Attenuation of Lung Inflammation and Pro-Inflammatory Cytokine Production by Resveratrol following Trauma-Hemorrhage

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Abstract

Although studies have demonstrated that resveratrol administration following adverse circulatory conditions is known to be protective, the mechanism by which resveratrol produces the salutary effects remains unknown. We hypothesized that resveratrol administration in males following trauma-hemorrhage decreases cytokine production and protects against lung injury. Male Sprague-Dawley rats underwent trauma-hemorrhage (mean blood pressure 40 mmHg for 90 min, then resuscitation). A single dose of resveratrol (30 mg/kg of body weight) or vehicle was administered intravenously during resuscitation. Twenty-four hours thereafter, tissue myeloperoxidase activity (a marker of neutrophil sequestration), cytokine-induced neutrophil chemoattractant (CINC)-1, CINC-3, intercellular adhesion molecule (ICAM)-1, and interleukin (IL)-6 levels in the lung and protein concentrations in bronchoalveolar lavage fluid were measured (n = 6 rats/group). One-way ANOVA and Tukey’s test were used for statistical analysis. Trauma-hemorrhage increased lung myeloperoxidase activity, CINC-1, CINC-3, ICAM-1, and IL-6 levels and protein concentrations in bronchoalveolar lavage fluid. These parameters were significantly improved in the resveratrol-treated rats subjected to trauma-hemorrhage. The salutary effects of resveratrol administration on attenuation of lung injury following trauma-hemorrhage are likely due to reduction of pro-inflammatory mediators.

Key Words: hemorrhagic shock, chemokine, adhesion molecule, cytokine

Introduction

A large numbers of studies have demonstrated that the enhanced secretion of pro-inflammatory cytokines by mast cells, dendritic cells, and macrophages is an important factor in the initiation and perpetuation of inflammation in different tissues (16). These cytokines recruit other immune cells including neutrophils, thereby increasing leukocyte trafficking and lung injury (22, 26, 28). Neutrophils can release superoxide anions and proteolytic enzymes, which diffuse across the endothelium and injure parenchymal cells, or alternatively, neutrophils can leave the microcirculation and migrate to and adhere to matrix proteins or other cells (4, 12). Intercellular adhesion molecule (ICAM)-1 is known to play a major role in the firm adhesion of neutrophils to the vascular endothelium. ICAM-1 is constitutively present on the surface of endothelial cells, and is markedly up-regulated following trauma-hemorrhagic shock (3). In addition to adhesion...
molecules, chemokines such as cytokine-induced neutrophil chemoattractant (CINC)-1, and CINC-3 are also potent chemotactic factors for neutrophils (11).

Hemorrhagic shock results in excessive production of pro-inflammatory mediators, such as cytokines and chemokines, which plays a significant role in the development of multiple organ dysfunctions under those conditions (25). Studies have shown that neutrophils are activated following hemorrhagic shock (26) and that lung injury is associated with an increased neutrophil accumulation in the lung after hemorrhagic shock (25). The activated neutrophils appear to infiltrate the injured lung in parallel with increased expression of adhesion molecules on endothelial cells and elevated local chemokines/cytokines levels following hemorrhagic shock (26). Furthermore, trauma-hemorrhagic shock increases ICAM-1 level in the lung (26, 28). Moreover, the levels of the chemokines, CINC-1 and CINC-3, are elevated in the lung after trauma-hemorrhage (26, 28). Interleukin (IL)-6 also appears to be an essential component of the inflammatory cascade that is associated with lung injury in hemorrhagic shock (24). Moreover, IL-6-deficient mice showed less neutrophil infiltration and organ damage, as compared with wild-type mice under those conditions (17).

Resveratrol (trans-3,4',5-trihydroxystilbene), an extract of red wine, has been shown to be protective following shock-like states in males (1, 19). Previous studies have shown that resveratrol can reduce neutrophil and cytokine production in vivo in a rodent model of lipopolysaccharide-induced airway inflammation (2). Neutrophils play an important role in lung inflammation in low-flow states (26, 28). In addition, lung is the critical organ for inflammation following trauma-hemorrhagic shock (26, 28). Therefore, we hypothesized that resveratrol administration following trauma-hemorrhage attenuates lung injury and cytokine production. To test the hypothesis, we examined the effects of resveratrol treatment on lung injury by measuring lung chemokine and cytokine production following trauma-hemorrhage.

Materials and Methods

The current study was approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital. All animal experiments were performed according to the guidelines of the Animal Welfare Act and The Guide for Care and Use of Laboratory Animals from the National Institutes of Health.

Trauma-Hemorrhage Procedure

A non-heparinized rat model of trauma-hemorrhage was used in this study (27). Briefly, male Sprague-Dawley rats (275-325 g) obtained from the National Science Council were housed in an air-conditioned room under a reversed light-dark cycle and allowed 1 week or more to adapt to the environment. Before experiment, they were fasted overnight but were allowed water ad libitum. The rats were anesthetized by isoflurane (Attane, Minrad Inc., Bethlehem, PA, USA) inhalation prior to the induction of soft tissue trauma via 5-cm midline laparotomy. The abdomen was closed in layers, and catheters were placed in both femoral arteries and the right femoral vein (polyethylene [PE-50] tubing; Becton Dickinson & Co., Sparks, MD, USA). The wounds were bathed with 1% lidocaine (Elkins-Sinn Inc., Cherry Hill, NJ, USA) throughout the surgical procedure to reduce postoperative pain. Rats were then allowed to awaken, and bled to and maintained at a mean blood pressure of 40 mmHg. This level of hypotension was continued until the animals could not maintain mean blood pressure of 40 mmHg unless additional fluid in the form of Ringer’s lactate was administered. This time was defined as maximum bleed-out, and the amount of withdrawn blood was noted. Following this, the rats were maintained at mean blood pressure of 40 mmHg until 40% of the maximum bleed-out volume was returned in the form of Ringer’s lactate. The animals were then resuscitated with four times the volume of the shed blood over 60 min with Ringer’s lactate. The time required for maximum bleed out was 45 min, the volume of maximum bleed out was 60% of the calculated circulating blood volume (23), and the total hemorrhage time was 90 min. Thirty min before the end of the resuscitation period, the rats received resveratrol (30 mg/kg, intravenously) (2) or an equal volume of the vehicle (~0.2 ml, 10% DMSO, Sigma, St. Louis, MO, USA). The catheters were then removed, the vessels ligated, and the skin incisions closed with sutures. Sham-operated rats underwent the surgical procedure, which included a laparotomy in addition to the ligation of the femoral artery and vein, but neither hemorrhage nor resuscitation was carried out. The rats were then returned to their cages and were allowed food and water ad libitum. The animals were sacrificed at 24 h after the end of resuscitation.

Preparation of Lung Tissue and Collection of Bronchoalveolar Lavage Fluid (BALF)

At 24 h after the completion of fluid resuscitation or sham-operation, the animals were anesthetized with isoflurane and then sacrificed. The chest was opened and the left side of lung was obtained after clamping the hilum. Excess blood was blotted and the left upper lobe of the lung was stored at -80°C until analyzed. The trachea was then cannulated and bronchoalveolar lavage fluid (BALF) was carried out...
with 5 ml of phosphate buffered saline (37°C). The BALF was centrifuged at 1,200 x g at 4°C for 7 min. The supernatant was collected and stored at -80°C until analyzed (25).

**Measurement of Myeloperoxidase (MPO) Activity**

MPO activity in homogenates of the whole lung was determined as described previously (25, 26). All reagents were purchased from Sigma (St. Louis, MO, USA). Briefly, equal weights (100 mg wet weight) of lung from various groups were suspended in 1 ml buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0) and sonicated at 30 cycles, twice, for 30 sec on ice. Homogenates were cleared by centrifuging at 2,000 x g at 4°C, and the supernatants were stored at -80°C. Protein content in the samples was determined using the Bio-Rad (Hercules, CA, USA) assay kit. The samples were incubated with a substrate o-dianisidine hydrochloride. This reaction was carried out in a 96-well plate by adding 290 µl 50 mM phosphate buffer, 3 µl substrate solution (containing 20 mg/ml o-dianisidine hydrochloride), and 3 µl H2O2 (20 mM). A sample (10 µl) was added to each well to start the reaction. Standard MPO (Sigma, St. Louis, MO, USA) was used in parallel to determine MPO activity in the sample. The reaction was stopped by adding 3 µl sodium azide (30%). Light absorbance at 460 nm was read. MPO activity was determined by using the curve obtained from the standard MPO.

**Determination of CINC-1, CINC-3, ICAM-1, and IL-6 Levels**

Lung CINC-1, CINC-3, ICAM-1, and IL-6 levels were determined using ELISA kits (R&D, Minneapolis, MN, USA) according to the manufacturer’s instructions and as described previously (25). Briefly, the samples were homogenized in PBS (1 : 10 weight : volume) (pH 7.4) containing protease inhibitors (Complete Protease Inhibitor Cocktail, Boehringer Mannheim, Germany). The homogenates were centrifuged at 2,000 x g for 20 min at 4°C and the supernatant was assayed for CINC-1, CINC-3, and ICAM-1 levels. An aliquot of the supernatant was used to determine protein concentration (Bio-Rad Dc Protein Assay, Bio-Rad, Hercules, CA, USA).

**Protein Assay in Lung Lavage**

Cell-free BAL fluid was evaluated for total protein content (Bio-Rad Dc Protein Assay, Bio-Rad, Hercules, CA, USA) (25).

**Statistical Analysis**

Results are presented as means ± SEM (n = 6 rats/group). The data were analyzed using one-way analysis of variance and Tukey’s test, and differences were considered significant at P < 0.05.

**Results**

Alteration in BALF Total Protein Content In sham-operated rats was found. No significant differences in BALF total protein content was found between vehicle- and resveratrol-treated groups (Fig. 1, A and B). Trauma-hemorrhage significantly increased total protein content in BALF. Resveratrol treatment attenuated the trauma-hemorrhage-induced increase in BALF total protein content; however, the levels remained higher than shams.

Alteration in Lung MPO Activity

Lung MPO activity in sham-operated or trauma-hemorrhaged rats with and without resveratrol treatment is shown in Fig. 2. In sham-operated rats, resveratrol did not alter lung MPO activity. Trauma-hemorrhage resulted in a significant increase in lung MPO activity in vehicle-treated rats. Furthermore, resveratrol treatment attenuated the increase in lung MPO activity.

Alteration in Lung IL-6 Levels

As shown in Fig. 3, lung IL-6 levels were not influenced by resveratrol administration in sham rats, as compared with shams receiving vehicle (Fig. 3). Trauma-hemorrhage significantly increased lung IL-6 levels, as compared with sham rats. Resveratrol
administration following trauma-hemorrhage, however, significantly reduced the elevated lung IL-6 levels.

**Alteration in Lung CINC-1, CINC-3, and ICAM-1 Expressions**

Trauma-hemorrhage significantly increased CINC-1 and CINC-3 expressions in the lung (Fig. 4, A and B). However, treatment with resveratrol prevented the trauma-hemorrhage-induced increase in CINC-1 and CINC-3 expressions. In addition, lung ICAM-1 levels increased significantly in vehicle-treated rats following trauma-hemorrhage (Fig. 4C). Resveratrol administration following trauma-hemorrhage prevented the increase in lung ICAM-1 levels.

**Discussion**

The lung is considered to be a critical organ in the development of the delayed organ dysfunction in patients suffering from traumatic injuries and severe blood loss (28). Multiple organ failure or dysfunction secondary to a systemic inflammatory response remains the major cause of mortality and morbidity (24). Neutrophils are the principal cells involved in host defense against acute bacterial and fungal infections (14) and thus these cells have a protective effect. However, under conditions such as those described in this study, the infiltration of these cells may cause tissue damage (26). Neutrophils movement and migration are mediated by multiple adhesion molecules on the neutrophils and endothelial cell surfaces and chemotactic factors. Initially, neutrophils interact with
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endothelial selectins, resulting in neutrophil rolling along the endothelial surface. This rolling process appears to allow neutrophil to become activated ("primed") by chemokines and other mediators secreted by the endothelium, resulting in their firm adhesion to endothelial adhesion molecules via the β1-integrins (9) and β2-integrins (21). Among adhesion molecules, ICAM-1 is an important mediator in the firm adhesion of neutrophils to the vascular endothelium and is strongly upregulated following trauma-hemorrhagic shock (3). With regard to chemokines, rat CINC-1 and CINC-3 are members of the IL-8 family, and are potent chemotactic factors for neutrophils (11). Chemotaxis of neutrophils is an important functional response to chemokines and is a key event in the recruitment of neutrophils in inflammation. Previous studies have showed that antibodies to CINCs reduced the influx of neutrophils and their activation in rat models of IgG immune complex-induced lung injury (20) and lipopolysaccharide-induced inflammation (10). Our previous studies also indicate that CINC-1 and CINC-3 levels correlated with tissue MPO activity, a marker of neutrophil content, following trauma-hemorrhage (26, 28).

Resveratrol is a naturally occurring plant antibiotic known as phytoalexins, found in many plants, nuts, and fruits, and is abundant in grapes and red wine (1). There is now considerable evidence demonstrating a role for resveratrol in mediating the production of pro-inflammatory cytokines (8, 29). The release of various cytokines from macrophages and lymphocytes, such as IL-6 (28), INFγ, IL-2, TNF-α, and IL-12 (8), has been shown to be inhibited by resveratrol. Ferrero et al. (6) has reported that resveratrol reduced granulocyte and monocyte adhesion to endothelial cells. Other groups have published on the inhibitory effect of resveratrol on the expression of the adhesion molecule molecules ICAM-1, VCAM-1 (5), and E-selection (18) on endothelial cells. A common link between the inhibitory effects of resveratrol mentioned above could be its ability to inhibit factors involved in gene transcription like MAPK, c-JNK, AP-1, and NF-kB (15). The cytokines IL-1, IL-6, and TNF-α are important early mediators in the lung during trauma-hemorrhage (25), and are required for expression of adhesion molecules and chemokines (13). The ability of resveratrol to modulate expression of inflammatory cytokines as well as adhesion molecules and chemokines suggests a role for resveratrol in the regulation of lung inflammation. Although we did not perform histology in this study, recent findings from our colleagues suggest that both MPO activity and histology follow a similar trend at 4 h after trauma-hemorrhage (7). However, it remains unknown whether similar changes occur in histology in the lung at 24 h after trauma-hemorrhage and resuscitation. The present study is the first to examine the protective effects of resveratrol in the lung following trauma-hemorrhage and to indicate that resveratrol administration following trauma-hemorrhage decreased CINC-1, CINC-3, and ICAM-1 levels. The dose for administration of resveratrol in the present study is obtained from a previous study (2). In addition, salutary effects of resveratrol in attenuation of lung injury after trauma-hemorrhage was lesser when resveratrol was administered in lesser dosages (i.e., 3 or 10 mg/kg) and similar when resveratrol was administered in dosage of 5 or 10 times (data not shown).

In conclusion, our study indicates that resveratrol administration ameliorates lung injury and IL-6 production following trauma-hemorrhage. The improvement in lung injury following resveratrol administration is likely due to a reduction of lung neutrophil accumulation associated with down-regulation of CINC-1, CINC-3, and ICAM-1 following trauma-hemorrhage. Furthermore, the suppression in lung cytokine production by resveratrol appears to contribute to the decrease in lung expressions of chemokine and adhesion molecule. Since resveratrol administration following trauma-hemorrhage decreased lung injury and cytokine production, this agent appears to be a novel adjunct for improving the depressed lung function in male animals following adverse circulatory conditions.

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References