10% Standard Fetal Bovine Serum (HyClone, Logan, Utah). Cells were grown to 70-80% confluence in a water-jacketed humidified incubator with 5% CO2 set at 37˚C (NAPCO 8000WJ, Thermoscientific, Marietta, OH). Experiments were performed using both myoblasts and myotubes. For preparation of myotubes, the medium was exchanged with fresh medium containing 2% Donor Equine Serum (HyClone, Logan, Utah) and allowed to differentiate for 5 days into multi-nucleated fibers. All cultures received fresh media 18 h preceding the heat treatment. A second water-jacketed humidified incubator with 5% CO2 was pre-set to an environmental temperature (T_{ENV}) of 42, 41, or 40.5°C (Forma Scientific 3154, Marietta, OH). T_{ENV} within the incubator was monitored using an YSI thermistor, accurate to 0.01°C. Once myoblasts and myotubes were placed in the incubator, it took ≈33 min for the T_{ENV} to reach 42˚C; this steady temperature was then retained for 60 min. Cells were harvested either immediately after heat treatment (1 h) or after recovery periods at 37˚C of 1 h, 2 h, and 5 h. Control cells (CON) were treated identically but kept at 37˚C for the

entire experiment. Measurements of media temperature in the baths that were heated required approximately 10-15 min delay of return of the media temperature to 37°C. In some experiments the cultured cells were treated with heat shock factor (HSF) inhibitors, ATP receptor inhibitors or NF-κB inhibitors prior to exposure to heat. Both sham controls with solvent vehicles and unheated cells treated with these agents were tested simultaneously. The doses and procedures for these agents are described in the Results section and Figure legends.

For mRNA measurements, cells were lysed in RNA-Isol Lysis reagent (5' Prime) and immediately stored at -80˚C. Additionally, the supernatant for the CON and 6 h samples were stored at -80˚C for later multiplex protein analysis or LDH determination. Additional experiments using myotubes were performed at T_{ENV} of 40.5°C and 41°C.

RNA Isolation and Real-Time PCR Analysis

Cells were harvested and lysed in RNA-ISOL Lysis Reagent according to the manufacturer's instructions. Briefly, RNA was separated from protein and DNA by the addition of bromochloropropane and precipitation in isopropanol. After a 75% ethanol wash and resuspension in DEPC H2O, purity of RNA samples was quantified using spectrophotometry. Total mRNA (1 ug) was then reverse transcribed using Verso cDNA Synthesis Kit (Thermo Scientific). Preformulated TaqMan Gene Expression Assays were purchased from Applied Biosystems for the following mouse genes: Interleukin-6 (IL-6) (Mm00446191_m1), Glyceraldehyde-3-Phosphate (GAPDH) (Mm99999915_g1), Heat Shock Protein 1A (HSP72) (Mm01159846_s1), Beta Actin (ACTB) (Mm00607939_s1), hypoxanthine guanine phosphoribosyl transferase (HPRT) (Mm01545399_m1), and Tumor Necrosis Factor (TNF-α) (Mm00443258_m1). Relative

quantitative real time reverse-transcription polymerase chain reaction (RT-PCR) was performed using the TaqMan Gene Expression Master Mix (Applied Biosystems), and reactions were performed in duplicate using 96-well optical plates on a StepOnePlus Real-Time PCR System (Applied Biosystems). Each target passed a validation experiment, thus the efficiencies between target and endogenous controls were approximately equal (<0.1). Candidates for housekeeping genes, GAPDH, HPRT, and ACTB, were tested for stability over various experimental treatments. GAPDH was used as the endogenous control to normalize the samples; GAPDH has been a commonly used stable housekeeping gene with C2C12 cells (47, 48). Relative quantitation was done using the ΔΔCT method, where CT is the threshold cycle, and all untreated samples were normalized to 1.

Cytokine Assay

Measurements of cytokines and chemokines (G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC, MCP-1, MIP-1α, RANTES, TNF-α) and soluble receptors (sGP130, sIL-6R, sTNFR1, sTNFR2, sIL-1R1, and sIL-1R2) were performed on cellular supernatant samples and from EDTA-treated plasma samples from the anesthetized hyperthermic mice. The platform used was the MILLIPLEX MAP Mouse Cytokine/Chemokine - Premixed 22-Plex Assay and a custom Mouse Soluble Cytokine Receptor Panel (Millipore, Billerica, MA). The tests were performed according to the manufacturer's protocols. Briefly, supernatant and antibody-coated beads were added to a 96-well primed filter plate and allowed to incubate overnight at 4°C. Following three washes, biotinylated detection antibodies were allowed to incubate for 1 h at room temperature (RT), after which SAV-phycoerythrin was allowed to incubate for 30 min at RT. All incubations occurred during

gentle shaking. Following three washes, beads were resuspended in sheath fluid and reactivity acquired using a Luminex 200 IS system with Xponent software (Millipore). Concentrations in pg/ml were determined using a standard curve, 5-parameter logistics and Milliplex analyst software (Viagene). Acquisition and analysis has been optimized for multiple parameter measurements.

IL-6 ELISA and LDH Assays

A mouse IL-6 ELISA (eBioscience) was used to quantify IL-6 released into the supernatant of ex vivo muscle bath and cell culture experiments. Briefly, frozen samples of physiological buffer from in-vitro experiments were brought to room temperature; samples were placed on a microwell plate, coated with a monoclonal anti-mouse IL-6 antibody. A biotin-conjugated anti-mouse IL-6 antibody is added to bind to mouse IL-6 captured by the plate coated antibody. Streptavidin-Horse Radish Peroxidase (HRP) is added to bind to the biotin-conjugated anti-mouse IL-6 antibody. A substrate solution, reactive with HRP, is then added to the wells and a colored product is formed in proportion to the amount of mouse IL-6 present. Lactate dehydrogenase activity assays were performed to evaluate cytotoxicity in the cell culture experiments. One hundred microliter samples of cell supernatant were tested using a commercially available kit (Cayman Chemical) per the manufacturer's instructions.

Statistics

Values of central tendency were expressed as means ± SE for normally distributed data and when noted, were expressed as medians for skewed distributions. Data was usually expressed as a log function, base 2, to be able to highlight the treatment effects, which often covered a broad range of response, and/or to transform skewed data to more normal distributions. Unless otherwise noted, the 42˚C cellular experiments were

repeated on three separate days with 6 samples taken from each day. The 40.5˚C and 41˚C cell experiments, as well as the signaling inhibitor experiments were performed on 4 separate days with 2 samples taken each day. For anesthetized heat stroke studies there were n=7 for matched controls and n=11 for heat stroke animals. For in-vitro animal studies, two groups of 8 total mice were used for paired CON and HEAT samples and 4 mice were used for contractile experiments (total n=12). Samples for RT-PCR were run in duplicate and analyzed by multi-way ANOVA; post hoc analysis was performed by comparing individual means using mean contrasts (SAS JMP® software). Non-parametric statistics (Wilcoxin or Kruskal Wallis for multiple groups) were used on sample populations that were not normally distributed. A minimum statistical significance was set at a $P<0.05$.

In cytokine/chemokine, multiplex analysis (Luminex), all targets were evaluated for outliers, using Grubb's outlier test (182). If one outlier was found per experimental group then that outlier was removed from analysis; if multiple outliers were found, then all data was included. Furthermore, target proteins that were predominantly found to be at minimum detectable limits were excluded. Each target, with consideration to its experimental group, was tested for normal distribution. If not normally distributed, nonparametric Wilcoxin tests were used to determine P values for differences between groups. P values for all 15 targets were adjusted using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) (9). FDR's of 0.15, 0.10, and 0.05 were compared and reported where appropriate.

Results

Response of Cultured Skeletal Muscle Cells to Acute Hyperthermia

Mean IL-6 mRNA expression was elevated ≈4-fold in myoblasts and ≈14-fold in myotubes, immediately following 1 h of 42˚C exposure (Fig. 2-1A). Surprisingly, in myotubes, but not myoblasts, IL-6 mRNA expression was at its greatest level at 5 h post-heat exposure, showing a late phase response (≈35-fold). Overall, mature myotubes had a greater IL-6 mRNA response than did myoblasts, at all time-points. TNF-α mRNA (Fig. 2-1B) was suppressed in response to heat. In myotubes, suppression was most significant at 1 h and 2 h into recovery, whereas in myoblasts, TNF-α mRNA was significantly decreased at 2 h post-heat. To test whether these effects could have been a reflection of cell damage from the heat exposure, LDH activity was measured in the media from the muscle baths in heated and sham control experiments. The distribution of LDH measurements from the heated experiments was nonparametric. The medians were 1.33 in control vs. 1.83 μU/mg lysate protein in heated baths (n = 8 independent samples/group, P = 0.56), suggesting no significant cell damage due to 1 h of 42°C heat treatment and recovery (data not shown).

Peak HSP72 mRNA was observed at 1 h post-heat (Fig. 2-1C). As with IL-6 (Fig. 2-1A), and TNF-α (Fig. 2-1B), the HSP72 response was greatest in myotubes. HSP72 mRNA was up regulated ≈1400- and 400-fold in myotubes and myoblasts, respectively. The HSP72 responses were transient; within 5 h after heat, HSP72 mRNA was no longer significantly different from baseline.

As shown in Figure 2-2, a lower level of hyperthermia (41°C) also elicited an IL-6 response and significantly inhibited TNF-α mRNA, being qualitatively similar but at a

lower magnitude than the responses to 42°C (Fig 2-1). IL-6 mRNA was increased ≈2.25- and ≈2.65-fold immediately following heat exposure (1 h) and 1 h into recovery (2 h), respectively. TNF-α mRNA was decreased ≈2.5-fold, immediately following heat (1 h). HSP72 mRNA was also increased ≈14.5- (1 h), ≈10.5- (2 h), and ≈5.8-fold (3 h). Following 40.5°C (Fig. 2-2A) exposure, IL-6 mRNA was no longer up-regulated, but rather down-regulated slightly ≈1.45- and ≈2.15-fold following 1 and 2 h (3 h) of recovery. However, TNF-α and HSP72 mRNA behaved qualitatively the same as they did after 41°C; i.e. TNF-α mRNA decreased ≈2.2-fold (1 h) and HSP72 mRNA increased ≈9.65- (1 h), ≈5.95- (2 h), ≈2.5-fold (3 h).

Effects of Hyperthermia on Protein Production in Supernatants of C2C12 Cells

Multiplex kits were used to measure protein expression released into the C2C12 culture supernatants in response to heat. These are the same experimental series in which mRNA was measured (Fig. 2-1), but samples were only obtained after a 5 h recovery period, thus representing accumulated protein over 18 hours. Therefore, the values measured after one hour of heat and 5 hours of recovery are over and above the accumulated protein arising from 18 hours of incubation at 37°C. Figure 2-3 shows absolute concentrations within the supernatants for both IL-6 and TNF-α. IL-6 was significantly elevated in myotube culture media, but IL-6 protein expression was orders of magnitude lower in myoblasts and often below the detection limit of the assay (Fig. 2-3A). In contrast, TNF-α protein production was undetectable in nearly all experiments.

Figure 2-4 compares changes in the remaining cytokines, chemokines, and soluble cytokine receptors measured with the Luminex assay. Changes are expressed as the “average difference” between samples from heated cells and the mean protein

concentration of the unheated control cells. Only samples in which over half of the measurements exceeded the “minimum detectable concentration” and showing at least a 0.1 fold change are shown. Samples that were down regulated are shown on the left (white vertical bars) and up-regulated on the right (dark shaded vertical bars). In myoblasts (Fig. 2-4A), only IP-10 of the 22 cytokines and chemokines was significantly up-regulated in response to heat. In myotubes (Fig. 2-4B), similar elevations of the same chemokines were seen in myoblasts but none were statistically significant. Using a separate soluble cytokine receptor kit (Fig. 2-4C) we also tested myotube supernatants for a variety of soluble receptors. Only sTNFR2 reached statistical significance and was down-regulated by heat exposure. In summary, of the many cytokines studied, IL-6 was the only cytokine significantly affected by heat. Of the chemokines and soluble receptors, only IP-10 in myoblasts and sTNFR2 in myotubes were significantly impacted by heat exposure.

Ex Vivo Experiments: Comparison of IL-6, TNF-α, and HSP72 mRNA Isolated Mouse Soleus

To test whether similar heat exposures induce IL-6 and TNF-α responses in intact muscles, soleus muscles were isolated and studied in tissue baths, ex vivo. In Group 1, both soleus muscles were rapidly excised from mice asphyxiated with CO2. In this experimental group the muscles were stimulated with twitch contractions during adjustment of Lo, and were also stimulated with 1 twitch/min throughout the remaining protocol. The effects of treatment are seen in Table 1. Two comparisons are made. First, mRNA expression, as measured at the end of the experiments, was compared to expression in fresh untreated soleus (using the background housekeeping genes as a reference). An unexpected finding was that isolation and incubation of soleus muscles

at 37°C bath temperature for 2 h resulted a >70-fold elevation in mRNA for IL-6, and a >7-fold elevation in TNF-α mRNA; whereas, HSP72 mRNA was unaffected. One hour exposure at 41°C hyperthermia and 1 hour of recovery at 37°C resulted in a significant and nearly 3-fold additional elevation in IL-6 mRNA which was ≈210 fold above fresh soleus. The baseline TNF-α mRNA was nearly halved by heat treatment. HSP72 was stimulated 45-fold by heat.

Because of the unexpected results in Group 1 studies, new experiments (Group 2) were done to try to eliminate the background elevation in IL-6 mRNA. These animals were anesthetized during isolation, thus allowing the muscles to be perfused right up until the final extraction step. Furthermore these muscles were never stimulated before or during the protocol, they were stretched to an “estimated” Lo (1 g), and were studied in a small but standard tissue bath. Again, isolation and exposure to the ex vivo conditions of the muscle bath resulted in a >35-fold elevation in IL-6 mRNA, and similar response of both TNF-α and HSP72 as were seen in Group 1. The effects of heat exposure were qualitatively similar to Group 1 when compared to matched controls, i.e. 1 h of heat treatment elevated IL-6 mRNA nearly 4-fold, over and above the strong background effect of muscle isolation.

IL-6 protein release from the muscle was studied from the buffer solutions at the end of the ex vivo experiments, Fig. 2-5a. In Group 1, the sham control tissue baths contained an average of 152 $\pm$ 27 pg/ml at the end of the experiment, a value that represents approximately 30 pg/mg of muscle/hour, assuming an average soleus weight of 10 mg. Although 1 h exposure at 41°C resulted in a small elevation in IL-6 protein in 5 of 7 tissue pairs, this did not reach statistical significance ($P = 0.14$). In contrast,

Group 2 animals that were not electrically stimulated at any time showed no measurable IL-6 protein in the baths in either control or heat. The data in Fig 2-5, are normalized to the same bath size. The larger baths in Group 2, in terms of the sensitivity of the assay, could not account for the differences in response.

Parallel experiments were done in Group 3 to quantify the effects of 1 hr 41°C exposure on contractile properties, Figure 2-5C. Force significantly declined by 10-20% in the hyperthermia group at twitch, 50, 80, and 150 Hz, when measured 1 h after return to 37°C. There was no measurable loss in force at any frequency over two hours at 37°C in the sham controls (Fig. 2-5B).

In Vivo Experiments: Comparison of IL-6, TNF-α, and HSP72 mRNA in Soleus and Plasma from Normothermic Versus Hyperthermic Exposure

In order to determine if IL-6 or TNF-α cytokines are elevated in a physiologically intact model of hyperthermia, anesthetized mice underwent a heat protocol that gradually elevated core temperature over a period of several hours, as described previously (136). Soleus muscles were rapidly removed at the end of a 30 min recovery following attainment of the peak core temperature of 42.4°C. The mRNA levels were then compared with sham control muscles from mice exposed to the same protocol but kept at 37°C. IL-6, TNF-α, and HSP72 mRNA levels were evaluated, as shown in Fig. 2-6. The median IL-6 mRNA was elevated 5.4-fold (Fig. 2-6A), TNF-α was elevated 4.3-fold (Fig. 2-6B) and HSP72 was elevated 63-fold (Fig. 2-6C). Therefore, heat exposure, at levels known to induce heat stroke, stimulates IL-6 gene expression in skeletal muscle tissue of intact mice. However, unlike in isolated tissue or cells, TNF-α mRNA was also stimulated.

Plasma cytokines were also measured at the 30 min time point of recovery, as well as at 2 h after peak temperature was attained. These values were compared against samples from sham control animals at these time points (Fig. 2-7). Note the median plasma IL-6 was elevated to 477 pg/ml after 30 min of recovery and 1314 pg/ml after 2 h of recovery, demonstrating a very robust circulating IL-6 response to heat. In contrast, TNF-α was elevated to 6.3 pg/ml at 30 min recovery, but was indistinguishable from sham controls at 2 h of recovery.

Mechanisms of Heat-Induced IL-6 mRNA Message

Throughout the previous experiments, the individual responses of both HSP72 and IL-6 mRNA to heat exposure (in vivo, ex vivo, and in vitro) were highly correlated (Fig. 2-8). In contrast, in the tissue bath isolation experiments the mRNA responses of IL-6 and HSP72 were completely uncoupled (Table 2-1). This suggested the possibility that some independent component of IL-6 mRNA regulation may be tied to the same signaling pathways in heat that regulate HSP72, most likely via heat shock factors (HSFs). To test this hypothesis two different HSF inhibitors, quercetin (Sigma) and KNK437 (Calbiochem) (100 μM for each, dissolved in 0.1% DMSO vehicle) were given to C2C12 myotubes, 6 h prior to heat exposure. Results were compared to control (37°C) treated with vehicle and control cells treated with the inhibitors. Results are shown in Fig. 2-9. As shown, quercetin treatment had a modest but insignificant effect on heat-induced HSP72 expression and IL-6 expression when compared to sham control 37°C, cells also treated with quercetin but without heat. In contrast, KNK437, significantly inhibited both HSP72 mRNA and completely eliminated IL-6 mRNA expression in response to heat exposure when compared against sham cells that were

unheated. Of note, these inhibitors had background influences on non-heated cells, independent of heat, i.e. KNK437 increased background mRNA expression 4.2 ± 0.7 fold and quercetin increased expression 2 ± 0.5 fold. In contrast, both drugs significantly reduced background HSP72 mRNA expression in unheated/sham control cells (not shown).

Furthermore, we identified ATP/purinergic receptor activation and the NF-κB pathway as another candidate mechanistic pathway, possibly driving the up regulation of IL-6. In electrically stimulated C2C12 cells the most potent stimulus for IL-6 production is ATP/purinergic receptor activation (21). We blocked purinergic receptors with the inhibitor, suramin (Sigma) (100uM, dissolved in 0.9% saline). Additionally, the promoter region of the IL-6 gene has a NF-κB binding site and thus an important mediator for IL-6 gene activation (110). We used the cell-permeable inhibitor peptide SN50 to block this pathway (18μM, dissolved in 0.9% saline). Interestingly, inhibition of ATP receptors and NF-κB translocation tended to further stimulate heat-induced IL-6 and HSP72 mRNA formation. Therefore, these experiments make it unlikely that either the purinergic receptor or the NF-κB pathways are important mechanisms for heat-induced IL-6 mRNA production.

Discussion

The results of this study are consistent with the hypothesis that heat is a novel stimulus for IL-6 production in skeletal muscle. Moreover, the response is proportional to the extent of heat exposure, paralleling the expression of HSP72 mRNA at different temperatures across all models and showing sensitivity to pharmacological inhibition of HSP transcription in cell culture. Our results support a potential role of skeletal muscles

as “heat stress sensors” that respond to hyperthermia exposure by producing elevated cytokines and chemokines, particularly IL-6, while suppressing other pro-inflammatory cytokines such as TNF-α. This pattern of response for muscle IL-6 mRNA is retained in conditions of heat stroke in the intact animal. However, in contrast, TNF-α is stimulated in these conditions, possibly from co-stimuli, such as endotoxin or catecholamines, known to be present in the circulation in heat stroke (1, 62) These observations may have important implications in understanding the integrated inflammatory responses observed during conditions of high fever, exertional hyperthermia, malignant hyperthermia and heat stroke.

Possible Mechanisms for Heat-Induced IL-6 Formation

The idea that IL-6 expression may be another arm of the integrated cellular response to heat was first proposed by Hasselgren and colleagues, who showed that heat stress increases IL-6 mRNA in the intestinal mucosa of mice (190) and when acute heat stress is applied to human enterocytes as a co-stimulus with IL-1β, IL-6 production is potentiated (140). HSF-1 has been proposed to have a role in partially opening the chromatin structure of the IL-6 promoter, which makes the promoter region more susceptible for activators or repressors to bind to it (81). Additionally, through the use of indirect activators of the heat shock transcription factors, IL-6 mRNA and protein expression were shown to be increased in the absence of hyperthermia, via HSF activation. Our data are entirely consistent with these findings and extend these studies to show that skeletal muscle may have an equally sensitive heat-induced IL-6 response.

Although signal transduction pathways for IL-6 gene regulation are extremely complex and include a wide variety of transcriptional regulators, as reviewed in (144), the simplest potential link between heat and IL-6 production would be via HSFs. When

activated, HSFs form trimers, move to the nucleus and interact with heat shock elements (HSE) on the DNA, inducing a myriad of physiological responses, many of which are not directly related to expression of heat shock proteins (58). Work in human intestinal epithelial cell lines has shown that the IL-6 gene promoter on the 5'UTR contains several possible HSEs (149). We tested for these same sequences in mouse using a blast search (NCBI Blast®) of the IL-6 gene on chromosome: 5; NC_000071.5 (30339701..30346508)), but were unable to locate the same HSE sequences identified in man within the 5' or 3' flanking regions (201). However, we did find one of the most potent HSE motifs (HSE3P, (201)) within the 2nd intron of the mouse IL-6 gene (numbering from 5'), beginning at nucleotide of 30340319:

nTTCnnGAAnnTTCn (common form of the HSE3P type, HSE motif; (201))

tTTCtaGAAaaTTCa (sequence in the 2nd intron of IL-6 gene in the mouse)

Such intronic regulatory sequences for HSF binding are common in many genes (58) and several have been identified as essential regulatory sites for transcriptional control of heat shock proteins (25, 103). Therefore, there is strong potential for direct control of transcription of IL-6 via HSF-HSE interactions as originally proposed by Pritts et al. (201).

We further tested the link between HSF and IL-6 using common pharmacological blockers of HSF-HSE interactions, quercetin (a bioflavanoid) and KNK437 (a benzylidene lactam derivative) (78, 203). Only KNK437 significantly inhibited heat-induced IL-6 and HSP72 mRNA formation, whereas quercetin substantially blocked neither HSP72 nor IL-6 mRNA in this cell line. KNK437 has an advantage over quercetin in that it has no known antioxidant characteristics (203) and therefore it is a more specific test of the HSF-specific interactions on IL-6. Caution is warranted in

interpretation of the KNK437 and quercetin experiments since they stimulated background IL-6 mRNA in non-heated cells, but in our experience treatment of myotubes with almost any solvent or mediator in modest concentrations elevates background IL-6. It appears to be an extremely sensitive regulatory system to external stimuli.

IL-6 and HSPs may share other common signaling pathways. For example, hyperthermia results in striking elevations in muscle ROS (210). ROS can stimulate both IL-6 (100) and HSP72 formation (61), possibly through the influence of damaged proteins on HSF induction. As an alternative hypothesis we considered the possibility that activation of ATP/purinergic receptors may be responsible for temperature-induced IL-6 mRNA stimulation. Activation of the ATP/purinergic receptors by electrical stimulation cause elevation in IL-6 mRNA in isolated C2C12 cells (21). Our rationale was that some phenotypes, such as erythrocytes, demonstrate a temperature-sensitive release of ATP in the ranges used in our study, which is believed to be an important vasodilatory signal in the skin during hyperthermia (91). However, blockade of the purinergic receptors did not depress, but rather enhanced heat-induced IL-6 mRNA formation. The NF-κB pathway is another candidate pathway, it is a major signal by which IL-6 is transcribed in immune cells such as macrophages and lymphocytes (168) as well as in contracting rodent skeletal muscle (87). The cell permeable inhibitor peptide SN50, known to inhibit translocation of the NF-κB active complex into the nucleus, was used to inhibit NF-κB activation. Similar to purinergic receptor blockade, IL-6 expression was not depressed, but enhanced.

Skeletal muscle is an ideal candidate to function as a heat sensor. It is a tissue of interest in exertional heat stress because of its capability to generate heat above core temperature. For example, during intense exercise in hot environments, skeletal muscle is ≈1°C above core temperature, often reaching as high as 41°C in humans (59, 157) and as high as ≈44°C in rats (18). Since victims of severe heat stroke generally exhibit core temperatures ≥42°C, and severe fevers from infection or drug overdose can reach 41°C (97, 166), the range of temperatures at which we have observed significant elevations in muscle IL-6 mRNA (>41°C, Fig. 2-5) have broad physiological and pathophysiological relevance.

IL-6 Production in Ex Vivo Soleus Muscles

In the isolated soleus muscles, overall effects of heat on IL-6 and TNF-α mRNA were nearly identical to those obtained in the isolated cell culture experiments. This suggests that the isolated C2C12 cell experiments are a relevant model for transcriptional regulation in response to heat. However, in every other way, the ex vivo soleus model presented a wide range of complexities and challenges in interpretation. First, we observed that the process of isolation and bath exposure had a large impact on both IL-6 (35 to 70-fold) and TNF-α mRNA (7.5 fold) expression, but no effect on HSP72 mRNA. Note that other muscle cytokine investigators have made similar observations with respect to cytokine up-regulation in ex vivo muscle preparations (161). Based on the stability of the HSP72 mRNA, our results suggest that isolation had no influence on gene transcription or housekeeping genes per se, but rather induced a non-specific pro-inflammatory stimulus that activated both TNF-α and IL-6 transcription or affected the stability of the mRNA. We eliminated three possibilities that might have caused this: endotoxin (by giving polymixin B in all buffers), asphyxia during isolation

(by maintaining circulation until immediately before resection) and muscle stimulation (by eliminating stimulation during incubation). The only untested factors that we hypothesize could account for these persistent effects are: 1) mechanical manipulation of the muscle, as proposed previously (161), 2) the high O2 partial pressures in the baths, which could induce oxidative stress and possibly stimulate ROS-sensitive inflammatory pathways such as via NF-κB (130), 3) the low O2 partial pressures that would likely be present in the core of the soleus muscles when hung in the tissue baths (12) and 4) the use of a low volume tissue bath that could result in exaggerated up regulation through an autocrine mechanism (24). We feel that these stresses may be unavoidable in isolated whole muscle preparations, making it a difficult model to apply to questions regarding cytokine regulation.

The protein production in the isolated muscle experiments also yielded interesting and unexpected results. In the first experiment, Group 1, the muscles were intermittently stimulated during incubation and the background IL-6 concentrations in the baths were extremely high in both control and heated muscle. We hypothesized that this was due to the overwhelming effect of isolation on IL-6 mRNA that simply washed out any independent effects of heat. In the second series, the muscles were not stimulated and though IL-6 mRNA was still greatly elevated due to isolation, there was no evidence of any IL-6 secretion under any condition. The only variable that we can identify that could account for these differences is the fact that Group 1 underwent intermittent stimulations. We hypothesize that in the intact muscle, transcriptional regulation and protein synthesis/protein secretion are not coupled to the same signaling systems. That is, mRNA and possibly protein production occur in response to one

stimulus (heat, etc.) but secretion requires a second hit. There are ample precedents for this. For example, HSP72, another protein secreted in a variety of stress conditions, is ubiquitously up regulated in response hyperthermia, but only secreted upon a second stimulus characterized by high intracellular Ca^{2+} (89). Although little is known about the secretory pathways for IL-6 in skeletal muscle, in human mast cells, IL-6 is packaged in 40-80 nm vesicles which must undergo some form of exocytosis (92). There are both Ca^{2+} -dependent (105) and Ca^{2+} independent (92) secretory pathways for these vesicles. Therefore, we speculate that in the intact, unstimulated muscle, the lack of elevations in intracellular Ca^{2+} or perhaps other secretory mediators resulted in no net IL-6 secretion despite high levels of mRNA and possibly protein. So, why was there secretion in cultured myotubes? One possibility is that in many isolated cultured cells, hyperthermia induces marked elevations in intracellular Ca^{2+} (126). However, in adult differentiated skeletal muscle, the tight Ca^{2+} regulation afforded by the mature sarcoplasmic reticulum may have prevented hyperthermia- induced Ca^{2+} accumulation, thus requiring superimposed muscle stimulation to induce exocytosis of the IL-6 vesicles. Further experimentation will be necessary to explore these speculations.

Regulation of IL-6 and TNF-α During In Vivo Heat Stroke

In the model of heat stroke used in this study, we demonstrated that there is a robust and very rapid elevation in circulating IL-6, and a small transient elevation in TNF-α. This pattern, seen in nearly all models of heat stroke (15, 16, 107, 154) resembles the responses of mRNA seen in the soleus muscles (Table 1), but differs from the responses seen in isolated muscle, where TNF-α was inhibited in heat. TNF-α is strongly stimulated by endotoxin, being a principle mediator of the innate immune response (10). Elevations in circulating endotoxin are commonly found during or

following heat stroke, arising from intestinal barrier dysfunction (165). We hypothesize that the differing responses in muscle TNF-α expression in whole animal heat stroke, versus isolated muscle during heat, reflect the overriding influence of other signals such as endotoxin or catecholamines that stimulate TNF-α (48, 102, 169). Since IL-6 is known to inhibit TNF-α expression in cell preparations (160, 200), it is also possible that the reduction of TNF-α mRNA seen in isolated muscle or cells could have occurred via an IL-6 autocrine inhibitory signaling pathway.

Significance

In this study we have identified heat as a stimulus for IL-6 expression in mouse skeletal muscle. However, considering the host of other stress stimuli which promote muscle IL-6 production that were previously mentioned (ROS, NO, Ca2+, muscle isolation, endotoxin, glycogen depletion, catecholamines, ATP, exhausting exercise, etc.), it is logical to hypothesize that skeletal muscle may be poised to act as a whole organism “stress sensor” and that the response to heat facilitates other stress signals driving IL-6 expression. In this way it could play a role as a sentinel for detecting and integrating multiple stress signals and responding appropriately by producing endocrine proteins, such as IL-6, that are designed to protect the organism from harm. Alternatively, skeletal muscle’s sensitivity to local stress may be oriented toward initiating repair and remodeling mechanisms within the muscle microenvironment. We hypothesize that IL-6, which remains the most predominant myokine yet discovered, has a unique role to play. Recent evidence has shown that very low levels of IL-6 protect mammals from life threatening conditions induced by severe hemorrhagic shock (4, 125, 129) and the fact that IL-6 knockout animals are more susceptible to heat stroke (108) provides theoretical support for this hypothesis.

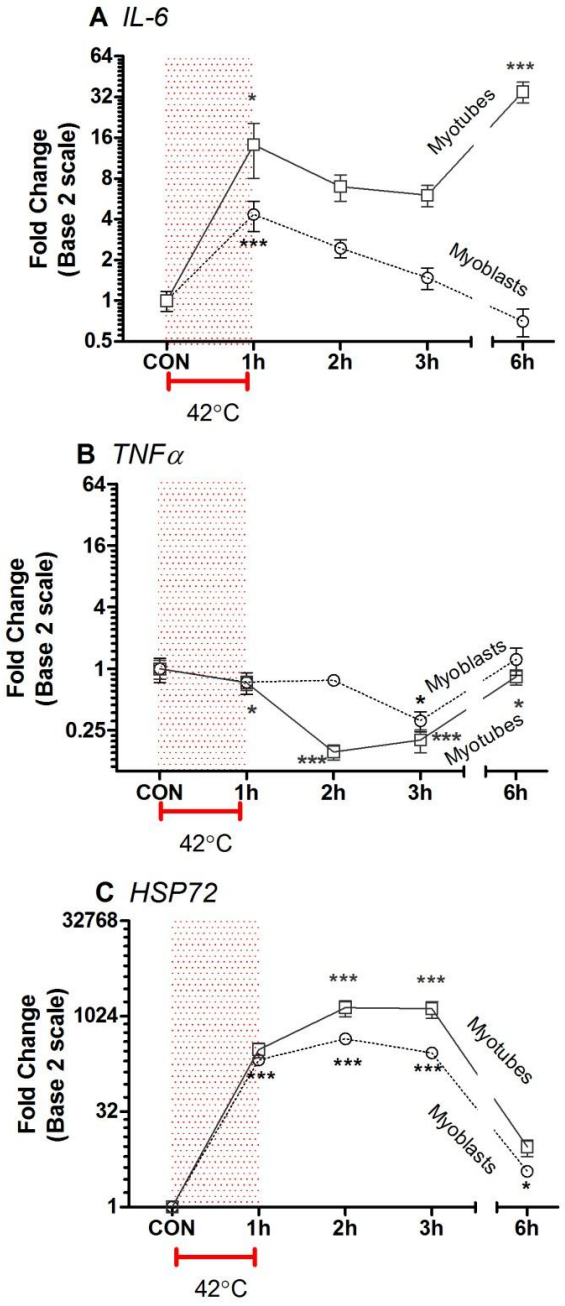


Figure 3-1. C2C12 myoblast and myotube IL-6, TNF-α, and HSP72 mRNA responses over time, following 42°C for 1h. Myoblasts (○) and differentiated myotubes (□) were grown as described in METHODS, maintained at 37°C or exposed to 42°C for 1h and harvested immediately (1h) or allowed to recover for 1h (total 2h), 2h (3h), or 5h (6h). Statistical tests were post-ANOVA least squares contrasts between means. Results are from multiple cultures in three independent experiments; * P<0.05; **P<0.01; *** P<0.0001

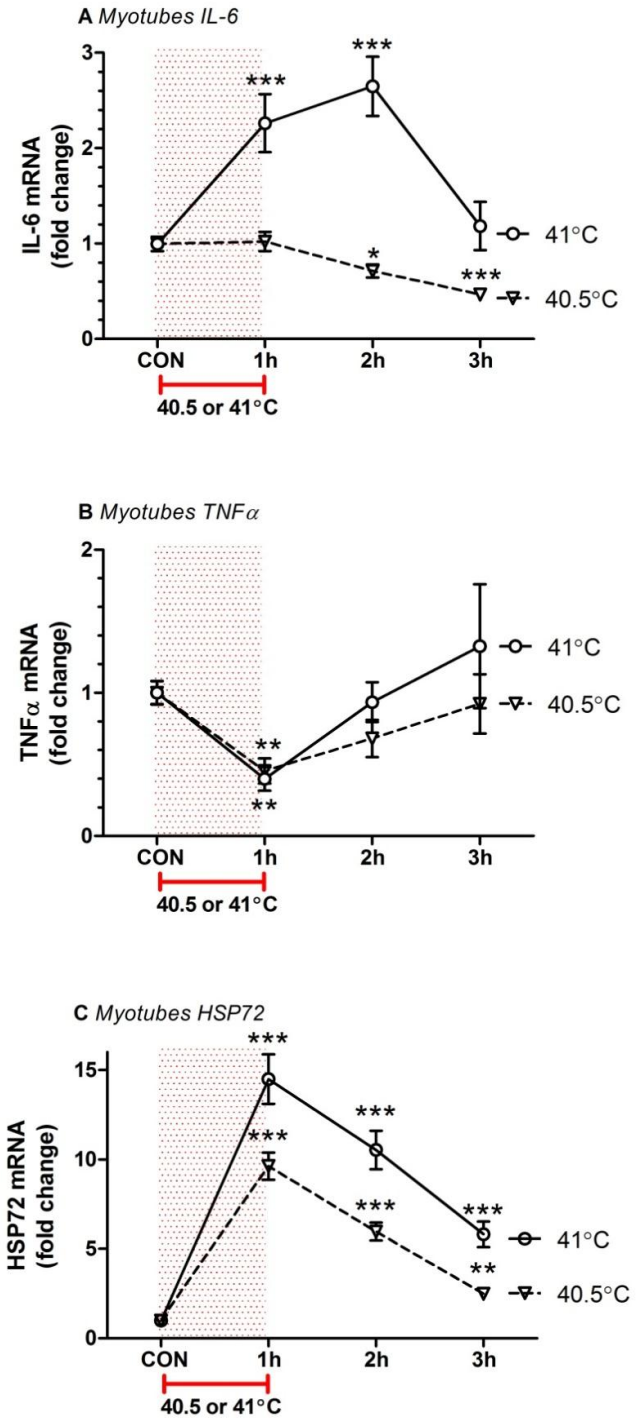


Figure 3-2. Response of C2C12 myotubes to lower levels of hyperthermia, 41°C and 40.5°C 1h. Differentiated C2C12 myotubes were treated as shown in Fig 3-1, except heat exposure was at either 41° (solid lines) or 40.5°C (broken lines) Statistical tests were post-ANOVA least squares contrasts between means. (Results are from two samples each in four separate independent experiments; * P<0.05; **P<0.01; ***P<0.0001).

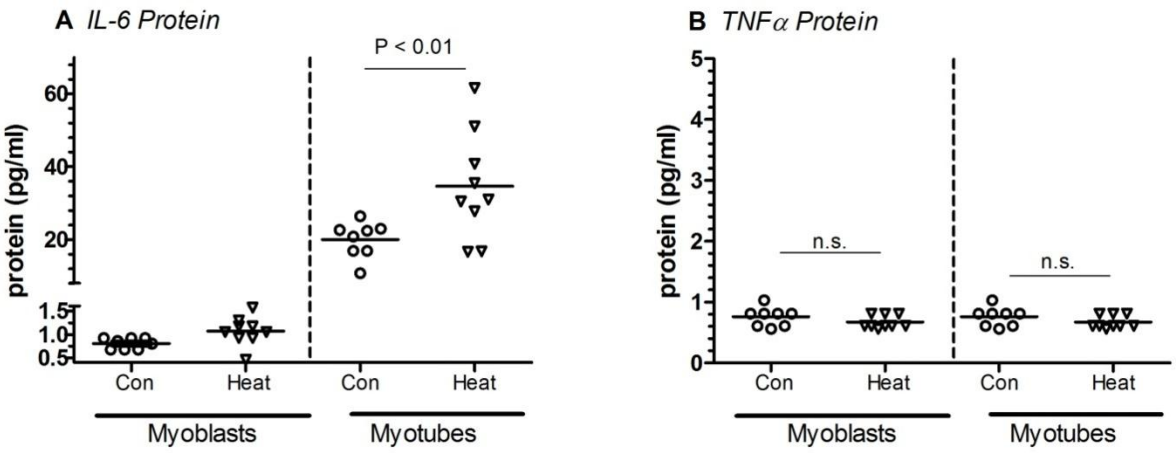


Figure 3-3. IL-6 and TNF-α supernatant protein in C2C12 myoblast and myotube cell culture: measured at 5 h recovery following 1 h of 42°C exposure. Assays are from multiplex protein measurements. Non-paired T, ** $P < 0.01$, ($n = 9$).

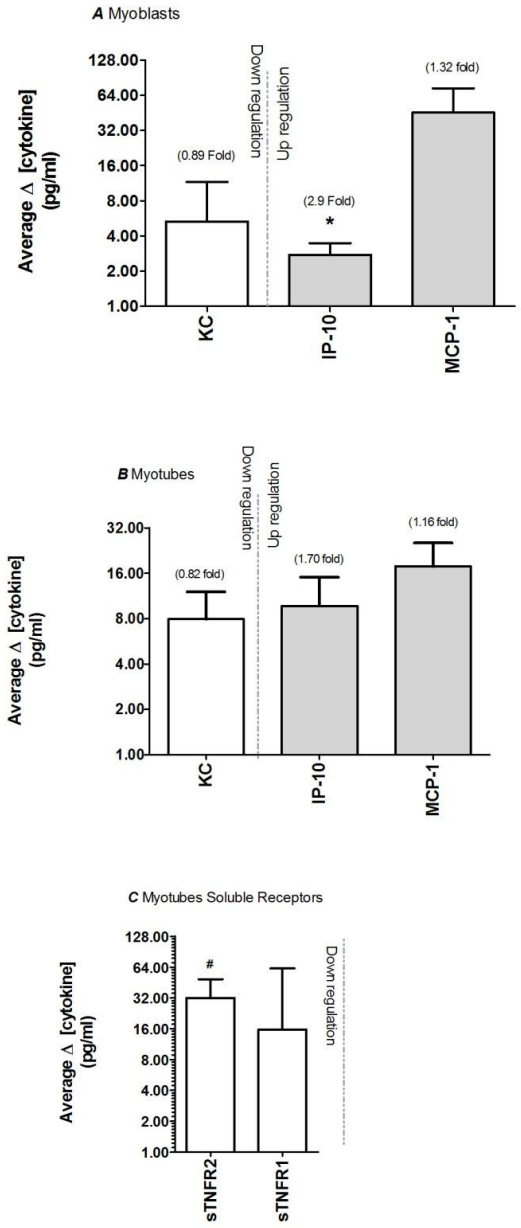


Figure 3-4. Changes in cytokine and chemokine protein expression within cellular supernatant of heated (42°C) cells compared to unheated C2C12 cells. Note: open bars are used to represent reductions in expression; colored bars represent increases in expression. Numbers in brackets are fold changes from control. A) Myoblasts, B) Myotubes. Because of many nonparametric sample populations, Wilcoxin statistical tests were performed to determine P-value. Results were adjusted using the Benjamini-Hochberg post-test to control the False Discovery Rate (FDR) for multiple testing. Results from multiple cultures in three independent experiments. All statistically differences are expressed at the $P<0.05$ level and designated as significant when FDR is set to 0.1(*), or 0.05 (#).

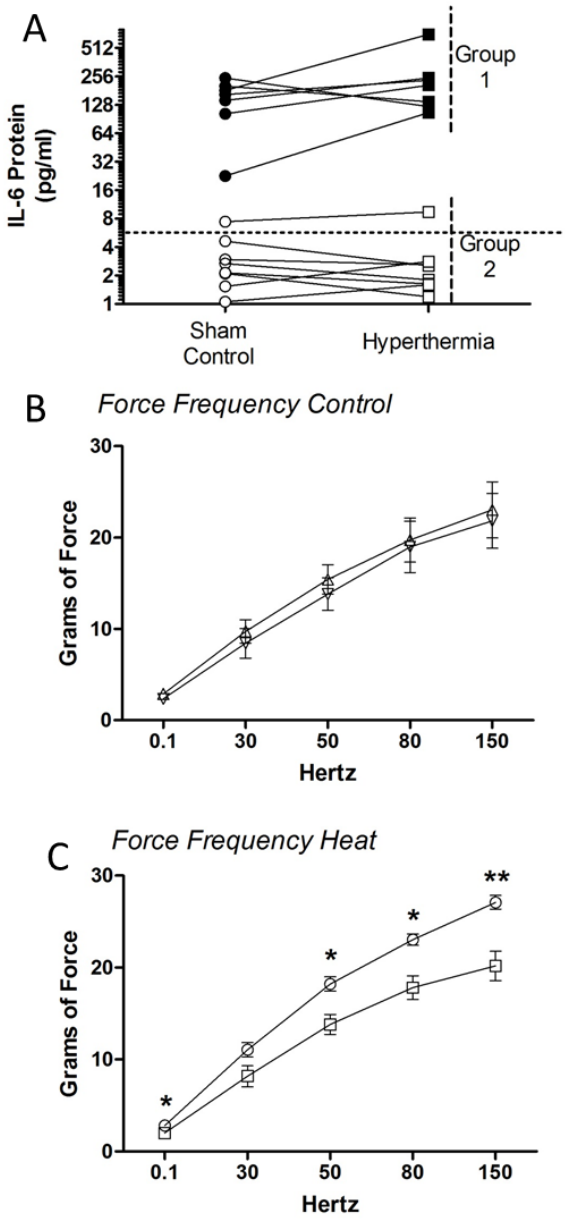


Figure 3-5. Ex Vivo Soleus protein and contractile function. A) Effects of heat on IL-6 protein release from isolated soleus muscles. Group 1, muscles were electrically stimulated at 1 twitch/sec and exposed to 1 h of 41°C followed by 1 h 37°C. Sham controls measured at 2 h of 37°C. Group 2 muscles were not stimulated electricaly. Heat did not induce a signficant elevation in protein in the baths in either experiment. Results are normalized to a standardized size muscle bath (4 ml). B) Changes in force frequency in isolated soleus in control experiments before and after 2 h bath exposure, C) O = Force frequency measurements at baseline; □ = Force-frequency after 1 h 41°C followed by 1 h 37°C. N = 4 in each group. Statistical differences using paired T-test (N=8 per group; *P<0.05; ** P<0.01).

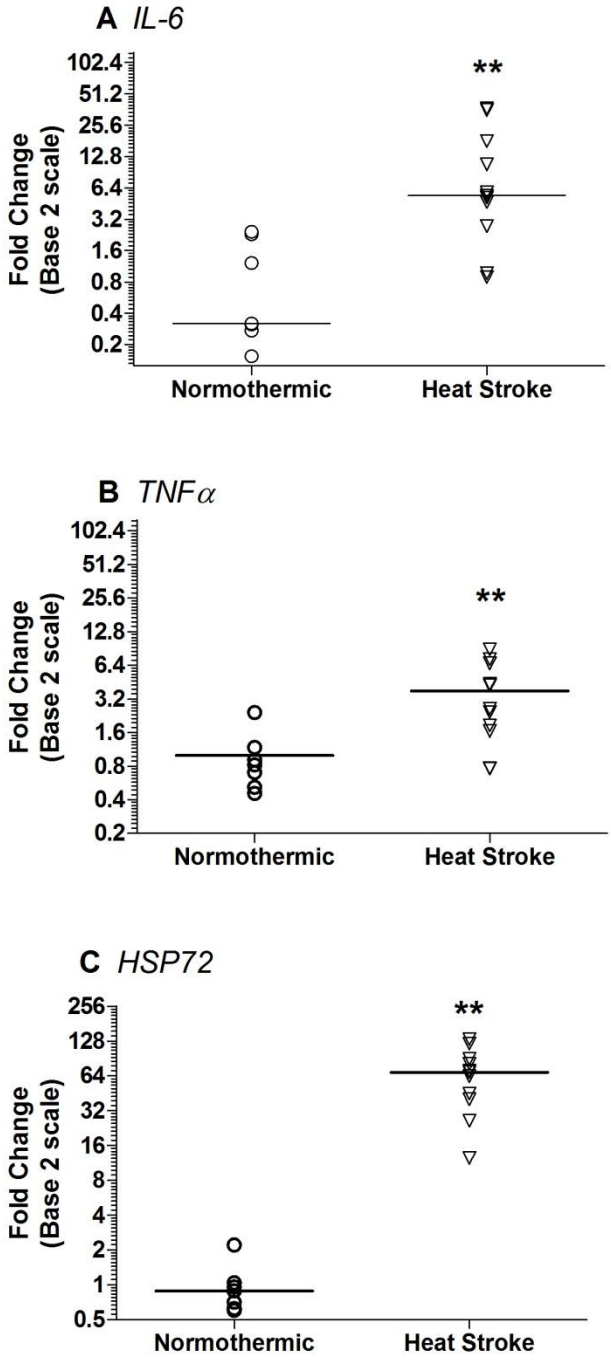


Figure 3-6. mRNA responses in in vivo soleus from anesthetized mice exposed to heat stroke conditions: The soleus was excised and analyzed for mRNA using real-time RT-PCR. A) IL-6; B) TNF-α; and C) HSP72 mRNA levels were elevated in the heat stroke (HS) mice compared to mice maintained at 37°C (CON) (N=7 and 11 for CON and HS, respectively; **P<0.01 Wilcoxon test).

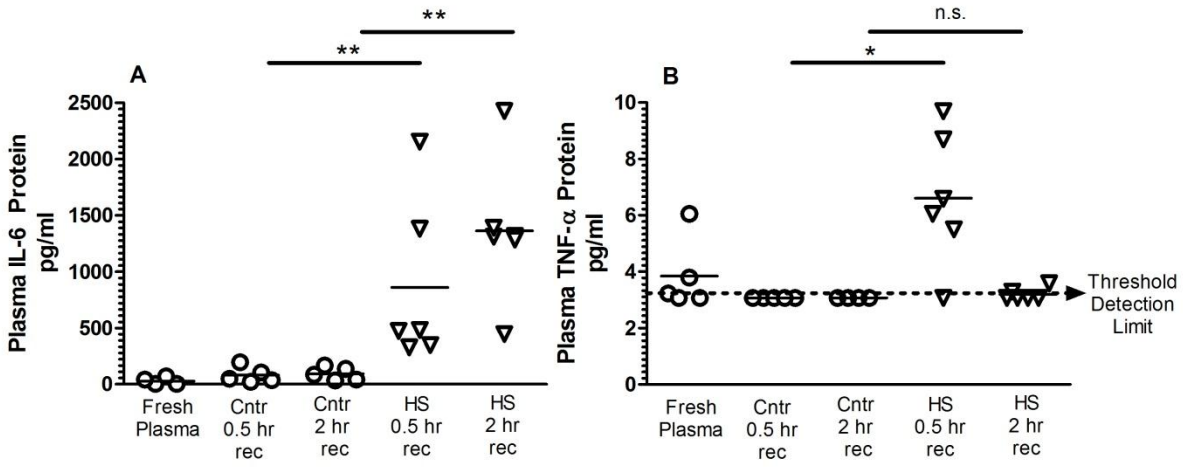


Figure 3-7. Circulating cytokine levels: A) Plasma IL-6 and B) Plasma TNF-α taken from newly anesthetized control mice (fresh plasma), time matched sham control mice 30 min after the protocol or 2 h after the protocol, and in animals exposed to a standardized heat stroke protocol, taken to 42.4°C. Plasma samples were obtained at 30 min and 2 hr after recovery at 37°C. Statistical analysis: Nonparametric Newman Kuhls, followed by post-hoc Wilcoxin, *P<0.05, **P<0.01

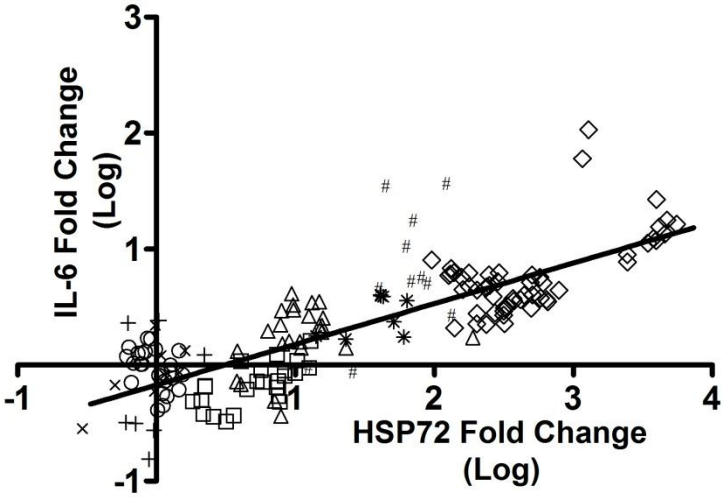


Figure 3-8. Plot of IL-6 and HSP72 mRNA changes from intact skeletal muscle and C2C12 cells. CON Myotubes (○), 40.5 oC Myotubes (□), 41°C Myotubes (Δ), 42°C Myotubes (◊), In-vivo soleus CON (X), In-vivo soleus in HEAT (*), anesthetized soleus CON (+), anesthetized soleus HS (#). (Best-fit Slope=0.3490, Slope significantly different from zero P<0.0001; r2 =0.6335)

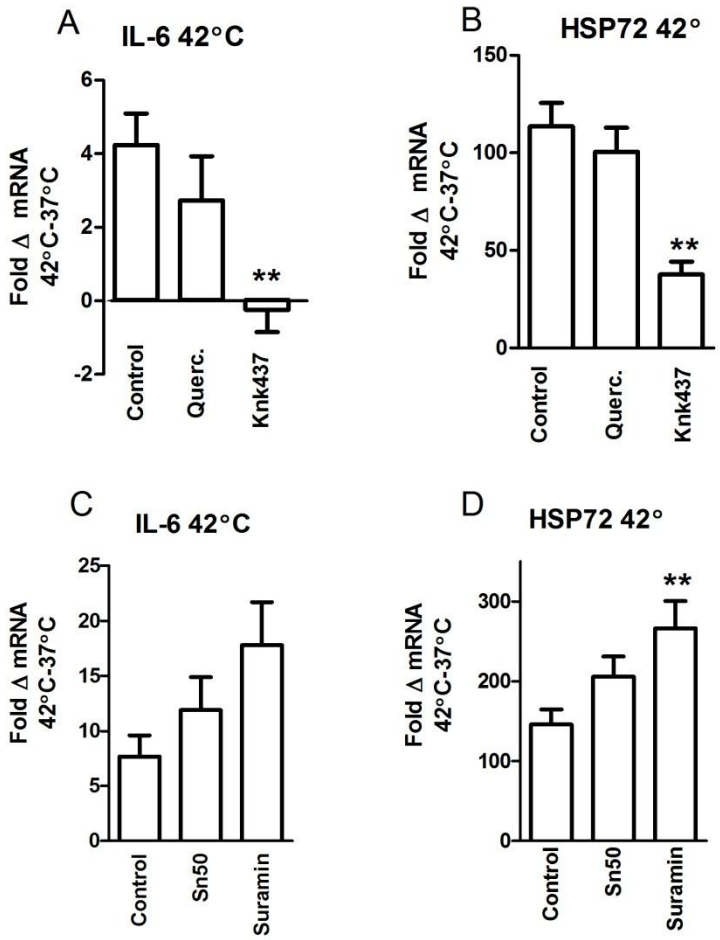


Figure 3-9. Pharmacological inhibition of HSF, ATP receptors and NF-κB signaling: A and B) Responses of IL-6 and HSP72 mRNA to pharmacological inhibition of HSF. C and D) Responses of IL-6 and HSP72 mRNA to pharmacological inhibition of ATP/purinergic receptors (suramin) and NF-κB (sn50). ** = $P < 0.01$, (Results are from two samples each obtained in four separate experiments, i.e. 8 cultured wells for each sample).

Table 3-1. Effects of muscle isolation and heat treatment on IL-6, TNF-α and HSP72 mRNA

	Fresh Soleus	Sham Control (2 h 37°C)		Heat (1 h 42°C + 1 h 37°C)	
		Group 1	Group 2	Group 1	Group 2
IL-6	1.0 ± 0.4	72.7 ± 15.2 **	35.3 ± 9.4 **	210.1 ± 28.0 ** +++	126.6 ± 12.1 ** +++
TNF-α	1.0 ± 0.2	7.2 ± 1.4 **	7.5 ± 2.2 **	3.5 ± 0.7 ** +	2.1 ± 0.5 ** +
HSP72	1.0 ± 0.2	1.1 ± 0.2	2.1 ± 0.5	45.7 ± 6.5 ** +++	45.1 ± 4.1** +++

n = 8/grp except fresh soleus where n = 7

Stats: Kruskal-Wallis followed by Wilcoxin Post hoc

* = Compared to fresh soleus

+ = Heat compared to sham control (paired data)

*** or +++ = $P < 0.001$, ** or ++ $P < 0.01$, * + $P < 0.05$

CHAPTER 4
HEAT SHOCK FACTOR-1 AND ACTIVATOR PROTEIN-1 RESPONSE ELEMENT ENHANCE INTERLEUKIN-6 GENE EXPRESSION TO STRESS STIMULI IN HEAT SHOCKED SKELETAL MUSCLE C2C12 MYOTUBES

Background

Interleukin-6 (IL-6) is a pleiotropic cytokine that has an integral role in the inflammatory response. It is a dominant signal seen in the circulation during and following stress conditions like: hyperthermia (68, 108, 192), hemorrhagic shock (185), burns (65) and trauma (119). The functional role of IL-6 during acute stress is poorly understood, but ample evidence supports positive, protective and restorative roles (4, 108, 112, 125, 129). Additionally, IL-6 serves as a primary mediator initiating the acute phase response (APR); serving critical roles responding to infection, hemorrhage, heat stroke, malignancy, or severe trauma (70). The cellular source of IL-6 seen during and after acute stress remains poorly understood.

Skeletal muscle has been identified as an endocrine organ due to its capacity to produce and secrete a variety of cytokines (myokines), such as IL-6 (144, 175). To date, myokine production has largely been viewed in the context of exercise. Much of what we know has centered on the ability of muscles to generate and secrete IL-6 and other myokines in response to exercise or metabolic challenges (144, 145). However, numerous observations suggest that skeletal muscle may also produce IL-6 in response to certain categories of internal or external stress exposure (191), including: contraction (75, 134, 146, 175), endotoxin (46), inflammatory cytokines (47, 114, 186), catecholamines (48, 77, 101), low glycogen (95, 173), ATP and adenosine (21), intracellular Ca^{2+} (2, 90), surgical manipulation (161), reactive oxygen species (ROS) (100), nitric oxide (NO) (117, 176), and heat (192). Most of these stimuli occur during

conditions of “stress” at either the tissue or the systemic level and therefore, IL-6 has many of the characteristics of a physiological stress hormone (191).

The heat shock response, mediated by heat shock factors (HSFs), is another quintessential stress response used by cells to cope with many physiological stresses. Several reports have linked IL-6 production during exercise in humans with elevated core temperatures (153, 172). Furthermore, we reported that heat stress stimulates IL-6 production in skeletal muscle and that IL-6 transcription can be blocked by the application of HSF inhibitors (192). However, whether direct HSF activation is responsible for elevation in IL-6 transcription or some indirect factor related to the pharmacological inhibition of HSF during heat is not known.

The present study had the following goals: 1) to test the hypothesis that HSF-1 regulates IL-6 transcription in muscle myotubes, in the presence or absence of heat; 2) to test the role of hyperthermia and/or HSF-1 activation as a co-stimulus or potentiator of IL-6 gene expression in response to other unrelated stress stimulants i.e. epinephrine (EPI) and lipopolysaccharide (LPS) (47, 48); 3) to test the hypothesis that HSF-1 accounts for the hyperthermia-induced effects on IL-6 gene regulation. We pursued a master regulatory pathway that could account for different integrative influences of hyperthermia on EPI- and LPS-stimulated IL-6 formation. Of the pathways tested, the results are consistent with a potential role for heat-stimulated elevations in activating transcription factor-3 (ATF-3) as a modulator of the integrated hyperthermia response of IL-6.

Materials and Methods

Chemicals and Reagents

Dulbecco's Modification of Eagle's Medium (DMEM) (Mediatech Inc, Mannassas, VA), Lipopolysaccharide (LPS) derived from Escherichia coli 026:B6 and L-Epinephrine (Sigma Chemical, St. Louis, MO), MG-132 (Z-Leu-Leu-Leu-CHO) (American Peptide Company, Sunnyvale, CA), Isol-RNA Lysis Reagent (5'-Prime, Inc.), Dimethyl Sulfoxide (DMSO) (Acros Organics, NJ, USA), Dulbecco's Phosphate Buffered Saline (DPBS), Standard Fetal Bovine Serum (FBS) (HyClone, Logan, Utah), Horse Serum (Lonza, Walkersville, MD), Tissue Protein Extraction Reagent (T-PER), HALT protease and phosphatase inhibitor cocktail, Verso cDNA Synthesis Kit (Thermo Scientific, Inc.; Rockford, IL), Bio-Rad protein assay (Bio-Rad Laboratories, USA), BD OptEIA Mouse IL-6 ELISA kit (BD Biosciences, San Jose, CA), Taqman Advanced Fast Master Mix, Opti-MEM (Life Technologies; Carlsbad, CA), FuGENE HD Transfection Reagent, Dual-Luciferase Reporter Assay System (Promega Corp.; Madison, WI).

Cell Culture

The C2C12 mouse myoblast cell line was purchased from the American Type Culture Collection (Mannassas, VA) and cultured in a water-jacketed humidified incubator set at 37˚C in 5% CO2- 95% atmospheric air (NAPCO 8000WJ, Thermoscientific, Marietta, OH). Laboratory cell stocks, passages 3-10, were routinely tested for mycoplasma infection using the method described by Zakharova et al (205). Cells were uniformly seeded to 6-well cell culture plates (Corning Inc, Corning, NY). Cultures were grown in DMEM containing 4.5 g/L of glucose, L-glutamine and sodium pyruvate with 10% FBS. Once cells reached near confluence, differentiation was induced by switching to fresh differentiation medium (DMEM 2% horse serum) and

differentiated for 5 days into multi-nucleated fibers (myotubes). Media was routinely exchanged every 1-2 days.

Plasmids and Reporter Gene Assays

Expression plasmids for the dominant-negative (d.n.) HSF-1 mutant, constitutively active (c.a.) HSF-1 mutant and empty vector (EV) were courteously obtained from Dr. Richard Voellmy (University of Miami, Miami, FL). The wild type (WT) full-length mouse IL-6 promoter/luciferase reporter containing all regulatory elements -1277 nucleotides from the start site (mIL-6.Luc) and mouse IL-6 promoter/luciferase reporter gene plasmid with a mutation in the putative AP-1 binding site (mIL-6.Luc AP-1) were a gift from Dr. Gail Bishop (University of Iowa, Iowa City, Iowa) and have previously been used and described (6). Plasmid DNA was amplified and isolated from bacterial cultures using Endotoxin-Free Maxi Prep Kits (Qiagen, Valencia, CA), precipitated in ethanol and resuspended in Tris-EDTA (TE) buffer for transfections in culture.

Myoblasts were transfected with plasmid DNA at 60-80% confluence using FuGENE HD Transfection Reagent at a 3.5:1 ratio of reagent to total DNA. Sixteen hours following transfection, muscle cells were differentiated into myotubes by incubation in differentiation medium. For all studies, 5-day differentiated myotubes were treated as described in cellular experiments (below) in differentiation media for 6 h. For reporter experiments, cells were harvested in 500 ul of passive lysis buffer (PLB), and luciferase activity was determined by normalizing firefly luciferase activity to Renilla luciferase activity using a Dual-Luciferase Reporter Assay. Luciferase activity of cell lysates was measured in relative light units (RLU) (Biotek Synergy 2 platform; Winooski, VT) and normalized (normalized RLU = RLU firefly luciferase/RLU Renilla luciferase).

Cellular Experiments

A variety of experiments were conducted to evaluate C2C12 cells responses to varying stress stimuli: EPI and LPS at varying temperatures or with various pharmacological interventions. All results were obtained from on three to five separate experiments to ensure reproducibility.

Heat Treatment

Myotubes were supplemented with EPI (100 ng/mL) or LPS (1000 ng/mL) and maintained at 37˚C or acutely treated in a second water-jacketed humidified CO2 incubator pre-set to an environmental temperature (T_{ENV}) of 40.5, 41, or 42˚C (Forma Scientific 3154, Marietta, OH). T_{ENV} within the incubator was monitored using an YSI thermistor, accurate to 0.01°C. Once cells were placed in the incubator it took ≈20 min for the T_{ENV} to reach the desired experimental temperature. Cells were harvested following the culmination of heat treatment (1 h) or after heat with recovery periods at 37˚C for 1 or 2 h; control cells (CON) were kept at 37˚C for the entirety of the experiment. Cells were lysed in Isol-RNA Lysis reagent and stored at -80˚C immediately.

In a separate experiment, myotubes were dosed with LPS (1000 ng/ml) or EPI (100 ng/ml). Treated cells were either maintained at 37°C for the entire experiment (6 h) or exposed to an experimental temperature (40.5, 41, or 42°C) for the first hour and 37°C for the remaining 5 h. The cellular supernatant was collected and the myotubes were lysed then stored at -80°C awaiting analysis.

Pharmacological Induction of Heat Shock

The proteasome inhibitor MG-132 was solubilized in DMSO, control cells were incubated in corresponding concentrations of DMSO (0.5% vol/vol). Cells were

pretreated for 1 h with MG-132 (10 uM) or vehicle. The concentration of MG-132 was based on previous studies in which they induced the heat shock response (149, 208). After preincubation for 1 h with MG-132 or DMSO, LPS (1000 ng/ml) or EPI (100 ng/ml) was added to the culture medium. Treatment of C2C12 cells with these concentrations of EPI and LPS have resulted in maximal or near-maximal IL-6 production (47, 48). Cells were harvested for RT-PCR at 1, 2 and 3 h and for protein analysis at 6 h.

RNA Isolation and Real-Time RTPCR Analysis

Cells were lysed in ISOL-RNA Lysis Reagent according to the manufacturer's instructions. Briefly, RNA was separated from protein and DNA by the addition of bromochloropropane and precipitation in isopropanol. After a 75% ethanol wash and resuspension in DEPC H2O, purity of RNA samples was quantified using spectrophotometry. Total mRNA (1 ug) was then reverse transcribed using Verso cDNA Synthesis Kit. Preformulated TaqMan Gene Expression Assays were purchased from Applied Biosystems for the following mouse genes: Interleukin-6 (IL-6) (Mm00446191_m1), Activating Transcription Factor -3 (ATF-3) (Mm00476032_m1), Glyceraldehyde-3-Phosphate (GAPDH) (Mm99999915_g1), Heat Shock Protein 1A (HSP72) (Mm01159846_s1), Beta Actin (ACTB) (Mm00607939_s1), and Hypoxanthine Guanine Phosphoribosyl Transferase (HPRT) (Mm01545399_m1). Relative quantitative real time reverse-transcription polymerase chain reaction (RT-PCR) was performed using the TaqMan Fast Advanced Master Mix, and reactions were performed in duplicate using 96-well optical plates on a StepOnePlus Real-Time PCR System (Applied Biosystems). Candidates for housekeeping genes, GAPDH, HPRT, and ACTB, were tested for stability over various experimental treatments as previously shown (192). GAPDH was used as the endogenous control to normalize the samples; GAPDH

has previously been used as a stable housekeeping gene for C2C12 cells treated with EPI and LPS (47, 48) and in heated cells (192). The changes in IL-6, ATF-3 and HSP72 mRNA were independent of changes in the level of mRNA for GAPDH. Relative quantitation was done using the ΔΔCT method, where CT is the cycle threshold, and all untreated samples (CON) were normalized to 1.

Protein Extraction and Quantification

Cells were washed with DPBS without calcium or magnesium and lysed for 15-20 mins at 4°C by the addition of 350 ul of T-PER lysis buffer containing HALT protease and phosphatase inhibitor cocktail. The lysate was transferred to clean microcentrifuge tubes and stored at -80°C for subsequent analysis. Total protein was quantified using the Bio-Rad protein assay using bovine gamma globulin (IgG) as a standard.

ELISA

A mouse BD OptEIA IL-6 ELISA kit was used to evaluate IL-6 protein. Briefly, 96-well clear flat bottom non-treated polystyrene microtest plates (BD Falcon, Franklin Lakes, NJ) were incubated with anti-mouse IL-6 capture antibody overnight at 4°C. The next day, plates were washed and blocked with 200 ul of assay diluent (PBS + 10% heat-inactivated FBS) for 1 h. Frozen samples of cellular supernatant were brought to room temperature, diluted (if necessary), and samples were plated. The working detector was prepared by adding Streptavidin-Horse Radish Peroxidase (HRP) conjugate to the biotinylated anti-mouse IL-6 antibody. The working detector binds to the IL-6 captured by the plate coated antibody. A substrate solution, reactive with HRP, is then added to the wells and a colorimetric product was formed in proportion to the amount of mouse IL-6 present. Concentrations of samples in pg/ml were interpolated

using a 5-parameter logistic standard curve, using Prism Software 5.0 (GraphPad Software, Inc.).

Statistics

Values of central tendency were expressed as means ± SE. Cellular experiments were repeated 3-5 separate times with 2-3 samples taken from each experiment (total n=8-10). Data were analyzed by t test and multi-way analysis of variance (ANOVA) where appropriate; post hoc analysis was performed by comparing individual means using mean contrasts (SAS JMP® Pro 10 software). A minimum statistical significance was set at a $P<0.05$. Statistical significance is denoted in asterisk form: * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$), and **** ($P<0.0001$).

Results

Hyperthermia Stimulates IL-6 Gene Expression, Role of HSF-1

IL-6 mRNA expression was consistently increased as a function of heat intensity at temperatures of 41°C or greater (Fig. 4-1A), confirming our previous studies (192). To address the question as to whether the IL-6 response was due to the activation of HSF-1 or whether it reflected other stress-induced pathways, such as via ROS (137), etc. we first tested whether stimuli other than hyperthermia that are known to activate HSF-1 would also upregulate IL-6. We employed a commonly used approach of activating the heat shock response by inhibiting the proteasome, which results in accumulation of damaged/misfolded proteins (208). Differentiated myotubes were pretreated with MG-132 (10 uM) 1 h, a treatment previously shown to activate HSFs (208) and sham control myotubes were treated with the vehicle (DMSO). Myotubes were then harvested for real time RT-PCR 1, 2, and 3 h after MG-132 pretreatment. As shown in Fig. 4-1B, MG-132 treatment induced the heat shock response (evidenced by

increased HSP72) in as little as 1 h following pretreatment. Regulation of HSP72 is mediated by the activation of HSF-1 (131, 132). Treatment with MG-132 also resulted in rapid accumulation of IL-6 mRNA during exposure (Fig. 4-1C), thus demonstrating that hyperthermia is not necessary for the IL-6 response, but rather that signals related to accumulation of damaged proteins are sufficient for IL-6 activation.

For the next series of experiments we acquired an IL-6 luciferase reporter construct (6) to evaluate IL-6 transcriptional regulation. Myoblasts were co-transfected with the mouse IL-6 promoter/luciferase reporter (mIL-6.Luc) and with a Renilla luciferase control reporter vector (pRL-TK). In all remaining experiments the Renilla reporter was incorporated with transfection. After transfection, the myoblasts were differentiated into myotubes over 5 days and then subjected to the hyperthermia treatment (42°C for 1 h, followed by 5 h of 37°C recovery). The results are shown in Fig. 4-1D, where the luciferase activity of cell lysates is expressed in relative light units (RLU) compared to the RLUs of the sham-transfected controls which were always set to 100%. Hyperthermia (42°C 1 h) stimulated mouse IL-6 luciferase promoter activity , as shown in Fig. 4-1D, in a manner similar to the results in wild type IL-6 mRNA measurements (Fig. 4-1A); therefore verifying the sensitivity of this IL-6 reporter to heat treatment.

The direct role of HSF-1 was then tested using co-transfection of plasmids in which the activity of HSF-1 was manipulated. Results were compared against cells co-transfected with empty vectors (EV) for these modified HSF-1 plasmids. To test the direct effect of HSF-1 in the absence of hyperthermia, we overexpressed HSF-1 with a constitutively active form of the HSF-1 plasmid (c.a. HSF-1). When tested in continuous

basal conditions (37°C), a significant increased IL-6 promoter activity was observed (Fig. 4-1E), confirming a regulatory role of HSF-1 on IL-6 transcription within the reporter region. We then tested the effects of a dominant negative form of HSF-1 (d.n. HSF-1), which functions to out-compete endogenous HSF-1, preventing it from effective trimerization and DNA binding. The knockdown of HSF-1 under basal (37°C) or heated (42°C 1 h) conditions significantly decreased the activity of the IL-6 promoter (Fig. 4-1F). Interestingly, when comparing the influence of the d.n. HSF-1 on the IL-6 promoter activity in these two conditions, a significantly greater "relative" influence of d.n. HSF-1 on the promoter activity was seen at 37°C compared to its influence at 42°C. This effect would be predicted to hold true in an "absolute" sense as well, because the overall responses of the luciferase reporter activity at 42 °C are predictably higher than at 37°C (Fig 4-1D). These results suggest that HSF-1 must play an integral role regulating IL-6 during basal conditions. Furthermore, based on the differential responses during heat exposure either the d.n.HSF-1 could not completely knock down endogenous HSF-1 activity during hyperthermia or other signaling pathways are contributing to IL-6 mRNA stimulation in hyperthermia.

Heat Potentiates IL-6 Gene Expression in Myotubes Treated with Lipopolysaccharide

Based on previous studies regarding the influence of HSFs on DNA confirmation and regulation (81), we hypothesized that the impact of hyperthermia and/or HSF-1 activation on IL-6 regulation may be extremely important as a "co-stimulus" with other stress-induced signals. This would have important implications in conditions of bacterial infection and fever, thus combining both hyperthermia and endotoxin (LPS) stimuli. Therefore, in this series we designed experiments to test the interaction

between the effects of heat and LPS in skeletal muscle myotubes. Myotubes were treated with LPS (1 ug/ml) and exposed to 1 h of 37, 40.5, 41, or 42°C environmental temperatures. After 1 h, cells were harvested or returned to 37°C for 1 or 2 h of recovery and then harvested. IL-6 mRNA was potentiated in myotubes co-treated with 41° or 42°C, immediately following heat (Fig. 4-2A). Interestingly, treatment with the intermediate temperature (41°C) resulted in the greatest potentiation (≈6 fold above the response of LPS alone), showing maximal potentiation after 1 h of 41°C heat and 1 h of 37°C recovery (2h), even beyond that of 42°C at any time point (Fig. 4-2A). The data illustrates that the influence of hyperthermia on the response to LPS at 42°C, though still present, is attenuated compared to the amplifying effect seen at lower temperatures (41°C).

Surprisingly, the effect of treatment with the proteasome inhibitor, MG-132, designed to mimic heat shock, when combined with LPS, resulted in decreased IL-6 mRNA response compared to LPS treatment alone (Fig. 4-2B). However, similar to the effects of heat treatment and LPS on mRNA (Fig. 4-2A), the addition of heat to myotubes transfected with the IL-6 luciferase reporter show potentiation of the IL-6 promoter response (Fig. 4-2C). The overexpression of HSF-1 also resulted in potentiated LPS-induced IL-6 promoter activity (Fig. 4-2D). Meanwhile, knockdown of HSF-1 with the d.n.HSF-1 mutant significantly attenuated the IL-6 promoter activity that occurred in response to LPS treatment (Fig. 4-2E). This was present in both basal and heated conditions, with a more predominant attenuation of the relative LPS response in basal conditions (Fig. 4-2E). These results demonstrate that HSF-1 directly potentiates

the LPS-driven IL-6 formation and that additional factors other than HSF-1 must play a role in IL-6 regulation during proteasome inhibition with MG-132.

Heat Potentiates IL-6 Gene Expression in Myotubes Treated with Epinephrine

The catecholamines, epinephrine and norepinephrine, are well known stimuli of IL-6 formation in muscle myotubes (48). To study the interactions of heat as a co-stimulant for this stress signal, we treated cells with EPI (100 ng/ml), with and without hyperthermia. Heat (both 41 and 42°C) greatly potentiated EPI-stimulated IL-6 mRNA (Fig. 4-3A). Unlike the interactions with LPS, the highest intensity of heat (42°C) resulted in the greatest potentiation effect, observed across all 3 h of evaluation (Fig. 4-3A). Also, in contrast to LPS, MG-132 inhibition of the proteasome potentiated the EPI-induced IL-6 mRNA response at both 2 and 3 h post -EPI treatment (Fig. 4-3B). Analysis of IL-6 promoter activity confirmed the potentiation of IL-6 in cells treated with heat (42°C) and EPI (Fig. 4-3C). Interestingly, the overexpression of HSF-1 combined with EPI treatment in cells maintained at 37°C yielded no significant changes in IL-6 promoter activity (Fig. 4-3D). However, the knockdown of HSF-1 under basal and heated conditions decreased IL-6 promoter activity (Fig. 4-3E). In contrast to the previous stimuli, greater knockdown of IL-6 promoter activity was seen in unheated myotubes compared to 37°C myotubes (Fig. 4-3E). These results show that in general, hyperthermia and/or HSF-1 appear to amplify the mRNA responses of myotubes to EPI. Importantly, there are clearly distinct differences in the nature of these interactions compared to those of LPS and hyperthermia.

Heat Induces ATF-3 mRNA; Epinephrine Co-Stimulation Attenuates Heat-Induced ATF-3 mRNA

Previous studies have identified activating transcription factor -3 (ATF-3) as a critical modulator of the interactions between the IL-6 response to LPS and HSF-1 (181). It is considered a “stress gene” and is one of the most transcriptionally active genes in acute exposure to hyperthermia (202). Since it is a negative regulator of LPS-induced IL-6 transcription, we hypothesized that it could be responsible for the differential effects of heat on LPS vs. EPI at high temperatures. To explore this possible mechanism we measured the effects of heat, LPS and EPI on ATF-3 mRNA expression. Myotubes were maintained at 37°C for 3h or exposed to 42°C for 1 h followed by 37°C for 2 h. Cultures were stimulated with heat alone, or co-stimulated with LPS (1 ug/ml) or EPI (100 ng/mL). Hyperthermia was a potent stimulus of ATF-3 mRNA, but EPI or LPS alone had no influence on ATF-3 mRNA (Fig. 4-4). However, when LPS and hyperthermia were given simultaneously, ATF-3 remained elevated but when myotubes were exposed to hyperthermia in the presence of EPI, there was no significant elevation in ATF-3 and it remained at basal levels. Therefore, the results are consistent with the hypothesis heat-induced signals such as ATF-3 may contribute inhibitory effects on IL-6 mRNA production, and that EPI stimulation suppresses this response. This data stands as a working hypothesis for the differential effects of hyperthermia on LPS vs. EPI-stimulated IL-6 mRNA.

The AP-1 Binding Site within the Mouse IL-6 Promoter is a Mediator to Heat Stimulated IL-6 Promoter Activity

Based on the observations from figures 4-1F, 4-2E, and 4-3E, where suppression of HSF-1 did not completely inhibit the responses of the IL-6 promoter to superimposed hyperthermia, we hypothesized that there is likely to be other parallel signaling

pathways besides HSF-1 that influence IL-6 regulation in hyperthermia. In previous work we observed no significant effect of pharmacological inhibition of NF-κB or purinergic receptor activity in hyperthermia (192), both important pathways of IL-6 transcriptional regulation that could be affected by hyperthermia, see review (191). However, another candidate site for heat-induced IL-6 regulation is the stress-induced AP-1 element. Proteins that activate the AP-1 site have previously been shown to be stimulated following heat shock (123). To test the hypothesis that activation of the AP-1 promoter site is also an integral component of heat-induced IL-6 transcription, C2C12 myoblasts were transfected with the mIL-6.Luc or a mIL-6.Luc with a mutated AP-1 binding site (mIL-6.Luc AP-1) (6). Results shown in Fig. 4-5, expressed again in RLU, but normalized to percentage of response of the intact mIL-6.luc promoter activity in the particular experimental condition (i.e. % IL-6 promoter activity=activity of mIL-6.Luc AP-1 mutant / activity of mIL-6.Luc) . During basal (37°C) conditions, there were no significant changes between the RLU activity of mIL-6.Luc vs. mIL-6.Luc AP-1 transfected myotubes, even in cells treated with LPS or EPI (Fig. 4-5A). However, with the addition of hyperthermia, the relationship between mIL-6.Luc and mIL-6.Luc AP-1 significantly changed (Fig. 4-5B). Heated mIL-6.Luc AP-1 myotubes showed significant reductions in IL-6 promoter activity compared to heated myotubes transfected with intact mIL-6.Luc (Fig. 4-5B). There was also a significant change in IL-6 promoter activity between heated EPI or LPS treated mIL-6.Luc AP-1 myotubes compared to those transfected with the intact mIL-6.Luc promoter (Fig. 4-5B).

These results are consistent with the hypothesis that in addition to HSF-1, hyperthermia stimulates IL-6 mRNA formation via activation of the AP-1 element of the

promoter regions of the IL-6 gene. An unexpected finding was that in the absence of hyperthermia, the AP-1 site appears to be unable to respond to EPI or LPS stimulation. The AP-1 site appears to require simultaneous hyperthermia to sensitize it to these stimuli. Furthermore, the consistent findings for both EPI and LPS in heat do not reveal any differential transcriptional response to hyperthermia, suggesting other mechanisms are involved.

Heat Differentially Regulates IL-6 Protein Expression

To test if IL-6 protein production is also potentiated by heat exposure, we treated C2C12 myotubes with EPI or LPS and exposed them to either 37, 40.5, 41, or 42°C for 1 h followed by 5 h of recovery at 37°C. Fig. 4-6A-C shows the amount of IL-6 protein released into the culture supernatant and Fig. 4-6D-F shows IL-6 protein extracted from the lysed myotubes. Heat stimulated IL-6 protein secretion after 42°C exposure (Fig. 4-6C), but this could not be detected with our assays at 40.5°C or 41°C (Fig. 4-6A and B). There was no effect of hyperthermia exposure alone on the IL-6 protein stored in the lysate at any temperature (Fig. 4-6D-F). Examining the effect of LPS and EPI alone, at 37°C, they both increased IL-6 secretion (Fig. 4-6A-C). Interestingly, at 37°C there was no appreciable or consistent increase in IL-6 protein found within the cellular lysate in response to EPI (4-6D-F), but EPI always stimulated secretion into the supernatant (Fig 4-6A-C). Meanwhile, LPS treated cultures at 37°C showed consistent upregulation of IL-6 protein within the lysate and secreted into the supernatant (Fig. 4-6D-F).

Co-stimulation with EPI and hyperthermia resulted in a further elevation in protein release at both 41°C and 42°C (Fig. 4-6B and C). In contrast, 42°C 1 h plus LPS resulted in a partial suppression of IL-6 protein release and suppression of IL-6 protein

accumulated within the supernatant (Figs 4-6C and F). These results demonstrate that in a manner in some ways similar to the responses seen for IL-6 mRNA to EPI and heat at 42°C, there is a differential effect of hyperthermia on the expression of IL-6 protein in response to EPI vs. LPS. That is, LPS-induced IL-6 protein production is suppressed by 42°C hyperthermia whereas EPI-induced IL-6 protein production is stimulated by 42°C hyperthermia.

Pharmacological Induction of the Heat Shock Response via Proteasome Inhibitor MG-132 Differentially Regulates IL-6 Protein Expression

To test if interactive effects of pharmacological heat shock induction on EPI and LPS stimulation seen with IL-6 mRNA (Fig 4.2B vs. 4.2C) are also observed with IL-6 protein, we pretreated C2C12 cells with MG-132 for 1 h and administered EPI or LPS for an additional 6 h. As shown in Fig 4-7A, MG-132 increases secreted IL-6 protein and potentiates the signal when co-stimulated with EPI. The same upregulation, though small, is seen in the cellular lysate (Fig. 4-7B). However, both intracellular and secreted LPS-induced IL-6 protein is attenuated by pretreatment with MG-132 (Fig. 4-7A, B). These results parallel the outcome of these treatments on mRNA responses and also parallel the responses seen with hyperthermia treatment and illustrate that hyperthermia per se is not an essential stimulus for these effects, but rather the presence of degraded or unfolded protein may be all that is required.

Intracellular Ca^{2+} is not an Important Regulator of Epinephrine or Lipopolysaccharide-Induced IL-6 Protein or Secretion

Adrenergic stimulation has been shown to be an important mediator of protein secretion in a number of settings (63, 88, 163, 194), but to the best of our knowledge the relationship was never investigated with IL-6. To possibly explain the differences in

the secretion of protein in response to hyperthermia + EPI vs. hyperthermia + LPS, we tested the hypothesis that the differences could be accounted for by the different influence of these treatments on intracellular Ca2+. Adrenergic stimulation is known to produce an intracellular Ca2+ current in other phenotypes (63) and it triggers the release of exosomes (159) and the secretion of proteins (63). To test this hypothesis, myotubes were pre-treated with the intracellular Ca2+ chelator (BAPTA-AM) for 2 h, and then treated with EPI or LPS. Intracellular Ca2+ chelation had no significant effect on the ability of myotubes to produce IL-6 in the lysate or secrete IL-6 in response to EPI or LPS (Fig. 4-8). The results are not consistent with a hypothesis that the unique effect of EPI on protein secretion is via differences in intracellular Ca2+ currents induced by EPI.

The Timing of Heat Shock Effects Lipopolysaccharide-Induced IL-6, but not Epinephrine-Induced IL-6

In the experiments described in Fig. 4-1 to 4-8, cells were supplemented with EPI or LPS and then immediately heat shocked. However, in a previous study Takii et al. heat shocked mouse embryonic fibroblasts and macrophages were pre-treated with heat and then treated with LPS (181). Their results differ from ours in that they observed that heat shock consistently suppresses LPS-induced IL-6 at the mRNA and protein level (181). In order to determine if the differences in response in the two studies are due to the timing of heat shock vs. the differences in phenotype, we repeated the order of the procedures in the experiments of Takii et al. (181). Myotubes were heat shocked at 42°C for 1 h (pre-HS) and then immediately supplemented with EPI or LPS and returned to 37°C 2 h (gene analysis) or 37°C 6 h (protein analysis). When myotubes were then stimulated with EPI, the IL-6 mRNA and secreted protein

were amplified as we have seen previously (Figs. 4-9A, B; respectively). However, for LPS treatment, there was neither potentiation nor attenuation of IL-6 mRNA (Fig. 4-9A) or secreted IL-6 protein in these conditions (Fig. 4-9B). These results are not consistent with an overall inhibitory effect of hyperthermia on IL-6 mRNA seen by Takii et al. (181) in contrast to the co-heat shocked and LPS supplemented groups (Figs. 4-2, 4-6), but do suggest that the timing of the HS exposure may be critical to the overall response to LPS such that the expected amplification of the mRNA by heat is missing.

Heat Attenuates Lipopolysaccharide and Epinephrine Co-Treated Myotubes

Previously it has been shown that the combination of EPI and LPS results in greater IL-6 protein production in myotubes (48), a finding that we have also observed (data not shown). Experiments were designed to test the effect of heat (42°C 1 h) on IL-6 mRNA and protein in LPS and EPI treated myotubes. Myotubes were maintained at 37°C for 3 h, exposed to EPI plus LPS under basal condition or EPI plus LPS and simultaneous heat shock (42°C 1 h 37°C 2 h). Cells co-stimulated with EPI and LPS under basal conditions increased IL-6 mRNA ≈188-fold (Fig. 4-10A), well above any increases in IL-6 mRNA we have previously seen under any condition (Fig. 4-1, 4-2, 4-3). Interestingly, the IL-6 mRNA signal was attenuated in these myotubes treated with EPI and LPS and simultaneous heat shock (Fig. 4-10A). These results suggest the possibility that the influence of hyperthermia may be IL-6 stimulus-intensity dependent. That is, at very high levels of IL-6 stimulation (perhaps regardless of the pathway), hyperthermia and its related signaling pathways effectively switches from an “amplifier” of the IL-6 response to an inhibitor. Figure 4-10B illustrates that accompanying these effects on IL-6 mRNA this triple stimulus effectively elevates ATF-3 mRNA 21.8-fold and clearly any influence of EPI in inhibiting this response is not effective. These results

are consistent with the role a possible role of ATF-3 as controlling this master switch. Heat also attenuated the IL-6 protein induced by the triple stimulus within the supernatant and the lysate (Fig. 4-10C, D).

Discussion

The current study provides evidence to support a critical role for the transcription factor HSF-1 in IL-6 gene regulation in skeletal muscle cells and that it is a primary signaling component of the response of the IL-6 gene to hyperthermia. However, hyperthermia also appears to stimulate other regulatory elements that affect IL-6 transcription, most notably the signaling pathways that activate the AP-1 regulatory element on the promoter region of the IL-6 gene. Therefore, hyperthermia stimulates IL-6 via multiple signaling pathways.

Hyperthermia had distinctly different influences on the IL-6 mRNA and IL-6 protein responses to hyperthermia, when coupled with co-stimuli, EPI vs. LPS. Hyperthermia uniformly amplified the IL-6 mRNA and protein responses during EPI exposure but these effects may not entirely be due to HSF-1, since constitutively active HSF-1 had no impact on the IL-6 promoter response to EPI.

The interactive responses to LPS were more complex. Whereas overall, the mRNA responses to LPS were greatly amplified by co-treatment with hyperthermia, these responses were enormous but were attenuated at the highest temperature (42°C). Furthermore, IL-6 protein production and secretion were always inhibited during co-treatment with hyperthermia at any temperature and proteasome inhibition inhibited IL-6 mRNA and protein secretion responses to LPS. These results suggest that LPS stimulation has unique interactions with hyperthermia that are both stimulatory and

inhibitory. Alternatively, the results are consistent with a molecular switch that when IL-6 is activated at very high transcription rates or at high temperatures, the effects of hyperthermia or HSF-1 on IL-6 transcription and translation move from largely facilitatory to inhibitory. A candidate for such a switch is ATF-3 which is a known inhibitory signal regulating IL-6. We demonstrate that hyperthermia differentially regulates ATF-3, such that when EPI but not LPS are available, hyperthermia inhibits ATF-3. The study also excluded other possible mechanisms for differential regulation of IL-6 mRNA production and IL-6 secretion by LPS and EPI including the proposed differential influence of these stimuli on intracellular Ca2+ and the possibility that each may influence the AP-1 site differently in the presence of hyperthermia.

Skeletal Muscle and Heat Stress

Skeletal muscle operates in a dynamic temperature range during physiological conditions (18, 59). Muscle can be exposed to high body temperature due to a breakdown in the body's ability to thermoregulate, but can also generate its own heat. The exothermic metabolic reaction driving muscle contraction yields muscle temperatures often about 1°C above core temperature and as high as 41°C in human (157) or 44°C in rats (18). Skeletal muscle is able to respond to heat alone (192), but perhaps its greatest impact can be seen in an exertional setting, such as exercise, physical activity or work. During conditions of extreme exertion, muscle not only generates heat, but it is also susceptible to internal (i.e. oxidants, unfolded or damaged proteins, and energy imbalance) and external stress signals (i.e. pathogen or damage associated molecular patterns, catecholamines, ATP and adenosine, and inflammatory cytokines) (191).

Hyperthermia alone contributes to IL-6 production in muscle (192), but temperatures greatest effect may be as a co-stimulus, priming the muscle to be more responsive to other stress signals. This potential is clearly demonstrated in Figures 4-1a, 4-2a, 4-3a. Heat upregulates IL-6 mRNA (Fig. 4-1A), moreover heat potentiates the signal of LPS (Fig. 4-2a) and EPI (Fig. 4-3a) compared to their respective signals during basal conditions (37°C). EPI treated C2C12 myotubes exhibit IL-6 mRNA potentiation that is proportional to temperature (i.e. 37°C<40.5°C<41°C<42°C), particularly at the 2 h time point. Meanwhile, LPS treated cells did not demonstrate this same pattern; IL-6 mRNA was greatest at 41°C. The reasoning for this discrepancy is not clear. One potential explanation is the differences in the signaling pathways of LPS- and EPI-induced IL-6.

Comparison of Epinephrine and Lipopolysaccharide Stress Signaling

Frost and colleagues (47, 48) outlined the basic EPI and LPS signaling pathways in C2C12 cells. In C2C12 myocytes EPI activates β1/2 –adrenergic receptors to stimulate IL-6 synthesis via mitogen-activated protein kinases (MAPK), JNK and p38 MAPK, but not MEK/ERK (48). JNKs are stress activated kinases known to contribute to the inflammatory response (82). JNKs are capable of phosphorylating many transcription factors belonging primarily to C/EBP, NF-KB, and AP-1 families (99).

In muscle, pathogen associated molecular patterns (PAMPS) such as LPS bind to TLRs-2 and -4 initiate an IL-6 transcriptional response via MAPKs (JNK, p38, and MEK/ERK) (47, 50). Additionally, LPS stimulation increases IKB-α protein degradation, but EPI does not (48). NF-KB signaling starts with the degradation of IKB-α, which functions to inhibit NF-KB from translocation into the nucleus. There is dispute as to the importance of the NF-KB signaling pathway in LPS-induced IL-6 synthesis in skeletal

muscle. Pharmacological NF-KB inhibitors have proven both effective and ineffective in attenuating LPS-induced IL-6 in C2C12 cells (47, 50). However, the most direct evidence illustrating the importance of NF-KB in LPS-induced IL-6 formation comes from a subsequent study by Frost et al. in which they attenuated IL-6 promoter activity by mutating the NF-KB response element (50). Based on the work of Frost and Lang's group it is likely that LPS-induced IL-6 signaling is mediated by transcription factors NF-KB and JNK in C2C12 cells (47, 50). Therefore, key differences between EPI and LPS induced signaling include: detection receptors (β-adrenergic vs. TLRs), MAPKs (LPS also signals through MEK), and NF-KB (mediator of LPS signaling).

EPI and LPS can have direct effects on cells by binding to their respective receptors and signaling a response, but also may stimulate secondary mediators such as inflammatory cytokines or ROS that can indirectly signal. The sequelae of an acute stress event could be a part of the differential regulation driving IL-6 synthesis and possibly account for the differences between EPI and LPS signaling. For example, LPS administration results in the upregulation of proinflammatory cytokines (IL-1B and TNF-α) in skeletal muscle (102) and ROS in myocytes (178, 179, 204), all are known to stimulate IL-6 in C2C12 cells (47, 100, 114).

HSF-1 and IL-6 Gene Regulation

Previously, we have found that heat stress independently stimulates IL-6 production in skeletal muscle and that IL-6 transcription can be blocked by the application of the heat shock factor inhibitor KNK437 (192). Others have also shown a link between hyperthermia, HSPs and IL-6 formation in epithelial cells (140), as well as a role for HSFs in IL-6 mRNA and protein expression in the absence of hyperthermia (149), which suggests some interaction between HSFs and the IL-6 gene. Furthermore,

we have identified a potential heat shock element (HSE) binding site in the promoter region of mouse (191), similar to that in human (81). We have also found sequences outside the 5' UTR promoter region in the human and mouse genomes that fit closely to the canonical HSE sequence, though the physiological function of these sites has yet to be verified (191). Since the mIL-6.luc reporter was sensitive to HSF activation, it is likely that a HSE sequence sensitive to HSF-1 must lie within the 0 to -1266 region of the promoter, the length of the construct.

In the present study, we further contribute to growing evidence that HSF-1 is an important regulator of IL-6. First, we show that pharmacological induction of heat shock via proteasome inhibition induces IL-6 in myotubes (Fig. 4-1B, C). Additionally, myotubes containing the c.a. HSF-1 plasmid increased basal IL-6 promoter activity (Fig. 4-1E) and potentiated the IL-6 promoter in myotubes co-stimulated with LPS (Fig. 4-2D). There was no effect of HSF-1 overexpression on EPI-induced IL-6 promoter activity (Fig. 4-3D). Additionally, we used a d.n. HSF-1 mutant to outcompete and inhibit endogenous HSF-1 activity. Myotubes containing the d.n. HSF-1 have an attenuation of temperature-, EPI-, and LPS-induced IL-6 promoter activity in basal and heated cells (Fig. 4-1F, 4-2E, 4-3E). Interestingly, the percentage of IL-6 activity is greater in heat shocked cells than basal cells (Fig. 4-1F, 4-2E, 4-3E).

These results suggest that HSF-1 is an important regulator of IL-6 promoter activity during basal and heated conditions regardless of treatment. However, because heated HSF-1 knockdown myotubes show an increase in relative IL-6 promoter activity compared to unheated myotubes suggests that HSF-1 is not the sole signal regulating IL-6 in heat shocked cells. Although unlikely, we acknowledge the possibility that heat

increases endogenous HSF-1 activity and is thus able to compete with the dysfunctional d.n. HSF-1 plasmid.

As a transcriptional regulator of IL-6, HSF-1 is believed to be an important co-factor functioning to partially open the chromatin structure of the promoter region, allowing for increased exposure of the promoter region to activators and repressors (81). In addition to IL-6 gene regulation, HSF-1 inhibits the gene regulation of other cytokines such as IL-1β, illustrating an extensive regulatory role of inflammation. HSF-1 has a direct interaction with C/EBP (also known as nuclear factor of IL-6) an essential regulator of IL-1β transcription (199). From the findings of Inouye et al. (81) and Takii et al. (181) we would expect HSF-1 to open the chromatin structure increasing exposure of the IL-6 promoter region and IL-6 suppression through the interaction of HSF-1 and ATF-3. The IL-6 promoter possesses ATF/CREB binding sites within close proximity to the NF-KB binding sites; therefore, ATF-3 may modulate NF-KB related transcription (56). Alternatively, ATF-3 may indirectly regulate IL-6 by inhibiting positive regulators of IL-6 such as C/EBPδ (113, 184). However, our results are not in agreement with this. We speculate the timing of HSF-1 and IL-6 induction may play a role, our results offer some support to this sentiment (Fig. 4-9). For example, if HSF-1 is activated at a time when IL-6 has already been activated, than HSF-1's effect on the chromatin structure increasing access to the IL-6 promoter may allow temporary increased access of positive IL-6 regulators. Additionally, some stress stimuli, such as EPI, may inhibit the induction of negative regulators of IL-6, as we show (Fig. 4-4). This still would not explain why we consistently see a heat induced IL-6 expression (192).

The AP-1 Binding Site within the Mouse IL-6 Promoter is Functionally Important to Heat Stimulated IL-6 Promoter Activity

JNK has been shown to be an essential regulator of EPI and LPS-induced IL-6 production. One function of JNK is to phosphorylate AP-1 transcription factors. Using an in-vivo rat sepsis model, it has been shown that in skeletal muscle there is an in early upregulation and late downregulation of NF-KB, meanwhile, AP-1 binding activity is increased at all time-points (147). AP-1 is a shared term referring to transcription factors composed of Jun, Fos, and ATFs. These AP-1 transcription factors bind to a common DNA site within the IL-6 promoter known as the AP-1 binding site (93). Recent evidence suggests that the activation of the JNK/AP-1 signal transduction pathway is a major player in skeletal muscle's ability to produce IL-6. JNK has been shown to be the driving mechanism of muscle-derived IL-6 in contraction (193), EPI (48), LPS (47), and proinflammatory cytokines stimulation (47).

The AP-1 regulatory site within the mouse IL-6 promoter was mutated to test the importance of AP-1 regulatory site to heat, EPI, and LPS-induced IL-6 promoter activity. Under basal conditions there were no differences between mIL-6.Luc and mIL-6.Luc AP-1 promoter activities (Fig. 4-5A) suggesting that EPI and LPS-induced IL-6 promoter activity is not regulated by JNKs phosphorylation of AP-1 proteins. When IL-6 promoter activity was compared between mIL-6.Luc and mIL-6.Luc AP-1 during heated conditions the promoter activity was downregulated in cells with the AP-1 site mutation (Fig.4-5B). Our results support that heat shock activates the AP-1 complex (123). One potential mechanistic explanation is that HSF-1 binds to the IL-6 promoter region to open the chromatin structure increasing exposure of the AP-1 regulatory site to the activated AP-

1 complex stimulated by heat. The AP-1 binding site is an alternative regulatory site controlling IL-6 gene regulation in conditions of heat.

It is surprising that there was no role of the AP-1 regulatory binding site within the IL-6 promoter region for regulating IL-6 promoter activity in EPI and LPS treated cells despite the experimental evidence for a role of JNK by others (47, 48). Our observations combined with those of Frost et al. (47, 48) suggest that JNK is a key mediator of EPI and LPS-induced IL-6 via a mechanism independent of the AP-1 regulatory site. A similar observation has been reported in tubular epithelial cells in which multiple leukocyte-derived factors were used to induce IL-6 via a JNK mechanism, but the mutation of the AP-1 binding site in the IL-6 promoter did not affect gene transcription (66). Our data and others suggest that the JNK interacts with other IL-6 regulatory signaling pathways not involving AP-1, possibly C/EBP or NF-KB.

Heat and Negative IL-6 Regulator, ATF-3

Interestingly, others have shown heat shock to inhibit LPS-induced IL-6 mRNA and protein expression in mouse embryonic fibroblasts and macrophages via HSF-1 dependent increased expression of ATF-3, a negative regulator of IL-6 (181). Our results aren't entirely different, we also see attenuation of LPS-induced IL-6 protein, but first we see an increase in IL-6 mRNA. We speculate that our conflicting results could be because of several reasons. First, there are likely some phenotypic differences between mouse embryonic fibroblasts and macrophages versus muscle cells. Second, although many of our experimental parameters are the same, one key difference is the timing of the administration of LPS. We chose to treat cells with LPS immediately before heat shock, while Takii et al. supplemented LPS immediately following heat shock (181). We reason that fever can be the result of bacterial infection and therefore

LPS should be present at the time of heat, although in terms of heat stroke or extreme exertion HSFs are likely present before endotoxin enters the circulation via breakdowns of the intestinal barrier. Despite these inconsistencies, the greater picture is clear, LPS-induced IL-6 protein is downregulated following heat shock, underlining the supreme regulatory control this system has.

In Fig. 4-4, we measure ATF-3 mRNA and see increased expression due to heat and heat plus LPS, consistent with reported results (181). However, we also show that EPI attenuates the heat-induced increase in ATF-3 mRNA. Transcription regulation of ATF-3 is regulated by the early growth response gene (Egr)-1 (14). Egr-1 is induced by growth factors and stress (i.e. heat shock) mediated through different subgroups of MAPKs, which in-turn differentially regulate Egr-1 (111). The MAPKs are major regulatory mechanisms involved in heat, LPS and EPI signaling, as we have described above (Comparison of epinephrine and lipopolysaccharide stress signaling). For example, MEK1 is an upstream regulator of ERK1/2 and subsequently Egr-1. Heat and LPS cause rapid activation of three distinct MAPKs, ERK, JNK, and p38 MAPK (47, 141). EPI does not signal through MEK/ERK and appears to signal predominantly through JNK (48, 133). Therefore, stressors that signal through MEK/ERK could provide a negative feedback mechanism to regulate IL-6 transcription.

Pharmacological Induction of the Heat Shock Response

We used the proteasome inhibitor MG-132 to induce heat shock in myotubes (Fig. 4-1B). MG-132 hinders proteasome function lending to the accumulation of abnormal proteins initiating the heat shock response (96, 149, 208). The pre-treatment of cells with MG-132 alone stimulated IL-6 (Fig. 4-1C, 4-7). In addition to the heat shock response MG-132 also inhibits NF-KB (50), which could explain the decreased IL-6 in

MG-132 and LPS co-treated myotubes (Fig. 4-2B, 4-7). NF-KB is an integral mechanism to stimulate IL-6 in LPS-treated myotubes (50). The NF-KB pathway has no known effect on EPI-induced IL-6. Therefore, MG-132 could co-signal with EPI to signal IL-6 without hindrance. Myotubes co-treated with MG-132 and EPI potentiates IL-6 (Fig. 4-3B, 4-7) possibly via HSF-1. Ultimately, caution is warranted when interpreting data from pharmacological inhibitors, many have multiple effects, but this experiment suggests that heat shock can stimulate/potentiate IL-6 independent of environmental heat.

Lastly, MG-132's attenuation of LPS-induced IL-6 could be a consequence of experimental timing. The timing of the induction of heat shock has different outcomes on IL-6 gene expression. For example, in Fig. 4-1, we administer EPI or LPS immediately prior to placement into the experimental incubator, but in Fig. 4-9 we heat shock cells for 1 h prior to the administration of EPI or LPS. Heat shock prior to LPS treatment has no significant effect on IL-6 mRNA or protein (Fig. 4-9). In MG-132 experiments (Fig. 4-5), there is a 1 h pre-treatment and a slower developing heat shock. Consequently, the pharmacologically-induced is distinct from the temperature-induced heat shock response time course, potentially altering signaling. Fascinatingly, if true, the timing of heat or the presence of stress stimuli preceding heat compared to stress events that may be the result of hyperthermia could be the difference between IL-6 potentiation and attenuation. The importance of the timing of stress stimuli and gene regulation in this case may be overstated because of heat shock-induced translational arrest, to be discussed.

Translational Control

In the present study we show an increase in IL-6 mRNA potentiation to LPS or EPI stimuli when combined with heat (Fig. 4-1, 4-2, and 4-3). Based on this potentiation, we hypothesized that IL-6 protein would also be upregulated due to the increased pool of IL-6 mRNA available for translation. Our data supports our hypothesis for the EPI treated cells, treatments of 41 or 42°C upregulated secreted IL-6 protein (Fig. 4-6B, C). Contrastingly, cells co-stimulated with heat and LPS attenuated IL-6 protein in the supernatant (Fig. 4-6B, C) and lysate (Fig. 4-6E, F).

The reasoning behind the different outcomes of protein production is poorly understood. Initially we hypothesized that our dosage of LPS could be a maximal dose and be an impediment on the system of translation with the addition of heat stress. Therefore, we performed additional experiments with lower concentrations of LPS (10-1000 ng/ml) (data not shown), but the outcome was the same. At any concentration of LPS in which we saw increased IL-6 protein expression heat attenuated the secreted IL-6 protein.

Elucidating the differences in translational control of our two different stress stimuli is beyond the scope of this study, but is something we hope to expound on at a later time. There are several different principles to discuss starting with the importance of the heat shock response. During heat shock there is a drastic change in the pattern of proteins synthesized because the survival of cells at higher temperatures is dependent on the presence of HSPs (139). Priority is placed on the production of HSPs, during heat shock non-HSPs are suppressed and HSPs are induced (34). To accomplish this, the heat shock response is regulated at the transcriptional, post-transcriptional and translational levels (34, 139). In heat shocked cells the ability of ribosomes to initiate

translation of normal mRNA is suppressed (139). Eukaryotic protein synthesis is primarily controlled at the level of translation initiation (33). Translation initiation is mediated by the eukaryotic initiation factors (eIF) eIF-2 and eIF-4F. Heat shock phosphorylates eIF-2 inhibiting overall protein synthesis (37, 164). eIF-4F, a second site of protein synthesis regulation, is a cap-binding protein deficient in heat shocked cells and represents a likely mechanism for the preferential translation of HSP mRNAs, as the translation of HSP mRNAs is hardly affected by the eIF-4F activity (139). The production of HSPs is likely unaffected because HSP mRNA translation is resistant to the inactivation of cap-binding initiation factors, which suppresses the translation of non-HSP mRNAs (138). In mammals, translational inhibition typically occurs at 40-41°C (138), the range in which the present study was performed.

The attenuation of IL-6 protein from the heat shocked LPS-stimulated cells follows the logic laid out above. Despite there being a greater IL-6 mRNA pool to pull from in the heated group, IL-6 protein is downregulated, likely a result of overall translational inhibition. However, the experiments performed in this study were matched; the cells that received EPI or LPS encountered the exact same environmental conditions. IL-6 protein was potentiated in the heat shocked EPI stimulated cells. Epinephrine predominantly signals through β1/2-adrenergic receptors (48). The activation of β-adrenergic receptors recruits ERK and mammalian target of rapamycin (mTOR) to facilitate long term potentiation of translation initiation (55). β-adrenergic agonist treatment of mouse hippocampal slices results in the activation of eIF-4E and eIF-4E kinase (Mnk1), as well as the inhibition of translation repressor 4E-BP (55). Initiation factor eIF-4E and its ability to form the eIF-4F initiation complex is a limiting step in

mRNA recognition and ribosomal recruitment. The eIF-4E initiation complex is an attaching site for translation factors and is under the control of inhibitory binding protein 4E-BP (143). Therefore, EPI treatment via β-adrenergic stimulation may preserve protein translation during heat shock.

IL-6 Secretion

Currently, it is unknown how IL-6 trafficking and secretion is regulated in skeletal muscle. Therefore, we must refer to limited secretory work done in other cell phenotypes to acknowledge that IL-6 protein secretion is regulated (128). In macrophages, newly synthesized IL-6 accrues in the Golgi complex and exits in tubulovesicular carriers via specific soluble N-ethylmaleimide sensitive fusion protein (NSF) attachment protein receptor (SNARE) protein they fuse with the recycling endosome (118). The recycling endosome can then compartmentalize proteins, whereas IL-6 is segregated from other proteins and can be independently secreted (118). Thus, IL-6 secretion can be regulated and differentially secreted from other cytokines. We believe that skeletal muscle could regulate IL-6 secretion through similar pathways.

Again, little is known about IL-6 secretory pathways, especially in skeletal muscle. We have observed very little IL-6 present in the cellular lysate of EPI treated cells, even during times of significant IL-6 upregulation within the supernatant (Fig. 4-2). We speculate that in addition to EPI stimulating IL-6 in muscle (48), it may also function to enhance IL-6 secretion. The notion that adrenergic stimulation is important in protein secretion is not unfounded, it has been shown to be an important mediator of protein secretion (63, 88, 163, 194), but never IL-6. A likely pathway of adrenoreceptor stimulation mediated protein release is through Ca2+ influx (63). Ca2+ is an

intracellular signal observed to trigger the release of exosomes (159) and other proteins (63). Intracellular Ca2+ as a mediator of IL-6 release is particularly interesting in skeletal muscle because transient increases in Ca2+ regularly occur during muscle contraction. In a series of experiments (Fig. 4-11), we pre-treated cells with the intracellular Ca2+ chelator BAPTA-AM or vehicle and treated cells with EPI or LPS. The sequestration of intracellular Ca2+ had no significant impact on IL-6 protein produced or secreted as a result of EPI or LPS.

Significance

When one considers the multiple important biological roles of IL-6, both locally within skeletal muscle (144, 145) and systemically (70, 174, 200), these findings could have important clinical significance. Our findings, in the present study and previously (191, 192), and that of others (140, 149) have shown a role of HSF-1 in IL-6 potentiation. Alternatively, others have shown an inhibitory effect of HSF-1 on IL-6 regulation (181). One such explanation is the use of alternative stimuli. LPS induced IL-6 was attenuated by HSF-1 (181), meanwhile, HSF-1 potentiates IL-6 when stimulated with EPI or IL-1β (140), although endogenous IL-1β should be suppressed by HSF-1 itself (199) and in the present study we see a role for HSF-1 in LPS-induced upregulation of IL-6 mRNA. As speculated upon, the timing of the presence of HSF-1 and IL-6 regulators could explain some of these inconsistencies. Therein lies the conundrum of HSF-1's role in IL-6 regulation, one can't definitively describe the role of HSF-1 in IL-6 regulation because similar to the "chicken-or-the-egg" one can come up with countless examples of why HSF-1 would be upregulated prior to and subsequent to sequelae upregulating IL-6. Some of these discrepancies can be cleaned up by overlooking gene regulation and focusing on protein expression. The organism seems

to know how to differentially translate proteins during dire situations such as heat shock (139). Fascinatingly, if taking this broad approach, the results of this study show preferential expression of IL-6 during stimulation with adrenergic stimuli (acute stress "fight or flight" mimetic) compared to endotoxin (bacterial infection mimetic), but with LPS undermining the effects of EPI when given together. Although presently the picture remains cloudy, we have identified HSF-1 as well as the AP-1 binding site within the IL-6 promoter region as regulator of IL-6 gene expression in the C2C12 mouse myoblast cell line. Further research is needed, but HSF-1 regulation may be a potential target to regulate pro-inflammatory cytokines, such as IL-6.

Figure 4-1. Heat shock stimulates IL-6 mRNA and promoter activity. Myotubes were grown as described in METHODS, A) maintained at 37°C or exposed to 40.5, 41 or 42°C for 1 h and harvested or allowed to recover 37°C for 1 or 2 h; pre-treatment with MG-132 (10 uM) for 1 h followed by an additional 1, 2, and 3 h increases HSP72 and IL-6 mRNA (B and C). C2C12 myoblasts were transfected with the following gene plasmids: D) mouse IL-6 promoter luciferase reporter (mIL-6.Luc), pRL-TK (Renilla), E) mIL-6.Luc, pRL-TK, and constitutively active (c.a.) HSF-1 or its empty vector (EV), F) mIL-6.Luc, pRL-TK, dominant negative (d.n.) HSF-1 and or EV. After transfection, myoblasts were differentiated for 5 days into myotubes, experiments were then carried out. Briefly, myotubes were kept under basal conditions 37°C 6 h or heated 42°C 1 h and 37°C 5 h (D); myotubes containing c.a. HSF-1 or EV were maintained at 37°C (E); and myotubes containing d.n. HSF-1 or its EV were kept under basal (37°C 6 h) or heated (42°C 1 h and 37°C 5 h) conditions. Luciferase activity in cell lysates was measured and normalized (normalized relative light units (RLU) = RLU firefly luciferase/RLU Renilla luciferase). Luciferase activity of the cells maintained at 37°C (D) or transfected with the mIL-6.Luc and EV (E and F) for each respective experiment were set at 100% activity, and the luciferase activity of the c.a. or d.n. HSF-1 plasmids is shown as a percentage of IL-6 promoter activity. Results from multiple cultures, four or five independent experiments, two samples per experiment; multi-way ANOVA testing the effects of temperature with in each treatment group A) heat ($P=0.0007$), and at individual time points statistical tests were post-ANOVA least squares contrasts between means * ($P<0.05$), ** ($P<0.01$), ***($P<0.001$), and **** ($P<0.0001$).

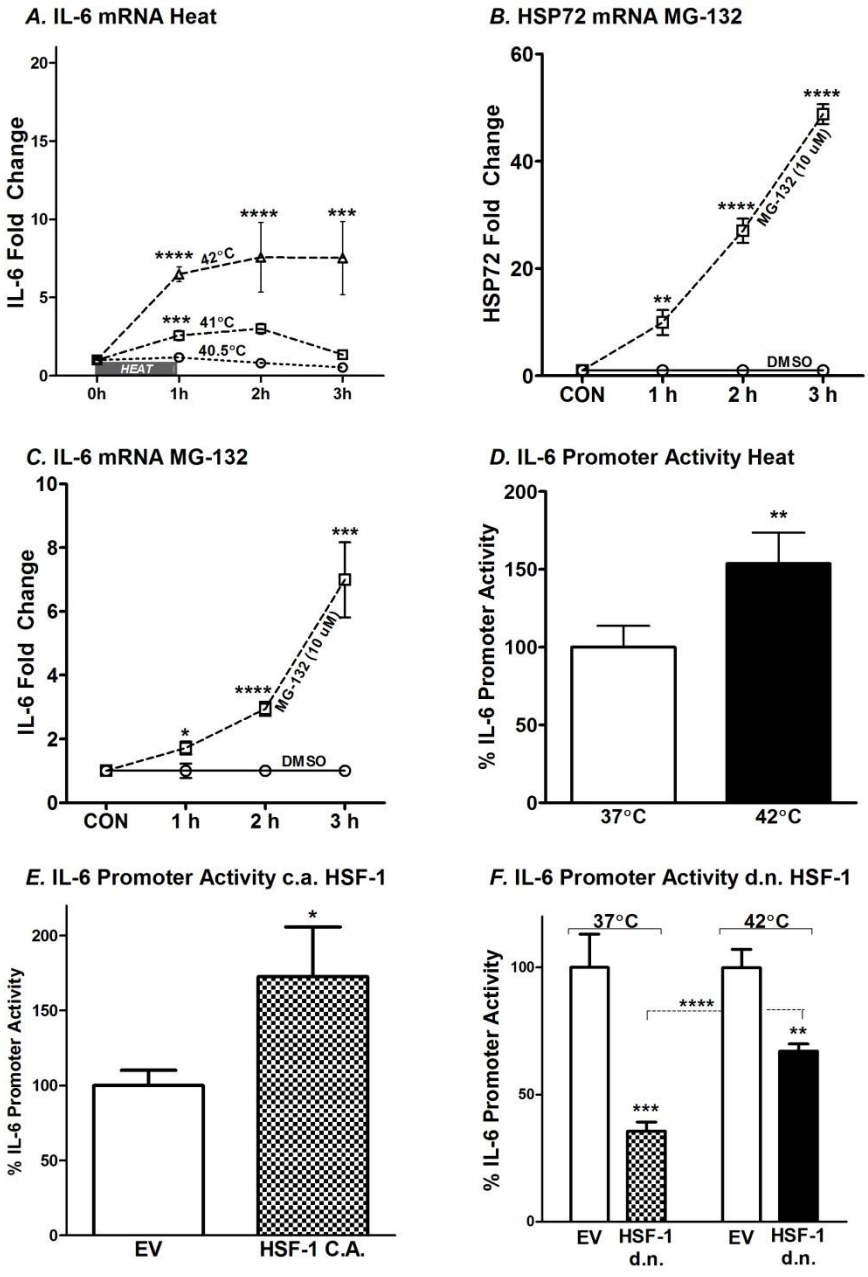
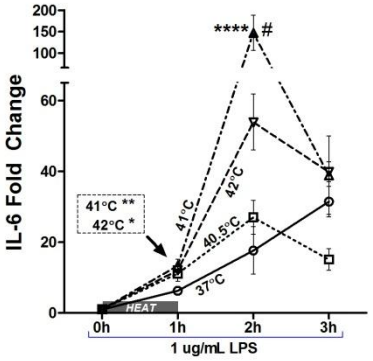
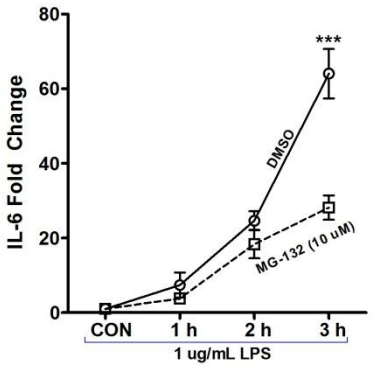

A. IL-6 mRNA Heat
IL-6 Fold Change
42°C
41°C
40.5°C
HEAT
0h
1h
2h
3h
B. HSP72 mRNA MG-132
HSP72 Fold Change
MG-132 (10 uM)
DMSO
CON
1 h
2 h
3 h
C. IL-6 mRNA MG-132
IL-6 Fold Change
MG-132 (10 uM)
DMSO
CON
1 h
2 h
3 h
D. IL-6 Promoter Activity Heat
% IL-6 Promoter Activity
37°C
42°C
E. IL-6 Promoter Activity c.a. HSF-1
% IL-6 Promoter Activity
EV
HSF-1 C.A.
F. IL-6 Promoter Activity d.n. HSF-1
% IL-6 Promoter Activity
37°C
42°C
EV
HSF-1 d.n.
EV
HSF-1 d.n.

Figure 4-2. Heat potentiates IL-6 mRNA and promoter activities in response to LPS. Experimental parameters were the same as described in Fig. 4-1, except myotubes were co-stimulated with LPS (1 ug/ml) at a time-point matched with the onset of heat treatment (42°C 1 h). A) Heat (41 and 42°C) and LPS potentiates IL-6 mRNA immediately upon removal from heat. The greatest potentiation occurred 2 h after the onset of 41°C and was significantly greater than cells co-treated with 42°C (# $P<0.05$). B) MG-132 attenuated IL-6 mRNA 3 h after LPS treatment. Heat (C) and HSF-1 overexpression (D) potentiated LPS stimulated IL-6 promoter activity. E) HSF-1 knockdown decreased LPS stimulated IL-6 promoter activity during basal and heated conditions, but IL-6 promoter activity was greater in heated myotubes compared to unheated. Results from multiple cultures, four or five independent experiments, two samples per experiment; multi-way ANOVA testing the effects of temperature with in each treatment group A) LPS ($P=0.0013$), and at individual time points statistical tests were post-ANOVA least squares contrasts between means * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$), and **** ($P<0.0001$).

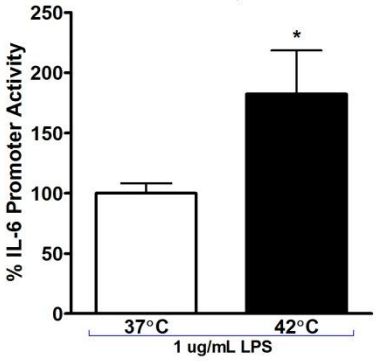
A. IL-6 mRNA Heat + LPS



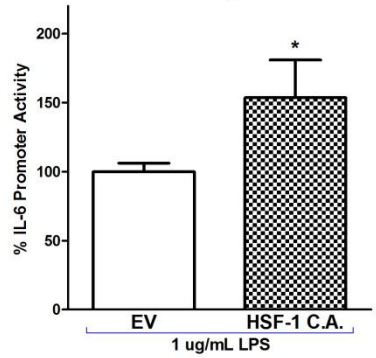
B. IL-6 mRNA MG-132 + LPS



C. IL-6 Promoter Activity Heat + LPS



D. IL-6 Promoter Activity LPS c.a. HSF-1



E. IL-6 Promoter Activity LPS d.n. HSF-1

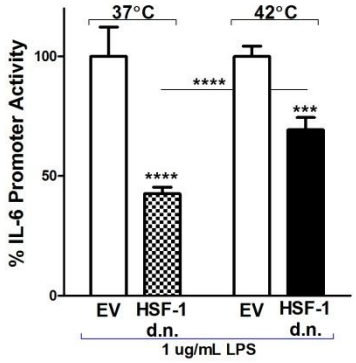
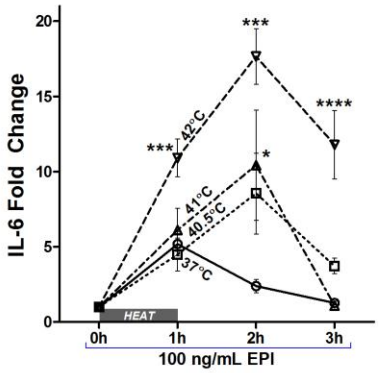
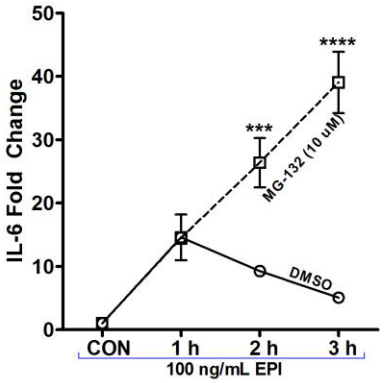


Figure 4-3. Heat potentiates IL-6 mRNA and promoter activities in response to EPI. Experimental parameters were the same as described in Fig. 4-1, except myotubes were co-stimulated with EPI (100 ng/ml) at a time-point matched with the onset of heat treatment (42°C 1 h). A) Heat (42°C) and EPI potentiates IL-6 mRNA immediately upon removal from heat. The greatest potentiation occurred 2 h after the onset of heat (41 and 42°C) and a prolonged IL-6 mRNA potentiation 3h after the onset of heat (42°C). B) MG-132 pretreatment and EPI potentiates IL-6 mRNA 2 and 3 h after EPI treatment. Heat (C) potentiated EPI stimulated IL-6 promoter activity and HSF-1 overexpression (D) had no effect. E) HSF-1 knockdown decreased EPI induced IL-6 promoter activity during basal and heated conditions, but IL-6 promoter activity was greater in heated myotubes compared to unheated. Results from multiple cultures, four or five independent experiments, two samples per experiment; multi-way ANOVA testing the effects of temperature with in each treatment group A) EPI ($P<0.0001$), and at individual time points statistical tests were post-ANOVA least squares contrasts between means * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$), and **** ($P<0.0001$).

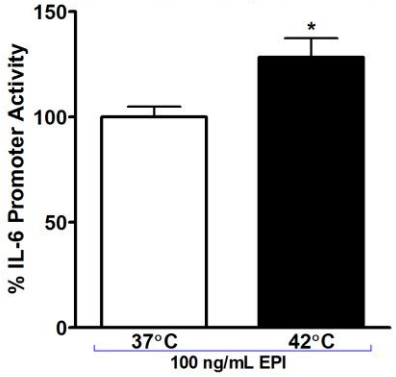
A. IL-6 mRNA Heat + EPI



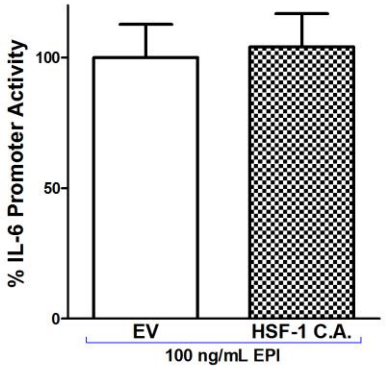
B. IL-6 mRNA MG-132 + EPI



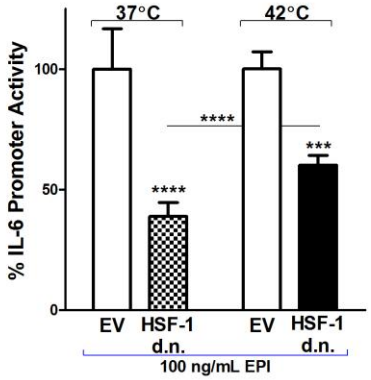
C. IL-6 Promoter Activity Heat + EPI



D. IL-6 Promoter Activity EPI c.a. HSF-1



E. IL-6 Promoter Activity EPI d.n. HSF-1



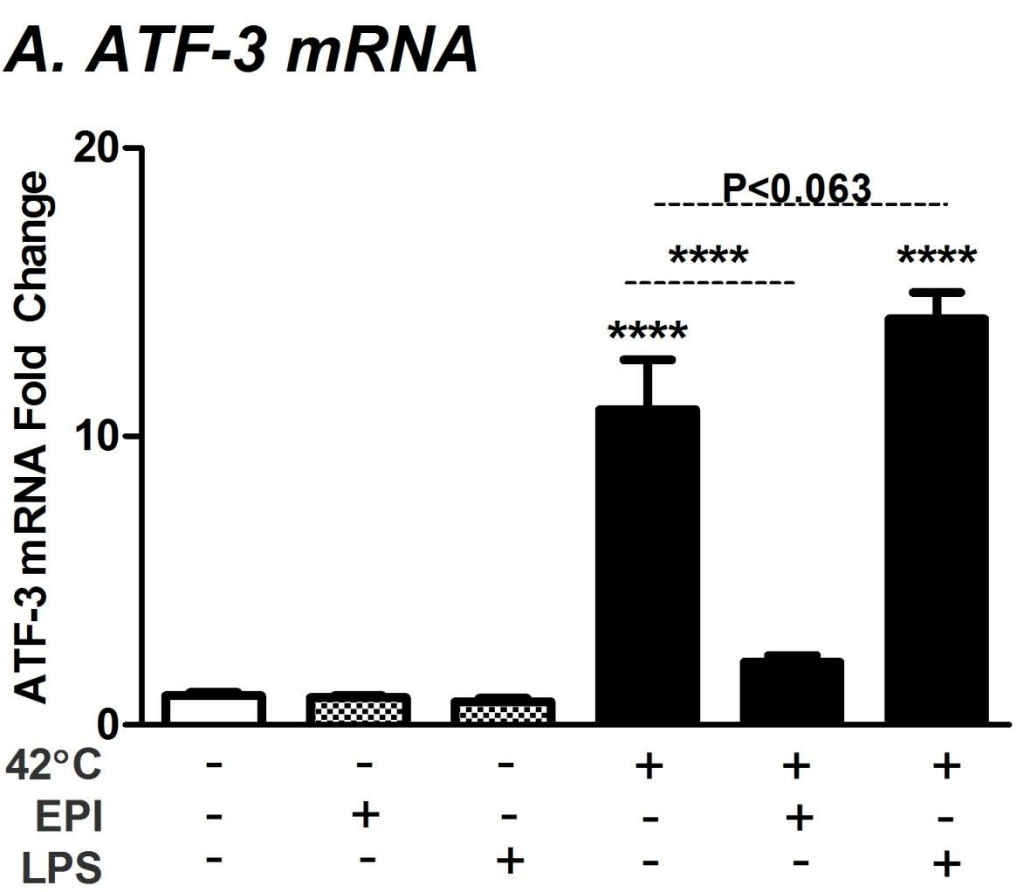


Figure 4-4. Heat induces ATF-3 mRNA and epinephrine co-stimulation attenuates heat induced ATF-3 mRNA. Myotubes were maintained at 37°C 3 h or exposed to 42°C for 1 h and recovery for 2 h at 37°C. Select cultures were co-stimulated with EPI or LPS. Cells were harvested for real-time RTPCR. ATF-3 mRNA was significantly upregulated in heat shocked cells and nearly potentiated when heat was co-stimulated with LPS (P=0.063). ATF-3 mRNA was entirely attenuated by EPI supplementation. Results from multiple cultures, three independent experiments, two-to-three samples per experiment; statistical tests were post-ANOVA least squares contrasts between means. ANOVA, * (P<0.05), ** (P<0.01), *** (P<0.001), and **** (P<0.0001).

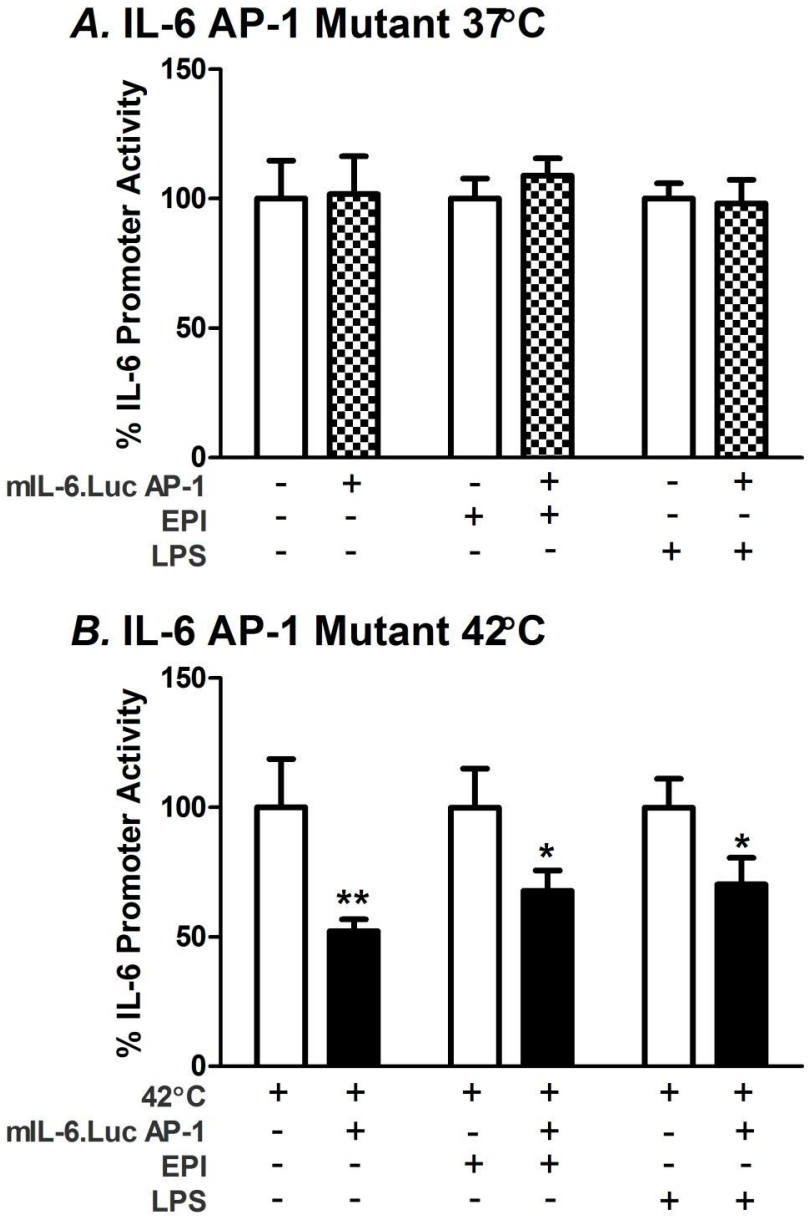


Figure 4-5. Mutation of the AP-1 regulatory site within the mouse IL-6 promoter reduces heat-stimulated IL-6 promoter activity. C2C12 myoblasts were transfected with the wild type mIL-6.Luc promoter or mIL-6.Luc with a mutated AP-1 binding site (mIL-6.Luc AP-1) plus the pRL-TK plasmid. After transfection, myoblasts were differentiated for 5 days into myotubes. Luciferase activity was measured (RLU), normalized, and calculated as a percentage of maximal promoter activity as described in Fig. 4-1. Mutation of the AP-1 regulatory site has no effect on CON, EPI or LPS treated IL-6 promoter activity during unheated conditions (A), but reduces heat stimulated and heat co-stimulated IL-6 promoter activity (B). Results from multiple cultures, five independent experiments, two samples per experiment; ANOVA, * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$), and **** ($P<0.0001$).

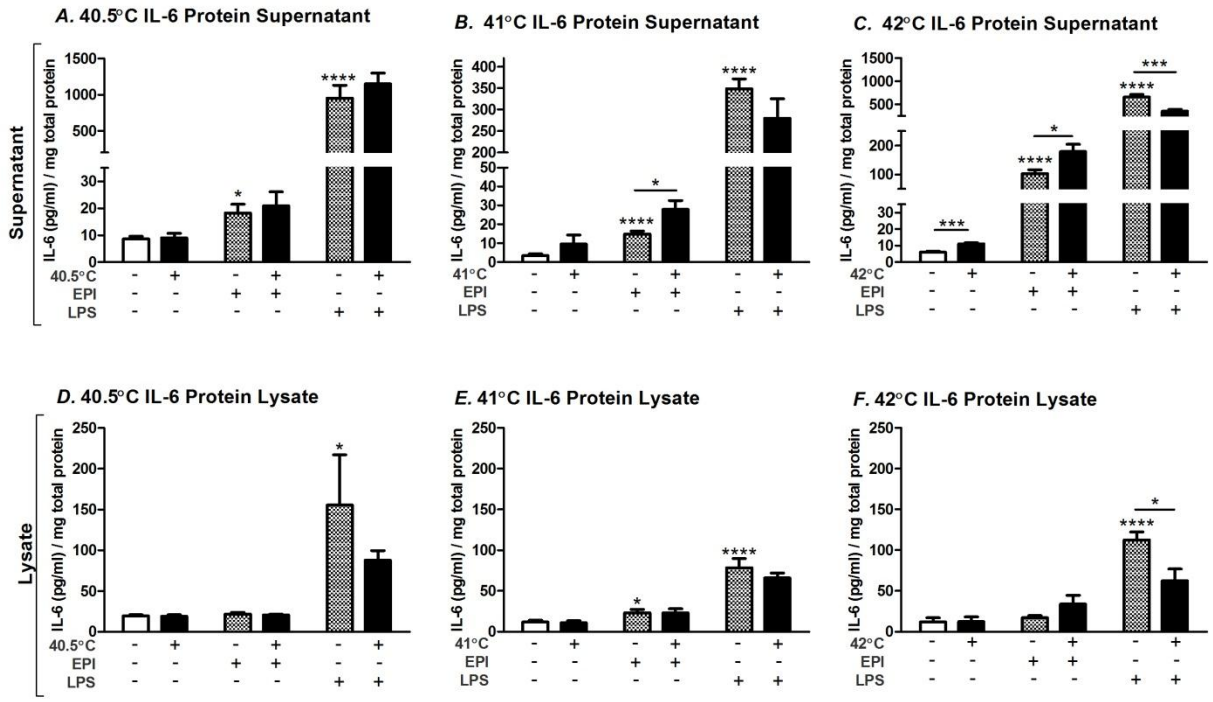


Figure 4-6. Heat regulates IL-6 protein in a temperature intensity based manner and differentially regulates IL-6 protein based on co-stimuli. Myotubes were maintained at 37°C or exposed to 40.5°C (A, D), 41°C (B, E) or 42°C (C,F) for 1 h and 37°C 5h. Select cultures were co-stimulated with EPI (100 ng/mL), or LPS (1 ug/mL). Supernatants were sampled (A-C) and cells were lysed (D-F) for IL-6 protein analysis and total protein quantification. When co-stimulated with EPI at temperatures ≥41°C secreted IL-6 protein was potentiated. At high temperatures (42°C) myotubes co-treated with LPS showed attenuated IL-6 protein within the supernatant and lysate. Results from multiple cultures, four independent experiments, two samples per experiment; ANOVA * (P<0.05), *** (P<0.001), and **** (P<0.0001).

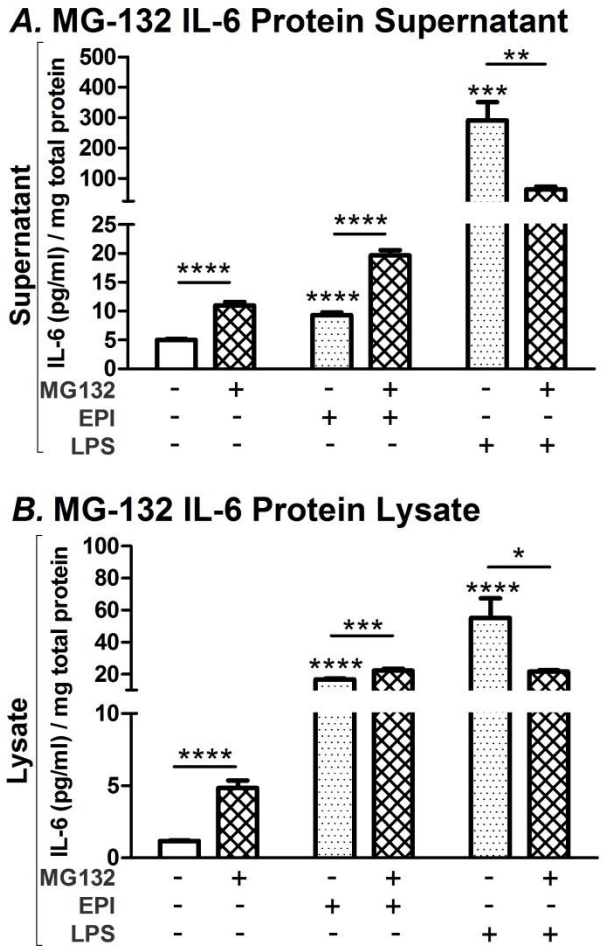


Figure 4-7. Pharmacological induction of the heat shock response regulates IL-6 protein. Myotubes were pre-treated with the proteasome inhibitor MG-132 (10 uM) or vehicle (DMSO) for 1 h. Myotubes were then untreated or co-treated with EPI (100 ng/ml) or LPS (1ug/ml) for 6 h. Supernatants were sampled (A) and cells were lysed (B) for IL-6 protein analysis and total protein quantification. Results mimic that of heated myotubes, MG-132 upregulates IL-6 protein, potentiates IL-6 protein when co-stimulated with EPI, and attenuates IL-6 protein when co-stimulated with LPS. Results from multiple cultures, four independent experiments, two samples per experiment; ANOVA, * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$), and **** ($P<0.0001$).

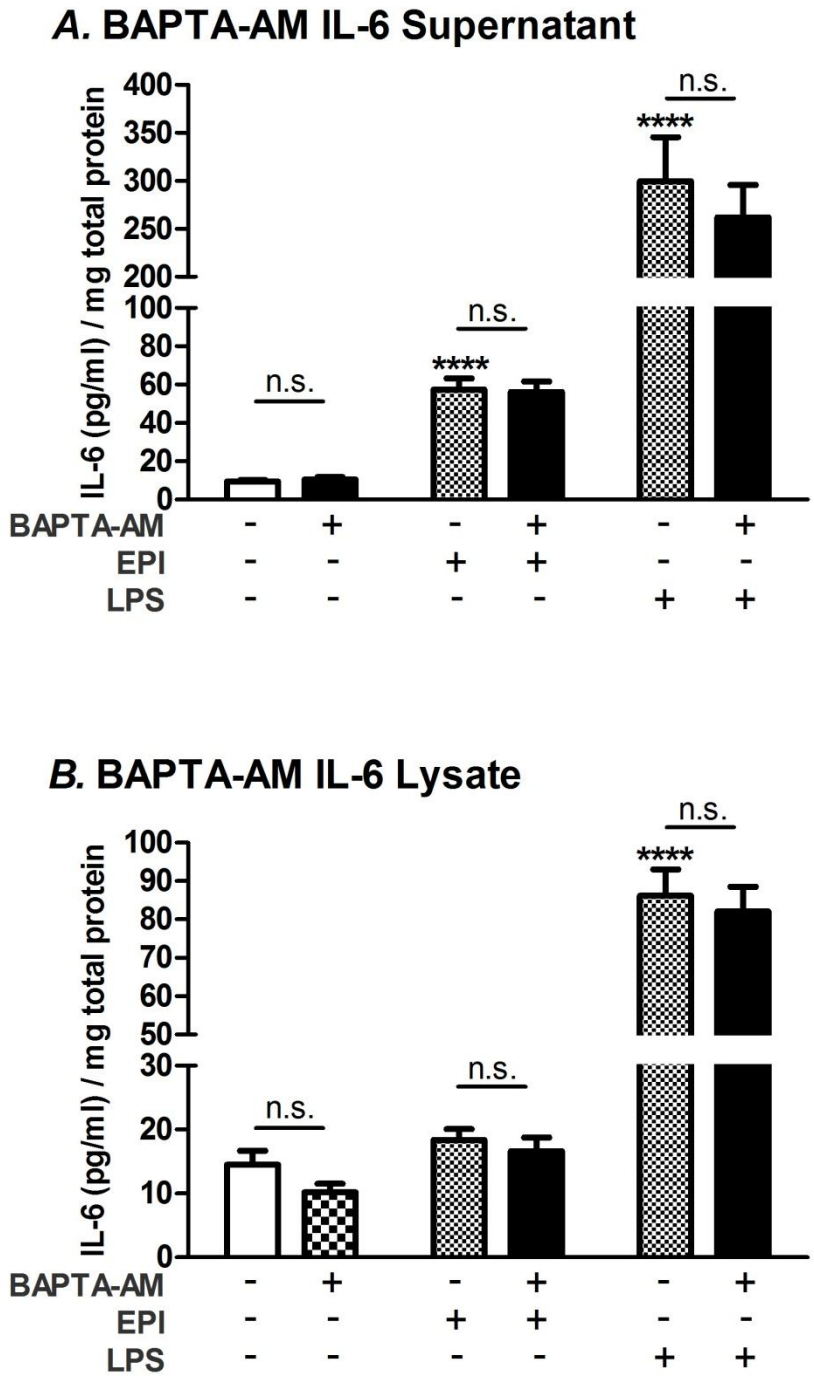


Figure 4-8. Intracellular calcium is not an important regulator of epinephrine or LPS induced IL-6 or its secretion. C2C12 myotubes were pre-incubated with an intracellular Ca2+ chelator (BAPTA-AM) for 2h. Then myotubes were treated with EPI or LPS, after 6 h supernatant was sampled and cells were lysed for IL-6 protein and total protein assays. ANOVA, * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$), and **** ($P<0.0001$).

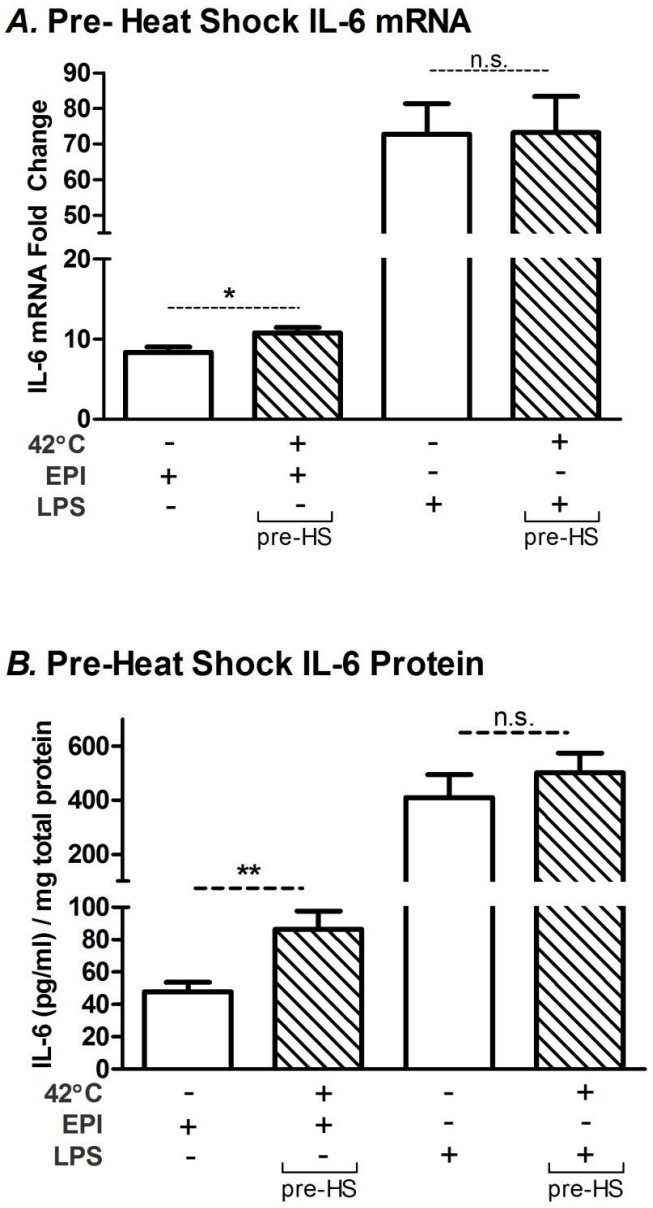


Figure 4-9. Effects of heat shock prior to epinephrine or LPS supplementation. Myotubes were maintained at 37°C or exposed to 42°C (A, B) for 1 h (pre-HS), then treated with EPI (100 ng/mL) or LPS (1 ug/mL) and returned to 37°C. Cells were lysed 2 h (for real-time RT-PCR) after drug supplementation and supernatants were sampled for secreted IL-6 protein (ELISA) and cells lysed for total protein quantification. When co-stimulated with EPI after heat shock IL-6 mRNA and secreted protein were potentiated. No significant changes were seen in myotubes treated with LPS between basal and pre-HS groups. Results from multiple cultures, four independent experiments, two samples per experiment; ANOVA * ($P<0.05$), *** ($P<0.001$), and **** ($P<0.0001$).

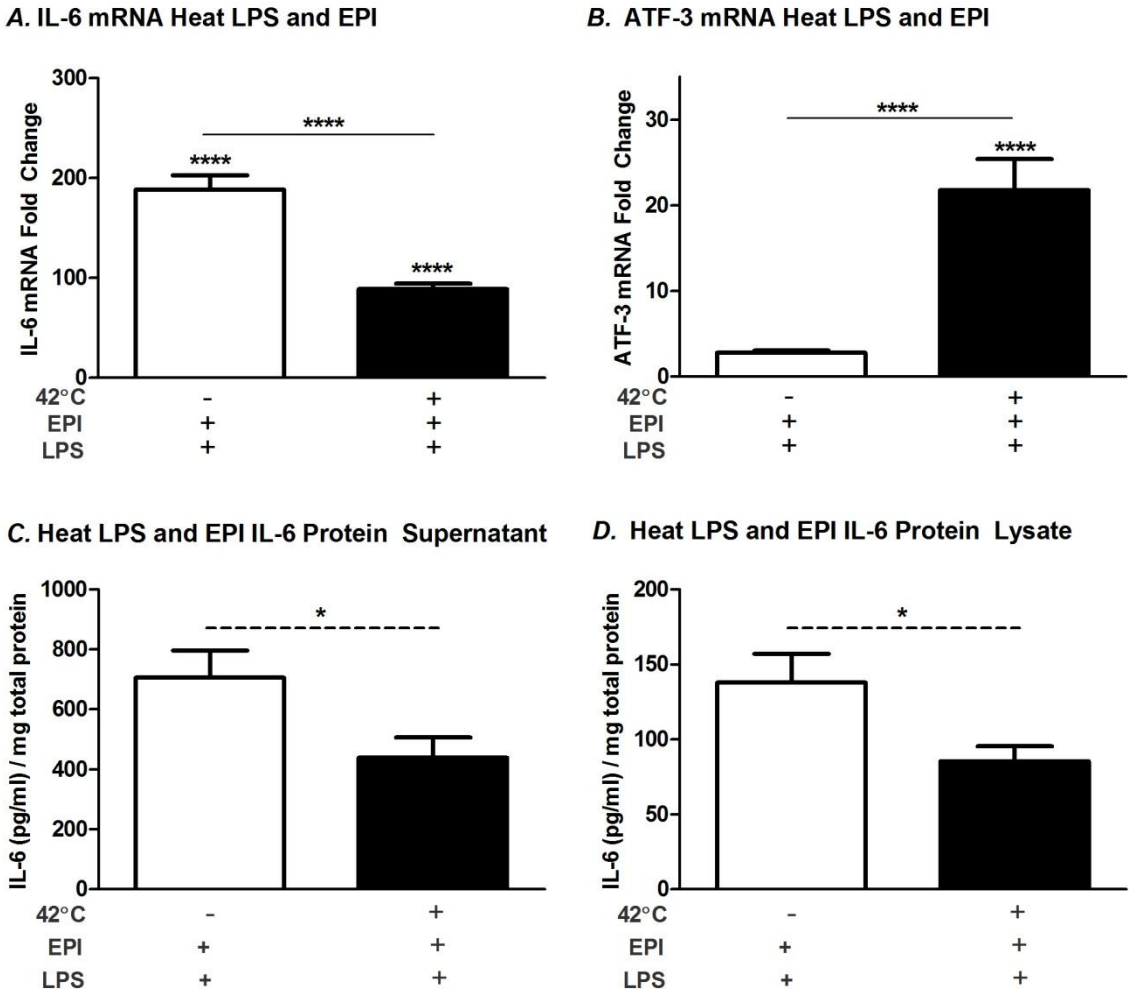


Figure 4-10. The effect of heat on epinephrine plus LPS co-stimulated myotubes. Myotubes were maintained at 37°C 3 h, exposed to EPI plus LPS under basal conditions, EPI plus LPS and simultaneous heat shock, or pre-heat shocked (pre-HS) (42°C 1h) then treated with EPI and LPS. Cells were harvested for real-time RTPCR 2h after drug treatment initiation. A) IL-6 mRNA was significantly upregulated in cells co-treated with EPI and LPS. The addition of a simultaneous heat shock attenuates IL-6 mRNA, but not a pre-HS treatment. B) ATF-3 mRNA was unchanged in EPI and LPS co-treated cells, but significantly upregulated in heated shocked cells, but with greatest ATF-3 mRNA expression occurring in the simultaneous HS group. C, D) IL-6 protein within the supernatant and the lysate were significantly attenuated in HS, EPI plus LPS treated cells. Results from multiple cultures, four independent experiments, two samples per experiment; statistical tests were post-ANOVA least squares contrasts between means. * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$), and **** ($P<0.0001$).

CHAPTER 5
CONCLUSIONS

Overview of Major Findings

The main focus of this dissertation was to evaluate mechanisms by which skeletal muscle might function as a stress sensor and endocrine organ, specifically in conditions of hyperthermic stress. The choice of heat stress was based on the fact that the heat shock response is a quintessential, evolutionarily conserved stress response that is an underlying component of a variety of physiological stresses that result in protein damage or degradation. In the present study, we identify hyperthermia as a novel stimulus of muscle derived IL-6 mediated by the transcription factor HSF-1 and the AP-1 binding site within the IL-6 promoter. When hyperthermia is applied as a co-stimulus in the presence of EPI or LPS IL-6 mRNA expression is potentiated. Heat shock via proteasome inhibition also stimulates IL-6 production in the absence of heat. In addition to being important regulatory components of heat-induced IL-6, HSF-1 and the AP-1 regulatory site appear to serve as master regulators of heat-induced IL-6 mRNA potentiation by EPI and LPS.

Response of Skeletal Muscle to Acute Hyperthermia

The present study has shown an IL-6 response proportional to the intensity of heat exposure, performing similarly to that of HSP72 mRNA at different temperature intensities. C2C12 myoblasts, C2C12 myotubes, ex vivo muscle, and in vivo muscle preparations all behaved similarly to acute heat exposure. One notable difference was seen between models, in conditions of heat stroke in the anesthetized intact animal, TNF-α mRNA was stimulated, whereas in the ex vivo and cell culture models TNF-α mRNA was suppressed. This difference could have been predicted because of the

presence of co-stimuli in the intact system, such as endotoxin or catecholamines, known to be present in the circulation during heat stroke (1, 62). This difference is noteworthy because TNF-α can stimulate IL-6 (47) and the presence of TNF-α prior to IL-6 is representative of the programmed cytokine response to endotoxin, which is distinct from the exercise-induced cytokine pattern (144). We have hypothesized that heat is an important component of exercise-induced cytokine induction in skeletal muscle because similar exercise loads in cool environments show an attenuated cytokine response (153, 172).

Furthermore, we evaluated the changes in cytokine/chemokine (G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC, MCP-1, MIP-1α, RANTES, TNF-α) and soluble receptor (sGP130, sIL-6R, sTNFR1, sTNFR2, sIL-1R1, and sIL-1R2) expression of the cellular supernatant of heated (42°C) and control cells. IL-6 was most markedly upregulated; the only other significant changes in secreted protein were the upregulation of IP-10 and downregulation of sTNFR2. IP-10 can function as a chemoattractant of immune cells and has a role in muscle's inflammatory response (27). We considered the upregulation of IP-10 a potential marker of damage and followed up with muscle function and cytotoxicity tests. Heat (41°C 1 h) was detrimental to force production using the described ex vivo muscle system (Fig. 3-5C), therefore we did not continue to increase the temperature intensity because of decay of specific force. Furthermore, ourselves and others (161) have observed that the excision of muscle is a dynamic inflammatory event itself and we have discontinued the use of this model. Using our cellular model, our most controlled method, we observed no significant change in cell cytotoxicity at our

greatest temperature intensity (42°C 1 h), suggesting no significant cell death. We believe that muscle responds to heat stress with IL-6 and IP-10 independent of muscle damage.

Hyperthermia is a Unique Stimulus for IL-6 Formation in Skeletal Muscle.

Specific aim 1 was designed to determine if hyperthermia is an additional and unrecognized stimulus for muscle IL-6 production. The major finding of this aim was that C2C12 myoblasts, myotubes, ex-vivo muscle preparations, and in-vivo soleus muscles all produce IL-6 in response to hyperthermia. Therefore, we were able to successfully answer aim 1; skeletal muscles produce IL-6 in response to heat. However, there were limitations to the experiments performed. Experiments using C2C12 myotubes allow for the most direct answer to our proposed question because they specifically test hyperthermia as a solitary stimulus (Figs. 3-1A, 3-2A). The use of ex-vivo and in-vivo models increase the translational relevance of this work, but there were experimental flaws within each model. For example, the isolation of the soleus from the animal for the ex-vivo experiments was a strong stimulus of IL-6 and any of our ensuing treatments were likely a superimposed stimulus in addition to muscle isolation (Table 3-1) (161).

The in-vivo model was also limited because it is difficult to isolate the independent variable and determine if hyperthermia was the stimulus or if it was some circulatory factor that resulted as a consequence of the heat stroke protocol. In addition to IL-6 as the principle outcome measure; TNF-α and HSP72 mRNA were evaluated in all models. TNF-α is a pro-inflammatory cytokine that is stimulated by endotoxin, but can also stimulate IL-6 (47). In the isolated models (cell culture and ex-vivo soleus) IL-6 elevation occurred independent of increases in TNF-α mRNA, but when using the intact

system TNF-α mRNA increased. Therefore, additional stimuli such as endotoxin were likely present in the circulation when using the in-vivo model (67). HSP72 mRNA was predominantly used as a positive control to ensure the heat treatment was intense enough to induce heat shock. These findings are the first to show that physiologically relevant hyperthermia stimulates IL-6 in skeletal muscle.

Mechanisms of Heat-Induced IL-6 Formation:

Heat Shock Factor-1

Specific aim 2 was designed to determine what molecular mechanisms regulate heat-induced muscle IL-6 mRNA. The major finding of this aim was that C2C12 myotubes require HSF-1 during basal and heated conditions to properly regulate the IL-6 gene. In the present study, the experimental results show that IL-6 and HSP72 mRNA levels increased as a function of the intensity of hyperthermia (Figs. 3-1, 3-2) and that the individual responses of HSP72 and IL-6 mRNA were highly correlated across all models (Fig. 3-8). This correlation suggests that some independent component of IL-6 mRNA regulation may be tied to the same signaling pathways in heat that regulate HSP72, further implicating HSF-1 as a potential facilitator of IL-6 induction.

In a series of experiments designed to test the importance of HSF-1 as a mediator of IL-6 regulation in myotubes we further demonstrate the importance of HSF-1. Using indirect activators (MG-132) of heat shock transcription factors, IL-6 mRNA and protein expression were shown to be increased in the absence of hyperthermia (Figs. 4-1B, C). Pharmacological inhibition (KNK437) of HSF-1 attenuated both the HSP72 and IL-6 mRNA response to heat in myotubes as well (Fig. 3-9A, B). Our data are consistent with previous findings (140). The idea that IL-6 expression may be another arm of the integrated cellular response to heat was first proposed by Hasselgren and colleagues.

They showed that heat stress increases IL-6 mRNA in the intestinal mucosa of mice (190) and when acute heat stress is applied to human enterocytes as a co-stimulus with IL-1β, IL-6 production is potentiated (140).

Furthermore, to more specifically test the importance of HSF-1, myotubes were transfected with a c.a. HSF-1 plasmid to overexpress HSF-1 or a d.n. HSF-1 plasmid to knockdown HSF-1. Overexpression of HSF-1 stimulated IL-6 promoter activity (Fig. 4-1E). The knockdown of endogenous HSF-1 inhibited IL-6 promoter activity (Fig. 4-1F). Therefore, we were able to successfully answer aim 2; HSF-1 is an important regulator of the IL-6 gene in C2C12 myotubes.

Results further identify HSF-1 as an important regulator of IL-6 promoter activity and for the first time in the C2C12 skeletal muscle cell phenotype. However, we have not yet been able to directly test HSF-1's interaction with the IL-6 promoter. In future studies, we wish to use chromatin immunoprecipitation analysis (CHiP) to identify in-vivo HSE binding sites. Then we can directly test the functionality of canonical HSEs within the IL-6 promoter by performing site-directed mutagenesis of the functional sites. Mutation of functional HSE regulatory sites within the IL-6 promoter region would enable us to directly test the regulatory role of HSF-1.

Alternative Pathways

We have further identified HSF-1 as an important mediator of IL-6 gene regulation. HSF-1 may not be the direct stimulus of IL-6. HSEs have been identified within the IL-6 promoter and intronic regions (Fig. 2-3); the functionality of these HSEs has yet to be tested. HSF-1 has been proposed to have a role in partially opening the chromatin structure of the IL-6 promoter, which makes the promoter region more susceptible for activators or repressors to bind to it (81). Therefore, HSF-1 may just be an important

co-factor. The signal transduction pathways that regulate the IL-6 gene are complex (Fig. 2-2) and multiple signals could be activated by heat stress.

Aside from HSF-1, we identified several other candidate regulatory pathways of hyperthermia-induced IL-6 mRNA: ATP/purinergic receptors, NF-κB, and the AP-1 family of proteins. ATP/purinergic receptors have been shown to be involved in IL-6 gene regulation in C2C12 cells (21), and other cell phenotypes (erythrocytes) demonstrate a temperature-sensitive release of ATP (91). The NF-κB pathway is a major signal by which IL-6 is transcribed in immune cells such as macrophages and lymphocytes (168), as well as in contracting rodent skeletal muscle (87). Others have shown heat shock to activate the AP-1 complex (123), offering a potential or contributing mechanism in addition to HSF-1. The pharmacological inhibition of ATP/purinergic receptors (suramin) and NF-κB (sn50) further stimulated heat-induced IL-6, leading us to conclude that these pathways are unlikely to be involved (Fig. 3-9).

The AP-1 site in the IL-6 promoter region was tested for heat-induced effects. The percentage of IL-6 promoter activity in heat shocked cells was significantly down with the mutation of the AP-1 binding site (Fig. 4-5). JNKs are stress activated protein kinases that can phosphorylate AP-1 transcription factors, but also C/EBP and NF-κB regulatory families (99). The mechanism by which heat shock induces AP-1 activation is poorly described, but interactions with MAPKs (such as JNK) represent an attractive candidate (94). JNK has been implicated in contraction (193), EPI (48), LPS (47), and proinflammatory cytokines stimulation (47) of IL-6 production in skeletal muscle. However, experiments have not always tested downstream of JNK for its interaction with different regulatory sites within the IL-6 promoter region.

The involvement of AP-1 transcription factors is a new potential regulator of hyperthermia-induced IL-6 that could function together or entirely separate from HSF-1. For example, HSF-1 could open the chromatin structure of the IL-6 promoter increasing exposure to AP-1 transcription factors activated by hyperthermia increasing promoter activity. Alternatively, HSF-1 may be an important component of IL-6 promoter activity, but not a stimulus. We show that HSF-1 knockdown decreases IL-6 promoter activity during basal and heated conditions. Others have shown that HSF-1 is necessary for maximal induction of IL-6 promoter activity (81). The AP-1 mutation only affects the promoter's activity during heated conditions leading us to speculate that it is an important component of the heat-induced signal. Revisiting specific aim 2, we have tested attractive candidates for hyperthermia-induced IL-6 gene regulation and identified HSF-1 and the AP-1 regulatory site in the IL-6 promoter as having regulatory roles. However, questions remain, such as testing HSF-1 for a direct interaction with the IL-6 promoter and identifying what phosphorylates the AP-1 transcription factors during hyperthermia.

Hyperthermia/HSF-1 Stimulates and Enhances IL-6 Gene Expression to Stress Stimuli in C2C12 Cells

Specific aim 3 was designed to test the role of hyperthermia and HSF-1 activation as a co-stimulus or potentiator of IL-6 gene expression in response to distinct stress stimulants: LPS and EPI. To date, much of what we know has centered on the ability of muscles to generate and secrete IL-6 and other myokines in response to exercise or metabolic challenges (144, 145). As we continue to expand muscle's role as an endocrine organ, specifically as an acute stress sensor, it is important to consider the integrative effects that are likely present during acute stress. We have identified two

external stimuli, endotoxin (LPS) and catecholamines (EPI), which are likely present during different acute stress events, but that function distinctly. The major finding of this aim was that hyperthermia and HSF-1 potentiate the IL-6 gene when co-stimulated with EPI or LPS.

Physical or psychological stress initiates a physiological response marked by the increased secretion of catecholamines, EPI and norepinephrine, from the sympathetic nervous system and adrenal medulla. EPI acts through α- and β-receptors to elicit cardiovascular and metabolic effects, and serves to activate components of the innate immune system. EPI functions to promote an anti-inflammatory cytokine milieu by stimulating IL-1RA, IL-10, IL-6, and inhibiting pro-inflammatory cytokines TNF-α and IL-1β (148, 177). Endotoxin and other foreign pathogens initiate an inflammatory cascade by way of TLRs. TLRs function to recognize microbial structures, like endotoxin, and can amount an innate immune response without prior antigen exposure (180). It functions to promote a pro-inflammatory cytokine milieu by stimulating TNF-α and IL-1β (144).

Despite resulting in different inflammatory outcomes EPI and LPS are not completely dissimilar and provide interesting physiologically relevant stimuli to compare and contrast. EPI and LPS are independently capable of producing IL-6 in C2C12 cells and primarily signal through the same mediator, JNK (47, 48). In myotubes treated with EPI or LPS immediately prior to heat IL-6 gene expression was potentiated (Fig. 4-2A, 4-3A). Furthermore, in HSF-1 knockdown experiments, the transfection of a d.n. HSF-1 plasmid resulted in the attenuation of the IL-6 promoter in heated cells treated with EPI and LPS (Fig. 4-2E, 4-3E). The overexpression of HSF-1 enhanced IL-6 promoter

activity alone and when co-stimulated with LPS, but there was no effect in heat and EPI co-treated cells. The overexpression of HSF-1 represents a complicated model. HSF-1 is a transient signal, constitutive activation may result in prolonged activation of stress pathways (5), which may result in the activation of different regulators of these activated pathways. Therefore, the constitutively active HSF-1 plasmid may not elicit a response representative of a transiently induced HSF-1 signal.

Alternatively, as we have discussed in “mechanisms of heat-induced IL-6 formation: alternative pathways” multiple pathways (i.e. HSF-1 and AP-1) are activated by hyperthermia. The overexpression of a single transcription factor (HSF-1) is a different signaling event than heat shock; alternative pathways (i.e. AP-1) may not be activated. Results suggest that basal HSF-1 is an important sentinel of IL-6 regulation no matter the stimulus or conditions; alternatively the overexpression of HSF-1 differentially regulates IL-6 promoter activity based on the stimulus (LPS or EPI). Although HSF-1 may represent a key mediator of IL-6 promoter activity during hyperthermia it is not the only activated pathway therefore it is important to test both.

Proteasome Inhibitor MG-132 Induces Heat Shock and IL-6 in Myotubes

The inhibition of the proteasome leads to the accumulation of abnormal proteins initiating the heat shock response (96). One hour after MG-132 pretreatment we see increases in HSP72 and IL-6 mRNA (Fig. 4-1B, C). By inhibiting the proteasome the degradation of IKB-α is also inhibited, IKB-α suppresses NF-KB activation (47). Therefore, MG-132 induces one of our candidate pathways (HSF-1), but also secondarily inhibits another (NF-KB). Pre-treatment with MG-132 followed by stimulation with EPI or LPS has opposite potentiating and attenuating effects on IL-6 mRNA, respectively. The attenuation of IL-6 mRNA in MG-132 pretreated myotubes

stimulated with LPS is likely the result of the inhibition of the NF-KB pathway, a principle mediator LPS-induced IL-6 (50). The NF-KB pathway is not a mediator of EPI-induced IL-6 (48).

Heat Upregulates ATF-3 mRNA, Epinephrine Attenuates Heat-Induced ATF-3

Others have shown that heat shock before LPS-treatment suppresses IL-6 mRNA by increasing the expression of ATF-3, a negative regulator of IL-6, and in turn resulting in attenuated IL-6 protein in heat shocked cells (181). In experiments in which we pre-heated myotubes before LPS-treatment we show no differences in IL-6 mRNA or protein compared to non-heated LPS supplemented myotubes. Interestingly, when examining ATF-3 mRNA expression our results were consistent, heat alone and heat plus LPS stimulate ATF-3 mRNA. Furthermore, the addition of EPI to cells prior to heat inhibits ATF-3 mRNA (Fig. 4-4); however, the combination of heat, LPS and EPI still results in ATF-3 mRNA. Transcription regulation of ATF-3 is regulated by Egr-1. Egr-1 is regulated via different subgroups of MAPK. Heat and LPS cause rapid activation of three distinct MAPKs: ERK, JNK, and p38 MAPK (47, 141). EPI does not signal through MEK/ERK and could explain why EPI doesn't stimulate ATF-3 (48, 133). Stressors that signal through MEK/ERK could provide a negative feedback mechanism via Egr-1/ATF-3. ATF-3's regulation of IL-6 is poorly understood, it may function to close the chromatin structure limiting access of the promoter (56). Additionally, ATF-3 and NF-κB binding sites are in close proximity suggesting ATF-3 may mediate NF-κB related transcription of IL-6 (184). Interestingly, HSF-1 is required for ATF-3 signaling (181). These results support heat as an important regulator of transcription and suggest that EPI, likely via β-adrenergic stimulation, has the power to override the attenuation of

IL-6 mRNA transcription by heat (Fig. 4-3), but not heat plus LPS induced ATF-3 mRNA.

Our results are not entirely consistent with previous work (181); we see no downregulation of the IL-6 gene with the combination of heat and LPS treatment despite seeing the same consistent changes of ATF-3. An alternative hypothesis is that the phenotypic differences of muscle cells versus embryonic fibroblasts and macrophages could account for some of these differences. One phenotypic difference is the ability of muscle to generate heat, the increased exposure to heat may alter its thermo-tolerance. Furthermore the timing of heat shock is important. Outcomes are different if heat shock occurs at the same time as LPS treatment or if LPS is supplemented immediately after heat shock (Fig. 4-9). We have shown consistent upregulation of IL-6 mRNA in myotubes stimulated with LPS then immediately heated (Fig. 4-2A). Despite these inconsistencies of IL-6 mRNA in heated cells treated with LPS, IL-6 protein is attenuated. This further demonstrates the remarkable control of the heat shock response to regulate transcription and translation.

Differential Regulation of IL-6 Protein in Heated Myotubes

Based on our observation that IL-6 mRNA was stimulated in heated cells and potentiated in heated cells co-treated with EPI or LPS, we hypothesized an upregulation of IL-6 protein because of the increased pool of IL-6 mRNA available for translation. To our surprise, IL-6 protein was upregulated in cells heated alone or heated and co-treated with EPI, but not heated and co-treated with LPS (Fig. 4-6). The attenuation of protein expression in heat is not completely unexpected; during heat shock there is a drastic change in the pattern of proteins synthesized. Priority is placed on the production of HSPs. During heat shock, non-HSPs are suppressed and HSPs are

induced (34). The survival of cells at higher temperatures is dependent on the presence of HSPs (139). Therefore, although, IL-6 mRNA has behaved much like HSP mRNA (Fig. 3-8) it is not surprising that we would see decreased protein expression.

The preferential production of HSPs can be accomplished at multiple levels. The heat shock response is regulated at the transcriptional, post-transcriptional and translational levels (34, 139). Knowing that we see potentiation of IL-6 mRNA, but not protein during heated conditions across multiple time-points suggests post-transcriptional or translational regulation. However, post-transcriptional increased mRNA decay is not a likely mechanism used to regulate the synthesis of non-HSPs, like IL-6 (72). Eukaryotic protein synthesis is primarily regulated at the level of translation initiation (33). eIF-2 and eIF-4F are two heat shock sensitive translation initiation factors (37, 139, 164). Certain mRNAs, such as HSPs, are able to be translated during heat shock because they are resistant to the inactivation of cap-binding initiation factors (138).

Attenuated IL-6 protein production, despite a greater pool of mRNA to translate in the heated groups is likely a result of this overall translational inhibition. If our experimental conditions result in this overwhelming translational inhibition, how does the addition of EPI potentiate IL-6 protein in heated myotubes? One potential explanation is a link made between β-adrenergic stimulation and the potentiation of translation initiation (55). EPI predominantly signals through β1/2-adrenergic receptors (48) and their activation recruits ERK and mammalian target of rapamycin (mTOR) to facilitate long term potentiation of translation initiation (55). Therefore, stimuli that signal through

β-adrenergic receptors, like EPI, may be able to preserve protein translation during heat shock.

The EPI treatment may have preserved protein translation in heat shock conditions when it is usually suppressed, however, this does not help to explain how heat alone was able to upregulate IL-6 protein expression. Lastly, an additional hypothesis is that there is a negative feedback loop of IL-6 regulation. LPS is a greater stimulus of IL-6 production than EPI or heat. The addition of any co-stimuli to LPS may be enough to trigger a negative-feedback mechanism. A IL-6 negative feedback mechanism exists via suppressor of cytokine signaling (SOCS)-3 (28).

Limitations and Future Directions

The contributions of this dissertation were the crucial first steps towards investigating a new physiological role for skeletal muscle as a stress sensor. I feel that the experiments in this dissertation provide the framework necessary to grow into multiple experiments and directions. I will discuss three chief limitations of this research that need to be addressed and close with future directions of interest.

First, there is evidence to suggest that skeletal muscle can contribute to changes in systemic cytokine levels during exercise (175); however, the evidence isn't entirely convincing and little work has been done since to confirm skeletal muscle's contribution. Furthermore, although exercise is a form of stress, to the best of our knowledge skeletal muscle's contribution to circulating cytokine production during acute stress events has not been tested. To best answer this limitation we have worked on developing an isolated hind-limb closed circuit perfusion system. Much to our dismay, this project has been met by great challenges and remains a work in progress.

A second limitation is that much of this work is cause and effect. We first asked the question; does skeletal muscle produce IL-6 in response to heat? We provide strong evidence that heat stimulates IL-6 production, but based on the results presented, no definitive statement can be made. Heat stress is a major physiological stress; the stimulus for IL-6 production could be part of the sequelae following a major stress event and not be the direct result of the initial heat event. For example, heat stress stimulates oxidants, which could then stimulate IL-6 production. To account for some of these limitations we used multiple stimuli such as environmental heat, pharmacological heat shock induction, and the overexpression and knockdown of a major heat-induced transcription factor (HSF-1). Furthermore, when evaluating stress response special care needs to be taken to account for experimental limitations. Therefore, we used multiple models C2C12 myoblasts and myotubes, ex-vivo muscle preparations, and in-vivo whole animal model.

Third, in the present work, we observed that heat induces skeletal muscle IL-6 production. We tested the importance of HSF-1 to IL-6 induction. Pharmacological induction and inhibition of HSF-1 stimulated and attenuated IL-6 mRNA. The overexpression and knock down HSF-1 with constitutively active and dominant negative plasmids increased IL-6 promoter activity. Our results suggest that HSF-1 is playing a major regulatory role in the presence or absence of heat and regardless of the principal IL-6 stimulus. These data provide the first evidence that HSF-1 is a regulator skeletal muscle IL-6, but they do not directly test the interaction.

Our principal hypothesis is that heat stress yields an accumulation of mis- or unfolded proteins that induce the heat shock response, activating HSF-1 and stimulating

skeletal muscle IL-6. We are interested in taking our novel hypothesis and applying them to new physiological questions. Based on the results of this dissertation, we have principally become interested in protein stress. Protein stress is a broad term to collectively refer to the increased oxidation of proteins, increased accumulation of mis-folded proteins, increased mitochondrial protein turnover, and any failures in protein synthesis or degradation. There are 2 major signaling cascades downstream of protein stress known to stimulate IL-6 production: 1) HSF-1 activation and 2) the "unfolded protein response" (UPR). The endoplasmic reticulum (ER) is the organelle responsible for folding and assembling the majority or proteins the cell displays or secretes (206). It responds to the accumulation of unfolded proteins by activating the UPR to help maintain homeostasis in the ER (73, 74). The UPR protects the ER's protein folding environment, similar to how the heat shock response protects the cytosolic protein folding environment. The concept of protein stress initiating an inflammatory response in skeletal muscle is a new exciting area of research with broad implications on sarcopenia, muscular dystrophy, tumor development and cancer biology.

Significance and Conclusions

The present study presents a case for skeletal muscle as a stress sensor. The proposed function of skeletal muscle as a stress sensor would presumably provide the whole organism with a preconditioning message. For example, the increased stress-induced production and secretion of IL-6 from skeletal muscle could act on the liver to initiate the acute phase response (8, 99). The advantage of skeletal muscle as an endocrine organ starts with muscles prevalence and distribution throughout the body. Muscle makes up about 40% of body weight in man and has the ability to sample regionally and systemically (85). If the function or circulation of one organ or region was

cutoff due to trauma, muscles in other regions are available. As we have explained, muscle is well equipped to recognize and respond to external stress signals, as well as internal stress.

Until now, muscle cytokine production has largely been viewed in the context of exercise (144, 145). For our model we chose heat stress because it is a unique stress event, hyperthermia results in the upregulation of circulating IL-6 (154). In addition to muscles producing heat during contraction or exercise, circulating IL-6 levels during exercise fluctuate with core temperature (153, 172). Our identification of heat as a stimulus for IL-6 expression in mouse skeletal muscle supports a potential role of temperature and muscle-derived IL-6 production in changes of circulating IL-6 during exercise. The addition of heat to the host of other stress stimuli which promote muscle IL-6 production that were previously mentioned (endotoxin (46), inflammatory cytokines (47, 114, 186), catecholamines (48, 77, 101), low glycogen (95, 173), ATP (21), intracellular Ca2+ (2, 90), surgical manipulation (161), ROS (100), and NO (117, 176)), further supports the notion that one of skeletal muscle's functions is to detect stress.

We have found that heat can both stimulate and potentiate IL-6 mRNA when other co-stimuli are present. The ability of heat to potentiate the IL-6 signal adds to its relevance as a stress sensor, as the heat shock response is the quintessential stress response used by cells to cope with physiological stress. Furthermore, through the use of pharmacological inhibitors and knockdown experiments, we have identified HSF-1 or its downstream stimuli as a differential regulator of IL-6 at the transcriptional and translational level in C2C12 cells. The finding that HSF-1 is the mechanism by which heat upregulates IL-6 offers future exciting avenues of research. Despite its name,

HSF-1 is not dependent on heat to be activated. It is activated in any condition that results in the accumulation of denatured proteins expanding the relevance of this work.

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BIOGRAPHICAL SKETCH

Steven Stanley Welc is the son of Susan and Richard Welc, the brother of Jennifer Welc, and the husband of Tracy Welc. He was born in Flemington, New Jersey. Steven graduated from Hunterdon Central Regional High School in Flemington, NJ and attended the University of North Carolina Wilmington in Wilmington, NC. He received his Bachelor of Arts degree in physical education and health with a concentration in exercise science from University of North Carolina Wilmington, where he was also awarded outstanding student in the department of health and human applied science. Following undergraduate school, Steven began his work as a doctoral student in Dr. Thomas L. Clanton's laboratory at the University of Florida in Gainesville, FL. As a doctoral student, his research focused on skeletal muscle as an acute stress sensor. Steven received his Ph.D. from the University of Florida in the winter of 2012.