Augmented central nitric oxide production inhibits vasopressin release during hemorrhage in acute alcohol-intoxicated rodents

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Whitaker AM, Sulzer JK, Molina PE. Augmented central nitric oxide production inhibits vasopressin release during hemorrhage in acute alcohol-intoxicated rodents. Am J Physiol Regul Integr Comp Physiol 301: R1529–R1539, 2011. First published August 17, 2011; doi:10.1152/ajpregu.00035.2011.—Acute alcohol intoxication (AAI) attenuates the AVP response to hemorrhage, contributing to impaired hemodynamic counter-regulation. This can be restored by central cholinergic stimulation, implicating disrupted signaling regulating AVP release. AVP is released in response to hemorrhage and hyperosmolality. Studies have demonstrated nitric oxide (NO) to play an inhibitory role on AVP release. AAI has been shown to increase NO content in the paraventricular nucleus. We hypothesized that the attenuated AVP response to hemorrhage during AAI is the result of increased central NO inhibition. In addition, we predicted that the increased NO tone during AAI would impair the AVP response to hyperosmolality. Conscious male Sprague-Dawley rats (300–325 g) received a 15-h intragastric infusion of alcohol (2.5 g/kg + 300 mg/kg·h) or dextrose prior to a 60-min fixed-pressure hemorrhage (−40 mmHg) or 5% hypertonic saline infusion (0.05 ml/kg·min). AAI attenuated the AVP response to hemorrhage, which was associated with increased paraventricular NO content. In contrast, AAI did not impair the AVP response to hyperosmolality. This was accompanied by decreased paraventricular NO content. To confirm the role of NO in the alcohol-induced inhibition of AVP release during hemorrhage, the nitric oxide synthase inhibitor, nitro-L-arginine methyl ester (L-NAME; 250 μg/5 μl), was administered centrally prior to hemorrhage. L-NAME did not further increase AVP levels during hemorrhage in dextrose-treated animals; however, it restored the AVP response during AAI. These results indicate that AAI impairs the AVP response to hemorrhage, while not affecting the response to hyperosmolality. Furthermore, these data demonstrate that the attenuated AVP response to hemorrhage is the result of augmented central NO inhibition.

hypovolemia; osmolality; ethanol; mean arterial blood pressure; L-NAME

TRAIUMATIC INJURY RANKS AS the number one cause of death for the 1- to 44-yr-old age group and the 5th leading cause of death overall (18). Acute alcohol intoxication (AAI) contributes to the increased risk of traumatic injury, with intoxicating blood alcohol levels present in more than 40% of injured patients (11, 37, 49). Patients with positive blood alcohol concentrations at the time of arrival to the emergency department compared with nonintoxicated individuals (16, 49). Results from clinical studies suggest that low mean arterial blood pressure (MABP) at the time of arrival into the emergency room is a predictor of poor patient outcome from traumatic injury and blood loss (25, 27); therefore it is critical to understand the mechanisms contributing to the greater hypotension seen in the alcohol-intoxicated trauma patient.

Studies from our laboratory have shown that AAI decreases basal MABP, exacerbates hypotension during hemorrhage, and attenuates blood pressure recovery during fluid resuscitation (43). The greater hypotension during AAI is associated with an inappropriate neuroendocrine response characterized by attenuated hemorrhage-induced rises in circulating AVP and catecholamines (43). Additional in vitro studies using isolated aortic and mesenteric arterioles have shown that the vascular responsiveness to pressor agents is not altered during AAI, indicating that the blunted neuroendocrine activation is most likely the principal mechanism contributing to impaired hemodynamic counter-regulation to hemorrhagic shock (42). Moreover, central cholinergic stimulation by intracerebroventricular administration of neostigmine, an ACh esterase inhibitor, restores the AVP response to hemorrhage during AAI, suggesting that AAI interferes with the central signaling mechanisms regulating AVP release during hemorrhagic shock (39).

AVP is a critical counter-regulatory hormone contributing to the recovery of blood pressure during hemorrhagic shock. Administration of a V1 receptor antagonist during hemorrhage exacerbates hypotension in response to blood loss and attenuates the restoration of blood pressure during the resuscitation period (7, 19). This suggests that an intact AVP response is critical to blood pressure control during hemorrhage. Several clinical case reports, as well as animal studies, have provided supporting evidence for the importance of AVP release for blood pressure recovery following hemorrhagic shock (12, 44, 57, 70). Compared with norepinephrine as a vasopressor following hemorrhage, AVP-treated animals have a more sustained increase in MABP and improved survival (70). Clinical and animal studies have demonstrated efficacy of AVP administration in improving blood pressure recovery in subjects unresponsive to fluid resuscitation or other vasopressors following severe blood loss (44, 57). Taken together, these reports indicate that AVP is a critical counter-regulatory hormone in response to blood loss; thus, the current study focused on the mechanisms involved in the impaired release of AVP in response to hemorrhage during AAI.

AVP is produced primarily in the magnocellular neurons of the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus and is secreted into systemic circulation from the posterior pituitary in response to decreases
in circulating blood volume and increases in plasma osmolality (19, 24, 53, 68). The present study aimed to examine how AAI affects the AVP response to decreases in circulating blood volume and increases in plasma osmolality. Under normovolemic and isoosmotic conditions, AVP release from the magnocellular neurons is tonically inhibited by nitric oxide (NO) (29, 30, 31). Alcohol intoxication increases NO content in the PVN (55). Thus, we hypothesized that increased inhibitory nitricergic tone contributes to the alcohol-induced attenuation of the AVP response to hemorrhage and would impair the AVP response to hyperosmolality.

MATERIALS AND METHODS

Animal Preparation

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center (LSUHSC) and were in accordance with the National Institutes of Health guidelines. Specific pathogen-free adult male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC), weighing 225–250 g at the time of arrival, were housed in the Division of Animal Care at LSUHSC and were exposed to a 12:12-h light-dark cycle and fed a standard rat diet (Purina Rat Chow,Ralston Purina, St. Louis, MO) ad libitum for 1 wk prior to surgery.

Surgical Preparation

Intracerebroventricular cannula placement. For central drug administration, a guide cannula was implanted in the lateral ventricle 7 days prior to vascular surgery, as previously described by our laboratory (39). Animals weighing 300–325 g were anesthetized with ketamine/xylazine (90 mg/kg and 9 mg/kg, respectively) and positioned in a stereotaxic apparatus. The overlying skin was cleared, and a hole was drilled in the skull (stereotaxic coordinates for guide cannula: 0.3 mm posterior to bregma, 1.3 mm lateral from midline, and 4.25 mm below the skull’s surface). A 22-gauge stainless-steel guide cannula was inserted into the right lateral ventricle of the brain, secured using dental cement, and anchored to the skull with three stainless-steel screws. A wire dummy cannula was used to seal the guide cannula until the time of the experiment. Following cannula placement, animals were returned to clean individual cages and allowed to recover for 7 days and provided food and water ad libitum prior to vascular/gastric catheter placement. Cannula placement was assessed by dipsogenic response to ANG II (100 ng/rat; Sigma, St. Louis, MO) on the day of vascular/gastric catheter placement.

Vascular/gastric catheter placement. Animals were anesthetized with an intramuscular injection of ketamine/xylazine (90 mg/kg and 9 mg/kg, respectively). Using sterile procedures, polyethylene catheters (PE-50; BD Diagnostic Systems, Sparks, MD) were inserted into the left carotid artery and advanced to the aortic arch and into the right jugular vein (43). An additional catheter (PE-50; BD Diagnostic Systems) was inserted into the antrum of the stomach, and secured with a purse-string suture. Gastric catheters were used for alcohol or dextrose administration. The catheter was flushed with saline, sealed, and routed subcutaneously to the nape of the neck where they were exteriorized through a small incision and secured with tape. After surgery, the animals were placed in individual cages, allowed to recover from anesthesia, and given food and water ad libitum for 2 or 3 days before alcohol or dextrose administration.

Alcohol and Dextrose Administration

The evening prior to the experiment, animals were randomly selected to receive an intragastric bolus of 30% alcohol (2.5 g/kg) followed by a 15-h constant infusion of alcohol (300 mg·kg⁻¹·h⁻¹) or isocaloric/isoosmotic 52% dextrose (12 g/kg) via the gastric catheter connected to an infusion pump (Harvard Apparatus PHD 2000 syringe pump; Holliston, MA). Animals were conscious and unrestrained, and they were restricted of food and water throughout the duration of the alcohol or dextrose infusion. This method of alcohol administration was selected because it resembles the route of alcohol consumption in humans, mimics a binge-drinking episode that frequently precedes a traumatic injury and presentation to the emergency department, and achieves intoxicating levels of blood alcohol (270 ± 30 mg/dl) (20, 37).

Experimental Protocols

All animals were subjected to the same animal procedures, surgical procedures, and alcohol or dextrose administration, prior to initiating the experimental protocols. Animals were conscious and unrestrained throughout the duration of the experiments.

Study I. Impact of AAI on NO content and nitric oxide synthase activity in the hypothalamic PVN and SON. To determine whether AAI increases NO content and nitric oxide synthase (NOS) activity in the PVN and SON, two experimental groups were used: dextrose-treated and alcohol-treated animals (n = 7–10). At the completion of the dextrose or alcohol infusion, conscious animals were decapitated, and the whole brain was immediately excised and flash-frozen in liquid nitrogen. The hypothalamus was isolated using the optic chiasm on the ventral surface as a landmark. Briefly, the whole brain was placed on its dorsal surface, and a 1-mm slice cut posterior to anterior margin of the optic chiasm. The PVN was isolated from the section with a scalpel. The SON was removed using a 1-mm diameter neuropunch. Nitrate/nitrite content and NOS activity were determined in the PVN and SON.

Study II. Impact of AAI on NO inhibitory signaling mechanisms modulating the AVP response to hemorrhagic shock. To characterize the time course of the effects of AAI on the AVP response to hemorrhage, animals were subjected to a fixed-pressure hemorrhage at the completion of the alcohol or dextrose infusion (n = 7–10), as previously described by our laboratory (42). Briefly, the carotid catheter was exteriorized and connected to a pressure transducer (MacLab, Colorado Springs, CO) for continuous blood pressure recording. Blood was withdrawn from the carotid catheter into a heparinized syringe to achieve a MABP of ~40 mmHg. Additional blood was removed to maintain MABP between 40 and 55 mmHg for 60 min. Arterial blood samples (1.0 ml) were obtained at baseline and at 5, 20, and 60 min during the hemorrhage for determination of plasma osmolality and AVP levels. At the completion of the hemorrhage, conscious animals were decapitated, and whole brains were immediately excised and flash-frozen in liquid nitrogen. The PVN and SON were isolated for determination of nitrate/nitrite content and NOS activity.

Study III. Impact of AAI on NO inhibitory signaling mechanisms modulating AVP in response to a hyperosmotic challenge. To determine whether AVP release is blunted in response to increased plasma osmolality, alcohol- and dextrose-treated animals were subjected to a hyperosmotic challenge (n = 8–10). At the completion of the 15-h alcohol or dextrose infusion, animals received a continuous small volume intravenous infusion of 5% hypertonic saline (0.05 ml·kg⁻¹·min⁻¹) for 60 min (1.2 ml total volume over the 60-min period). A 5% hypertonic saline solution was chosen on the basis of preliminary studies demonstrating its ability to produce a significant increase in plasma osmolality that was sustained throughout the infusion period with no change in MABP. Blood samples (1.0 ml) were taken at baseline and at 5, 20, and 60 min during the hypertonic saline infusion, and the volume was replaced with isotonic saline. At the completion of the hypertonic saline infusion, conscious animals were decapitated and whole brains were immediately excised and flash-frozen in liquid nitrogen. The PVN and SON were isolated for determination of nitrate/nitrite content and NOS activity.
Study IV. Effect of intracerebroventricular nitro-l-arginine methyl ester on hemodynamic and AVP response to hemorrhage during AAI.

To determine whether central NOS inhibition would restore AVP levels in response to hemorrhage during AAI, animals were randomly divided into sham (nonhemorrhaged) or hemorrhage groups. There were a total of eight treatment groups (n = 5–8 per group) in this experiment: dextrose/vehicle/sham, dextrose/vehicle/hemorrhage, dextrose/nitro-l-arginine methyl ester (l-NAME)/sham, dextrose/l-NAME/hemorrhage, alcohol/vehicle/sham, alcohol/vehicle/hemorrhage, alcohol/l-NAME/sham, and alcohol/l-NAME/hemorrhage. Following the dextrose or alcohol infusion, animals were randomly selected to receive an injection (5 μl icv) of vehicle (sterile water), or the NOS inhibitor, l-NAME (Sigma; 250 μg/5 μl) 15 min prior to a fixed-pressure hemorrhage (as described above). This dose was selected because it has been shown to produce maximal NOS inhibition in the brain for up to 6 h (31, 50). MABP was recorded continuously during hemorrhage. Arterial blood samples were obtained at baseline (prehemorrhage) and at 5, 20, and 60 min during the hemorrhage period for determination of blood alcohol levels and circulating AVP levels. At the completion of the hemorrhage, conscious animals were decapitated, and the whole brains were immediately excised and flash-frozen in liquid nitrogen. The PVN and SON were isolated for determination of nitrate/nitrite content and NOS activity.

Analytical Procedures

Blood sample analysis. Arterial blood samples (1 ml) were collected in chilled heparinized syringes and placed in tubes containing 10 μl/ml of aprotinin (Sigma, St. Louis, MO). Blood was centrifuged for 10 min at 10,000 rpm and 4°C. Plasma was collected and stored at −80°C for hormone analysis. Blood alcohol levels were determined using an amperometric oxygen electrode (Analox Instruments Limited, London, UK). Plasma osmolality was measured using a vapor pressure osmometer (5500 vapor pressure osmometer; Wescor, Logan, UT).

Arginine vasopressin measurements. Circulating AVP levels were determined in plasma extracts. Briefly, plasma samples were acidified with an equal volume of 1% trifluoroacetic acid in water (buffer A). C-18 SEP-Columns (Waters, Milford, MA) were pretreated with buffer B (60% acetonitrile + 40% buffer A) followed by buffer A. The acidified plasma was loaded onto the pretreated column and washed three times with buffer A. The peptides were eluted with 3 ml of buffer B and evaporated to dryness using a Speed Vac concentrator and condensation trap. The residue was reconstituted using the radioimmunoassay kit buffer provided by the AVP kit. Plasma AVP levels were measured using a rat-specific radioimmunoassay kit (Phoenix Pharmaceuticals, Burlingame, CA) with a coefficient of variation of 9% (interassay) and 1% (intra-assay). The range of detection for the AVP radioimmunoassay was 10–1280 pg/ml.

Brain tissue analysis. The hypothalamic brain sections were weighed and stored at −80°C until analysis. On the day of the assay, the brain tissue was homogenized in PBS (100 μl/mg tissue). The samples were centrifuged for 10 min at 10,000 rpm, and the supernatant was collected. NOS activity in the supernatant was measured using a standard assay kit and is expressed as micromoles of NO produced per milligram protein per minute (Oxford Biomedical Research, Oxford, MS). Protein was measured using Pierce BCA standard protein assay (Thermo Fisher Scientific, Rockford, IL). For determination of NO content, the supernatant was filtered using a 30-kDa molecular weight cut-off filter (Millipore, Billerica, MA). Stable NO metabolites (nitrate/nitrite) in the filtered supernatant were measured using a NO colorimetric assay kit (Cayman Chemical, Ann Arbor, MI), which is based on the biochemical conversion of l-arginine to l-citrulline by NOS.

Statistical Analysis

All data are expressed as means ± SE with the number of animals per group indicated in the figure legends. Statistical analysis of differences in MABP, osmolality, NO content, NOS activity, and AVP levels were determined by two-way ANOVA with or without repeated measures using Sigma Plot 11.0 statistical software. Pairwise multiple comparisons were determined using the Holm-Sidak method. Statistical significance was set at P < 0.05.

RESULTS

Impact of AAI on NO Content and NOS Activity in the Hypothalamic PVN and SON

In the PVN, AAI produced a significant 70 ± 10% increase in NO content compared with dextrose-treated controls at the end of the 15-h infusion (33 ± 12 μmol/mg tissue vs. 9 ± 4 μmol/mg tissue; Fig. 1A). There were no differences in NOS activity between alcohol- or dextrose-treated animals (0.34 μmol NO-mg protein−1·min−1 vs. 0.29 μmol NO-mg protein−1·min−1; Fig. 1B). In the SON, AAI produced a significant increase in NO content (28 ± 6 μmol/mg tissue vs. 12 ± 2 μmol/mg tissue) compared with dextrose-treated controls (Fig. 1C). In addition, NOS activity was significantly increased (33 ± 4%) in alcohol-treated animals in the SON compared with dextrose-treated controls (Fig. 1D).

Impact of AAI on NO Inhibitory Signaling Mechanisms Modulating the AVP Response to Hemorrhagic Shock

AAI decreased the total amount of blood removed to achieve a fixed pressure of ~40 mmHg compared with dextrose-treated animals (57 ± 2% vs. 65 ± 3%). No differences were noted between dextrose- and alcohol-treated animals in plasma osmolality during hemorrhage (Fig. 2A). There were no differences between alcohol- and dextrose-treated animals in baseline values of circulating AVP (27 ± 1 pg/ml and 28 ± 3 pg/ml, respectively; Fig. 2B). Hemorrhage produced a significant increase in AVP levels (81 ± 17 pg/ml vs. 27 ± 1 pg/ml; P < 0.05) in dextrose-treated animals at the completion of hemorrhage (T = 60 min) compared with baseline values (Fig. 2B). This response was significantly attenuated by alcohol compared with dextrose-treated hemorrhaged animals (45 ± 5 pg/ml vs. 81 ± 17 pg/ml; respectively).

There were no differences in NO content in the PVN at the end of hemorrhage in dextrose-treated animals compared with baseline values (9 ± 4 μmol NO/mg tissue vs. 9 ± 3 μmol NO/mg tissue; Fig. 1A). NO content in the PVN of intoxicated animals were markedly elevated 62 ± 2% compared with dextrose-treated animals at the end of hemorrhage (Fig. 1A). NOS activity was significantly higher in the PVN of both dextrose- and alcohol-treated animals at the end of hemorrhage compared with values obtained at the completion of the infusion (Fig. 1B). Furthermore, NOS activity in alcohol-treated animals was 62 ± 5% higher than dextrose-treated animals at the completion of hemorrhage (Fig. 1B). In the SON, there were no differences between dextrose- or alcohol-treated animals in NO content at the end of hemorrhage; however, NOS activity was significantly elevated in both dextrose- and alcohol-treated animals compared with baseline values (Fig. 1C, D).
Impact of AAI on NO Inhibitory Signaling Mechanisms
Modulating AVP in Response to a Hyperosmotic Challenge

Hypertonic saline (5%; 0.05 ml·kg⁻¹·min⁻¹) infusion resulted in an immediate (T = 5 min) 6 ± 0.04% increase in plasma osmolality in both dextrose- and alcohol-treated animals, which was sustained throughout the duration of the infusion (Fig. 2C). There were no differences in baseline values of circulating AVP levels between dextrose- and alcohol-treated animals (22 ± 1 pg/ml vs. 24 ± 1 pg/ml, respectively; Fig. 2D). Hypertonic saline produced a significant increase in AVP in both dextrose controls (26 ± 0.05%) and alcohol-treated animals (27 ± 0.04%) at 5 min of the hypertonic saline infusion compared with treatment-matched baseline values (Fig. 2D). This was maintained throughout the infusion. There were no differences between dextrose- and alcohol-treated animals in the AVP response to the hypertonic saline infusion.

In response to the hypertonic saline infusion, there were no differences in NO content in dextrose-treated animals compared with baseline values in the PVN; however, hypertonic saline produced a significant 74 ± 5% decrease in NO content in the PVN of alcohol-treated animals compared with baseline values (Fig. 1C). There were no differences in NOS activity in the SON in dextrose-treated animals compared with baseline values; however, there was a significant increase in NOS activity in alcohol-treated animals compared with treatment-matched baseline values (Fig. 1D). There were no differences noted in NO content or NOS activity in the PVN or SON of the hypothalamus at the completion of the hypertonic saline infusion in alcohol-treated animals compared with dextrose-treated controls.

Effect of ICV L-NAME on Hemodynamic and AVP Response to Hemorrhage During AAI

All animals were subjected to a similar degree of hypotension during the fixed-pressure hemorrhage (Fig. 3A). MABP averaged 113 ± 3 mmHg at the start of hemorrhage (T = 0 min) and decreased to an average of 55 ± 1 mmHg in the dextrose/vehicle-treated animals. An average of 72 ± 3% of the total blood volume per kilogram of body weight was removed from dextrose/vehicle-treated animals during the 60-min hemorrhage period to achieve a fixed pressure of ~40–55 mmHg. MABP averaged 112 ± 3 mmHg at the start of hemorrhage (T = 0 min) and decreased to an average of 51 ± 0.8 mmHg in the alcohol/vehicle-treated animals. A significantly lower amount of blood (62 ± 3% of the total blood volume) was removed from alcohol/vehicle-treated animals during the hemorrhage period. MABP averaged 124 ± 8 mmHg at the start of hemorrhage (T = 0 min) and decreased

Fig. 1. Effects of acute alcohol intoxication, hemorrhage, and hypertonic saline on NO content and NOS activity in the PVN and SON: A: nitrate/nitrite (µmol/mg tissue) in the PVN. B: NOS activity (µmol NO·mg protein⁻¹·min⁻¹) in the PVN. C: nitrate/nitrite (µmol/mg tissue) in the SON. D: NOS activity (µmol NO·mg protein⁻¹·min⁻¹) in the SON measured in dextrose-treated (white bars) and alcohol-treated (black bars) animals at baseline (the completion of the alcohol infusion), end of hemorrhage or end of hypertonic saline infusion (n = 4–10). Data are presented as means ± SE and were analyzed using a two-way ANOVA. *P < 0.05 vs. dextrose-treated animals; #P < 0.05 vs. treatment-matched baseline values.
to an average of 57 ± 3 mmHg in the dextrose/L-NAME-treated animals. MABP averaged 115 ± 3 mmHg at the start of hemorrhage (T = 0 min) and decreased to an average of 52 ± 2 mmHg in the alcohol/L-NAME-treated animals. Intracerebroventricular L-NAME administered 15 min prior to hemorrhage did not alter the total amount of blood removed to achieve a target MABP of 40–55 mmHg in dextrose/L-NAME-treated animals; however, it significantly increased the total amount of blood removed during the fixed-pressure hemorrhage (72 ± 1% vs. 62 ± 3% total blood volume) in alcohol/L-NAME-treated animals compared with alcohol/vehicle-treated animals (Fig. 3B). There were no significant differences in heart rate among the groups (data not shown).

Hemorrhagic shock produced a marked 89 ± 0.3% increase in circulating AVP levels at the end of the hemorrhage period (T = 60 min) in dextrose/vehicle-treated animals compared with baseline values (Fig. 4). This was not further enhanced by intracerebroventricular administration of L-NAME (92 ± 0.2% increase from baseline values). AAI (alcohol/vehicle-treated animals) produced a marked 45 ± 2% increase in NO content (10 ± 2 μmol/mg tissue vs. 5 ± 0.5 μmol/mg tissue) and a 28 ± 6% increase in NOS activity (0.7 ± 0.08 μmol·mg protein⁻¹·min⁻¹ vs. 0.5 ± 0.12 μmol·mg protein⁻¹·min⁻¹) in the PVN compared with dextrose/vehicle-treated controls (Fig. 5, A and B). Intracerebroventricular administration of the NOS inhibitor, L-NAME, produced a significant decrease in NO content at the end of hemorrhage in both dextrose controls (41 ± 3%) and alcohol-treated animals (44 ± 10%) compared with treatment-matched (alcohol or dextrose)/vehicle-treated animals. This was associated with a decrease in NOS activity in dextrose- (35 ± 8%) and alcohol/L-NAME-treated animals (61 ± 5%) compared with treatment-matched (dextrose or alcohol)/vehicle-treated animals. Furthermore, L-NAME decreased NO content in dextrose- (38 ± 4%) and alcohol/vehicle-treated (56 ± 6%) sham animals. This was associated with a decrease in NOS activity in dextrose- (30 ± 11%) and alcohol-treated (52 ± 7%) sham animals. In the SON, there were no differences in NO content between dextrose/vehicle- and alcohol/vehicle-treated animals (14 ± 2 μmol/mg tissue vs. 20 ± 4 μmol/mg tissue, respectively); however, there was a significant 14 ± 6% increase in NOS activity in alcohol/vehicle-treated animals compared with dextrose/vehicle-treated control animals (Fig. 5, C and D). ICV administration of the NOS inhibitor, L-NAME, produced a significant 56 ±
6% decrease in NO content in the SON of alcohol/L-NAME-treated animals compared with alcohol/vehicle-treated animals; however, there were no differences in NOS activity.

**DISCUSSION**

The present study examined the effects of AAI on the nitrergic regulatory mechanisms modulating AVP release in response to hemorrhage and hyperosmolality. In agreement with previous findings from our laboratory, these results demonstrate that AAI markedly attenuates the AVP response to hemorrhagic shock (43). Furthermore, we observed a significant increase in NO content, a known inhibitor of AVP release, in the hypothalamic PVN and SON at the completion of the alcohol infusion. NO content remained significantly elevated in the PVN of alcohol-intoxicated animals at the completion of hemorrhage. In contrast, AAI did not impair the AVP response to an osmotic challenge. This was accompanied by a significant decrease in NO content in the PVN of alcohol-intoxicated animals. To confirm the role of NO in the alcohol-induced inhibition of AVP release during hemorrhage, the NOS inhibitor L-NAME was administered intracerebroventricularly prior to hemorrhage. Our results clearly demonstrate that central NOS inhibition restores the AVP response to hemorrhage during AAI. These data suggest that the alcohol-induced impairment of the AVP response to hemorrhage is the result of augmented central NO inhibition.

NO has been shown to play an important role in regulating AVP release. NO is locally produced from the conversion of L-arginine to L-citrulline by NOS (1, 63). In the central nervous system, NO functions as an important signaling molecule regulating neuroendocrine function in the hypothalamus (29, 30, 31, 55). Studies have shown that NO is present in the magnocellular neurons in the PVN and SON of the hypothalamus, the key sites of AVP production (9, 29, 30, 36). AVP is also produced to a lesser extent in the parvocellular neurons, where it functions to potentiate the actions of corticotropin-releasing hormone on the corticotroph cells of the anterior pituitary (2, 13). Magnocellular and parvocellular neurons are differentially regulated by NO (5, 34, 59, 61). NO exerts inhibitory effects on the magnocellular neurons, it has been shown to stimulate parvocellular neurons (5, 34, 59, 61). Patch-clamp recordings from magnocellular and parvocellular neurons show that administration of the NO donor, N-acetyl-S-nitroso-D-penicillamine, produces reversible membrane depolarization in parvocellular neurons, while it inhibits the activity of magnocellular neurons (5, 34, 59). Alcohol intoxication has been shown to selectively affect NO production in different brain regions (4, 55, 66). AAI has been shown to decrease NO production in various cortical neurons and increase NO production in circumventricular organs (4, 66). Other studies examining the effects of AAI on NO production...
have shown that a single acute intraperitoneal injection of alcohol (4.5 g/kg) results in a significant increase in NO content in the plasma, anterior pituitary, and PVN of the hypothalamus (55). Furthermore, AAI increases NOS activity in the anterior pituitary and PVN (55). In the present study, we observed an increase in NO content and NOS activity in the PVN and SON in response to AAI. In addition, NO content and NOS activity in the PVN remained elevated at the end of hemorrhage in alcohol-intoxicated animals.

Taken together, these observations led us to hypothesize that the alcohol-induced impairment in the AVP response to hemorrhage was the result of accentuated NO inhibitory tone in the PVN. To test this hypothesis, we administered the NOS inhibitor L-NAME intracerebroventricularly 15 min prior to hemorrhage at a dose of 250 μg/5 μl L-NAME. L-NAME was selected because it is a nonselective NOS inhibitor (inhibiting neuronal NOS, endothelial NOS, and inducible NOS) allowing for complete NOS inhibition. The dose was selected on the basis of observations by Kadekaro et al. (31), demonstrating significant NOS inhibition in the PVN immediately following intracerebroventricular administration of 250 μg/5 μl L-NAME (31). Additional studies have demonstrated an onset of NOS inhibition within 15 min after administration and effects that last up to 6 h in several brain structures following intracerebroventricular administration of L-NAME; therefore, we chose to wait 15 min following intracerebroventricular administration of L-NAME prior to initiating the fixed-pressure hemorrhage (50). To confirm NOS inhibition in our model, NO content and NOS activity were measured in the PVN and SON at the completion of hemorrhage. In the present study, we observed a 41% decrease in NO content and a 35% decrease in NOS activity in the PVN of dextrose/L-NAME-treated animals compared with dextrose/vehicle-treated animals at the end of hemorrhage (75 min after L-NAME administration). In alcohol-treated animals, intracerebroventricular administration of L-NAME resulted in a 44% decrease in NO content and a 61% decrease in NOS activity in the PVN compared with alcohol/vehicle-treated animals at the completion of the hemorrhage period. NO content in the SON, however, was not altered by intracerebroventricular L-NAME administration. A potential reason for the contrasting effects between the PVN and SON could be limited access of the drug to the SON. Additionally, PVN NO content could have been modulated by afferent inputs to this region from areas that were also subjected to high concentrations of the drug. In dextrose-treated animals, NOS inhibition did not further increase circulating AVP levels following hemorrhage, suggesting that these animals were maximally responding to the blood loss. This is consistent with studies done by Kadekaro et al. (31), demonstrating no further increase in AVP in response to hemorrhage following administration of L-NAME. In contrast, central NOS inhibition during AAI was accompanied by restoration of the AVP response to hemorrhage, strongly suggesting that NO plays a role in the blunted hemorrhage-induced rise in AVP during AAI. One limitation of these studies is that intracerebroventricular drug administration does not allow for specificity of the effects mediated by different brain regions. However, the combined findings of a lack of an increase in NO content in the SON at the completion of hemorrhage in alcohol-treated animals, and the restoration of AVP response to hemorrhage following central NOS inhibition during AAI, strongly suggest a role for NO in the blunted AVP response to hemorrhage during AAI.

Fig. 5. Effects of ICV L-NAME on NO content and NOS activity in the PVN and SON: Effects of ICV L-NAME (250 μg/5 μl) or vehicle (5 μl sterile water) on nitrate/nitrite content (μmol/mg tissue) in the PVN (A), NOS activity (μmol NO·mg protein^{-1}·min^{-1}) in the PVN (B), nitrate/nitrite content (μmol/mg tissue) in the SON (C), and NOS activity (μmol NO·mg protein^{-1}·min^{-1}) in the SON (D) measured in dextrose-treated (white bars) and alcohol-treated (black bars) animals at the end of hemorrhage (n = 6–8). Data are presented as means ± SE and were analyzed using a two-way ANOVA. *P < 0.05 vs. dextrose-treated animals. #P < 0.05 vs. treatment-matched baseline values.
of hemorrhage and the failure of 1-NAME administration to
decrease NOS activity in the SON lead us to speculate that the
blunted AVP response to hemorrhage during AAI is primarily
the result of increased NO production in the PVN. Future
studies utilizing intraparaventricular nucleus administration of
1-NAME are warranted to isolate the specific brain region
mediating the blunted AVP response to hemorrhage during
AAI.

In contrast to the SON, which is composed entirely of
magnocellular neurons, the PVN includes multiple populations
of neurons, including magnocellular and parvocellular neuro-
secretory neurons and autonomic-related neurons with connec-
tions to the dorsal vagal complex, rostral ventrolateral medulla,
and nucleus tractus solitarius (64). The magnocellular neurons
of PVN and SON are primarily innervated by A1 noradrenergic
neurons projecting from the ventrolateral medulla, whereas the
parvocellular neurons of the PVN receive input from the A1
region, A2 region, and locus coeruleus (64, 65). Magnocellular
neurons have been identified as a primary cellular source of
NO (3, 9, 23, 41); however, within the PVN, other neurons
may contribute to the production of NO. Using a combination
of immunohistochemistry and tract-tracing techniques, Li and
Chen (33) and Stern (59) identified ~25% of the autonomic
neurons of the PVN projecting to the dorsal vagal complex, as
well as the rostral ventrolateral medulla as NO-producing
neurons. In addition, astrocytes and microglia have been re-
ported to release large amounts of NO (47). Thus, it is possible
that the increased NO content in the PVN may be the result of
NO produced from several sources, in addition to that pro-
duced by the magnocellular neurons.

The mechanisms by which alcohol modulates NO produc-
tion in the PVN are currently unknown. Potential sources
include AAI-induced alterations in inflammatory state and
NOS expression and activity and disruption of central renin-
angiotensin signaling. AAI modulates the inflammatory re-
sponse to hemorrhage reflected as an attenuated tissue cytokine
response following blood loss (22, 43) and suppressed stimu-
lated cytokine response by peripheral blood mononuclear cells
isolated within 24 h after hemorrhage. The proinflammatory
cytokine IL-6 has been shown to inhibit NOS expression (52)
and stimulate the activity of AVP-producing neurons (48). IL-6
and NO are coexpressed in AVP-producing neurons in the
PVN and SON (21). Thus, decreased tissue inflammation may
have contributed to the inhibitory effects of alcohol on the
hemorrhage-induced rise in AVP. Alcohol could also produce
direct effects on NOS expression and activity. The effects of
alcohol are dose dependent and exhibit differential modulation
of NOS isoforms (66). The mechanism involved in alcohol
modulation of NOS in the PVN is currently unknown, but it
warrants further investigation. An alternative and novel hyp-
thesis is that the increased NO content in the PVN during
AAI and hemorrhage may be the result of alcohol-induced
alterations in the renin angiotensin system (RAS), particularly
that of ANG II. In addition to the effects mediated by the AT1
receptor, ANG II is also converted to ANG (1–7) by angioten-
sin-converting enzyme (ACE) 2, a recently discovered compo-
nent of the RAS (71). ACE 2 has been identified in many
tissues, including the brain, and has been implicated in cardio-
vascular function, regulation of blood pressure, and baroreflex
sensitivity through production of ANG (1–7) (14, 62, 71).
ANG (1–7) acts through the Mas receptor to stimulate NO
production through an Akt-dependent pathway (10, 26, 51, 73).
Studies have shown that overexpression of ACE 2 produces a
significant increase in brain NO production (17). In addition,
intracerebroventricular administration of ANG (1–7) has been
shown to increase NO in brain tissue (73). These findings
suggest that ANG (1–7) stimulates NO production. Recent
findings from our laboratory (unpublished data) show that AAI
increases ACE 2 activity in the PVN. We speculate that the
increase in NO production in the PVN and SON during AAI is
due to increased central ANG (1–7) activation of the Mas
receptor, thus contributing to the attenuated AVP release in
response to blood loss.

A key finding from these studies is that while AAI attenu-
ated the AVP response to hemorrhage, AAI did not impair the
AVP response to increases in plasma osmolality. However, it is
important to note that the basal AVP levels in this study are
higher than previous studies. We speculate that this is likely
due to the prior surgical procedures to which the animals had
been subjected. At the time of the baseline AVP measurement
in our studies, the animals had been subjected to two separate
surgical procedures (intracerebroventricular, vascular/gastric),
as well as an overnight 15-h dextrose/alcohol infusion. The
animals were deprived of food and water throughout the
duration of the infusion, and we speculate that this may also
have contributed to the high baseline AVP values observed.
While this is a limitation to this study, time-matched sham
control animals subjected to the same surgical procedures and
alcohol/dextrose administration have been included for all
experiments. The baseline values for our sham animals at this
time point are very similar to the experimental groups. Never-
theless, when stimulated, alcohol impaired the AVP response
to hemorrhage, but not hyperosmolality. This difference may
be the result of the different mechanisms modulating AVP
release in response to decreases in blood volume and increases
in plasma osmolality. A study by Thrasher and Keil (68)
demonstrated that an intact arterial baroreceptor system is
crucial in mediating a normal AVP response to hypovolemia.
AAI has been shown to produce a dose-dependent impairment
of baroreflex sensitivity; which is primarily centrally mediated
(15, 72). A potential mechanism of the alcohol-induced inhi-
bition of AVP release in response to blood loss is increased
GABA-ergic tone. The magnocellular neurons are modulated
by GABA-ergic afferents which inhibit baroreceptor-mediated
AVP release (60). The effects of NO on AVP release have been
demonstrated to be mediated by increased GABA-ergic syn-
aptic activity (6, 60). Using whole-cell patch clamp recordings,
Bains and Ferguson (6) and Stern and Ludwig (60) dem-
On the 10.220.33.1 on October 30, 2017 http://ajpregu.physiology.org/ Downloaded from http://ajpregu.physiology.org/
inhibited by blockade of the GABA system (74). It is well documented that alcohol stimulates GABA-ergic neurotransmission (32, 35, 38). Thus, one could speculate that alterations in NO in the PVN during AAI and hemorrhage may attenuate AVP release indirectly via NO-mediated stimulation of GABA-ergic activation. In addition to GABA, local administration of sodium nitroprusside, a NO donor, has been shown to increase the release of several additional neurotransmitters, including aspartate and glutamate and, in turn, decrease MABP (28). These findings suggest that the predominant NO-mediated neurotransmission is one of inhibitory tone, suggesting a net inhibition of AVP release and sympathetic activation. Alcohol-mediated GABAergic stimulation is likely to synergize with NO-mediated GABAergic activation to suppress AVP release. Nevertheless, NO-mediated neurotransmission is not limited to a GABAergic mechanism.

In contrast to baroreceptor-mediated AVP release, osmotic stimulation of AVP release is regulated by the magnocellular neurons of the PVN and SON, which are osmosensitive neurons and have the ability to sense changes in osmolality, as well as the ability to regulate neuronal systems involved in maintaining body fluid homeostasis (8, 56). Hypertonicity causes cell shrinkage and activation of ion channels, resulting in membrane depolarization and AVP release (8). Studies have shown that the AVP response to osmotic stimulation is not inhibited by NO (29, 30). Furthermore, in the present studies, we see a significant decrease in NO content, an inhibitor of AVP, in the PVN of alcohol-treated animals following a 5% hypertonic saline infusion. However, the mechanisms involved in the hypertonic saline-mediated decrease in NO content are currently unknown.

Perspectives and Significance

Because low MABP at the time of arrival into the emergency room is a predictor of poor patient outcome from traumatic injury and blood loss (25, 27), it is important to have an understanding of the mechanisms contributing to the greater hypotension seen in the alcohol-intoxicated trauma patient. Studies from our laboratory have shown that the greater hypotension during hemorrhage in AAI is not due to a decrease in circulating blood volume (unpublished observations) or impaired vascular responsiveness to pressor agents, but is primarily the result of a blunted neuroendocrine response (42, 43). Additional studies have shown that central pharmacologic activation of sympathetic outflow can restore the AVP response and improve blood pressure recovery during hemorrhage and fluid resuscitation in AAI (39), suggesting that alcohol interferes with signaling mechanisms regulating AVP release. Results from this study, as well as reports from the literature, demonstrate that NO, an inhibitor of AVP release, is increased by AAI (55). Furthermore, we have demonstrated that central NOS inhibition restores the AVP response to hemorrhage during AAI. These data provide evidence that NO inhibits AVP release during hemorrhage in AAI. Additionally, we have shown that AVP release in response to an osmotic challenge is not impaired by AAI, and hypertonic saline is able to decrease NO content in the PVN. To translate these findings to a clinical approach, additional studies examining the ability of hypertonic saline fluid resuscitation to restore AVP levels and improve MABP in the alcohol-intoxicated hemorrhaged host are warranted.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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