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Hemorrhagic Shock and Tissue Injury Drive Distinct Plasma Metabolome Derangements in Swine

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Abstract

Background—Tissue injury and hemorrhagic shock induce significant systemic metabolic reprogramming in animal models and critically injured patients. Recent expansions of the classic concepts of metabolomic aberrations in tissue injury and hemorrhage opened the way for novel resuscitative interventions based on the observed abnormal metabolic demands. We hypothesize that metabolic demands and resulting metabolic signatures in pig plasma will vary in response to isolated or combined tissue injury and hemorrhagic shock.

Methods—A total of 20 pigs underwent either isolated tissue injury, hemorrhagic shock, or combined tissue injury and hemorrhagic shock referenced to a sham protocol (n=5/group). Plasma samples were analyzed by UHPLC-MS.

Results—Hemorrhagic shock promoted a hypermetabolic state. Tissue injury alone dampened metabolic responses in comparison to sham and hemorrhagic shock, and attenuated the

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The authors have declared no conflicts of interest.

Author Contribution

EM, AB, CS, HM designed the study.

GN, EG, EP, MF, HM performed surgeries on the animals, collected samples.

NC, JR TN, MW, KH and AD set up the metabolomics assays, performed metabolomics analyses.

AD prepared figures and tables, wrote the paper.

NC and AD revised the paper.

All the Authors critically contributed to the finalization of the manuscript and approved the final version that has been submitted for consideration of publication.

hypermetabolic state triggered by shock with respect to energy metabolism (glycolysis, glutaminolysis and Krebs cycle). Tissue injury and hemorrhagic shock had a more pronounced effect on nitrogen metabolism (arginine, polyamines and purine metabolism) than hemorrhagic shock alone.

Conclusion—Isolated or combined tissue injury and hemorrhagic shock result in distinct plasma metabolic signatures. These findings indicate that optimized resuscitative interventions in critically ill patients is possible based on identifying the severity of tissue injury and hemorrhage.

Keywords

Metabolism; Succinate; UHPLC-MS; Trauma; Hemorrhagic Shock

Background

Trauma is a major cause of disability and mortality in the United States and the leading cause of productive life years lost for individuals under 65¹, CDC data – June 2016. Uncontrolled hemorrhage and traumatic brain injury dominate early (<72 hrs from admission) death, while late deaths (>72 hours from admission) are primarily a result of coagulation defects and inflammatory states secondary to the initial injury. Inflammatory responses encompass a broad variety of disease including infectious complications, acute respiratory distress and multiple organ failure² and have been recently related to mechanisms involving small molecule metabolites³. Early metabolic changes predispose the body for inflammatory^{4,5} and coagulopathic⁶ complications, but the mechanisms remain poorly understood. Despite advances in damage control surgery and resuscitation, patients continue to have substantial delayed morbidity^{7,8}.

Metabolic changes generated during trauma were initially described by Cuthbertson as an early hypometabolic state followed by a hypermetabolic state, also known as the “ebb” and the “flow” phases⁹. With advances in technology subsequent investigators have approached this relevant question using mass spectrometry (MS) to provide a comprehensive description of metabolic pathways and their response to tissue injury. MS-based methods allow the simultaneous detection of hundreds of small molecules, which include metabolites from well described and crucial metabolic pathways. Using this approach, rodent models of hemorrhagic shock have been described to produce hyperglycemia, increased glycolysis, lipid mobilization, proteolysis, amino acid catabolism and impaired redox homeostasis^{5,10–12}. Similar observations were reported in critically ill patients^{4,13}.

Prior experimental and retrospective clinical work has sought to define the plasma metabolome of trauma patients, but has not differentiated the key components of tissue injury (TI) and hemorrhagic shock (HS). These two entities have been shown to produce opposite effects on coagulation pathways in animal models, and it is reasonable to anticipate that they produce differential effects on the metabolome¹⁴. Additionally, while the HS model has been shown to mirror metabolic derangements observed in response to ischemia/hypoxia, the role of TI alone on the metabolic sequelae observed in trauma patients has not been hitherto elucidated.

Assessment of pathologic changes of the global metabolome in response to TI and shock can help identify translatable targeted therapies to reduce morbidity and mortality. For example early administration of plasma rather than normal saline improves survival and metabolic recovery in a rodent model of profound HS¹¹. Recent investigations have highlighted the critical role of small molecule metabolites such as succinate (a citric acid cycle intermediate) as both a metabolic marker of tissue hypoxia and mitochondrial uncoupling, similar to lactate, as well as a driver of ischemia reperfusion injury through mitochondrial ROS^{15,16}. Additionally, succinate has been linked to coagulopathy and ARDS through its role in IR injury^{3,17,18}.

In this study we examined the dynamic changes in porcine plasma metabolome responses to HS or TI, either isolated or in combination (TI+HS), in comparison to sham controls (no TI or HS). The goal of this study was to determine the relative contribution of TI and HS to the overall plasma metabolome in a model relevant to critical care medicine. In previous studies utilizing rodent models, where minimum to no trauma was induced, neither laparotomy alone or anesthesia were sufficient to elicit the metabolic derangement that was instead observed upon mild and severe hemorrhage^{5,10,12,19}. These models however did not include severe TI, and may have thus underestimated the relative contribution of traumatic injury to the etiology of metabolic alterations observed following hemorrhage in rodents and humans^{4,13}. To test this hypothesis and expand on our previous observations in rodent models, we employed a porcine model of TI, in the presence or absence of HS. This represents a better translational model for higher mammals and thus for clinical translatability²⁰. We hypothesized that the study of differential models of TI and HS would produce distinct metabolic signatures in plasma.

Materials and Methods

2.1 Animal model

The animal protocol was approved by the University of Colorado International Animal Care and Use Committee. A total of 20 male outbred immature swine (50–55 kg; n = 5 per group) underwent anesthesia with ketamine (20mg/kg) (VETone, Boise, ID, USA) and acepromazine (0.2mg/kg) (VETone, Boise, ID, USA) and xylazine (2mg/kg) (Akorn, Decatur, IL, USA), then were maintained on general anesthesia using isoflurane (0.5–2%) in room air, by mask. Animals underwent tracheostomy, femoral artery cannulation to measure blood pressure and induce hemorrhage. After tracheostomy, the animals were placed on mechanical ventilation to maintain O₂ saturation > 90%. Upon completion of the experiment, the animal was euthanized by exsanguination followed by cardiectomy. An overview of the animal model is displayed in Figure 1. Each group (sham, TI, HS, and TI +HS) consisted of 5 animals. The plasma samples at each time point were pooled before analysis.

2.2 Sham

Blood draws were performed at baseline, after 30, 60 and 90 min (consistent with the last time point of the TI group) to determine whether baseline values in the absence of HS or TI would result in plasma metabolic changes due to anesthesia and supine positioning.

2.3 Tissue Injury

Pigs underwent a severe TI model consisting of unilateral femur fracture with muscle crush, laparotomy with bowel crush, and clamshell thoracotomy with multiple surgeons operating simultaneously. Femur fracture was accomplished with a mid-thigh cut down to expose of the mid shaft periosteum of the femur. Direct blunt force of the bone was used to cause displacement and cortical disruption of the femur. A xiphoid to pubis laparotomy and clamshell thoracotomy were performed in a hemostatic manner. The bowel was crushed using 1 cm intervals over a 30 cm span of proximal jejunum. Blood draws were performed at baseline, and 30, 60 and 90 minutes after TI.

2.4 Hemorrhagic Shock

Pigs underwent hemorrhage to a mean arterial pressure of 25mm Hg for 30 minutes, followed by clamshell thoracotomy to access the descending aorta and 30 minutes of descending thoracic aortic cross clamping. No resuscitation fluids were given. Blood draws were performed at baseline, after 30 minutes of hemorrhage, after 30 minutes of descending thoracic aortic cross clamping and 30 minutes or at time of death after releasing aortic cross clamp.

2.5 Combined Tissue Injury and Hemorrhagic Shock

Pigs underwent simultaneous TI, followed by the HS protocol. Blood was drawn at time points as described in the hemorrhagic shock model.

2.6 Blood samples

Whole blood was collected through direct venous puncture of the femoral vein with a 21 gauge needle during each time point, and plasma was recovered through centrifugation (2500 g for 10 min at 4°C). Plasma samples from 5 individual animals were pooled at each time point prior to analysis.

2.7 Metabolomics analyses

Metabolomics analyses were performed as previously reported^{4,10}. In brief, plasma samples were extracted in methanol, acetonitrile, and water at a ratio of 5:3:2 and agitated for 30 minutes at 4°C. Following centrifugation at 10,000g the supernatant was collected stored at -80°C until analysis with an UHPLC system. Metabolites were assigned using Maven software (Princeton, NJ, USA).

2.8 Statistical analysis

Relative quantitation was performed by exporting integrated peak area values into Excel (Microsoft, Redmond, CA, USA)). Hierarchical clustering analysis and partial least square-discriminant analysis (PLS-DA) for intra- and intergroup comparisons were performed through the software GENE-E (Broad Institute, Cambridge, MA, USA), as previously reported.²¹ Integrated peak area values in arbitrary units were graphed through GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA) and figure panels were assembled through Photoshop CS5 (Adobe, Mountain View, CA, USA).

Results

A total of 168 metabolites were monitored in plasma from pigs ($n = 5$ per group) undergoing HS, TI, or a combination of TI+HS in comparison to sham animals (no trauma, no shock) (Supplementary Table 1). The model is summarized in Figure 1. Hierarchical clustering analysis and partial least square discriminant analysis (PC scores and loading values are provided in Supplementary Table 2) were performed to cluster metabolites following similar trends over the time course analyses in either group (a vectorial version of this figure is provided in Supplementary Figure 1). Three main clusters of metabolites were identified: i) metabolites increasing after HS and further increasing after TI+HS (Figure 2.left); ii) metabolites increasing only in the TI+HS group (Figure 2.top right); iii) metabolites increasing after HS and decreasing after TI+HS (Figure 2.bottom right).

Metabolites in these three clusters are either part of glycolysis, the tricarboxylic acid cycle (TCA cycle), glutaminolysis (Figure 3), nitrogen metabolism (arginine catabolism, urea cycle, creatine metabolism and polyamines – Figure 4) and purine metabolism and oxidation (Figure 5).

Shock primed increases in plasma glycemia, while similar glycolytic rates were observed in shock and combined treatments in terms of lactatemia (Figure 3). Notably, TI alone resulted in the depression of plasma lactate levels compared to sham (Figure 3). Similarly, plasma accumulation of TCA cycle intermediates was the lowest in animals undergoing TI alone, while it was increased after HS and partially attenuated (at levels higher than in sham animals) in the combined TI and HS group (e.g. glutamine, alpha-ketoglutarate, malate, fumarate - Figure 3). On the other hand, combined TI and HS had the greatest effect on the time-course arginine consumption, creatine and polyamine (putrescine, spermidine, spermine) accumulation in plasma, followed by HS, while no significant effect was observed in response to TI alone or the sham procedure (Figure 4).

Similarly, increases in purine metabolism – involving the whole cascade from nicotinamide/adenosine/guanosine to inosine, hypoxanthine, urate and hydroxyisourate/allantoate - were primed by HS and further exacerbated by the combination of TI and HS (Figure 5). Conversely, TI alone resulted in lower levels of purine oxidation products hypoxanthine, urate and hydroxyisourate, compared to the sham protocol (Figure 5).

Discussion

Recent advancements in the field of metabolomics have opened the opportunity to revisit the classic concept of metabolic modulation in trauma and HS⁹. Understanding the metabolic sequelae to trauma and hemorrhage has relevant implications in defining the severity of injury in the intensive care setting (e.g. by measuring base deficit, strong anion gap and plasma lactate levels^{22–24}). At the same time, metabolic approaches are key in defining effective resuscitation strategies to meet the increased physiologic demands of critically injured patients by providing specific metabolic substrates such as glutamine^{12,25–27} and arginine^{28–32}, either alone or in combination with omega-3 fatty acids and nucleotides³³. Recently, we applied metabolomics technologies to delineate the metabolic derangements in

hemorrhaged rodents and critically ill patients to identify shared perturbations of the post-shock metabolic pathways observed in the animal model and the clinical setting^{4,6,10,13}. After confirming the potential clinical relevance of the rodent model, we moved to defining the time course development of metabolic derangements in plasma after early mild hemorrhage and late severe HS¹⁰. These observations from our and other groups suggest the potential significance of TCA cycle intermediates accumulating in post-shock plasma with respect to metabolic acidosis^{10,13,34–36}. Recent observations indicate a role for ischemic hypoxia-derived succinate in inflammation^{3,16}, and for post-shock plasma succinate levels in fibrinolysis in rodents and humans⁶. Consequently, we performed stable isotope tracing experiments with heavy glucose and glutamine *in vivo* in rodents to determine the metabolic origin of these TCA cycle compounds^{5,12}. Labeling propagation in metabolites downstream to glucose and glutamine oxidation revealed a role for a metabolic blockade at complex II of mitochondria in mediating the accumulation of TCA cycle intermediates, especially succinate, in post-shock animals^{5,12}. Based on these data and from recent insights in the understanding of the mechanisms that underlie ischemia/reperfusion injury^{37,38}, we postulated that plasma accumulation of carboxylic acids *in vivo* is at least in part explained by the uncoupling of normal electron flow in the respiratory complex chain of mitochondria under conditions of hemorrhagic hypoxia due to the lack of oxygen as the final acceptor of electrons in complex IV. However, it is unclear whether the metabolic derangements observed in these models and clinical patients are explained by HS alone or are a result of the combined effect of TI and HS. Therefore, we employed a porcine model of poly-trauma and HS, previously characterized through nuclear magnetic resonance (NMR)-based metabolomics technologies³⁶, to disentangle the relative contribution of TI and HS.

We show here for the first time that HS primes metabolic derangements that are exacerbated by the addition of TI, especially with respect to nitrogen unbalance (arginine consumption and generation of polyamines) and purine catabolism. This is relevant in the light of the increasingly appreciated role of adenosine (and purines in general) in mediating early responses to shock¹⁰, provoking hypotension³⁹, stimulating Na/K ATPase⁴⁰, promoting a transient “hypometabolic” state (ebb phase) by attenuating sympathetic activation⁴¹, a phenomenon that confers a partial protection from HS-induced lung injury⁴². Similarly, polyamines accumulate in response to hemorrhage^{5,10} and traumatic brain injury⁴³, markers of nitrogen metabolism unbalance that have been shown to correlate with increased fibrinolysis¹⁴. While the mechanisms remain unclear, it has been suggested that heparin anticoagulant reverses polyamine (e.g. spermine) induced coagulation defects in the presence of glycosaminoglycans⁴⁴. Alternative mechanisms involve coagulation factor XIIIa, an enzyme with transglutaminase activity that bridges fibrin complexes to form an insoluble clot. The activity of this enzyme is partially inhibited by the higher than physiological concentrations of polyamines, which can be used as alternative substrates by factor XIIIa resulting in the formation of weaker clots⁴⁵.

Notably, energy related metabolic pathways such as glycolysis and TCA cycle were dampened by TI in comparison to sham animals. Similarly, TI +HS attenuated the HS-dependent increase in the plasma level of metabolites such as glucose, glutamine, alpha-ketoglutarate, malate and fumarate. Though mechanistic studies are necessary, this observation is relevant in that it suggests that tissue injury promotes a “hypometabolic” state,

likely triggered by the release of soluble metabolic mediators. While previous studies have described the alterations of the plasma and mesenteric lymph proteomes after severe trauma and hemorrhage^{46–48}, it will be interesting to determine whether TI alone is characterized by specific proteomics signatures (e.g. damage associated molecular patterns – DAMPS, histones, etc) in comparison to shock alone or TI/HS. Additionally, metabolic enzymes released with TI in the bloodstream may play moonlighting functions⁴⁹, such as the metabolic enzyme α -enolase participating in inflammatory cascades⁵⁰.

In this study we employed a large animal model of TI and HS to disentangle the relative contribution of the systemic metabolic derangement observed in post-shock animal models and critically ill patients. While HS has a general effect on promoting a hypermetabolic state, TI promotes nitrogen imbalance (at the amino acid and purine catabolism level) while dampening energy metabolism (glycolysis and TCA cycle). This observation holds relevant clinical implications, as it suggests that metabolic resuscitation should be personalized on the basis of the severity of TI and HS. Follow up studies with non-human primate models of trauma and hemorrhage⁵¹ or clinical samples will further elucidate the impact of TI and HS on metabolism and will likely provide insights into the mechanism of metabolic derangements following TI and HS. Despite encouraging results, this study is preliminary in that a limited number of animals were investigated for a time-course analysis that stops at 90 min after TI and/or HS. Future studies will be necessary to investigate additional time points for prolonged analyses. Even though discovery-mode metabolomics based on relative quantitative approaches is an rapidly expanding area of investigation in the field of intensive care medicine, future targeted quantitative approaches and metabolic flux analyses *in vivo* will likely contribute additional complementary insights on the comparability of the swine model to what observed in civilian and military patients undergoing TI and HS, as well as to disentangle the relative contribution of specific metabolic substrates (e.g. glutamine, arginine, aspartate) in fueling the metabolic derangements observed here.

In summary, the current work identifies clear patterns of metabolic changes associated with TI, HS, and TI+HS. Notably, TI alone is characterized by a hypometabolic state which is in stark contrast to TI+HS which results in a hypermetabolic state. Thus if injured patients demonstrate similar patterns of altered metabolism they may benefit from targeting resuscitation to recovery of specific metabolic pathways based on the severity of TI and the presence or absence of HS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A schematic representation of the swine model of poly-trauma and hemorrhage employed in this study. Model injury components are listed from 1 through 6 (top right corner).

Figure 2.

Heat maps of time course (0, 30, 60 and 90 min) metabolic changes in sham, shock, tissue injury (TI) and tissue injury combined with hemorrhage to MAP 25 mmHg (T+HS). Metabolite levels were Z-score normalized across all observations and median values were plotted following the color scheme in the bottom right corner (low: blue, high: red). Metabolites affected by shock alone (left), only by TI+HS (top right) or primed by shock but dampened by tissue injury (bottom right) are shown.

Figure 3.

Time course analysis of glycolysis, TCA cycle and glutaminolysis markers in plasma of pigs undergoing shock (green), tissue injury (blue) or a combination of the two (red) in comparison to sham pigs (no tissue injury, no shock). Time points shown here are at 0, 15, 30, 45 and 60 min (0 min time point = after 30min from shock). Lines are representative of median values for each metabolite in each group.

Figure 4.

Time course analysis of nitrogen metabolism (arginine, urea cycle, creatine and polyamine metabolism) markers in plasma of pigs undergoing shock (green), tissue injury (blue) or a combination of the two (red) in comparison to sham pigs (no tissue injury, no shock). Time points shown here are at 0, 15, 30, 45 and 60 min (0 min time point = after 30min from shock). Lines are representative of median values for each metabolite in each group.

Figure 5.

Time course analysis of purine metabolism and oxidation markers in plasma of pigs undergoing shock (green), tissue injury (blue) or a combination of the two (red) in comparison to sham pigs (no tissue injury, no shock). Time points shown here are at 0, 15, 30, 45 and 60 min (0 min time point = after 30min from shock). Lines are representative of median values for each metabolite in each group.