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Mitochondrial dysfunction in rat splenocytes following hemorrhagic shock☆

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ABSTRACT

The regulation of mitochondrial function is critical in cellular homeostasis following hemorrhagic shock. Hemorrhagic shock results in fluid loss and reduced availability of oxygen and nutrients to tissues. The spleen is a secondary lymphoid organ playing a key role in 'filtering the blood' and in the innate and adaptive immune responses. To understand the molecular basis of hemorrhagic shock, we investigated the changes in splenocyte mitochondrial respiration, and concomitant immune and metabolic alterations. The hemorrhagic injury (HI) in our rat model was induced by bleeding 60% of the total blood volume followed by resuscitation with Ringers lactate. Another group of animals was subjected to hemorrhage, but did not receive fluid resuscitation. Oxygen consumption rate of splenocytes were determined using a Seahorse analyzer. We found a significantly reduced oxygen consumption rate in splenocytes following HI compared to sham operated rats. The mitochondrial stress test revealed a decreased basal oxygen consumption rate, ATP production, maximal respiration and spare respiratory capacity. The mitochondrial membrane potential, and citrate synthase activity, were also reduced in the splenocytes following HI. Hypoxic response in the splenocyte was confirmed by increased gene expression of Hif1α. Elevated level of mitochondrial stress protein, hsp60 and induction of high mobility group box1 protein (HMGB1) were observed in splenocytes following HI. An increased inflammatory response was demonstrated by significantly increased expression of IL-6, IFN-β, Mip-1α, IL-10 and NFκbp65. In summary, we conclude that splenocyte oxidative phosphorylation and metabolism were severely compromised following HI.

1. Introduction

Severe hemorrhage leads to dysregulation of multiple biochemical pathways leading to cell apoptosis, organ damage and mortality. Severe hemorrhage, which often occurs with traumatic injuries, causes whole body hypoxia, metabolic perturbations and systemic inflammatory response [1–4]. Patients who survive the initial traumatic insult remain susceptible to multiple organ failure and death [5,6]. Tissue damage occurs in many organs, including liver, intestine, lungs, heart and spleen depending on the severity of hemorrhagic injury (HI), and these alterations persist for a prolonged period of time despite fluid resuscitation [7–9]. HI in the human as well as in animal models triggers a systemic inflammatory response and decreased splenic function [8,10]. Studies from other laboratories demonstrated a declined phagocytic function for splenic macrophage, reduced splenic blood flow and declined lymphokine release following HI [11–13]. Previous studies demonstrated alterations in a number of genes related to mitochondria and glucose oxidation following HI in animal models [14,15]. Mitochondrial functional preservation is critical in energy homeostasis following HI.

Hypoxia may be implicated as one of the causes for the increased tissue and systemic inflammatory responses seen in traumatic injuries [16,17]. Furthermore, the decreased oxygen availability in tissues result in declined OXPHOS activity and ATP production. To further confirm the dysregulation of mitochondrial metabolism in HI, our laboratory and others have demonstrated that agents which potentiate mitochondrial function can improve organ function and survival following HI in experimental models of hemorrhagic shock [18–20]. However, the understanding of the role of mitochondria in HI is still evolving and the effect of HI on splenocyte mitochondrial respiration remains unknown. In this study we sought to determine the alteration of mitochondrial respiration in splenocytes, and to further investigate the changes to molecular mediators of stress and inflammation following hemorrhagic shock in a rat model.

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2. Materials and methods

2.1. Animals and hemorrhagic injury procedures

Male Sprague Dawley (Charles River Laboratory Wilmington, MA, USA) rats were used. The animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Augusta University. The animals were subjected to sham or hemorrhagic injury (HI) as described before [18]. The animals were anesthetized with 2.5% isoflurane, a midline laparotomy was performed, and the incision closed to induce soft tissue trauma. Both femoral arteries and one femoral vein were cannulated (PE-50 tubing) and one artery was connected to a blood pressure analyzer (Digi-Med; Micro-Med Inc., Louisville, KY, USA) while hemorrhage was performed through the other artery. The resuscitation fluid was administered through the femoral vein. All surgical sites were bathed with bupivacaine. Sham animals were not subjected to bleeding or resuscitation. The animals in the HI groups were bled for 45 min, maintaining the low MAP of 40 ± 5, until 60% of circulatory blood volume was withdrawn. The animals were maintained at this low pressure for another 45 min. The HI animals were divided into two groups; one group was resuscitated with Ringer's lactate for 1 h while the other group was not resuscitated. The animals that were resuscitated were observed for 2 h, euthanized and tissues collected. The animals in the group that were not resuscitated were euthanized when their mean arterial pressure (MAP) dropped below 30 mm Hg.

2.2. Isolation of splenocytes

A part of each animal's spleen was harvested and submerged in RPMI 1640 (Thermo Scientific, Chicago, IL). Red blood cells were lysed using lysis buffer (Thermo Scientific, Chicago, IL) and vigorous trituration. Remaining splenocytes were centrifuged and resuspended in RPMI 1640 supplemented with 10% FBS.

2.3. Mitochondrial respiration

The Seahorse XFp Analyzer (Seahorse Biosciences, North Billerica, MA) was used according to the manufacturer's protocol to measure oxygen consumption rate (OCR) of the isolated rat splenocytes. Splenocytes were plated in XFp base medium, minimal DMEM (Seahorse Biosciences, North Billerica, MA) supplemented with 1 mM pyruvate, 2 mM glutamine, 10 mM glucose (Sigma) at a density of 3×10^5 cells per well in specialized XFp miniplates pretreated with Cell Tak (Fisher). Plates were spun and then incubated for 30 min at 37 °C prior to loading into the Seahorse analyzer. Three baseline OCR measurements were taken for each well in the first 35 min, and then the following mitochondrial inhibitors were sequentially injected: oligomycin (1 μM), carbonycyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) (0.3 μM), antimycin A and rotenone (1 μM). Three OCR values were automatically calculated after each injection by the Seahorse XFp software. Data were obtained as the mean value ± SEM for each time point in pmol per minute (n = 3 replicates per treatment group). For each experimental Sham and HI animal pair, OCR values of animals in HI group were normalized to the sham.

2.4. TMRE staining

Both sham and HI splenocytes were stained with 1 μM TMRE for 30 min alongside a baseline control first treated with 20 μM FCCP for 10 min. Fluorescence was read at Ex/Em = 549/575, and TMRE fluorescence was normalized to the sham values for each experiment.

2.5. Western blot analysis

The spleen tissue was homogenized in Pierce RIPA lysis buffer (Thermo Scientific, Chicago, IL) containing 25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA). Protein samples were resolved on SDS polyacrylamide gel, transferred to PVDF membrane, blocked using 5% (w/v) nonfat dried milk or 5% BSA in Tris-buffered saline containing 25 mmol/L Tris-HCl (pH 7. 4), 0.13 mol/L NaCl, 0.0027 mol/L KCl and 0.1% Tween 20 for 1 h at room temperature (RT) and then incubated with respective antibodies overnight at 4 °C or for 1 h at RT. The membranes were probed with antibodies to GAPDH, VDAC, HMGB1, heat shock protein 60 (hsp60) (Thermo Scientific). The membranes were washed and incubated with horseradish peroxidase conjugated secondary antibody to mouse IgG or rabbit IgG (Cell Signalling) followed by enhanced western lightning plus-ECL (PerkinElmer). The protein bands were detected by autoradiography and quantitated by densitometry using the ImageJ software (NIH, Rockville, MD).

2.6. Real-time polymerase chain reaction

Total RNA was isolated from spleen tissue using the TRIzol method as previously described [21]. 300 ng of total RNA isolated was reverse transcribed to cDNA using reverse transcription kit (Promega). Quantitative real-time PCR was performed using Agilent Technologies Stratagene Mx3000P real-time PCR machine. The primer sequences are provided in Supplementary Table 1. The thermal cycling conditions were 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 50 s. Results are expressed as a ratio of expression to beta-actin and normalized to the values obtained for samples in sham group.

2.7. Citrate synthase activity measurement

Citrate synthase activity was determined using a Citrate Synthase Activity Assay Kit (Sigma, St Louis, MO) according to the manufacturer's instructions. Briefly, 20 mg of spleen tissue was homogenized in 200uL citrate synthase assay buffer. The citrate synthase activity level was then determined using a coupled enzyme reaction, which results in a colorimetric (412 nm) product proportional to the enzymatic activity present. The enzyme activity values were then normalized to the total protein content of the spleen homogenate and was further normalized to sham values.

2.8. Statistics

Multi-group comparisons were carried out and significance determined by one-way ANOVA followed by Tukey's test using GraphPad software (Graphpad Prism, WA). Two-group comparisons for significance were done by Mann–Whitney nonparametric test using GraphPad software. A p value < 0.05 is considered significant.

3. Results

3.1. Hemorrhagic injury and fluid resuscitation

A sub-group of rats were subjected to HI and following a hypotensive period, Ringer's lactate was administered for fluid resuscitation. The fluid resuscitation resulted in a significant increase in MAP (Fig. 1A). Consistent with the blood loss (Fig. 1B) and contributed to by the consequential hypoxia, plasma lactate levels were significantly elevated at 2 h following the end of resuscitation (Fig. 1C). However, in another sub-group, when animals were not resuscitated by the fluid, none of them survived > 1 h after the shock period (Fig. 1D).

3.2. Mitochondrial oxygen consumption and ATP production following HI

In order to determine mitochondrial functional integrity following HI, we tested the OCR of splenocytes isolated from rats subjected to HI