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Glutamine metabolism drives succinate accumulation in plasma and the lung during hemorrhagic shock

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Abstract

Background—Metabolomic investigations have consistently reported succinate accumulation in plasma after critical injury. Succinate receptors have been identified on numerous tissues, and succinate has been directly implicated in post-ischemic inflammation, organ dysfunction, platelet activation and the generation of reactive oxygen species, which may potentiate morbidity and mortality risk to patients. Metabolic flux (heavy isotope labeling) studies demonstrate that glycolysis is not the primary source of increased plasma succinate during protracted shock. Glutamine is an alternative parent substrate for ATP generation during anaerobic conditions; a

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Author contribution

A.L.S., A.D'A., E.E.M., A.B. conducted the literature search. A.L.S., A.D'A., E.E.M., A.Ban., C.C.S., K.C.H., M.F., A.B., and E.D.P. designed the study. A.L.S., M.F., and A.Bac. contributed to the animal model procedure. A.D'A., J.A.R., and M.J.W. collected mass spectrometry data, which K.C.H. interpreted. M.F. and A.Bac. contributed to data collection. A.L.S. and A.D'A. performed data analysis. A.L.S., A.D'A., E.E.M., A.Ban. C.C.S., J.A.R., M.J.W., H.B.M., and E.D.P. contributed to data interpretation. A.L.S. wrote the manuscript, for which A.D'A. and J.A.R. prepared figures. A.D'A., E.E.M., A.Ban., C.C.S., and E.D.P. participated in critical revision of the manuscript.

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biochemical mechanism that ultimately supports cellular survival but produces succinate as a catabolite. We hypothesize that succinate accumulation during hemorrhagic shock is driven by glutaminolysis.

Methods—Sprague-Dawley rats were subjected to hemorrhagic shock for 45 min (Shock, n=8), and compared to normotensive shams (Sham, n=8). At 15 min, animals received intravenous injection of 13C515N2-glutamine solution (iLG). Blood, brain, heart, lung and liver tissues were harvested at defined time points. Labeling distribution in samples was determined by ultra high pressure liquid chromatography-mass spectrometry (UHPLC-MS) metabolomic analysis. Repeated measures ANOVA with Tukey comparison determined significance of relative fold-change in metabolite level from baseline.

Results—Hemorrhagic shock instigated succinate accumulation in plasma and lungs tissues (8.5 vs. 1.1 fold increase plasma succinate level from baseline, Shock vs. Sham, p=0.001; 3.2 fold higher succinate level in lung tissue, Shock vs. Sham, p=0.006). Metabolomic analysis identified labeled glutamine and labeled succinate in plasma (p=0.002) and lung tissue (p=0.013), confirming glutamine as the parent substrate. Kinetic analyses in shams showed constant total levels of all metabolites without significant change due to iLG.

Conclusion—Glutamine metabolism contributes to increased succinate concentration in plasma during hemorrhagic shock. The glutaminolytic pathway is implicated as a therapeutic target to prevent the contribution of succinate accumulation in plasma and the lung to post-shock pathogenesis.

Keywords

metabolomics; metabolopathy; glutaminolysis; succinate; mass spectrometry

Background

Trauma and hemorrhagic shock deprive tissues of oxygen and delay waste removal, provoking biochemical adaptation for acute cell survival. The resultant loss of equipoise potentiates post-injury inflammation, coagulopathy and the catabolic state, contributing to morbidity, including multiple organ failure (MOF), and mortality. (1–6) While the overall incidence of MOF after trauma is decreasing, the rates of MOF-related resource utilization, morbidity and death are unchanged. (7) Thus, patients initially surviving injury remain at risk of poor outcome precipitated by post-injury systemic derangements. Investigation has spanned decades, but the comprehensive metabolic response to trauma and shock remains incompletely defined, limiting the identification of superior prognostic biomarkers and treatment targets to predict and prevent post-shock pathogenesis. (1–3)

An increasing body of evidence submits that non-lactate metabolites, accumulating in plasma due to hypoxia-induced metabolic imbalance, are important contributors to post-injury pathophysiology. (8, 9) Physiochemical measurements of metabolic change after trauma broadly account for non-lactate anions and are more accurate at diagnosing acid-base disorders than conventional measures such as base deficit and the anion gap. (10) These physiochemical assessments can better predict mortality in certain patient populations, including those with normal serum lactate levels on admission. (11–14) Mass spectrometry

(MS) metabolomic approaches have identified the specific non-lactate anions that significantly increase or decrease in plasma and tissues, documenting consistent accumulation of succinate following protracted shock in human trauma patients and animal models. (15–17) Additionally, investigations into succinate-mediated cellular signalling have corroborated that succinate may not simply be an inert byproduct of anaerobic metabolism, but rather may have downstream cellular, tissue and systemic metabolic effects. (18, 19) Succinate receptors have been identified on numerous tissues, (20) and succinate has been directly implicated in post-ischemic inflammation, organ dysfunction, platelet activation and the generation of reactive oxygen species. (8, 9, 21, 22)

The purpose of our investigation is to use MS-based metabolomics in an animal model to determine the metabolic pathways contributing to succinate accumulation in plasma and tissues in the shock state. While glycolysis can generate succinate physiologically, labeling studies have previously demonstrated that glucose is not the primary source of increased succinate concentration in plasma during protracted shock. (17) (Figure 1) Thus, treatments exclusively targeting the glycolytic pathway during shock would be ineffective in preventing succinate-mediated pathogenesis. Here we investigate an alternative hypothesis: During hypoxia, oxygen debt prevents oxidative phosphorylation for cellular ATP production. Cell survival mechanisms require an immediate, compensatory, metabolic adaptation to produce high-energy phosphates and maintain cellular integrity. (9, 23) Alternate metabolic pathways, such as glutamine metabolism (glutaminolysis), can produce ATP under hypoxic conditions to secure cell survival, but generate succinate as a catabolic product. (9, 21) (Figure 1b) Thus, we hypothesize that succinate accumulation in plasma and tissues during protracted hemorrhagic shock derives from glutaminolysis.

Methods

Animal model

Animal experiments were performed under protocol approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver. All animals were maintained in the accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals*. Animals were housed under barrier-sustained conditions with 12 hour light-dark cycles and allowed free access to food and water before use.

Sprague-Dawley rats (n=16) weighing 407 g to 515 g (Harlan Labs, Indianapolis, IN) were anesthetized with 50 mg/kg Pentobarbital sodium via intraperitoneal injection at the beginning of the model and re-dosed intravenously (1 mg/kg) as necessary during the procedure. 1% Lidocaine injection was administered at all incision sites for analgesia. A tracheostomy was performed and a tracheostomy tube was placed. The right femoral artery and vein were cannulated, and femoral artery catheters were connected to Pro-Paq devices (Protocol Systems, Beaverton, OR) to monitor heart rate and mean arterial pressure (MAP). Core body temperature was monitored rectally and normothermia ($>36^{\circ}\text{C}$) was maintained with a heat lamp. iSTAT (Abbott Laboratories, Abbott Park, IL) arterial blood gas and blood chemistry panels were obtained at baseline (B), 15 min and 45 min time points to monitor respiratory and metabolic acid/base status. 0.5 mL Blood samples in heparinized tubes were drawn at B for metabolomics. Nonlethal hemorrhagic shock was induced via controlled

hemorrhage from the femoral artery to a MAP of 28–30 mmHg (*Shock*, n=8). At 15 min into shock, animals received a 1mL/kg intravenous infusion of $^{13}\text{C}_5$ - $^{15}\text{N}_2$ -glutamine (no. CNLM-1275-H-0.1, Cambridge Isotope Laboratories, Inc., Tewksbury, MA) in sterile water (35 mM) at a rate of 0.5 mL/min (iLG). The volume of labeled glutamine solution administered to animals was concentrated to prevent a volume bolus that could alter hemodynamics during hemorrhagic shock. The dose administered represents an approximate 10% total increase in endogenous circulating glutamine levels. In preliminary sham animals this dose was determined to have no effect on steady-state metabolism while still ensuring adequate substrate for MS detection of isotopologue signals throughout the experiment. No additional blood was drawn after iLG until the 30 min time point to allow for drug circulation (duration of 15 min). 0.3 mL Blood samples in heparinized tubes were then drawn at serial time points (30, 35, 40, 45 min) for metabolomic kinetic analysis. Animals remained in shock for a total of 45 min then were sacrificed. Brain, heart, lung and liver tissue was harvested after sacrifice. Blood samples were centrifuged at 5,000 *g* for 15 min at 4°C. Plasma was removed and centrifuged again at 12,600 *g* for 6 min at 4°C. Plasma and tissue samples were flash frozen in liquid nitrogen and stored at –80°C prior to batched metabolomics analysis. Sham control animals (*Sham*, n=8) remained normotensive throughout the duration of the experiment. Shams were anesthetized and underwent tracheostomy, blood vessel cannulation, iSTAT, iLG, blood sampling and organ harvesting at identical time points to those of shocked animals.

Metabolomics analyses

Rat plasma samples (20 μL) were extracted using methanol:acetonitrile:water (5:3:2) at 1:50 dilution, as previously reported. (15) Frozen rat organ samples were milled with a mortar and pestle in the presence of liquid nitrogen, then weighed to the nearest 0.1 mg and extracted in ice-cold lysis/extraction buffer at a concentration of 10 mg/mL. Samples were agitated vigorously at 4°C for 30 min and centrifuged at 10,000 *g* for 15 min at 4°C. Protein and lipid pellets were discarded, while supernatants were stored at –80°C prior to batch metabolomics analyses.

Metabolomics analyses were performed as previously reported. (17) Sample extracts (10 μL for plasma samples, 20 μL for organ extracts) were injected onto a Thermo Vanquish ultra-high performance liquid chromatography system [Thermo, San Jose, CA, USA] and run on a Kinetex XB-C18 column (150 $\times$ 2.1 mm, 1.7 μm particle size – Phenomenex, Torrance, CA, USA) at 25°C and a flow rate of 250 $\mu\text{L}/\text{min}$ (mobile phase: 5% acetonitrile, 95% 18 m Ω H₂O, 0.1% formic acid – 3 min isocratic run and 9 min linear gradient, as reported). (24) The UHPLC system was coupled online with a Thermo Q Exactive mass spectrometer (Thermo, Bremen, Germany), scanning in Full MS mode (2 μscans) at 70,000 resolution in the 60–900 *m/z* range. Electrospray ionization was achieved using 4 kV spray voltage, 15 sheath gas, and 5 auxiliary gas, and was operated in negative and positive ion mode (separate runs). Calibration was performed before each analysis using positive or negative ion mode calibration mixes (Pierce – Thermo Fisher, Rockford, IL, USA) to ensure sub ppm error of the intact mass.

Metabolite assignments were performed using Maven (25) (Princeton, NJ, USA) after conversion of .raw files to .mzXML format using MassMatrix (Cleveland, OH, USA). Maven allows for peak picking, feature detection and metabolite assignment against the KEGG pathway database. Assignments were confirmed against chemical formula determination, as gleaned from isotopic patterns and accurate intact mass, and validated against experimental retention times for >650 standard compounds (Sigma Aldrich, St. Louis, MO, USA; MLSMS, IROATech, Bolton, MA, USA). Isotopologue distributions, corrected for natural abundance, and flux analyses from $^{13}\text{C}_5$ - $^{15}\text{N}_2$ -glutamine were performed through the software Maven as previously reported. (26)

Statistical analysis

Comparison of hemodynamic measures and iSTAT blood gas analysis values between the two groups were performed using Student's *t*-test. Integrated peak area values for each metabolite, including the isotopologue distributions, were exported into .csv files and used to determine the relative fold-change in unlabeled metabolite level from baseline, or heavy isotope (labeled) level from natural abundance (<1%), to each time point. Repeated-measures ANOVA with Tukey comparison was used to compare fold-changes at matched time point between Sham and Shock animals. Statistical significance was accepted at $p < 0.05$. Values are presented as means $\pm$ standard errors of the mean. Statistical analyses were performed and graphed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Figure panels were assembled using Photoshop CS6 (Adobe, Mountain View, CA, USA).

Results

Hemorrhagic shock model produces acid/base imbalance consistent with anaerobic metabolism

(Table 1) At baseline, all animals were normotensive with base deficit values, obtained by iSTAT, within physiologic normal range. In the shock cohort, hemorrhagic shock was induced within 15 min of the start of controlled hemorrhage and was maintained for the duration of the model. Sham animals remained normotensive for the duration of the model. Base deficit was significantly increased at 15 min and 45 min in shocked animals when compared to shock baseline and sham controls. Sham animals were free from acidosis, assessed by base deficit, throughout the experiment.

Hemorrhagic shock alters metabolite levels in plasma

Batched MS-based metabolomic analyses were performed on plasma samples from shock and sham models ($n=8$ /group). Baseline metabolic profiles were similar between the two groups. Kinetic analyses of sham animals showed that total metabolite levels remained in steady-state, compared to baseline, for the duration of the experiment without significant change due to tissue manipulation during the procedure or labeled glutamine administration. Conversely, 30 min into hemorrhagic shock, plasma levels of glutamine, glutamate, succinate, fumarate and malate increased several-fold from baseline (1.4 fold increase in plasma glutamine at 30 min, $p=0.004$; 3.4 fold increase in plasma glutamate at 40 min, $p=0.016$; 8.5 fold increase in plasma succinate at 30 min, $p=0.022$; 10.5 fold increase in

plasma fumarate at 30 min, $p=0.004$; 18.6 fold increase in plasma malate at 30 min, $p=0.019$; Sham M+0 vs. Shock M+0). This trend continued for the duration of the model. (Figure 2)

Glutaminolysis contributes to succinate accumulation in plasma during hemorrhagic shock

Baseline (pre-iLG) heavy-isotope labeling distribution for all metabolites was consistent with natural abundance. Upon correction for natural abundance, analysis following iLG identified labeled $^{13}\text{C}_5$ - $^{15}\text{N}_2$ -glutamine (M+5+2) and its catabolic products, glutamate (M+5+1), α -ketoglutarate (M+4), succinate (M+4), fumarate (M+4), malate (M+4), oxaloacetate (M+4) and citrate (M+5). (Figure 3a) We administered equivalent levels of labeled glutamine to both models, with equivalent rates of progressive decrease—representing comparable glutaminolysis (30 min, $p=0.932$; 35 min, $p=0.802$; 40 min, $p=0.763$; 45 min, $p=0.595$; Sham plasma glutamine M+5+2 vs. Shock plasma glutamine M+5+2). (Figure 2) During shock, levels of labeled glutamate and succinate were significantly higher at 30 min when compared to sham controls, a trend that continued to the 45 min time point (30 min, $p=0.01$, Sham plasma glutamate M+5+1 vs. Shock plasma glutamate M+5+1; 30 min, $p=0.002$, Sham plasma succinate M+4 vs. Shock plasma succinate M+4). However, levels of labeled fumarate and malate were significantly lower in shocked animals than in shams at matched time points (30 min, $p=0.01$, Sham plasma fumarate M+4 vs. Shock plasma fumarate M+4; 30 min, $p<0.001$, Sham plasma malate M+4 vs. Shock plasma malate M+4). (Figure 2) In 7 of 8 shocked animals, distribution of labeled fumarate and malate was consistent only with natural abundance, indicating no labeling derived from iLG. This catabolic pattern suggests succinate as the final metabolite of glutaminolysis during shock. (Figure 3b)

Succinate accumulates in the lung during hemorrhagic shock but does not accumulate in the brain, heart or liver

Batched MS metabolomic analyses were also performed on homogenized brain, heart, liver and lung tissue collected after sacrifice from shock and sham models ($n=8/\text{group}$). At the conclusion of 45 minutes of hemorrhagic shock, total succinate (M+0) had accumulated in lung tissue when compared to sham animals at that time point (3.2 fold increase, $p=0.006$; Sham lung succinate M+0 vs. Shock lung succinate M+0). However, total succinate levels were lower in shocked brain, heart and liver tissues than those from sham animals, although only brain and liver differences were statistically significant (1.6 fold decrease in brain, $p=0.02$; 6.3 fold decrease in liver, $p=0.003$; Sham succinate M+0 vs. Shock succinate M+0). (Figure 4a) Fumarate and malate accumulated in all tissues after shock (>1.35 fold increase in fumarate in all tissues, $p<0.01$; >1.36 fold increase in malate all tissues, $p<0.01$; Sham M+0 vs. Shock M+0).

Glutaminolysis contributes to succinate accumulation in the lung during hemorrhagic shock

We were unable to confirm that there were no pre-iLG labeling distributions of succinate, fumarate and malate in the organ tissues due to the confines of the model (tissue harvest required sacrifice). Levels of labeled glutamine (M+5+2) were similar between sham and

shock models in all four tissues (data not shown). However, labeled succinate, fumarate and malate were not observed in brain or heart tissues from either model. We also detected significantly lower levels of labeled succinate and fumarate in shocked liver tissue than sham tissue (succinate in liver, $p=0.009$; fumarate in liver, $p=0.037$; Sham M+4 vs. Shock M+4). (Figure 4b) However, labeled succinate and fumarate did accumulate in shocked lung tissue (succinate in lung, $p=0.013$; fumarate in lung, $p=0.027$; Sham M+4 vs. Shock M+4). (Figure 4b) Labeled malate was higher in both liver and lung tissues following shock when compared to sham (malate in liver, $p=0.037$; malate in lung, $p=0.027$; Sham M+4 vs. Shock M+4).

Discussion

Early metabolomic reports have demonstrated that succinate, an intermediate substrate of the citric acid cycle (CAC), is consistently elevated after protracted shock in human trauma patients and animal models of trauma and hemorrhagic shock. (15–17) Accumulating succinate could have wide influence on post-shock cell signaling and tissue pathophysiology as succinate G-protein coupled receptors (GPR91) have been identified on numerous cell types. (20) Recent investigations have demonstrated that succinate controls the generation of mitochondrial reactive oxygen species under conditions of ischemia/reperfusion, and plays a key role in cellular immunity, promoting pro-inflammatory events. (8, 18, 19, 21) Even in the absence of sepsis, this type of systemic inflammatory response after injury underlies the development of acute respiratory distress syndrome (ARDS) and MOF. (1) Elevated succinate levels in plasma may also have an acute impact on trauma-induced coagulopathy (TIC). Succinate receptors on platelets can independently facilitate platelet activation, yet little is known regarding their subsequent function and resulting clot strength. (22) Animal models have demonstrated a correlation between elevated succinate in plasma and hyperfibrinolysis after shock, suggesting a succinate-mediated mechanism driving dysfunctional platelet-fibrin interactions. (15) Thus, metabolic pathways that contribute to ischemic succinate accumulation may represent therapeutic targets for prevention of early and late post-shock pathogenesis.

Parent compounds and relevant enzymatic pathways that generate succinate after trauma remain undefined. (17) This knowledge gap limits our ability to manipulate succinate metabolism in animal models in order to better understand its clinical effect and investigate treatment targets for human trauma patients. Previous reports by this group and others have suggested that succinate is a catabolite of glutaminolysis under hypoxic conditions. (9, 17, 21) In this study, we investigate this theory by administering a low dose (<10% of circulating glutamine concentration) of heavy isotope labeled $^{13}\text{C}_5$ - $^{15}\text{N}_2$ -glutamine solution (iLG) to rats during protracted hemorrhagic shock. The dose of labeled glutamine was determined such that it would have minimal effect on metabolism while guaranteeing detection of relevant downstream metabolites, and was not considered a significant glutamine load. Labeled glutamine is not intended to drive metabolism, rather it is utilized to visualize metabolic flux during shock. (17)

We have previously published that this hemorrhage model engenders a sufficient hypoxic exposure to induce anaerobic metabolism and prime post-shock pathologies. (27, 28) Intra-

operative increases in base deficit in shocked rats confirm anaerobic metabolism early in the model and prior to iLG. (29) (Table 1) Therefore, our metabolic flux analysis represents glutamine metabolism in the anaerobic state, and ultimately confirm that glutaminolysis contributes to succinate accumulation in plasma and the lung during hemorrhagic shock in our animal model.

As part of an initial analysis, MS-based metabolomics of total metabolite levels detail the broad accumulation of CAC intermediates in plasma following shock. (Figure 2) Importantly, neither tissue manipulation during the procedure nor drug administration alter steady-state metabolism, confirming that hypoperfusion, with cellular hypoxia, alone instigates metabolic change in our model. These results align with an increasing body of metabolomics-based evidence reporting elevated levels of CAC intermediates in shocked plasma from both animal models and severely injured human trauma patients. (15–17, 30) Together with our intra-operative base deficit measurements, these results also support theories suggesting non-lactate anions are contributing to clinically relevant acid/base derangement. (8, 9, 29)

Shock-induced metabolic change within the CAC is predictable and anticipated. (9, 31) During hypoxia, mitochondrial uncoupling at the electron transport chain (ETC) disables oxidative phosphorylation and obstructs reversible CAC reactions, requiring immediate, compensatory metabolic adaptation for sustained energy production and cellular survival. (8) (Figure 3b) Alternate pathways, such as glutamine metabolism, become preferential as they facilitate substrate-level phosphorylation for critical ATP production. (9, 21) While recent metabolomic analyses of injured patients and animal models have reported the consequences of these biochemical changes—substrate accumulation and depletion—they have been largely descriptive in nature. Very little *in vivo* experimental evidence exists investigating the underlying biochemical mechanics during hemorrhagic shock. (17)

Recently, we used similar stable isotope labeling experiments to demonstrate that glucose does not contribute substantially to increases in plasma succinate levels in the shock state. (17) Our results presented in this investigation confirm that glutamine is a major biochemical precursor for succinate accumulation in plasma during shock. (Figure 2) Isotope labeling demonstrates that (an)aerobic metabolism does not alter the rate of glutaminolysis, but dramatically influences its catabolic results. Carbon/nitrogen tracing in sham animals reveals widespread distribution of label consistent with steady-state metabolism of glutamine. However, we identify significantly higher levels of labeled glutamate and succinate in plasma during hemorrhagic shock, implicating glutaminolysis as a primary contributing pathway to succinate generation in the shock state. Importantly, labeled fumarate and malate are not identified during shock, while unlabeled plasma levels (M+0) are elevated, confirming that fumarate and malate are not catabolic products of glutaminolysis during protracted shock. This metabolic signature supports recent investigations into complex biochemical adaptations within the ETC during hypoxia. (8) Evidence presented in those reports suggests succinate dehydrogenase (SDH)—complex II of the ETC—becomes unidirectional during hypoxia, preventing the metabolism of succinate to fumarate. This results in a substrate bottleneck at SDH and succinate accumulates as a metabolic end point of glutaminolysis. (8, 32) (Figure 3b)

Lung injury after trauma remains a critical risk factor for MOF-related morbidity and mortality. (7) For decades, the post-injury systemic inflammatory response has been implicated in the pathogenesis of ARDS and MOF, but the initial priming/activating events have not been conclusively determined. (1, 7, 33, 34) During ischemia/reperfusion events, succinate controls the generation of reactive oxygen species and can function as a signaling molecule to initiate the inflammatory response. (8, 21) Succinate accumulation has also been recently implicated in pulmonary hypertension both in *in vitro* and *in vivo* models. (35, 36) In our analysis, total succinate levels are elevated in lung tissue, corroborating succinate as a potential inflammatory mediator capable of priming the lung for a “second-hit” after injury. (1) (Figure 4a) Labeling in succinate (M+4) in lung tissue also confirms that succinate accumulated in the lung derives from glutamine. (Figure 4b)

Unlike lung tissue, succinate is not elevated but lowered in shocked brain, heart and liver tissues by the end of the model, suggesting tissue-specific metabolic adaptation and pathologic effects from accumulating metabolites. Tissue samples were obtained after animal sacrifice and are confined to a single time point. This limits broad extrapolation of results as organs may demonstrate a variable sensitivity and metabolic adaptation to depth and duration of shock. Additional models with tissue harvest at sequential time points through shock would better characterize substrate flux in an organ specific manner. Low succinate concentration after 45 minutes of protracted, non-resuscitated hemorrhage does not necessarily protect these tissues from later pathologies resulting from exposure to elevated circulating succinate generated by other cells and tissues.

There are other potential limitations inherent in the design of this study. The short time course of the animal model excludes resuscitation, limiting a complete understanding of glutaminolysis and succinate metabolism beyond initial hemorrhagic shock. How and where succinate concentration increases or normalizes following various resuscitation strategies could impact pathogenesis in specific tissue beds. Thus, the metabolic and systemic consequences of succinate flux after reperfusion are essential to translating potential resuscitation and treatment approaches to trauma patients surviving initial injury. Also, while rodent models of trauma and hemorrhagic shock afford tightly controlled experimental conditions and minimize genetic variability, we acknowledge that they are imperfect at approximating human biology and physiology. (37–39) Important results from animal experiments ultimately require confirmation with investigation in the human trauma population.

Conclusion

Our investigative aim is to identify treatment targets against succinate-mediated pathogenesis after critical injury. To this end, the purpose of our study was to define the underlying biochemical mechanism contributing to elevated succinate levels in plasma and tissues following protracted shock. Our results confirm that glutaminolysis contributes to succinate accumulation in plasma and the lung following hemorrhagic shock in an animal model. Shock-induced increases in plasma levels of fumarate or malate are not derived from glutamine, a metabolic pattern supporting mitochondrial uncoupling with succinate as the final metabolite for glutaminolysis during shock.

While previous investigations maintain that glutaminolysis is an acute cellular survival pathway, resulting metabolic imbalance instigates immediate and later systemic derangements. These consequences may ultimately be fatal. Future investigation will build upon the mechanistic details presented in this study to inhibit enzymes (e.g. glutaminase, glutamine dehydrogenase) within the glutaminolysis pathway in order to conclusively characterize the pathologic effects of succinate in plasma and tissues after protracted shock, and confirm early, pre-hospital, treatment targets to prevent these outcomes.

References

1. Moore FA, Moore EE. Evolving concepts in the pathogenesis of postinjury multiple organ failure. *Surg Clin North Am.* 1995; 75(2):257–77. [PubMed: 7899997]
2. Moore FA, Haenel JB, Moore EE, Whitehill TA. Incommensurate oxygen consumption in response to maximal oxygen availability predicts postinjury multiple organ failure. *J Trauma.* 1992; 33(1): 58–65. discussion -7. [PubMed: 1344026]
3. Lenz A, Franklin GA, Cheadle WG. Systemic inflammation after trauma. *Injury.* 2007; 38(12): 1336–45. [PubMed: 18048040]
4. Moore FA, McKinley BA, Moore EE, Nathens AB, West M, Shapiro MB, Bankey P, Freeman B, Harbrecht BG, Johnson JL, et al. Inflammation and the Host Response to Injury, a large-scale collaborative project: patient-oriented research core--standard operating procedures for clinical care. III. Guidelines for shock resuscitation. *J Trauma.* 2006; 61(1):82–9. [PubMed: 16832253]
5. Desborough JP. The stress response to trauma and surgery. *Br J Anaesth.* 2000; 85(1):109–17. [PubMed: 10927999]
6. Weekers F, Giulietti AP, Michalaki M, Coopmans W, Van Herck E, Mathieu C, Van den Berghe G. Metabolic, endocrine, and immune effects of stress hyperglycemia in a rabbit model of prolonged critical illness. *Endocrinology.* 2003; 144(12):5329–38. [PubMed: 12960028]
7. Sauaia A, Moore EE, Johnson JL, Chin TL, Banerjee A, Sperry JL, Maier RV, Burlew CC. Temporal trends of postinjury multiple-organ failure: still resource intensive, morbid, and lethal. *J Trauma Acute Care Surg.* 2014; 76(3):582–92. discussion 92–3. [PubMed: 24553523]
8. Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL, Logan A, Nadtochiy SM, Ord EN, Smith AC, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature.* 2014; 515(7527):431–5. [PubMed: 25383517]
9. Chinopoulos C. Which way does the citric acid cycle turn during hypoxia? The critical role of alpha-ketoglutarate dehydrogenase complex. *Journal of neuroscience research.* 2013; 91(8):1030–43. [PubMed: 23378250]
10. Kaplan LJ, Cheung NH, Maerz L, Lui F, Schuster K, Luckianow G, Davis K. A physicochemical approach to acid-base balance in critically ill trauma patients minimizes errors and reduces inappropriate plasma volume expansion. *J Trauma.* 2009; 66(4):1045–51. [PubMed: 19359913]
11. Kaplan LJ, Kellum JA. Initial pH, base deficit, lactate, anion gap, strong ion difference, and strong ion gap predict outcome from major vascular injury. *Crit Care Med.* 2004; 32(5):1120–4. [PubMed: 15190960]
12. Kaplan LJ, Kellum JA. Comparison of acid-base models for prediction of hospital mortality after trauma. *Shock.* 2008; 29(6):662–6. [PubMed: 18180695]
13. Martin M, Murray J, Berne T, Demetriades D, Belzberg H. Diagnosis of acid-base derangements and mortality prediction in the trauma intensive care unit: the physicochemical approach. *J Trauma.* 2005; 58(2):238–43. [PubMed: 15706182]
14. Sen S, Wiktor A, Berndtson A, Greenhalgh D, Palmieri T. Strong ion gap is associated with mortality in pediatric burn injuries. *J Burn Care Res.* 2014; 35(4):337–41. [PubMed: 24823334]
15. D'Alessandro A, Moore HB, Moore EE, Wither MJ, Nemkov T, Gonzalez E, Slaughter A, Fragos M, Hansen KC, Silliman CC, et al. Early hemorrhage triggers metabolic responses that build up during prolonged shock. *American journal of physiology Regulatory, integrative and comparative physiology.* 2015 ajpregu 00030 2015.

16. Peltz ED, D'Alessandro A, Moore EE, Chin T, Silliman CC, Sauaia A, Hansen KC, Banerjee A. Pathologic metabolism: An exploratory study of the plasma metabolome of critical injury. *J Trauma Acute Care Surg*. 2015; 78(4):742–51. [PubMed: 25807403]
17. D'Alessandro A, Slaughter AL, Peltz ED, Moore EE, Silliman CC, Wither M, Nemkov T, Bacon AW, Fragoso M, Banerjee A, et al. Trauma/hemorrhagic shock instigates aberrant metabolic flux through glycolytic pathways, as revealed by preliminary (13)C-glucose labeling metabolomics. *Journal of translational medicine*. 2015; 13:253. [PubMed: 26242576]
18. Aguiar CJ, Rocha-Franco JA, Sousa PA, Santos AK, Ladeira M, Rocha-Resende C, Ladeira LO, Resende RR, Botoni FA, Barrouin Melo M, et al. Succinate causes pathological cardiomyocyte hypertrophy through GPR91 activation. *Cell communication and signaling: CCS*. 2014; 12:78. [PubMed: 25539979]
19. de Castro Fonseca M, Aguiar CJ, da Rocha Franco JA, Gingold RN, Leite MF. GPR91: expanding the frontiers of Krebs cycle intermediates. *Cell communication and signaling: CCS*. 2016; 14(1):3. [PubMed: 26759054]
20. Ariza AC, Deen PM, Robben JH. The succinate receptor as a novel therapeutic target for oxidative and metabolic stress-related conditions. *Frontiers in endocrinology*. 2012; 3:22. [PubMed: 22649411]
21. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, Frezza C, Bernard NJ, Kelly B, Foley NH, et al. Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. *Nature*. 2013; 496(7444):238–42. [PubMed: 23535595]
22. Hogberg C, Gidlof O, Tan C, Svensson S, Nilsson-Ohman J, Erlinge D, Olde B. Succinate independently stimulates full platelet activation via cAMP and phosphoinositide 3-kinase-beta signaling. *J Thromb Haemost*. 2011; 9(2):361–72. [PubMed: 21143371]
23. Lukyanova LD, Kirova YI. Mitochondria-controlled signaling mechanisms of brain protection in hypoxia. *Frontiers in neuroscience*. 2015; 9:320. [PubMed: 26483619]
24. D'Alessandro A, Nemkov T, Hansen KC, Szczepiorkowski ZM, Dumont LJ. Red blood cell storage in additive solution-7 preserves energy and redox metabolism: a metabolomics approach. *Transfusion*. 2015
25. Clasquin, MF.; Melamud, E.; Rabinowitz, JD. LC-MS data processing with MAVEN: a metabolomic analysis and visualization engine. In: Baxevanis, Andreas D., et al., editors. *Current protocols in bioinformatics/editorial board*. Vol. Chapter 14. 2012. p. 1
26. D'Alessandro A, Amelio I, Berkers CR, Antonov A, Vousden KH, Melino G, Zolla L. Metabolic effect of TAP63alpha: enhanced glycolysis and pentose phosphate pathway, resulting in increased antioxidant defense. *Oncotarget*. 2014; 5(17):7722–33. [PubMed: 25229745]
27. Moore EE, Moore FA, Franciose RJ, Kim FJ, Biffl WL, Banerjee A. The postischemic gut serves as a priming bed for circulating neutrophils that provoke multiple organ failure. *J Trauma*. 1994; 37(6):881–7. [PubMed: 7996599]
28. Stringham JR, Moore EE, Gamboni F, Harr JN, Fragoso M, Chin TL, Carr CE, Silliman CC, Banerjee A. Mesenteric lymph diversion abrogates 5-lipoxygenase activation in the kidney following trauma and hemorrhagic shock. *J Trauma Acute Care Surg*. 2014; 76(5):1214–21. [PubMed: 24747451]
29. Rutherford EJ, Morris JA Jr, Reed GW, Hall KS. Base deficit stratifies mortality and determines therapy. *J Trauma*. 1992; 33(3):417–23. [PubMed: 1404512]
30. Cohen MJ, Serkova NJ, Wiener-Kronish J, Pittet JF, Niemann CU. 1H-NMR-based metabolic signatures of clinical outcomes in trauma patients--beyond lactate and base deficit. *J Trauma*. 2010; 69(1):31–40. [PubMed: 20622576]
31. Krebs HA. The citric acid cycle: A reply to the criticisms of F. L. Breusch and of J. Thomas. *Biochem J*. 1940; 34(3):460–3. [PubMed: 16747181]
32. Chouchani Edward T, Pell Victoria R, James Andrew M, Work Lorraine M, Saeb-Parsy K, Frezza C, Krieg T, Murphy Michael P. A Unifying Mechanism for Mitochondrial Superoxide Production during Ischemia-Reperfusion Injury. *Cell metabolism*.
33. Moore EE, Moore FA, Harken AH, Johnson JL, Ciesla D, Banerjee A. The two-event construct of postinjury multiple organ failure. *Shock*. 2005; 24(Suppl 1):71–4. [PubMed: 16374376]

34. Yi J, Slaughter A, Kotter CV, Moore EE, Hauser CJ, Itagaki K, Wohlaue M, Frank DN, Silliman C, Banerjee A, et al. A “Clean Case” of Systemic Injury: Mesenteric Lymph After Hemorrhagic Shock Elicits a Sterile Inflammatory Response. *Shock*. 2015
35. Garcia-Morales LJ, Chen NY, Weng T, Luo F, Davies J, Philip K, Volcik KA, Melicoff E, Amione-Guerra J, Bunge RR, et al. Altered Hypoxic-Adenosine Axis and Metabolism in Group III Pulmonary Hypertension. *American journal of respiratory cell and molecular biology*. 2015
36. Yang L, Yu D, Fan HH, Feng Y, Hu L, Zhang WY, Zhou K, Mo XM. Triggering the succinate receptor GPR91 enhances pressure overload-induced right ventricular hypertrophy. *International journal of clinical and experimental pathology*. 2014; 7(9):5415–28. [PubMed: 25337184]
37. van der Worp HB, Howells DW, Sena ES, Porritt MJ, Rewell S, O’Collins V, Macleod MR. Can animal models of disease reliably inform human studies? *PLoS medicine*. 2010; 7(3):e1000245. [PubMed: 20361020]
38. Perel P, Roberts I, Sena E, Wheble P, Briscoe C, Sandercock P, Macleod M, Mignini LE, Jayaram P, Khan KS. Comparison of treatment effects between animal experiments and clinical trials: systematic review. *Bmj*. 2007; 334(7586):197. [PubMed: 17175568]
39. Tompkins RG. Genomics of injury: The Glue Grant experience. *J Trauma Acute Care Surg*. 2015; 78(4):671–86. [PubMed: 25742245]

Figure 1.

The citric acid cycle and relevant metabolic contributions. Solid black arrows indicate primary pathway; dotted arrows indicate alternate pathways. αK_g, α-ketoglutarate; ATP, adenosine triphosphate; NADH, nicotinamide adenine dinucleotide; 2HG, 2-hydroxyglutarate. (a) Aerobic metabolism under physiologic conditions. (b) During ischemia, glutaminolysis provides α-ketoglutarate to facilitate substrate-level phosphorylation and secure cellular survival. Succinate is produced as a catabolic product.

Figure 2.

Batched MS-based metabolomic analyses were performed on plasma samples from shock and sham models. Baseline metabolic profiles were similar between groups. In shams, unlabeled (M+0, blue) metabolite levels remained in steady state for the duration of the experiment without significant change due to instrumentation or iLG, while hemorrhagic shock instigated broad metabolite accumulation. Plasma abundance of labeled glutamine (M+5+2, red) following iLG was consistent between models at all time points and demonstrated progressive metabolism. Shock generated labeled glutamate (M+5, red; M+5+1, green) and succinate (M+4, red) accumulation, but did not increase fumarate (M+4, red) or malate (M+4, red) abundance compared to sham. ($*p<0.05$, $**p<0.001$, $n=8/\text{group}$) $M+X+Y$, molecular ion peak + no. of (labeled) ^{13}C + no. of (labeled) ^{15}N

Figure 3.

Batched MS-metabolomic analyses were performed on plasma samples from shock and sham models. (a) Schematic of isotopologue analysis following iLG identifying catabolic products of labeled $^{13}\text{C}_5-^{15}\text{N}_2$ -glutamine (M+5+2) metabolism. (b) Hypoxia-driven uncoupling of the electron transport chain prevents succinate metabolism to fumarate in order to facilitate substrate-level phosphorylation and generate ATP/NADH. (15–17)) $M+X+Y$, molecular ion peak + no. of (labeled) ^{13}C + no. of (labeled) ^{15}N

Figure 4.

Batched MS-metabolomic analyses were performed on homogenized brain, heart, liver and lung tissue collected after sacrifice from shock and sham models. (a) After 45 min of hemorrhagic shock, total succinate (M+0, blue) had accumulated in lung tissue. Total succinate levels were lower in shocked brain, heart and liver tissues when compared to sham controls. Fumarate and malate accumulated in all tissues after shock. (b) Isotopologue analysis identified labeled succinate in shocked lung tissue when compared to shams, but did not identify labeled (M+4, red) succinate in shocked liver tissue. (* $p < 0.05$, ** $p < 0.001$, $n = 8/\text{group}$) M+X+Y, molecular ion peak + no. of (labeled) ^{13}C + no. of (labeled) ^{15}N

Table 1

Characteristics of rats in sham and shock models (*mean ± SEM*).

Characteristic	Sham (n=8)	Shock (n=8)	Sham vs. Shock
Weight (g)	447.4 ± 7.3	447.4 ± 14.3	0.969
Percent blood loss	11.5% ± 0.82	35.1% ± 2	<0.001
Mean arterial pressure (mmHg)	<i>p value Change from baseline</i>		
Baseline	110 ± 6.7	106 ± 5.1	0.931
15 min	108 ± 6.7	29 ± 0.9	<0.001
45 min	70 ± 7.3	21 ± 5.2	<0.001
Base deficit (mEq/L)	<i>p value Change from baseline</i>		
Baseline	1.5 ± 0.8	1.3 ± 0.4	0.25
15 min	2.8 ± 1.0	10.9 ± 1.6	<0.001
45 min	3.4 ± 1.0	16.6 ± 2.5	0.001