Invited Paper, Best Paper Award

A systems biology approach to heat stress, heat injury and heat stroke

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ABSTRACT

Heat illness is a major source of injury for military populations in both deployed and training settings. Developing tools to help leaders enhance unit performance while reducing the risk of injury is of paramount importance to the military. Here, we review our recent systems biology approaches to heat stress in order to develop a 3-dimensional (3D) realistic thermoregulation model, identify the molecular basis and mediators of injury, and characterize associated biomarkers. We discuss the implications of our work, future directions, and the type of tools necessary to enhance force health protection in the future.

Keywords: Heat stress, Transcriptomics, Proteomics, Systems biology, Protein aggregation, Metabolomics, Energetics, Computational modeling

1. HEAT ILLNESS IN THE U.S. ARMED FORCES

Hyperthermia, heat cramps, heat exhaustion, heat injury, and heat stroke represent a continuum of disorders termed heat illness [1-3]. External or internal heat sources, drugs, and/or disease impair heat dissipation, leading to elevated core temperature (Tc) [4-6]. Military operations place military personnel at greater risk of developing heat-related illnesses. Dehydration, circulatory collapse, and severe hyperthermia can eventually lead to heat stroke, defined as neurological dysfunction associated with a body temperature higher than 40°C (104°F) in humans [3, 7, 8].

Over 2000 heat-related injuries requiring hospitalization, including 324 cases of heat stroke, occurred throughout the U.S. Armed Forces during 2013 [4]. In a five-year retrospective study, 10,319 cases of heat injury required medical resources to treat, including 1872 cases of heat stroke [9, 10]. The actual incidence is projected to be considerably higher when considering undocumented instances that never reach triage [9, 10]. While sudden death primarily occurs in individuals with pre-existing cardiac abnormalities, organ failure secondary to rhabdomyolysis, or acute onset of organ failure may occur as the result of heat injury/stroke [6]. Systemic inflammatory response syndrome (SIRS) is also a primary cause of organ dysfunction related to heat stroke [3, 11, 12], leading to severe encephalopathy, rhabdomyolysis, acute renal failure, acute respiratory distress syndrome, myocardial injury, hepatocellular injury, intestinal ischemia, pancreatic injury, and hemorrhagic complications [3].

Assuming $6200 per day and 3.2 day average hospital stay [13], 2500 annual cases of heat injuries and heat stroke per year could cost the DoD $50 million per year (USD). Other costs include lost duty days, and the training investment associated with the Service member upon discharge for medical reasons. Further, the 30-year mortality rates from heart, kidney, and liver failure are increased by 40% in Service members with a history of heat stroke [5, 6]. Indeed, there is an urgent need for computational models and molecular markers of susceptibility to avoid scenarios that put Service members at greater risk, and biomarkers of early organ injury and recovery to improve diagnosis, treatment, and return to duty decisions.

2. A SYSTEMS BIOLOGY APPROACH TO HEAT ILLNESS

U.S. Army Medical Research and Materiel Command (USAMRMC) supports several systems biology studies, particularly in the areas of coagulopathy, infection, and post-traumatic stress disorder. Complex physiological disorders, such as heat illness, also lend themselves to a systems biology approach. Two common elements of systems biology include computational models and integrative approaches. Classical physiological studies of heat

Advances in Global Health through Sensing Technologies 2015, edited by Šárka O. Southern, Isaac R. Rodriguez-Chavez, Claudia Gärtner, Jonathan D. Stallings, Proc. of SPIE, Vol. 9490, 949009 · © 2015 SPIE · CCC code: 0277-786X/15/$18 · doi: 10.1117/12.2183870

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
31 AUG 2015	**Research**	**00-01-2014 to 00-05-2015**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
A systems biology approach to heat stress, heat injury and heat stroke	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Jonathan Stallings; Danielle Ippolito	5e. TASK NUMBER **X1**
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
U.S.Army Center for Environmental Health Research,568 Doughten Drive,Fort Detrick,MD,21702-5010	**15-64**
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, MD, 21702-5010	**USAMRMC**
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES
Published in Proceedings of SPIE, Vol. 9490 949009-1

14. ABSTRACT
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15. SUBJECT TERMS
heat stress, transcriptomics, proteomics, systems biology, protein aggregation, metabolomics, energetics, computational modeling

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**12**	

illness, however, have relied on *in vivo* monitoring of a single core temperature to evaluate transient temperature measurements in animals. In some cases, these two dimensional heat curves are 'modeled,' but fail to provide predictive information about the temperature distribution and/or specific risks to individual organs that could not be otherwise determined through those classical means. Further, models do not exist to integrate molecular responses with a computational, predictive temperature distribution over a long period of time in multiple organs [14-16], or to provide organ-specific predictions about molecular responses to injury. Here, we describe our integrated computational and pan-omics approaches to develop new tools to protect Service members from heat-related injury.

2.1 Computational thermal regulation model

Recently, Reifman's group described an anatomically accurate, three-dimensional rat model of thermoregulation during heat stress [17], later verified in independent studies [18]. Rats were placed at 37°C until their core temperature reached 41.8°C (defined as $T_{c,Max}$) and compared to time-matched, unheated control animals. Depending on cellular metabolism and heat exchange with blood perfusion, visceral organs have different temperatures even under normothermic conditions [19]. Since thermal injury contributes to inflammatory responses [2, 3, 20], the evaluation of temperature distribution and identification of hot spots provided novel predictions about organ-specific thermal injury, organ dysfunction [20-22], and better understanding of injury progression.

The model combined a comprehensive description of heat transfer parameters with an accurate representation of the rat anatomy to demonstrate that monitoring the differential thermal response in each body organ is critical to assess the actual condition of the animal during heat stress. The model was the first to describe the differential circadian response to heat stress. The computational model can be used to simulate heat-stress scenarios to optimize experimental protocols at reduced cost with fewer experimental animals. Novel heat stress management techniques (e.g., localized cooling) could be designed and tested *in silico*, and in turn provide testable prediction that could be used to refine the model as appropriate. Since brain cooling shows promise in the recovery from heat stroke in rats [23], *in silico* designs and tests could accelerate methods to cool other organs at high risk for injury, such as liver and kidney [24, 25].

2.1 Utility of the computational model

Molecular indicators of heat injury can be integrated with computational models to identify and molecularly characterize 'hot spots' in spatial temperature distribution, suggesting predictive and diagnostic biomarkers of systemic inflammation and organ injury after heat stress. Combining imaging techniques with the physiological models and molecular indicators of injury may facilitate real time analysis of estimating tissue damage during heat-stress, progression of organ dysfunction, and/or recovery. The model could also simulate the efficacy of preventative measures (e.g., customized apparel) for decreasing incidence of heat illness among military operatives.

In summary, the model successfully predicted the thermal response in rats throughout heat stress, demonstrating the critical importance of monitoring the spatial distribution of temperature during heat stress. Quantification of the heat load in various organs by determining otherwise immeasurable indices (e.g., average temperature rise, maximum temperature, and volume fraction of organ reaching the fatal temperature) can be determined. Thus, the model complements experimental data and can be used for further understanding the complex processes involved in thermoregulation. Finally, the model enables identification of organ-specific risks during heat stress, potentially aiding in the development of improved clinical strategies for injury prevention and management.

3. PAN-OMIC PROFILES

3.1 Transcriptomics and proteomics

Bioinformatically mining the global changes in gene response induced by heat-stress in liver, lung, kidney, and heart at $T_{c,Max}$ suggested molecular corroboration of published physiological HSP response models at the pathway level [26]. Further analysis of our pan-omic profiles supported protein misaggregation as a molecular mechanism of heat-induced cardiac injury. Our results were the first to molecularly link protein misaggregation to *in vivo* heat stress-

induced injury and/or recovery. Analysis of heat-injured heart tissue by transcriptomics and quantitative global, mass spectrometry-based proteomics usingisotope tags for relative and absolute quantitation [iTRAQ] demonstrated enrichment in KEGG pathways consistent with protein folding disorders, mitochondrial dysfunction, and perturbation in cellular energetics (e.g., oxidative phosphorylation, Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, antigen processing and presentation, and cardiac muscle contractility) [26, 27].

Animals with histological evidence of heat-induced heart injury had a large shift in relative abundance of proteins with high supersaturation scores, suggesting increased abundance of proteins with a higher propensity to aggregate after heat stress. By 48 hours, the increased propensity to aggregate was more pronounced in animals with histopathological evidence of heat injury than those without cardiac injury. Thus, disruption in protein folding and presumptive aggregation could contribute to the persistent subcellular indicators of energy crisis and regulation of apoptosis observed in heat-injured animals. Under pathological conditions, pathogenic aggregates can form from misfolded proteins (e.g., Alzheimer's Disease) [28-33].

The propensity of a protein to form pathogenic aggregates can be accurately calculated from extrinsic and intrinsic factors influencing the propensity of a protein to aggregate, especially amino acid sequence [34, 35]. Even single point mutations can affect aggregation of a peptide or protein in its unfolded state [36]. Predictive algorithms can determine the propensity of unstructured polypeptides to aggregate, the absolute aggregation rate, and the most aggregation-prone regions of a sequence [28, 36-43]. Ciryam et al [27] demonstrated that certain pathways known to be associated with proteotoxic stress (e.g., Alzheimer's, Parkinson's, Huntington's Diseases) or large functional complexes (i.e., ribosome, proteosome, and oxidative phosphorylation) also have higher average propensities to aggregate. Inappropriate changes in protein conformation are clearly linked to disease through several distinct mechanisms involving improper degradation, improper localization, dominant-negative mutations, amyloid accumulation, and gain of toxic function [32].

Similarly, the consensus heat-stress response (cHSR) common to heart, kidney, liver, and lung was enriched for genes related to protein folding and the regulation of apoptosis, including many genes not previously linked to the *in vivo* heat stress response. In the ER, unfolded proteins stimulate the unfolded protein response (UPR) [32]. HSPs act as cellular chaperones in response to misfolded proteins [44-46], and stress-induced expression of HSPs has been implicated in many diseases [30-32, 47-49]. Global repression of translation accompanies the increased expression of HSPs and other stress-responsive genes [26, 50, 51] in our pan-omic studies.

Stress-dependent changes in RNA splicing, particularly in post-translationally spliced genes, can occur after heat shock [52-54]. Any heat-stress induced splicing events would necessarily alter the calculation of supersaturation scores and the propensity to aggregate by altering intrinsic characteristics of expressed proteins. Currently, we are evaluating alternatively spliced genes (see enriched pathways in Figure 1) and developing weighted scores for supersaturation and propensity to aggregate based on the relative abundance of RNA splice variants.

3.2 Circulating metabolomics

Integrating proteomics and transcriptomics data is complicated by disparities in gene and protein expression of biological and technical origin [55]. Biological variables include spatial and temporal differences in gene and protein expression patterns. Technical hurdles in proteomics data acquisition include large dynamic range of protein expression resulting in protein data gaps requiring compensation by complex statistical paradigms [55]. Complementing large scale proteomics and transcriptomics data sets with metabolomics analyses affords the advantage of predicting modulation in enzyme regulation in metabolic networks. Change in biochemical networks affords unique insight into the global metabolic response of an organism to an insult. Biochemical inter-relationships provide a system-wide assessment of an organism's energy consumption, lipid transport and storage, protein and nucleotide degradation, distribution of nutrients, and fatty acid storage, and the regulation of all of these processes by circulating hormone release in response to stimuli [56]. Recent technological advances in accurate-mass instrumentation have resulted in reliable reconstruction of an organism's biochemical networks in response to a stressor or disease state from various accessible biofluids, including plasma and serum [57].

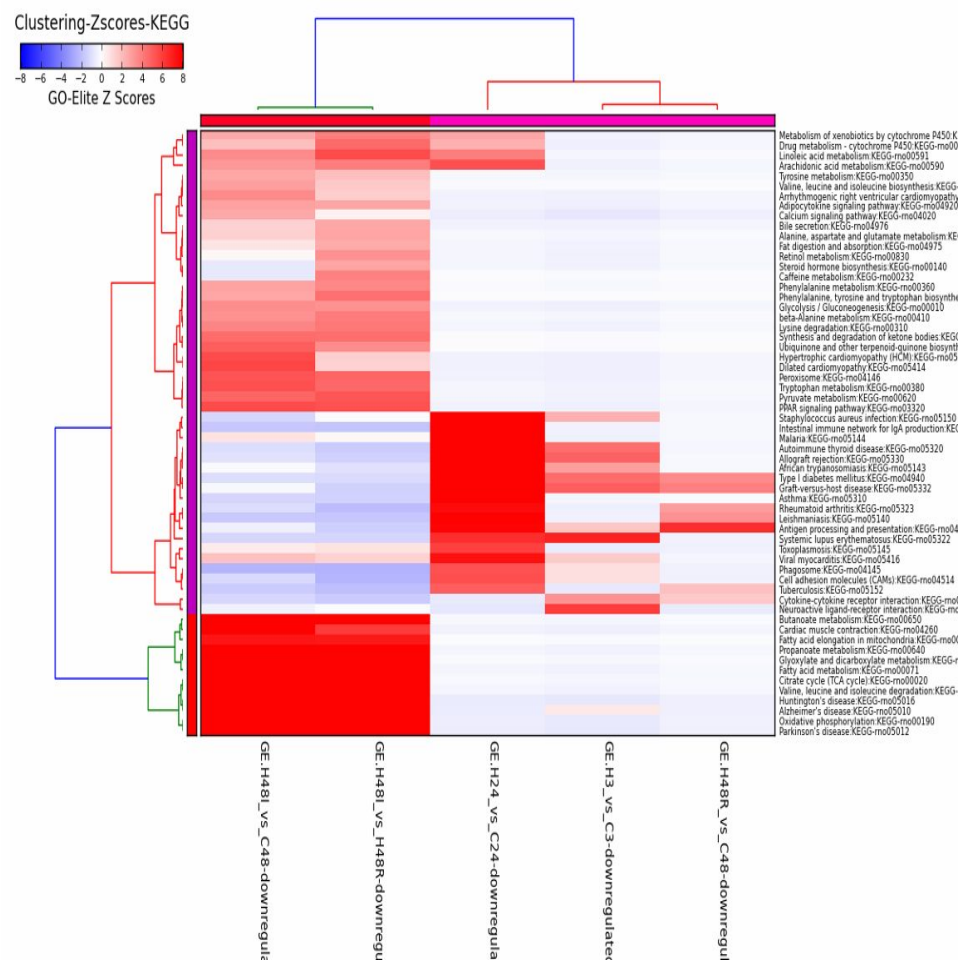

Figure 1. Key KEGG pathways involved in heat stress-induced heart injury, based on gene microarray and splicing data. Analysis of down regulated genes in heat-stressed animals with heart injury at 48 h (H48I) versus heat stressed animals that recovered at 48 h (H48R) or control animals (C48) demonstrated significant enrichment of KEGG pathways related to energy metabolism, oxidative phosphorylation, and protein folding disorders.

Assessing global plasma metabolomics profiles in relationship to gene and protein expression in specific organs affords insight into global biochemical perturbations linked to specific organ histopathology. Tissue-specific damage can be inferred from a global metabolomics analysis of plasma metabolite concentrations. Changes in metabolites comprising a given biochemical network are linked to the physiological functions governed by the networks with the most significant changes [58]. Integrating metabolomic profiling of plasma with proteomic and transcriptomic analysis provides a powerful predictive tool for gauging dynamic responses to genetic modification and pathophysiological stimuli. Quantifying perturbations in the global metabolic response to heat illness and subsequent organ injury affords unique insight into the coordination among biochemical, gene, and protein responses in the organism. Further, recent technological advancements allow unprecedented accuracy in global assessment of organism response to environmental stressors [59]. The metabolome is exquisitely sensitive to subtle changes in physiology (e.g., stress, changes in temperature, food intake, etc.). Biochemical network analysis must overcome this biological sensitivity by accurately discriminating normal physiological fluctuation from pathological distress. Assessing change in metabolic networks instead of individual metabolites can differentiate normal physiological variation from pathological perturbation.

The pathogenesis of heat illness is multifactorial and subject to significant inter-individual variability in magnitude of response to heat exposure. Heat illness derives from prolonged dysregulation of multiple, interconnected biochemical and physiological networks [60]. The clinical presentation of overt heat illness often does not manifest for 48 hours post-exposure, and may persist for several months [61]. In rat models of heat stress and thermoregulation [17, 18, 26, 62, 63], global changes in protein and gene networks associated with energy production implicate proteolysis, energy crisis, and protein aggregation in the pathogenesis of heat illness.

Plasma biochemical changes were compared with tissue protein/gene expression changes in rats with histopathological evidence of heat-induced injury, unheated controls, and uninjured, heat-stressed controls. Global plasma metabolomics analysis indicated biochemical profiles unique to heat stress, heat stress without injury, and heat injury in cardiac tissue. Perturbations in biochemical networks controlling energy production and use, lipid mobilization and storage, and cell death were comparable to transcriptomics and proteomics network analysis [26]. Metabolic perturbations in the plasma reflected perturbations in the protein signal transduction cascades.

The metabolic pathway analysis supports perturbation in metabolic networks affecting energetics and cell death [26]. Acceleration of pyrimidine degradation was hastened at $T_{c,Max}$, most notably reflected in an increase in 3-ureidopropionate and a concomitant increase in the metabolic products 5,6-dihydrouracil and uracil. By 48 hours, the elevation in 3-ureidopropionate was accompanied by a *decrease* in metabolic biproducts, suggesting upregulation in dihydropyrimidinase, the enzyme catalyzing the reaction with persistent nucleotide degradation suggesting cell death. Increased acetylation and sulfation indicated an increase in post-translational modifications engaging protein degradation, supporting a persistent metabolic crisis precipitating cellular death. These increases were especially pronounced in plasma from animals with cardiac injury, especially N-acetylcytidine, N-acetylthreonine, 4-acetylpheonlsulfate, and 4-vinylphenolsulfate. Elevated glutathione metabolism (e.g., increased oxidized glutathione) supported persistent redox stress initiated at time of injury ($T_{c,Max}$) and persisted to 48 hours.

Perturbations in urea cycle components (e.g., arginine, citrulline, ornithine, urea) coupled with changes in concentrations of other amino acids (e.g., asparagine, proline, and the branched chain amino acids isoleucine, valine, and leucine) supported persistent degradation of amino acids with a concomitant decrement in urea excretion, both indicators of cellular death and/or autophagy. Heat-induced perturbations in TCA cycle intermediates (e.g., citrate, malate, succinate, and α-ketoglutarate), β-oxidation components (e.g., hexanoylcarnitine, palmitoylcarnitine, stearoylcarnitine, and oleoylcarnitine), co-factors for amino acid oxidation (e.g., pyroxidal and pyroxidate) and ketone bodies (β-hydroxybutyrate) collectively indicated compromised mitochondrial function and deficits in cellular respiration. Persistent elevation in cholesterol-derived steroidal hormones (e.g., corticosterone) suggested increased inflammatory and stress responses persisting to 48 hours after heat stress.

Finally, a persistent and pronounced decrement in plasma bile acids supported liver dysfunction and decreased intestinal uptake of bile acids necessary for cholesterol production. Hierarchical clustering analysis supported the following indicators of heat stress and/or persistent cardiac injury at 48 hours: 5,6'-dihydrouracil and 3-ureidopropionate (nucleic acid catabolism), ornithine (urea cycle/energetics), N1-methyladenosine and pyroxidate (amino acid oxidation and degradation), taurodeoxycholate (bile acid), 7-HOCA and corticosterone (steroidal stress hormones regulating cholesterol synthesis and fatty acid transport), and sulfated glutathione (redox stress).

Metabolites, gene expression, and protein profiles collectively indicate a crisis in cellular energetics in response to heat stress [26, 64]. All profiles converge on mitochondrial oxidative phosphorylation as a key metabolic pathway dysregulated in response to heat-related illness. Oxidative phosphorylation represents the final biochemical process in cellular respiration necessary for the physiological process of cardiac muscle contraction [56, 65]. Comparative analysis of protein, gene, and metabolic indicators indicates considerable overlap of protein, gene, and metabolic contributors to energetics and contraction, the primary functional consequence of energy production in the heart (Figure 2). In oxidative phosphorylation, 36/44 (81.8%) of the proteins matched transcript changes. In cardiac muscle contraction, protein-gene matches were 73.9% (17/23).

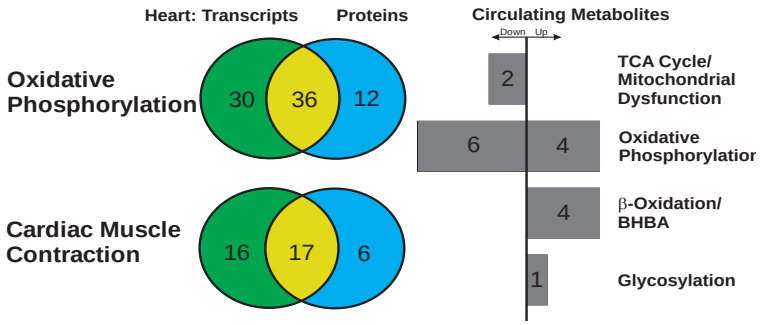

Figure 2. Venn diagrams show congruence between tissue transcriptomics and proteomics with respect to KEGG pathways that were enriched in the studies. When evaluating the circulating metabolites, several alterations coincide with the KEGG pathways.

A physiological model integrating the plasma metabolomics data supports system-wide deficit in energy stores required for adequate cardiac function (see Figure 3). This model is supported at the pathway level by transcriptomics and proteomics analyses [26]. Combined analysis of tissue transcriptomics, tissue proteomics, and plasma metabolomics suggests that heat stress fundamentally compromises the mobilization of fuel sources available to cardiac tissue from liver, muscle, and fat. Cardiac tissue is particularly susceptible to shortages of metabolic precursors for energy production (e.g., ketones, lactate, glucose, amino acids, and fatty acids). The heart muscle's cycle of contraction and relaxation places the heart in a continuous state of aerobic work. The cellular composition of cardiomyocytes reflects this energy demand. More than half of the cellular volume of cardiomyocytes is mitochondria. Although the heart requires a continuous supply of energy, it does not store glycogen or lipids in large quantities. Instead, the heart relies on uptake of free fatty acids, glucose, and ketone bodies from the circulation, originating from the liver and lipid stores. Deficits in liver and lipid mobilization of energy stores in injured cardiac tissue suggest a fundamental change in lipid and amino acid distribution and glycolysis, supporting a model of diminished energy stores available for normal cardiac functioning. Metabolic evidence of a shortage of energy reserves from the circulation combined with protein aggregation and proteolysis within the cardiac tissue itself are plausible mechanisms for the observed effects in the histopathology of these animals [26].

4. INTEGRATING PHYSIOLOGICALLY BASED MODELS WITH OMICS KNOWLEDGE

Our work in heat-stressed animals provides the basis for developing a computational physiological model of heat stress and recovery, an assessment of global gene, protein, and metabolomic responses, and a computationally derived aggregation score that could be integrated with future heat-stroke–induced organ injury experiments. Top-down integration of heat-induced changes in metabolomics, proteomics, and transcriptomics (to include small RNA) will provide the foundation for a computationally based linkage to human 3D thermoregulation models. Anchoring these systemic stress responses to the physiological model of heat stroke will provide further insight into predicting risk, severity, and timing of organ injury in response to hyperthermia. Such a model will potentially accelerate the development of strategies to improve prevention, classification, and treatment of heat-related illnesses.

Computational modeling could potentially relate the changes in molecular indicators of cardiac injury to a functional physiological consequence. The Cardiac Physiome project seeks to computationally relate ATP generation to contraction and relaxation during each heart beat [66]. Models of cardiomyocyte contraction define the variables of glycolysis, citric acid cycle, fatty acid oxidation, and oxidative phosphorylation needed to produce ATP, the molecular unit of energy shuttled into the sodium/potassium and myosin ATPase pumps fueling cardiac muscle contraction [67, 68]. If expression changes in genes, proteins, and metabolites could computationally predict a quantifiable decrease in the number of molecules of ATP produced, then computational models could predict the impact of the molecular changes on cardiac function [66-68].

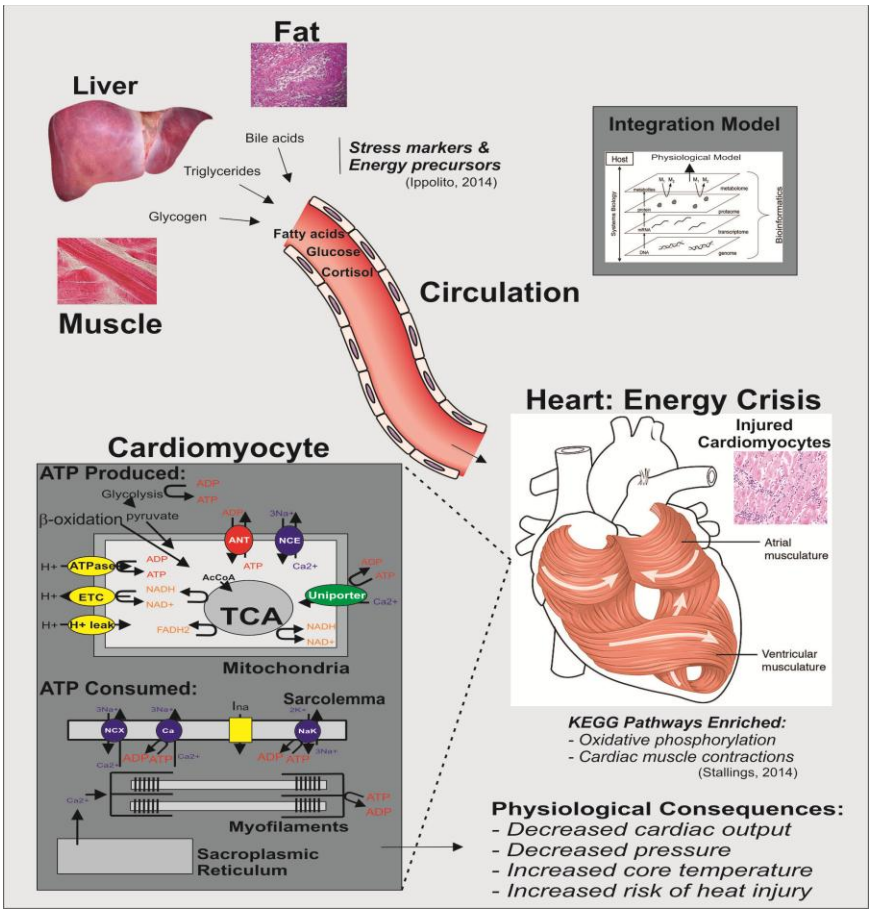

Figure 3. Theoretical Model. Precursors from muscle, fat and liver are altered in the circulating metabolome. As a result, there is an energy crisis in the heart, as evidenced by histopathology showing cardiomyocyte degeneration, and enriched KEGG pathways including oxidative phosphorylation. Quantitatively estimating subsequent reduction in available ATP resulting from reduced availability of circulating glucose and triglycerides for glycolysis and β-oxidation in the cardiomyocyte can estimate deficits ATP available for the sodium/potassium ATPase. As postulated above, the net reduction in available ATP could predict decrements in cardiac output, pressure and other physiological parameters that could be anchored to the thermoregulation model.

Metabolites, gene expression, and protein profiles collectively implicate a crisis in cellular energetics in response to heat stress (Figures 1 - 2). Glycolysis, TCA cycle, and fatty acid β-oxidation converge on mitochondrial oxidative phosphorylation as a key metabolic pathway dysregulated in response to heat-related illness. Oxidative phosphorylation represents the final biochemical process in cellular respiration necessary for the physiological process of cardiac muscle contraction [56, 65] . Thus, the observed metabolic dysregulation of a significant proportion of representative biochemical mediators in glycolysis, TCA cycle, fatty acid β-oxidation, and oxidative phosphorylation indicates a limiting supply of NADH and ultimately ATP for operating the calcium, sodium/potassium and myosin ATPase pumps providing the energetic input for cardiac myocyte contractility (Figure 3).

The pan-omics data indicate downregulation in all four of these energetics processes. Each of these processes yields NADH and/or FADH for entry into the oxidative phosphorylation respiratory chain to produce ATP. NADH and FADH are converted in stoichiometric ratios to ATP, which is directly converted to quantifiable units (kJ) of energy when expended in powering the calcium, myosin and sodium/potassium ATPase pumps. Quantitatively estimating a percent decrement in available ATP can be used to computationally model subsequent deficits in calcium, myosin, and sodium/potassium pump activity and, by extension, cardiac output [66] (Figure 3). The cardiac

sodium/potassium ATPase, for example, requires 5.7 kJ of energy from ATP per mole of solute transported, where the change in free energy for transport, ΔG_t , is described by the following equation:

$$\Delta G_t = RT \ln (C_2/C_1),$$

where R is the gas constant 8.315 kJ/mol ● K, T is the absolute temperature, and C_1 and C_2 are the solute concentrations on either side of the traversed membrane.

Hydrolysis of ATP to ADP + inorganic phosphate is highly exorgenic, generating 30.5 kJ/mol of available energy. ATP produced by the cardiac mitochondria supplies the calcium, myosin, and sodium/potassium ATPases fueling cardiac muscle contraction. Thus, altered abundance of genes, proteins, and metabolites in biochemical pathways governing fundamental energetic processes is the most direct relationship between changes in molecular endpoints and measurable changes in cardiac physiology. The hypothalamic-pituitary axis and lipid distribution from the liver and skeletal muscle indirectly affect the availability of ATP for cardiac muscle function. Adrenal glucocorticoids and circulating triglycerides regulate availability of fuel sources taken up from the circulation by the heart and converted to ATP in the cardiomyocyte cytosol and/or mitochondria.

Metabolomic analysis indicates that cortisol expression was significantly elevated in response to heat stress and heat injury [63]. Cortisol increases in response to stress-activated neuro-endocrine hypothalamic-pituitary axis [69]. Cortisol also regulates lipid and glucose transport from liver to heart, and could potentially be incorporated into a model predicting the availability of fuel sources in cardiac tissue [70, 71].

Metabolomics analysis, corroborated by proteomics and transcriptomics profiles, also implicates an imbalance in lipid transport with cardiac injury [63]. Circulating triglycerides are taken up by the heart as fuel sources. Fatty acid β-oxidation is highly exorgenic. One molecule of palmytoyl-CoA yields 131 ATP as direct products of β-oxidation itself or the subsequent entry of NADH into the TCA cycle and, ultimately, the electron transport chain [56]. Lipid transport has been successfully modeled in lipidmetabolism-related aging [72]. Defining reductions in triglycerides can render a quantifiably verifiable model of impaired lipid transport in heat-injured animals [73, 74]. Quantitatively estimating subsequent reduction in available ATP resulting from reduced availability of circulating triglycerides for β-oxidation in the cardiomyocyte can estimate deficits ATP available for the myosin, calcium, and sodium/potassium ATPases. As postulated above, the net reduction in available ATP could predict decrements in cardiac output (see Figure 3).

Ultimately, models linking molecular changes with measurable physiological and functional outputs such as cardiac output and contractility can theoretically be incorporated into the existing thermoregulation model. The resulting computational framework could be used to directly or indirectly link spatial and temporal thermal “hot spots” with molecular changes predictive of histopathological and functional organ damage. Research is underway in our laboratory to integrate physiological and molecular endpoints into the thermoregulation model of heat stress and injury.

5. COMPETING INTEREST AND DISCLOSURE STATEMENT

Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the “Guide for Care and Use of Laboratory Animals” (NRC 2011) as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council in facilities that are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. The authors have declared that they have no competing interests. The views, opinions, assertions, and/or findings contained herein are those of the authors and should not be construed as official US Department of Defense or Department of the Army position, policy, or decision, unless so designated by other official documentation. Citations of commercial organizations or trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations. This paper has been approved for public release with unlimited distribution.

6. ACKNOWLEDGMENTS

The research was supported by the Military Operational Medicine Research Program, US Army Medical Research and Materiel Command, Fort Detrick, Maryland.

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