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Angiotensin (1-7) contributes to nitric oxide tonic inhibition of vasopressin release during hemorrhagic shock in acute ethanol intoxicated rodents

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Abstract

Aims—Acute ethanol intoxication (AEI) attenuates the arginine vasopressin (AVP) response to hemorrhage leading to impaired hemodynamic counter-regulation and accentuated hemodynamic stability. Previously we identified that the ethanol-induced impairment of circulating AVP concentrations in response to hemorrhage was the result of augmented central nitric oxide (NO) inhibition. The aim of the current study was to examine the mechanisms underlying ethanol-induced up-regulation of paraventricular nucleus (PVN) NO concentration. Angiotensin (ANG) (1-7) is an important mediator of NO production through activation of the Mas receptor. We hypothesized that Mas receptor inhibition would decrease central NO concentration and thus restore the rise in circulating AVP levels during hemorrhagic shock in AEI rats.

Main Methods—Conscious male Sprague Dawley rats (300-325 g) received a 15h intra-gastric infusion of ethanol (2.5g/kg + 300mg/kg/h) or dextrose prior to a fixed-pressure (~40mmHg) 60 minute hemorrhage. The Mas receptor antagonist A-779 was injected through an intracerebroventricular (ICV) cannula 15 min prior to hemorrhage.

Key Findings—PVN NOS activity and NO were significantly higher in AEI compared to DEX-treated controls at the completion of hemorrhage. ICV A-779 administration decreased NOS activity and NO concentration, partially restoring the rise in circulating AVP levels completion of hemorrhage in AEI rats.

Significance—These results suggest that Mas receptor activation contributes to the NO-mediated inhibitory tone of AVP release in the ethanol-intoxicated hemorrhaged host.

Keywords

AVP; NO; ethanol; mean arterial blood pressure; A-779

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Conflicts of Interest:

The authors declare that there are no conflicts of interest.

Introduction

Traumatic injury accounts for approximately 37 million emergency department visits per year (National Trauma Institute; 2009). Ethanol intoxication increases the risk of traumatic injury and disturbs hemodynamic stability with ethanol-intoxicated trauma patients presenting with greater hypotension (Bilello et al., 2011, Fabbri et al., 2001, Shih et al., 2003). Clinical studies have demonstrated that a low mean arterial blood pressure (MABP) is a predictor of poor patient outcome (Heckbert et al., 1998). Previous studies from our laboratory using a rodent model of acute ethanol intoxication (AEI) preceding hemorrhagic shock demonstrated that AEI attenuates the neuroendocrine counter-regulatory response to hemorrhage and aggravates hemodynamic instability throughout the resuscitation period (Molina et al., 2004). Moreover, attenuation of arginine vasopressin (AVP) release during hemorrhage was shown to play a major role in the impaired hemodynamic counter-regulation during AEI (Whitaker et al., 2011). The ethanol-induced impairment of AVP levels during hemorrhage was demonstrated to result predominantly from augmented central nitric oxide (NO) inhibitory tone (Whitaker, Sulzer, 2011). Central NO synthase (NOS) inhibition effectively restored the rise in circulating AVP levels and hemodynamic stability in AEI animals, suggesting that NO is a key mediator of the blunted AVP response to hemorrhagic shock. The mechanisms involved in ethanol-mediated modulation of NO production in the paraventricular nucleus (PVN), the primary site of AVP production in the brain, remained unknown.

NOS concentration and NO production are regulated by several neuroendocrine modulators (Stern et al., 2003). Among these, the renin angiotensin system (RAS), particularly through the actions of angiotensin (ANG) II can lead to up-regulation of PVN NO content. ANG II has been shown to stimulate AVP release by binding to the AT₁ receptor located on the PVN as well as on A1 noradrenergic afferents innervating the PVN (Chiodera et al., 1998), (Guggilam et al., 2008, Mouw et al., 1971, Qadri et al., 1993). This is particularly important during hemorrhage as demonstrated by a greater decrease in MABP and a markedly attenuated AVP response to hemorrhage following central administration of Losartan, an AT₁ receptor antagonist (Velasco and Rocha-e-Silva, 1989). Additionally, conversion of ANG II to ANG (1-7) by angiotensin converting enzyme (ACE) 2 has been identified as an important pathway for ANG II mediated effects. ACE2 is present in many tissues including the brain (Danilczyk et al., 2003, Feng et al., 2010). Over expression of ACE2 produces a significant increase in brain NO content (Feng, Xia, 2010). Further support for the relevance of this pathway was provided by studies showing that central administration of ANG (1-7) has been shown to increase brain NO concentration (Zhang et al., 2008) and to stimulate NO production through activation of the Mas receptor (Heitsch et al., 2001, Sampaio et al., 2007, Zhang, Lu, 2008). Hence, we speculated that increased ANG (1-7) could be a potential mechanism underlying ethanol-mediated increase in PVN NO concentration. We hypothesized that ethanol-induced increase in PVN ACE2-mediated conversion of ANG II to ANG (1-7) leads to an increase in PVN NO concentration that, in turn, inhibits AVP release during hemorrhagic shock. The aim of these studies was to demonstrate that blockade of the Mas receptor would decrease central NO content, restore the AVP response to blood loss, and improve MABP recovery in AEI hemorrhaged rats.

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 Institute of Health guidelines. Specific-pathogen free adult male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing 225-250 g at the time of

arrival to the institution were housed in the Division of Animal Care at LSUHSC. Animals were exposed to a controlled environment with a 12h light/12h dark cycle and fed a standard rat diet (Purina Rat Chow, Ralston Purina, St. Louis, MO) *ad libitum* for 1 week prior to surgery.

Surgical Preparation

Intracerebroventricular (ICV) cannula placement—A guide cannula was implanted in the lateral ventricle for central drug administration 7 days prior to vascular surgery as previously described by our laboratory (Molina, Zambell, 2004). Animals weighing 300-325 g at the time of the ICV surgery were anesthetized with an intramuscular injection of ketamine/xylazine (90 mg/kg and 9 mg/kg, respectively) and the surgical area shaved and cleaned with exodine surgical scrub followed by isopropyl alcohol. The animals were positioned in a stereotaxic apparatus and the overlying skin was cleared and a hole drilled in the skull (stereotaxic coordinates for guide cannula: 0.3 mm posterior to bregma, 1.3mm lateral from midline, and 4.25mm below the skull's surface). A 22-gauge stainless steel guide cannula was inserted into the right lateral ventricle of the brain. The cannula was secured using dental cement and anchored to the skull with three stainless steel screws. A wire dummy cannula was inserted into the cannula to seal the guide cannula until the time of the experiment. Following surgery, animals were returned to clean individual cages and allowed to recover for 7 days. Animals were provided food and water *ad libitum* prior to vascular/gastric catheter placement. Correct 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—On the day of the surgery, animals were anesthetized with an intramuscular injection of ketamine/xylazine (90 mg/kg and 9 mg/kg, respectively). Catheters which consisted of PE50 tubing (BD Diagnostic Systems, Sparks, MD) were inserted into the left carotid artery and advanced to the aortic arch and into the right jugular vein using aseptic surgical procedures as previously described by our laboratory (Greiffenstein et al., 2007, Molina, Zambell, 2004). An additional gastric catheter (PE50; BD Diagnostic Systems, Sparks, MD) was inserted into the antrum of the stomach, and secured with a purse-string suture. Gastric catheters were used for ethanol or dextrose administration. All catheters were flushed with normal saline, sealed, and exteriorized subcutaneously to the nape of the neck and secured with tape. After surgery, the animals were placed in individual cages and given food and water *ad libitum* for 2-3 days before ethanol or dextrose administration.

Ethanol and Dextrose Administration

The evening prior to the experiment at approximately 5 pm, gastric catheters were exteriorized and connected to an infusion pump (Harvard apparatus PHD 2000 syringe pump; Holliston, MA). Animals were randomly selected to receive either an intra-gastric bolus of 30% ethanol (2.5 g/kg) followed by a 15 hour maintenance infusion of ethanol (300 mg/kg/hr) or iso-caloric/iso-volumic 52% dextrose (12 g/kg). Animals were conscious, unrestrained, and restricted of food and water throughout the duration of the 15 hour infusion. This method of ethanol administration resembles the route of ethanol consumption in humans mimicking a binge drinking episode that frequently precedes a traumatic injury (Gmel et al., 2006, Savola et al., 2005) and achieved intoxicating levels of blood ethanol (270 ± 30 mg/dl) in this study. Previous studies in our laboratory achieved blood ethanol concentrations of 255 ± 15 mg/dl.

Fixed-Pressure Hemorrhagic Shock

At the completion of the 15 hour infusion, conscious and unrestrained animals were randomized to sham or hemorrhage groups and subjected to fixed-pressure hemorrhage as previously described by our laboratory (Molina, Zambell, 2004). Briefly, the carotid catheter was exteriorized and MABP was monitored continuously throughout the hemorrhage period (Powerlab, AD Instruments, Colorado Springs, CO). Blood was removed through the carotid catheter to decrease MABP to ~40 mmHg and maintain MABP between 40-55 mmHg during the 60 minute hemorrhage period.

Experimental Protocols

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

Study I. Impact of AEI on Renin Angiotensin System in response to hemorrhage—

The aim of this study was to characterize how AEI affects the various hormones and enzymes of the central and peripheral RAS involved in MABP control, AVP release and NO production (angiotensinogen, renin, ACE2). Animals were subjected to a fixed-pressure hemorrhage at the end of the ethanol or dextrose infusion (n=6-10 per group). Arterial blood samples were obtained at baseline and at the completion of the 60 min hemorrhage period for determination of circulating concentrations of angiotensinogen concentration, pro-renin/renin concentration and ACE2 activity. At the completion of the hemorrhage period, conscious animals were decapitated and whole brains immediately excised and flash-frozen in liquid nitrogen. The PVN and SON were isolated for determination of ACE2 activity, nitrate/nitrite content and NOS activity. The hypothalamus was isolated using the optic chiasm on the ventral surface as a landmark (Stern, Li, 2003). Briefly, the whole brain was placed on its dorsal surface and a 1 mm slice was cut just posterior and anterior to the optic chiasm isolating the hypothalamus. The PVN was isolated from the section with a scalpel. The SON was isolated with a 1 mm neuropunch on each side of the optic chiasm. The brain sections were weighed, submerged again in liquid nitrogen and stored at -80°C until analysis.

Study II. Impact of ICV ANG (1-7) on NO content in the PVN—To demonstrate that ANG (1-7) could indeed stimulate NO production in the PVN as well as to examine the ability of A-779 to inhibit the Mas receptor, normotensive animals were administered ICV injections of either vehicle (5 µl sterile water), ANG (1-7) (50 pmol/5µl) or A-779 (50 pmol/5µl) + ANG (1-7) (50 pmol/5µl) (n=5-8 per group). The doses of ANG (1-7) and A-779 were selected based on reports in the literature showing the ability of microinjection of ANG (1-7) into the caudal ventrolateral medulla to decrease MABP and A-779 to block the ANG (1-7)-mediated decrease in MABP (Cangussu et al., 2009). MABP was recorded for 60 minutes. At the end of the 60 minutes, animals were euthanized, brains excised and tissues saved for measurements of nitrate/nitrite content in the PVN.

Study III. Effect of central Mas receptor inhibition on PVN NO content, hemodynamic stability and circulating AVP levels in response to hemorrhage during AEI—To determine whether central Mas receptor inhibition would decrease PVN NO content and restore the rise in AVP circulating levels in response to hemorrhage during AEI, animals were randomly divided into sham (non-hemorrhaged) or hemorrhage groups. The Mas receptor antagonist A-779 was injected ICV 15 min prior to hemorrhage. There were a total of 8 treatment groups (n=4-7 per group) in this experiment: dextrose/vehicle/sham, dextrose/vehicle/hemorrhage, dextrose/A-779/sham, dextrose/A-779/hemorrhage, ethanol/vehicle/sham, ethanol/vehicle/hemorrhage, ethanol/A-779/sham, and ethanol/A-779/hemorrhage.

hemorrhage. MABP was recorded continuously during hemorrhage. Arterial blood samples were obtained at baseline (pre-hemorrhage) and at the completion of the hemorrhage period for determination of blood ethanol concentration, circulating AVP and ANG II concentrations. At the completion of the 60 minute hemorrhage period, conscious animals were decapitated and the whole brains immediately excised and flash-frozen in liquid nitrogen. The PVN and SON were isolated from the hypothalamus as previously described by our laboratory (Whitaker, Sulzer, 2011) for determination of ANG (1-7) concentration, nitrate/nitrite content, and NOS activity.

Analytical Procedures

Blood sample analysis—Arterial blood samples (1 ml) were collected from the carotid catheter into chilled heparinized syringes. Blood samples were placed in tubes containing 10 μ L/ml of aprotinin (Sigma, St. Louis, MO) and centrifuged for 10 minutes at 10,000 rpm and 4°C. Plasma was collected and stored at -80°C. Blood ethanol levels were determined using an amperometric oxygen electrode (Analox Instruments Limited, London, UK).

Arginine vasopressin measurements—Circulating AVP levels were determined in plasma extracts as previously described by our laboratory (Whitaker, Sulzer, 2011). Briefly, plasma samples were acidified with 1% trifluoroacetic acid in water (buffer A) and centrifuged for 10 minutes at 10,000 rpm and 4°C. C-18 SEP-Columns (Waters Corporation, Milford, MA) were washed with 1 ml buffer B (60% acetonitrile + 40% buffer A) followed by 3 washes with 3 ml buffer A. The acidified plasma was loaded onto the pre-treated 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 stored at -20°C until analysis. On the day of the assay, the residue was reconstituted using the buffer provided by a rat-specific radioimmunoassay kit (Phoenix Pharmaceuticals, Burlingame, CA). The range of detection for the AVP RIA was 10-1280 pg/ml.

Renin Angiotensin System—Plasma angiotensinogen was measured using a solid phase sandwich ELISA kit (IBL, Japan) with a range of detection of 0.08-5 ng/ml. Total pro-renin/renin were measured using a total antigen assay (Molecular Innovations, Novi, MI) with a range of detection of 0.1-100 ng/ml. Circulating ANG II concentration was determined in plasma extracts produced as described above under 'arginine vasopressin measurements'. Plasma ANG II concentrations were measured using a rat-specific ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA). The range of detection for the ANG II enzyme-linked immunoassay was 0-25 ng/ml.

Brain tissue analysis—On the day of the assay, the brain tissue was homogenized in ice cold PBS (100 μ L/mg tissue). The samples were centrifuged for 10 minutes at 10,000 rpm and the supernatant collected. NOS activity in the supernatant was measured using a commercially available assay kit (Oxford Biomedical Research, Oxford, MS) and values expressed as μ moles NO produced/mg protein/minute. This kit can be used to accurately measure as little as 1 pmol/ μ L NO utilizing very small sample size (~5 μ L). Protein was measured using Pierce BCA standard protein assay (Thermo Fischer Scientific, Rockford, IL). For determination of NO content, the supernatant was filtered using a 30 kDa molecular weight cut off filter (Millipore, MA). Stable NO metabolites (nitrate/nitrite) in the filtered supernatant were measured using a NO colorimetric assay kit (Cayman Chemical, Ann Harbor, MI). ANG (1-7) was measured using a rat-specific ANG (1-7) ELISA kit (NovaTeinBio, Cambridge, MA) with a range of detection of 1.56-50 ng/ml. ACE2 activity was measured using the SensoLyte™ 390 ACE2 Activity Assay Kit (AnaSpec Inc. Fremont, CA) which utilizes the Mc-Ala/Dnp fluorescence resonance energy transfer (FRET) peptide.

In the intact FRET peptide the fluorescence of Mc-Ala is quenched by Dnp. ACE2 cleaves the FRET peptide into two separate fragments and the fluorescence of Mc-Ala is recovered, and can be monitored at excitation/emission = 330/390 nm. This assay can detect sub-nanogram levels of ACE2 activity utilizing very small sample size (~10 μ L).

Statistical Analysis

All data are expressed as mean $\pm$ SEM with the number of animals per group indicated in the figure legends. Statistical analysis of differences in MABP, NO content, NOS activity, ANG (1-7) content and hormone levels were determined by two-way analysis of variance (ANOVA) with or without repeated measures using Sigma Plot 11.0 statistical software. Pair-wise multiple comparisons were determined using the Holm-Sidak method. Statistical significance was set at $p < 0.05$.

Results

Impact of AEI on RAS

Hemorrhage did not result in significant changes in circulating levels of angiotensinogen from those recorded at baseline in dextrose-treated animals (5071 ± 637 ng/ml vs. 5556 ± 530 ng/ml). AEI (270 ± 30 mg/dl) produced a significant increase in circulating levels of angiotensinogen both at baseline (7923 ± 1102 ng/ml vs. 5071 ± 637 ng/ml) and at the completion of hemorrhage (8698 ± 877 ng/ml) when compared to those of dextrose-treated controls (Figure 1a). No differences in baseline concentration of circulating pro-renin/renin (39 ± 13 ng/ml vs. 40 ± 5 ng/ml) were observed between dextrose and ethanol animals. Hemorrhage produced a significant increase in pro-renin/renin concentration in dextrose-treated animals as compared to treatment-matched baseline values (39 ± 13 ng/ml vs. 69 ± 19 ng/ml). Hemorrhagic shock produced an increase in circulating pro-renin/renin concentration (40 ± 5 pg/ml vs. 50 ± 9 pg/ml in ethanol-treated animals. However, this increase failed to reach statistical significance (Figure 1b). ACE2 activity in plasma was similar in dextrose- and ethanol-treated animals at baseline. Following hemorrhage, ACE2 activity was increased in both dextrose and ethanol-treated animals, but reached statistical significance only in ethanol-treated animals (0.1 ± 0.01 μ M Mca/mg protein/min vs. 0.08 ± 0.02 μ M Mca/mg protein/min; Figure 1c).

No differences in renin activity were observed in the PVN or SON of dextrose- and ethanol-treated animals (Figure 2a). However, ACE2 activity in the PVN of ethanol-treated animals was higher than that of dextrose-treated hemorrhaged animals (3.6 ± 0.5 μ M Mca/mg protein/min vs. 2.2 ± 0.3 μ M Mca/mg protein/min) at the completion of hemorrhage (Figure 2b).

ICV ANG (1-7) stimulates NO production

To examine the ability of ICV ANG (1-7) to stimulate NO production in the PVN as well as to confirm the ability of A-779 to block the Mas receptor, ANG (1-7) was administered ICV alone or in combination with A-779, a Mas receptor antagonist. ICV administration of ANG (1-7) resulted in a significant increase in NO content in the PVN compared to that of vehicle-treated animals (25 ± 3 μ mol/mg tissue vs. 11 ± 3 μ mol/mg tissue; Figure 3b). This increase in central NO content was associated with a decrease in MABP (~8% decrease from baseline) 30-60 minutes post-administration of central ANG (1-7). Central administration of the Mas receptor antagonist, A-779, prior to ANG (1-7) prevented the ANG (1-7)-mediated increase in PVN NO content and the decrease in MABP, confirming the ability of A-779 to block the Mas receptor (Figure 3a).

Central Mas receptor inhibition improves hemodynamic and AVP responses to hemorrhage during AEI

We predicted that the increased PVN ACE2 activity observed in ethanol-treated animals would lead to increased ANG (1-7) production. The rise in ANG (1-7) would in turn be expected to contribute to the ethanol-accentuated PVN NO production through activation of the Mas receptor. To demonstrate that the AEI-induced NOS-derived NO in the PVN was the result of ANG (1-7) stimulation of the Mas receptor, the Mas receptor antagonist A-779, was administered centrally prior to hemorrhage. All animals were subjected to a similar degree of hypotension during the fixed-pressure hemorrhage. MABP averaged 105 ± 4 mmHg at baseline (T=0 minutes) and decreased $40 \pm 1\%$ during the hemorrhage period in dextrose/vehicle-treated animals (Figure 4a). An average of $66 \pm 3\%$ of the total blood volume per kilogram of body weight was removed from dextrose/vehicle treated animals during the 60 minute hemorrhage period to achieve a fixed-pressure of ~ 40 -55 mmHg (Figure 4b). MABP averaged 109 ± 4 mmHg at baseline (T= 0 minutes) and decreased $44 \pm 2\%$ in the ethanol/vehicle-treated animals. A significantly lesser amount of blood ($60 \pm 2\%$ of the total blood volume) was removed from ethanol/vehicle-treated animals during the hemorrhage period. MABP averaged 113 ± 8 mmHg at baseline (T=0 minutes) and decreased $46 \pm 2\%$ during the hemorrhage period in the dextrose/A-779-treated animals. MABP averaged 109 ± 4 mmHg at baseline (T=0 minutes) and decreased $45 \pm 2\%$ during the hemorrhage period in the ethanol/A-779-treated animals. ICV A-779 administered 15 minutes prior to hemorrhage did not alter the total amount of blood removed to achieve a target MABP of 40-55 mmHg in dextrose/A-779-treated animals. However, ICV A-779 pretreatment significantly increased the total amount of blood removed during the 60 minute fixed-pressure hemorrhage period ($68 \pm 1\%$ vs. $60 \pm 2\%$ total blood volume) as compared to the amount removed from the ethanol/vehicle-treated animals.

Hemorrhagic shock produced a significant increase in circulating levels of AVP at the end of the hemorrhage period (T=60 minutes) in dextrose/vehicle-treated animals compared to baseline values (Figure 5a). This was not further enhanced by ICV administration of A-779. As previously demonstrated, AEI (ethanol/vehicle-treated animals) attenuated the AVP response to hemorrhage as compared to that of dextrose/vehicle-treated animals (41 ± 6 pg/ml vs. 65 ± 6 pg/ml). ICV administration of A-779 prior to hemorrhage in ethanol-treated animals partially restored circulating levels of AVP to values similar to those obtained in dextrose/hemorrhaged animals. There were no differences in baseline values of ANG II among the groups. Hemorrhage produced a significant increase in circulating ANG II in dextrose/vehicle-treated animals and ethanol/A-779-treated animals (Figure 5b).

AEI (ethanol/vehicle-treated controls) resulted in a marked increase in NO content (44 ± 10 vs. 26 ± 5 μ M NO/mg tissue) and NOS activity (0.26 ± 0.04 vs. 0.18 ± 0.03 μ M NO/mg protein/min) in the PVN compared to the response in dextrose/vehicle-treated controls (Figure 6a and 6b). ICV administration of the Mas receptor antagonist, A-779, decreased NOS activity (66%, $p < 0.05$) and NO concentration (27%, $p = 0.06$) in the PVN SON. The results from the previous studies demonstrated a significant increase in ACE2 activity at the end of hemorrhage; however, it was unknown whether this contributed to increased hypothalamic ANG (1-7) concentration. Therefore, ANG (1-7) content was measured in the PVN at the completion of hemorrhage. AEI increased ANG (1-7) 58% ($p < 0.05$) in ethanol/vehicle-treated animals (Figure 6c). A similar response was observed in ethanol/A-779-treated animals (46% increase $p = 0.06$) as compared to dextrose-treated controls.

Discussion

The present study sought to expand on the findings that enhanced central nitroergic tone contributes to the attenuated circulating AVP concentration during hemorrhage in acute

alcohol intoxicated rodents. In agreement with previous findings from our laboratory (Molina, Zambell, 2004, Whitaker, Sulzer, 2011), the results from this study demonstrate that AEI significantly attenuates the rise in circulating AVP levels and enhances PVN NO concentration in hemorrhaged animals. In addition, our results show enhanced PVN ANG 1-7 and substantial Mas-mediated upregulation of NOS in AEI hemorrhaged animals. Our findings show that ANG (1-7)-mediated NO upregulation partially contributes to the inhibitory tone on AVP release during hemorrhage in AEI.

ANG II acts through the AT₁ receptor to produce vasoconstriction and AVP release. In addition, ANG II is also converted to ANG (1-7) by Angiotensin Converting Enzyme 2 (ACE2), a recently identified component of the renin angiotensin system (RAS) (Danilczyk, Eriksson, 2003, Xia and Lazartigues, 2008). ACE2 is expressed in many tissues including the brain and has been associated with regulation of cardiovascular function, blood pressure and baroreflex sensitivity through production of ANG (1-7) (Diz et al., 2008, Stewart et al., 2008, Xia and Lazartigues, 2008). In contrast to the predominately vasoconstrictor effects of ANG II, ANG (1-7) promotes vasodilation (Xia and Lazartigues, 2008). In the brain, ANG (1-7) is co-localized with NOS in the PVN and SON of the hypothalamus (Calka and Block, 1993). ANG (1-7) stimulates NO production through activation of the Mas receptor through a PI3K/Akt-dependent pathway (Heitsch, Brovkovich, 2001, Sampaio, Souza dos Santos, 2007, Zhang, Lu, 2008). Studies have demonstrated that ANG (1-7) binding to the Mas receptor rapidly induces PI3K/Akt activation (Sampaio, Souza dos Santos, 2007). Activation of this pathway induces Ser1177 phosphorylation ultimately resulting in an increase in NOS activity (Calka and Block, 1993). Additional *in vitro* studies have shown a concentration-dependent increase in NO release from endothelial cells upon addition of ANG (1-7) (Heitsch, Brovkovich, 2001). Our results show that ICV administration of ANG (1-7) resulted in a significant increase in the content of PVN NO. Our previous studies had shown that increase PVN and SON NO production during AEI plays an important role in the inhibition of AVP release during hemorrhagic shock in AEI. Taken together the results from the present study suggest that increased central ANG (1-7) activation of the Mas receptor leads to NO upregulation which in turn suppresses AVP release in AEI hemorrhaged rats. Support for this observation has been provided by reports in the literature. Over-expression of ACE2 produces a significant increase in brain NO production (Feng, Xia, 2010). Furthermore, ICV administration of ANG (1-7) has been shown to increase NO content in brain tissue (Zhang, Lu, 2008).

The present studies show significant ethanol-induced alterations in both central and peripheral components of the RAS at the completion of hemorrhage. AEI significantly increased circulating angiotensinogen concentration. Angiotensinogen, a protein produced by the liver, is cleaved by renin into ANG I, the immediate precursor of ANG II. Most salient is the observation that PVN ACE2 activity was markedly higher in AEI animals at the completion of hemorrhage. ACE2 converts ANG II to the NO-stimulating peptide, ANG (1-7) (Diz, Garcia-Espinosa, 2008, Stewart, Lazartigues, 2008, Xia and Lazartigues, 2008); and in fact, PVN ANG (1-7) levels were also higher in AEI rats at completion of hemorrhage. Furthermore, this increase in ANG (1-7) was paralleled by increased PVN NO concentration. We predict this to have been causal, based on our observations that central ANG (1-7) administration produced an increase in PVN NO content. The contribution of ANG (1-7)-mediated NO production was demonstrated by central administration of A-779, a Mas receptor antagonist prior to hemorrhage. Our results showed that central Mas receptor inhibition decreased NO content 27% and NOS activity 66% in the PVN and partially restored the AVP response to hemorrhage during AEI. Taken together, these results suggest that ANG (1-7) partially contributes to enhanced PVN NO production in AEI.

Additional mechanisms are likely at play in the accentuated NO production seen during AEI. One possible mechanism is through AEI-induced dysregulation of tissue inflammation. AEI modulates the inflammatory response to hemorrhage, both at the tissue level (Greiffenstein, Mathis, 2007, Greiffenstein and Molina, 2008, Molina, Zambell, 2004), and in peripheral blood mononuclear cells isolated within 24 hours post hemorrhage. The pro-inflammatory cytokine interleukin (IL)-6 has been shown to inhibit NOS expression (Saura et al., 2006). IL-6 and NO are co-expressed in AVP-producing neurons in the PVN and SON (González-Hernández et al., 2006). One could speculate that AEI-induced modulation of tissue inflammation may have contributed to the inhibitory effects of ethanol on the hemorrhage-induced rise in AVP. Unpublished observations in our laboratory have shown a decrease in IL-6 in the PVN following hemorrhage in ethanol-intoxicated rats. Additionally, studies by Kovacs and others demonstrated that AEI inhibits macrophage IL-6 production (Goral, 2004). An additional possibility that was not examined is a direct effect of ethanol on NOS expression and activity. Ethanol has been shown to exert dose-dependent differential modulation of NOS isoforms (Syapin, 1998). The mechanism involved in ethanol modulation of NOS in the PVN is currently unknown, and warrants further investigation.

The experimental approach using a Mas receptor antagonist to examine the contribution of ANG (1-7)-mediated NO production to the attenuated circulating concentration of AVP during hemorrhage in the intoxicated host did not take into account the potentially attenuated ANG II-stimulated AVP release. An optimal approach would have been the use of central ACE2 inhibition utilizing MLN-4760 preventing the conversion of ANG II to ANG (1-7). This would have restored ANG II stimulatory effects on AVP release, and simultaneously decreased ANG (1-7)-mediated NO production. Studies by Diz and others have successfully utilized this inhibitor in the brain demonstrating that MLN-4760 when administered directly into the nucleus of the solitary tract can reduce baroreceptor reflex sensitivity (Diz, Garcia-Espinosa, 2008). While optimal, this ACE2 inhibitor is not commercially available. DX600, another ACE2 inhibitor which is commercially available is used predominately in *in vitro* studies and has not been successfully characterized *in vivo* (Huang et al., 2003). Thus, additional studies utilizing central ACE2 inhibitors to target the both ANG II-stimulatory and NO-inhibitory mechanisms regulating AVP release would allow for a more complete understanding of the mechanisms controlling AVP release that are affected by ethanol intoxication in the hemorrhaged host.

Conclusions

The results from these studies indicate that ANG (1-7)-mediated NO production partially contributes to the accentuated PVN nitrergic inhibitory tone during AEI. Additionally, these results provide further evidence for a key role for increased PVN NO inhibitory tone as a significant mechanism responsible for the blunted hemorrhage-induced rise in circulating AVP levels in AEI. Removal of the accentuated NO inhibitory tones in the PVN restores AVP levels and hemodynamic stability in ethanol-intoxicated animals (Whitaker, Sulzer, 2011). Moreover, these studies provide potential targets for development of specific resuscitation strategies designed to improve clinical outcomes in the ethanol-intoxicated hemorrhaged host. Future investigations aimed at developing therapeutic strategies to target the disrupted central signaling pathways involved in the impaired counter-regulatory response to hemorrhage are warranted to try to improve outcome in the ethanol-intoxicated hemorrhaged host.

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Figure 1. Impact of AEI on circulating components of the renin angiotensin system in response to hemorrhage

A. Plasma angiotensinogen (ng/ml), B. Plasma pro-renin/renin (pg/ml) and C. Plasma ACE 2 activity ($\mu\text{M Mca/min}$) measured at baseline and at the completion of hemorrhage in dextrose-treated (white bars) and ethanol-treated (black bars) animals (n=6-10). Data are presented as mean $\pm$ SEM and were analyzed using a two-way ANOVA with repeated measures. * $p < 0.05$ vs. treatment-matched baseline values; # $p < 0.05$ vs. time-matched dextrose-treated animals.

Figure 2. AEI increases ACE 2 activity in the PVN post-hemorrhage

A. Renin activity ($\mu\text{M Mca/mg protein/min}$) and B. ACE 2 activity ($\mu\text{M Mca/mg protein/min}$) measured in the PVN and SON at the completion of hemorrhage in dextrose-treated (white bars) and ethanol-treated (black bars) animals ($n=6-10$). Data are presented as mean $\pm$ SEM and were analyzed using a two-way ANOVA with repeated measures. * $p<0.05$ time-matched dextrose-treated animals.

Figure 3. Central ANG (1-7) administration increases NO content in the PVN and decreases MABP

A. MABP (mmHg) and B. Nitrate/nitrite content ($\mu\text{mole/mg tissue}$) in the PVN and SON measured in animals treated with ICV vehicle, ANG (1-7) or A-779 + ANG (1-7) ($n=5-8/\text{group}$). Data are presented as mean $\pm$ SEM and were analyzed using a two-way ANOVA with or without repeated measures. * $p<0.05$ ANG (1-7) vs. vehicle-treated animals; # $p<0.05$ ANG (1-7) vs. A-779 + ANG (1-7).

Figure 4. Central Mas receptor inhibition improves hemodynamic stability during hemorrhage in AEI

Effects of ICV A-779 or vehicle on A. Mean arterial blood pressure (MABP) and B. Total blood volume removed in dextrose-treated and ethanol-treated animals ($n=5-7$). Data are presented as mean $\pm$ SEM and were analyzed using a two-way ANOVA with or without repeated measures. * $p<0.05$ vs. basal MABP; @ $p<0.05$ vs. dextrose/vehicle-treated animals # $p<0.05$ vs. ethanol/vehicle-treated animals.

Figure 5. Central Mas receptor inhibition partially restores circulating AVP concentration in response to hemorrhage during AEI

Effects of ICV A-779 or vehicle on A. AVP (pg/ml) and B. ANG II (pg/ml) circulating concentration in dextrose-treated and ethanol-treated animals (n=4-6). Data are presented as mean $\pm$ SEM and were analyzed using a two-way ANOVA. $P=0.06$ and $p=0.09$ vs. time-matched baseline values. * $p<0.05$ vs. time-matched baseline values; # $p<0.05$ vs. dextrose/vehicle-treated animals.

Figure 6. Central Mas receptor inhibition decreases NOS activity in the PVN

A. Nitrate/nitrite content ($\mu\text{mole/mg}$ tissue), B. NOS activity ($\mu\text{mole NO/mg protein/min}$) and C. ANG (1-7) content (pg/mg protein) in the PVN measured in dextrose-treated (white bars) and ethanol-treated (black bars) animals at the end of hemorrhage ($n=5-7$). Data are presented as mean $\pm$ SEM and were analyzed using a two-way ANOVA. * $p < 0.05$ vs. dextrose-treated animals; # $p < 0.05$ vs. treatment-matched baseline values.