9-1-2015

Coordination Between Autophagy and the Heat Shock Response: Evidence From Exercise in Animals and Humans

Nathan H. Cole

Nathan H. Cole
Candidate

Health, Exercise, & Sports Sciences
Department

This thesis is approved, and it is acceptable in quality and form for publication:

Approved by the Thesis Committee:

Christine M. Mermier, Chairperson

Karol Dokladny

Orrin B. Myers

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by

NATHAN H. COLE

B.S. University Studies, University of New Mexico, 2013

THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE
PHYSICAL EDUCATION
CONCENTRATION:
EXERCISE SCIENCE

The University of New Mexico
Albuquerque, New Mexico

July, 2015

Acknowledgments

I would like to thank Dr. Christine Mermier for her unwavering guidance, support, generosity, and patience (throughout this project, and many that came before it); Dr. Orrin Myers for all his assistance in moving from the numbers to the meaning (not to mention putting up with my parabolic model); Dr. Karol Dokladny for breaking trail in the lab, and for reminding me at the outset not to loose my joy and enthusiasm somewhere in the literature; and Dr. Pope Moseley for providing the conceptual framework upon which this project was built, as well as the opportunity to pursue it.

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Abstract

Proteins play a critical role in nearly every biological activity. In consequence, organismal health and homeostasis often hinges on the ability of intracellular regulatory systems to sustain the quality and function of these diverse, structurally complex macromolecules. Correct protein function depends on correct form, and during periods of destabilizing cellular stress, protein quality is managed in part by the heat shock response, which acts to support, isolate, and reform new or damaged proteins, and in part by the autophagic recycling of abnormal proteins, cytotoxic protein aggregates, and terminally damaged organelles. We conducted a pooled analysis of available research in humans and rodents regarding heat shock and autophagic activity through the unique proteostatic challenges presented by acute exercise and the post-exercise progression from catabolism to anabolism. This analysis reinforces a model of regulatory coordination between these protein management pathways, offering interspecies support for an Hsp70-moderated transition away from the presiding catabolic influence of autophagy in the immediate post-exercise window, toward an anabolic phase of restoration and remodeling. This relationship has already been demonstrated with direct human cellular research, and may help shed light on the molecular underpinning of epidemiological associations between health and physical activity. Differential responses were also observed in these two primary proteostatic systems according to exercise intensity and tissue of origin, which may have important implications for research design, and perhaps eventually for exercise prescription.

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Abbreviations

AA – amino acid
ADP – adenosine diphosphate
Akt – Protein kinase B
AT – anaerobic threshold
Atg – autophagy-related protein
ATP – adenosine triphosphate
cm – centimeter
cm/min –centimeters per minute
CMA – chaperone mediated autophagy
DNA – deoxyribonucleic acid
FoxO – forkhead box O protein
GXT – graded exercise test
h – hour
HSP – heat shock protein
HSR – heat shock response
Hsc70 – heat shock 70 kDa protein 8, HSPA8
Hsp60 – 60 kDa heat shock protein
Hsp70 – 70 kDa heat shock protein
Hsp90 – 90 kDa heat shock protein
IGF-1 – insulin-like growth factor 1
kDa – kilodalton
kg – kilogram
km – kilometers
km/h – kilometers per hour
LAMP-2A – lysosome-associated membrane protein type 2A
LC3 – microtubule-associated protein 1 light chain 3
LT – lactate threshold
METs – metabolic equivalents (1 MET = 3.5 ml/kg/min $\dot{V}O_2$)
METhour – METs * exercise duration (h)
m – meters
min – minute
ml – milliliter
mTOR – mammalian target of rapamycin
m/min – meters per minute
m/s – meters per second
MVC – maximal voluntary contraction
NEF – nucleotide exchange factor
PBMC – peripheral blood mononuclear cell
PI3P – phosphatidylinositol 3-phosphate
r – Pearson product-moment correlation coefficient
R^2 – coefficient of determination
ROS – reactive oxygen species
s – second
ULK1 – Unc-51-like kinase 1
ULK2 – Unc-51-like kinase 2
UPS – ubiquitin−proteasome system
W – watts

Chapter 1
Review of Literature

The purpose of this chapter is to:

1) Outline proteomics, alongside the general importance of, and challenges to, intracellular proteostasis.
2) Summarize the respective contributions of autophagy and the heat shock response to cellular proteostasis, as well as the regulatory pathways understood to coordinate their operation.
3) Evaluate available research regarding the significance of autophagic and heat shock processes to whole-organism health and disease.
4) Place a special focus on the coordination of these processes in mediating the unique proteostatic challenges presented by physical activity in skeletal muscle.

Proteins, Proteomics, and Proteostasis

As advancing technology and intense research effort have combined to enhance our understanding of genetics in recent decades,[1] academic interest has begun to shift from a narrow focus on the genetic blueprint, a sometimes-overwhelming compendium containing all necessary information for the lifecycle of a single organism, to a complementary study of the proteins expressed from those instructions, and how these proteins interact on a macro scale.[1,2] The specific complement of proteins expressed by a genome at the whole organism, tissue, or cellular level has come to be known as a proteome, while proteomics refers to any post-genomic study of such a protein assembly, from the structural to interactional level.[1,2] Central to this emerging field of research is an investigation into the various mechanisms by which these protein ecosystems are able to maintain an adaptive, functional equilibrium in the face of acute and chronic cellular stressors.[3] Maintaining the operational status quo of intracellular protein systems through a responsive cycle of synthesis, maintenance, and elimination is a process termed protein homeostasis, or proteostasis, and is accomplished by a tightly-regulated network of protein management pathways responsible for overseeing the conformational integrity, interactive capability, physical location, overall concentration, and eventual catabolism of

individual proteins comprising the proteome.[3,4] This formidable level of regulatory oversight is fundamentally necessitated by the intricacy, delicacy, and diversity of protein species; as well as the dynamic environment in which they operate, and the complexly evolving needs of the physiological system they support.[3,4]

Unlike DNA molecules, which are intrinsically stabilized by the structural symmetry of their complementary base pairs, and only required to perform localized folding (coiling), proteins are manufactured in an inherently disordered arrangement, and often require complex folding maneuvers to obtain a functional conformation.[5] Moreover, most mammalian cells rely on more than 10,000 individual protein varieties for normal function, each synthesized as linear polypeptide chains that can extend to several thousand amino acids in length.[5] In 1969, Cyrus Levinthal noted that even a simple, theoretical protein consisting of 150 amino acids contains enough potential variation about the individual peptide bonds in its primary sequence to allow an estimated 10^{300} disparate secondary conformations, while completely denatured simple proteins observed *in vitro* are reliably able to reestablish a fully-functional, native conformation in a matter of seconds.[6] Given that these polypeptide chains could not possibly evaluate all, or even most, of the available configurations in a space of seconds, the speed at which they are able obtain a correct, complex structural organization from their most disordered state has been termed Levinthal's paradox, and progress toward elucidation has required the cooperative effort of molecular biologists, chemists, physicists, and mathematicians.[5]

More than forty years later, theories regarding protein folding have evolved considerably, but remain central to molecular biology.[5,7] At a basic level, the scientific community has established that linear peptide chains are driven toward progressively

more stable intermediate configurations by the formation of non-covalent bonds and sequestering, hydrophobic interactions between individual amino acids at various points in the peptide sequence.[5] Given optimal conditions, folding is theorized to proceed along these lines until the primary protein backbone approaches its lowest free-energy state, representing the molecule's most stable, or 'native' configuration.[4,7,8] In real world conditions, conformational entropy, kinetic or enthalpic barriers, and the disruptive influences of a crowded, fluctuating intracellular environment often present nascent proteins with considerable opposition to both the obtainment and maintenance of a stable, functional native state.[4,5,7,8]

These ineluctable forces acting to confound the optimal progression of protein folding lead to a 'rugged' free energy landscape, as polypeptide chains are often diverted to sub-optimal intermediate forms, which are then required to overcome substantial entropic and/or enthalpic barriers before returning to the path toward a stable, native configuration, **Figure 1**.[5,7-9] These misfolded intermediates are not only lacking proper function, they also frequently expose unshielded hydrophobic residues, leaving them more vulnerable to damaging non-native interactions with other nearby proteins and intermediate forms.[9] Left unchecked, these non-native interactions can lead to the formation of dangerous protein aggregates, the accumulation of which has been associated with a growing number of pathologies, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis.[3,9]

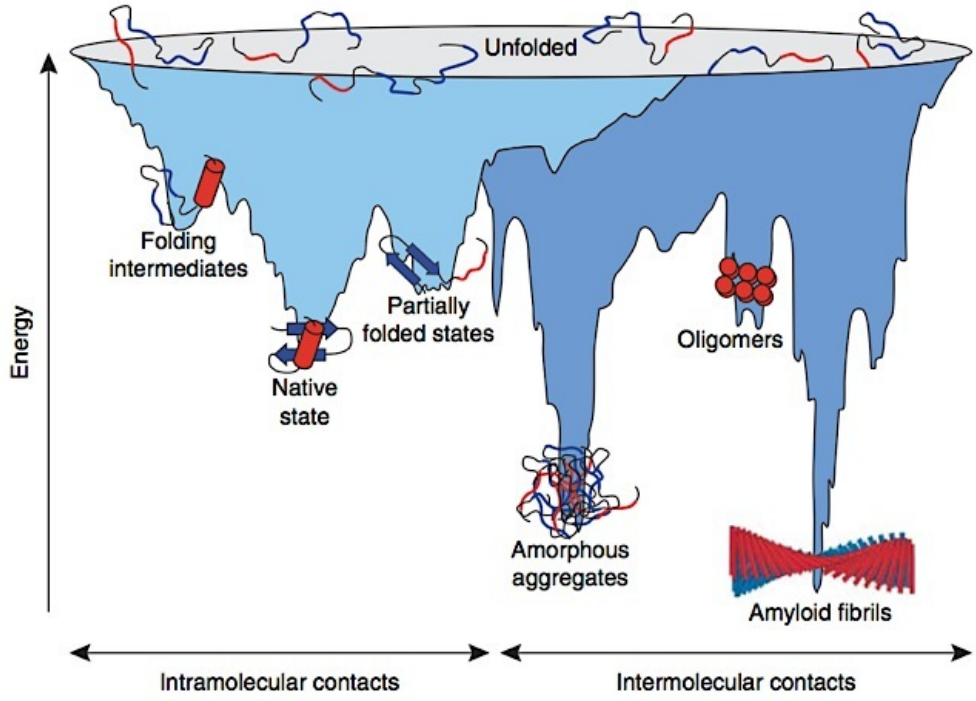


Figure 1: The rugged free-energy landscape of protein folding and aggregation.
Graphic reprinted without permission from Vabulas et al. 2010.[9]

Protein Management Systems

Considering the *in vivo* challenges confronting partially folded proteins in their journey toward the native state, as well as the logistical importance of these nascent forms achieving functional status, and the danger to nearby molecules that intermediate conformations can represent; it is not surprising that a network of ancillary molecules has developed to help smooth progress through the transitional phases of protein folding, while intracellular proteolytic systems have developed to sequester and destroy cytotoxic aggregate structures. In fact, numerous pathways and hundreds of individual proteins participate in maintaining these aspects of proteostasis.[4] Some of the most noteworthy and best characterized of these proteostatic mechanisms include the system of molecular chaperones, which facilitate proper protein folding while counteracting aggregation, and the process of autophagy, which is one of the primary intracellular mechanisms

responsible for the breakdown of irreparably damaged or aggregated protein structures.[4,10]

Molecular Chaperones

The molecular chaperones, as a class, are any of a structurally unrelated group of proteins found to interact with partially-folded intermediate conformations to limit further non-native interaction, enhance stability, or otherwise facilitate a correct progression to the native state without becoming incorporated in the final structure, **Figure 2**.[4]

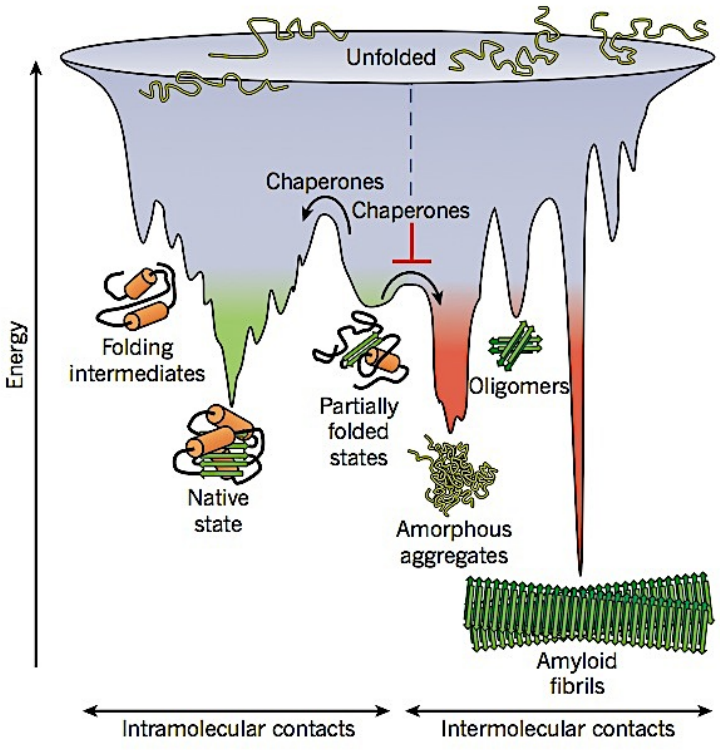


Figure 2: Molecular chaperones facilitate proper protein folding and counteract aggregation.
Graphic reprinted without permission from Hartl et al. 2011.[4]

These ancillary proteins are evolutionarily ancient and highly conserved, with varieties expressed in every form of life.[9,11] Approximately 20-30% of all proteins in

modern mammalian cells seem to be altogether incapable of correct *de novo* folding without the assistance of these facilitators.[4] In line with a broad course of development, numerous classifications or families of molecular chaperones exist, operating in conjunction to support the protein folding process in different ways.[4,9] Many chaperones have come to be known as heat shock proteins, or HSPs, because they are inducible by heat treatment, though this can be misleading, as any comparable form of cellular stress leading to sufficient protein damage will also upregulate the expression of these chaperones, including cold exposure, shifts in pH, osmolality fluctuations, or the stress of vigorous exercise in muscle tissue.[4,9,12] These HSPs are often classified according to their molecular weight in kilodaltons, with the Hsp70 (70 kDa heat shock proteins), Hsp90 (90 kDa heat shock proteins), and chaperonin families being the most prominently involved in the *de novo* folding of newly synthesized polypeptides and the amelioration of proteins that have become damaged.[4,9] These each depend on ATP as well as regulatory interactions with various cofactors and supporting HSP families to perform repetitive, transient binding and enclosing maneuvers when exposed to unshielded hydrophobic residues in nearby intermediate structures.[4,9] Of the three families, the chaperonins and Hsp90 typically act in a more specialized capacity downstream of Hsp70.[9] As such, 20% of proteins folding *de novo* are estimated to require the assistance of Hsp70, while an estimated 10% are passed by Hsp70 to the chaperonins for further modification, and only a small but vital fraction are eventually transferred Hsp90.[4,9]

The Hsp70 Family

The Hsp70 family is present in all cells except certain archaea, and includes both stress-inducible Hsp70, and the constitutively expressed homologue Hsc70 (heat shock

70 kDa protein 8, or HSPA8).[9,11] These proteins are not identical, and have been reported to differentially impact certain cellular processes, but are generally accepted to function similarly in their roles regarding protein folding and transport.[13] The Hsp70 class employs a less complicated interaction with folding intermediates than the other major chaperone families, and offers a broad first line of protection against misfolding or aggregation, while also often playing a cooperative transport role linking to other chaperone systems and proteolytic pathways.[4,9,14]

When free *in situ*, Hsp70 adopts an 'open' conformation with the allosteric binding of ATP, and becomes available for interaction with partially-folded polypeptides.[4,9,11] Transient binding of Hsp70 is then initiated as a ß-sandwich segment within the molecule recognizes an approximately seven-residue exposed segment presenting concentrated hydrophobic residues in nearby intermediate structures.[4,9,11] With the hydrolysis of ATP, the ß-sandwich segment performs a conformational shift, temporarily trapping a portion of the polypeptide chain.[4,9,11] The speed of the ATPase reaction is accelerated by Hsp40, also called DnaJ, a supportive Hsp family that can recruit Hsp70 to viable substrates, and may also assist with transporting damaged proteins to catabolic pathways.[4,15] Nucleotide exchange factors (NEFs) later catalyze the removal of ADP from the Hsp70 complex, allowing ATP to rebind and release the trapped portion of the polypeptide chain, leading to a cyclical opening and closing sequence in the continued presence of exposed substrate.[4,9,11]

The closed conformation of Hsp 70 protects vulnerable hydrophobic portions of the intermediate structure from more damaging non-native interactions leading to aggregation, and the release period often proves sufficient for fast folding proteins to

establish their native state.[4,11] Moreover, the repeated binding sequence that occurs when folding remains stalled provides continued protection for a flexible period, allowing transport of intractable or irreparable structures, and offering time for damaged or *de novo* intermediates to recover from temporary kinetic confinement or environmental stressors.[4,9]

The Chaperonins

Unlike simpler structures, complex proteins with slower folding trajectories may not be able to attain a stable conformation with this level of assistance alone.[9] These can be transferred to the chaperonins for more specialized attention by Hsp70 or intermediates like prefoldin.[4,9] The chaperonins are widely distributed, and divided into two classes: class 1, called the Hsp60 family or GroEL, are expressed in eukaryotic mitochondria and bacteria, while class 2, called TRiC or CCT, are expressed in eukaryotic cytosol and some archaea.[4,9] As large (~800-900 kDa) double-ringed structures, the chaperonins act alone (TRiC), or in conjunction with supportive Hsp10/GroES chaperones (Hsp60/GroEL), to partially or fully enclose intermediate structures, curtailing non-native interaction and creating a stable folding environment.[4,9] The chaperonins are capable of globally encapsulating structures up to 60 kDa, though some TRiC substrates are considerably larger, indicating that these chaperonins may assist with the folding of individual domains in larger protein structures.[4,9]

In a series ATP-dependent conformational shifts, the chaperonins construct a cage-like cavity, the interior of which is prominently lined with polar residues to create a net negative charge.[9,16] The polar surfaces of the enclosure's inner walls attract ordered water molecules, which is hypothesized to facilitate folding by forcing polypeptide chains

to bury hydrophobic residues.[9,16] The cavity remains stable for the time required to hydrolyze ATP, approximately 10-15 seconds in the case of Hsp60/GroEL, but potentially much longer for TRiC, offering a complex proteins a substantial period for uninterrupted folding.[4,9,16] As with Hsp70, enclosure and release occurs repeatedly in the continued presence of exposed hydrophobic substrate, leading to a cycle that may help dislodge maladroit folding patterns and resolve kinetic trapping within the molecule in a process called 'iterative annealing'.[9,17] As successive cycles of chaperonin confinement then also limit the conformational disorder of encapsulated polypeptides, these chaperones can assist partially-folded intermediates in overcoming both entropic and enthalpic folding barriers, in a process that may considerably extend the range of environmental conditions in which some complex proteins are able to successfully reach a native conformation.[4,9,17]

The Hsp90 System

Compared with other major molecular chaperone pathways, the Hsp90 system has proved difficult to elucidate.[4,18] This is because Hsp90 not only acts to stabilize protein folding, but also seems to sit at the center of a proteostatic network with diverse and critical influence, having been reported to impact apoptosis, cell-cycle signaling, mitotic signal transduction, telomere upkeep, vesicular transport, innate immunity, and targeted proteolysis.[4,9,18] Like Hsp70 and the chaperonins, the pincher-like dimer structure of Hsp90 performs cyclic, ATP-driven conformational shifts to assist protein folding.[4,9,18] Unlike most nucleotide-binding proteins, however, interaction with ATP does not fasten Hsp90 into a rigid conformation, instead shifting the conformational equilibrium toward a more closed operative conformation.[18] The unusual structural flexibility is likely

necessitated by diversity of Hsp90's interactions, which involve numerous substrates, facilitators, and co-chaperones.[4,18] Moreover, unlike other chaperones, Hsp90 does not seem to bind directly with fully denatured polypeptide chains, instead apparently restricting interaction to partially-formed intermediate states, suggesting that Hsp90 has a specialized role assisting certain complex proteins with successful stabilization late in their folding trajectories.[18] Many of these targets are critical signal transducers whose inherent instability is central to their roles as molecular switches, and Hsp90's ability to stabilize these complex molecules may have acted as a vital evolutionary capacitor, by allowing cells to successfully integrate unstable mutant proteins into complex cell signaling networks.[4,19]

The Heat Shock Response: Regulatory Control

As has been mentioned, cellular stress leading to significant protein denaturing instigates an upregulation of many molecular chaperones, including Hsp70 and Hsp90.[4,9,20] Main transcriptional control of this stress response has been traced to so called heat shock factors (HSFs), three of which are expressed in mammals.[9,20,21] Of these three, HSF1 appears to be the primary regulator of stress-induced HSP transcription, sometimes operating in conjunction with HSF2. HSF2 is poorly characterized compared to HSF1, and seems to play a role in cellular differentiation and development, but can also be activated when the ubiquitin-dependent proteolytic system is inhibited, indicating an induced response to the buildup of damaged proteins.[20] Little is also known about HSF4, which is tissue specific, and has only been seen to play a role in the maintenance and development of specialized sensory tissues.[9,21]

In an elegant negative feedback loop, HSF1 is held inert in the cytosol during routine cellular operation by the very chaperones it helps to regulate.[9,20] As stress denatured proteins attract Hsp90 and Hsp70 away from inactive complexes with HSF1 monomers, the monomers are released into the cytosol.[9,20] HSF1 monomers then trimerize and progress to the nucleus, where the trimer is modified and activated, initiating transcription of numerous HSP genes, including those responsible for Hsp70 and Hsp90.[9,20] As the concentration of damaged proteins diminishes, unoccupied Hsp70 and Hsp90 begin to accumulate, eventually binding and inactivating HSF1, **Figure 3.**[9,20]

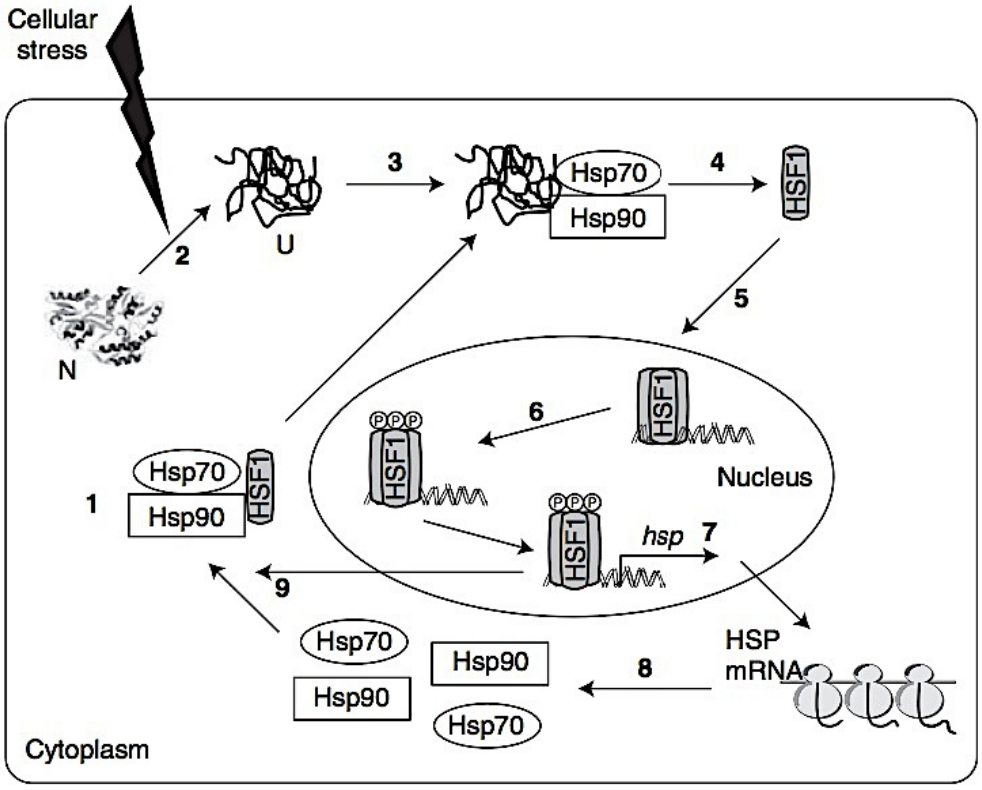


Figure 3: Transcriptional regulation of heat shock proteins by HSF1.
(1) Under normal conditions HSF1 exists as an inert monomer in a complex with Hsp90 and Hsp70. (2) Cellular stress increases the amount of denatured proteins in the cytoplasm. (3) Denatured proteins bind to Hsp70 and Hsp90, resulting in the displacement of HSF1 (4). (5) HSF1 then trimerizes and translocates to the nucleus where it undergoes a series of posttranslational modifications, including phosphorylation (6). (7) Activated HSF1 trimer induces the transcription of a number of hsp genes, resulting in the translation of HSPs, including Hsp90 and Hsp70 (8). (9) The increased cellular concentration of these chaperones in turn inactivates HSF1 by binding to monomeric or trimeric forms of HSF1.
N, native protein; U, unfolded protein.
Graphic reprinted without permission from Vabulas et al., 2010.[9] Caption quoted directly.

FOXO (forkhead box O protein) overexpression has also been demonstrated to upregulate inducible HSPs including Hsp40, Hsp70, and Hsp90 in *drosophila* muscle, while Hsp70 overexpression in rat soleus has been shown to directly downregulate FOXO activity.[22,23] This may represent another negative feedback cycle, and, given FOXO's central role as a proteolytic regulator, hints at complex regulatory layering in need of further illumination.[22-24]

The Intracellular Paths to Proteolysis

Of course, despite the best efforts of molecular chaperones, many damaged proteins cannot be successfully refolded, and the safe disposal of these structures becomes a priority. To accomplish this, the autophagic pathways operate in conjunction with the ubiquitin–proteasome system (UPS), and perhaps the calpain proteases, to coordinate the intracellular degradation of aggregated protein structures and terminally damaged proteins or organelles, as well as the timely removal of proteins playing a transient regulatory capacity, or those found to be in surplus.[25-27] These pathways are each tightly regulated, as either over- or under-activation of these systems has been linked to a wide spectrum of pathology, from arthritis, myopathy, and neurodegenerative disease, to diabetes, cancer formation, and immune dysfunction.[25-27]

The calpain family is a group of cytosolic proteases activated by interaction with calcium ions.[25,28] This pathway is ancient and ubiquitously distributed, but remains poorly understood, largely because members of the calpain family seem to occupy specialized regulatory roles.[28,29] Accordingly, the calpain system seems to display only limited proteolytic capability, and is able to directly recognize specific substrates rather than

relying on molecular tagging systems, as is the case for autophagy and the UPS.[28,29] As such, the role of this pathway is likely oriented more toward cellular signaling and regulation than straightforward protein degradation, with protease capabilities being employed for bio-modulation of existing protein structures, rather than full-scale protein dissasembly.[28,29]

The ubiquitin–proteasome system is, in contrast, a robust and well-characterized proteolytic pathway, acting in complement to autophagy as a principal regulator of intracellular protein degradation in eukaryotic cells.[26,30,31] This system is understood to selectively and individually target misfolded or malformed polypeptides, whereas autophagy degrades larger protein aggregates and organelles, often in a less specific fashion.[32] Additionally, while autophagic systems reduce protein substrate to individual amino acids, the UPS pathway renders proteins into short (3-25 AA) peptide sequences, which may later undergo further lysosomal degradation.[31]

Proteins are selectively marked for UPS-mediated catabolism through binding with ubiquitin, a 76-AA ancillary protein acting as a molecular tagging system.[30-32] While a single ubiquitination seems sufficient to allow targeted proteins entry into the UPS pathway, the binding of multiple ubiquitin molecules, either individually or as polyubiquitin chains, may help regulate subsequent processing.[31] Ubiquitinated proteins are then transported, somewhat mysteriously, to a large, barrel-like (~2.5 MDa) protease organelle known as a 'proteasome', where actual proteolysis occurs.[30,31] Proteasomes, 26S in eukaryotes, are widely distributed throughout the nucleus and cytoplasm, also allowing the UPS to play a substantial role in overseeing cell signaling, by acting to rapidly degrade various transient regulatory proteins.[31,32]

Autophagy, and the lysosome-dependent pathways

The umbrella term autophagy means 'self eating', and can be applied to numerous separate lysosome-dependent cellular processes playing different roles in protein catabolism.[26] These processes are novel in comparison to the ancient chaperones, and are only known to exist in eukaryotic cytoplasm, though they are ubiquitous in eukaryota.[33] Currently three primary types of autophagy pathways are best characterized, these being more specifically described as macroautophagy, microautophagy, and chaperone-mediated autophagy.[26,34] This interpretation is clearly limited, however, as more than a dozen autophagic processes have been delineated, based largely on the type of structures being degraded and how they are selected, along with minor regulatory and mechanistic variations.[35,36]

Macroautophagy

Macroautophagy is a bulk catabolic process limited to eukaryotic cytosol, which operates by engulfing a portion of the cytoplasm in a double-membrane vesicle called an autophagosome.[33,34,37] Engulfment then leads to indiscriminate degradation of the autophagosome and its contents when this structure subsequently fuses with a specialized organelle containing catabolic enzymes, called a lysosome, **Figure 4**.[34,37] The high capacity of this proteolytic pathway is ideally suited for urgent, short-term energy provision, and macroautophagy is upregulated during even relatively brief periods of nutrient deprivation (1-3 hours), representing the primary intracellular pathway responsible for protein catabolism in conditions of cellular energy-deficit.[34] This process is also upregulated in times of cellular stress, being crucial in the removal of large, pathogenic protein aggregates, damaged organelles, or even invading microbes.[33,34]

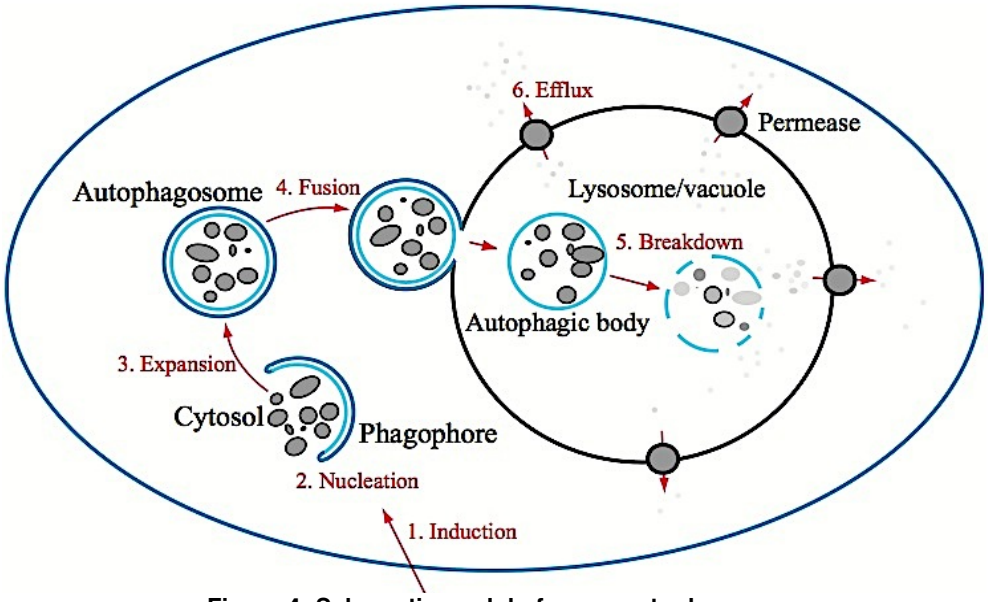


Figure 4: Schematic model of macroautophagy.
Autophagy occurs at basal levels and can be induced (1) by certain environmental or intracellular cues. The process begins with the nucleation step (2) in which a membrane core of unknown origin, termed the phagophore or isolation membrane, sequesters a portion of cytoplasm. The phagophore expands (3), probably through the vesicle-mediated addition of membrane (not shown) to generate the double-membrane autophagosome. Upon completion, the autophagosome outer membrane fuses (4) with the lysosome, releasing the inner single-membrane vesicle. The autophagic body is broken down (5) by lysosomal hydrolases and the resulting macromolecules are released back into the cytosol via membrane permeases (6) for reuse in the cytosol in catabolic or anabolic reactions. The steps of cargo recognition and packaging needed for specific types of autophagy are not depicted.
Graphic reprinted without permission from Mizushima et al., 2007.[34] Caption quoted directly.

Macroautophagy is a complex, multistage process, beginning with the activation of various autophagy-related proteins, known as Atgs.[36,37] In mammals, induction of macroautophagy is thought to depend on the activation of Atg1, comprising Unc-51-like kinases 1 or 2 (ULK1 or ULK2), which then forms a complex with Atg13.[36,37] Activation of these proteins initiates a nucleation process, as requisite proteins and lipids are conscripted and locally concentrated for assembly of the autophagosome membrane, called a phagophore in the early stages of construction.[36,37] The mechanisms underlying this nucleation remain somewhat uncertain, but successful phagophore formation in mammals is known to require phosphatidylinositol 3-phosphate (PI3P), acting together

with Atg14, Beclin 1, and a variety of other ancillary proteins to form several regulatory complexes, acting either to promote or inhibit continuation of the process, depending on arrangement.[36,37]

Membrane expansion and autophagosome completion then depend on ubiquitin-like conjugation systems centered on Atg8, called LC3 in mammals, and Atg12.[36,37] LC3 and Atg12 are ubiquitin-like proteins which are conjugated with phosphatidylethanolamine and Atg5, respectively, in the developing autophagosome membrane through a series of reactions catalyzed by Atg7, Atg10, Atg3, and Atg4.[37] Once the autophagosome is successfully matured, it finds and fuses with a lysosome to form a so called 'autolysosome', in a process leading to the degradation of the autophagosome structures and contents by lysosomal hydrolases.[36,37] Finally, the macromolecular end-products are released back into cytosol through specialized permeases in the lysosomal membrane, where they become available for catabolic energy provision or novel anabolic processes.[34,37]

Chaperone-Mediated Autophagy

Chaperone-mediated autophagy (CMA) is a more selective process, in which individual proteins are targeted through specific amino acid motifs, and delivered directly to the lysosome.[26,38] This is the secondary autophagic pathway upregulated in response to nutrient deprivation, being induced subsequent to macroautophagy in longer (8-10 hour) periods of starvation.[34,38] This system is also capable of regulatory modulation in a manner similar to the UPS, through selective degradation of specific regulatory proteins.[38] Aside from these specialist roles, CMA also provides an efficient pathway for

the catabolism of surplus, damaged, or aberrant proteins, and is upregulated in response to denaturing stressors.[38]

CMA begins with the assistance of constitutively-expressed Hsc70, which recognizes and binds CMA-designated protein substrates through a specific amino acid motif, usually beginning with glutamine, which is then followed successively by a positively charged residue, a hydrophobic residue, a negatively charged residue, and finishes with either a positive or hydrophobic finale.[35,38] These motifs are often uncovered in protein structures following damage, misfolding, or multi-unit protein disassembly, and because this unit is based on a sequence of electrical charges, an incomplete motif can be completed through post-translational modification, with charges added to existing partial sequences by phosphorylation or acetylation.[38]

Once successfully bound, the Hsc70/protein complex proceeds directly to the lysosome, where the protein moiety interacts with the cytosolic portion of lysosome-associated membrane protein type 2A (LAMP-2A).[38] Once this occurs, the LAMP-2A monomer begins to assemble a complex for translocation of the substrate into the lysosomal lumen, while the waiting protein is unfolded prior to entry.[35,38] Both of these processes are facilitated by the molecular chaperones, with Hsp90 stabilizing the LAMP-2A complex during formation, and Hsc70 working with co-chaperones to unfold the candidate protein for translocation.[35,38] Once the protein has been sufficiently unfolded, it is transferred across the membrane by the LAMP-2a complex, which is subsequently disassembled to allow binding of novel substrate.[35,38]

Microautophagy

Despite decades of research, microautophagy remains the most mysterious of the major autophagic pathways, particularly in mammals.[39,40] While much of the evidence regarding microautophagy comes from yeast, the process is generally characterized as a direct invagination of the lysosomal membrane, which encloses a small portion of the nearby cytosol to form a self-contained 'endosome' that is then degraded along with its constituents upon arrival at the lysosomal lumen.[39,40] Much regarding the regulatory control and mechanistic underpinning of microautophagy remains unclear, though it appears capable of both bulk and selective substrate uptake.[39,40] While evidence suggests that this pathway is operational in mammals, its level of physiological importance in comparison to macroautophagy and CMA remains in need of elucidation.[39,40]

Autophagy: Regulatory Control

Macroautophagy is the most common and best characterized of the autophagic processes, and will serve as the focus of following sections, being referred to hereafter simply as autophagy.[33,37] Significant progress has been made in recent years to advance empirical insight into the regulatory control of autophagic pathways, though many questions remain to be answered.[37,41] Transcriptional control of autophagy is orchestrated by a complex series of dynamically interactive metabolic signaling pathways, ultimately overseen by protein kinase B, (also known as Akt and PKB), and adenosine monophosphate-activated protein kinase (AMPK) through their downstream effectors, mammalian target of rapamycin (mTOR), and FoxO.[42,43] Akt responds to growth factors such as insulin and insulin-like growth factor 1 (IGF-1), and generates signals favoring anabolism and energy expenditure.[43] Conversely, AMPK is sensitive to nutrient scarcity,

and activates pathways favoring catabolism and energy conservation.[44] These regulators can be thought of as acting in coordinated opposition to maintain a careful balance between protein synthesis and degradation in response to constantly fluctuating metabolic signals, **Figure 5**.[10]

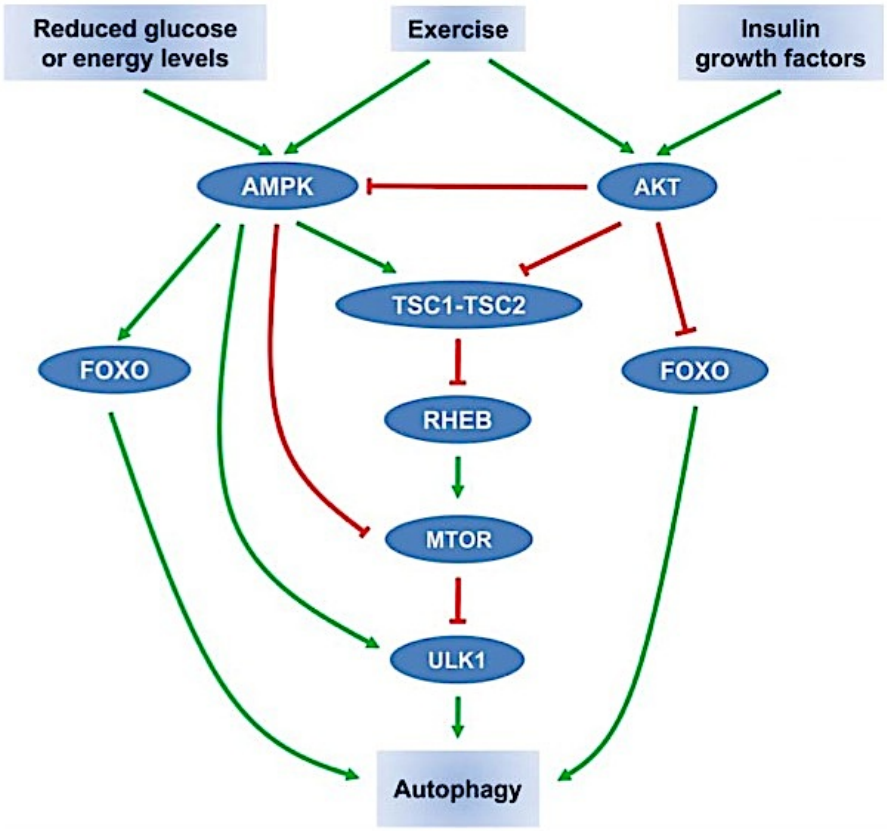


Figure 5: Overview of autophagic regulatory pathways.
Green lines indicate activation, red lines indicate inhibition.
Adapted and reprinted without permission from Dokladny et al. 2015.[10]

As mentioned previously, induction of autophagy is initially thought to be instigated through the activation of Atg1, called ULK1 in mammals.[36,44] Under conditions promoting catabolism and energy conservation, activation of ULK1 through direct phosphorylation by AMPK leads to an upregulation of autophagy.[44] Under conditions favoring anabolism, activation of Akt leads to an upregulation of mTOR activity, precipitating the inhibitory phosphorylation of ULK1 by mTOR complex 1,

curtailing autophagy.[44] AMPK activation also prompts an increase in FoxO activity, while FoxO is corresponding inhibited by activated Akt.[45] FoxO, in turn, is responsible for the transcriptional induction of numerous autophagy-related genes, including those accountable for several proteins regulating nucleation and expansion of the phagophore, such as microtubule-associated protein 1 light chain (LC3), Becilin-1, and Bnip3.[42,43] Some research suggests that this inhibition of FoxO is actually the major pathway by which Akt downregulates autophagic activity, and much remains to be determined regarding the nuanced integration of input ultimately governing these multifarious signaling pathways.[43]

Implications for Health and Disease:

Dysregulation of these proteostatic systems has serious implications for whole organism health and homeostasis.[3,4,26,38,46-52] Knockout research in animals has revealed that the ability to successfully navigate proteostatic challenges proves indispensable from the earliest stages of growth and development, with several projects reporting that mice bred without the ability to express vital autophagy-related proteins such as Atg5 and Atg7 are unable to survive beyond a few days of their birth.[53,54]

Moreover, mounting evidence indicates that these protein management systems are central to the survival and normal function of individual tissues, particularly those composed of post-mitotic cells.[34,52] Mature, non-dividing cells like neurons and skeletal muscle fibers are finite in adult mammals, and their survival depends on the ability of these proteostatic mechanisms to control protein quality throughout the life cycle.[34,52]

Mice born with defects specifically preventing normal autophagic activity in skeletal muscle have been repeatedly shown to experience severe muscle wasting, with

inspected muscle fibers exhibiting severely disorganized muscle structure, alongside an assemblage of protein aggregates and damaged or defective organelles, the accumulation of which eventually leads to cell death.[55-57]

Several studies in cell cultures have shown that inhibition of autophagy in neuron rapidly leads to an accumulation of aggregated protein structures, including the tau aggregates associated with Alzheimer's disease and similar neurodegenerative disorders, eventually causing cytotoxicity and cell death.[58,59] In contrast, research in *drosophila* has provided evidence that upregulation of autophagy accelerates the destruction of both these mutant tau aggregates and the aberrant huntingtin proteins associated with Huntington's disease, reducing cellular toxicity.[60]

In humans, numerous devastating myopathies have been traced to genetic defects impairing intramuscular autophagic pathways.[61-63] Danon disease has been shown to be precipitated by lack-of-function mutations in the gene responsible for LAMP-2, which plays a crucial role in chaperone mediated autophagy.[61,64] Pompe disease is caused by the inherited deficiency of a key lysosomal enzyme, acid α-glucosidase, which leads to an accumulation of the intended substrate, glycogen, within the lysosome, impairing normal lysosomal function.[62,65] Similarly, X-linked myopathy with excessive autophagy (XMEA) is tied to mutations in the VMA21 gene, which lead to deficient activity in lysosomal hydrolases, and pathological accumulation of intended substrate.[63]

More generally, a normal age-related decline in autophagic activity has been linked to sarcopenia, the ubiquitous loss of skeletal muscle mass with age.[47,66] Conversely, enhanced autophagy function is being increasingly linked to life span

enhancement, and has been shown to exert direct control over longevity in numerous test organisms, including *S. cerevisiae*, *C. elegans*, and *D. melanogaster*.[48,49]

Similarly, research in *C. elegans* reported that inhibition of HSF1, the master regulator of heat shock induction, led to accelerated aging and life span reduction, while HSF1 overexpression promoted longevity and reduced cytotoxic protein aggregation.[46] Moreover, overexpression of molecular chaperones in the Hsp70 network has been repeatedly shown to ameliorate pathogenic protein structures associated with several prominent degenerative disorders.[67-69] In *drosophila*, overexpression of Hsp70 was found to alleviate the neurotoxicity usually associated with abnormal polyglutamine expansion in protein structures linked to Machado-Joseph disease and seven other human neurodegenerative disorders.[70] Similar research, again in *D. melanogaster*, found that engineered overexpression of human Hsp70 was able to completely prevent the drastic neurotoxicity otherwise observed with induced expression of α-synuclein, a protein implicated in the development of Parkinson's disease.[67] In multiple cellular studies, Hsp70 overexpression was also able to counteract the aggregation of mutant huntingtin proteins associated with Huntington's disease, suppressing formation of soluble oligomers, and mitigating toxicity.[68,69]

Skeletal Muscle: unique challenges to proteostasis

Skeletal muscle makes up 40-50% of the human body, and plays central roles in physical mobility, protein storage, and metabolic homeostasis, which extend the fundamental importance of this tissue to general health, longevity, and quality of life.[52,66,71] Muscle tissue also presents a number of unique challenges to protein management systems, not the least of these being the cell-wide stressors provoked by

exercise.[52] Physical activity can rapidly expend local energy substrate, while simultaneously generating heat, mechanical damage, reactive oxygen species (ROS), and pH shifts, all of which act in concert to produce widespread protein denaturement.[52,72] Moreover, muscle tissue remains capable of enormous adaptive plasticity throughout adulthood, demanding wholesale remodeling of cell-wide protein architecture, while intramuscular protein catabolism provides a responsive pool of amino acids available for organism-wide emergency energy provision and crucial anabolic reactions.[34,72,73]

The normal performance of these important functions depends in large part on the protein management systems, which mop up or refurbish damaged proteins following exercise, degrade available protein to provide substrate for anabolic adaptation or catabolic energy production, and generally facilitate the complex proteostatic maintenance of this particularly protein-centered tissue.[34,52]

A growing number of investigations have empirically demonstrated that both heat shock and autophagic processes can be strongly induced by the stress of acute exercise in animals,[57,74-78] and humans.[79-84] Somewhat surprisingly, other projects have failed to observe increased expression of major markers relating to these processes, such as LC3 and Hsp70, following acute exercise,[85-88] with research providing indications of a differential response in these proteostatic systems according to tissue type,[89,90] and exercise intensity.[91,92]

Summary

Given the apparent importance of these proteostatic mechanisms to general health and longevity, it seems clear that developing a more comprehensive understanding of

their operation and regulation will help provide crucial insights into numerous formidable pathologies, and may even eventually illuminate the fundamental aging process.[3,4,47,49,70]

The upregulation of proteostatic systems in response to exercise also offers an intriguing mechanistic connection to the health and longevity benefits often associated with routine exercise, [93] while providing a unique opportunity to observe the interplay of these systems in response to routine cellular insult, potentially shedding light on regulatory pathways.[10]

It seems clear that further efforts to illuminate the individual roles and cooperative interaction of these proteostatic systems are strongly warranted, and investigators may be able to gain valuable insight through observing the unified action of these pathways as they manage a dynamic proteostatic balance through the complexly evolving demands of acute exercise and subsequent transition to recovery and repair.

Chapter 2
Introduction

Proteins play a critical role in nearly every biological activity, and intracellular homeostasis depends in large part on the ability of regulatory systems to sustain the quality and function of these diverse, structurally complex macromolecules.[3,4] Dysregulation of these systems can have serious consequences, and investigations into a number of challenging, progressive pathologies have become increasingly concentrated on the mechanisms by which healthy protein ecosystems are able to maintain an adaptive, functional equilibrium in the face of acute and chronic cellular stress.[3,4,50,51,94]

Unraveling these mechanisms is a daunting task, as perpetuating the operational status quo of intracellular protein systems is accomplished through a fluctuating cycle of synthesis, maintenance, and elimination involving numerous interactive pathways and hundreds of individual proteins.[2-4] The importance of the task is difficult to overstate, however, as this tightly-regulated network of protein management systems ultimately influence almost every aspect of cellular function, overseeing the conformational integrity, interactive capability, physical location, overall concentration, and timely catabolism of the individual proteins upon which functionality depends.[3,4] This formidable level of regulatory oversight is fundamentally necessitated by the intricacy, delicacy, and diversity of protein species; as well as the dynamic environment in which they operate, and the complexly evolving needs of the physiological system they support.[3,4]

Basic Concepts

Proteins are synthesized in linear polypeptide chains, requiring complex folding maneuvers in order to attain the specific 3-dimensional conformation required for normal function.[5,7] Cellular stress can also damage and deform successfully folded proteins, which then must re-attain a stable, 'native' configuration to resume proper function.[5,7] Misfolded or transitional protein states often expose unshielded hydrophobic regions, which increases risk of damaging non-native interaction with nearby structures, and promotes aggregation.[5,7]

Considering the logistical importance of these nascent forms achieving functional status, and the danger to nearby molecules that intermediate protein conformations can represent; it is not surprising that a network of ancillary molecules has developed to help smooth progress through the transitional phases of protein folding, while intracellular proteolytic systems have been established to sequester and destroy aberrant proteins and cytotoxic aggregate structures.[4,26] Stabilization and proper folding of damaged and *de novo* proteins is overseen by the molecular chaperones,[4] while intracellular proteolysis is accomplished through the coordinated efforts of the ubiquitin–proteasome system (UPS), and the lysosome-dependent autophagic processes.[30] Regulatory control of these systems is complex, dynamic, and only partially understood, though mounting evidence suggests that cross-talk between these protein management pathways plays an important role in maintaining proteostasis.[4,10,22,30,37,80]

Protein Management Systems

The molecular chaperones, as a class, are any of a structurally unrelated group of proteins found to interact with partially-folded intermediate proteins to limit aggregation,

enhance stability, or otherwise facilitate a correct progression to the native state without becoming incorporated in the final structure.[4] A number of these chaperones, often called heat shock proteins (HSPs) are transcriptionally induced by denaturing forms of cellular stress leading to an increased intracellular concentration of damaged proteins, in what has been termed the heat shock response (HSR).[20,21] The critical importance of this ancient pathway is underscored by its universal distribution in known organisms, and the conferred ability to stabilize complicated protein structures has been implicated as an evolutionary capacitor.[9,11,19] In modern mammalian cells, approximately 20-30% of all proteins seem to be incapable of correct *de novo* folding without the assistance of these facilitators.[4]

Despite the best efforts of these molecular chaperones, many damaged proteins cannot be successfully refolded, and the safe disposal of these structures becomes a priority. To accomplish this, the autophagic pathways operate in conjunction with the ubiquitin–proteasome system (UPS), to coordinate the intracellular degradation of terminally damaged proteins or organelles and aggregated protein structures, as well as the timely removal of proteins playing a transient regulatory capacity, or found to be in surplus.[26,27,30] These pathways are each tightly controlled, with abnormal activity being linked to a spectrum of pathology, from myopathies and neurodegenerative diseases, to diabetes, cancer formation, and immune dysfunction.[26,50,51,94]

The ubiquitin–proteasome system operates selectively to individually target misfolded or malformed proteins through a molecular tagging system, whereas autophagy is able to degrade larger protein aggregates and organelles, often in a less specific fashion.[32] Additionally, while autophagic systems reduce protein substrate to individual

amino acids, the UPS pathway renders proteins into short (3-25 AA) peptide sequences, which may later undergo further lysosomal degradation.[31]

The umbrella term autophagy can be applied to numerous distinct, lysosome-dependent cellular processes playing different roles in protein catabolism.[26] These processes are novel in comparison to the primordial chaperones, and are only known to exist in eukaryotic cytoplasm, though they are ubiquitous in Eukaryota.[33] Currently three primary types of autophagy pathways are best characterized, these being more specifically described as macroautophagy, microautophagy, and chaperone-mediated autophagy.[26,34]

Macroautophagy is a bulk catabolic process, which operates by engulfing a portion of the cytoplasm in a double-membraned vesicle called an autophagosome.[33,34,37] Engulfment then leads to indiscriminate degradation of the autophagosome and its contents when this structure subsequently fuses with a specialized organelle containing catabolic enzymes, called a lysosome.[34,37] The high capacity of this pathway is ideally suited for urgent, short-term energy provision, and macroautophagy is upregulated during even relatively brief periods of nutrient deprivation (1-3 hours), as well as during periods cellular stress, being crucial in the removal of large, pathogenic protein aggregates, damaged organelles, or even invading microbes.[33,34]

Though microautophagy remains the most mysterious of the major autophagic pathways, the process appears capable of both bulk and selective substrate uptake, and is characterized by a direct invagination of the lysosomal membrane, which encloses a small portion of the nearby cytosol to form a self-contained ‘endosome’ that is then degraded along with its constituents upon arrival at the lysosomal lumen.[39,40]

Chaperone-mediated autophagy (CMA) is a more selective process, in which individual proteins are targeted through specific amino acid motifs, and delivered directly to the lysosome by the molecular chaperone Hsc70 (HSPA8), where a binding with LAMP-2 precipitates transport across the lysosomal membrane.[26,38] This system is also capable of regulatory modulation in a manner similar to the UPS, through selective degradation of specific regulatory proteins.[38]

Macroautophagy is the most common and best characterized of the autophagic processes, and will serve as the focus of following sections, being referred to hereafter simply as autophagy.[33,37]

Implications for Health and Disease

Successfully navigating proteostatic challenges proves critical to normal growth and development, as mice bred without the ability to express vital autophagy-related proteins like Atg5 and Atg7 die within days of birth.[53,54] Mice born with defects specifically preventing autophagy in skeletal muscle experience severe muscle wasting, with muscle fibers exhibiting disorganized muscle structure, alongside an assemblage of protein aggregates and damaged or defective organelles, the accumulation of which eventually leads to cell death.[55-57]

In humans, devastating myopathies have been traced to genetic defects impairing the muscular autophagic pathways, with Danon disease precipitated by a lack of LAMP-2,[61,64] Pompe disease caused by the lack of a key lysosomal enzyme,[62,65] and XMEA (X-linked myopathy with excessive autophagy) tied to deficient activity in lysosomal hydrolases.[63] Moreover, a typical age-related decline in autophagic activity has been

linked to sarcopenia, the inevitable loss of skeletal muscle mass with age,[47,66] while enhanced autophagy may directly promote longevity.[48,49]

Similarly, animal models show that inhibition of HSF1, the transcription factor responsible for stress-induced molecular chaperones, leads to accelerated aging, while HSF1 overexpression promotes longevity.[46] Additionally, overexpression of heat shock proteins in the 70 kDa (Hsp70) network, which is regulated by HSF1, have been shown to ameliorate both pathogenic protein oligomers associated with Huntington's disease, and protein aggregates related to Parkinson's disease, in cell and animal models.[67-69]

Skeletal Muscle: unique challenges to proteostasis

Proteostatic mechanisms have a particularly important role in post-mitotic cells such as neurons and skeletal muscle fibers, as these cells are finite in adult mammals, and their survival depends on the ability to control protein quality.[34,52] This is of fundamental importance to whole-body homeostasis, as skeletal muscle is the most abundant tissue in mammals, making up 40-50% of the human body, and serves a central role in health, function, and metabolic regulation.[52,66,71]

Muscle tissue also presents a number of unique challenges to these protein management systems, not the least of these being the cell-wide stressors provoked by exercise, which can rapid expend local energy substrate, while simultaneously generating heat, mechanical damage, reactive oxygen species (ROS), and pH shifts leading widespread protein denaturement.[52,72] Moreover, muscle tissue remains capable of enormous adaptive plasticity throughout adulthood, demanding wholesale remodeling of cell-wide protein architecture, while muscular protein catabolism provides a responsive

pool of amino acids for organism-wide emergency energy provision and crucial anabolic reactions.[34,72,73]

Regulatory Interactions

Transcriptional control of stress-inducible chaperones has been traced to so called heat shock factors (HSFs).[9,20] Of these, HSF1 appears to be the primary regulator of stress-induced heat shock response.[9,20] In an elegant negative feedback loop, HSF1 is held inert in the cytosol during routine cellular operation by the very chaperones it helps to regulate.[9,20] As stress denatured proteins attract Hsp90 and Hsp70 away from inactive complexes with HSF1 monomers, the monomers are released into the cytosol.[9,20] HSF1 monomers then trimerize and progress to the nucleus, where the trimer is modified and activated, initiating transcription of numerous HSP genes, including those responsible for Hsp70 and Hsp90.[9,20] As the concentration of damaged proteins diminishes, unoccupied Hsp70 and Hsp90 begin to accumulate, eventually binding and inactivating HSF1.[9,20]

Transcriptional control of autophagy is currently thought to be regulated by a complex series of interactive metabolic signaling pathways, ultimately overseen by protein kinase B (Akt), and adenosine monophosphate-activated protein kinase (AMPK) through their downstream effectors, mammalian target of rapamycin (mTOR), and FoxO (forkhead box O proteins).[42,43] Akt responds to growth factors such as insulin and insulin-like growth factor 1 (IGF-1), and generates signals favoring anabolism and energy expenditure.[43] Conversely, AMPK is sensitive to nutrient sensitivity, and activates pathways favoring catabolism and energy conservation.[44] These regulators can be thought of as acting in coordinated opposition to maintain a careful balance between protein synthesis and degradation in response to continuously fluctuating metabolic signals.[10]

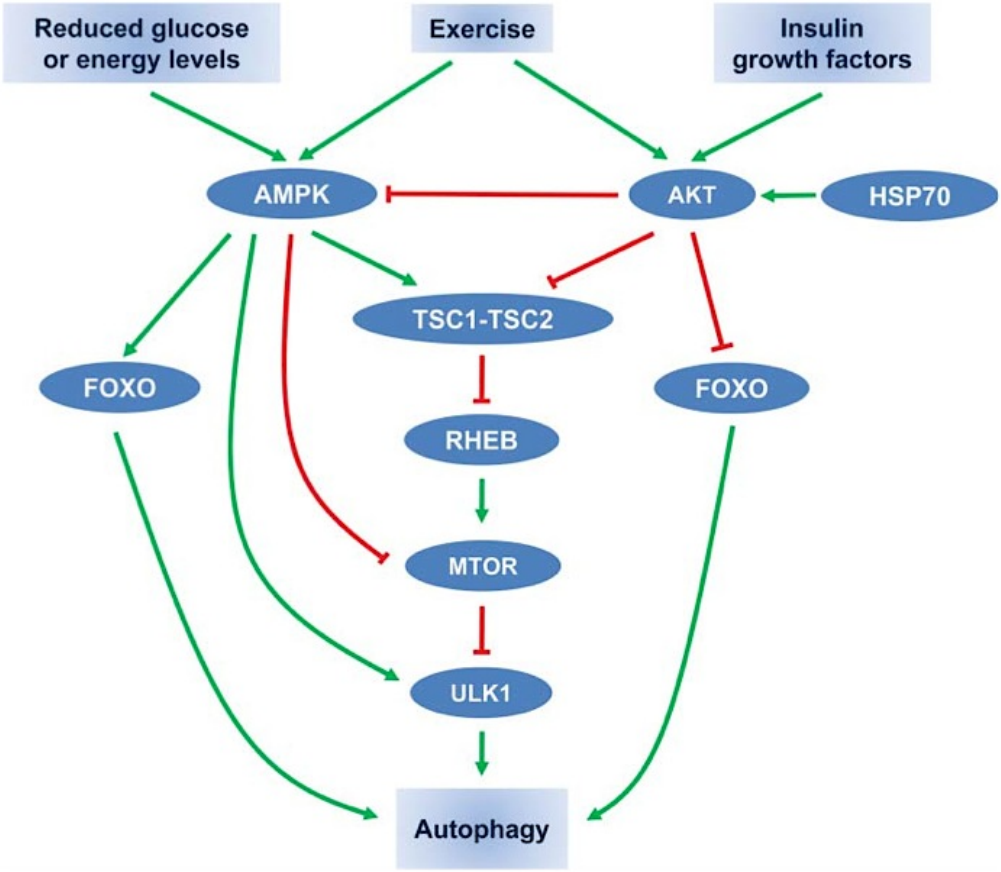


Figure 6: Schematic overview of Hsp70 participation in the regulation of autophagy
Green lines indicate activation, red lines indicate inhibition.
Graphic reprinted from Dokladny et al., 2015.[10]

Interactive Pathways: coordination and control

Recent evidence suggests that the comparatively ancient molecular chaperones are able to directly influence autophagic activity through Hsp70-induced phosphorylation of Akt, leading to a downregulation of autophagy, **Figure 6**.[80] In cell models, overexpression of Hsp70 lead to increased concentrations of phosphorylated Akt, and prevented normal activation of autophagy during caloric restriction, while HSF1 blockade intensified autophagic activity.[80] Other researchers have shown that overexpression of Hsp70 can also directly downregulate FoxO3a,[22] which is known to

oversee autophagy induction *in vivo*,[24,45] and operates downstream of Akt.[43] Preliminary comparative analysis of the time-course response in autophagic and heat shock systems following exercise also supports a model of regulatory coordination between these two proteostatic systems.[10]

Observation of this interplay led researchers to propose that these two systems provide an integrated response to the proteostatic challenges posed by exercise.[10] As a unique instigator of both damaging cellular stress and generative adaptation, physical exercise offers a singular model through which to observe the interaction of these pathways in the metabolic transition from catabolism to anabolism.[10] Clearly, the autophagic systems play a critical role in recycling expendable proteins for energetic catabolism during exercise, as well as in cleansing the cell of damaged protein structures in the immediate post-exercise window; however, these destructive processes must soon be attenuated to allow a successful transition into adaptive restoration and remodeling. As such, the suggested model posits that Hsp70 acts as a molecular switch following physical activity, manifesting a shift in regulatory dominance away from the cleansing catabolic processes, and toward the restorative influence of Akt-mediated anabolism.[10]

A number of investigations have demonstrated that both the HSR and autophagic processes can be strongly induced by the stress of acute exercise in animals,[57,74-78] and humans.[79-84] Somewhat surprisingly, other projects have failed to observe increased expression of major markers relating to these processes, such as microtubule-associated protein 1 light chain 3 (LC3) and Hsp70, following acute exercise,[85-88] with research providing indications of a differential response in these proteostatic systems according to tissue type,[89,90] and exercise intensity.[91,92]

In order to further explore the time-course response of these systems following acute exercise, we undertook a systematic review of the literature in an effort to illuminate the individual roles and cooperative interaction of these systems in managing a dynamic proteostatic balance through the complexly evolving demands of acute exercise and subsequent transition to recovery and repair.

This project supplements previous work by incorporating research from rodent models to augment available human data, and by providing a detailed analysis of the impact of exercise intensity. Metabolic equivalents of task (METs) were assigned to each exercise performed in human research, and METhours were calculated based on the duration of activity, allowing us to compare the correlation between various measures of intensity and the magnitude of proteostatic responses. Despite limited available data, an exploration of tissue-specific responses was also attempted.

Chapter 3
Methods

Literature Search

Published, peer-reviewed data germane to this project was accumulated using 27 electronic research repositories including Medline, PubMed, ScienceDirect, Wiley Online Library, and Worldcat databases. The following keywords were used to search, alone or in various combinations including Boolean commands: acute, exercise, physical activity, autophagy, macroautophagy, heat shock, LC3, Atg1, Hsp, Hsp70, Hsp72. Article titles were then manually evaluated for relevance, with the full text of propitious selections downloaded and reviewed to determine if all necessary inclusion criteria had been met. Articles selected for inclusion were then cross-referenced in an attempt to uncover any pertinent research not otherwise revealed in the electronic search process. Data sets from individual articles were then cross-checked for originality. In cases where multiple articles had been published based on the same set of data, a single selection was made based on completeness of the information presented, and the duplicates were subsequently disregarded.

Study Selection

Only peer-reviewed articles presenting original research and published in the English language prior to May, 2015, were considered for inclusion in this project. Available research from all healthy human and rodent subjects was utilized, with no set limits on age, sex, or training status. Focus was primarily on skeletal muscle, though limited available research prompted the inclusion of data from cardiac muscle, as well as PBMCs (peripheral blood mononuclear cells). Intracellular expression of Hsp70 was used as an indicator of the stress-inducible heat shock response, while expression of the

mammalian Atg1 homologue, LC3, was used as an index of autophagic activity. In order to help shed light on the time course of these responses following acute exercise, minimum necessary data for inclusion in this project consisted of a baseline measure of Hsp70 or LC3, as well as at least one measure of the same variable recorded at a reported time subsequent to a single, quantified bout of physical activity, and otherwise unaffected by the research intervention.

Variables Considered

The primary variables considered in this project were LC3, Hsp70 (70 kDa or 72 kDa, intracellular only), the duration of exercise performed, the intensity of exercise performed (in approximated METs), and a combinatory measure representing both the duration and intensity of exercise (METhours). Normalized phosphatidylethanolamine-conjugated LC3 (LC3-II) was the primary measure available in existing autophagy research,[57,74,75,79-81,87,95-97] though the ratio of conjugated to unconjugated (LC3-II/LC3-I) was also used in several included projects.[57,85,98]

METs and METhours: fixing intensity

Metabolic equivalents of task (METs) are a measure of exercise intensity corresponding to a volumetric rate of oxygen consumption ($\dot{V}O_2$). 1 MET is generally accepted to represent $\dot{V}O_2$ = 3.5 ml/kg/min, and is intended to approximate a basal or resting level of metabolic activity.[99,100] MET values for human subjects were estimated by comparing available descriptions of the exercise performed with published data regarding oxygen consumption during physical activity of similar modality and intensity, largely located through the Compendium of Physical Activities.[101] Descriptions of the exercise protocol utilized in each study included in this project can be found alongside

estimated MET values, and the specific reference used to estimate METs, in **Tables 1 - 4,** located in the appendix **(p. 59-68)**. METhours were then calculated by straightforward multiplication of the estimated METs and the reported exercise duration, as such: **METs * exercise duration (h) = METhours**.[100]

Animal subjects were separated into high or low intensity groupings based on natural lines of separation apparent in the data, essentially representing a separation between the animals that were exercised to exhaustion, and those that were not. Divisions between intensity groupings in human data were based on estimated METhours, separated into 50% or 25% increments of the estimated METhour range, according to data availability.

Statistics and Analyses

To homogenize data for a pooled comparative analysis, all post-exercise measures of Hsp70 and LC3 were converted to a percentage of their corresponding baseline measures **[(post measure / baseline) * 100]**. The temporal progression of Hsp70 and LC3 responses following exercise was then established, by plotting the data against time from initiation of exercise according to various grouping strategies. The time course response of autophagic and heat shock systems was then estimated by fitting a parabolic trendline to each resulting plot, with the starting point set to 100 (our homogenized baseline).

In an effort to clarify which aspect of physical exercise is most important to the induction of heat shock and autophagic activity, correlations were explored between the magnitude of Hsp70 and LC3 responses and the available measures of exercise intensity, namely METs, METhours, and exercise duration. To examine the heat shock response,

correlations with each intensity measure were performed for all human Hsp70 data collected in the first 24 and 48 hours post-exercise, in order to capture the projected upslope of heat shock activity. Regarding the autophagic response, correlations with each intensity measure were limited to the maximal measured autophagy per study group, in an effort to equalize the impact of each individual study, given the small set of available research projects.

All figures were developed and correlations performed using Microsoft Excel (Microsoft Office Excel for Mac 2011; Seattle, WA, USA).

Chapter 4
Results

Study Characteristics

Regarding research in humans, 6 autophagy articles, with 104 total subjects (men = 85, women = 19),[79-81,85,87,95] and 28 heat shock articles, with 265 total subjects (men = 228, women = 37),[12,80,82-84,86,88,103-122,124,125] were identified as meeting inclusion criteria, with one article exploring both, see **ref 80**.[80] An additional 6 articles investigating autophagy,[57,74,75,96-98] as well as 19 articles examining heat shock,[76-78,89,126-140] were identified as meeting inclusion criteria with data from research in rodents. A full list of these articles alongside the primary variables extracted can be found in **Tables 1 - 4** in the appendix **(p. 59-68)**.

Time Course Responses

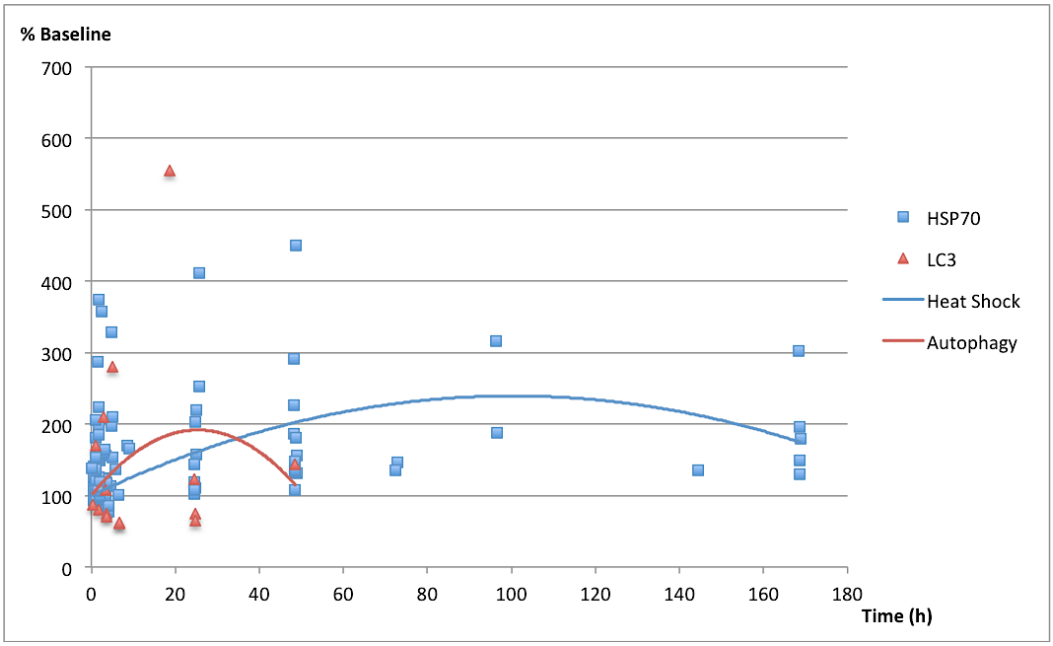


Figure 7: Projected time course of autophagic and the heat shock activity following acute exercise in humans.
Autophagy time to peak: 25.4 h; peak amplitude: 194%
Heat shock time to peak: 100.5 h; peak amplitude: 240%
The X-axis indicates time from the start of exercise, representing the sum, in hours, of the reported exercise duration and period of time until post-exercise data collection; the Y-axis represents the relative intensity of HSP70 protein expression or LC3 protein expression. Each point indicates a mean group measure of expressed LC3 or Hsp70 proteins reported by a single study at the corresponding time, normalized to baseline.

As indicated by measures of expressed LC3 and Hsp70 proteins, the projected time course of autophagic and heat shock activity following acute exercise in humans can be seen in **Figure 7**. Similarly, time course projections for autophagy and heat shock processes following acute exercise in rodents can be seen in **Figure 8**, again based on measures of expressed LC3 and Hsp70 proteins. Two studies reporting unusually high Hsp70 measures in rodents post-exercise (1,000-6,000% baseline) were excluded as outliers.[89,138]

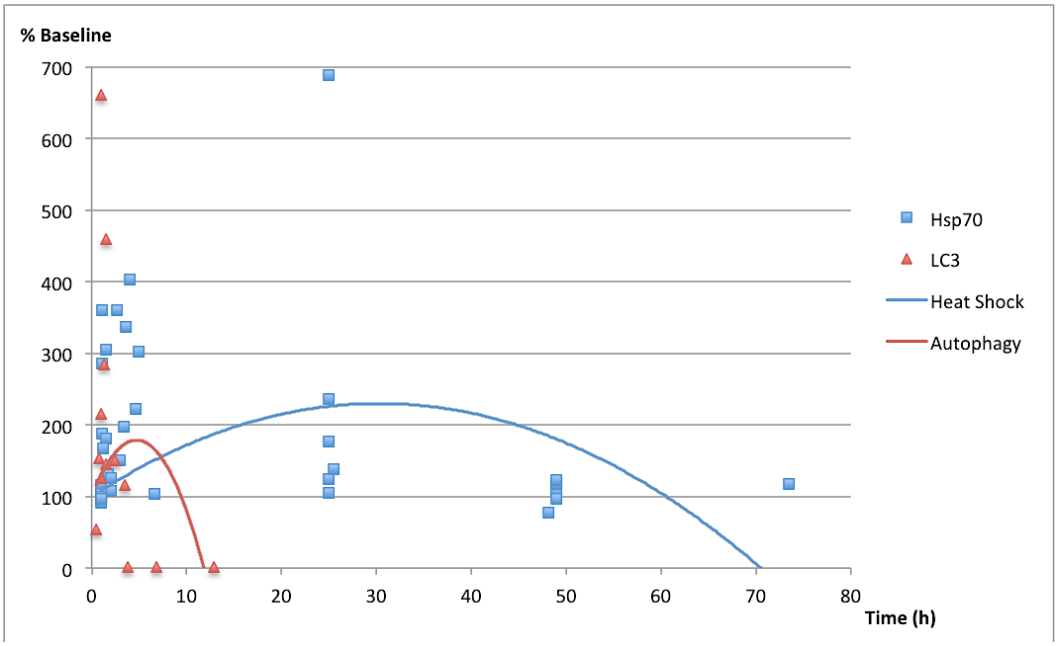


Figure 8: Projected time course of autophagic and the heat shock activity following acute exercise in rodents.
Autophagy time to peak: 4.7 h; peak amplitude: 182%
Heat shock time to peak: 30.1 h; peak amplitude: 231%
The X-axis indicates time from the start of exercise, representing the sum, in hours, of the reported exercise duration and period of time until post-exercise data collection; the Y-axis represents the relative intensity of HSP70 protein expression or LC3 protein expression. Each point indicates a mean group measure of expressed LC3 or Hsp70 proteins reported by a single study at the corresponding time, normalized to baseline.

In humans, the response of both autophagic and heat shock systems to acute exercise seemed to vary by tissue type, with the induced expression of both LC3 and Hsp70 tending to be higher in PBMCs than muscle tissue, as can be seen for autophagy in **Figure 9**, and heat shock systems in **Figure 10**.

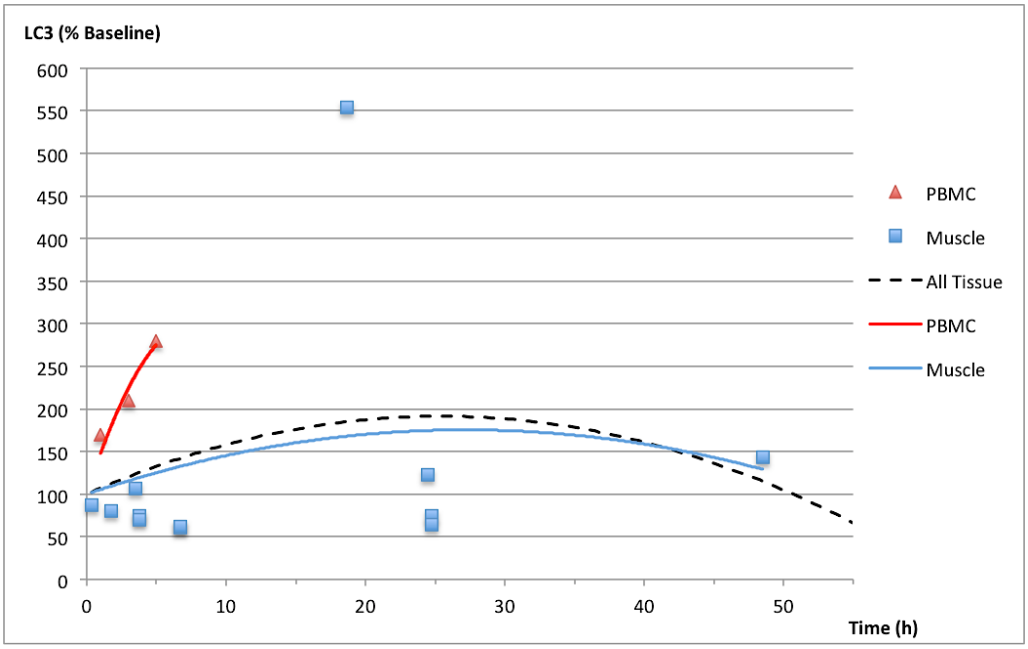


Figure 9: Indications of a differential autophagic response in humans by tissue type. The X-axis indicates time from the start of exercise, representing the sum, in hours, of the reported exercise duration and period of time until post-exercise data collection; the Y-axis represents the relative intensity of LC3 protein expression. Each point indicates a mean group measure of expressed LC3 protein reported by a single study at the corresponding time, normalized to baseline.

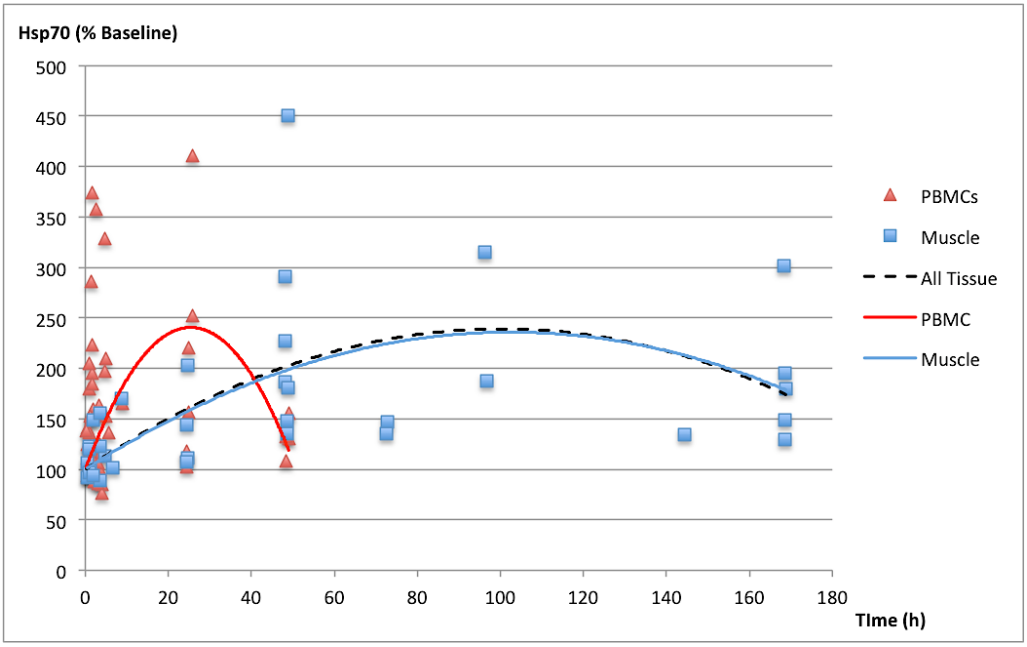


Figure 10: Indications of a differential heat shock response in humans by tissue type. The X-axis indicates time from the start of exercise, representing the sum, in hours, of the reported exercise duration and period of time until post-exercise data collection; the Y-axis represents the relative intensity of Hsp70 protein expression. Each point indicates a mean group measure of expressed Hsp70 protein reported by a single study at the corresponding time, normalized to baseline.

A clear differential in the magnitude of Hsp70 and LC3 induction following acute exercise can also be observed according to the intensity of the exercise performed. **Figure 11** represents LC3 data from both animals and humans, divided into high and low intensity groups. In both rodents and humans, higher intensity exercise was seen to elicit higher levels of LC3 expression.

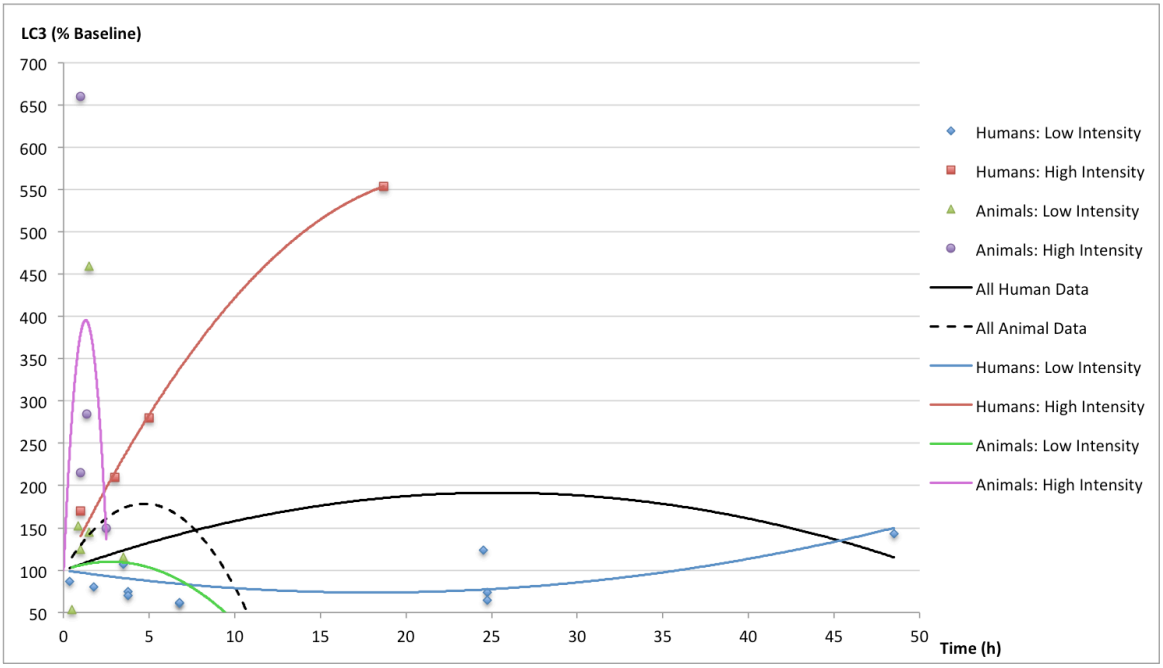


Figure 11: Exercise intensity modulates the acute autophagic response in animals & humans
The X-axis indicates time from the start of exercise, representing the sum, in hours, of the reported exercise duration and period of time until post-exercise data collection; the Y-axis represents the relative intensity of LC3 protein expression. Each point indicates a mean group measure of expressed LC3 protein reported by a single study at the corresponding time, normalized to baseline.

Figure 12 provides a similar representation of Hsp70 data, which also displays an increased response to higher intensity exercise in both humans and rodents. Sufficient human data were available to further explore the scaling of heat shock induction relative to exercise intensity, by grouping Hsp70 according to a quartile division of the METhours range available in our dataset, as seen in **Figure 13**.

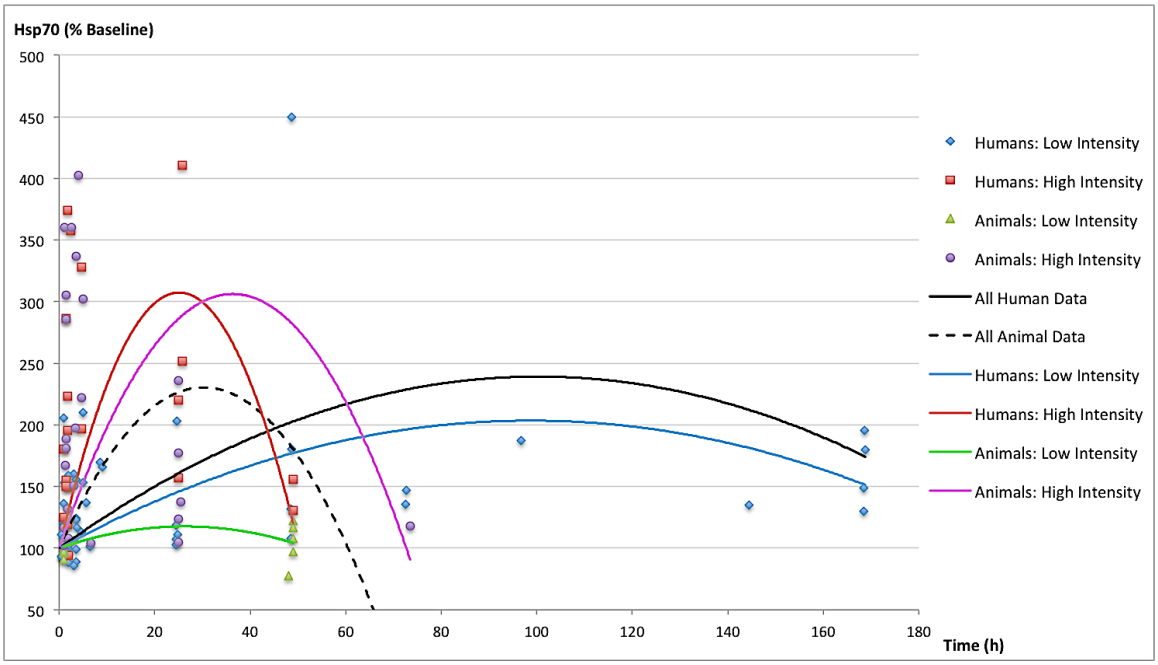


Figure 12: Exercise intensity modulates the acute heat shock response in animals & humans
The X-axis indicates time from the start of exercise, representing the sum, in hours, of the reported exercise duration and period of time until post-exercise data collection; the Y-axis represents the relative intensity of Hsp70 protein expression. Each point indicates a mean group measure of expressed Hsp70 protein reported by a single study at the corresponding time, normalized to baseline.

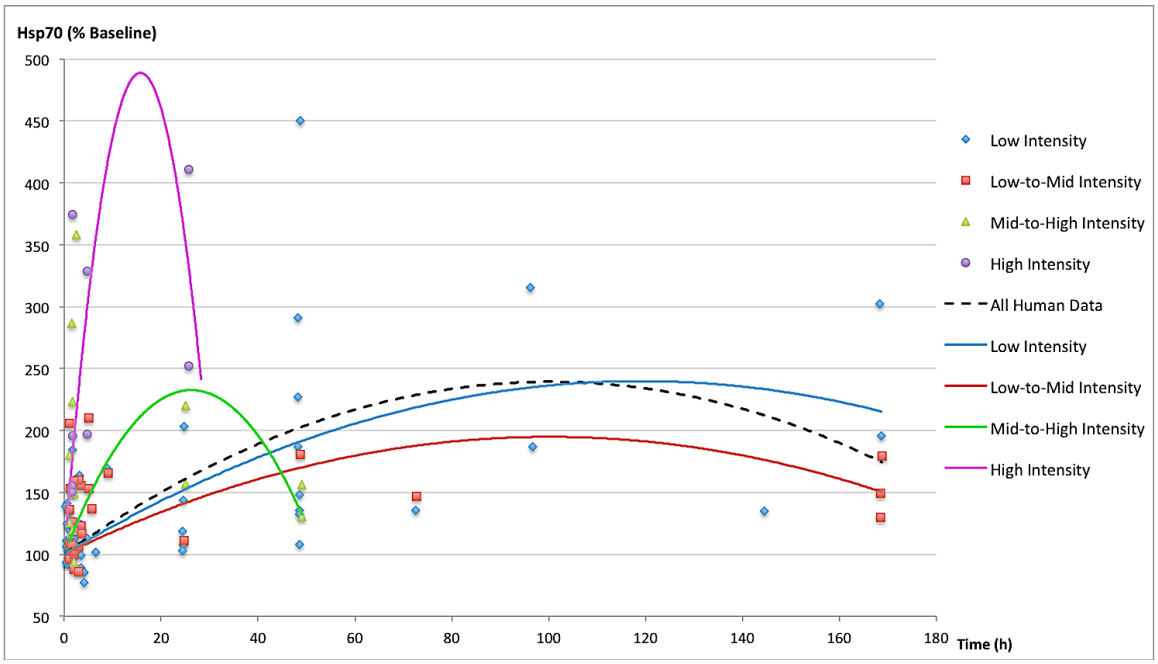


Figure 13: Scaling of the acute heat shock response according to exercise intensity in humans
The X-axis indicates time from the start of exercise, representing the sum, in hours, of the reported exercise duration and period of time until post-exercise data collection; the Y-axis represents the relative intensity of Hsp70 protein expression. Each point indicates a mean group measure of expressed Hsp70 protein reported by a single study at the corresponding time, normalized to baseline. Each intensity group represents 25% of the range in METhours observed in this study (1.48 to 20.53 METhours).

Correlations with Measures of Exercise Intensity

METhours were found to act as the best correlate for human Hsp70 activity during the first 24 hours post exercise (r = 0.61), considerably outperforming either METs (r = 0.09), or exercise duration (r = 0.37) alone. This relationship between METhours and Hsp70 activity was attenuated by 48 hours post exercise (r = 0.41), but remained stronger than the relationship with either METs (r = 0.03), or exercise duration (r = 0.25) alone.

The maximum LC3 response reported for each study included in this project was also best predicted by METhours (r = 0.92), though exercise duration performed almost identically (r = 0.91), and both offered a sizable improvement over METs alone (r = 0.74). These relationships were found to be significantly impacted by a single study involving >18 hours of nearly continuous exercise,[81] with exclusion of this study from calculations strengthening the correlation between METhours and maximum measured autophagy (r = 0.97), while concomitantly weakening the relationship with exercise duration (r = 0.51), and improving the relationship METs alone (r = 0.83).

Graphical representations of these relationships can be found with other supplemental information in the appendix **(p. 53-58)**.

Chapter 5
Discussion

Primary Findings

The results of our analysis reveal that rodents and humans display striking similarities in the successive peaking behavior of autophagic and heat shock systems following acute exercise. The time course response of these systems was considerably accelerated in rodents as compared to human subjects, yet the ratio of time to estimated peak activity for these pathways (time-to-peak autophagy : time-to-peak HSR) was conspicuously similar between models, at 0.16 for animals and 0.25 for humans, particularly given the simplistic predictive methodology employed. In addition, the magnitude of the projected peak activity was similar for both responses in both models, with projected peak autophagy reaching 182% of baseline in animals and 194% of baseline in humans, while peak heat shock activity was projected to reach 231% and 240% of baseline in animals and humans, respectively. This general homogeneity across species lends welcome credence to the veracity of our simplistic predictive model, offering an indication that these rough estimations are reliable, if not altogether accurate in imposed symmetry.

Our projections reinforce the suggested regulatory model, indicating that autophagic activity dominates the proteostatic environment in the immediate post-exercise window, but then diminishes rapidly as HSP expression escalates, returning to baseline well before peak heat shock activity. Consistency in the relationship between time-to-peak activity level in these systems, despite the interspecies differential in absolute time-to-peak activity, adds particular cogency to the proposed paradigm, as

enzyme kinetics are understood to vary between species,[141] yet the sustained relationship we were able to observe helps to negate the possibility that any connection detected previously was an artifact of coincidental timing.

These analyses were also able to detect a differential in tissue-specific responses following exercise, particularly in Hsp70 expression. This has important ramifications for research attempting to extrapolate the easily-obtained data from peripheral blood-borne immune cells to predict proteostatic responses in muscle or other tissues, which can be more difficult to sample, and indicates that results from muscle tissue may not accurately represent proteostatic activity in immune cells. Our pooled indications of a tissue-specific response are in agreement with several individual projects,[34,89,90] and are conceptually quite sensible, as the mechanism and extent of proteostatic challenges are likely to vary considerably from tissue to tissue, according to the nature of the insult.

This is particularly true with regard to exercise and skeletal muscle, as the cellular stress and proteostatic challenge that is imposed by exercise will vary in degree and localization according to the intensity and modality of the exercise performed, being utmost in the specific muscles, and the specific muscle fibers, most activated by a particular exercise.[72] In support of this notion, recently published research has shown that exercise training leads to increased autophagy in a fiber-type specific fashion, with aerobic exercise training preferentially upregulating autophagy in aerobic muscle fibers.[142]

Indications of a divergence in proteostatic activity along these lines has important implications for research design and implementation, arguing for considered care in the

selection of tissue type and measurement location according to the nature of proteostatic challenge induced, and the physiological system(s) of interest.

The scaled response to exercise intensity that we observed in both the autophagic and heat shock systems has also been reported elsewhere in the literature,[90,91] and, like the tissue-specific responses we observed, is conceptually plausible given current understanding of the relationships between muscle damage, anabolic stimulus, and exercise intensity.[143,144] Essentially, higher exercise intensities provoke a greater proteostatic disturbance in the activated muscle fibers by escalating production of heat, reactive oxygen species, and hydrogen ions, leading to increased protein damage and increased demand for the autophagic and heat shock pathways.[72,90,91] At the same time, higher intensity exercise is known to produce a greater anabolic stimulus, upregulating muscle growth and remodeling subsequent to activity.[144]

We saw a clear distinction in the projected activity level of each proteostatic system according to exercise intensity in both humans and rodents, with evidence of layering in the Hsp70 response for human subjects according to several intensity levels, hinting at a titrated heat shock response. Interestingly, the low intensity grouping in human autophagy research was projected to experience decreased autophagic activity following exercise, while low intensity activity seemed more than sufficient to activate the heat shock response. This may indicate that the anabolic stimulus of exercise, likely effected through Akt/mTOR and mediated by Hsp70, was able to overwhelm the concomitant activation of catabolic processes like autophagy at a particular level of intensity, as has been suggested elsewhere.[79] If verified through further research, this

phenomenon may be of considerable interest to athletes focused on optimizing muscular anabolism.

Regular participation in physical activity is frequently linked to benefits in health and longevity.[93] Similarly, autophagic activity and the HSR have both also been independently linked to life span.[46,48,49] Accordingly, the scaled upregulation of these systems in response to exercise of increasing intensity helps establish another plausible mechanistic link to the long term benefits observed with routine activity, and may help shed light on the dose-response relationship. In line with Selye's general adaptation syndrome,[145] some extreme exercise intensities may actually overwhelm available proteostatic mechanisms, while minimal effort activity may provoke insufficient cellular insult to activate these systems, with either case likely leading to suboptimal adaptation and exercise benefit. Indeed, recent epidemiological research suggests that the most avid participants in physical activity face morbidity and mortality outcomes comparable to the most sedentary members of the population.[93] This offers evidence of an upper limit to exercise benefit, while the risks of chronic inactivity have long been established, and continue to be illuminated.[146] Exercise-mediated activation of these proteostatic pathways offers a plausible route of inquiry into the molecular behaviors underlying these epidemiological observations, and may eventually help shed light on the optimal level of activity required to obtain certain health benefits.[52]

Given the modulating effect that exercise intensity seems to exert over autophagy and the HSR, the strong performance of METhours as a correlate of proteostatic responses has definite value for researchers developing exercise interventions intending to stimulate these systems. Some of the research currently available may not have been

able to precipitate the intended proteostatic challenge, simply because the exercise intervention selected proved of insufficient intensity.[143] Moreover, care must be taken in attempting to directly compare the response of these systems from research efforts employing different exercise protocols. Finally, considering that our combinatory metric, METhours, was able to robustly outperform exercise duration and raw MET estimates as a correlate for both Hsp70 and autophagic activity, it is important to carefully evaluate both duration and absolute intensity when designing exercise protocols intended to stimulate these systems.

Limitations

LC3 as a measure of autophagic activity:

While the use of LC3 expression as a sole marker for autophagic activity is widespread in existing literature, the evaluation of this measure has come to be complicated by an increasing number of known limitations.[147-149] The concentration of conjugated LC3 has been shown to correlate well with the total number of intracellular autophagosomes; however, a portion of this marker is also destroyed with the autophagosome upon reaching the lysosome, which seriously complicates the interpretation of measured LC3 concentrations in times autophagic flux.[149] Moreover, LC3 has been shown to readily interact with aggregated protein structures, independent of autophagy.[147] While some authorities have recommended using the LC3-II/LC3-I ratio, this method also presents difficulties, as LC3 immunoreactivity has been found to increase upon conjugation, leading to exaggerated comparative measures of LC3-II.[149] Unfortunately, previously published work cannot be expected reflect modern wisdom, and LC3 proved to be the only measure of autophagic activity with sufficient prevalence

in the available literature to allow a pooled analysis; however, these values and the resultant projections should be interpreted with care.

Estimating intensity

The use of metabolic equivalents as an estimate of intensity is a widely accepted scientific convention with considerable known limitations. 1 MET is intended to approximate a standard level of resting energy consumption, though the accepted value of $\dot{V}O_2$ = 3.5 ml/kg/min seems to have been derived from the resting measures of a single 70 kg, 40 year-old man.[150] Subsequent research using larger groups supports the idea that this value is significantly lower for most people.[150] Moreover, exercise intensity is relative to individual athletic conditioning, and modality-specific training status.[100,151] The Compendium of Physical Activities was utilized to locate the majority of research data used to fix intensity in this project, and the creators of the Compendium highlight that while the resource confers a valuable ability to estimate exercise cost for large groups, extrapolation cannot precisely estimate energy cost for individuals.[100] Given that the METhours estimated in this project considerably outperformed exercise duration as a correlate to measurements of autophagic and heat shock activity, the value of this group approximation capacity seems clear. Despite this apparent utility, the estimations of intensity employed herein likely contain considerable inaccuracy, and our relevant conclusions must be cautiously interpreted.

Combined tissues

It is important to note that several of our projections include combined data from PBMCs and muscle tissue, despite our observation that these tissues display a divergent magnitude in the response of proteostatic systems following exercise. In light of relative

scarcity, all available data was pooled for analysis in this project, and it is our hope that future research efforts will allow for more specifically targeted analyses. In the meantime, these combinatory projections are limited by the incorporated variability.

Forced symmetry in the parabolic model

It is also important to understand that the basic parabolic model used herein to predict the activity of autophagic systems and the HSR is simplistic, reductive, and fundamentally acts to impose unnatural symmetry on complex biological processes. These projections should not be strictly interpreted, and are intended only to give a broad view of the focal proteostatic processes, in the hope that this will help to highlight the most obvious relationships, and call attention to gaps in existing research.

Recommendations

Clearly, further research into the regulatory coordination of these systems seems warranted, and this analysis helps illuminate areas in the existing research where data are lacking. We found that many measurement time points for Hsp70 and LC3 following acute exercise are duplicated with considerable redundancy, while limited data are available to cast light on the behavior of these systems in the 24 to 48 hour window post-exercise, with even less information available after 48 hours.

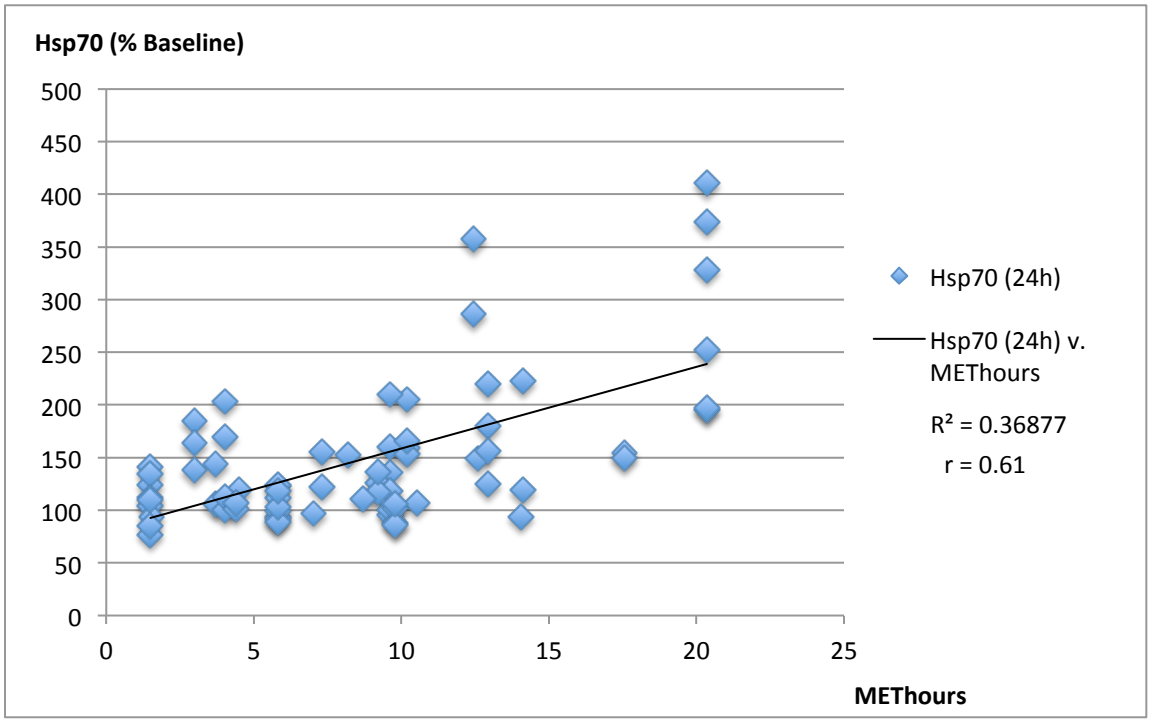
More research projects focusing on comparative activation of these systems following exercise of various intensities would also be helpful in determining the level of activity required to achieve robust activation of these systems, and animal research may allow us to explore an upper limit to healthy activation through physical activity. It may also be interesting to evaluate how exercise employing different intensities, and different quantities of muscle mass differentially impact the proteostatic processes in activated

tissues, as well as ancillary systems (e.g. vascular), that may be affected by the concentrated waste products of vigorous exercise.

Conclusion

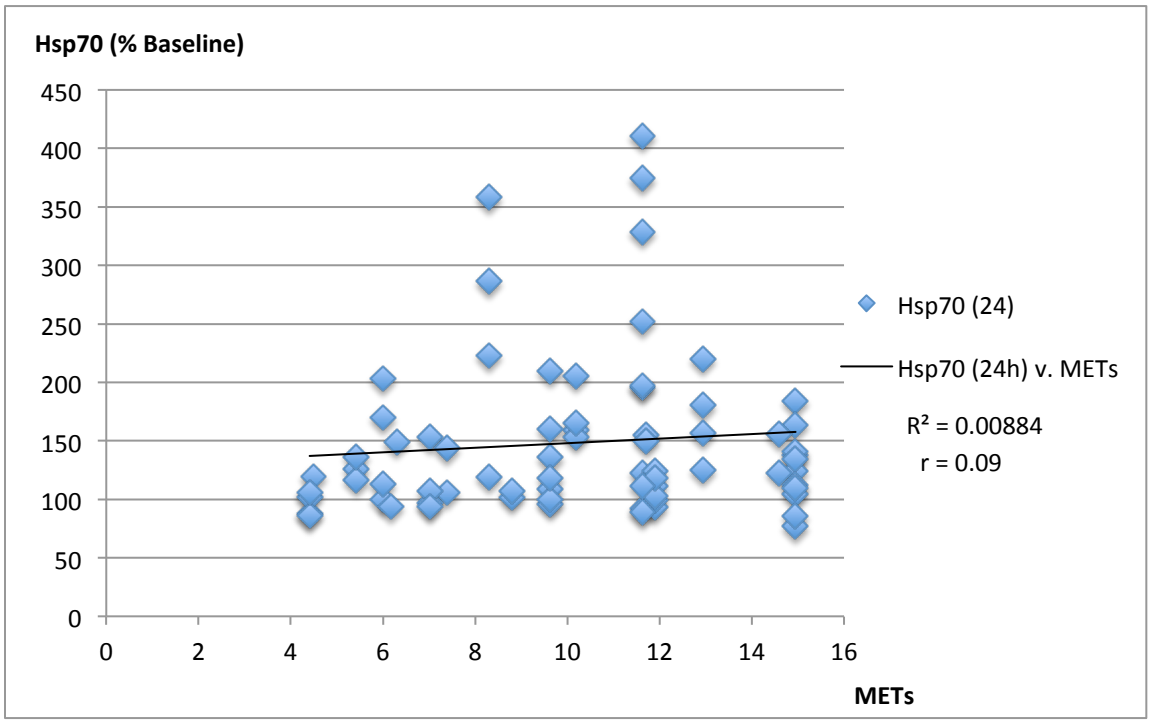
In summary, our pooled analysis reinforces the proposed regulatory model for coordination between heat shock and autophagic pathways, offering interspecies support for an Hsp70-moderated transition away from the presiding catabolic influence of autophagy in the immediate post-exercise window, eventually allowing the molecular chaperones to begin a phase of restoration and remodeling. This relationship has been demonstrated with direct humans cellular research following exercise,[80] and further investigation into the intricacies of this regulatory coordination seems highly meritorious. The differential responses we observed in these two primary proteostatic systems according to exercise intensity and tissue of origin may also have important implications for research design, and perhaps eventually for exercise prescription.

Appendix



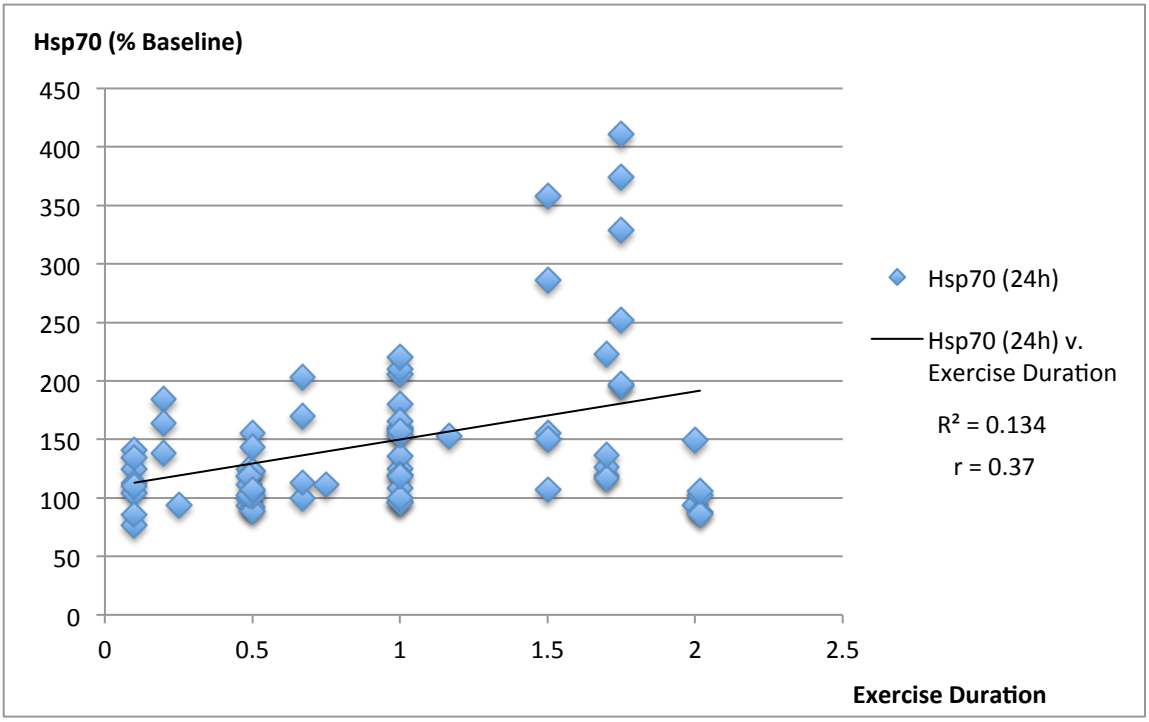
Supplemental Figure 1: Correlation between human Hsp70 protein expression in the 24h post-exercise window and estimated METhours

R^2 = coefficient of determination, r = Pearson product-moment correlation coefficient.



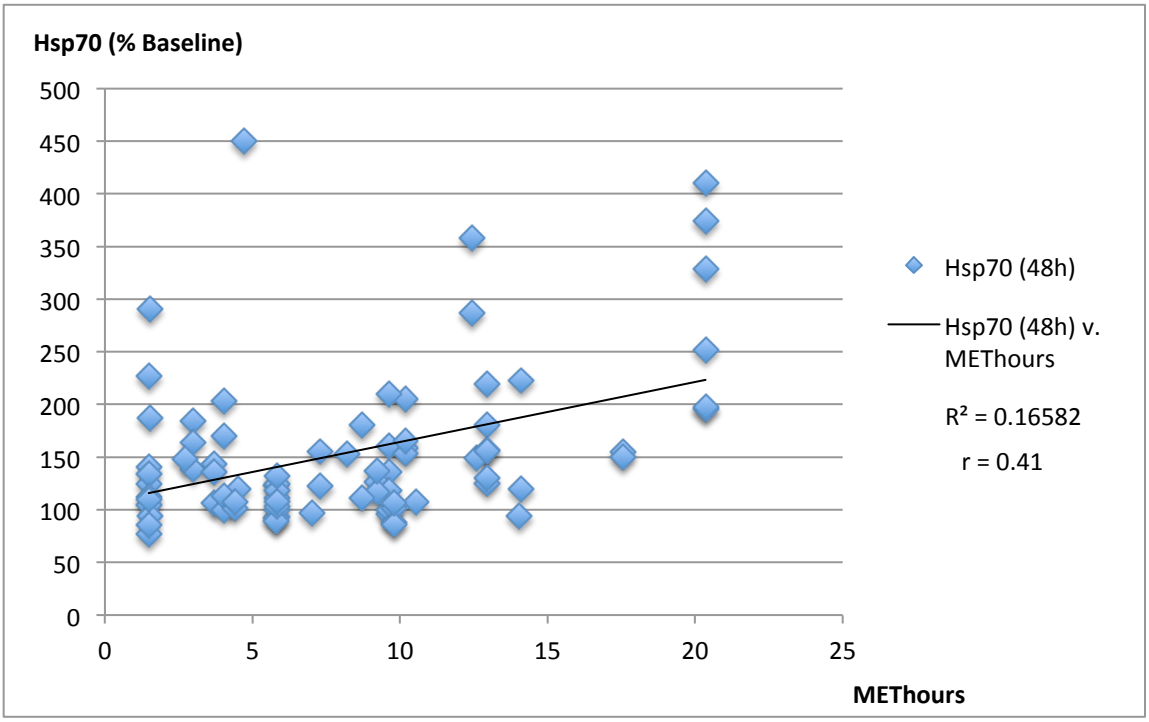
Supplemental Figure 2: Correlation between human Hsp70 protein expression in the 24h post-exercise window and estimated METs

R^2 = coefficient of determination, r = Pearson product-moment correlation coefficient.



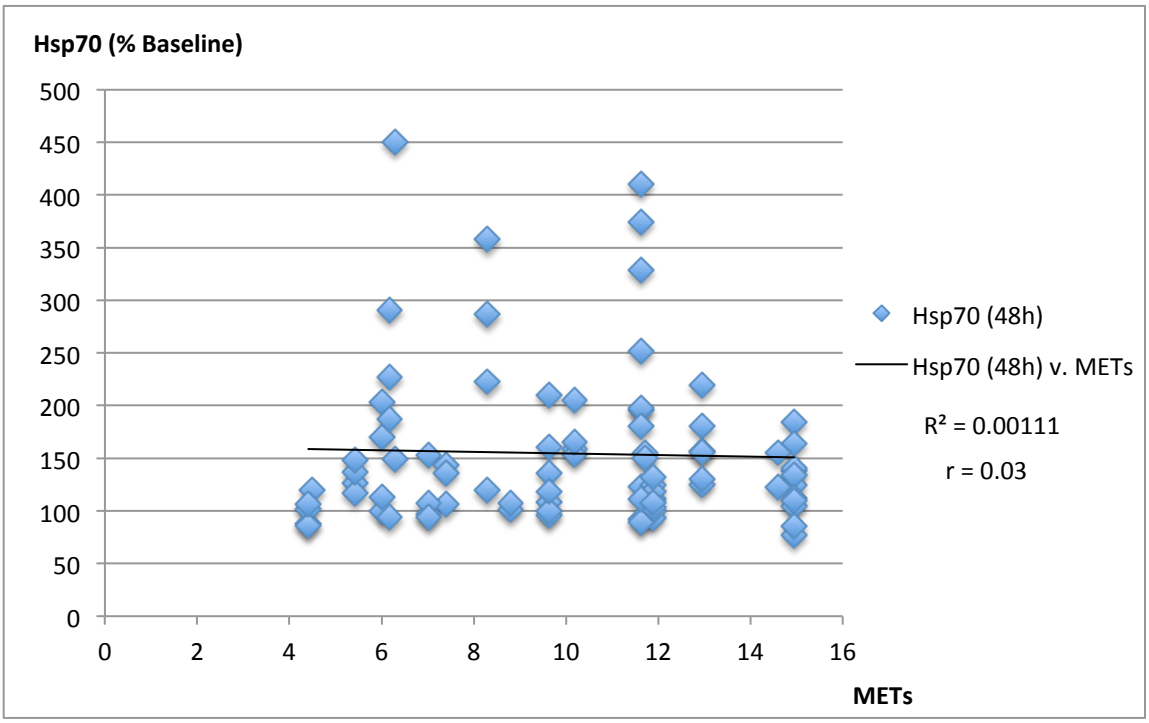
Supplemental Figure 3: Correlation between human Hsp70 protein expression in the 24h post-exercise window and exercise duration

R^2 = coefficient of determination, r = Pearson product-moment correlation coefficient.



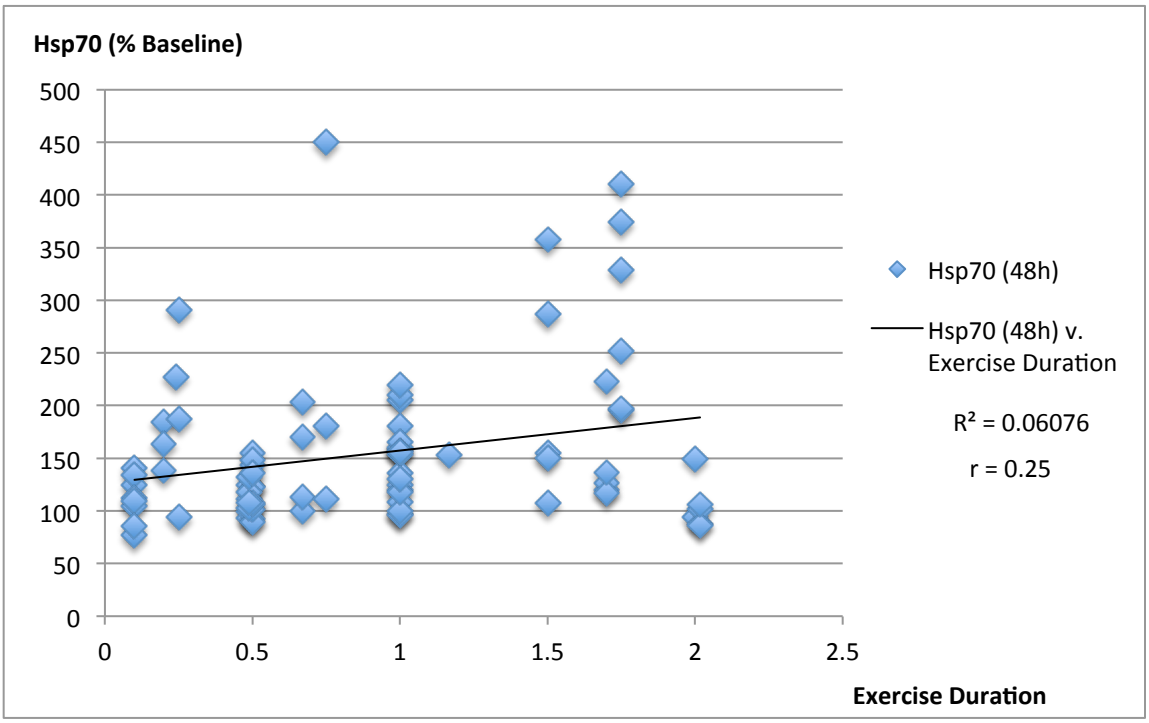
Supplemental Figure 4: Correlation between human Hsp70 protein expression in the 48h post-exercise window and estimated METhours

R^2 = coefficient of determination, r = Pearson product-moment correlation coefficient.



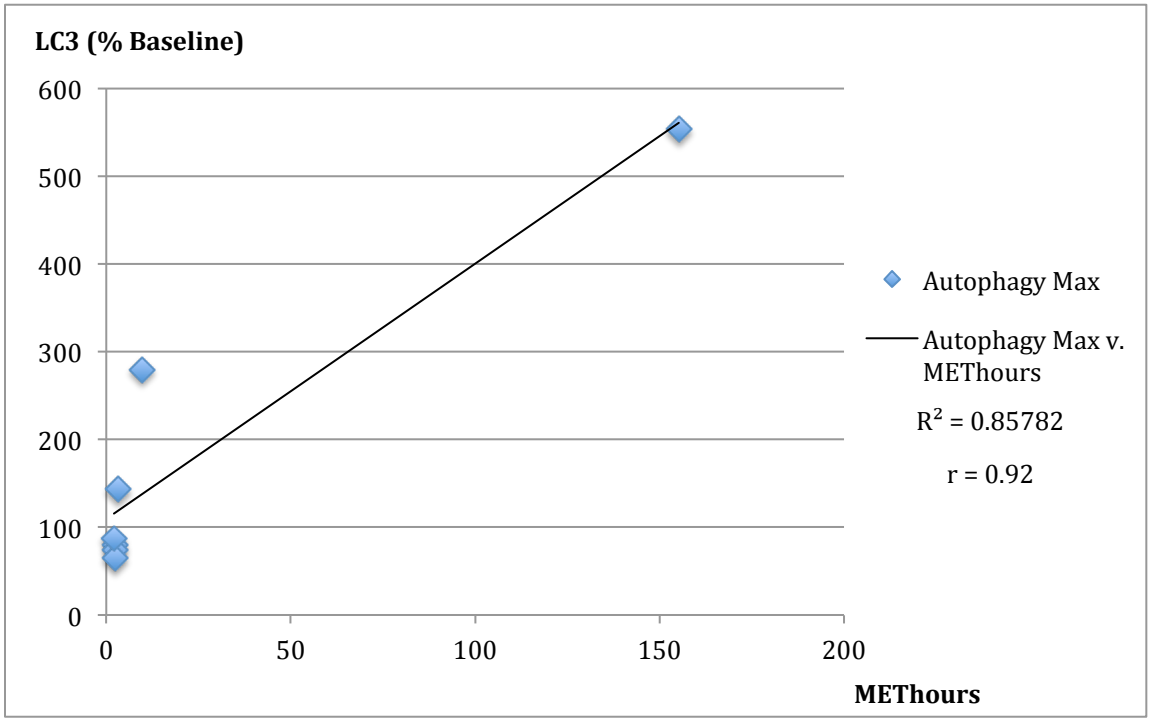
Supplemental Figure 5: Correlation between human Hsp70 protein expression in the 48h post-exercise window and estimated METs

R^2 = coefficient of determination, r = Pearson product-moment correlation coefficient.



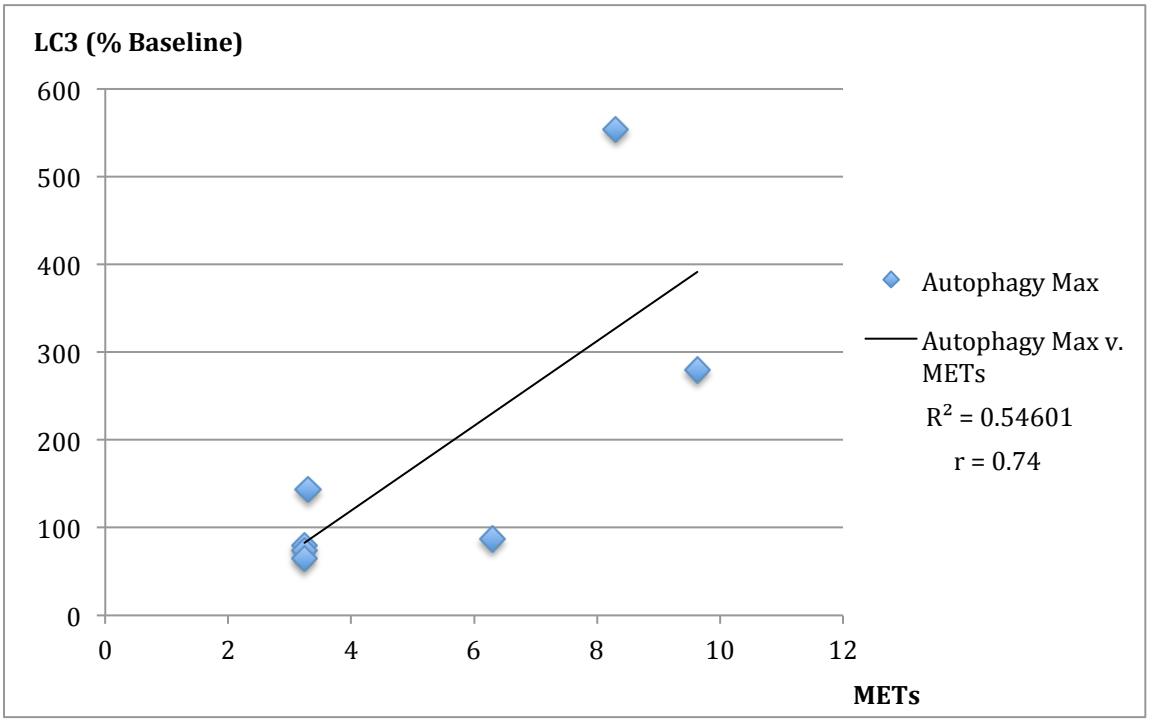
Supplemental Figure 6: Correlation between human Hsp70 protein expression in the 48h post-exercise window and exercise duration

R^2 = coefficient of determination, r = Pearson product-moment correlation coefficient.



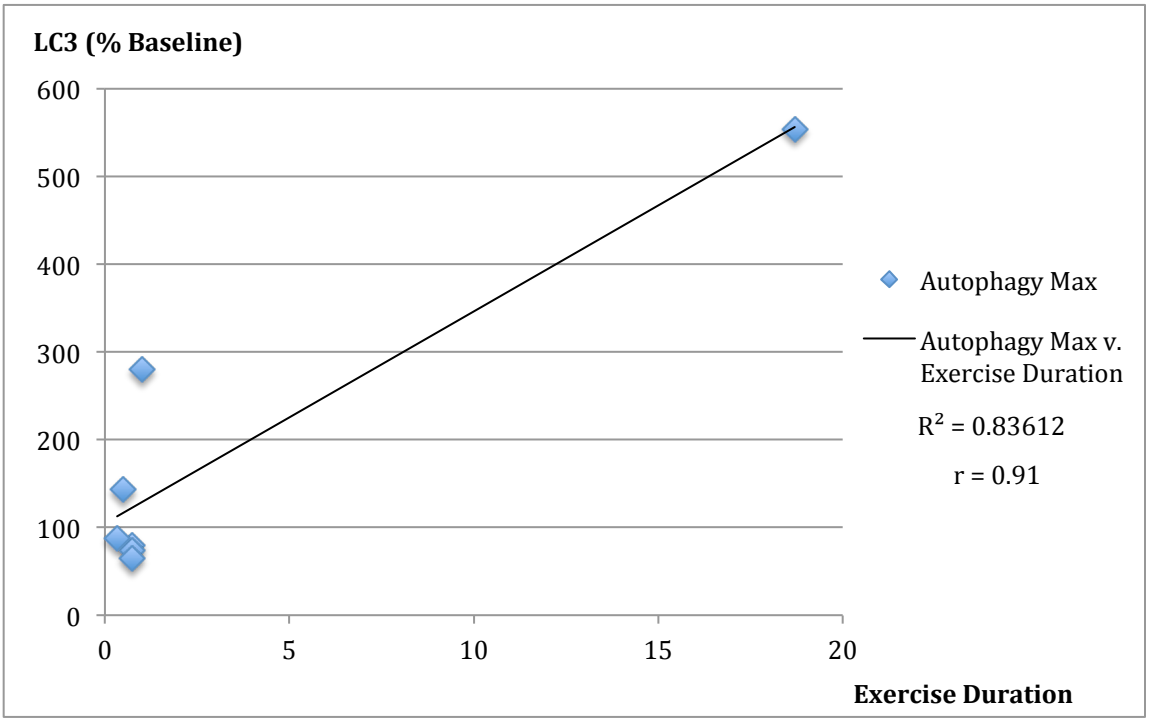
Supplemental Figure 7: Correlation between maximal human LC3 protein expression and estimated METhours

R^2 = coefficient of determination, r = Pearson product-moment correlation coefficient.



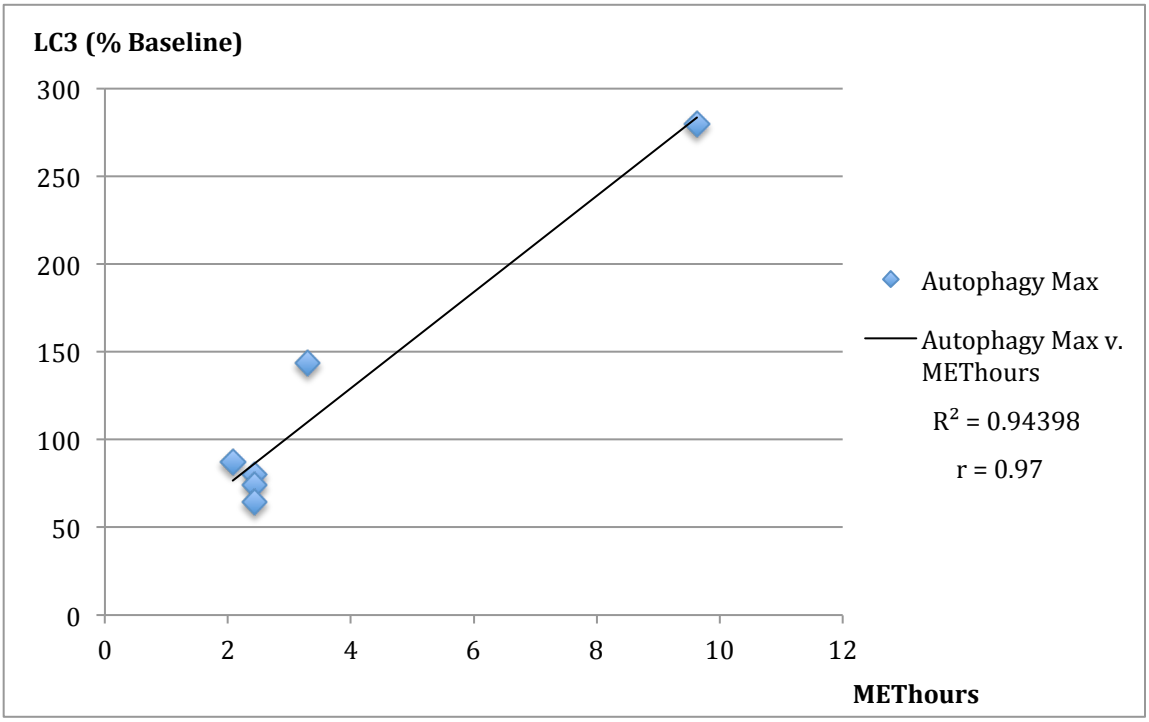
Supplemental Figure 8: Correlation between maximal human LC3 protein expression and estimated METS

R^2 = coefficient of determination, r = Pearson product-moment correlation coefficient.



Supplemental Figure 9: Correlation between maximal human LC3 protein expression and exercise duration

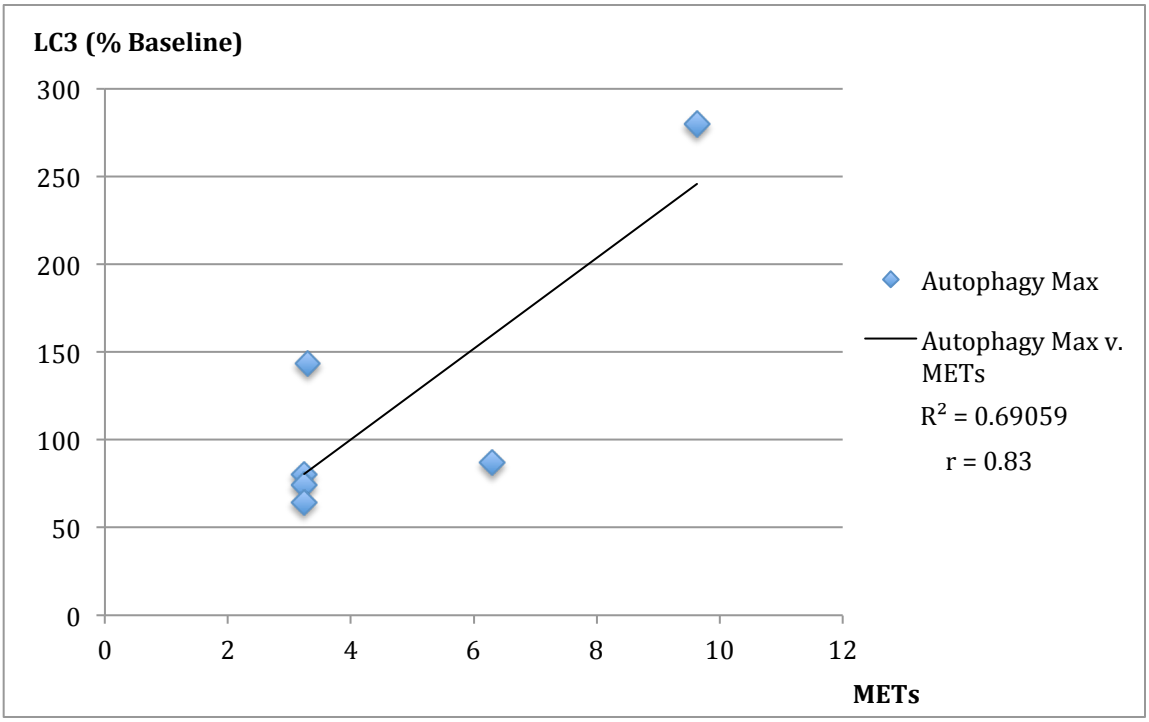
R^2 = coefficient of determination, r = Pearson product-moment correlation coefficient.



Supplemental Figure 10: Correlation between maximal human LC3 protein expression and estimated METhours

ref 81 excluded, see p. 44

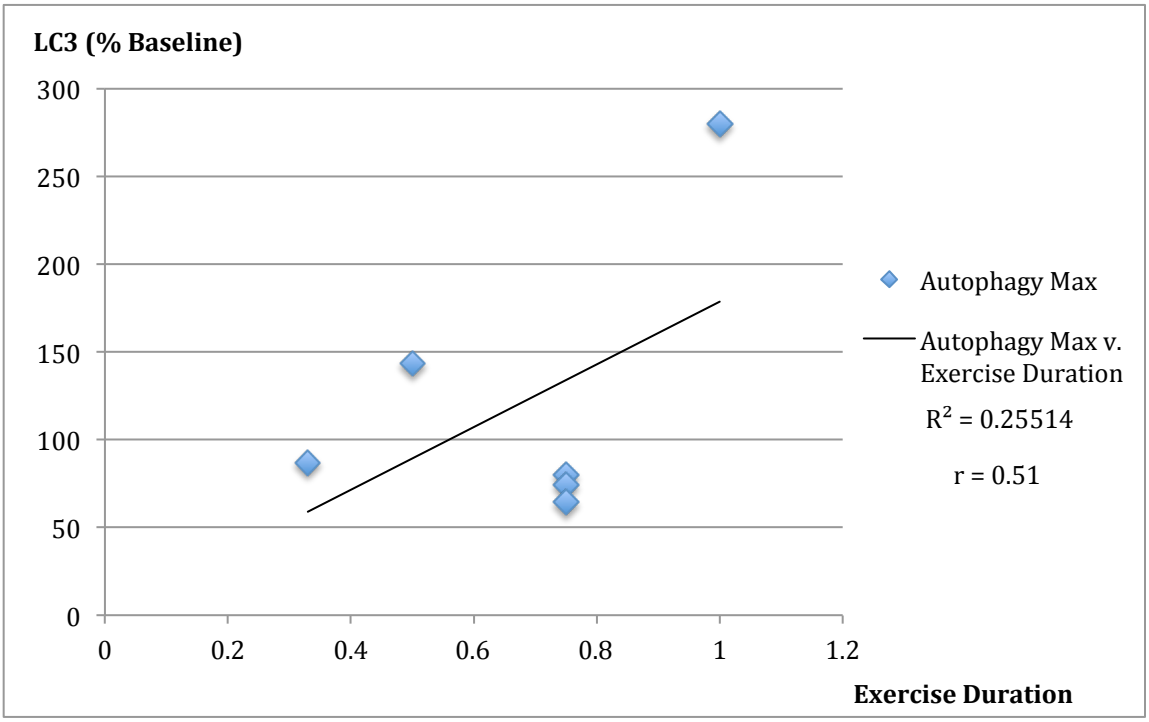
R^2 = coefficient of determination, r = Pearson product-moment correlation coefficient.



Supplemental Figure 11: Correlation between maximal human LC3 protein expression and estimated METs

ref 81 excluded, see p. 44

R^2 = coefficient of determination, r = Pearson product-moment correlation coefficient.



Supplemental Figure 12: Correlation between maximal human LC3 protein expression and exercise duration

ref 81 excluded, see p. 44

R^2 = coefficient of determination, r = Pearson product-moment correlation coefficient.

Table 1: Included Studies Pertaining to Autophagy in Humans

Author	Tissue	Exercise Protocol	Intensity Group	METs	METs Source	MET hours	Exercise Duration (h)	Collection Time (h)	Total Time (h)	n	LC3 (% baseline)	Measure
Glynn (2010) [95]	Vastus Lateralis	Leg extension: 10sets of 10reps @ 70% 1RM, 3 min RP	Low	3.24	Haddock and Wilkin (2006) [152]	2.43	0.75	1	1.75	13	80	LC3bII
Jamart (2012) [74]	Vastus Lateralis	Treadmill: variable speed (7.9 ± 0.7 km/h)	High	8.3	Sherrman, Morris et al. (1998) [153]	155.21	18.7	0	18.7	11	554	LC3bII
Dokladny (2013)[80]	PBMC	Treadmill: 60 min at 70 – 80% of VO2max	High	9.77	Welk, Blair et al. (2000) [154]	9.77	1	0	1	8	170	LC3-II/ß-actin
Dokladny (2013)[80]	PBMC	Treadmill: 60 min at 70 – 80% of VO2max	High	9.77	Welk, Blair et al. (2000) [154]	9.77	1	2	3	8	210	LC3-II/ß-actin
Dokladny (2013)[80]	PBMC	Treadmill: 60 min at 70 – 80% of VO2max	High	9.77	Welk, Blair et al. (2000) [154]	9.77	1	4	5	8	280	LC3-II/ß-actin
Fry (2013) [85]	Vastus Lateralis	Leg extension: 8 sets of 10 reps @ 70% 1RM, 3 min rest periods	Low	3.24	Haddock and Wilkin (2006) [152]	2.43	0.75	3	3.75	16	75	LC3b-II/LC3b-I
Fry (2013) [85]	Vastus Lateralis	Leg extension: 8 sets of 10 reps @ 70% 1RM, 3 min rest periods	Low	3.24	Haddock and Wilkin (2006) [152]	2.43	0.75	6	6.75	16	60.5	LC3b-II/LC3b-I
Fry (2013) [85]	Vastus Lateralis	Leg extension: 8 sets of 10 reps @ 70% 1RM, 3 min rest periods	Low	3.24	Haddock and Wilkin (2006) [152]	2.43	0.75	24	24.75	16	74.1	LC3b-II/LC3b-I
Fry (2013) [85]	Vastus Lateralis	Leg extension: 8 sets of 10 reps @ 70% 1RM, 3 min rest periods	Low	3.24	Haddock and Wilkin (2006) [152]	2.43	0.75	3	3.75	16	70	LC3b-II/LC3b-I
Fry (2013) [85]	Vastus Lateralis	Leg extension: 8 sets of 10 reps @ 70% 1RM, 3 min rest periods	Low	3.24	Haddock and Wilkin (2006) [152]	2.43	0.75	6	6.75	16	62	LC3b-II/LC3b-I
Fry (2013) [85]	Vastus Lateralis	Leg extension: 8 sets of 10 reps @ 70% 1RM, 3 min rest periods	Low	3.24	Haddock and Wilkin (2006) [152]	2.43	0.75	24	24.75	16	64.3	LC3b-II/LC3b-I

Table 1 (continued): Included Studies Pertaining to Autophagy in Humans

Author	Tissue	Exercise Protocol	Intensity Group	METs	METs Source	MET hours	Exercise Duration (h)	Collection Time (h)	Total Time (h)	n	LC3 (% baseline)	Measure
Masschelein (2013) [87]	Vastus Lateralis	Cycle ergometer: @ 50.7% of VO2max	Low	6.3	Lang, Latin et al. (1992) [155]	2.08	0.33	0	0.33	22	87	LC3b-II/LC3b-I
Ogborn (2015) [79]	Vastus Lateralis	Single leg press & extension: 4 sets each, 10 reps/set, @ 75% 1RM, 2 min rest periods	Low	3.3	Phillips and Ziuraitis (2004) [123]	1.65	0.5	3	3.5	18	107.14	LC3bII
Ogborn (2015) [79]	Vastus Lateralis	Single leg press & extension: 4 sets each, 10 reps/set, @ 75% 1RM, 2 min rest periods	Low	3.3	Phillips and Ziuraitis (2004) [123]	1.65	0.5	24	24.5	18	123.32	LC3bII
Ogborn (2015) [79]	Vastus Lateralis	Single leg press & extension: 4 sets each, 10 reps/set, @ 75% 1RM, 2 min rest periods	Low	3.3	Phillips and Ziuraitis (2004) [123]	1.65	0.5	48	48.5	18	143.53	LC3bII

Table 2: Included Studies Pertaining to the Heat Shock Response in Humans											
Author	**Tissue**	**Exercise Protocol**	**Intensity Groups**	**METs**	**METs Source**	**METhours**	**Exercise Duration (h)**	**Collection Time (h)**	**Total Time (h)**	**n**	**Hsp70 (% baseline)**
Ryan (1991) [88]	PBMC	Treadmill: 30-45min run @ 4-6 km/h, then 75-90 min walk @ 2-10º grade.	Low, Mid-Low	4.41	Crouter, Clowers et al. (2006) [156]	9.80	2.02	0	2.02	5	88
Ryan (1991) [88]	PBMC	Treadmill: 30-45min run @ 4-6 km/h, then 75-90 min walk @ 2-10º grade.	Low, Mid-Low	4.41	Crouter, Clowers et al. (2006) [156]	9.80	2.02	0	2.02	5	102
Ryan (1991) [88]	PBMC	Treadmill: 30-45min run @ 4-6 km/h, then 75-90 min walk @ 2-10º grade.	Low, Mid-Low	4.41	Crouter, Clowers et al. (2006) [156]	9.80	2.02	1	3.02	5	86
Ryan (1991) [88]	PBMC	Treadmill: 30-45min run @ 4-6 km/h, then 75-90 min walk @ 2-10º grade.	Low, Mid-Low	4.41	Crouter, Clowers et al. (2006) [156]	9.80	2.02	1	3.02	5	106
Puntschart (1996) [12]	Vastus lateralis	Treadmill: 30 min @ AT	Low, Low	11.63	Wulff, Cochrane et al. (1998) [157]	5.82	0.5	0.07	0.57	5	92.19
Puntschart (1996) [12]	Vastus lateralis	Treadmill: 30 min @ AT	Low, Low	11.63	Wulff, Cochrane et al. (1998) [157]	5.82	0.5	0.5	1	5	123.03
Puntschart (1996) [12]	Vastus lateralis	Treadmill: 30 min @ AT	Low, Low	11.63	Wulff, Cochrane et al. (1998) [157]	5.82	0.5	3	3.5	5	88.89
Fehrenbach (2000) [124]	Monocytes	Half marathon (21.1 km), self paced	High, High	11.63	Wulff, Cochrane et al. (1998) [157]	20.35	1.75	0	1.75	12	374.35
Fehrenbach (2000) [124]	Monocytes	Half marathon (21.1 km), self paced	High, High	11.63	Wulff, Cochrane et al. (1998) [157]	20.35	1.75	3	4.75	12	328.27
Fehrenbach (2000) [124]	Monocytes	Half marathon (21.1 km), self paced	High, High	11.63	Wulff, Cochrane et al. (1998) [157]	20.35	1.75	24	25.75	12	410.47
Fehrenbach (2000) [124]	Granulocytes	Half marathon (21.1 km), self paced	High, High	11.63	Wulff, Cochrane et al. (1998) [157]	20.35	1.75	0	1.75	12	195.7
Fehrenbach (2000) [124]	Granulocytes	Half marathon (21.1 km), self paced	High, High	11.63	Wulff, Cochrane et al. (1998) [157]	20.35	1.75	3	4.75	12	196.96
Fehrenbach (2000) [124]	Granulocytes	Half marathon (21.1 km), self paced	High, High	11.63	Wulff, Cochrane et al. (1998) [157]	20.35	1.75	24	25.75	12	251.9
Fehrenbach (2001) [111]	Monocytes	Treadmill: 60min @ 90% AT	High, Mid-High	12.94	Mercer, Dolgan et al. (2008) [158]	12.94	1	0	1	6	180.2
Fehrenbach (2001) [111]	Monocytes	Treadmill: 60min @ 90% AT	High, Mid-High	12.94	Mercer, Dolgan et al. (2008) [158]	12.94	1	24	25	6	220
Fehrenbach (2001) [111]	Monocytes	Treadmill: 60min @ 90% AT	High, Mid-High	12.94	Mercer, Dolgan et al. (2008) [158]	12.94	1	48	49	6	156
Fehrenbach (2001) [111]	Granulocytes	Treadmill: 60min @ 90% AT	High, Mid-High	12.94	Mercer, Dolgan et al. (2008) [158]	12.94	1	0	1	6	125.2
Fehrenbach (2001) [111]	Granulocytes	Treadmill: 60min @ 90% AT	High, Mid-High	12.94	Mercer, Dolgan et al. (2008) [158]	12.94	1	24	25	6	156.8

Table 2 (continued): Included Studies Pertaining to the Heat Shock Response in Humans

Author	Tissue	Exercise Protocol	Intensity Groups	METs	METs Source	METhours	Exercise Duration (h)	Collection Time (h)	Total Time (h)	n	Hsp70 (% baseline)
Fehrenbach (2001) [111]	Granulocytes	Treadmill: 60min @ 90% AT	High, Mid-High	12.94	Mercer, Dolgan et al. (2008) [158]	12.94	1	48	49	6	130.6
Niess (2002) [110]	Lymphocytes	Treadmill: GXT; 15min break; Run @ 110% AT	Low, Low	11.89	Wyndham, van Graan et al. (1971)[159]	5.83	0.49	0	0.49	9	111.28
Niess (2002) [110]	Lymphocytes	Treadmill: GXT; 15min break; Run @ 110% AT	Low, Low	11.89	Wyndham, van Graan et al. (1971)[159]	5.83	0.49	3	3.49	9	99.21
Niess (2002) [110]	Lymphocytes	Treadmill: GXT; 15min break; Run @ 110% AT	Low, Low	11.89	Wyndham, van Graan et al. (1971)[159]	5.83	0.49	24	24.49	9	102.84
Niess (2002) [110]	Lymphocytes	Treadmill: GXT; 15min break; Run @ 110% AT	Low, Low	11.89	Wyndham, van Graan et al. (1971)[159]	5.83	0.49	48	48.49	9	107.9
Niess (2002) [110]	Granulocytes	Treadmill: GXT; 15min break; Run @ 110% AT	Low, Low	11.89	Wyndham, van Graan et al. (1971)[159]	5.83	0.49	0	0.49	9	93.46
Niess (2002) [110]	Granulocytes	Treadmill: GXT; 15min break; Run @ 110% AT	Low, Low	11.89	Wyndham, van Graan et al. (1971)[159]	5.83	0.49	3	3.49	9	124.45
Niess (2002) [110]	Granulocytes	Treadmill: GXT; 15min break; Run @ 110% AT	Low, Low	11.89	Wyndham, van Graan et al. (1971)[159]	5.83	0.49	24	24.49	9	118.35
Niess (2002) [110]	Granulocytes	Treadmill: GXT; 15min break; Run @ 110% AT	Low, Low	11.89	Wyndham, van Graan et al. (1971)[159]	5.83	0.49	48	48.49	9	132.03
Thompson (2002) [83]	Biceps brachii	Preacher Curl: 2 sets x 50 eccentric MVCs @ 1/15 s, 2 min break	Low, Low	6.18	Ballor, Becquc et al. (1987) [160]	1.55	0.25	48	48.25	10	186.96
Khassaf (2003) [109]	Vastus lateralis	Cycle: single leg, 70% VO_2peak for 45 min	Low, Low	6.3	Lang, Latin et al. (1992) [155]	4.73	0.75	48	48.75	16	450
Thompson (2003) [108]	Biceps brachii	Preacher Curl: 2 sets x 50 eccentric MVCs @ 1/15 s, 2 min break	Low, Low	6.18	Ballor, Becquc et al. (1987) [160]	1.48	0.24	48	48.24	8	227
Thompson (2003) [108]	Vastus lateralis	Treadmill: 30 min downhill running (-10°)	Low, Low	5.42	Sherrman, Morris et al. (1998) [153]	2.71	0.50	48	48.5	8	148
Febbraio (2004) [107]	Vastus lateralis	Cycle: semirecumbant, 65% VO_2max for 120 min	High, Mid-High	6.3	Lang, Latin et al. (1992) [155]	12.60	2	0	2	6	149.31
Morton (2006) [106]	Vastus lateralis	Treadmill: 45 min running @ LT	Low, Mid-Low	11.63	Wulff, Cochrane et al. (1998) [157]	8.72	0.75	24	24.75	8	111.11
Morton (2006) [106]	Vastus lateralis	Treadmill: 45 min running @ LT	Low, Mid-Low	11.63	Wulff, Cochrane et al. (1998) [157]	8.72	0.75	48	48.75	8	180.34
Morton (2006) [106]	Vastus lateralis	Treadmill: 45 min running @ LT	Low, Mid-Low	11.63	Wulff, Cochrane et al. (1998) [157]	8.72	0.75	72	72.75	8	147
Morton (2006) [106]	Vastus lateralis	Treadmill: 45 min running @ LT	Low, Mid-Low	11.63	Wulff, Cochrane et al. (1998) [157]	8.72	0.75	168	168.75	8	179.48

Table 2 (continued): Included Studies Pertaining to the Heat Shock Response in Humans

Author	Tissue	Exercise Protocol	Intensity Groups	METs	METs Source	METhours	Exercise Duration (h)	Collection Time (h)	Total Time (h)	n	Hsp70 (% baseline)
Paulsen (2007) [122]	Vastus lateralis	Quadriceps: 300 unilateral, maximal, isokinetic, eccentric actions (10º/s)	Low, Low	6	Taylor, Jacobs et al. (1978) [161]	4.02	0.67	0.5	1.17	11	100
Paulsen (2007) [122]	Vastus lateralis	Quadriceps: 300 unilateral, maximal, isokinetic, eccentric actions (10º/s)	Low, Low	6	Taylor, Jacobs et al. (1978) [161]	4.02	0.67	4	4.67	11	112.94
Paulsen (2007) [122]	Vastus lateralis	Quadriceps: 300 unilateral, maximal, isokinetic, eccentric actions (10º/s)	Low, Low	6	Taylor, Jacobs et al. (1978) [161]	4.02	0.67	8	8.67	11	169.78
Paulsen (2007) [122]	Vastus lateralis	Quadriceps: 300 unilateral, maximal, isokinetic, eccentric actions (10º/s)	Low, Low	6	Taylor, Jacobs et al. (1978) [161]	4.02	0.67	24	24.67	11	203.12
Paulsen (2007) [122]	Vastus lateralis	Quadriceps: 300 unilateral, maximal, isokinetic, eccentric actions (10º/s)	Low, Low	6	Taylor, Jacobs et al. (1978) [161]	4.02	0.67	96	96.67	11	187.18
Paulsen (2007) [122]	Vastus lateralis	Quadriceps: 300 unilateral, maximal, isokinetic, eccentric actions (10º/s)	Low, Low	6	Taylor, Jacobs et al. (1978) [161]	4.02	0.67	168	168.67	11	195.57
Tupling (2007) [121]	Vastus lateralis	Single-leg isometric knee extention @ 50% MVC for 30 min	Low, Low	7.4	Ballor, Becquc et al. (1987) [160]	3.70	0.5	0	0.5	10	106.13
Tupling (2007) [121]	Vastus lateralis	Single-leg isometric knee extention @ 50% MVC for 30 min	Low, Low	7.4	Ballor, Becquc et al. (1987) [160]	3.70	0.5	24	24.5	10	143.63
Tupling (2007) [121]	Vastus lateralis	Single-leg isometric knee extention @ 50% MVC for 30 min	Low, Low	7.4	Ballor, Becquc et al. (1987) [160]	3.70	0.5	48	48.5	10	135.61
Tupling (2007) [121]	Vastus lateralis	Single-leg isometric knee extention @ 50% MVC for 30 min	Low, Low	7.4	Ballor, Becquc et al. (1987) [160]	3.70	0.5	72	72.5	10	135.39
Tupling (2007) [121]	Vastus lateralis	Single-leg isometric knee extention @ 50% MVC for 30 min	Low, Low	7.4	Ballor, Becquc et al. (1987) [160]	3.70	0.5	144	144.5	10	134.74
Yamada (2007) [120]	PBMC	Treadmill: 2 x 50 min bouts @ 56% VO_2max,15 min rest	High, High	8.3	Sherrman, Morris et al. (1998) [153]	14.11	1.7	0	1.7	8	119.22
Watkins (2008) [119]	Vastus lateralis	Cycling: 30 min @ 70% VO_2max	Low, Low	8.79	Achten and Jenkendrup (2003) [162]	4.40	0.5	6	6.5	10	101.42
Watkins (2008) [119]	Vastus lateralis	Cycling: 30 min @ 70% VO_2max	Low, Low	8.79	Achten and Jenkendrup (2003) [162]	4.40	0.5	24	24.5	10	107.17
Quadrilatero (2009) [86]	Vastus lateralis	Cycling: 2h @ 60% VO_2max	Low, Mid-Low	7.02	Achten and Jenkendrup (2003) [162]	7.02	1	0	1	8	96.7
Quadrilatero (2009) [86]	Vastus lateralis	Cycling: 2h @ 60% VO_2max	High, Mid-High	7.02	Achten and Jenkendrup (2003) [162]	14.04	2	0	2	8	93.7
Paulsen (2009) [84]	Biceps brachii	Eccentric elbow flexion: 180º @ 30º/s (14 sets x 5 reps, 30s between)	Low, Low	6.18	Ballor, Becquc et al. (1987) [160]	1.55	0.25	1	1.25	24	94.02
Paulsen (2009) [84]	Biceps brachii	Eccentric elbow flexion: 180º @ 30º/s (14 sets x 5 reps, 30s between)	Low, Low	6.18	Ballor, Becquc et al. (1987) [160]	1.55	0.25	48	48.25	24	290.98
Paulsen (2009) [84]	Biceps brachii	Eccentric elbow flexion: 180º @ 30º/s (14 sets x 5 reps, 30s between)	Low, Low	6.18	Ballor, Becquc et al. (1987) [160]	1.55	0.25	96	96.25	24	315.48

Table 2 (continued): Included Studies Pertaining to the Heat Shock Response in Humans											
Author	**Tissue**	**Exercise Protocol**	**Intensity Groups**	**METs**	**METs Source**	**METhours**	**Exercise Duration (h)**	**Collection Time (h)**	**Total Time (h)**	**n**	**Hsp70 (% baseline)**
Paulsen (2009) [84]	Biceps brachii	Eccentric elbow flexion: 180º @ 30º/s (14 sets x 5 reps, 30s between)	Low, Low	6.18	Ballor, Becquc et al. (1987) [160]	1.55	0.25	168	168.25	24	301.98
Vissing (2009) [118]	Biceps brachii	Stepping: 30 min @ 60 steps/min (concentric) ht:0.58m	Low, Mid-Low	14.6	Latin et al. (2001) [163]	7.30	0.5	3	3.5	14	155.58
Vissing (2009) [118]	Biceps brachii	Stepping: 30 min @ 60 steps/min (concentric) ht:0.58m	Low, Mid-Low	14.6	Latin et al. (2001) [163]	7.30	0.5	168	168.5	14	149.1
Vissing (2009) [118]	Biceps brachii	Stepping: 30 min @ 60 steps/min (eccentric) ht:0.58m	Low, Mid-Low	14.6	Latin et al. (2001) [163]	7.30	0.5	3	3.5	14	122.74
Vissing (2009) [118]	Biceps brachii	Stepping: 30 min @ 60 steps/min (eccentric) ht:0.58m	Low, Mid-Low	14.6	Latin et al. (2001) [163]	7.30	0.5	168	168.5	14	129.86
Magalhaes (2010) [117]	PBMC	Treadmill: 30 min @ 2.20 m/s, 1º grade	High, Mid-High	8.3	Sherrman, Morris et al. (1998) [153]	12.45	1.5	0	1.5	9	286.5
Magalhaes (2010) [117]	PBMC	Treadmill: 30 min @ 2.20 m/s, 1º grade	High, Mid-High	8.3	Sherrman, Morris et al. (1998) [153]	12.45	1.5	1	2.5	9	357.8
Amorim (2011) [115]	PBMC	Treadmill: 100 min @ 56% of VO_2peak (2 x 50min w/ 15min btw)	High, High	8.3	Sherrman, Morris et al. (1998) [153]	14.11	1.7	0	1.7	9	223
Hillman (2011) [125]	Monocytes	Cycling: 90 min @95% LT	High, High	11.71	Achten and Jenkendrup (2003) [162]	17.57	1.5	0	1.5	7	155
Hillman (2011) [125]	Monocytes	Cycling: 90 min @95% LT	High, High	11.71	Achten and Jenkendrup (2003) [162]	17.57	1.5	0	1.5	7	150
Kuennen (2011) [105]	PBMC	Treadmill: 2 x 50 min @ 1.75 m/s, 10 min break	Low, Mid-Low	5.42	Sherrman, Morris et al. (1998) [153]	9.21	1.7	0	1.7	8	125.9
Kuennen (2011) [105]	PBMC	Treadmill: 2 x 50 min @ 1.75 m/s, 10 min break	Low, Mid-Low	5.42	Sherrman, Morris et al. (1998) [153]	9.21	1.7	2	3.7	8	116.9
Kuennen (2011) [105]	PBMC	Treadmill: 2 x 50 min @ 1.75 m/s, 10 min break	Low, Mid-Low	5.42	Sherrman, Morris et al. (1998) [153]	9.21	1.7	4	5.7	8	136.7
Peart (2011) [116]	Monocytes	Cycling: 4 min self-paced "all-out"	Low, Low	14.95	Achten and Jenkendrup (2003) [162]	1.5	0.1	0.5	0.6	7	140.9
Peart (2011) [116]	Monocytes	Cycling: 4 min self-paced "all-out"	Low, Low	14.95	Achten and Jenkendrup (2003) [162]	1.5	0.1	1	1.1	7	134.3
Peart (2011) [116]	Monocytes	Cycling: 4 min self-paced "all-out"	Low, Low	14.95	Achten and Jenkendrup (2003) [162]	1.5	0.1	2	2.1	7	112.2
Peart (2011) [116]	Monocytes	Cycling: 4 min self-paced "all-out"	Low, Low	14.95	Achten and Jenkendrup (2003) [162]	1.5	0.1	4	4.1	7	76.9
Peart (2011) [116]	Lymphocytes	Cycling: 4 min self-paced "all-out"	Low, Low	14.95	Achten and Jenkendrup (2003) [162]	1.5	0.1	0.5	0.6	7	124.5
Peart (2011) [116]	Lymphocytes	Cycling: 4 min self-paced "all-out"	Low, Low	14.95	Achten and Jenkendrup (2003) [162]	1.5	0.1	1	1.1	7	104.4

Table 2 (continued): Included Studies Pertaining to the Heat Shock Response in Humans											
Author	**Tissue**	**Exercise Protocol**	**Intensity Groups**	**METs**	**METs Source**	**METhours**	**Exercise Duration (h)**	**Collection Time (h)**	**Total Time (h)**	**n**	**Hsp70 (% baseline)**
Peart (2011) [116]	Lymphocytes	Cycling: 4 min self-paced "all-out"	Low, Low	14.95	Achten and Jenkendrup (2003) [162]	1.5	0.1	2	2.1	7	109.9
Peart (2011) [116]	Lymphocytes	Cycling: 4 min self-paced "all-out"	Low, Low	14.95	Achten and Jenkendrup (2003) [162]	1.5	0.1	4	4.1	7	85.4
Taylor (2012) [114]	Monocytes	Cycling: 60 mi @ 90% LT	Low, Mid-Low	10.19	Achten and Jenkendrup (2003) [162]	10.19	1	0	1	8	205.4
Taylor (2012) [114]	Monocytes	Cycling: 60 mi @ 90% LT	Low, Mid-Low	10.19	Achten and Jenkendrup (2003) [162]	10.19	1	1	2	8	159.1
Taylor (2012) [114]	Monocytes	Cycling: 60 mi @ 90% LT	Low, Mid-Low	10.19	Achten and Jenkendrup (2003) [162]	10.19	1	4	5	8	153.2
Taylor (2012) [114]	Monocytes	Cycling: 60 mi @ 90% LT	Low, Mid-Low	10.19	Achten and Jenkendrup (2003) [162]	10.19	1	8	9	8	165.6
Dokladny (2013) [80]	PBMC	Treadmill: 60 min @ 70 – 80% of VO_2max	Low, Mid-Low	9.63	Wulff, Cochrane et al. (1998) [157]	9.63	1	0	1	8	136
Dokladny (2013) [80]	PBMC	Treadmill: 60 min @ 70 – 80% of VO_2max	Low, Mid-Low	9.63	Wulff, Cochrane et al. (1998) [157]	9.63	1	2	3	8	160
Dokladny (2013) [80]	PBMC	Treadmill: 60 min @ 70 – 80% of VO_2max	Low, Mid-Low	9.63	Wulff, Cochrane et al. (1998) [157]	9.63	1	4	5	8	210
de Matos (2013) [113]	Vastus lateralis	Cycling: 60 min @ 50% PPO	Low, Low	4.5	Lang, Latin et al. (1992) [155]	4.50	1	0	1	8	119.5
Peart (2013) [112]	PBMC	Cycling: 10 x 15s sprints @ 120% PPO, 45s @ 50W between	Low, Low	14.95	Achten and Jenkendrup (2003) [162]	2.99	0.2	0	0.2	7	138.3
Peart (2013) [112]	PBMC	Cycling: 10x15s sprints @ 120% PPO, w/ 45s @ 50W between	Low, Low	14.95	Achten and Jenkendrup (2003) [162]	2.99	0.2	1.5	1.7	7	184.3
Peart (2013) [112]	PBMC	Cycling: 10x15s sprints @ 120% PPO, w/ 45s @ 50W between	Low, Low	14.95	Achten and Jenkendrup (2003) [162]	2.99	0.2	3	3.2	7	163.7
Ruell (2014) [104]	Lymphocytes	Treadmill: 60 min @ 72% of VO_2max	Low, Mid-Low	9.63	Wulff, Cochrane et al. (1998) [157]	9.63	1	0	1	7	96.01
Ruell (2014) [104]	Lymphocytes	Treadmill: 60 min @ 72% of VO_2max	Low, Mid-Low	9.63	Wulff, Cochrane et al. (1998) [157]	9.63	1	1	2	7	99.9
Ruell (2014) [104]	Monocytes	Treadmill: 60 min @ 72% of VO_2max	Low, Mid-Low	9.63	Wulff, Cochrane et al. (1998) [157]	9.63	1	0	1	7	108.37
Ruell (2014) [104]	Monocytes	Treadmill: 60 min @ 72% of VO_2max	Low, Mid-Low	9.63	Wulff, Cochrane et al. (1998) [157]	9.63	1	1	2	7	118.32
Lee (2014) [103]	Monocytes	Cycling: 90-min @ 50% VO_2max	Low, Mid-Low	7.02	Achten and Jenkendrup (2003) [162]	10.53	1.5	0	1.5	12	107
Lee (2014) [103]	Monocytes	Cycling: 90-min @ 50% VO_2max	Low, Mid-Low	7.02	Achten and Jenkendrup (2003) [162]	8.19	1.17	0	1.17	12	153

Table 3: Included Studies Pertaining to Autophagy in Animals

Author	Tissue	Breed	Exercise Protocol	Intensity Groups	Exercise Duration (h)	Collection time (h)	Total Time (h)	LC3 (% baseline)
Grumati (2011) [57]	Tibialis Anterior	C57BL/6 wild-type mice	TM: 1 h @ 10cm/m, ↑ 2cm/m/2min until 40 cm/m	High	1	0	1	660.2
Ogura (2011) [96]	Cardiac muscle	Sprague–Dawley rats	TM: 30 min @ 30 m/min	Low	0.5	0	0.5	53.5
Ogura (2011) [96]	Cardiac muscle	Sprague–Dawley rats	TM: 30 min @ 30 m/min	Low	0.5	0.5	1	125
Ogura (2011) [96]	Cardiac muscle	Sprague–Dawley rats	TM: 30 min @ 30 m/min	Low	0.5	1	1.5	145
Ogura (2011) [96]	Cardiac muscle	Sprague–Dawley rats	TM: 30 min @ 30 m/min	Low	0.5	3	3.5	115.5
Kim (2012) [98]	Gastrocnemius	ICR mice	TM: 50 min @ 12.3 m/min, 5° grade	Low	0.83	0	0.83	152.7
Kim (2012) [98]	Gastrocnemius	ICR mice	TM: 50 min @ 12.3 m/min, 5° grade	Low	0.83	3	3.83	1.9
Kim (2012) [98]	Gastrocnemius	ICR mice	TM: 50 min @ 12.3 m/min, 5° grade	Low	0.83	6	6.83	1.7
Kim (2012) [98]	Gastrocnemius	ICR mice	TM: 50 min @ 12.3 m/min, 5° grade	Low	0.83	12	12.83	1.6
Jamart (2013) [74]	Homogenized Muscle	C57BL/6 wild-type mice	TM: 90 min @ 10m/min	Low	1.5	0	1.5	459.2
He (2013) [97]	Skeletal Muscle	C57BL/6 wild-type mice	TM: 40m @ 10 m/min + 30 min ↑ 1m/min/10min; 30 min max + ↑ 1m/min/5min until immobile	High	1.33	0	1.33	284.2
Vainshtein (2015) [75]	Homogenized Muscle	C57BL/6 wild-type mice	TM: Run to non-volitional exhaustion	High	1	0	1	215.6
Vainshtein (2015) [75]	Homogenized Muscle	C57BL/6 wild-type mice	TM: Run to non-volitional exhaustion	High	1	1.5	2.5	149.9

Table 4: Included Studies Pertaining to the Heat Shock Response in Animals								
Author	**Tissue**	**Breed**	**Exercise Protocol**	**Intensity Groups**	**Exercise Duration (h)**	**Collection time (h)**	**Total Time (h)**	**LC3 (% baseline)**
Salo (1991) [126]	Homogenized hind limb muscle	Sprague-Dawley rats	TM: 64.9 min @ 26.8 m/min, 10º	High	1.08	0	2	360
Skidmore (1995) [127]	Gastrocnemius	Sprague-Dawley rats	TM: 90 min @ 17m/min	High	1.5	0	2	188.3
Skidmore (1995) [127]	Soleus	Sprague-Dawley rats	TM:90 min @ 17m/min	High	1.5	0	2	285.9
Locke (1995) [128]	Myocardium	Sprague-Dawley rats	TM: 60 min @ 30 m/min	High	1	24	26	123.7
Locke (1995) [128]	Myocardium	Sprague-Dawley rats	TM: 102 min @ 24 m/min	High	1.7	0	2	131.7
Hernando (1997) [130]	Soleus	Wistar rats	TM: 60 min @ 27 m/min	High	1	1.65	3.65	359.9
Hernando (1997) [130]	Soleus	Wistar rats	TM: 60 min @ 27 m/min	High	1	2.61	4.61	336.6
Hernando (1997) [130]	Soleus	Wistar rats	TM: 60 min @ 27 m/min	High	1	3.64	5.64	221.9
Hernando (1997) [130]	Soleus	Wistar rats	TM: 60 min @ 27 m/min	High	1	5.65	7.65	103.8
Smolka (2000) [131]	Soleus	Wistar rats	TM: Undulating speed (10-30 m/min), -3º grade)	High	1.37	2	4	197.5
Paroo (2002) [132]	Myocardium	Sprague-Dawley rats	TM: 60 min @ 30 m/min	High	1	24	26	235.8
Paroo (2002) [132]	Myocardium	Sprague-Dawley rats	TM: 60 min @ 30 m/min	High	1	24	26	104.6
Milne (2005) [133]	Myocardium	Sprague-Dawley rats	TM: 60 min @ 30 m/min, 2º grade	High	1	0.5	2.5	305.6
Antunes (2006) [134]	Leukocytes	Wistar rats	TM: 60 min @ 10-30 m/min, -3º grade	High	1	0	2	116.6
Antunes (2006) [134]	Leukocytes	Wistar rats	TM: 60 min @ 10-30 m/min, -3º grade	High	1	1	3	108
Antunes (2006) [134]	Leukocytes	Wistar rats	TM: 60 min @ 10-30 m/min, -3º grade	High	1	2	4	150.7
Antunes (2006) [134]	Leukocytes	Wistar rats	TM: 60 min @ 10-30 m/min, -3º grade	High	1	3	5	402.2
Antunes (2006) [134]	Leukocytes	Wistar rats	TM: 60 min @ 10-30 m/min, -3º grade	High	1	4	6	302.4

Table 4 (continued): Included Studies Pertaining to the Heat Shock Response in Animals								
Author	**Tissue**	**Breed**	**Exercise Protocol**	**Intensity Groups**	**Exercise Duration (h)**	**Collection time (h)**	**Total Time (h)**	**LC3 (% baseline)**
Melling (2008) [135]	Myocardium	Sprague-Dawley rats	TM: 60 min @ 30 m/min, 2º grade	High	1	0	2	102.1
Melling (2008) [135]	Myocardium	Sprague-Dawley rats	TM: 60 min @ 30 m/min, 2º grade	High	1	24	26	177.3
Bombardier (2009) [136]	Soleus	Sprague-Dawley rats	TM: (intermittent) 5 min bouts @ 17 m/min, −13.5°, w/ 2 min rest periods for 90 total min	High	1.5	24	26	137.7
Touchberry (2011) [137]	Soleus	Wistar rats	Downhill(-16º): 5 min @ 18 m/min	Low	0.083	2	3	125.2
Touchberry (2011) [137]	Soleus	Wistar rats	Downhill(-16º): 5 min @ 18 m/min	Low	0.083	48	49	77.3
Milne (2012) [78]	Myocardium	Sprague-Dawley rats	TM: 60 min @ 30 m/min, 2º grade	High	1	0.5	2.5	181.3
Milne (2012) [78]	Myocardium	Sprague-Dawley rats	TM: 60 min @ 30 m/min, 2º grade	High	1	24	26	688.6
Lewis(2013)[139]	Vastus intermedius	Fischer 344 x Brown Norway Rats	TM : 60 min @ 16 m/min, 0º	Low	1	0	1	103.9
Lewis(2013)[139]	Vastus intermedius	Fischer 344 x Brown Norway Rats	TM : 60 min @ 16 m/min, 0º	Low	1	48	49	107.9
Lewis(2013)[139]	Vastus intermedius	Fischer 344 x Brown Norway Rats	TM : 60 min @ 16 m/min, -16º	Low	1	0	1	96
Lewis(2013)[139]	Vastus intermedius	Fischer 344 x Brown Norway Rats	TM : 60 min @ 16 m/min, -16º	Low	1	48	49	116.8
Lewis(2013)[139]	Vastus intermedius	Fischer 344 x Brown Norway Rats	TM : 60 min @ 16 m/min, 0º	Low	1	0	1	98.8
Lewis(2013)[139]	Vastus intermedius	Fischer 344 x Brown Norway Rats	TM : 60 min @ 16 m/min, 0º	Low	1	48	49	97.1
Lewis(2013)[139]	Vastus intermedius	Fischer 344 x Brown Norway Rats	TM : 60 min @ 16 m/min, -16º	Low	1	0	1	90.8
Lewis(2013)[139]	Vastus intermedius	Fischer 344 x Brown Norway Rats	TM : 60 min @ 16 m/min, -16º	Low	1	48	49	122.6
Bombardier (2013) [76]	Soleus	Sprague-Dawley rats	TM: (intermittent) 5 min bouts @ 17 m/min, −13.5°, w/ 2 min rest periods for 90 total min	High	1.5	72	74	118.0
Musumeci (2013) [77]	Rectus femoris	Sprague-Dawley rats	TM: 70-75min @ 30 m/min, 10º	High	1.21	0	2	167.2
Rossato (2014) [140]	Macrophages	Wistar rats	Swimming: 60 min, 5% overweight	Low	1	0	1	97.1

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