Downregulation of Angiotensin Subtype 1 Receptor in Rostral Ventrolateral Medulla During Endotoxemia


Abstract—We reported recently that an upregulation of the inducible nitric oxide synthase (iNOS) in the rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons are located, is a crucial determinant for the elicitation of cardiovascular depression during experimental endotoxemia. The current study evaluated the hypothesis that a downregulation of the molecular synthesis and functional expression of angiotensin subtype 1 receptor (AT1R) in the RVLM is consequential to this upregulated iNOS. In adult Sprague-Dawley rats maintained under propofol anesthesia, intravenous administration of Escherichia coli lipopolysaccharide (15 mg/kg) elicited a reduction, followed by an augmentation and a secondary decrease in sympathetic vasomotor outflow, together with progressive hypotension and bradycardia. There was also a progressive increase in iNOS mRNA and protein level in the ventrolateral medulla. This was followed by a significant downregulation of both mRNA and protein levels of AT1R in the ventrolateral medulla, alongside reduced efficacy of angiotensin II (50 pmol) to induce an increase in systemic arterial pressure, heart rate, or sympathetic vasomotor outflow on unilateral microinjection into the RVLM. Pretreatment with microinjection of a selective iNOS inhibitor, S-methylisothiourea (250 pmol) bilaterally into the RVLM significantly reversed the reduction in both synthesis and activity of AT1R. We conclude that a downregulation of molecular synthesis and functional expression of AT1R in the ventrolateral medulla is consequential to the overproduction of NO through upregulation of iNOS in the RVLM and may underlie the cardiovascular depression that takes place during experimental endotoxemia. (Hypertension. 2003;42:103-109.)

Key Words: receptors, angiotensin | nitric oxide synthase | central nervous system | hypotension | bradycardia | nervous system, sympathetic

Sepsis is associated with profound cardiovascular abnormalities characterized by hypotension, decreased systemic resistance, altered vascular reactivity to contractile agents, and a high mortality rate. One well-known mediator of sepsis-induced circulatory failure is nitric oxide (NO) produced through activation of the inducible NO synthase (iNOS) in macrophages at the peripheral vasculature. In addition, we reported recently that overproduction of NO by iNOS in the rostral ventrolateral medulla (RVLM) is also a crucial determinant for the reduction in sympathetic vasomotor outflow and fatal cardiovascular depression during experimental endotoxemia. Whereas we proposed that the underlying mechanisms include formation of the cytotoxic substance peroxynitrite and activation of GABAergic neurotransmission, the possibility exists for the engagement of other mediators in the RVLM.

As the medullary site of the sympathetic premotor neurons, the RVLM maintains arterial pressure by providing a tonic excitation to preganglionic sympathetic neurons in the spinal cord. It is also a target site where the brain renin-angiotensin system acts to regulate sympathetic outflow. Microinjection of angiotensin II (ANG II) into the RVLM elicits an increase in systemic arterial pressure (SAP) and sympathetic activity. Of the 2 subtypes of ANG II receptors, it is generally accepted that activation of the angiotensin subtype 1 receptor (AT1R) in the RVLM contributes mainly to the sympathoexcitatory effect of ANG II. Recent studies further suggest that this excitatory angiotensinergic action on sympathetic premotor neurons in the RVLM is tonically active under normotensive or hypertensive conditions.

Several reports showed that NO antagonizes the biological functions of ANG II. For example, NO reduces ANG II receptor binding sites or AT1R gene expression in smooth muscle cells. Of particular note is that NO downregulates AT1R and inhibits ANG II–induced migration of vascular smooth muscle cells during sepsis. It follows that a downregulation of the molecular synthesis and functional expression of AT1R in the RVLM induced by the overproduction of NO through iNOS activation may underlie the reduction in sympathetic vasomotor outflow during sepsis. The present study validated this hypothesis, based on an animal model of experimental endotoxemia.

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Methods

Animals
Eight-week-old male Sprague-Dawley rats (n=338) purchased from the Experimental Animal Center, National Science Council, Taiwan, were used. All experimental procedures were approved by our institutional animal care committee.

General Preparation
Rats received during the experiment continuous intravenous infusion of propofol (30 mg·kg⁻¹·h⁻¹), which provided satisfactory anesthetic maintenance while preserving the capacity of central cardiovascular regulation.19 Pulsatile and mean systemic arterial pressure (MSAP) as well as heart rate (HR) were recorded on a polygraph (Gould RS3400).4–6,19,20 Animals were mechanically ventilated to maintain end-tidal CO₂ to be within 4% to 5%, as monitored by a capnograph (Datex Normocap). All data were collected from animals with a maintained rectal temperature of 37±0.5°C.

Evaluation of Sympathetic Vasomotor Tone
The SAP signals were simultaneously subject to on-line power spectral analysis.4–6,19–21 We were particularly interested in the very low-frequency (0 to 0.25 Hz) and low-frequency (0.25 to 0.8 Hz) components in the SAP spectrum. Our laboratory demonstrated previously20 that these spectral components of SAP signals take origin from the RVLM, and their power density reflects the prevailing sympathetic neurogenic vasomotor tone.4–6,19–21

Microinjection of Test Agents Into the RVLM
Bilateral microinjection of test agents with the use of a glass micropipette, at a volume of 50 nL, was carried out stereotaxically and sequentially into functionally identified RVLM sites as detailed previously.4–6,19–21 Each animal received a single injection of a selective iNOS inhibitor,22 5-methylisothiourea (SMT; Tocris Cookson), together with multiple doses of ANG II (Sigma) or L-glutamate (Sigma). The doses used were the same as in our recent studies4–6,21–24 when these test agents were used for the same purpose as in the current study. Microinjection of artificial cerebrospinal fluid (aCSF) served as the vehicle and volume control.

Isolation of Total RNA and Reverse Transcription–Polymerase Chain Reaction
Isolation and extraction of total RNA from the ventrolateral part of medulla oblongata, at the level of the RVLM (0.5 to 2.5 mm rostral to the obex), and reverse transcription-polymerase chain reaction (RT-PCR) analysis of AT1R, AT2R, iNOS, or GAPDH mRNA were carried out as reported previously.21–24 The amount of mRNA product for AT1R, AT2R, or iNOS was analyzed by ImageMaster VDS analysis software (Amersham Pharmacia Biotech) and was expressed as the ratio to GAPDH mRNA product.

Protein Extraction and Western Blot Analysis
Western blot analysis was carried out with the use of a rabbit polyclonal antiserum against AT1R, AT2R, iNOS, or β-tubulin (Santa Cruz) as the primary antiserum.27,28 This was followed by incubation with horseradish peroxidase–conjugated goat anti-rabbit IgG (Jackson). Specific antibody-antigen complex was detected with enhanced chemiluminescence Western blot detection system (NEN Life Science Products). The amount of AT1R, AT2R, or iNOS protein was quantified by Photo-Print Plus software (ETS Vilber-Lourmat) and was expressed as the ratio to β-tubulin protein.

Experimental Scheme
Lipopolysaccharide (LPS, 15 mg/kg, serotype 0111:B4; Sigma) was administered intravenously to induce endotoxemia. Injection of the same amount of saline served as vehicle and volume control. Temporal changes in MSAP, HR, or power density of vasomotor components of the SAP signals were routinely followed for 8 hours. As we reported previously,4–29 these hemodynamic changes can be divided into 3 phases. We determined the expression profile of AT1R, AT2R, or iNOS, at mRNA or protein level, during Phase I and during early or late stages of Phases II and III endotoxemia. We also evaluated the cardiovascular responses to microinjection of ANG II or L-glutamate into the RVLM during these 5 phases. A causative role for NO produced by iNOS in these events was further investigated by pretreatment with bilaterally microinjected SMT into the RVLM.

Statistical Analysis
All values are expressed as mean±SEM. One-way or 2-way ANOVA with repeated measures was used to assess group means, as appropriate, to be followed by the Scheffé multiple range test for post hoc assessment of individual means. A value of P<0.05 was considered statistically significant.

Results

Temporal Changes in Cardiovascular Parameters During Experimental Endotoxemia
Intravenous injection of LPS (15 mg/kg) resulted in distinct temporal changes4–29 in MSAP, HR, or the power density of vasomotor components of SAP signals, our experimental index for sympathetic vasomotor outflow. The sequence of cardiovascular events can be divided into 3 phases (Figure 1). Phase I endotoxemia manifested a significant reduction in MSAP and the power density of vasomotor components of

Figure 1. Time course changes in MSAP, HR, or total power density of vasomotor components (0 to 0.8 Hz) in SAP spectrum in rat that received intravenous administration of LPS (15 mg/kg; at arrow) or saline. These animals received, in addition, bilateral microinjection into the RVLM of SMT (250 pmol) or aCSF, given immediately before LPS treatment. Values are mean±SEM, n=6 to 7 animals in each group. *P<0.05 vs corresponding (aCSF+saline) group; #P<0.05 vs corresponding (aCSF+LPS) in Scheffé multiple range test.
SAP spectrum, which occurred immediately after LPS administration. Phase II exhibited a rebound increase in MSAP, alongside a significant reversal of the depressed vasomotor spectral signals to an increase in power density. This was followed by Phase III, which was characterized by a significant secondary decrease in MSAP and the power density of the vasomotor components of SAP signals, together with bradycardia. At the dose of LPS used, Phase III endotoxemia persisted until termination of our experiment 8 hours after administration of the endotoxin. Of note was that the manifested cardiovascular depression during this phase was significantly attenuated on pretreatment with bilateral microinjection of a selective iNOS inhibitor, SMT (250 pmol), into the RVLM, delivered immediately before animals received LPS treatment.

**Downregulation of AT1R or Upregulation of iNOS in the Ventrolateral Medulla During Experimental Endotoxemia**

RT-PCR analysis of tissue samples from the ventrolateral medulla that contains the bilateral RVLM (Figure 2) showed that the basal level of AT1R mRNA was significantly higher than that of AT2R mRNA (*P*<0.05 in quadruplicate analyses). There was also a progressive reduction in AT1R but not AT2R mRNA expression in the ventrolateral medulla during experimental endotoxemia. Of note was that the significant...
downregulation of AT1R mRNA, first detected during late Phase II endotoxemia, persisted throughout Phase III. Superimposed on a low but detectable basal expression of iNOS mRNA in the ventrolateral medulla, there was also a significant augmentation of iNOS mRNA expression that commenced at Phase I, followed by a progressive increase during Phase II and III endotoxemia (Figure 2).

Western blot analysis revealed comparable changes, with a time lag of ~45 minutes, in protein expression of AT1R, AT2R, or iNOS in the ventrolateral medulla during experimental endotoxemia (Figure 3). A significant reduction in the protein level of AT1R but not AT2R was observed at Phase III endotoxemia, along with a progressive surge of iNOS protein in the ventrolateral medulla during Phase II and III endotoxemia.

### Reversal by iNOS Inhibitor of Downregulation of AT1R in the Ventrolateral Medulla During Experimental Endotoxemia

Bilateral microinjection into the RVLM of a selective iNOS inhibitor, SMT (250 pmol), delivered immediately before animals received LPS treatment significantly reversed the reduction of AT1R mRNA (Figure 4A) or protein (Figure 4B) expression in the ventrolateral medulla during endotoxemia. The same pretreatment, however, did not appreciably affect the basal mRNA or protein level of AT1R or AT2R in the ventrolateral medulla (data not shown).

### Reduced Cardiovascular Responses to ANG II During Experimental Endotoxemia and Their Reversal by iNOS Blockade

Unilateral microinjection of ANG II (50 pmol) into the RVLM promoted a significant increase in MSAP, HR, or the power density of the vasomotor components of SAP signals (Figure 5). Whereas those ANG II–induced cardiovascular responses remained essentially unaltered in saline-treated control subjects,
the magnitude of ANG II–promoted hypertension, tachycardia, or increase in sympathetic vasomotor outflow underwent a significant reduction that began at late Phase II and persisted throughout Phase III endotoxemia. Pretreatment with local application of SMT (250 pmol) into the RVLM again significantly reversed these progressive manifestations of reduced cardiovascular responses to ANG II (Figure 6).

**Lack of Changes in Cardiovascular Responses to L-Glutamate During Experimental Endotoxemia**

Unilateral microinjection into the RVLM of L-glutamate (1 nmol) resulted in an increase in MSAP, HR, or the power density of the vasomotor components of SAP signals (Figure 7). These cardiovascular excitatory effects of L-glutamate were not significantly altered over a period of 8 hours after administration of LPS.

**Discussion**

The current study demonstrated that a downregulation of both molecular synthesis and functional expression of AT1R in the RVLM, alongside reduction in sympathetic vasomotor tone, hypotension, or bradycardia, takes place during LPS-induced endotoxemia. We further showed that this downregulation of AT1R is consequential to the overproduction of NO through activation of iNOS in the RVLM during experimental endotoxemia.

**Figure 6.** Time course changes in MSAP, HR, or total power density of vasomotor components (0 to 0.8 Hz) in SAP spectrum in rat that received intravenous administration of LPS (15 mg/kg) or saline. These animals received in addition bilateral microinjection into the RVLM of SMT (250 pmol) or aCSF given immediately before LPS treatment, followed by ANG II (50 pmol/L) at various phases after LPS treatment. Values are mean±SEM, n=6 to 7 animals in each group. *P<0.05 vs corresponding (aCSF+ANG II+saline) group; #P<0.05 vs corresponding (aCSF+ANG II+LPS) group in Scheffé multiple range test.

**Figure 7.** Time course changes in MSAP, HR, or total power density of vasomotor components (0 to 0.8 Hz) in SAP spectrum in rat that received intravenous administration of LPS (15 mg/kg) or saline. These animals received in addition unilateral microinjection into the RVLM of L-glutamate (L-Glu, 1 nmol) or aCSF, given at various phases after LPS or saline treatment. Values are mean±SEM, n=6 to 7 animals in each group. *P<0.05 vs corresponding (aCSF+saline) group in Scheffé multiple range test.
In vitro observations on smooth muscle cells indicated that NO donors suppress AT1R expression at both mRNA and protein levels under normal conditions\(^{16,17}\) or during sepsis.\(^{18}\) The current study provided novel results to suggest that comparable cellular and molecular events took place at the RVLM during experimental endotoxemia. Similar to our previous observations,\(^{4,29}\) intravenous administration of LPS elicited a robust and progressive increase in iNOS mRNA and protein expression in the ventrolateral medulla. Intriguingly, this upregulation of molecular synthesis of iNOS was followed, after a time lag, by a decrease in both mRNA and protein levels of AT1R in the ventrolateral medulla, alongside reduced efficacy of ANG II to induce an increase in SAP, HR, or sympathetic vasomotor outflow on microinjection into the RVLM. More importantly, we established a causative relationship between the surge in iNOS-induced NO and reduction in AT1R expression in the RVLM during endotoxemia by demonstrating that pretreatment with microinjection of SMT bilaterally into the RVLM significantly reversed the downregulation of molecular synthesis and functional expression of AT1R at the ventrolateral medulla.

There are at least 2 ramifications of the downregulation of molecular synthesis and functional expression of AT1R at the RVLM during experimental endotoxemia. The first one relates to the reduction in sympathetic vasomotor outflow from the RVLM. The endogenous ANG II that originates from, for example, the paraventricular nucleus of hypothalamus,\(^{15}\) plays a critical role in the generation of tonic sympathetic vasomotor activity through activation of AT1R on RVLM neurons.\(^{12-15}\) On the other hand, we reported recently that NO produced by iNOS in the RVLM elicits sympathoinhibition\(^{4,5}\) through GABAergic neurotransmission.\(^{6}\) Furthermore, fatal cardiovascular depression associated with overproduction of NO in the RVLM during endotoxemia\(^{4,5}\) engages the formation of peroxynitrite.\(^{5}\) It follows that the progressive reduction in AT1R expression in the ventrolateral medulla that commenced at late stage of Phase II and persisted throughout Phase III endotoxemia would shift the balance in favor of sympathoinhibition, resulting in the significant cardiovascular depression seen during the last phase of experimental endotoxemia.

The second ramification relates to the maintained functional role of AT2R in the RVLM. We reported previously\(^{30}\) that ANG II exerts a tonic inhibition on baroreceptor reflex through an action on AT2R in the RVLM. Whereas this mode of action of ANG II is congruent with the sympathoexcitatory effect of the octapeptide through AT1R activation at the RVLM during hypertension,\(^{15}\) it is detrimental in the case of endotoxemia. We found that the molecular synthesis of AT2R at the RVLM did not undergo discernible changes during experimental endotoxemia. It is therefore conceivable that the maintained tonic inhibitory action of ANG II on the baroreceptor reflex through AT2R in the presence of retarded efficacy of the octapeptide to increase sympathetic vasomotor outflow because of the downregulated AT1R in the RVLM will exacerbate the impending cardiovascular depression during endotoxemia.

The mechanism that underlies transcriptional regulation of AT1R by NO during endotoxemia is not clear. Ichiki et al\(^{17}\) reported that downregulation of AT1R expression by NO may be attributable to a decrease in the activity of 2 unidentified DNA binding proteins that bind to the proximal promoter region of AT1R gene. In addition, both AP-1 and NF-κB in the upstream promoter region of the AT1R gene are potential consensus elements that may be regulated by NO.\(^{17,31}\) Whether these regulators are related to NO-induced downregulation of AT1R gene during endotoxemia, however, requires further delineation.

It is possible that the reduced cardiovascular excitatory effects of ANG II might be related to nonselective cytotoxicity because of the overproduction of iNOS-produced NO in the RVLM during experimental endotoxemia. This possibility of reduced responsiveness of RVLM neurons is deemed unlikely, since the increase in SAP, HR, or sympathetic vasomotor outflow induced by microinjection of l-glutamate into the RVLM was not appreciably altered during the entire course of endotoxemia. It is also possible that cardiovascular depression induced by NO overproduction in the RVLM during endotoxemia indirectly affects AT1R. In this regard, preliminary results indicated that reducing SAP to levels comparable to Phase III endotoxemia by infusion of nitroprusside (10 μg·kg\(^{-1}·h^{-1}\)) did not discernibly affect the cardiovascular responses to microinjection of ANG II (50 pmol) into the RVLM.

In conclusion, the current study revealed that a downregulation of the molecular synthesis and functional expression of AT1R in the RVLM induced by the overproduction of NO through iNOS activation may underlie the cardiovascular depression during endotoxemia.

**Perspectives**

Several studies\(^{16-18,31,32}\) reported the downregulation of AT1R activity in the peripheral blood vessels, heart, lung, kidney, and adrenal gland during sepsis. Together with our present novel findings of a downregulation of AT1R synthesis and activity in the RVLM by NO during experimental endotoxemia, it is likely that an interaction between ANG II and NO are of key importance in the manifestation of cardiovascular abnormality associated with sepsis. ANG II–NO interaction is also involved in the pathophysiology of abnormal vascular tone, vessel wall remodeling, renal injury, insulin resistance, and hyperlipidemia during hypertension.\(^{33}\) In addition, the RVLM is now known to be intimately related to the physiological processes of cardiovascular regulation and the pathological processes of sepsis and hypertension, with differential contributions by AT1R, AT2R, and NOS isoforms.\(^{4-6,9,10,12-18,21,29,30,32,33}\) As such, further delineation of the role of ANG II–NO interactions in, and the contribution of angiotensin subtype receptors or NOS isoforms to, central cardiovascular regulation at the RVLM should open a new vista in our search for cellular and molecular mechanisms that underlie hypertension and sepsis and novel therapeutic interventions.

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