Early hemorrhage triggers metabolic responses that build up during prolonged shock

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DESPITE DECADES OF ADVANCES in prehospital care, trauma remains the leading cause of death for individuals under the age of 40 (36). As much as 40% of injury-related mortality is attributed to uncontrollable hemorrhage (36), which in both civilian and military settings is the leading preventable cause of death after injury (40). Conspicuous factors associated with early mortality in trauma patients include trauma-induced coagulopathy, hypothermia, and metabolic acidosis, a series of mechanisms referred to as the “bloody vicious cycle” and later renamed as the “lethal triad” (14). These concepts laid the foundation for “damage control surgery”, an approach aimed at minimizing operating time as to control sources of significant bleeding and gastrointestinal contamination, while prioritizing early management of coagulopathy, hypothermia, and metabolic acidosis (46).

Early descriptions of metabolic responses to trauma were documented by Cuthbertson, who characterized two distinct phases: the “ebb” and the “flow” (9). The former corresponds to an early hypometabolic state that may serve a protective role aimed at reducing posttraumatic energy depletion. The latter is accompanied by an increased metabolic rate (including increased energy expenditure and oxygen consumption) (14, 18, 24, 29). Other overlapping stages have been described over the years, such as the “ischemia-reperfusion,” “leukocytic,” and “angiogenic” (2), although the boundaries between these phases are rather labile (4, 24). The incomplete understanding of the metabolic stages seen during trauma and hemorrhage has hampered the capacity to significantly improve resuscitative strategies.

Advances in fields such as proteomics (10) and metabolomics (6, 8, 32, 42) offer big strides toward the understanding of the complex biochemistry underpinning metabolic responses to trauma/hemorrhagic shock. Mass spectrometry-based metabolomics has emerged as a more sensitive analytical approach in early “ischemia-reperfusion injury phase” responses (6), allowing detection of thousands of molecular features corresponding to hundreds of small molecules (<1.5 kDa) representative of key metabolic pathways (e.g., glycolysis, Krebs cycle, and ATP/purine catabolism).

Just as the “omics” era has enabled a more sophisticated approach to understanding changes in metabolism, viscoelastic assays have greatly expanded our understanding of the role of fibrinolysis in trauma-induced coagulopathy. The more severe forms of fibrinolysis (>15% of clot lysis 30 min after reaching maximum clot strength) can predict mortality, while fulminant lysis (complete clot degradation within 30 min) is associated with 100% mortality (26). Metabolic acidosis and coagulopathy are, indeed, deeply intertwined (14, 34). Recent studies identifying postinjury fibrinolysis using thrombelastography...
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(TEG) have refined the degree of clot lysis that is associated with adverse outcomes from >15% to >3% (21). However, no observational evidence has been reported so far about the potential correlation of metabolome profiles and fibrinolysis. Identifying metabolites associated with fibrinolysis can lead to therapeutic interventions to attenuate impaired regulation preventing progression to hyperfibrinolysis.

In this study, we performed an ultra-high-performance liquid chromatography-mass spectrometric (UPLC-MS) analysis of the dynamic changes in the rat plasma metabolome following rapid hemorrhage and prolonged shock compared with sham controls. The purpose of this study is to document the metabolic responses of rapid near-lethal hemorrhage and prolonged hemorrhagic shock in a model that produces a hyperfibrinolytic coagulopathic phenotype. We hypothesize that metabolic changes that occur early during hemorrhage will increase during shock, in parallel to an increase in fibrinolysis (LY30) and plasma levels of the activator protein of fibrinolysis (tissue plasminogen activator).

METHODS

Animal model. The animal protocol (Fig. 1) was approved by the University of Colorado International Animal Care and Use Committee no. 90814. Juvenile male Sprague-Dawley rats (275–400 g) were induced with pentobarbital sodium followed by tracheostomy and femoral artery cannulation to measure blood pressure and induce hemorrhage. Prior to initiation of hemorrhagic shock, animals were allowed to recover from their initial surgery for airway and vascular access, including maintenance of normothermia (temperature >36°C), heart rate >240, and mean arterial pressure (MAP) >85 mmHg.

Sham. Blood draws were performed at baseline, after 1 and 30 min (consistent with the last time point of the shock group) to determine whether baseline values in the absence of hemorrhagic shock would result in plasma metabolic changes due to anesthesia and supine positioning. Blood samples obtained for the total duration of the experiment were less than 15% estimated blood volume (EBV).

Hemorrhage. Blood draws were adjusted for weight differences among rats, and the EBV was calculated from the animal weight employing the conversion of 0.06 ml of blood per gram conversion factor. The first blood sample obtained (baseline) consisted of 8% (± 1%) of the total EBV. This was determined to be the minimal amount of blood obtained at a single time point to run TEG and have residual plasma for metabolomics analyses. This baseline blood draw was the first of a series of blood draws to induce hemorrhagic shock to attain a MAP of 25 (±2) mmHg in 5 min. Blood volume (0.5 ml) from these time points was ≤3% EBV for the smallest animals. MAP was recorded at minute intervals correlating with 0.5-ml blood draws through minutes 1–5 with an additional blood draw (maximum 1.5 ml) if the animal’s blood pressure did not drop sufficiently over the prescribed serial blood draws.

Shock. Animals were kept at MAP of 25 for 30 min, since the time window from injury to arrival at our hospital for most severely injured patients who underwent emergency department thoracotomies was 20–30 min (interquartile range, data not shown), with an average of 24 min. The degree of shock was selected on the basis of previous work with rodents that did not show changes in coagulation when blood pressure was >30 mmHg (47). Scheduled blood draws of 0.5 ml were drawn at 10 min and 20 min after the goal MAP was achieved. Additional blood was removed between these time points if the MAP exceeded 28 mmHg. At the end of 30 min, a final blood draw of 8% EBV was obtained, which was lethal in 100% of animals within a 10-min time frame.

Blood samples, rodent thrombelastography, and tissue plasminogen activator measurement. Whole blood was collected in 3% citrated at a 1:10 ratio, based on our previous experience with rodents and TEG (48). Individual Eppendorf tubes were prefilled with citrate and marked to an appropriate fill level to ensure reproducible ratios of whole blood to citrate. Citrated native TEG assays were recalculated and run according to the manufacturer’s instructions on a TEG 5000 Thrombelastograph Hemostasis Analyzer (Haemonetics, Niles, IL). The following parameters were recorded from the tracings of the TEG: R time (minutes), angle (α, °), maximum amplitude (MA, mm), and lysis 30 min after MA (LY30, %).

Whole blood was activated for TEG analysis with recalcification. Blood not used for TEG was spun to plasma for metabolomics analysis. Whole blood was centrifuged at 6,000 g for 10 min at 4°C. Plasma was removed and spun at 12,500 g for 10 min at the same temperature to remove contaminating platelets and acellular debris. The remaining plasma was flash frozen in liquid nitrogen and stored at −80°C until analyzed.

The levels of tissue plasminogen activator (tPA) were measured via ELISA, as previously reported (48).

Metabolomics analyses. Plasma samples (10 μl) were immediately extracted in ice-cold lysis/extraction buffer (methanol:acetonitrile: water 5:3:2) at 1:25 dilutions. Samples were then agitated at 4°C for 30 min and then 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 metabolomics analyses.

Metabolomics analysis. Metabolomics analyses were performed as previously reported (12). Ten microliters of sample extracts were injected into an UPLC system (Ultimate 3000, Thermo, San Jose, CA) and run on a Kinetex XB-C18 column (150 × 2.1 mm, 1.7-μm particle size; Phenomenex, Torrance, CA) at 250 μl/min (mobile phase: 5% acetonitrile, 95% 18 mΩ H2O, 0.1% formic acid). The

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Fig. 1. An overview of the Sprague-Dawley rat animal model of hemorrhage adopted in this study.
UPLC system was coupled online with a QExactive system (Thermo, San Jose, CA), scanning in Full MS mode (2 μs/cans) at 70,000 resolution in the 60–900 m/z range, 4-kV spray voltage, 15 sheath gas, and 5 auxiliary gas, operated in negative and then positive ion mode (separate runs). Calibration was performed before each analysis against positive or negative ion mode calibration mixes (Piercenet; Thermo Fisher, Rockford, IL) to ensure subparts per million error of the intact mass. Metabolite assignments were performed using the software Maven (7) (Princeton, NJ), upon conversion of .raw files into .mzXML format through MassMatrix (Cleveland, OH). The software allows for peak picking, feature detection, and metabolite assignment against the KEGG pathway database. Assignments were further confirmed against chemical formula determination (as gleaned from isotopic patterns and accurate intact mass), and retention times against a library of 619 standard compounds (Sigma-Aldrich, St. Louis, MO; MLSMS, IROATech, Bolton, MA).

Statistical analysis. Relative quantitation was performed by exporting integrated peak area values into Excel (Microsoft, Redmond, CA) for statistical analysis (repeated-measures ANOVA with Tukey multiple-column comparison test, significance threshold for P values < 0.05) and partial least square–discriminant analysis (PLS-DA), calculated through the macro MultiBase (freely available at www.NumericalDynamics.com).

Hierarchical clustering analysis (HCA) was performed through the software GENE-E (Broad Institute, Cambridge, MA; freely available at http://www.broadinstitute.org/cancer/software/GENE-E/). Box and whisker plots were graphed through GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA), and figure panels were assembled through Photoshop CS5 (Adobe, Mountain View, CA).

RESULTS

Plasma metabolomics analyses were performed on seven Sprague-Dawley rats exposed to progressive hemorrhage (5 min: H1 through H5), or hemorrhagic shock for 10, 20, or 30 min (S10, S20; see Shock and Fig. 1). All of the rats involved in this study demonstrated systemic hyperfibrinolysis via TEG (LY30 >3%) after severe hemorrhagic shock (Supplemental Table S1). Overall, 118 metabolites were monitored throughout each stage, and results are reported in Supplemental Table S1, together with the metabolite name, KEGG pathway ID, super-pathway assignment (color-coded consistently throughout the paper), the polarity in which each metabolite has been detected, the experimentally observed mass-to-charge ratios, line plots showing the trends throughout each sample on the basis of the median values across each biological replicate, statistical analyses comparing late shock time points, specifically S10, S20, and Shock, to baseline values, together with fold-changes of median values from Shock rats against baseline parameters.

To highlight metabolic trends throughout the tested stages, results were elaborated for HCA, as reported in Fig. 2, whereby metabolites are grouped in a pathway-wise fashion (metabolite names are extensively reported in Supplemental Fig. S1, also including metabolite names and clustering patterns). Metabolite levels in each biological replicate during time course analyses were, thus, used to inform the elaboration of partial least square discriminant analyses (PLS-DA), as reported in Fig. 3. Sample group clustering followed a clockwise distribution (PC1 + PC2 accounting for almost the 50% of the total covariance, with PC1 explaining 45.8% of the covariance alone), a distribution that closely paralleled the phenotype (time-course measurements; Fig. 3A). The top 10 metabolites showing the highest covariances across the principal components are labeled in Fig. 3B. Lactate was another key contributor to metabolic group clustering in response to hemorrhagic shock. Other key compounds include metabolites involved in the tricarboxylic acid (TCA) cycle, such as citrate and malate, metabolites involved in lipid metabolism (acyl-conjugated carnitines), purine/nitrogen catabolism substrates and by-products (nicotinamide, urate, and the polyamine spermidine), and glutathione homeostasis/oxidative stress (glutamate and glutathione disulfide, GSSG).

Although metabolic trends were consistent across biological replicates (Fig. 2), hive plots of key metabolites were graphed (Fig. 4) as to highlight twofold quantitative changes (either increases or decreases) that could not be appreciated through a linear color-coding of normalized values as the one used for heat maps. The choice of these metabolites was informed by PLS-DA analyses (Fig. 3) and line plot analyses of robust Z-score-normalized linear metabolic changes across metabolic stages, both in each independent rat and in the overall set of samples assayed in this study (Supplemental Fig. S2). These changes were observed in the hemorrhage/shock samples, albeit not in the sham group (Supplemental Fig. S3). As a result, we could highlight rat-specific patterns for some of these key metabolites, such as lactate, malate, and uracil, twofold decreasing in a subset of biological replicates at earliest hemorrhage time points (H1 through H5), while increasing twofold after 10, 20, or 30 min from hemorrhage through hemorrhagic shock (S10, S20, Shock). Other metabolites like glutamate, GSSG, and urate increased twofold in all of the samples during early hemorrhage (H time points), even before significant hemorrhagic shock could ensue (S10, S20, Shock). Again, these changes were absent in the sham group and only began to be visible (though remaining still significantly lower than in the hemorrhage/shock samples) for some metabolites (e.g., urate) at the 45-min draw in the sham plasma samples.

A pathway-wise elaboration of the results was performed as to gain mechanistic insights of the dynamic metabolic changes secondary to hemorrhage and hemorrhagic shock. Results are schematized from Figs. 5–7, either providing a snapshot of key intermediates of energy metabolism (glycolysis and TCA cycle, Fig. 5), fatty acid mobilization, transamination, glutathione homeostasis, and urea cycle (clockwise order, Fig. 6), and purine metabolism (Fig. 7). Most of the changes observed in response to hemorrhagic shock (S10, S20, Shock) are preceded by consistent trends (mostly toward increase) in the levels of most metabolites, trends beginning during early hemorrhage (H1 through H5). However, biological variability (exemplified by the hive plot in Fig. 4) negatively affected the calculation of statistical significance at these early stages.

Energy metabolism. Progressive plasma accumulation of glucose and fructose was observed upon hemorrhage, although significantly increased hyperglycemia was observed only upon hemorrhagic shock. Consistently, glycolytic metabolites, including hexose-phosphates and late by-products pyruvate and lactate, increased progressively from H3–H4 hemorrhage time points as to become significant after late hemorrhagic shock. Of note, lactate did decrease twofold during early hemorrhage (H1–H3) in three rats, before increasing twofold at the immediately subsequent hemorrhage time point in all the biological replicates (Fig. 4).
In addition, lipolysis and β-oxidation of lipids were suggested by the progressive accumulation of glycerophospholipid breakdown products (N-methylethanolamine phosphate, glycero-3-phosphoethanolamine, ethanolamine phosphate), fatty acid-mobilizing acyl-carnitines (propanoyl-carnitine, butanoyl-carnitine), and ketone bodies (acetacetate and hydroxyisobutyrate, Supplemental Table S1). Changes in lipolysis and β-oxidation were evident even before hemorrhagic shock and were statistically significant after it. On the other hand, the levels of these metabolites did not change in the sham group (Supplemental Fig. S3).

Hypercatabolism was also indicated by increased levels of all TCA cycle intermediates, from citrate to ketoglutarate, succinate, fumarate, malate, and oxaloacetate in the shock group, but not in shams (Fig. 5, Supplemental Fig. S3). Plasma levels of the carboxylic acid citramalate increased after hemorrhagic shock, while they did not in the sham group (Fig. 5, Supplemental Fig. S3).

Amino acids: proteolysis, transamination, and glutathione homeostasis. In the present study, all of the amino acid levels, except glycine, increased in response to early hemorrhage and shock (but not in sham animals) (Fig. 6, Supplemental Table
S1). However, their relative fold-change increase was not distributed proportionally to their normal observed frequency in vertebrates (31), with glutamate and tyrosine increasing the most (5.6- and 3.48-fold change increase over the baseline levels; Supplemental Fig. S4). Glutamate anabolism intermediates aminobutanoate and succinate semialdehyde increased immediately after early hemorrhage (Supplemental Table S1). Increases in the levels of reduced and oxidized glutathione (GSH and GSSG) and 5-oxoproline were observed in the shock group only, although only the former started increasing during...

Fig. 3. Partial least square analysis of metabolic changes in rat plasma upon exposure to hemorrhage and hemorrhagic shock. In A, clusters are indicative of each sample group (from baseline to Shock). Cluster distribution followed a clockwise trend, paralleling the phenotype. In B, metabolites (variables) are graphed, contributing to maximize the covariance (mostly explained by PC1 = 45.8%, and to a lesser extent by PC2 = 1.6%) throughout all the samples (observations) in A. The top 10 metabolites contributing the most to covariance are highlighted in B.

Fig. 4. Hive plot comparative analysis of metabolic changes in rat plasma following hemorrhage/hemorrhagic shock. All identified metabolites are included on the horizontal axis (color codes and position in the bar are consistent with Table 2), while those accumulated (twofold, top axis) or depleted (0.5-fold change, bottom axis) in a particular time point compared with baseline values are indicated by a connecting arc. Connecting arcs are color-coded depending on the biological replicate (rat numbers are consistent with Tables 1–3 and Figs. 2 and Supplemental Fig. S1). The figure shows that a subset of key metabolites, including glutamate, lactate, malate, oxidized glutathione (GSSG), urate, and uracil undergo rat-specific changes (twofold increase or decrease) since earliest time points (H1 = 1 min after minor hemorrhage).
early hemorrhage (H3), and the latter started increasing only after prolonged hemorrhagic shock (Fig. 6).

**Urea cycle and purine catabolism/salvage.** Among all amino acids, arginine accumulation did not reach significance even upon late hemorrhagic shock (Fig. 6). Citrulline increased significantly upon late hemorrhagic shock (Fig. 6). The levels of urea cycle intermediates (e.g., arginine-succinate) did not increase significantly after hemorrhagic shock, while minor, but significant, late increase in the levels of ornithine was observed (Fig. 6). Polyamines accumulated since early stages of hemorrhagic shock, especially the late products of this pathway, spermidine and spermine, increasing up to 6.15- and 4.35-fold over the baseline values (Supplemental Table S1).

Purine catabolites urate, hydroxyisourate, and allantoin increased at H4 time points to become significant during prolonged hemorrhagic shock (Fig. 6). Nicotinamide increases were observed even later at S10, although they became significant only at S20 and Shock time points (Fig. 7). Immediate increases in the levels of adenosine followed a two-stage increase, with 1.4 increase \((P = 0.007)\) immediately after hemorrhage (H1), and 2.17-fold increase after prolonged shock \((P = 0.004)\). None of the metabolites described in this paragraph increased in sham animals (Supplemental Fig. S3).

**DISCUSSION**

Previous investigations on the metabolic response to trauma have consolidated the concept of trauma-induced hypercatabolism, resulting in an increased rate of proteinolysis of lean skeletal muscle sustained glycolysis, hepatic gluconeogenesis, and biosynthesis of acute-phase proteins (2, 3, 24, 45, 49). However, most of the evidence accumulated during the past decades has been based upon targeted biochemical assays, and no panoramic overview has been hitherto produced about the dynamic changes to the plasma metabolome induced by trauma/hemorrhagic shock. The advent of NMR-based metabolomics has allowed us to analyze tens of metabolites in a single analysis, thereby paving the way for a deeper understanding of the main metabolic changes secondary to traumatic injury (8, 32, 32, 42). Recent advances in the field of MS-based metabolomics have brought about a new era in the field of trauma metabolomics, even though reports available so far mainly focus on the “ischemia/reperfusion” stage (6). In the present study, we report the dynamic changes of the rat plasma metabolome in response to hemorrhage and sustained hemorrhagic shock, by exploiting a model of trauma-induced coagulopathy that results in the promotion of hyperfibrinolysis.
and poor prognosis. The model was designed to test the hypothesis that metabolic changes in response to traumatic injury could ensue during progressive hemorrhage or could, instead, be triggered by acute hemorrhagic shock. As a result, in the shock group, but not in the sham one (a control group to determine the effect of anesthesia and supine positioning), the dynamic alterations of the metabolome involved almost all the metabolic pathways tested in this study, including energy metabolism (glycolysis, β-oxidation of fatty acid, TCA cycle), amino acid metabolism (amino acid levels, urea cycle, polyamines, and nitrogen balance), nucleotide catabolism, one-carbon metabolism, and glutathione homeostasis. No toxic effect by anesthesia alone (e.g., lactate accumulation) was observed in the sham group.

Early increases in plasma glucose is suggestive of ongoing hepatic gluconeogenesis or ongoing glycolgenolysis, as to mobilize fast energy sources in response to hypoxia/local anoxia, even before actual hemorrhage shock has enough time to ensue, consistent with the concept of traumatic diabetes or trauma-induced insulin resistance. On the other hand, accumulation of TCA cycle intermediates after shock might result from local cellular breakdown (cell lysis) or mitochondrial uncoupling. These results are consistent with recent findings on ischemia/reperfusion injury in rat organs (6) and human plasma from trauma activation and emergency department thoracotomies subjects (38). These results are consistent with incommensurate oxygen demands by trauma patients to sustain mitochondrial metabolism (35). Accumulation of TCA cycle intermediates, especially succinate, has been reported to occur in response to hypoxia following ischemia through reverse TCA fluxing of carbon backbones from aspartate (6). Consistently, in our model, hypoxygenation should ensue in response to hemorrhage. On the other hand, reperfusion has been reported to promote reverse electron transport chain.
fluxes, resulting in reactive oxygen species generation and succinate depletion. Future expansions of the present model could be designed to understand whether resuscitation might result in a similar metabolic phenotype. Moreover, TCA cycle intermediates might also promote hypoxic responses by inhibiting the enzyme prolyl hydroxylase, thereby preventing degradation and promoting stabilization of hypoxia-inducible factor 1α (HIF-1α) (41).

Accumulation of carboxylic acids might contribute to non-lactate-dependent metabolic acidosis (16). In parallel, carboxylic acids, similar to citrate, might scavenge calcium ions, thereby affecting coagulation cascades. Succinate, for example, has been reported to affect platelet and neutrophil activity (33, 44). These considerations further underline the complex interplay between energy metabolism, acidosis, and coagulopathy at the metabolic level.

Citramalate is a carboxylic acid isobar to 2-hydroxyglutarate that unexpectedly increased in a shock-dependent fashion. This metabolite, produced by bacteria, has been recently found to be uptaken/pynocytosed by red blood cells (5). Hemolysis secondary to hemorrhagic shock might trigger release of this metabolite in the plasma and indicate as of yet uninvestigated ties between bacterial metabolism and bacterial metabolite-triggered responses to injury, even in the absence of sepsis.

Muscle proteolysis in response to trauma/hemorrhagic shock is known to promote the release of free amino acids for catabolic purposes (27, 37), as we here observe in response to shock, but not in shams. If plasma amino acid accumulation in response to hemorrhagic shock would be dependent in proteolysis alone, fold-change increases in amino acid levels should be proportional to their abundance in proteins. However, this did not seem to be the case upon a rough comparison of the shock/baseline amino acid ratios and the expected abundance of each amino acid in the proteome (as indirectly gleaned by the frequency of the codons for each amino acid in the vertebrate genomes). Amino acids like tyrosine and glutamate were extremely enriched, suggesting ongoing anabolic reactions toward their specific enrichment. Glutamate, a key amine group donor and excitatory neurotransmitter, can, indeed, be generated through glutamine deamination and, thereby, provide a carbon substrate for transamination reactions. In parallel, glutamate can be used as an amine group donor in transamination reactions to generate ketoglutarate, so as to fuel the TCA cycle. Thus, it is worth noting that glutamine levels increased to a lesser extent compared with other amino acids only upon hemorrhagic shock, albeit not in sham animals, suggesting ongoing glutaminolysis in response to hemorrhage/hemorrhagic shock. Enteral supplementation of glutamine is

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Fig. 7. An overview of purine metabolism in plasma samples from rats undergoing progressive hemorrhage and hemorrhagic shock. Results are graphed as box-plots indicating median values (line), mean values (+), and upper/lower quartile distributions for each group. Asterisks indicate significance upon repeated-measures ANOVA test (*P ≤ 0.1; **P ≤ 0.01; ***P ≤ 0.001).
one nutritional concept for severely injured patients in recent years (27, 37). Additionally, glutamine exerts an important nutritional effect serving as a principle fuel source for enterocytes and intestinal mucosa (24). However, recent evidence from labeling experiments on ischemia/reperfusion injury suggests that glutamine might serve a key role as a nitrogen donor, rather than as a carbon substrate (6).

Glutamine-derived glutamate might serve transamination purposes, thereby, compensating hypoxia-induced accumulation of pyruvate and oxaloacetate by promoting their conversion to alanine and aspartate, respectively. Anabolism of glutamate is further supported by the hemorrhage-dependent accumulation of synthesis intermediates aminobutanoate and succinate semialdehyde.

Hemorrhage-dependent accumulation of glutamate and cysteine was not accompanied by similar trends for glycine. While glycine can also fuel late glycolysis at the trioses stage, these three amino acids are the substrates for the biosynthesis of glutathione (GSH), hinting at alterations in the glutathione pool in response to hemorrhagic shock. Both GSH and GSSG levels increased in response to hemorrhage, suggesting an increased necessity to cope with oxidative stress. Of note, accumulation of 5-oxoproline was observed in shock rats (but not in sham animals), which is relevant in that oxoproline is a marker of glutathione turnover that has been associated with impaired GSH homeostasis in stored blood products (11). At the same time, 5-oxoproline can be converted back to glutamate by the enzyme oxoprolinase in an ATP-dependent fashion, contributing to the observed increase in glutamate. In the context of trauma, oxoprolinase might play a potential role as osmoprotectant and contribute to unexplained acidosis (15).

An arginine increase only after late shock is indicative of early arginine consumption, either through the urea cycle or as a substrate to promote nitric oxide generation by endothelial nitric oxide synthase (eNOS). Late citrulline increase supports the hypothesis of nitric oxide (NO) production by eNOS activity only after prolonged shock. NO generation would be relevant in that it would result in the promotion of vasodilation (to meet higher energy and oxygen demands in response to hemorrhage-induced hypercatabolism). NO would also further strengthen hypoxic responses by stabilizing HIF-1α (1), and promising beneficial effects have been documented for enteral arginine supplementation in rats undergoing trauma/hemorrhagic shock (43).

At the same time, aspartate accumulation is consistent with proteolysis, or partial impairment of the purine salvage reactions (deaminated IMP conversion back to AMP fueling the TCA cycle at the level of fumarate) (6). This is relevant in the light of the observed upregulation of purine catabolism in response to hemorrhage/hemorrhagic shock, resulting in the accumulation of IMP breakdown products inosine, hypoxanthine, and xanthine, and downstream metabolites urate, hydroxyisourate, and allantoin. Of note, urate conversion to hydroxyurate and allantoin is mediated by the rat enzyme uricase, which is nonfunctional in greater apes and humans, where no significant plasma accumulation of urate was observed in trauma patients (38). On the other hand, urate-to-allantoin conversion in humans might be driven by reactivating species-scavenging reactions (28). In the light of these data, here, we hypothesize that accumulation of urate, a potent antioxidant, might represent an adaptive response against hemorrhage-triggered ischemia.

Hemorrhagic shock also apparently fueled pathways branching from the urea cycle, namely polyamine synthesis pathway. These highly basic compounds might play a role as pH buffers, other than contribute to osmoprotection resulting from hemorrhage-induced hypovolemia, suggesting hitherto under investigated ties between conserved stress-response mechanisms in plants and mammals (20). At the same time, polyamine metabolism has been previously related to edema formation and necrotic formation after traumatic brain injury (13) and increased urinary polyamine excretion has been observed in patients with surgical and accidental trauma undergoing total parenteral nutrition (39). As the hemorrhaged rats tested in this study developed hyperfibrinolysis, it is relevant to note how polyamines have been previously associated with the modula-

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Fig. 8. A schematic representation of time course metabolic changes during early hemorrhage and prolonged hemorrhagic shock. Color codes for pathway arrows are consistent with pathway legends in Supplemental Table S2.
tion of blood coagulation and fibrinolysis (25). Further studies will address the specific hypothesis, as the hereby observed fold-change increase in polyamine levels in hemorrhaged rats is also observable in humans and affects coagulation cascades and clot stability.

Early shock increases in the levels of adenosine in response to hemorrhage document the insurgence of a key defensive protective auto/paracrine signaling cascade aimed at limiting cellular damage in response to adverse conditions, including hypoxia or ischemia (22). Indeed, adenosine release promotes vasodilation, stimulation of glycogen breakdown, and reduced neuronal excitability, other than neurotransmitter release to reduce neuronal energy requirements (22). Adenosine signaling via adenosine receptor A2B [A(2B)R] has been implicated in protection during acute kidney injury through the inhibition of neutrophil-dependent TNF-α release (23). While beneficial under control conditions, plasma adenosine increase in response to hypoxia has been observed to promote sickling of red cells in rats and humans through signaling via adenosine receptor A2B [A(2B)R] (50).

Late nicotinamide accumulation might result from the activity of poly-ADP-ribose polymerase, which can use nicotinamide adenine dinucleotides as substrates to trigger DNA damage and apoptosis/necrosis, and, in turn, partially deplete NAD/NADP reservoirs, thereby negatively influencing energy and antioxidant potential.

**Perspectives and Significance**

Dynamic changes in the rat plasma metabolome in response to progressive hemorrhagic shock were observed during early hemorrhage, before sustained hemorrhagic shock. Conversely, significant changes in early and late time points (30 min through shock) were not observed in the sham group. Specific phenotypes were observed, with half of the biological replicates showing trends toward early metabolic depression during hemorrhage, followed by hypercatabolism, while the other half showing immediate hypercatabolic reactions. While complementing and expanding recent evidence in humans (38), the appreciation of such metabolic phenotypes will pave the way for tailored resuscitative strategies. Testable hypotheses were generated, indicating a likely correlation of metabolic adaptation to hemorrhage (e.g., plasma elevation in the levels of succinate, urate, and polyamines), but not anesthesia and su- pine positioning, and the hyperfibrinolytic phenotype observed in the tested rats.

**Conclusion.** Almost immediate increases in the levels of adenosine were observed, suggesting a signaling role for this metabolite in early hemorrhage responses. In keeping with the literature, hypermetabolism corresponded to increased glycemia and glycolysis, accumulation of TCA cycle intermediates, and amino acid hypermetabolism corresponded to increased glycemia and glyco-

Most of the observed metabolites, including carboxylic acids, oxoproline and other acids (e.g., urates), increasing up to 36-fold in response to hemorrhage/hemorrhagic shock, albeit not in shams, might justify nonlactate metabolic acidosis in the clinical setting. Future studies will be necessary on a larger animal study/human cohort through absolute quantitation-based metabolomics. These studies will improve our understanding of the correlation between the levels of these metabolites and the severity of shock and investigate whether functional ties exist between hemostatic function (e.g., platelet activation, fibrinolysis, as suggested by the presented correlative evidence herein) or immune responses (e.g., neutrophil priming) and the levels of the metabolites affected by hemorrhage/hemorrhagic shock.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


