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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) is developed in many clinical situations and 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 hour) and reperfusion (3 hours). They were randomized into 2 groups (n=7/group) 30 min after ischemia: Vehicle (Veh) and VPA (300 mg/kg, IV). Primary end-point for this study was survival over 4 hours from the start of ischemia.

Experiment II: The histological and biochemical effects of VPA treatment on lungs were examined 3 hours (1 hr ischemia + 2 hrs reperfusion) after intestinal I/R injury (Veh vs. VPA, n = 9/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 cytokine 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, four-hour 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 vs. 7709 ± 1990 pg/ml, $p = 0.011$), and lung tissue concentrations of CINC (1188

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Conflict of Interest Statement

None of the authors have any conflicts of interest to declare

± 28 vs. 1298 ± 27 , $p < 0.05$), MPO activity (368 ± 23 vs. 490 ± 29 , $p < 0.05$) and 8-isoprostane levels (1495 ± 221 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.

Keywords

valproic acid; acute lung injury; ischemia reperfusion; inflammation; oxidative damage; intestine

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 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) can it 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.

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.

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 minutes. 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 ° that was measured with an indwelling rectal thermometer. Vascular clamp was removed to initiate reperfusion after 60 minutes of ischemia. Valproic acid (300 mg/kg) or vehicle (normal saline) were administered 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 2ml/kg). Animals in the vehicle group were given identical volume of water and normal saline without the VPA.

Experimental Design

This study included two experiments. In the first experiment, all animals underwent normothermic ischemia for 60 minutes as described, and 30 minutes after ischemia, they were randomized into 2 groups (n=7 per group): 1) Vehicle (Veh); 2) Valproic acid (VPA). They were followed until death, with 4-hours survival from the start of ischemia (1 hour ischemia+ 3 hours reperfusion) being the primary endpoint. In the second experiment, we evaluated the histological and biochemical changes in the lungs 3 hours after insult (1 hour ischemia+ 2 hours 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).

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].

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.

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 analyses were performed in triplicates.

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 NaF, 10% glycerol, 0.5% TritonX-100, and protease inhibitor cocktail. The homogenates were centrifuged at 1,500 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).

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 (50mg) was homogenized by sonication with 1 ml of a lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycine, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{mL}$ leupeptide, 28 $\mu\text{g}/\text{mL}$ aprotinin). The samples were centrifuged three times at 1,500 $\times$ g at 4°C for 15 min, and supernatants were analyzed for MPO levels reading at 450 nm.

8-Isoprostane

Tissue level of 8-isoprostane was used as a marker of lipid peroxidation. The concentration of 8-isoprostanes was measured using an 8-isoprostane enzyme immunoassay (EIA) kit (Cayman Chemical Co., Ann Arbor, MI, USA) as per the manufacturer's instructions. Briefly, plates were precoated with mouse monoclonal antibody and blocked with a proprietary formulation of proteins. After a wash, 50 μl of standard/tissue sample was added to the corresponding wells and incubated for 18 h at 4°C. The wells were washed five times with wash buffer. Ellman's reagent (200 μl) was added to each well and kept in the dark for 120 min for color development. The plates were read at 420 nm and the blank was subtracted. The concentration of 8-isoprostanes was calculated from a standard curve plotted with standard samples.

Data Analysis

Survival rates were compared by Kaplan-Meier log-rank test. Continuous data were summarized as means and standard deviation. Student's t-test was used to compare the differences between two groups. Mann-Whitney test was used for non-parametric data, and *p* value of less than 0.05 was considered to be statistically significant. Data were analyzed using SPSS for windows (version 15.0, SPSS Inc., Chicago, IL).

RESULTS

Survival Study

As shown in Fig 1, following intestinal I/R injury, all rats in the Veh control group died in less than 4 h (213 ± 48 min). However, VPA treated animals displayed significantly longer survival time (277 ± 72 min). The 4-hour survival rate (1 hr ischemia+ 3 hr reperfusion) in the VPA group was significantly higher, compared to the control.

Acute Lung Injury Study

As shown in Table 1 and Figure 2, the intestinal I/R increased the lung histological score (5.3 ± 0.6 ; Figure 2A and 2B). This injury was significantly attenuated by VPA treatment (3.4 ± 0.3 , *p* = 0.025 vs. the control group).

Lung cytokine-induced neutrophil chemoattractant—We have recently found that CINC could be an early biomarker for hemorrhagic shock induced acute lung injury (Fukudome et al., manuscript submitted to Surgery). To determine whether CINC can be

found in lungs after intestinal I/R injury, and whether VPA treatment can affect any possible alteration of CINC protein, we measured pulmonary CINC levels in control and VPA groups. As shown in Figure 3A, CINC levels in VPA group (1188 ± 28 pg/ml) were significantly ($p = 0.012$) lower than the control group (1298 ± 27 pg/ml).

Myeloperoxidase activity—MPO activity in lung homogenate was measured as a marker of neutrophil recruitment and damage to lung tissues. As shown in Figure 3B, the MPO was significantly lower in the VPA group than control group (490 ± 29 vs. 368 ± 23 ng/ml; $p=0.005$), which suggests that VPA decreases neutrophil migration into the lungs after the intestinal I/R injury.

8-isoprostane and IL-6—It has been reported that 8-isoprostane is a mediator of oxidant stress [17] and IL-6 is an important proinflammatory mediator for ALI [18]. Neutrophil lung recruitment/ infiltration has been correlated with levels of 8-isoprostane and IL-6 in a mouse model of pulmonary ischemia/reperfusion [19]. To find out whether protective effect of VPA on lungs are through this mechanism, we measured the levels of 8-isoprostane in lung and IL-6 in blood. In this experiment, we observed that intestinal ischemia induced tissue levels of 8-isoprostane in lung (2091 ± 177 pg/ml) and IL-6 in serum (7709 ± 1990). However, VPA treatment significantly decreased 8-isoprostane (1496 ± 221 pg/ml, $p = 0.030$) and IL-6 (952 ± 213 , $p = 0.011$). These results indicate that intestinal I/R can induce 8-isoprostane activity and IL-6 production, and treatment with VPA can inhibit these alterations (Figure 4).

Interleukine-10 (IL-10)—To determine effect of VPA on production of IL-10 (anti-inflammatory cytokine), we examined levels of IL-10 in blood. As shown in the upper panel of Figure 5, VPA treatment significantly decreased IL-10 production compared to the control group (81 ± 127 vs. 267 ± 162 pg/ml, respectively). Although VPA treatment decreased the levels of pro-inflammatory (IL-6) as well as the anti-inflammatory (IL-10), cytokines, this was more pronounced for IL-6. Thus, the ratio of IL-6 to IL-10 was significantly decreased in VPA group compared to the control group (lower panel of Figure 5), suggesting that the overall effect was anti-inflammatory.

DISCUSSION

In this study, we have demonstrated that VPA treatment improves survival in a rat model of intestinal I/R. We have also identified some of the underlying mechanisms for this protective effect: amelioration of ALI, inhibition of CINC, MPO and 8-isoprostane in lung, and reduction of IL-6 in serum. To the best of our knowledge, this is the first study that evaluates the effect of VPA on survival and distant organ damage (acute lung injury) after intestinal I/R.

It has been proposed that intestinal I/R is a critical triggering event in the development of distant organ dysfunction. The ischemia and reperfusion of intestines evokes rapid response including recruitment of neutrophils and release of proinflammatory cytokines. Lung is particularly susceptible to the damaging effects of increased neutrophils and cytokine activation following intestinal I/R [2, 20]. CINC is a rat chemokine known to be involved in neutrophil chemotaxis, and binds to CXCR2 receptor [21, 22]. CINC belongs to the IL-8 family for chemokines, and is homologous to the mouse keratinocyte-derived chemokine (KC), and to the human growth related oncogene alpha (GRO α) protein [21, 22]. Recently, we have discovered that CINC is detectable in the serum rapidly after global ischemia caused by massive blood loss in a rat model of hemorrhagic shock (Fukudome et al., manuscript submitted to Surgery). Because of the association between intestinal I/R and ALI and the central role of neutrophils, we decided to analyze the lung CINC in the present

study. Our data confirm that pulmonary CINC expression is elevated following intestinal I/R. The rise in lung CINC appeared to parallel a rise in lung MPO, which is a marker of pulmonary neutrophil infiltration and inflammation. Infiltration neutrophils into tissue is commonly assessed by changes in the activity of MPO, an enzyme restricted mainly to polymorphonuclear neutrophils [23]. Furthermore, this study investigated the effect of VPA on 8-isoprostane, anti-inflammatory cytokine IL-10, and proinflammatory cytokine IL-6. 8-isoprostane is a prostaglandin-like compound formed in vivo from the free radical-catalyzed peroxidation of arachidonate independent of the cyclooxygenase. In addition to being an important marker of oxidant stress, a number of reports have shown that 8-isoprostane also possesses potent biological activity and likely mediates certain aspects of oxidative injury [17]. IL-6 is one of potent proinflammatory cytokines and exerts toxic action on target cells in ischemia/reperfusion [24]. Serum levels of IL-6 are typically elevated in intestinal I/R models, and reflect increased mortality [25]. In this study, the lung tissue CINC concentration, MPO activity, and 8-isoprostane expression were increased in the Veh control group, in parallel with the serum levels of IL-6. However, a significant reduction in these proteins was observed in VPA treatment group. Surprisingly, IL-10, the anti-inflammatory cytokine was also decreased in this group. The extent of decrease, however, was much less, and even though we could not be sure of the meaning of IL-6/IL-10 ratio, it might suggest that the balance was in favor of an anti-inflammatory state [33]. Overall, our results demonstrated that VPA attenuates ALI by decreasing activity of CINC, MPO and 8-isoprostane in lung and levels of IL-6 in blood. The ability of VPA to modulate these proteins/enzymes may thus be a major mechanism responsible for the beneficial effects of the drug.

VPA, a well-known anti-epileptic drug, has been identified as a histone deacetylase inhibitor (HDACI) that regulates key cellular mechanisms by rapidly increasing the acetylation of nuclear and non-nuclear proteins [14, 26]. In stroke model, VPA meliorates neurological outcomes [10, 27]. In hemorrhagic shock models, we have recently demonstrated that VPA treatment improves survival and modulates various critical pathways (e.g. Akt survival pathway members, heat shock proteins, early/immediate kinases) through acetylation [13, 28]. These results suggested that the benefits of VPA might be mediated by anti-inflammatory effects of this HDACI through protein acetylation. In addition to the acetylation of proteins, anti-inflammatory effects of VPA might be due to a decrease in oxidative damage. The anti-oxidant property of VPA has been previously described in neuronal cells [29–31]. Treatment with VPA inhibits excitatory amino acid glutamate- and oxidant FeCl_3 -induced oxidative damage to lipid and protein in primary cultured rat cerebral cortical cells [30]. Also, VPA prevents oxidative damage induced by inhibition of mitochondrial complex I activity in SH-SY5Y cells [32].

Our study has some limitations. Firstly, to avoid excessive instrumentation we did not perform hemodynamic monitoring. Thus we could not identify the effects of VPA on hemodynamics. However, we have monitored the parameters of hemorrhagic shock in small and large animals in the previous studies, where the survival advantages in VPA treated animals are not due to better restoration of hemodynamic status but to direct activation of pro-survival pathways [13]. Therefore, we do not think that better preservation of blood pressure was the main reason for the improved survival and decreased ALI. Secondly, although the 4-hour survival rate was higher in VPA group compared to control group, none of the animals survived long-term due to intestinal gangrene (data not shown). However, we did not administer antibiotics nor performed life saving operations (e.g. resection of necrotic intestine), which are routinely done in clinical practice. The impressive finding is that despite lethal necrosis of the intestines animals survived for a period of time (4 h), which is clinically meaningful. Theoretically, this time is long enough to perform a life saving operation (or transfer to a different facility) for definitive treatment. Thus, pharmacological

treatment with HDACI could serve as a bridge to surgery, and potentially attenuate morbidity and mortality. Finally, for logistical reasons we could not measure many other cytokines and regulatory pathways that could have been involved. We are currently exploring a number of these pathways using chromatin immunoprecipitation, genomic DNA microarray, and high throughput proteomic techniques.

In summary, we have revealed for the first time that VPA treatment improves survival and attenuates acute lung injury in a rat model of intestinal I/R injury by decreasing neutrophil trapping, creating an anti-inflammatory environment and minimizing oxidative damage.

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Figure 1. Effect of VPA on 4 hour survival of animals after intestinal ischemia

Male Sprague-Dawley rats were subjected to intestinal ischemia and reperfusion. They were randomized into 2 groups ($n = 7/\text{group}$) 30 min after ischemia: Vehicle (Veh) control and VPA. Survival rates were recorded for ~4 hours. Kaplan-Meier curves were used for the comparison of survival rates between the Veh control and VPA groups. The symbol * indicates that a value significantly ($p < 0.05$) differs from the control group.

2A

2B

Figure 2. VPA treatment attenuated acute lung injury

Acute lung injury was scored as described in Materials and Methods and expressed as mean values $\pm$ SD ($n = 9$ per group). The symbol * indicates that a value significantly differs from control group ($p < 0.05$). Representative histological sections of a lung from animal groups of the vehicle control and VPA treatment. VPA group had less alveolar congestion, neutrophil infiltration, and alveolar wall thickness. Hematoxylin & eosin staining; original magnification, X40.

3A

3B

Figure 3. CINC and Myeloperoxidase (MPO) activity in lung (n=9 animals per group)

Levels of CINC and myeloperoxidase (MPO) were analyzed from lung tissues of rats treated with or without VPA after intestinal ischemia. Values represent the means $\pm$ SD (n=9). The symbol * indicates that a value significantly ($p < 0.05$) differs from the control group.

4A

4B

Figure 4. VPA decreased levels of 8-isoprostane in lungs and IL-6 in serum

The levels of 8-isoprostane were analyzed in lung tissue of rats treated with or without VPA. Values represent means $\pm$ SD (n=9). The symbol * indicates that a value significantly ($p < 0.05$) differs from the control group.

5A

5B

Figure 5. Effect of VPA on serum levels of IL-6, IL-10 and ratio of IL-6 to IL-10

The levels of serum IL-6 and IL-10 were measured in rats treated with or without VPA after ischemia. The ratio of IL-6 to IL-10 was calculated, and values represent the means $\pm$ SD (n=9). The symbol * indicates that a value significantly ($p < 0.05$) differs from control group.

Table 1

Acute lung injury scores

Median (min-max)	Control	VPA	P value
Alveolar congestion	3 (2–4)	2 (1–3)	.031
Hemorrhage	1 (0–2)	1 (0–1)	.208
Infiltration of neutrophil	1 (0–2)	1 (0–1)	.176
Thickness of alveolar wall/hyaline membrane	0 (0–1)	0 (0–0)	.304
Total scores	5 (3–8)	3 (2–5)	.025