THE EMERGING ROLE OF EXOSOMES IN STRESS PHYSIOLOGY

by

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Abstract

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The emerging role of exosomes in stress physiology

Thesis directed by Professor Monika Fleshner, Ph.D.

Acute activation of the stress response modulates an adaptive series of physiological changes to optimize an organism's survival. One component of the stress response involves an induction of the innate immune system through the release of Hsp72, an endogenous danger associated molecular pattern rapidly released into the blood following stressor exposure. The mechanism of Hsp72 release is unknown, but recent findings suggest Hsp72 is release via the non-classical exosome releasing pathway. This dissertation explores the emerging role of exosomes during stress-induced immunomodulation. Exosomes, biologically active nanoparticles released by most cells in the body, encompass a variety of proteins and microRNA (miRNA) that can be modified in response injury, infection, or disease. The goals in this dissertation as follows: (1) characterize Hsp72 in the plasma, peripheral tissues, brain, and cerebrospinal fluid following acute stressor exposure; (2) investigate potential targets for stress-sensitive markers associated with exosomes; (3) explore the potential pathways activated during the stress response responsible for modulating exosomes; and (4) examine how stress-modified exosomes impact bacterial inflammation. We provide evidence in this dissertation that exposure to an acute stressor modifies Hsp72 expression and the miRNA character of circulating plasma exosomes in the

absence of a pathogenic challenge or disease. Further, we identify sympathetic nervous system (SNS) activation as an important signal for part of the stress-evoked changes in circulating exosomes. Importantly, these stress-modified plasma exosomes contribute to enhanced host response during a bacterial challenge. Future research is necessary to determine both the cellular sources and targets of stress-modified exosomes.

Dedication

To Moni: I would like to express my deepest appreciation and gratitude to you for the opportunity to join your lab to pursue my degree. I cannot thank you enough for your experienced guidance, honest interactions, and positive influence. I am especially fortunate to have your support as I move out of the research sphere. I was right to pick you as a mentor.

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I. Introduction

Multicellular organisms have developed many biological strategies to maintain physiological homeostasis in the face of stress. The cumulative effect of acute stressor exposure, such as when a prey animal is the target of a predator, manifests itself through a series of behavioral and physiological changes to enhance survival. These stress-induced changes are commonly referred to as the "fight-or-flight" response. One component of this response involves the induction of innate immunity, which can prepare an organism under stress for a pathogenic challenge or injury sustained from the stress. While stress-induced immunomodulation is well established in stress physiology, the mechanisms driving this induction are currently under investigation.

The purpose of this dissertation is to investigate the molecular and cellular interactions between the acute stress response and the immune system. One mechanism examined involves the cellular secretion of small circulating nanoparticles known as exosomes. Exosomes are found in many biological fluids and aid in cell-to-cell communication. The second chapter provides a comprehensive literature review on exosome regulation of innate immunity and explores potential candidates for stress-modified proteins and microRNA (miRNA) that are both known to associate with exosomes and are capable of modulating innate immunity. In the third chapter, we demonstrate that exposure to an acute stressor modifies the proteomic and microRNA profile of exosomes in the plasma, which contribute to enhanced host defense to a bacterial challenge. The forth chapter reveals that removing exosomes present in fetal bovine serum, a critical component of various cell culture medias, impacts cellular responses to a bacterial

challenge, demonstrating the need for careful experimental design for *in vitro* analysis of exosome-mediated immunity. Chapter five adds to the current stress physiology literature by examining the impact of acute stressor exposure on cerebrospinal fluid concentrations of heat shock protein 72 (Hsp72), a highly stress-inducible protein capable of priming the innate immune system. A comprehensive analysis of brain and peripheral tissue Hsp72 is included. The sixth and final chapter of this dissertation evaluates the success of a National Science Foundation (NSF) sponsored program at recruiting women and underrepresented minorities into undergraduate research experiences and retaining their interest in scientific research.

The culmination of these studies illustrates the emerging role of exosomes in cytoprotection and immunity during the stress response. These findings open the door to further research on the immunomodulatory functions of stress-modified exosomes and their associated proteins and miRNAs. Furthermore, the results of the NSF survey reveal the value of undergraduate research experiences in both advancing research and increasing diversity in the sciences.

II. Exosomes: an emerging factor in stress-induced immunomodulation

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Abstract

Cells release small (40-100nm) vesicles known as exosomes constitutively, but their composition and function changes in response to a variety of physiological challenges, such as injury, infection, and disease. There have been many advances in understanding the immunological relevance of exosomes, but few studies have explored their role in stress physiology. There is clear evidence that exposure to a variety of acute stressors will prime the innate immune response, but the priming mechanisms are not fully understood. Since exosomes are emerging as important inflammatory mediators, they likely exhibit a similar role when an organism is exposed to an acute stressor. Here, we review our current knowledge of the basic properties and immunological functions of exosomes and provide emerging data supporting the role of stress-modified exosomes in regulating the innate immune response.

Introduction

Multicellular organisms contain a variety of unique vesicular particles that can be visualized by electron microscopy. These extracellular vesicular particles discovered in 1946[1] were commonly overlooked, considered to be nothing more than platelet "dust" and cellular debris[2,3]. It was not until the 1980's that researchers began to discover the functional properties of these vesicular particles, specifically their role in promoting cell-to-cell communication and modulating immunity. Current research interest in the field of extracellular vesicles focuses predominantly on a population of endosome-derived vesicles known as exosomes. In recent years, there has been a push to distinguish exosomes by their proteomic and genetic components because exposure to a variety of pathogens and disease states can modify the composition and function of these vesicles. The most striking

findings focus on the immunological relevance of exosomes, making them a novel target for immunotherapies. Understanding the mechanisms involved in exosomal modifications and their resulting immunological consequences provide valuable insight into the possibility of using exosomes as disease markers and therapeutic tools.

Although exosomes have recently emerged as important regulators of the immune response, much of the published research examines only their role in response to a variety of physiological challenges, such as injury, infection, and disease. Research has shown that certain environmental stressors can modify the composition of exosomes *in vitro*, such as heat[4,5], hypoxia[6], irradiation[7], and changes in conditioned media[8]. However, our understanding of how activation of the stress response in the absence of injury or disease can modify the profile and function of exosomes *in vivo* is rudimentary. The role of exosomes during the stress response is of interest because there is clear evidence that exposure to a variety of acute stressors primes the innate immune response, and the priming mechanisms of stress-induced immunomodulation are not fully understood.

While research on the acute stress response has been increasingly well characterized since Hans Selye's discovery of the general adaptation syndrome in 1956[9], there remains considerable uncertainty about the mechanisms involved in stress-modified immunity. Exposure to an acute stressor is known to prime the innate immune system through the release of the 72kD heat shock protein (Hsp72)[10–12] and recent studies demonstrate that stressor exposure can also modify microRNA (miRNA) associated with immunity. However, it is unclear how stress-induced Hsp72 and miRNA are released into the circulation and how they interact with innate immune cells. Various studies have

confirmed the presence of Hsp72 and miRNA on exosomes, but little research has confirmed their association with exosomes during activation of the stress response.

Based on our understanding that a) exosomes are known to modulate the immune system; b) exosomes are susceptible to environmental stressors; c) exposure to an acute stressor can prime innate immunity; and d) some stress-sensitive proteins and miRNA associate with exosomes, we predict that stressor exposure will alter the composition of exosomes in a way that will modulate immunity in an organism. Here, we review our current knowledge of the basic properties and immunological functions of exosomes and provide emerging data supporting the role of stress-modified exosomes in regulating the innate immune response.

Properties of exosomes

Exosomes, which range in size from 40-100 nm in diameter, are endosome-derived vesicles secreted from both hematopoietic and non-hematopoietic cells, tumor cells, and microbes. Exosomes were first described in 1983 by Rose Johnstone's research group as the specific group of membrane enclosed vesicles isolated from the cell culture supernatant of immature red blood cells known as reticulocytes[13]. This discovery was the unexpected result of recovering the shed transferrin receptor from maturing reticulocytes through high-speed ultracentrifugation. At that time, exosomes were regarded as secreted vesicles enabling cells to eliminate obsolete molecules. Since their discovery, exosomes have been discovered in mammalian blood[14–16], urine[17], human saliva[18], seminal fluid[19], breast milk[20], bronchoalveolar fluid[21], bile[8], cerebrospinal fluid[23,24], and amniotic fluid[25]. Furthermore,

researchers have discovered that exosomes exert a wide range of biological functions, spurring more research in the field of extracellular vesicles.

Biophysically, exosomes are similar to cytosol encapsulated in a phospholipid bi-layer enriched in phosphatidylserine, lipid rafts, ceramide, and sphingomyelin[26–31]. The genetic and proteomic content of exosomes is reflective of their cellular origin, but there are some common markers of exosomes secreted from various cellular sources. The transmembrane domain proteins known as tetraspanins, including CD9, CD63, CD81, and CD82, are present on nearly all exosomes and serve as reliable markers for identifying the presence of exosomes[17,32–40]. Rabs, annexins, and adhesion molecules, which contribute to exosome docking and fusion with the recipient cell[29,31,34,40,41], are also common to most exosomes. Additionally, there are various immunological molecules associated with exosomes, such as major histocompatibility complex (MHC)[21,35] and the T-cell co-receptor CD86[42].

A near universal characteristic of exosomes is the presence of short (20-25 nucleotides), non-coding RNAs known as microRNAs (miRNAs), which are known to regulate messenger RNA (mRNA) and various physiological processes[43–49]. miRNA post-transcriptionally block the expression of target genes by binding to the 3' end of mRNA. Interestingly, miRNA do not have to perfectly complement mRNA amino acid sequences to repress their transcription, thereby enabling a single miRNA to target hundreds of mRNA and modify protein expression.

Cellular proteins that are targeted for exosomes are first directed to endosomes through Ca^{2+}-dependent ubiquitination[50]. The endosome is then directed to a

multivesicular body (MVB) by the endosomal sorting complex required for transport (ESCRT). The ESCRT protein complex is critical for forming the MVBs and sorting the endosomal proteins. Following a modified ubiquitination step to avoid normal lysosomal targeting and a steady influx of Ca^{2+}, the MVB fuses with the cell's plasma membrane and releases its vesicular compartments into the extracellular space[29]. Once these vesicles are released from the cell, they are regarded as exosomes.

The mechanism of targeting miRNA into endosomes is not well understood. While exposure to various physiological challenges can modify miRNA content in exosomes, recent studies have demonstrated that these variations are the result of targeted selection, not random incorporation into the MVB[51]. Thus far, researchers suggest that cytosolic miRNA associates with RNA-induced silencing complexes (RISC) through Argonaut proteins, and the complex is transported to the MVB by the chaperone heat shock protein 90 (Hsp90)[52,53]. However, additional research on selective miRNA transport to endosomes is still needed.

When exosomes are secreted from the cell, there are several proposed mechanisms for how they may interact with their recipient cells. Thus far, exosomes are known to express surface adhesion molecules, which likely aid in exosome capture by the recipient cell. For example, exosomes released by dendritic cells bear intercellular adhesion molecule 1 (ICAM-1) and can bind to its ligand, lymphocyte function-associated antigen 1 (LFA-1), on the surface lymphocytes and leukocytes[54]. Additionally, the β1 and β2 integrins are also expressed on exosomes secreted by reticulocytes[55], dendritic cells[30,31], and T cells[39], which allows the exosomes to bind to cells expressing ICAM-1 or fibronectin. Once

bound to the recipient cell, exosomes can either transfer their contents to the cytosol of the recipient cell[51] or activate cells through surface receptors[35].

Conceptually, exosomes have several advantages over traditional intercellular communication involving soluble proteins. Instead of activating a cell through various separate signals, exosomes can provide a more comprehensive package of signals at once. An exosome can use appropriate adhesion molecules to target a lymphocyte, while simultaneously providing antigen packaged in MHC with co-stimulatory molecules all at a single time. Exosomes also enable the transfer of multiple miRNAs at once, targeting various mRNA in a cell simultaneously. Additionally, it is possible that vesicular packaging allows for a more efficient uptake of the associated contents due to actual docking and fusion with the host cell. The release of exosomes appears to be a highly conserved process of cell-to-cell communication with important implications for both normal and disease-associated physiological processes.

Additional advantages of exosomal transport include protection conferred by the lipid bi-layer as well as resistance to complement factors. Exosomes secreted by antigen presenting cells are resistant to complement-mediated lysis through the surface expression of the complement-inhibitory proteins CD55 and CD59[56,57]. In one study by Clayton et al., administration of CD55 neutralizing antibody to human sera resulted in the increased deposition of the complement protein C3b on exosomes, which induced exosome lysis[56]. Through exosome secretion, proteins and miRNA are protected from degradation as they circulate through the extracellular environment or across mucosal barriers, thus enabling long-distance cellular communication and obviating the need for direct cell-to-cell contact.

Exosome regulation of innate immunity

Interest in the immunological roles of exosomes has grown rapidly since the discovery that exosomes purified from B-cells and dendritic cells are enriched in MHC[36,58] and T-cell co-stimulatory molecules[36,42]. Since that discovery, researchers revealed that exosomes released from leukocytes, lymphocytes, tumor cells, and injured cells are capable of inducing inflammatory immune responses. There are a variety of mechanisms exosomes use to induce inflammatory responses and there is a surge of promising immunotherapies that have developed in light of these findings. Based on our understanding that exposure to an acute stressor primes innate immunity, this review will focus on discussing exosome interactions with the innate immune system and provide insight into their potential role during an acute stress response.

There are a variety of mechanisms exosomes utilize to promote innate immunity. For example, exosomes derived from pathogen-infected mammalian cells demonstrate a variety of inflammatory functions. Macrophages infected with *Mycobacterium avium (M. avium)* release exosomes rich in bacteria-associated glycopeptidolipids[59]. These lipid-rich exosomes express ligands that bind to surface Toll-like receptors (TLRs) on uninfected macrophages, stimulating transcription and secretion of the pro-inflammatory cytokine TNF-α through activation of the NF-κB pathway. Since the composition of exosomes is reflective of its source, it is likely that macrophages infected by similar pathogens secrete exosomes expressing pathogenic molecules that interact with uninfected immune cell through TLRs.

Anand et al. (2010) also examined the inflammatory roles of exosomes released from macrophages infected with mycobacteria [60]. Macrophages infected with either *Mycobacterium smegmatis* or *M. avium* release exosomes with greater surface expression of Hsp70, which then stimulate the NF-κB pathway through activation of the TLRs to transcribe TNF-α. Exosomes expressing high levels of Hsp70 can induce a pro-inflammatory response by promoting natural killer (NK) cell activity[61] and macrophage TNF-α release[62]. In addition, since Hsp70 is a highly stress-inducible protein, this study provides support for examining the role of exosomes in stress physiology.

Exosomes are also capable of the modulating innate immunity through miRNA regulation. Macrophages challenged with lipopolysaccharide (LPS) upregulate intracellular miR-155 and downregulate miR-125, resulting in enhanced immunity through IκB kinase ε (IKKε) and TNF-α activity[63]. These miRNA modifications also extend to C57BL/6 mice administered an intraperitoneal injection of LPS. While this study did not examine the role of exosomes in the LPS-challenged macrophages or mice, both miR-155[64] and miR-125b[51] have been shown to associate with exosomes. Other exosome-associated miRNA have also been linked to TLR mediated inflammation[43], pro-inflammatory cytokine expression[65], and macrophage differentiation[66]. Based on these findings, there are a variety of exosomal miRNA that are ideal candidates for examination during stress-enhanced innate immunity.

In summary, exosomes are important inflammatory mediators and their role in stress physiology is likely tied to their immune properties. Since the composition and functionality of exosomes are modified during a variety of physiological challenges, we propose that a similar reorganization occurs following exposure to a stressor. Exposure to

an acute stressor will likely modify the expression of stress-associated proteins and miRNA content in circulating exosomes that are capable of modulating the immune response. To understand how exposure to an acute stressor might modify exosomes in these ways, it is important to determine which proteomic and miRNA components of the stress response could be likely candidates for modulating immunity through exosome expression.

Exosomes in the acute stress response

How exposure to an acute stressor promotes immunity

Over time, multicellular organisms developed many biological strategies to maintain physiological homeostasis in the face of intrinsic or extrinsic stressors. When an organism is exposed to a stressor, or perceived threat, the body responds by initiating physiological changes that enhance the organism's survival. The changes that occur in response to an acute stressor are known as the acute stress response. Two major components of the acute stress response are activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS). The physiological changes that follow activation of the HPA axis and the SNS include redirection of oxygen and nutrients, glycogen breakdown in the liver, elevation in blood pressure and heart rate, piloerection, and pupil dilation. The acute stress response enables the organism to mobilize energy stores for enhanced mobility and vigilance to optimize their chance of survival.

Another adaptive feature of the acute stress response is enhanced immunity resulting from the release of catecholamines during activation of the SNS. The major catecholamines released during the acute stress response are norepinephrine (NE) from the sympathetic nerve terminals and epinephrine (E) form the adrenal medulla.

Catecholamines induce the release of danger-associated molecular patterns (DAMPs)[67], which in turn modulate the innate immune system by inducing macrophages and dendritic cells to secrete pro-inflammatory cytokines that enhance the elimination of pathogens and mobilization of antigen-presenting cells. This induction of pro-inflammatory cytokines can enhance an organism's immune response to injuries sustained from the stressor, promoting survival both during and after exposure to an acute stressor.

The secretion of DAMPs during the acute stress response is critical for the adaptive modulation of innate immunity. Similar to pathogen associated molecular patterns (PAMPs), DAMPs are recognized by cells of the immune system primarily through binding to TLRs. Binding to TLRs initiates the NF-κB pathway, which leads to the transcription and release of pro-inflammatory cytokines[68]. Unlike PAMPs, DAMPs are produced endogenously, in the absence of a pathogen. As a result, DAMPs are considered alarm signals that work to promote an organism's survival in preparation for tissue damage or exposure to a pathogen. Examples of DAMPs include high mobility group box 1[69,70], uric acid[68], and heat shock proteins (Hsps). One DAMP in particular, Hsp72, is reliably increased in the circulation following exposure to a variety of acute stressors, demonstrates potent pro-inflammatory functions, and associates with exosomes. These properties make Hsp72 an optimal candidate for examining the inflammatory role of exosomes during the acute stress response.

Hsp72: a critical protein in stress enhanced immunity

The Hsps are a family of highly conserved proteins found within most nucleated cells. Intracellular Hsps (iHsps) aid in folding newly synthesized proteins, facilitate intracellular

trafficking, and repair proteins or polypeptides denatured from exposure to a cellular stressor. When cells are exposed to a stressor, such as heat, iHsps contribute to cellular homeostasis and promote cell survival by maintaining the structural integrity of the cells' proteins [10,71–76].

Some Hsps, such as Hsp72, are found in the extracellular environment of organisms suffering from disease and are known to be immunologically active[77–79]. Circulating levels of Hsp72 *in vivo* are also elevated during exposure to a stressor in the absence of disease, such as tail shock[10,11,67], heat[4,5], predatory fear[80], and exercise[81,82]. These stress-induced elevations in Hsp72 are likely a critical immunomodulatory component of the normal stress response. When rats are subjected to tail shock stress and then immediately receive a subcutaneous injection of *E. coli*, stressed rats resolve their inflammation faster than non-stressed rats[10]. Micropunch analysis of the subcutaneous *E. coli* inflammatory sites in stressed rats revealed significantly elevated levels of Hsp72 and lower colony forming units of *E. coli* two hours following the bacterial challenge compared to non-stressed controls. Administration of a neutralizing Hsp72 antibody attenuated the stress-induced improvement to *E. coli* challenge[12], further indicating that stress-induced elevations of Hsp72 concentrate to inflammatory sites and contribute to bactericidal activity.

Functionally, stress-induced Hsp72 activates macrophages, dendritic cells, and neutrophils by binding to surface bound TLR4 and inducing the secretion of pro-inflammatory cytokines[11,83]. Additionally, Hsp72 increases inducible nitric oxide synthase in macrophages, thereby enhancing their bactericidal activity[10,84]. In this way, stress-

induced Hsp72 acts like a DAMP in that it is up-regulated in the absence of a pathogen and facilitates inflammation.

The mechanisms of Hsp72 release are currently under investigation. Stress can damage tissues and the resulting necrosis does induce cells to rupture and release their intracellular contents, but studies have observed Hsp72 in the absence of detectible cell death[78,85,86]. Researchers have also demonstrated that blockades of known protein transport pathways, such as the classical secretion pathway, has no significant effect on Hsp72 concentrations following exposure to a stressor[61,78,84]. It is also unlikely Hsp72 would be targeted for the classical secretion pathway because it lacks the necessary leader sequences and is elevated in the plasma within 10 minutes of stressor onset, a time frame that is too rapid for classical cellular secretion. However, several studies have demonstrated that Hsp72 can associate with exosomes[61,87,88], making them a potentially novel secretory pathway for the active release of stable Hsp72 during the stress response.

One of the first studies to recognize an increased expression of Hsp72 on the surface of exosomes in response to cellular stress was in human peripheral blood mononuclear cells challenged with 43°C heat stress[87]. Tumor cells exposed to heat shock in culture also increase exosomal expression of Hsp72, which can in turn promote NK cell activity and secretion of TNF-α from macrophages[5]. Additionally, exosomal Hsp72 can enable dendritic cells to eliminate tumor cells following their injection into allogeneic mice[89]. Collectively, these data illustrate that exosomes provide a secretory route for Hsp72 during the stress response. Given the known immunomodulatory functions of both Hsp72 and exosomes,

exosome-associated Hsp72 likely accounts for a portion of the stress-induced priming of the immune response.

MicroRNA: potential targets for stress enhanced immunity

miRNA are susceptible to modulation during a variety of physiological challenges, but multiple reports indicate that exposure to a wide variety of stressors in the absence of disease or injury are also capable of modifying miRNA (Table 2.1). For example, human fibroblast cells exposed to heat shock differentially express 123 miRNAs from basal culture conditions[90]. Restraint stress in CD1 mice modified miR-9 and -26-a/b in the frontal cortex, potentially enhancing synaptic efficacy and allowing frontal neurons to respond immediately to external stimuli[91]. In a similar study examining C57BL/6J/Cnrm mice, restraint stress reduced miR-135a and -124 expression, resulting in greater mineralcorticoid concentrations in the amygdala[92]. These stress modified miRNA are even transferrable to offspring. Rodgers et al. demonstrated that male mice subjected to chronic variable stressors during puberty or adulthood elicited robust changes in sperm miRNA, resulting in offspring with blunted glucocorticoid responses to acute restraint stress[93].

Of the studies examining the impact of stress on miRNA, two studies related miRNA modifications to immunological consequences. Social isolation stress in Sprague-Dawley rats reduced expression of miR-29 and -203 in wound tissue, resulting in delayed oral mucosal healing[94]. Exposure to acute exhaustive cycling exercise in competitive male rowers modulated circulating plasma miRNA involved in inflammation (miR-21 and -146a)[95]. In addition, miRNA modified by stressor exposure have demonstrated various immunological activities in separate studies (see Table 2.1). While none of these studies

miRNAs	Species / cell type	Stressor	Ref
miR-34, miR-137, miR-154, miR-672, miR-219-5p, miR-75, **miR-7a,** miR-34a, **miR-30a**, **miR-500**, **miR-185**, **miR-203**, miR-200a, miR-140, **let-7d**, **miR-125a-5p**, **miR-27b**, **miR-210**, miR-142, miR-434, miR-204, **miR-142-3p**, **miR-193**, miR-31, **miR-150**, miR-148b-3p, **miR-223**, **miR-23a**	Sprague–Dawley rat jejunum	40°C heat stress for 120 min	102
miR-135a, miR-124	C57BL/6/J/Cnrm mouse amygdala	2 hours of restraint	92
miR-9, **miR-26-a/b, let-7a**	CD1 mouse frontal cortex	Repeated restraint	91
miR-125b, miR-452, miR-133b, **miR-192**, miR-382, **miR-378**, miR-101, miR-424, **miR-22**, miR-138, miR-7, miR-376a, miR-31, **miR-222**, miR-33a, **miR-29b**, miR-606, **let-7c**, **let-7d**, miR-218, miR-196a, miR-204, miR-196b, miR-154, miR-1298, **miR-18a**, miR-487b	Human dermal fibroblasts	44°C heat stress for 40 min	90
miR-29, **miR-203**	Sprague-Dawley rat serum	Social Isolation	94
miR-193-5p, miR-204, **miR-29c**, **miR-30a**, **miR-30c**, miR-32, miR-375, **miR-532-3p**, miR-698	C57BL.6:129 F1 hybrid mouse sperm	Variable Stressors: constant light, fox odor, novel objects, cage changes, white noise, and restraint	93
miR-144, **miR-16**	Medical students' saliva and blood	Nationally administered medical exam	103
miR-18a	Fisher 344 paraventricular nucleus	Single and repeated restraint stress	104
miR-410, **miR-212**, **miR-29c**, miR-29b-2, **miR-708, let-7e,** miR-137, miR-22, miR-219-2, miR-99a	C57BL/6J, AKR/J, DBA/2J mouse forebrain	Sleep deprivation	105

Table 2.1. List of microRNA (miRNA) modified by stressor exposure. Recent publications characterize the diverse array of miRNA modified by stressor exposure. This table presents a summary of these publications and includes the species, cell type, and stressors analyzed, the miRNA significantly modified by the stressors, and the bibliographic information. MiRNA in bold have been demonstrated to associate with exosomes in separate studies[51].

specifically examine miRNA changes in exosomes, the observations presented here suggest that stress-modified regulation of miRNAs may be crucial components of exosome-based stress-enhanced immunity, which is in line with previous findings demonstrating the immunomodulating consequences of stress[5,11,12,96,97].

In vivo evidence of stress modified exosomes with immunological consequences

A recent study demonstrated that exposing Fisher 344 rats to inescapable tail shock modifies both Hsp72 expression and miRNA content in circulating plasma exosomes[64]. The exosomal miRNAs modified by tail shock, miR-142-5p and -203, have demonstrated immunomodulatory functions and likely contribute to stress-enhanced immunity by enabling cytokine-mediated survival signaling[98] and mediating leukocyte cytokine release[99,100]. In a separate study examining cardiac hypertrophy, researchers discovered that down regulation of miR-142-5p enhanced cytokine-mediated survival in cardiac myocytes through the IL-6 receptor, gp130[98]. One study by Ru et al. revealed that miR-203 directly targets suppressor of cytokine signaling 3 (SOCS3)[99] and blocks its role as an inhibitor of cytokine signal transduction through the Janus tyrosine kinase and signal transducer and activator of transcription factors (JAK/STAT) pathway[101]. Activation of SOCS3 terminates the innate immune response; therefore, a stress-induced reduction in exosomal miR-203 prevents activation of SOCS3 and its downstream suppression of pro-inflammatory cytokines. Another study by Wei et al. discovered that decreased expression of mir-203 in macrophage RAW264.7 cells reversed myeloid differentiation factor 88 (MyD88) repression, enabling downstream induction of TNF-α and IL-6[100]. Collectively, these findings suggest that acute stressor exposure modifies plasma exosomes capable of activating TLR4 through Hsp72 expression, up-regulating gene expression for gp130 by

repressing miR-142-5p, and suppressing SOCS3 activity and reversing MyD88 repression by down-regulating miR-203.

Furthermore, the stress-modified plasma exosomes were isolated and co-administered with subcutaneous injections of *Escherichia coli* (*E. coli*) to non-stressed Fisher 344 rats. Analysis of the inflammatory sites and body weights indicated that rats administered stress-modified exosomes had enhanced host defense to the bacterial challenge compared to rats that received non-stressed plasma exosomes. This study demonstrates for the first time that exposure to an acute stressor can modulate host immunity by modifying the composition of circulating exosomes, thus justifying continued research on the role of exosomes in stress physiology.

Potential mechanisms for exosome modulation during the acute stress response

The current literature suggests a novel role for circulating exosomes following exposure to an acute stressor, but there is little research examining which factors of the acute stress response could modulate exosomes. There are convincing data demonstrating that the stress-associated modulation of exosomal Hsp72 and miR-142-5p likely involves norepinephrine (NE) activation of the α_1-adrenergic receptors (α_1-ADR)[64]. SNS activation of the α_1-ADR initiates its G-protein coupled receptor (GPCR) to activate the effector enzyme phospholipase C (PLC). PLC catalyzes the breakdown of phosphatidylinositol biphosphate (PIP_2) into diacylglycerol and inositol triphosphate (IP_3). IP_3 binds to its receptor, IP_3R, on the endoplasmic reticulum, which in turn elevates cytosolic Ca^{2+}. As stated earlier, cytosolic Ca^{2+} is necessary for both increasing ubiquitination to target molecules to endosomes and for directing MVB fusion with the plasma membrane.

Blockade of the α_1-ADR with prazosin, an α_1-ADR antagonist, prior to inescapable tail shock prevents the stress-associated elevation in plasma Hsp72[67]. In contrast, administration of phenylephrine, an α_1-ADR agonist, in the absence of stress induces an elevation of plasma Hsp72 similar to levels seen in rats exposed to inescapable tail shock. Recent data indicate that administration of prazosin also reduces Hsp72 expression and reverses stress-modified miRNA alterations in plasma exosomes following exposure to tail shock stress[64]. Prazosin did not alter the rate of exosome release into the plasma, therefore, it is possible that blockade of the α_1-ADR alters the Ca^{2+} induced loading of miRNA and Hsp72 onto endosomes through decreasing ubiquitination, thereby modifying their expression on circulating exosomes. Since NE is released following exposure to acute stress and preferentially binds to α_1-ADR, we speculate that NE is critical for targeting proteins and miRNA during the acute stress response by inducing elevations in cytosolic Ca^{2+} to promote cargo loading on endosomes selected for the MVB (Figure 2.1). Stress-modified exosomes then migrate through the circulation and are readily available to modulate host immunity, either stimulating immune cells to initiate the innate immune response or to trigger local inflammation.

Concluding remarks

There is reasonable evidence demonstrating that exposure to acute stressors in the absence of injury, inflammation, or disease will modify the proteomic and miRNA composition of exosomes to enhance innate immunity. A review of the exosome literature strongly suggests that exosomes are important in modulating the immune response. Given the known immunological properties of Hsp72 and various miRNA, it is plausible that

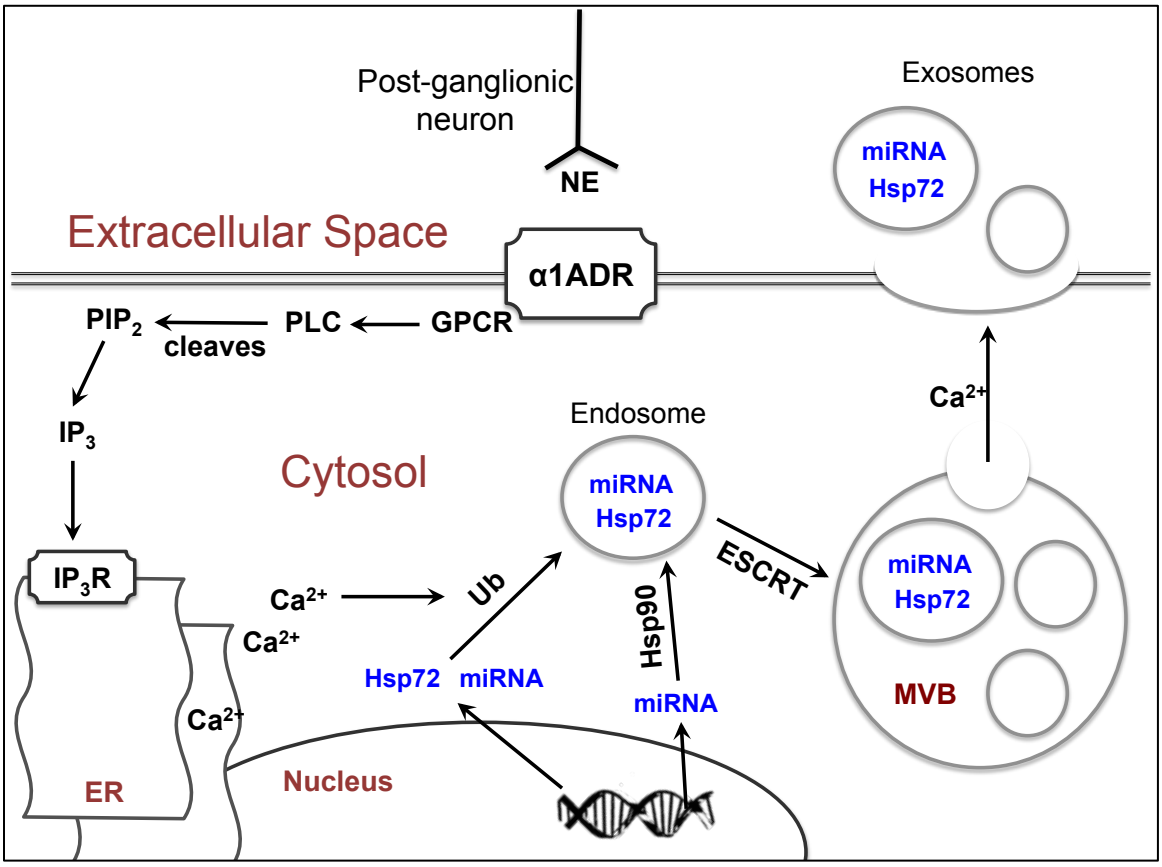

Figure 2.1. Proposed mechanism for exosome modulation during the stress response. Initiation of the stress response activates the sympathetic nervous system (SNS), inducing the release of catecholamines through post-ganglionic nerve fibers. The α_1-adrnergic receptor (α_1-ADR) preferentially binds to the catecholamine norepinephrine (NE). Recent studies have demonstrated that blockade of the α_1-ADR with the drug, Prazosin, attenuates Hsp72 and miRNA expression on plasma exosomes[64], making it a target of interest for understanding the impact of stress on plasma exosomes. The α_1-ADR is coupled to a G-protein coupled receptor (GPCR), which dissociates upon receptor activation and promotes the effector enzyme phospholipase C (PLC). PLC catalyzes the breakdown of phosphatidylinositol biphosphate (PIP_2) into inositol triphosphate (IP_3). IP_3 binds to its receptor, IP_3R, on the endoplasmic reticulum, which in turn elevates cytosolic Ca^{2+}. Cytosolic Ca^{2+} is increases ubiquination to target Hsp72 and select miRNAs to endosomes. Other miRNAs are targeted to the endosome through the chaperone protein heat shock protein 90 (Hsp90). The endosome is then directed to a multivesicular body (MVB) by the endosomal sorting complex required for transport (ESCRT). Through Ca^{2+} mediation, the MVB fuses with the cell's plasma membrane and releases its stress-modified endosome into the extracellular space, where they are then regarded as exosomes.

exposure to an acute stressor can enhance an organism's immune response through the secretion of modified exosomes.

Understanding the immunological role of exosomes will expand knowledge concerning the mechanisms of stress-enhanced immunity and their use in immunotherapy. From a clinical point of view, exposing cells to non-lethal stress to elevate Hsp72 expression on exosomes appears to enhance the immunogenicity of exosome-based vaccines, specifically cancer vaccines. Additionally, stress-modified miRNA can block mRNA transcription to modify an immunological response. There is great potential for future research on the immunological impact of stress modified exosomes, which may give further insight into the delicate balance between the stress response and immune function.

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III. Exposure to an acute stressor modifies the proteomic and microRNA profile of plasma exosomes and improves host defense to bacterial challenge

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Abstract

Exosomes, biologically active nanoparticles (40-100 nm) released by hematopoietic and non-hematopoietic cells, contain a variety of proteins and small, non-coding RNA known as microRNA (miRNA). Exposure to various pathogens and disease states modifies the composition and function of exosomes, but there are no studies examining *in vivo* exosomal changes evoked by the acute stress response. The present study reveals that exposing male Fisher 344 rats to an acute stressor modulates proteins and miRNA in plasma exosomes, specifically up-regulating heat shock protein 72 (Hsp72) and decreasing miR-142-5p and -203 expressions. The selected miRNAs and Hsp72 are associated with immunomodulatory functions and are likely a critical component of the stress response. Further, we demonstrate that some of these stress-induced modifications in plasma exosomes are initiated by sympathetic nervous system (SNS) activation of alpha-1 adrenergic receptors (ADRs), since drug-mediated blockade of the receptors significantly attenuates the stress-induced modifications of exosomal Hsp72 and miR-142-5p. Moreover, *in vivo* analysis reveals that administering rats stress modified plasma exosomes improves their inflammatory response to subcutaneous injections of *Escherichia coli* (*E. coli*) compared to rats administered non-stressed plasma exosomes or saline. Together, these findings demonstrate that activation of the acute stress response potentiates innate immunity by modifying the proteomic and miRNA profile of exosomes released into the circulation.

Introduction

The extracellular environment within multicellular organisms contains unique vesicular particles known as exosomes, which are 40-100 nm vesicles secreted into the

extracellular environment from both hematopoietic and non-hematopoietic cells[1,2]. Exosomes are similar to cytoplasm encased in a lipid bi-layer containing molecules unique to the cellular origin of the exosome. Interest in the immunological roles of exosomes has grown rapidly since the discovery that exosomes modulate immunity through expression of MHC class I and II molecules[3–5], activation of natural killer cells[6], modification of T cell activation[7,8], and induction of tolerance to oral antigens[9,10]. Exposure to a variety of pathogens or disease states, such as microbial infection[11–13] and cancer[14,15], alters the membrane and intra-vesicular composition of exosomes, as well as their function, providing valuable insight into their potential use as disease biomarkers and therapeutic tools. While a growing body of evidence demonstrates that the composition and functional relevance of exosomes can be modified during a variety of physiological challenges, to date there are no studies examining the characteristics and immunological consequences of plasma exosomes in stress physiology.

Exposure to stressors, whether acute or chronic, can exert adverse consequences on immune function. Research has demonstrated that stressor exposure can exacerbate cardiovascular disease[16], diabetes[17], obesity[18], Alzheimer's[19], and cancer[20]. Additionally, much of the earlier research in stress physiology examined the immunosuppressive impact of stress through restrained T cell dependent antibody responses[21] and suppression of anti-viral host-defense[22]. While the negative and immunosuppressive consequences of stress are well established in the literature, recent research demonstrates that exposure to acute stressors can also potentiate innate immunity. Exposure to acute stressors can evoke an exaggerated inflammatory cytokine and chemokine response under sterile conditions[23], enhance dermatological immunity[24], stimulate macrophage phagocytosis[25], and improve

host defense to subcutaneous *Escherichia coli* (*E. coli*) challenge[26]. These findings indicate that potentiation of innate immunity could be an adaptive feature of the acute stress response, possibly enhancing an organism's response to injuries sustained from the stressor. The mechanisms driving stress-modified immunity are still under investigation, but recent research on the immunological function of exosomes during disease make them a novel target for immunomodulation during the acute stress response.

To understand how exposure to an acute stressor might modify the immunological function of plasma exosomes, we examined components of the stress response that could be likely candidates for both associating with exosomes and modulating immunity. Of particular interest is the 72kD heat shock protein (Hsp72), a molecular chaperone abundant in the plasma following exposure to an intense and acute stressor[26]. When exposed to a stressor, cells rapidly synthesize intracellular Hsp72 to maintain cellular homeostasis by refolding denatured proteins and promoting cellular survival[26–30]. However, when cells release Hsp72 into the extracellular environment, it becomes immunologically active by stimulating leukocytes through toll-like receptors (TLRs) and inducing the secretion of pro-inflammatory molecules[23,31,32]. The mechanism of Hsp72 release into the circulation during stressor exposure is unclear, but several studies have demonstrated that Hsp72 can associate with exosomes in cell culture[33–36] and amniotic fluid[37]. Thus far, it is unknown whether stress-induced Hsp72 circulating in the plasma is associated with exosomes. Given the reported immunological properties of both exosomes and extracellular Hsp72, it is important to determine if stressor exposure modulates host immunity through the up-regulation of Hsp72 expression on plasma exosomes.

Growing data also indicate that exosomes contain small (20-25 nucleotides), non-coding RNA known as microRNA (miRNA)[38–42]. MicroRNA in plasma exosomes can elicit activity on distal cells upon their inclusion[38,43,44], potentially regulating target genes and modulating translation of messenger RNA (mRNA). Interestingly, a variety of environmental stressors, such as heat stress[45,46] and oxidative stress[47,48], can modify miRNA associated with TLR mediated inflammation[49], cytokine expression[50,51], and macrophage differentiation[52], making miRNA another target of interest in stress-enhanced immunity. To explore the impact of acute stress on exosomal miRNA in the plasma, we analyzed miR-142-5p, -150, -155, and -203 based on evidence of their differential expression in heat stressed rats[45], their role in cellular stress[53], their involvement in TLR-mediated immunity [54], and their association with macrophage differentiation[52,55].

In the present study, we hypothesized that exposure to an intense, acute stressor modulates protein and miRNA expression in plasma exosomes. Our findings confirm this hypothesis by demonstrating that stress modifies plasma exosomes through up-regulation of Hsp72 expression and down-regulation of two miRNAs. Pathway enrichment analyses of the stress-modified exosomal miRNAs target genes reveals functionally enriched pathways implicated in the stress response. Further, we identify sympathetic nervous system (SNS) activation of the α_1-adrenergic receptor (ADR) as an important signaling process to exosomal expression of Hsp72 and down-regulation of miR-142-5p. Lastly, we reveal that these stress-modified plasma exosomes significantly enhance host defense in an *in vivo* model of bacterial challenge. Rats subjected to subcutaneous injections of *E. coli* and stress-modified plasma exosomes have constrained inflammatory responses and lose less body weight compared to rats injected with *E. coli* co-administered with either non-stressed

plasma exosomes or saline. These are the first studies to demonstrate that activation of the stress response modifies plasma exosomes capable of regulating the inflammatory response to *in vivo* bacterial challenge.

Materials and Methods

Animals and housing

Adult male Fisher 344 rats (8-9 weeks old; Harlan, Indianapolis, IN) weighing approximately 230-275 grams were used in all experiments. Rats were singly housed in clear Nalgene plastic cages (48 × 27 × 20 cm) with microisolator tops in a specific pathogen-free barrier facility and allowed access to food and water *ad libitum* (Harlan Standard Lab Chow). Temperature and humidity remained constant and rats were maintained on a 12-hour light:dark cycle (lights on at 0700). Rats acclimated to these housing conditions for 1 week prior to any experimental manipulations and were handled daily. The care and use of the rats were in accordance with protocols approved by the University of Colorado Institutional Care and Use Committee.

Inescapable tail shock protocol and plasma collection

Inescapable tail shock (stress) was performed as previously described[56]. Briefly, rats were randomly selected to either remain in their home cages (control; n = 8) or placed in a Plexiglas restraining tube (23.4 × 7 cm; n = 8). Electrodes were placed across the tail, which protruded from the back of the restraining tube. Rats were exposed to 100, 1.5 mA, 5-second, intermittent, (average inter-trial interval of 60 +/- 25 s) inescapable tail shocks administered by an automated shock system (Precision Calculated Animal Shocker; Colbourn Instruments, Whitehall, PA). Immediately following stressor termination, rats

were sacrificed via rapid decapitation. Trunk blood was collected in EDTA-coated tubes (13 × 75 mm) and plasma was isolated at 3000 × *g* at 4°C for 15 minutes. This stressor procedure has been previously demonstrated to reliably and consistently elevate Hsp72 and inflammatory cytokines in the plasma[23,26,57].

Confirmation of activation of the stress response

Three parameters were used to confirm activation of the acute stress response in rats exposed to inescapable tail shock stress: spleen-to-body weight ratio, blood glucose, and plasma corticosterone. Spleens were aseptically removed and weighed immediately following sacrifice. Reduction in spleen weights are indicators of sympathetic nervous system activity[58]. Blood glucose, another marker of SNS activation[59], was measured from the trunk using an Accu-Chek Compact blood glucose monitoring system (Roche Diagnostics, Indianapolis, IN), immediately following sacrifice. Plasma corticosterone, a marker of hypothalamic-pituitary-adrenal (HPA) axis activation, was measured with the commercially available DetectX ELISA kit from Arbor Assays (Ann Arbor, MI) at a 1:200 dilution.

Exosome enrichment with ultracentrifugation

Exosomes were isolated from the plasma by differential centrifugation as previously described[60], with some modifications to optimize isolation from small volumes of plasma. Briefly, 600 µL of plasma was diluted to 1 mL with RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) and successively centrifuged at 2,000 × *g* for 10 minutes at 4°C and then 10,000 × *g* for 20 minutes at 4°C to remove cellular debris. The plasma was then run through a 0.22 µm filter (Millipore, Bedford, MA) to remove remaining debris. Exosomes were

pelleted from the filtered plasma supernatant by ultracentrifugation at 300,000 × *g* for 2 hours at 4°C. The supernatant was aspirated, labeled as "UC exosome depleted," and aliquoted, while the exosome pellet was washed three times in cold phosphate buffered saline (PBS). After washing, the exosome pellet was re-suspended in 150 µL of PBS and referred to as the "UC exosome enriched" fraction. It is important to note that this procedure does not discriminate between exosomes and other small vesicular structures or large protein aggregates. We did not apply a sucrose gradient to the UC exosome enriched fractions because the yields are too small for extensive analysis.

Exosome enrichment with ExoQuick precipitation

To isolate exosomes in the plasma from other small vesicular structures and large protein aggregates, plasma was treated with the commercial exosome precipitation reagent, ExoQuick (Systems Biosciences, Mountain View, CA), according to the manufacturer's instructions and under sterile hood conditions. Briefly, the plasma was divided into 250 µL aliquots and exosomes were precipitated out of the supernatant with the addition of 63 µL of ExoQuick™. Following an overnight incubation at 4°C, the plasma was centrifuged at 1,500 × *g* for 30 minutes at 4°C. The supernatant was aspirated off and labeled "exosome depleted." The remaining pellet that contained the exosomes was washed and re-suspended in 75µl of PBS and labeled "exosome enriched."

Exosome visualization

Exosome enriched fractions from Thromboplastin-D treated plasma (described below) and untreated plasma were deposited on the surface of Formvar-carbon grids, air-dried, stained with Uranyl acetate, and transmission election microscope (TEM) images

were recorded with a Phillips CM100 transmission electron microscope (FEI, Inc., Hillsboro, OR). For nanoparticle tracking analysis (NTA), fibrins and fibrinogens were removed from the plasma to prevent exosomes from adhering to one another. Briefly, 100 µL aliquots of the plasma were warmed to 37°C and treated with an equal volume of Thromboplastin-D (Thermo Scientific, Middletown, VA), also pre-warmed to 37°C, and incubated for 15 minutes. Fibrins and fibrinogens were pelleted from the plasma following centrifugation at 10,000 rpm for 5 minutes at room temperature. The supernatant was fractionated with ExoQuick™ treatment as described above. Thromboplastin-D treated plasma samples and exosome enriched samples were diluted 1:10,000 while exosome depleted samples were diluted 1:10 in sterile filtered PBS. NTA were performed using the LM10-HS instrument (NanoSight Ltd., Amesbury, UK) equipped with a green 532 nm laser in light scatter mode, with NTA 2.3 analytical software. The instrument was calibrated using commercially available 100 nm polystyrene beads (Polysciences, Warrington, PA) and sterile filtered PBS.

Exosome confirmation assays

Multiple markers (CD63, A33, IL-6) were measured in the exosome enriched and exosome depleted fractions from plasma treated with ExoQuick with commercially available ELISAs to determine successful exosome isolation. CD63 is a membrane tetraspanin common to exosomes from a variety of cellular sources[61–64]. Intestinal epithelial cells are known to release exosomes expressing A33, a protein not found in soluble form in the plasma[4,65,66]. Exosomes are not known to express IL-6; therefore, it should not be present in the exosome enriched fractions. All exosome fractions were run neat in the commercial A33 ELISA (Uscn Life Science, Wuhan, China) and the commercial

IL-6 ELISA (R&D Systems, Minneapolis, MN). Exosome fractions were diluted 1:20 for the commercial CD63 ExoELISA (System Biosciences, Mountain View, CA). Optical densities were measured using a SpectraMax Plus 354 plate reader (Molecular Devices, Sunnyvale, CA) and analyzed using four-parameter curve fit and software (SoftMax 5.4.1). Sample concentrations were then corrected by total protein using a commercially available BCA Protein Assay Kit (ThermoScientific, Rockford, IL) according to the manufacturer's instructions. Exosome enriched fractions were diluted 1:10 and exosome depleted fractions were diluted 1:25.

Additionally, an exosome assay developed by Logozzi et. al using two common markers for exosomes, the tetraspanin CD63 (clone AD1, BD Pharmingen, San Jose, CA) and the membrane transport protein Rab5b (clone A-20, Santa Cruz, CA), provides additional evidence for successful plasma exosome isolation[67]. All samples were run neat.

To establish the successful isolation of exosomes in the fractionated plasma following ultracentrifugation, we assessed the activity of acetylcholinesterase (AChE), an enzyme specific to these vesicles[68–70]. AChE activity was assessed using a commercially available kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions. The detection limit is 10 U/L, where U is one unit of enzyme that catalyzes the production of 1 μmole of thiocholine per minute. All samples were run neat.

Measurement of Hsp72

Hsp72 was measured with a commercially available ELISA kit from Enzo Biosciences (Plymouth Meeting, PA) according to the manufacturer's instructions. All exosome fractions were run neat and plasma samples were run 1:2. Since plasma exosome

precipitation changes the distribution of protein concentrations, values are analyzed in pg per mg of total protein.

CD63 Dynabead immunoprecipitation

To determine if CD63 depletion correlates with Hsp72 depletion in plasma from stressed rats, Thromboplastin-D plasma was treated with magnetic Dynabeads (Invitrogen, Oslo, Norway) coated in 50 µg/mL purified mouse anti-rat CD63 antibody (BD Pharmingen, San Jose, CA) according to manufacturer's instructions. Following CD63 isolation, the supernatant was divided into 200 µL aliquots and exosomes were precipitated out of the supernatant with the addition of 50µl of ExoQuick. Following an overnight incubation at 4°C, the ExoQuick treated plasma was centrifuged at 1500 × *g* for 30 minutes at 4°C. The supernatant was aspirated off and labeled "exosome depleted." The remaining pellet that contained the exosomes was re-suspended in 50µl of PBS and labeled "exosome enriched." Both fractions were immediately analyzed for BCA, CD63 and Hsp72.

Administration of α_1-adrenergic receptor antagonist

To determine whether blockade of the α_1-adrenergic receptor (ADR) impacts exosome associated Hsp72 and miRNA following stressor exposure, the α_1-ADR was inhibited using the antagonist, prazosin (Sigma-Aldrich, St. Louis, MO). Prazosin was administrated to the rats (n = 8/group) as previously described[56]. Thirty minutes prior to the tail shock stressor, rats either received a 2.0 mg/kg intra-peritoneal injection of prazosin dissolved in sterile, endotoxin-free water and subjected to heat, or no injection. Control rats were time-matched to stressed rats and received prazosin injections at concurrent times. Pilot studies indicate that saline injection has no impact on cytokine,

CD63 exosome, or Hsp72 concentrations compared to rats receiving no injection. Immediately following termination of the stressor, rats were sacrificed and plasma and spleens were collected. Spleens were weighed to examine the success of prazosin administrations, homogenized with the commercial tissue Hsp72 kit according manufacturer's instructions (Enzo Biosciences), and assayed for intracellular Hsp72. Lactate dehydrogenase (LDH), a marker of cell death, was measured in the plasma using a commercially available colorimetric assay from Bioo Scientific (Austin, TX) according to the manufacturer's instruction. All plasma samples were run neat. Plasma exosomes were isolated with ExoQuick and assayed for Hsp72, BCA, and CD63 as described above. Exosomal miRNA was subsequently analyzed as described below.

Measurement of miRNA

Plasma exosomes from separate rats were isolated and the RNA within was purified using SeraMir (System Biosciences). Isolated miRNA was reverse transcribed for qRT-PCR using the TaqMan® miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied BioSystems). MiRNA from stressed (n = 7), control (n = 7), stress + prazosin (n = 4), and control + prazosin (n = 4) rats were analyzed for changes in the levels of four miRNA (rno-miR-142-5p, miR-150, miR-155, and miR-203). Analysis was performed on the reverse transcript from 1.2ng RNA. All real time qPCR analysis was performed in a 96 well plate (Bio-Rad, Hercules, CA) format on the CFX96 (Bio-Rad) with TaqMan® probes. After a 10 minute 95°C melting step, samples were run at a 95°C melting step for 15 seconds and 60°C annealing and elongation step for 60 seconds for 40 cycles. Samples were normalized to miR-191 as a stable endogenous control [71]. Ct values were obtained using CFX manager 2.0 (Bio-Rad) and all fell within the limit of detection. Data

was deconvoluted using the ΔΔCt method with samples normalized from RNA of exosomes isolated from control rats.

KEGG and Wikipathway WebGestaldt pathway enrichment analysis

The miRNA database miRDB[72,73] was used to examine predicted gene targets of stress-modified exosomal miR-142-5p and miR-203. Pathway enrichment analyses were performed as previously described[74]. Briefly, 374 genes predicted to be targets of rno-miR-142-5p and 386 genes predicted to be targets of rno-miR-203 were uploaded into the WebGestaldt Bioinformatics system and subjected to KEGG[75] and Wikipathways[76] (Wiki) pathway analysis to identify the top functionally enriched pathway categories related to the targeted genes.

Bacterial culture and challenge

One day prior to *in vivo* bacterial challenge, frozen stock cultures of *E. coli* (ATCC 15746; American Type Culture Collection; Bethesda, MD) were rehydrated in 40 mL of brain-heart infusion (Difco Laboratories, Detroit, MI) and proliferated overnight in a humidified incubator at 37°C and 5% CO_2. Colony forming units (CFUs) were assessed by extrapolating optical densities (595 nm, Molecular Devices) of 100 µL/well *E. coli* in a 96-well plate from previously determined growth curves[77]. Cultures were centrifuged at 1200 × *g* for 15 minutes, the supernatant was discarded, and the *E. coli* was resuspended to 1 × 10^{10} CFUs/mL in either sterile injection saline, plasma exosomes from stressed rats, or plasma exosomes from control rats. Exosomes from stressed and control rats were diluted in sterile injection saline to equate protein concentrations (14.67 mg/mL) as determined by BCA analysis. Rats were shaved and administered dorsal subcutaneous injections of 2.5

× 10^9 CFUs of *E. coli* resuspended in 0.25 cc of one of three conditions: plasma exosome from stressed rats (stress exosomes, n = 9), plasma exosomes from control rats (control exosomes, n = 9), or sterile injection saline (n = 10). Four hours following the initial *E. coli* injection, rats received a 0.25 cc booster injection of their respective treatment (stress exosomes, control exosomes, or sterile injection saline; no *E. coli*) at the inflammatory site to amplify and sustain potential inflammatory changes.

In vivo inflammation assessment

Inflammation was assessed hourly on the day of injection from 10:00 to 19:00h by measuring the diameter of the inflammatory site with digital calipers as described previously[57,77]. The inflammatory site and body weights were monitored daily between 10:00 and 11:00h until resolution. All measurements were performed on the same dorsal region by an independent observer blind to the experimental conditions.

Statistical analyses

Two-way ANOVAs were run to analyze the effect of stress or exosome fraction on individual proteins. Data points were treated as outliers if they failed Grubb's test for outliers and were also recorded as affected by experimental procedures by the experimenter. A Pearson's product-moment correlation was performed to examine correlations between AChE and Hsp72 in ultracentrifuged plasma. Body weight and inflammatory diameter data from the *E. coli* challenge experiments were analyzed by repeated measures ANOVA. If significant interactions were present, then Fisher's protected least significant difference (PLSD) post-hoc analyses were conducted on the data. To determine functionally enriched pathways generated from the predicted genes modulated

by stress-modified miRNA, a correlation value and *p*-value associated with the strength of correlation was calculated for each functionally enriched pathway. All data are presented as means ± the standard error of the mean (SEM). $p<0.05$ was considered statistically significant.

Results

Visualization and characterization of plasma exosomes from F344 rats

Multiple qualitative and quantitative analyses were used to confirm successful exosome enrichment with the commercial ExoQuick precipitation reagent. TEM analysis of plasma samples from rats subjected to inescapable tail shocks (stress) confirmed both the presence of exosome-sized particles in the exosome enriched fractions and the ability of Thromboplastin-D, an anticoagulant, to block particle aggregation[78] and enhance visualization. Figure 3.1*A* shows exosome-sized particles, but the highly stained background and presence of larger, aggregated particles are likely due to fibrins and fibrinogens present in the sample. In contrast, the TEM image in Figure 3.1*B* reveals exosome-sized particles without aggregation, suggesting that treating plasma with Thromboplastin-D prior to ExoQuick fractionation removes aggregating or coagulation factors for optimal visualization. Further analysis with NTA revealed visual confirmation of particles 50 to 100 nm in size in plasma exosome enriched fractions isolated from stress and non-stressed rats (control), as well as successful enrichment of exosomes under identical scatter conditions for exosome enriched and depleted fractions (Figure 3.1*C*). Together, these visual observations indicate that ExoQuick treatment of the plasma successfully enriches exosome-sized particles.

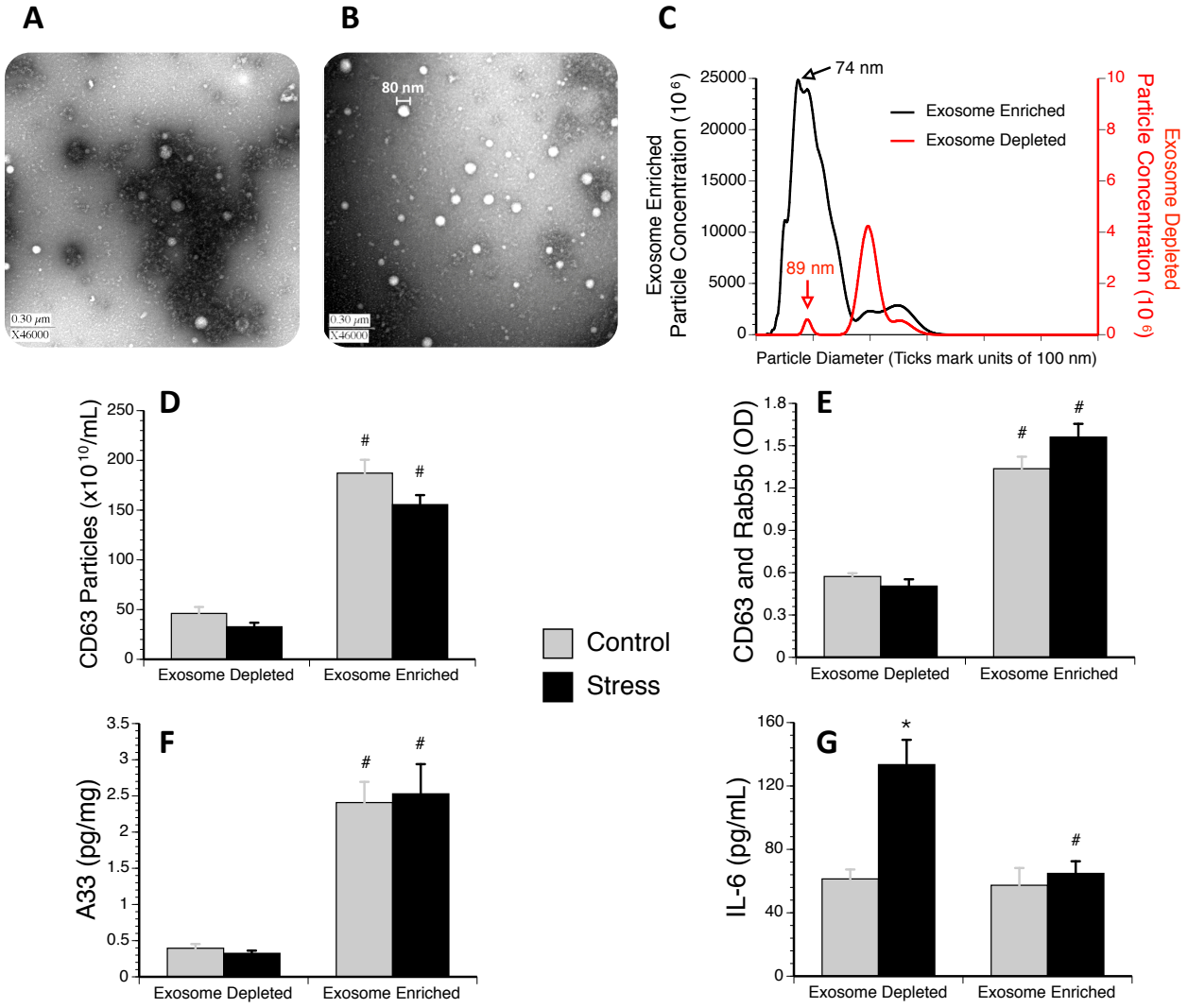

Figure 3.1. Confirmation of successful plasma exosome enrichment from male Fisher 344 rats. A. TEM demonstrates successful isolation of exosome-sized particles (~80nm) from the plasma with ExoQuick precipitation. **B.** TEM shows how pre-treating plasma with Thromboplastin-D prior to exosome isolation with ExoQuick removes clouding factors and prevents vesicle aggregation. **C.** Exosome enriched and exosome depleted plasma fractions from stressed rats pre-treated with Thromboplastin-D were characterized for size distribution and quantitated by NanoSight LM10, using light scatter from a 532 nm green laser. ELISA analyses reveal that ExoQuick exosome isolation successfully enriches exosomes as marked by known exosome markers. Exposing rats to tail shock stress (Stress) had no impact on the rate of plasma exosome release compared to non-stressed rats (Control) as measured by: **D.** the tetraspanin CD63, **E.** the adhesion molecule Rab5b, **F.** and the intestinal exosome marker A33. **G.** Analysis of IL-6 confirms activation of the stress response and specificity of ExoQuick for exosome associated proteins. These data suggest that stress does not impact the rate of exosome release into the plasma. Proteomic results are expressed in means ± SE; 6-8 rats/condition. * indicates significant difference when compared to control rats ($p < 0.05$). # indicates significant difference when compared to exosome depleted fraction ($p < 0.05$). Two-way ANOVA with Fisher PLSD post hoc test was used.

Subsequent ELISA analysis reveal that the exosome associated proteins CD63, Rab5b, and A33 were significantly expressed in the exosome enriched plasma fractions from both stressed and control rats, compared to their corresponding exosome depleted fractions (Figure 3.1*D-F*). IL-6, a stress responsive cytokine not known to associate with exosomes, was only significantly expressed in the exosome depleted plasma fraction from stressed rats (Figure 3.1*G*), both confirming activation of the stress response and successful isolation of proteins only known to associate with exosomes. These data also suggest that exposure to stress does not impact the rate of exosome release into the plasma.

Exposure to stress increases Hsp72 expression on plasma exosomes

To determine whether our stressor modifies protein expression on plasma exosomes, we examined the association between stress-inducible Hsp72 and plasma exosomes. Exposure to tail shocks successfully activated the acute stress response in rats as indicated by reduction in spleen weights (Figure 3.S1*A*), as well as elevations in plasma corticosterone (Figure 3.S1*B*) and blood glucose (Figure 3.S1*C*). Hsp72 is a highly stress-responsive protein and, consistent with prior observations, stressed rats had marked accumulation of Hsp72 in the plasma compared to control rats (Figure 3.2*A*)[23,56,57,79]. Quantification of Hsp72 following exosome enrichment after exposure to stress was responsible for elevations of Hsp72 in both the exosome depleted and exosome enriched plasma fractions compared to corresponding plasma fractions in the control group (Figure 3.2*B*). Statistical analyses also indicated a significant interaction between stress and exosome enrichment on Hsp72 concentrations. These data indicate that inescapable tail shock stress elevates Hsp72 expression in both the exosome depleted and exosome enriched plasma fractions, but the greatest concentration of Hsp72 is present in the

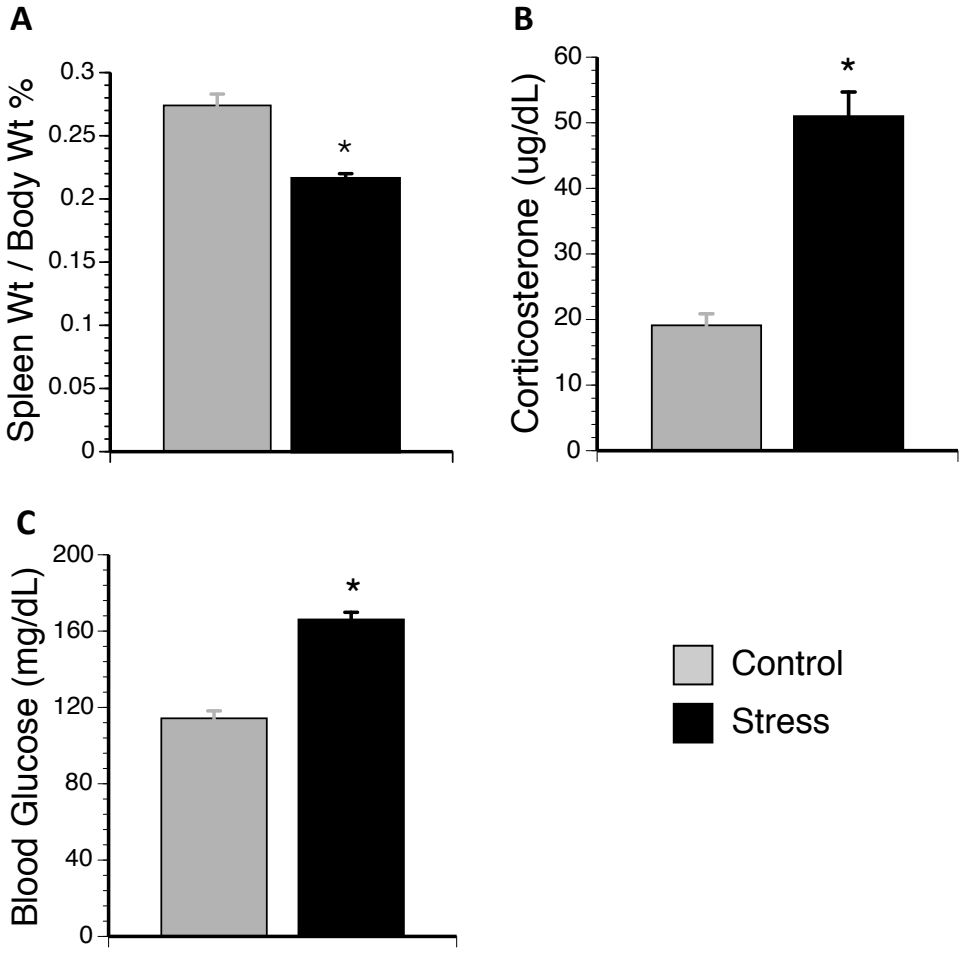

Figure 3.S1. Inescapable tail shock activates the stress response. Male Fisher 344 rats exposed to inescapable tail shock (Stress) activates the stress response as indicated by **A.** reduction in spleen weight, **B.** elevated plasma corticosterone, and **C.** elevated blood glucose compared to non-stressed controls. Results are expressed in means ± SE; 6-8 rats/condition. *Indicates significant difference when compared to control rats ($p < 0.05$). Two-way ANOVA was used.

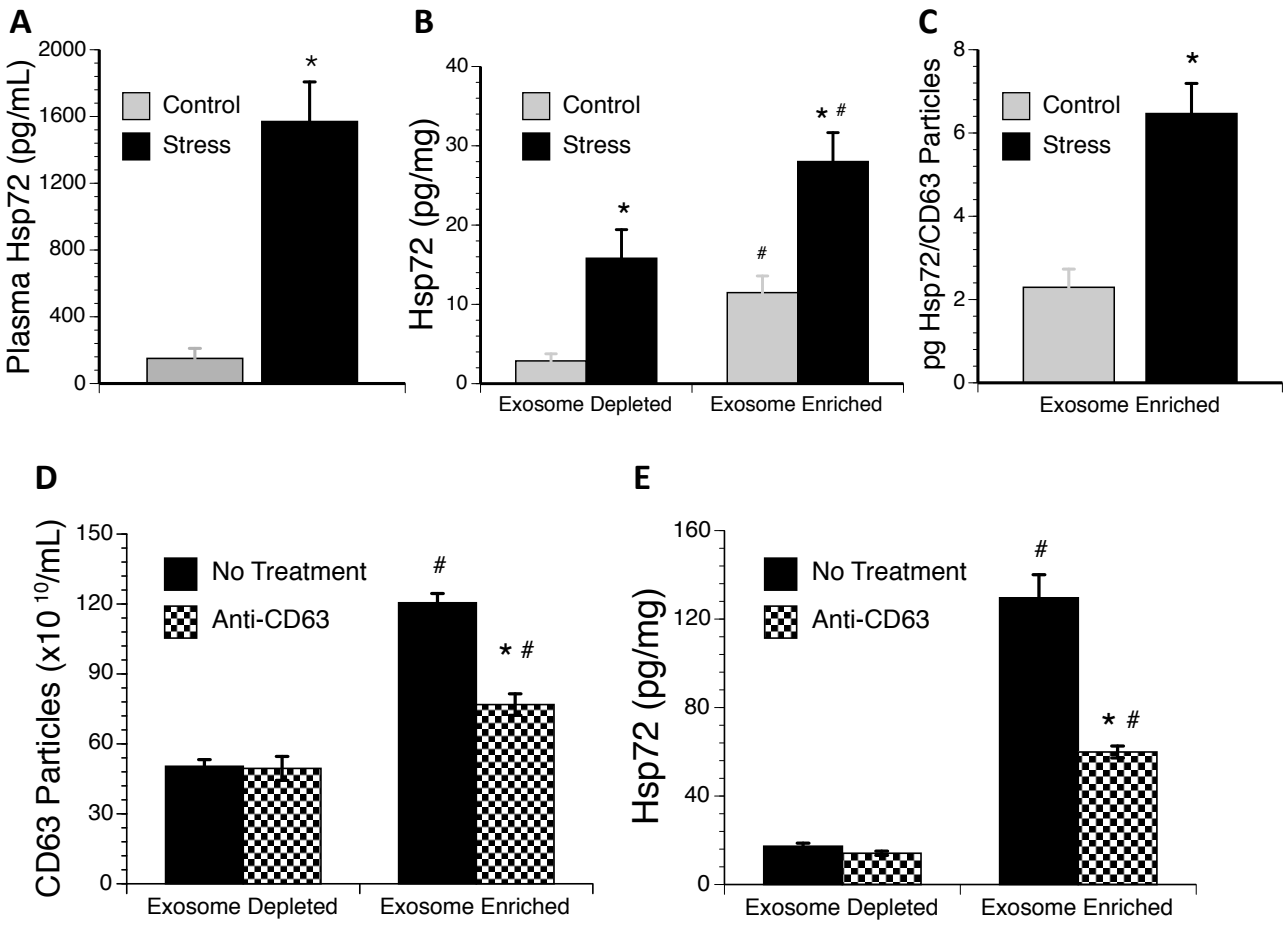

Figure 3.2. Exposure to an acute stressor modifies the proteomic profile of circulating plasma exosomes. A. Male Fisher 344 rats exposed to inescapable tail shocks (Stress) had elevated plasma concentrations of Hsp72 compared to non-stressed rats (Control). Following exosome enrichment with ExoQuick precipitation, **B.** concentrations of Hsp72 were the highest in the exosome enriched fractions from stressed rats. **C.** Correction with CD63 particles confirmed that exposure to stress increases Hsp72 expression in the exosome enriched fraction compared to control conditions. **D.** Following pre-treatment with Thromboplastin-D, plasma was divided and either treated with magnetic Dynabeads coated in CD63 antibodies (Anti-CD63) to immunoprecipitate CD63 particles from the plasma or left undisturbed (No Treatment) prior to ExoQuick precipitation. Anti-CD63 treatment attenuates CD63 expression in the plasma exosome enriched fractions from stressed rats compared no treatment conditions. **E.** Anti-CD63 treatment also attenuates Hsp72 in the plasma exosome enriched fractions from stressed rats compared to no treatment conditions. Results are expressed in means ± SE; 6-8 rats/condition. * indicates significant difference when compared to control rats ($p < 0.05$). # indicates significant difference when compared to exosome depleted fraction ($p < 0.05$). Two-way ANOVA with Fisher PLSD post hoc test was used.

exosome enriched fractions. Further analysis of the exosome enriched fractions revealed that stressed rats also express significantly greater concentrations of Hsp72 compared to control rats when corrected by CD63 exosomes instead of total protein (Figure 3.2*C*).

To further confirm that stress-induced Hsp72 is associated with plasma exosomes, particles expressing CD63, a tetraspanin common to exosomes released from most cells[61,64,67], were immunoprecipitated from Thromboplastin-D treated plasma from stressed rats prior to ExoQuick precipitation. Immunoprecipitation with magnetic Dynabeads coated in anti-CD63 antibodies successfully reduced CD63 expression in the exosome enriched fraction compared to untreated plasma (Figure 3.2*D*). As expected, CD63 immunoprecipitation had no impact on CD63 expression in the exosome depleted fraction. CD63 depletion also significantly decreased Hsp72 expression in the exosome enriched fraction from the plasma of stressed rats (Figure 3.2*E*), indicating that a significant portion of stress-induced plasma Hsp72 is complexed with CD63 positive exosomes.

Enriching exosomes by ultracentrifugation also confirmed the presence of Hsp72 on plasma exosomes. Confirmation of exosome isolation by ultracentrifugation was determined by measuring acetylcholinesterase (AChE) activity. Figure 3.S2*A* illustrates that AChE activity was significantly elevated in the exosome enriched fractions, and exposure to stress did not impact AChE activity. Subsequent Hsp72 analysis of the ultracentrifuged plasma fractions revealed that exposure to stress significantly elevated Hsp72 in both the exosome depleted and exosome enriched plasma fractions compared to corresponding plasma fractions in the control group (Figure 3.S2*B*). Simple Pearson product-moment correlation analysis revealed a positive correlation of AChE to Hsp72 in both the exosome

enriched and exosome depleted plasma fractions following exposure to stress (Figure 3.S2*C*). Together, analyses of both ExoQuick enriched exosomes and ultracentrifuged exosomes confirm that Hsp72 is associated with exosomes derived from the plasma of stressed rats, revealing that the proteomic profile of plasma exosomes are subject to modifications following organismal exposure to an intense and acute stressor.

Stress reduces exosome expression of miR-142-5p and -203

Plasma exosomes isolated from the α_1-ADR study were further analyzed with qRT-PCR for stress and inflammation related miRNA (miR-142-5p, -150, -155, and -203). There was no evidence of contamination as determined by a negative reverse transcription control (Figure 3.S3). The exosome samples isolated from stressed, stress + prazosin, and control + prazosin rats were compared to those from control rats and variations were observed. Specifically, miR-142-5p and -203 expressions were significantly reduced in stressed rats compared to exosomes isolated from control rats (Figure 3.3). To our knowledge, this is the first evidence that *in vivo* exposure to an acute stressor, in the absence of pathogens or disease, modifies miRNA content in plasma exosomes.

Differential gene expression and pathway enrichment analyses

Based on miRDB query, 374 genes are predicted targets of rno-miR-142-5p and 386 genes are predicted targets of rno-miR-203. These gene targets were uploaded into the WebGestaldt Bioinformatics system and subjected to KEGG and Wiki pathway analyses. Of these 760 genes, 736 were unambiguously mapped and included in the analyses. Table 3.1 exhibits the pathways identified by KEGG and Wiki modulated by the rno-miR-142-5p and

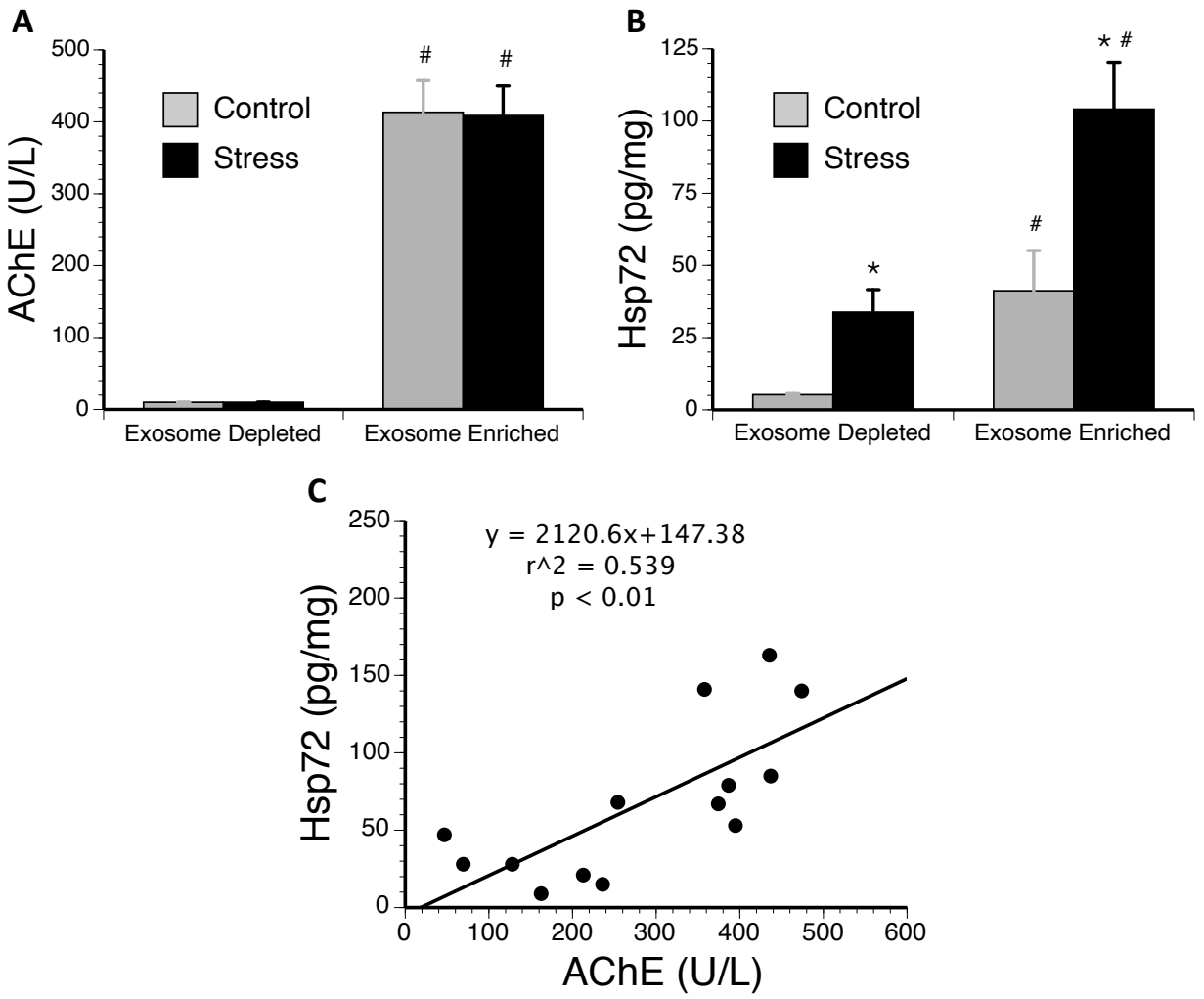

Figure 3.S2. Confirmation of successful plasma exosome isolation by ultracentrifugation. A. Assessment of acetylcholinesterase (AChE) activity indicates successful exosome enrichment with ultracentrifugation. **B.** ELISA analysis of the ultracentrifuged plasma exosome fractions demonstrates exposure to stress elevates Hsp72 expression in the exosome enriched and exosome depleted fractions, but the highest concentrations of Hsp72 are in the exosome enriched fractions from stressed rat plasma. Results are expressed in means ± SE; 7 rats/condition. * indicates significant difference when compared to control rats ($p < 0.05$). # indicates significant difference when compared to exosome depleted fraction ($p < 0.05$). Two-way ANOVA with Fisher PLSD post hoc test was used. **C.** Simple Pearson product-moment correlation analysis reveals a positive correlation of AChE to Hsp72 when both exosome fractions from stressed rats are combined.

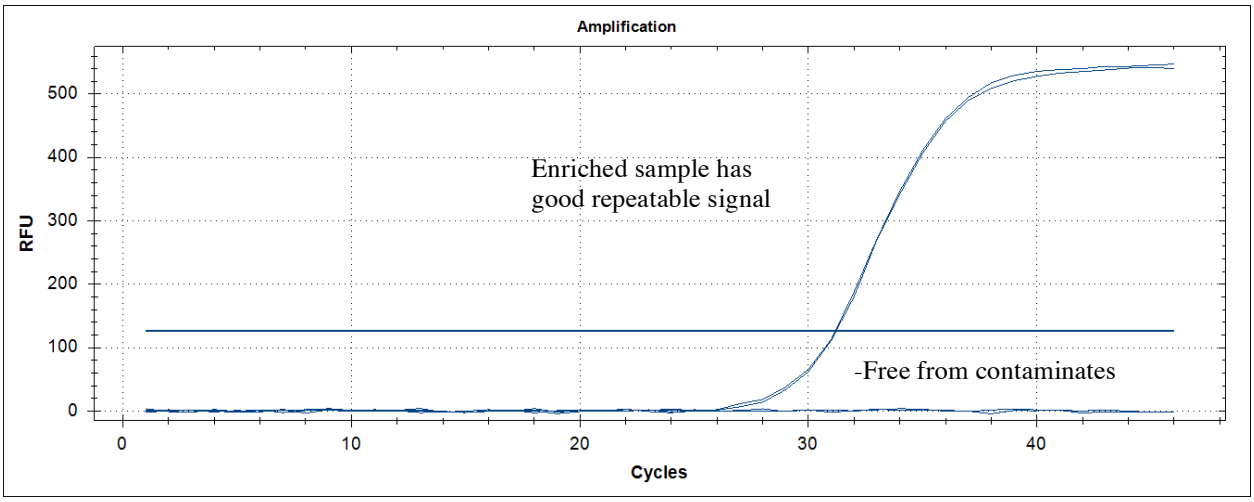

Figure 3.S3. Amplification of negative reverse transcription control with qRT-PCR reveals no evidence of contamination.

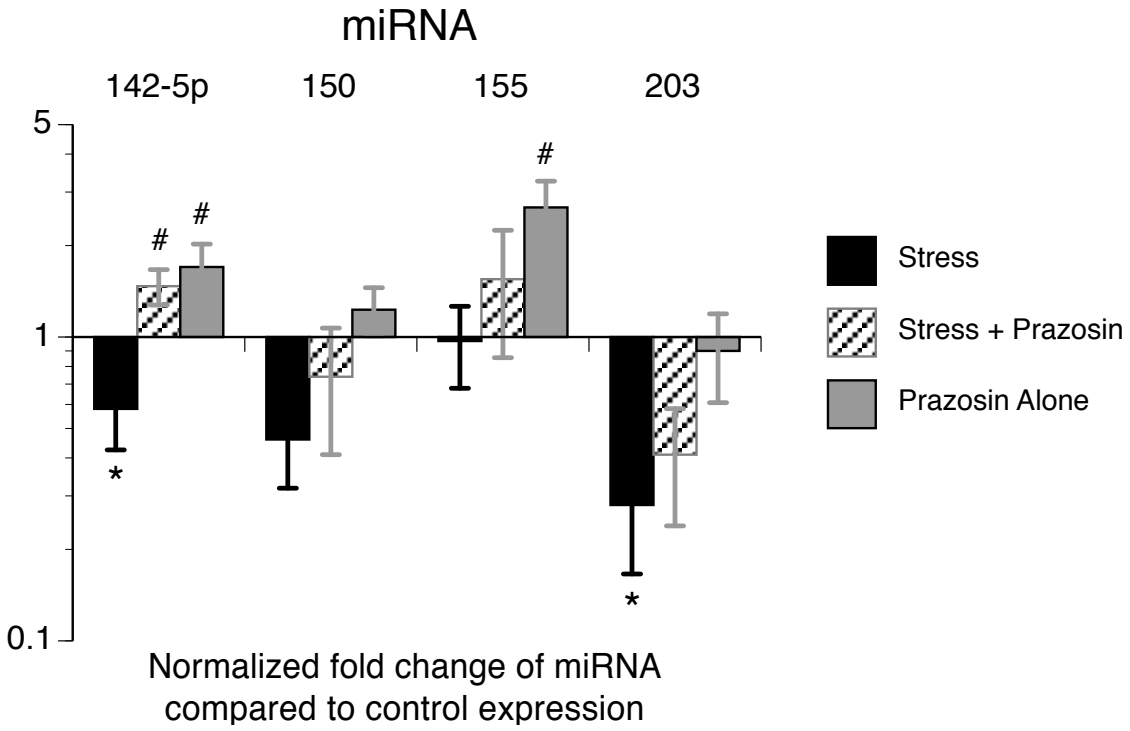

Figure 3.3. Impact of acute stressor and adrenergic regulation of plasma exosomes miRNA. Exposure to tail shock stress (Stress) significantly reduced plasma exosome expression of miR-142-52 and -203. Intraperitoneal administration of the α_1-adrenergic receptor antagonist prazosin (2.0 mg/kg) 30 minutes prior to Stress attenuated the stress-induced down-regulation of miR-142-5p, but not miR-203. * indicates significant fold change when compared to plasma exosomes from control rats in the absence of prazosin ($p < 0.05$). # indicates significant effect of prazosin ($p < 0.05$). Two-way ANOVA with Fisher PLSD post hoc test was used.

Bioinformatics Database *Functionally Enriched Pathway* **rno-miR-142-5p**	**Gene Count**	**Adj. P value**
KEGG		
Ubiquitin mediated proteolysis	9	0.0001
Pathways in cancer	10	0.0127
Spliceosome	6	0.0227
VEGF signaling pathway	4	0.0263
Renal cell carcinoma	4	0.0263
Wnt signaling pathway	6	0.0263
Carbohydrate digestion and absorption	3	0.0263
Cell cycle	5	0.0263
Gastric acid secretion	4	0.0263
Phosphatidylinositol signaling system	4	0.0263
WIKI		
Proteasome degradation	*6*	*0.0003*
mRNA processing	*7*	*0.0018*
Kit receptor signaling pathway	*5*	*0.0040*

rno-miR-203	**Gene Count**	**Adj. P value**
KEGG		
Metabolic Pathways	25	0.0018
TGF-beta signaling pathway	6	0.0034
Aldosterone-regulated sodium reabsorption	4	0.0140
Fc gamma R-mediated phagocytosis	5	0.0168
Insulin signaling pathway	6	0.0168
MAPK signaling pathway	8	0.0280
RNA degradation	4	0.0343
Hypertrophic cardiomyopathy (HCM)	4	0.0343
Phosphatidylinositol signaling system	4	0.0343
Focal adhesion	6	0.0343
WIKI		
MAPK signaling pathway	7	0.0215
Diurnally regulated genes with circadian orthologs	3	0.0430
NFE2L2	2	0.0430
P53 pathway	3	0.0430
mRNA processing	5	0.0430
Adipogenesis	5	0.0430
Insulin signaling	6	0.0430
Wnt signaling pathway	5	0.0430
Small ligand GPCrs	2	0.0463
TGF-beta receptor signaling pathway	6	0.0473

Table 3.1. KEGG and Wikipathways (Wiki) functionally enriched pathway categories generated from genes significantly differentially expressed in plasma exosomes from rats exposed to inescapable tail shock.

rno-miR-203. The number of genes in each pathway and a *p*-value associated with each functional pathway category is also reported.

Both KEGG and Wiki analysis revealed that genes differentially expressed in exosomes between stress and control rats were related to several functional categories including metabolic pathways (25 genes), mRNA processing (12 genes), cancer pathways (10 genes), ubiquitin mediated proteolysis (9 genes), mitogen-activated protein kinase (MAPK) signaling (8 genes), transforming growth factor-β (TGF-β) signaling pathway (6 genes), and TGF-β receptor signaling pathway (6 genes).

Blockade of α_1-adrenergic receptors attenuates stress-modified changes in Hsp72 and miR-142-5p expression

Previous work from our laboratory suggests that Hsp72 release into the extracellular environment is mediated by activation of the α_1-adrenergic receptors (ADRs)[56]. It is unknown, however, if α_1-ADR blockade impacts the release of exosome-associated Hsp72 in the plasma. Consistent with previous findings, administration of the α_1-ADR antagonist, prazosin, to stressed rats reduced plasma concentrations of Hsp72 compared to untreated rats exposed to the same stressor (Figure 3.4*A*) while attenuating stress-induced reductions in spleen weight (Figure 3.S4*A*) and boosting stress-induced elevations in blood glucose (Figure 3.S4*B*)[56,79]. Prazosin did not impact plasma Hsp72, spleen weight, or blood glucose concentrations in the control rats, confirming prazosin selectivity to sympathetic nervous system (SNS) activity[56,58,59]. Additionally, prazosin administration attenuated stress-induced cell death, as quantified by lactate dehydrogenase (LDH) (Figure 3*B*). Plasma exosome enrichment from stressed rats revealed that prazosin had no impact on CD63 (Figure 3.4*C*) or total protein concentrations

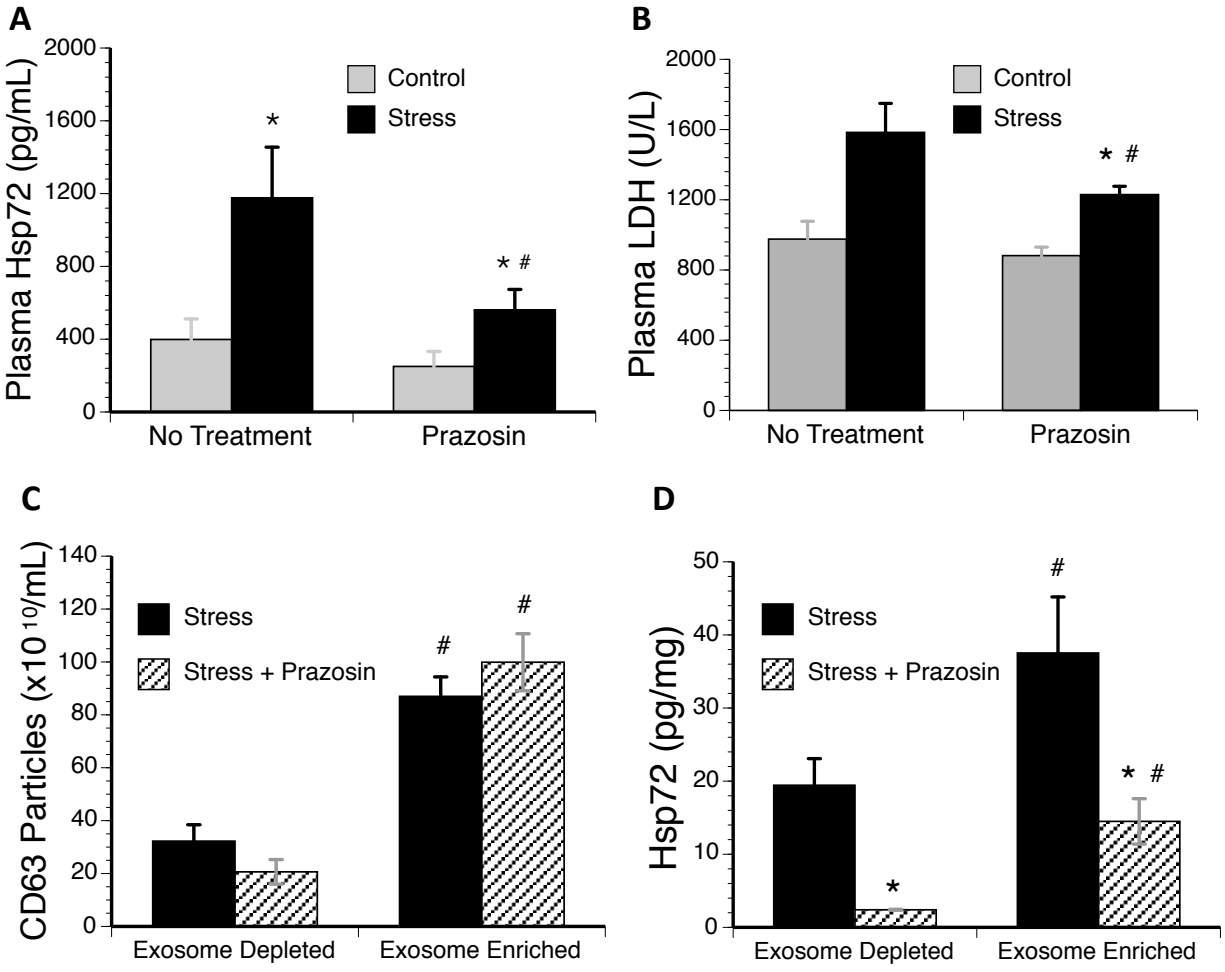

Figure 3.4. Adrenergic regulation of exosome expression of Hsp72. Adult male Fisher 344 rats were either injected intraperitoneally with the α_1-adrenergic receptor antagonist prazosin (2.0 mg/kg) 30 minutes prior to exposure to tail shock stress (Stress) or left undisturbed. Plasma analysis reveals that prazosin administration **A.** significantly attenuates stress-induced elevations of Hsp72 as well as **B.** lactate dehydrogenase activity, a marker of cell death. Exosomes were fractionated with ExoQuick in the stressed rats, revealing that prazosin administration **C.** had no effect on CD63 particle concentrations, but **D.** significantly attenuates Hsp72 concentration in both the exosome depleted and exosome enriched fractions. Results are expressed in means ± SE; 6-8 rats/condition. * indicates significant difference when compared to control rats ($p < 0.05$). # indicates significant difference when compared to no treatment group or exosome depleted fraction ($p < 0.05$). Two-way ANOVA with Fisher PLSD post hoc test was used.

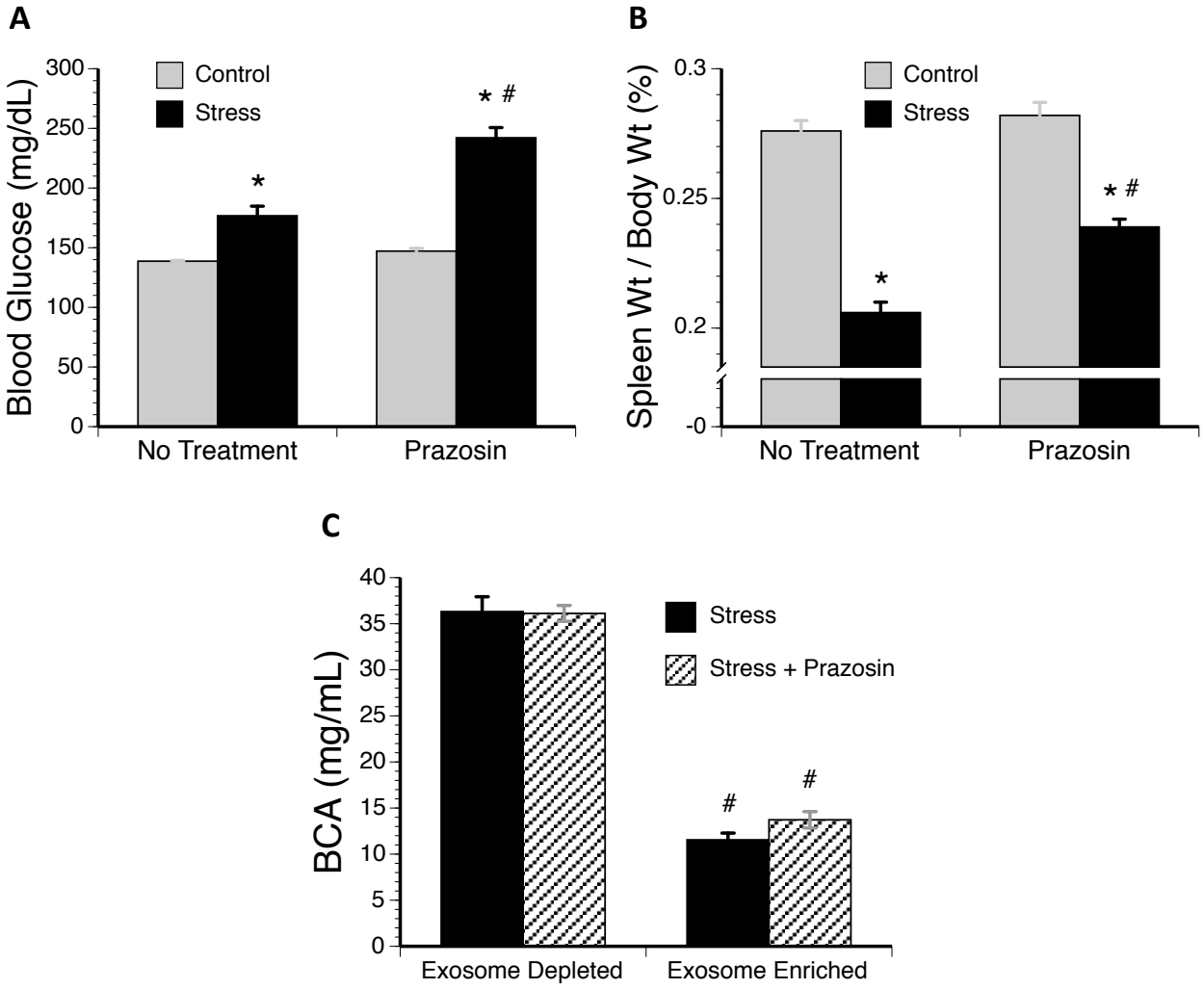

Figure 3.S4. Adrenergic regulation of blood glucose, spleen weight, and total protein. Adult male Fisher 344 rats were either injected intraperitoneally with the α_1-adrenergic receptor antagonist prazosin (2.0 mg/kg) 30 minutes prior to exposure to tail shock stress (Stress) or left undisturbed. Successful prazosin administration was confirmed by **A.** elevations blood glucose and **B.** elevations in spleen/body weight percentage in stressed rats. **C.** Prazosin administration in the stressed rats had no effect on exosome enriched or exosome depleted concentrations of BCA. Results are expressed in means ± SE; 6-8 rats/condition. * indicates significant difference when compared to control rats ($p < 0.05$). # indicates significant difference when compared to no treatment group or exosome depleted fraction ($p < 0.05$). Two-way ANOVA with Fisher PLSD post hoc test was used.

(Figure 3.S4*C*); however, α_1-ADR blockade significantly decreased Hsp72 expression in both the exosome enriched and depleted fractions (Figure 3.4*D*). These results suggest that direct activation of the α_1-ADRs during the stress response may be critical for either the exosomal loading or intracellular synthesis of Hsp72, but not exosome release into the circulation. Additionally, stress-induced reductions in miR-142-5p, but not miR-203, were prevented by prazosin administration (Figure 3.3), suggesting that SNS activation of the α_1-ADRs is also capable of modifying exosomal miRNA content in addition to Hsp72 expression.

Plasma exosomes from stressed rats facilitate in vivo host defense to subcutaneous E. coli challenge

To investigate one functional consequence of stress-modified plasma exosomes, we challenged unstressed rats with subcutaneous injections of *E. coli*, known to produce a rapid inflammatory response driven by the innate immune system[26,77], co-administered with either plasma exosomes from stressed rats, plasma exosomes from control rats, or saline. Host defense to subcutaneous *E. coli* challenge is primarily driven by activation of macrophage and neutrophil cells, which are enriched with Toll-like receptors (TLRs). Since Hsp72 is associated with TLR activation[28,80] and miR-142-5p and -203 are involved in inflammation-related signaling[50,81] and monocyte differentiation[52], this model of bacterial challenge is ideal for examining the impact of stress-modified plasma exosomes on host immunity. In addition, our previous work supports a role for stress-induced Hsp72 in anti-microbial responses and inflammation[26,77]. We observed that subcutaneous injections of *E. coli* produced a time-dependent increase in inflammatory swelling in all groups, but rats that received their *E. coli* injection with plasma exosomes isolated from stressed rats had a

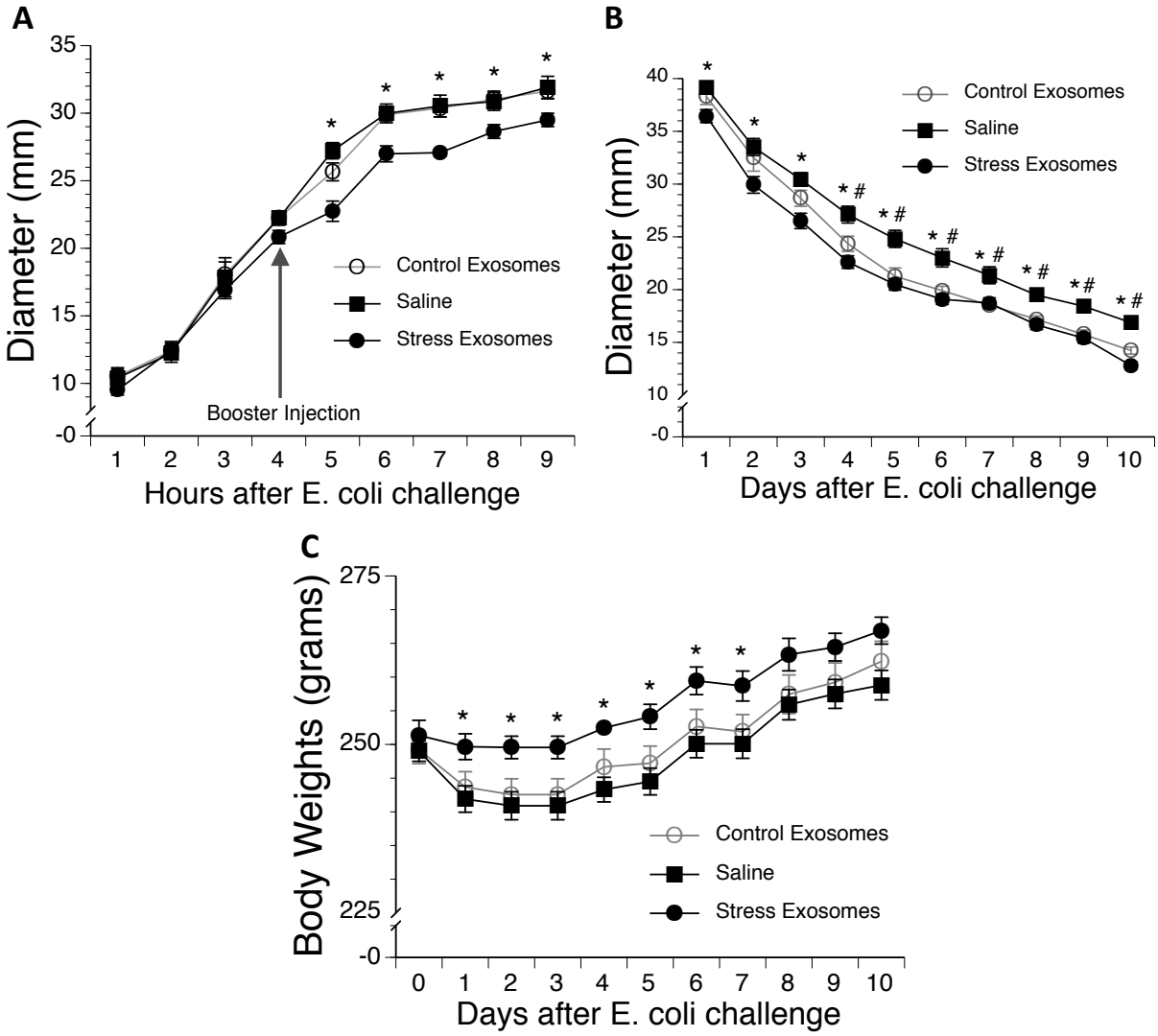

Figure 3.5. Inflammatory assessment following subcutaneous *E. coli* challenge. Adult male Fisher 344 rats were injected subcutaneously (s.c.) with *E. coli* (2.5×10^9 CFUs) suspended in 0.25 cc of either sterile saline, plasma exosomes from rats exposed to inescapable tail shocks (stress exosomes), or plasma exosomes from non-stressed rats (control exosomes). Four hours after s.c. *E. coli* administration, rats received a booster injection of their corresponding exosome treatment (0.25 cc) at the site of infection. The inflammatory site was measured hourly up to nine hours and then daily for 10 days. **A.** Rats administered stress exosomes had a significantly smaller inflammatory size than rats administered saline or control exosomes. **B.** This difference was sustained for 3 days, at which point rats from the control exosome group had inflammatory sizes that were also significantly smaller than the saline group. **C.** Rats receiving stress exosomes also weighed significantly more than rats from the saline and control exosome groups for up 7 days after *E. coli* administration. Results are expressed in means ± SE; 9-10 rats/condition. * indicates significant difference when between stress exosome and saline injected rats ($p < 0.05$). # indicates significant difference between control exosome and saline injected rats ($p < 0.05$). Repeated ANOVA with Fisher PLSD post hoc test was used.

significantly constrained response compared to rats administered saline or control plasma exosomes, as measured by the development of the inflammatory site (Figure 3.5*A*), resolution of the inflammatory site (Figure 3.5*B*), and reduction in weight loss (Figure 3.5*C*). Collectively, these data indicate that stress-modified plasma exosomes improve host response to subcutaneous bacterial challenge and suggest that exosomes may be a critical component to stress-enhanced immunity.

Discussion

As accumulating data reveal that a variety of disease states can change both the composition and function of exosomes, it is important to understand if similar modifications occur during the acute stress response and whether stress-modified exosomes facilitate or constrain immunity. In this series of experiments, we reveal that exposure to an intense and acute stressor in the absence of injury or disease alters the proteomic and miRNA composition of plasma exosomes. This stress-induced modification of plasma exosomes is partially mediated by SNS activation of α_1-ADRs. Additionally, stress-modified plasma exosomes injected into non-stressed rats challenged with subcutaneous *E. coli* exert immunomodulatory functions that improve host defense to bacterial challenge as measured by restriction in inflammation and attenuation of weight loss.

Our laboratory consistently observes a reliable increase in Hsp72 following exposure to a variety of acute stressors[23,56,82], which is a critical component of stress-enhanced immunity to subcutaneous *E. coli* challenge[26,57,77]. Determining the cellular release of Hsp72 during stressor exposure is important because a large body of research

has demonstrated their role in modulating immunity through direct activation of macrophages, dendritic cells, and neutrophils by binding to TLR2 or TLR4 and inducing the secretion of inflammatory cytokines[57,83]. Here we provide evidence that a significant concentration of stress-induced Hsp72 is released into the blood via an exosome release pathway. CD63 immunoprecipitation of plasma from stressed rats prior to exosome isolation corresponds to a marked decrease in Hsp72, further supporting its association with exosomes. Consistent with the exosome literature, our data indicate that exosome-associated Hsp72 is released, albeit at low levels, during basal states [33,34,37,84]. Stressor exposure also elevates Hsp72 in the exosome depleted plasma fractions, suggesting that a portion of extracellular Hsp72 is soluble, perhaps released into the blood after necrotic cell death[23]. Additionally, there is evidence that enzymes in the blood may cleave exosome associated proteins[85], potentially releasing soluble Hsp72 from stress-modified plasma exosomes. Together, these findings demonstrate that plasma exosomes in stressed rats express greater concentrations of Hsp72 compared to non-stressed control rats, and the presence of soluble Hsp72 could be the result of either stress-induced cell death or enzymatic cleavage of the exosomal proteins.

The data presented here also demonstrate that exposure to an acute stressor down-regulates miR-142-5p and -203 expression in plasma exosomes, likely affecting macrophage and dendritic cell-mediated immunity. Recent findings indicate that down-regulation of miR-142 promotes early normal macrophage differentiation[52]. Since host defense to subcutaneous *E. coli* challenge is primarily driven by macrophages and neutrophils[77,86], we predict that a reduction in plasma exosome miR-142 expression would promote monocyte differentiation into macrophages, followed by their immediate

migration to the site of infection and subsequent recruitment of neutrophils. The MD2/CD14 complex then binds lipopolysaccharide, a large molecule embedded in the outer membrane of *E. coli*, and associates with TLR4 on the macrophage surface, thus stimulating the release of pro-inflammatory cytokines and improving bacterial killing and clearance. Down-regulation of miR-142-5p is also known to enable cytokine-mediated survival signaling in response to haemodynamic stress through the IL-6 receptor, gp130[48], further supporting its immunological contribution during the stress response. Additionally, miR-203 is known to directly target suppressor of cytokine signaling 3 (SOCS3)[87] and block its role as an inhibitor of cytokine signal transduction through the Janus tyrosine kinase and signal transducer and activator of transcription factors (JAK/STAT) pathway[88]. Activation of SOCS3 terminates the innate immune response; therefore, stress-induced reductions in exosomal miR-203 could prevent activation of SOCS3 and its downstream suppression of pro-inflammatory cytokines. Down-regulation of miR-203 is also capable of inducing TNF-α and IL-6 synthesis by enabling myeloid differentiating factor 88 (MyD88) activation[89].

Pathway analyses of the 760 predicted mRNA targets of miR-142-5p and -203 revealed functionally enriched pathways that overlap with demonstrated targets of the stress response. For example, microarray analysis of genes within the dorsal raphe nucleus of Fisher 344 rats revealed that exposure to tail shock modulates genes involved in the TGF-β pathway[74], which is a prime target of miR-203. A recent study also revealed that the anti-inflammatory properties of TGF-β are repressed with increased expression of miR-203[51]. Therefore, stress-induced reductions in miR-203 likely promote elevations in TGF-β activity, which may be crucial for constraining stress-induced immunomodulation. Thus,

the sustained reduction in the *E. coli* inflammatory site of rats injected with stress exosomes may be linked to TGF-β activity. This is in line with preliminary data from our laboratory revealing elevations in plasma TGF-β concentrations two hours following tail shock termination (data not shown). KEGG analysis also revealed that miR-203 targets metabolic pathways, which is in line with cortisol mediation of metabolic pathways during the stress response[90]. Additional KEGG analysis of predicted gene targets for miR-142-5p revealed involvement with ubiquitin mediated proteolysis. Reductions in miR-142 expression are associated with elevations in ubiquitin proteasome activity[91], which is a cellular strategy for degrading proteins denatured by stress and repairing DNA damage[92]. Collectively, these observations suggest that stress-induced down-regulation of miR-142-5p and -203 and up-regulation of Hsp72 may be crucial components of exosome-based stress-induced immunomodulation and cytoprotection, which is in line with previous findings demonstrating the immunomodulating and cellular consequences of stress[57,77,93–95].

This study also reveals that exosomal expression of stress-induced Hsp72, and miR-142-5p rely on sympathetic nervous system release of norepinephrine (NE) and its subsequent activation of one of its target receptors, the α_1-ADR. Consistent with previous research, blockade of the α_1-ADR with prazosin prior to inescapable tail shock stress reduces stress-induced elevations of Hsp72 in plasma[56,79], and attenuates the stress-induced down-regulation of miR-142-5p. Since down-regulation of miR-142-5p is known to enable cytokine-mediated survival[48], prazosin administration should decrease associated inflammatory cytokine activity. Indeed, previous research demonstrates that pre-treatment with prazosin prior to tail shock stress attenuates stress-induced elevations in monocyte

chemotactic protein-1 (MCP-1)[96], IL-1β[97], and IL-6[97]. In line with these findings, administration of phenylephrine, an α_1-ADR agonist, in the absence of stress induces an elevation of plasma Hsp72 similar to levels seen in rats exposed to inescapable tail shock[56]; however, additional studies are needed to determine if stimulation of the α_1-ADR in the absence of an acute stressor modifies exosomal expression of Hsp72 and miRNAs. Interestingly, prazosin administration also decreased Hsp72 in the exosome depleted fraction of stressed rats. LDH assessment of the plasma reveals that prazosin administration attenuates cell death, which likely decreases the necrotic release of soluble Hsp72 into the circulation. While both NE and epinephrine (E) bind to α_1-ADRs, NE has a higher affinity for these receptors[79] and depletion of E through adrenalectomy has no effect on stress-induced Hsp72 in the circulation[56].

Based on these findings, we hypothesize that exposure to an intense, acute stressor modifies exosomal expression of Hsp72 and miR-142-5p by activating the SNS and inducing the release of NE from sympathetic nerve terminals. Stimulation of the α_1-ADRs by NE activates phospholipase C and elevates cytosolic Ca^{2+}. Fusion of the multivesicular body (MVB), the endocytic source of exosomes, to the plasma membrane is Ca^{2+} dependent; therefore, the surge in Ca^{2+} may facilitate exosome release. Alternatively, NE stimulation of alpha adrenergic receptors increases ubiquitination through Ca^{2+} flux[98,99], which is required for targeting cellular proteins to endosomes prior to fusing with the MVB. Interestingly, expression of multiple exosome markers, such as the tetraspanin CD63, the membrane transport protein Rab5b, and the intestinal epithelial exosome marker A33, were unchanged following exposure to inescapable tail shock. Additionally, CD63 expression is unaffected by prazosin, therefore it is unlikely that activation of the α_1-ADR

impacts exosome release, but rather their composition through ubiquitination. Thus, exposure to an acute stressor potentially modifies exosome-associated Hsp72 and miRNA in the plasma by increasing their rate of loading onto intracellular endosomes rather than impacting the rate of secretion. Conversely, α_1-ADR activation may be critical for Hsp72 synthesis or miRNA transcription. Future studies should elucidate the tissue origin of these stress modified exosomes and examine whether α_1-ADR blockade in stressed animals affects cytosolic expression of Hsp72 and miR-142-5p, thus indicating whether α_1-ADR activation is critical for their transcription and synthesis or their exosomal loading.

The release of Hsp72 and miRNAs through an exosomal pathway has several advantages. Most notably, exosomes provide a protective lipid bi-layer that can facilitate long distance communication between cells. These vesicles are also resistant to complement-mediated lysis through surface expression of the complement-inhibitory proteins CD55 and CD59[100,101]. From a stress physiology perspective, this form of cellular communication may be evolutionarily advantageous. For example, if an organism is subjected to a harmful stressor, such as a predator, the organism's cells could secrete stress-modified exosomes into the circulation prior to experiencing injury. When injury occurs, stress-modified exosomes are already in the circulation and available to facilitate the host immune response. Cytokine induction by resident leukocytes at the site of injury triggers the activation and expression of adhesion molecules on the adjacent vascular endothelium. Since exosomes are known to express intercellular adhesion molecules (ICAMs)[102], plasma exosomes could bind ICAM receptors on vascular endothelial cells and consequently leave the circulation and migrate to the injured tissue. At the site of infection, stress-modified exosomes could boost innate immunity through Hsp72-mediated TLR4

activation of macrophages and neutrophils, or transfer their content through clathrin-mediated endocytosis with a recipient cell[103], subsequently stimulating a pro-inflammatory cytokine response and enhancing the organism's chance of survival.

There are a variety of clinical applications for stress-modified exosomes that could potentially modulate immunity. For example, exposing cells to a non-lethal stressor, such as heat, to elevate Hsp72 expression and down-regulate miR-142-5p and -203 in exosomes could enhance the immunogenicity of exosome-based vaccines, specifically cancer vaccines[14,104,105]. Alternatively, since exosomes are capable of delivering their content to recipient cells[38,44], they could transfer their content to target cells, where Hsp72 could translocate to the target cell's cytosol and perform its cytoprotective and anti-apoptotic functions and miRNAs could modulate mRNA translation.

In summary, our data indicate that *in vivo* exposure to an acute stressor modifies the proteomic and miRNA expression of exosomes released into the plasma, likely impacting innate immune function through TLR association, monocyte differentiation, and cytokine secretion. Furthermore, our results suggest that SNS activation of α_1-ADRs is a critical component of some of these exosomal modifications. Given the known immunomodulatory and protective functions of Hsp72, miRNA, and exosomes, we speculate that modulation of plasma exosomes is a critical component of the stress response. Future studies should further identify the immunomodulatory factors and cellular sources of stress-modified exosomes in the plasma, which will challenge current paradigms concerning the mechanisms of stress-enhanced immunity and advance knowledge concerning their use in immunotherapy.

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IV. Exosomes in fetal bovine serum dampen primary macrophage IL-1β response to lipopolysaccharide (LPS) challenge

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Abstract

Small, biologically active nanoparticles known as exosomes (40-100 nm) released from cells into the extracellular environment are involved in multiple physiological processes. Exosomes are present in nearly all biological fluids, including bovine serum, a critical component of cell culture media. Exosomal function is often tested in cell culture experiments, but the media used to sustain and culture the cells contains fetal bovine exosomes that interfere with the exosomes of interest. Standard practice for *in vitro* analysis of exosomal function on cells entails the use of ultracentrifugation to remove bovine exosomes from fetal bovine serum (FBS). Depleting bovine exosomes from FBS ensures they are neither erroneously characterized nor competing with the experimental exosomes of interest. However, subjecting FBS to standard ultracentrifugation methods depletes microvesicles and large protein complexes in addition to bovine exosomes, potentially impacting cellular response to various challenges. To address the indiscriminating removal of various non-exosome particles with ultracentrifugation, there are now commercially available FBS specifically devoid of bovine exosomes. While there are a growing number of studies examining cellular responses to exosomes, there is no assessment on how exosome-depleted FBS impacts cellular responses to immunological challenges. In this study, we demonstrate that macrophages cultured with lipopolysaccharide (LPS) in the presence of exosome-depleted FBS have an exaggerated IL-1β response compared to macrophages cultured in medium supplemented with standard FBS. In contrast, macrophages cultured in exosome-depleted FBS demonstrate no change in IL-6, IL-10, TNF-α, monocyte chemotactic factor-1 (MCP-1), nitric oxide (NO), or lactose dehydrogenase (LDH) levels to the LPS challenge. Future studies using *in vitro* methods to

examine exosome function need to account for the immunological impact of fetal bovine exosomes in their culture conditions. The potential role of exosome-inflammasome interactions is also discussed.

Introduction

Exosomes are 40-100 nm biologically active vesicles secreted into the extracellular environment from both hematopoietic and non-hematopoietic cells[1,2]. Since their discovery in 1983[1], exosomes have been found in mammalian blood[3–5], urine[6], saliva[7], seminal fluid[8], cerebrospinal fluid[9,10], and breast milk[11]. The structure of exosomes are similar to cytoplasm encased in a lipid bi-layer containing proteins and microRNA (miRNA) unique to the cellular origin of the exosome. The content of exosomes can be transferred to recipient cells and modulate cellular function and messenger RNA (mRNA) transcription. Interest in the immunological roles of exosomes has grown rapidly since the discovery that exosomes purified from B-cells and dendritic cells are enriched in MHC class I and II molecules, as well as T-cell co-stimulatory molecules[12–14]. Additionally, exosomes are capable of natural killer cell activation, stimulation and inhibition of T cell activation, and induction of tolerance to oral antigens[12,15–18]. Exposure to a variety of pathogens or disease states, such as microbial infection[19,20] and cancer[12,21], modifies the composition and function of exosomes, providing valuable insight into their potential use as disease biomarkers and therapeutic tools.

Standard practice for the *in vitro* analysis of exosome function entails ultracentrifugation (approximately 110,000 $\times g$ for 15-24 hours at 4°C)[22,23] of fetal bovine serum (FBS), a critical component for various cell culture medias, to remove fetal bovine

exosomes. Removing exosomes from the FBS ensures those exosomes do not compete with the experimental exosomes of interest and are not erroneously characterized. However, standard ultracentrifugation methods also isolate microvesicles and large protein complexes, potentially impacting cell survival and response to various challenges. To address the indiscriminating removal of various non-exosome particles with ultracentrifugation, there are now commercially available FBS specifically devoid of fetal bovine exosomes.

While there are a growing number of studies examining cellular responses to exosomes, there is no assessment on how exosome-depleted FBS impacts *in vitro* cellular responses to pathogenic challenges. To identify the potential immunological relevance of fetal bovine exosomes in cell culture media, we challenged primary rat macrophages with lipopolysaccharide (LPS) in cell culture media supplemented with 10% of either standard FBS (x+ FBS) or exosome depleted FBS (x- FBS). LPS is a common pathogen associated molecular pattern embedded in the outer membrane of Gram-negative bacteria, such as *Escherichia coli* (*E. coli*), and associates with Toll-like receptor 4 (TLR4) on leukocytes to stimulate the release of pro-inflammatory cytokines and chemokines. These pro-inflammatory mediators improve bacterial killing and clearance and enhance cell survival. We then examined cytokine (IL-1β, IL-6, IL-10, TNF-α), monocyte chemotactic factor-1 (MCP-1), lactate dehydrogenase (LDH), and nitric oxide (NO) responses in the macrophages. Here, we demonstrate that macrophages cultured with LPS in the presence of x- FBS have an exaggerated IL-1β response compared to LPS-treated macrophages cultured in x+ FBS. Further, we observed a dose dependent decrease in the LPS-treated macrophage IL-1β response when x- FBS was supplemented with fetal bovine exosomes.

The selectivity of the IL-1β response in macrophages cultured in exosome-depleted FBS may implicate a critical interaction between exosomes and the inflammasome, a cytosolic multimeric complex that contributes to mature IL-1β processing. These data demonstrate that fetal bovine exosomes may have potent immunological consequences to cells in culture, which should be taken into consideration when examining exosomes in culture conditions.

Materials and Methods

Exosome visualization

Standard FBS (x+ FBS) and exosome-depleted FBS (x- FBS) were diluted 1:10,000 in sterile filtered phosphate buffered saline (PBS) and analyzed with nanoparticle tracking analysis (NTA). NTA were performed using the LM10-HS instrument (NanoSight Ltd., Amesbury, UK) equipped with a 532 nm laser at scatter mode. The instrument was calibrated using commercially available 100 nm polystyrene beads (Polysciences, Warrington, PA) and sterile filtered PBS.

Animals and housing

All experiments were performed on peritoneal macrophages isolated from adult male Fisher 344 rats (8-9 weeks old; Harlan, Indianapolis, IN) weighing approximately 230-275 grams. Rats were singly housed in clear Nalgene plastic cages (48 × 27 × 20 cm) with microisolator tops in a specific pathogen-free barrier facility and allowed access to food and water *ad libitum* (Harlan Standard Lab Chow). Temperature and humidity remained constant and animals were maintained on a 12-hour light:dark cycle (lights on at 0700). Animals acclimated to these housing conditions for one week prior to peritoneal

macrophage isolation and were handled daily. The care and use of the animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

Peritoneal macrophage isolation

Peritoneal macrophages were isolated from rats (n=10 per experiment) sacrificed by rapid decapitation as previously described[24]. Briefly, 20 mL of cold dissection medium (Iscove's medium [Gibco, Grand Island, NY] with 1% penicillin-streptomycin [Sigma, St. Louis, MO]) was administered to the peritoneal cavity and the abdomen was massaged for 20 seconds. Approximately 13 mL of fluid was recovered from the cavity and placed on ice until centrifugation at 1500 rpm for 10 minutes. The pelleted macrophages were resuspended to 1×10^6 cells/mL in dissection medium. Cell counts were performed with the Moxi Z automated cell counter (Orflo Technologies, Hailey, ID). The resuspended macrophages (500 µL/well) were added to sterile 24-well flat-bottom culture plates (Costar, Corning, NY) and incubated in a humidified 37°C incubator with 5% CO_2. Macrophages were allowed one hour to adhere prior to experimental FBS treatments and LPS.

FBS conditions and LPS treatments

Following macrophage adherence, the media was removed from all wells and adherent macrophages were washed twice with sterile filtered PBS. Peritoneal macrophages from each rat were then cultured in Iscove's media (Gibco) supplemented with 10% of either commercially available x- FBS (System Biosciences, Mountain View, CA) or x+ FBS. All FBS treatments originated from the same lot number to avoid lot-to-lot

variability. Macrophages were also concurrently cultured in the presence of 2.5 μg/mL *E. coli* derived LPS (*E. coli*, 0111:B4, Sigma) or no LPS (control). The total volume of the FBS condition and LPS or control treatment was 400 μL/well. Following incubation in a humidified 37°C incubator with 5% CO_2 for 24-hours, macrophage media was collected and assayed for nitric oxide (NO). The remaining media was stored at -20°C until analysis.

Exosome-depleted FBS supplementation with fetal bovine exosomes

To verify that changes in inflammatory proteins are a direct result of bovine exosome depletion, x- FBS was supplemented with multiple doses of the isolated fetal bovine exosomes (courtesy of System Biosciences). Exosome-depleted FBS was supplemented with 0, 10, 25, 50, 100, 150, or 200% of the original concentration of fetal bovine exosomes in standard FBS. Peritoneal macrophages from 10 rats were isolated and cultured as described above. Following adherence, macrophages were challenged with 2.5 μg/mL *E. coli* derived LPS and co-cultured with either x- FBS supplemented with various doses of bovine exosomes or x+ FBS. Macrophages were incubated with 400 μL per well of each treatment condition for 24-hours in a humidified 37°C incubator with 5% CO_2. Following incubation, media was collected and stored at -20°C until analysis.

Cytokine, chemokine, and nitric oxide (NO) quantification

Multiple markers of inflammation (IL-1β, IL-6, IL-10, TNF-α, MCP-1, and NO) were measured in the macrophage culture medias with commercially available ELISA kits according to the manufacturer's instructions. Media from LPS-treated macrophages were assayed at optimal dilutions for IL-1β (1:5), IL-6 (1:100), IL-10 (1:1), and TNF-α (1:50) in commercial ELISAs from R&D Systems (Minneapolis, MN). Additionally, LPS-treated

macrophage media was measured for MCP-1 (1:20) in a commercial ELISA from Invitrogen (Grand Island, NY). Nitric oxide (NO) was assessed in LPS-treated macrophage media at a 1:2 dilution with a commercially available assay kit (Cayman Chemical, Ann Arbor, MI). Macrophage media from control treatments (no LPS) were assayed without dilution in IL-1β, IL-6, IL-10, TNF-α, MCP-1, and NO. Optical densities were measured using a SpectraMax Plus 354 plate reader (Molecular Devices, Sunnyvale, CA) and analyzed using four-parameter curve fit and software (SoftMax 5.4.1).

Cell viability

To evaluate whether bovine exosome removal impacts macrophage viability, cell death was assayed with a commercially available lactate dehydrogenase (LDH) assay kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Optical densities were measured using the SpectraMax Plus 354 plate reader.

Statistical Analyses

Two-way ANOVAs were run to analyze the effect of LPS or FBS conditions on individual cytokines (IL-1β, IL-6, IL-10, TNF-α), the chemokine MCP-1, NO, and LDH. Data points were treated as outliers if they failed Grubb's test for outliers. A Pearson's product-moment correlation was performed to examine correlations between cytokines and cell death as measured by LDH. Data are presented as means ± the standard error of the mean. $P<0.05$ was considered statistically significant.

Results

Nanoparticle visualization of x- FBS and x+ FBS

NTA analysis with the Nanosight LM10 instrument visually confirmed that x- FBS had a significant reduction in exosome sized particles (50-100nm) compared to x+ FBS (Figure 4.1).

LPS stimulates cytokine, chemokine and NO response in macrophages

Macrophages cultured in 2.5 μg/mL LPS for 24 hours in x- and x+ FBS resulted in significant elevations of IL-1β ($p<0.001$; Figure 4.2*A*), IL-6 ($p<0.001$; Figure 4.2*B*), TNF-α ($p<0.001$; Figure 4.2*C*), MCP-1 ($p<0.001$; Figure 4.2*D*), and NO ($p<0.001$; Figure 4.3*A*), compared to non-LPS (control) treated macrophages in x- and x+ FBS conditions. These findings are consistent with previous work from our laboratory[24,25] and are indicative of a robust immunological response. Cytotoxicity as measured by lactate dehydrogenase revealed a main effect of LPS treatment ($p<0.05$; Figure 4.3*B*), but not FBS condition.

Commercial removal of fetal bovine exosomes exaggerates macrophage IL-1β response to LPS, independent of cell death

As illustrated in Figure 4.2*A*, macrophages cultured with x- FBS had significantly elevated levels of IL-1β following a 24-hour treatment with 2.5 μg/mL LPS compared to macrophages cultured in x+ FBS ($p<0.001$). However, x- FBS failed to impact IL-6, TNF-α, and MCP-1 in LPS-treated macrophages (Figure 4.2*B-D*). Additionally, FBS conditions did not significantly change LDH activity, demonstrating that the increase in IL-1β in the x- FBS condition is independent of cell death. Pearson's product moment correlation revealed no correlation between LDH and IL-1β, IL-6, TNF-α, or MCP-1.

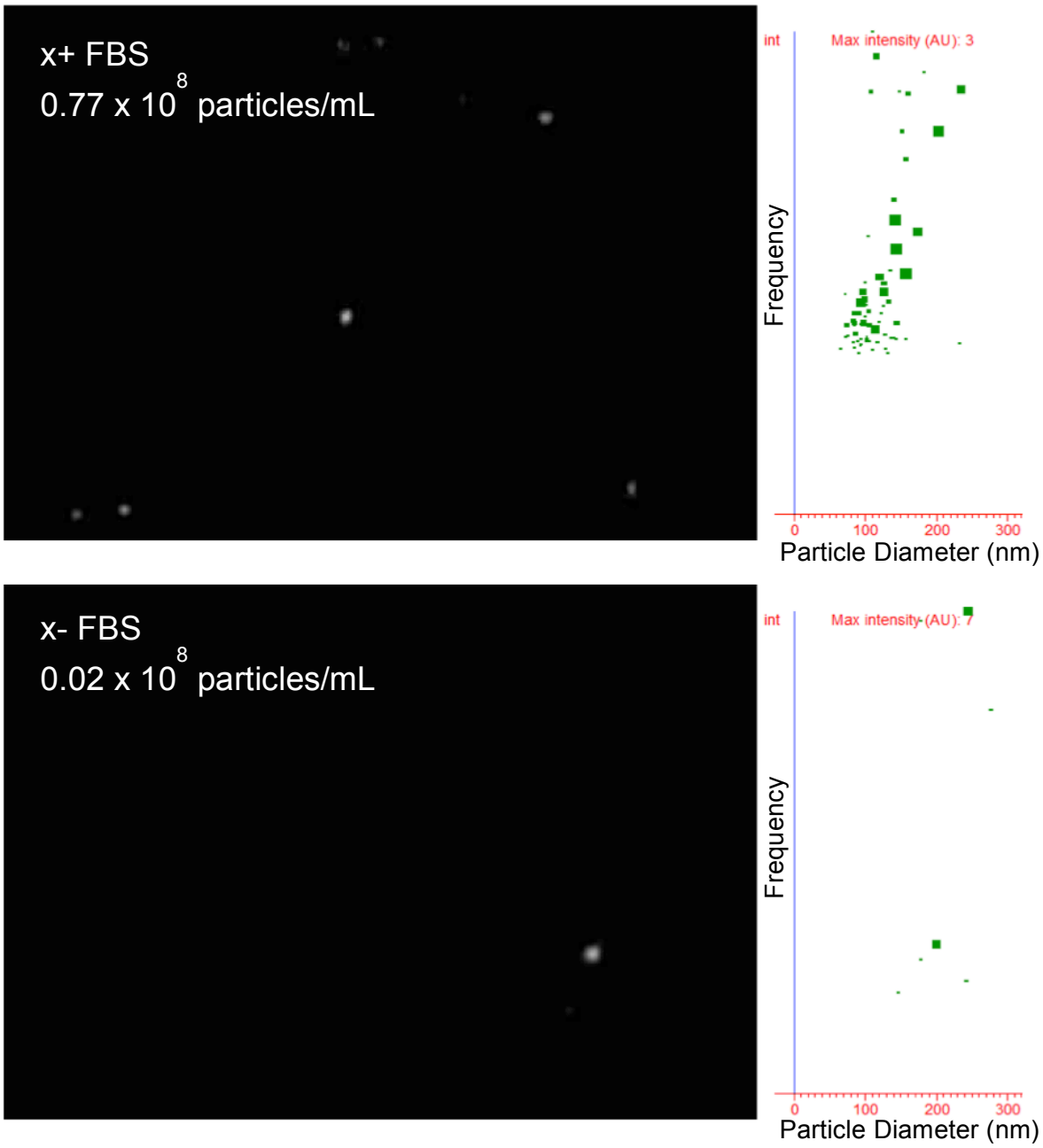

Figure 4.1. Visual analysis of nanoparticles in FBS conditions. Exosome-depleted (x- FBS) and lot-matched standard FBS (x+ FBS) were diluted 1:10,000 in sterile filtered PBS and analyzed with Nanoparticle Tracking Analysis (NTA). NTA confirmed that x- FBS contains significantly fewer exosome sized particles (40-100 nm) compared to the x+ FBS.

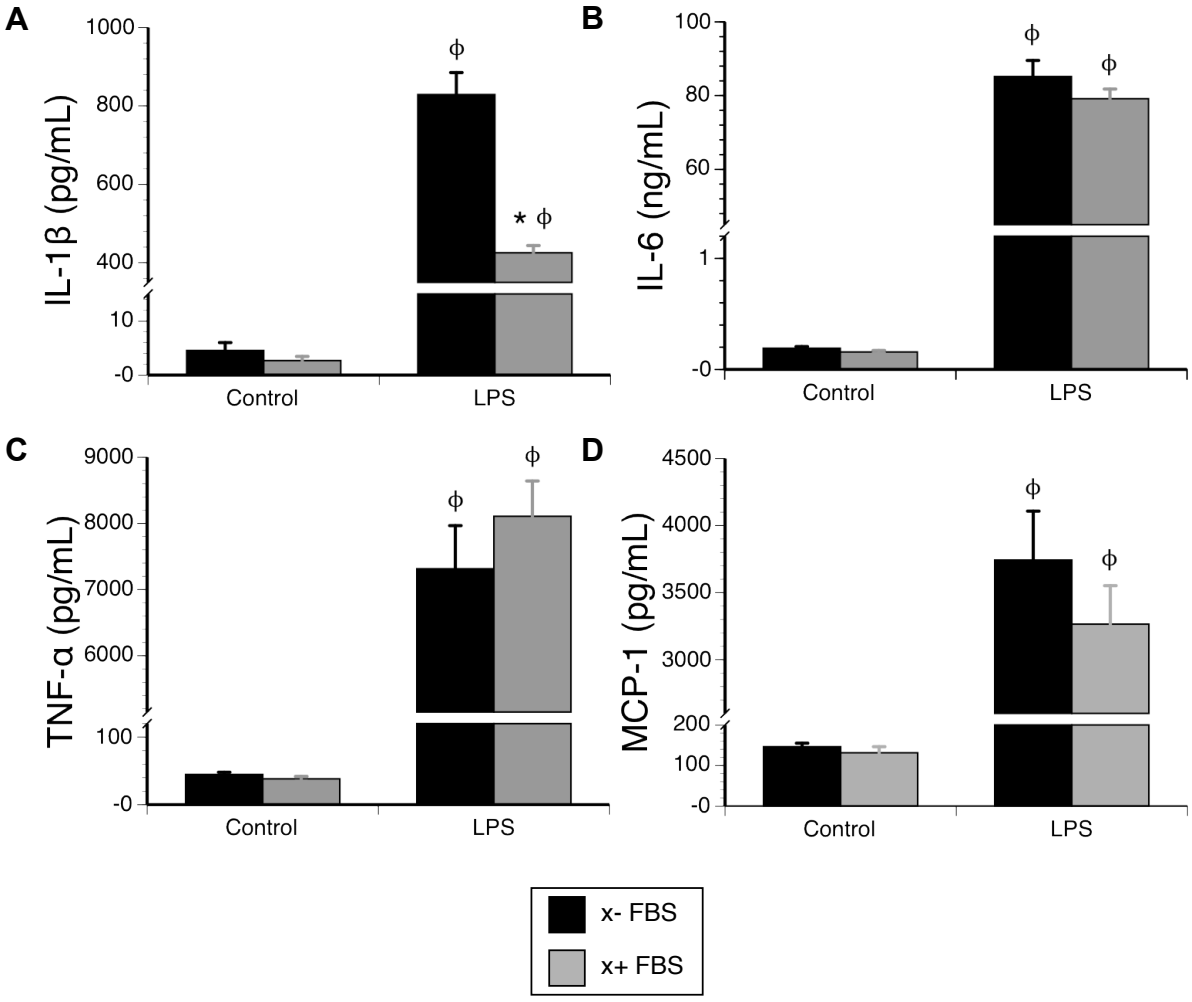

Figure 4.2. Impact of fetal bovine exosomes on macrophage cytokine and chemokine response to LPS. Primary macrophages from male Fisher 344 rats were challenged with 2.5 μg/mL of LPS (*E. coli*, 0111:B4) suspended in either standard FBS (x+ FBS) or lot matched FBS devoid of bovine exosomes (x- FBS). **A.** LPS challenge significantly increased macrophage IL-1β release, but the removal of bovine exosomes further increased this response. **B.** LPS challenge significantly increased macrophage IL-6, **C.** TNF-α, and **D.** MCP-1 concentrations, regardless of FBS culture conditions. Data represent means ± SEM. * indicates significant difference ($p < 0.05$) when compared to x- FBS conditions. ϕ indicates significant effect of LPS ($p < 0.05$) when compared to non-LPS treated cells (control). Two-way ANOVA with Fisher PLSD post hoc test was used.

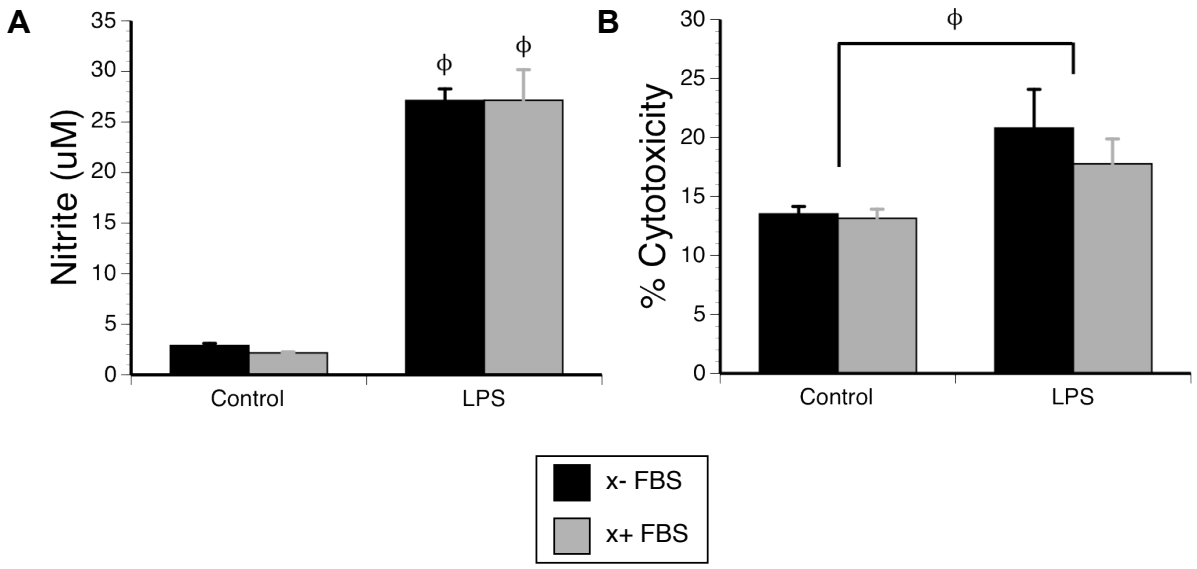

Figure 4.3. LPS challenge elevates nitrite and cytotoxicity levels in rat macrophages, regardless of FBS exosome condition. Primary macrophages from male Fisher 344 rats challenged with 2.5 μg/mL of LPS (*E. coli*, 0111:B4) suspended in either standard FBS (x+ FBS) or lot matched FBS devoid of bovine exosomes (x- FBS). **A.** LPS challenge significantly increased macrophage nitrite concentrations and **B.** cytotoxicity levels, regardless of FBS culture conditions. Data represent means ± SEM. ϕ indicates main effect of LPS ($p < 0.05$) when compared to non-LPS treated cells (control). Two-way ANOVA with Fisher PLSD post hoc test was used.

Supplementation with fetal bovine exosomes reduces macrophage IL-1β response to LPS

Supplementation of fetal bovine exosomes in x- FBS conditions decreased the macrophage IL-1β response to LPS treatment in a dose-dependent manner (Figure 4.4*A*). However, 200% bovine exosome supplementation in x- FBS failed to restore macrophage IL-1β concentrations observed in cells cultured in x+ FBS ($p<0.05$). FBS culture conditions did not significantly impact macrophage IL-6 response to LPS, but x- FBS supplementation with 50% of the original concentration of bovine exosomes did attenuate the response (Figure 4.4*B*). FBS culture conditions did not significantly impact macrophage TNF-α response to LPS, but x- FBS supplementation with 25-150% of the original concentration of bovine exosome significantly attenuated the response (Figure 4.4*C*). FBS conditions and bovine exosome supplementation had no impact on macrophage IL-10 response to LPS (Figure 4.4*D*).

Discussion

In this study, we identify a previously unknown immunological function of fetal bovine exosomes on rat macrophage response to LPS challenge. The commercial removal of fetal bovine exosomes exaggerates the macrophage IL-1β, but not IL-6, IL-10, TNF-α, MCP-1, NO, or LDH response to LPS. Additionally, when x- FBS is supplemented with increasing doses of fetal bovine exosomes, the macrophage IL-1β response is reduced, confirming an interaction between the exosomes and the IL-1β pathway.

IL-1β is unique from other inflammatory markers in that its synthesis and release is dependent on inflammasome activation of caspase-1, which cleaves the inactive pro-IL-1β

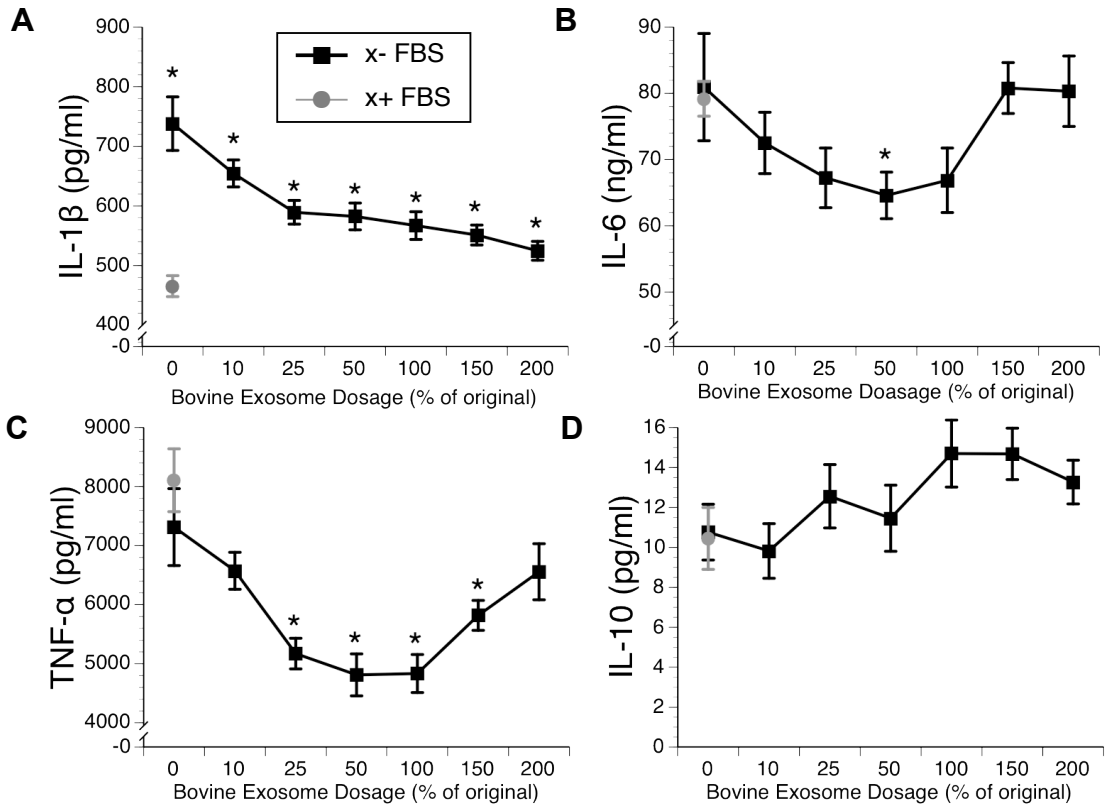

Figure 4.4. Depleting bovine exosomes from FBS correlates with an exaggerated IL-1β macrophage response when challenged with LPS, but other cytokines are unaffected. Primary macrophages from male Fisher 344 rats were challenged with 2.5 μg/mL of LPS (*E. coli*, 0111:B4) suspended in either standard FBS (x+ FBS), lot matched FBS devoid of bovine exosomes (x- FBS), or x- FBS supplemented with increasing doses of bovine exosomes. **A.** Macrophages cultured in x- FBS had a significantly elevated IL-1β response to LPS compared to macrophages cultured in x+ FBS. Supplementation with bovine exosomes greatly reduced macrophage IL-1β response to LPS, but supplementation with 200% of the original bovine concentration did not reduce IL-1β concentrations to levels observed with LPS-treated macrophages in x+ FBS culture conditions. **B.** FBS culture conditions did not significantly impact macrophage IL-6 response to LPS, but x- FBS supplementation with 50% of the original concentration of bovine exosomes did attenuate the response. **C.** FBS culture conditions did not significantly impact macrophage TNF-α response to LPS, but x- FBS supplementation with 25-150% of the original concentration of bovine exosome significantly attenuated the response. **D.** FBS conditions and bovine exosome supplementation had no impact on macrophage IL-10 response to LPS. Data is represents mean ± SEM. * indicates significant difference ($p < 0.05$) when compared to x+ FBS conditions. Two-way ANOVA with Fisher PLSD post hoc test was used.

into the mature and immunologically active IL-1β. Although little is known about exosome regulation of the inflammasome and caspase-1 pathway, a recent study has demonstrated that overexpression of the microRNA miR-223 can block Nod-like receptor family, pyrin domain containing 3 (NLRP3) accumulation in monocytes and inhibit inflammasome activation[26]. A separate study confirmed the presence of mir-223 in monocyte-derived microvesicles[27]. Therefore, fetal bovine exosomes may contain miR-223 or similar microRNA capable of regulating LPS-induced macrophage IL-1β release through the reduction of NLRP3 activity and the downstream decrease in caspase-1 activation.

Interestingly, supplementing x- FBS with 200% of the original concentration of bovine exosomes in x+ FBS still did not return the LPS-treated macrophage IL-1β response to levels observed in the LPS-treated macrophages in x+ FBS condition. Since a common property of exosomes is the expression of adhesion molecules, such as intercellular adhesion molecules (ICAMs)[28] and integrin[29], isolated bovine exosomes may have adhered to one another and resuspension in x-FBS may not have completely separated the particles. Alternatively, the commercial exosomes isolation method may also result in exosome aggregation. If some portion of the bovine exosomes is indeed aggregated, those particles may not be able to access their respective receptors on the macrophages. Additionally, aggregated exosomes may also behave differently from polydispersed exosomes. While there are no known studies examining the behavior of aggregated exosomes, work on protein aggregation reveals changes in their thermodynamic and kinetic parameters, often leading to toxicity[30]. It is therefore possible that aggregated exosomes may behave differently toward recipient cells, but based on the IL-6, TNF-α, and IL-10 concentrations in

the dose-response experiment, the fetal bovine exosomes did not exert toxic effects on the LPS-treated macrophages.

Although little is known about the profile and functional characteristics of fetal bovine exosomes, work on human placental and trophoblast exosomes suggests they have potent immunomodulatory functions. Pregnancy is characterized as a mild immunosuppressive state regulated by the placental release of molecules that prevent fetal rejection by the maternal immune system[31]. Human placental exosomes have been shown to down-regulate the natural killer (NK) cell receptor NKG2D on NK, CD8+, and γδ T cells, resulting in impaired cytotoxicity against the erythroid cell line K562[32]. Extracellular vesicles from the placenta of healthy, pregnant women, which can be identified by their expression of placental-type alkaline phosphatase, are also anti-inflammatory in that they suppress activation of CD4+ and CD8+ T cells by modulating surface expression of CD3-ζ and Janus kinase 3[33,34]. Additionally, extracellular vesicles secreted by syncytiotrophoblasts and trophoblasts express FasL throughout the pregnancy, promoting immunotolerance through lymphocyte apoptosis[33]. Exosomes derived from the serum of pregnant women were also found to both inhibit IL-2 production of activated T-cells and block autocrine-driven proliferation[34]. Together, these studies suggest the exosomal regulation of the immune system during fetal development may be important to both avoid excessive inflammation and rejection of the fetal allograft.

Since placental and trophoblast exosomes may provide a mechanism of fetal protection, exosomes derived from fetal bovine serum may act similarly and offer immunosuppressive, cross-species protection to cells in culture. While it is important to

remove these immunosuppressive bovine exosomes from FBS prior to analysis of experimental exosomes, researchers need to be aware that exosome-depleted culture conditions could significantly alter the immunological activity of their cells. Therefore, experimental design should incorporate careful analyses to evaluate whether changes in IL-1β activity are a direct result of bovine exosome depletion or the experimental exosome condition. Although our study clearly reveals an immunological impact of x- FBS on primary macrophages in culture, the alternate ultracentrifugation approach also suffers potential issues. Overnight ultracentrifugation not only removes exosomes, but also larger microvesicles and heavy protein complexes that could be essential for cell growth and survival. Commercially available exosome-depleted FBS may be a better alternative to ultracentrifuged FBS. Alternatively, future studies examining the immunological impact of exosomes in an *in vitro* model could consider supplementing media with synthetic or serum-free replacements.

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V. Stressor exposure elevates Hsp72 in the circulation, peripheral tissues, and hypothalamus, but not cerebrospinal fluid

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Abstract

Exposure to acute stressors elevates concentrations of the 72kDa heat shock protein (Hsp72) in plasma, peripheral tissues, and various brain regions, where it confers cytoprotection intracellularly and primes the innate immune system extracellularly. While recent research is uncovering the releasing mechanisms, potential sources, and functional consequences of Hsp72, it is still unknown if Hsp72 is detectable in the cerebrospinal fluid (CSF) and if it is sensitive to stressor exposure. The aim of the present study is to expand current research on stress-inducible changes on Hsp72 to include assessment of CSF, select brain regions, and peripheral tissues. Adult male Sprague-Dawley rats exposed to inescapable tail shock had increased Hsp72 concentrations compared to non-stressed controls in plasma, hypothalamus, subcutaneous adipose (SQ adipose), spleen, and mesenteric lymph nodes (MLNs). Stressor exposure did not, however, detectably increase Hsp72 in the CSF. These results suggest that circulating plasma Hsp72 does not cross the blood brain barrier (BBB) and further, that the elevated Hsp72 observed in the hypothalamus either is not rapidly released into the extracellular environment or is restricted to the brain parenchyma. Thus, stress-evoked increases in Hsp72 may function extracellularly in the periphery to prime innate immunity and intracellularly in the brain to confer cytoprotection.

Introduction

Over time, multicellular organisms have developed various biological strategies, such as increasing alertness, cognition, and mobility, to enhance survival in the face of intrinsic or extrinsic stressors. These series of changes are often referred to as the “fight-or-flight” response and are initiated by activation of the sympathetic nervous system (SNS)

and hypothalamic-pituitary-adrenal (HPA) axis. Exposure to acute stressors is also known to facilitate immunity through the release of danger associated molecular patterns (DAMPs)[1]. DAMPs act similarly to pathogen associated molecular patterns (PAMPs), but are endogenous molecules released in response to tissue or cellular damage rather than pathogen-derived molecules. When DAMPs are released from a cell into the extracellular space, they modulate local inflammatory and systemic innate immunity[2,3]. Activation of the immune response can prepare an organism under stress for a pathogenic challenge or injury sustained from the stressor, thus promoting the organism's survival.

One group of DAMPs that are reliably increased in cells exposed to various stressors is the heat shock proteins. When cells are exposed to a stressor, intracellular heat shock proteins function as classic cellular chaperones, contributing to cellular homeostasis and promoting cell survival by repairing proteins or polypeptides denatured from exposure to cellular stress[4]. One member of the heat shock protein family, the 72kDa heat shock protein (Hsp72), is also found in the extracellular environment following exposure to a variety of acute stressors, such as heat stress[5], predatory fear[6], exercise[7], and tail shock[8]. Extracellular Hsp72 can modulate activation of macrophages, dendritic cells, and neutrophils by binding to surface Toll-like receptors and inducing the NF-κB pathway, resulting in the transcription and release of pro-inflammatory cytokines[9,10]. Various *in vivo* studies have confirmed that Hsp72 is a functionally significant danger signal for the immune system that is capable in some contexts of initiating pro-inflammatory cytokine production through the activation of Toll-like receptors 2 and 4 (TLR2/4)[9] and improving bactericidal killing and clearance[8,11].

Stress-inducible increases of plasma Hsp72 are accompanied by similar elevations in various peripheral tissues, such as the spleen, liver, mesenteric lymph nodes (MLNs), heart, and adrenal glands[12]. Although Hsp72 is reliably elevated in a variety of tissues in response to acute stressors, it is still unclear which tissue is the source of circulating Hsp72. Previous research has demonstrated that removal of the spleen and adrenal glands[13] has no effect on plasma concentrations of Hsp72 and are therefore unlikely tissue sources. However, recent studies suggest that SQ adipose is both stress-responsive[14] and immunomodulatory[15], making it a novel candidate as an *in vivo* tissue source of extracellular Hsp72.

Exposure to an acute stressor can also elevate Hsp72 in the central nervous system (CNS). Rats exposed to either inescapable tail shock[16] or cat stress[6] express elevated concentrations of Hsp72 in the hypothalamus and dorsal vagal complex (DVC) of the brain, but it is unknown whether these regions release Hsp72 into the extracellular environment or if the stress-induced elevations are intracellularly restricted. In addition, there is evidence that acute stressor exposure can alter blood-brain barrier permeability[31], making it feasible that Hsp72 could extravasate from the blood into the CSF. If extracellular Hsp72 is elevated in the CSF following exposure to an acute stressor, it could interact with microglia in the brain and spinal cord, known to express TLR4[17], and prime/stimulate an immune response in the CNS in a similar manner to Hsp72 effects on peripheral monocytes. While activation of neuroimmunity during the acute stress response may be advantageous if the organism experiences an injury or pathogenic challenge to the CNS, unnecessary inflammation could damage healthy cells in the brain.

Given that extracellular Hsp72 can prime the innate immune response and Hsp72 is detectable in the brain, and to broaden our understanding of the stress response in the CNS, we tested if exposure to an acute stressor impacts the concentrations of Hsp72 in the CSF. Only two studies to date have measured Hsp72 in the CSF: one study detected its presence in exercising humans[18] and another found elevated concentrations in meningitis patients[19]. Here, we chose to examine the acute stress response in Sprague-Dawley rats as they contain sufficient volumes of CSF for Hsp72 analysis. Our findings indicate that Hsp72 is present in low concentrations in the CSF, but unaffected by exposure to a stressor. This suggests that Hsp72 is not stress-sensitive in the CSF, cannot cross the blood brain barrier to enter from peripheral blood into the CSF, or may not be released into the extracellular environment by cells in the hypothalamus. However, if the hypothalamus is capable of releasing Hsp72 into the extracellular space, it is possible that either the Hsp72 concentrations in the CSF was below our ELISA's detection limit or that extracellular Hsp72 does not diffuse after release, thus restricting any impact it may have to local microglia. Alternatively, elevated Hsp72 in the brain induced by stressor exposure may remain intracellular and function within the cell, contributing to cellular homeostasis and promoting cell survival by repairing proteins or polypeptides denatured by cellular stress[4].

Materials and Methods

Animals and housing

Adult male Sprague-Dawley rats (10-11 weeks old; n=9-11/group; Harlan, Indianapolis, IN) weighing approximately 325-375 grams were used in all experiments. Rats were pair housed in clear Nalgene plastic cages (48 × 27 × 20 cm) with microisolator

tops in a specific pathogen-free barrier facility and allowed access to food and water *ad libitum* (Harlan Standard Lab Chow). Temperature and humidity remained constant and rats were maintained on a 12-hour light:dark cycle (lights on at 0700). Rats acclimated to these housing conditions for 2 weeks prior to any experimental manipulations and were handled daily. The care and use of the rats were in accordance with protocols approved by the University of Colorado Institutional Care and Use Committee.

Inescapable tail shock protocol

Rats were randomly selected to either remain in their home cages (control), receive inescapable tail shocks (stress), or receive inescapable tail shocks and return to their home cages for two hours before sacrifice (stress + 2hrs). Shocks were administered as previously described[20]. Briefly, rats from both stress groups were placed in a Plexiglas restraining tube (23.4 × 7 cm). Electrodes were placed across the tail protruding from the back of the restraining tube. Rats received 100, 1.5 mA, 5-second, intermittent, (average inter-trial interval of 60 +/- 25 s) inescapable tail shocks administered by an automated shock system (Precision Calculated Animal Shocker; Colbourn Instruments, Whitehall, PA). Euthanasia was performed with isoflurane anesthesia (Phoenix Pharmaceuticals, St. Joseph, MO) followed by rapid decapitation following stressor termination, whereas rats from the stress + 2hrs groups were sacrificed two hours after stressor termination. Control rats were alternately sacrificed with rats in the stress and the stress + 2hrs groups. Blood glucose was measured from the trunk using an Accu-Chek Compact blood glucose monitoring system (Roche Diagnostics, Indianapolis, IN) immediately following sacrifice. This stressor procedure reliably and consistently elevates Hsp72 and glucocorticoids in the plasma[3,11,21].

Fluid and tissue collection

Cerebrospinal fluid (CSF) was collected from rats via acute intrathecal lumbar puncture as previously described[22]. Briefly, rats were anesthetized with isoflurane before inserting an 18-gauge cannula at spinal level L5. A sterile PE10 catheter was then inserted into the open end of the cannula to a 7.75 cm mark. CSF was aspirated from the lumbar area and immediately frozen in liquid nitrogen and stored at -80°C until analysis. Following CSF extraction, rats were sacrificed via rapid decapitation and trunk blood was collected in EDTA-coated tubes (13 × 75 mm) and plasma was isolated at 3000 $\times g$ at 4°C for 15 minutes. Brains, spleens, the left medial lobe of the liver, mesenteric lymph nodes (MLNs), and subcutaneous adipose (SQ adipose) were aseptically removed and weighed immediately. All tissues were immediately frozen in liquid nitrogen and stored at -80°C until processing and analysis.

Tissue processing

Prior to homogenization, brains were thawed and the frontal cortex, hypothalamus, and dorsal vagal complex (DVC) were dissected over ice as previously described[23]. The hypothalamus and DVC were chosen because previous studies demonstrated elevations of Hsp72 in these regions following stressor exposure[6,16]. The frontal cortex was chosen as a control region. Spleens (0.3 g), livers (0.3 g), MLNs (7-10 nodes), and brain regions were all processed with the extraction buffer provided in the commercially available tissue Hsp72 ELISA kit from Enzo Life Sciences (Plymouth Meeting, PA) according to the manufacturer's instructions. Briefly, all tissue samples were placed in 2.0 mL homogenization tubes with six 2.8 mm ceramic (zirconium oxide) beads. Homogenization tubes containing spleens, livers, or MLNs received 1.0 mL of Hsp72 extraction buffer and were placed in a Precellys-

24 bench-top tissue homogenizer (Bertin Corp., Rockville, MD) for two cycles at 5000 rpm for 20 seconds. Tubes containing the frontal cortex or DVC received 500 µL of Hsp72 extraction buffer, whereas the hypothalamus received 250 µL of the extraction buffer. Tubes containing brain regions were placed in the Precellys-24 tissue homogenizer for one cycle at 5000 rpm for 10 seconds. The homogenized tissues in extraction buffer were transferred to 1.5 mL microtubes and centrifuged at 21,000 $\times g$ for 10 minutes at 4°C. Supernatant was collected and assayed immediately.

SQ adipose tissues (0.32 g) were placed in 2.0 mL homogenization tubes containing six ceramic beads with 0.950 mL of homogenization buffer (84 mg sodium fluoride, 90 mg sodium pyrophosphate, 185 mg sodium orthovanadate, 1 mL RIPA buffer, 200 µL phenylmethanesulfonyl-fluoride (PMSF), 100 µL phosphatase inhibitor cocktail, and ¼ tablet complete protease inhibitor cocktail (Roche) diluted to 10 mL in deionized water). The tubes were placed in the Precellys-24 tissue homogenizer for two cycles at 5000 rpm for 50 seconds. The tubes were then spun at 10,000 $\times g$ for 10 minutes at 4°C .The homogenized adipose supernatant was carefully transferred to 1.5 mL microtubes and centrifuged at 21,000 $\times g$ for 45 minutes at 4°C. Supernatant was collected and assayed immediately.

ELISA analyses

Corticosterone, a marker of stress-induced HPA axis activation, was measured in the plasma at a 1:200 dilution with the commercially available DetectX Corticosterone Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI) according to the manufacturer's instructions. Hsp72 was measured with commercially available ELISA kits from Enzo Life

Sciences according to the manufacturer's instructions. Plasma samples (1:2 dilution) and CSF (no dilution) were analyzed in the Hsp72 High Sensitivity ELISA kit (Enzo Life Sciences). The tissues were analyzed in the tissue specific Hsp72 ELISA kit (Enzo Life Sciences). SQ adipose was diluted 1:2, whereas spleens, livers, MLNs, frontal cortex, hypothalamus, and dorsal vagal complex were diluted 1:4 in the tissue Hsp72 ELISA. Hsp72 values in tissue samples were corrected by total protein, as measured with the commercially available Pierce BCA Protein Assay Kit (Thermo Scientific, Rockport, IL) according to the manufacturer's instructions. The SQ adipose, MLNs, frontal cortex, hypothalamus, and dorsal vagal complex were diluted 1:10, while the livers and spleens were diluted 1:20. Optical densities for all assays were measured using a SpectraMax Plus 354 plate reader (Molecular Devices, Sunnyvale, CA) and analyzed using four-parameter curve fit and software (SoftMax 5.4.1).

CSF spiked with Hsp72

To verify that CSF does not contain matrix interference that prevents reliable detection of Hsp72 ELISA, undiluted CSF from non-stress control rats were spiked with 0, 0.2, 0.78 and 6.25 ng/mL of Hsp72 standard provided in the kit and quantified in the manufacturer's assay. Optical densities were measured using a SpectraMax Plus 354 plate reader and analyzed using four-parameter curve fit and software.

Statistical analyses

Two-way ANOVAs were run to analyze the effect of stress on Hsp72, corticosterone, blood glucose, and spleen weights. Data points were treated as outliers if they failed Grubb's test for outliers and were also recorded as affected by experimental procedures by

the experimenter. If significant interactions were present, then Fisher's protected least significant difference (PLSD) post-hoc analyses were conducted on the data. All data are presented as means ± the standard error of the mean (SEM). $P<0.05$ was considered statistically significant.

Results

Characterization of inescapable tail shock stress in Sprague-Dawley rats

Consistent with previous findings, exposure to tail shocks activated the acute stress response in Sprague Dawley rats as indicated by elevations in plasma corticosterone[24] ($F(2,27) = 23.414$, $p < 0.0001$; Figure 5.1*A*) and blood glucose[25] ($F(2,31) = 9.463$, $p < 0.001$; Figure 5.1*B*). Elevated plasma concentrations of corticosterone in the stress and stress + 2hrs groups, compared to rats from the control group, indicates activation of the HPA axis, a primary system involved in the acute stress response[26]. Rapid elevations in blood glucose is both a marker of HPA axis[27] and SNS activation[28], another system involved in the acute stress response. Rats from both the stress and stress + 2hrs groups exhibited a reduction in spleen-to-body weight percentage (Figure 5.1*C*) compared to the control group ($F(2,27) = 5.691$, $p < 0.01$), also indicating SNS activation[29].

Hsp72 in the plasma and CSF

Analysis of the Hsp72 standard spikes in undiluted CSF revealed there were no matrix interferences in the Hsp72 assay (Figure 5.2*A*). Rats in the stress group had significantly elevated concentrations of Hsp72 in the plasma ($F(2,27) = 6.697$, $p < 0.01$) compared to rats in the control and stress + 2hrs groups (Figure 5.2*B*). Plasma Hsp72 rapidly decreased to baseline levels two hours after stressor termination. Hsp72 was

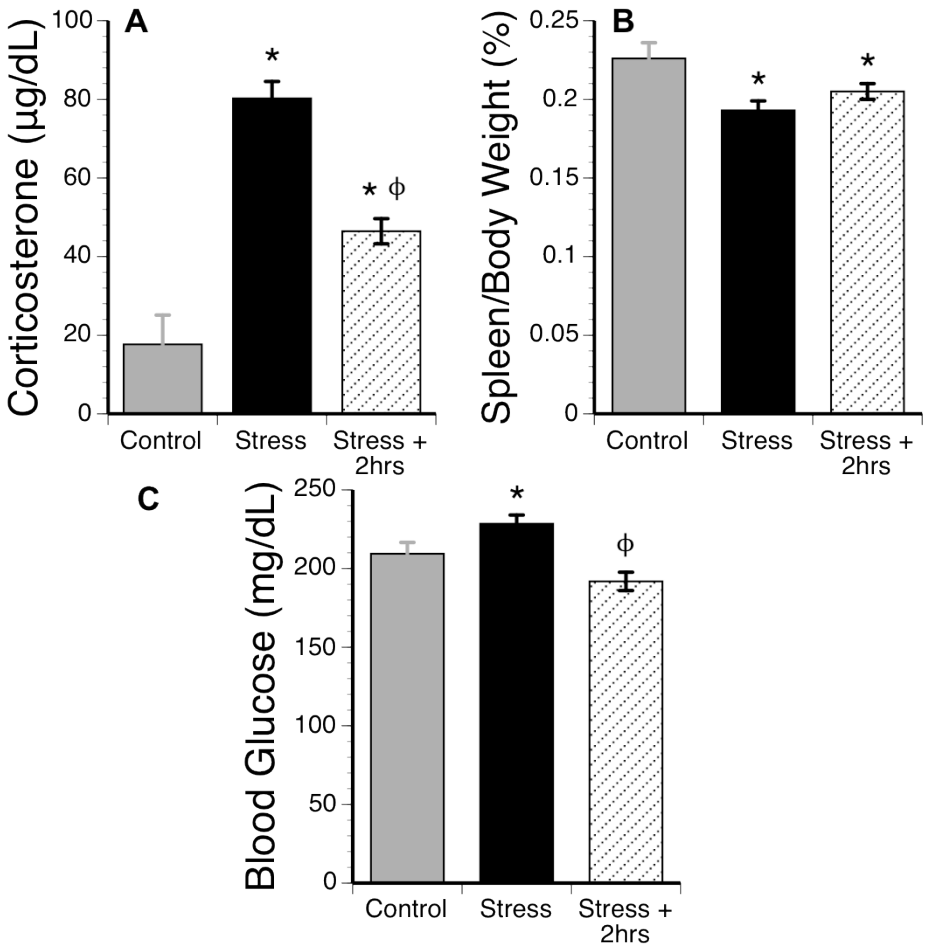

Figure 5.1. Pair-housed male Sprague-Dawley rats exposed to inescapable tail shock (Stress) activates the stress response. A Exposure to inescapable tail shock (Stress) elevated plasma corticosterone compared to the non-stressed (Control) group. Two hours of rest prior to sacrifice attenuated the stress-induced elevation of corticosterone, but concentrations were still significantly elevated compared to controls. **B.** Both stress conditions reduced spleen weight compared to controls. **C.** Rats exposed to Stress had elevated blood glucose compared to controls, but blood glucose returned to control levels two hours after stressor termination. Results are expressed in means ± SE; 6-8 rats/condition. * indicates significant difference when compared to control rats ($p < 0.05$). Φ indicates significant difference between Stress and Stress + 2hrs ($p < 0.05$). Two-way ANOVA was used.

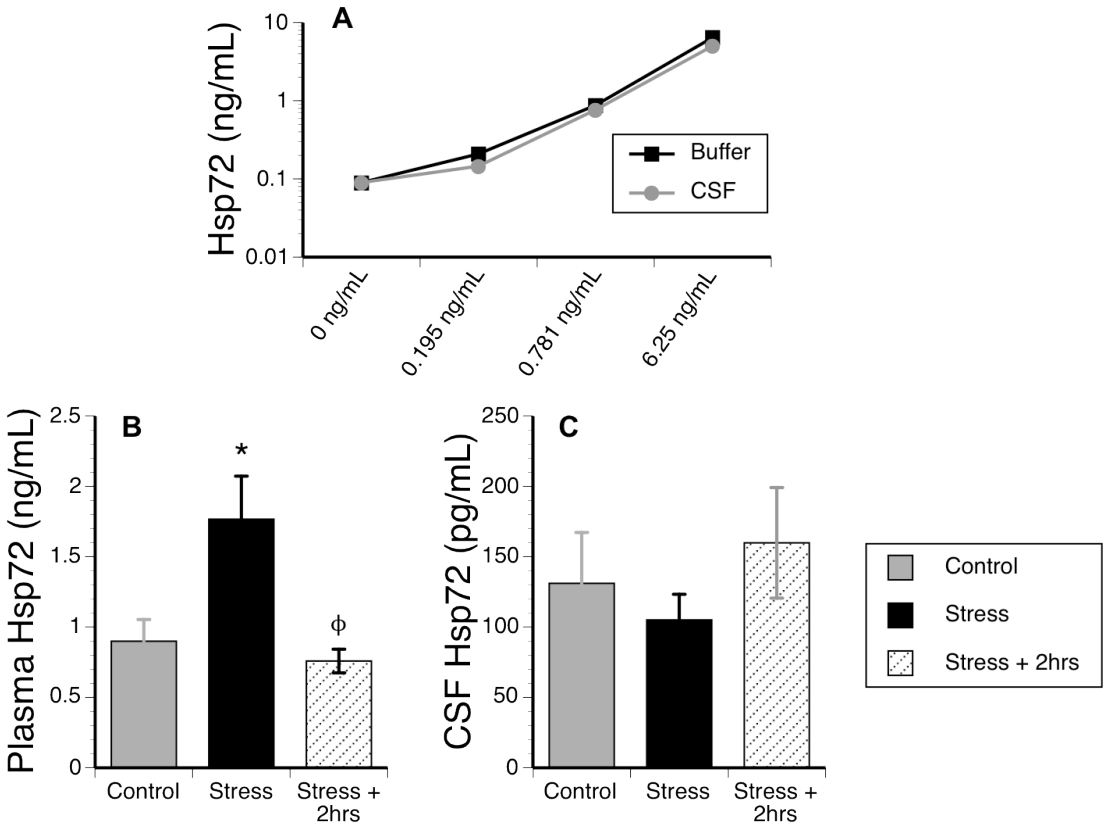

Figure 5.2. Extracellular Hsp72 in the plasma and CSF. ***A.*** Known concentrations of Hsp72 spiked into undiluted CSF were as detectable as equivalent spikes in the assay buffer provided by the commercial high sensitivity Hsp72 kit. **B.** Stressor exposure significantly elevates Hsp72 in the plasma, but concentrations return to baseline two hours after stressor termination. **C.** Stressor exposure has no impact on Hsp72 in the CSF. Results are expressed in means ± SE; 6-8 rats/condition. * indicates significant difference when compared to control rats ($p < 0.05$). Φ indicates significant difference between Stress and Stress + 2hrs ($p < 0.05$). Two-way ANOVA was used.

detectable in the CSF; however, stress had no detectable impact on Hsp72 concentrations in the CSF (Figure 5.2*C;* $F(2,21) = 6.697$, $p = 0.424$).

Hsp72 in the brain

Of the three brain regions examined in this study (hypothalamus, frontal cortex, and DVC), Hsp72 was only elevated in the hypothalamus following exposure to tail shock stress ($F(2,20) = 11.852$, $p < 0.001$; Figure 5.3*A*). Hsp72 concentrations were reliably elevated both immediately and 2 hours after stressor termination ($ps < 0.01$). In contrast, stress had no impact on Hsp72 concentrations in the frontal cortex or dorsal vagal complex.

Hsp72 in peripheral tissues

As illustrated in Figure 5.3*B*, exposure to tail shock stress increased Hsp72 in peripheral tissues that became statistically reliable two hours after stressor termination. There was a main effect of stress on Hsp72 in the SQ adipose ($F(2,28) = 3.776$, $p<0.05$), and post hoc analysis revealed that the elevation was only significant for rats in the stress + 2hrs group. Similarly, post hoc analyses illustrated that animals sacrificed two hours following stressor termination did have significantly elevated concentrations of Hsp72 in the spleens and MLNs ($p < 0.05$) compared to control measurements. Post hoc analysis of Hsp72 concentrations in liver only revealed a significant elevation in the stress + 2hrs group compared to the stress group ($p < 0.05$).

Discussion

In this study, our data demonstrates that concentrations of Hsp72 were elevated in blood but not in CSF immediately or 2 hours after acute stressor exposure, lending support to the conclusion that Hsp72 does not cross the blood brain barrier to enter the CSF from

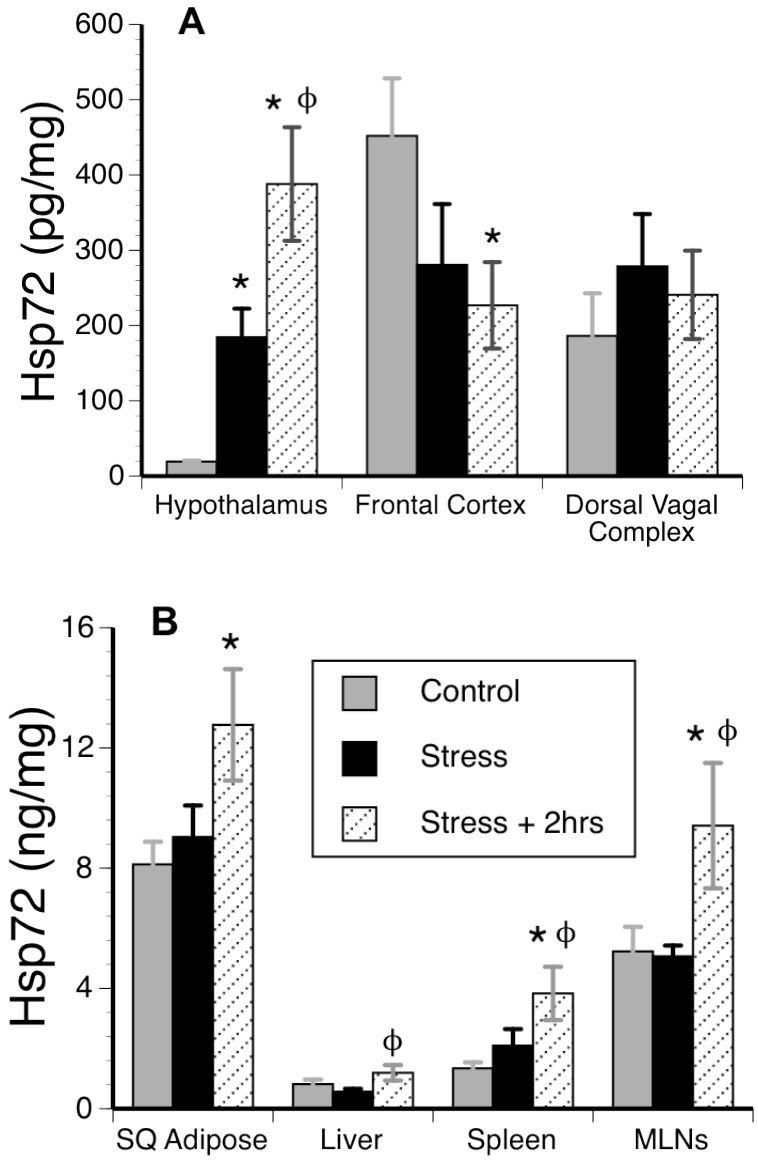

Figure 5.3. Hsp72 in the brain and peripheral tissues. A. Exposure to inescapable tail shock (Stress) elevated Hsp72 in the hypothalamus and continued to rise two hours after stressor termination. Stress had no impact on Hsp72 concentrations in the dorsal vagal complex or frontal cortex, but Hsp72 was significantly decreased for the Stress + 2hrs group in the frontal cortex. **B.** Stressor exposure significantly elevates Hsp72 in the SQ adipose, spleen, and MLNS two hours after stressor termination. Results are expressed in means ± SE; 6-8 rats/condition. * indicates significant difference when compared to control rats ($p < 0.05$). Φ indicates significant difference between Stress and Stress + 2hrs ($p < 0.05$). Two-way ANOVA was used.

the peripheral blood. In addition, stressor exposure increased Hsp72 concentrations in the brain, but not in the CSF. There are several possible explanations for these findings. First, Hsp72 may be released from brain tissue; however, it is not detectable until later than two hours after stressor exposure. The increase in hypothalamic Hsp72 is greater after 2 hours than it is immediately after stressor termination. Thus two hours following stressor termination may be too soon to measure changes in Hsp72 concentrations in the CSF. Second, the site for CSF extraction may have been too far from the location of extracellular Hsp72 in the brain. There is evidence for regional specificity of CSF composition[30]. Third, hypothalamic Hsp72 may be released into the extracellular space and not diffuse from the site, thus locally restricting its function. Finally, Hsp72 increases in hypothalamus may remain intracellular and function to protect the cells from cellular stress.

We examined the impact of inescapable tail shock stress on Hsp72 concentrations in the periphery of pair-housed, male Sprague-Dawley rats. Consistent with previous studies, exposure to inescapable tail shock stress elevated plasma corticosterone[24] and blood glucose.[25] Importantly, this was the first known study to demonstrate elevated Hsp72 in the plasma, SQ adipose, spleen, MLNs, and hypothalamus of Sprague-Dawley rats subjected to inescapable tail shock stress. Previous studies in our laboratory primarily examined the impact of inescapable tail shock in Fisher 344 rats on Hsp72 concentrations in the plasma, peripheral tissues, and the brain. The current experiment extends these findings to another strain, demonstrating the ubiquity of stress-induced Hsp72.

If extracellular Hsp72 is restricted to the periphery during the stress response, it is likely that its ability to activate the innate immune system is also restricted to the

periphery. This restriction may be a critical adaptation to avoid unnecessary inflammation in CNS response to a stressor. While activation of the immune system serves to promote healing and recovery after an insult, unchecked inflammation in the CNS could produce damaging effects, such as demyelination or direct neuronal damage. The neuroprotective effects of elevated concentrations of intracellular Hsp72 have been demonstrated in the hippocampus, which prevented memory deficits associated with general anesthesia[33]. Therefore, restricting Hsp72 to the hypothalamus during stressor exposure could confer neuroprotection without activating the neuro-immune system.

This study also investigated various markers for activation of the acute stress response, such as elevated plasma corticosterone, increased blood glucose, and reduced spleen weights. While exposure to the inescapable tail shock stressor successfully activated the acute stress response in pair-housed male Sprague-Dawley rats as measured by these markers, corticosterone and glucose concentrations were significantly higher than typically observed in this species[24,25,34]. However, isoflurane anesthesia has been shown to elevate baseline concentrations of plasma corticosterone and blood glucose[35], which could explain their elevated concentrations in this experiment.

Circulating Hsp72, plasma corticosterone, blood glucose, and spleen weights rapidly approached baseline levels two hours following stressor termination. Interestingly, while Hsp72 concentrations are highest in the plasma immediately following termination of the stressor, tissue concentrations of Hsp72 are only significantly elevated two hours following termination of the stressor. There are several potential explanations for the delayed elevation of tissue-specific Hsp72. Plasma Hsp72 could migrate out of the periphery into

the tissues. If the SQ adipose, spleen, and MLNs are not the source of extracellular Hsp72, it is possible that they are instead the target tissues. Recent research from our laboratory has demonstrated that plasma Hsp72 is associated with exosomes, which are 50-100 nm cellular particles released into the extracellular environment. These exosomes could target and transfer their Hsp72 content to the recipient tissues and confer cellular cytoprotection. Alternatively, the delayed elevation of Hsp72 in the peripheral tissues may indicate that they are the cellular source of Hsp72. During stressor exposure, these tissues could synthesize Hsp72 and release it into the plasma to immediately prime the innate immune system for injury or a pathogenic insult. Following stressor termination, these tissues may discontinue the active release of Hsp72 into the plasma before terminating synthesis, possibly to confer cytoprotection to their own cells.

Based on the results from this study, it is difficult to determine whether stress-induced elevations of Hsp72 in the peripheral tissues are the result of migration or synthesis. Therefore, future studies should block stress-induced elevations in plasma Hsp72 and examine the impact on tissue-associated Hsp72. Previous studies suggest that catecholamines are necessary for the stress induced elevations of circulating Hsp72. Johnson et al. demonstrated that blockade of α_1-adrenergic receptors (α_1-ADRs), a target for norepinephrine, attenuates circulating Hsp72 in the plasma[13]. If activation of the α_1-ADRs is necessary for the release of Hsp72 into the circulation, animals administered an α_1-ADR antagonist prior to inescapable tail shock should have elevated concentrations of tissue-associated Hsp72 compared to saline-injected stressed animals. However, if α_1-ADRs attenuate plasma Hsp72 by blocking its cellular synthesis, then animals administered the α_1-ADR antagonist prior to inescapable tail shock stress should exhibit basal

concentrations of Hsp72 in the peripheral tissues. Preliminary studies from our lab on male Fisher 344 rats exposed to inescapable tail shock stress reveal that administration of the α_1-ADR antagonist blocks stress induced elevations of Hsp72 in the spleen and subcutaneous adipose, but not the MLNs, suggesting that different tissues have various roles in the stress response[36].

Together, these findings add to the current literature on stress-induced elevations of Hsp72 in the periphery and CNS. To our knowledge, this is the first study to examine the impact of acute stressor exposure on Hsp72 in CSF. While Hsp72 is detectable in the CSF of male Sprague-Dawley rats, exposure to inescapable tail shock stress did not modify those concentrations despite robust elevations in the hypothalamus. Restricting Hsp72 in the CNS to the brain may be advantageous to prevent neurological damage associated with activation of the neuro-immune system.

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VI. Evaluation of the Research Experiences for Undergraduates (REU) sites program

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Introduction

The Research Experiences for Undergraduates (REU) program, sponsored by the National Science Foundation (NSF), supports the meaningful participation by undergraduate students in most areas of research in science and engineering. NSF provides funding for both standalone programs (known as REU Sites) and for individual students conducting research under an NSF-funded project (known as REU supplements). The REU program is a major component of NSF's effort to broaden the participation of individuals, especially those from underrepresented groups, in science and engineering research. In order to gain insights for designing REU Sites and to determine the extent to which these programs contribute to the diversity goals of the NSF, we gathered information during the summers of 2006 through 2009 on REU Sites funded or co-funded by the NSF's Directorate for Biological Sciences. We believe the findings reported here represent the first comprehensive program-wide evaluation of an REU Sites program.

NSF established the REU Sites Program in 1987, in an effort to increase students' interest in science and to promote the participation of diverse groups in research careers. In Fiscal 2010, NSF used an estimated over $80 million of its budget to support REU programs (NSF Solicitation 09-598). Undergraduate students' active participation in research is one of the most effective methods of attracting and retaining them in the sciences, and several studies have shown student benefits from an REU-type experience[1–4]. The Directorate for Biological Sciences (BIO) funds several REU Sites programs that provide innovative and exciting research experiences for undergraduates in many areas of the biological sciences. For purposes of this article, all subsequent reference to the REU program specifically refers to the Sites funded or co-funded by BIO.

Survey methods

Each summer from 2006 to 2009, a survey was sent electronically to the Principal Investigators (PIs) conducting an REU Sites program. Their responses were collected, and the data compiled and analyzed. The survey was designed to identify the participant groups' racial and ethnic profiles, academic status, gender, fields of interest, and disability status. Included in the 2008 and 2009 surveys were questions to evaluate enrichment activities, recruitment methods, and measures of program success at each site. We should note that the response rate from PIs was close to 100 percent for each of the four years of the survey.

For purposes of the REU program, underrepresented minorities are defined as those who self-identify as African Americans, Hispanics, Native Americans (including Alaska Natives and Pacific Islanders), and persons with disabilities. Our survey also kept track of Asian, White, and female participants. Students must be U.S. citizens, U.S. nationals, or permanent residents, and be enrolled in an undergraduate degree program to be eligible to participate in the program. It is important to note that data were collected from the REU Program Directors or PIs, and not from the student participants.

Results and discussion

Number of Applications and Acceptance Rate

The summer 2006 data provided the first glimpse of the overwhelming demand for this program. In that year, PIs collectively received 13,671 student applications for the 106 Sites that conducted a program. In the subsequent three summers, the number of Sites increased slightly but not in proportion to the number of applications (Figure 6.1). In 2009,

the large number of applications (21,193) resulted in the lowest acceptance rate (acceptance rate is defined here as the ratio of the total number of students who participated to the total number of applications received). These data suggest that the number of available opportunities for undergraduates interested in an REU has not kept up with the demand. The survey does not provide data on how many applications, on average, a student sends to the Sites. It is therefore currently impossible to infer the extent to which the average number of applications sent by individual applicants influences the acceptance rate.

Demographic Characteristics of Student Participants

From 2006 to 2009, the percentage of participants belonging to an underrepresented group participating in REU has increased (see Table 6.1), from 43% in 2006 to 49% in 2009. Figure 6.2 shows the racial and ethnic distribution for summer 2009 participants, which shows that students from all underrepresented groups participate. The distribution for the other three summers is similar, with some observable trends. While the proportions of Native Americans, Pacific Islanders, and persons with disabilities have remained constant over the four years, the proportion of Hispanic students has steadily risen while those of African Americans have fluctuated slightly during the same period. This data is consistent with the growing numbers of Hispanic students matriculating in college[5]. The gender distribution has remained relatively constant over the four years, with a predominance of females (between 62-64% over the 4 years). Over the four years, the percentage of non-traditional students, defined as 30 years or older, has remained low at 2-4%. These findings indicate that the REU program has been successful in recruiting a diverse group of students with a large number of underrepresented students, and that the

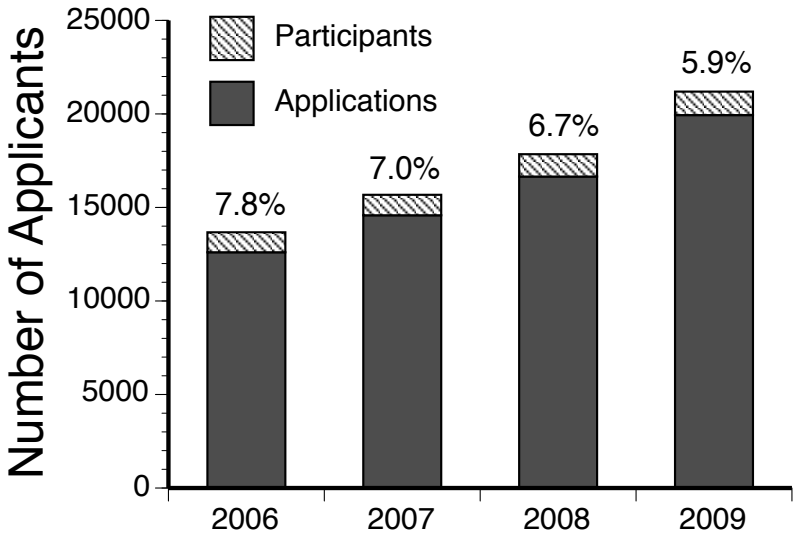

Figure 6.1. Total number of REU Sites' applications and participants from 2006 through 2009. Applications to REU sites within the Biological (BIO) Directorate of the NSF have steadily increased from 2006 through 2009, while acceptance rates to the programs have declined, as indicated by the percentage values above the bars.

Demographics by Year Total, *n*	**2006** *1068*	**2007** *1095*	**2008** *1196*	**2009** *1256*
% African American	17	18	15	17
% Hispanic	18	18	21	22
% Native American	5	5	5	6
% Pacific Islander	2	2	2	2
% Persons with Disabilities	1	1	1	1
% Asian	6	7	6	6
% White	52	48	50	46
% Male	36	38	36	37
% Female	64	62	64	63

Table 6.1. The Demographic profile of the REU Sites' participants from 2006 through 2009. The total number of REU student participants from an underrepresented minority group (those who self-identify as African American, Hispanic, Native American, or Pacific Islander) has increased from 2006 through 2009 within the BIO directorate. While proportions of Native Americans, Pacific Islanders, and persons with disabilities have remained constant over the four years, the proportion of Hispanic participants has steadily risen. Additionally, the majority of these participants over the four-year period are female. These data indicate that the BIO REU program successfully recruits a diverse group of students.

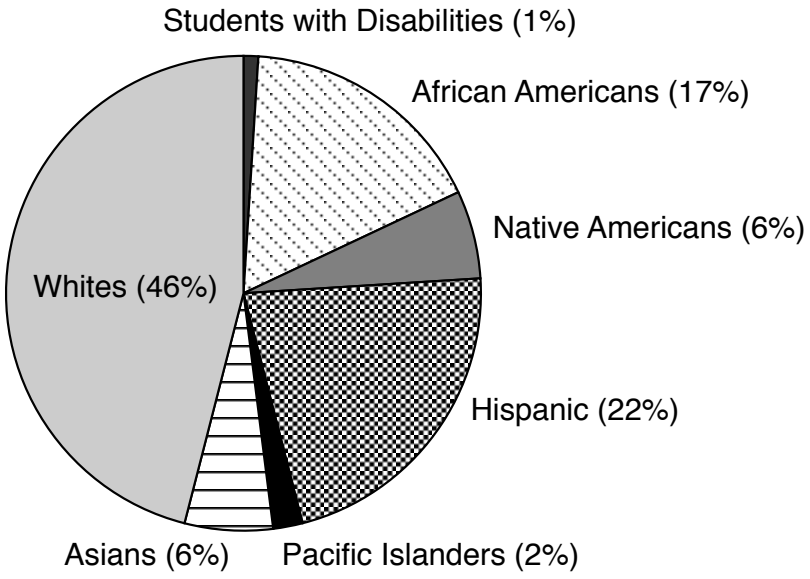

Figure 6.2. Distribution of 2009 REU participants. This graphic represents the racial and ethnic distribution of REU participants in BIO from 2009. These distributions closely resemble the racial and ethnic distributions from 2006 through 2008.

various efforts of PIs to reach these populations have been effective.

Academic Background of Student Participants

Students who have just completed their freshman year participate in low numbers – an average of 10% from 2006-2009, although an increasing trend was seen over the four years (from 9% in 2006 to 11% in 2009). The largest group consisted of students who had just completed their junior year (57%), with decreasing trend over the years (58% in 2006 compared to 53% in 2009). The data indicate that the majority of participants were either rising juniors or seniors. Several REU programs require completion of specific science courses and these data reflect the growing participation of students who are more advanced in their academic standing and who have completed the prerequisite courses.

In 2008 and 2009, PIs were asked to indicate the number of participants who come from PhD granting institutions, and the data show that the majority of students are recruited from non-PhD institutions (61% and 57%, respectively). This result is consistent with the goal of the program to provide opportunities to students whose access to research is limited. In addition, the percentage of students recruited from outside the host institution has remained consistently high (between 82-85%), while the percentage of students with prior REU experience has remained low (from 5-9%). These data show that, in general, PIs have succeeded in recruiting students whose tenure at an REU program could make a significant impact on their career choice. In general, these students are from institutions with limited research activities and who are getting exposure to the REU program for the first time in their academic career.

Recruitment Methods

For the 2008 and 2009 summer REU programs, PIs were asked to indicate their top one or two most effective recruitment methods. The choices included (1) the Internet (website, email, etc.); (2) direct mailings (letters, postcards); (3) media (flyers, newspapers, magazines, TV, radio); (4) conferences, meetings, networking, site visits; (5) campus recruitment office or equivalent; or (6) other, in which case they are asked to specify. In both years, PIs reported that the Internet (n=114 and 117, respectively for 2008 and 2009) and conferences (n=61 and 57, respectively for 2008 and 2009) were the most effective recruitment tools. REU PIs reported that their participants discovered their program through their own website linked to the NSF website. Through the NSF website, students are able to find additional information about individual programs and access an online application. Other effective recruitment methods included word of mouth through a fellow student or faculty member (n_{2008}=16; n_{2009}=30) and direct mailings (n_{2008}=21; n_{2009}=26).

Enrichment Activities and Other Program Features

To enable student participants to become independent thinkers and effective communicators, the REU programs implement a variety of enrichment activities. According to the survey data from 2008 and 2009, the most common feature of an REU program are scheduled lab meetings, seminars, workshops, and an end-of-program symposium where student participants prepare a poster or an oral presentation of their work. The PIs also indicated that networking and social events are part of the program (97% and 99%, respectively for 2008 and 2009). These activities are commonly viewed as enhancing student skills in communication and critical thinking, and helping them to develop a cohort

experience. In addition, a significant number of programs include journal club, GRE preparatory courses, and field trips. A surprising finding is that less than 20% of the PIs indicated that their program included ethics training (20 of 124 programs in 2008 and 24 of 131 in 2009). In general, the program enhancement activities, done jointly with an intensive research lab experience, allow the students to derive greater benefits than just joining a lab. In fact, many programs recognize these additional benefits and include non-REU Site participants, such as students supported by an REU supplement, in the REU program activities.

Over the course of four years, the average number of weeks for the program has remained constant at ten. Very few programs are conducted in foreign locations and the number has decreased over time (from seven to four, respectively, for 2006 and 2009). The surveys also revealed the main fields of research undertaken by all of the REU participants (see Table 6.2). Although the combined 2,452 REU participants from 2008 and 2009 represent over 19 subfields within the biosciences, the majority of the participants conduct research or use tools in Molecular and Cellular Biology (n=191). The next four most represented fields in the REU program include Animal Biology (n=164), Microbiology (n=147), and Environmental and Conservation Science (n=150). Overall, the REU programs are increasingly becoming multidisciplinary, involving projects in Chemistry/Biochemistry (n=126), Geosciences (n=54), and other disciplines. These data reflect the broad range of scientific interests represented in the REU program.

Sub-Fields of Research Sponsored by BIO	Number of REU participants
Animal Science	164
Bioinformatics	90
Biotechnology	60
Chemistry	126
Chemisrty/Biochemistry	27
Environmental Science	150
Evolution	81
Genomics	109
Geosciences	54
Math and Computational Biology	29
Microbiology	147
Molecular and Cellular Biology	191
Nanotechnology	21
Organismal Biology	113
Physics	19
Plant Sciences	136
Social, Behavioral, and Economic	28

Table 6.2. Main sub-fields of research pursued by REU participants in BIO Directorate from 2008 to 2009. The subfields within the BIO directorate with the most student participants includes molecular and cellular biology, animal science, and environmental science. The subfields with the fewest student participants are physics, nanotechnology, and chemistry/biochemistry; however, these fields may have a greater number of participants under other NSF Directorates.

Preferred Measurements of Program Success

Each REU includes some type of program evaluation that PIs use to continue to improve their program and to determine their relative effectiveness in providing a high quality research experience. In addition, PIs track the career progress of former participants. When asked to list up to three metrics they use to measure program success, the 2009 PIs mentioned graduate school attendance (n=66) and participant general satisfaction of the program (n=66) most frequently. Getting a student's name on a publication was the next most used metric (n=47), followed by completion of research reports (n=32). The PIs also use faculty or mentor evaluations (n=30), professional conference attendance (n=26), entrance into a science or education career (n=24), completion of a degree (n=16), and increased confidence (n=10) as metrics to gauge program success. Data from the 2008 survey revealed that PIs also viewed graduate school attendance (n=77), student publication/presentation (n-64) and participant general satisfaction of the program (n=54) as the top three choices. All of the surveyed REU sites supported in 2008 and 2009 utilized multiple metrics to assess program success.

Summary and conclusions

The data collected from these surveys reveal very interesting results.

1) The REU Sites programs supported by the NSF Directorate for Biological Sciences engage a very diverse group of undergraduates, with over 40% participation by groups traditionally underrepresented in science in each of the year of study. In summer 2009, approximately half of the participants were members of an underrepresented group. It is interesting to note that all racial and ethnic groups are

represented, including non-traditional students (30 years or older) and persons with disabilities. Among the racial and ethnic groups, our data show increasing participation by Hispanic students, a result that is consistent with the growing numbers of Hispanic students matriculating in college[5]. In order to ensure that disabled students are able to participate in the REU program, a small supplemental funding is typically provided by NSF. PIs report that many of their former participants track into graduate programs in the biosciences. Participation in an REU-type program is becoming increasingly important for students applying for competitive graduate programs as well as for those seeking funding opportunities such as the NSF Graduate Research Fellowship.

2) Although PIs can recruit from their own students, the number of students who come from the host institution is relatively small. The majority of students who participate are those who come from outside the host institution (85% on average) and those who come from a non-PhD institution (approximately 60%). In addition, many PIs are selecting students whose participation would be more likely to impact the students' career choice. Only 5-9% of students in the program have had a prior REU experience.
3) There is an overwhelming demand for the REU program. With over 21,000 applications received collectively by the Sites in 2009, PIs are finding that sorting through myriads of applications from qualified students can be a challenge. Other than increasing funding for the program, creative ways need to be developed in order to manage the large number of applications and the alarmingly low acceptance rates. In very unusual and limited situations, PIs have requested

additional supplement funding from NSF to support students they find difficult to reject.

4) The predominant participant is female, reflecting the large numbers enrolled in undergraduate programs in biology[6]. More students are being accepted who have met a required set of courses, and the majority of REU participants are rising juniors or seniors. In biology, a small yet significant number (10%) are students who have just completed high school or their freshman year in college. This young group is seen as more likely to be influenced by the REU experience, as many of these students have not yet decided on a career.

5) A typical REU Sites program is ten weeks long, with a strong faculty/mentor - student interaction outside of the research activity. PIs report that their programs include such enrichment activities as seminars, field trips, student symposia, and others. Many programs offer opportunities for cross-disciplinary research, and in some instances, biology majors work side by side with their non-biology peers. A surprising finding was the small number of PIs who reported ethics and the responsible conduct of research (RCR) as part of their program. RCR training is now required for REU Sites whose NSF awards started after January 2010, a requirement consistent with the America COMPETES Act (ACA, Pub. L. No. 110-69).

6) There are very few REU Sites in foreign countries, and the number of these programs has decreased over the four-year period of the study. Although a few Sites send isolated students abroad, the number of programs whose primary activities are based abroad has dwindled from seven in 2006 to four in 2009. This trend is not consistent with the increasing globalization of science.

7) PIs report that the Internet – their own website which is linked to the NSF REU website -is an effective method for recruiting students. Individual program websites provide students with information about the programs, and allow them to seek programs that are relevant to their interests. A few PIs use their websites for receiving online applications, making it possible for students to easily apply to them. The NSF REU website also provides an email contact for the PI, Program Director or Coordinator.
8) Another surprising finding is that despite the huge recruitment success of REU PIs, diversity was not one of the metrics they considered for measuring success of their own program. Although PIs collectively are doing an outstanding job at recruiting a diverse set of participants, they view participant progression into graduate programs and student publications and presentations as important metrics for measuring success of their program. A large number of PIs also use general program satisfaction by students (and sometimes by mentors) as a metric of success.

PIs interested in seeking funding from NSF should note the general characteristics of the REU program described in this article and design their proposal in the context of increasing the numbers and diversity of students applying to the program (including possibly encouraging applications from veterans and persons with disabilities). Potential PIs should also consider the need to introduce younger audiences (high school graduates and freshmen) to the program. The increasing trends toward cross-disciplinary research, more quantitative biology, and international/global collaborations should also be considered. Proposals that provide cutting-edge research projects for students, especially in a collaborative environment, would be consistent with the direction in which biosciences

are heading with the recommendations of studies done by the National Research Council[7,8] and the American Association for the Advancement of Science[9].

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VII. General Discussion

The results presented in this dissertation further knowledge on the immunological impact of exosomes during the acute stress response. We demonstrate that exposure to an acute stressor can modulate proteomic and genetic components of exosomes, which in turn are capable of enhancing an organism's response to a bacterial challenge. Although the third chapter only examines three exosomal markers altered by stressor exposure, chapter two reveals there are many stress-associated microRNA (miRNA) capable of impacting an immune response. Future research should examine if these candidate miRNA associate with exosomes and contribute to immunomodulation during the stress response.

Exosomes are emerging as potential therapeutic tools, therefore selective modulation of miRNA and proteomic content could modulate the induction of the immunity during various disease states and infections. However, as exosome research progresses, researchers examining the immunological role of exosomes in cell culture conditions need to recognize the potential immunosuppressive role of fetal bovine serum (FBS) exosomes. While removing FBS exosomes is critical for careful analysis of exosome function, this can result in an exaggerated IL-1β response to a standard immunological challenge.

The fifth chapter suggests that the immunological role of extracellular heat shock protein 72 (Hsp72) is restricted to the periphery. While Hsp72 is rapidly induced in the hypothalamus following acute stressor exposure, concentrations are unchanged in the cerebrospinal fluid. Thus, the restriction of extracellular Hsp72 to the blood may be advantageous for priming innate immunity while intracellular Hsp72 in the peripheral tissues and hypothalamus can confer cytoprotection during stress. However, additional

research is necessary to clarify whether there is regional specificity for Hsp72 in the central nervous system.

Lastly, survey analyses of the Research Experiences for Undergraduates (REU) program demonstrates that there is high demand by students for paid undergraduate lab experiences. Additionally, the REU program is successfully meeting the Congressional mandate to improve gender and ethnic diversity in the sciences. Undergraduate research assistants are becoming a valuable asset to academic laboratories across the country and the REU program enables students with challenging economic conditions to participate meaningfully in National Science Foundation funded research projects.

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