Journal of Surgical Research **176,** 171–177 (2012)
doi:10.1016/j.jss.2011.05.039

Role of Akt-Dependent Pathway in Resveratrol-Mediated Cardioprotection after Trauma-Hemorrhage

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Originally submitted February 8, 2011; accepted for publication May 24, 2011

Background. **Resveratrol has been shown to have protective effects for patients in shock-like states, and Akt (protein kinase B) is known to play a role in pro-inflammatory events in response to injury. The aim of this study is to determine whether resveratrol provides cardioprotection mediated *via* an Akt-dependent pathway in trauma-hemorrhaged animals.**

Methods. **Male Sprague-Dawley rats underwent trauma-hemorrhage and resuscitation. A single dose of resveratrol (30 mg/kg body weight) with or without a PI3K inhibitor (wortmannin) or vehicle was administered intravenously during the resuscitation. Two hours after either the trauma-hemorrhage or sham operation, the cardiac output, the positive maximal pressure increase of the left ventricle ($+dP/dt_{max}$), and the negative maximal pressure decrease of the left ventricle ($-dP/dt_{max}$) were measured. Cardiac myeloperoxidase (MPO) activity, interleukin (IL)-6, and intercellular adhesion molecule (ICAM)-1 levels, Akt activity, and apoptosis were measured. One-way ANOVA and Tukey's test were used for statistical analysis.**

Results. **Cardiac output and $\pm dP/dt_{max}$ decreased significantly after trauma-hemorrhage. Administration of resveratrol significantly improved these cardiac function parameters. Trauma-hemorrhage increased cardiac MPO activity, IL-6 levels, and ICAM-1 levels, and these parameters were significantly improved in the resveratrol-treated rats subjected to trauma-hemorrhage. Although trauma-hemorrhage decreased cardiac Akt phosphorylation (p-Akt), resveratrol treatment following trauma-hemorrhage prevented the same decrease in cardiac p-Akt. The increase in cardiac apoptosis was attenuated in rats that received resveratrol. Co-administration of wortmannin prevented the beneficial effects of resveratrol on the attenuation of pro-inflammatory responses and cardiac injury after trauma-hemorrhage.**

Conclusion. **Resveratrol attenuates cardiac injury following trauma-hemorrhage, which is, at least in part, due to its anti-inflammatory effects *via* Akt-dependent pathways.**

Key Words: **resveratrol; trauma-hemorrhage; protein kinase B (Akt); pro-inflammatory mediators.**

INTRODUCTION

Trauma-hemorrhage results in the excessive production of pro-inflammatory mediators that play a significant role in the development of multiple organ dysfunctions in those conditions [1]. Studies have shown that neutrophils are activated following trauma-hemorrhage and that cardiac injury is associated with an increased neutrophil accumulation in the heart [2–4]. Neutrophils can release superoxide anions and proteolytic enzymes that diffuse across the endothelium and injure parenchymal cells; alternatively, neutrophils can exit the microcirculation, migrate, and adhere to matrix proteins or other cells [5, 6]. Intercellular adhesion molecule (ICAM)-1 is known to play a major role in the firm adhesion of neutrophils to the vascular endothelium and is markedly up-regulated following trauma-hemorrhagic shock [7]. Furthermore, there is convincing evidence that interleukin (IL)-6 plays a significant role in organ injury and is required for the expression of adhesion molecules [8, 9].

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0022-4804/$36.00

Resveratrol has been shown to have cardioprotective effects during ischemia reperfusion [10], and our studies have shown that resveratrol can attenuate hepatic injury after trauma-hemorrhage [9]. The PI3K/Akt pathway is known to be an endogenous negative feedback or compensatory mechanism, which serves to limit proinflammatory and chemotactic events in response to injury [11–13]. Inhibition of the PI3K/Akt pathway with the PI3K inhibitor wortmannin increases serum cytokine levels and decreases the survival of mice subjected to sepsis [12, 14]. In addition, the PI3K/Akt pathway is known to play a pivotal role in the ability of neutrophils to undergo chemotaxis [15, 16]. Furthermore, the involvement of PI3K in cell migration is supported by the ability of selective PI3K inhibitors, such as wortmannin, to mitigate neutrophil chemotaxis [16]. Studies have also shown that activation of the PI3K pathway protects organs or cells against ischemia-reperfusion injury and hypoxia through suppression of the apoptosis machinery [17]. Resveratrol has been shown to be protective in male rats after experimental induction of shock-like states [9, 18]. Nevertheless, it remains unclear whether Akt plays a role in resveratrol-mediated cardioprotection following trauma-hemorrhage. We hypothesized that the beneficial effects of resveratrol following trauma-hemorrhage are mediated by an Akt-related pathway. To test this hypothesis, we treated animals with resveratrol alone and in combination with the PI3K inhibitor wortmannin after trauma-hemorrhage. The effects of these treatments were then examined with respect to cardiac function as well as cardiac MPO activity, IL-6, ICAM-1, phospho (p)-Akt/Akt levels, and apoptosis following trauma-hemorrhage.

MATERIALS AND METHODS

Animals

This 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.

Rat Trauma-Hemorrhage and Resuscitation Model

A non-heparinized rat model for trauma-hemorrhage and resuscitation was used in this study [19]. 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 were allowed 1 wk or more to adapt to the environment. Before the experiment, rats were fasted overnight but were allowed water *ad libitum*. The rats were anesthetized using isoflurane (Attane; Minrad Inc., Bethlehem, PA) inhalation prior to the induction of soft tissue trauma *via* a 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 and Co., Sparks, MD). The wounds were bathed with 1% lidocaine (Elkins-Sinn Inc., Cherry Hill, NJ) throughout the surgical procedure to reduce postoperative pain. Rats were then allowed to awaken, subjected to bleeding and maintained at a mean blood pressure of 40 mmHg. This level of hypotension was continued until the mean blood pressure could not be maintained without the use of additional Ringer's lactate replacement fluid. This time point was defined as the maximum bleed-out time, and the amount of blood withdrawn was noted. Next, the rats were maintained at a 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 Ringer's lactate with four times the volume of the shed blood over a period of 60 min. The time required for maximum hemorrhage was about 45 min, the volume of maximum hemorrhage was about 60% of the calculated circulating blood volume, and the total hemorrhage time was about 90 min [20]. Thirty minutes before the end of the resuscitation period, the rats received resveratrol (30 mg/kg, intravenously) [9], resveratrol plus the PI3K inhibitor wortmannin (1 mg/kg, intravenously at the beginning of resuscitation), wortmannin or an equal volume of the vehicle (~ 0.2 mL, 10% DMSO; Sigma, St. Louis, MO, USA) [8]. The catheters were then removed, the vessels ligated, and the skin incisions closed with sutures. Sham-operated animals underwent a surgical procedure that included a laparotomy in addition to ligation of the femoral artery and vein, but neither hemorrhage nor resuscitation was performed. The animals were then returned to their cages and were given food and water *ad libitum*. The animals were sacrificed 2 h after the end of the resuscitation or sham operation. There were eight rats in each group.

Measurement of Cardiac Output and *In Vivo* Heart Performance

Two hours after either the sham operation or trauma-hemorrhage and resuscitation, animals were re-anesthetized using sodium pentobarbital; cardiac output, maximal rate of left ventricular pressure increase ($+dP/dt_{max}$), and maximal rate of left ventricular pressure decrease ($-dP/dt_{max}$) were measured, as described previously [21].

Measurement of Myeloperoxidase (MPO) Activity

The cardiac MPO activity in homogenates of left ventricle tissue was determined as described previously [9, 22]. Frozen tissue samples were thawed and suspended in phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma). Samples were sonicated on ice, centrifuged at 12,000 *g* for 15 min at 4°C, and an aliquot was transferred to phosphate buffer (pH 6.0) containing 0.167 mg/mL o-dianisidine hydrochloride and 0.0005% hydrogen peroxide (Sigma). The change in absorbance at 460 nm was measured spectrophotometrically for 5 min. MPO activity was calculated using a standard curve generated using human MPO (Sigma), and values were normalized to protein concentration.

Measurement of IL-6 and ICAM-1 Levels

IL-6 and ICAM-1 levels in the heart were determined using ELISA kits (R&D, Minneapolis, MN) according to the manufacturer's instructions as described previously [22]. Briefly, the samples were homogenized in PBS (1:10 weight:volume; pH 7.4) containing protease inhibitors (Complete Protease Inhibitor Cocktail; Boehringer Mannheim, Germany). Homogenates were centrifuged at 2000 *g* for 20 min at 4°C, and the supernatant was assayed for IL-6 and ICAM-1 levels. An aliquot of the supernatant was used to determine protein concentration (Bio-Rad D_C Protein Assay; Bio-Rad, Hercules, CA).

Western Blot Assay

Rat heart tissues were homogenized in a buffer as described previously [23]. The homogenates were centrifuged at 12,000 *g* for 15 min at 4°C, analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were then transferred