Central acetylcholinesterase inhibition improves hemodynamic counter-regulation to severe blood loss in alcohol-intoxicated rats

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Abbreviated title: ICV neostigmine improves outcome from hemorrhage

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ABSTRACT

Acute alcohol intoxication results in impaired hemodynamic counter-regulation to blood loss and is associated with an attenuated hemorrhage-induced release of catecholamines and arginine vasopressin (AVP). We speculated that restoration of the neuroendocrine response to hemorrhage would improve mean arterial blood pressure (MABP) recovery during acute alcohol intoxication. Previously we have demonstrated that intracerebroventricular (ICV) choline, a precursor of acetylcholine, transiently increases sympathetic nervous system (SNS) outflow, but is not capable of improving neuroendocrine and hemodynamic compensation to hemorrhage in alcohol-treated rats. We hypothesized that prolongation of the observed effect via ICV administration of neostigmine, an acetylcholinesterase inhibitor, would enhance SNS outflow, restore the neuroendocrine response, and in turn improve hemodynamic responses to hemorrhage during acute alcohol intoxication. ICV neostigmine (1 μg) increased MABP, catecholamines, and AVP within 5 min, and reversed hypotension due to both 40% hemorrhage and intragastric alcohol (30% w/v; 2.5 g/kg) administration in chronically-catheterized male Sprague-Dawley rats (225-250g). Acute alcohol intoxication prior to 50% hemorrhage decreased basal MABP, accentuated hypotension mid-hemorrhage, suppressed the hemorrhage-induced release of norepinephrine and AVP, and prevented restoration of MABP to basal levels post-fluid resuscitation with lactated Ringers. ICV neostigmine (0.5 μg) produced a sustained increase in MABP beginning at 30 min of hemorrhage, which persisted throughout fluid resuscitation in both control and alcohol-treated animals. ICV neostigmine enhanced epinephrine responses and restored the hemorrhage-induced release of norepinephrine and AVP in alcohol-treated rats. These results demonstrate that inhibition of acetylcholinesterase in the central nervous system enhances SNS outflow, restores the neuroendocrine response to severe blood loss, and thereby
improves hemodynamic counter-regulation during acute alcohol intoxication. Taken together, this study provides evidence for a central (and not peripheral) role of alcohol in impairing hemodynamic stability during hemorrhagic shock.
KEYWORDS

Hemorrhage
Ethanol
Neostigmine
Mean arterial blood pressure
Sympathetic nervous system
INTRODUCTION

According to the Center for Disease Control and Prevention, trauma ranked as the fifth leading cause of death in the United States, representing 4.8% of all deaths in 2005 (20). Trauma usually consists of a combined insult of tissue injury and hemorrhagic shock, the latter of which results in severe tissue hypoperfusion that increases the risk of end organ damage and death (9). Fluid resuscitation reduces immediate mortality following hemorrhagic shock by improving tissue perfusion, however long term mortality rates remain 22% (27).

Acute alcohol intoxication increases the risk of traumatic injury (21,36). Nearly half of all injured victims that enter emergency departments across the United States test positive for blood alcohol (6, 24, 38), with blood alcohol levels (BAL) frequently exceeding 80 mg/dL, the legal limit in most states (14, 24, 37, 38). Alcohol-intoxicated trauma victims enter the emergency department more hypotensive than their sober counterparts (41). Clinical data suggest that mean arterial blood pressure (MABP) at the time of admittance into the emergency department is one of the most critical indicators of a patient’s outcome and survival from traumatic injury and blood loss (15); therefore, the greater hypotension noted in alcohol-intoxicated trauma victims is likely to contribute to their increased morbidity and mortality. Previously we have demonstrated that acute intoxication produced by intragastric administration of alcohol, decreases baseline MABP, accentuates hypotension throughout hemorrhage, and blunts the pressor response to fluid resuscitation, regardless of the dose (1.75, 5, and 8 g/kg) and frequency of alcohol administration (single dose, three-day binge, and 15-h constant infusion, respectively) (12, 25, 26, 30, 33). This impaired hemodynamic compensatory response to hemorrhage in alcohol-intoxicated rats is associated with suppression of catecholamine (epinephrine and norepinephrine) and arginine vasopressin (AVP) responses (30). Taken
together, these observations led us to hypothesize that the inappropriate release of these vasoactive hormones during hemorrhage is the principle mechanism by which alcohol acts to impair MABP recovery following blood loss. Thus, we hypothesized that restoration of the neuroendocrine response would improve hemodynamic counter-regulation to hemorrhagic shock in alcohol-treated rats.

We aimed to dissect central effects of acute alcohol intoxication on the counter-regulatory responses to hemorrhage from systemic effects. The results from recent studies demonstrated that intracerebroventricular (ICV) administration of choline, a precursor of acetylcholine, immediately stimulates sympathetic nervous system (SNS) outflow in both control and alcohol-intoxicated animals, reflected in an increase in MABP, plasma epinephrine, norepinephrine, and AVP (26). However, these effects were transient and not prolonged enough to improve hemodynamic stability following hemorrhagic shock in alcohol-intoxicated animals. The objective of the current studies was to produce sustained SNS activation by administering neostigmine, an acetylcholinesterase inhibitor, centrally. When injected directly into the central nervous system via the right lateral ventricle (i.e., ICV), neostigmine is confined to the brain, and functions by inhibiting the breakdown of acetylcholine into acetate and choline by acetylcholinesterase, therefore increasing acetylcholine availability (44). Thus, any effects elicited by injection of this drug reflect a centrally-mediated response, which enables isolating central from systemic mechanisms.

To test our hypothesis, we initially confirmed that ICV neostigmine administration enhances SNS outflow, and that in turn, the augmented catecholamine and AVP responses contribute to the pressor effects induced by central acetylcholinesterase inhibition. We then determined whether ICV neostigmine was capable of independently reversing hemorrhage- and
alcohol-induced hypotension, before subjecting animals to a protocol that involved both alcohol and hemorrhage. Our results showed that ICV neostigmine administration produced an immediate activation of SNS outflow reflected in increased MABP, heart rate, plasma catecholamines, AVP, and glucose. In addition, we provide strong evidence that the pressor response elicited by ICV neostigmine in both normotensive and alcohol-intoxicated, hypotensive animals is due to enhanced catecholaminergic and vasopressinergic activity. Taken together, these data suggest that central acetylcholinesterase inhibition restores the neuroendocrine response and improves hemodynamic compensatory responses to blood loss in alcohol-intoxicated rats. Moreover, these data provide evidence that the alcohol-induced impairment in hemodynamic counter-regulation to hemorrhagic shock is elicited through attenuation of centrally-mediated SNS activation.
MATERIALS AND METHODS

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at Louisiana State University Health Sciences Center and were performed in accordance with the guidelines of the National Institutes of Health. Specific pathogen-free, adult male Sprague-Dawley rats (225-275g) arrived to the institution and were allowed one week to acclimate to their surroundings. During this time, they were caged in pairs, allowed standard rat chow (2018 Teklad Global 18% Protein Rodent Diet, Harlan, US) and water ad libitum, and housed in a controlled-temperature (22°C) and controlled-illumination (12-h light/dark cycle) environment.

Surgical Procedures

Intracerebroventricular cannula placement: Animals were anesthetized with ketamine/xylazine (90 mg/kg and 9 mg/kg, respectively) in order to stereotaxically implant guide cannulas into the right lateral ventricle as previously described by our laboratory (25). Following cannula placement, animals were returned to individual cages, allowed 7-9 days to recover from surgery, and provided with food and water ad libitum. Cannula placement and patency were tested on the day of vascular and gastric surgery using ICV administration of 5 μL of angiotensin II (20 ng; Sigma, St. Louis, MO), which induced an immediate thirst response. Only animals that exhibited angiotensin II-induced polydipsia reflecting accurate placement of the cannula in the lateral ventricle were used in these studies.

Vascular and gastric catheter placement: Sterile catheters were placed into the left carotid artery and right jugular vein, as well as the antrum of the stomach, of anesthetized animals (ketamine/xylazine; 90 mg/kg and 9 mg/kg, respectively) using aseptic surgical
procedures as previously described by our laboratory (33). The catheters were routed subcutaneously through a trocar and exteriorized at the nape of the neck. Following this surgical procedure, the animals were returned to their individual cages, provided food and water *ad libitum*, and allowed to recover for 2-3 days.

**Alcohol administration**

On the day prior to hemorrhage at approximately 4:00 pm, conscious and unrestrained animals received an intra-gastric bolus of 30% ethyl alcohol (2.5 g/kg) followed by a 15-hour constant infusion of alcohol (~300 mg/kg/hour). The total dose received over the alcohol administration period was approximately 7 g/kg, achieving blood alcohol levels (BAL) of 192 ± 21 mg/dL. Time-matched control animals received isocaloric/isovolumic 52% dextrose (12.2 g/kg). During the 15-hour overnight infusion, animals had no access to rat chow.

**Fixed-Volume Hemorrhagic Shock**

Conscious and unrestrained animals were randomized to sham or hemorrhage groups and subjected to fixed-volume hemorrhage as previously described by our laboratory (25). Briefly, half the circulating blood volume (calculated as 6% of the animal’s body weight in grams) was removed over 60 minutes, with the bulk of the blood (40% of circulating blood volume) removed within the first 10 minutes. Animals were not heparinized at any time throughout the experimental protocol. MABP was monitored throughout the active blood loss period using Powerlab (Powerlab, AD Instruments, Colorado Springs, CO). Blood (1.5 mL) was removed from time-matched sham animals at pre- and post-hemorrhage time points (T = 0 and 60 minutes, respectively).

**Fluid Resuscitation**
At the end of the 60-minute hemorrhage period, an intravenous (IV) bolus of warmed (37°C) lactated Ringers equal to 40% of the total blood volume removed was returned to the animals, followed by a constant infusion of lactated Ringers of two times the blood volume removed. In total, 2.4 times the blood volume removed was returned to the animals in the form of lactated Ringers. MABP was monitored throughout the fluid resuscitation period. Blood (1.5 mL) was removed from hemorrhaged and time-matched sham animals at the end of the fluid resuscitation period (T = 120 minutes) and was stored for future analysis. The animals were returned to clean individual cages, allowed water and food ad libitum, and monitored for survival. At the end of the one week observation period, animals were euthanized by an intravenous injection of sodium pentobarbital (125 mg/kg) followed by exsanguination.

**Experimental Protocols**

All animals were subjected to surgical procedures (ICV cannula and/or vascular and gastric catheter placement) and allowed 1-3 days to recover before initiating the experimental protocol. In addition, animals were conscious and unrestrained throughout all of the following experimental protocols.

*Study 1—ICV neostigmine modulates sympathetic nervous system activation:* To establish an optimal dose of neostigmine, we performed a dose response curve using 0.1, 0.2, 0.3, 1, 2, and 3 μg of neostigmine (n = 5-12 animals per group). For ICV neostigmine methyl sulfate (Sigma, St. Louis, MO) administration, an injection cannula (25-gauge, 11.5 mm stainless steel tubing) was connected to a Hamilton microsyringe (10 μL) with polyethylene tubing (25-30 cm) and was inserted through a guide cannula previously fixed into the skull. Neostigmine (5 μL) was infused slowly over an 8-10-second period to normotensive animals.
In order to determine whether ICV neostigmine (1 μg) increases SNS activity, neostigmine was injected to normotensive animals and then MABP was monitored for 60 minutes (n = 3 animals per group). Other outcome measures including heart rate, and plasma epinephrine, norepinephrine, AVP, glucose, and insulin were recorded at 5 and 10 minutes after neostigmine injection.

Study 2—Pressor response of ICV neostigmine is elicited through peripheral catecholamines and/or AVP: In order to determine the contribution of increased vasoactive hormone release to the pressor response of ICV neostigmine, prazosin hydrochloride, an α₁-adrenergic receptor antagonist (0.5 mg/kg; Sigma, St. Louis), [β-mercapto-β,β-cyclpentamethylenepropionyl1, O-me-Tyr², Arg⁸]-vasopressin, a selective V₁a receptor antagonist, (10 μg/kg; Sigma, St. Louis), both antagonists, or isotonic saline were administered intravenously 5 minutes prior to ICV neostigmine (1 μg) administration (n = 3-12 animals per group). MABP was monitored for 30 minutes following ICV neostigmine administration.

Study 3—ICV neostigmine impact on MABP during acute alcohol intoxication: Animals were administered alcohol through the gastric catheter as described above (see Alcohol Administration). To determine whether ICV neostigmine reverses hypotension associated with acute alcohol intoxication, neostigmine (1 μg) was injected thirty minutes following the cessation of the 15-hour alcohol infusion (n = 4-5 animals per group). Blood pressure was recorded for 60 minutes.

Study 4—ICV neostigmine impact on MABP response to hemorrhage: Animals were subjected to a modified version of the fixed-volume hemorrhage protocol described above (see Hemorrhagic Shock). Briefly, 40% of the estimated circulating blood volume was removed within 10 minutes. In order to determine whether ICV neostigmine reverses hypotension
associated with blood loss, neostigmine (1 μg) or sterile water (5 μL) was injected at the end of blood withdrawal (T = 10 minutes; n = 4-6 animals per group). MABP was monitored for 120 minutes. Animals were not administered resuscitation fluids at any time following blood loss. At the end of the MABP monitoring period, animals were returned to their individual cages, provided food and water ad libitum and monitored daily for one week to establish survival.

**Study 5—ICV neostigmine effects on hemodynamic and neuroendocrine responses to hemorrhage during acute alcohol intoxication:** Alcohol administration was initiated the evening prior to fixed-volume hemorrhagic shock and fluid resuscitation (Figure 1). In order to determine the effects of central acetylcholinesterase inhibition on compensatory responses to hemorrhagic shock during acute alcohol intoxication, neostigmine (0.5 μg) or sterile water (5 μL) was administered via the ICV cannula 10 minutes into the hemorrhage and fluid resuscitation protocol. There were a total of eight treatment groups in this experiment (n = 6-9 animals per group as indicated in the figure legend): dextrose/water/sham, dextrose/water/hemorrhage, dextrose/neostigmine/sham, dextrose/neostigmine/hemorrhage, alcohol/water/sham, alcohol/water/hemorrhage, alcohol/neostigmine/sham, and alcohol/neostigmine/hemorrhage.

**Blood Sample Analysis**

Arterial blood samples were collected in chilled heparinized syringes and aliquots of blood were placed in tubes containing 20 μL/mL of catecholamine preservative composed of 9% ethylenediaminetetraacetic acid, 6% glutathione and deionized water at a pH of 6.0-7.4. Blood samples were centrifuged for 15 min at 10000 rpm for plasma separation. Blood alcohol levels and plasma glucose levels were measured using an amperometric oxygen electrode and the respective kits (Analox Instruments Limited, London, England).
**Catecholamine measurements:** Blood samples were quantified for circulating epinephrine and norepinephrine levels using a high-performance liquid chromatography system consisting of a chromatographic analyzer with a catecholamine column and an electrochemical detector (Bioanalytic Systems, West Lafayette, IN) as previously described by our laboratory (25). The interassay coefficient of variability for catecholamines was 25%.

**Arginine vasopressin measurements:** AVP levels were determined in extracted plasma samples using a commercially available human, rat, mouse and ovine-specific radioimmunoassay (Phoenix Pharmaceuticals, Belmont, CA) as previously described by our laboratory (30). Briefly, plasma samples were acidified using 1% trifluoroacetic acid (called Buffer A) in water. C-18 SEP-Columns (Waters Corporation, Milford, MA) were pre-treated with 1 mL of 60% acetonitrile plus 40% Buffer A (called Buffer B) followed by Buffer A alone (3 mL, 3X). The supernatant was then loaded into the pre-treated column and washed with Buffer A (3 mL, 2X) and then Buffer B (3 mL, 1X). Finally the eluant was evaporated to dryness using the Speed Vac Concentrator and Condensation Trap (Sarant, Farmindale, NY) with Duo-Seal Vacuum pump (Sargert-Welch Scientific Company, Skoki, IL) and the residue was reconstituted using radioimmunoassay buffer provided by the kit. Levels were determined using manufacturer’s instructions. The radioimmunoassay reliable detection range was 10-1280 pg/mL of AVP and had 100% specificity for [Arg^8]-vasopressin (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH$_2$), the form of vasopressin found in most mammals.

**Plasma insulin measurements:** Insulin levels were determined in 100 μL of non-extracted plasma samples using a commercially available rat-specific radioimmunoassay (LINCO Research, St. Charles, MO). The radioimmunoassay reliable detection range was 0.1-10 ng/mL of insulin and had 100% specificity for rat insulin.
Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM) with the number of animals used per group indicated in the figure legends. Statistical analysis of MABP and hormones was accomplished using one- or two-way analysis of variance (ANOVA) with or without repeated measures (also indicated in figure legends). Statistical analysis of survival curves was accomplished using the Gehan-Breslow test. All pair-wise multiple comparisons were completed with the Holm-Sidak method. Statistical significance was set at P < 0.05.
RESULTS

**ICV neostigmine modulates sympathetic nervous system activation.** The dose response to ICV neostigmine (0.1, 0.2, 0.3, 1, 2, and 3 μg) was established and 1 μg was selected based on its ability to produce an immediate (within 5-10 minutes) increase (13 ± 3%; P < 0.001) in MABP in normotensive animals that was sustained for 60 minutes. The peak MABP response occurred at 10 minutes (20 ± 4%; P < 0.001). In addition, this dose of ICV neostigmine produced an immediate (within 5 minutes) increase in heart rate (14 ± 4%; P < 0.001), plasma glucose (37 ± 7%; P = 0.007), and circulating levels of epinephrine (399 ± 100%; P = 0.008), norepinephrine (91 ± 26%; P = 0.020), and AVP (378 ± 89%; P = 0.015) but did not significantly affect plasma levels of insulin (Table 1). IV neostigmine (1 μg) was incapable of producing a pressor response in normotensive animals confirming that these observed effects were centrally-mediated (data not shown).

**Pressor response to ICV neostigmine is elicited through peripheral catecholamine and/or AVP release.** Basal MABP in normotensive animals pre-treated with either prazosin alone or both antagonists together was reduced within 5 minutes, whereas intravenous [β-mercaptopo-β,β-cyclopentamethylenepropionyl1, O-me-Tyr2, Arg8]-vasopressin alone did not alter basal MABP in normotensive animals (Table 2). Both prazosin and [β-Mercapto-β,β-cyclopentamethylenepropionyl1, O-me-Tyr2, Arg8]-vasopressin blunted the pressor response to ICV neostigmine in the first ten minutes after injection. The antagonists in combination abolished the pressor effect of ICV neostigmine (Figure 2). MABP in control groups (IV saline + ICV water, IV prazosin + ICV water, and IV V1a antagonist + ICV water) remained unchanged throughout the time course; however, the IV V1a antagonist + ICV water group had a decrease in MABP at 30 post ICV water injection.
Impact of ICV neostigmine on MABP during acute alcohol intoxication. Acute alcohol intoxication lowered basal MABP by 12 ± 4% (P < 0.001) 30 minutes following administration. ICV neostigmine completely reversed the drop in MABP caused by acute alcohol intoxication (Figure 3). ICV water did not alter alcohol-induced hypotension.

Impact of ICV neostigmine on MABP response to hemorrhage. Removal of 40% of the estimated circulating blood volume produced a 33 ± 9% (P < 0.001) drop in MABP (Figure 4). ICV neostigmine injected immediately following blood loss (T = 10 minutes) was capable of reversing hemorrhage-induced hypotension without any fluid resuscitation. MABP remained elevated at 120 minutes post-hemorrhage. ICV water did not affect MABP in hypotensive animals. Survival was significantly improved in neostigmine-treated animals (100% versus 25% in water controls; P = 0.027) one week post-hemorrhage, despite the absence of fluid resuscitation.

Effects of ICV neostigmine on hemodynamic counter-regulation to hemorrhagic shock in alcohol-intoxicated animals. Removal of 40% of the estimated circulating blood volume produced a decrease in MABP to 60 ± 6 and 51 ± 5 mmHg within 10 minutes in control and alcohol-treated animals, respectively (Figure 5). Mid-hemorrhage (T = 21-36 minutes after initiating blood removal), alcohol-treated animals (alcohol/water group) were more (29 ± 2%; P < 0.001) hypotensive than dextrose controls (dextrose/water group), although both groups had approximately the same amount of blood removed at that time. Fluid resuscitation did not restore MABP to basal levels in dextrose controls (17 ± 5% from baseline; P = 0.01) or alcohol-treated animals (20 ± 5% from baseline; P = 0.01).

ICV neostigmine administered at 10 minutes into hemorrhage reversed hypotension and returned MABP to basal levels post fluid resuscitation in both dextrose controls.
(dextrose/neostigmine group) and alcohol-treated animals (alcohol/neostigmine group),

Nevertheless, alcohol-treated animals remained more hypotensive than dextrose controls (P = 0.040) at 120 minutes. MABP remained constant in dextrose controls and alcohol-treated sham animals. ICV neostigmine increased MABP by 13% in all sham animals as described above.

Mortality at 48 hours post-hemorrhage was 29% and 33% in dextrose controls and alcohol-treated animals, respectively. Survival of animals administered ICV neostigmine was 100% in both dextrose controls and alcohol-treated animals at 48 hours post-hemorrhage. At one week post-hemorrhage, survival of animals administered ICV neostigmine was 83% in both dextrose controls and alcohol-treated animals, compared to 71% in the dextrose/water group and 67% in the alcohol/water group.

Effects of ICV neostigmine on the neuroendocrine response to hemorrhage during acute alcohol intoxication. Hemorrhagic shock produced a marked 542 ± 156% (P = 0.007) and 522 ± 195% (P = 0.022) increase in circulating levels of epinephrine at the end of hemorrhage (T = 60 minutes) in dextrose controls and alcohol-treated animals, respectively (Figure 6). ICV neostigmine did not alter post-hemorrhage epinephrine levels in dextrose controls, but further enhanced the hemorrhage-induced increase in alcohol-treated animals (1193 ± 300%; P = 0.004).

Hemorrhagic shock produced a significant 123 ± 41% increase (P = 0.023) in circulating levels of norepinephrine at the end of hemorrhage (T = 60 minutes) in dextrose controls, which was prevented by alcohol (Figure 6). ICV neostigmine did not alter the hemorrhage-induced increase in plasma norepinephrine in dextrose controls, but restored the hemorrhage-induced increase in alcohol-treated animals.

Hemorrhagic shock increased circulating levels of AVP by 125 ± 58% (P = 0.064), which was prevented by alcohol (Figure 6). ICV neostigmine did not alter post-hemorrhage plasma
AVP in dextrose controls, but restored the hemorrhage-induced increase in alcohol-treated animals.
DISCUSSION

The results presented here are the first to demonstrate that inhibition of acetylcholinesterase in the central nervous system is capable of improving hemodynamic compensation and outcome from hemorrhagic shock in alcohol-intoxicated rats. These findings indicate that the improved counter-regulation occurs through enhanced release of vasoactive hormones and that central nervous system nicotinic receptors contribute to the anticholinesterase-induced pressor effect. Overall, our results demonstrate that increasing central acetylcholine availability enhances sympathetic neurotransmission in alcohol-intoxicated animals following hemorrhage. Moreover, they strongly suggest that alcohol exerts its detrimental effects on hemodynamic counter-regulation to severe blood loss through central mechanisms.

Initially we speculated that both central and peripheral mechanisms contribute to impairment of hemodynamic counter-regulation to blood loss during acute alcohol intoxication. Mechanisms that may explain this alcohol-induced phenomenon include a decreased circulating blood volume prior to hemorrhage, an impaired vascular responsiveness to vasoactive substances, and/or a blunted neuroendocrine response to hemorrhage. Preliminary findings suggest that blood volume is not markedly altered in our model of alcohol intoxication (unpublished observations). In addition, recent studies from our laboratory have provided strong evidence that vascular responsiveness is not significantly altered in alcohol-intoxicated animals (28). However, our previous studies have consistently demonstrated that the impaired hemodynamic counter-regulation to hemorrhage in alcohol-intoxicated animals is associated with suppression of catecholamine (epinephrine and norepinephrine), and arginine vasopressin (AVP) responses to severe hemorrhage (MABP maintained at 40 mmHg for 60 minutes) (30).
Taken together, these observations led us to hypothesize that the inappropriate release of vasoactive hormones during hemorrhage is the principle mechanism by which alcohol acts to impair MABP recovery following blood loss. MABP is centrally-regulated in the short-term through baroreceptor (32) and cholinergic mechanisms (3). Both pathways are thought to affect SNS neurotransmission. The baroreceptor-mediated physiological response to hemorrhage is the result of a decrease in perfusion pressure during blood loss causing baroreceptors in the aortic arch and carotid sinus to decrease their nerve firing rate. In turn, this results in enhanced sympathoexcitatory mechanisms through increased neurotransmission of cholinergic neurons in the rostral ventrolateral medulla (5, 32). Cholinergic pathways are independently involved in the central control of MABP through SNS activation as well (3, 18, 34, 35). The activation of sympathetic responses plays a central role in orchestrating hemodynamic counter-regulation to blood loss— increasing systemic vascular resistance and cardiac output which ultimately contributes to restoration of MABP (7). SNS activation produces an increase in the release of epinephrine and norepinephrine from the adrenal medulla, and additional increases in circulating levels of norepinephrine from sympathetic postganglionic noradrenergic nerve terminals (8, 43). Baroreceptor signaling and cholinergic pathways contribute to direct increases in the release of AVP from magnocellular neurons of the supraoptic nucleus and paraventricular nucleus (32, 35). AVP contributes to maintaining hemodynamic homeostasis by increasing systemic vascular resistance (through V₁ receptors), predominantly in the splanchnic circulation, as well as through increasing renal water reabsorption (through V₂ receptors) (19, 29). Together, epinephrine, norepinephrine, and AVP are three of the main vasoactive hormones that regulate cardiovascular function, particularly in response to hypotension. Our previous work suggests that alcohol alters
signaling involved in the activation of these pathways and blunts the release of these hormones, resulting in impaired hemodynamic counter-regulatory responses to hemorrhagic shock.

In the present studies, we chose to pharmacologically enhance SNS activation with neostigmine, an acetylcholinesterase inhibitor of the carbamate family of cholinomimetic agents that does not cross the blood brain barrier (4). Acetylcholinesterase inhibitors have been demonstrated to reverse the hypotension associated with hemorrhagic shock as well as improve survival in rats (2, 13, 39, 42). Our results verify reports in the literature (11, 16, 17, 22, 23) that demonstrate the ability of neostigmine to immediately activate the SNS, indicated by increases in MABP, sympathoadrenal activation and a decrease in insulin, when injected directly into the central nervous system (Table 1). We determined that 1 μg of neostigmine ICV was sufficient to increase MABP at least 20 mmHg. A dose of 0.5 mg was the minimally effective dose that was able to increase MABP by a similar degree. Based on those observations, and because of the observed side effects (e.g., shaking, salivation) that occurred once the drug was administered ICV at the higher dose, the dose of neostigmine was reduced from 1 to 0.5 μg in the final study. An increase in MABP, heart rate, plasma catecholamines, AVP, and plasma glucose and a decrease in plasma insulin were interpreted as an indication of activation of SNS activity. SNS activation inhibits insulin release from the pancreatic β cell, whereas parasympathetic nervous system activation stimulates insulin release, particularly during hyperglycemia (1). Thus, we measured insulin levels as an additional confirmation that central neostigmine administration as well as systemic physostigmine injection result in activation of the sympathetic nervous system. The same dose of neostigmine was administered IV to rule out the possibility that leakage of ICV administered drug would contribute to the measured effects. This dose of neostigmine administered systemically did not affect any of the parameters monitored. In addition, we have
confirmed that the pressor response produced by ICV neostigmine is partially mediated through enhanced catecholamine and AVP release (Figure 2) by blocking $\alpha$-adrenergic and AVP $V_1$ receptors with prazosin and $[\beta$-mercapto-$\beta,\beta$-cyclpentamethylenepropionyl$^1$, O-me-Tyr$^2$, Arg$^8$]-vasopressin, respectively. The doses for the receptor antagonists used in these studies were based on reports in the literature indicating that they are effective in inhibiting pressor responses of ICV U-46619, a thromboxane A$_2$ analog (45).

Previously we have demonstrated that a model of severe hemorrhagic shock, which involves removal of approximately 55-65% of estimated blood volume to maintain a target MABP of approximately 40 mmHg for 60 minutes, results in a blunted release of epinephrine, norepinephrine, and arginine vasopressin in alcohol-intoxicated animals (30). In this study, we used a more moderate hemorrhage model where only 50% of blood was removed from both dextrose controls and alcohol-treated animals. Because this produces a greater hypotension in alcohol-treated animals, a greater vasopressor release was expected. However, the data shows that despite the greater hypotension seen in acute alcohol intoxication, alcohol-intoxicated animals had a similar hemorrhage-induced rise in epinephrine and AVP levels, and an attenuated rise in norepinephrine levels. Thus, although the overall levels would not appear to be markedly blunted, the relative attenuated response to a greater hypotensive insult in alcohol-intoxicated animals confirmed our previous findings. Furthermore, ICV neostigmine was effective in enhancing and restoring the hemorrhage-induced increase in catecholamines and AVP, respectively, and this improved hemodynamic counter-regulation in alcohol-treated animals (Figure 6). Taken togeter, these observations lend strong support for our hypothesis implicating attenuated neuroendocrine activation as a central mechanism responsible for impaired hemodynamic stability in alcohol-intoxicated hemorrhaged animals.
It is important to note that the model of alcohol administration used in these studies reflects a human binge drinking episode, described as the consumption of five or more drinks over a period of time sufficient to elevate blood alcohol levels above intoxicating levels (31). According to the National Survey of Drug Use and Health, binge drinking has been shown to increase the risk of traumatic injury and other alcohol-related deaths in an otherwise healthy population (10, 40). Therefore, the alcohol model and blood alcohol levels achieved in these studies (192 ± 21 mg/dL) are clinically relevant.

The current studies allowed us to determine the effects of acute alcohol intoxication on outcome to severe hemorrhage. Because the ability to compensate hemodynamically following blood loss correlates with improved tissue perfusion and better outcome, MABP is an appropriate outcome measure. However, this may be thought of as a limitation of the study because heart rate and cardiac output could provide a more complete indication of tissue perfusion. It may be critical to make these measurements being that ICV neostigmine administration leads to an increase in MABP, which we speculate is due to increased SNS activity which could potentially lead to compromised blood flow to organs. Future studies are warranted to determine the effect of central acetylcholinesterase inhibition on blood flow to specific organ beds following hemorrhage using more direct measures.

ICV administration of drugs allows direct administration into the central nervous system, so that the effects of the drugs on the central nervous system can be observed. However, one limitation is the lack of neuroanatomical specificity of the effects elicited. Additional studies are warranted to identify the exact location of action by administration of the drugs into specific brain regions and/or ablation of certain brain regions.

**Perspectives and Significance**
Results from these studies and those reported in the literature indicate that ICV neostigmine enhances central cholinergic activity, activates the SNS, and improves catecholaminergic and vasopressinergic responses to hemorrhagic shock in alcohol-intoxicated rats. Restoring the neuroendocrine response through ICV neostigmine reverses hypotension during and following hemorrhagic shock in alcohol-intoxicated animals. These results suggest that the inappropriate neuroendocrine response during hemorrhagic shock is a central mechanism involved in hemodynamic instability in alcohol-intoxicated animals. Moreover, they provide preclinical evidence for a novel approach to management of the alcohol-intoxicated traumatic injury victim that may be advantageous over present management and care. An approach to reduce the volume of resuscitation fluid necessary to restore MABP and tissue perfusion following severe hemorrhage would likely result in improved outcomes, and decreased cost of management of the trauma/hemorrhage victim. Central administration of pharmacotherapies during fluid resuscitation in the clinical setting is not a practical approach to treat a trauma/hemorrhage victim. Therefore, in order to translate our findings to a clinically relevant scenario, studies using intravenous injection of a centrally-acting acetylcholinesterase inhibitor are warranted. Additional investigation on the impact of these drugs on tissue and organ injury, as well as their impact on metabolic and inflammatory responses to hemorrhage, are also warranted.
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GRANTS

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Measures of SNS activity following central acetylcholinesterase inhibition via ICV neostigmine administration

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>5 minutes</th>
<th>10 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP (mmHg)</td>
<td>125 ± 4</td>
<td>142 ± 4*</td>
<td>150 ± 5*</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>374 ± 18</td>
<td>426 ± 16*</td>
<td>364 ±18</td>
</tr>
<tr>
<td>Epinephrine (pg/mL)</td>
<td>204 ± 29</td>
<td>615 ± 145</td>
<td>1019 ± 205*</td>
</tr>
<tr>
<td>Norepinephrine (pg/mL)</td>
<td>233 ± 48</td>
<td>445 ± 60*</td>
<td>243 ± 30</td>
</tr>
<tr>
<td>AVP (pg/mL)</td>
<td>7.64 ± 1.0</td>
<td>36.6 ± 6.8*</td>
<td>47.0 ± 9.1*</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>127 ± 6.2</td>
<td>174 ± 8.3*</td>
<td>214 ± 2.6*</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>1.03 ± 0.2</td>
<td>0.82 ± 0.2</td>
<td>0.93 ± 0.02</td>
</tr>
</tbody>
</table>

**Table 1:** Hemodynamics and plasma hormone and glucose levels in normotensive rats (n = 3) following ICV neostigmine (1 μg) administration. MABP: mean arterial blood pressure; AVP: arginine vasopressin. Data are presented as mean ± SEM and were analyzed using one-way ANOVA with repeated measures. *P versus basal level (P < 0.05 is significant).
Effect of antagonist pre-treatment on basal MABP

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Following pre-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prazosin</td>
<td>115 ± 4</td>
<td>92 ± 4*</td>
</tr>
<tr>
<td>V1a receptor antagonist</td>
<td>124 ± 3</td>
<td>125 ± 4</td>
</tr>
<tr>
<td>Both antagonists</td>
<td>138 ± 7</td>
<td>86 ± 6*</td>
</tr>
</tbody>
</table>

Table 2: Effect of intravenous α₁-adrenerceptor antagonist (prazosin; 0.5 mg/kg) and/or intravenous V1a receptor antagonist ([β-mercapto-β,β-cyclopentamethylenepropionyl¹, O-methyl-Tyr², Arg⁸]-vasopressin; 10 μg/kg) pretreatment on mean arterial blood pressure (MABP; mmHg) in normotensive rats at baseline (n = 8-12). MABP recordings were measured at 5 minutes following antagonist administration. This graph contains the absolute basal values of MABP data represented in Figure 2. Data are presented as mean ± SEM and were analyzed using one-way ANOVA with repeated measures. *P versus pre-treatment time point (P < 0.05 is significant).
FIGURE LEGENDS

Figure 1  **Experimental design:** Investigating the impact of acute alcohol intoxication on hemorrhagic shock. Star represents administration of bolus of alcohol (2.5 g/kg). Details are outlined in *Materials and Methods*.

Figure 2  **Effects of catecholamine and vasopressin receptor blockade on the pressor response to ICV neostigmine:** Intravenous $\alpha_1$-adrenerceptor antagonist (prazosin; 0.5 mg/kg) and/or intravenous $V_{1a}$ receptor antagonist ([\(\beta\)-mercaptop-\(\beta\),\(\beta\)-cyclopentamethylenepropionyl\textsuperscript{1}, O-me-Tyr\textsuperscript{2}, Arg\textsuperscript{8}\)]-vasopressin; 10 $\mu$g/kg) pretreatment prior to 1 $\mu$g neostigmine ICV or ICV water (n = 3-12). The absolute basal MABP for the rats used in this study is located in Table 2. Groups are presented as IV pre-treatment + ICV treatment (e.g., IV saline + ICV water). Data are presented as mean ±SEM and were analyzed using two-way ANOVA with repeated measures. *P versus T = 0 min; †P versus both IV antagonists + ICV neostigmine group; ‡P versus IV prazosin + ICV neostigmine group; §P versus IV $V_{1a}$ antagonist + ICV neostigmine group (P < 0.05 is significant).

Figure 3  **Effects of ICV neostigmine on MABP in alcohol-treated rats:** ICV neostigmine (1$\mu$g) reverses alcohol-induced hypotension (n = 4-5).

MABP: mean arterial blood pressure. Data are presented as mean ± SEM and were analyzed using two-way ANOVA with repeated measures. *P
versus pre-alcohol time point; ^P versus water group (P < 0.05 is significant).

**Figure 4**  
**Effects of ICV neostigmine on MABP following moderate blood loss:**  
ICV neostigmine (1 µg) administered 10 minutes following 40% blood loss (no fluid resuscitation) reverses hemorrhage-associated hypotension (n = 4-6). MABP: mean arterial blood pressure. Data are presented as mean ± SEM and were analyzed using two-way ANOVA with repeated measures. ^P versus T = 0 minutes; ^P versus water group (P < 0.05 is significant).

**Figure 5**  
**Impact of ICV neostigmine on hemodynamic response to hemorrhage during acute alcohol intoxication:** Effects of neostigmine (0.5 µg) or sterile water (5 µL) on mean arterial blood pressure (MABP) in dextrose controls and alcohol-treated animals subjected to 50% hemorrhage and fluid resuscitation (n = 7-9). Data are presented as mean ± SEM and were analyzed using two-way ANOVA with repeated measures. ^P versus dextrose/water group; ^P versus alcohol/water group; @P versus T = 0 time point (P < 0.05 is significant).

**Figure 6**  
**ICV neostigmine improves neuroendocrine response to hemorrhage during acute alcohol intoxication:** Effects of neostigmine (0.5 µg) or sterile water (5 µL) on plasma epinephrine, norepinephrine and arginine vasopressin (AVP) in pg/mL in dextrose controls (left column) and alcohol-treated animals (right column) subjected to 50% hemorrhage and fluid resuscitation (n = 7-9). Data are presented as mean ± SEM and were
analyzed using two-way ANOVA with repeated measures. *P versus basal; †P versus water/hemorrhage group (P < 0.05 is significant).
REFERENCES


