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Experimental paper

Effect of valproic acid on acute lung injury in a rodent model of intestinal ischemia reperfusion☆

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ABSTRACT

Objectives: Acute lung injury (ALI) can develop during the course of many clinical conditions, and is associated with significant morbidity and mortality. Valproic acid (VPA), a well-known anti-epileptic drug, has been shown to have anti-oxidant and anti-inflammatory effects in various ischemia/reperfusion (I/R) models. The purpose of this study was to investigate whether VPA could affect survival and development of ALI in a rat model of intestinal I/R.

Methods: Two experiments were performed. *Experiment I*: Male Sprague-Dawley rats (250–300 g) were subjected to intestinal ischemia (1 h) and reperfusion (3 h). They were randomized into 2 groups ($n = 7$ per group) 30 min after ischemia: Vehicle (Veh) and VPA (300 mg/kg, IV). Primary end-point for this study was survival over 4 h from the start of ischemia. *Experiment II:* The histological and biochemical effects of VPA treatment on lungs were examined 3 h (1 h ischemia + 2 h reperfusion) after intestinal I/R injury (Veh vs. VPA, $n = 9$ per group). An objective histological score was used to grade the degree of ALI. Enzyme linked immunosorbent assay (ELISA) was performed to measure serum levels of interleukins (IL-6 and 10), and lung tissue of cytokine-induced neutrophil chemoattractant (CINC) and myeloperoxidase (MPO). In addition, the activity of 8-isoprostane was analyzed for pulmonary oxidative damage.

Results: In Experiment I, 4-h survival rate was significantly higher in VPA treated animals compared to Veh animals (71.4% vs. 14.3%, $p = 0.006$). In Experiment II, ALI was apparent in all of the Veh group animals. Treatment with VPA prevented the development of ALI, with a reduction in the histological score (3.4 ± 0.3 vs. 5.3 ± 0.6, $p = 0.025$). Moreover, compared to the Veh control group the animals from the VPA group displayed decreased serum levels of IL-6 (952 ± 213 pg/ml vs. 7709 ± 1990 pg/ml, $p = 0.011$), and lung tissue concentrations of CINC (1188 ± 28 pg/ml vs. 1298 ± 27 pg/ml, $p < 0.05$), MPO activity (368 ± 23 ng/ml vs. 490 ± 29 ng/ml, $p < 0.05$) and 8-isoprostane levels (1495 ± 221 pg/ml vs. 2191 ± 177 pg/ml, $p < 0.05$).

Conclusion: VPA treatment improves survival and attenuates ALI in a rat model of intestinal I/R injury, at least in part, through its anti-oxidant and anti-inflammatory effects.

1. Introduction

Intestinal ischemia and reperfusion (I/R) injury occurs in the setting of various clinical situations, such as necrotizing enterocolitis, midgut volvulus, intussusception, mesenteric ischemia, hemorrhagic and septic shock.[1] Intestinal I/R injury has been shown not only to cause local damage to the bowel but also to release numerous mediators in the circulation that can cause multiple organ failure including acute lung injury (ALI).[2] Among these mediators, reactive oxygen species (ROS) play a critical role in the development of ALI. Administration of anti-oxidants has been shown to decrease this injury in various models including hemorrhagic shock, intestine I/R, and sepsis.[3] Similarly, activated neutrophils in the circulation have been identified as important inducers of distant organ injury, especially ALI.[4] Cytokine-induced neutrophil chemoattractant (CINC) is a potent neutrophil chemotactic factor. An increase in CINC expression promotes neutrophils aggregation in the lung leading to severe inflammatory reaction and exuberant free radical generation, which eventually culminates in the development of ALI.[5,6]

Numerous strategies have been tried to attenuate neutrophil mediated inflammatory damage to the lung, without much

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success.[7–9] Valproic acid (VPA), used as anti-epileptic agent for decades, has recently been identified to have cell protective, anti-inflammatory, and anti-apoptotic properties after being tested in various ischemia reperfusion models.[10–14] However, these properties of VPA have not been evaluated in the setting of intestinal I/R injury. We hypothesized that VPA administration may mitigate the deleterious effects of intestinal I/R injury and improve survival by decreasing the post-inflammatory ALI.

In the present study, we investigated two possible effects of VPA in a rat model of intestinal I/R injury: (1) whether treatment with VPA improves short term survival; and (2) whether it can attenuate immune-mediated acute lung injury, as measured by alteration in pulmonary histology and tissue levels of CINC, MPO and 8-isoprostane, and circulating interleukin-6 levels.

2. Materials and methods

All the research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals. The study adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, and was approved by the Institutional Animal Care and Use Committee.

2.1. Animal preparation and operation

Male Sprague-Dawley rats weighing 250–300 g were housed in a controlled environment with free access to food and water before the experiment. They were anesthetized with ketamine/xylazine and subjected to a midline laparotomy and isolation of the superior mesenteric artery (SMA). A micro-bulldog clamp was applied across the proximal SMA (at the level of origin from aorta), and the abdominal incision was closed. Start of ischemia (application of the clamp) was taken as time 0 min. A 24 gauge catheter was inserted into the tail vein for drug or fluid infusion. All procedures were performed under a heating pad to maintain the body temperature of 36–38 °C that was measured with an indwelling rectal thermometer. Vascular clamp was removed to initiate reperfusion after 60 min of ischemia. Valproic acid (300 mg/kg) or vehicle (normal saline) were administrated over 5 min intravenously starting at 30 min after intestinal ischemia after randomization. A person who did not participate in the major procedure (GJ) prepared valproic acid or vehicle in random order which was given to the main operator who delivered it in a blinded fashion. Valproic acid (Calbiochem, San Diego, CA) was dissolved in distilled water and diluted in normal saline (total volume 2 ml/kg). Animals in the vehicle group were given identical volume of water and normal saline without the VPA.

2.2. Experimental design

This study included two experiments. In the first experiment, all animals underwent normothermic ischemia for 60 min as described, and 30 min after ischemia, they were randomized into 2 groups ($n = 7$ per group): (1) Vehicle (Veh), and (2) Valproic acid (VPA). They were followed until death, with 4-h survival from the start of ischemia (1 h ischemia + 3 h reperfusion) being the primary endpoint. In the second experiment, we evaluated the histological and biochemical changes in the lungs 3 h after insult (1 h ischemia = 2 h reperfusion) in the VPA and vehicle treated animals ($n = 9$ per group). This time point was selected to ensure adequate sample size in the control group (rapid decline in survival with a longer reperfusion period).

2.3. Tissue sample collection

At the end of the observation period, blood samples were withdrawn for the measurement of circulating cytokines. The left lung was rapidly removed and cut into small pieces, and was snap frozen in liquid nitrogen and stored at −80 °C. A rapid tracheal infusion method for routine lung fixation was used to preserve the right lung for histological evaluation as previously described.[15]

2.4. Acute lung injury (ALI) scoring

The ALI scoring was performed by a board certified pathologist (KBL) blinded to the treatment assignment of the samples. The method for objective quantification of the injury has been previously described.[16] In brief, ALI was classified into 4 categories based on the severity of alveolar congestion and hemorrhage, infiltration of neutrophils in the air spaces or vessel walls, and the thickness of alveolar wall/hyaline membrane formation. The severity of each category was graded from 0 (minimal) to 4 (maximal) and the total score was calculated by adding the scores in each of these categories. In each animal, 4 separate lung sections were graded to generate the mean score.

2.5. Cytokine measurements

Serum concentrations of IL-6 and IL-10 were determined with commercially available enzyme linked immunosorbent assay (ELISA) kits (R&D Systems Inc., Minneapolis, MN). The concentration of cytokine was measured by optical densitometry at 450 nm in a SpectramaxPlus 384 microplate reader (Molecular Devices, Sunnyvale, CA). All of the analyzes were performed in triplicates.

2.6. Lung cytokine-induced neutrophil chemoattractant (CINC)

CINC was quantified in homogenized lung tissue using commercially available ELISA kit (R&D Systems Inc., Minneapolis, MN). Briefly, the samples were homogenized in 0.5 ml of lysis buffer containing 50 mmol/l HEPES, 10 mmol/l sodium pyrophosphate, 1.5 mmol/l $MgCl_2$, 1 mmol/l EDTA, 0.2 mmol/l sodium orthovanadate, 0.15 M NaCl, 0.1 M sodium fluoride, 10% glycerol, 0.5% TritonX-100, and protease inhibitor cocktail. The homogenates were centrifuged at 1500 g for 15 min at 4 °C, and the supernatant was assayed for CINC levels. CINC concentrations were quantified in 50 μl of lung tissue supernatant according to the manufacture's instructions by measuring optical densitometry values at 450 nm in a SpectramaxPlus 384 microplate reader (Molecular Devices, Sunnyvale, CA).

2.7. Myeloperoxidase activity (MPO)

MPO activity in lung tissue was determined using the Myeloperoxidase Assay Kit (Cell Sciences Inc., Canton, MA) according to the manufacturer's instructions. In brief, lung tissue (50 mg) was homogenized by sonication with 1 ml of lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycine, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptide, 28 μg/ml aprotinin). The samples were centrifuged three times at 1500 × *g* at 4 °C for 15 min, and supernatants were analyzed for MPO levels reading at 450 nm.

2.8. 8-Isoprostane

Tissue level of 8-isoprostane was used as a marker of lipid peroxidation. The concentration of 8-isoprostanes was measured using