Effects of the Kv7 voltage-activated potassium channel inhibitor linopirdine in rat models of hemorrhagic shock

Sean P. Nassoïy¹, Favin S. Babu¹, Heather M. LaPorte¹, Kenneth L. Byron², and Matthias Majetschak^{1,2,3,*}

¹Burn and Shock Trauma Research Institute, Department of Surgery, Loyola University Chicago, Stritch School of Medicine

²Department of Molecular Pharmacology and Therapeutics, Loyola University Chicago, Stritch School of Medicine

Abstract

Recently, we demonstrated that Kv7 voltage-activated potassium channel inhibitors reduce fluid resuscitation requirements in short-term rat models of hemorrhagic shock. The aim of the present study was to further delineate the therapeutic potential and side effect profile of the Kv7 channel blocker linopirdine in various rat models of severe hemorrhagic shock over clinically relevant time periods. Intravenous administration of linopirdine, either before (1 or 3 mg/kg) or after (3 mg/kg) a 40% blood volume hemorrhage, did not affect blood pressure and survival in lethal hemorrhage models without fluid resuscitation. A single bolus of linopirdine (3 mg/kg) at the beginning of fluid resuscitation after hemorrhagic shock transiently reduced early fluid requirements in spontaneously breathing animals that were resuscitated for 3.5 hours. When mechanically ventilated rats were resuscitated after hemorrhagic shock with normal saline (NS) or with linopirdine-supplemented (10, 25 or 50 µg/mL) NS for 4.5 hours, linopirdine significantly and dose-dependently reduced fluid requirements by 14%, 45% and 55%, respectively. Lung and colon wet/dry weight ratios were reduced with linopirdine (25/50 µg/mL). There was no evidence for toxicity or adverse effects based on measurements of routine laboratory parameters and inflammation markers in plasma and tissue homogenates. Our findings support the concept that linopirdine-supplementation of resuscitation fluids is a safe and effective approach to reduce fluid requirements and tissue edema formation during resuscitation from hemorrhagic shock.

Keywords

Linopirdine; Kv7 voltage-activated potassium channels; endothelial permeability; fluid shifts; fluid resuscitation; hemodynamics

*Corresponding author: Matthias Majetschak, M.D., Ph.D., Burn & Shock Trauma Research Institute, Loyola University Chicago, Stritch School of Medicine, 2160 S. 1st Avenue, Maywood, IL 60153, Phone: (708) 327-2472, Fax: (708) 327- 2813, mmajetschak@gmail.com.

³contact address after May 1st, 2018: Department of Surgery, University of South Florida Morsani College of Medicine. DR. MATTHIAS MAJETSCHAK (Orcid ID : 0000-0002-4086-0887)

Introduction

Injuries account for 9% of the world's deaths [1]. In the United States trauma is the leading cause of death up to the age of 45 years and the 5th leading cause of death in the overall population [2]. Hemorrhagic shock is the major cause of potentially preventable death after accidental injuries [3]. While fluid resuscitation is an essential component in the treatment of patients with traumatic-hemorrhagic shock, large volume fluid resuscitation is associated with development of tissue edema and clinical signs of fluid overload, such as development of acute respiratory distress syndrome, abdominal compartment syndrome or hyperchloremic metabolic acidosis, which significantly increases mortality and morbidity of critically ill trauma patients [4–9]. Although evidence suggests that the early use of vasopressors for hemodynamic support in patients with hemorrhagic shock might be deleterious [10, 11], pressure-support resuscitation of hemorrhagic shock with arginine vasopressin has been discussed as a possible strategy to improve outcomes [12–14]. Furthermore, the European guidelines on management of major bleeding and coagulopathy following trauma recommend a restricted volume replacement strategy to achieve target blood pressure until bleeding can be controlled and administration of vasopressors in addition to fluids to maintain target arterial pressure in the presence of life-threatening hypotension [15]. The latter recommendation, however, was graded 1C, which is a strong recommendation with low-quality or very low-quality evidence [15]. Thus, new drugs that stabilize hemodynamics, reduce resuscitation fluid requirements and lack adverse effects that are associated with vasopressor treatment could have the potential to significantly improve outcomes in patients with traumatic-hemorrhagic shock. Recently, we provided initial evidence that inhibitors of Kv7 voltage activated potassium channels could fill this gap [16, 17]. Kv7 voltage-activated potassium channels are important regulators of the membrane potential in excitable cells, such as cardiac and vascular myocytes [18–21]. Kv7 channels function to stabilize resting membrane potential and suppress cell excitability [19]. Recent studies suggested that pharmacological treatments that suppress the activity of vascular Kv7 channels can promote vasoconstriction and increase peripheral vascular resistance [18]. We showed that intravenous administration of Kv7 channel inhibitors administered as single bolus injections or as supplements to crystalloid resuscitation fluids dose-dependently reduce fluid requirements to maintain hemodynamics in short-term models of fixed blood pressure hemorrhagic shock in rats [16, 17]. Because further characterization of the therapeutic potential and possible side-effect profile of Kv7 channel inhibition after hemorrhagic shock is required to enable transition of this new therapeutic approach into clinical practice, the aim of the present study was to perform a detailed efficacy testing of Kv7 channel inhibition in various preclinical models of hemorrhagic shock. Thus, in the present study, we tested the effects of the selective Kv7 channel inhibitor linopirdine utilizing well-characterized lethal and non-lethal hemorrhagic shock models in spontaneously breathing and mechanically ventilated rats over clinically relevant time periods. We selected linopirdine for these studies as it has previously undergone safety pharmacology testing in humans and has also been evaluated as a cognition-enhancing drug in clinical trials [22–24]. Although linopirdine failed to improve cognitive function in Alzheimer's disease, no adverse side effects, except elevated alanine transferase levels, were noted during oral administration for up to six months [22–24].

Results

Series 1: Linopirdine pre- and post-treatment in lethal hemorrhagic shock without fluid resuscitation

In the first set of experiments, linopirdine (1 and 3 mg/kg) or vehicle were administered 15 min before hemorrhage. There were no differences in MAP between groups at baseline (Fig. 1A). All animals tolerated 40% BV hemorrhage and blood pressures were indistinguishable between groups during the experimental procedure (Figs. 1A/B). Median survival times during additional 2% BV hemorrhages that were performed in 15 min intervals were 180 min, 206 min and 183 min in animals treated with vehicle, 1 mg/kg and 3 mg/kg linopirdine, respectively (Fig. 1C, $p > 0.05$).

In the second set of experiments, linopirdine (3 mg/kg) or vehicle were administered 15 min after the start of the hemorrhage period (Fig. 2). Baseline MAPs were comparable among groups. Although MAP was slightly higher in animals after linopirdine treatment, these differences did not reach statistical significance. Median survival times were 173 min for vehicle-treated animals and 188 min for animals treated with 3 mg/kg linopirdine (Fig. 2C, $p > 0.05$).

Series 2: Linopirdine treatment of spontaneously breathing rats during resuscitation from hemorrhagic shock

Next, we tested the effects of a single i.v. dose of 3 mg/kg linopirdine at the beginning of fluid resuscitation from 30 min of hemorrhagic shock in spontaneously breathing animals. Physiological parameters were comparable between the vehicle- and linopirdine-treatment groups at baseline (Fig. 3). There were no differences in hemorrhage volumes to achieve the MAP target of 25 mmHg during the shock period (Figs. 3A/B). All animals could be resuscitated with normal saline to a resuscitation MAP target of 70 mmHg (Fig. 3B) and hematocrit values were comparable between the groups (Fig. 3C). In all animals lactate plasma concentrations increased to 9–10 mmol/L at the end of the shock period and normalized with fluid resuscitation (Fig. 3D). $\text{PaO}_2/\text{FiO}_2$ (P/F) ratios and serum creatinine concentrations were indistinguishable between vehicle-treated and linopirdine-treated animals throughout the resuscitation period. With linopirdine treatment, however, fluid requirements to maintain MAP at 70 mmHg were significantly reduced during the early resuscitation period and reached fluid requirements of vehicle-treated animals at the end of the resuscitation period (Fig. 3G).

Series 3: Linopirdine treatment of mechanically ventilated rats during resuscitation from hemorrhagic shock

As in series 2, physiological parameters were comparable between the vehicle- and linopirdine-treatment groups at baseline (Fig. 4). While the hemorrhage volumes to achieve the MAP target of 40 mmHg during the shock period were comparable in animals resuscitated with normal saline (NS, 47.2 ± 1.5 % TBV hemorrhage) and NS supplemented with 25 $\mu\text{g/mL}$ (47.7 ± 1.8 % TBV hemorrhage) and 50 $\mu\text{g/mL}$ (47.2 ± 1.7 % TBV hemorrhage) linopirdine, animals that were subsequently resuscitated with NS supplemented with 10 $\mu\text{g/mL}$ linopirdine reached the MAP target of 40 mmHg with lower hemorrhage

volumes ($41.5 \pm 1.3\%$ TBV hemorrhage, $p < 0.05$ vs. all other groups, Fig. 4A/B). All animals could be resuscitated to the resuscitation MAP target of 60 mmHg until the end of the experiment. Hematocrit values, lactate levels and P:F ratios were indistinguishable between all groups throughout the experiments. At the end of the resuscitation period, serum creatinine concentrations were significantly higher in animals that were resuscitated with NS supplemented with 25 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ linopirdine, as compared with animals that were resuscitated with NS alone and NS supplemented with 10 $\mu\text{g/mL}$ linopirdine (serum creatinine (mg/dL): NS alone – 1.14 ± 0.13 ; NS supplemented with linopirdine 10 $\mu\text{g/mL}$ – 1.18 ± 0.15 ; 25 $\mu\text{g/mL}$ – 1.66 ± 1.63 ($p < 0.05$ vs. NS alone); 50 $\mu\text{g/mL}$ – 1.60 ± 0.24 ($p < 0.05$ vs. NS alone)).

While differences in fluid requirements between animals resuscitated with NS alone and animals resuscitated with NS supplemented with 10 $\mu\text{g/mL}$ linopirdine were not significantly different when the entire observation period was analyzed, fluid requirements were significantly reduced from $t = 105$ min until $t = 300$ min in animals resuscitated with NS supplemented with 25 $\mu\text{g/mL}$, and from $t = 70$ min until $t = 300$ min in animals resuscitated with NS supplemented with 50 $\mu\text{g/mL}$ linopirdine (Fig. 4G). As compared with animals resuscitated with NS alone, cumulative fluid requirements were reduced by $14 \pm 7\%$, $45 \pm 6\%$ and $55 \pm 3\%$ when resuscitation was performed with NS supplemented with 10 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ linopirdine, respectively. Furthermore, initial fluid requirements ($t = 30 - 90$ min) to achieve the resuscitation MAP target after the shock period were significantly reduced with all three doses of linopirdine (mL/kg: normal saline alone – 119 ± 21 ; 10 $\mu\text{g/mL}$ – 80 ± 8 ; 25 $\mu\text{g/mL}$ – 69 ± 7 ; 50 $\mu\text{g/mL}$ – 56 ± 8 ; $p < 0.0001$ vs. NS alone for all). From 90 – 240 min, additional fluid requirements remained constant at 0.7 ± 0.01 mL/kg min⁻¹ in animals resuscitated with NS alone and at 0.47 ± 0.01 and 0.37 ± 0.01 mL/kg min⁻¹ with NS supplemented with 25 and 50 $\mu\text{g/mL}$ linopirdine, respectively ($p < 0.001$ vs. NS alone). During the last hour of resuscitation ($t = 240 - 300$ min) fluid requirements increased 2.9-fold to 1.95 ± 0.06 mL/kg min⁻¹ in animals resuscitated with NS alone, 1.76-fold to 0.83 ± 0.03 mL/kg min⁻¹ in animals resuscitated with 25 $\mu\text{g/mL}$ linopirdine and 1.8-fold to 0.69 ± 0.01 mL/kg min⁻¹ in animals resuscitated with 50 $\mu\text{g/mL}$ linopirdine ($p < 0.05$ vs. NS alone for both). Fluid requirements in animals resuscitated with NS supplemented with 10 $\mu\text{g/mL}$ linopirdine were comparable with animals treated with higher doses of linopirdine from $t = 90 - 180$ min, and thereafter increased to resuscitation fluid requirements of animals treated with NS alone.

We then compared tissue wet-dry weight ratios, routine laboratory parameters and plasma and tissue concentrations of inflammation markers between groups. Due to a technical failure, we lost the tissue and plasma samples from one animal in the 50 $\mu\text{g/mL}$ linopirdine group. Because all physiological parameters in animals resuscitated with 25 and 50 $\mu\text{g/mL}$ linopirdine were indistinguishable at all time points ($p > 0.05$ for at all time points) and all other measured parameters were comparable, we compared animals resuscitated with NS alone, animals resuscitated with NS supplemented with 10 $\mu\text{g/mL}$ linopirdine and animals resuscitated with NS supplemented with 25/50 $\mu\text{g/mL}$ linopirdine.

The wet-dry weight ratios of lung, skeletal muscle, small intestine and colon are shown in Fig. 5. While the comparison of lung wet-dry weight ratios by ANOVA indicated significant

differences among groups, post-hoc testing did not reach a level of statistical significance for the comparison of animals treated with NS saline alone and animals treated with NS supplemented with 25/50 µg/mL linopirdine (Fig. 5A). Nevertheless, lung wet-dry weight ratios were significantly lower with NS supplemented with 25/50 µg/mL linopirdine, as compared with NS supplemented with 10 µg/mL linopirdine ($p < 0.05$), and post-hoc testing for linear trend among groups ($p < 0.05$) confirmed a trend towards reduced wet-dry weight ratios in lungs from animals resuscitated with NS supplemented with 25/50 µg/mL linopirdine. While there were no significant differences between groups for skeletal muscle (Fig. 5B, post-test for linear trend: $p > 0.05$) and small intestine (Fig. 5C, post-test for linear trend: $p > 0.05$) wet-dry weight ratios, resuscitation with NS supplemented with 25/50 µg/mL linopirdine significantly reduced colon wet-dry weight ratios (Fig. 5D, post-test for linear trend: $p < 0.05$).

Tab. 1 shows the analyses of various routine laboratory parameters and inflammation markers in plasma at the end of the experiments ($t = 300$ min). Except for total plasma protein concentrations, which were significantly higher in animals resuscitated with 25/50 µg/mL linopirdine, there were no statistically significant differences between any of the other parameters among the groups. Similarly, measurements of inflammation markers and superoxide dismutase activities, a key anti-oxidative enzyme, in tissue extracts did not reveal significant differences among groups (Tab. 2). Gross necropsies did not show signs of ischemia or any other abnormalities after linopirdine treatment. Furthermore, none of the animals in series 1–3 showed evidence of neurological effects of linopirdine, such as seizures, during the experiments.

Discussion

In the present study, we further characterized the effects of Kv7 channel blockade with linopirdine in various rat models of hemorrhagic shock. There are several new findings from the present study. First, a single bolus injection of linopirdine treatment does not improve blood pressure and survival time during lethal hemorrhagic shock in the absence of fluid resuscitation. Second, the fluid-sparing effects of a single bolus injection of linopirdine at the beginning of crystalloid resuscitation after hemorrhagic shock are short-lived (< 2 hours). Third, supplementation of crystalloid resuscitation fluids with linopirdine dose-dependently reduces fluid requirements and third-spacing of fluid over clinically relevant time periods without noticeable adverse effects and toxicity.

While the plasma half-life of linopirdine is 0.4 – 3.2 hr after oral administration in humans, a half-life of 0.6 hr has previously been determined after intravenous injection in rats [23, 25]. Thus, the observation that linopirdine treatment 15 min before hemorrhage did not affect blood pressure and survival time in a lethal hemorrhagic shock model without fluid resuscitation could be explained by the removal of large proportions of the drug during the subsequent hemorrhage period. The inefficacy of linopirdine to increase blood pressure or survival time when administered after 40% TBV hemorrhage without subsequent fluid resuscitation, however, indicates that it does not enhance shock tolerance and lacks relevant vasopressor actions. The latter is consistent with the previous observations that systemic vasopressor effects of linopirdine in normal rats are minimal [17, 26].

We have shown previously that a single bolus injection of 3 mg/kg of linopirdine prior to crystalloid resuscitation after hemorrhagic shock significantly reduces fluid resuscitation requirements over the next 45 min; higher doses of linopirdine did not improve its fluid-sparing effects [17]. The findings from the present study confirm these observations and now demonstrate that the fluid-sparing effects of linopirdine wear off within three plasma half-lives. In combination with our previous observation that supplementation of resuscitation fluids with linopirdine also dose-dependently reduces fluid requirements and the cumulative dose of drug administered during short-term experiments [17], these data point towards continuous infusion of linopirdine or supplementation of resuscitation fluids with linopirdine as an optimized resuscitation protocol for longer time periods.

To address the therapeutic potential of linopirdine-supplemented resuscitation fluids in clinically more relevant scenarios, we then resuscitated mechanically ventilated animals for 4.5 hours. In these experiments, a higher dose of isoflurane was required to prevent spontaneous breathing, which has been reported to impair cardiac contractility, systemic vascular resistance and blood pressure in rats [27–30]. Consistent with these hemodynamic consequences of isoflurane, we observed in pilot experiments that mechanically ventilated animals could not survive the hemorrhage and resuscitation protocols that we used in spontaneously breathing animals. Accordingly, we adjusted the MAP targets for mechanically ventilated animals during the shock and resuscitation periods to 40 mmHg and 60 mmHg, respectively. Despite the reduced severity of hemorrhagic shock and the lower MAP target during resuscitation in mechanically ventilated rats, fluid requirements to achieve the resuscitation target with NS alone were more than three-fold higher than in spontaneously breathing animals, indicating significant impairment of hemodynamic stability. Under these conditions, supplementation of resuscitation fluids with linopirdine dose-dependently reduced cumulative fluid requirements over a 4.5 hour resuscitation period by half. The significantly increased fluid requirements during the last hour of resuscitation in animals treated with NS alone demonstrate that these animals were not stably resuscitated. As hematocrit values, however, remained constant from $t = 30$ –300 min, these data suggest progressive development of capillary leakage as the major contributing factor for increased resuscitation fluid requirements. While animals treated with NS supplemented with 25 and 50 $\mu\text{g/mL}$ of linopirdine also showed increased fluid requirements during the late resuscitation period, the magnitudes of these increases were significantly reduced, as compared with animals treated with NS alone. In combination with the observations that hematocrit values were indistinguishable between groups, that linopirdine treatment resulted in higher plasma protein concentrations and reduced wet-dry weight ratios in organs that are prone to fluid shifts during resuscitation from hemorrhagic shock, i.e. lung and large intestine, our findings suggest that linopirdine stabilizes endothelial permeability during the inflammatory response to hemorrhagic shock and resuscitation. Recent evidence suggests that Kv7 channels are expressed in the coronary endothelium and contribute to the paracrine regulation of vascular reactivity [31, 32]. Thus, our observations may point towards another role of endothelial Kv7 channels, the regulation of vascular endothelial barrier function during inflammation. The molecular mechanisms underlying these effects, however, remain to be determined.

While oral linopirdine treatment was found to be reasonably safe in humans [22–24], information on adverse effects after intravenous administration is currently not available. Consistent with previous reports on intravenous linopirdine administration in rodents [17, 33, 34], detailed physiological monitoring and measurements of numerous routine laboratory parameters of metabolism, organ function and inflammation in the present study did not provide evidence for acute toxicity associated with intravenous linopirdine after hemorrhagic shock. Linopirdine, however, has been reported to inhibit vasodilator responses in isolated renal arteries of rats [35–37], and another Kv7 channel blocker, XE991, was found to constrict the renal vasculature and to reduce renal blood flow *in vivo* in rats [38], raising the possibility that glomerular filtration might be reduced by linopirdine treatment. Thus, we cannot exclude that the increases in creatinine concentrations at the end of the experiments in animals resuscitated with 25 and 50 µg/mL linopirdine reflect impairment of renal function. Although these increases could also be explained by dilutional effects due to the large differences in fluid requirements between groups, possible adverse effects of linopirdine on renal function should be considered and closely monitored in future pre-clinical studies in other disease models.

Taken together, the findings of the present study support the concept that linopirdine-supplementation of resuscitation fluids is a safe and effective approach to reduce fluid requirements and tissue edema formation during resuscitation from hemorrhagic shock. Linopirdine has previously been described to antagonize kynurenine induced hypotension, a natural tryptophan metabolite implicated in the pathophysiology of sepsis [39], and to protect against myocardial ischemia-reperfusion injury [40]. Thus, it might be speculated that the efficacy profile of linopirdine extends beyond hemorrhagic hypovolemic shock. Considering that linopirdine has a short systemic half-life after intravenous injection and its fluid-sparing effects wear off within three plasma half-lives, linopirdine appears to be a drug that is easily controllable if adverse effects would occur. Our findings justify further efficacy testing of linopirdine in other preclinical fluid resuscitation models, such as large animal models or other models of hemorrhagic shock, septic shock or ischemia-reperfusion injury. If successful, such data on the repurposing of linopirdine as a resuscitation fluid supplement could quickly lead to the transition towards clinical trials.

Methods

All procedures were performed according to National Institutes of Health Guidelines for Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Loyola University Chicago. Male Sprague-Dawley rats (300 – 350 g) were purchased from Harlan (Indianapolis, IN, USA). All doses of linopirdine were selected based on our previous studies [17].

Series 1: Linopirdine pre- and post-treatment in lethal hemorrhagic shock without fluid resuscitation

The lethal hemorrhagic shock model was as described in detail previously, with slight modifications [41, 42]. Rats were anesthetized with 1.5% isoflurane/100% oxygen administered by nose-cone inhalation. At this dose, rats did not respond to noxious stimuli,

but were able to breathe spontaneously. The femoral artery was cannulated with a 24-gauge BD angiocath shielded IV catheter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) to allow for monitoring of arterial blood pressure, and blood withdrawal. The femoral vein was then cannulated with 1.5-french tubing for fluid and drug administration. Core body temperature was maintained at 37°C using warming lamps and a Far Infrared Warming Pad (Kent Scientific Corporation, Torrington, CT, USA). Hemodynamics were continuously monitored with the surgivet invasive blood pressure monitor (Med-Electronics, Beltsville, MD, USA) and blood pressures values were recorded every 5 min. Animals were then hemorrhaged 40% of their total blood volume (TBV) over a 10 minute period, followed by 2% TBV hemorrhage every 15 minutes until death. Death was defined as asystole or loss of a pulse pressure. In pre-treatment experiments, animals received 0.5 mL NS (n = 3, vehicle), 1 mg/kg linopirdine (n = 5) or 3 mg/kg linopirdine (n = 3) in 0.5 mL NS 15 minutes prior to the initiation of hemorrhage. In post-treatment experiments, animals received 0.5 mL NS (n = 3) or 3 mg/kg linopirdine in 0.5 mL NS (n = 4) 15 min after start of the hemorrhage. At the end of the experiments, animals were euthanized (isoflurane inhalation, bilateral pneumothorax).

Series 2: Linopirdine treatment of spontaneously breathing rats during resuscitation from hemorrhagic shock

We utilized a model of fixed blood pressure hemorrhage, as described in detail previously [17, 41]. Animals were anesthetized and instrumented as in Series 1. Animals were hemorrhaged to a mean arterial blood pressure (MAP) of 25 mmHg for 30 min. At the end of the 30 min shock period, 0.5 mL NS (n=6) or 3 mg/kg linopirdine (n=6) in 0.5 mL NS were injected as an intravenous bolus injection. This treatment was followed by resuscitation with NS to a MAP of 70 mmHg until t = 240 min. At the end of resuscitation period, rats were euthanized (isoflurane inhalation, bilateral pneumothorax).

Series 3: Linopirdine treatment of mechanically ventilated rats during resuscitation from hemorrhagic shock

Animals were oro-tracheally intubated with a 16-gauge EXEL disposable safelet angiocatheter (EXELINT International, Los Angeles, CA, USA) and mechanically ventilated with a SomnoSuite small animal anesthesia system (Kent Scientific Corporation, Torrington, CT, USA). Animals were ventilated with a positive end expiratory pressure (PEEP) of 2 mmHg, a fraction of inspired oxygen (FiO₂) of 1.0 and anesthetized with isoflurane at 2.5%. Tidal volumes were titrated to maintain normal PaCO₂ (35–45 mmHg). Animals were instrumented as in Series 2 and then hemorrhaged to a MAP of 40 mmHg for 30 min. Animals were then resuscitated with NS (n=5) or with NS supplemented with 10 µg/mL (n=3), 25 µg/mL (n=3), or 50 µg/mL (n=3) of linopirdine to a MAP of 60 mmHg until t=300 min. At the end of resuscitation, rats were euthanized (isoflurane inhalation, bilateral pneumothoraces). Gross necropsy was performed, and tissue from lung, small intestine (jejunum), colon and skeletal (pectoral) muscle were collected, snap-frozen in liquid nitrogen and stored at –80°C until further processing.

Arterial blood gases and routine laboratory parameters

Arterial blood gases and routine laboratory parameters were analyzed using the Element point of care veterinary blood gas, electrolyte and critical care analyzer and the DryChem 7000 veterinary chemistry analyzer (Cuattro Veterinary USA, Loveland, CO, USA).

Wet Weight to Dry Weight Ratios

The ratio of the tissue wet weight to dry weight was determined gravimetrically, as previously described [43, 44].

Tissue extract preparation—Snap frozen tissues were homogenized 1:5 weight/volume in a 1/10 phosphate buffered saline, 1% sodium orthovanadate, 0.5% phenylmethylsulfonyl fluoride and centrifuged at 16100 x g at 4°C for 30 min. The supernatants were aliquoted and stored at -80°C until further processing.

Superoxide dismutase (SOD) activity—SOD activity, an important antioxidant defense system in cells, was measured in tissue extracts utilizing the SOD Assay kit-WST (Sigma Aldrich) according to the manufacturer's recommendation and expressed as SOD/mg protein.

Enzyme linked immunosorbent assays (ELISA)—Concentrations of cytokines (tumor necrosis factor (TNF) α , interleukin (IL)6 and IL10) were measured in plasma and tissue extracts utilizing the DuoSet ELISA Development kits (R&D Systems) according to the manufacturer's recommendations. Tissue extract cytokine concentrations are expressed as pg/mg protein.

Protein quantification

Protein concentrations were measured with the Bio-Rad RC DC Protein Assay kit as per manufacturer's recommendations.

Data analyses and statistics

Data are described as mean $\pm$ standard error (SE). Data were analyzed with one-way analysis of variance (ANOVA) and Dunnett's multiple comparisons test or with two-way ANOVA and Bonferroni post-hoc tests to correct for multiple testing, as appropriate. Survival was plotted using the Kaplan-Meier method and survival between groups was compared with the log-rank test. Average fluid requirements over specific time periods were analyzed by linear regression analyses. A two-tailed $p < 0.05$ was considered significant. Data analyses were calculated with the GraphPad Prism program (GraphPad Software).

Acknowledgments

This research was supported by the National Institute of General Medical Sciences (Awards R01GM107495 and T32GM008750) of the National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Loyola University Chicago has filed a patent application for the use of Kv7 channel inhibitors for fluid resuscitation (United States Patent Application 20170304281). SPN, KLB and MM are the inventors.

References

1. World Health Organization (WHO). WHO Library Cataloguing-in-Publication Data. 2014. Injuries and violence: the facts 2014.
2. Heron M, Hoyert DL, Murphy SL, Xu J, Kochanek KD, Tejada-Vera B. Deaths: final data for 2006. *Natl Vital Stat Rep.* 2009; 57:1–134.
3. Kauvar DS, Lefering R, Wade CE. Impact of hemorrhage on trauma outcome: an overview of epidemiology, clinical presentations, and therapeutic considerations. *J Trauma.* 2006; 60(6 Suppl):S3–11. [PubMed: 16763478]
4. Smith JB, Pittet JF, Pierce A. Hypotensive Resuscitation. *Curr Anesthesiol Rep.* 2014; 4:209–215. [PubMed: 25294973]
5. Madigan MC, Kemp CD, Johnson JC, Cotton BA. Secondary abdominal compartment syndrome after severe extremity injury: are early, aggressive fluid resuscitation strategies to blame? *J Trauma.* 2008; 64(2):280–285. [PubMed: 18301187]
6. Bougle A, Harrois A, Duranteau J. Resuscitative strategies in traumatic hemorrhagic shock. *Ann Intensive Care.* 2013; 3:1. [PubMed: 23311726]
7. Malbrain ML, Marik PE, Witters I, Cordemans C, Kirkpatrick AW, Roberts DJ, Van Regenmortel N. Fluid overload, de-resuscitation, and outcomes in critically ill or injured patients: a systematic review with suggestions for clinical practice. *Anaesthesiol Intensive Ther.* 2014; 46:361–380. [PubMed: 25432556]
8. Handy JM, Soni N. Physiological effects of hyperchloraemia and acidosis. *Br J Anaesth.* 2008; 101:141–150. [PubMed: 18534973]
9. Gupta B, Garg N, Ramachandran R. Vasopressors: Do they have any role in hemorrhagic shock? *J Anaesthesiol Clin Pharmacol.* 2017; 33:3–8. [PubMed: 28413267]
10. Sperry JL, Minei JP, Frankel HL, West MA, Harbrecht BG, Moore EE, Maier RV, Nirula R. Early use of vasopressors after injury: caution before constriction. *J Trauma.* 2008; 64:9–14. [PubMed: 18188092]
11. Collier B, Dossett L, Mann M, Cotton B, Guillaumondegui O, Diaz J, Fleming S, May A, Morris J. Vasopressin use is associated with death in acute trauma patients with shock. *J Crit Care.* 2010; 25:173e9–14.
12. Cohn SM, Blackburn LH, Landry DW, Proctor KG, Walley KR, Wenzel V. San Antonio Vasopressin in Shock Symposium report. *Resuscitation.* 2010; 81:1473–1475. [PubMed: 20655650]
13. Anand T, Skinner R. Arginine vasopressin: The future of pressure-support resuscitation in hemorrhagic shock. *J Surg Res.* 2012; 178:321–329. [PubMed: 22480832]
14. Cossu AP, Mura P, De Giudici LM, Puddu D, Pasin L, Evangelista M, Xanthos T, Musu M, Finco G. Vasopressin in hemorrhagic shock: a systematic review and meta-analysis of randomized animal trials. *Biomed Res Int.* 2014; 2014:421291. [PubMed: 25254206]
15. Rossaint R, Bouillon B, Cerny V, Coats TJ, Duranteau J, Fernandez-Mondejar E, Filipescu D, Hunt BJ, Komadina R, Nardi G, et al. The European guideline on management of major bleeding and coagulopathy following trauma: fourth edition. *Crit Care.* 2016; 20:100. [PubMed: 27072503]
16. Majetschak M, Byron KL, Nassoiy SP. Methods of using potassium channel inhibitors (blockers) for fluid resuscitation. United States Patent Application. 20170304281. Publication date: October 26, 2017
17. Nassoiy SP, Byron KL, Majetschak M. Kv7 voltage-activated potassium channel inhibitors reduce fluid resuscitation requirements after hemorrhagic shock in rats. *J Biomed Sci.* 2017; 24:8. [PubMed: 28095830]
18. Mackie AR, Byron KL. Cardiovascular KCNQ (Kv7) potassium channels: physiological regulators and new targets for therapeutic intervention. *Mol Pharmacol.* 2008; 74:1171–79. [PubMed: 18684841]
19. Haick JM, Byron KL. Novel treatment strategies for smooth muscle disorders: Targeting Kv7 potassium channels. *Pharmacol Ther.* 2016; 165:14–25. [PubMed: 27179745]

20. Miceli F, Soldovieri MV, Martire M, Tagliatalata M. Molecular pharmacology and therapeutic potential of neuronal Kv7-modulating drugs. *Curr Opin Pharmacol*. 2008; 8:65–74. [PubMed: 18061539]
21. Wulff H, Castle NA, Pardo LA. Voltage-gated potassium channels as therapeutic targets. *Nat Rev Drug Discov*. 2009; 8:982–1001. [PubMed: 19949402]
22. Rockwood K, Beattie BL, Eastwood MR, Feldman H, Mohr E, Pryse-Phillips W, Gauthier S. A randomized, controlled trial of linopirdine in the treatment of Alzheimer's disease. *Can J Neurol Sci*. 1997; 24:140–145. [PubMed: 9164692]
23. Pieniaszek HJ Jr, Fiske WD, Saxton TD, Kim YS, Garner DM, Xilinas M, Martz R. Single-dose pharmacokinetics, safety, and tolerance of linopirdine (DuP 996) in healthy young adults and elderly volunteers. *J Clin Pharmacol*. 1995; 35:22–30. [PubMed: 7751410]
24. van Dyck CH, Lin CH, Robinson R, Cellar J, Smith EO, Nelson JC, Arnsten AF, Hoffer PB. The acetylcholine releaser linopirdine increases parietal regional cerebral blood flow in Alzheimer's disease. *Psychopharmacology*. 1997; 132:217–226. [PubMed: 9292621]
25. Rakestraw DC, Bilski DA, Lam GN. Determination of linopirdine and its N-oxide metabolites in rat plasma by liquid chromatography. *J Pharm Biomed Anal*. 1994; 12:1055–1061. [PubMed: 7819380]
26. Joshi S, Sedivy V, Hodyc D, Herget J, Gurney AM. KCNQ modulators reveal a key role for KCNQ potassium channels in regulating the tone of rat pulmonary artery smooth muscle. *J Pharmacol Exp Ther*. 2009; 329:368–376. [PubMed: 19151245]
27. Reddy AK, Namiranian K, Lloyd EE, Bryan RM, Taffet GE, Hartley CJ. Effect of isoflurane on aortic impedance in mice. *Conf Proc IEEE Eng Med Biol Soc*. 2009; 1104–1105. [PubMed: 19964748]
28. Yang C-F, Chen M-C, Chen T-I, Cheng C-F. Dose-dependent effects of isoflurane on cardiovascular function in rats. *Tzu Chi Medical Journal*. 2014; 26:119–122.
29. Riha H, Papousek F, Neckar J, Pirk J, Ostadal B. Effects of isoflurane concentration on basic echocardiographic parameters of the left ventricle in rats. *Physiol Res*. 2012; 61:419–423. [PubMed: 22670699]
30. Sano Y, Ito S, Yoneda M, Nagasawa K, Matsuura N, Yamada Y, Uchinaka A, Bando YK, Murohara T, Nagata K. Effects of various types of anesthesia on hemodynamics, cardiac function, and glucose and lipid metabolism in rats. *Am J Physiol Heart Circ Physiol*. 2016; 311:H1360–H1366. [PubMed: 27694213]
31. Goodwill AG, Fu L, Noblet JN, Casalini ED, Sassoon D, Berwick ZC, Kassab GS, Tune JD, Dick GM. KV7 channels contribute to paracrine, but not metabolic or ischemic, regulation of coronary vascular reactivity in swine. *Am J Physiol Heart Circ Physiol*. 2016; 310:H693–704. [PubMed: 26825518]
32. Chen X, Li W, Hiett SC, Obukhov AG. Novel Roles for Kv7 Channels in Shaping Histamine-Induced Contractions and Bradykinin-Dependent Relaxations in Pig Coronary Arteries. *PLoS One*. 2016; 11:e0148569. [PubMed: 26844882]
33. Streng T, Christoph T, Andersson KE. Urodynamic effects of the K⁺ channel (KCNQ) opener retigabine in freely moving, conscious rats. *J Urol*. 2004; 172:2054–2058. [PubMed: 15540788]
34. Mackie AR, Brueggemann LI, Henderson KK, Shiels AJ, Cribbs LL, Scrogin KE, Byron KL. Vascular KCNQ potassium channels as novel targets for the control of mesenteric artery constriction by vasopressin, based on studies in single cells, pressurized arteries, and in vivo measurements of mesenteric vascular resistance. *J Pharmacol Exp Ther*. 2008; 325:475–83. [PubMed: 18272810]
35. Chadha PS, Zunke F, Zhu H-L, Davis AJ, Jepps TA, Olesen SP, Cole WC, Moffatt JD, Greenwood IA. Reduced KCNQ4-Encoded Voltage-Dependent Potassium Channel Activity Underlies Impaired β -Adrenoceptor-Mediated Relaxation of Renal Arteries in Hypertension. *Hypertension*. 2012; 59:877–884. [PubMed: 22353613]
36. Stott JB, Barrese V, Greenwood IA. Kv7 Channel Activation Underpins EPAC-Dependent Relaxations of Rat Arteries. *Arterioscler Thromb Vasc Biol*. 2016; 36:2404–2411. [PubMed: 27789473]

37. Stott JB, Barrese V, Jepps TA, Leighton EV, Greenwood IA. Contribution of Kv7 channels to natriuretic peptide mediated vasodilation in normal and hypertensive rats. *Hypertension*. 2015; 65:676–682. [PubMed: 25547342]
38. Salomonsson M, Brasen JC, Braunstein TH, Hagelqvist P, Holstein-Rathlou NH, Sorensen CM. K(V)7.4 channels participate in the control of rodent renal vascular resting tone. *Acta Physiol*. 2015; 214:402–414.
39. Sakakibara K, Feng GG, Li J, Akahori T, Yasuda Y, Nakamura E, Hatakeyama N, Fujiwara Y, Kinoshita H. Kynurenine causes vasodilation and hypotension induced by activation of KCNQ-encoded voltage-dependent K(+) channels. *J Pharmacol Sci*. 2015; 129:31–37. [PubMed: 26318674]
40. Hedegaard ER, Johnsen J, Povlsen JA, Jespersen NR, Shanmuganathan JA, Laursen MR, Kristiansen SB, Simonsen U, Botker HE. Inhibition of KV7 Channels Protects the Rat Heart against Myocardial Ischemia and Reperfusion Injury. *J Pharmacol Exp Ther*. 2016; 357:94–102. [PubMed: 26869667]
41. Bach HH, Wong YM, Tripathi A, Nevins AM, Gamelli RL, Volkman BF, Byron KL, Majetschak M. Chemokine (C-X-C motif) receptor 4 and atypical chemokine receptor 3 regulate vascular alpha(1)-adrenergic receptor function. *Mol Med*. 2014; 20:435–447. [PubMed: 25032954]
42. Bach HH, Laporte HM, Wong YM, Gamelli RL, Majetschak M. Proteasome inhibition prolongs survival during lethal hemorrhagic shock in rats. *J Trauma Acute Care Surg*. 2013; 74:499–507. [PubMed: 23354244]
43. Garcia-Covarrubias L, Manning EW 3rd, Sorell LT, Pham SM, Majetschak M. Ubiquitin enhances the Th2 cytokine response and attenuates ischemia-reperfusion injury in the lung. *Crit Care Med*. 2008; 36:979–982. [PubMed: 18209671]
44. Geng Q, Romero J, Saini V, Baker TA, Picken MM, Gamelli RL, Majetschak M. A subset of 26S proteasomes is activated at critically low ATP concentrations and contributes to myocardial injury during cold ischemia. *Biochem Biophys Res Commun*. 2009; 390:1136–1141. [PubMed: 19944202]

Figure 1. Linopirdine pre-treatment in lethal hemorrhagic shock without fluid resuscitation
 Arrows: Time points of drug administration. Open squares: vehicle (n = 3). Grey squares: 1 mg/kg linopirdine (n = 5). Black squares: 3 mg/kg linopirdine (n = 3). Data are mean $\pm$ SE.
A. Mean arterial blood pressure (MAP), mmHg. **B.** Percent total blood volume (% TBV) hemorrhage. **C.** Kaplan-Meier survival curves.

Figure 2. Linopirdine post-treatment in lethal hemorrhagic shock without fluid resuscitation
 Arrows: Time points of drug administration. Open squares: vehicle (n = 3). Black squares: 3 mg/kg linopirdine (n = 4). Data are mean $\pm$ SE. **A.** Mean arterial blood pressure (MAP), mmHg. **B.** Percent total blood volume (% TBV) hemorrhage. **C.** Kaplan-Meier survival curves.

Figure 3. Linopirdine treatment of spontaneously breathing rats during resuscitation from hemorrhagic shock

Arrows: Time points of drug administration. Open squares: vehicle ($n = 6$). Black squares: 3 mg/kg linopirdine ($n = 6$). Data are mean $\pm$ SE. **A.** Percent total blood volume (% TBV) hemorrhage. **B.** Mean arterial blood pressure (MAP), mmHg. **C.** Hematocrit (%). **D.** Blood lactate concentrations (mmol/L). **E.** $\text{PaO}_2/\text{FiO}_2$ (P/F) ratios (mmHg). **F.** Blood creatinine concentrations (mg/dL). **G.** Resuscitation fluid requirements (mL/kg). Significant differences ($p < 0.05$) between groups are labeled.

Figure 4. Linopirdine treatment of mechanically ventilated rats during resuscitation from hemorrhagic shock

Arrows indicate the start of fluid resuscitation with normal saline (NS) alone (open squares, $n = 5$) and with NS supplemented with 10 (open circles, $n = 3$), 25 (grey circles, $n = 3$) and 50 (black circles, $n = 3$) $\mu\text{g/mL}$ linopirdine. Data are mean $\pm$ SE. **A.** Percent total blood volume (% TBV) hemorrhage. **B.** Mean arterial blood pressure (MAP), mmHg. **C.** Hematocrit (%). **D.** Blood lactate concentrations (mmol/L). **E.** $\text{PaO}_2/\text{FiO}_2$ (P/F) ratios (mmHg). **F.** Blood creatinine concentrations (mg/dL). *: $p < 0.05$ vs. NS alone. **G.** Resuscitation fluid requirements (mL/kg). Significant differences between groups are labeled. *: $p < 0.05$ for 25 $\mu\text{g/mL}$ linopirdine vs NS alone. #: $p < 0.05$ for 50 $\mu\text{g/mL}$ linopirdine vs. NS alone.

Figure 5. Wet weight-dry weight (W/D) ratios after linopirdine treatment

W/D ratios are expressed as percent of the W/D ratio of animals resuscitated with normal saline alone. Open bars: NS alone (vehicle, $n = 5$). Light grey bars: NS supplemented with 10 $\mu\text{g/mL}$ linopirdine ($n = 3$). Dark grey bars: NS supplemented with 25 and 50 $\mu\text{g/mL}$ linopirdine ($n = 5$). Data are mean $\pm$ SE. **A.** Lung. **B.** Skeletal muscle. **C.** Small intestine. **D.** Colon. Significant differences between groups are labeled.

Table 1

Plasma routine laboratory parameters and cytokine concentrations.

Analyte	NS alone	NS supplemented with linopirdine	
		10 µg/mL	25/50 µg/mL
Protein (g/dL)	2.3 ± 0.05	2.4 ± 0.28	2.8 ± 0.07 *
Bilirubin (mg/dL)	0.6 ± 0.08	0.7 ± 0.07	0.7 ± 0.05
GGT (U/L)	<10	<10	<10
ALT (U/L)	108 ± 17	115 ± 26	110 ± 15
Cholesterol (mg/dL)	<50	<50	<50
Na ⁺ (mmol/L)	149.7 ± 0.5	147.7 ± 0.9	147.1 ± 0.5
K ⁺ (mmol/L)	6.8 ± 0.4	7.0 ± 0.7	6.9 ± 0.2
Ca ²⁺ (mmol/L)	5.4 ± 0.09	6.1 ± 0.14	6.8 ± 0.24
Cl ⁻ (mmol/L)	126 ± 0.4	128 ± 0.8	123 ± 1.5
TNFα (pg/mL)	11 ± 2	3 ± 1	8 ± 3
IL-6 (pg/mL)	16 ± 11	56 ± 41	20 ± 6
IL-10 (pg/mL)	24 ± 5	57 ± 37	47 ± 22

Analytes were measured in plasma obtained at t = 300 min from animals after resuscitation with normal saline (NS) alone (n = 5) and animals resuscitated with NS supplemented with 10 µg/mL (n = 3) or 25/50 µg/mL (n = 5) linopirdine. GGT: gamma-glutamyl transferase. ALT: alanine aminotransferase. TNF: tumor necrosis factor. IL: interleukin.

*
p<0.05 vs. NS alone.

Table 2

Cytokine tissue concentrations and superoxide dismutase activities

	NS supplemented with linopirdine		
Analyte	NS alone	10 µg/mL	25/50 µg/mL
<i>Lung</i>			
TNFα (pg/mg)	3.2 ± 0.9	3.1 ± 1.5	2.6 ± 1.1
IL-6 (pg/mg)	38 ± 9	213 ± 124	106 ± 37
IL-10 (pg/mg)	9 ± 3	14 ± 2	13 ± 2
SOD (U/mg)	15 ± 1.1	17 ± 1.2	13 ± 2.6
<i>Skeletal muscle</i>			
TNFα (pg/mg)	27 ± 8	22 ± 5	32 ± 6
IL-6 (pg/mg)	88 ± 14	98 ± 27	85 ± 24
IL-10 (pg/mg)	81 ± 24	90 ± 27	75 ± 15
SOD (U/mg)	12 ± 1.5	13 ± 1.7	11 ± 2.1
<i>Small intestine</i>			
TNFα (pg/mg)	1.1 ± 0.9	3.1 ± 1.7	1.3 ± 0.6
IL-6 (pg/mg)	15 ± 9	11 ± 6	5 ± 4
IL-10 (pg/mg)	19 ± 6	19 ± 7	14 ± 7
SOD (U/mg)	17 ± 1.3	16 ± 1.3	14 ± 1.4
<i>Colon</i>			
TNFα (pg/mg)	27 ± 11	9 ± 6	14 ± 4
IL-6 (pg/mg)	56 ± 26	109 ± 35	51 ± 8
IL-10 (pg/mg)	73 ± 34	80 ± 41	55 ± 18
SOD (U/mg)	25 ± 1.1	22 ± 4	14 ± 4

Analytes were measured in tissue extracts from animals after resuscitation with normal saline (NS) alone (n = 5) and animals resuscitated with NS supplemented with 10 µg/mL (n = 3) or 25/50 µg/mL (n = 5) linopirdine. TNF: tumor necrosis factor. IL: interleukin. SOD: superoxide dismutase.

* p<0.05 vs. NS alone.

There were no statistically significant differences among groups.