Effect of valproic acid upon skeletal muscle subjected to prolonged tourniquet application

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ABSTRACT
Background Valproic acid (VPA), a histone deacetylase inhibitor, has shown improved outcomes when used as a pharmaceutical intervention in animal studies of hemorrhage, septic shock, and combined injuries. This study was designed to investigate the ability of VPA to mitigate ischemia–reperfusion injury produced by prolonged tourniquet application to an extremity.

Methods The ischemia–reperfusion model in anesthetized rats was established using hemorrhage and a 3-hour tourniquet application. VPA was administered intravenously prior to tourniquet wear and removal. Ischemia–reperfusion injury was evaluated by investigating pathway signaling, immune modulation of cytokine release, remote organ injury, and skeletal muscle function during convalescence.

Results We found that VPA sustained Protein kinase B (Akt) phosphorylation and Insulin-like growth factor signaling and modulated the systemic release of interleukin (IL)-1β, tumor necrosis factor alpha, and IL-6 after 2 hours of limb reperfusion. Additionally, VPA attenuated a loss in glomerular filtration rate at 3 days after injury. Histological and functional evaluation of extremity skeletal muscle at 3, 7, and 21 days after injury, however, demonstrated no significant differences in myocytic degeneration, necrotic formation, and maximal isometric tetanic torque.

Conclusions Our results demonstrate that VPA sustains early prosurvival cell signaling, reduces the inflammatory response, and improves renal function in a hemorrhage with prolonged ischemia and reperfusion model. However, these do not translate into meaningful preservation in limb function when applied as a pharmaceutical augmentation to tourniquet wear.

Level of evidence IV.

INTRODUCTION
Casualty demographics compiled under the Joint Theater Trauma System describe a fivefold increase in vascular injury during the Iraq and Afghanistan wars when compared with previous conflicts. Extremity wounds compose 54% of all recorded combat wounds directly resulting from hostile enemy action.1,2 Casualties with compressible limb injury rapidly exsanguinate, prompting the fielding of battlefield tourniquets with guidelines to use them for hemorrhage control. Since implementation, comparative studies of tourniquet recipients versus those who did not receive a tourniquet show significantly improved survival outcomes for the recipients.3 The extent of tissue injury and dysfunction distal to tourniquet application is directly related to the magnitude and duration of an ischemic episode. Tourniquet-induced skeletal muscle ischemia for periods beyond 3 hours shows not only the onset of ischemia-based injury but also exacerbated reperfusion injury and potential limb dysfunction.4,5 Current evacuation strategy typically enables expeditious transition between echelons of care circumventing the majority of tourniquet-based ischemic injury, though an evolving operational environment may produce scenarios which exceed the ischemic threshold of permanent injury. Perpetuating tourniquet use as a field strategy when evacuation to definitive care is delayed benefits from investigation of supplemental pharmaceutical strategies to mitigate ischemia–reperfusion injury. Early hemorrhage control sustains patient survival in the prehospital setting, and this intervention may be pharmacologically augmented in the future to create a prosurvival phenotype to mitigate long-term tissue damage.

Valproic acid (VPA), the most widely prescribed antiepileptic drug worldwide, was first synthesized in 1882 as an analog of valeric acid and was approved for administration in 1967.6,7 Recently, VPA has gained renewed attention as a histone deacetylase inhibitor capable of reversing widespread deacetylation changes when supplied at dosages (150 mg/kg to 400 mg/kg) well above those required for its intended use as an antiepileptic drug.8 Epigenetic modulation of gene expression is...
an essential mechanism to cell survival and tissue homeostasis. Modification of DNA by methylation and associated histones by acetylation, methylation, ubiquitination, and sumoylation allows a cell to dynamically alter gene expression in response to a host of stimuli. Maintenance of histone acetylation is accomplished by histone acetyltransferases and histone deacetylases, which catalyze the addition and removal of acetyl groups. Perturbation of their activity manifests during ischemia and hemorrhagic shock. Cardiac and cerebral ischemia have been shown to produce a ∼40% decrease in total histone acetylation. Animal studies investigating hemorrhagic shock combined with traumatic brain injury or sepsis have shown improved survival with VPA treatment in an otherwise fatal model of polytrauma. Specifically, VPA has been shown to upregulate signaling pathways that suppress the induction of apoptosis including serine/threonine protein kinase (Akt), glycogen synthase kinase-3β, antipapoptotic members of the BCL2 family, mitogen-activated protein kinases (ERK/JNK), and nuclear factor κB (NF-κB).20 21 Increased NF-κB has been shown to reduce levels of interleukin (IL)-6, which is an inflammatory response acts concurrently with injury to local tissues. A commercially available pneumatic digit tourniquet (Model DC 1.6, D.E. Hokanson, Bellevue, WA) was placed on the left hindlimbs that had been elevated and suspended from the operating table. Tourniquet pressure was increased from 250 mm Hg to 300 mm Hg using a rapid cuff inflator equipped with a pressure transducer (Model E20, D.E. Hokanson, Bellevue, WA); this pressure was maintained for a period of 3 hours. Tourniquet-induced ischemia in rat skeletal muscle during this duration has been previously shown to produce an initial complete loss of function and an approximate 80% recovery in the absence of hemorrhage. Ten minutes prior to tourniquet release, a second dose of VPA was administered. At 3 hours of total tourniquet time, the tourniquet pressure was released, and volume-based fluid resuscitation (15 mL/kg body weight, 1 mL/kg/min) with Hextend was administered via the tail vein. Rats designated for Akt pathway signaling, cytokine release, and tibialis anterior wet/dry quantitation remained anesthetized for a 2-hour observation period after reperfusion of the hindlimb. Rats designated for GFR and muscle function quantitation after injury regained consciousness and were returned to their cages singly housed. At 3, 7, and 21 days after injury, these rats were again anesthetized. GFR quantitation occurred at 3 days after injury, and muscle function was evaluated at all three time points. In all groups, blood samples were collected from the initial hemorrhage and immediately prior to euthanasia with plasma samples stored at −80°C; immediately after euthanasia, tibialis anterior muscles of ischemic and non-ischemic limbs were harvested, weighed, and processed in accordance with sampling requirements under each methodology. In anticipation of attrition associated with 42.5% total blood volume hemorrhage, 10 rats were allocated to each experimental group. Experiments whose endpoint was designated at 2 hours of reperfusion were performed prior to all other experimental groups and proceeded until a predetermined threshold of n = 4 had been met. Rats within these groups used preservation of phosphorylated Akt in skeletal muscle as a key metric to substantiate VPA dosage efficacy before continuation with experimentation at later time points. Total rats used within these groups and mortality are as follows: VPA high dosage: n = 7/2, low dosage: n = 7/1, and saline control: n = 7/2. One rat was excluded from the VPA high-dosage group due to observed respiratory distress at the time of operation in accordance with a priori exclusion criteria. Experimental timelines for each methodology and their respective endpoints are shown in figure 1.
Rats in experimental groups with endpoints of 3, 7, and 21 days proceeded until a minimum predetermined threshold of n=6, as determined by muscle function as a primary outcome, had been met. Total rats used within these groups and mortality are as follows: 3 days (VPA n=9/2, saline n=8/1), 7 days (VPA n=8/2, saline n=8/1), and 21 days (VPA n=10/3, saline n=7/1). No rats were excluded from the experimental groups at endpoints of 3, 7, and 21 days.

VPA dosing and quantitation
VPA sodium salt prepared in 0.5 mL of 0.9% saline (400 mg/kg or 300 mg/kg) was administered by standard infuse/withdraw PHD 22/2000 syringe pumps (Harvard Apparatus) during a 10 min interval at concentrations shown to inhibit histone deacetylation in rats in a dose-optimization study. Delivery of VPA to skeletal muscle distal to tourniquet application preischemia and post reperfusion injury employed a split-dosing strategy with a 2:3 proportion (267 or 200 µg/kg) of the total dose administered 10 min prior to tourniquet inflation and 1:3 proportion (133 or 100 µg/kg) of the total dose administered 10 min prior to tourniquet release based on previous studies employing a similar dosing strategy.[30]

CMA 20 high Molecular Weight (MW) cut-off/10 mm membrane length/100 kD cut-off microdialysis probes (CMA microdialysis, Kista, Sweden) were implanted bilaterally within the tibialis anterior muscles, and a CMA 402 dual syringe microdialysis pump perfused the probe lumens with isotonic T1 perfusion fluid containing 25 µM VPA dosing and quantitation are as follows: 3 days proceeded until a minimum predetermined threshold of 3, 7, or 21 days proceeded until a minimum predetermined threshold of 3, 7, or 21 for glomerular filtration rate quantitation and peak isometric force production of the tibialis anterior muscles (B). IL-1, IL-6, tumor necrosis factor-α, tumour necrosis factor alpha.

Figure 1. Experimental timelines and methodologies. Animals underwent hemorrhage of 42.5% total blood volumes with a 3-hour tourniquet application and subsequent fluid resuscitation. Tissues were harvested for systemic IL-1β, TNF-α, IL-6, and local serine/threonine protein kinase pathway activity at 2 hours post reperfusion (A) or proceeded to days 3, 7, or 21 for glomerular filtration rate quantitation and peak isometric force production of the tibialis anterior muscles (B). IL-1, IL-6, tumor necrosis factor-α, tumour necrosis factor alpha.

Muscle function
Tibialis anterior muscle function was quantified in vivo using procedures previously shown.32 Rats were placed in a supine position with hind limbs stabilized in a custom jig and paw secured to a footplate. The extensor digitorum longus muscle was severed to delineate force output from the tibialis anterior muscle only during measurement. The footplate was connected to a servomotor-controlled force-displacement transducer (Model 300 series, Aurora Scientific, Aurora, ON, Canada), and muscles were activated via their motor nerve using a subcutaneous electrode. Relative force measurements were connected to a physiological stimulator, and the crural muscles were activated with a stimulus voltage adjusted to elicit peak torque. The maximum output force of the target muscle, peak

isometric tetanic force, will be quantified and represents a relevant outcome for clinical translation of experimental therapies. The servo motor input and force and displacement transducer outputs were controlled and acquired, respectively, using a PC equipped with a data acquisition board (National Instruments, Austin, TX) and custom designed LabVIEW (National Instruments, Austin, TX)-based software program.

**Kidney function**
Transdermal monitoring of GFR using the NIC-Kidney Device and FITC–sinistrin (MediBeacon, St. Louis, MO) quantified disparities in acute kidney injury in animals receiving 400 mg/kg VPA and saline vehicle control. FITC–sinistrin dissolved in sterile isotonic saline was administered via carotid artery catheter at 7 mg/100 g of body weight with injections of ≤0.5 mL. Monitoring of transdermal FITC–sinistrin fluorescence continued for a duration of 2 hours post injection.

Data downloaded from the NIC-Kidney Device was processed by MediBeacon Studio software, and the elimination kinetics of FITC–sinistrin was used to calculate GFR. Baseline GFR quantitation was performed prior to injury and after injury quantification occurred at 72 hours post ischemia–reperfusion injury. Decrements in GFR between baseline and values after injury were evaluated for significance between VPA-treated and vehicle control animals.

**Akt pathway activity and cytokine release**
Tissue lysate prepared from snap-frozen tibialis anterior muscles collected after 3 hours of pneumatic tourniquet application and 2 hours of reperfusion or uninjured contralateral limbs were prepared in accordance with manufacturer recommendations for the pAkt and Akt Pathway Total 7-Plex kit (Invitrogen, Carlsbad, CA). Total and phosphorylated Akt pathway proteins were quantified concurrently on a Bio-Plex 200 System (Bio-Rad, Hercules, CA). Circulating cytokines in plasma samples were assayed by RayBiotech (Atlanta, GA) using a Quantibody Rat Cytokine Array 67 kit.

**Histology**
Tibialis anterior muscles fixed in 10% buffered formalin were processed for light microscopy using standard procedures for H&E staining. Samples were blinded to treatment group and procedural limb then evaluated by a histopathologist using a predetermined histological grading scale. Features evaluated by histological grading included skeletal muscle and predominant muscle changes, intermyocyte hemorrhage and edema, fibrosis, foreign body reaction, and inflammation as indicated by the presence of neutrophils, histiocytes, and lymphocytes.

Tibialis anterior sections designated for wet: dry ratio quantitation were immediately weighed on dissection and snap-frozen in liquid nitrogen until a subsequent 48-hour lyophilization where dry weight was achieved. Levels of skeletal muscle edema over uninjured tissue are expressed as a comparative ratio of wet versus dry tissue weights.

**Statistical analysis**
Contralateral non-ischemic limbs served as controls for experiments quantifying muscle function and local signaling changes, whereas rats receiving saline alone served as controls for experiments quantifying systemic inflammation levels and GFR. Comparison of tetanic force output between the experimental and non-ischemic contralateral limb were evaluated for significance by one-way analysis of variance test with post hoc multiple comparisons and Bonferroni correction at alpha=0.05. Akt pathway analyte, cytokines, GFR, and aggregate injury scores were evaluated for significance using Student’s t-test using GraphPad Prism V7.03 (GraphPad Software, San Diego, CA).

For these comparisons, non-parametric statistical tests were used due to the relatively small sample sizes of each group (n=4–7 animals per tourniquet application). Data are presented as mean with SE of the mean or scatter plot unless otherwise noted.

**RESULTS**
To verify VPA penetration into soft tissues distal to tourniquet application prior to cessation of blood flow and during the reperfusion period, free VPA was recovered from the interstitial space of both tibialis anterior muscles throughout the 3-hour ischemic period and for 2 hours after restoration of blood flow. Interstitial pharmacokinetics of VPA showing equivalent peak concentrations within experimental and contralateral limbs prior to tourniquet application are shown in figure 2.

**Akt pathway signaling**
After 2 hours of reperfusion, total Akt from skeletal muscle distal to tourniquet placement trended toward a decline when compared with contralateral limbs. This loss approached attenuation by high-dose VPA but did not surpass a threshold of significance. Ischemia–reperfusion injury reduced phosphorylated Akt in skeletal muscle of tourniquet-injured limbs to 27.88% when compared with contralateral limbs in animals receiving saline control. In contrast, tourniquet-injured limbs in animals treated with 400 mg/kg VPA showed 101.66% of the quantified phosphorylated Akt within contralateral limbs and, surprisingly, animals receiving 300 mg/kg VPA showed a 113.99% proportion of phosphorylated Akt compared with uninjured contralateral limbs (figure 3). Whereas concentrations of phosphorylated insulin-like growth factor receptor remained relatively equivalent in VPA and saline control animals, phosphorylated insulin receptor substrate-1 (IRS-1) of tourniquet-injured limbs as a proportion of contralateral limbs showed an 89.28% increase in VPA treated animals versus those receiving saline vehicle control. Downstream of Akt signaling activation, phosphorylated p70S6K showed a 35.31% increase in VPA-treated animals over saline alone in tourniquet-injured skeletal muscle (figure 4).

**Cytokine release and kidney function**
Animals receiving 400 mg/kg VPA compared with those receiving saline vehicle control showed a 91.47% reduction in plasma levels of IL-1β, a 73.76% reduction in TNF-α, and a 45.41% reduction in IL-6 (figure 5). GFR decrement was calculated as a decline from values quantitated prior to hemorrhage and tourniquet application to 72 hours after injury. Animals receiving 400 mg/kg VPA showed a 19.21% improvement in GFR reduction when compared with animals receiving saline vehicle control (p=0.0424; VPA=−7.0%±6.5, saline=−26.2%±7.3).

**Muscle function and histological analysis**
Peak tetanic force quantitation of in vivo tibialis anterior muscles showed nearly an absence of response to stimuli at 3 days after injury and no significant difference in animals receiving VPA and saline vehicle control. At 7 days after injury, a detectable force output was established with no differences in treatment groups. A mean decrement in tetanic force output in tourniquet-injured limb versus contralateral limb of 35.05% (VPA) and 57.03% (saline vehicle control) was shown 21 days after injury. Despite recorded differences in mean values in functional limb recovery...
between treatment groups at this time point, variance of quantified tetanic force output values within each group prevented interpreting these values as surpassing a threshold of significance (figure 6).

There was no significant difference with VPA treatment in elevated tibialis anterior weight to dry weight ratios (p=0.8634; VPA=10.8%±0.96, saline=10.5%±1.49) after 2 hours of reperfusion. Representative images from uninjured skeletal muscle and tourniquet-injured limbs throughout convalescence are shown in figure 7. Aggregate injury scores in both VPA-treated and saline-treated animals showed no difference between groups (p=0.3734; tourniquet: VPA=0.2±0.20, saline=0.4±0.24; uninjured: VPA=2.8±0.37, saline=3.2±0.20).

**DISCUSSION**

There are two main findings of this study. First, VPA administration at concentrations relevant to previous rat studies significantly attenuated reductions in Akt pathway signaling and suppressed an acute inflammatory response in our injury model. Second, these beneficial effects did not translate into improvements in physiological function of the skeletal muscle of the affected limb.

Akt pathway activity was examined based on previous observations that VPA treatment at these concentrations successfully modulated protein expression and signaling, increased Akt phosphorylation, and improved survival outcomes in rat models of hemorrhagic shock. Given the mechanistic overlap in hypovolemic shock manifesting as a global ischemic event and the localized ischemia distal to tourniquet application occurring typically concurrent to significant blood lost, VPA was selected as a candidate for this study with Akt phosphorylation as an indicator of a prosurvival phenotype that allows for comparison with previously published findings.

There was a trend toward loss of total Akt after ischemia–reperfusion injury but high variability between experimental animals prevented meeting a threshold of statistical significance.
when administering high dosages of VPA versus saline vehicle control (p=0.0905). Despite a possibly depleted pool of Akt protein available for signal transduction, VPA treatment at both low and high dosage concentrations ameliorated the reduction of quantified phosphorylated Akt in tourniquet-injured limbs when compared with contralateral limbs. Akt phosphorylation is therefore reduced by ischemia–reperfusion injury, but VPA acts to restore this signaling pathway.

Akt activation integrates a host of transmembrane receptor–ligand-mediated stimuli whose influential pathways include insulin and insulin-like growth factor. Delineating a singular mechanism of Akt activation was not within the scope of this study; however, a preserved activation of IRS-1, whose upstream regulation of Akt is well characterized,35 suggests that this may be a means of maintaining Akt signaling in the context of ischemia–reperfusion injury. Likewise, phosphorylated p70s6K protein acts as an indicator of mammalian target of rapamycin (mTOR) pathway activity, whose activation downstream of Akt promotes cell growth, proliferation, and inhibits autophagy.36 Given reports of autophagy inhibition with VPA treatment after injury37 and the preservation of p70s6K signaling within this study, an autophagy-mediated mechanism producing a decrement in total Akt is suggested.

Acute ischemia and subsequent reperfusion injury modulating release of inflammatory mediators is well characterized.38 39 VPA suppression of inflammatory mediator release has been shown in animal models of hemorrhagic shock and septic shock,17 22 and our results likewise demonstrate an amelioration of systemic cytokine levels by VPA in the combined injury model studied herein.

Furthermore, kidney function as assessed by GFR measurement at 72 hours after injury was decreased in saline-treated rats but was restored by VPA administration. This finding is similar to previous demonstrations of sustained survival of renal cells and preservation of renal function by VPA in animal models of hemorrhagic or septic shock.24 To our knowledge, this is the first study that demonstrates a beneficial effect of VPA on renal function in a hemorrhage model that includes clinically relevant hemorrhage control measures (eg, prolonged tourniquet application) that produce significant tissue damage.

Figure 3  Total and phosphorylated Akt protein in rat tibialis anterior muscles. (A) Total Akt measured in tourniquet-injured limbs receiving saline vehicle control compared with contralateral limbs in animals receiving VPA at 400 mg/kg. (B) Total Akt protein in tourniquet-injured limbs as a proportion of contralateral limbs in animals receiving VPA at low (300 mg/kg), high (400 mg/kg), or saline vehicle control. (C) Phosphorylated Akt protein in tourniquet-injured limbs quantified as a percentage of contralateral limbs was significantly less in animals receiving saline vehicle control when compared with animals receiving low and high dosages of VPA. **p<0.01, ***p<0.001. Akt, serine/threonine protein kinase; VPA, valproic acid.

Figure 4  Akt pathway activity. Phosphorylated Akt pathway proteins in tibialis anterior muscles subject to 3 hours of ischemia and 2 hours of reperfusion quantified as a percentage of contralateral limbs. (A) Animals treated with 400 mg/kg valproic acid showed no significant difference in phosphorylated insulin-like growth factor receptor-1, (B) whereas phosphorylated insulin receptor substrate-1 (C) and phosphorylated p70s6K were significantly increased. *P<0.05. Akt, serine/threonine protein kinase.
Characterized intravenous route and using Hextend as fluid molecular target. Additionally, our administering VPA via a more mechanism of cellular sequestration and procession to the drug’s muscles distal to tourniquet application, however, suggest a decreasing quantities of VPA within the interstitial fluids of.

Extending our study throughout the duration of convalescence, however, did not demonstrate that an early preservation of Akt signaling nor immune modulation translated into meaningful functional improvements of the limb. Simultaneous with evaluation of cell signaling, edema within tibialis anterior muscles after reperfusion of both treatment and control groups was elevated to comparable levels. Physiological events appreciable by light microscopy, including myocyte degeneration and loss, the formation of necrosis, and eventual myocyte repopulation, proceeded on course regardless of treatment. Substantiating these observations were an absence of significant skeletal muscle functional recovery over non-treated limbs when quantitatively force output under in vivo stimulated tetanic contraction. These latter findings greatly limit the utility of VPA as a pharmaceutical augmentation to prolonged tourniquet application.

This study is not without limitations. Tourniquet application inherently creates conditions where intravenously dosed drugs become sequestered from the circulating blood. Differences in pharmacokinetic profiles between limbs are evident in figure 1. Decreasing quantities of VPA within the interstitial fluids of muscles distal to tourniquet application, however, suggest a mechanism of cellular sequestration and procession to the drug’s molecular target. Additionally, our administering VPA via a more characterized intravenous route and using Hextend as fluid resuscitation are less translatable to field care scenarios. Hextend was recently removed from military clinical practice guidelines, and our future studies will reflect more current resuscitative practices. Inclusion of intravenous administration was selected to facilitate detection of improved outcomes which, once verified, would prompt refinement of delivery methods. Furthermore, this study sought to test conditions where a 3-hour tourniquet application would be required for hemorrhage control, which produced a persistent functional deficit that did not attenuate with VPA administration. These data do not preclude a therapeutic benefit of VPA administration with a less profound ischemic injury when a tourniquet is applied for a shorter duration.

CONCLUSIONS
Tourniquet application for extended durations and associated reperfusion injuries cause a substantial loss in prosurvival cellular signaling manifesting in tissue loss. These findings build on previous research regarding hemorrhagic shock to determine if signaling and functional preservation are possible in the extreme conditions where tourniquet application produces complete cessation of blood flow for an extended duration. Despite compelling evidence that VPA treatment sustains Akt signaling, suppresses an acute inflammatory response, and attenuates loss in kidney function after prolonged tourniquet application with hemorrhage and subsequent resuscitation, these findings do not translate into a preservation in skeletal muscle function after injury. VPA therefore may not be appropriate as a pharmacological adjunct to prolonged tourniquet application.

Contributors RM: guarantor, conceptualization, methodology, and writing; LM: methodology; CV: project administration; GG: validation and data curation; JW: supervision and funding acquisition.

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Competing interests None declared.
Patient consent for publication Not applicable.

Ethics approval This study was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and in accordance with the principles of the Guide for the Care of and Use of Laboratory Animals. All procedures were performed in a laboratory accredited by the Association for the Accreditation of Laboratory Animal Care following a protocol approved by the Institutional Animal Care and Use Committee.

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