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Central NOS inhibition differentially affects vasopressin gene expression in hypothalamic nuclei in septic rats

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ABSTRACT

Our aim was to investigate the effect of central NOS inhibition on hypothalamic arginine vasopressin (AVP) gene expression, hormone release and on the cardiovascular response during experimental sepsis. Male Wistar rats were intracerebroventricularly injected with the non-selective NO synthase (NOS) inhibitor (L-NAME) or aminoguanidine, a selective inhibitor of the inducible isoform (iNOS). After 30 min, sepsis was induced by cecal ligation and puncture (CLP) causing an increase in heart rate (HR), as well as a reduction in median arterial pressure (MAP) and AVP expression ratio (AVP_R), mainly in the supraoptic nucleus. AVP plasma levels (AVPp) increased in the early but not in the late phase of sepsis. L-NAME pretreatment increased MAP but did not change HR. It also resulted in an increase in AVPp at all time points, except 24 h, when it returned to basal levels. AVP_R, however remained reduced in both nuclei. Aminoguanidine pretreatment resulted in increased MAP in the early phase and higher AVP_R in the supraoptic, but not in the paraventricular nucleus, while AVPp remained elevated at all time points. We *suggest* that increased central NO production, mainly inducible NOS-derived, reduces AVP gene expression differentially in supraoptic and paraventricular nuclei, and that this may contribute to low AVP plasma levels and hypotension in the late phase of sepsis.

1. Introduction

Arginine vasopressin (AVP) is mainly synthesized in magnocellular neurons of the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei and plays an important role in body fluid homeostasis and regulation of arterial blood pressure. The parvocellular neurons of the PVN also synthesize AVP, which acts synergistically with corticotrophin-releasing hormone in the regulation of corticotrophin secretion (Chrousos, 1995).

Clinical and experimental sepsis studies have reported high AVP plasma levels in the initial phase of sepsis. In the late phase, however, hormone levels are inappropriately low, despite hypotension, thus contributing to the vasodilatory shock (Correa et al., 2007; Landry et al., 1997; Oliveira-Pelegrin et al., 2009; Pancoto et al., 2008; Sharshar et al., 2002).

The reasons underlying low AVP secretion in the late phase of sepsis remain uncertain (Holmes et al., 2001; Rocha et al., 2006). Nevertheless, some hypotheses have been suggested, such as impaired baroreflex sensitivity (Holmes et al., 2001; Pancoto et al., 2008; Rocha et al., 2006), depletion of neurohypophyseal hormone content (Holmes et al., 2001; Oliveira-Pelegrin et al., 2009; Rocha et al., 2006; Sharshar et al., 2002), and overproduction of NO (Carnio et al., 2006; Correa et al., 2007; Giusti-Paiva et al., 2002; Holmes et al., 2001; Rocha et al., 2006).

During sepsis, the complex interaction between host and infectious agents results in high production of iNOS-derived NO (Correa et al., 2007; Feihl et al., 2001; Wong et al., 1996). The elevated NO levels in the late phase of sepsis have been shown to be related to deleterious effects of this syndrome (Correa et al., 2007; McCann et al., 2000; Oliveira-Pelegrin et al., 2009; Pancoto et al., 2008). In the central nervous system, NO has been suggested to participate in the modulation of AVP secretion, showing either stimulatory or inhibitory effects (Carnio et al., 2006; Giusti-Paiva et al., 2005; Ota et al., 1993). In the initial phase of cecal ligation and puncture (CLP)-induced experimental sepsis, when the NO levels are close to basal levels, we observed increased neuronal activity in the SON and PVN (Correa et al., 2007), as well as in medullar autonomic structures (Bruhn et al., 2009). These autonomic structures are related to the control of cardiovascular functions and have noradrenergic afferent pathways to the SON and PVN (Cunningham and Sawchenko, 1991). Noradrenaline has been

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described to exhibit short-term interaction with the nitrergic system in SON and PVN magnocellular neurons of the rat hypothalamus. The inducible NOS (iNOS) isoform seems to be constitutively expressed in these neurons, in addition to the neuronal NOS (nNOS) isoform (Grange-Messent et al., 2004). The noradrenergic input into magnocellular neurons from medullar autonomic structures seems to be apt at up-regulating the expression of nNOS and iNOS encoding genes, and at stimulating enzymatic activity of both NOS isoforms. These findings indicate that the stimulation from noradrenergic input occurring during hypotension may increase central NO production (Grange-Messent et al., 2004). While apparently beneficial at first sight, it is noteworthy that high concentrations of NO can have deleterious effects on neurons (Sharshar et al., 2003b).

In previous studies we could show that in the late phase of sepsis the high levels of NO derived from iNOS activity were accompanied by decreased neuronal activity in autonomic structures, as well as in the SON and PVN (Bruhn et al., 2009; Correa et al., 2007) this indicating that high levels of NO may impair AVP synthesis in this phase (Oliveira-Pelegrin et al., 2009; Rocha et al., 2006). Furthermore a recent study using semiquantitative methodologies revealed differences in hormone content between SON and PVN in clinical and experimental septic shock, but did not detect apparent differences in AVP mRNA content (Sonneville et al., 2010), suggesting that translation of AVP mRNA and/or transport of the peptide may be differentially regulated in SON and PVN magnocellular neurons, and that this may account for the inappropriate AVP release in septic shock. In order to better understand the modulation of AVP expression we herein investigated by means of a quantitative PCR analysis AVP transcript levels in the SON and PVN during CLP-induced sepsis and the possible role of centrally produced NO in the regulator circuitry of AVP gene expression. We hypothesized that the high NO levels would contribute to the down regulation of AVP gene expression during sepsis.

2. Material and methods

2.1. Animals

Male Wistar rats (200–250 g) provided by the Animal Facility of the Campus of Ribeirão Preto, University of São Paulo, were housed in controlled temperature (25 ± 1 °C) and photoperiodic (12:12 h light: day cycle) conditions, with food (Nuvilab CR-1, NUVITAL) and tap water available *ad libitum*. All experimental protocols were approved and performed according to the guidelines of the Ethics Committee of the University of São Paulo (CEUA)-Campus Ribeirão Preto. Humane endpoints in shock research (Nemzek et al., 2004) were used as criterion to euthanize CLP animals in severe distress, immediately before or soon after the time points defined in this study.

2.2. Drugs

The non-selective inhibitor of NOS isoforms, L-N^G-nitroarginine methyl ester (L-NAME) and the iNOS selective inhibitor, aminoguanidine (AG) were obtained from Sigma Chemical (St. Louis, MO, USA) and dissolved in pyrogen-free sterile saline at concentrations of 250 μg/μL. The vehicle saline was used for control injections.

2.3. Lateral ventricle cannulation procedure

Animals were anesthetized by intraperitoneal injection of ketamine–xylazine (100 and 14 mg/Kg, respectively) and fixed in a Kopf stereotaxic frame. A stainless guide cannula (0.7 mm outer diameter) was introduced into the right lateral ventricle (coordinates: A: −0.8 mm, L: 1.4 mm, and D: 3.2–3.7 mm). The cannula was attached to the bone with stainless screws and acrylic cement. A tight-fitting stylet was kept inside the guide cannula to prevent occlusion.

2.4. Cecal ligation and puncture surgery

Animals were randomly assigned to one of two groups, CLP group and sham-operated group. All experiments were performed at the same time of day (9:00 AM). Induction of severe sepsis was performed by a CLP model as previously described (Oliveira-Pelegrin et al., 2009). Briefly, rats were anesthetized with a short-acting anesthetic agent (tribromoethanol; 2.5%, 250 mg/kg i.p.; Acros Organics). Under sterile surgical conditions the animals were subjected to a midline laparotomy, and the cecum was carefully isolated to avoid damage to the blood vessels. The cecum was then ligated below the ileocecal valve without causing bowel obstruction and punctured ten times with a 16-gauge needle allowing the fecal contents to spill into the peritoneum. Sham-operated animals were submitted to laparotomy, the cecum was manipulated but neither ligated nor punctured. The abdominal cavity was closed in two layers, and all animals received a subcutaneous injection of saline (20 ml/kg body weight). The animals were allowed to recover in their cages with free access to food and water.

2.5. Experimental protocol

Intracerebroventricular (i.c.v.) injections were performed using a 10 μL Hamilton syringe and a dental injection needle (200 μm outer diameter; Mizzy, Brazil) connected to a PE-10 tube. The volume of each injection was 1.0 μL for all protocols. Injections were performed over a period of 1 min, and an additional minute was allowed to elapse before the injection needle was removed from the guide cannula to avoid reflux. The animals were kept in their boxes during the entire procedure. They received an i.c.v. injection of L-NAME (250 μg/μL) or AG (250 μg/μL) or saline 30 min before surgeries. The dose of the drugs used, the timing of pretreatment, and the route of administration were based on data from a previous study (Giusti-Paiva et al., 2002). In a first set of experiments, femoral cannulated animals were used to determine mean arterial pressure (MAP) and heart rate (HR) before and after experimental procedures (CLP or sham surgeries). In a second set, sham-operated or CLP-induced sepsis animals were decapitated at 4, 6, 20 or 24 h for blood and brain collection. Based mainly on the biphasic pattern of vasopressin secretion seen in previous studies (Correa et al., 2007; Oliveira-Pelegrin et al., 2009) we defined the period from 4 to 6 h as early phase of sepsis, and that from 20 to 24 h as late phase. Hematocrit was measured by centrifugation, plasma osmolality by freezing-point depression (Precision System, INC.) and plasma AVP levels were determined by specific radioimmunoassay (RIA). The SON and PVN regions were carefully microdissected from the hypothalamus for total RNA extraction and processed for quantitative polymerase chain reaction (qPCR) to evaluate AVP relative expression ratios in these nuclei.

2.6. Blood pressure and heart rate measurement

The day before the experiment, the animals had a polyethylene catheter inserted into the femoral artery and filled with 0.3% heparin in sterile saline. The animals were kept in individual boxes. On the day of the experiment, the arterial catheter was connected to a pressure transducer (BSL SS13L) and a unit of data acquisition (BSL MP35 System, BIOPAC Systems, Inc., CA, USA) to record MAP and HR of conscious and freely moving rats. The data were converted and analyzed using software BSL PRO® v.3.6.7 (BIOPAC Systems, Inc., CA, USA). After a period of system stabilization the basal MAP and HR were determined. During the experiment, the MAP and HR records were made every 15 min up to 6 h (initial phase) and at the following day between 22 h and 24 h (late phase) after surgeries. The results were expressed as deviation from basal MAP and HR.

2.7. Vasopressin measurement

After the plasma extraction using acetone and petroleum ether the plasma AVP levels were measured by double antibody-specific radioimmunoassay as previously described (Correa et al., 2007; Oliveira-Pelegrin et al., 2009; Pancoto et al., 2008). The assay sensitivity, intra-assay, and inter-assay coefficients of variations were 0.9 pg/mL, 7.7% and 11.9%, respectively.

2.8. Tissue collection, RNA extraction and reverse transcription

Rats were decapitated and the brain was rapidly removed from the cranium, frozen in dry ice and stored at −80 °C until required. For cryostat sectioning, the brains were placed in a brain matrix (Insight Equipment LTDA, Ribeirao Preto, Brazil) and cut based on the rat brain atlas coordinates (Swanson, 1998) and having the optic chiasm as anatomical landmark for reproducibility of the dissection. One or two coronal sections of approximately 1-mm thickness were removed from the hypothalamic region. The SON and PVN were carefully punched out by using a stainless steel needle of 1200 μm and 1400 μm diameter, respectively. The tissue punches were placed in 1 mL of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) for homogenization and total RNA extraction. All samples were treated with RNase-free DNAse I (Invitrogen, Carlsbad, CA, USA) to remove any contaminating genomic DNA. The purity and quantification of total RNA isolated were assessed by spectrophotometry using a NanoDrop® ND-1000 system (NanoDrop Technologies). First strand cDNA synthesis was carried out using the following protocol. Two micrograms of total RNA, 1 μL of oligo$(dT)_{12-18}$ primer (0.5 μg/μL, Invitrogen, Carlsbad, CA, USA) and 1 μL of dNTP mix (10 mM) (Invitrogen, Carlsbad, CA, USA) were incubated at 69 °C for 5 min and chilled on ice. Subsequently, 4 μL of 5× First Strand Buffer, 2 μL of DTT (0.1 M) and 1 μL of RNAseOUT™ Ribonuclease Inhibitor (Invitrogen, Carlsbad, CA, USA) were added and the samples incubated for 2 min at 42 °C. Next, SuperScript™ II Reverse Transcriptase (200U, Invitrogen, Carlsbad, CA, USA) was added and the reaction incubated at 42 °C for 50 min followed by 15 min at 70 °C. cDNA was stored undiluted at −20 °C until analysis. All cDNA samples were diluted 1:5 with DEPC-treated water before being used as templates in quantitative PCR assays.

2.9. Primer design

Gene-specific primers (AVP: (+) atc cga cat gga gct gag ac, (−) aaa aac cct ctc gac act cg; GAPDH: (+) tca cca cca tgg aga agg c, (−) gct aag cag ttg gtg gtg ca; ACTb: (+) tgt cac caa ctg gga cga ta, (−) ggg gtg ttg aag gtc tca aa; 18S: (+) acg gaa ggg cac cac cag ga, (−) cac cac cac cca cgg aat cg) were designed based on GenBank sequences (supplementary material). The reference gene (GAPDH, ACTb and 18S) primer combinations had already been validated in previous studies (Bonefeld et al., 2008; Gouraud et al., 2007). Desalinated and highly purified primers were synthesized commercially (Invitrogen).

2.10. Quantitative real-time PCR (qPCR)

Quantitative real-time PCR studies were performed using SYBR® Green methodology (Applied Biosystems) in a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany). qPCR reactions, performed in 96-well 0.2 mL thin-wall PCR microplates (Axygen) sealed with film, consisted of 8 μL of SYBR Green PCR Master Mix, 2.5 μL of each forward and reverse primer (10 μM), and 2 μL of 1:5-diluted template cDNA in a total volume of 20 μL. Cycling was performed using the optimized conditions in a four-step experimental run protocol: (i) denaturation program (2 min at 50 °C and 10 min at 95 °C); (ii) amplification and quantification program repeated 40 times (15 s at 95 °C, 35 s at 58 °C, 60 s at 60 °C); melting curve program (60–95 °C with a heating rate of 0.5 °C/s and a continuous fluorescence measurement); (iv) cooling program down to 4 °C. Melting curves were established after thermocycling to verify that the used pairs of primers produced a gene-specific product. Melting temperatures (T_M) for each PCR product are shown in supplementary material. The qPCR assays for each studied gene included a negative control (without cDNA template) and a positive control consisting of naïve animal cDNA. All samples were run in triplicate.

Standard curves for each pair of primers (AVP, GAPDH, ACTb, 18S) were prepared by serial 1:10 dilutions of positive control cDNA samples. Amplification efficiencies were calculated according to the equation: $E = 10^{(-1/\text{slope})} - 1$ (Bustin et al., 2009). Amplification efficiencies (E), slopes, *y*-intercepts and correlation coefficients were obtained directly from the Mastercycler ep realplex Software (supplementary material) showing similar E-values and correlation coefficients for all analyzed genes.

2.11. Statistical analysis

The data are presented as mean ± SEM. Statistical analysis of MAP and HR was performed by repeated measure two-way ANOVA and a *post hoc* Student–Newman–Keuls (SNK) test. RIA data were analyzed by logit transformation of the raw data. Hematocrit, plasma osmolality and plasma nitrate results were statistically analyzed by two-way ANOVA, followed by a *post hoc* Student–Newman–Keuls (SNK) test. The Relative Expression Software Tool — Multiple Condition Solver (REST-MCS©-version 2) (Pfaffl et al., 2002) was used to analyze mRNA relative expression ratios by using quantification cycle (Cq) values determined at the threshold, which was considered significant when 10 times standard deviation above the noise of the baseline. $P \leq 0.05$ was used as criterion for significance.

3. Results

All animals submitted to CLP-induced experimental polymicrobial sepsis developed the typical early clinical signs of sepsis, such as lethargy, piloerection and diarrhea. Sham-operated animals remained active in their cages, as expected.

3.1. Effect of central injection of L-NAME and AG on hematocrit, plasma osmolality changes in CLP rats

In the vehicle pretreated group, the CLP-induced sepsis caused a significant increase ($F_{(1,50)} = 11.3$, $P = 0.002$) in hematocrit, both at 4 and 20 h, but did not affect plasma osmolality at any time (Table 1). In animals pretreated with L-NAME there was no difference in hematocrit between sham and CLP groups (Table 1). Animals that underwent AG pretreatment showed increased hematocrit values at 6 h ($F_{(1,50)} = 21.2$, $P < 0.001$) after CLP, yet later on, at 20 h, the values were significantly lower when compared to the saline group ($F_{(6,76)} = 2.5$, $P = 0.032$) (Table 1). Plasma osmolality was not affected by any of the pretreatment protocols (Table 1).

3.2. Effect of central injection of L-NAME and AG on AVP mRNA expression and release

The relative expression ratio (*R*) of AVP mRNA was calculated on the basis of PCR efficiency (E) and quantification cycle deviation (ΔCq) (Pfaffl, 2001) by using a REST-MCS software (Pfaffl et al., 2002). Three different reference genes of (GAPDH, ACTB and 18S) were used for calculating the normalization factor in the analysis of the AVP relative expression ratio. Naïve animals were used as controls.

In the SON we observed a significant decrease ($F_{(7,24)} = 134.4$; $P < 0.001$) in relative AVP expression for all time points after CLP. Although pretreatment with L-NAME attenuated the decrease in AVP expression ratio observed in the saline group ($F_{(11,36)} = 27.6$; $P < 0.001$), these levels were still significantly lower than those for their respective

Table 1
Effect of intracerebroventricular pretreatment with nitric oxide synthase inhibitors on hematocrit and plasma osmolality.

Time (h)	4	6	20	24
Hematocrit (%)				
Saline + Sham	39 ± 1 (8)	39 ± 2 (7)	39 ± 3 (8)	37 ± 1 (6)
Saline + CLP	46 ± 2 (8)*	44 ± 3 (8)	47 ± 2 (6)*	37 ± 2 (7)[a]
L-NAME + Sham	39 ± 2 (8)	39 ± 2 (7)	42 ± 1 (7)	39 ± 1 (8)
L-NAME + CLP	41 ± 2 (8)	44 ± 2 (8)	42 ± 3 (6)	42 ± 2 (8)
AG + Sham	41 ± 1 (8)	38 ± 1 (8)	35 ± 1 (6)[b]	35 ± 1 (7)[b]
AG + CLP	44 ± 1 (10)	47 ± 1 (7)*	38 ± 2 (6)[c,+]	39 ± 2 (6)[c]
Osmolality (mOsm/kg)				
Saline + Sham	281 ± 3 (8)	282 ± 3 (8)	283 ± 4 (8)	279 ± 3 (8)
Saline + CLP	277 ± 3 (8)	277 ± 7 (8)	290 ± 3 (6)	277 ± 6 (8)
L-NAME + Sham	284 ± 4 (8)	286 ± 5 (8)	284 ± 6 (7)	291 ± 2 (8)
L-NAME + CLP	285 ± 4 (8)	278 ± 3 (8)	277 ± 3 (7)	280 ± 5 (8)
AG + Sham	280 ± 3 (9)	279 ± 2 (8)	288 ± 6 (7)	281 ± 4 (8)
AG + CLP	284 ± 3 (10)	281 ± 5 (8)	276 ± 4 (9)	288 ± 2 (9)

Measurements are expressed as mean ± S.E.M for each group. Number of animals (n) in parentheses. Statistical analysis performed using ANOVA following by SNK test. CLP, cecal ligation and puncture. AG, Aminoguanidine.
* $P<0.05$ compared to sham group.
\+ $P<0.05$ compared to vehicle pretreated group.
[a] $P<0.05$ compared to other time points within the group.
[b] $P<0.05$ compared to 4 h within the group.
[c] $P<0.05$ compared to 4 and 6 h within the group.

sham groups, both at 4 and 6, as well as at 20 h ($F_{(7,24)}=144.1$; $P<0.001$). Conversely, AG pretreatment led to an increase ($F_{(7,24)}=16.2$; $P<0.001$) in AVP expression ratio at 4 and 24 h in the SON, compared to saline and L-NAME pretreatment ($P<0.001$) (Fig. 1, *top panel*). In the sham group, both L-NAME and AG pretreatments affected AVP expression in the SON, yet they did so in an almost inverse manner when we compared the early and late phases.

In the PVN, CLP caused a reduction in AVP mRNA expression ratio ($F_{(7,24)}=2.7$; $P=0.031$) at 4 h when compared with the sham group. L-NAME pretreatment when compared with saline ($F_{(11,36)}=23.8$, $P<0.001$) and sham ($F_{(7,24)}=8.5$; $P<0.001$) groups caused a decrease in AVP expression ratio at 4 h, 6 h and 24 h after CLP. Pretreatment with AG caused a smaller decrease in the AVP expression ratio at 4 and 20 h after CLP when compared to L-NAME groups ($P<0.001$). However, at 6 and 24 h there was a reduction compared to sham group ($F_{(7,24)}=68.1$, $P<0.001$). Additionally, at 24 h this reduction was significantly accentuated if compared to saline and L-NAME groups ($P<0.001$) (Fig. 1, *middle panel*).

Plasma AVP levels in saline treated rats increased significantly ($F_{(3,36)}=5.0$; $P=0.005$) 6 h after CLP, but by 20 h and 24 h they returned to basal levels. L-NAME injection increased AVP plasma levels at 4, 6 and 20 h ($F_{(3,36)}=5.7$; $P=0.003$) after CLP with significant difference from saline group at 4 and 20 h ($F_{(6,56)}=5.0$; $P<0.001$). However, at 24 h the AVP plasma levels also were basal in this L-NAME group. Pretreatment with AG caused a mild but sustained increase in AVP plasma levels in both sham and CLP groups. In the sham group the AVP plasma levels were higher than saline and L-NAME groups at 4, 6 and 24 h ($F_{(6,56)}=2.6$; $P=0.028$), while in the CLP animals there was a reduction compared to saline group at 6 h and to L-NAME group at 4 and 20 h (Fig. 1, *bottom panel*).

3.3. Effect of central injection of L-NAME and AG on blood pressure and heart rate

All animals showed similar basal blood pressure (87.2 ± 1.8 mm Hg) and heart rate (366.5 ± 6.1 bpm). CLP animals pretreated with saline (vehicle) injection showed a significant decrease ($F_{(38,454)}=2.8$, $P<0.001$) in blood pressure during the initial (until 6 h) and late (22 to 24 h) phase of sepsis. The pretreatment with L-NAME and AG increased ($F_{(76,454)}=1.5$, $P=0.006$) with a similar pattern the MAP in the initial phase of sepsis. However, in the late phase of sepsis, despite the apparent improvement promoted by L-NAME and AG there was

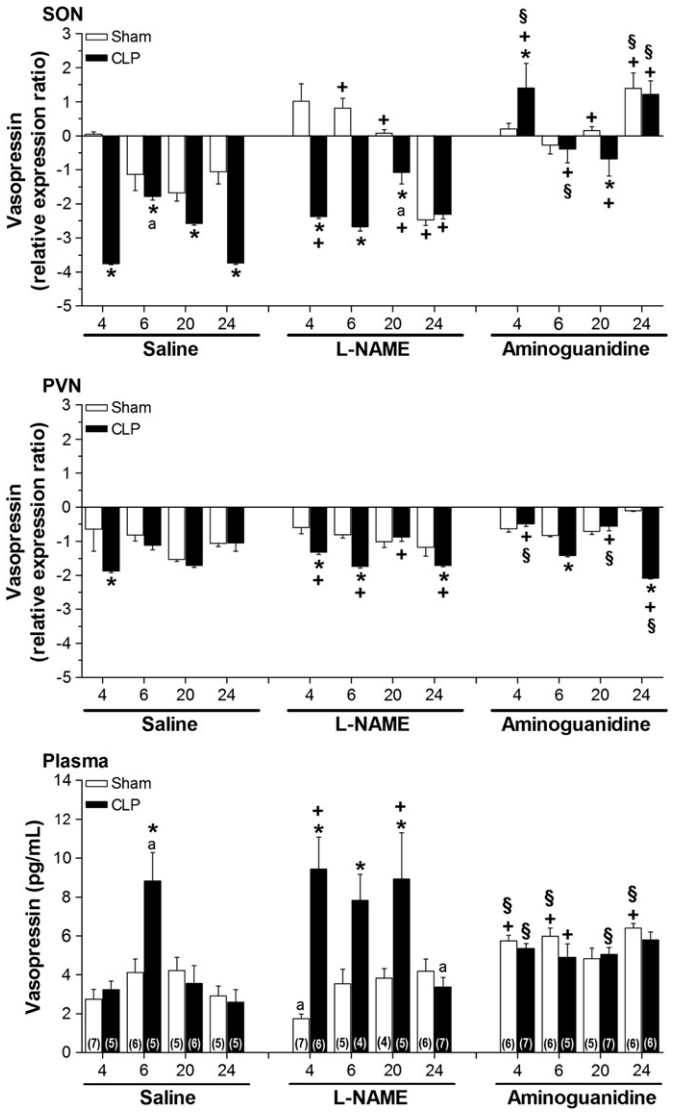

Fig. 1. Effect of intracerebroventricular pretreatment with the nitric oxide synthase inhibitors (L-NAME and Aminoguanidine, 250 μg) on vasopressin gene expression and release in animals submitted to sham or CLP. *Top panel*: Vasopressin relative expression ratio in the supraoptic nucleus (SON, $n=4$ each group). *Middle panel*: Vasopressin relative expression ratio in the paraventricular nucleus (PVN, $n=4$ each group). *Bottom panel*: Vasopressin plasma levels (n in parentheses). The data show means ± S.E.M. Statistical analysis were performed using ANOVA following by SNK test. *$P<0.05$ compared to sham group. $^{+}P<0.05$ compared to vehicle pretreated group. $^{\S}P<0.05$ compared to L-NAME pretreated group. $^{a}P<0.05$ compared to other time points within the group.

no statistical differences between the experimental groups (Fig. 2, *top panel*).

The increased heart rate after CLP ($F_{(38,362)}=9.8$, $P<0.001$) was not altered by L-NAME or AG i.c.v. pretreatment (Fig. 2, *bottom panel*). The sham groups showed similar pattern of blood pressure and heart rate alterations during all period independent of the pretreatment (data not shown).

4. Discussion

In our experiments we observed that CLP-induced sepsis resulted in a decreased AVP relative gene expression ratio (AVP_R) mainly in the SON. Moreover, we observed herein for the first time that an intracerebroventricular injection of nitric oxide inhibitors attenuated this decrease, or even increased gene expression. Therefore we suggest that the observed compromised AVP gene expression may be

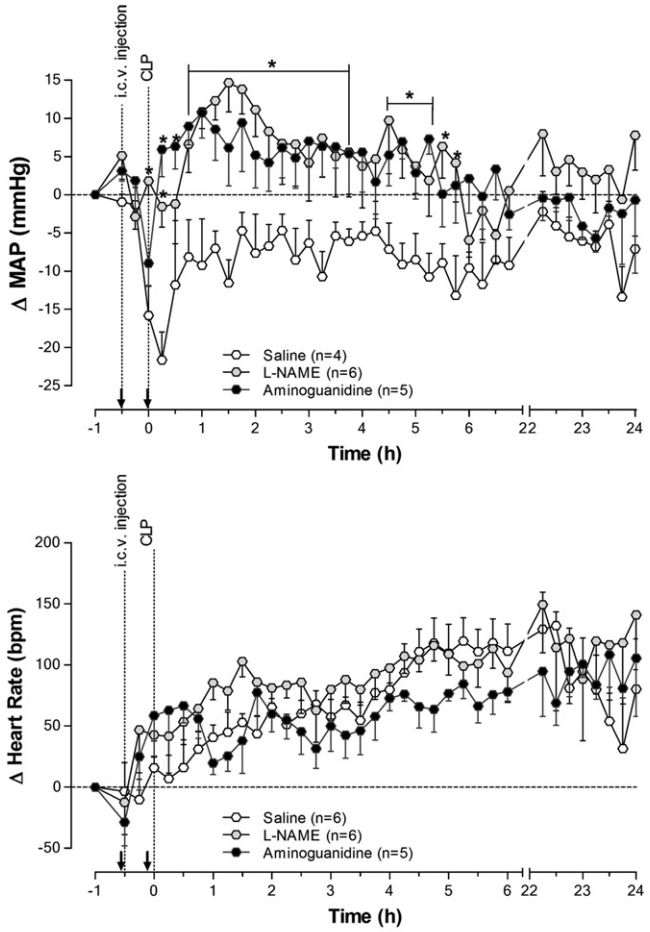

Fig. 2. Effect of intracerebroventricular pretreatment with the nitric oxide synthase inhibitors (L-NAME and Aminoguanidine, 250 μg) on blood pressure and heart rate of animals submitted to CLP. *Top panel*: Variation of mean arterial pressure (MAP) from basal. *Bottom panel*: Variation of heart rate from basal. Arrows indicate the time of the injection and CLP surgery. The data show means ± S.E.M of the deviating from basal. Number of animals (n) in parentheses. Statistical analysis performed using ANOVA following by SNK test. *$P<0.05$ compared to saline group.

caused by a central overproduction of NO, which in turn contributes to the inappropriately low basal AVP plasma levels in the late phase of sepsis.

4.1. Physiological alterations during sepsis

Hypotension and hypovolemia are the major changes usually observed in clinical and experimental sepsis (Correa et al., 2007; Holmes et al., 2001; Landry et al., 1997; Levy et al., 2003; Oliveira-Pelegrin et al., 2009; Pancoto et al., 2008; Rocha et al., 2006) and are important stimuli for AVP secretion (Cunningham and Sawchenko, 1991). As in previous studies (Correa et al., 2007; Landry et al., 1997; Oliveira-Pelegrin et al., 2009; Pancoto et al., 2008; Sharshar et al., 2003a), the septic animals showed hypovolemia and hypotension, as well as increased plasma AVP levels in the early phase, independent of osmotic changes. A strong neuronal activation in the SON and PVN (Correa et al., 2007) and in autonomic structures involved in cardiovascular regulation was previously reported and could, at least partially, be responsible for the increased plasma AVP levels observed in this phase.

As we did not see brain activation in these structures in the late phase (Bruhn et al., 2009) we consider that this may be a cause for the basal plasma AVP levels observed in clinical and experimental studies (Correa et al., 2007; Landry et al., 1997; Oliveira-Pelegrin et al., 2009; Pancoto et al., 2008; Sharshar et al., 2003a). From results obtained by pretreating septic animals intraperitoneally with aminoguanidine we hypothesized that the overproduction of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) in the late phase of sepsis may prevent hypothalamic activation, thus blunting vasopressin secretion and contributing to hypotension, irreversible shock and death (Correa et al., 2007). As there may be a difference between peripheral and central effects of aminoguanidine on NO production, and the abovementioned study only considered peripheral effects, we herein chose an intracerebroventricular injection protocol to further investigate this question.

There are contradictory reports about the presence of iNOS under 'normal' physiological conditions in the central nervous system. While an *in situ* hybridization study indicated absence of iNOS mRNA (Wong et al., 1996) others reported constitutive enzyme expression and activity in magnocellular neurons of the rat hypothalamus (Grange-Messent et al., 2004).Under normal conditions, NO present in magnocellular neurons would be synthesized not only by nNOS but also by the iNOS (Grange-Messent et al., 2004), and this basal NO production may exert tonic inhibition of vasopressinergic neurons. This could explain the low AVP_R which we observed herein in the hypothalamic nuclei of sham saline pretreated animals, as already suggested by investigators using other experimental models (Giusti-Paiva et al., 2005; Liu et al., 1997; Southam and Garthwaite, 1993). During sepsis, however, the expression and activation of the iNOS isoform in the hypothalamus increase, apparently mediated by stimulatory effect of noradrenergic input from the brainstem, which may have been caused by hypotension and/or mediated by IL-1β (Grange-Messent et al., 2004; Wong et al., 1997). Moreover, after central iNOS induction, the enzyme remains active for 4 to 24 h and produces NO in nanomolar concentrations, which are about 100 times higher than those generated by constitutive NOS (Feihl et al., 2001; Moncada et al., 1991; Wong et al., 1996).

We believe that in the early phase of sepsis, when central NO production is only moderately elevated, stimulatory noradrenergic input from the brainstem to magnocellular neurons is preserved. Furthermore, there is evidence that mRNAs encoding neuropeptides can be transported to axonal terminals in association with secretory granules (Grinevich et al., 2001, 2003) and this migration appears to be stimulated by such noradrenergic input (Xu et al., 2005). Taken together, this may explain why, during the early phase of sepsis, hypothalamus activation and plasma AVP levels are high (Correa et al., 2007) while, at the same time, there is a marked decrease in AVP_R, mainly in the SON.

In contrast, the high levels of NO production in the late phase can impair the stimulatory effects of the catecholamines (Vacher et al., 2003), thus explaining the attenuated decrease in AVP_R. Together with the previously reported impairment of hypothalamic activation and decreased plasma AVP levels, this suggests that hormonal synthesis is compromised and could be responsible for the decreased AVP secretion in this phase (Correa et al., 2007).

Recently, a decreased AVP protein content in the SON was reported in the late phase of experimental and human sepsis (Sonneville et al., 2010). Since vasopressin is mainly synthesized in the SON (Sawchenko and Swanson, 1983) this alteration could explain the depletion in neurohypophyseal content (Oliveira-Pelegrin et al., 2009; Sharshar et al., 2002).

The PVN and SON differ in their responses to noradrenergic input in physiological conditions. *In vitro* experiments showed that while in the SON there is an increase in iNOS enzymatic activity following noradrenergic stimulation, there is no such activation in the PVN (Grange-Messent et al., 2004). The differential activity of iNOS may explain why AVP gene expression in the PVN is less compromised than in the SON. This is in accordance with results indicating an increased AVP content in the PVN (Oliveira-Pelegrin et al., 2009), without correlated changes in AVP mRNA levels during sepsis (Sonneville et al., 2010). These investigators suggest that there is a

regulatory mechanism preserving functionality of the hypothalamus-hypophysis adrenal axis under septic conditions. From such a perspective, all these findings are complementary and suggest that the contribution of PVN in the alteration of AVP secretion may be minor than that of the SON.

4.2. Effect of non-selective inhibition of NOS isoforms by L-NAME injection

L-NAME blocks NOS enzyme activity without affecting the expression of NOS isoforms (Abu-Soud et al., 1994). Central inhibition of NOS by L-NAME during endotoxemia by LPS resulted in an increase in AVP release, suggesting that high concentrations of NO may have an inhibitory effect in this condition (Giusti-Paiva et al., 2003). In our experiments, the central injection of L-NAME promoted changes in AVP plasma levels as well as gene expression in both SON and PVN. In the early phase of sepsis, L-NAME pretreatment resulted in an increase in AVP plasma levels and attenuated the decrease in AVP_R in both nuclei. At 24 h however, AVP plasma levels were basal and AVP gene expression was similar to the sham group. Another reason for the inadequately low AVP plasma levels in the late phase of sepsis could be an inhibitory effect of NO directly on the neurohypophyseal hormone release (Ahern et al., 1999). The neuroendocrine changes observed after NOS inhibition by L-NAME in CLP animals are concomitant with the reversion in hypotension accompanied by normal hematocrit, both suggesting hypovolemia normalization. Thus, these results show that, at least in the initial phase of polymicrobial sepsis, the non-selective inhibition of NOS isoforms may contribute to the restoration of blood pressure, mainly by increasing AVP release.

4.3. Effect of selective inhibition of inducible NOS isoform by aminoguanidine injection

Aminoguanidine has a higher affinity for the iNOS enzyme than for the constitutive NOS isoforms. In our experiments, AG did not only attenuate the CLP-induced decrease but even caused an increase in AVP gene expression in the SON. In the PVN, its effect was less pronounced, showing only an attenuation in the decrease of AVP gene expression at 4 and 20 h after CLP. Interestingly, the intracerebroventricular injection of AG equalized AVP plasma levels throughout the two phases of sepsis. The main argument for the selective inhibition of iNOS by AG is its effect on blood pressure (Nilsson, 1999). In our experiments, blood pressure increased during the first 6 h, when compared to the vehicle group, similar to previous results following intraperitoneal AG injection (Correa et al., 2007). The effect observed in the early phase of sepsis was similar to that seen by administration of the non-selective NOS isoform inhibitor L-NAME. Nevertheless, no effect was observed in the late phase of sepsis. Furthermore, iNOS knockout mice also showed improvement in blood pressure after endotoxemia by intravenous injection of LPS (Carnio et al., 2005). These results indicate that NO produced both centrally and peripherally by iNOS has an important role in cardiovascular regulation, especially in the initial phase of sepsis.

Although polymicrobial sepsis induced by CLP may not fully represent a typical clinical situation, it closely resembles the diversity of infectious agents and the progression of peritoneal contamination with mixed flora in the presence of devitalized tissue like perforated appendicitis and diverticulitis (Deitch, 2005; Garrido et al., 2004; Hubbard et al., 2005). In this study, using this experimental model and NOS isoform inhibitors we observed for the first time a differential involvement of NO on the AVP relative expression ratio in the SON and PVN hypothalamic nuclei during polymicrobial sepsis.

In conclusion, we suggest that the increase in the mainly iNOS-derived central NO levels, may differentially inhibit AVP gene expression in both SON and PVN, and this inhibition could lead to the impairment of vasopressin synthesis and contribute to basal AVP plasma levels and to hypotension in the late phase of sepsis. Nevertheless, a more definitive picture would require determining nitric oxide levels and/or iNOS activity in the brain.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneuroim.2010.06.019.

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