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TITLE: Prevention of Organ Injury in Exertional Heat Stroke: Preclinical Evaluation of a New Class of NSAIDs

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14. ABSTRACT

In the first year we have completed a study of >128 adult male and female mice. Each mouse was exercise trained for a period of 3 weeks and then exposed to exertional heat stroke (EHS), running on a forced running wheel in a 37.5°C/35% RHG environmental chamber. Mice ran until they became unconscious (core temperature of 42.2°C in both sexes), neurological symptoms and organ injury resembling human EHS. Blood and tissue samples were collected at 0.5 h, 3 h, 24 h 4d,9d and 14d of recovery. Heart tissue and plasma were submitted for metabolomic and lipidomic analysis (awaiting results). Blood samples were submitted for cytokine analyses, metabolic hormone analyses and corticosteroids. Female mice were significantly more resistant to EHS, running longer, at higher running velocities and greater heat loads. Female mice had significantly higher levels of corticosterone (>2 fold) and greater levels of metabolic hormones associated with adipose tissue. Analyses of metabolic hormones and histology in both sexes suggest transient injury or "stunning" to the pancreas.

15. SUBJECT TERMS
Sex differences, exertional heat stroke, multi-organ injury, heat stress, metabolic hormones

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1. INTRODUCTION

Exertional heat stroke (EHS) is a serious medical problem in the U.S. Armed Forces, both during basic training and deployment operations. In the 2016 Medical Military Surveillance Report [23(3)], there were 417 cases of heat stroke (largely EHS) and 2,350 cases of heat injury reported in the previous year. The rate of heat injury in active component members was 0.35/100 person years in males and 0.16/100 in females. The incidence rate of heat injury in males, however, were nearly identical. The reasons for these sex differences are not known. The Military needs solutions to determine when warfighters are fit to return to duty without further risk of EHS or other complications and whether there are long term consequences of EHS that can be identified and treated. We have developed the first relevant preclinical EHS model in mice that resembles the condition in humans. It is our aim to utilize this model to solve a series of problems related to EHS, to identify biomarkers that will translate to the conditions experienced by Warfighters, to evaluate the influence of common drugs and agents that may amplify the deleterious effects of EHS, and to develop treatment and prevention strategies that are applicable to the needs of military medicine. Ultimately our goal is to save lives and suffering of US Military personnel.

There are four basic purposes of this project 1) To identify relevant biomarkers that could be helpful to the US Military in identifying effective and complete recovery from exertional heat stroke and in identifying risk factors for long term complications of EHS. 2) Determine if there are significant differences in the response to EHS between males and females. 3) To determine if non-steroidal anti-inflammatory drugs (NSAIDs) impose additional risk factors for complications of EHS, and 4) To evaluate a new line NSAIDs that may offer a safe line of protection from organ injury in EHS.

2. KEYWORDS

Sex differences, exertional heat stroke, multi-organ injury, heat stress, metabolic hormones, non-steroidal anti-inflammatory drugs, biomarkers

3. ACCOMPLISHMENTS

What were the major goals of the project?

Year 1: 2 Months: Complete approval of IACUC protocols, coordinate the data collection schedule between 3 centers, set up of new equipment and attain approval of Environmental Risk Assessment.

6 Months: Study EHS in male mice: surgical implantation of transmitters, recovery, exercise training and collection of data from 56 mice exposed to EHS or exercise control. Mice will be studied in groups of 8, implanted 2 weeks apart.

2 Months: Submission of samples and analytical and morphological tests of organ and tissue injury, submission of samples for immunological studies, metabolic hormone studies, metabolomics and proteomics analyses and integration of data from 3 centers.

PROGRESS: All of year one goals have been completed except for final analysis of results.

Year 2: 6 Months: Study EHS in female mice: surgical implantation of transmitters, recovery, exercise training and collection of data from 56 female mice exposed to EHS or exercise control.

3 Months: Submission and analyses of samples for multiplex (Luminex) determination cytokines and metabolic hormones, development and testing of new assays for detection of targeted biomarkers from plasma and analyses of organ injury using histopathological analyses.

2 Months. Complete analysis and initial reports of metabolomics and proteomics, comparison of males and females and outcome of cytokine and metabolic hormone measurements.

PROGRESS: All samples planned for year one have been collected. We have submitted samples for metabolomic/lipidomic analyses and will continue to evaluate results as they come in.

Year 3: 4 Months: Completion of testing the impact of ibuprofen on organ injury in male and female mice during EHS in 48 mice. Submission of plasma samples for cytokine analyses and tissues for analysis of histopathological injury.

3 Months: Completion of testing for the impact of the predominant COX2 inhibitor, diclofenac vs. its H_2S-analog (ATB-337) on organ intestinal injury and damage to other organ systems following EHS in 32 male mice. Submission of plasma samples for cytokine analysis and multiple organ injury and for measurement of metabolomics and eicosanoid products.

3 Months: Completion of testing for the impact of the more predominant COX1 inhibitor, naproxen vs. its H_2S-analog (ATB-346) on organ intestinal injury and damage to other organ systems following EHS in 32 male mice. Submission of plasma samples for cytokine analysis and multiple organ injury and for measurement of metabolomics and eicosanoid products.

2 Months: Complete analysis of samples from mice, integrate data collection from the 3 laboratories and prepare final reports and manuscripts of experimental outcomes.

PROGRESS: These studies have not been started but are in the planning stages to begin on Jan 3, 2017

What was accomplished under these goals? In the past year we completed the entire cohort of male and female mice exposed to EHS and collected samples at 6 time points up to 14 days of recovery. We also collected samples from controls. All samples have been submitted or are currently being submitted for secondary analysis by our co-investigators at the USARIEM and USACEHR for metabolomics, lipidomics, metabolic hormone analyses. We are completing further analyses of targeted biomarkers and histological tissue analyses at UF, which are partially complete and ongoing.

Highlighted findings:

- Surprisingly, females exhibited a significant resistance to EHS compared to age-matched male mice (example, Fig 1), which parallel the observations see in humans in the field. Whether the underlying physiology is the same in humans and mice is not known, but we hope to identify features in the male and female mice that will allow us to move on to hypothesis testing in humans. Grouped data are shown in Fig. 2. Female mice

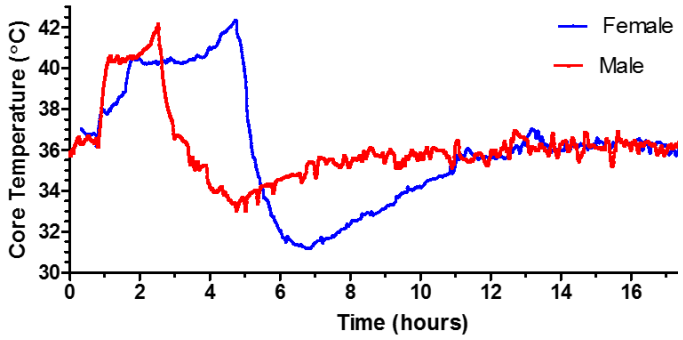


Fig. 1. Typical core temperature profiles of male (red and female mice during EHS trials.

ran nearly 50% longer in the heat than males (Fig. 2A), they endured a much greater heat load (2B), and lost a greater percentage of their body weight, (2C), suggesting that they experienced a greater heat stress. Interestingly, the average max core temp (Tc,max) achieved was almost identical between males and females, as was the minimum temp seen during the hypothermic recovery phase (2D).

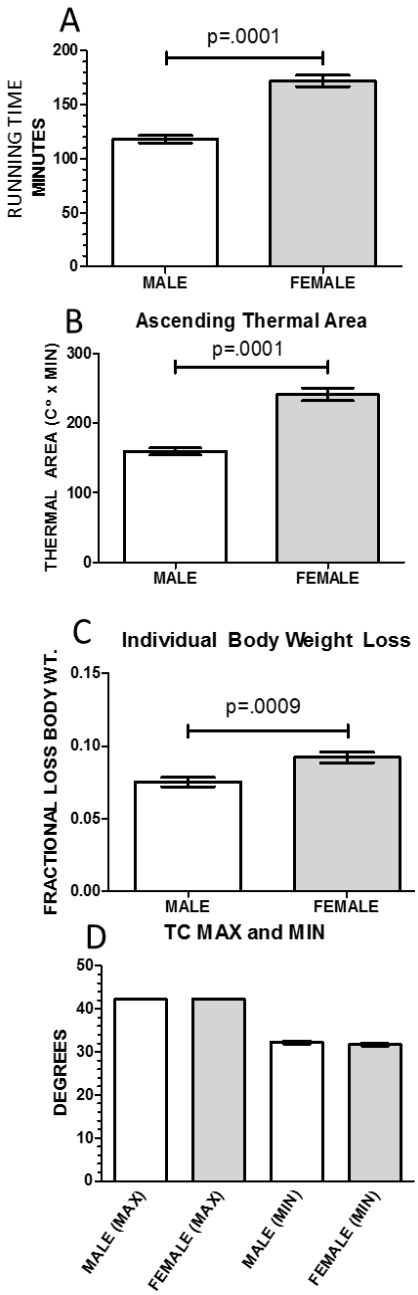


Fig. 2. Comparisons of ♂ & ♀mice of A) running time in the heat, B) thermal load, C) Fractional loss of body weight loss and D) max and min core temperatures

- We have identified a unique pattern of plasma cytokine responses to EHS that are contrastingly different than those seen in passive heat stroke models (data not shown, see appendix material). This study in males is the topic of a recently accepted publication in the Journal of Applied Physiology found in the Appendix.

- Based on preliminary histological evaluations, currently underway, we have observed transient pancreatic injury that appears evident at approximately 0.5-3 h but is largely resolved at the 24 h of recovery (Fig. 3, example).

- Analyses of metabolic hormones and blood glucose also suggest a hormonal dysfunction in the hours following EHS. These are characterized by reductions of pancreatic hormones c-peptide, insulin and glucagon (Fig. 4). The reductions in c-petpide and insulin are expected in the short term because of hypoglycemia, but did not recover completely by 24 h. Unexpectantly, there were no corresponding elevations in glucagon secretion (not shown) which is stimulated by hypoglycemia in a heathy pancreas. Therefore, we suspect pancreatic dysfunction due to ischemia, heat or possibly inflammation are limiting pancreatic responsiveness. Significant elevations in corticosterone were observed at 0.5 h, followed by suppression at 3 h. This is in contrast to passive heat stroke, as described by Leon et al in 2006, where it continues to rise up to the 3 h time point of recovery.

- Significant differences were observed in several metabolic hormones between female and males at the 3 h time point. resistin was elevated > 2 fold and corticosterone > 1.7 fold in females compared to males (not shown). Both of these hormones have been shown to have the capacity to improve heat tolerance in animal models and are candidates for further hypothesis testing.

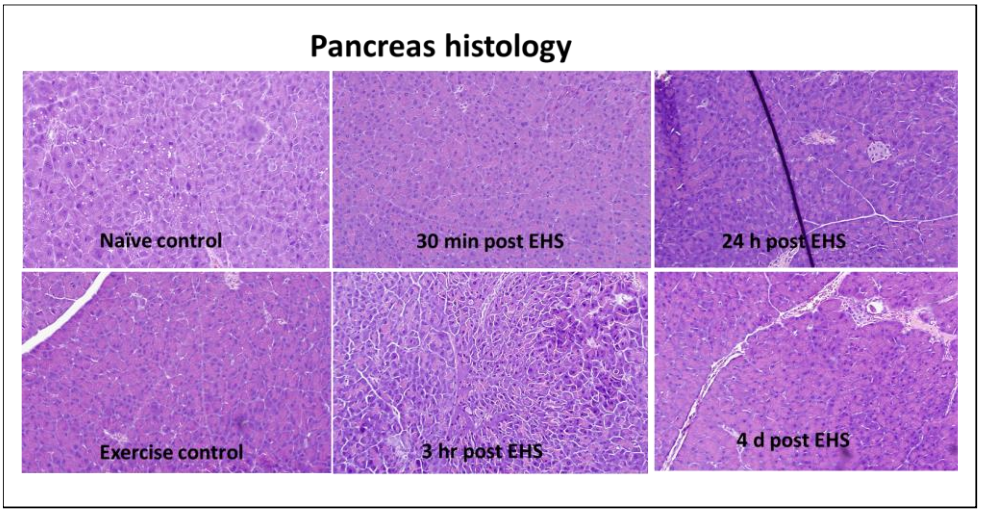


Fig. 3. Histology examples of pancreas from male mice during recover from EHS

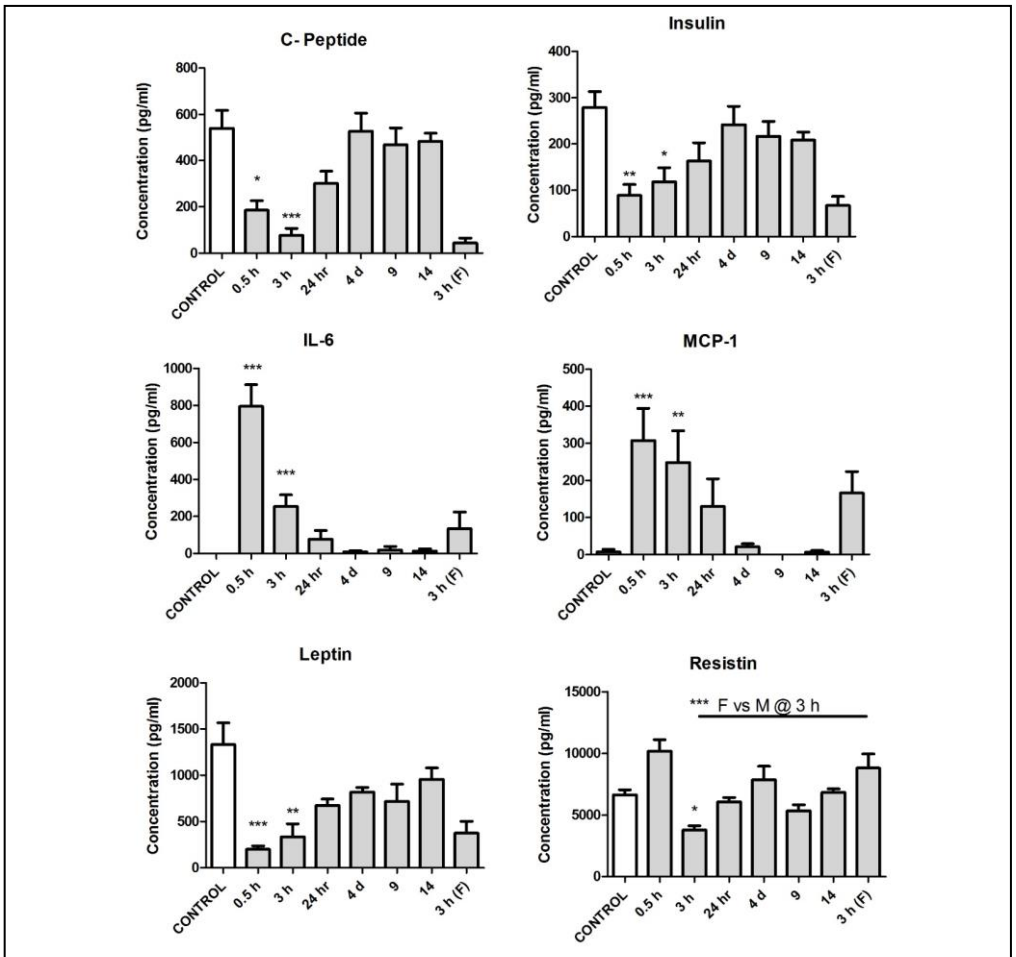


Fig. 4. metabolic hormone markers during recovery from heat stroke over 14 days. Female data at 3 h is represented by the last bar on the right.

- Mice exhibited hypoglycemia, as expected, during and within 3 h of EHS, but profound hyperglycemia at 24 h and beyond. We have some concern that hyperglycemia was evident up to 14 days, but also appeared in control mice under identical but non-thermal stress conditions, suggesting that this may be secondary effects of disruption of the sleep cycle and/or stress induced by transport of the cages to our facility overnight. Further studies are planned to determine if the hyperglycemia is present when the animals are left in the vivarium during recovery. This is a point of current evaluation.

- Significant muscle injury was seen in the soleus muscles that included apparent infiltration of inflammatory cells and centralization of the myonuclei (an indication of repair following injury). We have not seen evidence of blatant injury in the tibialis muscle (a fast fiber containing muscle) or in the diaphragm. We are now exploring responses of other limb muscles.

What opportunities for training and professional development did the project provide?

Because of this support we were able to provide training opportunities for Alex Mattingly MS, who was supported for part of the year on the project. We were also able to use this support to employ two MS students in our Department, Christian Garcia and Gerard Robinson. Both students, who are minority students, have been inspired to go on next year for their Ph.D. They both have first author abstracts for the Experimental Biology Meeting in Chicago of 2017. Finally, we provided training for our postdoc, Dr. Orlando Laitano. He has only been partially funded by this project but has not only provided the senior guidance in the lab but also developed a new line of research (funded by our endowment) which is looking at the molecular sources of rhabdomyolisis in heat, with and without coexisting hypertonic stress (relevant to heat stress and heat injury in the US Military). This is related to this study but not supported by this project.

How were the results disseminated to communities of interest?

- The P.I. presented the preliminary findings of these studies at Ft Detrick on October 19-20 at the Extreme Environments Research in Progress Review.
- Two abstracts have been submitted for presentation at the 2017 Experimental Biology Meetings in Chicago.

  Gerard P. Robinson, Michelle A. King, Alex J. Mattingly, Christian K. Garcia, Orlando Laitano, David Van Steenbergen, Lisa R. Leon, Thomas L. Clanton "**Major Metabolic Hormone Responses to Exertional Heat Stroke in Mice." FASEB J.**

  Christian K. Garcia, Gerard P. Robinson, Alex J. Mattingly, Orlando Laitano, David Van Steenbergen, Michelle A. King, Lisa R. Leon, Thomas L. Clanton "**Differences in tolerance to exertional hyperthermia between male and female mice" FASEB J**

- In Nov, 2016, a new manuscript was accepted for publication based on the work that led to this project and one that represents the ongoing collaboration between USAIREM and UF. King, MA, Leon, LR, Morse, DA, Clanton, TL. "**Unique cytokine and chemokine responses to exertional heat stroke in mice."** J Appl Physiol (in press)

What do you plan to do during the next reporting period to accomplish the goals and objectives:

We are currently doing more sample collection in an additional cohort of animals (still within the IAUCUC guidelines and animal numbers) in order to collect some additional samples for a new biomarker we have proposed in collaboration with investigators at USACEHR (Rahsa Hammamieh) that involve long term epigenetic markers in the bone marrow/leukocytes. We are also taking additional brain samples for histological evaluation to compare to frozen samples taken in our first two cohorts.

We will begin the NSAID studies beginning in January, which will include the influence of EHS on gut and organ injury using ibuprophen, naproxen and a H2S containing naproxen.

We will work with USACEHR on the metabolomic and lipidomic analysis of the heart and plasma (Danielle Ipollito). The results of those studies should be completed in the next few weeks but will require weeks to months to analyze. We will depend on the expertise of our collaborators at USACEHR who we are working with closely on that project.

4. IMPACT

Impact on the Field. This model has become extremely refined and predictable and we believe it will stand the test of time as the first go-to model for preclinical studies in EHS research. We continue to be surprised by new findings that are not expected from other models such as passive heat stroke.

Impact on other Disciplines: We have confidence that biomarkers we can identify may be applicable across other fields, particularly with respect to studies underway on epigenetic markers. We also are of the opinion that our work identifies a unique “stress induced immune response” which can be separated from classic innate immunity. This may ultimately impact the field of immunology.

Impact on technology transfer: Nothing to report

Impact on Society beyond science and technology: It is possible that our work will impact the evaluation and treatment of exertional heat stroke patients. However, at this time, it is premature to predict how this will be manifest.

5. CHANGES OR PROBLEMS

We have had no significant problems performing this work and because of excellent help in the laboratory we are ahead of schedule in data collection. We have much work to do on analysis, as expected.

Our expenditures are in line with expectations.

There have been no changes in terms of care and use of animal subjects.

6. PRODUCTS:

- abstracts submitted for presentation at the 2017 Experimental Biology Meetings in Chicago.

Gerard P. Robinson, Michelle A. King, Alex J. Mattingly, Christian K. Garcia, Orlando Laitano, David Van Steenbergen, Lisa R. Leon, Thomas L. Clanton "**Major Metabolic Hormone Responses to Exertional Heat Stroke in Mice." FASEB J.**

Christian K. Garcia, Gerard P. Robinson, Alex J. Mattingly, Orlando Laitano, David Van Steenbergen, Michelle A. King, Lisa R. Leon, Thomas L. Clanton "**Differences in tolerance to exertional hyperthermia between male and female mice" FASEB J**

- New Manuscript in press:

King, MA, Leon, LR, Morse, DA, Clanton, TL. **Unique cytokine and chemokine responses to exertional heat stroke in mice.** J Appl Physiol (in press)

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Individuals who have worked on the project.

Personnel at UF.

Name: Thomas Clanton Ph.D.
Project Role: P.I.
Researcher Identifier Orchid: 0000-0003-0600-7150
Nearest person-month worked 3.5 Person Months
Contribution to project : All aspects of the project.
Funding support: Univ of Florida

Name: Orlando Laitano, Ph.D.
Project Role: Postdoctoral fellow
Researcher Identifier
Nearest person-month worked: 2 Person Months
Contribution: Data collection, planning design of experiments, directing other lab personnel.
Funding support: Rest of support from the National Institutes of Health

Name: Alex Mattingly, MS
Project Role Senior Graduate Student/Research Assistant
Researcher Identifier
Nearest person-month worked 3 Person Months
Contribution: Oversees surgeries, data collection and managing activities and training of other personnel.
Funding support: Univ of Florida Research Assistantship.

Name: Christian Garcia
Project Role: Graduate student research assistant
Researcher Identifier
Nearest person-month worked 8 Person Months
Contribution: Ran most of the training and EHS experiments, collected specimens, animal care, histology
Funding support: Entirely from this award.

Name: Gerard Robinson
Project Role: Graduate student research assistant
Researcher Identifier
Nearest person-month worked: 6 Person Months
Contribution: Ran training and EHS experiments, collected specimens, animal care, histology
Funding support: Entirely from this award.

Name: Deborah Morse
Project Role Technician
Researcher Identifier
Nearest person-month worked: 2 Person Months
Contribution: Assisted in animal care, performed some biochemical experiments, laboratory management
Funding support: Supported in part by three other large NIH grants from the PI and two other investigators.

***Has there been a change in the active other support of the PI since the last reporting period*.**

The PI (Clanton) has received an NIH RO1.

National Institutes of health RO1 NIGMS 1R01GM118895-01 July 2016-June 2020.
"Functional role of skeletal muscle in the innate immune response to sepsis"
PI: Clanton (13% Effort)
$197,000 direct costs/year for 4 years. Total Award: $1,182,000
This project uses transgenic animals developed in our laboratory to explore the role of muscle cytokines and chemokines in the immunological response to septic shock. There is no overlap with this project

What other organizations were involved as partners:

Organization Name: USARIEM (Lisa Leon, primary contact)
Location of Organization: Natick MA.
Contribution to the Project:
Evaluate samples for metabolic hormones and cytokine expression.
Collaboration and planning of experiments, writing manuscripts and data analysis.

Organization Name: USACEHR (Danielle Ippolito, primary contact)
Location of the Organization: Frederick MD
Contribution to the Projects: Evaluate samples for metabolomic, lipidomic and proteomic markers. Lead the team in interpretation of the big data obtained with these tests.

APPENDIX MATERIAL

1. Quad Chart for 4th Quarter 2015-2016
2. Publication in press. King et al.
3. Abstracts submitted Garcia et al., Robinson et al.

Prevention of Organ Injury in Exertional Heat Stroke: Preclinical evaluation of a new class of NSAIDs

Log Number: #14267001 FY 16

W81XWH-15-2-0038 BAA Extramural Medical Research

PI: Thomas L Clanton **Org: University of Florida**

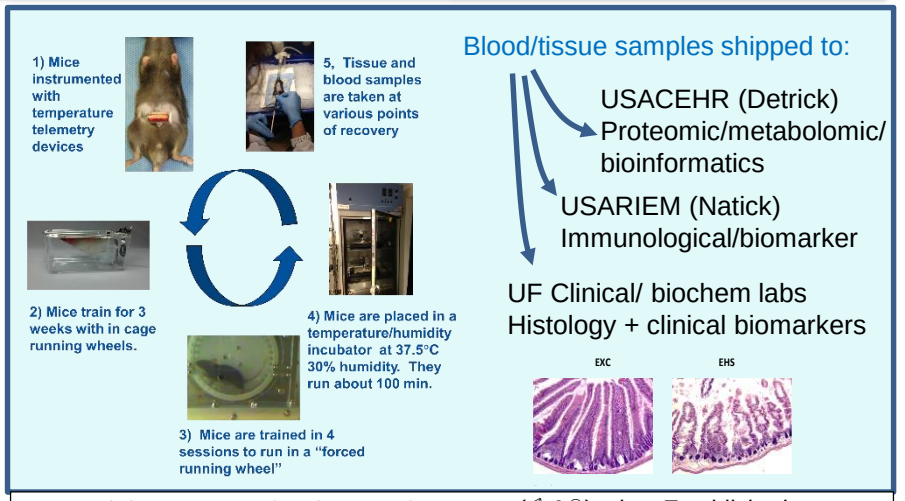
Award Amount: $358,418.00 (1st yr including IDC)

Study/Product Aim(s)

- to define the time course of multi-organ injury, repair and recovery of metabolic control in exertional heat stroke (EHS)
- to determine sex differences in susceptibility to EHS in mice
- to identify metabolomic and proteomic biomarkers that define underlying disorder in EHS
- to test the impact of commonly used NSAIDs on susceptibility to organ injury in EHS
- to test the effectiveness of new H2S-containing NSAIDs on reducing intestine and organ damage in EHS

Approach

Instrumented and exercise-trained mice (♂ &♀) run on a running wheel within an incubator (37.5°C) until symptom limited (neurological). Samples of blood and various organ systems are taken at intervals up to 14 days and prepared for proteomic, metabolomic and genomic analysis. In upcoming experiments, the animals will be given different varieties of NSAID to determine susceptibility to organ injury.



Accomplishments: Completed EHS studies on >60 (♂ &♀) mice. Established new protocols and directions for epigenetic testing, trained 3 new employees and are processing tissue samples from 8 organ systems for histology.

Timeline and Cost

Activities CY	15	16	17	18
Collection of tissues from EHS studies in male and female mice				
Proteomic/metabolomic/and immunological analysis of samples				
Test effects of common NSAIDs on organ injury in EHS				
Effects of new generations of H2S containing NSAIDs in EHS				
Estimated Budget ($K)		**$325K**	**$265K**	**$268K**

Note: October start date in '15 so budget listed in cy 16'

Updated: First submission 11/30/2016

Goals/Milestones

CY15 Goal – ☑ purchase equipment, train personnel begin EHS

CY16 Goals – ☑ Complete male EHS and control experiments with 6 time points of EHS recovery ☐ Submit male samples to USACEHR and USARIEM ☐ Complete female mice EHS studies

CY17 Goal –☐ submit remaining EHS samples to USACEHR and USARIEM ☐ Begin studies of effects of predominant NSAIDs on organ injury in EHS. ☐ Begin studies of NSAID-H2S drug studies

CY18 Goal –Complete NSAID-H2S studies and analyze and write up data

Comments/Challenges/Issues/Concerns

- Experimental model working well, there are no major problems.
- Have completed data collection for entire first and 2nd year. Samples have been analyzed by USARIEM and submitted to USACEHR
- Beginning NASAID studies in Jan 2017

Budget Expenditure to Date

Projected Expenditure: $401,799

Actual Expenditure: $231,193 (as of 11/30/16)

Differences in tolerance to exertional hyperthermia between male and female mice

Christian K. Garcia, Gerard P. Robinson, Alex J. Mattingly, Orlando Laitano, David Van Steenbergen, Michelle A. King, Lisa R. Leon, Thomas L. Clanton

The Department of Applied Physiology and Kinesiology, University of Florida, Gainesville FL, US Army Research Institute of Environmental Medicine, Thermal and Mountain Medicine Division, Natick MA.

Surveillance reports suggest possible reductions in heat stroke susceptibility in female vs. male active component members of the US Armed Forces, but whether these differences reflect behavior or underlying biology is unknown. Previous studies in mice have shown that females exhibit markedly better resistance to moderate, passive heat. However, whether heat tolerance translates to acute settings or to exertional heat stroke (EHS) has not been tested. In this study, we compared responses in male and female mice to an established model of EHS. Exercise trained mice (3 wks) were maintained at 37.5°C (35% RH) and ran using a preprogrammed incremental protocol on a forced running wheel. The EHS end point was defined as loss of consciousness. Female mice on average ran longer than males (177 vs. 124 min; p=0.0001), and were exposed to greater heat loads (241 vs. 160 °C • min; p=0.0001). Male and female mice ran to nearly identical average peak core temperatures, both 42.2°C (n.s.). There were no differences in the minimum temperature during post EHS hypothermia 32°C (n.s.) or the time to reach the minimum temperature. However, females lost a greater % body weight (9.2% vs 7.5% $p < 0.001$), demonstrated significantly higher levels of circulating corticosterone (286 vs 183 ng/ml, $p = 0.001$, 3 h) and higher levels of resistin polypeptide (8891 vs. 3781 pg/ml, $p = 0.004$, 3 h). These results demonstrate that female mice have greater resistance to EHS during exercise in hyperthermia. Possible mechanisms include greater body surface to mass ratio in females vs. males (3.3 vs. 3.2 m^2/kg; p=0.0001), greater aerobic conditioning in females (characteristic of mice), or a hormonally or genetically induced resistance to hyperthermia. Though controversial, marked elevations in circulating corticosterone and resistin in females have the capacity to contribute to improved heat tolerance. We conclude that female mice are significantly more resistant to EHS than male mice. Inherent thermal tolerance in female mice may provide an evolutionary advantage because metabolic rate and heat production have been shown to double during pregnancy and lactation. *Author views not official US Army or DoD policy. W81XWH-15-2-0038*

Major Metabolic Hormone Responses to Exertional Heat Stroke in Mice.

Gerard P. Robinson, Michelle A. King, Alex J. Mattingly, Christian K. Garcia, Orlando Laitano, David Van Steenbergen, Lisa R. Leon, Thomas L. Clanton

The Department of Applied Physiology and Kinesiology, University of Florida, Gainesville FL, US Army Research Institute of Environmental Medicine, Thermal and Mountain Medicine Division, Natick MA.

Disordered glucose metabolism has been shown to be a strong prognostic indicator of poor outcomes in heat stroke. In mice, within the first few hours of recovery from exertional heat stroke (EHS), glucose is reduced; however, by 24 h and for up to 4 days, sustained hyperglycemia has also been observed. To gain perspective on the underlying mechanisms that contribute to these responses we looked at circulating metabolic hormones responsible for glucose regulation. Male mice (n= 8, per group) ran in forced running wheels within an enclosed climatic chamber at 37.5°C/35% relative humidity (RH) until loss of cognitive function (approx. 2 h). Animals were sacrificed at 0.5h, 3h, 24h, 4d, 9d or 14d post-EHS. Plasma samples were collected and metabolic hormones analyzed using Luminex multiplex technology or ELISA (corticosterone). Hormones secreted by the pancreas, amylin ($p=0.02$), c-peptide ($p=0.003$), and insulin ($p=0.003$) were markedly suppressed at 0.5 h and continued to be suppressed at 3 h. Expected glucagon responses were absent during this period. Cytokines that influence glucose metabolism or uptake, IL-6 and MCP-1, displayed a significant increase at 0.5 and 3 h ($p=0.0004$, $p=0.0008$ respectively), with peak concentrations appearing at 0.5 h. Resistin (secreted by white adipose) was also significantly elevated at 0.5 h ($p=0.003$) and then decreased to low levels at 3h ($p<0.002$). Corticosterone was significantly elevated at the 0.5 h ($p=0.0015$) and 3 h time points ($p=0.0009$). These results may reflect, in part, hypoglycemia seen following exercise in the heat but are also consistent with transient organ dysfunction, specifically in the liver (i.e. failure to elevate glucose) and/or pancreas (suppression of insulin secretion without compensating glucagon response). *Author views not official US Army or DoD policy. Supported by the Department of Defense W81XWH-15-2-0038*

Unique cytokine and chemokine responses to exertional heat stroke in mice

[1]Michelle A. King, [2]Lisa R. Leon, [1]Deborah A. Morse [1]Thomas L. Clanton

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Running Title: Heat stress-induced cytokine responses in mice

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Abstract

In heat stroke, cytokines are believed to play important roles in multi-organ dysfunction and recovery of damaged tissue. The time course of the cytokine response is well defined in passive heat stroke (PHS), but little is known about exertional heat stroke (EHS). In this study we used a recently developed mouse EHS model to measure the responses of circulating cytokines/chemokines and cytokine gene expression in muscle. A very rapid increase in circulating IL-6 was observed at max core temperature (Tc,max) that peaked at 0.5 h of recovery and disappeared by 3 h. IL-10 was not elevated at any time. This contrasts with PHS where both IL-6 and IL-10 peak at 3 h of recovery. KC, G-CSF, MIP-2, MIP-1β and MCP-1 also demonstrated near peak responses at 0.5 h. Only G-CSF and KC remained elevated at 3 h. Muscle mRNA for innate immune cytokines (IL-6, IL-10, IL-1β, but not TNFα) were greatly increased in diaphragm and soleus compared to similar measurements in PHS. We hypothesized that these altered cytokine responses in EHS may be due to a lower Tc,max achieved in EHS or a lower overall heat load. However, when these variables were controlled for, they could not account for the differences between EHS and PHS. We conclude that moderate exercise, superimposed on heat exposure, alters the pattern of circulating cytokine and chemokine production and muscle cytokine expression in EHS. This response may comprise an endocrine reflex to exercise in heat that initiates survival pathways and early-onset tissue repair mechanisms.

Key Words: IL-6, CXCL1, G-CSF, exercise, hyperthermia

NEW AND NOTEWORTHY

Immune modulators called cytokines are released following extreme hyperthermia leading to heat stroke. It is not known whether exercise in hyperthermia, leading to exertional heat stroke (EHS), influences this response. Using a mouse model of EHS, we discovered a rapid accumulation of interleukin-6 and other cytokines involved in immune cell trafficking. This response may comprise a protective mechanism for early induction of cell survival and tissue repair pathways needed for recovery from thermal injury.

INTRODUCTION

Exertional heat stroke (EHS) is a life threatening condition where the body is no longer able to dissipate the heat load produced during physical exertion. This can lead to extreme elevations in core temperature (Tc), central nervous system dysfunction and subsequent multi-organ damage (7). This condition affects seemingly healthy individuals, such as military personnel, occupational workers, and athletes, making this illness even more enigmatic. While EHS is distinct from passive heat stroke (PHS) (35), the etiologies of both conditions are still poorly understood, and though multi-organ dysfunction is common in both (35, 38, 39, 53), the extent to which they share underlying mechanisms is not known. Despite efforts to prevent multi-organ damage via rapid cooling, many individuals still succumb to multi-organ failure. Furthermore, for those individuals who survive the initial heat injury, forty percent are more likely to die earlier in life than their matched counterparts (62). To develop clinical interventions as well as prevent long-term organ damage, it is important to understand the underlying causes responsible for multi-organ injury.

The multi-organ dysfunction that occurs as a consequence of heat stress has been suggested to be the result of excessive inflammatory processes, where cytokines serve as important mediators (38, 56). The local response to tissue damage involves the production of cytokines at the injury site, which, with the help of chemokines, function in attracting lymphocytes, neutrophils, and monocytes to aide in the healing process (69). PHS models, as well as hyperthermia itself, display an acute rise in cytokines with dominant elevations in interleukin-6 (IL-6), interleukin 10 (IL-10), interleukin -1β (IL-1β) and a lesser rise in tumor necrosis factor alpha (TNFα) (12, 30,

39). Importantly, the circulating cytokine pattern following PHS is unique from that seen following exposure to endotoxin or acute exercise (39, 49, 64, 67). However, the circulating cytokine pattern following EHS has yet to be determined.

One of the distinct differences between PHS and EHS is the role of the exercising muscle. Exercising muscle is not only the main contributor to increases in Tc during physical activity, but also has the ability to act as an endocrine organ, contributing cytokines, particularly IL-6, to the circulation (49, 58). Furthermore, skeletal muscle has been shown to be responsive to heat stress following PHS (64). However, the role of the skeletal muscle in contributing to the circulating cytokine profile is not known in EHS.

To understand the acute cytokine responses to EHS, our objective was to determine the pattern of cytokines and chemokines expressed in the circulation as well as the expression of select cytokines in skeletal muscle throughout the course of EHS and recovery. Because there may be a cumulative effect of hyperthermia, exercise, and other potential factors such as endotoxemia or release of catecholamines, we hypothesized that the stress-induced cytokine response to EHS would be greater in magnitude in EHS, but follow a similar time course as that observed in PHS. We predicted that the additional stress of exercise would exacerbate the associated cytokine and chemokine profile.

Methods

Animal Care

All animal protocols were approved by the University of Florida Institutional Animal Care and Use Committee. Ninety-five mice were used for data collection in this

study. A subset of these mice had been used previously to determine multi-organ dysfunction in EHS (35). All were C57BL/6J males (Jackson Laboratories, Bar Harbor, ME) weighing an average of 29.1 ± 3 SD g, approximate 4 months of age. Mice were housed in groups until they were implanted with telemetry devices, after which they were individually housed in 7.25”W x 11.75”D x 5H” cages, lined with Harlan corn cobb bedding, maintained on a 12 h on by 12 h off light cycle at 20-22°C/30-60% RH. A standard chow diet (Teklad LM-485m Envigo, Madison, WI) and water were provided ad libitum until the EHS protocol. Experiments were performed in the morning of the light cycle (approximately 0700-1000).

Animal Preparation and Training

As described previously (35), under isoflurane anesthesia, mice were implanted with temperature telemetry transmitters (TA-E-Mitter, Starr life Sciences, Oakmont, PA), for monitoring Tc. The mice were allowed to recover with subcutaneous buprenorphine injections every 12 h for 48 h and then recovered undisturbed for >2 weeks. Following this recovery period, exercise wheels and enrichment huts (Silent Spinner and Small Animal Igloo Hideaways, PETCO, San Diego, CA) were introduced into the cages for 3 weeks. During this period, mice had ad libitum access to the running wheel throughout the day and night. On the third week, additional exercise training/acclimation was implemented to familiarize the mice to the environmental chamber in the laboratory (Thermo-Forma 3940 Incubator, Thermo-Fisher, Waltham, MA) and to the customized forced running wheel system (Lafayette Model 80840, Lafayette, Ind.). The first exercise session in the chamber consisted of 15 min of free-wheeling, where the mouse was free to run and explore their surroundings. This was followed by a short recovery period (<5 minutes). Then mice were started at an initial speed of 2.5 m/min, then increased by 0.3

m/min, every 10 min, for 60 min. Training sessions on the next two consecutive days consisted of only the incremental protocol for 60 min. At the fourth and final session the same protocol was used but exercise time and incremental speed were elevated until the animals exhibited fatigue. Fatigue was defined as refusal to run or walk on the wheel for > 5 s. No shock or any other manual stimuli were used to maintain running speed.

Exertional Heat Stroke

Following the last training session, mice were given two days of rest, with free access to the running wheel in their cages. The evening before or the morning of the EHS test, mice were brought to the laboratory in their own cage. Tc was monitored with a data acquisition system, averaged over 30 sec intervals (VitalView, Starr Life Sciences). After at least 2 hours of resting data in the environmental chamber, each mouse was monitored until Tc dropped to < 37.5°C for > 15 min. At this time, the environmental temperature (Tenv) and relative humidity (RH) were increased to 37.5°C, 50%RH, water, food, and the cage lid were removed leaving only the wire rack exposed. This Tenv was based on a previous work where we studied EHS at three different Tenv (between 37.5-39.5) and RHs (35-90%) (35). At this temperature, the animals' exertional heat production had the greatest contribution to overall heat load and therefore had the greatest potential for distinguishing differences from PHS. As soon as the environmental chamber equilibrated to the target Tenv (approximately 1h), the chamber was opened and the animal quickly placed in the running wheel. The forced running wheel protocol was then initiated. The mouse's behavior was monitored continuously in real time with a video camera. Running speed began at 2.5 m/min and increased 0.3 m/min every 10 min until the mouse reached a Tc of 41°C, which served

as threshold beyond which the running speed was kept constant (Fig. 1A,B). The end point of the EHS test was “symptom limited” as nearly all mice (≈98%) displayed a sudden loss of consciousness and collapse. However, reaching a Tc of 42.5°C was also considered a humane end point, but was a rare occurrence. At the end of the protocol, Tenv was adjusted back to room temperature, the chamber door opened, and the mouse carefully watched until it regained consciousness. At this time, it was weighed and returned to its home cage. Tc continued to be monitored for a 24h recovery or until sacrifice at an earlier time point (described below). The 12-hr light-dark cycle was maintained in the environment during the recovery period.

EHS Experiments

Five groups of mice were studied (n=6-9 per group) to determine the time course of cytokine expression. Mice were sacrificed at: 80 min into the protocol (which was set to be ≈0.5 h before $T_{c,max}$), at $T_{c,max}$, 0.5, 3, and 24 h post $T_{c,max}$. At each time point blood and tissue samples were collected. Five other groups of sham controls (EXC) were treated identically without heat exposure and tissues sampled at the same times. These mice were exercised at the average time and intensity of the EHS mice (max speed: 5.2 m/min, duration: 113 min) but with the environmental chamber maintained at 25°C and 50% RH (35).

For sample collection, the mice were placed under isoflurane anesthesia and blood samples obtained by transthoracic cardiac stick. Soleus (sol), gastrocnemius (gastroc) and diaphragm (dia) were removed for later biochemical or histological analyses. Thoracotomy and heart removal were performed under deep anesthesia.

Tissue and blood samples were obtained from another group of naïve control mice (NC) that did not undergo surgery, any exercise training, any specific enrichment and no exercise or heat interventions (n=6).

PHS experiments:

Two more groups of animals were exposed to a passive heat stroke (PHS) protocol. One set (n=6) was exposed to 39.5°C at 30% RH, identical to previous approaches described by Leon and colleagues (40) except that the endpoint for these passive heat stroke mice was Tc of 42.1°C, rather than 42.4-42.7°, which was used for previous studies (40, 64). This end point temperature was used because it was the average $T_{c,max}$ acquired by the EHS mice in this study. This was done to determine if differences in response of EHS could be attributed to the lower peak Tc reached. We only took samples at the 3 h time point in these mice because this corresponds to a time when there is marked cytokine expression in PHS, but a time when there is almost no circulating cytokine expression in EHS.

Another set of mice (PHSm; “m” for matched) (n=6) underwent a passive heating protocol that, designed to mimic the shortened thermal area (heat load) experienced in EHS groups. Thermal area was calculated as defined by Leon et al. (40), adapted from Hubbard et al. (32). Mathematically this equals approximately the area under the curve of the temperature profile for all points at which Tc was >39.5°C (units = °C•min). To obtain a very similar thermal area in PHSm, the environmental temp was elevated to 43.5°C /50% RH, determined by trial and error in a group of test mice. These mice were also studied at the single time point of 3 hours post Tc max for the same reasons identified in PHS mice.

Plasma Cytokine Measurements

Blood was collected, using heparin as the anticoagulant, spun at 2,000 RCF and plasma (250 µl) was pulled off the buffy coat, aliquoted and stored at -80°C. Plasma cytokines and chemokines were determined using a Luminex system, employing MILLIPLEX MAP Mouse cytokine/chemokine- premixed 25 plex assay kits which include the antibodies for the following analytes: G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES, and TNF-α. The test was performed according to the manufacturer's protocols, as described elsewhere (67).

RNA isolation, reverse transcription, and real-time PCR

In order to determine innate immune cytokine expression in skeletal muscle the soleus, diaphragm, and gastroc muscles were dissected and flash frozen at the -0.5 h, Tc max, 0.5 h, 3h, and 24h time points. As previously described (67) RNA was separated from DNA by bromochloropropane and precipitation in isopropanol. After a 75% ethanol wash and re-suspension in DEPC water, purity of RNA was quantified by spectrophotometry. Total mRNA was reverse transcribed using a Verso CDNA Synthesis Kit. Pre-formulated Taqman Gene Expression assays were used for IL-1β, IL-6, IL-10, and TNFα. Relative quantitative real time reverse transcription polymerase chain reaction (RT-PCR) was done using TaqMan Gene Expression Master Mix on a StepOnePlus. Hypoxanthine phosphoribosyltransferase (HPRT) was used as a housekeeping gene based on previous studies in which we observed the gene to be stable in hyperthermic myofibers and tissues (67). Changes in target gene expression

were independent of changes in the level of mRNA for HPRT. Relative quantitation was calculated using the ΔΔCT method as described previously (31).

Statistical Analyses

Statistical analyses were performed using SAS JMP (Cary, NC) and Graphpad Prism (La Jolla, CA). The large majority of cytokine and mRNA data were non-parametric and therefore, Kruskal-Wallis was used for all ANOVA analyses. Post hoc tests were done with Dunn's multiple comparison test for nonparametric comparisons. Central tendency and variance of data were expressed as medians +/- 25-75% quartiles because of the nonparametric nature of the data sets. To determine the probability of type I error due to multiple comparisons, the Benjamini-Hochberg procedure for estimating false discovery rate was applied (6), using a cutoff of 0.15 as an acceptable false discovery rate.

Results

Plasma Cytokine and chemokine responses to EHS

We sampled plasma cytokines and chemokines at time intervals denoted on a typical EHS Tc profile in Fig. 1A. Cytokines such as IL-1β , IL-6, IL-10, and TNFα which are classically involved in the innate immunity, are elevated following heat stroke (10, 11, 39, 64). However, in this model of EHS, only IL-6 was significantly elevated at any time point over the course of EHS, reaching a peak at +0.5 h into recovery (Fig. 2A). This response was suppressed by 3 h and remained undetectable at 24 h. Sham exercise controls displayed no significant changes in IL-6 nor any of the cytokines measured in this study, at any time (Fig. 2B).

As shown in Fig. 3A, plasma chemokines, MCP-1, MIP-1β, and MIP-2 followed a similar trajectory seen for IL-6, where peak concentrations occurred at 0.5 h of recovery, disappearing by 3 h (Figure 2A). G-CSF and KC were also significantly elevated at 0.5 h but showed sustained or increasing levels at 3 h. G-CSF is not structurally classified as a chemokine, but works synergistically with many other chemokines like KC to mobilize immune cells (68). All chemokines returned to control values by 24 h. There were no significant elevations in these chemokines in sham exercise controls (Fig. 3B). All other cytokines and chemokines tested with the multiplex array showed no significant elevation during EHS (data not shown). Refer to Table 1 for functional and structural classifications of responsive chemokines observed in this study.

Passive heat stroke experiments

Previous PHS studies have shown that circulating IL-6 and IL-10 reach a peak response at 3 h of recovery (39, 64), with little or no response at $T_{c,max}$ and only modest responses at ≈0.5 h of recovery (64). To understand the origins of this delay in the PHS cytokine profile compared to the EHS profile, we tested several possible experimental mechanisms related to heat exposure.

First, because our EHS animals achieved an average symptom-limited $T_{c,max}$ of only 42.1°C (-0.3 to -0.6°C lower than the $T_{c,max}$ in studies by Leon and colleagues (39, 64)), we repeated the standard PHS experiment in mice, but stopped exposure when Tc reached 42.1°C. A typical temperature profile for this group (PHS) compared to EHS is shown in Fig. 4. Second, the PHS protocol resulted in an increased thermal area compared to EHS, averaging 409 ± 71°C•min in this series, compared to 146 ± 30 SD °C•min in EHS. Therefore, we hypothesized that the altered cytokine response to EHS

might reflect differences in the overall thermal load between PHS and EHS. To test this, we studied a second group of PHS animals (PHSm) in which the thermal area was matched, using an elevated T_{env} in the chamber (43.5°C). This resulted in an average thermal area = 148 ± 20 SD °C • min (n.s. from EHS). A typical thermal profile for PHSm experiments is also shown in Fig 4. We tested only the 3 h time point in these experiments because it represented a time when EHS cytokine responses were nearly absent in EHS but reached peak concentrations in PHS.

Comparisons of cytokines and chemokines between sham exercise controls (EXC), EHS, PHS and PHSm animals at the 3 h recovery point are illustrated in Fig 5. In Fig. 5 (A-C) are cytokine/chemokine responses to PHS that showed no response in EHS or EXC but were significantly elevated in PHS and PHSm (i.e., IL-6, MIP-2 and RANTES). In Fig 5 (D-F) are cytokines/chemokines for which there were no responses in EHS, EXC or PHSm, but there were significant elevations in PHS. Both G-CSF and KC (not shown) were significantly elevated in PHS and/or PHSm, and were not significantly different from EHS (not shown). Elevations during EHS in these two chemokines are shown in Fig. 3.

Skeletal muscle innate immune cytokine gene expression

Skeletal muscle mRNA expression of IL-6, IL-10, IL-1β, and TNFα were evaluated over the course of the EHS and EXC protocol through 24 hours of recovery. The primary rationale was that significant muscle injury is associated with EHS but not PHS, based on plasma creatine kinase measurements (35) and unpublished observations of hind limb motor dysfunction during recovery. In addition, in a previous study, the same approach was used in PHS at the similar time points, making

295 comparison possible (64). Therefore, measuring the mRNA expression of important
296 inflammatory cytokines in muscle can provide an indication of the timing of ongoing
297 damage and repair processes in the muscle.
298 The results are summarized in Fig. 6 using samples from the whole
299 gastrocnemius (white bar), soleus (light grey bar) and diaphragm (dark grey bar).
300 Results are expressed as fold change compared to samples taken from “naive controls”
301 that did not undergo surgery or acute exercise and were not exercise trained or
302 exposed to heat. Note the tendency in early time points (-0.5 h to $T_{c,max}$) for cytokine
303 mRNA to be suppressed prior to reaching $T_{c,max,}$ (discussed below). There was very little
304 mRNA response at any time point in gastroc; however, sol and dia, elevations in
305 cytokine gene expression (IL-6, IL-1β, and IL-10) peaked at 0.5 h after $T_{c,max}$. IL-6
306 mRNA was also evident in Dia at $T_{c,max}$. These elevations in mRNA are 3-10 times
307 higher than seen in comparable conditions and times during PHS (64). Note that TNFα
308 mRNA was not significantly elevated at any time point. Furthermore, in exercise
309 controls, exercised to match EHS, and trained identically, there were no significant
310 elevations in muscle cytokine gene expression at any time point.
311 Based on the plasma cytokine results we hypothesized that moderate acute
312 exercise or the exercise training protocol itself may be responsible for suppression of
313 cytokines. To test this, we compared our EXC group (which received enrichment and
314 training sessions as previously described) to mice that were exposed to a single bout of
315 moderate exercise, matched in timing and intensity to the EHS experiments. This
316 experimental bout was preceded by only a familiarization trial the day prior, identical to
317 the 60 minute incremental training session that EXC mice received. We then measured

inflammatory cytokine gene expression at 0.5 h of recovery because this time point displayed the greatest cytokine response in plasma. As shown in Fig. 7A, exercise suppressed IL-6, IL-1β and IL-10 mRNA in the gastroc and sol, but not in the dia. Comparable trends were seen in the EXC (i.e. trained) animals but fewer time points were statistically significant (Fig. 7B). The data are consistent with acute, moderate exercise inducing an acute inhibition of inflammatory cytokine gene expression in skeletal muscle.

Discussion

We have demonstrated that EHS results in cytokine/chemokine responses in plasma and skeletal muscle that are uniquely different in the timing, magnitude and/or species compared to passive models of heat stroke. Contrary to our original hypothesis where we proposed the combined effects of exercise and hyperthermia would amplify the IL-6 induced response, circulating IL-6 emerges rapidly, reaching a peak level at 0.5 h of recovery and disappearing by 3 h, a point in time when the magnitude of circulating IL-6 is highest in PHS. Similar responses were seen for MIP-1β, MCP-1 and MIP-2; whereas, G-CSF and KC increased rapidly but remained elevated at 3 h of recovery. At that time point, they were not different in magnitude from PHS. There was no evidence for elevations in circulating IL-10 at any time during recovery from EHS, whereas this is routinely elevated during recovery from PHS [(39, 64), Fig 4].

Exploration of possible environmental variables related to the timing and magnitude of heat exposure failed to provide a suitable explanation for these phenomena. Therefore, the data suggest that the predominant experimental factor

driving the rapid and unique cytokine/chemokine responsiveness of EHS is related to the influence of moderate, forced exercise, performed during hyperthermia. Neither matched exercise alone nor matched heat exposure alone could reproduce this pattern.

Possible origins of the cytokine/chemokine response pattern in EHS

There are several underlying stimuli that are thought to interact to produce the pattern of cytokine production seen in heat stroke that may be differentially affected by exercise in heat. One frequently mentioned stimulus is endotoxin or other pathogen-associated-molecular-patterns (PAMPs) released into the circulation from a leaky intestinal barrier (29, 56). However, the pattern of cytokines seen in the plasma during EHS is not typical of known cytokine responses to PAMPs, e.g. there is an absence of circulating TNFα, IL-1β, or IL-12, at any time point. It appears more likely that the response is driven by a “stress-induced cytokine response” in which IL-6 is a predominant element. We have previously described this concept in the context of PHS in mice (64) where we observed altered expression of cytokine genes and toll like receptor isoforms that are uniquely different from the responses seen in the classic innate immune response. Its theoretical origins are based on observations of the response of isolated skeletal muscles to variety of forms of cellular and systemic stress mediators (63, 65, 66).

Other possible influences that may contribute to the uniqueness of the PHS response include effects of intense endurance exercise alone, which produce rapid elevations IL-6 and a variety of other cytokines and chemokines (47, 48). However, in paired exercise controls, there were no significant elevations in cytokines or chemokines. This may have been due to the moderate intensity of exercise. It is

365 possible that hyperthermia amplified the exercise-induced IL-6 (52) as it does with other
366 stimuli (66), but the exercise alone cannot account for the response.
367 Muscle injury is another potential factor. Local cytokines and chemokines
368 produced following injury play important roles in tissue regeneration and repair (24, 59).
369 Muscle injury was likely present in this model since elevations in plasma creatine kinase
370 are present in this model of EHS but not PHS (35). In addition, Fig. 5 suggests ongoing
371 inflammatory gene expression in both limb and diaphragm muscle during the recovery
372 period that exceed by many fold what is seen in PHS (64). The responses appear to be
373 local because mRNA for cytokines such as IL-1β and IL-10 are greatly upregulated in
374 muscle but these do not appear elevated in blood during the course of recovery. In
375 addition, previous reports of the timing and magnitude of the circulating cytokine
376 responses in the blood following muscle injury appear to be too small and slow to
377 account for observations seen in EHS (59, 61).
378 Because the EHS animals received training sessions and had access to running
379 wheels prior to EHS, this may have modified the cytokine responses during heat stroke.
380 Previous studies have shown that endurance exercise training alters or dampens
381 immune responsiveness (25, 45). It takes only two weeks of voluntary wheel running in
382 C57BL/6J mice to induce significant increases in heart-body mass ratio and percentage
383 of oxidative fibers (1), suggesting that endurance training was likely in the mice provided
384 running wheels. Resolving this variable will require a different approach, since mice
385 unaccustomed to wheel running have more difficulty completing the EHS protocol and
386 likely would experience much higher levels of psychological stress.

One important difference in the cytokine profile in EHS compared to PHS was the absence of circulating IL-10, at any time point. This was unexpected, since increases in circulating IL-10 are one of the most predictive circulating cytokines seen in human patients in heat stroke (9) and in animal models in PHS (10, 39, 64). Furthermore, IL-6 has been shown to be an important stimulus for IL-10 production (57), and intense exercise alone stimulates IL-10 (46). One possible explanation may reflect the effects of "forced" exercise on immune modulators such as corticosterone. In mice, during forced swimming exercise, corticosterone levels exceed 800 ng/ml within 5 min, approximately half of the value seen in parallel experiments in mice exposed only to passive heat (42°C) (26). In the mouse model for PHS, corticosterone has been shown to exceed 400 ng/ml, but this value is reached after ≈ 3 h of recovery (39). Though we did not measure plasma glucocorticoids in this setting, it is possible that forced running resulted in an early stress-induced surge in glucocorticoids that may have suppressed global cytokine gene expression. This could also explain the apparent suppression of muscle cytokine mRNA seen immediately after forced running (Fig. 6A & B). Almost all cytokines and chemokines are suppressed by glucocorticoids, including IL-10 (19). Interestingly, one cytokine not affected appreciably by glucocorticoids is G-CSF (13), which turned out to be one of the most profoundly expressed plasma cytokines in EHS, rising rapidly in the circulation but continuing to rise up to 3 h.

A second important and unexpected finding was the very rapid emergence of IL-6, which was elevated in the plasma, at or shortly before $T_{c,max}$ (Fig. 1). This would seem to be too fast to reflect *de novo* protein synthesis, particularly when there appears to be simultaneous suppression of IL-6 mRNA (at least in muscle, Fig. 6). Most of the

410 circulating chemokines also emerged during this time frame (Fig. 2). One possible
411 mechanism is that these cytokines/chemokines were pre-stored in microvesicles or
412 endosomes and were then released early in EHS. In mouse limb muscle, IL-6 is stored
413 in such microvesicles and then released within 25 min from the beginning of an exercise
414 protocol (37). Microvesicle or exosome release has also been shown in some systems
415 to be facilitated by heat stress or by co-stimulation with other cytokines like IL-1β (18,
416 72). For example, in tumors, heat stress is a powerful stimulus for release of exosomes
417 that contain many of the same CCL- and CXC- chemokine species we describe here
418 (18). In theory, triggered release of pre-stored cytokines in this manner could supersede
419 opposing immunosuppressive influences of glucocorticoids produced in the stress of
420 exercise in the heat. This could be a kind of fail-safe, acute endocrine stress response
421 from tissues that could be important in recovery from acute illness.
422 Because of the large role muscle plays in exercise we have focused on it as a
423 source of circulating cytokines in EHS. However, it is highly plausible that other organs
424 make significant contributions to the cytokine profile seen in EHS. Tissue damage
425 resulting from heat stress may impart damage to the liver, kidney, heart, spleen, lung,
426 small intestine, and brain as well as the skeletal muscle (8, 10, 23, 27, 35, 43). When
427 these organs are damaged, they may release cytokines or resident macrophages,
428 dendritic cells, endothelial cells, or astrocytes may participate in the inflammatory
429 response to injury. Therefore, although we did not directly measure other organs as
430 potential sources of circulating cytokines, it is likely that they contribute to the cytokine
431 profile seen in plasma.
432

Functional significance of the pattern of cytokine/chemokine production in EHS. In this model all experimental animals survived up to two weeks or to the point of sample collection. After a few hours of recovery, they show a remarkable ability to return to near-normal behavior, despite evidence of underlying organ damage (35). One of the primary functions of both cytokines and chemokines, besides defending against pathogens, is to participate in the process of wound healing and damage repair (69). This occurs, in part, through recruitment of peripheral blood mononuclear cells (PBMCs) and other immune cells into damaged tissue (59), but also by stimulation, recruitment and mobilization of stem cell or progenitor cell populations in the bone marrow or other tissues (5, 42, 50).

In a previous study (51) we demonstrated that in PHS, early injection of low levels of recombinant IL-6 enabled anesthetized mice to withstand hyperthermic temperatures for longer periods of time, to have protection from intestinal injury and to demonstrate suppression of pro-inflammatory cytokines in the circulation. The protective influence of IL-6 in similar acute, life threatening conditions, or the loss of protection in knockout studies, has now been well established in a number of models, including hemorrhagic shock (2), sepsis (4, 41), acute pancreatitis (21), ischemic heart injury (22) and liver failure (20). Several mechanisms have been proposed, but include pre- or post-conditioning through JAK/STAT3 signaling, promoting cell survival (22, 44, 55), upregulation of MnSOD in critical organs such as liver (14), activation of acute phase response in liver (15) and stimulation of anti-inflammatory cytokines and cytokine receptors (60). We hypothesize that the early secretion of IL-6 and possibly chemokines

456 in this model of EHS may have played an overall protective role in supporting survival
457 and protection from multi-organ injury.
458 The specific sets of chemokines secreted may also have contributed to
459 recovery from heat injury. There are two broad categories, as shown in Fig. 2/Table 1:
460 The CCL- chemokines (i.e MCP-1|CCL2 and MIP-1β|CCL4) and CXCL-chemokines (i.e.
461 MIP-2|CXCL2 and KC|CXCL1). The CCL-chemokines are important for stimulating
462 chemotaxis of monocytes out of the bone marrow and into injured tissues to begin the
463 process of repair (28), and CCL4 has an additional role in stimulating migration of
464 natural killer (NK) lymphocytes (28), which are important in surveillance and ultimate
465 clearing of heavily damaged cells (16, 33). CXCL-chemokines primarily trigger release
466 of neutrophils and other immune cells from bone marrow and also function as a
467 chemotactic stimulus for movement of neutrophils into damaged tissues (28). The
468 cytokine, G-CSF stimulates granulopoiesis in the bone marrow and works in synergy
469 with MIP-2 and KC to increase several types of circulating leukocytes (68). As
470 importantly in this setting, G-CSF is a critical stimulus for mobilization of adult stem cells
471 from the bone marrow (5) . Although IL-6, in combination with its soluble receptor, has
472 been shown to contribute to promotion of progenitor cells (50), its role in this process is
473 not as clearly understood. Some of the chemokines seen in EHS, may act like IL-6, and
474 may also have direct protective effects of tissues exposed to stressful conditions, e.g.
475 CXCL1 (3), G-CSF(36). IL-6 does have extensive effects on immune cell trafficking that
476 include transition from innate to acquire immunity (34) and stimulation of lymphocyte
477 movement across the endothelium and into tissues (17).

The marked elevation in circulating GCS-F is consistent with human data during short term hyperthermia (41.8°C) where circulating GCS-F rapidly increases in the circulation (54). It is also very modestly increased during exercise in some studies (71) or not at all in others (70), though there may be a closer association with muscle damage than there is with exercise (70). The source of GCS-F in this setting is not known but muscle fibers have been shown to be capable of secreting GCS-F following LPS exposure (70).

In summary, we have demonstrated that EHS displays a unique pattern of circulating cytokines and cytokine gene expression in muscle that is unlike that seen in PHS, sepsis, or intense exercise. This response is characterized by the greatest elevations in IL-6, and several chemokines, at the beginning of the recovery period. We verified that this pattern of expression is not simply a result of exposure to lower peak Tc or exposure to decreased thermal loads, but by elimination, appears to be an effect arising from acute exercise superimposed on heat.

Clinical and Integrative Perspectives

It is apparent from this data that exercise, whether acute or chronic, can play a unique role in the overall immune responsiveness to severe hyperthermia exposure. The data are consistent with the existence of an exercise- and hyperthermia-induced, rapid physiological response system that is geared toward initiating survival pathways and recruitment of immune cells involved in rapid wound healing and repair from thermal injury. One would expect that different exercise intensities, levels of exercise training and the timing of exposure of exertion vs. hyperthermia would likely impact the

background immune responsiveness and clinical outcomes in conditions in which EHS can occur.

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Figure Captions

Figure 1. A: Typical core temperature profile for the EHS protocol, showing the intervals of blood/tissue collections relative to peak core temp ($T_{c,max}$). **B:** The average forced running wheel time course, starting at 2.5 m/min, with 0.3 m/min until 40.5°C, then held at steady state exercise until $T_{c,max}$.

Figure 2. Effects of EHS on common cytokines of innate immunity. A. The responses of common innate immune cytokines to EHS. **B.** Cytokine responses to sham exercise controls. Significance from naïve control, P < *=0.05, **0.01,***0.001 (post hoc tests). B-H procedure for multiple ANOVAs = FDR < 15%. Bars = Medians, Tables below = 25%-75% quartiles.

Figure 3. **Effects of EHS on chemokines and related cytokines. A.** The responses during and following EHS. B**.** Responses to sham exercise controls. Post hoc significance from naïve control: P < *=0.05, **0.01,***0.001 (post hoc tests). B-H procedure for multiple ANOVAs = FDR < 10% Bars = Medians, Tables below = 25%-75% quartiles. B-H procedure for multiple ANOVAs = FDR < 15%.

Figure 4. Typical Tc profiles for EHS, PHS and PHSm. Shaded areas represent the thermal areas (Time • Temp above 39.5°C).

Figure 5. Comparison of cytokines and chemokines significantly different at 3 h between EHS and models of PHS. PHSm = PHS at thermal area matched to EHS. *

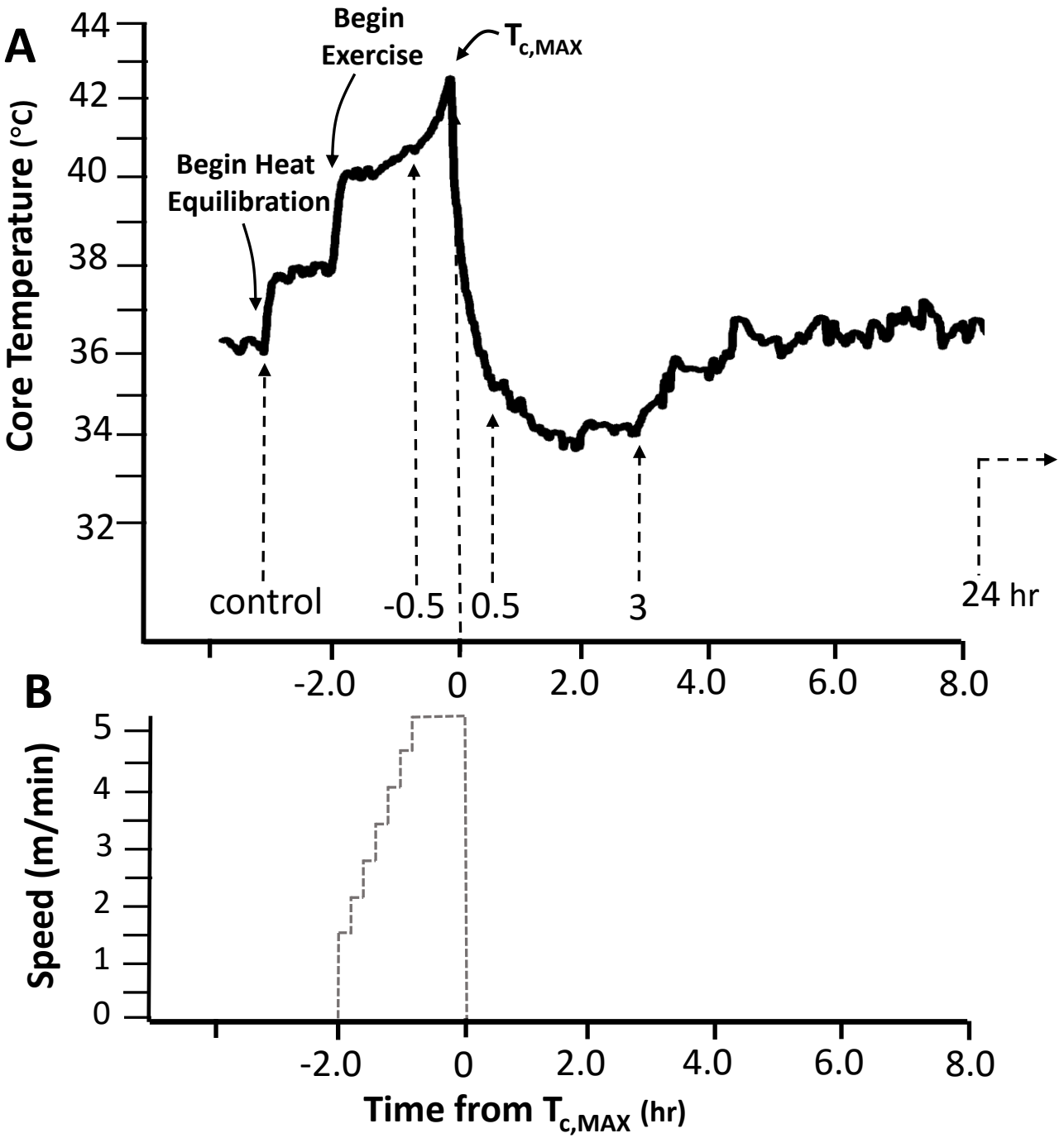
<0.05, ** <0.01, *** 0.001, Kruskall Wallis, Dunn's Post hoc comparisons. Bars = Median with 25-75% quartiles. B-H procedure for multiple ANOVAs = FDR < 15%.

Figure 6. Fold changes in innate immune cytokine mRNA in EHS gastroc, soleus and diaphragm muscle. All changes reported relative to naïve control mouse muscle. Kruskal-Wallis ANOVA, Dunns post hoc: * P < 0.05, **<0.01,***<0.001. Medians +/- 25-75% Quartiles. B-H procedure for multiple ANOVAs = FDR < 15%.

Figure 7. Effects of a single bout of exercise (matched to EHS) on innate immune cytokine gene expression in muscle. Samples collected at 0.5 h post $T_{c,max}$. **A.** Untrained mice without cage running wheels or exercise training, **B.** Response of EXC mice. Medians +/- 25-75% quartiles. FDR = 0.15 using B-H procedure.

Table 1. Functional-structural classes of chemokines/related cytokines observed in heat stroke

Com. Abbrev	Name	Structure Name	Human Homologue	Observed in	Primary Functions
MCP-1	Monocyte chemoattractive Factor-1	CCL2	Human MCP-1	EHS/PHS	Induces migration of monocytes and other immune cells
MIP-1β	Macrophage inflammatory protein-1β	CCL4	Human MIP-1β	EHS/PHS	Induces migration of monocytes and other immune cells
RANTES	Regulated on activation, normal T cell expressed and secreted	CCL5	Human RANTES	PHS	Stimulates T cells, basophils and eosinophils
IP-10	Interferon-γ induced Protein-10	CXCL10	Human IP-10	PHS	Induces migration of neutrophils, macrophages and other immune cells.
MIP-2	Macrophage inflammatory protein-2	CXCL2	Human MIP-2 (90%- IL-8 homologue)	EHS/PHS	Induces migration of neutrophils, macrophages and other immune cells.
KC	Keratinocyte chemoattractant	CXCL1	IL-8 (similar to MIP2)	EHS/PHS	Stimulates hematopoeitic and other stem cells and migration, similar to MIP-2
G-CSF	Granulocyte colony stimulating factor.	CXC synergist	Human G-CSF	EHS/PHS	Not a chemokine but synergistic with CXCL1 and CXCL2 Stimulating hematopoietic and stem cell release


A
Core Temperature (°C)
44
42
40
38
36
34
32
Begin Exercise
$T_{c,MAX}$
Begin Heat Equilibration
control
-0.5
0.5
3
24 hr
-2.0
0
2.0
4.0
6.0
8.0
B
Speed (m/min)
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4
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1
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-2.0
0
2.0
4.0
6.0
8.0
Time from $T_{c,MAX}$ (hr)

A
EHS
Plasma cytokines (pg/ml)
IL-6 * * *
IL-6 * *
IL-6
IL-10
IL-1β
TNFα
Control
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0.5 hr
3 hr
24 hr
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3 3
2 2
4 24
2 2
3 3
3 3
18 100
2 77
3 4
3 4
51 100
2 3
3 3
3 3
3 23
2 2
3 3
3 3
2 3
2 2
3 3
3 3

B
Exercise control
Plasma cytokines (pg/ml)
IL-6
IL-10
IL-1β
TNFα
Control
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3 3
3 3
2 5
2 2
3 3
3 3
2 3
2 2
3 3
3 3

A
EHS
Plasma chemokines (pg/ml)
400
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300
250
200
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50
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MCP-1
MIP-1β
MIP-2
G-CSF
KC
MCP-1 **
MIP-1β ***
MIP-2 **
G-CSF *
KC ***
G-CSF **
KC *
Control
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25%Q 75%Q
3 13
3 6
2 62
30 88
5 57
3 24
3 3
5 66
43 87
10 210
3 23
3 3
55 142
54 209
19 117
32 99
66 147
84 258
177 296
266 693
7 38
3 4
3 46
190 600
47 422
2 5
3 3
2 2
37 81
30 117
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Exercise Control
Plasma chemokines (pg/ml)
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MIP-1β
MIP-2
G-CSF
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2 62
30 88
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4 18
3 3
3 10
37 222
30 300
6 4
3 26
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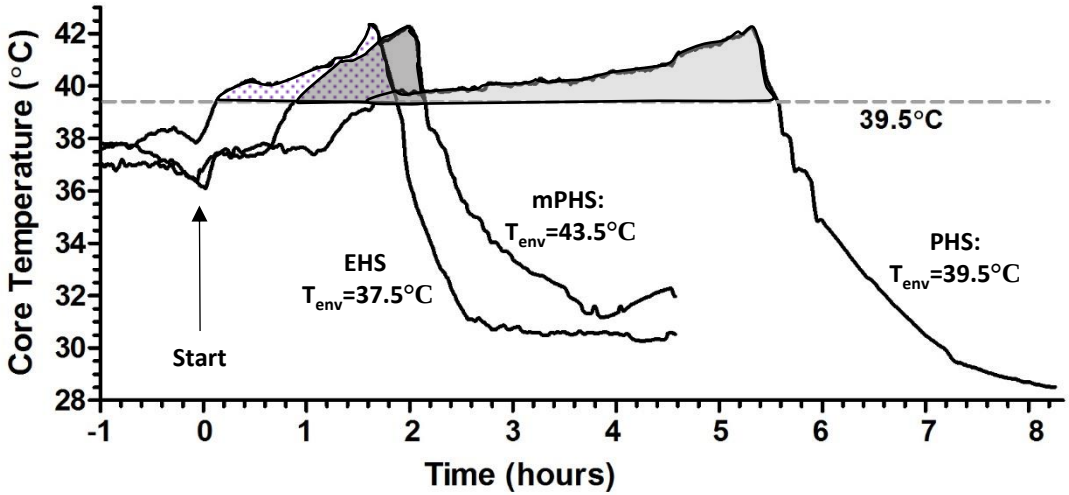
Core Temperature (°C)
42
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34
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30
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-1
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1
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3
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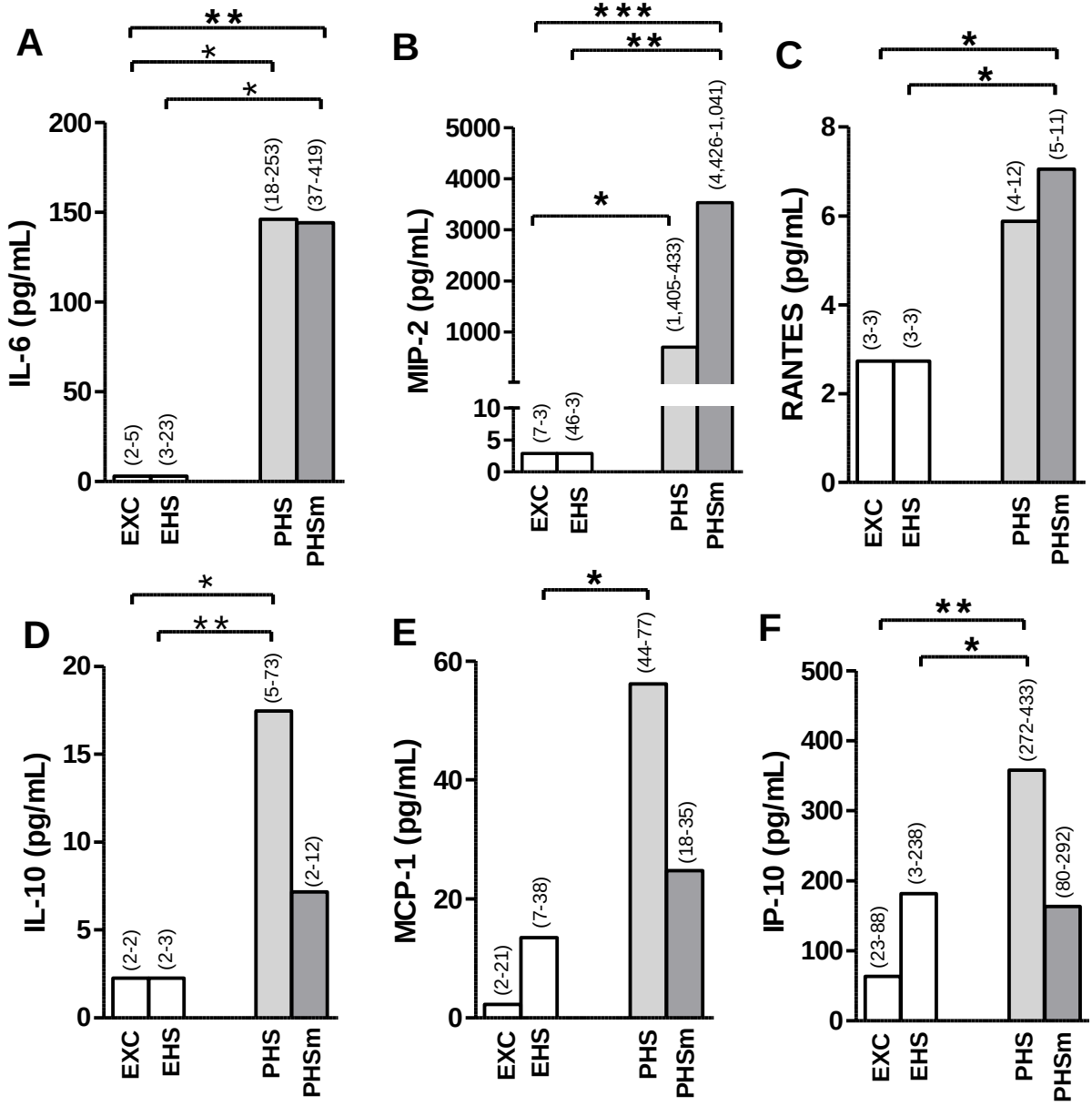
Time (hours)
39.5°C
mPHS:
T_{env}=43.5°C
PHS:
T_{env}=39.5°C
EHS
T_{env}=37.5°C
Start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IL-6 (pg/mL)
200
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(18-253)
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EXC
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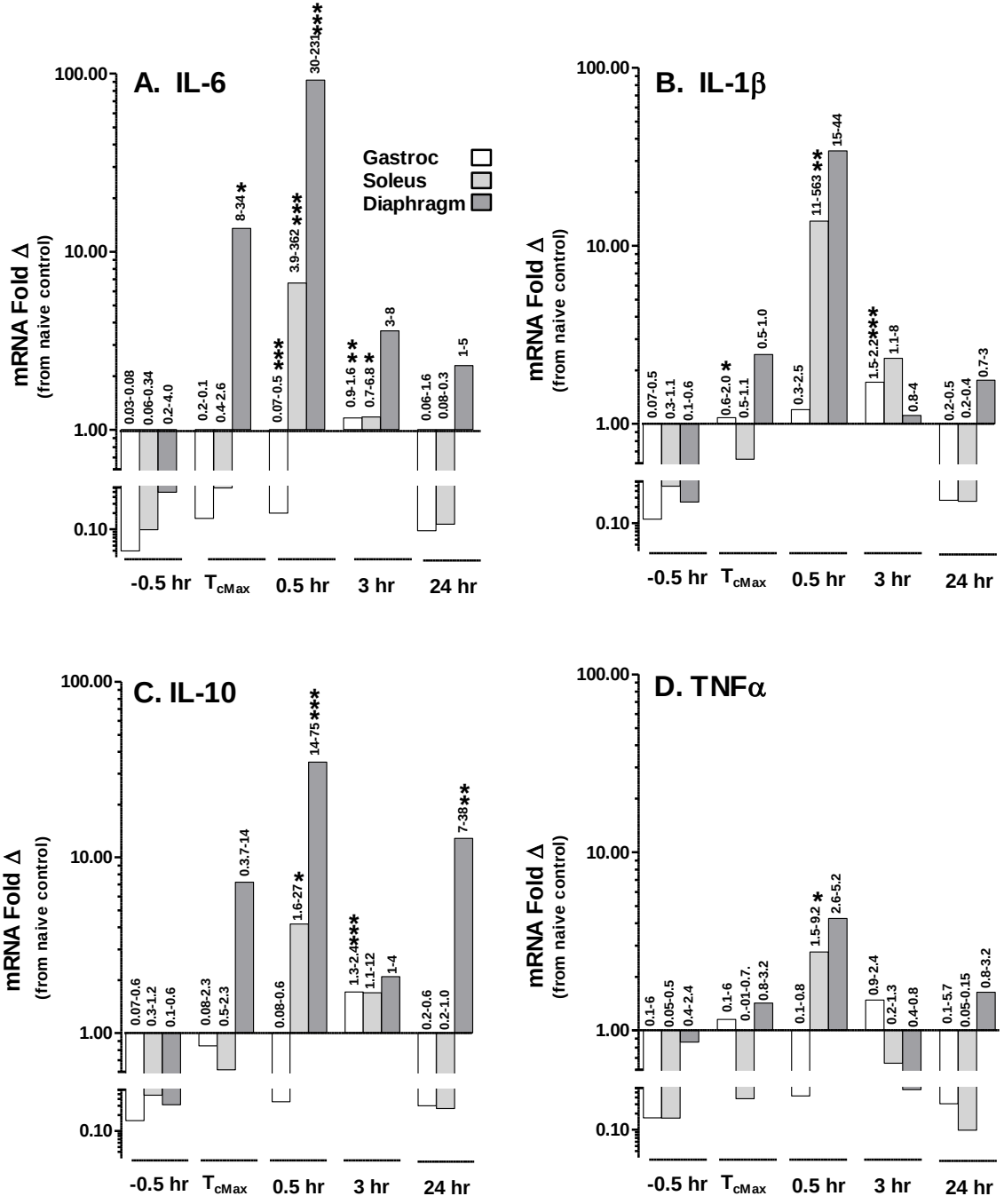
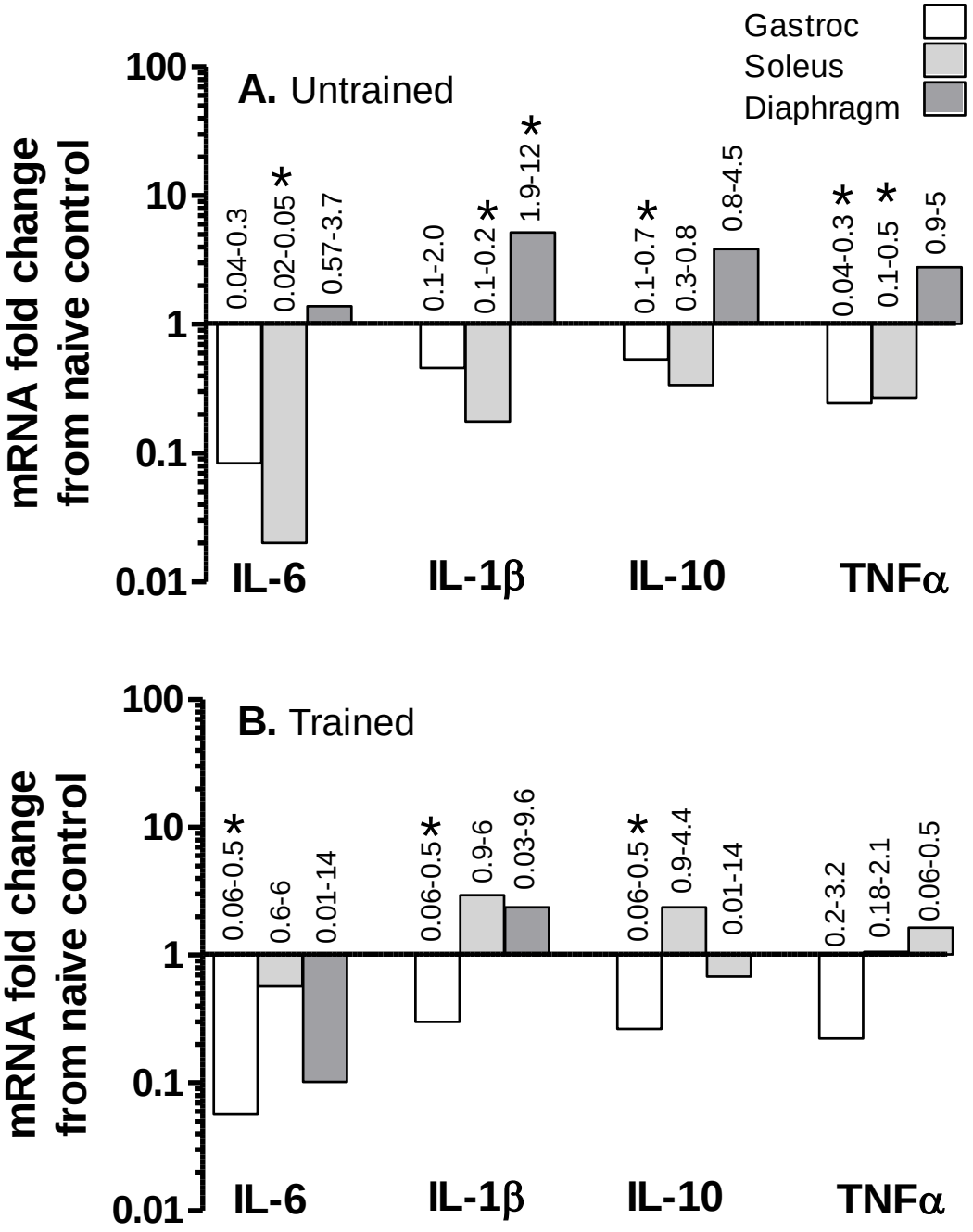
PHSm
B
MIP-2 (pg/mL)
5000
4000
3000
2000
1000
10
5
0
***
**
*
(7-3)
(46-3)
(1,405-433)
(4,426-1,041)
EXC
EHS
PHS
PHSm
C
RANTES (pg/mL)
8
6
4
2
0
*
*
(3-3)
(3-3)
(4-12)
(5-11)
EXC
EHS
PHS
PHSm
D
IL-10 (pg/mL)
20
15
10
5
0
*
**
(2-2)
(2-3)
(5-73)
(2-12)
EXC
EHS
PHS
PHSm
E
MCP-1 (pg/mL)
60
40
20
0
*
(2-21)
(7-38)
(44-77)
(18-35)
EXC
EHS
PHS
PHSm
F
IP-10 (pg/mL)
500
400
300
200
100
0
**
*
(23-88)
(3-238)
(272-433)
(80-292)
EXC
EHS
PHS
PHSm


A. IL-6
B. IL-1β
C. IL-10
D. TNFα
mRNA Fold Δ
(from naive control)
100.00
10.00
1.00
0.10
Gastroc
Soleus
Diaphragm
0.03-0.08
0.06-0.34
0.2-4.0
0.2-0.1
0.4-2.6
8-34 *
0.07-0.5 ***
3.9-362 ***
30-231 ***
0.9-1.6 **
0.7-6.8 *
3-8
0.06-1.6
0.08-0.3
1-5
0.07-0.5
0.3-1.1
0.1-0.6
0.6-2.0 *
0.5-1.1
0.5-1.0
0.3-2.5
11-563 **
15-44
1.5-2.2 ***
1.1-8
0.8-4
0.2-0.5
0.2-0.4
0.7-3
0.07-0.6
0.3-1.2
0.1-0.6
0.08-2.3
0.5-2.3
0.3.7-14
0.08-0.6
1.6-27 *
14-75 ***
1.3-2.4 ***
1.1-12
1-4
0.2-0.6
0.2-1.0
7-38 **
0.1-6
0.05-0.5
0.4-2.4
0.1-6
0.-01-0.7.
0.8-3.2
0.1-0.8
1.5-9.2 *
2.6-5.2
0.9-2.4
0.2-1.3
0.4-0.8
0.1-5.7
0.05-0.15
0.8-3.2
-0.5 hr
T cMax
0.5 hr
3 hr
24 hr


Gastroc
Soleus
Diaphragm
A. Untrained
B. Trained
mRNA fold change from naive control
100
10
1
0.1
0.01
0.04-0.3
0.02-0.05 *
0.57-3.7
0.1-2.0
0.1-0.2 *
1.9-12 *
0.1-0.7 *
0.3-0.8
0.8-4.5
0.04-0.3 *
0.1-0.5 *
0.9-5
0.06-0.5 *
0.6-6
0.01-14
0.06-0.5 *
0.9-6
0.03-9.6
0.06-0.5 *
0.9-4.4
0.01-14
0.2-3.2
0.18-2.1
0.06-0.5
IL-6
IL-1β
IL-10
TNFα