A TALE OF TWO SIRTUINS: THE IMPACT OF SIRT1 AND SIRT3 ON THE PATHOPHYSIOLOGY OF SHOCK

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TARGETING SIRTUINS TO IMPROVE OUTCOMES IN SHOCK

Dedication

To my husband, Michael Root, MD, PhD – the smartest scientist and most dedicated teacher I know. Without a doubt, you are my greatest advocate and staunchest supporter; and without you, none of this would have been worth it. Thank you for being my best friend.

To my children, Sarah and Zachary – your love for science is contagious. Never quit seeking answers from the universe. Asking “why” is another way of saying “I love you”.

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When I decided to go back to graduate school, I went with a singular purpose – to become a better scientist in order to improve my patient's outcomes. As a surgical resident, I became acutely aware that although patients "successfully" survived their traumatic injuries, they frequently developed multi-organ dysfunction despite what seemed to be appropriate care. This was both frustrating and perplexing; and eventually compelled me to seek more formal training in translational research.

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ABSTRACT

TARGETING SIRTUINS TO IMPROVE OUTCOMES IN SHOCK

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Both acute blood loss and severe infection activate common cellular pathways leading to shock – a pathologic condition characterized by systemic inflammation, oxidative stress, and mitochondrial dysfunction. Sirtuins, a highly conserved group of NAD-dependent enzymes, play a critical role in cellular survival and many of the benefits associated with sirtuin activation are thought to be secondary to decreased inflammation, reduced oxidative stress, and improved mitochondrial physiology. As such, we hypothesized that sirtuin pathways play a crucial role in shock and could be could be targeted to improve outcomes following acute blood loss and severe infection. In a series of in vivo and in vitro experiments recapitulating hemorrhagic shock and severe sepsis, we explored the impact of sirtuin activation on inflammation, mitochondrial function, and survival. Following decompensated hemorrhagic shock, resuscitation with resveratrol, a SIRT1 activator, significantly improved renal mitochondrial function and decreased oxidative damage. Similarly, resuscitation with nicotinamide mononononucleotide (NMN), a key biosynthetic NAD precursor, was found to mitigate inflammation, support cellular energetics and improve both physiologic resilience and survival. In contrast, impaired expression of either SIRT1 or SIRT3 resulted in a pro-inflammatory phenotype with accelerated mortality in sepsis. Interestingly, deletion of SIRT1 did not significantly

worsen the degree of mitochondrial dysfunction observed in septic liver, but was associated with decreased CI and CII respiration in kidney. Deletion of SIRT3 did not significantly impact the degree of mitochondrial dysfunction observed in either liver or kidney. Taken together, these data strongly suggest that SIRT1 and SIRT3 play a key role in the pathophysiology of shock. Although further research is needed to determine if SIRT1 and SIRT3 overexpression improves outcomes or if pharmacologically manipulating NAD metabolism in conjunction with sirtuin activation provides added benefit, targeting sirtuins appears beneficial in hemorrhagic and septic shock.

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CHAPTER 1:

Of Shock and Sirtuins

"Shock is the manifestation of the rude unhinging of the machinery of life"

Samuel V. Gross, 1872

"The pulse, however, does not respond; it grows feebler, and finally disappears, and this 'momentary pause in the act of death' is soon followed by the grim reality."

John Collins Warren, 1895

In 1743, the French Army surgeon Henri Francois Le Dran coined the term "choquer" or "shock" to describe the progressive clinical failure he observed in dying soldiers, noting that many of those that survived their initial wounding would die hours to days later with "purulent matter".(65) It is now recognized that both acute blood loss and severe infection, or sepsis, activate common cellular pathways leading to shock –a pathological condition characterized by cardiovascular collapse and systemic inflammation. During shock, decreased tissue perfusion leads to cellular hypoxia, mitochondrial dysfunction, and bioenergetic failure. Concurrently, both damaged tissues and microbial products activate the immune system through Toll-like receptors; thereby, increasing NFκB signaling and the systemic release of pro-inflammatory cytokines.(17) These cytokines not only enhance the recruitment of leukocytes to damaged or infected tissues, they further exacerbate hypotension, cellular hypoxia, and oxidant stress.(208) Without prompt intervention, shock progresses from cellular injury to organ failure, and ultimately, to death.

Depending on the etiology of the shock state, initial therapy includes prompt hemorrhage control, or antibiotics, along with aggressive intravenous fluids and blood products resuscitation in order to restore perfusion pressure. Reperfusion, however, unleashes a storm of reactive oxygen species (ROS), inflammatory cytokines, and toxic

metabolites – all of which can further activate systemic inflammation. In response to oxidant damage, cytokine induced neutrophil chemoattractant (CINCs) recruit neutrophils and the pro-inflammatory cytokine IL6 up-regulate vascular expression of intracellular adhesion molecule (ICAM) 1, an endothelial molecule that promotes the egress of neutrophils from the circulation into tissues.(237) With increased accumulation of tissue neutrophils, more ROS and proteolytic enzymes are released leading to increased tissue damage and subsequently more inflammation (Figure 1). Importantly, the severity of shock, and its concomitant inflammatory response, directly contribute to the development of multi-organ failure (MOF).(149,208) Clinically, MOF develops in up to 50% of hypotensive trauma patients and is a universal finding in patients with septic shock.(6,23,56,63,168)

Although only 12% of the U.S. population is older than 65 years of age, this demographic accounts for more than 60% of all sepsis cases and over 80% of sepsis-related deaths.(5,160) While the elderly frequently have comorbid conditions that can exacerbate the course of sepsis, an increasing body of clinical and experimental data suggests that age itself is an independent risk factor for sepsis-related death.(160,203,231,241,242) With age, two key pro-inflammatory cytokines, IL6 and TNFα, are frequently elevated and are thought to contribute to increased oxidative stress, mitochondrial dysfunction, and a progressive decline in physiologic function.(28) During sepsis, however, this cytokine pattern becomes even more exaggerated and exceeds that found in younger patients.(29,130) Because MOF accounts for nearly 50% of all deaths in the intensive care unit (161), there is increasing interest in developing therapies that treat shock and target the cellular pathways implicated in organ failure

Sirtuins, a highly conserved group of NAD-dependent enzymes, play critical roles in cellular survival and may be the key to better outcomes in shock. Sirtuins were first

described in *S. cerevisiae* after the Silent information regulator 2 (*SIR*2) gene was found to confer stress resistance and promote longevity.(117) Since then, seven sirtuins have been identified in humans; each with a discrete subcellular localization and specific targets.(236) Sirtuins primarily regulate biologic function by removing acyl groups (most commonly acetyl) from lysine residues or by functioning as ADP-ribosyltransferases.(102) Because the deacetylation reaction requires NAD as a substrate, sirtuin activity can be directly influenced by the nutritional and redox state of the cell. Moreover, because nearly every metabolic enzyme is modified by lysine acetylation, sirtuins can directly control and coordinate a variety of pathways in times of cellular stress.(283) Although incompletely characterized, many of the pro-survival benefits associated with sirtuin activation are thought to be secondary to, decreased inflammation, reduced oxidative stress, and improved mitochondrial physiology (Figure 2).(233) As such, ***we hypothesized that sirtuin pathways play a key role in shock and could be targeted to improve outcomes following acute blood loss and severe infection.***

SIRTUIN 1 (SIRT1)

SIRT1 is the best characterized mammalian *SIR*2 homologue.(73) Located in the nucleus and cytoplasm, SIRT1 is known to deacetylate over 30 target proteins and is capable of transcriptionally influencing a number of cellular processes ranging from gluconeogenesis to apoptosis.(236) Because SIRT1 absolutely requires NAD as cofactor, it can respond to metabolic perturbations and nutrient stress by modulating survival pathways. Indeed, calorie restriction is potent SIRT1 activator and has been shown to extend lifespan in a variety of organisms including yeast, worms, mice and rats.(50,92,146) Interestingly, short-term calorie restriction also appears to improve survival following sepsis induced by cecal ligation and puncture (CLP) and is associated

with decreased inflammation following hepatic ischemia-reperfusion injury in mice.(90,190,194) When SIRT1 was pharmacologically inhibited, however, the physiologic benefits of calorie restriction following reperfusion were lost. Taken together these data strongly suggest that activating SIRT1 pathways could mitigate the pathophysiology of shock.

SIRT1 AND INFLAMMATION

NFκB is considered to be the central mediator of the human immune response and its activity increases significantly during hemorrhagic and septic shock.(179,213) As a prominent downstream target of SIRT1, NFκB activity can be modulated to temper inflammation.(269) By deacetylating the RelA subunit of NFκB; SIRT1 inhibits the transcription of over 100 target genes involved in the inflammatory response, including IL6, TNFα, CINC, cyclooxygenase -2, and ICAM-1.(179,269) In contrast, deletion of SIRT1 results in a marked increase in NFκB activation along with increased cytokine and ICAM 1-expression following lipopolysaccharide challenge.(77)

SIRT1 AND OXIDATIVE STRESS

Although reactive oxygen species (ROS) are routinely produced in low quantities during normal cellular activity, significant oxidative stress is a hallmark of shock and reperfusion injury. During shock, ROS are created by a number mechanisms including dysfunctional mitochondria, hypoxanthine recycling by xanthine oxidase, and by nicotinamide adenine dinucleotide phosphate oxidase (NOX) during neutrophil activation.(148) If left unchecked, ROS can cause oxidative damage to nucleic acids and proteins leading to further mitochondrial dysfunction, cell damage, and leukocyte recruitment. Mitochondrial derived ROS can also serve as signaling molecules to up-regulate inflammation. In addition to directly activating NFκB and increasing the

transcription of pro-inflammatory cytokines, ROS can enhance the cleavage and subsequent release of mature cytokine by activating the NLRP3 inflammasome.(30,81,286) Finally, oxidative stress from vascular NOX can exacerbate shock and local damage by causing endothelial dysfunction, which in turn leads to platelet activation, increased vascular permeability, and loss of vasoreactivity.(21,275) Therapies that either provide antioxidants or enhance endogenous antioxidant systems have been shown to decrease free radical damage, decrease inflammation, and improve organ function following hemorrhagic and septic shock.(22,68,187)

SIRT1 plays a critical role in mitigating oxidant damage. By deacetylating and activating forkhead box O3 (FOXO3), SIRT1 augments the transcription of key antioxidant enzymes such as superoxide dismutase and catalase.(128) SIRT1 can also enhance antioxidant defenses by increasing the activation and expression of PGC1α. Along with its co-regulator NRF2, PGC1α promotes increases the transcription of antioxidant genes including superoxide dismutase, heme oxygenase-1, and glutathione reductase.(226) Without SIRT1, however, the expression of these key antioxidants is significantly impaired and tissues are more susceptible to oxidant stress and cellular death. When subjected to ischemia-reperfusion injury, SIRT1 null hearts experienced considerably more tissue infarction and damage than wild-type controls. In contrast, SIRT1 transgenic hearts demonstrated substantially higher levels of superoxide dismutase and were more protected following ischemia-reperfusion, with less infarcted myocardium and better contractility.(105)

SIRT1 AND MITOCHONDRIAL BIOGENESIS

Mitochondria are subcellular organelles that utilize oxygen to create the majority of the available ATP via oxidative phosphorylation. Any significant decrease in the

availability of oxygen, or perturbation in the electron transport chain, however, can lead to decreased ATP levels and cellular stress. As previously described, reperfusion of hypoxic tissues generates ROS, which, in turn, damages mitochondrial proteins and further impairs oxidative phosphorylation in key organs. During hemorrhagic and septic shock, arterial smooth muscle cells can also experience mitochondrial dysfunction leading to low ATP levels. As a result K_{ATP} channels in vascular smooth muscle beds open, leading to hyperpolarization, persistent hypotension, and on-going poor tissue perfusion.(254)

Despite adequate resuscitation, persistent mitochondrial dysfunction may contribute to ongoing cellular injury and organ failure following shock. In fact, patients who develop organ failure are more likely to demonstrate early evidence of mitochondrial dysfunction when compared to similarly injured patients.(33) Notably, septic patients also develop mitochondrial dysfunction despite fluid resuscitation and antibiotics. When skeletal muscle biopsies were taken within 24 hours of ICU admission, non-surviving septic patients were found to have significantly decreased complex I activity and lower tissue ATP levels when compared to both sepsis survivors and healthy controls. Moreover, the severity of septic shock directly correlated with decreased muscle ATP levels.(24) The ability to generate sufficient ATP maybe further compromised during sepsis by an overall decrease in mitochondrial mass.(34,35,232) Indeed, patients with MOF were found to have 30 to 50% less mitochondrial protein in both intercostal and thigh muscle relative to non-septic controls.(70,71)

SIRT1 promotes mitochondrial biogenesis and oxidative phosphorylation by activating PGC1α. In turn, PGC1α increases the transcription of mitochondrial proteins and enzymes by coactivating a number of transcription factors, including NRF1 and 2, ERRα, PPARα, and TFAM. In fact, the overexpression of PGC1α leads to a pronounced

increase in mitochondrial mass in otherwise healthy mice.(205) Although PGC1α expression is increased during hypoxia and in response to ROS,(258) Raju and colleagues demonstrated that cardiac PGC1α levels fall dramatically in severe hemorrhagic shock and are associated with impaired mitochondrial function and recovery.(8,111,112) Resuscitation with the SIRT1 activator, resveratrol, preserved PGC1α protein levels and increased expression of key mitochondrial transcription factors. Importantly, mitochondrial function was also enhanced and correlated with both improved cardiac contractility and better survival. When animals were co-treated with sirtinol, a SIRT1 antagonist, the mitochondrial benefits of resveratrol were entirely lost, suggesting that resveratrol's ability to preserve PGC1α function is SIRT1 dependent. In a similar study, Biel et al found that SIRT1 null hepatocytes were significantly more likely to die than wild-type following ischemia-reperfusion. In contrast, genetic overexpression or pharmacological activation of SIRT1 significantly improved mitochondrial stability, suppressed defective autophagy, and decreased tissue damage.(18)

SIRTUIN 3 (SIRTS)

Although three sirtuins (SIRT3, SIRT4, and SIRT5), are located in the mitochondria, only SIRT3 functions as a deacetylase and there is growing appreciation that this sirtuin, in particular, plays a crucial role in regulating mitochondrial metabolism and oxidative stress.(1,89,97,250) In fact, in SIRT3 knock out mice, several enzymes in the Kreb's cycle and multiple complexes in the electron transport chain are hyperacetylated, suggesting that SIRT3 may directly regulate their activity.(1,69,114)

During calorie restriction, SIRT3 expression is enhanced and increased mitochondrial NAD serves as a potent stimulus for activation of SIRT3 pathways.(14,97) In response to a perceived energy deficit, SIRT3 coordinates a switch from glycolysis to

fatty acid oxidation.(97) Additionally, SIRT3 activates acetyl-CoA synthase 2 to increase levels of mitochondrial acetyl CoA and deacetylates hydroxyl methylglutaryl CoA synthase 2 to increase the production of ketone bodies, a key alternative fuel for the heart, kidney and brain.(88,215,260) Given the entry of acetyl CoA into the Kreb's cycle is impaired during critical illness (138), activating SIRT3 pathways could provide an alternative means of supporting energy metabolism.

SIRT3 serves as a major regulator of oxidative stress and can modulate both the generation and detoxification of ROS. Not only can SIRT3 can modulate the flow, and thus potentially the leak, of electrons through complexes I, II, and III, SIRT3 can deacetylate and down-regulate enzymes that form low levels of ROS such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and aconitase.(1,14,69,209) SIRT3 also mitigates oxidative stress by directly activating the antioxidant enzyme manganese superoxide dismutase (SOD2) and by increasing the concentration of reduced glutathione through its interactions with isocitrate dehydrogenase 2, with a subsequent increase in NADPH levels.(191,221) While SIRT3 knockout mice appear phenotypically normal, these animals demonstrate evidence of impaired complex I function with increased oxidative damage, reduced tissue ATP levels, and markedly elevated levels of pro-inflammatory cytokines. Overexpression of SIRT3, on the other hand, rescues mitochondrial function and minimize oxidative damage.(1)

Finally, there is increasing data to suggest that SIRT3 can prevent oxidant-induced apoptosis. During shock, damage from ROS can lead to mitochondrial membrane depolarization and activation of the membrane permeability transition pore (mPTP).(59,137,141) SIRT3 is known to deacetylate and stabilize cyclophilin D, a key component of the mitochondrial permeability transition pore (mPTP). When subjected to calcium challenge, mitochondria from aged SIRT3 null mice were significantly more

prone to swelling; a phenomenon that was completely rescued by the mPTP inhibitor cyclosporine A.(87) Similarly, in a cisplatin induced acute kidney injury, SIRT3 overexpression mitigated ROS-induced mitochondrial membrane depolarization and decreased autophagy.

Although there are limited studies to date specifically investigating the role of SIRT3 in either hemorrhagic or septic shock, SIRT3 appears play a vital role in mitigating inflammation, oxidant damage, and organ dysfunction.(284) Indeed, when the leukocyte gene expression profile in 172 blunt trauma patients presenting in hemorrhagic shock was investigated, the inability to increase SIRT3 expression was strongly associated with poor outcomes including infection, organ failure and death.(216)

This thesis explores the hypothesis that ***sirtuin pathways play a crucial role in shock and could be could be targeted to improve outcomes.*** Using a model of decompensated hemorrhagic shock in rats, we investigated the impact of pharmacologically activating SIRT1 during resuscitation. Specifically, we found that that nicotinamide monononucleotide (NMN) supported cellular energetics and enhanced physiologic resilience following hemorrhagic shock. NMN is the immediate biosynthetic precursor to NAD, and thus, provides an essential cofactor for sirtuin activation (Chapter 2). Similarly, we report that resuscitation with resveratrol, a naturally occurring SIRT1 activator, significantly improves mitochondrial function and decreases oxidative damage (Chapter 3). In separate and ongoing investigations, we explore the impact of manipulating SIRT1 and SIRT3 in a murine model of severe sepsis; noting that deletion of either sirtuin results in a pro-inflammatory phenotype with accelerated mortality (Chapter 4 and 5, respectively).

CHAPTER 2:

Nicotinamide Mononucleotide (NMN), a Key NAD Precursor, Improves Cellular Metabolism and Physiologic Resilience in Hemorrhagic Shock

INTRODUCTION

Hemorrhagic shock is a physiologic condition that occurs with rapid blood loss and is characterized by profound vasoconstriction, tissue hypoperfusion, and cellular hypoxia. In response to decreased oxygen tension, there is a dramatic decline in oxidative phosphorylation with increased anaerobic metabolism in an attempt to preserve cellular energy status.(42) Without prompt and adequate resuscitation, hemorrhagic shock progresses from cellular dysfunction to organ failure and, ultimately, to death.(183)

Nicotinamide adenine dinucleotide (NAD) is a ubiquitous molecule whose ability to accept and donate electrons via interconversion with NADH is key to cellular metabolism and energy generation. NAD is required at the GAPDH-dependent step in glycolysis and provides reducing equivalents to complex I of the electron transport chain to drive oxidative phosphorylation. NAD also serves as an essential co-substrate for a wide variety of enzymes involved in cellular resilience, including sirtuins and poly (ADP ribose) polymerases.(39) During hemorrhagic shock, the tissue concentration of NAD falls rapidly in proportion to the severity of the injury.(262) This decreased availability of NAD is further exacerbated by a redox shift in favor of NADH, which is no longer efficiently reoxidized to NAD by the mitochondrial electron transport chain under hypoxic conditions.(259) In other contexts, it is well established that NAD depletion leads to mitochondrial dysfunction and cell death (3,248) , and enhancing NAD has been shown to improve tissue function.(38,78,83) Given the central role that NAD plays in cellular energy metabolism, signaling, and a host of other biochemical reactions (39),

resuscitative strategies that restore NAD could prove therapeutically useful in hemorrhagic shock.

While NAD can be synthesized de novo from dietary tryptophan, nicotinic acid (niacin), or from other intermediates in the synthesis pathways, the majority of cellular NAD comes from the recycling of liberated nicotinamide. Nicotinamide is converted to nicotinamide mononucleotide (NMN) in a rate-limiting step catalyzed by the enzyme nicotinamide phophoribosyltransferase (NAMPT). NMN is then converted to NAD by one of three NMN adenylyltransferase isoforms (NMNAT1-3).(16)

Chaudry and colleagues first described using NAD precursors during the resuscitation of hemorrhagic shock in 1976.(44) Although the infusion of NAD, nicotinamide and nicotinic acid each increased NAD concentrations in both liver and kidney tissue, treatment did not restore tissue ATP levels and did not improve survival. As such, these investigators concluded that “these infusions have no salutary effects” and research investigating the impact of NAD metabolism during hemorrhagic shock remained relatively stagnant in the following decades. However, it is possible that the minimal resuscitation protocol used in Chaudry’s pioneering experiments may have limited the potential benefit of NAD supplementation. Indeed, recently, very high dose oral niacin (1080 mg/kg) has been shown to improve lung injury following hemorrhagic shock.(110) When given at the time of resuscitation, niacin restored lung NAD concentrations to baseline values, mitigated inflammation, and transformed a uniformly lethal model into one with 30% survival. Thus, the use of NAD precursors such as NMN warrants further investigation.

Although the benefit of NMN supplementation has not been investigated in trauma, there is a growing body of literature to suggest that NMN could prove therapeutically useful. Providing exogenous NMN bypasses the need for NAMPT in the salvage

pathway and has been shown to increase tissue NAD rapidly.(270) This is particularly important given the fact that NAMPT may be suppressed in the setting of ischemia-reperfusion and depressed NAMPT activity could contribute to the decreased NAD observed in hemorrhagic shock.(104) Moreover, the NAMPT-catalyzed reaction is energetically costly, and thus providing its product directly could be especially helpful when cells are energetically stressed.(31) Importantly, there is evidence that NMN can acutely mitigate the effects of ischemia-reperfusion injury. In a murine model of cardiac infarction, pretreatment with NMN 30 minutes before ischemia significantly increased basal levels of NAD, prevented a decline in NAD post-ischemic insult, and reduced infarct size.(266) As such, using NMN as resuscitative adjunct to support metabolism could improve cellular resilience.

In this study, we investigate the metabolic and mitochondrial impact of exogenous NMN on hemorrhagic shock. Our findings suggest that NMN preserves oxidative phosphorylation, enhances physiologic reserve, and improves survival after severe shock.

RESULTS

Pretreatment and resuscitation with NMN during fixed pressure hemorrhagic shock reduces lactic acidosis

To investigate the physiologic impact of NMN during hemorrhagic shock and resuscitation, we subjected NMN pretreated rats (400mg/kg/day X 5 days, oral) and controls to a fixed pressure shock followed by resuscitation with and without NMN (400 mg/kg, intravenous) (Figure 3A). NMN and control animals were maintained at a mean arterial blood pressure (MAP) of 40 mmHg for 90 minutes. Both groups shed a similar percentage of blood volume and had similar reductions in circulating hemoglobin (Figure

3B, E). Although the volume of fluid required to maintain the shock state for 90 minutes and the blood pressure following resuscitation were statistically indistinguishable between the groups, NMN treated rats received slightly less volume overall, indicating that they were not resuscitated more completely than controls (Figure 3C,D). Importantly, despite experiencing the same degree and duration of blood loss, NMN treated rats had significantly lower serum lactate than did controls during hemorrhagic shock and resuscitation (Figure 3E). As a byproduct of anaerobic metabolism, elevated lactate levels reflect ongoing tissue hypoperfusion and correlate with both the severity and survivability of shock.(196) Moreover, lactate is used to gauge the success of resuscitation in humans.(27) Thus, our findings suggest that NMN partially mitigates a key metabolic derangement induced by hemorrhagic shock.

NMN increases NAD and preserves bioenergetics

Hemorrhagic shock results in significantly depressed pyridine and adenine nucleotide pools, which can negatively impact the function of vital organs.(43) We tested whether exogenous NMN could preserve NAD and ATP levels in kidney and liver tissues following shock. In sham animals, NMN significantly increased NAD levels in both kidney and liver (Figure 4A, 2B). Following shock, NAD levels were nearly 3 fold higher in NMN treated animals compared to shock control animals. NADH was also significantly higher in NMN treated animals following shock. While the decline in NAD during hemorrhagic shock was less dramatic than in previous reports(44), we observed robust depression of the NAD/NADH ratio (Figure 4A, B). Importantly, NMN completely mitigated the decrease in NAD concentration and the NAD/NADH redox ratio in both tissues.

Hemorrhagic shock and resuscitation are also known to deplete ATP reserves, likely as a consequence of mitochondrial dysfunction, which results in increased reliance on

anaerobic metabolism. As expected, ATP levels were significantly decreased following shock and resuscitation in both kidney and liver (Figure 4C). NMN treatment substantially rescued ATP levels in the kidney, whereas liver stores were more depressed and could not be restored (Figure 4C).

NMN preserves NAD dependent mitochondrial respiration

Given the restoration of ATP in kidney and decrease in lactate accumulation, we wondered if NMN treatment could positively impact mitochondrial respiration. We found that hemorrhagic shock and resuscitation impaired complex I (CI)-dependent respiration in both tissues when fatty acids or a standard pyruvate/glutamate/malate mixture were provided as substrates (Figure 5A, B). No defect was apparent when electrons were supplied directly to complex II via succinate or to complex IV via TMPD/ascorbate. Since CI accepts electrons from NADH, these results suggest a deficiency in the NAD pool, an upstream factor involved in the generation of NADH, or in CI itself. Consistent with the possibility that NAD(H) is the limiting factor, treatment with NMN completely preserved CI-dependent respiration following hemorrhagic shock (Figure 5A,B).

To more directly test the hypothesis that mitochondrial NAD depletion causes the defect in CI-dependent respiratory capacity, we next sought to determine NAD levels in isolated mitochondria following shock. To our knowledge, the mitochondrial NAD pool, which is separate from the nuclear and cytosolic pool,(267) has not previously been examined in hemorrhagic shock. Although NMN significantly increased mitochondrial NAD in both kidney and liver, we were surprised to discover that hemorrhagic shock did not decrease mitochondrial NAD. In fact, in contrast to whole tissue NAD, mitochondrial levels were maintained in kidney (Figure 5C) and significantly increased in the liver of control animals following shock (Figure 5D). NMN treatment further augmented mitochondrial NAD concentrations (Figure 5C, D). Thus, the size of the mitochondrial

NAD pool per se cannot account for the changes in mitochondrial respiration. NADH was not reproducibly detected in isolated mitochondria, most likely due to collapse of the redox potential during isolation.

NMN and hemorrhagic shock influence the expression of enzymes in the NAD salvage pathway

To better understand the impact of hemorrhagic shock on total and mitochondrial NAD levels, we examined the expression patterns of enzymes in the NAD salvage pathway. Metabolic stress influences the expression of NAMPT and increasing NAMPT can mitigate ischemic injury in a variety of models.(104,199,238,266) The expression of NAMPT and the downstream enzymes NMNAT1-3, however, have not previously been examined in hemorrhagic shock. Following shock, there was a trend toward increased NAMPT gene expression in both kidney and liver (Figure 6A,E). Of the three NMNAT isoforms, NMNAT1 (nuclear) and NMNAT3 (putatively mitochondrial) were readily detected while NMNAT2 (which has been reported in the Golgi complex and axons) was not.(16) Hemorrhagic shock and resuscitation had minimal impact on the renal expression of NMNAT1, but modestly increased expression of NMNAT3. In contrast the expression of both enzymes was reduced in liver tissue (Figure 6 C, D, G, H). Although NMN had no effect on NAMPT or NMNAT gene expression in sham operated rats, it enhanced the gene expression of both NAMPT and NMNAT3 relative to controls following hemorrhagic shock (Figure 6A,D,E,H). Shock, however, was not associated with any clear change in NAMPT protein expression at the end of resuscitation (Figure 6B,F). These results indicate that NMN can influence NAD metabolism in part via enzyme expression independently from its direct contribution to synthesis.

NMN mitigates inflammation following hemorrhagic shock and resuscitation

Hemorrhagic shock and resuscitation results in a pro-inflammatory state characterized by elevated cytokine levels, oxidative stress, and insulin resistant hyperglycemia. Circulating levels of IL6, a pro-inflammatory cytokine, strongly correlate with mortality and directly targeting IL6 improves survival, suggesting that inflammation mediated by this pathway is highly relevant to the pathological effects of hemorrhagic shock.(165,227) Given the key role that NAD plays in modulating sirtuin-dependent inflammatory pathways,(150) we hypothesized that NMN might dampen the inflammatory response following hemorrhagic shock and resuscitation. Indeed, pretreatment and resuscitation with NMN significantly reduced systemic IL6 cytokine levels and tended to decrease TNFα, while substantially ameliorating shock-induced hyperglycemia (Figure 7A, B). Interestingly, basal TNFα was significantly reduced in sham-treated animals, suggesting that NMN can have a potent effect on this pathway, although the repression was largely overcome during shock. At the tissue level, NMN also significantly improved the post-resuscitation cytokine profile in the kidney (Figure 7C). Whether these changes in gene expression reflect immune cell infiltration or the activation of inflammatory pathways in resident cells is not yet clear. In contrast, NMN had no impact on the induction of inflammatory genes in liver (Figure7D).

NMN improves mitochondrial function in isolated hepatocytes

Because NMN improves the inflammatory status, its beneficial effects on mitochondrial function may be secondary to either decreased inflammation or direct protective effects in targets tissues. In order to determine whether NMN directly impacts cellular mitochondrial function, we exposed primary hepatocytes to plasma harvested from both sham and shocked rats. Consistent with the hypothesis that circulating inflammatory factors can induce mitochondrial dysfunction, primary hepatocytes treated with shocked plasma had significantly depressed CI-dependent respiration. When

hepatocytes were co-cultured with NMN, however, this CI defect was completely prevented (Figure 8A).

Given that plasma from shocked and sham animals may have varying concentrations of many different pro-inflammatory cytokines, we also treated primary hepatocytes with a fixed concentration of IL6. In these experiments, co-treatment with NMN again completely prevented mitochondrial dysfunction (Figure 8B). Thus, NMN can preserve mitochondrial function in a cell-autonomous manner, although the reduction in circulating IL6 also likely contributes to its net benefit *in vivo*.

Interestingly, treatment with IL6 did not significantly impact NAD levels in our primary hepatocytes. With the addition of NMN, however, cytokine exposed cells nearly doubled their NAD over baseline suggesting that inflammation enhances either NMN uptake or the enzymatic activity of NAD biosynthesis pathways, or decreases NAD turnover (Figure 8C).(206)

The impact of NMN on organ function

In order to determine if NMN preserved organ function, blood samples were taken from sham and shocked animals at the end of resuscitation and assayed for markers of liver, kidney and cardiac dysfunction. Although hemorrhagic shock and resuscitation resulted in a significant increase in markers of liver and kidney damage, values remained within the normal range with only a trend toward decreased injury in NMN treated animals (Table 1). The fact that these injury markers remained relatively low is likely a reflection of the early time point examined. Even at this early time point, however, hemorrhagic shock and resuscitation was associated with an increased in creatine kinase activity that was mitigated by NMN. Creatine kinase levels can result from either skeletal or cardiac muscle damage. Acute blood loss does not generally lead to skeletal muscle damage in the absence of crush injury and is associated more

strongly with cardiac injury.(118) Thus, NMN may have cardioprotective effects that warrant further investigation in this model.

NMN enhances physiologic reserve and decreases mortality

Based on improvements in inflammatory status, lactate metabolism, mitochondrial function, and trends toward decreased injury biomarkers, we hypothesized that NMN would enhance physiologic reserve and improve survival following hemorrhagic shock. Using a decompensated shock model, we performed a second set of experiments to evaluate physiologic reserve, as indicated by the overall time animals could be maintained in severe shock without resuscitation (Figure 9A). Pretreatment with NMN increased the time animals could sustain severe shock by nearly 25%, despite equal or greater blood loss when compared to controls (Figure. 9B, C). In spite of enduring this more strenuous injury, animals treated with NMN maintained lower lactate levels and exhibited improved survival (Figure 9D,F). As such, pre-treatment and subsequent resuscitation with NMN substantially improved the ability of rats to tolerate hemorrhagic shock.

DISCUSSION

In the United States, traumatic injury is the leading cause of death in those under the age of 45, with nearly 40% of patients who develop hemorrhagic shock dying within the first 24 hours.(93,122) Because both the severity of blood loss and duration of hypoperfusion correlate directly with organ failure and mortality, current resuscitative strategies focus on rapidly repairing the injury, restoring blood volume and improving perfusion pressure. If the shock state is too severe or prolonged, however, cellular metabolism remains depressed and resuscitation efforts fail to improve clinical

outcomes. Developing therapies to restore and support cellular metabolism following hemorrhagic shock could potentially mitigate organ dysfunction and improve survival.

During prolonged hypoperfusion there is a dramatic decline in NAD availability due to both a reduction in the size of the NAD pool as well as a shift toward the reduced form (NADH). Given the essential roles that NAD plays in glycolysis, oxidative phosphorylation, and pathways that promote cellular resilience, we investigated the possible benefit of using NMN to restore NAD levels as a treatment for hemorrhagic shock.(41,172,270) We specifically focused on the impact on bioenergetics in kidney and liver because these organs are at a high risk of failure following acute blood loss.(15,91) Pretreatment and subsequent resuscitation with NMN increased NAD levels, restored the NAD/NADH ratio, improved mitochondrial function and mitigated inflammation. Importantly, NMN also enhanced whole organism physiologic resilience during hemorrhagic shock and improved survival following resuscitation. As such, NMN holds promise as a therapeutic adjunct and warrants further clinical investigation.

One of the most prominent metabolic effects of using NMN to restore NAD was the improvement in mitochondrial function. During hemorrhagic shock, there is a progressive defect in mitochondrial respiration when NAD-linked substrates such as isocitrate, α ketoglutarate and β hydroxybutyrate are used, suggesting that severe blood loss preferentially inhibits complex I.(42,193) Although NMN has been shown to improve overall mitochondrial respiratory capacity in chronic models of aging and Alzheimer's disease,(153,167) our research provides the first data demonstrating NMN completely preserves CI-dependent mitochondrial respiration following hemorrhagic shock. We initially hypothesized that this rescue was secondary to NMN's ability to mitigate a decline in mitochondrial NAD. Our data, however, do not support this hypothesis. In fact, in contrast to the decline in total tissue NAD that we observed,

mitochondria harvested from control animals actually had preserved or *increased* levels of NAD following hemorrhagic shock. Mitochondrial NAD levels were further enhanced in NMN treated animals. Although surprisingly little is known about the concentration of mitochondrial NAD following hemorrhagic shock, our findings do support Hift and Strawitz's description of increased "light absorbing material" in isolated liver mitochondria harvested from dogs in "irreversible hemorrhagic shock". Writing in 1961, these investigators attributed the increased spectrophotometric absorption peak noted at 265 to 270mμ to "nucleotide material".(94) In retrospect, this likely represented NADH, with a UV absorption peak of 259 nm. Although the precise mechanisms accounting for the generation and maintenance of the mitochondrial NAD pool remain a subject of debate, the finding of increased mitochondrial NAD suggests either augmented cytoplasmic to mitochondrial transfer or enhanced mitochondrial NAD recycling.(39) In either case, the ability to increase mitochondrial NAD following hemorrhagic shock may reflect an adaptive response to cellular stress, and suggests that the depletion of cytosolic NAD may be even greater than indicated by bulk tissue measurements.

Given the fact that mitochondrial NAD levels are preserved during hemorrhagic shock, there must be another mechanism by which NMN benefits mitochondrial function. CI is prone to oxidant damage and dysfunction, but can be rescued with the induction of antioxidant defenses.(40,84,173,182,228) Increasing nuclear/cytosolic NAD has been shown to modulate the response to cellular stress by up-regulating anti-inflammatory and anti-oxidant pathways.(211) In particular, SIRT1 uses NAD as an obligatory co-substrate to deacetylate the RelA/p65 subunit of NFκB in macrophages leading to decreased expression of pro-inflammatory cytokines such as IL6 and TNFα.(207) SIRT1 may also decrease the damage associated with ischemia-reperfusion by deacetylating and activating PGC1α. In turn, PGC1α increases the expression of antioxidant enzymes

as well as SIRT3, a mitochondrial NAD-dependent deacetylase that mitigates mitochondrial oxidative stress by directly activating isocitrate dehydrogensase 2 and manganese superoxide dismutase.(191,277) In support of a potential role for SIRT1, we and others have shown that resuscitation with resveratrol, a SIRT1 activator, improves mitochondrial function, attenuates inflammation, mitigates oxidant damage, and improves organ function following hemorrhagic shock.(8,188,252,253) Similarly, Jeung et al demonstrated that high dose oral niacin (another NAD precursor and SIRT1 activator) attenuates systemic inflammation and is associated with decreased pulmonary NFkB expression and oxidative stress in a rodent model of hemorrhagic shock. In this study, NMN mitigated both the systemic and tissue specific inflammatory response.(110) Importantly, changes in systemic inflammation were not purely responsible for improved mitochondrial function because NMN also preserved CI-dependent mitochondrial respiration in permeabilized hepatocytes exposed to a controlled dose of inflammatory cytokines. Thus, NMN also appears to have a cell-autonomous anti-inflammatory impact.

Interestingly, while NMN rescued NAD levels and mitochondrial function in both kidney and liver, it only preserved ATP in the kidney. The total amount of ATP, however, is influenced by both production and consumption. During hemorrhagic shock, ATP consumption is significantly decreased in the kidney because hypotension leads to decreased glomerular filtration and thus a decreased need for ATP dependent resorption in the distal nephron. In contrast, ATP consumption in liver actually escalates during hemorrhagic shock because increased sodium influx requires increased Na+-K+ ATPase activity.(9) Thus, failure to rescue ATP in the liver may not reflect a tissue difference in how NMN impacts mitochondrial function, but rather a difference in the degree of energetic stress experienced by these two tissues during shock.

Very little is known about the NAD salvage pathway in hemorrhagic shock. We wondered if decreased expression of either NAMPT or NMNAT contributed to the NAD depletion during shock, or alternatively, whether any compensatory response would be present. Although ischemia-reperfusion injury decreases NAMPT expression in cardiac myocytes,(104) hemorrhagic shock appears to increase the expression of NAMPT mRNA in liver and tended to increase it in kidney. Surprisingly, NMN supplementation further enhanced NAMPT mRNA expression in both tissues following resuscitation, but not in sham NMN treated controls. We were also interested in determining if the expression of NMNAT1-3 changed following hemorrhagic shock. NMNAT2 was not reliably detected, but we were able to quantify transcripts for NMNAT1 and NMNAT3, which convert of NMN to NAD in the nucleus/cytoplasm and mitochondria respectively. Both NMNAT1 and NMNAT3 were suppressed with shock in the liver, suggesting that NAD biosynthetic capacity may be decreased in this organ during acute blood loss. In contrast, NMNAT1 was not affected and NMNAT3 was increased in the kidney. Intriguingly, NMN enhanced NMNAT3 expression following shock in both organs. The induction of transcripts related to NAD biosynthesis, particularly when precursors are available, likely reflects an adaptive response to cellular stress and may explain why our isolated hepatocytes only dramatically increased NAD in the presence of both IL6 and NMN.(267) Thus, hemorrhagic shock leads to organ-specific changes in the expression of genes related to NAD biosynthesis, and we reveal that in the setting of severe injury, the direct contribution of NMN to the NAD pool may be augmented by the induction of biosynthetic enzymes.

While encouraging, our study has a number of limitations. First, we investigated pretreating and subsequently resuscitating hemorrhagic shock with NMN. This approach suggests that NMN may be useful when significant surgical blood loss (e.g.

cardiac or vascular surgery) or traumatic injury is anticipated (e.g. combat). However, it will be important to determine whether NMN can be used post-injury to treat unexpected bleeding events or trauma. We are currently planning experiments to test the benefit of using NMN only during resuscitation and are encouraged by recent data from Yamamoto *et al* demonstrating that NMN limits myocardial ischemia when given during reperfusion.(266) Second, we did not investigate the optimal duration of therapy. This is particularly important because the duration of increased NAD and the timing of SIRT1 activation may influence how acute inflammation progresses to a more chronic hypo-inflammatory state.(150) Finally, although we did see a trend toward improved organ function following hemorrhagic shock in NMN treated animals, studying early changes in mitochondrial function required sacrificing rats at a time point that was not optimal for these assays; future studies over an extended time course may strengthen these findings.

Together, our findings suggest that NMN holds significant promise as an adjunct to resuscitation. In our severe hemorrhagic shock model, NMN supported cellular metabolism by increasing tissue NAD levels, preserving the cellular redox ratio, and enhancing CI-dependent mitochondrial respiration. NMN also appeared to have anti-inflammatory properties, blunting both the systemic inflammatory response as well as the cellular effects of cytokine exposure. Importantly, NMN's biochemical and immunologic benefits translated to improved physiologic resilience. NMN treated animals were able to tolerate longer periods of hypoperfusion with improved survival. Given these encouraging preclinical findings, future research investigating NMN's therapeutic potential in injured patients is warranted.

CHAPTER 3:

Resveratrol Improves Mitochondrial Function but Increases the Risk of Hypoglycemia Following Hemorrhagic Shock

Hemorrhagic shock remains a common cause of death in severely injured patients with a significant number of deaths occurring as the result of multi-organ failure (MOF) days to weeks after the initial injury.(168,240) Because impaired tissue oxygen delivery and depletion of cellular energy stores during traumatic clearly contribute to the development of MOF and early mortality in trauma patients,(61,170,171) current resuscitation strategies focus on replacing circulating blood volume in an attempt to restore adequate tissue perfusion.

While re-establishing tissue oxygenation is clearly vital, there is increasing evidence to suggest that mitochondrial dysfunction also plays a significant role in the development of MOF.(120,129) Under normal physiologic conditions, mitochondria provide almost 95% of the available adenosine triphosphate via oxidative phosphorylation; and oxygen utilization by the mitochondrial electron transport system is tightly coupled to oxygen availability.(115) “Decoupling” can occur, however, when mitochondrial complexes are modified or there is decreased availability of NADH leading to decreased electron flow.(4) Decoupling not only results in decreased ATP production, it is associated with the generation of damaging free radical oxygen species.(107) With prolonged hemorrhagic shock, there is a progression from decreased oxidative phosphorylation to irreversible, structural damage to the mitochondria.(214,234) With resuscitation previously hypoxic mitochondria cannot effectively transfer electrons; resulting in a

deluge of reactive oxygen species (ROS) upon reperfusion and increased damage to mitochondrial proteins.(57)

Importantly, mitochondrial dysfunction persists even if adequate tissue oxygen and perfusion are restored. Using near-infrared spectroscopy (NIRS) in an a rabbit model of severe hemorrhagic shock, Rhee *et al* noted that despite normalization of blood pressure, cardiac output and tissue oxygen delivery, mitochondrial cytochrome a,a3 oxidation remained significantly decreased in splanchnic and skeletal muscle beds.(192) In a similar experiment, decoupling of the hepatic cytochrome a,a3 redox state from oxygen delivery was associated with increased early mortality following hemorrhagic shock.(32) Clinically, shock-induced mitochondrial dysfunction has also been linked to the development of MOF. In a prospective study of 24 severely injured patients monitored continuously with NIRS, Cairns *et al* noted that patients who developed MOF demonstrated a significantly higher incidence of mitochondrial decoupling early in the course of their resuscitation when compared to those who did not develop organ dysfunction (89% vs 13%, $p<0.05$).(33)

Mitochondrial dysfunction also contributes to the phenomenon of post-injury hyperglycemia. In addition to damaging proteins, ROS can influence insulin signaling by directly oxidizing the insulin receptor or indirectly by activating various protein tyrosine phosphatases.(47) Importantly, the development of post-injury insulin resistance and hyperglycemia can be minimized by blocking the production of ROS.(280) Because hyperglycemia is an independent risk factor for adverse outcomes, including renal failure and death in trauma patients,(135,169) resuscitation strategies that either mitigate mitochondrial dysfunction or improve glycemic control could prove beneficial.

Resveratrol, a naturally occurring polyphenol found in red wine, has been shown to promote mitochondrial function, reduce oxidative damage, and improve glycemic control

in a variety of disease states.(134,154,243) Although many of resveratrol's biological effects are thought to be mediated by SIRT1 – a highly conserved NAD-dependent deacetylase, its mechanism as a direct SIRT1 activator has been called into question.(13,20,116,178) In previous studies evaluating resveratrol, fluorophore-tagged substrates were used to assess SIRT1 activity. However, when non-tagged peptides were used, SIRT1 activity was not enhanced, suggesting that resveratrol may be binding to the unnatural fluorophore.(116,178) More recently, however, the Sinclair group investigated the relationship between resveratrol, and other sirtuin activating compounds (STACs), using non-fluorescent techniques. In addition to discovering that resveratrol allosterically interacts with specific hydrophobic motifs shared by SIRT1 substrates, they found that a glutamine in SIRT1's N-terminal domain was essential for activation.(106) Resveratrol allosterically lowers the Km for binding peptide substrates and NAD, thereby increasing SIRT1's activity nearly 10 fold even when SIRT1 and NAD levels are limited. Resveratrol can also enhance SIRT1 by activating AMPK (12,279) which in turn enhances the expression of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in the NAD salvage pathway.(74)

Although there is a growing body of literature to suggest that resveratrol significantly reduces inflammation and mitigates organ dysfunction in rodent models of hemorrhagic shock (111,239,273,276), there is little data regarding its impact on mitochondrial function or glucose regulation following acute blood loss. Given resveratrol's interaction with SIRT1 can promote mitochondrial function a number of ways (Figure 2) (198,205,226), we hypothesized that resuscitation with resveratrol would ameliorate shock-induced mitochondrial dysfunction and improve hyperglycemia.

RESULTS

Physiologic and laboratory parameters

In contrast to previous studies, resuscitation with resveratrol (RSV) did not significantly improve the mean arterial blood pressure (MAP) in our model (8). In fact, blood pressure during the 18 hours post-resuscitation remained significantly lower than baseline values in both groups (Figure 10A). As expected, our model of decompensated hemorrhagic shock did result in severe lactic acidosis as well as a significant anemia given the crystalloid-only resuscitation (Figure 10B, Table 2).(222) Nonetheless, when compared to lactated Ringer's (LR) alone, resuscitation with LR+RSV resulted in significantly less lactate production (10.2 ± 3.0 *vs.* 6.9 ± 3.3mmol/L, $p < 0.05$). At 18 hours, lactate levels in both groups had normalized. Although clinical assays of acute kidney injury (AKI), such as BUN and creatinine, were not elevated above normal values in either group post resuscitation, resveratrol did significantly decrease in serum neutrophil gelatinase-associated lipocalin (NGAL) levels (Table 2). NGAL is a 25kd protein that is covalently bound to matrix metalloproteinase 9 in neutrophils. Following epithelial injury, the expression of NGAL is markedly induced and appears to be a more sensitive biomarker for AKI than serum creatinine.(54,98) Elevations in NGAL precede changes in serum creatinine and can be used to diagnose AKI up to 48 h prior to a clinical change in creatinine or urine output. Given this sensitivity, changes in NGAL are thought to reflect AKI in real time, and following NGAL levels may allow for the institution of earlier, and more effective, renoprotective therapies.(98)

While previous studies have reported that resveratrol significantly decreases IL6 in various tissues following hemorrhagic shock(239,261,272,273), only one has reported a decrease in serum TNFα.(111) In our study, we did not appreciate a significant decrease in serum levels of either TNFα or IL6. Given that tissue cytokines can

contribute to local damage, it is possible that we may have observed more of a significant difference had we directly assayed renal tissue.

Resveratrol supplementation during resuscitation restored mitochondrial function following hemorrhagic shock and decreased mitochondrial ROS production.

Using high-resolution respirometry, we observed that hemorrhagic shock significantly decreased the respiratory capacity of all mitochondrial complexes in both kidney and liver (Figure 11A,B). Following resuscitation, resveratrol significantly improved CII and CIV-dependent respiratory capacity in the kidney and robustly restored the respiratory capacity of all complexes in liver. Notably, resveratrol was associated with sustained improvement in respiratory capacity 18 hours post-resuscitation. resveratrol supplementation also significantly decreased mitochondrial ROS production following resuscitation (Figure 11C, D). Although treatment with resveratrol continued to mitigate ROS production in the liver 18 hours post-resuscitation, the generation ROS in the kidney normalized by 18 hours and levels did not differ between resuscitation strategies.

Resveratrol treatment during resuscitation ameliorated mitochondrial oxidative stress following hemorrhagic shock and resuscitation

Resuscitation with resveratrol resulted in a significant increase in the mRNA expression of superoxide dismutase 2 (SOD2) and catalase (CAT) in kidney tissue when compared to resuscitation with LR alone (Figure 12B). In contrast, severe hemorrhagic shock was associated with a marked increase in the expression of cyclooxygenase 2 (COX-2) mRNA that was not influenced by either resuscitative strategy (Figure 12A).

Oxidative damage to mitochondrial proteins increased dramatically following severe hemorrhagic shock and improved with resveratrol treatment during resuscitation. 4-hydroxynonenal, a measure of lipid peroxidation from reactive oxygen species, increased robustly following severe shock (Figure 12B). Resveratrol supplementation

significantly ameliorated the degree of lipid peroxidation observed. We also measured the degree of nitrosative stress. While 3-nitrotyrosine levels increased with LR resuscitation, resveratrol was not associated with a significant improvement in nitrosative stress when compared to LR (Figure 12C).

Resveratrol supplement increased the mRNA expression of SIRT1 and the NAD/NADH ratio in kidney

Severe shock resulted in a dramatic decline in the mRNA expression of SIRT1. Resuscitation with resveratrol, however, restored expression of SIRT1 to the levels seen in the sham group (Figure 13A). Although hemorrhagic shock and subsequent resuscitation with LR alone did not significantly alter NAD or NADH concentrations, resuscitation with resveratrol significantly decreased tissue NADH levels (160.6 ± 21.3 *vs.* 265.7 ± 24.5 nmol/mg protein) and nearly doubled the NAD/NADH ratio (Figure 13B).

Resuscitation with resveratrol enhances pyruvate dehydrogenase and α-ketoglutarate dehydrogenase activity following hemorrhagic shock

Hemorrhagic shock is known to decrease the activity of key enzymes in the Krebs cycle including pyruvate dehydrogenase.(42) In order to determine if resveratrol enhanced the activity of Krebs cycle enzymes, we measured the rate of NAD conversion to NADH spectrophotometrically. As expected, after hemorrhagic shock, the activity of all enzymes measured decreased significantly in the kidney (Figure 14). Resuscitation with resveratrol, however, robustly enhanced the activity of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. Future experiment will determine if this improved activity is associated with a change in acetylation status.

Resveratrol increased transcription of PGC1-α in kidney, but did not promote mitochondrial biogenesis

To gain further insight into the potential impact of resveratrol on mitochondrial biogenesis following hemorrhagic shock, we evaluated citrate synthase activity as a proxy for mitochondrial content in the kidney, but did not observe a significant change at any time point (Figure 15A). We also investigated the expression of mitochondrial transcriptional regulators including PGC1α. Notably, while the mRNA expression of PGC1α was significantly elevated in the resveratrol L resuscitation group, resveratrol did not restore the expression of mitochondrial transcription factor A (TFAM) (Figure 15B). Interestingly, the expression of nuclear respiratory factor (NRF) -1 was not significantly altered by either hemorrhagic shock or by resuscitation, whereas resuscitation significantly up-regulated the expression of NRF-2 regardless of strategy.

Resveratrol significantly lowered blood glucose and improved insulin resistance following resuscitation, but was associated with decreased serum insulin levels

As previously described (280), hemorrhagic shock resulted in severe hyperglycemia, hyperinsulinemia, and increased insulin resistance. Notably, resuscitation with resveratrol lead to a significant reduction in blood glucose that persisted up to 90 minutes post-resuscitation (Figure 16A). In fact, resveratrol was associated with glucose levels that were significantly lower than sham operated animals with roughly 33% of the resveratrol animals developing true hypoglycemia (glucose < 70mg/dl). No animal in any other group developed such low glucose levels. By 18 hours, the hypoglycemia had resolved and groups were not significantly different.

Although resveratrol resulted in lower glucose levels, this was not caused by an increase in insulin secretion. In fact, following resuscitation, resveratrol treated animals had significantly lower serum insulin levels as well as improved insulin resistance as measured by the Ln HOMA-IR index.(113) All glycemic parameters, however, were similar between treatment groups 18 hours post resuscitation (Figure 16A-C).

Resveratrol increases plasma glucagon-like peptide-1 (GLP-1) levels following resuscitation

Decompensated hemorrhagic shock resulted in significant hyperglycemia and was accompanied by elevated glucagon and corticosterone levels (Figure 16F, G). Glucagon-like peptide-1, a gut-derived hormone known to enhance insulin secretion in response to glucose, was also elevated (Figure 16E). Interestingly, when compared to resuscitation with LR, resveratrol supplementation resulted in significantly higher levels of GLP-1 (Figure 16E). Resveratrol did not significantly influence either glucagon or corticosterone secretion; and at 18 hours, all hormone levels were similar regardless of resuscitation strategy.

Resveratrol supplementation preserved the active form of IRS1 in both liver and kidney tissues

Insulin receptor substrate-1 (IRS1) plays a key role in insulin signaling pathways and is activated upon tyrosine phosphorylation (pY612 IRS1). Following resuscitation with LR, the expression level of pY612 IRS1 in liver and kidney tissue was significantly reduced and could contribute to post-injury hyperglycemia. Resveratrol, however, preserved the expression of phosphorylated IRS1 immediately post-resuscitation (Figure 17A,B). At 18 hours, however, the expression level of pY612 IRS1 did not differ between treatment strategies.

DISCUSSION

Resveratrol, a naturally occurring polyphenol with known antioxidant, anti-inflammatory, and anti-glycemic properties, has been shown to be protective in various models of human disease.(111,244,274) Given hemorrhagic shock is frequently complicated by mitochondrial dysfunction, exuberant ROS production, and

hyperglycemia, we hypothesized that adding resveratrol to standard fluid resuscitation would prove beneficial.(120,280) In our model of decompensated hemorrhagic shock, resveratrol supplementation significantly ameliorated mitochondrial dysfunction, decreasing both the production of mitochondrial ROS and the subsequent damage from lipid peroxidation. Resveratrol also decreased serum glucose levels and improved insulin sensitivity by enhancing GLP-1 secretion and by preserving IRS1 phosphorylation. Finally, our data suggests that resveratrol benefits mitochondrial function in the acute setting, not by promoting mitochondrial biogenesis, but by activating SIRT1 and PGC1α-mediated antioxidant pathways.

Currently, the initial approach to treating hemorrhagic shock is volume replacement with crystalloid fluids in order to increase the circulating intravascular volume, restore blood pressure, and maintain organ perfusion.(224) Crystalloid resuscitation, however, does not prevent systemic inflammation or oxidative stress.(2,136) Moreover, we have previously reported that resuscitation with lactated Ringer's does not restore mitochondrial respiratory capacity in vital organs such as the heart, liver and kidney.(119) Similarly, in this study, resuscitation with lactated Ringer's did not restore mitochondrial respiratory capacity or mitigate mitochondrial oxidative stress in the kidney. Because mitochondrial dysfunction can further exacerbate cellular damage by reducing aerobic ATP production and increasing the generation of reactive oxygen species (ROS) (129), resuscitative fluids that preserve mitochondrial function or augment antioxidant capacity could prove beneficial.

Resveratrol has previously been shown to be protective in several models of critical illness. In septic animals, treatment with resveratrol prevented oxidative damage in lymphocytes (7), preserved tissue morphology in the lung and kidney (125), mitigated acute lung injury and prevented myocardial depression.(142,220) Resveratrol has also

been shown to be beneficial in several trauma-hemorrhage models. Resuscitation with resveratrol not only improves cardiac output (239), but has been shown to decrease hepatic injury and inflammation following hemorrhagic shock.(276) As such, resveratrol may provide a useful therapeutic adjunct to standard fluid resuscitation following traumatic injury.

Although the mechanism underlying resveratrol's benefit remains controversial (235), resveratrol appears to attenuate injury by activating SIRT1 dependent pathways. SIRT1, a NAD dependent "survival" enzyme, deacetylates and activates PGC1α; a key regulator of mitochondrial function and metabolism.(127,251) In turn, PGC1α impacts two different pathways that may be critical to cell survival following ischemia-reperfusion (Figure 1). Firstly, PGC1α is required for the induction of many ROS-detoxifying enzymes, including SOD2 and catalase.(145,226) Secondly, PGC1α induces mitochondrial biogenesis by binding to NRFs and enhancing their activity.(244) NRFs subsequently increase the expression of TFAM, an enzyme directly responsible for the transcription of nuclear-encoded mitochondrial proteins.(80,226) As an upstream regulator of PGC1α, strategies that enhance SIRT1 expression or activity may also play an important role in regulating oxidative stress and mitochondrial biogenesis *in vitro*. Importantly, the antioxidant and mitochondrial effects of resveratrol also appear to be SIRT1 mediated. In cell culture models, knocking down SIRT1 not only blocked the protective effects of resveratrol on mitochondrial oxidative stress (265), it also prevented resveratrol-induced up-regulation of mitochondrial biogenesis factors.(55)

In this study, adding resveratrol to standard resuscitation with lactated ringer's dramatically restored mitochondrial function while mitigating oxidative damage. While resveratrol has been shown to enhance the enzymatic activity of succinate dehydrogenase, a protein that constitutes complex II on the inner mitochondrial

membrane (134), in our study, resveratrol also appears to dramatically restore CIV activity. Although it is possible that resveratrol could directly impact CIV activity, it is more likely that the benefit observed stems from resveratrol's known antioxidant effects.(244) Both ROS and lipid peroxidation products can effectively inhibit CIV activity following ischemia-reperfusion.(46,174) In this current study, resuscitation with resveratrol resulted in decreased ROS production and 4-hydroxynonenal damage following hemorrhagic shock. Thus, the salutary effect of resveratrol on mitochondrial function could be in part secondary to decreased CIV inhibition by oxidant byproducts.

Resveratrol also appears to enhance antioxidant defenses following hemorrhagic shock. In this study, resveratrol significantly increased the expression of both SOD2 and catalase following hemorrhagic shock, which could have contributed to the observed decrease in mitochondrial oxidative stress. Alternatively, resveratrol supplementation in our study effectively prevented the reduction in CII and CIV activity following hemorrhagic shock, which may have also contributed to the observed decrease in ROS production. Thus, the improvement in oxidative damage observed with resveratrol may be multifactorial – resuscitation with resveratrol may either increase antioxidant defenses, decrease mitochondrial ROS production, or both.

In contrast to previous studies demonstrating augmented mitochondrial biogenesis (55,134,244), resveratrol did not seem to promote mitochondrial biogenesis in our acute hemorrhagic shock model. Although the expression of PGC1α was enhanced, resveratrol had no effect on the other transcriptional factors regulating mitochondrial biogenesis (e.g. NRF-1, NRF-2 and TFAM) and did not change mitochondrial content as measured by citrate synthase activity. Since NRF-1, NRF-2 and TFAM are downstream targets of PGC1α, further studies with longer observation are needed to determine if resveratrol has any long-term effects on mitochondrial biogenesis. Finally, because

resuscitation with resveratrol increased the transcript levels of SIRT1, PGC1α, and the downstream targets, SOD2 and catalase, we conclude that the acute benefits of resveratrol appear to be mediated via an antioxidant, rather than mitochondrial biogenesis pathway.

Resveratrol may also improve mitochondrial function by favorable impacting cellular redox potential.(147,197) In this study, resveratrol supplementation significantly decreased the NADH concentration and nearly doubled the NAD to NADH ratio. It is possible that this favorable change in the NAD to NADH ratio have influenced SIRT1 activity in our model.(201)

We also found that resveratrol significantly enhanced the activity of two key Krebs cycle enzymes, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. By converting pyruvate to acetyl CoA, pyruvate dehydrogenase serves a key mediator between glycolysis and oxidative phosphorylation. α-ketoglutarate dehydrogenase (αKDH), on the other hand, serves as the Krebs cycle rate-limiting enzyme and can be reversibly inhibited by ROS. As such, resveratrol's ability to increase αKDH may be secondary to its antioxidant effects. Moreover, using resveratrol during resuscitation potentially promoted the entry of NADH into electron transport.

Although resveratrol is known to have anti-glycemic effects in chronic models of obesity(12), its impact on glucose metabolism in hemorrhagic shock has not been previously reported. Hyperglycemia and acute insulin resistance frequently develop in injured patients and are associated with worse clinical outcomes.(123) Although a number of mechanisms have been implicated, mitochondrial dysfunction appears to play a key role.(140,280) Mitochondrial-derived ROS may promote insulin resistance by serving as a second messenger and down regulating the insulin signaling pathway.(47,124) Indeed, blocking the increase in ROS after injury prevents the acute

development of insulin resistance and reduces the severity of hyperglycemia.(280) In our study, resveratrol supplementation not only decreased mitochondrial ROS production, it preserved the expression of tyrosine phosphorylated IRS1 following hemorrhagic shock. Because only tyrosine phosphorylated IRS1 is capable of activating downstream insulin signaling pathways,(86) it is likely that the observed decrease in insulin resistance with resveratrol was, in part, secondary to decreased oxidant stress and preserved of insulin signaling.

Resveratrol may also improve glycemic control by stimulating the secretion of glucagon-like peptide (GLP-1).(58) GLP-1 is a gut-derived hormone that enhances insulin sensitivity by inducing glucose-dependent insulin secretion while suppressing glucagon release.(278) Although we observed an increase in GLP-1 following resuscitation with resveratrol, we did not see a concomitant increase in insulin levels. GLP-1, however, has been shown to have independent insulin-like effects on glucose metabolism in rat liver, muscle and fat.(157) Even in depancreatized animals, GLP-1 can still potentiate glucose utilization in peripheral tissues and has been shown to increase the expression and phosphorylation of IRS1.(76,204) As such, the increased secretion of GLP-1 observed in our resveratrol treated rats may have contributed to both lower glucose and increased phosphorylated IRS1 expression. How resveratrol increases GLP-1 secretion, however, remains to be determined.

Consistent with previous studies, hemorrhagic shock in our model resulted in significantly elevated TNF-α and IL-6 levels which may have directly contributed to the development of hyperglycemia and insulin resistance.(158,264) Although resveratrol has been shown to mitigate inflammation and reduced blood TNF-α, IL-6, and IL-1β levels in diabetic rats treated with enteral resveratrol for 30 days (180), cytokine levels did not decrease with a single dose of resveratrol in our study. Increased dosing

regimens and longer observation periods maybe needed to determine if resveratrol improves the systemic cytokine profile in hemorrhagic shock.

While the potential benefit of using resveratrol to treat hemorrhagic shock is intriguing, there may be some pitfalls. Activation of SIRT1 appears to be beneficial during reperfusion, but some of its actions may interfere with the normal response to acute hypoxia. SIRT1 deacetylates and inhibits the activities of hypoxia inducible factor 1α (HIF1α) and HIF2α.(64,144) Acetylated HIF1α can activate a number of adaptive metabolic responses including a switch from oxidative phosphorylation to anaerobic metabolism, which could promote cell survival under conditions of hypoperfusion.(60) Similarly, while deacetylating and down-regulating its pro-inflammatory response appears helpful, NFκB also coordinates the response to cellular stress and the up-regulation of its activity in response to ROS may help cells resist apoptosis during hemorrhagic shock and resuscitation.(179,213) Resveratrol can also inhibit cyclooxygenase, thereby decreasing the production of thromboxane. Thromboxane is a potent inducer of platelet aggregation and vasoconstriction, therefore, it is at least theoretically possible that resveratrol could negatively impact hemostasis in a bleeding trauma patient.

Our study has several limitations that will need to be addressed before resveratrol can be translated into clinical care. First, we only explored the impact of resveratrol following acute blood loss. Further studies are needed to determine if treating hemorrhagic shock with resveratrol improves mitochondrial function or prevents organ dysfunction in the long-term. Additionally, we only tested one dose of resveratrol. It is possible that alternative dosing regimens may preferentially influence downstream targets of SIRT1 or PGC1α. We also only explored the impact of resveratrol on two tissue types. Because activating SIRT1 has been reported to both increase and

decrease inflammation in different tissues (79,177), it is possible that resveratrol may also have tissue-specific effects. Finally, we did not use a hyperinsulinemic-euglycemic clamp to assess the impact of resveratrol on insulin sensitivity. While this is considered the gold-standard method of assessing insulin sensitivity, the frequent blood samples required would have negatively affected the hemorrhage model. Moreover, the continuous insulin and glucose infusions required may have potentially affected mitochondrial function. Instead, we used the Ln HOMA-IR index which has been shown to correlate well with the hyperinsulinemic-euglycemic clamp method and is frequently used as a clinical surrogate of insulin sensitivity.(45) Lastly, we did not treat hypoglycemic animals. Glucose administration may provide additional benefit during resuscitation with resveratrol, but this requires further study.

In this work we explored the effects of resveratrol on mitochondrial injury following hemorrhagic shock and resuscitation. Resveratrol supplementation led to a restoration of mitochondrial function, alleviated oxidative stress, and improved hyperglycemia following hemorrhagic shock. These mitochondrial-protective effects appear to be mediated via stimulation of a SIRT1-PGC1α-antioxidant pathway rather than by mitochondrial biogenesis. Further work is needed, however, to determine if the antioxidant benefits observed are mediated by interactions between SIRT1 and PGC1α. Additionally, we will need to determine if the restoration of electron transport and Krebs cycle enzymatic activity, as well as enhanced glycemic control, is secondary to decreased oxidative stress or the result of resveratrol-induced post-translational modifications. Nonetheless, adding resveratrol to standard fluid resuscitation appears to be beneficial and warrants further investigation.

CHAPTER 4:

Deleting SIRT1 Promotes a Pro-Inflammatory Phenotype in Sepsis

Sepsis, the physiologic response to overwhelming infection, is a major healthcare problem in the United States with significant human and economic costs.(139) Each year, more than 750,000 patients are hospitalized with sepsis resulting in an estimated $17 billion in healthcare costs.(5) Despite antibiotics and supportive care, sepsis is the most frequent cause of death in the intensive care unit and continues to claim more than 215,000 lives annually.(131) Although sepsis is characterized by a profound systemic inflammatory state in response to infection, most patients with sepsis do not die of the inciting infection *per se*. Rather, despite eradication of the underlying infection, the majority of non-survivors die days to weeks later after developing progressive organ failure and profound muscular weakness. Importantly, sepsis-induced organ failure appears to occur even with adequate tissue perfusion and in the absence of significant tissue necrosis or apoptosis.(19,101,202,249)

Recent laboratory and clinical investigations suggest that organ dysfunction may develop in sepsis, in part, because pro-inflammatory mediators directly damage the mitochondria. In both animal and clinical studies, sepsis results in early mitochondrial ultrastructural abnormalities as well as functional impairment. (26,53,101,164,256) Sepsis-induced mitochondrial dysfunction subsequently leads to increased reactive oxygen species, bioenergetic failure and ultimately cellular dysfunction.(52,200,218) Notably, the degree of mitochondrial dysfunction observed closely correlates with the severity of clinical disease.(24,25) When skeletal muscle biopsies were taken within 24 hours of ICU admission, non-surviving septic patients were found to have significantly depressed complex I activity and lower tissue ATP levels when compared to both sepsis

survivors and healthy controls. Moreover, the severity of septic shock directly correlated with the decrease in muscle ATP levels.(24) The ability to generate sufficient cellular ATP may be further compromised because sepsis results in a loss of mitochondrial mass.(34,232) Indeed, patients with MOF were found to have 30 to 50% less mitochondrial protein in both intercostal and thigh muscle relative to non-septic controls.(70,71) Ultimately, the ability to survive sepsis, may depend on the capacity to recover mitochondrial function.(217)

SIRT1, a highly conserved NAD deacetylase, can activate a number of key survival pathways during cellular stress and may hold the key to preserving mitochondrial function in sepsis (Figure 1). SIRT1 can promote mitochondrial resilience by suppressing NFκB-mediated inflammation, by enhancing the transcription of antioxidant defenses including SIRT3, and by promoting mitochondrial biogenesis.(126,128,226) Indeed, treating sepsis with the SIRT1 activator, resveratrol, appears to dramatically decrease cytokine levels, reduce oxidant injury, minimize mitochondrial damage, and improve both organ function and survival.(99,125,210,220)

The benefit of activating SIRT1 in sepsis, however, may not be so clear-cut and may depend on the both the model and tissue type. While several studies have shown that deleting or inhibiting SIRT1 in macrophages results in a strongly pro-inflammatory phenotype (207,268,271), others studies have suggested that SIRT1 either has little impact on the immune response or that its inhibition decreases inflammation.(51,67,156) Moreover, pharmacologically inhibiting SIRT1 during the adaptive phase of sepsis has been shown to improve survival in a murine model of polymicrobial sepsis.(245) Clearly, a better understanding of the functional role, potential tissue specificity, and possible temporal influence of SIRT1 during sepsis is needed in order to reconcile these very divergent findings.

In an attempt to understand the impact of SIRT1 on the pathophysiology of sepsis, we conducted a serious of in vitro and in vivo experiments that recapitulated a severe infection using inducible SIRT1 knock out mice, as well as various SIRT1 activators and inhibitors.

RESULTS

Survival rates after CLP vary depending model severity

Cecal ligation and puncture (CLP) is considered the “gold standard” model of sepsis. There are many variables, however, that can affect the severity and consistency of the model.(195) In order to determine if we were achieving reliable and reproducible results, a series of severity experiments were performed in 3 month old C57BL/6J male mice (Jackson Laboratories). By altering both the length of cecum ligated (full vs ½ length) and the gauge of needle used (23g vs 25g), we were able to demonstrate a graduated difference in survival over 7 days based on model severity (Figure 18A). Animals in the severe sepsis model had 100% mortality, whereas moderate and mild sepsis carried 50% and 90% mortality respectively. Importantly, physiologic variables known to reflect sepsis such as temperature and serum glucose also varied with severely septic animals developing more pronounced hypothermia and hypoglycemia (Figure 18C,D).(176) When endpoints of euthanasia were calculated (Table 3), differences in clinical appearance and behavior also correlated with severity of sepsis (Figure 18B).

Sepsis is associated with decreasing SIRT1 expression over time in liver

Although SIRT1 expression has been shown to decrease promptly following endotoxin exposure in mice (282), CLP is associated with a more gradual and less robust cytokine response. As such, we sought to determine if CLP impacted SIRT1 expression in liver and kidney tissue of sham and CLP treated mice were harvested at 0,

3, 9, 18, and 24 hours. SIRT1 expression over time was determined by qPCR. Given SIRT1 absolutely requires NAD, we also evaluated the impact of sepsis on the expression of NAMPT, a critical enzyme involved in the NAD salvage pathway.

Our preliminary data suggests that SIRT1 transcripts in liver decreased significantly within 18 hours whereas NAMPT expression was markedly elevated at 9 hours (Figure 19A). In contrast, SIRT1 expression in the kidney did not change, although NAMPT decreased significantly by 24 hours (Figure 19B).

Pharmacologic manipulation of SIRT1 influences survival and physiology

Sepsis is characterized by an initial hyperinflammatory phase, followed by a hypoinflammatory adaptive phase that if left unchecked may progress to a state of prolonged immunosuppression.(100) Recent work by the McCall group suggests that SIRT1 plays a crucial role in the epigenetics of sepsis and can be manipulated during the adaptive phase to improve survival.(150,151) Based on our preliminary findings that SIRT1 gene expression in liver declines significantly by 18 hours, we elected to start pharmacologic interventions 6 hours after CLP. Starting therapy at this delayed time also makes clinical sense because it can take several hours for a patient's infection to progress to a diagnosis of sepsis. Following CLP, intraperitoneal resveratrol significantly improved survival when compared to wild type controls (Figure 20A). In contrast, animals treated with the SIRT1 inhibitor Ex-527 demonstrated increased early mortality. At 30 hours, however, there appeared to be an inflection point where SIRT1 inhibition may extend survival. Interestingly, at 24 hours EX-527 also significantly improved body temperature with a trend toward lower serum glucose (Figure 20B, C). These findings appear to support the theory that enhancing SIRT1 may be beneficial early during sepsis, whereas its inhibition may be protective during the adaptive phase.(245) Additional studies, however, are needed and will be performed to confirm these findings.

Deletion of SIRT1 negatively impacts survival and physiology in sepsis

In order to determine if deleting SIRT1 negatively impacts clinical outcomes in polymicrobial sepsis, 3-4 month old inducible SIRT1 knock out (S1KO) mice and their wild type littermates were treated with tamoxifen 2 weeks prior to CLP. Despite using the same technique to induce "severe sepsis", our in-house bred mice demonstrated significantly less overall mortality than in mice purchased from Jackson Labs. Although determining the reason for this difference is outside the scope of this current project, animals bred in different locations may have different intestinal flora and we suspect differences in mortality may be secondary to differences in the gut microbiome.(95) Animals from different venders not only demonstrate significant differences in the viable counts of bacteria in their feces, there are striking differences in their cecal microbiota when PCR-derived amplicons from bacterial 16S rRNA genes are evaluated by denaturing gradient gel electrophoresis.(96,108)

Nonetheless, when compared to WT mice from the same cohort, S1KO mice were significantly more likely to die, with the majority of deaths occurring within the first 48 hours ($p=0.026$, Figure 21A). Overall, S1KO mice also appeared clinically less well with a trend toward higher Endpoints of Euthanasia scores and significantly more weight loss over the 5 day course (Figure 21B,C). Physiologically, S1KO mice appeared "more septic" (176) with a lower core body temperature and worse hypoglycemia early during the course of the experiment (Figure 21D,E C,D).

Deletion of SIRT1 negatively impacts mitochondrial function the kidney 5 days post CLP

Given S1KO mice appeared more "septic" following CLP, we hypothesized that deleting SIRT1 would be associated with significantly worse mitochondrial function 5 days post-CLP. Surprisingly, when compared to WT septic mice, deleting SIRT1 only

significantly impaired complex II dependent respiration in the kidney (Figure 22D). Interestingly, complex II, or succinate dehydrogenase, activity is directly influenced by SIRT3.(49) Given that sepsis has recently been shown to decrease SIRT3 expression in the kidney (284), it is possible that deleting SIRT1 further impedes the recovery of SIRT3, thus contributing to the decreased CII activity observed in S1KO sepsis survivors. In order to address this question, we will be looking at the acetylation status of succinate dehydrogenase, as well as SIRT1 and SIRT3 protein expression in WT and S1KO mice 5 days post CLP. Additionally, we will perform control experiments in sham WT and S1KO mice in order to assess baseline mitochondrial content, respiration, and sirtuin expression.

Deletion of SIRT1 negatively impacts physiologic variables and organ function 36 hours post-CLP

Septic S1KO and WT mice were treated with resveratrol (20mg/kg IP every 6hrs) and sacrificed at 36 hours post-CLP in order to determine if SIRT1 status influenced physiologic variables or organ function. Although all septic animals were more hypothermic and hypoglycemic than sham controls, septic S1KO mice tended to have lower temperatures and serum glucose values when compared to septic WT mice (Figure 23A, B). Moreover, septic S1KO mice also looked less clinically well, with a trend toward higher endpoints of euthanasia scores (Figure 23C). Notably, SIRT1 activation with resveratrol did not improve these physiologic variables. While deletion of SIRT1 negatively impacted renal function (Figure 23D), its impact on liver function was variable and not statistically significant (Figure 23E). Again, the use of resveratrol did not improve organ function. In order to determine if the negative trends observed in S1KO mice are significant, additional experiments will be performed to increase the number of biologic replicates in all groups.

Deletion of SIRT1 has minimal impact on liver mitochondrial function following sepsis

It is well known that sepsis negatively impacts mitochondrial function.(217) In order to determine the impact of SIRT1 on mitochondrial respiratory capacity and redox status, S1KO and WT mice were randomized to sham treatment, CLP or CLP with resveratrol (20mg/kg IP every 6hrs). At 36 hours post-CLP, liver tissue homogenates were assayed. Sepsis did not significantly change mitochondrial content in liver, but did decrease CI, II, and IV respiratory capacity in WT mice (Figure A-C). S1KO mice had significantly decreased CI activity at baseline, however, deleting SIRT1 did not make the degree of sepsis-induced mitochondrial dysfunction worse. In fact, following sepsis, WT and S1KO had similar CI, II, and IV dependent respiration regardless of resveratrol treatment (Figure 24).

Preliminary experiments suggest that although the redox state does not significantly change in WT animals with sepsis, resveratrol tended to decrease NADH and thereby increasing the NAD/NADH ratio. In contrast, S1KO mice may have decreased NAD pools at baseline (Figure 24D)

Deletion of SIRT1 significantly impacts kidney mitochondrial function following CLP

Given our finding that SIRT1 KO mice were more susceptible to sepsis-induced renal dysfunction, we wondered if deleting SIRT1 was associated with increased mitochondrial dysfunction. As previously described, S1KO and WT mice were randomized to sham treatment, CLP or CLP with resveratrol (20mg/kg IP every 6hrs) and euthanized 36 hours later. Despite similar citrate synthase activity, S1KO mice demonstrated significantly worse fatty acid respiration, as well as decreased CI and CII dependent respiration, when compared to WT septic mice (Figure 25 A-C). There was a trend

toward improved CI function in WT animals treated with resveratrol (Figure 25C). Renal redox status was not influenced by SIRT1 expression remained stable regardless of resveratrol treatment and was not influenced by SIRT1 expression (Figure 25D).

Manipulation of SIRT1 modifies the inflammatory response in vivo and in vitro

There is controversy regarding whether or not SIRT1 is pro or anti-inflammatory. While SIRT1 inhibition has been shown to attenuate the production of pro-inflammatory cytokines in lipopolysaccharide (LPS)-–stimulated macrophages and in septic animals,(67,156) pharmacologic SIRT1 activation with resveratrol has similarly been shown to be protective in sepsis.(125,210,220) In order to determine if SIRT1 tempers the inflammatory response in sepsis, we conducted a series of *in vivo* and *in vitro* experiments. IL 6 levels were measured serially by ELISA in WT and S1KO mice subjected to CLP. Although baseline values were similar, IL6 levels were significantly more elevated in S1KO mice early during the course of sepsis. Because macrophages are a key source of IL6 during sepsis, we looked at the temporal expression of IL6 in cultured bone marrow derived macrophages (BMDMs) treated with LPS. BMDMs harvested from SIRT1 KO mice had a more robust inflammatory response with significantly higher gene expression of IL6 than WT cells (Figure 26B). In order to confirm gene expression translates to higher protein expression, however, we plan to measure both cellular IL6 protein levels by immunoblot as well as secreted IL6 levels by ELISA in LPS-stimulated BMDMs in future studies.

SIRT1 can also modulate inflammation by enhancing the expression of superoxide dismutase (SOD2) and catalase (CAT)(128,226), thereby decreasing the impact of ROS. Surprisingly, after LPS-stimulation, SIRT1 KO BMDMs actually had higher SOD2 and CAT expression than WT cells (Figure 26C, D). Very preliminary data suggests a similar pattern of gene expression in LPS stimulated BMDM treated with the SIRT1 inhibitor,

EX-527 (Figure 26E). This suggests that SOD2 and CAT expression are not exclusively controlled by SIRT1 expression and increased expression can be activated in times of oxidative stress. This increased expression of antioxidants may reflect an overall increased burden of ROS in S1KO cells. We will test this hypothesis in future experiments by measuring the generation of ROS after LPS exposure in WT, S1KO, and pharmacologically manipulated BMDMs.

Myeloid deletion of SIRT1 is pro-inflammatory during sepsis.

Although we demonstrated that deleting SIRT1 was pro-inflammatory using both *in vivo* and *in vitro* septic challenges, it is possible that nonspecific SIRT1 deletion in the whole organism, and not just an enhanced inflammatory response, could account for differences in mortality. Moreover, the impact of myeloid-specific SIRT1 deletion on inflammation remains controversial; thus differences in the inflammatory response could theoretically be driven by SIRT1 deletion in the immune cells or in key organs such as the kidney or liver. (51,207) In order to determine if deleting SIRT1 significantly influenced the immune response in sepsis, SIRT1 was deleted in macrophages and granulocytes using LysMCre. When subjected to CLP, the S1KO LysMCre mice had significantly higher serum IL6 levels than their WT littermates at 12 hours. S1KOLysMCre also had increased 5 day mortality. Although we have fewer mice in this experiment, none of the WT mice died in these experiments which would be highly unusual. Previously WT mice in our S1KO cohort experienced an 80% mortality rate. This discrepancy is concerning and we will repeat these experiments in order to validate that deleting SIRT1 in myeloid cells increases mortality.

CONCLUSIONS

In this ongoing study, we report that deleting or inhibiting SIRT1 results in a pro-inflammatory phenotype both *in vivo* and *in vitro*. Using a polymicrobial model of intra-abdominal sepsis, whole body deletion of SIRT1 negatively impacted both survival and physiology. The influence of SIRT1 on mitochondrial function during sepsis, however, appears to be organ dependent. While deleting SIRT1 did not alter mitochondrial function in the liver, it did significantly impair both mitochondrial capacity in the kidney and was associated with decreased renal function following CLP. We further investigated the role of SIRT1 on the on the immune response by stimulating WT and S1KO bone marrow derived macrophages with LPS. In this cell culture model of sepsis, SIRT1 deletion significantly increased IL6 gene expression and may be associated with increased generation of ROS. Similar findings were appreciated when WT BMDMs were treated with the SIRT1 inhibitor EX527. Preliminary data suggest that specifically deleting SIRT1 in macrophages and granulocytes recapitulates the pro-inflammatory phenotype observed in our whole body SIKO model. Although future experiments are planned to confirm our preliminary findings, SIRT1 appears to play a vital role in sepsis and a could potentially be a viable therapeutic target.

CHAPTER 5:

Deleting SIRT3 Enhances the Inflammatory Response in Sepsis

As the major mitochondrial deacetylase, sirtuin 3 (SIRT3) directly modulates cellular energetics and.is poised to play a pivotal role in coordinating the mitochondrial response to sepsis. In addition to regulating fatty acid oxidation and several of enzymes in the Kreb's cycle, SIRT3 can deacetylate complexes I, II, and III (102); thus influencing the flow electrons through the electron transport chain as well as the generation of damaging ROS. SIRT3 can also directly mitigate oxidative stress by activating superoxide dismutase and by increasing the concentration of reduced glutathione.(191,221) While SIRT3 knockout mice appear phenotypically normal, these animals demonstrate evidence of complex I inhibition as well decreased basal ATP levels. Overexpression of SIRT3, on the other hand, rescues mitochondrial function and minimize oxidative damage.(1). Recently, deletion of SIRT3 in C2C12 myoblasts was noted to decrease respiratory capacity, increase reactive oxygen species and increase insulin resistance. Interestingly, a similar phenotype is observed in septic patients and in LPS treated C2C12 cells.(62,72,155) In order to determine if SIRT3 could be a potential therapeutic target in sepsis, we are currently investigating the impact of modulating SIRT3 both *in vitro* and *in vivo*.

RESULTS

Deleting SIRT3 increases early mortality but doesn't significantly impact morbidity

Given the central role SIRT3 plays in mitochondrial metabolism, we anticipated that deleting SIRT3 would significantly impact the physiologic response to polymicrobial sepsis. Surprisingly, S3KO mice and their WT littermates responded similarly to the

septic insult. Although the deletion of SIRT3 is reported to have minimal impact on standard biometrics (1), S3KO mice in our cohort weighed less at baseline than their WT littermates. Both genotypes lost weight at the same rate following CLP, however, S3KO mice lost a smaller total percentage of body weight (Figure 28 A, B). After CLP, S3KO and WT mice had similar core body temperatures and serum glucose levels (Figure 28 C, D). Moreover, in animals that survived 5 days post-CLP, SIRT3 status did not significantly impact organ function (Figure 28E). While the overall mortality at 5 days was similar, S3KO animals died earlier than WT mice (Figure 28F).

Deleting SIRT3 has minimal clinical impact within the first 36 hours post-CLP

Given the increased early mortality in S3KO mice, we wondered if SIRT3 deletion was associated with worse clinical physiology or increased organ dysfunction. In order to address this question, septic mice were assessed every 6 hours starting 12 hours post-CLP. S3KO and WT mice had similar core body temperatures and looked clinically similar with nearly identical endpoints of euthanasia scores (Figure 29A, C). Although S3KO mice had a modest, but statistically significant, increase in their serum glucose starting at 24 hours, they were by no means hyperglycemic (Figure 29B). Both genotypes also had a similar degree of organ dysfunction 36 hours post-CLP (Figure 29D).

SIRT3 status has minimal impact on mitochondrial function in sepsis

Using high resolution respirometry, we interrogated mitochondrial respiratory capacity in both liver and kidney whole homogenates at 36 hour and 5 days post-CLP. Despite its central role in mitochondrial metabolism, SIRT3 status did not significantly impact mitochondrial content or respiratory function in our CLP model at these time points. In fact, septic S3KO and WT mice had identical respirometry profiles. In the liver, CLP resulted in decreased CI and CIV activity at 36 hours and persistent CIV

dysfunction at 5 days, regardless of genotype (Figure 30B, C). While the kidney demonstrated significant mitochondrial dysfunction 5 days post-CLP, this dysfunction did not appear to be influenced by SIRT3 status either (Figure 30 E, F). Thus, these data suggest that S3KO and WT likely share a common pathophysiology that leads to the same degree of mitochondrial dysfunction observed at the time points measured. It is possible, however, that we missed earlier differences in mitochondrial function by waiting until 36 hours. Indeed, Zhao and colleagues recently reported that S3KO mice demonstrated increased sepsis-induced acute kidney injury 24 hours post-CLP.(284) Regardless, it does not appear that differences in mitochondrial respiratory capacity account for the increased early mortality observed in our SIRT3 cohort. It is possible that another tissue that we didn't examine, like macrophages or cardiac tissue, might have mitochondrial dysfunction that could account for the differences in mortality.

Deleting SIRT3 is pro-inflammatory

In order to determine if SIRT3 status impacted the acute inflammatory response, we serially measured serum IL6 levels in S3KO and WT mice subjected to CLP. S3KO mice demonstrated significantly higher IL6 levels that peaked 6 hours post CLP. Interestingly, S3KO and WT mice had similar IL6 levels starting 12 hours post CLP; and this similarity in cytokine profile may help explain why we didn't we observe differences in their clinical physiology (Figure 31 A. Figure 29A, C). We then looked at LPS stimulated BMDMs in order to determine if SIRT3 status influenced the temporal expression of pro-inflammatory cytokines. Although the sample size was small, these preliminary data suggest that IL6 expression may peak earlier in S3KO cells (Figure 31B). WT and S3KO BMDM were then pretreated with honokiol, a purported SIRT3 activator (185) for one hour prior to LPS stimulation in order to determine if SIRT3 activation would dampen the inflammatory response. In these preliminary studies,

honokiol appears to decrease IL6 and TNFα expression. These results, however, did not reach significance in our small sample size. Somewhat surprisingly, there was a trend toward decreased IL6 expression in honokiol treated S3KO cells suggesting that honokiol may have off –target anti-inflammatory (or antioxidant) effects (Figure 31 D, E). Indeed, pretreatment with honokiol significantly reduced SOD2 expression in S3KO, but not in WT cells co-cultured with LPS (Figure 31F). Additional experiments are planned in order to explore if SIRT3 status changes the temporal expression of pro-inflammatory cytokines *in vitro.* In addition, we will determine if SIRT3 overexpression mitigates the inflammatory response in CLP and *in vitro.* Finally, we will determine what role ROS play in the S3KO pro-inflammatory phenotype by co-culturing cells with the antioxidant N-acetylcysteine.

SIRT3 expression dramatically declines during sepsis in multiple tissues

We were struck with how similar septic S3KO and WT mice were in terms of physiology and mitochondrial function following CLP. Given these similarities, we wondered if WT animals adopted a S3KO phenotype as a natural response to sepsis and if this transformation was necessary in order to mount an inflammatory response. In order to begin to address this hypothesis, we cultured WT BMDMs with LPS and measured SIRT3 mRNA overtime. We also subjected WT mice to CLP and measured the temporal expression of SIRT3 in liver and kidney. Our preliminary data suggest that during sepsis, SIRT3 is down regulated in multiple tissues (Figure 32) which may explain why septic S3KO and WT share the same phenotype at later time points.

CHAPTER 6:

Conclusions and Future Directions

Although hemorrhagic and septic shock differ significantly in their underlying etiologies, both conditions are associated with a pathologic state of systemic inflammation, vascular collapse, mitochondrial dysfunction, and ultimately multiple organ failure (MOF). Given MOF remains the most common cause of in-hospital morbidity following shock and accounts for 50-80% of all deaths in the intensive care unit (48,66,175), there is increasing interest in developing therapies that target shock-induced pathways. Sirtuins play a critical role in modulating inflammation, oxidative damage, and mitochondrial function during cellular stress. As such, we hypothesized that manipulating SIRT1 and SIRT3 would significantly impact the pathophysiology of shock.

SIRT1

SIRT1 is a highly conserved NAD-dependent deacetylase that plays a critical role in cellular resilience and longevity.(102) Given its absolute requirement for cytosolic NAD, SIRT1 directly responds to cellular stress and hypoxia by providing transcriptional regulation of key pro-survival pathways.(11,282) SIRT1 enhances mitochondrial biogenesis by deacetylating the transcriptional co-activator PGC1α, decreases oxidative stress by activating the FOXO3 dependent expression of superoxide dismutase and catalase, and suppresses NFκB-mediated inflammation by deacetylating the p65 subunit of the NFκB complex.(37,121,198) SIRT1 also deactivates and down regulates the expression of poly (ADP-ribose) polymerase (PARP) – a NAD consuming enzyme that triggers cell death in the setting of oxidant-mediated DNA damage. Given its ability to modulate the inflammatory response, mitigate oxidative damage and promote cell

survival, SIRT1 has become a key therapeutic target for many diseases characterized by impaired mitochondria and inflammation.(12,36,134)

SIRT1 ACTIVATION IN HEMORRHAGIC SHOCK

Using a model of decompensated hemorrhagic shock, we investigated the impact of activating SIRT1 during resuscitation using two different pharmacologic agents - resveratrol and nicotinamide mononucleotide (NMN). Resveratrol, a polyphenol found in red wine, is thought to increase SIRT1 activity either directly by allosteric interactions or indirectly by activating AMPK to increase NAD levels.(12,102,103,181,243) When given during resuscitation, we found that resveratrol enhanced mitochondrial enzymatic activity, restored oxidative phosphorylation, mitigated oxidant damage, and preserved renal function.(252,253) Similarly, we found that NMN, a naturally occurring NAD precursor, preserved mitochondrial function, mitigated inflammation by decreasing the generation of IL6, enhanced physiologic reserve and improved survival following hemorrhagic shock.

Both resveratrol and NMN, however, have pleiotropic effects. Thus, the mechanistic benefit of SIRT1 activation following hemorrhagic shock remains encouraging but unproven. In order to better understand if targeting SIRT1 preserves mitochondrial function or mitigates inflammation following hemorrhagic shock, we will conduct complimentary experiments using inducible SIRT1 knockout (S1KO) and S1KO LysMCre mice. By directly altering SIRT1 expression in both tissue and immune cells, we will be able to evaluate the impact of SIRT1 signaling on bioenergetics, mitochondrial function, and inflammation in hemorrhagic shock. Future experiments will also include SIRT1 transgenic knock in (S1Tg) in order to determine if overexpression of SIRT1 improves physiologic function. Additionally, we will resuscitate SIKO mice with

resveratrol and NMN in order to determine if their benefits in hemorrhagic shock are SIRT1 dependent.

SIRT1 ACTIVATION IN SEPTIC SHOCK

Although there is some debate regarding the potential benefit (or harm) of activating SIRT1 in sepsis (207,268,271), deleting SIRT1 in our polymicrobial model was clearly pro-inflammatory and associated with worse clinical outcomes. Septic S1KO mice demonstrated higher levels of the inflammatory cytokine IL6, were more hypothermic, had worse renal mitochondrial function, and died sooner than their WT counterparts. While preliminary experiments using SIRT1 LysMCre mice suggest that many of these findings may be secondary to a pro-inflammatory phenotype rather than a primary tissue defect, we are conducting additional experiments given an unexpectedly high survival rate in the WT littermates. Additionally, in order to determine if augmenting SIRT1 activity is anti-inflammatory, S1Tg mice will be subjected to CLP. In addition to measuring serial IL6 levels, the deacetylation status of tissues prone to developing MOF (eg. lungs, liver, and kidney) will be compared to our septic SIKO and WT mice. We will also determine if SIRT1 overexpression improves survival.

In our sepsis survival experiments, pharmacologic activation of SIRT1 using resveratrol decreased overall mortality. At first glance, these findings appear to contradict those reported by Vachharajani et al. Using a similar CLP model, these authors report that SIRT1 inhibition with EX527 administered 24 hours *after* the septic insult improved survival.(245) Interestingly, EX527 appeared to have a biphasic response in our model. When given 6 hour hours after cecal puncture, EX527 was associated with increased mortality. However, after 24 hours, EX527 was associated with improved clinical parameters, including serum glucose and temperature, with a

trend toward improved late mortality. Given that sepsis has a biphasic inflammatory response, it is possible that SIRT1 activation improves the early hyperinflammatory phase, whereas SIRT1 inhibition promotes the adaptive phase of sepsis. Clearly, a better understanding of the functional role, potential tissue specificity, and temporal influence of SIRT1 activation during sepsis is needed.

SIRT1 EXPRESSION IN CRITICALLY ILL PATIENTS

Both injury and sepsis trigger an immune response that directly correlates with both the frequency and severity of complications(63,109,159). In a recent analysis of 167 severely injured patients, Xiao et al noted that >80% of the leukocyte transcriptome was altered within 12 hours of injury.(263) In patients whose post-injury course was complicated by the development of MOF or infection, both the magnitude and time to resolution of pro-inflammatory gene expression were significantly prolonged. Patients with complicated courses also had decreased expression of genes involved in mitochondrial function and oxidative phosphorylation. Interestingly, Xiao and colleagues noted a significant decrease in SIRT1 expression in all severely injured patients. Although the relationship between SIRT1 and inflammation has not been previously explored in critically ill patients, SIRT1 activity in peripheral blood mononuclear cells (PBMC) has been shown to correlate with severity of inflammation in chronic conditions such as rheumatoid arthritis.(257) Because SIRT1 activity is known to modulate inflammation, we will test the hypothesis that PBMC SIRT1 activity is suppressed in critically ill patients following hemorrhagic and septic shock. Moreover, we further speculate that decreased SIRT1 activity will predict the development of MOF.(282)

TARGETING NAD IN SHOCK

NAD functions as an essential cofactor in a number of cellular reactions and is

absolutely essential for both SIRT1 activity and maximal oxidative capacity. During hemorrhagic shock and resuscitation, tissue NAD levels fell significantly in our model; potentially creating a competition for the limited amounts of NAD between poly (ADP-ribose) polymerase-1 (PARP1), an NAD dependent enzyme activated during cellular stress, and SIRT1.(10,44,219,230)(53-57) If PARP1 is preferentially activated, the stressed cell goes on to die; whereas if SIRT1 is activated, adaptive survival pathways are stimulated. Because the Km of PARP1 for NAD is lower than that of SIRT1, and SIRT1 itself is a repressor of PARP1, falling NAD levels can favor activating PARP1; this would exacerbate the loss of SIRT1 activity and make it less effective at inhibiting inflammation or promoting cell survival. Moreover, it has been shown that repleting NAD by overexpressing NAMPT can protect against the damage associated with PARP overexpression in a SIRT1-dependent manner.(281) Thus, the availability of NAD plays a major role in determining the fate of the cell and strategies to preserve or increase NAD could tip the balance in favor of SIRT1 activation. Indeed, supplementation with NMN in our hemorrhagic shock model significantly enhanced tissue NAD levels, restored mitochondrial function, and improved survival.

In addition to providing precursor molecules, cellular NAD levels could be enhanced by directly inhibiting PARP1 activity. PARP1 has previously been shown to play a key role in the development of multiple organ damage following acute blood loss.(255) With hemorrhagic shock and resuscitation, PARP1 is dramatically activated and correlates with the development of intestinal injury, cardiac failure and acute lung inflammation. Genetically knocking out PARP, however, significantly mitigates the development of organ damage and nearly doubles the mean survival time.(143) Similarly, pharmacologic inhibiting PARP at the time of resuscitation appears to preserve organ function and improve cardiac function in variety of animal models.(162,229) It is unclear,

however, if these benefits are simply related to inhibiting PARP, or if they also preserve NAD levels and activate SIRT1 pathways.

Although we did not observe a decline in tissue NAD in our CLP model, nor did we investigate the use of NMN, it is possible that supplementing NAD precursors could prove beneficial in sepsis. To date, niacin is the only NAD precursor that has been investigated in sepsis.(133) When given to endotoxemic rats, high dose niacin significantly increased NAD levels in lung tissue and downregulated the NFκB pathway. As a result, animals receiving niacin developed significantly less pulmonary inflammation and were more likely to survive than placebo treated animals. All tissues in sepsis, however, may not respond equally to increased intracellular NAD and there is some concern that enhancing NAD in immune cells could promote the release of pro-inflammatory cytokines in a SIRT6 dependent manner.(247)

Although our results investigating the relationship between NAD and SIRT1 in shock are highly encouraging, many important questions remain. The optimal timing for delivery of NAD precursors and SIRT1 activators remains unknown. It is also unclear if strategies to prevent PARP activation can be used to concomitantly augment NAD levels and promote SIRT1 activation. These questions are particularly relevant given the suggestion that SIRT1 activation may have both positive and negative effects at different times during the inflammatory response.(245,246) Lastly, the potential for synergy between interventions that allosterically activate SIRT1 and those that restore its cosubstrate, NAD, remains completely unexplored.

SIRT3

There is an increasing appreciation that SIRT3 plays a significant role in age-related diseases and as the major mitochondrial deacetylase, is poised to play a pivotal role in

coordinating the mitochondrial response to sepsis.(163) SIRT3 mitigates oxidative stress, increasing both the availability and activity of MnSOD and isocitrate dehydrogenase 2. SIRT3 may further minimize oxidative stress by promoting the oxidation of fatty acids.(186) During fatty acid oxidation, some electrons shuttle from NADH directly to the electron-transferring flavoprotein,(82) thereby bypassing complex I, a key site of free radical generation that is frequently inhibited during shock. SIRT3 may also modulate oxidative phosphorylation by deacetylating multiple complexes in the electron transport chain.(102,184) Understanding how SIRT3 impacts the pathophysiology of sepsis could potentially lead to more targeted therapies and improved outcomes.

When subjected to CLP, S3KO mice in our study demonstrated a pro-inflammatory phenotype with serum IL6 levels that peaked earlier in the course of sepsis. Septic S3KO mice also had a significantly higher mortality than their WT littermates. Recently Zhao et al reported similar findings and suggest that when challenged with a septic insult, SIRT3 expression in the kidney decreases. SIRT3 deletion was also associated with a significant upregulation of the NLRP3 inflammasome that was rescued with SIRT3 overexpression.(284)

Somewhat surprisingly, SIRT3 deletion did not significantly worsen the mitochondrial respiration observed in whole homogenates of septic liver or kidney, nor did it significantly impact tissue NAD levels. It is possible that deleting SIRT3 resulted in mitochondrial dysfunction at an earlier time point or that mitochondria from a different tissue (e.g., cardiac or immune cells) may be more impaired in our S3KO cohort. We also subjected WT mice to CLP and measured the temporal expression of SIRT3 in liver and kidney. Our preliminary data suggest that during sepsis, SIRT3 is down regulated in

multiple tissues which may explain why septic S3KO and WT share the same phenotype at later time points.

Given their striking physiologic similarities at 36 hours, we wondered if WT animals adopt a S3KO phenotype as a natural response to sepsis and if this transformation was necessary in order to mount an inflammatory response. In order to address this hypothesis in future experiments, we will culture bone marrow derived macrophages harvested from WT, S3KO and transgenic overexpressing SIRT3 mice (S3OE) with LPS. In addition to measuring SIRT3 mRNA expression over time, we will quantify mitochondrial derived reactive oxygen species as well as the activation of antioxidants and the inflammasome. S3KO macrophages will also be co-cultured with the antioxidant N-acetylcysteine in order to determine if reactive oxygen species are the pro-inflammatory driver. Finally, we will compare how the systemic and tissue-based inflammatory response to CLP changes over time in WT, S3KO, and S3OE mice.

SUMMARY

SIRT1 and SIRT3 play critical roles in mitigating the pathophysiology of shock and represent attractive therapeutic targets. Impaired expression of either SIRT1 or SIRT3 appears to promote a pro-inflammatory phenotype, whereas pharmacologic manipulation of either SIRT1 or the availability of their co-substrate NAD decreases inflammation, improves mitochondrial function, and enhances physiologic resilience.

CHAPTER 7:

Materials and Methods

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania and were conducted in accordance with the guidelines established by the National Institutes of Health. All chemicals and cell culture solutions were obtained from Sigma Aldrich or ThermoFisher Scientific unless otherwise stated.

ANIMALS

Rodents were housed with constant temperature, humidity, and a timed 12-hour light/dark cycle. Regardless of experimental procedure, all animals were allowed access to standard rodent chow (PMI Rodent Diet, ASAP, Quakertown, PA) and water *ad lib*.

For hemorrhagic shock experiments, male Long Evans rats (250-300g) were purchased (Charles River Laboratories, Raleigh, NC) and allowed to acclimate at least 3 days. For NMN experiments, animals were randomized to either plain drinking water or water with NMN (400mg/kg/day, Metro International Biotech, LLC, Cambridge, MA) for five days prior to surgery. For resveratrol experiments, animals received standard drinking water. For sepsis experiments, male C57BL/6J mice (3 month old) were purchased from Jackson Laboratories.

Inducible whole-body SIRT1 knockout mice were generated by breeding floxed SIRT1$^{\Delta ex4}$ mice with tamoxifen-inducible cre ERT2 mice.(189) Heterozygous SIRT1$^{\Delta ex4}$ with ERT2 were cross matched and 10 weeks old homozygous SIRT$^{\Delta ex4}$ERT2 mice and wild type littermates were treated with tamoxifen (0.1mg/g) for 5 days by gavage. Two to four weeks later, animals were subjected to CLP (or sham) procedures or underwent bone marrow harvest.

SIRT1$^{\Delta ex4}$ LysMCre mice were generated by crossing floxed SIRT1 $^{\Delta ex4}$mice with LysM Cre mice (Lyz2 $^{tm(cre)Ifo}$ purchased from Jacskon Laboratories in the C57BL/6J background. These mice were also treated with tamoxifen (0.1mg/g) for 5 days by gavage in order to directly compare with the inducible whole-body SIRT1 knockout cohort.

SIRT3 knock out mice were originally generated by Lombard et al (152) and were backcrossed to the C57BL/6 background for 10 generation prior experiments.

EXPERIMENTAL PROCEDURES

NMN and Hemorrhagic Shock

Spontaneously breathing Long Evans rats were anesthetized using vaporized isoflurane by mask (2-4%) and underwent sterile placement of femoral vascular catheters (PE50, Braintree Scientific, Inc., Braintree, MA). Mean arterial pressure (MAP) was recorded throughout the experimental protocol (Digi-Med Signal Analyzers, Louisville, KY). A sterile prepped 5-cm midline laparotomy was performed to simulate soft tissue trauma. All surgical sites were bathed in 1% lidocaine (APP Fresenius Kabi, Lake Zurich, IL) and closed in layers. Animals received 0.25% buprenorphine (0.05mg/kg, subcutaneously, Reckitt Benkiser Healcare Ltd, Hull, England). Animals were then placed in a plexiglass restraining apparatus and allowed to fully emerge from anesthesia (~30 minutes).

Animals were randomized to either fixed pressure hemorrhagic shock (n=9-12 per treatment group) or sham controls (n=5 per treatment group). Shocked animals were passively bled via the femoral artery and maintained at a MAP of 40 mmHg for 90 minutes. If the MAP fell below a 40 mmHg, small boluses (0.2 ml) of intravenous lactated Ringer's (LR) were provided to maintain the MAP. At 90 minutes, animals were

intravenously resuscitated with 4 times the shed blood volume in LR ± sterile filtered NMN (400mg/kg) over 60 minutes (60R). Sham animals were gently restrained for 90 minutes and then were given 5ml of LR ± sterile filtered NMN (400mg/kg) over 60 minutes.). Blood samples were taken at Baseline, 90 minutes and 60R (Figure 3). Following resuscitation, animals were again anesthetized with isoflurane (2-4%). Liver and kidney tissues were rapidly harvested and either immediately clamp frozen in liquid nitrogen or prepared for mitochondrial assays. Animals were euthanized under anesthesia.

In a separate survival experiment, animals were randomized to water ± NMN (400mg/kg/day) for 5 days prior to surgery. Animals underwent general anesthesia with vascular access, laparotomy, and full reversal as described. Animals were then randomized to either decompensated hemorrhagic shock (223) (n=11 per treatment) or served as sham controls (n=3 per treatment). Shocked animals were passively bled via the femoral artery and maintained at a MAP of 40 mmHg. When the blood pressure could no longer be maintained without fluid infusion (Decompensation), a MAP of 40 mmHg was sustained by incrementally infusing 0.2 cc boluses of LR until 40% of the total shed volume had been returned in the form of boluses (Severe Shock). Animals were then resuscitated with four times the shed volume in LR ± NMN (400mg/kg) over 60 minutes and followed for an additional 48 hours (Figure 9). The total time from initiation of bleeding to the start of resuscitation represents each animal's ability to tolerate the shock and reflects individual physiologic resilience. Animals received buprenorphine (0.05mg/kg, every 8 hours) and a subcutaneous LR bolus (50 ml/kg) ± NMN (400 mg/kg) at 24 hours. Blood samples were taken at Baseline, Severe Shock, 60R, 24 and 48 hours. At 48 hours, any surviving animals were anesthetized using isoflurane (2-4%) by mask followed by tissue harvest and euthanasia.

NMN and Primary Hepatocyte Harvest

Primary hepatocytes were isolated as previously described using a modified two-step perfusion method.(166) Animals (n=4) were anesthetized with isoflurane (2-4%). The abdomen was prepped and draped in sterile fashion. The inferior vena cava was cannulated with a 24g angiocath and the portal vein was cut. The liver was then infused with 300 ml of liver perfusion media (Invitrogen, 17701-038) at 17ml/kg/minute to desanguinate followed by 400 ml of liver digestion media (Krebs Ringer bicarbonate buffer (KRBB) + 20 mM HEPES (pH 7.4), 500μM $CaCl_2$, collagenase (≥20,000 units)/elastase (30 units) (Worthington, LK002066), and DNAseI (200units, Worthington LK003170). After the perfusion, liver was removed, disrupted to release cells using cell scrapers. The cell suspension was then filtered through 70μm filter and centrifuged at 50g for 5 minutes at 4°C, washed once in KRBB and precipitated in 25% Percoll gradient at 120g for 5 minutes at 4°C. Cells were resuspended in hepatocyte media (M199, NaHCO3 (2.2 gm/L), glutamine 0.1g/L, 0.25% BSA, 10% Fetal Bovine Serum (Hyclone) and 1% penicillin-streptomycin Using pre-coated collagen 6 well plates, cells were plated at $1x10^6$ cells/well and incubated for 12 hours (37°C, 5% CO_2).

In order to obtain sham and shock plasma for hepatocyte co-culture experiments, animals were anesthetized as described, underwent vessel cannulation, and allowed to awaken and stabilize prior to bleeding. Sham animals (n=9) were rapidly bled into heparinized collection tubes (BD Vacutainer) and lactate was confirmed to be ≤2 mmol/L. The shock animals (n=9) were bled to MAP of 40 mmmHg for 90 minutes, and then exasanguinated into heparinized tubes. Collected blood was immediately centrifuged (1300 rcf, 10 min, 4°C) and plasma was immediately stored at -80C.

Resveratrol and Hemorrhagic Shock

After vascular line placement, laparotomy and full reversal of anesthesia, male

Long Evans rats were bled to a MAP of 40mmHg and subjected to decompensated hemorrhagic shock as previously described. When 40% of the shed volume had been returned in the form of LR boluses (Severe Shock), animals were resuscitated with four times the shed volume in LR with or without resveratrol (30 mg/kg, 50mg/ml in 50% DMSO, Orchid Pharmaceuticals, Lalilab, Durham NC), over 60 minutes and then followed for 18 hours.(220) Animals (n=6 per group) were sacrificed prior to hemorrhage (Sham), at Severe Shock, following LR Resuscitation (LR) or resveratrol+LR Resuscitation (LR + RSV), 18 hours after LR Resuscitation (18hr LR) or LR + resveratrol Resuscitation (18hr LR +RSV). Animals were re-anesthetized at each time point (2% isoflurane) and blood samples and tissues were harvested.

Cecal Ligation and Puncture (CLP) Sepsis Model

CLP is considered the gold standard in sepsis research because it closely mimics the pathophysiology observed in patients and is highly reproducible.(195) In this model, the cecum is exposed via a midline laparotomy under 2% isoflurane. Using a 4.0 silk, the cecum is ligated just below the ileocecal valve and punctured twice with a 23 gauge needle. The cecum is returned and the abdominal incision is closed in 2 layers with 4.0 vicryl. Cecal perforation subsequently progresses to polymicrobial peritonitis, bacteremia, systemic inflammation, multi-organ failure and eventually death. Control animals also undergo laparotomy, however, the cecum is only manipulated and not injured.

Post-CLP, all animals immediately receive normal saline (75µl/g) and buprenorphine (0.1mg/kg) subcutaneously. Animals received subcutaneous fluids (normal saline;75µl/g) and antibiotics (ceftriaxone 25mg/kg; flagyl 12.5mg/kg) every 12 hours starting 12 hours post-CLP for the duration of the experiment. Buprenorphine (0.05mg/kg) was provided subcutaneously every 12 hours for the first 48 hours post

CLP. Depending on the protocol, resveratrol (20mg/kg in solutol), EX-527 (10mg/kg in solutol) or solutol as vehicle was given intraperitoneally starting at 6 hours post-CLP and then given every 12 hours for the duration of the experiment.

Blood Chemistries and Inflammatory Cytokines

Blood was assayed for arterial blood gases, lactate, hemoglobin, and blood urea nitrogen (BUN) using point of care veterinary cartridges (i-STAT, Abbot Point of Care Inc., Princeton, NJ). Serum creatinine aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine kinase were analyzed spectrophotometrically using commercially available kits (Pointe Scientific, Canton, MI). Serum IL6 TNFα, and neutrophil gelatinase-associated lipocalin (NGAL) levels were measured by enzyme-linked immunosorbent assays (Invitrogen, ThermoFisher Scientific and Bioporto Diagnostics, Hellerup, Denmark). Each sample was run in duplicate with known standards and according to the manufacturer's guidelines.

Blood glucose was determined at each time point using a standard glucometer for rodents (AlphaTRAK, Abbott Laboratories, IL). In resveratrol experiments, blood glucose levels post resuscitation were measured every 15 minutes for 1.5 hours and then at 120, 240 and 360 minutes. Hypoglycemia was defined as blood glucose lower than 70mg/dl.(285)

NAD/NADH Assays

NAD was extracted from frozen tissue samples (50mg) isolated mitochondria (100µg), or primary hepatocytes ($1X10^6$ cells) in ice-cold 0.6M perchloric acid. Tissues were homogenized at 20 Hz for 1 minute by tissue lyser (Qiagen). Mitochondria and cells were vortexed hard for 30-45 seconds. After centrifugation for 10 minutes (15,000g, 4°C), the clear supernatant was removed and diluted 1:100 in ice-cold 100mM sodium phosphate buffer, pH 8. Using a modified Graeff and Lee enzymatic cycling

assay (85), NAD was measured in a 96-well format. In brief, 5µl of NAD standards and diluted tissue extracts were mixed with 95µl of cycling mixture (0.2% ethanol, 0.11/ml alcohol dehydrogenase, 1.1mg/ml diaphorase, 20 µM resazurin, 10µM flavin mononucleotide, 10mM nicotinamide, and 0.1mg/ml BSA in 100mM phosphate buffer, pH 8.0). NAD concentration was determined based on the rate of resorufin accumulation using spectrophotometry (excitation at 544nm, emission at 590 nm, Synergy H1, Biotek).

NADH was extracted from frozen tissue (50mg) and isolated mitochondria (100µg) in ice-cold extraction buffer (0.25N KOH in 50% ETOH). Samples were homogenized and centrifuged as described. The supernatant was removed and heated at 55°C for 10 minutes to hydrolyze free NAD. After dilution in ice-cold 100 mM sodium phosphate buffer (1:50), samples were assayed using the described enzymatic cycling assay. The protein concentrations from the NAD and NADH extraction pellets were determined by using a Pierce BCA Protein Assay kit.

Primary Hepatocytes:

Primary hepatocytes were washed with phosphate buffered saline (PBS) 12 hours after plating. Cells were treated with sterile filtered plasma (0.22µm) harvested under shock and sham conditions, with and without NMN (100nM) for 24 hours. Cell survival was assessed by trypan blue exclusion (89-96% survival) and did not differ between groups.

In a separate experiment, hepatocytes were washed with PBS after 12 hours after initial plating and the media was changed to Dulbecco's Modified Eagle's Medium, 10% FBS, with 1% penicillin–streptomycin. Cells were treated with and without recombinant rat IL6 (20ng/ml, Sigma SRP4145) ± 100nM NMN for 1 hour. Survival ranged from 96-98% and did not differ between treatment groups. Cells were immediately assessed

using high resolution respirometry or were treated with 0.6M perchloric acid and stored at -80° C until assayed for NAD.

ATP Determination

ATP from frozen tissues (50mg) was measured using a commercially available ATP determination kit (Life Technologies) according to the manufacturer's instructions.

Isolation of Mitochondria

Liver and kidney tissues (4-6 g) were immediately immersed in ice-cold mitochondrial isolation buffer (MIB) (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA, final pH adjusted to 7.2 using KOH, and freshly supplemented with 0.5% fatty acid-free BSA). Tissue was homogenized in MIB with 5% BSA and mitochondria were isolated using differential centrifugation as previously described.(252,253). The final mitochondrial pellet was resuspended in MIB or treated with 0.6M perchloric acid for NAD measurements. Protein concentration was measured by Pierce BCA Protein Assay.

Mitochondrial Respiratory Capacity Using High Resolution Respirometry:

A standard substrate/inhibitor titration protocol was used for functional analysis of mitochondrial respiratory function.(252,253) Freshly isolated mitochondria (0.15 mg) were resuspended in respiration medium (110mM mannitol, 0.5mM EGTA, 3mM $MgCl_2$, 20mM taurine 10mM KH_2PO_4, 60mM K lactobionate, 0.3mM DTT, and 0.1% BSA (fatty acid free), adjusted to pH of 7.1 with KOH)(132). Oxygen consumption was measured using high-resolution respirometry at 37°C with constant stirring (Oxygraph-2k Oroboros Instruments, Innsbruck, Austria). Following stabilization (3-5 minutes), real-time oxygen concentration and flux data were continuously collected (DatLab software 4.3, Oroboros Instruments, Innsbruck, Austria). After the basal respiration rate was recorded, complex I (CI)-dependent respiration was induced by adding 10 mM glutamate, 5 mM malate and

1 mM ADP to the respiration chamber. In order to determine complex II (CII)-dependent respiration, rotenone (0.5 μM), a selective inhibitor of CI, was added followed by 10 mM of succinate. Antimycin A (5 μM) was then added to inhibit complex III (CIII); followed by TMPD (0.5 mM) and ascorbate (2 mM) as artificial substrates for complex IV. This protocol was completed within 60 minutes.

Similarly, isolated hepatocytes ($2.5x10^5$ cells) were resuspended in respiration buffer and analyzed using high-resolution respirometry. After stabilization, baseline oxygen consumption was recorded and cells were permeabilized digitonin (3 μg) and allowed to stabilize for 10 minutes. Cells were then subjected to the substrate/inhibitor titration protocol used for isolated mitochondria.

Citrate Synthase Activity

Citrate synthase activity is commonly used as a quantitative enzyme marker for the presence of intact mitochondria. Citrate synthase activity was determined according to the method described by Srere and Matsuoka (225), which couples coenzyme A to 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) (212). Kidney and liver tissue (100 μg) were suspended in an assay buffer that included 0.1 mM DTNB (in 1M Tris buffer, pH 8.0), 0.3 mM acetyl coenzyme A and 0.05% Triton X-100. Following the addition of 1 mM oxaloacetate, citrate synthase activity was determined spectroph otometrically by measuring the absorbance of thio-nitrobenzoic acid at 412 nm at 37°C.

Total Production of Mitochondrial-Derived ROS

Isolated mitochondria (10 μg) were suspended in 1 ml of buffer (250 mM sucrose, 20 mM 3-[N-morpholino] butane sulfonic acid, 10 mM Tris-base, 100 μMPi [K], 0.5 mM Mg^{2+}, pH 7.0; 30°C) containing CI substrates (malate/glutamate, 2.5/2.5 mM) and 2',7'-dichlorodihydro fluorescein diacetate (10 μM). Antimycin A was subsequently added to inhibit CIII and measure the inherent ROS production. Background

fluorescence was determined and was subtracted from all readings. After incubation at 30°C for 1 hour, the fluorescent signal from dichlorofluorescein (DCF) was detected (excitation 488 nm, emission 525 nm) and quantified using a Modulus Microplate Reader (Turner Biosystems, Sunnyvale, CA).

Measurements of plasma metabolic hormones

Blood (1.0 ml) was collected prior to sacrifice and plasma was stored at -80°C until analysis. Insulin, corticosterone, total GLP-1and glucagon were determined by the Radioimmunoassay and Biomarkers Core (Penn Diabetes Research Center, University of Pennsylvania) using commercially available ELISA and radioimmunoassay kits (Insulin and corticosterone ELISA -ALPCO Diagnostics, Windham, NH; total GLP-1 and glucagon radioimmunoassay -EMD Millipore, Billerica, MA). All samples were performed in duplicate.

Homeostatic model assessment (HOMA)- insulin resistance (IR) index

HOMA is a mathematical model of the relationship between glucose and insulin that can be used to estimate insulin resistance and β cell function. The natural Log (Ln) HOMA-IR index is used clinically to characterize abnormal glucose tolerance and assess insulin resistance using the equation: Ln (insulin level (μU/ml) × glucose (mg/dl) /405). (113)

Immunoblotting

For western blot analysis, frozen liver and kidney tissue was lysed in RIPA buffer supplemented with phosphatase inhibitors (PhosSTOP, Roche), protease inhibitors (Complete, Roche), nicotinamide (1mM) and trichostatin A (1μM) using a tissue lyzer (Qiagen). Lysates were centrifuged for 15 minutes (15,000g, 4°C). Lysates were denatured in 25% laemmli buffer + BME at 95°C for 5 minutes and were resolved on 4-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Gels were

transferred to polyvinylidene fluoride membranes (Millipore) blocked with nonfat soy milk for 5 minutes.(75) Individual membranes were probed using anti-NAMPT (1:5000, SC67020, Santa Cruz), anti-phospho-Y612-IRS-1(1:1000; Invitrogen Biosource International, Carlsbad, CA), anti-actin antibodies (1:10.000, Abcam), in tris-buffered saline with 0.1% Tween20.

Mitochondrial damage by reactive oxygen and nitrogen species was assessed by measuring 4-hydroxynonenal and 3-nitrotyrosine by Western blot (Abcam, Cambridge,MA). Briefly, mitochondrial protein (20 µg) was loaded in a 4-12% polyacrylamide gel and separated by electrophoresis (Invitrogen, San Diego, CA). Proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Richmond, CA). After the membranes were blocked for 1 hour at room temperature (10 mmol/L Tris, 150 mmol/L NaCl, and 0.05% Tween-20 supplemented with 5% dry milk), blots were incubated with the respective primary antibodies at 1:1000 dilution overnight at 4°C. After washing, membranes were incubated with peroxidase-linked donkey anti-rabbit or sheep anti-mouse IgG secondary antibodies (Amersham, Buckinghamshire, UK) at 1:5,000 dilution for 1 hour at room temperature.

Proteins of interest were detected by chemiluminescence using horseradish peroxidase conjugated secondary antibodies and Western Lightening Plus ECL (Perkin Elmer). Images were captured using a Bio-Rad imaging station and quantified using Image J (National Institutes of Health, Bethesda, MD)

Gene Expression

Liver and kidney RNA was extracted from frozen tissue using Trizol (Sigma-Aldrich) with ethanol precipitation. RNA (1µg) was used to create cDNA using a High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems) according to the manufacter's recommendation. Real-time polymerase chain reaction was performed using an Applied

Biosystems 7900HT system with SYBR green (Applied Biosystems). Two technical replicates per sample were obtained and relative mRNA expression levels were calculated using the ΔΔCT method normalized to actin as a housekeeping gene. Primers can be found in Table 4.

Statistical Analysis

Results are expressed as mean ± standard error of the mean. Comparison between two groups was performed using Students *t* or Mann Whitney test depending on normality of data distribution. One-way analysis of variance was used to compare 3 or more groups with a post hoc Students *t* or Mann Whitney test if statistically significant ($p<0.05$). Survival was analyzed using Kaplan-Meier curves with a log rank Mantel-Cox test. A chi square test was used to evaluate survival at 24 hours. All statistical analysis were performed using Prism 7 (GraphPad Software, Inc).

TABLES

Table 1: Clinical variables in hemorrhagic shock treated with NMN.

Organ Function Assay	Control Sham	NMN Sham	Control Shock	NMN Shock
Aspartate Aminotransferase (IU/L)	34.6 ± 6.2	34.4 ± 4.2	66.9 ± 4.9*	57.9 ± 4.8§
Alanine Aminotransferase (IU/L)	14.1 ± 1.7	11.6 ± 2.2	34.8 ± 5.9*	26.6 ± 4.8§
Creatinine (IU/L)	19.2 ± 1.3	22.9 ± 0.9	37.3 ± 4.8*	33.6 ± 4.8§
Blood Urea Nitrogen (mg/dL)	22.6 ± 2.0	18.2 ± 2.3	26 ±1.2	27.4 ± 0.6§
Creatine Kinase (U/L)	197 ± 53	220 ± 36	613 ± 81*	351 ± 42§♯

Results analyzed by one-way ANOVA followed by Student's t test
*$p<0.05$ Control Sham vs Control Shock
§$p<0.05$ NMN Sham vs NMN Shock
♯$p<0.05$ Control Shock vs NMN Shock

Table 2. Physiologic and laboratory parameters in hemorrhagic shock treated with resveratrol.

Characteristics	Sham (*n*=6)	Severe Shock (*n*=6)	LR (*n*=6)	LR+RSV (*n*=6)
MAP (mmHg)	111 ± 2	41 ± 1[a]	78 ± 5[a,b]	86 ± 2[a,b]
HR (bpm)	440 ± 12	419 ± 8[a]	438 ± 23	456 ± 15
Lactate (mmol/L)	1.3 ± 0.3	17.2 ± 1.3[a]	10.2 ± 1.2[a,b]	6.9 ± 1.3[b,c]
pH	7.40 ± 0.01	7.05 ± 0.09[a]	7.20 ± 0.04[a,b]	7.32 ± 0.03[b]
PCO2 (mmHg)	47 ± 4	17 ± 2[a]	28 ± 1[a,b]	33 ± 1[b]
PO2 (mmHg)	85 ± 4	128 ± 4[a]	113 ± 5[a]	105 ± 2[b]
$HCO3^-$ (mmol/L)	29.1 ± 2.0	5.4 ± 1.6[a]	11.3 ± 1.1[a,b]	17.2 ± 1.4[b,c]
BUN (mg/dl)	23 ± 2	29 ± 2[a]	25 ± 1	30 ± 3
Creatinine (mg/dl)	0.28 ± 0.03	0.63 ± 0.03[a]	0.53 ± 0.05[a]	0.57 ± 0.06[a]
Na^+ (mmol/L)	135 ± 1	128 ± 1[a]	130 ± 3	138 ± 1[bc]
K^+ (mmol/L)	4.5 ± 0.3	6.4 ± 0.6[a]	5.0 ± 0.4[b]	5.4 ± 0.4[b]
Cl^- (mmol/L)	103 ± 1	101 ± 1[a]	100 ± 2	112 ± 3[b,c]
Hemoglobin (g/L)	12.3 ± 0.4	5.0 ± 0.5[a]	3.8 ± 0.1[b]	4.1 ± 0.3
NGAL (ng/ml)	60.5 ±8.2	73.3 ±23.2	107.8 ± 28.5[a,b]	53.6 ± 1.0 [b,c]

LR = Lactated Ringer's solution; RSV = Resveratrol; MAP = Mean Blood Pressure; HR = Heart Rate in beats/min; BUN = Blood Urea Nitrogen. NGAL = neutrophil gelatinase-associated lipocalin. Values are mean ± SEM. *n* = 6.

[a]*p*<0.05 *versus* Sham.
[b]*p*<0.05 *versus* Severe Shock.
[c]*p*<0.05 *versus* LR Resuscitation.

Table 3. Endpoints of Euthanasia Scoring System
Adapted from the University of Pennsylvania Institutional Animal Care and Use

Physical Appearance	
0	Normal
1	Lack of grooming
2	Rough coat, nasal/ocular discharge
3	Very rough coat, abnormal posture, enlarged pupils
Clinical Signs	
0	Normal
1	Small changes of potential signficance
2	Body temperature change of 1-2 ˚C , respiratory rate ↑up to 30%
3	Body temperature change of >2 ˚C , respiratory rate ↑up to 50%, or markedly ↓
Unprovoked Behavior	
0	Normal
1	Minor changes
2	Abnormal, reduced mobility, decreased alertness, inactive
3	Unsolicited vocalizations, self-mutilation, ether very restless or immobile
Stimulated Behavior	
0	Normal
1	Minor depression/exaggeration of response
2	Moderately abnormal responses
3	Violent reactions, or comatose

Committee Guidelines on Adopted from the University of Pennsylvania's Institutional Animal Care and Use Committee's guide for Humane Endpoints for Laboratory Animals

Table 4. Primers used for qPCR

Transcript	Forward Primer	Reverse Primer
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
IL6	TAGTCCTTCCTACCCCAACTTCC	TTGGTCCTTAGCCACTCCTTC
TNFα	CTGTGCCTCAGCCTCTTCTC	ACTGATGAGAGGGAGCCCAT
NAMPT	TCTGGAAATCCGCTCGACAC	TATCCACTCCGTCCCCTTGA
NMNAT1	CAGAGCATCCGCTACTTGGT	ATCGGGTGGAATGGTTGTGT
NMNAT2	TCTGACTGGATCAGGGTGGA	ATGGTGCTCTAACACACTGC
NMNAT3	CCTGCGTTTGTTTGAGGTGG	ATGATGCCGTTTCCATCCACT
SIRT1	CAGGTTGCAGGAATCCAAA	CAAATCAGGCAAGATGCTGT
PGC1-α	CCAGTCTACGGCTGTTTGGT	TGGAAGAACAGATGTGCCCC
NRF1	ACAGATAGTCCTGTCTGGGGAAA	TGGTACATGCTCACAGGGATCT
NRF2	TGAAGTTCGCATTTTGATGGC	CTTTGGTCCTGGCATCTCTAC
TFAM	GTTTCGTGCGGGTTTGTGAA	GAAACTGCAATGGCTCTGCC
SOD2	GCCTGCACTGAAGTTCAATG	ATCTGTAAGCGACCTTGCTC
CAT	ACCCTCTTATACCAGTTGGC	GCATGCACATGGGGCCATCA
COX-2	ATTCTTTGCCCAGCACTTCA	ATCATCAGACCAGGCACCA

FIGURES

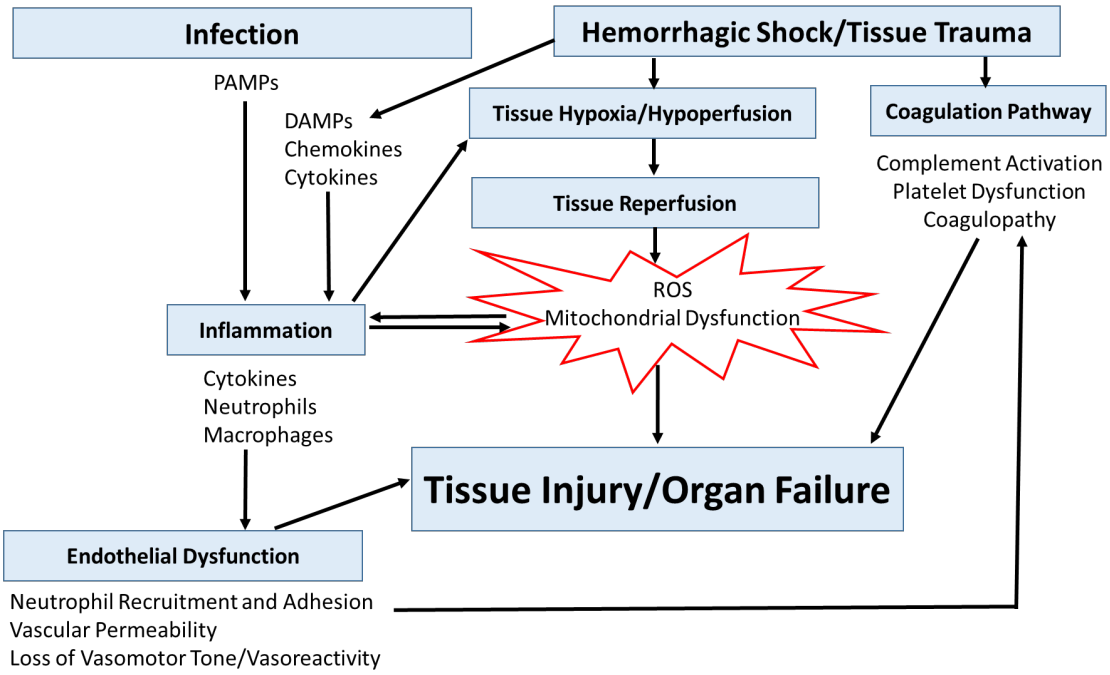


Figure 1: Pathways activated in the resuscitation of shock states.

Traumatic injury with acute blood loss results in vasoconstriction, activation of the coagulation pathway, release of DAMPs (damage associated molecular patterns), and the secretion of chemokines and cytokines which activate the immune response. Similarly, infection releases PAMPs (pathogen associated molecular patterns) which activate the immune system. Both types of shock are associated with tissue hypoxia and hypoperfusion. Decreased oxygen and fuel substrates restrict ATP generation and lead to reduced NAD levels. With reperfusion, there is massive generation of ROS (reactive oxygen species) and mitochondrial dysfunction, which in turn further activates the immune system, injures the endothelium and causes more tissue damage.

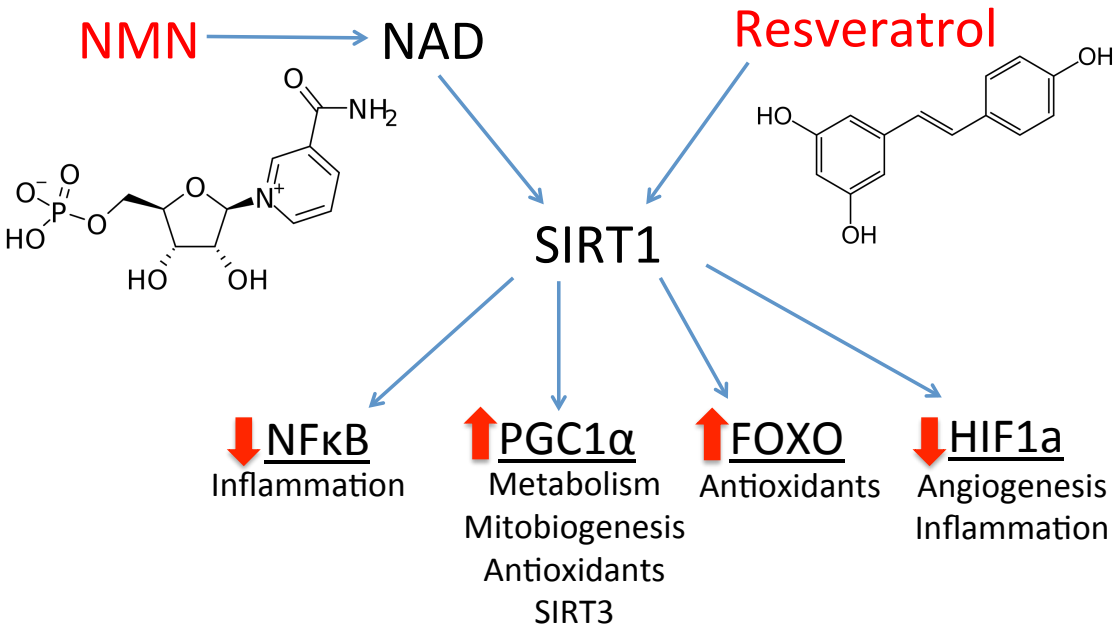


Figure 2: SIRT1 targets and activators.

SIRT1 can interact with a number of targets involved in the pathophysiology of shock. Specifically, SIRT1 can inhibit NFκB and decrease inflammation. SIRT1 deacetylates PGC1α (perioxisome proliferator-activated receptor gamma co-activator 1-alpha), a key transcriptional co-activator that promotes mitochondrial biogenesis, coordinates a switch to fatty acid metabolism and decreases oxidative stress. PGC1α also increases the transcription of SIRT3, the major mitochondrial deacetylase. Interactions with FOXO FOXO (forkhead box O) increases the expression of key antioxidant enzymes such as manganese superoxide dismutase and catalase. Nicotinamide mononucleotide (NMN) can be directly converted to NAD, an essential activator of SIRT1. Resveratrol also can increase SIRT1 activity either by increasing NAD or by allosteric interactions. SIRT1 inhibits NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), thereby downregulating the transcription of pro-inflammatory cytokines.

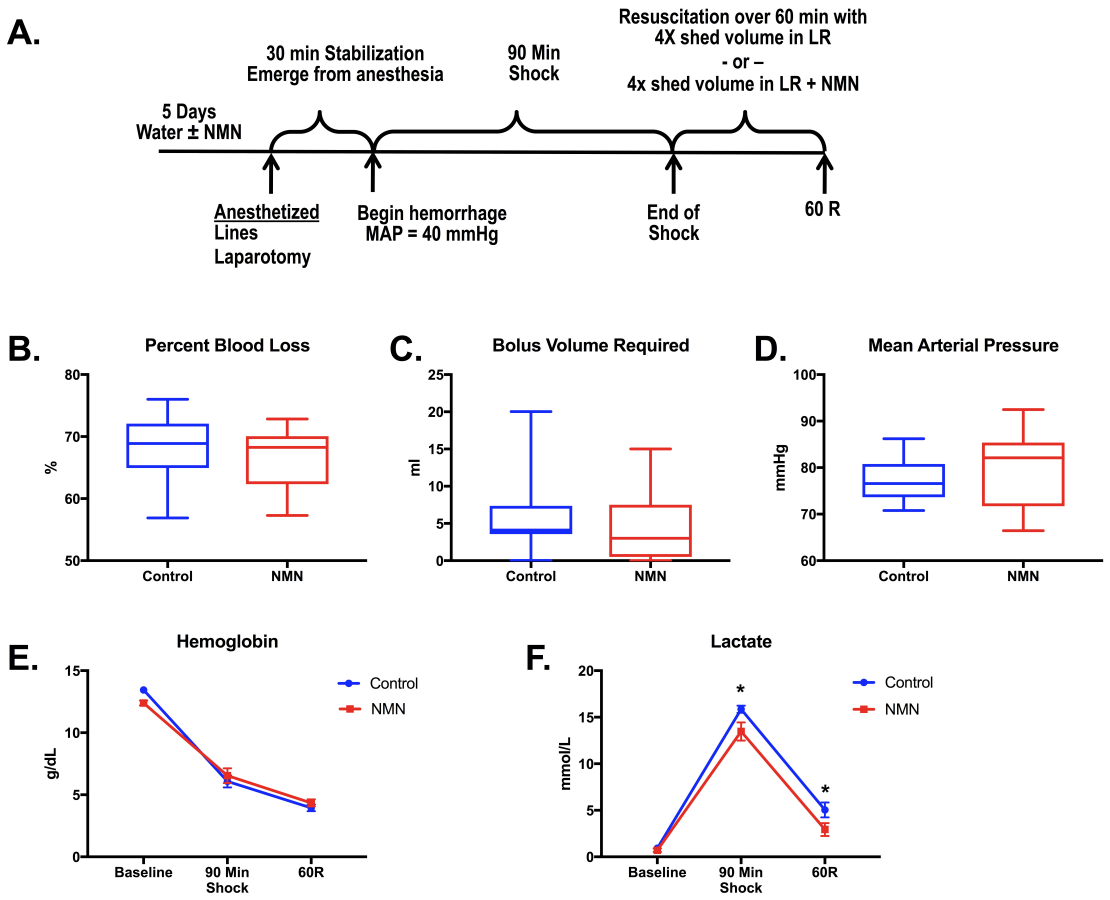


Figure 3: Fixed pressure hemorrhagic shock design and experimental variables.

(**A**) Animals were randomized to water ± NMN (400 mg/kg/day) for 5 days (n=9 -12 per group). Animals were bled to a mean arterial blood pressure (MAP) of 40mmHg for 90 minutes and then resuscitated with 4X the shed volume in lactated Ringer's ± NMN (400mg/kg) over 60 minutes. Control animals did not differ from NMN treated in terms of percentage of total blood volume shed (**B**), the volume of LR needed to maintain a MAP of 40 mmHg for 90 minutes (**C**), or the MAP after 60 minutes of resuscitation (**D**). Hemoglobin was similar between groups (**E**). NMN treated animals had significantly lower lactate levels during shock and following resuscitation (**F**). Data were analyzed by Student's t test or Mann Whitney as appropriate. * $p<0.05$.

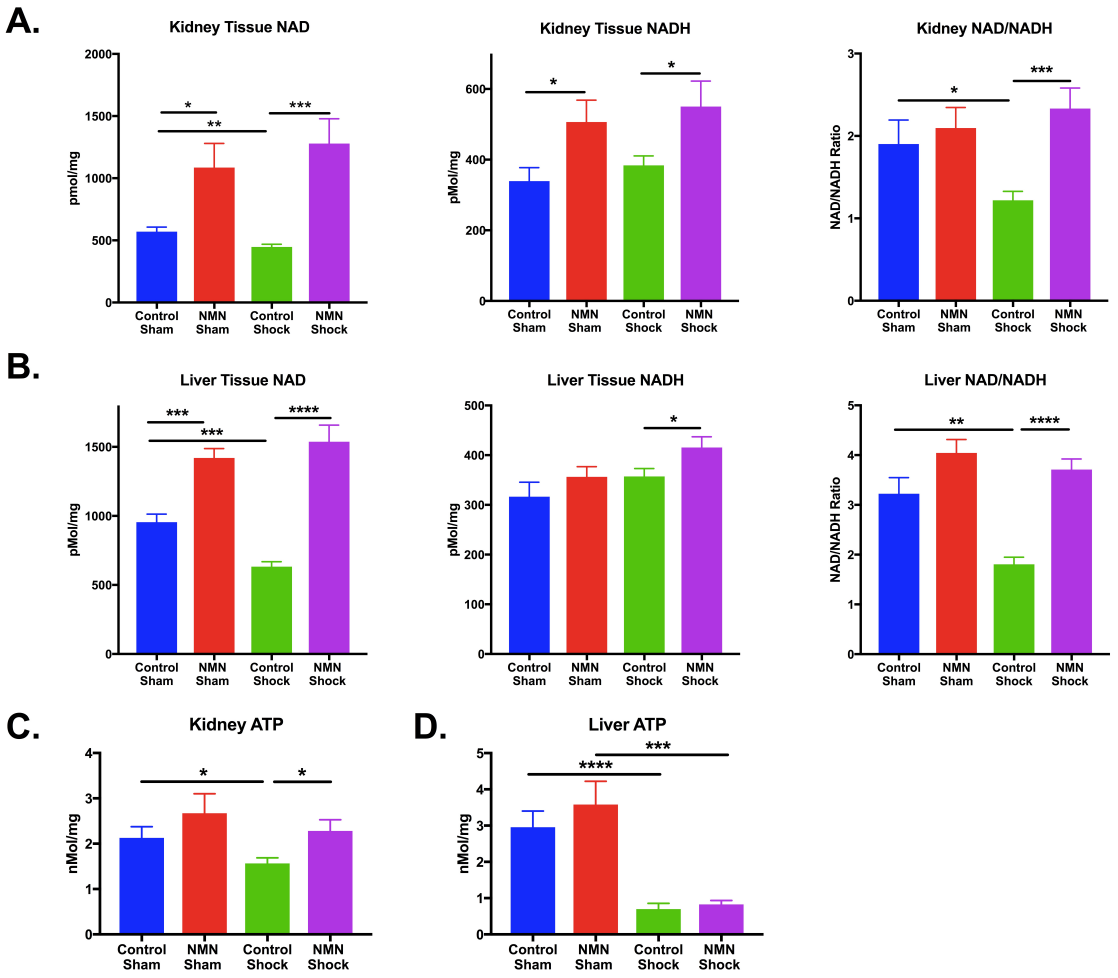


Figure 4: NMN increases NAD and NADH, and preserves renal ATP following resuscitation from hemorrhagic shock.

NAD, NADH, and ATP levels were measured in extracts from snap frozen tissues. In the kidney, NMN increased the NAD and NADH levels in both sham (n=5-6 per treatment group) and shocked animals (9-12 per treatment group), and prevented the sharp decline in NAD/NADH ratio observed in shocked animals (**A**). In the liver, NMN increased NAD levels in both sham and shocked animals, but only increased NADH significantly in shocked animals, while again preserving the NAD/NADH ratio (**B**). ATP levels declined in both tissues following hemorrhagic shock and resuscitation (**C, D**). Treatment with NMN, however, completely prevented this decline in renal tissue (**C**). Data were analyzed by one way ANOVA, followed by Student's t test or Mann Whitney as appropriate. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

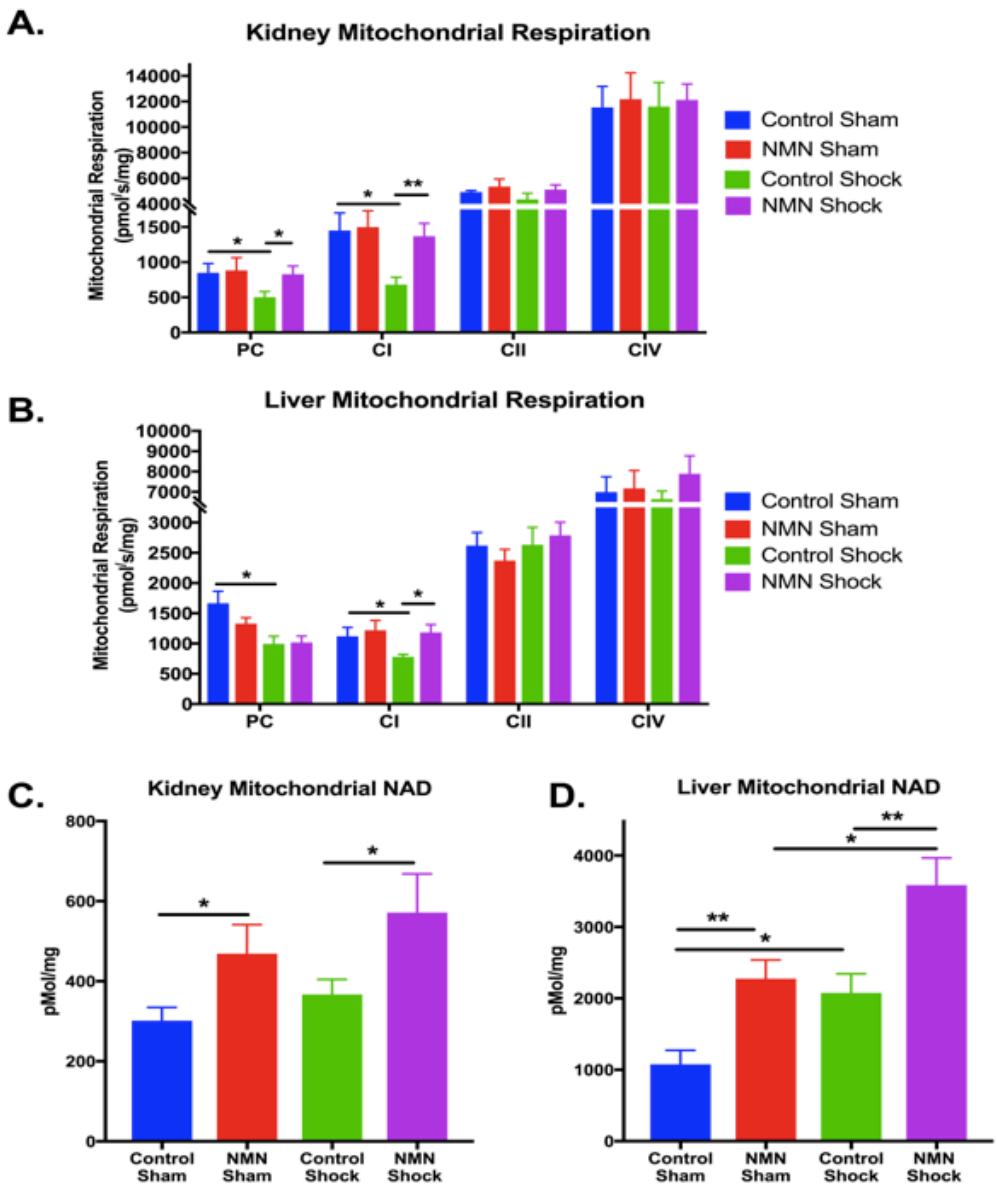


Figure 5: NMN preserves Complex I-dependent mitochondrial respiration and enhances mitochondrial NAD content.

Following hemorrhagic shock and resuscitation, mitochondria from kidney and liver tissue were freshly isolated and evaluated using high-resolution respirometry. In both tissues, a defect in respiration was noted when NAD-dependent substrates such as palmatoylcarnitine (PC) and pyruvate/glutamate/malate (CI) were used. Pretreatment, and subsequent resuscitation, with NMN completely restored mitochondrial respiration in kidney (**A**) and preserved respiration with CI substrates in liver (**B**). Without NMN treatment, NAD content was preserved following resuscitation in kidney (**C**) and increased in liver (D). NMN augmented mitochondrial NAD levels in both the sham and shocked states (**C, D**). (n=5-6 per treatment group). Data were analyzed by one-way ANOVA, followed by Student's t test or Mann Whitney as appropriate.

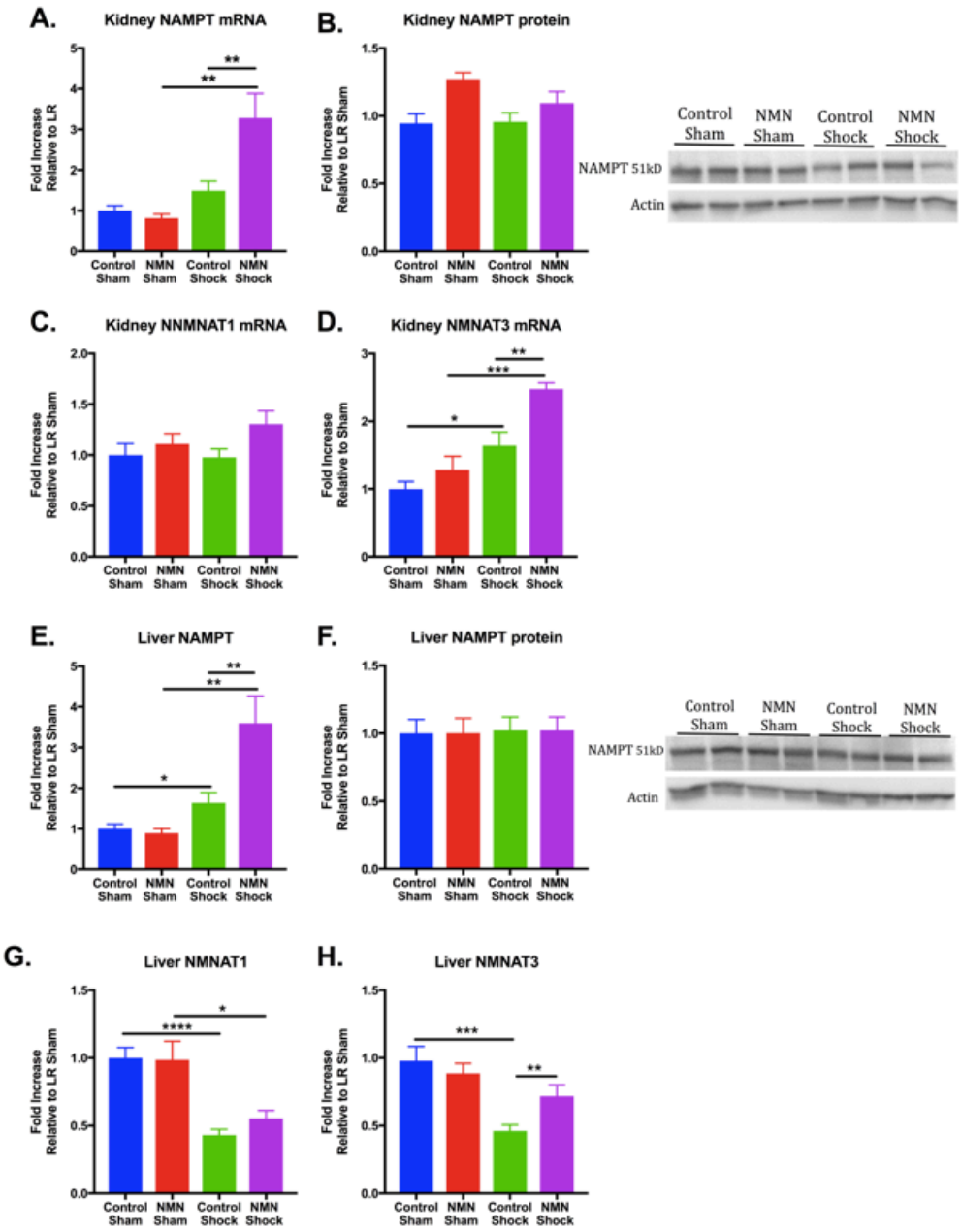


Figure 6: NMN increases NAMPT and NMNAT3 following hemorrhagic shock.

Following hemorrhagic shock, there was a trend toward increased NAMPT mRNA that was further enhanced with NMN treatment in kidney (**A**) and liver (**E**). NAMPT protein expression was not affected by shock in either tissue (**B, F**). NMNAT1 expression was not affected in kidney (**C**), but declined in liver and was not affected by NMN (**G**). NMNAT3 expression was increased in kidney and decreased in liver following shock, but in both tissues was enhanced by NMN treatment only in animals that had been shocked (**D, H**). (n=5-12 per treatment group) Data were analyzed by one-way ANOVA, followed by Student's t test or Mann Whitney as appropriate. *p<0.05, **p<0.01, ***p<0.001.

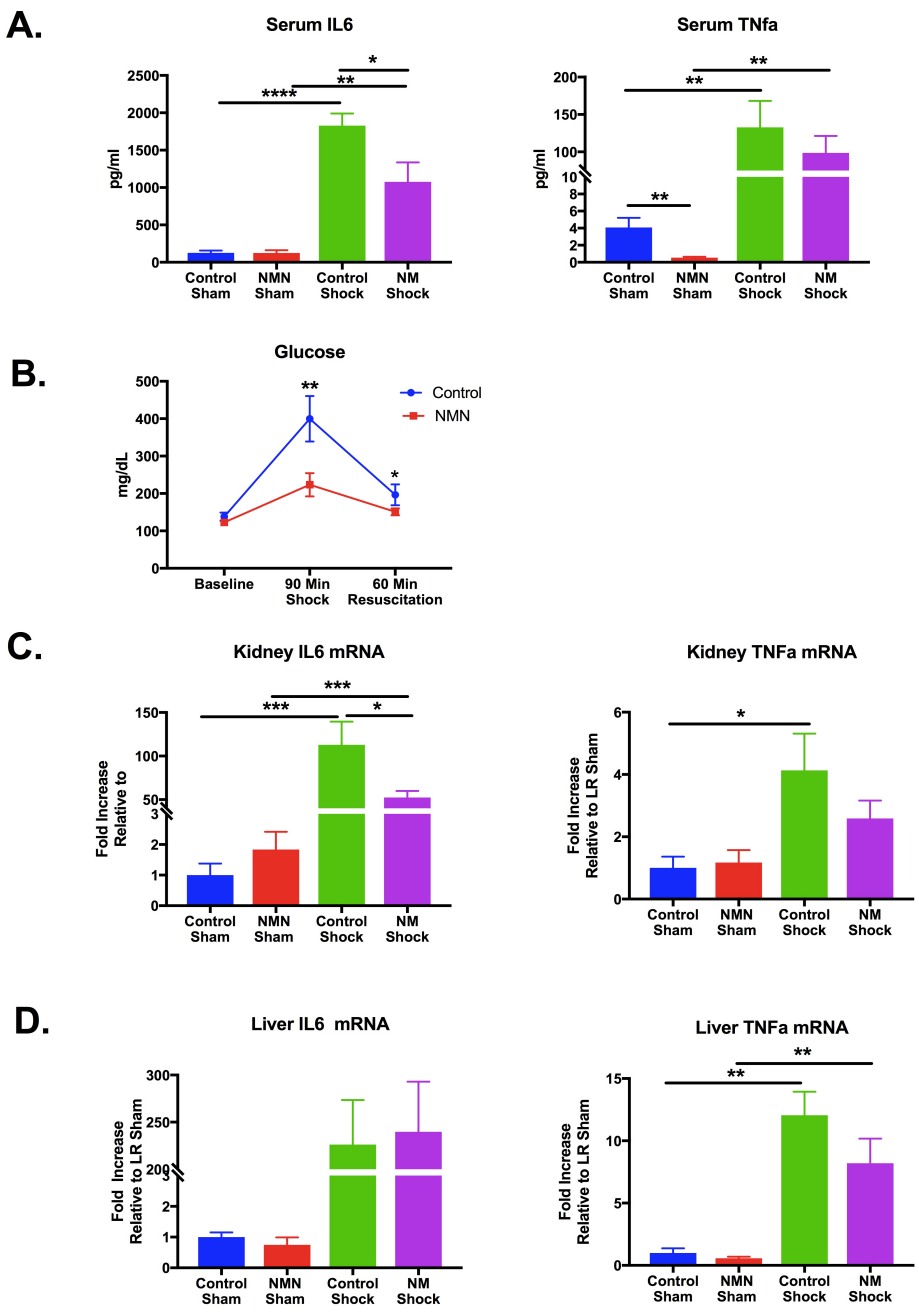


Figure 7: NMN mitigates inflammation following hemorrhagic shock.

Serum IL6 and TNFα were determined by ELISA. Serum levels of both pro-inflammatory cytokines increased with hemorrhagic shock, whereas NMN significantly lowered IL6 with a trend toward improved TNFa levels (**A**). The decrease in cytokines correlated with improved hyperglycemia following shock (**B**). Inflammatory cytokine expression was measured by qPCR in kidney and liver tissues. NMN partially mitigated IL6 mRNA expression in kidney, but not in liver (**C, D**). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

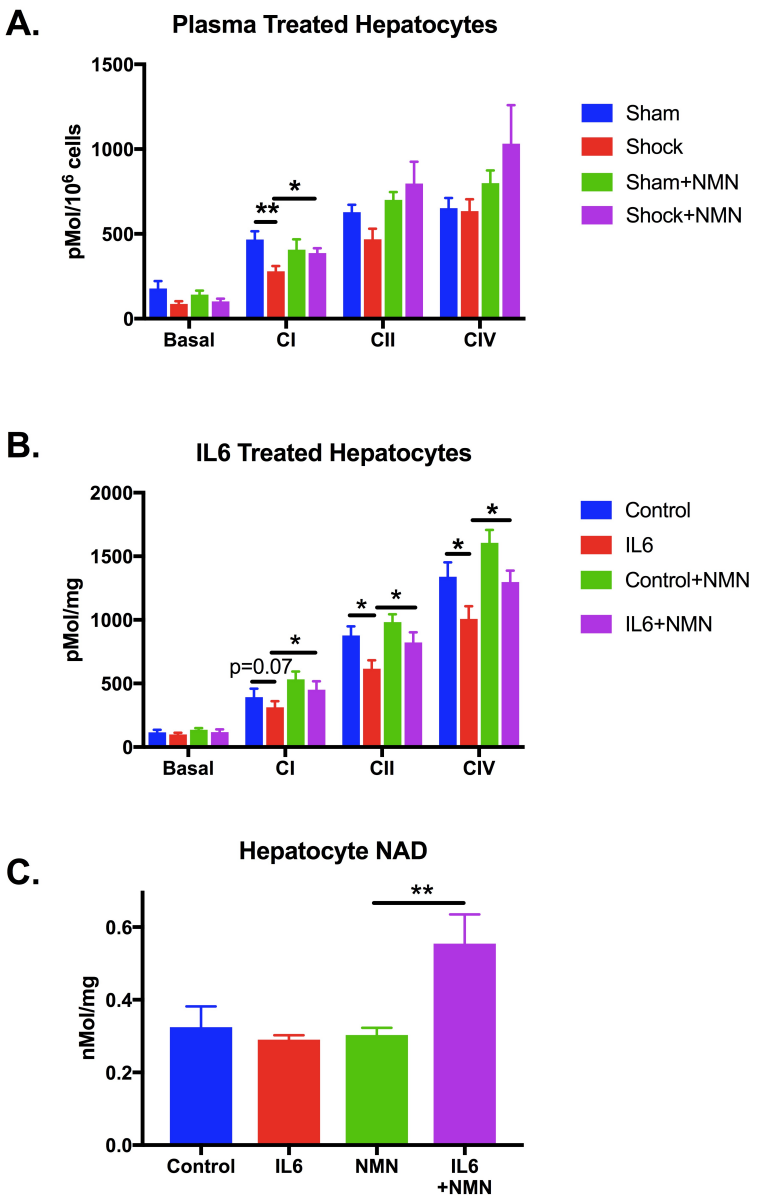


Figure 8: NMN rescues mitochondrial function and increases NAD in isolated hepatocytes after cytokine exposure.

After co-culture for 24 hours with plasma harvested from animals in hemorrhagic shock, digitonin-permeabilized isolated hepatocytes exhibited a defect in complex I dependent respiration that was mitigated by concurrent treatment with NMN (100μM) (**A**). When treated with IL6 (20ng/ml) for 1hour, isolated hepatocytes developed global mitochondrial dysfunction with decreased respiratory capacity at every complex that was mitigated by co-treatment with NMN (100μM) (**B**). NMN increased NAD levels only in cells that were co-treated with IL6 (**C**). (n=6-8 per group)*$p<0.05$, **$p<0.01$.

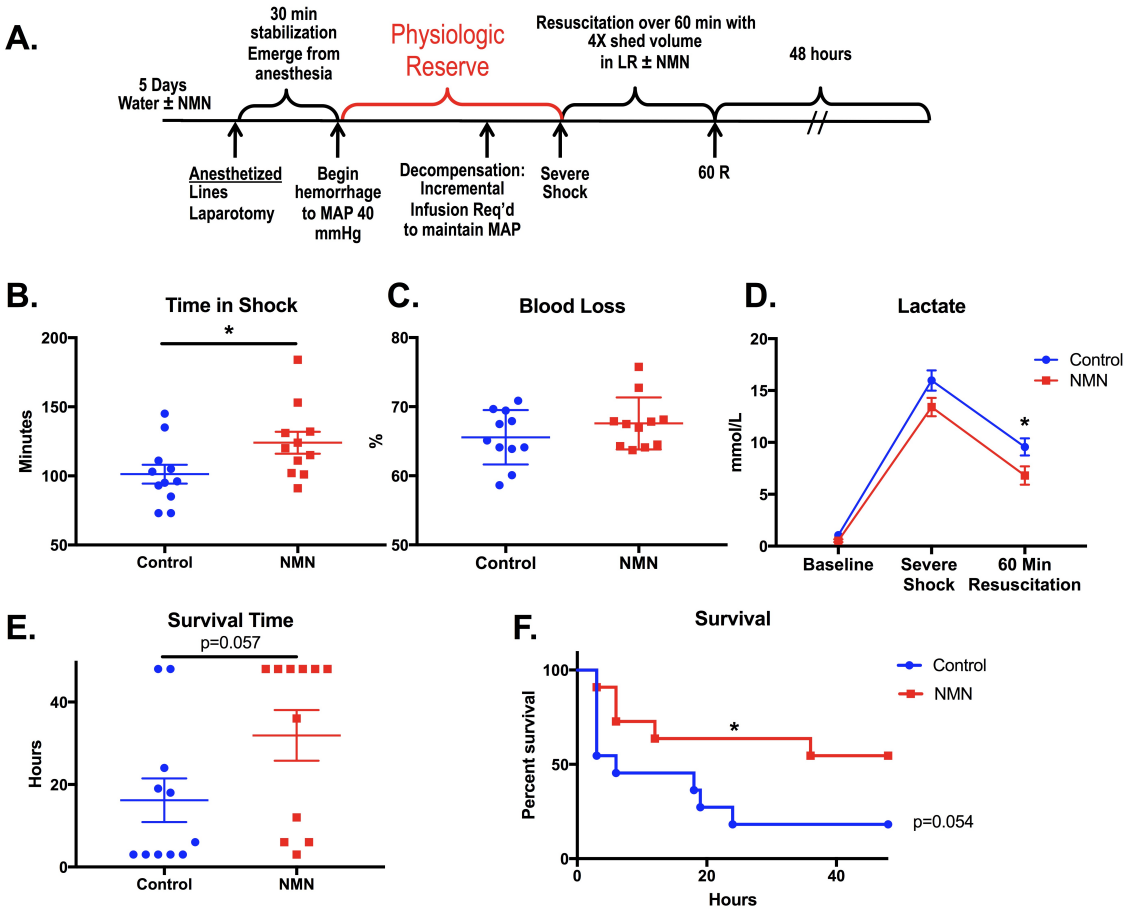


Figure 9: NMN enhances tolerance to hemorrhagic shock.

After randomization to water ± NMN (400 mg/kg/day) for 5 days, animals were bled to a mean arterial blood pressure (MAP) of 40mmHg and then maintained at 40 mmHg with incremental fluid boluses until 40% of the shed blood volume had been returned. Animals were then resuscitated with 4X the shed volume in lactated Ringer's ± NMN (400mg/kg) over 60 minutes and observed for 48 hours (n=11 per treatment group)(**A**). Animals pretreated with NMN were able to sustain a shock state longer than controls (**B**) but with a similar percentage of blood loss (**C**). NMN treated animals had lower blood lactate concentrations following resuscitation (**D**) and despite having spent more time in shock, were significantly more likely to be alive at 24 hours than were controls (**E, F**). *p<0.05.

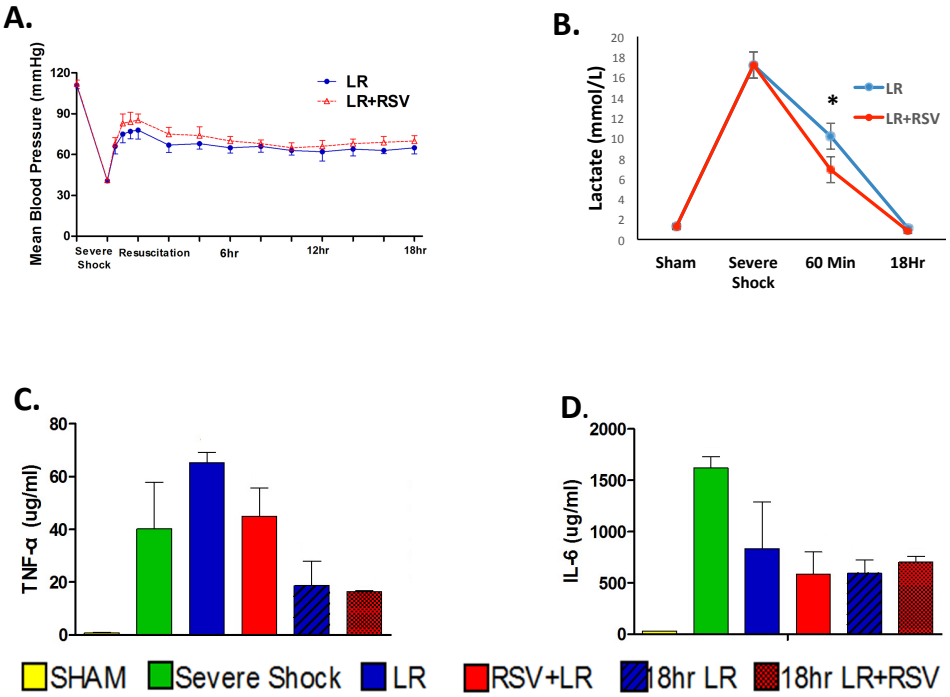


Figure 10: Hemodynamics and lactate values during resuscitation with resveratrol.

Male Long Evan's rats were subjected to a decompensated model of hemorrhagic shock and then resuscitated with 4X the shed volume in lactated Ringer's solution (LR) ± resveratrol (RSV; 30mg/kg) over 60 minutes. (**A**) RSV did not influence mean arterial blood pressure. (**B**) Lactate levels were significantly lower in the RSV treated group at 60 minutes, but returned to baseline in both groups by 18 hours. RSV treatment did not significantly decrease the levels of TNF-α or IL-6 when compared to LR resuscitation alone (**C,D**). TNF-α and IL-6 were measured by ELISA. SS= severe shock, R = after resuscitation. Values are mean ± SEM, n = 6 per group. *$p<0.05$.

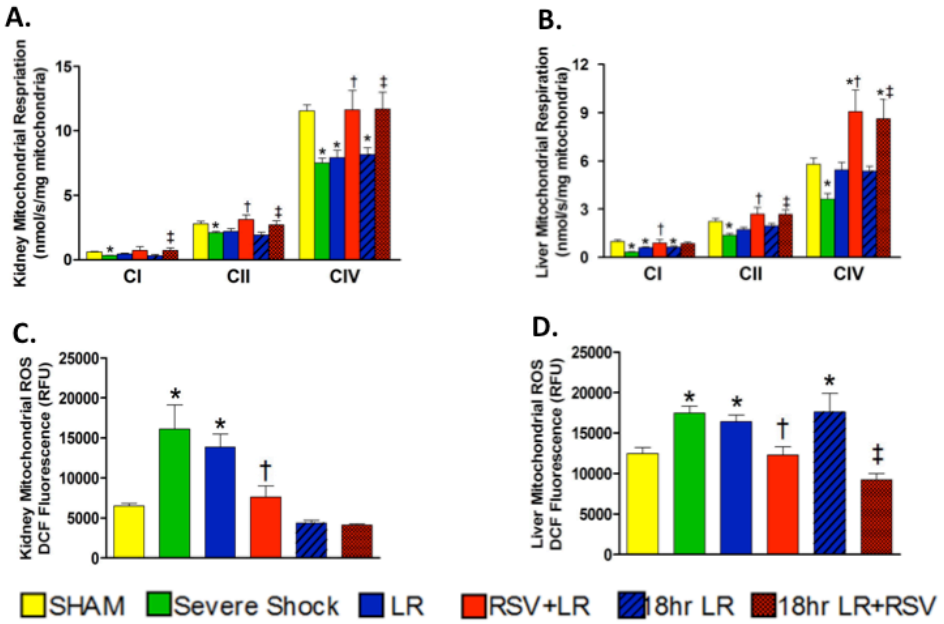


Figure 11. RSV supplementation during resuscitation restores mitochondrial function and decreases the production of ROS.

The respiratory capacity of individual complexes in isolated intact mitochondria was assessed by high-resolution respirometry. Following hemorrhagic shock, mitochondrial CI, CII, and CIV in the liver and kidney demonstrated decreased activity. (**A**) In the liver, RSV supplementation during resuscitation robustly restored the respiratory function of all complexes and significantly improved CII- and CIV- dependent respiratory capacity over baseline at 18 hours after resuscitation. (**B**) RSV supplementation significantly restored kidney CII- and CIV-dependent respiration following resuscitation and improved the respiratory capacity of all mitochondrial complexes 18 hours later. Mitochondrial ROS production was detected by measuring the fluorescent signal from dichlorofluorescein. RSV supplementation significantly reduced liver (**C**) and kidney (**D**) mitochondrial ROS production following resuscitation. *LR*, resuscitation with LR solution; *LR + RSV*, resuscitation with LR solution plus RSV (30 mg/kg); *18hr LR*, 18 hours after resuscitation with LR solution; *18hr LR + RSV*, 18 hours after resuscitation with LR solution plus RSV (30 mg/kg). Values are mean ± SEM. n = 6. *$p < 0.05$ versus sham; †$p < 0.05$, LR versus LR + RSV; ‡$p < 0.05$, 18hr LR versus 18hr LR + RSV.

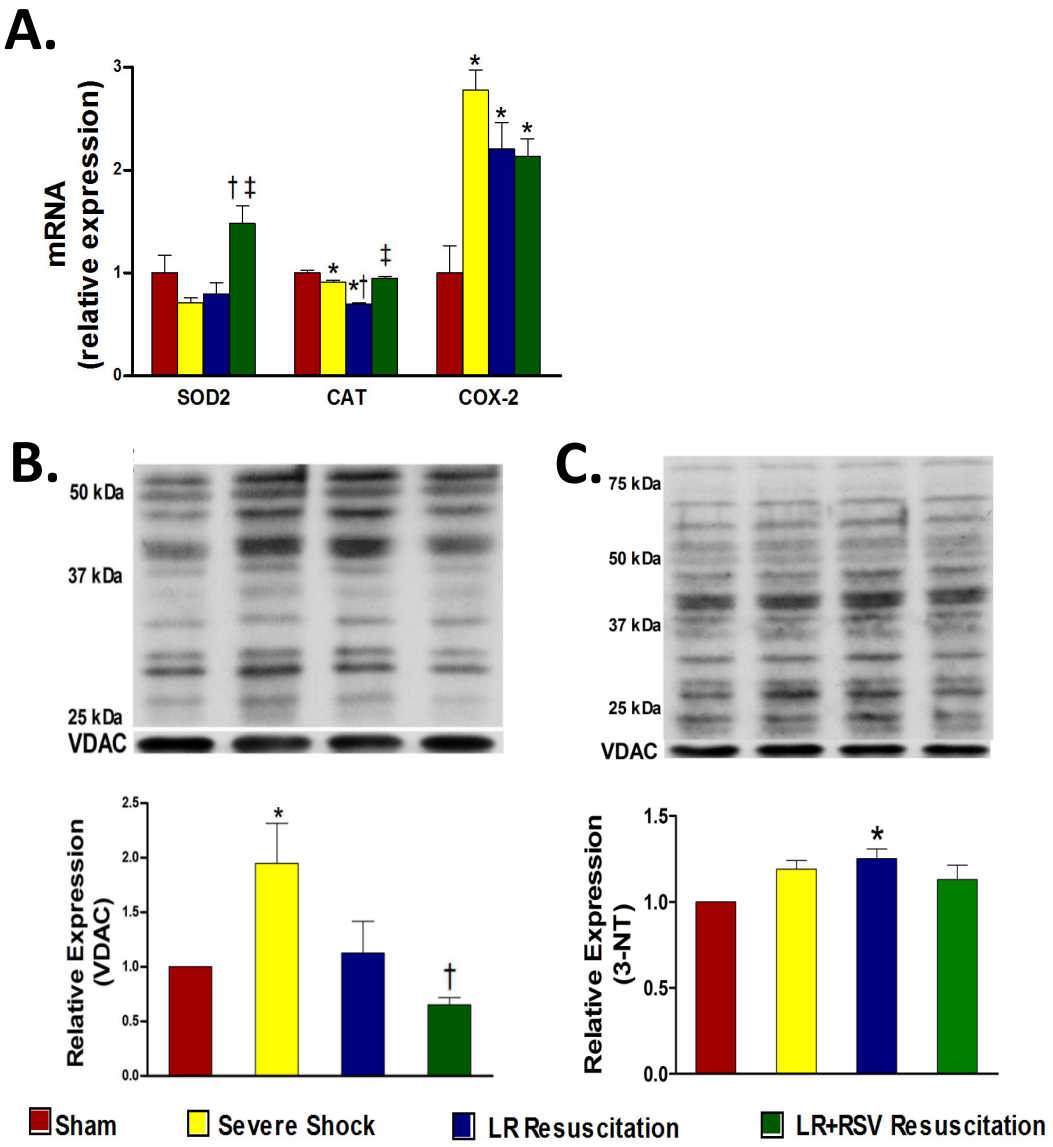


Figure 12: Resveratrol treatment during resuscitation ameliorates renal mitochondrial oxidative stress following hemorrhagic shock and resuscitation.

(**A)** LR+RSV resuscitation significantly increased the mRNA expression of superoxide dismutase 2 (SOD2) and catalase (CAT) in kidney tissue when compared to LR resuscitation. (**B)** 4- hydroxynonenal (4-HNE) was measured by western blots a marker of mitochondrial lipid peroxidation. 4-HNE levels robustly increased following Severe Shock and were significantly reduced with LR+RSV resuscitation. (**C)** Expression levels of 3-nitrotyrosine (3-NT) in mitochondria were determined by western blot. 3-NT levels increased with LR resuscitation, but did not increase with LR+RSV resuscitation. LR = Lactated Ringer's solution; RSV = Resveratrol; COX = cyclooxygenase. Values are mean ± SEM. n = 6, *p<0.05 *versus* Sham; †p<0.05 *versus* Severe Shock; ‡p<0.05 *versus* LR Resuscitation.

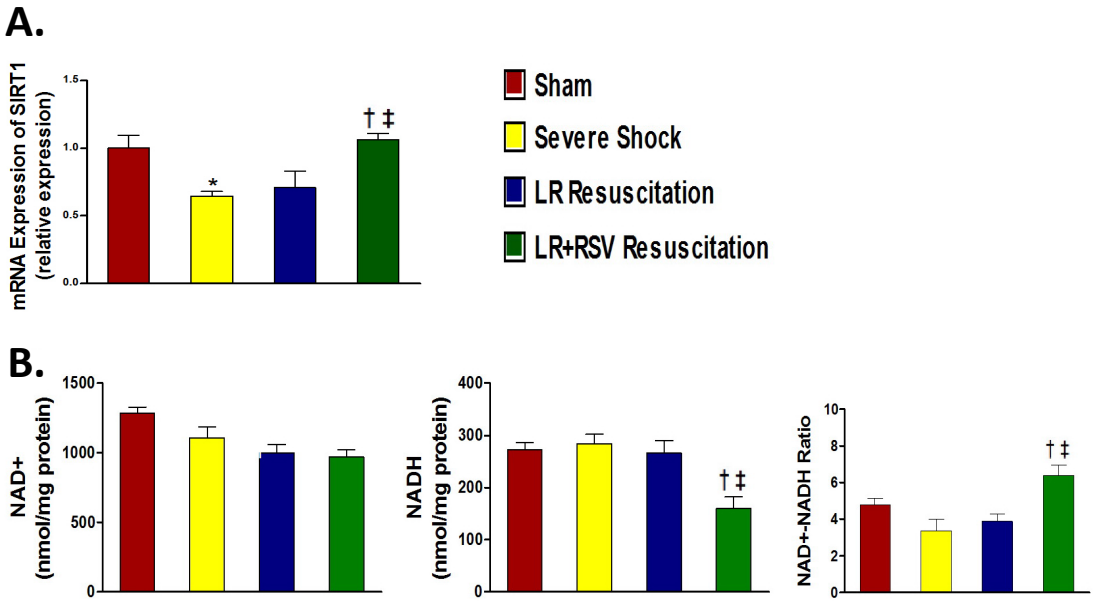


Figure 13: Resveratrol supplementation enhances the mRNA expression of Sirtuin 1 (SIRT1) and increases the nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinucleotide dehydrogenase (NADH) ratio in kidney.

(**A**) The decline in the mRNA expression level of SIRT1 after severe shock and LR resuscitation was reversed with RSV administration during resuscitation. (**B**) NAD+, NADH concentration and NAD+-NADH ratio in kidney tissue were obtained by detecting the fluorescent signal of resazurin. LR+RSV resuscitation significantly decreased the NADH concentration and nearly doubled the NAD/NADH ratio when compared to LR resuscitation. LR = Lactated Ringer's solution; RSV = Resveratrol. Values are mean ± SEM. n = 6, *p<0.05 *versus* Sham; †p<0.05 *versus* Severe Shock; ‡p<0.05 *versus* LR Resuscitation.

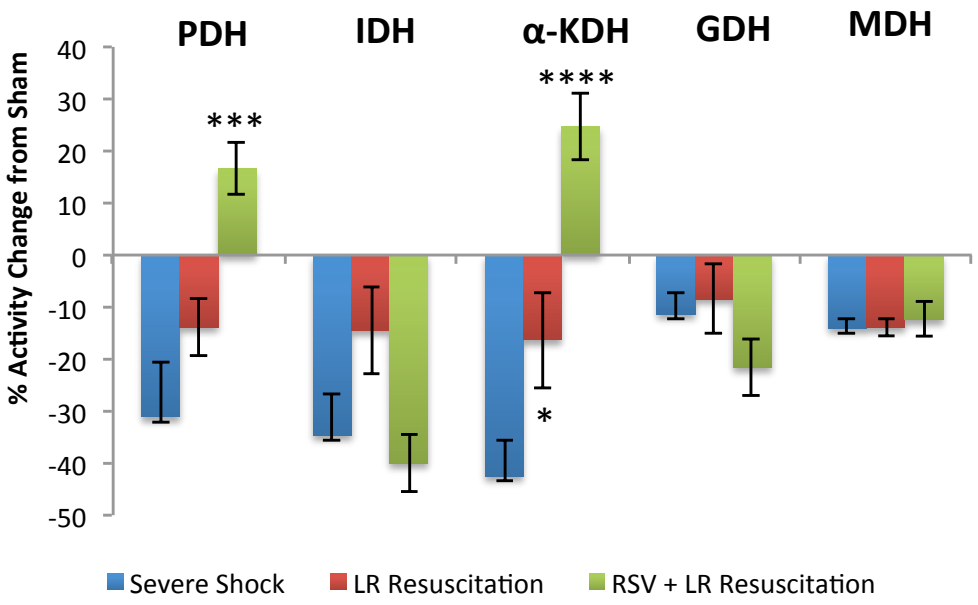


Figure 14: Resuscitation with RSV enhances pyruvate dehydrogenase and α-ketoglutarate dehydrogenase activity following hemorrhagic shock.

Kidney tissue harvested after 60 minutes of resuscitation with lactated Ringer's (LR) or LR + resveratrol (30mg/kg). Enzymatic activity of Kreb cycle enzymes was assessed by measuring by the spectrophotometric reduction of NAD+ to NADH. All enzyme activities decreased with severe shock. When compared to LR, RSV significantly enhanced the activity of PHD and a-KDH, but was associated with decreased IDH activity. PDH = pyruvate dehydrogenase, IDH = isocitrate dehydrogenase, a-KDH= alpha ketoglutarate dehydrogenase, GDH = glutamate dehydrogenase, MDH = malate dehydrogenase. Values are mean ± SEM. n = 6 per time point. *$p<0.05$, ***$p<0.001$,****$p<0.0001$; Severe Shock vs LR or LR+RSV.

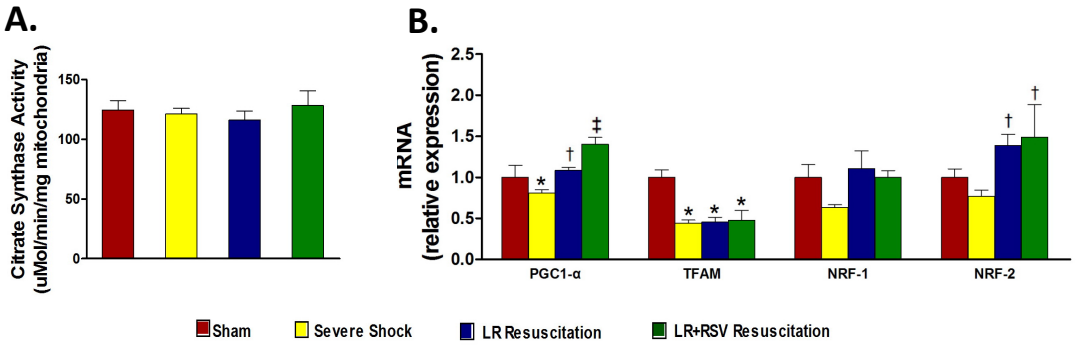


Figure 15: Mitochondrial content and expression of mitochondrial biogenesis factors in the kidney following hemorrhagic shock and resuscitation.

Citrate synthase activity was determined spectrophotometrically based on coenzyme A coupled to 5, 5′-dithiobis-2-nitrobenzoic acid. (**A**) Citrate synthase activity, a proxy for mitochondrial content, did not significantly change with hemorrhagic shock or with either resuscitative strategy. (**B**) mRNA expression of peroxisome proliferator-activated receptor gamma coactivator 1- alpha (PGC1-α) was significantly elevated in the LR+RSV group compared to the LR resuscitation only group. The expression of other factors involved in mitobiogenesis such as mitochondrial transcription factor A (TFAM), nuclear respiratory factor (NRF) 1 and 2, however, were not affected by RSV. LR = Lactated Ringer's solution; RSV= Resveratrol. Values are mean ± SEM. n = 6, *p<0.05 versus sham; †p<0.05 *versus* Severe Shock; ‡p<0.05 *versus* LR Resuscitation.

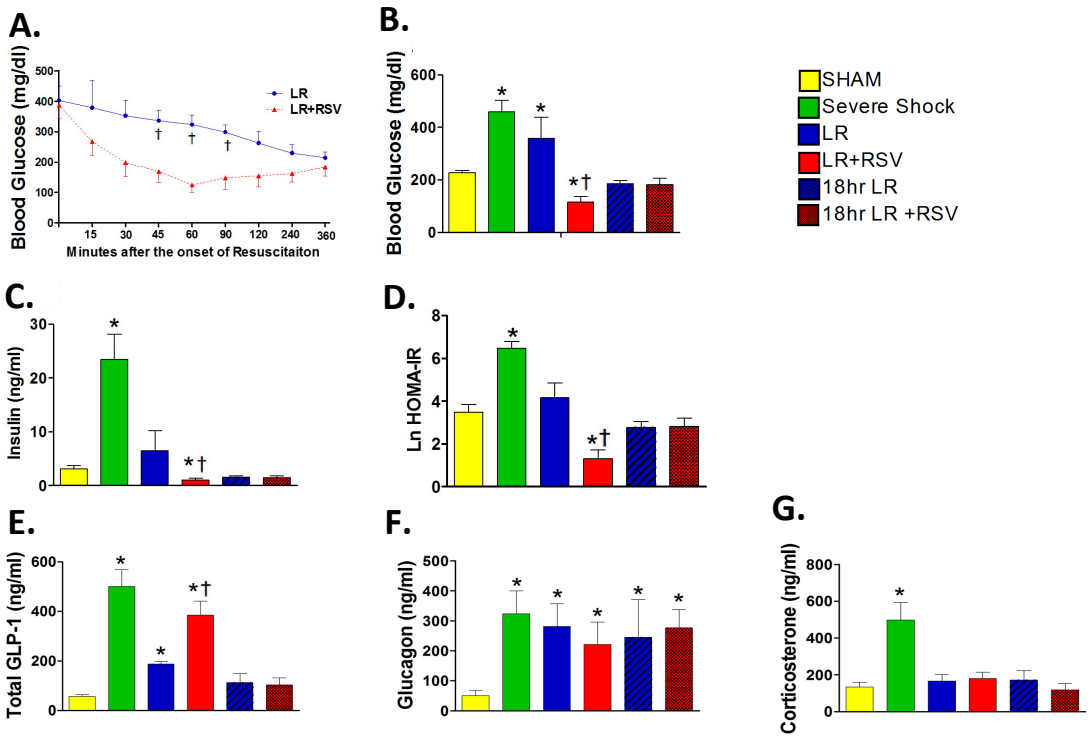


Figure 16: Resuscitation with RSV lowers blood glucose and improves insulin resistance.

Decompensated hemorrhagic shock resulted in severe hyperglycemia, hyperinsulinemia, and a significantly elevated Ln HOMA-IR index. (**A, B**) RSV was associated with significantly lower blood glucose values 45, 60, and 90 minutes after the onset of resuscitation and animals treated with LR + RSV were more hypoglycemic than those in the sham group. (**C**) RSV supplementation resulted in significantly decreased plasma insulin levels, as determined by ELISA. (**D**) RSV significantly improved insulin resistance as calculated by the Ln HOMA-IR index (Ln [insulin × glucose/405]). (**E**) Compared with LR alone, LR + RSV significantly increased GLP-1 levels following hemorrhagic shock but did not influence glucagon or corticosterone values (**F, G**). GLP-1and glucagon were measured by radioimmunoassay; corticosterone was measured by ELISA. LR= lactated Ringer's solution, RSV = resveratrol (30mg/kg); Ln-HOMA-IR index = the natural Log homeostatic model assessment –insulin resistance index; GLP-1= glucagon like peptide-1 Values are mean ± SEM. n = 6. *$p < 0.05$ versus sham; †$p < 0.05$, LR versus LR + RSV.

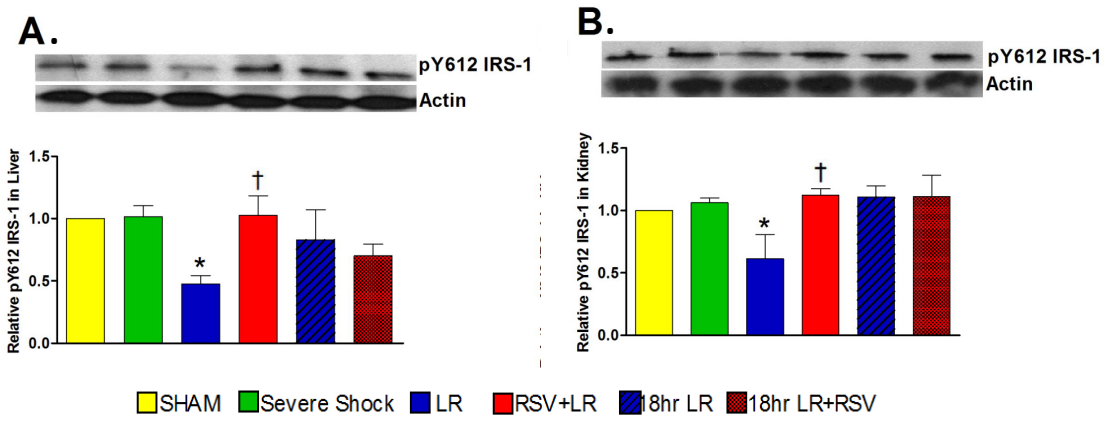


Figure 17: Resveratrol (RSV) restores activation of IRS-1 in both liver and kidney.

The phosphorylated tyrosine 612 of IRS-1 (pY612 IRS-1) is the active form of IRS-1 that participates in insulin signaling. Western blotting for this modification indicates that hemorrhagic shock and resuscitation with only LR are associated with significantly decreased phosphorylation of IRS-1 in liver and kidney tissues. Resuscitation with LR + RSV robustly preserved the phosphorylation of IRS-1 in both the liver (*A*) and the kidney (*B*). *LR*, resuscitation with LR solution; *LR + RSV*, resuscitation with LR solution plus RSV (30 mg/kg); *18hr LR*, 18 hours after resuscitation with LR solution; *18hr LR + RSV*, 18 hours after resuscitation with LR solution plus RSV (30 mg/kg). Values are mean ± SEM. n = 4–5. *$p < 0.05$ versus sham; †$p < 0.05$, LR versus LR + RSV.

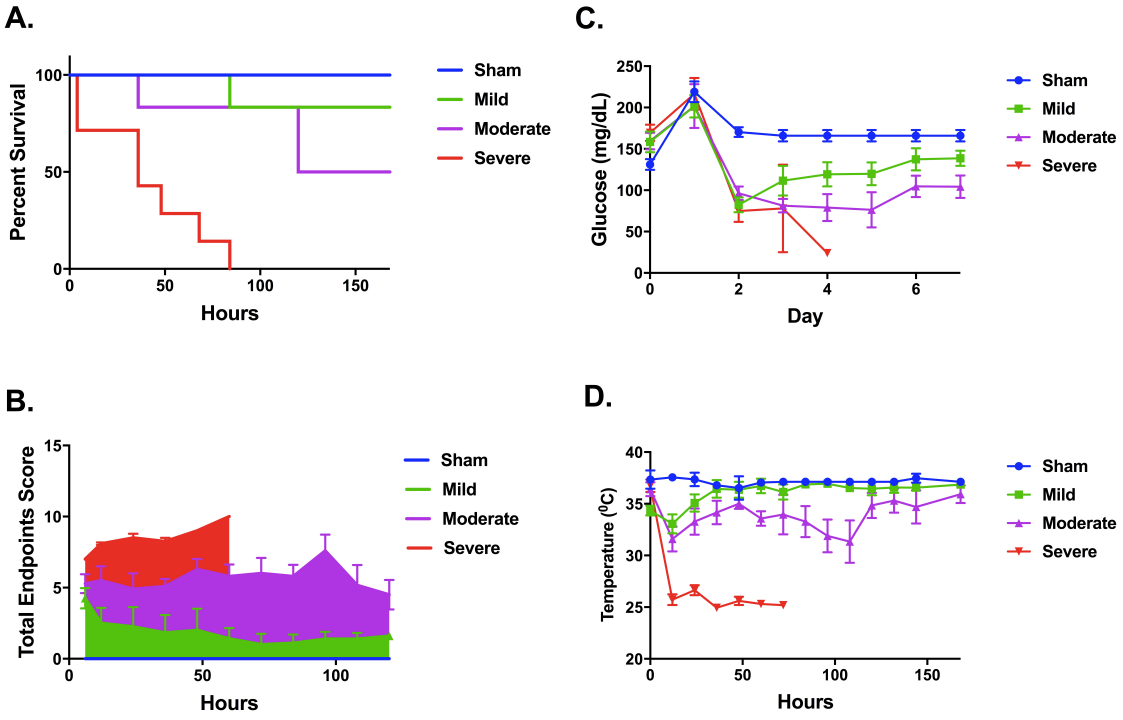
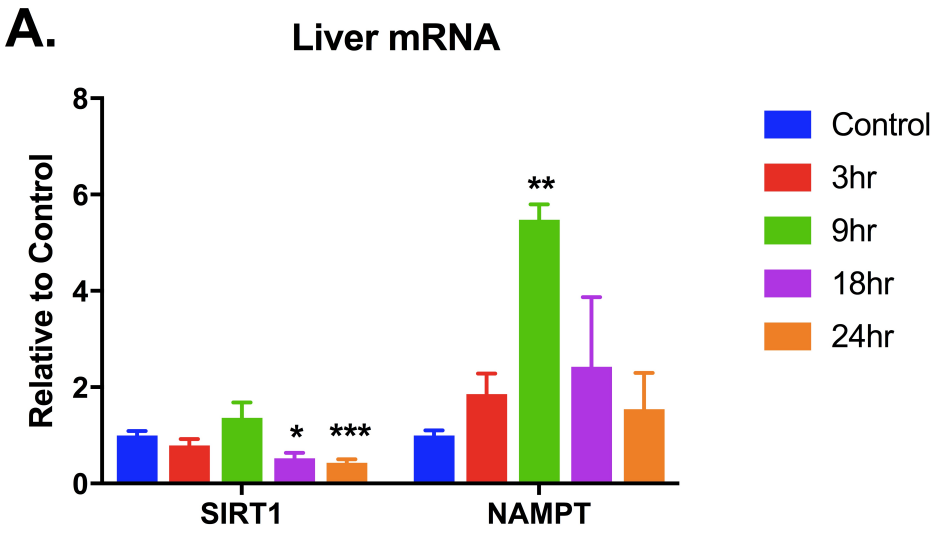


Figure 18: Severity of cecal ligation and puncture (CLP) impacts mortality and physiology.

Male C57BL/6J mice (12 weeks, n=6-9/group) were subjected to sham surgery or CLP (mild, moderate, or severe) and followed for 7 days. (**A)** Mortality varied from 0% (Sham) to 100% (Severe). (**B**). The severity of sepsis clearly impacted the Endpoints of Euthanasia Score at every time point. Both glucose and temperature were negatively influenced by the severity of sepsis with clear differences between groups (**C** and **D**).



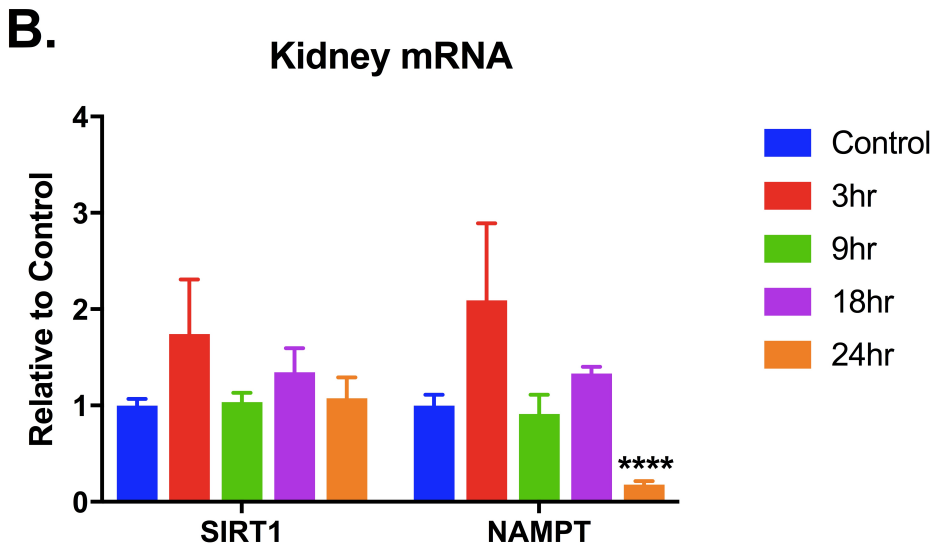


Figure 19: The temporal expression of SIRT1 in liver and kidney after CLP.

Male C57BL/6J mice (12 weeks, n=2-3/group) were subjected to sham surgery or severe CLP and sacrificed at 3, 9, 18 and 24hrs. (**A**) In liver, sepsis was associated with decreased SIRT1 and increased NAMPT gene expression that peaked at 9 hours. (**B**) In kidney, sepsis did not significantly change SIRT1 expression, but did result in significantly lower expression of NAMPT at 24 hours. Data were analyzed by one-way ANOVA, and if significant, reanalyzed with a Student's t-test or Mann Whitney as appropriate. *$p<0.05$, ***$p<0.0005$.

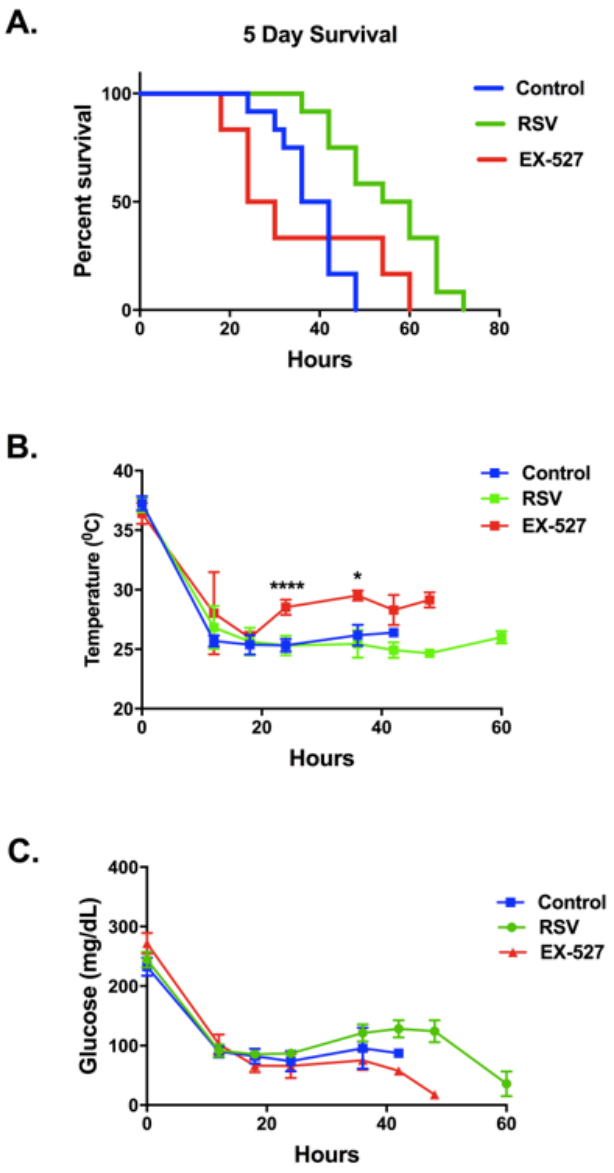


Figure 20: Pharmacologic manipulation of SIRT1 influences survival and physiology in severe sepsis.

Six hours after CLP, mice received either placebo (n =12), resveratrol (20 mg/kg; n=12), or EX-527(10 mg/kg; n=6) by IP injection. Therapy continued every 12 hours until death. Although this model was 100% lethal, resveratrol treated mice lived longer than either placebo (p=0.0005) or EX-527 treated animals (p=0.054) by log rank Mantel Cox analysis. When compared to controls and resveratrol, SIRT1 inhibition with EX-527 significantly improved temperature starting at 24 hours (**B**) with a trend toward improved glucose levels (**C**). Data were analyzed by one-way ANOVA, and if significant, reanalyzed with a Student's t-test or Mann Whitney as appropriate. *p<0.05, ****p<0.0001.

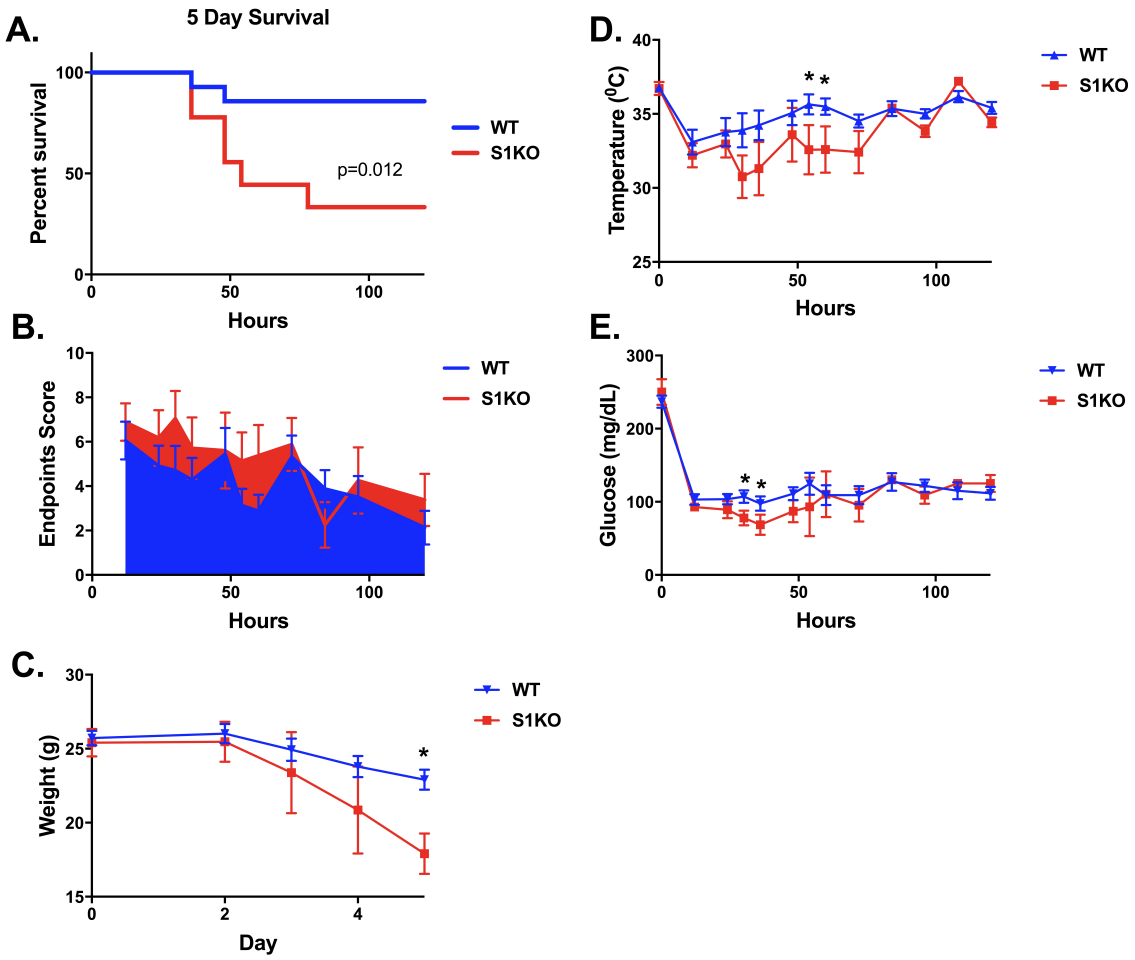


Figure 21: SIRT1 deletion increases the severity and mortality of sepsis.

Wild-type (WT, n=12) and S1KO (n=9) male mice were subjected to severe sepsis and followed for 5 days. Temperature, glucose, and endpoints of euthanasia were assessed every 6 hours. (**A**) S1KO mice had significantly worse 5 day survival as determined by log rank Mantel Cox analysis (p=0.012) as well as (**B**) a trend toward higher Endpoints of Euthanasia scores. (**C**) Over the course of 5 days, SIKO mice lost significantly more weight than WT mice. Physiologically, S1KO mice tended to be more hypothermic and more hypoglycemic early during sepsis than WT mice. Data was analyzed by one-way ANOVA, and if significant, reanalyzed with a Student's t-test or Mann Whitney as appropriate. *p<0.05

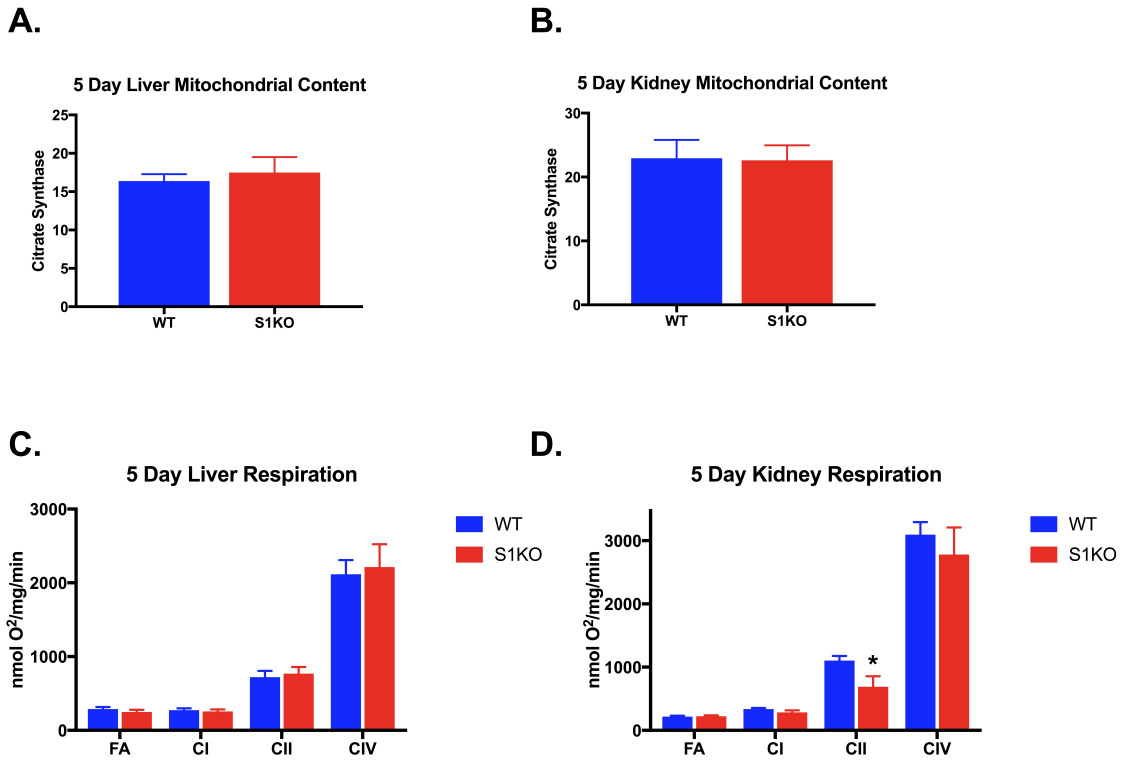


Figure 22: The impact of SIRT1 deletion on mitochondrial respiratory capacity in liver and kidney tissue 5 days post-CLP.

Five days after CLP, surviving WT (6) and S1KO (n=3) mice were sacrificed and mitochondrial function was measured in liver and kidney tissue homogenates. Mitochondrial content and respiration in whole tissue homogenates were measured 5 days after CLP in WT (n=6) and S1K0 (n=3) survivors. SIRT1 status did not significantly impact citrate synthase activity, a proxy for mitochondrial content, in either liver (**A**) or kidney tissue (**B**). Mitochondrial respiratory capacity was assessed in whole tissue homogenates using high resolution respirometry. (**C**) Although, SIRT1 deletion did not influence liver mitochondrial function, (**D**) it was associated with decreased CII-dependent respiration in kidney tissue. FA= fatty acid dependent respiration; CI = complex I; CII =complex II; CIV = complex IV. Data were analyzed using a Student's t-test or Mann Whitney as appropriate. *$p<0.05$

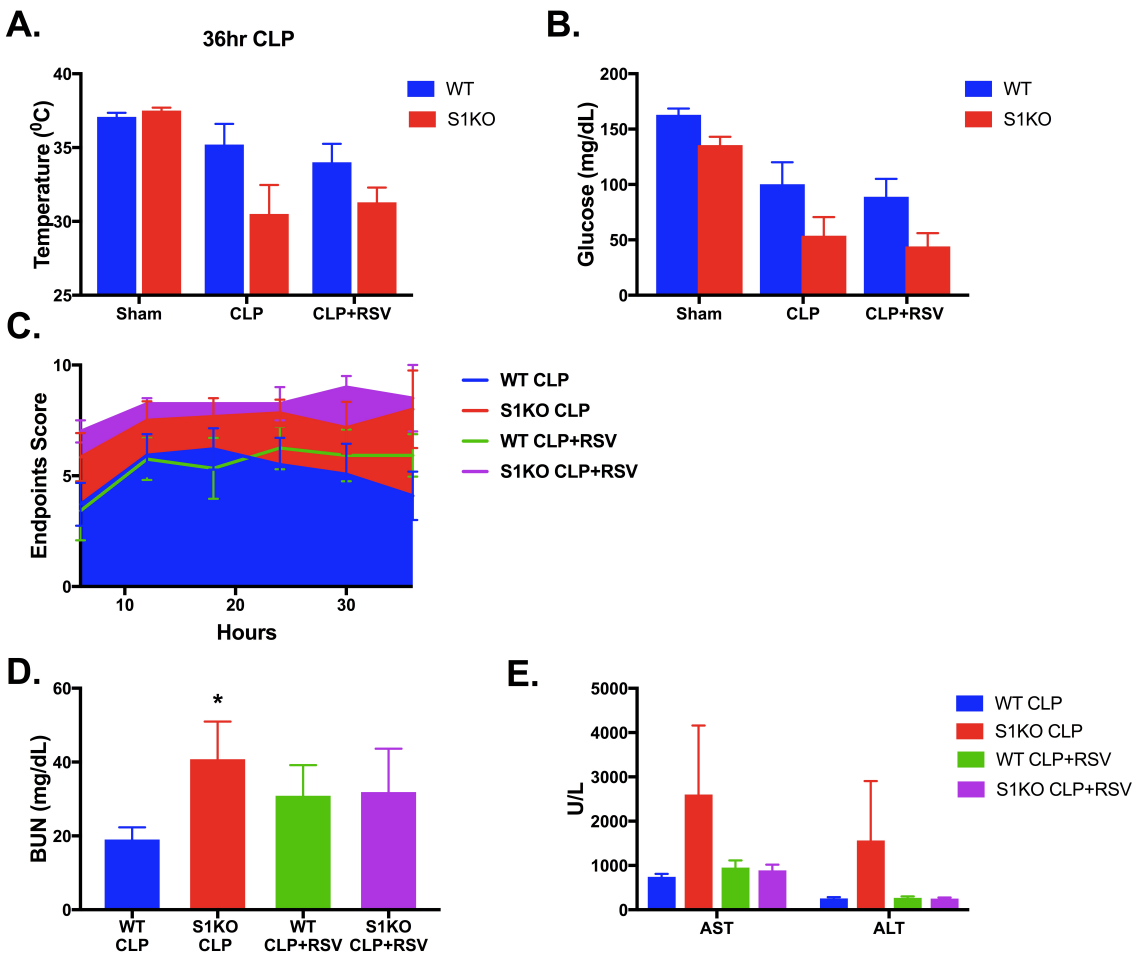


Figure 23: SIRT1 deletion negatively impacts physiologic variables and organ function 36 hours post-CLP.

WT and S1KO mice were subjected to a sham procedure (n= 3, 2), CLP (n= 5, 4) or CLP with RSV (20mg/kg, n=6, 4) and sacrificed 36 hours later. Physiologic variables (temperature, serum glucose) were measured and endpoints of euthanasia were scored. Organ function was assessed by measuring serum blood urea nitrogen (BUN), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). Although not statistically significant, S1KO mice tended to be more hypothermic (**A**), more hypoglycemic (**B**), and less clinically well with higher Endpoints of Euthanasia scores (**C**) when compared to WT mice. (**D**) S1KO mice had more renal dysfunction with significantly higher BUN levels. (**E**) S1KO mice demonstrated significant variability in terms of liver dysfunction. Resveratrol treatment (RSV) had minimal impact on any variable measured. Comparisons were made between WT and S1KO animals within each treatment group using Student's t test or Mann Whitney as appropriate. *$p<0.05$

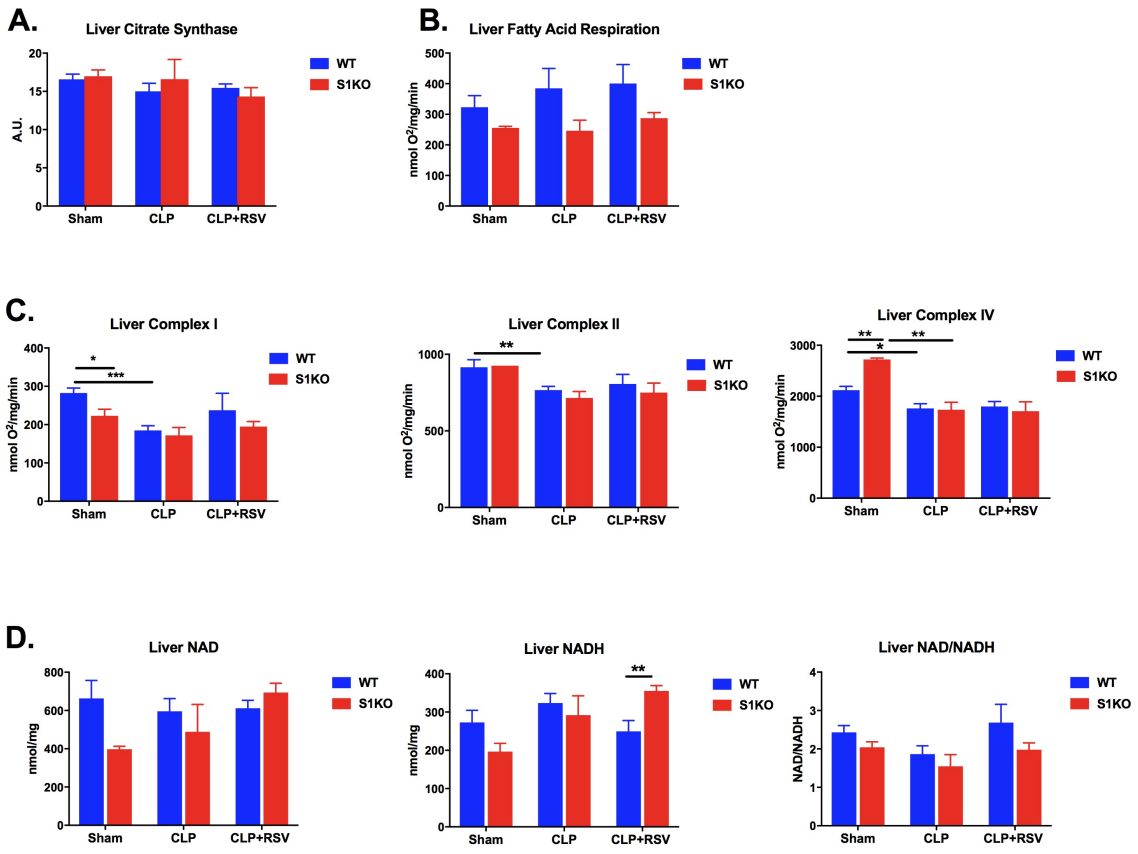


Figure 24: Liver mitochondrial function and NAD content at 36 hours post-CLP.

WT and SIKO mice were subjected to a sham procedure (n= 3, 2), CLP (n= 5,4) or CLP with resveratrol (RSV; 20mg/kg, n=6,3). Mitochondrial function was measured in liver whole homogenates 36 hours after CLP. (**A**) Mitochondrial content did not differ between groups when assessed by citrate synthase activity. (**B**) Using high-resolution respirometry, fatty acid dependent respiration trended higher in WT animals, but did not reach statistical significance. (**C**) SIRT1 KO sham animals had significantly lower CI, but higher CIV respiratory capacity than WT shams. Although S1KO and WT animals had similar respiratory capacity following CLP, sepsis significantly reduced CI, II, and IV dependent respiration in WT animals. RSV had minimal impact on respiratory capacity, although there was a trend toward improved CI-dependent respiration in WT CLP animals. (**D**) NAD and NADH were not significantly different between WT and S1KO animals, although RSV was associated with increased NADH levels in S1KO animals. RSV= resveratrol; FA= fatty acid dependent respiration; CI = complex I; CII =complex II; CIV = complex IV. Data was analyzed by one-way ANOVA and comparisons were made between WT and S1KO animals within each treatment group using Student's test or Mann Whitney as appropriate. *p<0.05, **p<0.01

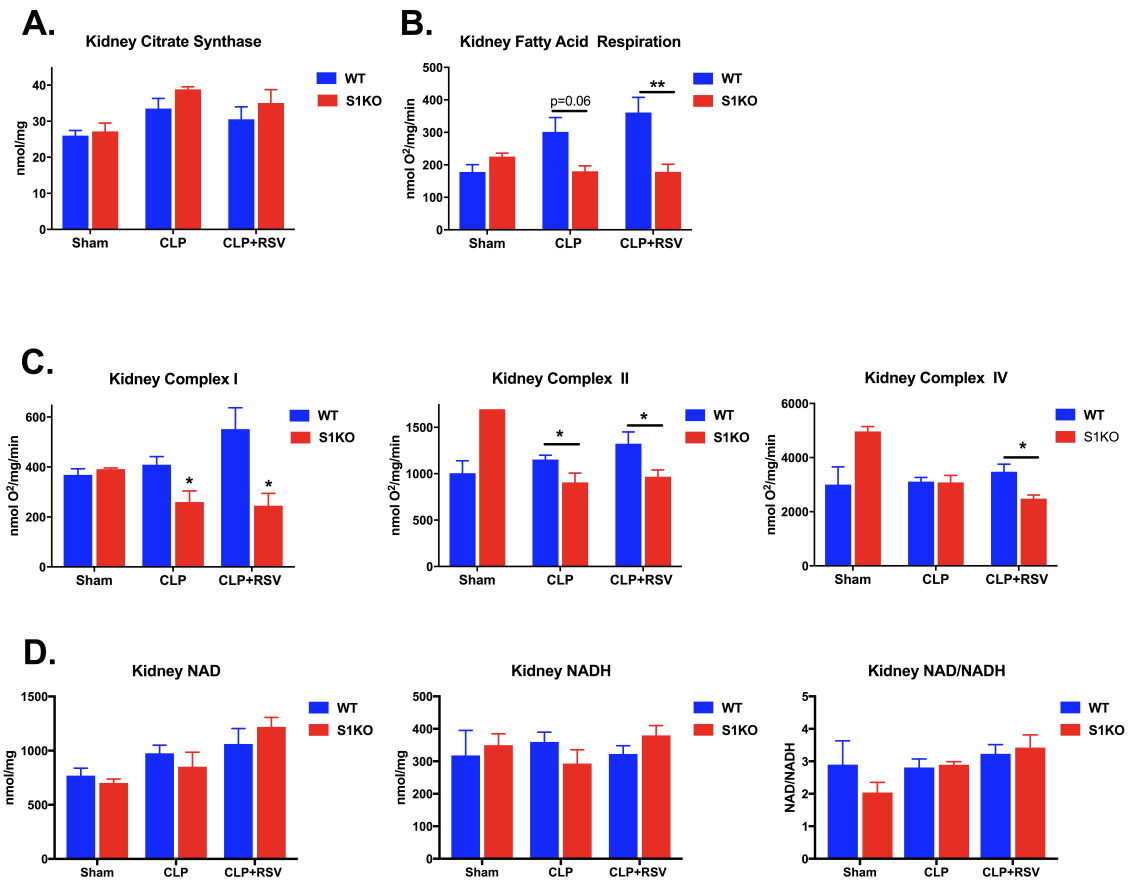


Figure 25: SIRT1 deletion significantly impacts kidney mitochondrial function and NAD content 36 hours post-CLP.

WT and SIKO mice were subjected to a sham procedure (n= 3,2), CLP (n= 5,4) or CLP with RSV (RSV; 20mg/kg, n=6,3). 36 hours after CLP, mitochondrial function was measured in renal whole homogenates using high resolution. (**A**) Mitochondrial content was similar between WT and S1KO mice as measured by citrate synthase activity. Following CLP, (**B**) S1KO mice demonstrated decreased fatty acid respiration, as well as decreased CI and CII dependent respiration when compared to WT septic mice (**B**, **C**). There was a trend toward improved CI dependent respiration in WT animals treated with RSV. FA= fatty acid dependent respiration; CI = complex I; CII =complex II; CIV = complex IV. Data were analyzed by one-way ANOVA and comparisons were made between WT and S1KO animals within each treatment group using Student's test or Mann Whitney as appropriate. *p<0.05.

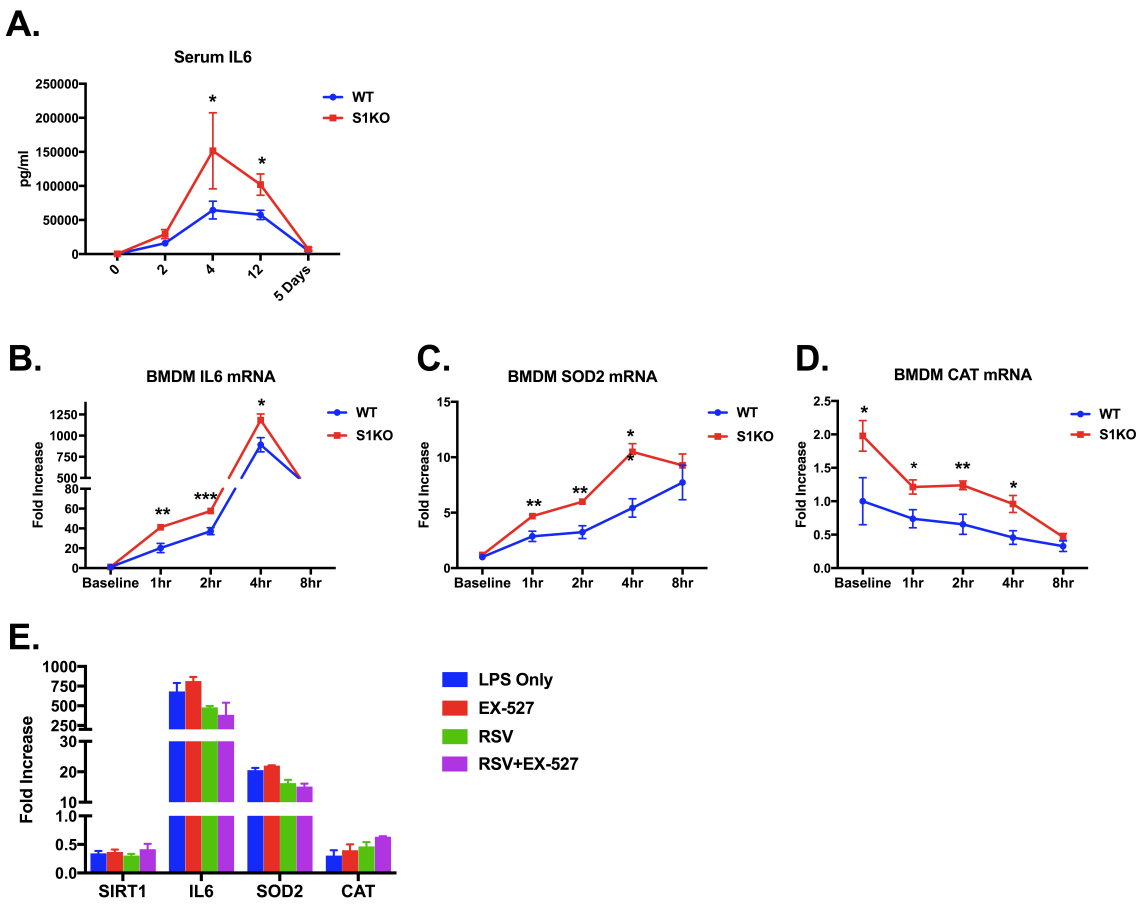


Figure 26: SIRT1 deletion is pro-inflammatory during sepsis.

Blood samples were collected from CLP-treated WT (n=7) and S1KO (n=9) mice at baseline, 2 hours, 4 hours and 5days. (**A**) SIRT1 deletion resulted in significantly higher serum IL6 levels in septic mice. Cultured bone marrow derived macrophages (BMDM) harvested from WT and S1KO mice were treated with LPS (1 µg/ml) and the gene expression of IL6 was followed over time (n=5 per group/time). The expression of two antioxidants, SOD2 and catalase, were also measured. (**B,C**) SIRT1 deletion resulted in significantly higher IL6 and SOD2 expression. (**D**) Catalase mRNA expression was significantly higher in S1KO BMDM, although its expression decreased with LPS exposure over time in both groups. (**E**) WT BMDM (n=2 per group) were pretreated for 1 hour with vehicle, EX-527 (1µM), RSV (50µM), or both EX-527+ RSV and co-cultured with LPS (1 µg/ml) for 8 hours. Gene expression of SIRT1 fell with LPS exposure and was not affected by pharmacologic interventions. Expression of IL6 and SOD2 increased with EX-527 and fell with RSV. In contrast, expression of CAT decreased with LPS exposure, but was partially rescued by RSV. Data were analyzed by one-way ANOVA and comparisons were made between WT and S1KO animals at each time point using Student's test or Mann Whitney as appropriate. *p<0.05, **p<0.01, ***p<0.001.

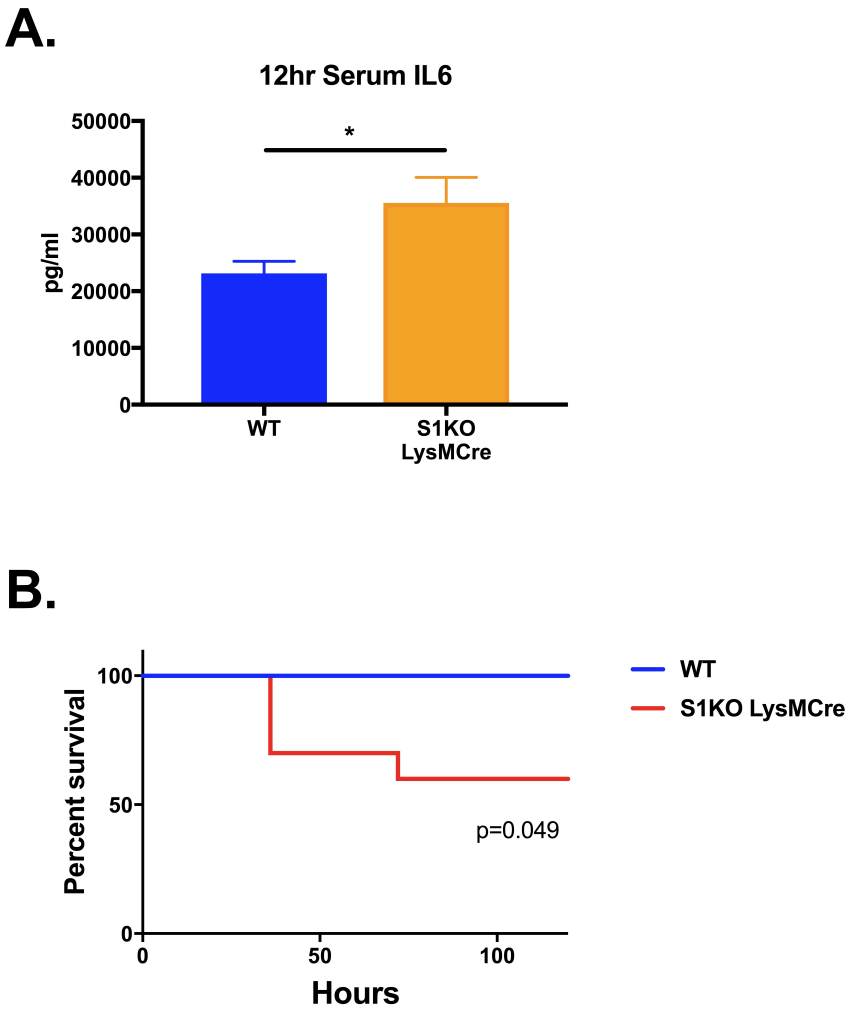


Figure 27: Myeloid deletion of SIRT1 is pro-inflammatory in CLP model of sepsis.

WT (n=8) and SIRT1-LysMCre (n=10) were subjected to CLP and followed for 5 days. (**A**) When compared to WT littermates, S1KO LysMCre mice had significantly higher serum IL6 levels 12 hours after CLP and (**B**) increased 5 day mortality.

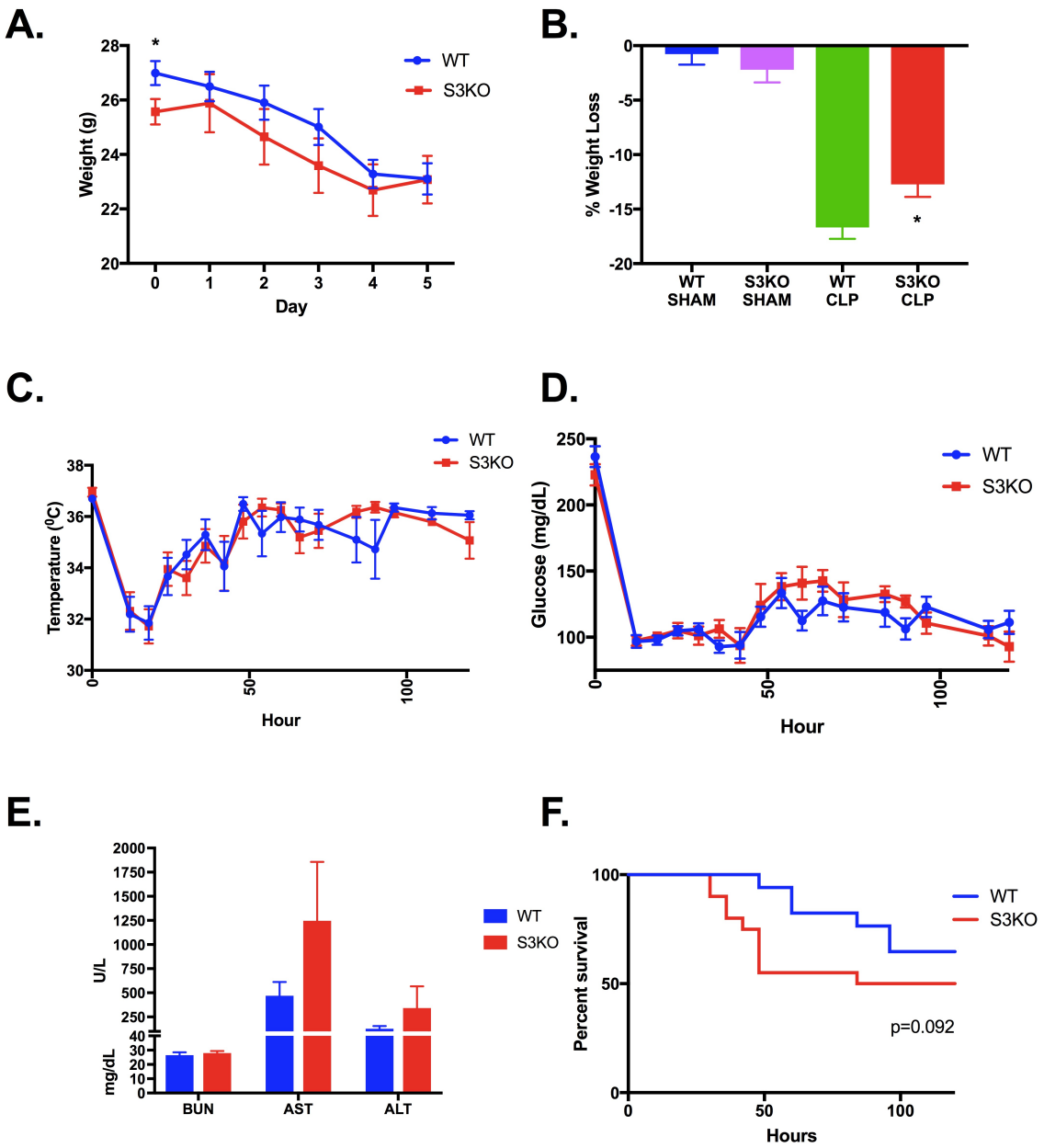


Figure 28: The impact of deleting SIRT3 on physiology and mortality over 5 days.

WT (n=17) and S3KO mice (n=20) were subjected to CLP and followed for 5 days. (**A**) At baseline S3KO mice weighed less than their WT littermates. (**B**) WT survivors lost a larger percentage of body weight than S3KO mice. (**C,D**) Groups did not differ in terms of temperature or serum glucose over time. (**E**) On day 5, organ function in surviving animals was assessed by serum blood urea nitrogen (BUN), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). (**F**) S3KO mice had increased mortality within the first 48 hours, but overall mortality was not significantly different at 5 days by log rank analysis. All other data were analyzed by Mann Whitney or Student's t tests depending on normality of data. *p<0.05.

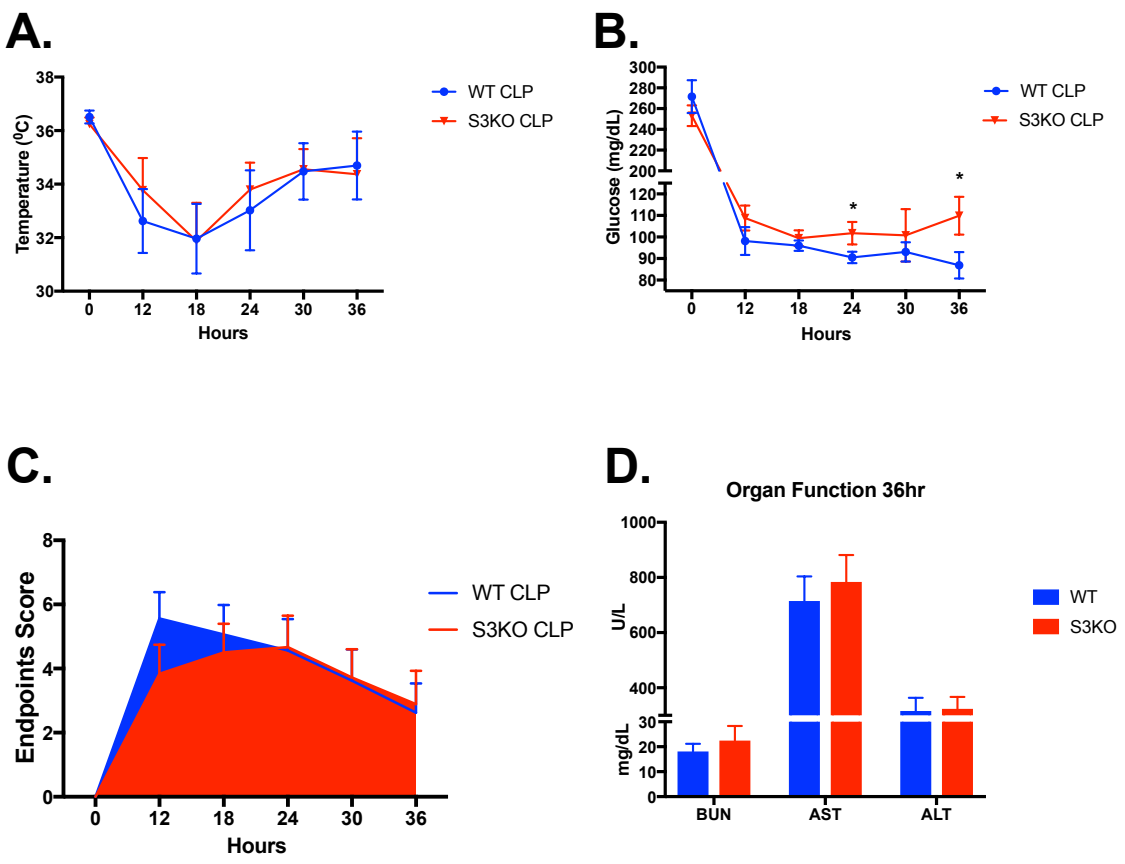


Figure 29: The impact of deleting SIRT3 on clinical variables and organ function 36 hours post CLP.

WT (n=8) and S3KO mice (n=9) were subjected to CLP and followed for 36 hours. (**A**) Both WT and S3KO mice developed significant hypothermia 18 hours post CLP. (**B**) Although glucose levels fell significantly in both groups, S3KO mice were significantly less hypoglycemic than WT animals at 24 and 36 hours. (**C**) WT and S3KO animals had similar endpoints of euthanasia scores and (**D**) did not differ in terms of renal (BUN) or liver (AST, AST) function tests. Data were analyzed by one-way ANOVA followed and if significant were reanalyzed by Mann Whitney or Student's t tests depending on normality of data. *$p<0.05$.

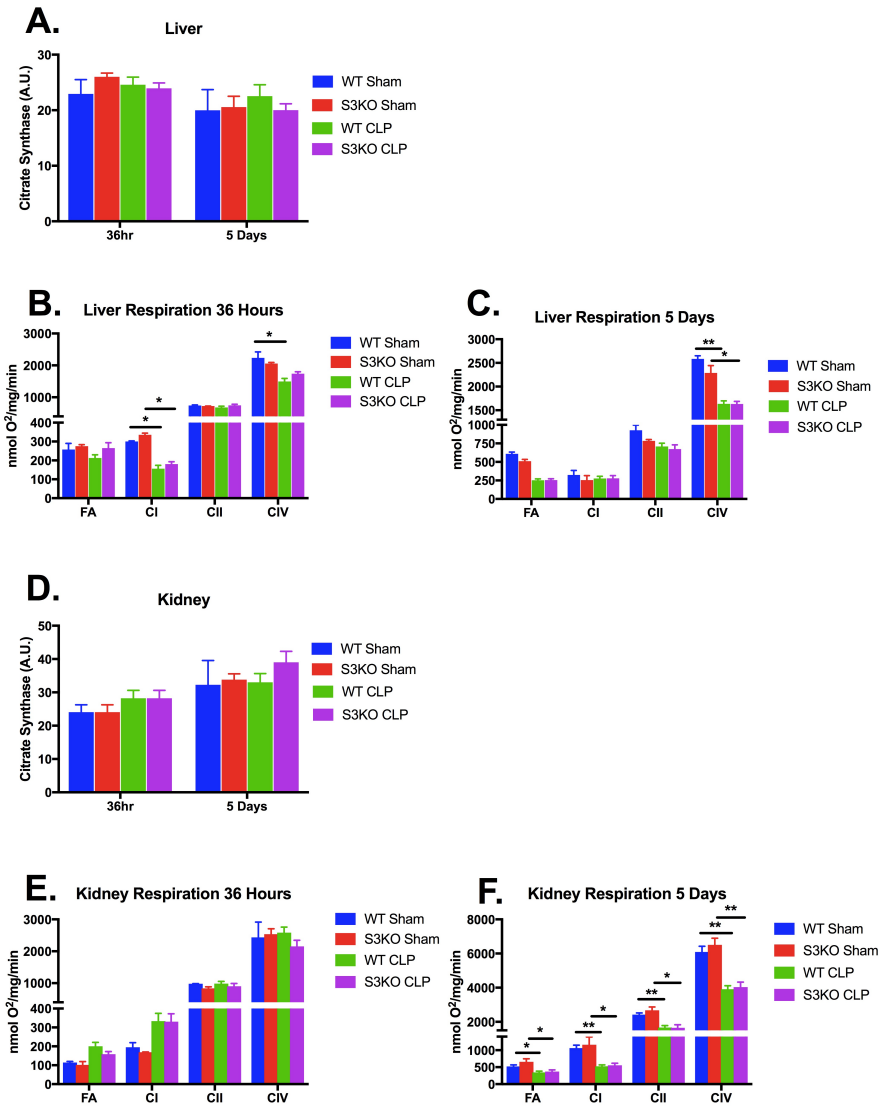


Figure 30: Deleting SIRT3 does not significantly impact mitochondrial respiration in liver or kidney following sepsis.

WT (n=10,9) and S3KO (n=6,10) were subjected to either 36 hour or 5 days of CLP. Sham animals (n=2-3 per group) underwent surgery without CLP. Whole homogenates of liver and kidney were used to measure mitochondrial content and complex dependent respiration. (**A**) In liver, mitochondrial content as measured by citrate synthase activity did not differ between groups either at 36 hours or 5 days post-CLP. (**B**) Both genotypes developed a similar CI deficiency by 36 hours and (**C**) exhibited a CIV defect at 5 days post. (**D**) Kidney mitochondrial content was similar at each time point. (**E**) Interestingly, there was a trend toward increased FA and CI dependent respiration at 36 hours in both WT and S3KO mice. (**F**) At 5 days, all complexes demonstrated significantly decreased respiratory capacity regardless of SIRT3 status. FA= Fatty acid; CI=Complex I, CII=Complex II, CIV = Complex IV. Data were analyzed between groups by Mann Whitney or Student's t tests depending on normality of data. *p<0.05, **p<0.01.

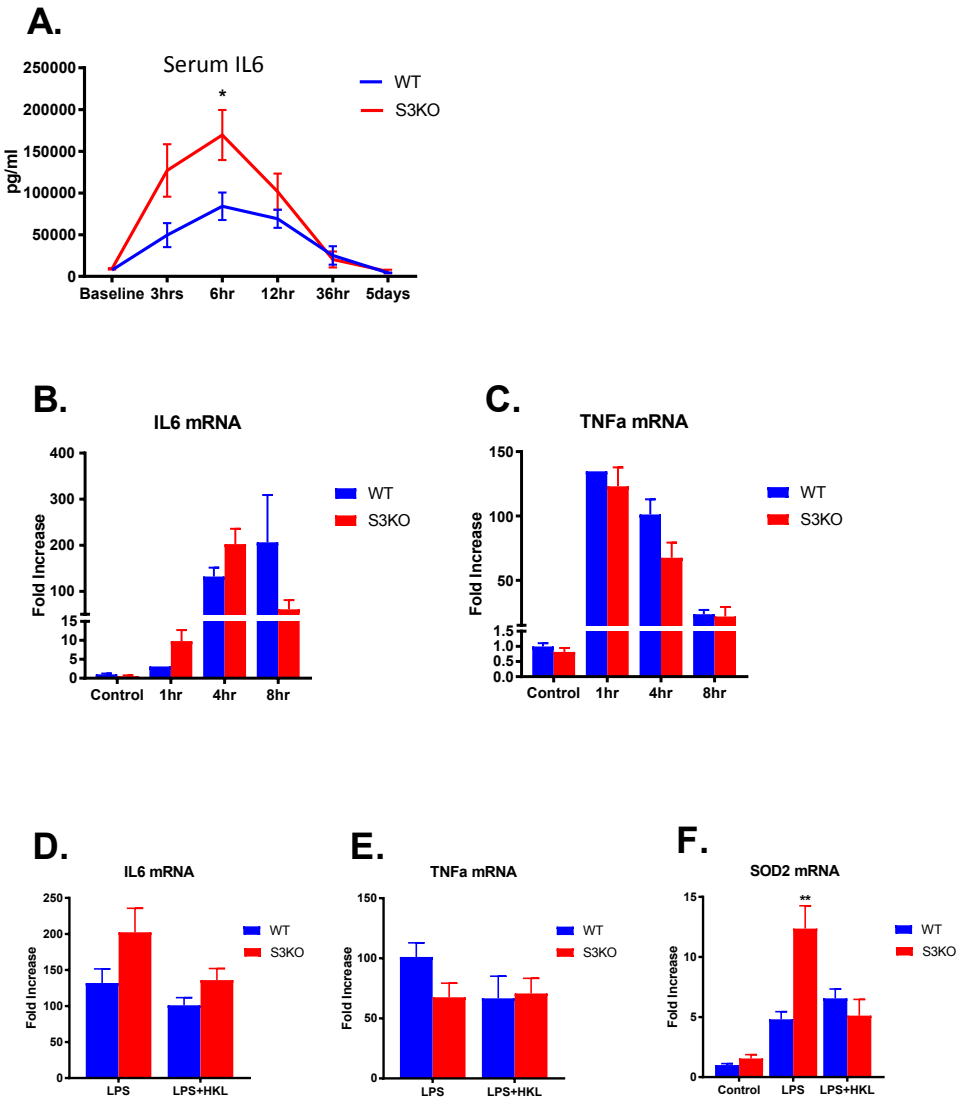


Figure 31: Deleting SIRT3 is pro-inflammatory.

WT (n=10) and S3KO (n=9) mice were subjected to CLP and followed for 5 days. Serum IL6 was measured by ELISA at baseline, 3, 6, 12 and 36 hours with a final sample taken at 5 days. (**A**) IL6 levels peaked at 6 hours and were significantly higher in S3KO mice. At 36 hours, IL6 levels were similar. (**B**) WT and S3KO bone marrow derived macrophages (n=2-5/time point) were co-cultured with LPS (1µg/ml) and assayed at different time points for cytokine gene expression. IL6 expression trended higher in S3KO BMDMs until 8hrs. (**C**) TNFα mRNA expression was similar. WT and S3KO BMDMs (n=3/group) were co-cultured with LPS (1µg/ml) and the SIRT3 activator, honokiol (HKL; 10µM) for 4 hours. (**D,E**) There was a trend toward increased IL6 and decreased TNFα mRNA expression in S3KO BMDMs. (**F**) S3KO BMDMs had increased SOD2 expression that decreased with HKL treatment. Data were analyzed between

groups by Mann Whitney or Student's t tests depending on normality of data. *p<0.05, **p<0.01.

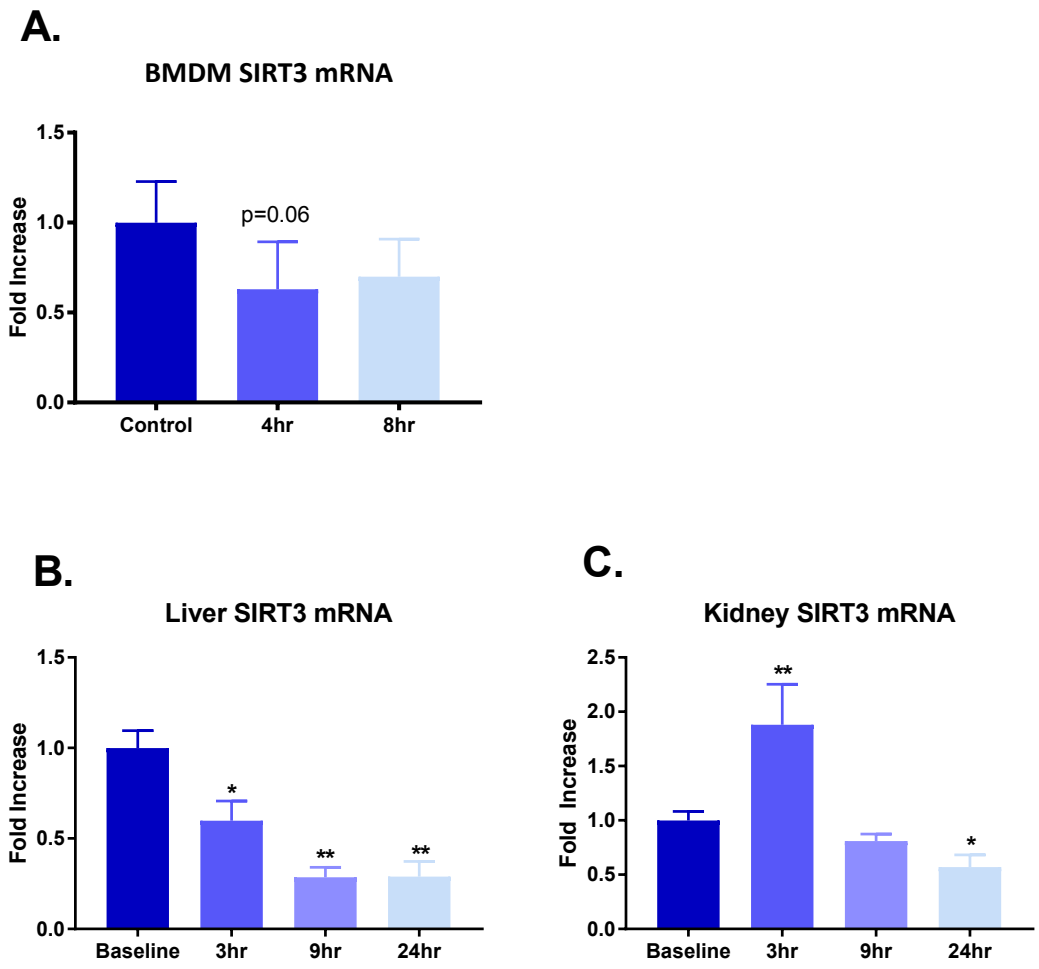


Figure 32: SIRT3 mRNA expression declines in multiple tissues during sepsis.

WT BMDM (n=3 per time point) were co-cultured with LPS (1µg/ml). (**A**) mRNA expression of SIRT3 decreased and approached significance after 4 hours of exposure. Male C57BL/6J mice (12 weeks, n=2-3/group) were subjected to sham surgery or severe CLP and sacrificed at 3, 9, and 24hrs. (**B**) There was a significant and progressive decline in liver SIRT3 mRNA over time. (**C**) Kidney SIRT3 mRNA expression increased significantly by 3 hours, but then progressively declined.

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