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Angiotensin Type 2 Receptors in the Intermediolateral Cell Column of the Spinal Cord: Negative Regulation of Sympathetic Nerve Activity and Blood Pressure

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Abstract

BACKGROUND—Our previous study demonstrated that AT2R in brainstem nuclei participated in the regulation of sympathetic outflow and cardiovascular function. However, the functional significance of AT2R in the intermediolateral cell column (IML) of the thoracic spinal cord in normal rats remains elusive. We hypothesized that AT2R activation in the IML exert a sympatho-inhibitory effect.

METHODS and RESULTS—Using Western-blot analysis, immunohistochemical staining and quantitative Real-Time PCR, both AT1R and AT2R expression were detected in the spinal cord. The highest AT2R protein expression was found in the IML, while AT1R expression didn't display regional differences within the gray matter. Microinjection of AngII into the IML dose-dependently elevated mean blood pressure (MAP, employing a transducer-tipped catheter) and renal sympathetic nerve activity (RSNA, using a pair of platinum-iridium recording electrodes), which was completely abolished by Losartan, and attenuated by TEMPOL and apocynin. Activation of AT2R in the IML with CGP42112 evoked hypotension (MAP: -21 ± 4 mmHg) and sympatho-inhibition (RSNA: $73 \pm 3\%$ of baseline), which were completely abolished by PD123319 and L-NAME. Blockade of AT2R in the IML with PD123319 significantly increased MAP (11 ± 1 mmHg) and sympathetic nerve activity (RSNA: $133 \pm 13\%$ of baseline). Moreover, PD123319 significantly enhanced the AngII induced pressor response. Furthermore, in isolated IML neurons, CGP42112 treatment augmented potassium current and decreased resting membrane potential by employing whole-cell patch clamp.

Conclusion—In the normal condition, AT2R in the IML tonically inhibit sympathetic activity through an NO/NOS dependent pathway and subsequent potassium channel activation.

Keywords

Intermediolateral cell column; Angiotensin type 1 receptor; Angiotensin type 2 receptor; arterial blood pressure; renal sympathetic nerve activity

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Introduction

The intermediolateral cell column (IML) of the spinal cord wherein are located the sympathetic preganglionic neurons (SPNs) plays an important role in determining sympathetic outflow and blood pressure [1, 2]. A high density of Angiotensin II (Ang II)-containing axon terminals in the IML of thoracic spinal cord [3-5] and Angiotensin type 1 receptor (AT1R) protein in the cell bodies of SPNs [6] has been demonstrated, suggesting that in the spinal cord, Ang II may act as a neurotransmitter or neuromodulator to regulate sympathetic outflow. Indeed, electrophysiological evidence from spinal cord slice preparations of rats have suggested that Ang II treatment evokes a membrane depolarization of IML neurons and increases the number of excitatory postsynaptic potentials (EPSPs) through AT1R signaling [7]. Moreover, in intact rats, intrathecal application [8, 9] or IML microinjection [10] of Ang II significantly increases sympathetic nerve activity and blood pressure by activating AT1R's. However, the functional significance of Angiotensin type 2 receptors (AT2R) within the IML of the spinal cord in the regulation of sympathetic outflow and cardiovascular activity is unknown.

AT1R and AT2R are G-protein-coupled receptors [11]. AT2R counteracts AT1R in most biological effects of Ang II [12], including the effects on neuronal electrophysiological characteristics [13, 14]. It was demonstrated that, activation of the AT1R increased neuronal excitability by depolarization through potassium channel inhibition, while AT2R suppressed neuronal excitability by an opposite mechanism [13, 14]. Even though the AT2R has long been believed to be abundantly expressed only in fetal life [8, 9, 15, 16], our recent data suggested that this notion is not always true. In contrast to the conventional concept, we found higher AT2R protein expression in adult rats compared to fetal and neonates [17], suggesting a functional role of the AT2R in mature animals. Indeed, activation of the AT2R by microinjection of the agonist, CGP42112 into the rostral ventrolateral medulla (RVLM) [18] or overexpression of AT2R by gene transfer into the RVLM [19], decreased blood pressure and suppressed sympathetic outflow. A similar inhibitory effect on blood pressure and norepinephrine excretion has also been recently observed following chronic intracerebroventricular (i.c.v.) infusion of Compound 21, a non-peptide AT2R agonist, in conscious rats [15, 20]. In the present study, we hypothesized that AT2R activation in the IML would reduce sympathetic outflow and blood pressure. We further determined a potential mechanism for this response.

Methods

Male Sprague-Dawley rats weighing between 250 to 300 g (8-12 weeks of age), were used in these experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center and were carried out under the guidelines of the American Physiological Society and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

1. Acute whole animal experiments

Each rat was anesthetized with urethane (800 mg/kg ip) and α -chloralose (40 mg/kg ip). Supplemental doses of anesthesia were administered at 1/10 of the initial dose per hour. Body temperature was maintained with a heating pad. A midline incision in the neck was made, and the trachea was cannulated to facilitate mechanical ventilation.

Recording of blood pressure and heart rate—Through a midline incision in the neck, the right common carotid artery was exposed and catheterized with a transducer-tipped catheter (model SPR-524, Millar Instruments; Houston, TX) for the measurement of pulsatile and mean arterial pressure (MAP). Heart rate (HR) was derived from the arterial

pressure pulse using a PowerLab model 16S and Chart 7 software (ADInstruments; Colorado Springs, CO). A femoral vein was cannulated with a polyethylene-20 catheter for administration of supplemental anesthesia and saline.

Recording of renal sympathetic nerve activity—The left kidney, renal artery, and nerves were exposed through a left retroperitoneal flank incision. The renal sympathetic nerves were identified, dissected free of the surrounding connective tissue, and was placed on a pair of platinum-iridium recording electrodes. When an optimal signal to noise ratio was achieved, the electrode and the renal nerve were covered with a fast setting silicone (Kwik-Sil, World Precision Instruments; Sarasota, FL). The signal was amplified with a Grass direct current preamplifier (model P18D, Astro-Med; West Warwick, RI) with the low-frequency cutoff set at 30 to 100 Hz and high-frequency cutoff at 1 to 3 kHz. The amplified discharge was imported to a computer system with other parameters. The raw nerve activity, integrated nerve activity, arterial pressure, and HR were recorded on a PowerLab data-acquisition system (model 16S, ADInstruments) and stored on disk until analyzed.

IML Microinjection—The rats were placed in a stereotaxic instrument to which a rat-spinal unit was attached (Narishige, Japan). The 5th and 12th thoracic vertebrae were rigidly fixed in the spinal unit. The dorsal surface of the spinal cord was exposed by laminectomy and irrigated with warm (37°C) paraffin oil to prevent it from drying. Microinjections were made from a quadruple-barrel glass micropipette and performed by a 4-channel pressure ejector (PM2000B, WPI). One barrel was filled with 2% Pontamine sky blue for marking the injection site, one barrel was filled with 1.77 nmol/20nL L-glutamate for the functional identification of IML, and the other two barrels were filled with test agents (Ang II, PD123319, losartan, CGP42112, TEMPO and apocynin, all of which were purchased from Sigma, St. Louis, MO). The microinjection coordinates for the IML was 0.4 - 0.6 mm lateral to the midline and 0.7 mm ventral to the dorsal surface of the spinal cord. The volume of the microinjection was determined using the marker-lines on the micropipette to observe the movement of the meniscus in the micropipette. Injections (10-20 nL) were made over a 10-s period.

2. Whole-cell patch clamp experiment

Dissociation of SPNs in the IML—Primary cultures of SPNs in the IML were prepared from spinal cord of adult male Sprague-Dawley rats (250-300g). Briefly, the thoracic spinal cord was isolated and the IML was separated from the gray matter, and then stored in pre-cooled 15ml centrifuge tubes with 1ml of Hibernate A supplemented with B27/GlutaMAX. The tissue pieces were washed twice with cooled Hibernate A with GlutaMAX and transferred to clean 15 ml centrifuge tubes containing Hibernate A with papain (2mg/ml) and GlutaMAX. The tissue pieces were incubated in 37°C for 20 minutes. The digestion were stopped by adding fetal bovine serum (FBS, final concentration was no less than 10%), followed by adding Hibernate A supplemented with B27/GlutaMAX to 40ml. The cells were filtered with a 70um filter and centrifuged at 1000 rpm for 5 min, followed by three washes with the cell culture medium. Dissociated neurons were seeded on 24-well culture plates or culture dishes (pretreated with Poly-D-Lysine) according to the protocol and maintained in culture medium. These acute isolated neurons consisted of 90% microtubule-associated protein 2 (MAP2)-immunoreactive (IR) neurons of which 85% were choline acetyltransferase (ChAT)-positive neurons.

Whole cell patch-clamp to measure potassium current and resting membrane potential—The whole cell patchclamp technique was used to determine the effect of Ang II on voltage-gated potassium current and resting membrane potential (RMP) in adult SPNs

from the IML of spinal cord. This experiment was carried out using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Measurements of the potassium current were performed in episodic-stimulation mode. For the potassium current measurement, patch pipettes were filled with (in mM) 135 KCl, 5 EGTA, 10 HEPES, 2 MgCl₂, 0.25 CaCl₂, 1 ATP, 0.1 GTP and 15 glucose, pH 7.2. The extracellular solution consisted of the following composition (in mM): 134 NaCl, 5.4 KCl, 2 MgCl₂, 10 HEPES, 10 glucose, 1.35 CaCl₂, 0.3 NaH₂PO₄ and 0.3 CdCl₂, pH 7.4. Na⁺ channels were blocked by TTX (3 μM). Cell membrane capacitance was determined by integrating the capacitive current evoked by a voltage step from 0 to 5 mV and dividing the resulting charge by the voltage step. Currents were not leak subtracted. Current traces were sampled at 10 kHz and filtered at 5 kHz. Holding potential was -80 mV. Current-voltage relations were elicited by test potentials over the range of -80 mV to +80 mV, with duration of 400 ms in 20 mV increments (5 s between steps). Peak currents were measured for each test potential. Measurements of the resting membrane potential were performed in gap-free mode. The patch pipettes were filled with (in mM) 145 potassium aspartic acid, 2.2 EGTA, 5 HEPES, 5 NaCl, 1.95 CaCl₂, 2 MgCl₂ and 10 glucose, pH 7.3. The extracellular solution consisted of the following composition (in mM): 137 NaCl, 5.4 KCl, 1 MgCl₂, 10 HEPES, 10 glucose and 2 CaCl₂, pH 7.4. P-clamp 10.2 programs (Axon Instruments) were used for data acquisition and analysis. All experiments were done at 22°C.

3. Histochemistry and Molecular Biology

Immunohistochemical staining of spinal cord section—Rats were perfused by transcardiac perfusion using chilled 4% paraformaldehyde. The spinal cord segments from T7 to L2 were removed, immersed in 4% paraformaldehyde for 1-2 h, and then dehydrated in 30 % sucrose in phosphate-buffered saline (PBS) overnight. The free-floating sections encompassing the T11 segment of spinal cord were frozen and sectioned at 30 μm on a cryostat. The tissue sections were permeabilized for 30 minutes at room temperature with a solution containing 0.3% Triton X-100 dissolved in PBS. The sections were blocked by using block solution containing 10% normal goat serum (NGS), 0.3% Triton X-100 in PBS at room temperature for 2 hours. The sections were incubated with primary antibody (mouse anti-MAP2: 1:250, Convince Inc; goat anti-AT1R: 1:250, Santa Cruz; rabbit anti-AT2R, 1:250, Abcam) in 10% NGS, 0.3% Triton X-100 in PBS at 4°C overnight. Following 3 washes with PBS, the tissue was exposed to secondary fluorescent antibody for 2hr. After washing with PBS for 3 times, the tissue sections were mounted with anti-fade reagent with DAPI at room temperature. The slides were examined with a laser confocal microscope (Leica TSC STED).

Immunocytochemical staining of isolated SPNs from the IML of spinal cord—Dissociated SPNs were plated on the cover slip. After 4 hours, the cells were fixed with 4% paraformaldehyde for 3 hours. The cells were permeabilized and blocked with solution containing 10% normal goat serum (NGS), 0.3% Triton X-100 in PBS at room temperature for 2 hours. The cells were incubated with primary antibody (chicken anti-MAP2: 1:250, Convince.Inc; mouse anti-ChAT: 1:250, Millipore; goat anti-AT1R: 1:250, Santa Cruz; rabbit anti-AT2R, 1:250, Abcam) in 10% NGS, 0.3% Triton X-100 in PBS at 4°C overnight. Following 3 washes with PBS, the cells were exposed to secondary fluorescence antibody for 2hr. After washing with PBS for 3 times, the cells were mounted on slides with anti-fade reagent with DAPI at room temperature. The slides were examined with a laser confocal microscope (Leica TSC STED).

Western Blot Analysis—In a separate group of the T7 to L2 spinal cord was removed and immediately frozen on dry ice, blocked in the coronal plane and sectioned at 0.5 mm thickness in a cryostat. The anterior column, posterior column, IML, and white matter were

punched using 21-g needles, and homogenized in RIPA buffer. Protein extraction from homogenates was used to analyze AT1R and AT2R expression by Western blot. The concentration of protein extracted was measured using a protein assay kit (Pierce; Rockford, IL) and adjusted to the same with equal volumes of 2X 4% SDS sample buffer. The samples were boiled for 5 min following by loading on a 10% SDS-PAGE gel (10 µg protein /30 µL per well) for electrophoresis using a Bio-Rad mini gel apparatus at 40 mA/ gel for 45 min. The fractionized protein on the gel was transferred onto a PVDF membrane (Millipore) and electrophoresed at 300 mA for 90 min. The membrane was probed with primary antibodies (rabbit anti-AT1R: 1:1000, Santa Cruz; rabbit anti-AT2R, 1:1000, Abcam) and secondary antibody (goat anti-rabbit IgG-HRP, Santa Cruz, 1:2500), and then treated with enhanced chemi-luminescence substrate (Pierce; Rockford, IL) for 5 min at room temperature. The bands in the membrane were visualized and analyzed using UVP BioImaging Systems.

Quantitative Real-Time PCR—RNA was extracted using TRIZOL Reagent (Invitroen) according to the manufacturer's instructions. The RNA quality and quantity were verified using NanoDrop 2000 (Thermo). Total RNA was reverse-transcribed using the iScript cDNA synthesis kits (Bio-RAD) according to manufacturer's protocols. Quantitative RT-PCR assays were carried out using SsoFast Eva Green Supermix RT-PCR kit (Bio-RAD). Relative quantification was analyzed using PTC-200 (Bio-RAD) and Chromo 4 continuous fluorescence detector (Bio-RAD). In each experiment, GAPDH RNA was amplified as a reference standard. Expression data for AT1R and AT2R was first normalized against GAPDH RNA as CT, and then the relative expression in different regions of the spinal cord was compared to IML using the CT method for quantification in Opticon Monitor software (Bio-RAD). Relative fold changes were determined as RQ values. The primers were designed using online software at <https://www.genscript.com/ssl-bin/app/primer> and synthesized in the Eppley Cancer Institute Molecular Biology Core Laboratory on the campus of the University of Nebraska Medical Center. Please see Table 1 for details of gene-specific primers.

Statistical Analyses

All data are described as the mean ± SEM. The integrated RSNA before agent intervention was set as a 100 % of baseline. The change in RSNA induced by a given agent was described as a % of baseline. A one way ANOVA was used followed by the Newman-Keuls analysis. Statistical analysis was done with the aid of Sigmaplot software. P < 0.05 was considered statistically significant.

Results

Expression of AT1R and AT2R

Based on retrograde labeling data in rats, the distribution of SPNs in the IML innervating the kidney spans from the T6 to L1 segments of the spinal cord, mostly concentrating in T11 [21]. Therefore we focused on the T11 segment in these studies. Figure 1 illustrates the protein and mRNA expressions of AT1R and AT2R in T11 segments of the spinal cord. Panel A shows immunofluorescence images illustrating the distribution of neurons (MAP2 as a neuronal marker) and the expression of AT1R and AT2R. MAP2 fluorescence converges at the grey matter, confirming this region as a major location of neuron cell bodies in the spinal cord. In addition, grey matter also exhibits a stronger AT1R immunoreactive signal than white matter, suggesting that neuronal cell bodies express more AT1R protein than axons. AT2Rs are also highly expressed in grey matter. Interestingly, the IML expresses the highest AT2R protein in the spinal cord. The Western blot results shown in panel B confirm the immunohistochemistry findings. Panel C shows the results of AT1R

and AT2R gene expression, which demonstrates no significant difference in mRNA levels of these two receptors among the four detected spinal cord regions.

Effect of Ang II on sympathetic nerve activity and blood pressure

Microinjection of 200, 400 and 800 pmol Ang II (in 20 nL normal saline) unilaterally into the IML at T11 resulted in dose-dependent, increased ipsilateral renal sympathetic nerve activity and systemic blood pressure. The rationale for choosing the dose of Ang II is based upon our previous study [18]. The peak responses of blood pressure and sympathetic nerve activity appeared approximately 1-3 min after Ang II treatment (RSNA peak: $143 \pm 3\%$, $163 \pm 18\%$, and $207 \pm 29\%$ of baseline; MAP peak: 10 ± 1 mmHg, 15 ± 2 mmHg, and 20 ± 2 mmHg for 200, 400, and 800 pmol Ang II, respectively). The pressor response to the high dose Ang II lasted longer than 30 min. Ang II did not significantly change HR. Ang II into the anterior column, posterior column, or white matter, or microinjection of normal saline into the IML did not evoke significant cardiovascular or sympathetic responses (data not shown). Based on the above dose-dependent responses, we used 400 pmol as the primary dose of Ang II in all of the following experiments.

Pretreatment of T11 segment with 4 nmol Losartan for 10 minutes, an AT1R blocker, completely abolished the Ang II-induced pressor response (MAP: 4 ± 1 mmHg vs. 15 ± 2 mmHg, $P < 0.05$) and sympatho-excitation (RSNA: $103 \pm 3\%$ vs. $163 \pm 18\%$, $P < 0.05$). However, Losartan itself in the IML slightly decreased blood pressure (MAP - 4 ± 1 mmHg) and increased RSNA ($110 \pm 2\%$ of baseline), which did not reach statistical significance.

Since reactive oxygen species (ROS) was involved in AngII-induced pressor effect in RVLM, we next want to examine the role of ROS in AngII-induced response in spinal cord by using pharmacological approach. As shown in Figure 2, exposure of T11 segment with superoxide anion scavenger (TEMPO, 0.1 μ mol) and the NAD(P)H oxidase inhibitor (apocynin, 3 nmol) completely abolished the Ang II-evoked hypertension and sympatho-excitation. TEMPO or apocynin themselves has no effect on blood pressure and sympathetic activity (data not shown).

Effect of AT2R on sympathetic nerve activity and blood pressure

Having determined the high expression of AT2R in IML, we sought to the physiological function of AT2R on sympathetic nerve activity and blood pressure. Microinjection of CGP42112, a specific peptide AT2R agonist, into the IML evoked a decrease in both blood pressure (MAP: -21 ± 4 mmHg) and sympathetic nerve activity (RSNA: $73 \pm 3\%$ of baseline), suggesting inhibition of sympathetic outflow (Figure 3). The decrease in blood pressure and RSNA were inhibited by pretreatment with the AT2R antagonist PD123319. Next, as a measure to assess the role of NO pathway in AT2R-mediated inhibition of sympathetic outflow, pretreatment of T11 segment with L-NAME, a NOS inhibitor, resulted in attenuation of this response.

AT2R activation inhibits the response to AT1R activation

To clarify the potential crosstalk between AT1R and AT2R in the IML in the regulation of blood pressure and sympathetic outflow, we evaluated the effects of PD123319 alone and PD123319 plus Ang II. PD123319 alone in the IML evoked a significant increase in both blood pressure (MAP: 11 ± 1 mmHg) and sympathetic nerve activity (RSNA: $133 \pm 13\%$ of baseline) (Figure 4a). On the other hand, following pretreatment with PD123319, Ang II evoked a bigger pressor response than Ang II alone (MAP: 30 ± 3 mmHg vs. 15 ± 2 mmHg, $P < 0.05$). Ang II following PD123319 induced a sympatho-excitatory response which was smaller than Ang II alone (Figure 4b and 4c).

Effects of Ang II on electrophysiological characteristics of isolated IML neurons

Based on the premise that activation of potassium channel was involved in AT2R-mediation regulation of blood pressure in RVLM, paraventricular nucleus (PVN) and SON, we rationalized that this channel could likely be involved in AT2R-mediated physiological response in spinal cord. We examined the effect of AT2R activation on potassium current and resting membrane potential in acutely dissociated IML neurons from adult rats using whole-cell patch clamp. As shown in Figure 5A and B, acute isolated IML neurons were SPNs as evidenced by ChAT immunofluorescence staining (SPNs marker [22-24]) immunoreactivity which are co-localized with MAP2 (mature neuron marker) staining. Furthermore, these neurons are also express AT1R and AT2R (Figure 5C).

We next examined the effect of AT2R activation on the potassium current. As shown in Figure 6, activation of AT2R in acute isolated SPNs with CGP42112 perfusion significantly increased potassium current and lowered resting membrane potential, both of which were blocked by AT2R antagonist PD123319.

Discussion

Cardiovascular and autonomic function are tightly controlled by multiple specific regions of the central nervous system, including the PVN and median preoptic (MnPO) in the hypothalamus, nucleus tractus solitarius (NTS), RVLM, caudal ventrolateral medulla (CVLM), and locus coeruleus (LC) in the brainstem, and IML in the spinal cord [25-27]. Ang II has been documented to participate in this regulatory process within most of above brain areas [28]. Very few reports however, have examined the influences of Ang II on blood pressure and sympathetic nerve activity in the IML. The only published study in whole animals was reported by Lewis and Coote in 1993 [10], in which they demonstrated a sympathoexcitation via activating AT1R by microinjection of Ang II into the IML. The functional significance of AT2Rs in the IML, on the other hand, is completely unknown. In the current study, we found that: (1) Both AT1R and AT2R proteins are expressed in the spinal cord, with higher levels in the grey matter and lower levels in white matter. There was no difference in AT1R expression between different regions of grey matter, while AT2R expression was higher in IML compared to the anterior and posterior columns. (2) Activation of AT1Rs in the IML evoked a hypertension and sympatho-excitation, which were mediated by NAD(P)H oxidase derived ROS. (3) Stimulation of AT2R in the IML, on the other hand, induced a hypotension and sympatho-inhibition through the NO/NOS signaling pathway. (4) Blockade of AT2Rs in the IML by PD123319 increased blood pressure and RSNA. In addition, AT2R blockade enhanced Ang II induced sympatho-excitation. (5) In acutely dissociated IML neurons, activation of AT2R facilitated potassium current and induced a hyperpolarization. These results reveal a sympatho-inhibitory effect of AT2R in the IML via a NO/NOS signaling pathway. Moreover, this inhibitory effect possesses a “tonic” characteristic, implying an important counterbalancing role of endogenous AT2R against AT1R in the IML to maintain a normal sympathetic tone under physiological conditions.

The major actions of Ang II are mediated by two 7 transmembrane, G protein-coupled receptors, the AT1R and AT2R [11]. Currently, the AT2R is believed to be abundantly expressed in fetal tissue and acts as the predominant Ang II receptor subtype prenatally. After birth, however this receptor is dramatically reduced or even disappears and the AT1R becomes dominant [29]. The AT2R therefore, has been thought to be of little functional significance in adulthood [30-33]. However, recently published data from this laboratory call this concept into question. We showed that AT2R protein is expressed to a higher degree in brain, liver, and kidney of adult rats compared to that in fetuses [17]. In the mouse brainstem, we found a gradual increase in AT2R protein expression during progression from

fetal to adult life [34]. In the current study, we found AT2R expression in the spinal cord of adult rats and substantial AT2R protein expression in the IML (Figure 1), strongly suggesting a critical role of the AT2R within the IML in adulthood.

Our previous studies have documented an inhibitory effect of stimulation of AT2Rs in the brain on sympathetic outflow. In conscious rats, chronic intracerebroventricular infusion of the non-peptide AT2R agonist, Compound 21, decreased arterial blood pressure and norepinephrine excretion [20]. In anaesthetized rats, microinjection of CGP42112, the classic AT2R agonist, directly into the RVLM evoked hypotension and sympatho-inhibition [18]. In the present study, we found that activation of AT2Rs in the IML by CGP42112 decreased blood pressure and RSNA, which was abolished by pretreatment with PD123319, an AT2R antagonist, and L-NAME, a NOS inhibitor. These results document a negative regulation of sympathetic outflow by AT2Rs in the IML through NO/NOS pathway, similar to that in the brain [18-20]. More importantly, blockade of the AT2R in the IML by PD123319 increased blood pressure and RSNA, suggesting a tonic inhibitory influence of AT2Rs in the IML under physiological conditions. This tonic effect appears to be unique to the IML because it was not observed in the RVLM [18]. Therefore AT2Rs may play a more important role in the IML to oppose AT1Rs than that in other central areas.

AT1R and AT2R display distinctly opposing effects in most physiological processes [12]. For example, in cultured neurons, Ang II augments neuronal excitability by suppressing potassium currents through AT1R and inhibits neuronal excitability by an opposing process through the AT2R [35-38]. In anaesthetized rats, stimulating AT1R in the RVLM significantly increases blood pressure and RSNA and activation of AT2R within this brainstem region evokes a hypotension and sympatho-inhibition [18]. AT1R and AT2R have been demonstrated to possess a similar affinity for Ang II [39, 40], and these two receptors are often co-expressed in most organs and tissues [39, 41]. The integrated responses to Ang II can be attributed, in part, to combined AT1R and AT2R-mediated actions. Due to the uneven expression levels between AT1R and AT2R however, the biological effects of Ang II are primarily dependent on activation of the AT1R. In the present study, microinjection of Ang II into the IML dose-dependently increased blood pressure and RSNA, suggesting that the AT1R is the predominant receptor subtype in this spinal region. Indeed, pretreatment with Losartan completely abolished these effects induced by Ang II. In the current study, we further analyzed the downstream signaling pathway of AT1R stimulation. Pretreatment with TEMPO (a superoxide anion scavenger) or apocynin (an NAD(P)H oxidase inhibitor) attenuated the hypertension and sympatho-excitation induced by Ang II in the IML, suggesting an involvement of NAD(P)H derived superoxide in this process, a well-documented mechanism underlying Ang II effect in other brain regions [42-44]. Even though the AT1R generally acts as the predominant Ang II receptor subtype in adult animals, this does not mean that AT2Rs do not have a significant influence. In rats with chronic heart failure, we demonstrated a contribution of decreased AT2R expression and function in the RVLM to sympathoexcitation, possibly due to the loss of an opposing action from AT2R against AT1R [18]. This opposing effect of AT2R in the RVLM, however does not appear to play a role in maintaining normal sympathetic drive under physiology conditions because blocking AT2R did not alter baseline RSNA in this setting. In contrast, in the current study, we found hypertension and sympatho-excitation induced by the microinjection of PD123319 into the IML in normal rats (figure 4), suggesting a crucial role of AT2R in the IML to maintain sympathetic tone under physiology conditions. Indeed, blockade of AT2R in the IML potentiated the Ang II-evoked pressor response, implying an opposing function of AT2R signaling to that of AT1R in the IML in blood pressure control. However, the sympatho-excitation induced by Ang II following PD123319 pretreatment was smaller than that by Ang II itself, possibly due to an activation of some sympatho-inhibitory reflexes, such as the arterial baroreceptor reflex.

The SPNs in the IML are a crucial relay to bridge between sympathetically relevant brain regions and the peripheral sympathetic nervous system [45]. The somas and dendrites of SPNs receive direct projections from neurons in supraspinal regions. Axons of SPNs exit the spinal cord to innervate post-ganglionic neurons in sympathetic ganglia and chromaffin cells in the medulla of the adrenal gland [46, 47]. On the other hand, the SPNs in the IML also receive direct synaptic input from local interneurons in the IML or other sites of the spinal cord, which mediate dorsal root-afferent signaling [48] or supraspinal descending impulses to the SPNs [47, 49]. Within this complex neuronal network, various neurochemicals are involved in the transmitting or modulation of excitatory or inhibitory drive to SPNs [45]. Based on the present study, we cannot determine whether Ang II in the IML functions as a neurotransmitter or neuromodulator acting on SPNs presynaptically or postsynaptically, or act simply as a regulatory hormone acting on the neuronal membrane receptors to exhibit its influence on sympathetic outflow. However, the reported electrophysiological evidence supports the hypothesis that Ang II induces both presynaptic and postsynaptic effects on IML neurons [7, 10]. In acutely dissociated IML neurons of adult rats in the present study, we found an AT2R mediated facilitation of potassium current and a decrease of resting membrane potential (Figure 6), suggesting a postsynaptic effect, at least as one mechanism of Ang II influence on SPNs. Indeed, we found ample AT2R protein expression in SPNs. Even though substantial histological and functional evidences have demonstrated profound influences of Ang II on glutamatergic and GABAergic synaptic transmission in the central nerve system, whether Ang II should be considered as a neurotransmitter is still controversial [50].

Study limitations

In the patch-clamp experiments, the acute isolated SPNs were applied, in which the SPNs were in stress status and the response may be different from the physiologic condition. Actually, we found that the acute isolated SPNs neurons only fired single action potentials with limited culture duration (data not shown). Although studies using dissociated adult cells have not been as extensively investigated due to the difficulties associated with long-term culturing adult CNS neurons, further studies are required to examine the electrophysiological characteristics of long-term cultured SPNs from IML.

Another limitation was in the specificity of anti-AT1-R and anti-AT2-R antibodies, further studies are required to verify the specificity of these antibodies by immunostaining in AT1-R or AT2-R knockout mice.

Conclusion

In conclusion, in the IML of the spinal cord, AT2Rs mediate a sympatho-inhibition and hypotension via a NO/NOS signaling pathway. Importantly, this negative regulation by AT2Rs is tonically active and therefore may play a critical role in maintaining normal sympathetic tone by counteracting the sympatho-excitatory effects of AT1R stimulation. In this study, we also found that in the IML, NAD(P)H oxidase-derived ROS is a major pathway mediating AT1R-induced increases in blood pressure and sympathetic outflow.

Perspectives

Even though Ang II has been well documented to be involved in sympathetic regulation within various regions of the central nervous system under both physiological and pathological conditions [51], the functional significance of Ang II and its receptors in the IML of the spinal cord remain largely unexplored. The present study reveals two interesting phenomena: (1) AT2R protein expression is higher in the IML compared to the other regions of spinal cord. (2) AT2Rs tonically suppress baseline RSNA. Given the critical contribution

of central Ang II to sympatho-excitation in many cardiovascular diseases, such as chronic heart failure [52], hypertension [53], and diabetes [54], it is important to clarify the pathological significance of Ang II and its receptors, especially the AT2R, in the IML. The altered expression and function of AT2R in the IML may contribute to the sympatho-excitation in the above mentioned diseases. For instance, renal nerve ablation (renal sympathetic denervation) is a novel strategy to treat resistant hypertension [55]. Our findings suggested that the AT2R in the IML has a postsynaptic effect which can inhibit the sympathetic outflow. As a result, manipulation of AT2R expression or activity in this spinal site by genetic or chemical methods may synergize with the effects of renal nerve ablation.

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Fig 1. Expression of AT1R and AT2R in spinal cord

A) Confocal immunofluorescent images showing AT1R and AT2R expression in the T11 segment of the spinal cord. **B)** Western-blot showing AT1R and AT2R protein expression in different regions of the spinal cord. (a) Representative blots and (b) mean data of blot density. #P < 0.05 vs white matter, *P < 0.05 vs IML; n = 3 in each group. **C)** Gene expression of AT1R and AT2R in different regions of the spinal cord. All genes were examined by real-time RT-PCR with gene-specific primers. $\Delta C(t)$ (a) and relative fold change to IML (b) were calculated with CT value (see details in Materials and Methods). n = 3 in each group.

Fig 2. Effect of Ang II on sympathetic nerve activity and blood pressure in the IML

A) Representative original recording of MAP and RSNA showing the responses to Ang II (a), Ang II following TEMPO (b), and Ang II following apocynin (c) in the IML. **B)** Mean data showing change of mean arterial pressure (a), sympathetic nerve activity (b), systolic pressure (c), diastolic pressure (d) and heart rate (e) after treatment with saline, Ang II, Ang II following TEMPO, or Ang II following apocynin, respectively. * $P < 0.05$ vs Saline group, # $P < 0.05$ vs Ang II-treated group; $n = 5$ in each group.

Fig 3. Effect of AT₂R on sympathetic nerve activity and blood pressure

A) Representative original recording of MAP and RSNA showing the responses induced by CGP42112 (a), CGP42112 following PD123319 (b), and CGP42112 following L-NAME (c) in the IML. **B)** Mean data showing change of mean arterial pressure (a), sympathetic nerve activity (b), systolic pressure (c), diastolic pressure (d) and heart rate (e) after treatment of saline, CGP42112, CGP42112 following PD123319, or CGP42112 following L-NAME, respectively. * $P < 0.05$ vs Saline group, # $P < 0.05$ vs Ang II-treated group; $n = 5$ in each group.

Fig 4. AT2R activation inhibits the response to AT1R activation

A) Representative original recording of MAP and RSNA showing the responses induced by PD123319 (a), Ang II (b), and Ang II following PD123319 (c) in the IML. **B)** Mean data showing change of mean arterial pressure (a), sympathetic nerve activity (b), systolic pressure (c), diastolic pressure (d) and heart rate (e) after treatment of saline, PD123319, Ang II or Ang II following PD123319, respectively. * $P < 0.05$ vs Saline group, # $P < 0.05$ vs Ang II-treated group; $n = 5$ in each group.

Fig 5. Expression of AT1R and AT2R in acute isolated SPNs

Immunostaining of AT1R (A) or AT2R (B) are colocalized with MAP2 and ChAT-positive staining. AT1R and AT2R are colocalized in these neurons (C). DAPI is nuclear marker; MAP2 is mature neuronal marker; ChAT is the specific SPN marker. Scale bars, 20µm.

Fig 6. Effects of Ang II on electrophysiological characteristics of isolated IML neurons

A) Representative whole cell patch-clamp recording showing that the transient K⁺ current was enhanced by CGP42112 treatment (100 nM). **B)** Peak K⁺ current measured in response to a test pulse from -80 to +80 mV. **C)** Complete current-voltage relationships from vehicle, CGP42112, PD123319, and PD123319 + CGP42112 treatments. **D)** Resting membrane potential. Values are means ± SE. *P < 0.05 vs vehicle group. #P < 0.05 vs CGP42112 group. n = 10 in each group.

Table 1
Gene-specific primers for real-time RT-PCR

Name of genes (Accession No.)	Forward primers	Reverse primers	Amplicon Size (nt [*])
Rat AT1R (NM_030985)	CCCAAGTCCACACATCAAAG	GCAAGGCAGACTGTATGGAA	129
Rat AT2R (NM_012494)	AGCAGAAACATCACCAGCAG	GGAATTGCTTCCAAATGCTT	113
Rat GAPDH (AF106860)	TCAAGAAGGTGGTGAAGCAG	AGGTGGAAGAATGGGAGTTG	111

*
nt, nucleotide numbers.