6-2016

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Abstract

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Keywords

hyperthermia, inflammation, heat stroke, NF-kB, AP-1

Disciplines

Agriculture | Animal Experimentation and Research | Animal Sciences | Musculoskeletal, Neural, and Ocular Physiology

Comments

This is a manuscript of an article published as Ganesan, Shanthi, Carmen Reynolds, Katrin Hollinger, Sarah C. Pearce, Nicholas K. Gabler, Lance H. Baumgard, Robert P. Rhoads, and Joshua T. Selsby. "Twelve hours of heat stress induces inflammatory signaling in porcine skeletal muscle." *American journal of physiology-Regulatory, integrative and comparative physiology* 310, no. 11 (2016): R1288-R1296. doi: 10.1152/ajpregu.00494.2015. Posted with permission.

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Articles in PresS. Am J Physiol Regul Integr Comp Physiol (March 23, 2016). doi:10.1152/ajpregu.00494.2015

Twelve hours of heat stress induces inflammatory signaling in porcine skeletal muscle

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Abstract

Heat stress causes morbidity and mortality in humans and animals and threatens food security by limiting livestock productivity. Inflammatory signaling may contribute to heat stress-mediated skeletal muscle dysfunction. Previously we discovered increased circulating endotoxin and intramuscular oxidative stress and TNFα protein abundance but not inflammatory signaling following 24 and 72 hours of heat stress. Thus, the purpose of this investigation was to clarify the role of inflammatory signaling in heat stressed skeletal muscle. Crossbred gilts (n=8/group) were assigned to either thermal neutral (24° C), heat stress (37° C), or pair-fed thermal neutral (24° C) conditions for 12 hours. Following treatment, animals were euthanized and the semitendinosus red (STR) and white (STW) were recovered. Heat stress did not alter inflammatory signaling in STW. In STR, relative heat shock protein abundance was similar between groups as was nuclear content of HSF1. In whole homogenate, relative abundance of the NF-κB activator IKKα was increased by heat stress though abundance of NF-κB was similar between groups. Relative abundance of phosphorylated NF-κB was increased by heat stress in nuclear fractions. AP-1 signaling was similar between groups. While there were few differences in transcript expression between thermal neutral and heat stress, 80 and 56% of measured transcripts driven by NF-κB or AP-1, respectively, were increased by heat stress compared to pair-fed thermal neutral. Heat stress also caused a reduction in IL-6 transcript and relative protein abundance. These data demonstrate that short-term heat stress causes inflammatory signaling through NF-κB in oxidative, but not glycolytic, skeletal muscle.

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Introduction

Heat-related complications continue to be a major human health concern and lead to potentially life-threatening conditions ranging from heat exhaustion to heat stroke and death (28). These may also lead to longer-term health problems including cardiovascular (14, 48) and kidney disease (13). These detrimental health effects were made clear during a 2006 heat wave where there were 655 heat-related deaths in California as well as 1,620 more hospitalizations, and more than 16,000 additional emergency room visits during a two week period at an estimated cost of approximately $5.4 billion (16). Further, nearly 50,000 Europeans died because of heat stress in 2003 and in 2015 approximately 1,800 Indians and 1,200 Pakistani died during a heat wave. In addition to human health concerns, heat stress also negatively affects animal production and welfare. For example, the U.S. swine industry loses approximately $1 billion annually due to heat stress (1, 43). While there are numerous factors contributing to these economic losses, a significant component results from a reduction in efficient growth and a failure to maximize lean tissue accretion (2). Despite the broad negative impacts of heat stress the cellular and molecular changes induced by heat stress in skeletal muscle are not well characterized. This mechanistic understanding is a prerequisite for developing effective countermeasures and therapeutic interventions in both animal agriculture and human medicine.

The detrimental effects of heat stress are in stark contrast to acute exposures to heat, termed therapeutic hyperthermia. Indeed, 30 minutes of heat exposure has been shown to decrease muscle atrophy (26, 40), augment regrowth (41, 47), and maintain insulin sensitivity in skeletal muscle (10, 11). Moreover, studies intending to model heat stroke, generally include heat exposure of less than two hours and components of exercise and have distinct outcomes different from prolonged heat exposure (4, 29, 53). Inflammation appears to be a central

component in these studies where changes in circulating cytokines as well as alterations in skeletal muscle indicate interlukin-6 (IL-6) is a key factor in this response (51). In addition, changes appear to be due to activator protein 1 (AP-1) but not nuclear factor kappa-B (NF-kB) signaling (44, 50, 51).

Heat stress is unique in that exposure to heat is longer than two hours and does not include a deliberate exercise component. Under these conditions 12 hours of heat stress increased circulating endotoxin (i.e. lipopolysaccharide, LPS) and decreased circulating tumor necrosis factor alpha (TNFα) in pigs (34). Findings of increased circulating endotoxin were confirmed following longer term heat stress in multiple species (8, 17, 31). Interestingly, intramuscular TNFα protein abundance was increased following 24 hours of heat stress raising the possibility of TNFα migration from the vasculature into tissues (25). Increased oxidative stress was also detected in these muscles (25). Increased circulating endotoxin, intramuscular TNFα, or oxidative stress would ostensibly initiate an inflammatory response via NF-κB (24). Additionally, the pro-inflammatory c-Fos/c-Jun heterodimer, AP-1, has been implicated in during heat stroke in skeletal muscle (49). Despite this compelling rationale to expect increased inflammatory signaling following heat stress, we were previously unable to detect increased inflammatory signaling in intestinal tissue (32) and in oxidative or glycolytic skeletal muscle following 24 or 72 hours of heat stress (25). Our previous findings of increased circulating endotoxin following 12 hours of heat stress (34) serves to provide a plausible mechanism triggering this inflammatory response. The purpose of this investigation was to clarify the role of inflammatory signaling in heat-stressed skeletal muscle with a focus on short-term heat stress. We hypothesized that short-duration heat stress would induce inflammatory signaling in oxidative and glycolytic skeletal muscle.

Materials and Methods

Animal treatments. All procedures were reviewed and approved by the Iowa State University Institutional Animal Care and Use Committee. A detailed approach and data from these animals have been previously published (3, 30, 34). Briefly, 24 individually penned crossbred gilts (65 ± 3 kg body weight (BW), mean ± SE) were selected by BW and were allocated to one of three treatments: (1) thermal neutral *ad-libitum* (TN; 24° C, 40% humidity, n=8); (2) heat stress *ad libitum* conditions (HS; 37° C, 40% humidity, n=8); or (3) pair-fed thermal neutral (PFTN; feed intake matched to heat stress counterparts and reared in thermal neutral conditions, n=8). The pair-fed thermal neutral group is an energetic control and was included because an immediate effect of heat stress is reduced feed intake (36). Therefore, to differentiate between direct and indirect effects of heat stress, we used both thermal neutral (*ad libitum* access to food) and pair-fed thermal neutral (reduced access to food) models to eliminate the potentially confounding effects of different feed intake. All pigs had *ad libitum* access to water and were fed the same corn and soybean based diet throughout the study period. Animals were monitored and rectal temperature, respiratory rate (breaths per minute) and feed intake recorded for every 2 hours throughout the study period. Body weight was recorded at the beginning and end of the treatment. Following a 12 hour application of the environmental heat treatment, gilts were sacrificed using barbiturate overdose (Fatal Plus dosed at 1 mL/4.5 kg body weight). The semitendinosus was collected and divided into red (STR) and white (STW) portions. Muscle samples were frozen in liquid nitrogen for further analyses.

QRT-PCR. STR and STW were powdered on dry ice. For mRNA extraction, 50 mg of muscle (n=8/group) was homogenized in Trizol (Invitrogen, Carlsbad, CA). Insoluble materials were removed from the homogenate by centrifugation at 1,500 x g for 10 minutes at 4° C.

Supernatant was mixed with 1 volume of ethanol and applied to a Direct-zol Mini Prep column (Zymo, Irvine, CA) then treated with DNase to prevent DNA contamination. RNA concentration was determined using an ND-1000 Spectrophotometer (λ = 260/280nm; NanoDrop Technologies, Inc., Wilmington, DE). Synthesis of 1 μg of RNA to cDNA was carried out using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). To assess transcript expression, we used Fluidigm protocol B (Fast Gene Expression for Analysis Using EvaGreen) and Eppendorf PCR Master Cycler (Quantitect SYBR Green PCR kit: Qiagen) for subsequent measures targeting cytokine expression. For the Fluidigm protocol, a pool of all primers (Table 1) and PerfeCTa master mix (Quanta Biosciences, Gaithersburg, MD) were used to pre-amplify 62.5 ng of cDNA from each sample. Pre-amplified samples were treated with Exonuclease 1 (New England BioLabs, Ipswich, MA) and diluted 1:10 using EvaGreen supermix (Bio-Rad, Hercules, CA) and DNA Binding Dye (Fluidigm, South San Francisco, CA). Forward and reverse primers were prepared using loading agent (Fluidigm, South San Francisco, CA). Once Dynamic Array 48.48 IFC (Fluidigm) was primed, samples and primers were loaded using IFC controller MX (Fluidigm). After loading, the array was transferred to the BioMark HD (Fluidigm) and run using a GE 48x48 PCR+Melt plate v2.pcl program. For the Eppendorf PCR master cycler, we synthesized new primers for cytokines and used the following program: 15 minutes hold at 95^{o} C and 45 cycles of denaturing at 95^{o} C for 15 seconds, annealing at 58^{o} C for 15 seconds, and extension at 72^{o} C for 20 seconds at which point data were acquired. Data were analyzed using the $\Delta\Delta$CT method with 18S as our reference gene ($CT_{sample} - CT_{18S} = \Delta CT$; $\Delta CT_{sample} - \Delta CT_{highest\ \Delta CT\ value} = \Delta\Delta CT$). All statistical analyses were performed on the ΔCT values and data are reported as fold change ($2^{\Delta\Delta CT}$).

Immunochemistry. Fifty milligrams of STR and STW muscle (n=8/group) were powdered on dry ice and homogenized in 1.5 mL of protein extraction buffer (10 mM sodium phosphate, pH 7.0, and 2% SDS). To obtain the whole homogenate the samples were then centrifuged at 1,000 x g for 15 minutes at 4° C and the supernatant collected. Protein concentration was measured using a BCA Microplate Protein Assay Kit (Pierce, Rockford, IL) according to manufacturer's instructions.

Nuclear fraction protein was isolated using a NE-PER Nuclear and Cytoplasmic Extraction Kit according to manufacturer's instructions (Thermo scientific, Rockford, IL). Briefly, 20 mg of STR muscle (n=8/group) was powdered on dry ice and homogenized with 200 µl of cytoplasmic extraction reagent I (CERI). Pellets were then harvested using 11 µl of CERII by centrifugation and suspended in 100 µl of Nuclear Extraction Reagent (NER). Samples were vortexed for 15 seconds every 10 minutes, for a total of 40 minutes and then centrifuged at 1,500 x g for 10 minutes. The resultant supernatant contained the protein found in the nuclear fraction and the protein concentration was measured using a BCA kit.

Homogenates were diluted to 4 mg/ml in loading buffer (62.5 mM Tris, pH 6.8, 1.0% SDS, 0.01% bromophenol blue, 15.0% glycerol, and 5% β-mercaptoethanol). 40 µg of protein were loaded into each well in 4-20% precast gradient gel (Bio-Rad, Hercules, CA) and proteins were separated at room temperature for 10 minutes at 60 V followed by 60 minutes at 120 V. Afterward, proteins were transferred (60 minutes, 100 V) to a nitrocellulose membrane (Bio-Rad, Hercules, CA) with a pore diameter of 0.2 µm. Membranes were blocked for 1 hour in 5% milk in Tris-buffered saline containing 0.2% Tween 20 (TTBS). Membranes with protein from whole homogenate were incubated with each of the following: HSP72 (primary 1:1000, cat. no NB120-2788, Novus Biological, Littleton, CO; secondary 1:5000), HSP27 (primary 1:1000, cat.

no ADI-SPA-800, ENZO life sciences, Farmingdale, NY; secondary 1:2000), pHSP27 (primary 1:1000, cat. no 2406, Cell Signaling Technology, Danvers, MA; secondary 1:1500), HSP90 (primary 1:1000, cat. no 4877, Cell Signaling Technology; secondary 1:1500), TNFα (primary 1:400, cat. no ab6671, Abcam, Cambridge, MA; secondary 1:5000), NF-κB (primary 1:5000, cat. no ab7970, Abcam, secondary 1:5000), phospho-NF-κB p65 (primary 1:1000, cat. no MA5-15160, Thermo Scientific, Rockford, IL; secondary 1:2000), IKBα (primary 1:1000, cat. no SC-371, Santa Cruz Biotechnology, Dallas, TX; secondary 1:2000), IKKα (primary 1:1000, cat. no SAB4500258, Sigma Aldrich, St Louis, MO; secondary 1:5000), SAPK/JNK (primary 1:1000, cat. no 9252s, Cell Signaling Technology, secondary 1:2000) or phospho-SAPK/JNK (primary 1:1000, cat. no 4668s, Cell Signaling Technology; secondary 1:2000), IL-1β (primary 1:1000, cat. no 12703, Cell Signaling Technology; secondary 1:1000), IL-2 (primary 1:1000, cat. no 12703, Cell Signaling Technology; secondary 1:1000), IL-6 (primary 1:1000, cat. no ab6672, Abcam; secondary 1:2000) overnight at 4^{o} C. Membranes with protein from the nuclear fraction were incubated with antibodies to NF-κB (primary 1:5000, cat. no ab7970, Abcam; secondary 1:5000), phospho-NF-κB p65 (primary 1:1000, cat. no 3033, Cell Signaling Technology; secondary 1:2000), AP-1 (primary 1:1000, cat. no A5968, Sigma Aldrich; secondary 1:3000), HSF1 (primary 1:1000, cat. no 4356; Cell Signaling Technology; secondary 1:3000) or phospho-HSF1 (primary 1:5000; cat. no ab76076, Abcam; secondary 1:3000) overnight at 4^{o} C.

Following three washes in TTBS, membranes were incubated with species-specific secondary antibodies (in dilutions as mentioned above, Anti-rabbit: cat. no 7074, Anti-mouse: cat. no 7076, Cell Signaling Technology) for 1 hour at room temperature. Membranes were washed 3 times in TTBS and incubated in enhanced chemiluminescence detection substrate (ECL Plus, GE Healthcare Bio-Sciences, Pittsburgh, PA) for 5 minutes and then membranes

were exposed to X-ray film. Densitometry of the appropriate bands was performed using Carestream 5.0 molecular imaging software (Carestream Health, New Haven, CT). Optical density was normalized to TN values. To confirm equal loading all membranes were stained with Ponceau S and the resultant images captured and lane optical densities quantified. In all instances resultant Ponceau S signal was similar between groups.

Statistics. To determine the effect of heat stress, data from thermal neutral, heat stress and pair-fed thermal neutral animals were compared using One-way ANOVA followed by Newman-Keuls post hoc test (GraphPad Prism, version 5.04). Statistical significance was set at $p<0.05$. Values are reported as mean ± SEM unless otherwise noted.

Results

We have previously reported the phenotype of 12 hour heat stress pigs and have published successful application of a heat load and heating curve (34). The rectal temperatures in thermal neutral and pair-fed thermal neutral were 39.2 ± 0.12° C and 39.1 ± 0.12° C, respectively, and in heat stressed pigs were 41.5 ± 0.13° C ($p<0.05$) (34). In addition, respiratory rate in thermal neutral and pair-fed thermal neutral were 42.18 ± 7.16 breaths/minute and 40.25 ± 6.85 breaths/minute respectively, and in heat stress was 142.24 ± 6.94 breaths/minute ($p<0.05$) (34). Over the course of the 12 hour treatment period heat stress pigs consumed 0.11 ± 0.03 kg feed and pair-fed thermal neutral pigs consumed 0.18 ± 0.04 kg feed while thermal neutral pigs consumed 1.00 ± 0.14 kg feed ($p<0.05$). Hematocrit and hemoglobin were within normal limits following 12 hours of heat stress indicating animals were similarly hydrated (34).

To determine the extent to which heat stress led to a heat shock response we measured protein abundance of heat shock protein (HSP) 72, 27 and 90. In oxidative muscle, we could not detect differences in the relative abundance of HSP72, HSP27, phosphorylated HSP27 and HSP90 compared to thermal neutral and pair-fed thermal neutral animals (Figure 1A). Heat shock factor (HSF) plays an important role in the regulation of HSP abundance, thus we measured heat shock factor (HSF) 1 in the nuclear fraction and found that relative abundance of HSF1 and phospho-HSF1 protein abundance was similar between groups (Figure 1B).

Next, we measured markers of NF-κB and AP-1 pathway activation. Relative abundance of these measures was similar between both thermal neutral groups for all measures despite differences in feed intake. In oxidative muscle, relative protein abundance of NF-κB, phosphorylated NF-κB, and the NF-κB inhibitor, IKBα were similar between groups. However, in heat stressed animals the NF-κB activator IKKα was increased by 159% compared to thermal

neutral pigs and 118% compared to pair-fed thermal neutral pigs (Figure 2A). Because there is previous evidence of increased AP-1-mediated inflammatory signaling during heat stroke (21, 49), we also measured abundance of the upstream activator of AP-1, MAPK c-Jun kinase (JNK), and discovered that relative protein abundance of total and phosphorylated JNK were similar between groups (Figure 2B).

To further investigate the potential for inflammatory signaling in heat-stressed skeletal muscle, we isolated nuclear fractions so that the abundance of transcription factors within the nuclei could be quantified. Heat stress increased nuclear NF-κB by 60 and 74% compared to thermal neutral and pair-fed thermal neutral, respectively ($p<0.05$). In addition, phosphorylated NF-κB also increased by 140 and 62% compared to thermal neutral and pair-fed thermal neutral, respectively ($p<0.05$; Figure 3). However, nuclear abundance of AP-1 was similar between groups (Figure 3).

To more thoroughly explore the role of NF-κB and AP-1-altered downstream gene regulation, we measured expression of transcripts driven by NF-κB or by AP-1 signaling via QRT-PCR (n=8/group). While there were few differences in transcript expression between thermal neutral and heat stress in oxidative muscle, 80% ($p<0.05$) of transcripts driven by NF-κB (90% at $p<0.1$; Table 2) and 56% ($p<0.05$) of transcripts driven by AP-1 (81% at $p<0.1$; Table 3) were increased in heat stressed pigs compared to the pair-fed thermal neutral pigs. Of interest, restricted food intake caused a broad numerical reduction in the expression of nearly all transcripts measured (thermal neutral vs pair-fed thermal neutral), however, these generally failed to reach statistical significance (Tables 2 and 3).

Since increased circulating endotoxin was previously found in these animals (34), we also measured transcript expression and relative protein abundance of cytokines in muscle. Heat

stress increased transcript expression of IL-1β by 520% ($p<0.05$) compared to thermal neutral and by 127% ($p<0.05$) compared to pair-fed thermal neutral. IL-15 was increased by 332% ($p<0.05$) compared to thermal neutral and 57% ($p<0.05$) compared to pair-fed thermal neutral. Heat stress decreased TNFα by 72% ($p<0.05$) compared to thermal neutral and 105% ($p<0.05$) compared to pair-fed thermal neutral, IL-2 ($p<0.05$) by 99% compared to thermal neutral, IL-10 by 109% ($p<0.05$) compared to pair-fed thermal neutral, and IL-6 by 84% ($p<0.05$) compared to thermal neutral and 86% ($p<0.05$) compared to pair-fed thermal neutral (Figure 4A). Despite reduction in transcript expression, relative protein abundance of TNFα was increased 80% ($p<0.05$) by heat stress compared to thermal neutral and by 96% compared to pair-fed thermal neutral. Consistent with transcript changes heat stress decreased ($p<0.05$) IL-6 protein abundance by 40% compared to pair-fed thermal neutral. Relative protein abundance of IL-1β and IL-2 were similar between groups (Figure 4B).

In glycolytic muscle, relative protein abundance of all measures was similar between groups (Figure 5). Transcript expression of NF-κB and AP-1-driven genes was largely unchanged by heat stress or reduced food intake (Tables 2 and 3). However, *DDIT4L* and *FBXO32* mRNA expression was increased by heat stress compared to thermal neutral ($p<0.05$; Tables 2 and 3).

Discussion

Prolonged exposure to elevated heat loads can result in heat stress, which is detrimental to human (23) and animal health and performance (1, 43). Aside from cooling and rehydration, there is no other consistently applied protocol available to mitigate heat stress-mediated injuries (15). Our previous work demonstrated that heat stress increased oxidative stress but not inflammatory signaling in porcine skeletal muscle after one day of heat exposure (25). Given the strong rationale to suspect inflammatory signaling including increased free radical injury, TNFα, and circulating endotoxin, in this investigation we hypothesized that heat stress would increase inflammatory signaling in skeletal muscle. We found that 12 hours of heat stress increased NF-κB signaling but not AP-1 signaling. In addition, heat stress led to increased expression of transcripts driven by NF-κB and AP-1 signaling as well as inflammatory cytokines.

Increased circulating endotoxin (31) and intramuscular TNFα (25) could serve as triggers of an inflammatory response. Previously, we reported increased endotoxin and decreased TNFα in blood following 12 hours of heat stress (34). Here, we report increased intramuscular TNFα protein abundance with discordant transcript expression supporting our previous observations following 24 hours of heat stress (25, 32). These data support our previous speculation (25) that circulating TNFα may migrate from the vasculature into skeletal muscle. To determine the extent to which inflammatory signaling contributed to the altered intracellular environment, we measured NF-κB pathway activation. While NF-κB abundance in whole homogenate was similar between groups, relative abundance of IKKα was increased following 12 hours of heat stress. IKKα is essential for the phosphorylation of IkB and translocation of NF-κB to the nucleus (37, 46). In addition to increased nuclear NF-κB content we also discovered that expression of transcripts driven by NF-κB was increased. Of note, these changes were predominately found

between the pair-fed thermal neutral and the heat stress groups indicating that these are heat stress-induced changes independent from decreased food intake. Supporting this, numerical changes between thermal neutral and heat stress were of the same direction but smaller magnitude. Increased NF-κB signaling may be due to increased intestinal permeability as heat stress of greater than 6 hours results in leaky gut (33, 34) whereas heat stress for 2 and 4 hours was insufficient to increase gut permeability in pigs (33). The role of heat stress-mediated inflammatory signaling following 2-6 hours of heat stress exposure is unknown but is currently being explored by our group. Consistent with an interaction of intestinal permeability and NF-κB signaling, preliminary findings are supportive of NK-κB pathway activation at only the six hour time point in oxidative skeletal muscle (unpublished observations). When modeling heat stroke using a heating paradigm of approximately two hours, NF-kB signaling does not appear to contribute to muscle dysfunction however, AP-1 appears to play a major role (50).

Our findings of increased expression of AP-1 driven transcripts following 12 hours of heat stress was surprising as measures of AP-1 pathway signaling and nuclear abundance of AP-1 were similar between groups. It seems likely that elevated transcript expression is a remnant of previous pathway activation. Such a hypothesis is not without precedent as studies focused on heat stroke have found increased AP-1 activation in both soleus and diaphragm in mice (49).

Apart from inflammation, increased NF-κB and AP-1 -driven transcripts regulate a variety of cellular functions. Apoptosis signaling was increased by heat stress suggesting an interaction of heat stress and apoptosis in skeletal muscle. In support of this previous work demonstrated that heat stress causes apoptosis in umbilical vein endothelial cell (9) and in swine skeletal muscle satellite cells (7).

In addition to transcripts driven by either NF-κB or AP-1, we also specifically measured transcript expression and relative protein abundance of several cytokines. Consistent with our hypothesis of a pro-inflammatory environment IL-1β transcript expression was increased along with several inflammatory cytokines; however, these changes did not lead to increased protein abundance. Of particular interest was the reduction in IL-6 transcript and relative protein abundance following heat stress compared to thermal neutral animals. It has been previously established that during heat stroke IL-6 is exported from skeletal muscle (50, 51) and provides protection to other organ systems (12, 18-20, 27, 35). Indeed, failure to adequately maintain increased IL-6 production may be a key factor that distinguishes heat stroke from heat stress and may also help to explain the multi organ dysfunctions that occur during heat stress. Further, the reduction in IL-6 may be related to elevated TNFα, as TNFα is inversely correlated with IL-6 (39, 51).

These data provide an initial description of the chronology of heat stress-mediated inflammatory signaling in skeletal muscle. Together with our previous data it appears that inflammatory signaling is increased following 12 hours of heat stress then return to baseline, following 24 hours of heat stress. Elevated abundance of HSP would provide a simple means by which to inhibit inflammatory signaling (42), however, HSP72 and other HSPs (HSP27, pHSP27 and HSP90) were similar between groups. In a proteomics experiment making use of the same tissues as in this investigation we found a modest (~10%) HSP response including increased expression of mitochondrial HSP70, HSP27, HSP20 and α-B-crystallin (3). Several of these proteins can blunt AP-1 inflammatory signaling, among other functions (5, 6, 45). This raises the possibility of a differential capacity of HSPs to squelch inflammatory pathway activities such that AP-1 signaling is more sensitive to HSPs than is NF-κB.

The lack of heat stress-mediated changes in glycolytic (STW) muscle was striking, though is in agreement with our previous observations following 24 and 72 hours of heat stress (25). A proteomic assessment of these tissues, however, detected some modest heat stress-mediated alterations in protein abundance related primarily to HSP expression, metabolism, and antioxidants (3). Given the widespread changes seen in STR, but not STW, the mitochondria are implicated as central figures in heat stress-mediated cellular dysfunction. Further implicating mitochondria is the metabolic shift away from oxidative phosphorylation as pyruvate entry into the mitochondria appears blunted during heat stress (1, 3, 52). It is unclear if mitochondria are a proximal or distal cause of heat stress-mediated cellular dysfunction as mitochondrial malfunction would be expected following oxidative stress or loss of Ca^{2+} homeostasis, which have been reported or may be anticipated during heat stress (22, 25, 38).

Perspective and Significance

In summary, following 12 hours of heat stress, NF-κB signaling was enhanced in oxidative skeletal muscle as indicated by increased abundance of NF-κB activator IKKα, increased NF-κB abundance in the nucleus, increased expression of NF-κB driven transcripts and increased expression of inflammatory cytokines. Elevated expression of AP-1 driven transcripts is likely due to the previous activation of AP-1 signaling that has subsided or was effectively inhibited following 12 hours of heat stress. As we were unable to detect elevated AP-1 content in the nuclear fraction our expectation is that AP-1-mediated transcript expression will soon cease. Likewise, given our findings following 24 hours of heat stress we also expect that NF-κB signaling will also be inactivated. In conclusion, 12 hours heat stress caused inflammatory signaling in oxidative muscles. These data also confirm our previous results that oxidative muscle is more sensitive to heat stress than is glycolytic muscle.

Acknowledgements

This work was supported by USDA grants 2014-67015-21627 (JTS) and 2011-6700330007 (LHB). The authors thank Jermilia Charles, Shannon Cruzen, and Martin Curry for technical assistance.

Conflict of interest

The authors have no conflicts to declare.

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Table 1. Sequences of primer pairs used for QRT-PCR.

Target gene	Forward primer	Reverse primer
18S	aaacggctaccacatccaag	tcgcggaaggatttaaagtg
BNIP3	cgcacacagtgttggagaaa	tccctcctcctctccatgtaa
BTG2	ttttcagcggggctctcc	agcccttggatggcttttca
CAT	acaaagacctgcggaccaa	agcttcccgttgccatca
CST3	acaactgtcccttccccaac	ttccaggggacggtgtaaac
DDIT4L	agttgctagaccgtagcttcc	ttgggttcagggacaacgtaa
EIF4EBP1	tggagtgtcggaactcacct	atcacccacagggctggt
FBXO32	gaaggacatgctgaacagcaaa	agtacttcctttgtgaacatagatcca
FLOT1	gctatcatggcccacatgac	tctgaggaggccactttgaa
GHR	gagcccatttgcatgtgaa	caccgttagcccaaatattcc
GNB2L1	ttctcacccaacagcagcaa	gcttgcagttagccaagttcc
HBXIP	ttggagcagcacttggag	cccagattgagtccttgtgaa
HNRPDL	cggattgagcccagatacttca	ggctcttcgtctgtgtacgtaa
HSPA2	acgacaaaggtcgtctaagca	ggcctcatcttccgacttgta
HSPA9	acaggaacaccaccattcca	ctccacttgagtctgtccatca
IGFBP3	cagcgctacaaggtcgacta	gtctcgcgcttggactca
IL-1β	ccaaagagggacatggagaa	ttatatcttggcgcccttg
IL-2	tgcagctcttgtgttgcatt	agcatcctggagagatcagc
IL-6	aggaacccagctatgaactcc	agtagccatcaccagaagca
IL-8	gaaatcacaggtgcccagt	tgcaagttgaggcaagaaga
IL-10	tgtgccctatggtgttcaac	ctttgtcacactccggaagc
IL-15	cagaagcaacctggcagcacg	acgcgtaactccacgagaaagca
LIPE	ctggatgtgcacttctggaaa	gccgatgccatgtttgcta
MCT4	gtgtgtgtgaatcgctttgg	gaccccagtggtgaggtaga
PHB2	agtgtggtggccaagttca	gtcagctcccttcggatcaa
RAB3D	agcgagttcgaaggcagaa	tggagggtgggctggaa
RNF4	gccttggaggcagaacctata	accacgggctctaaagattca
SOD1	atggtgggccaaaggatca	gatgtacacagtggccacac
SP1	agaggcataaacgcacacac	atgctttgacaggtggtcac
TET2	gcctcagcacgtacaaaaca	ctggtcctgaaagtcgcaaaa
TNFα	ctggccccttgagcatca	gggcttatctgaggtttgagac
TPX2	gaaggcacaaacttggaagca	ttggacgagccttgaagca

Table 2. Relative abundance of NF-κB regulated transcript expressions in STR and STW. The transcript expressions were measured using QRT-PCR with fluidigm dynamic array for NF-κB driven transcript in STR and STW. Values are fold change ± SE; n=8/group. * indicates significantly different from TN ($p<0.05$); # indicates significantly different from HS ($p<0.05$). Gene function is based on the Gene cards ®. STR - semitendinosus red, STW - semitendinosus white, TN - thermal neutral, HS - heat stress, PFTN - pair fed thermal neutral.

Gene Name	Description	Function	STR			STW		
			TN	HS	PFTN	TN	HS	PFTN
BTG2	B-cell translocation gene 2	Anti-proliferative	1.00 +/- 0.31	2.00 +/- 0.87	0.49 +/- 0.18 #	1.00 +/- 0.31	1.24 +/- 0.58	1.04 +/- 0.42
CAT	Catalase	Antioxidant	1.00 +/- 0.36	1.60 +/- 0.47	0.55 +/- 0.28	1.00 +/- 0.25	0.62 +/- 0.20	0.77 +/- 0.18
EIF4EBP1	Eukaryotic translation initiation factor 4E binding protein 1	Translation repression	1.00 +/- 0.26	2.21 +/- 0.54	0.50 +/- 0.18 #	1.00 +/- 0.22	0.84 +/- 0.21	0.97 +/- 0.19
GNB2L1	Guanine Nucleotide Binding Protein (G Protein), Beta Polypeptide 2-Like	Translational repression	1.00 +/- 0.25	1.85 +/- 0.37	0.53 +/- 0.23 #	1.00 +/- 0.24	0.70 +/- 0.19	0.85 +/- 0.17

PHB2	Prohibitin 2	Transcriptional repression	1.00 +/- 0.31	2.07 +/- 0.47	0.53 +/- 0.21 #	1.00 +/- 0.25	0.66 +/- 0.19	0.99 +/- 0.14
IGFBP3	Insulin-like growth factor-binding protein 3	Insulin signaling	1.00 +/- 0.33	2.78 +/- 0.57*	0.44 +/- 0.12 #	1.00 +/- 0.25	1.02 +/- 0.28	1.02 +/- 0.20
LIPE	Lipase, Hormone-Sensitive	Converts cholesteryl esters to free cholesterol	1.00 +/- 0.30	3.30 +/- 1.09	0.63 +/- 0.22 #	1.00 +/- 0.22	0.91 +/- 0.40	1.13 +/- 0.45
RNF4	E3 Ubiquitin Ligase	Mobilize the stored fats; Protein modification; protein ubiquitination	1.00 +/- 0.26	1.98 +/- 0.50	0.66 +/- 0.24 #	1.00 +/- 0.22	0.84 +/- 0.23	1.11 +/- 0.19
TPX2	Targeting protein for Xklp2	Spindle assembly factor	1.00 +/- 0.31	1.98 +/- 0.38	0.67 +/- 0.33 #	1.00 +/- 0.24	0.83 +/- 0.23	1.10 +/- 0.29

Table 3. Relative abundance of AP-1 regulated transcript expressions in STR and in STW. The transcript expressions were measured using QRT-PCR with fluidigm dynamic array for AP-1 related transcripts in STR and STW muscle. Values are fold change ± SE; n=8/group. * indicates significantly different from TN ($p<0.05$); # indicates significantly different from HS ($p<0.05$. Gene function is based on the Gene cards ®. STR - semitendinosus red, STW - semitendinosus white, TN - thermal neutral, HS - heat stress, PFTN - pair fed thermal neutral.

Gene Names	Description	Function	STR			STW		
			TN	HS	PFTN	TN	HS	PFTN
BNIP3	BCL2/Adenovirus E1B 19kD-Interacting Protein 3	Apoptosis	1.00 +/- 0.20	2.27 +/- 0.66	0.48 +/- 0.13#	1.00 +/- 0.32	1.14 +/- 0.50	1.24 +/- 0.35
DDIT4L	DNA Damage-inducible Transcript 4-like protein	Inhibits cell growth	1.00 +/- 0.37	5.45 +/- 1.72	0.49 +/- 0.24#	1.00 +/- 0.24	1.96 +/- 0.23*	1.31 +/- 0.17
FBXO32	F-box only protein 32	Muscle atrophy, Proteasomal degradation	1.00 +/- 0.81	6.84 +/- 2.43	0.58 +/- 0.38#	1.00 +/- 0.46	2.99 +/- 0.75*	1.53 +/- 0.48
FLOT1	Flotillin 1	Scaffolding protein	1.00 +/- 0.32	1.95 +/- 0.82	0.46 +/- 0.18	1.00 +/- 0.34	1.05 +/- 0.30	1.09 +/- 0.31
GHR	Growth Hormone Receptor	Modulator/inhibitor of GH signaling	1.00 +/- 0.33	2.50 +/- 0.51	1.51 +/- 0.24#	1.00 +/- 0.28	0.80 +/- 0.22	0.84+/- 0.18

CST3	Cystatin C	Proteinase inhibitor	1.00 +/- 0.25	1.52 +/- 0.30	0.56 +/- 0.20#	1.00 +/- 0.33	0.52 +/- 0.11	0.67 +/- 0.11
HBXIP	Hepatitis B virus X-interacting protein	Cell proliferation	1.00 +/- 0.26	1.78 +/- 0.33	0.51 +/- 0.21#	1.00 +/- 0.19	0.67 +/- 0.19	0.93 +/- 0.11
MCT4	Proton-linked monocarboxylate transporter	Cell proliferation	1.00 +/- 0.06	0.82 +/- 0.25	0.41 +/- 0.10*	1.00 +/- 0.35	0.73 +/- 0.32	0.83 +/- 0.20
HNRPDL	Heterogeneous Nuclear Ribonucleoprotein D-Like	Transcriptional regulator	1.00 +/- 0.36	1.35 +/- 0.26	0.52 +/- 0.23	1.00 +/- 0.23	0.69 +/- 0.16	1.01 +/- 0.07
HSPA2	Heat Shock 70kDa Protein 2	Molecular chaperone	1.00 +/- 0.26	2.04 +/- 0.90	0.62 +/- 0.30	1.00 +/- 0.31	0.72 +/- 0.20	0.74 +/- 0.16
HSPA9	Heat Shock 70kDa Protein 9	Molecular chaperone	1.00 +/- 0.24	2.50 +/- 0.49	0.58 +/- 0.22#	1.00 +/- 0.25	0.87 +/- 0.21	1.04 +/- 0.14
RAB3D	Ras-related protein Rab-3D	Protein transport	1.00 +/- 0.25	7.13 +/- 5.78	0.64 +/- 0.21	1.00 +/- 0.19	0.80 +/- 0.26	0.74 +/- 0.10
SOD1	Superoxide Dismutase 1	Antioxidant	1.00 +/- 0.25	1.88 +/- 0.28	0.57 +/- 0.22#	1.00 +/- 0.23	0.99 +/- 0.23	1.00 +/- 0.11
SP1	Sp1 Transcription Factor	Transcription factor	1.00 +/- 0.27	2.12 +/- 0.65	0.60 +/- 0.18	1.00 +/- 0.21	0.99 +/- 0.21	1.18 +/- 0.12
TET2	Tet Methylcytosine Dioxygenase 2	DNA demethylation	1.00 +/- 0.29	1.92 +/- 0.48	0.53 +/- 0.19#	1.00 +/- 0.16	0.81 +/- 0.26	0.83 +/- 0.17

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Figure Legends

Figure 1. Effect of heat stress on heat shock responses in STR. Following 12 hours of heat stress, HSP72, HSP27, pHSP27, HSP90 protein abundance in whole homogenate (A) and HSF1 or phosphorylated HSF1 protein abundance in nuclear fraction (B) were measured by Western blot. Representative blots are included. Ponceau S stain (PS) was used as a loading control. Values are mean ± SE; n=8/group. STR - semitendinosus red, TN - thermal neutral, HS - heat stress, PFTN - pair fed thermal neutral.

Figure 2. Effect of heat stress on inflammatory signaling in STR whole homogenate. Following 12 hours of heat stress, TNFα, NF-κB pathway (A) and JNK (B) pathway protein expression for STR were measured by Western blot in in whole homogenate. Representative blots are included. Ponceau S stain (PS) was used as a loading control. Values are mean ± SE; n=8/group. * indicates significantly different from TN; # indicates significantly different from HS. STR - semitendinosus red, TN - thermal neutral, HS - heat stress, PFTN - pair fed thermal neutral.

Figure 3. The relative abundance of transcription factors in STR nuclear fraction. In the STR transcription factor for NF-κB, and AP-1 pathway protein abundance were measured by Western blot in the nuclear fraction. Sample blots are included. Ponceau S stain (PS) was used as a loading control. Values are mean ± SE; n=8/group. * indicates significantly different from TN; # indicates significantly different from HS. STR - semitendinosus red, TN - thermal neutral, HS - heat stress, PFTN - pair fed thermal neutral.

Figure 4. Effect of heat stress on inflammatory cytokines in STR. Following 12 hours of heat stress, transcripts expression for inflammatory cytokines was measured by QRT-PCR in STR (A). Values are fold change ± SE; n=8/group. Relative protein abundance was measured by

Western blot in whole homogenate (B). Sample blots are also included. Ponceau S Stain (PS) was used as a loading control. Values are mean ± SE; n=8/group. * indicates significantly different from TN; # indicates significantly different from HS. STR - semitendinosus red, TN - thermal neutral, HS - heat stress, PFTN - pair fed thermal neutral.

Figure 5. Effect of heat stress on inflammatory signaling in STW whole homogenate. Relative protein abundance was measured by Western blot in whole homogenate. Sample blots are also included. Ponceau S Stain (PS) was used as a loading control. Values are mean ± SE; n=8/group. STW - semitendinosus white, TN - thermal neutral, HS - heat stress, PFTN - pair fed thermal neutral.

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Figure 1. Effect of heat stress on heat shock responses in STR

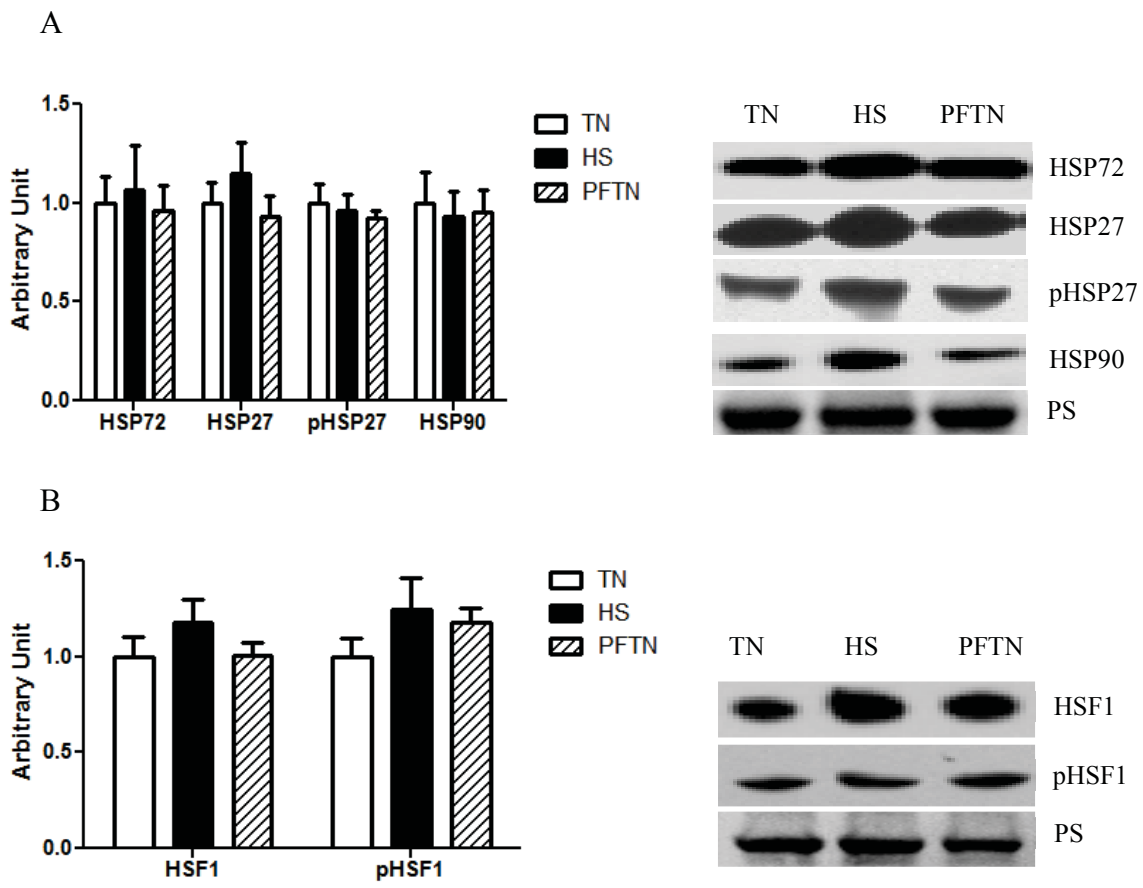

Figure 2. Effect of heat stress on inflammatory signaling in STR whole homogenate

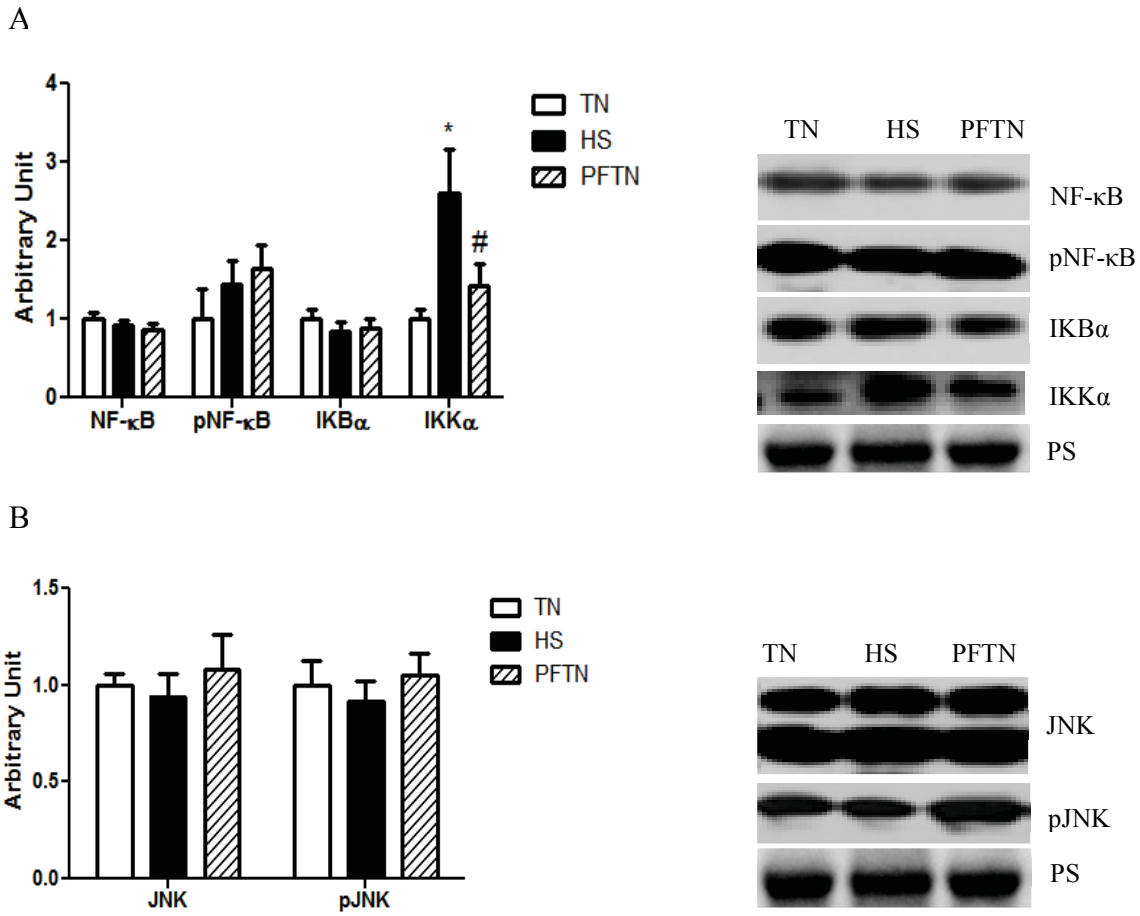

Figure 3. The relative abundance of transcription factors in STR nuclear fraction

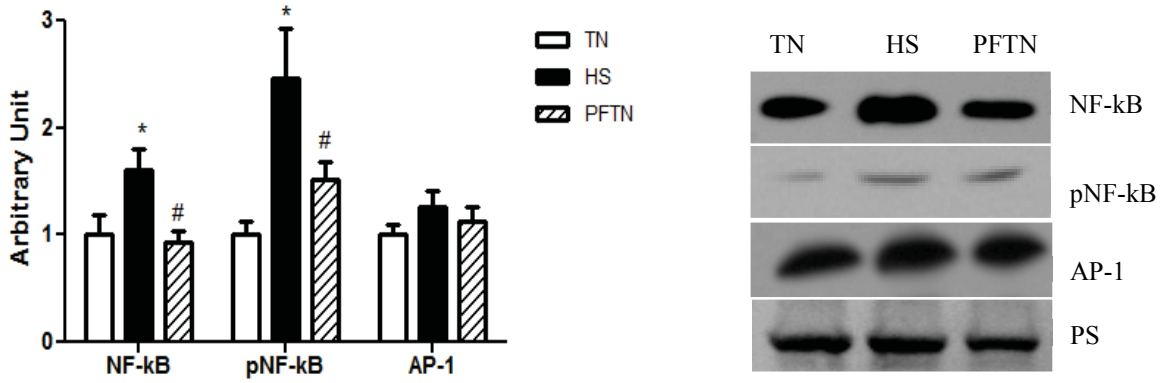

Figure 4. Effect of heat stress on inflammatory cytokines in STR

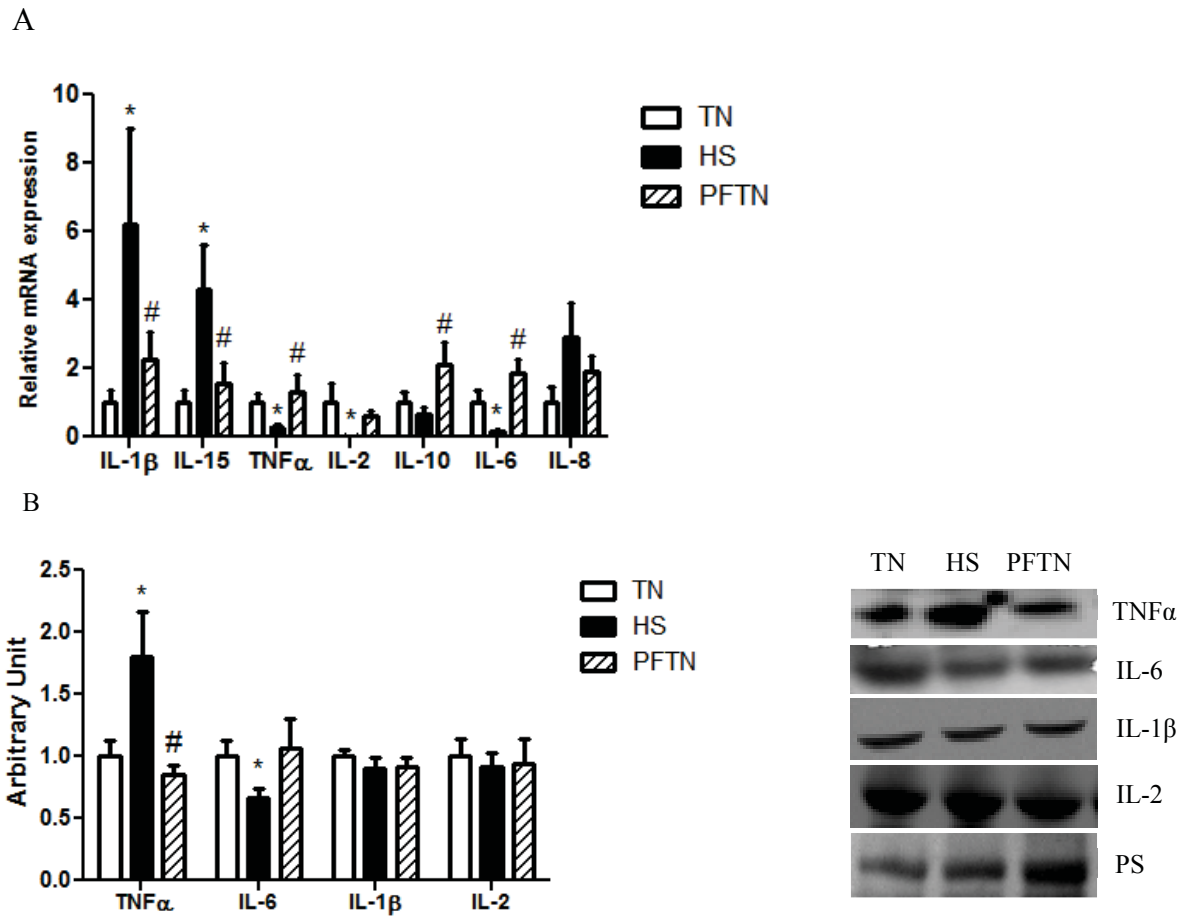

Figure 5. Effect of heat stress on inflammatory signaling in STW whole homogenate