Sympathoexcitation by central ANG II: Roles for AT$_1$ receptor upregulation and NAD(P)H oxidase in RVLM

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Gao, Lie, Wei Wang, Yu-Long Li, Harold D. Schultz, Dongmei Liu, Kurtis G. Cornish, and Irving H. Zucker. Sympathoexcitation by central ANG II: Roles for AT$_1$ receptors and NAD(P)H oxidase in the rostral ventrolateral medulla (RVLM). Am J Physiol Heart Circ Physiol 288: H2271–H2279, 2005. First published January 6, 2005; doi:10.1152/ajpheart.00949.2004.—Chronic heart failure is often associated with sympathoexcitation and blunted arterial baroreflex function. These phenomena have been causally linked to elevated central ANG II mechanisms. Recent studies have shown that both mRNA and protein expression of AT$_1$ receptors (83.2%; 

P 0.05; protein, 85.1%; 

P 0.05), and O$_2$ production (83.2%; 

P 0.05) in the RVLM. In addition, impaired baroreflex control of HR (Gain$_{max}$ reduced by 48.2%; 

P 0.05), and RSNA (Gain$_{max}$ reduced by 53.6%; 

P 0.05) by ANG II was completely abolished by losartan. Losartan significantly decreased baseline RSNA (~49.5%; 

P 0.05) and increased baroreflex control of HR (Gain$_{max}$ increased by 64.8%; 

P 0.05) and RSNA (Gain$_{max}$ increased by 67.9%; 

P < 0.05), but had no significant effects on mRNA and protein expression of AT$_1$ receptor and NAD(P)H oxidase subunits and O$_2$ production in the RVLM. These data suggest that in normal rabbits, NAD(P)H oxidase-derived ROS play an important role in the modulation of sympathetic activity and arterial baroreflex function by subchronic central treatment of exogenous ANG II via AT$_1$ receptors.

baroreflex; renal sympathetic nerve activity; free radicals; angiotensin II type 1 receptor; rostral ventrolateral medulla; reactive oxygen species

CHRONIC HEART FAILURE is often associated with sympathoexcitation (24, 40, 41, 63) and impaired arterial baroreflex function (15, 50, 54, 55). These phenomena have been causally linked to elevated central ANG II (14, 16, 39, 56). Indeed, increases in central ANG II have been shown to increase sympathetic outflow and blunt arterial baroreflex responses in normal animals (4, 57). Conversely, central administration of losartan has been shown to reduce sympathetic outflow and enhance baroreflex sensitivity in rats with chronic heart failure (14, 34). Low-dose, chronic suppressor infusion of ANG II into conscious rabbits is capable of resetting the arterial baroreflex (3, 4). However, high-dose, chronic central ANG II infusions in conscious rabbits did reduce the baroreflex control of heart rate (HR) (17).

Recent evidence indicates that NAD(P)H oxidase-derived reactive oxygen species (ROS) are important mediators of ANG II signaling (22, 61). ANG II not only augments ROS formation and increases oxidative activity but also upregulates mRNA and protein expression of most NAD(P)H oxidase subunits both in vitro (44) and in vivo (38). In addition, ROS have been shown to play an important role in various physiological and pathophysiological processes (25, 47) in the central nervous system. ROS have been linked to regulation of sympathetic nerve activity. For example, treatment with the cell membrane-permeable superoxide dismutase (SOD) mimetic tempol decreased renal sympathetic nerve activity (RSNA) in both normotensive and hypertensive rats (58, 59). Conversely, the SOD inhibitor diethyldithiocarbamate has the opposite effect (48).

AT$_1$ receptors are widely distributed in the central nervous system from the forebrain to the brain stem (28). The rostral ventrolateral medulla (RVLM) contains a high density of these receptors (1) and is a major site of sympathoexcitation in response to ANG II administration into the cerebrospinal fluid (23). The RVLM not only plays a critical role in the generation and maintenance of sympathetic nerve activity (11, 12) but also is an essential part of the central baroreflex pathway (10, 18).

Therefore, we hypothesized that centrally administered ANG II may act via an AT$_1$ receptor mechanism to activate sympathetic outflow and impair arterial baroreflex function by stimulation of NAD(P)H oxidase and ROS in the RVLM. The purpose of this study was to determine whether central subchronic infusion of ANG II stimulates O$_2$ production and expression of NAD(P)H oxidase subunits and AT$_1$ receptors in the RVLM. Second, we identified the effects on sympathetic nerve activity of ANG II type 1 (AT 1) receptors in the rostral ventrolateral medulla (RVLM). The aims of this study were to determine whether central subchronic infusion of ANG II in normal animals has effects on O$_2$ production and expression of NAD(P)H oxidase subunits as well as ANG II type 1 (AT 1) receptors in the rostral ventrolateral medulla (RVLM). Twenty-four male New Zealand White rabbits were divided into four groups and separately received a subchronic intracerebroventricular infusion of saline alone, ANG II alone, ANG II with losartan, and losartan alone for 1 wk. On day 7 of intracerebroventricular infusion, mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA) values were recorded, and arterial baroreflex sensitivity was evaluated while animals were in the conscious state. We found that ANG II significantly increased baseline RSNA (161.9%; 

P < 0.05), mRNA and protein expression of AT 1 receptors (mRNA, 66.7%; 

P < 0.05; protein, 85.1%; 

P < 0.05), NAD(P)H oxidase subunits (mRNA, 120.0–200.0%; 

P < 0.05; protein, 90.9–197.0%; 

P < 0.05), and O$_2$ production (83.2%; 

P < 0.05) in the RVLM. In addition, impaired baroreflex control of HR (Gain$_{max}$ reduced by 48.2%; 

P < 0.05) and RSNA (Gain$_{max}$ reduced by 53.6%; 

P < 0.05) by ANG II was completely abolished by losartan. Losartan significantly decreased baseline RSNA (~49.5%; 

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P < 0.05), but had no significant effects on mRNA and protein expression of AT 1 receptor and NAD(P)H oxidase subunits and O$_2$ production in the RVLM. These data suggest that in normal rabbits, NAD(P)H oxidase-derived ROS play an important role in the modulation of sympathetic activity and arterial baroreflex function by subchronic central treatment of exogenous ANG II via AT 1 receptors.

baroreflex; renal sympathetic nerve activity; free radicals; angiotensin II type 1 receptor; rostral ventrolateral medulla; reactive oxygen species

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METHODS

Animals. Experiments were carried out on 24 male New Zealand White rabbits that weighed 3.5–4.2 kg. These experiments were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and conformed to the Guidelines for the Care and Use of Experimental Animals of the American Physiological Society and the National Institutes of Health. Rabbits were housed in individual cages under controlled temperature and humidity with a 12:12-h dark-light cycle and were fed standard rabbit diet (sodium content, 0.31%; Harlan Teklad) with water available ad libitum.

Subchronic lateral cerebral ventricle infusion. The skull was exposed through an incision on the midline of the scalp. After the bregma was identified, a 19-gauge stainless steel cerebroventricular cannula was implanted into the right lateral cerebral ventricle, 4 mm lateral to the bregma and 6 mm below the cerebrum surface, and fixed tightly to the skull with super-glue adhesive and dental cement. The position of the cannula in the lateral cerebral ventricle was confirmed by the staining of all four ventricles after injection of 0.1 ml of Evans blue dye at the end of the experiments. An osmotic minipump (model 201; Durieck; Cuptertino, CA) filled with isotonic saline (1 μl/h), ANG II (80 ng·μl⁻¹·h⁻¹), ANG II (80 ng·μl⁻¹·h⁻¹) plus losartan (20 μg·μl⁻¹·h⁻¹), or losartan alone (20 μg·μl⁻¹·h⁻¹) was implanted subcutaneously in the back of neck and connected to the cerebroventricular cannula. The infusion was continued for 7 days.

Arterial pressure and HR recording. While animals were under anesthesia and aseptic conditions, a catheter connected to a radio-telemetry unit (Data Sciences International; St. Paul, MN) was inserted into the descending aorta via a branch of the right femoral artery for direct measurement of arterial pressure (AP) with rabbits in the conscious state. HR was derived from the AP pulse using a PowerLab (model 8S; ADInstruments; Colorado Springs, CO) data-acquisition system.

RSNA recording. RSNA-recording electrodes were implanted as described previously (32). In brief, while animals were under anesthesia and under sterile conditions, the left kidney was exposed retroperitoneally, and a branch of renal nerve was separated from the renal plexus and the surrounding connective tissues. A pair of stainless-steel, stranded Teflon-coated recording electrodes were placed around the nerve branch. The nerve-electrode junction was insulated electrically from the surrounding tissues and covered with fast-setting silicone (Kwik-Sil; World Precision Instruments; Sarasota, FL). A ground wire was secured to a nearby muscle. The recording electrodes and ground wire were tunneled under the skin and exteriorized in the midscapular area. During the experiment, the electrical signal from the electrode was amplified with a Grass P55 preamplifier (Grass Instruments; West Warwick, RI) with high- and low-frequency cutoffs of 1,000 and 100 Hz, respectively. The output from the Grass amplifier was directed to the PowerLab system, which sampled at 1,000 samples/s. The signal was also full-wave rectified and integrated. The average rectified signal (RC filtered; time constant, 0.5 s) was then recorded and stored for later analysis. The frequency of nerve discharge was counted by using a window discriminator and rate meter. The cursor of the window discriminator was set just above the electrical noise. Both frequency and integrated nerve activity were recorded continuously along with the raw nerve activity. The outputs of RSNA used in this experiment are percent of maximum (% max) and represent the maximum RSNA induced by nasopharyngeal stimulation with cigarette smoke, which has been shown to be a suitable and reliable method of comparing RSNA baroreflex curves under a variety of different conditions (5).

Evaluation of arterial baroreflex function. Evaluation of the arterial baroreflex was carried out as previously described (31). In brief, rabbits were studied in a quiet, dimly lit room while they stood in a Plexiglas box. AP, HR, and RSNA values were recorded using a PowerLab system. Intravenous infusions were administered via a lateral ear vein. After the animal had adjusted to the environment and all hemodynamics were stable, an intravenous infusion of sodium nitroprusside (SNP) was started at a rate of 100 μg·kg⁻¹·min⁻¹ at 0.5 ml/min. When AP reached its nadir (usually 40–50 mmHg), the SNP infusion was stopped, and a phenylephrine (PE) infusion was started at a rate of 80 μg·kg⁻¹·min⁻¹ at 0.5 ml/min. The PE infusion continued until AP reached ~110 mmHg. The baroreflex was analyzed over the pressure range from lowest to highest pressure. This infusion rate increased AP at a rate of ~1 mmHg/s.

The HR, MAP, and RSNA data were acquired every 2 s from the thresholds to the saturation points. A sigmoid logistic function was fit to the data using a nonlinear regression program (SigmaPlot 8.0; Jandel). Four parameters can be derived from the following equation: HR or RSNA = A/(1 + exp[B(MAP – C)]) + D, where A is the HR or RSNA range, B is the slope coefficient, C is the pressure at the midpoint of the range (BP50), and D is the minimum HR or RSNA. The peak slope (or maximum gain) was determined by taking the first derivative of the baroreflex curve and was calculated using the equation Gainmax = A1 × A2 × 4π, where A1 is the range and A2 is the average slope.

O₂ production in RVLM. O₂ production was measured by the lucigenin enhanced chemiluminescence (ECL) method (TD-20/20 luminometer; Turner Designs; Sunnyvale, CA). Total protein concentration was determined using a bichinchoninic acid protein-assay kit (Pierce; Rockford, IL). NADPH (100 μM) and dark-adapted lucigenin (5 μM) were added into 0.5-ml microcentrifugal tubes just before reading. Light emission was recorded over 10 min, and values were expressed as mean light units per minute per milligram of protein.

Preparation of RVLM tissue. At the end of the experiment, each rabbit was euthanized with pentobarbital sodium. The brain was removed and immediately frozen on dry ice, blocked in the coronal plane, and sectioned into 300-μm-thick slices using a cryostat. The RVLM was punched according to the method of Palkovits and Brownstein (42) for the analysis of O₂ production and mRNA and protein of AT1 receptor and NAD(P)H subunits.

RT-PCR analysis of AT1 receptor and NAD(P)H subunit mRNA in RVLM. Total RNA of the RVLM was isolated using the RNeasy Mini Kit RNA Isolation System (Qiagen; Valencia, CA), after which cDNA was synthesized by means of Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen Life Technologies; Carlsbad, CA) according to the manufacturers’ instructions. RNA was treated in parallel in the presence or absence of reverse transcriptase. PCR amplification was performed by means of a PTC-100 programmable thermal controller (MJ Research; Watertown, MA) as follows: 1 cycle at 95°C for 15 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min. The primer pairs were as follows: AT1 receptor, 5′-TTGGAACAGCTTTGGCGT-3′ and 5′-GCCAGCCGACGCAAATAA-3′; gp91(phox) (GenBank accession no. S59041), gp91(phox) (GenBank accession no. AF323788), p67(phox) (GenBank accession no. AF323787), p47(phox) (GenBank accession no. AF324409), p40(phox) (GenBank accession no. AF323790), and p22(phox) (GenBank accession no. AF323787) with β-actin (GenBank accession no. AF483949) as an internal control. The primer pairs were as follows: AT1 receptor, 5′-TTTGGAACAGCTTTGGCGT-3′ and 5′-GCCAGCCGACGCAAATAA-3′; gp91(phox), 5′-GCTTGGTG-GCTGTGATAAGCA-3′ and 5′-CCTCCTGATCTGTTGCCTCA-3′; p67(phox), 5′-AACTCATGGTGGACAAAGG-3′ and 5′-GGTCT-TCCAGGAAGGCTTCTG-3′; p47(phox), 5′-ACGAGAGTGGTTTG-GTTC-3′ and 5′-TAGCAGGTACGTCTCCTCTT-3′; p40(phox), 5′-CACTGGGAAACGCAAAGT-3′ and 5′-CTGTTGAGGCTCTC-3′; and 5′-ATCACGGAGCAGATGACT-3′ and 5′-GATCGTGACATCGAG-3′ and 5′-GTGTCATTCTGCTTGG-3′. The amplification products were visualized on 2% agarose gels by the use of ethidium bromide and were sequenced so that their identities could be confirmed. The bands were analyzed using UVP BioImaging Systems.
Western Blot analysis of AT1 receptor and NAD(P)H subunit protein in RVLM. The RVLM was homogenized with the homogenizer in RIPA buffer. Protein extraction from homogenates was used for Western blot analysis for rabbit AT1 receptor, gp91phox, p67phox, p47phox, and p40phox. Protein concentration was measured using a protein assay kit (Pierce). Samples were adjusted to the same concentrations of protein, mixed with equal volumes of 2×4% SDS sample buffer, boiled for 5 min, and then loaded onto a 7.5% SDS-PAGE gel (5 μg protein/30 μl per well) for electrophoresis using a Bio-Rad minigel apparatus at 40 mA (for each gel) for 45 min. The fractionized proteins on the gel were electrophoretically transferred onto the polyvinylidene difluoride membrane (Millipore) at 300 mA for 90 min. The membrane was probed with primary antibody (1:1,000 dilutions of rabbit anti-human AT1 receptor polyclonal antibody and goat anti-human gp91phox, p67phox, p47phox, and p40phox polyclonal antibodies; Santa Cruz) and secondary antibody (1:2,500 dilutions of goat anti-rabbit IgG-horseradish peroxidase and rabbit anti-goat IgG-horseradish peroxidase; Santa Cruz) and then treated with enhanced chemiluminescence substrate (Pierce) for 5 min at room temperature. The bands in the membrane were visualized and analyzed using UVP BioImaging Systems.

**RESULTS**

Cardiovascular and sympathetic effects of ANG II and losartan. Basal MAP, HR, and RSNA values obtained before any treatments were similar among the four experimental groups (Table 1). The intracerebroventricular infusion of ANG II significantly increased RSNA (115.3%; P < 0.05), and losartan completely abolished the effects of ANG II on RSNA (P < 0.01). Although there was a tendency for the intracerebroventricular infusion of ANG II to increase MAP and HR, this did not reach statistical significance. The intracerebroventricular infusion of losartan significantly decreased baseline RSNA (–49.5%; P < 0.05) but had no effects on MAP and HR (Table 1). Original 7-day recordings of RSNA, MAP, and HR after intracerebroventricular infusion of saline (Fig. 1A) or ANG II (Fig. 1B) show that the frequency and integrated RSNA values appear to be greater after ANG II treatment than after saline treatment. Mean data for baseline RSNA (expressed as a percent of maximum nerve activity) after infusion are shown in Table 2. As can be seen, RSNA was significantly increased in the ANG II group compared with the saline group (control), RSNA in the ANG II plus losartan group was significantly lower than that from the ANG II group, and RSNA from the losartan group was significantly lower than that from the saline group (control).

Baroreflex effects of infusion of ANG II and losartan. Original 7-day recordings of AP show the changes induced by PE infusion after SNP infusion and the attendant HR and RSNA responses after intracerebroventricular infusion of saline (Fig. 2A) or ANG II (Fig. 2B). It is evident that the reflex bradycardia and RSNA response to PE after ANG II treatment were depressed compared with saline treatment.

Composite arterial baroreflex curves for the control of HR and RSNA in the four groups of rabbits are shown in Fig. 3, and the other parameters that describe MAP-HR and RSNA-HR baroreflex curves are shown in Table 2. As can be seen, rabbits that received intracerebroventricular infusion of ANG II exhibited depressed baroreflex control of HR. This depression was due primarily to a reduction in the range of HR induced by altering AP and the minimum HR achieved during increases in AP (Table 2). The rabbits that received intracerebroventricular infusion of ANG II plus losartan had restored baroreflex function compared with the ANG II group. However, the rabbits that received intracerebroventricular infusion of losartan alone exhibited enhanced baroreflex control of HR that was due primarily to an increase in the average slope of the baroreflex function (Table 2).

Regarding the baroreflex control of RSNA, intracerebroventricular infusion of ANG II blunted the sensitivity with a significant decrease in the average slope and increase in the minimum RSNA achieved during increases in AP (Table 2). The BP50 value also was significantly elevated in this case and was completely abolished by losartan. On the other hand, rabbits that received intracerebroventricular infusion of losartan alone exhibited enhanced baroreflex control of RSNA that was due primarily to the increase in range of the baroreflex curve (Table 2).

**mRNA expression of AT1 receptor and NAD(P)H oxidase subunits in RVLM after ANG II and losartan infusions.** The mRNA expression of AT1 receptor and NAD(P)H oxidase subunits in the RVLM was assessed by RT-PCR. As shown in Fig. 4B, intracerebroventricular infusion of ANG II for 7 days significantly increased AT1 receptor expression by 66.7%, p40phox expression by 120.0%, p47phox and p67phox expression by 200.0%, and gp91phox expression by 175.0%. We failed to detect mRNA expression for p22phox in the RVLM of either the saline- or ANG II-treated group. However, as a positive control, we observed mRNA expression of p22phox in liver and kidney of these rabbits (data not shown). In the rabbits treated with ANG II plus losartan, mRNA expression of AT1 receptor and NAD(P)H oxidase subunits in the RVLM was significantly

**Table 1. Baseline MAP, HR, and RSNA values before and after intracerebroventricular infusion in conscious rabbits**

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP, mmHg</th>
<th>Heart Rate, beats/min</th>
<th>RSNA, % of maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 7</td>
<td>Day 0</td>
</tr>
<tr>
<td>Saline</td>
<td>71.4±5.5</td>
<td>70.9±7.1</td>
<td>203.3±16.5</td>
</tr>
<tr>
<td>ANG II</td>
<td>70.7±6.7</td>
<td>79.3±5.8</td>
<td>201.4±18.8</td>
</tr>
<tr>
<td>ANG II + losartan</td>
<td>71.9±5.8</td>
<td>71.2±6.7</td>
<td>197.7±17.6</td>
</tr>
<tr>
<td>Losartan</td>
<td>73.3±6.1</td>
<td>72.4±5.7</td>
<td>203.5±15.7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 rabbits/group. MAP, mean arterial pressure; HR, heart rate; RSNA, renal sympathetic nerve activity. *P < 0.05 compared with saline group; †P < 0.01 compared with ANG II group; ‡P < 0.05 compared with saline group.
Fig. 1. Effects of subchronic intracerebroventricular infusion of ANG II on renal sympathetic nerve activity (RSNA). Original recordings of RSNA, arterial blood pressure (AP), mean arterial pressure (MAP), and heart rate (HR) after intracerebroventricular infusion of saline (A) or ANG II (B) for 7 days in conscious rabbits. Note the increased RSNA without a significant change in AP and HR by ANG II infusion compared with saline infusion. Freq, frequency; bpm, beats per minute.

Fig. 2. Original recordings of AP changes induced by intravenous infusion of phenylephrine and attendant RSNA and HR reflex responses after 7 days of intracerebroventricular infusion of saline or ANG II. Note the suppressed reflex RSNA response and bradycardia responses to phenylephrine-induced pressor effect in ANG II-treated (B) compared with saline-treated (A) rabbits.
decreased compared with the ANG II group and almost the same as the saline group (Fig. 4B). In addition, mRNA expression of AT1 receptor and NAD(P)H oxidase subunits in the RVLM of losartan-treated rabbits was not significantly different from saline-treated rabbits (Fig. 4B). Figure 4A is a representative RT-PCR image that shows the upregulation of AT1 receptor, p40\textsuperscript{phox}, p47\textsuperscript{phox}, p67\textsuperscript{phox}, and gp91\textsuperscript{phox} mRNA expression in RVLM of ANG II-treated rabbits (Fig. 4A) compared with saline-treated animals (controls, Fig. 4C).

Protein expression of AT1 receptor and NAD(P)H oxidase subunits in RVLM after infusion of ANG II and losartan. As shown in Fig. 5B, protein expression for AT1 receptor, p40\textsuperscript{phox}, p47\textsuperscript{phox}, p67\textsuperscript{phox}, and gp91\textsuperscript{phox} was significantly increased in rabbits treated with ANG II compared with rabbits treated with

Table 2. Arterial baroreflex curve parameters after central administration of ANG II and losartan

<table>
<thead>
<tr>
<th>Group</th>
<th>Lower Plateau, beats/min</th>
<th>Range, beats/min</th>
<th>BP(_{50}), mmHg</th>
<th>Average Slope, beats/min mmHg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP-HR Baroreflex Curves</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>61.9 ± 2.8</td>
<td>311.2 ± 23.3</td>
<td>79.6 ± 3.8</td>
<td>0.07 ± 0.1</td>
</tr>
<tr>
<td>ANG II</td>
<td>198.3 ± 32.4(^a)</td>
<td>182.2 ± 15.3(^b)</td>
<td>91.4 ± 8.3</td>
<td>0.06 ± 0.1</td>
</tr>
<tr>
<td>ANG II + losartan</td>
<td>56.3 ± 3.9(^c)</td>
<td>295.6 ± 17.7(^d)</td>
<td>83.2 ± 4.7</td>
<td>0.08 ± 0.2</td>
</tr>
<tr>
<td>Losartan</td>
<td>62.2 ± 4.1</td>
<td>311.8 ± 21.4</td>
<td>80.4 ± 5.8</td>
<td>0.12 ± 0.1</td>
</tr>
</tbody>
</table>

| MAP-RSNA Baroreflex Curves |
|-----------------------------|--------------------------|------------------|-------------------|---------------------------------------|
| Saline                      | 0.13 ± 0.1               | 99.5 ± 3.4       | 76.8 ± 5.1        | 0.27 ± 0.2                            |
| ANG II                      | 11.6 ± 0.9\(^c\)         | 87.7 ± 4.1       | 98.5 ± 6.6\(^d\) | 0.12 ± 0.1                            |
| ANG II + losartan           | 2.3 ± 0.8\(^d\)          | 103.3 ± 9.5      | 81.2 ± 7.2        | 0.24 ± 0.1\(^d\)                     |
| Losartan                    | 0.26 ± 0.1\(^e\)         | 118.2 ± 7.8      | 79.3 ± 7.4        | 0.32 ± 0.2                            |

Values are means ± SE; \(n = 6\) rabbits/group. BP\(_{50}\), midpoint of pressure range. \(^a\)\(P < 0.01\) and \(^b\)\(P < 0.05\) compared with saline group; \(^c\)\(P < 0.01\) and \(^d\)\(P < 0.05\) compared with ANG II group; \(^e\)\(P < 0.05\) compared with saline group.
saline. In the rabbits treated with ANG II plus losartan, the protein expression of AT1 receptor and NAD(P)H oxidase subunits in RVLM was significantly decreased compared with the ANG II-treated group and almost the same as the saline-treated group (Fig. 5B). In addition, the protein expression of AT1 receptor and NAD(P)H oxidase subunits in the RVLM of losartan-treated rabbits was not significantly different from the saline-treated rabbits (Fig. 5B).

**DISCUSSION**

The major findings of this study are that subchronic intracerebroventricular infusion of ANG II increases mRNA and protein levels of NAD(P)H oxidase components concomitant with increased local O$_2$/$\text{H}_2$O$_2$ production in the RVLM, elevations in basal RSNA, and impaired arterial baroreflex function. These results strongly suggest that NAD(P)H oxidase-derived ROS play an important role in the modulation of sympathetic activ-
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Fig. 6. Mean data of NAD(P)H-dependent O$_2^-$ production in RVLM after 7 days of intracerebroventricular infusion of saline, ANG II, ANG II plus losartan, or losartan alone measured by lucigenin chemiluminescence. MLU, mean light units. *P < 0.01; n = 6 rabbits/group.

Strong evidence is accumulating to suggest that central ROS are involved in the action of ANG II in the regulation of cardiovascular activity and function of central autonomic networks. For example, the adenoviral vector-mediated overexpression of SOD abolishes the cardiovascular effects of intracerebroventricular-injected ANG II in mice (61), and chronic systemic infusion of ANG II in mice causes a gradually developing hypertension that is correlated with marked elevations in O$_2^-$ production specifically in the subfornical organ (62). Moreover, in the nucleus of the solitary tract, the essential inhibition to a greater extent when the animals were under conditions of chronic overproduction of ROS, which further supports our hypothesis that ROS in the RVLM plays an important role in modulation of central autonomic nervous system activity and cardiovascular function by central ANG II. However, the mechanisms by which ROS excites the RVLM neurons are still unknown. Some studies (2, 29) indicate that ROS may directly activate Ca$^{2+}$ channels to enhance Ca$^{2+}$ currents and therefore might depolarize neurons and increase excitation and spontaneous activity.

The main source of ANG II-derived ROS is NAD(P)H oxidase (20, 37), an enzyme first described in phagocytes. NAD(P)H oxidase is composed of two membrane-bound subunits (gp91phox and p22phox), several cytoplasmic subunits (p40phox, p47phox, and p67phox), and the small G protein Rac 1a (26). After stimulation of AT$_1$ receptors by ANG II, the cytoplasmic subunits bind to the membrane subunits and activate the enzyme, which results in production of O$_2^-$ (21, 30, 49). In the present study, RT-PCR and Western blots showed a significant enhancement of most NAD(P)H oxidase subunit mRNA and protein in the RVLM from ANG II-infused vs. vehicle-infused and ANG II plus losartan-treated rabbits that correlated positively with changes in O$_2^-$ production. This would be expected to contribute to higher NAD(P)H oxidase O$_2^-$-generating activity, because increases in even individual components have been shown to enhance NAD(P)H oxidase activity in cell-free assays (13). Previous reports by other groups have shown that prolonged subcutaneous or intraperitoneal infusion of ANG II significantly increased renal cortical (6) or aortic (7, 38) NAD(P)H oxidase subunit expression. The data reported here extend those findings to rabbit RVLM, which is a specific central region that not only maintains sympathetic vasomotor outflow and plays a key role in controlling the baroreceptor reflex (9) but also has a high density of AT$_1$ receptors (1) and is a major site of sympathoexcitation in response to ANG II administered into the cerebrospinal fluid (23). This suggests close relationships between the brain renin-ANG II system, central redox signaling, and the regulation of cardiovascular function and autonomic nerve activity.

In the present study, we failed to detect mRNA expression for p22phox in the RVLM of rabbits. This does not appear to be due to a technical problem, because as a positive control, we did observe mRNA expression of p22phox in liver and kidney of these rabbits. However, this is not to say that NAD(P)H oxidase works well even without p22phox. One possibility, we believe, for the lack of p22phox in the RVLM of rabbit is the existence of some yet-unknown p22phox homolog, similar to what is observed in smooth muscle cells that lack gp91phox. Recent studies have identified the existence of several gp91phox homologs such as Nox1 and Nox4 (27).

The ANG II receptor is a central component of the renin-angiotensin system. Activation of ANG II receptors mediates a variety of effects from contraction of vascular smooth muscle to secretion of hormones including the effects on NAD(P)H oxidase and ROS. Thus regulation of their expression is important in cardiovascular and central responsiveness to ANG II. The existence of two types of ANG II receptors, AT$_1$ and AT$_2$, has been demonstrated. Although little is known about the function of AT$_2$ receptors, AT$_1$ receptors mediate many of the functions described. The other finding of the present study is that subchronic intracerebroventricular infusion of ANG II significantly increased the mRNA and protein expression of AT$_1$ receptors in the RVLM of rabbits, which might give an
explanation for our previous finding that in the chronic heart failure animal model, elevated ANG II levels were often concomitant with overexpression of AT1 receptors. Porter (43) also found that 1 wk of intracerebroventricular ANG II infusion produced a significant increase in brain AT1 receptor protein (via Western blot) and mRNA (via relative RT-PCR) expression. Moellehoff et al. (36) showed an increase in AT1 receptor number in some brain regions in rats after repetitive intracerebroventricular injections of ANG II (by immunohistochemical staining). The exact molecular mechanisms by which the expression of AT1 receptors in brain was upregulated by its ligand are still unclear. On the one hand, a number of factors including aldosterone are suggested to modulate the expression of AT1 receptor protein and mRNA (46). Chronic ANG II infusion increases plasma aldosterone and then induces transcription factors through mineralocorticoid receptors, and this is followed by an increase in AT1 receptor expression (51). ANG II also activates various nuclear transcription factors including activator protein-1, the signal transducers and activators of transcription family of transcription factors, cAMP response-element binding protein, and nuclear factor-κB (45), some of which are involved in the transcription of AT1 receptor genes (8).

In the present study, we also found that subchronic intracerebroventricular infusion of ANG II significantly increased basal RSNA, which may be due to the stimulation of ANG II on NAD(P)H oxidase and ROS in RVLM as described above, and contributed to the impaired baroreflex control of HR and RSNA observed in the same experiments. Many studies have solidly supported the idea that sympathetic activity and/or renin-angiotensin system activity can antagonize arterial baroreflex function in both humans and experimental animals (19, 33, 52). This was in agreement with the work of Gaudet et al. (17), who described diminished sensitivity of the cardiac baroreflex in conscious normotensive rabbits after long-term central administration of ANG II at subpressor doses. Considering these data, the increased NAD(P)H oxidase expression and ROS production in RVLM is critical for the effects of intracerebroventricular ANG II infusion on basal sympathetic activity and arterial baroreflex function. In addition, we still found that subchronic intracerebroventricular infusion of the AT1 receptor antagonist losartan produced enhanced baroreflex control of both HR and RSNA concomitant with suppressed baseline sympathetic activity but without any change of AT1 receptor protein and NAD(P)H oxidase subunit expression or O2 production in RVLM, which indicates that in conscious normal rabbits, there was tonic activation of these receptors from endogenous ANG II on arterial baroreflex function and sympathetic activity but no effect on AT1 receptor and NAD(P)H oxidase subunit expression.

In summary, our results demonstrate that in normal rabbits, subchronic intracerebroventricular infusion of ANG II upregulated AT1 receptor and NAD(P)H oxidase subunit expression and increased O2 production in RVLM mediated by central AT1 receptors and contributed to the enhanced sympathetic outflow and impaired arterial baroreceptor reflex control of HR and RSNA induced by this ANG II treatment. Endogenous ANG II, however, regulates the sympathetic activity and artery baroreflex function independent of the NAD(P)-ROS pathway in RVLM.

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REFERENCES

ROS and Sympathoexcitation Induced by Ang II


