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Protein markers related to vascular responsiveness after hemorrhagic shock in rats

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ABSTRACT

Background: Vascular hyporesponsiveness is an important pathophysiological feature of some critical conditions such as hemorrhagic shock. Many proteins and molecules are involved in the regulation of the pathologic process, however the mechanism has still remained unclear. Our study was intended to look for the related protein markers involved in the regulation of vascular reactivity after hemorrhagic shock.

Methods: Differential in-gel electrophoresis and tandem mass spectrometry were applied to quantify the differences of protein expression in the superior mesenteric arteries from hemorrhagic shock and normal rats.

Results: A total of 2317 differentially expressed protein spots in the superior mesenteric arteries of rats before and after hemorrhagic shock were found, and 146 protein spots were selected for tandem mass spectrometry identification. Thirty-seven differentially expressed proteins were obtained, including 3 uncharacterized proteins and 34 known proteins. Among them, heat shock protein beta-1 and calmodulin were the known proteins involved in the occurrence of vascular hyporesponsiveness. Bioinformatics analysis results showed that 18 proteins were related to vasoconstriction, 11 proteins may be involved in other vascular functions such as regulation of angiogenesis and endothelial cell proliferation.

Conclusions: The changes of vascular responsiveness after hemorrhagic shock in rats may be associated with the upregulation or downregulation of previously mentioned protein expressions. These findings may provide the basis for understanding and further study of the mechanism and treatment targets of vascular hyporeactivity after shock.

1. Introduction

Hemorrhagic shock is the leading cause of death in traumatic injuries [1]. Vascular hyporesponsiveness is one of the most common complications of serious conditions such as trauma and hemorrhagic shock. Reduced vascular reactivity plays an important role in the development and outcome of shock. Restoring vascular reactivity may help stabilize hemodynamics and improve organ perfusion [2]. Previous studies indicated that the vascular hyporesponsiveness after hemorrhagic shock is related to many proteins such as protein kinase C (PKC) and Rho kinase [2–5]. Although previous studies raised some

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signaling pathways for vascular hyporeactivity, those studies mainly depended on traditional pharmacology and pathophysiological methods, which are severely limited [4,6–14].

DIGE proteomics uses differential in-gel electrophoresis (DIGE) to analyze differential expression proteins between control and target samples. It is aimed to improve the reproducibility of protein analysis, reduce the inter-gel variability, and facilitate the spot identification and matching, thus increasing the number of analyzable spots [15–17]. This method may lead to the identification of vascular hyporesponsiveness-specific protein markers and provide a basis for developing new methods for early diagnosis and treatment of vascular hyporesponsiveness. In this study, we applied the DIGE proteomics technique to identify vascular responsiveness-related protein markers so as to further investigate the mechanisms of shock-induced vascular hyporeactivity and provide new treatment targets for vascular dysfunction after severe trauma and hemorrhagic shock.

2. Materials and methods

For detailed materials and methods, see Supplemental materials and methods.

2.1. Animal management and sample preparation

This study was approved by the Research Council and Animal Care and Use Committee of Research Institute of Surgery, Daping Hospital, the Third Military Medical University. All experiments were conformed to the guidelines of ethical use of animals (eighth edition 2011, Washington, DC, National Academy, USA). Sixty Sprague–Dawley rats weighing 200–250 g were randomly divided into six groups as follows: normal control with (A1) or without endothelium (B1), 30 min shock with (A2) or without endothelium (B2), and 3 h shock with (A3) or without endothelium group (B3). Thirty minutes and 3 h shock selected were based on our previous findings that these two times are the vascular hyperresponsiveness and hyporesponsiveness time after shock [18–20]. The shock model we used was constructed with a fixed mean arterial blood pressure in combination with a fixed maintaining time, the mean arterial blood pressure was reduced to 40 mm Hg and was maintained at this level for 30 min or 3 h by withdrawal or infusion of the shed blood (The detailed method in Supplemental materials and methods). After completion of the shock model, a laparotomy was performed, and superior mesenteric arteries (SMAs) were obtained from the shocked rats. After removal of endothelium in B1, B2, and B3 groups, SMAs were immediately stored in liquid nitrogen for subsequent protein extraction and proteomics research.

2.2. Differential in-gel electrophoresis

Samples were minimally labeled with Cy3 or Cy5 fluorescent dyes (50 μg of protein per 400 pmol of dye, GE Healthcare, Pittsburgh, PA) for 30 min at 4°C based on the manufacturer's instructions. To minimize the system and inherent biological variation, half of the samples from each group were labeled with Cy3 and the other half of the samples were labeled with Cy5. An internal standard was prepared by mixing equal amounts of all samples analyzed and was labeled with Cy2 fluorescent dye (GE Healthcare). Sample multiplexing was also randomized to produce unbiased results (Table).

Immobilized pH gradient (IPG) strips (24 cm, pH3-l0 NL; GE Healthcare) were loaded with 50 μg of each Cy2-, Cy3-, and Cy5-labeled sample in a buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% DTT (GE Healthcare), and 1% (v/v) IPG buffer (pH3-l0 NL; GE Healthcare). First-dimension isoelectric focusing (IEF) was carried out in a Protean IEF cell (Bio-Rad, Hercules, CA) at 70 kV/h. Second-dimension separation was performed on 12% gel (24 cm × 20 cm), which was cast in low fluorescence glass plates on an Ettan DALT VI system (GE Healthcare).

After electrophoresis, fluorescence images of the gels were acquired on a Typhoon 9410 Variable Mode Imager (GE Healthcare). Cy2, Cy3, and Cy5 images for each gel were scanned at 488/520-, 532/580-, and 633/670-nm excitation and/or emission wavelengths, respectively, at 100 dpi resolution, thus obtaining a total of 27 images (9 × 3). After imaging the three fluorophores for each gel, the images were imported to the DeCyder version 6.5 image analysis software (GE Healthcare) for spot detection, spot matching (intragel), and determination of statistically significant biological variation (intergel), 1.5-fold difference in abundance, with a confidence of P values <0.05 considered significant.

2.3. In-gel digestion and tandem mass spectrometry protein identification

Tandem mass spectrometry (matrix assisted laser desorption ionization-time of flight-time of flight mass spectrometry) can identify proteins through the ratio of specific mass and charge of peptide (M/Z). Preparative IEF was performed with increased protein amount loaded into IPG strips for spot picking. Each gel was loaded with 1250 μg protein from each sample. IEF and equilibration of gel strip and second dimension sodium dodecyl sulfate polyacrylamide gel Electropheresis were performed as the same as described previously, except that the last phase of the IEF was carried out at 120 kV/h. After sodium dodecyl sulfate polyacrylamide gel Electropheresis, the gel was stained with Colloidal Coomassie Blue G-250. Significant spots were excised and in-gel digested with trypsin (Sigma-Aldrich, Saint Louis, MO) at 37°C overnight. After concentration and

Table – Two-dimensional DIGE experimental design*.

Gel number	Cy2 (10 μL)	Cy3 (10 μL)	Cy5 (10 μL)
Gel 1	Pooled standard	A1	A3
Gel 2	Pooled standard	B1	A1
Gel 3	Pooled standard	A1	B2
Gel 4	Pooled standard	A3	A2
Gel 5	Pooled standard	B3	B1
Gel 6	Pooled standard	A2	B2
Gel 7	Pooled standard	A3	B3
Gel 8	Pooled standard	B2	B1
Gel 9	Pooled standard	B3	A2

*Each gel was loaded with 50 μg of the Cy2-labeled protein pool from all samples as an internal standard, 50 μg of Cy3-labeled sample, and 50 μg of Cy5-labeled sample as indicated.