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The regulation of interleukin-6 implicates skeletal muscle as an integrative stress sensor and endocrine organ

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Abstract

Skeletal muscle has been identified as an endocrine organ owing to its capacity to produce and secrete a variety of cytokines (myokines) and other proteins. To date, myokines have primarily been studied in response to exercise or metabolic challenges; however, numerous observations suggest that skeletal muscle may also release myokines in response to certain categories of internal or external stress exposure. Internal stress signals include oxidative or nitrosative stress, damaged or unfolded proteins, hyperthermia or energy imbalance. External stress signals, which act as indicators of organismal stress or injury in other cells, employ mediators such as catecholamines, endotoxin, alarmins, ATP and pro-inflammatory cytokines, such as tumour necrosis factor- α and interleukin-1 β . External stress signals generally induce cellular responses through membrane receptor systems. In this review, we focus on the regulation of interleukin-6 (IL-6) as a prototypical stress response myokine and highlight evidence that *IL-6* gene regulation in muscle is inherently organized to respond to a wide variety of internal and external stressors. Given that 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 hypothesize that it may comprise a fundamental component of the organism's acute stress response.

In this review, we 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 centred on the ability of muscles to generate and secrete cytokines in response to exercise or metabolic challenges (Pedersen & Febbraio, 2008, 2012). These cytokines have more recently been termed 'myokines', defined as 'cytokines and other peptides that are produced, expressed and released by muscle fibres and exert paracrine, autocrine or endocrine effects' (Pedersen & Febbraio, 2008). 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

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(IL-6) production in skeletal muscle and that *IL-6* transcription can be blocked by application of heat shock factor (HSF) inhibitors (Welc *et al.* 2012). 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 focuses on IL-6 as a model signal that represents a potential efferent arm of the ability of skeletal muscle to contribute to the stress response of the organism. We explore experimental evidence of how various stimuli known to accompany acute 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 evaluate the promoter region of the *IL-6* gene to outline the relationships between known signalling pathways and their links to stress signals that can be detected by muscle. Finally, we briefly review the potential physiological significance of IL-6 in stressed tissue and in the whole organism.

An overview of cytokine responses to stress: comparison with exercise

In many forms of stress there are striking changes in circulating cytokines that 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 haemorrhagic shock (Fink, 2003), hyperthermia (Pedersen & Febbraio, 2008), burns (Deitch, 1990) and trauma (Ding *et al.* 2004) have all been shown to induce intestinal barrier dysfunction and to produce cytokine responses. It is, however, unknown whether 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 (Bosenberg *et al.* 1988). One of the arguments against endotoxin as the stimulus for exercise-induced cytokine expression is that the time line of the release pattern and the relative concentrations of specific cytokines are distinctly different in exercise compared with endotoxin exposure (Pedersen & Febbraio, 2008). For example, the endotoxin response is characterized by an early rise in tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) and a later elevation of IL-6, interleukin-10 (IL-10) and interleukin-1 receptor antagonist (IL-1RA), whereas in exercise, pro-inflammatory cytokines (TNF- α and IL-1 β) are blunted or absent, but there are marked elevations in circulating IL-6, IL-1RA and IL-10 (Pedersen & Febbraio, 2008).

Interleukin-6 has been one of the more predictable and dominant cytokines expressed following endurance exercise (Drenth *et al.* 1995). 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 (Steensberg *et al.* 2000). Additional experiments that demonstrated 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 (reviewed by Pedersen & Febbraio, 2008). 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 the interleukins IL-1 β , IL-8, IL-10, IL-15 and IL-18, as well as TNF- α , high-mobility group box-1 protein (HMGB1) and fibronectin type III domain containing 5 (FNDC5)-Irisin among many others, as recently reviewed by Pedersen & Febbraio (2012). 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, it is clear that it is not a necessary prerequisite for a cytokine response to intense exercise.

Like exercise, the cytokine response to some stress exposures differs from endotoxin exposure. For example, in hyperthermia the circulating TNF- α and IL-1 β 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 (Robins *et al.* 1995). In haemorrhagic shock, IL-6 and TNF- α elevations tend to coincide, but with a predominant IL-6 response (Mok & Moore, 2008). These patently different cytokine profiles following stress more closely resemble those seen following endurance exercise than those seen after endotoxin exposure.

Interleukin-6 is a dominant signal seen in the circulation during and following stress conditions such as hyperthermia (Welc *et al.* 2012). For example, in a mouse model of heat stroke, plasma IL-6 can exceed 1000 pg ml⁻¹, measured 2 h after heat exposure (Welc *et al.* 2012). Equally high levels of IL-6 are seen 2 h following haemorrhagic shock in mice (Isayama *et al.* 2012). However, although there is ample evidence that skeletal muscles upregulate IL-6 and other myokines in stress, as we shall discuss in the following sections, 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 Fig. 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, mechanical stress, thermal stress, osmotic stress and oxidative stress (as reviewed by Wright *et al.* 2009), as well as nitrosative stress (Vassilakopoulos *et al.* 2003) and energy imbalance (Gleeson, 2000). However, muscle is also exposed and is sensitive, via membrane receptor systems, to blood-borne mediators of stress, 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. 1). In this review, we 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: pathogen-associated molecular patterns and damage-associated molecular patterns

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; Takeda, 2005). Toll-like receptors are evolutionarily conserved receptors that were originally identified to function in recognizing microbial structures such as endotoxin, now referred to in general as pathogen-associated molecular patterns (PAMPs; Takeda, 2005). The ability of the innate immune system to mount an immune response to a pathogen, without prior exposure to an antigen, is mediated largely through TLRs. Toll-like receptors 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 that make them responsive to a wide range of different microbial components and related substances (Lemaitre *et al.* 1996). Frost *et al.* (2002) have characterized the interactions between bacterial endotoxin, lipopolysaccharide (LPS), TLR4 and cytokine production in skeletal muscle. 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 (Frost *et al.* 2006).

Skeletal muscle is potentially exposed to multiple types of PAMPs. As mentioned, in addition to infection, many acute stress events, even strenuous exercise, can result in release of endotoxins or inflammatory mediators from the intestine (Bosenberg *et al.* 1988). Given that muscle represents a high percentage of body mass, approximately 40% in man, it has an enormous potential for sensing blood-borne changes, particularly in high metabolic states, such as in exercise, where both capillary cross-sectional area and turnover of interstitial fluid are elevated manyfold. In a classic experiment by Mathison *et al.* (1980) 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.

In addition to detecting the presence of invading micro-organisms, 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, HMGB1, free nucleic acids from released DNA or RNA, mitochondrial membranes (which resemble bacterial membranes) and oxidized lipids (Ulevitch, 2004). They all initiate signalling events via subsets of TLRs. For example, although 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 (Johnson & Fleshner, 2006), most probably via TLR activation (Fang *et al.* 2011). β -Defensins are another type of TLR ligand

(Funderburg *et al.* 2011) that consists of antimicrobial peptides produced by skeletal muscle and other tissues (García *et al.* 2001). 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 (Bustin, 2002). Finally, lipids are oxidized in the presence of reactive oxygen species (ROS) or reactive nitrogen species, which are often elevated in stress, producing a variety of oxidized membrane-like products, some of which can signal via TLRs (Zmijewski *et al.* 2005).

Both PAMPs and DAMPs bind to TLRs and signal through a number of proteins, such as myeloid differentiation primary response gene 88, TRI-domain-containing adapter molecule interferon- β and interleukin-1 receptor-associated kinase, ultimately leading to the activation of downstream transcription factors. Figure 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 by Broderick *et al.* 2007). Although TLRs can signal through a variety of pathways, all TLRs signal via mobilization of the P60/P65 elements of the nuclear factor- κ B (NF- κ B) protein, which translocate into the nucleus and activate the NF- κ B response element on the promoter region of *IL-6* (Patel *et al.* 2012). Another pathway that appears to be critical and perhaps obligatory for IL-6 signalling from some TLRs, such as TLR3 and TLR4, is via ‘interferon regulatory factor’ (IRF) activation, particularly IRF-3, -5 or -7 (Patel *et al.* 2012). Finally, nearly all TLRs can activate the *IL-6* promoter region via mitogen-activated protein kinases (MAPKs), specifically c-Jun N-terminal kinase (JNK) and p38 MAPK (Patel *et al.* 2012).

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 activator protein 1 (AP-1). Activator protein 1 can then bind to its transcription site within the promoter region to stimulate *IL-6* transcription (Matsuzawa & Ichijo, 2005; Fig. 2). This pathway is probably the most common stress-activated transcriptional pathway for *IL-6*. Besides TLRs, it is activated by oxidative stress, toxins, hyperosmolarity, ischaemia–reperfusion, ceramide, catecholamine activation and endoplasmic reticular (ER) stress (i.e. the unfolded protein response, UPR; Nishina *et al.* 2004). 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 (Whitham *et al.* 2012).

Catecholamines

Stressful conditions initiate a well-coordinated physiological response marked by increased secretion of the catecholamines adrenaline and noradrenaline 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. Adrenaline 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 (van der Poll *et al.* 1996) and pleiotropic IL-6 (Steensberg *et al.*

2001b). Additionally, catecholamines inhibit pro-inflammatory cytokines, such as TNF- α and IL-1 β (van der Poll *et al.* 1996).

Both α - and β -adrenergic receptors are expressed on skeletal muscle cells (van Alphen *et al.* 1963). Adrenaline predominantly binds β_1 - and β_2 -adrenergic receptors and is a strong stimulant for IL-6 synthesis in skeletal muscle (Frost *et al.* 2004). Although less studied, noradrenaline, which binds α -receptors, also stimulates IL-6 formation in C2C12 muscle cells, over a similar dosage range to adrenaline (Frost *et al.* 2004). The β -receptor density on muscle is proportional to the percentage of slow-twitch fibres, and the affinity of the receptors for their antagonists is higher in fast- than in slow-twitch or mixed fibres (Martin *et al.* 1989). In rats, infusion of adrenaline, at a dosage sufficient to achieve plasma concentrations comparable to sepsis, yielded a 40-fold and 15-fold increase of IL-6 mRNA and IL-6 protein, respectively, in skeletal muscle (Frost *et al.* 2004). Others have found that only supraphysiological doses of adrenaline (>100 nM) stimulate IL-6 gene expression in skeletal muscle (Holmes *et al.* 2004); however, that study exposed intact soleus muscles to adrenaline for only 1 h and did not test noradrenaline. 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 (Welc *et al.* 2012), which tends to minimize the true physiological responses when treated samples are compared against sham controls.

As the most common second messenger system for catecholamine stimulation in most cell systems is cyclic AMP (cAMP), which is upstream of cyclic adenosine monophosphate response element (CRE) signalling on the IL-6 promoter region (Fig. 2), one would think that this would comprise the most likely signalling pathway for catecholamines; however, there is no evidence for this at this time. The primary pathway appears to be through the MAPK signalling described in the previous subsection for TLR activation, i.e. JNK/AP-1 (Frost *et al.* 2004; Fig. 2).

ATP and adenosine

Several lines of evidence have shown that extracellular ATP, ADP or adenosine causes stimulation of IL-6 formation and/or release in skeletal muscle (Buvinic *et al.* 2009) or in other cell phenotypes. Both ATP and its metabolites are released into the extracellular medium after pathophysiological events including hypoxia, cell swelling, shear stress or inflammation (Schwiebert & Zsembery, 2003). The fact that cellular damage is a source of extracellular ATP (Ryten *et al.* 2004) has resulted in ATP sometimes being included in the list of 'alarmins', although purinergic receptors for ATP or adenosine do not signal via the same mechanisms as TLRs (Rao & Pober, 2008).

Muscle also releases considerable quantities of ATP, AMP and adenosine during contraction (Buvinic *et al.* 2009). 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 (Lynge *et al.* 2001). Purinergic P1 receptors are stimulated by adenosine, whereas P2 receptors are stimulated by ATP and/or ADP (Ralevic & Burnstock,

1998), and skeletal muscle sarcolemma is generally equipped with both categories of receptors (Lyngé & Hellsten, 2000).

The roles of P1 adenosine receptors in IL-6 regulation in adult skeletal muscle are not well understood. In human and rat primary cultured muscle cells, the effects of adenosine on IL-6 can be inhibited by a general MAPK inhibitor, but which MAPK pathway is involved is not clear (Høier *et al.* 2010). In cardiac fibroblasts exposure to adenosine stimulates *IL-6* mRNA via the p38/AP-1 pathway and via protein kinase C- δ , which is upstream of p38 (Fig. 2; Feng *et al.* 2010). In cultured myotubes and in rat muscle cells extracellular adenosine stimulates G-protein-coupled cAMP activity, but the complete link with IL-6 regulation and P1 receptor activity has not been clearly established (Henning *et al.* 1993).

The P2 receptors can activate a number of physiological responses in muscle cells. The P2X receptors mediate rapid and selective permeability to several cations (Ralevic & Burnstock, 1998), whereas P2Y receptors reduce Cl^- conductance (Voss, 2009) and stimulate G-protein-coupled activation of phospholipase C and protein kinase C (Ralevic & Burnstock, 1998). Via these multiple pathways, P2 stimulation mobilizes 'slow-release' intracellular Ca^{2+} stores, which are then linked in several ways to *IL-6* transcription (Buvinic *et al.* 2009). 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; Shigemoto-Mogami *et al.* 2001).

Inflammatory cytokines, TNF- α and IL-1 β

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-1 β . Skeletal muscle contains both IL-1 β and TNF- α receptors, which are present in normal conditions, but are generally upregulated only in diseased or damaged muscle (Zhang *et al.* 2000). The response of cultured muscle cells and intact skeletal muscle (Frost *et al.* 2003) to inflammatory cytokines emulates the response seen in the circulation, in that marked increases in IL-6 are produced.

As shown in Fig. 2, the skeletal muscle IL-6 response to TNF- α and IL-1 β is mediated in part through the JNK/AP-1 signalling network discussed previously (Frost *et al.* 2003). However, in addition, CCAAT enhancer binding protein C/EBP binding site (Hungness *et al.* 2002) and NF- κB signalling (Li *et al.* 1998) 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 signalling via glucocorticoids. As shown in Fig. 2, glucocorticoids inhibit *IL-6* via the intracellular glucocorticoid receptor and glucocorticoid-responsive elements (GREs) on the promoter region (Barnes, 1998); therefore, IL-6 works in a global feedback loop where stress/inflammatory signals stimulate IL-6 production and anti-inflammatory signals from steroid release feed back to suppress IL-6 release.

'Internal' stress signals and IL-6 regulation

Reactive oxygen species

Interleukin-6 is elevated in cultured myotubes but not myoblasts in response to exposure to ROS (Kosmidou *et al.* 2002), and in humans IL-6 release from contracting skeletal muscle is inhibited by supplementation with the antioxidants vitamins C and E (Fischer *et al.* 2004). *In vivo*, short-term vitamin E supplementation reduces *IL-6* mRNA and IL-6 protein production in skeletal muscle and attenuates LPS-induced increases in NF- κ B activation (Huey *et al.* 2008). Also, reduced oxidative stress with *N*-acetylcysteine decreases extracellular signal-regulated kinase 1/2, p38 and NF- κ B signalling proteins and also reduces IL-6 formation (Sigala *et al.* 2011). These data suggest that ROS are an active component of internal stress-induced IL-6 signalling.

Reactive oxygen species are generated in a variety of different stress-like conditions in skeletal muscle (as reviewed by Wright *et al.* 2009). 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. Reactive oxygen species do not have to be formed endogenously within the cell; they can also come from external sources, but nevertheless most ROS are short-lived, local signals, ideal for early signalling of local, intracellular stress. One potential stress signalling 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 signalling (Kosmidou *et al.* 2002; Fig. 2). Recent studies of intact muscle have also suggested that the primary redox-sensitive pathway is via p38 MAPK (Sigala *et al.* 2011).

Another redox regulated signalling pathway potentially involved in *IL-6* transcriptional regulation is the NF- κ B family of proteins. There is considerable support for the concept that one of the chief ways in which ROS influence most transcriptional targets in oxidative stress is via NF- κ B signalling (Ji *et al.* 2004). In addition, a number of cell culture studies using different phenotypes have shown that NF- κ B activation can stimulate *IL-6* transcription (Craig *et al.* 2000); however, most recent studies have ruled out NF- κ B as the predominant signalling link between ROS and *IL-6* both in intact skeletal muscles (Sigala *et al.* 2011) and in cardiac fibroblasts, in favour of a p38/AP-1 mechanism (Sano *et al.* 2001).

Reactive nitrogen species

Nitric oxide and other reactive nitrogen species may also play roles in the ability of muscle to respond to acute stress. All three NO synthases (NOSs) have been detected in skeletal muscle in various conditions (Buchwalow *et al.* 2005), but the neuronal isoform (nNOS) is abundantly expressed in fast fibres and is most prevalent along the sarcolemma of fast-twitch fibres (Kobzik *et al.* 1994). Production of NO is significantly elevated in muscle during contraction (Kobzik *et al.* 1994; Pye *et al.* 2007), hypoxia (Javeshghani *et al.* 2000), endotoxin exposure (Frost *et al.* 2004), ischaemia-reperfusion (Thiemermann *et al.* 1997), crush injury (Rubinstein *et al.* 1998) and by exposure to inflammatory cytokines (Williams *et al.* 1994). Nitric oxide may mediate redox-sensitive transcriptional control via several pathways (Fig. 2) (Steensberg *et al.* 2007), as well as by modification of proteins within the

cytoplasm or nucleus. Pharmacological inhibition of NO during exercise attenuates increases in *IL-6* mRNA (Steensberg *et al.* 2007).

The gene regulatory link between NO and *IL-6* transcription has not been entirely determined in skeletal muscle, but results from other cellular phenotypes have pointed to multiple pathways. In mononuclear cells, NO stimulates guanylyl cyclase to form cGMP, which can then activate NF- κ B (Siednienko *et al.* 2011; Fig. 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 ($<10 \mu\text{M}$), common to normal physiological signalling, stimulate *IL-6* (Siednienko *et al.* 2011). Recent reports in osteoblasts have also demonstrated the importance of cyclic adenosine monophosphate response element-binding protein (CREB)/cyclic adenosine monophosphate response element (CRE) signalling via cGMP for NO-stimulated *IL-6* formation (Broderick *et al.* 2007; Fig. 2).

Another possible link for both reactive nitrogen species and ROS with *IL-6* is through their influence on Ca^{2+} signalling and cytosolic Ca^{2+} concentrations. There are many well-known effects of ROS and reactive nitrogen species on Ca^{2+} regulation that are dose dependent. In general, both reactants, at low concentrations, stimulate Ca^{2+} release from the sarcoplasmic reticulum or activate pathways responsible for elevating cytosolic Ca^{2+} via intracellular stores (Favero *et al.* 1995). 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 (Holmes *et al.* 2004).

Hyperthermia

During intense exercise in hot environments, muscle operates about 1°C above core temperature, often reaching as high as 41°C in humans (Saltin *et al.* 1972), and can reach $\sim 44^{\circ}\text{C}$ in rats in hyperthermic exercise (Brooks *et al.* 1971). Muscle therefore 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 (Welc *et al.* 2012). Interleukin-6 mRNA was upregulated at temperatures $>41^{\circ}\text{C}$ 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, HSPs and *IL-6* formation in intestinal epithelial cells (Parikh *et al.* 1998). 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 (Pritts *et al.* 2002). The stimulus is therefore likely to be 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 (Welc *et al.* 2012).

As a transcriptional regulator, HSF-1 is an important cofactor that functions to partly open the chromatin structure of the *IL-6* promoter region, allowing other activators and repressors to bind to it more efficiently (Inouye *et al.* 2007). Additionally, HSF-1 affects the gene regulation of other cytokines, such as $\text{TNF-}\alpha$ and $\text{IL-1}\beta$. Heat shock factor-1 also has an

apparent direct interaction with the C/EBP (sometimes called 'nuclear factor (NF) of IL-6'), an essential regulator of *IL-1 β* transcription (Xie *et al.* 2002) and of *IL-6* transcription (Fig. 2). However, in contrast to the observed physiological effects of heat on IL-6, HSF-1 inhibits C/EBP binding to its promoter region and would therefore potentially suppress IL-6 (Fig. 2).

Heat shock factors primarily bind to heat shock elements (HSEs) on the DNA. Heat shock factor 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 (Yamamoto *et al.* 2009; Guertin *et al.* 2012; Fig. 3). The effectiveness of these HSE sites for binding HSF trimers has been studied in *in vivo* conditions (Guertin *et al.* 2012). In both humans and mice, putative HSE sequences have been identified on the *IL-6* gene. For example, Pritts *et al.* (2002) identified three potential HSEs within the *IL-6* promoter, although these sequences are still unproven and are atypical of most HSEs (Yamamoto *et al.* 2009). More classical sequences are present on the human promoter at about -660 nucleotides from the origin (Fig. 3; Inouye *et al.* 2007). This HSE binding site on *IL-6* has been confirmed for the human gene (Inouye *et al.* 2007). We have identified a similar potential site in the mouse gene at -964 nucleotides from the start site (Fig. 3). Notably, few effective HSEs exactly fit the canonical sequence (Fig. 3), and relaxation of nucleotides at several locations is most often seen for *in vivo* binding sites (Guertin *et al.* 2012). 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. 3) and lie within the second intron (Figs 2 and 3), about 200 nucleotides from the end of the second exon. Although 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 (Lange *et al.* 1997; Cooper *et al.* 2000).

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 monocyte chemotactic protein-1, is the 'unfolded protein response' (UPR; Gargalovic *et al.* 2006). The UPR is initiated when the endoplasmic reticulum, where most proteins are synthesized, is placed under stress such that proteins being produced remain incomplete or unfolded. This sets up a series of signalling pathways through activation of three primary signalling proteins, i.e. protein kinase RNA-like endoplasmic reticulum kinase (PERK) inositol requiring enzyme 1 alpha (IRE1 α) Activating transcription factor 6 (ATF6). These proteins release or activate second messengers, such as Activating transcription factor 4 (ATF4) and the X-box-binding protein-1, among others (Wouters & Koritzinsky, 2008). The UPR pathways are upregulated in a variety of muscle disorders and challenges, as well as following chronic loading and unloading of living muscle, and are therefore highly relevant to muscle pathophysiology (Deldicque *et al.* 2012).

Cytokine regulation in response to UPR is not yet well studied in skeletal muscle, but in endothelial cells and in purified B cells *IL-6* transcription is greatly stimulated by UPR via ATF4 and X-box-binding protein-1 (Iwakoshi *et al.* 2003). The signalling systems for how

UPR elevates *IL-6* transcription appear to be via JNK/AP-1, NF- κ B and/or Ccaat-enhancer-binding proteins (C/EBP; Deldicque *et al.* 2012); however, the predominant linkage between UPR and *IL-6* transcription in skeletal muscle remains unexplored.

Energy imbalance

Pedersen & Febbraio (2008) suggest that the cytokine response of muscle to contraction is not inflammatory, but centred on the ongoing metabolic needs of the muscle, initiating shifts in metabolic pathways in target organs that favour energy availability. Muscle *IL-6* gene expression (Keller *et al.* 2001) and protein release (Steensberg *et al.* 2001a) are intensified during times of low intramuscular glycogen. Additionally, glucose ingestion during exercise attenuates release of IL-6 protein from leg muscle (Febbraio *et al.* 2003).

Low glycogen depots are detrimental to moderate and intense exercise performance (Hargreaves & Richter, 1988). The drop in glycogen storage during conditions of continued energy demands could be perceived as an 'internal stress', in which the muscle is being asked to function in conditions that are 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, (Febbraio *et al.* 2002), suggesting the involvement of HSFs. In addition, low glycogen during contraction leads to the phosphorylation and transfer of p38 MAPK to the nucleus (Chan *et al.* 2004), which can upregulate *IL-6* via the AP-1 site (Fig. 2). Thus, several signalling pathways are likely to be 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. Given that 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 signalling molecules that can be used without greatly compromising the metabolism of contracting muscle cells.

Although it is likely that an array of signalling proteins could be produced by skeletal muscle in response to stress (Pedersen & Febbraio, 2008, 2012), 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-1 β and other pro-inflammatory cytokines from inflammatory cells (Xing *et al.* 1998). It also promotes formation of anti-inflammatory cytokines, such as IL-1RA and IL-10, as well as production

of corticosteroids (Febbraio *et al.* 2003). 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, haemorrhage, heat stroke, malignancy or severe trauma (Heinrich *et al.* 1990).

Interleukin-6 also has many protective and restorative roles throughout the body, particularly in acute stress settings. For example, in haemorrhagic shock, IL-6 supplementation protects the lung from injury, protects the intestine from ischaemia and prevents circulatory collapse and cell apoptosis (Meng *et al.* 2000; Alten *et al.* 2008; Moran *et al.* 2009). In heart muscle, IL-6 and associated Signal transducer and activator of transcription 3 (STAT3) signalling pathways are critical for preconditioning and postconditioning stimuli that protect the ischaemic or damaged heart (Dawn *et al.* 2004). In heat stroke, IL-6-deficient mice have much higher mortality (Leon, 2007). Interleukin-6 has neuroprotective functions in the brain during hypoxia (Biber *et al.* 2008) and it promotes wound healing in a variety of tissues (McFarland-Mancini *et al.* 2010). Its strong effects on mobilization of energy stores in the liver, muscle and other organs during intense exercise (Pedersen & Febbraio, 2012) 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 adrenaline and noradrenaline, chronic or excessive exposure to IL-6 during inappropriate times can promote and even cause serious illnesses. As a stress hormone, therefore, IL-6 has its place and time, and it functions in a delicate balance between its life-saving and life-threatening impacts on whole-body homeostasis.

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New findings

What is the Topic of this review?

This review discusses the regulation of the interleukin-6 (IL-6) gene and how it is uniquely set up to respond to stress signals in skeletal muscle. We propose that skeletal muscle is a 'stress sensor' that responds by releasing highly active endocrine and paracrine proteins, such as IL-6.

What advances does this highlight?

Recent discoveries on the regulation of the IL-6 gene in various stress exposures have resulted in the emergence of a new potential role for skeletal muscle in life-threatening stress conditions. Understanding the relationship between IL-6 secretion and stress may provide new insights into normal adaptive responses to disease, deconditioning, inflammation and ageing.

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

Abbreviations: P2X, Purinergic receptor type 2 X; P2Y, Purinergic receptor type 2 Y; P1, Purinergic receptor type 1; IL-6, Interleukin-6; TNF-alpha, Tumor Necrosis Factor-alpha; IL-1Beta, Interleukin-1Beta; UPR, Unfolded Protein Response; ER, Endoplasmic Reticulum; NO, Nitric Oxide; ROS, Reactive Oxygen Species; Ca^{2+} , Calcium; TLR 2, Toll-Like Receptor 2; TLR 4, Toll-Like Receptor 4; beta, Beta-adrenergic receptor; alpha, Alpha-adrenergic receptor; HSF, Heat Shock Factor; cGMP, cyclic guanosine monophosphate; cAMP, cyclic adenosine monophosphate; PAMPS, Pathogen associated molecular patterns; DAMPs, Damage associated molecular patterns; ATP, Adenosine Triphosphate.

Figure 2. Acute stress stimuli and their relationship to the interleukin-6 promoter region
 Note that these regulatory sites are not accurately placed in their respective location along the gene. Abbreviations: HSF, Heat Shock Factor; HSE, Heat Shock Element; GRE, Glucocorticoid Response Element; NO, Nitric Oxide; cGMP, Cyclic guanosine monophosphate; ATP, Adenosine Triphosphate; Ca^{2+} , Calcium; cAMP, Cyclic adenosine monophosphate; PKA, Protein Kinase A; CaMK, Calcium/Calmodulin-dependent Protein Kinase; CREB, cyclic adenosine monophosphate response element-binding protein CRE, cyclic adenosine monophosphate response element; PKC, Protein Kinase C; ROS, Reactive Oxygen Species; JNK, c-Jun N-terminal kinases; p38 MAPK, p38 mitogen-activated protein kinase; AP-1, Activator Protein-1; ERK, Extracellular signal-regulated kinases; C/EBP, Ccaat-enhancer-binding proteins; CHOP, Ccaat-enhancer-binding protein homologous protein; ER, Endoplasmic reticulum; UPR, Unfolded protein response; TNF-alpha, Tumour Necrosis Factor-alpha; IL-1Beta, Interleukin-1Beta; NF-KB, Nuclear Factor Kappa Beta; PAMPS, Pathogen associated molecular patterns; DAMPS, Damage associated molecular patterns; LPS, Lipopolysaccharide; IRF, Interferon regulator factor.

CANONICAL HSE SEQUENCE		nTTCnnGAAnATTCn	
Within the IL-6 promoter			
HUMAN	-660 FROM ORIGIN	AGTTCACCCGACTTCGT	REVERSE 3' to 5' confirmed
MOUSE	-964 FROM ORIGIN	TGTTGTTGAAGTTTCAT	REVERSE 3' to 5'
Intronic Sites on the IL-6 gene			
MOUSE	+218 from exon 2	ATTCTAGAAAATTCAC	Forward 5' to 3'
HUMAN	+192 from exon 2	CTTCTGGAATGCAG	Forward 5' to 3'

Figure 3.
Potential matches of the canonical heat shock element (HSE) sequence for heat shock factor binding in the human and mouse interleukin-6 gene