NAD(P)H Oxidase Inhibition Attenuates Neuronal Chronotropic Actions of Angiotensin II

Chengwen Sun, Kathleen W. Sellers, Colin Sumners, Mohan K. Raizada

Abstract—It is well established that the central cardiovascular effects of angiotensin II (Ang II) involve superoxide production. However, the intracellular mechanism by which reactive oxygen species (ROS) signaling regulates neuronal Ang II actions remains to be elucidated. In the present study, we have used neuronal cells in primary cultures from the hypothalamus and brain stem areas to study the role of ROS on the cellular actions of Ang II. Ang II increases neuronal firing rate, an effect mediated by the AT1 receptor subtype and involving inhibition of the delayed rectifier potassium current (I_{Kv}). This increase in neuronal activity was associated with increases in NADPH oxidase activity and ROS levels within neurons, the latter evidenced by an increase in ethidium fluorescence. The increases in NADPH oxidase activity and ethidium fluorescence were blocked by either the AT1 receptor antagonist losartan or by the selective NAD(P)H oxidase inhibitor gp91ds-tat. Extracellular application of the ROS scavenger, Tempol, attenuated the Ang II–induced increase in neuronal firing rate by 70%. In addition, gp91ds-tat treatment resulted in a 50% inhibition of Ang II–induced increase in firing rate. In contrast, the ROS generator Xanthine-Xanthine oxidase significantly increased neuronal firing rate. Finally, Ang II inhibited neuronal I_{Kv}, and this inhibition was abolished by gp91ds-tat treatment. These observations demonstrate, for the first time, that Ang II regulates neuronal activity via a series of events that includes ROS generation and inhibition of I_{Kv}. This signaling seems to be a critical cellular event in central Ang II regulation of cardiovascular function. (Circ Res. 2005;96:659-666.)

Key Words: angiotensin • neurohumoral control of circulation • cellular signal transduction • hypertension • ion channel

The brain renin-angiotensin system (RAS) plays a critical role in the central control of cardiovascular functions. Angiotensin II (Ang II), the key peptide of the RAS pathway, interacts with the angiotensin type 1 receptors (AT1,R) located within the cardiovascular sensitive brain nuclei of the hypothalamus and brain stem to regulate blood pressure (BP), sympathetic outflow, fluid balance, baroreflexes, and secretion of neurohormones. Tight regulation of this complex hormonal system is important in the maintenance of normal cardiovascular function, as its dysregulation leads to hypertension, heart failure, and other related cardiovascular diseases. Thus, it is not surprising that tremendous investigative efforts have been put forward to define the neuronal and physiological circuits involved in central Ang II actions. In spite of significant progress in this area, understanding of the cellular and molecular mechanisms involved in these physiological actions of Ang II remains to be completely elucidated.

Studies from two independent research groups have been the main sources for our current understanding of the cellular mechanisms of Ang II actions in brain neurons. Our group has focused on elucidating the signal transduction mechanisms involved in the regulation of neuronal activity and neuromodulation by Ang II. We have demonstrated that the stimulation of neuronal AT1,R initiates a cascade of signaling events involving PKC, CaMK II, MAP kinase, MARCKs, and PI3 Kinase that regulates both evoked responses and enhanced effects of Ang II. The evoked responses include increases in neuronal firing rate and neurotransmitter release and regulation of potassium and calcium currents, whereas the enhanced effects include transcriptional and translational regulation of norepinephrine neuromodulation involving MAP kinase and PI3 kinase.

Davisson’s group has taken a distinct approach and studied the role reactive oxygen species (ROS) in Ang II actions in the brain. Their studies have established that redox signaling is important in Ang II–mediated regulation of cardiovascular functions. Involvement of neuronal ROS in Ang II–induced hypertension and sympathetic excitation of myocardial infarction-induced heart failure has been demonstrated. Collectively, studies from both groups have led us to hypothesize that evoked responses of Ang II in neurons involve ROS. Thus, our objective in the present study was to determine whether Ang II–induced increases in neuronal activity involves ROS.

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659
Materials and Methods

Animals and Chemicals

Adult male Wistar-Kyoto (WKY) rats were obtained from Charles River Laboratories (Wilmington, Mass). Rats were housed individually and kept on a 12:12-hour light/dark cycle in a climate-controlled room. Rat chow (HarlanTekland) and water were provided ad libitum. One-day-old WKY rat pups were used for the preparation of primary neuronal cultures. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Florida.

Dihydroethidium (DHE) was purchased from Molecular Probes, Inc. DMEM was obtained from Gibco; crystallized trypsin (1×) was from Cooper Biomedical. The selective NAD(P)H oxidase inhibitor, gp91ds-tat ([H]KKRKQRRR-CSTRIRRQL(NH2)) and its control, scrambled gp91ds-tat ([H]KKRRQKRRR-CLITRRQR(NH2)) were synthesized by Taufs University Core Facility. Poly-L-lysine (MW 150,000), Ang II, PD123319, ATP, GTP, HEPES, and others were purchased from Sigma.

Surgery and ICV Injection

Radiotelemetric pressure transducers (Transoma Medical) consisting of a fluid-filled catheter attached to a PA-C40 transmitter were implanted into the abdominal aorta of anesthetized rats. Before implantation, the aorta was clamped and the catheter was then inserted and secured with medical adhesive. Rats were allowed to recover 1 week after surgery. Blood pressure (BP) and heart rate (HR) were recorded using the DATAQUEST ART 2.0 software (Transoma Medical).

For ICV injection, rats were implanted with a 22-Ga guide cannula (Plastics One) into the right lateral ventricle (anteroposterior [AP]: 1.3 mm; mediolateral [ML]: 1.5 mm, 4.5 mm below skull). Beginning 1 week after surgery, Ang II (30 ng) and gp91ds-tat (200 to 800 ng) in 2 μL of artificial cerebrospinal fluid were injected into the right lateral ventricle of unrestrained rats using a 28-Ga, 4.5-mm internal cannula. BP and HR were recorded every minute for an average of 10 seconds before and after injections using radiotelemetry. Water intake was measured as described previously.

Preparation Primary Neuronal Cells in Culture

Brain areas containing the hypothalamus and brain stem were dissected from one-day-old WKY rats. Cells were dissociated and plated in poly-L-lysine precoated 35-mm diameter tissue culture dishes. Cultures were enriched with neurons and grown for 10 to 14 days in DMEM with 10% PDHS as described previously. Neuronal cultures that contain 90% neurons and 10% astroglia were used for all in vitro experiments. This protocol allows the neurons to recover from the isolation procedure, develop extensive network of neurites, and express neuron-specific properties comparable to those seen in the in vivo situation.

Determination of Intracellular ROS

ROS generation was determined using the oxidant-sensitive fluorogenic probe Dihydroethidium (DHE) essentially as described elsewhere. Briefly, neuronal cells were incubated with 100 nmol/L DHE for 30 minutes at 37°C. This is followed by incubation of cultures with PBS or 100 nmol/L Ang II for 5 minutes. In another set of neurons, cells were pretreated with 5 μmol/L gp91ds-tat or scrambled-gp91ds-tat for 10 minutes before Ang II treatment to assure complete inhibition of the enzyme. Eithidium fluorescence within neurons was detected by a fluorescent microscope (Nikon Inc.), and its intensity in individual cells was analyzed using Quantity One Software (Bio Rad). Each treatment condition was run in triplicate within experiments, and each set of experiments was performed using three separate culture dishes.

Measurement of NAD(P)H Oxidase Activity

The lucigenin-derived chemiluminescence method was used to measure Ang II–induced NAD(P)H oxidase activity in neuronal cultures. Neuronal cells were treated with (100 nmol/L) Ang II or PBS for 5 minutes, washed in ice-cold PBS, cells scraped, and then sonicated for 1 second. Ten minutes before recording luminescence, NAD(P)H (100 μmol/L) and lucigenin (5 μmol/L) were added and light emission was recorded during the next 10 seconds by a Microbetajet Luminometer. Data were presented as count/min per mg protein.

Electrophysiological Recordings

Spontaneous action potentials (APs) from neurons were recorded with the whole-cell voltage clamp configuration in current clamp mode. Cells were bathed in Tyrode’s solution containing (in mmol/L) 140 NaCl, 5.4 KCl, 2.0 CaCl2, 2.0 MgCl2, 0.3 NaH2PO4, 10 HEPES, and 10 dextrose; pH adjusted to 7.4 with NaOH. The patch electrodes (resistances from 3 to 4 MΩ) were filled with an internal pipette solution containing (in mmol/L) 140 KCl, 4 MgCl2, 10 HEPES, 10 NaCl, 10 Na2ATP, 10 Na2GTP, and 0.1 EDTA.

Figure 1. Effect of gp91ds-tat on Ang II–induced increase in BP, HR, and water intake in WKY rats. BP and HR were recorded using radiotelemetry after ICV injection of gp91ds-tat (800 ng) before ICV injection of Ang II (30 ng). A, Representative time course trace of MAP response after Ang II treatment. Arrows indicate saline and Ang II injections. X-axis depicts time in minutes relative to Ang II injection (as time 0). B, Representative time course traces of MAP response of gp91ds-tat followed by Ang II treatment. Arrows indicate gp91ds-tat (gp) and Ang II (Ang) injections. X-axis depicts time in minutes relative to Ang II injection (as time 0). C, Quantitation of change in MAP in mm Hg (left) and HR in bpm (right). White bars represent Ang II alone and black bars are peak change of MAP or HR at each condition. Data are mean±SE (n=5). *Significantly different from Ang II injection alone (P<0.05). D, Control rats (Con) or rats pretreated with gp91ds-tat (gp, 800 ng) or it scrambled-gp91ds-tat for 10 minutes before Ang II treatment. Arrows indicate saline and Ang II injections. X-axis depicts time in minutes relative to Ang II injection (as time 0). B, Representative time course traces of MAP response of gp91ds-tat followed by Ang II treatment. Arrows indicate gp91ds-tat (gp) and Ang II (Ang) injections. X-axis depicts time in minutes relative to Ang II injection (as time 0). C, Quantitation of change in MAP in mm Hg (left) and HR in bpm (right). White bars represent Ang II alone and black bars are peak change of MAP or HR at each condition. Data are mean±SE (n=5). *Significantly different from Ang II injection alone (P<0.05). D, Control rats (Con) or rats pretreated with gp91ds-tat (gp, 800 ng) or it scrambled-gp91ds-tat for 10 minutes before Ang II treatment. Arrows indicate saline and Ang II injections. X-axis depicts time in minutes relative to Ang II injection (as time 0). B, Representative time course traces of MAP response of gp91ds-tat followed by Ang II treatment. Arrows indicate gp91ds-tat (gp) and Ang II (Ang) injections. X-axis depicts time in minutes relative to Ang II injection (as time 0). C, Quantitation of change in MAP in mm Hg (left) and HR in bpm (right). White bars represent Ang II alone and black bars are peak change of MAP or HR at each condition. Data are mean±SE (n=5). *Significantly different from Ang II injection alone (P<0.05). #Significantly different from Ang II alone.
The recording pipettes had resistances of 3 to 4 MΩ observed. Mean data represent I0.3 NaH2PO4, 10 dextrose, 0.3 CdCl2, and 0.001 tetrodotoxin; pH7.4.

MgCl2, 10 HEPES, 5 EGTA, 4 Dextrose, 0.1 GTP, and 1 ATP; pH 7.2. Standard recording conditions for whole-cell configuration of the patch clamp technique.20 Neurons P/H9024

The resting membrane potential was measured as the number fully developed APs (depolarization beyond 0 mV) per second (Hz).

For the inside-out patch-clamp recordings, the bath solution facing the cytoplasmic side contained (in mmol/L) 134 NaCl, 5.4 KCl, 1.35 CaCl2, 2 MgCl2, 10 HEPES, 0.3 NaH2PO4, 10 dextrose, 0.3 CdCl2, and 0.001 tetrodotoxin; pH7.4. The recording pipettes had resistances of 3 to 4 MΩ and were filled with a solution containing (in mmol/L) 135 KCl, 0.25 CaCl2, 2 MgCl2, 10 HEPES, 5 EGTA, 4 Dextrose, 0.1 GTP, and 1 ATP; pH 7.2. Standard recording conditions for Ik, were achieved by stepping from a holding potential of −40 to +10 mV for every 10 seconds. Under these conditions, only delayed rectifier K+ current (Ik) was observed. Mean data represent I0.3, measured at 50 ms after the initiation of the pulse. Ik was expressed as current density (current divided by its capacitance), and all recordings were performed at room temperature.

For the inside-out patch-clamp recordings, the bath solution facing the cytoplasmic side contained (in mmol/L) 137 KCl, 10 NaCl, 2 MgCl2, 5 EGTA, 0.1 GTP, and 3 ATP at pH 7.2. The patch-pipette solution contained (in mmol/L) 140 KCl, 2.4 CaCl2, 1.3 MgCl2, 10 HEPES, and 0.001 tetrodotoxin at pH 7.4. The overall frequency response was set at 2 kHz and digitized at 10 kHz. Single K+ channel current was recorded at a membrane potential of −40 mV, and the results were expressed as open-state probability (NPo).

**Data Analysis**

All data are expressed as mean±SE. Comparisons between experimental groups were performed using ANOVA followed by a Newman-Keuls test. Differences were considered significant at P<0.05.

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**Results**

**gp91ds-tat, a NAD(P)H Oxidase Inhibitor, Attenuates the Central Effects of Ang II In Vivo**

Our objective in the first series of experiments was to establish the in vivo efficacy of gp91ds-tat to test the role of NAD(P)H oxidase–derived ROS in Ang II actions. ICV administration of 800 ng gp91ds-tat elicited no effects on basal BP or HR in WKY rats (BP: 108±2 versus 107±3 mm Hg; HR 312±6 versus 329±8 bpm control versus gp91ds-tat treatment, n=5). However, pretreatment with gp91ds-tat (800 ng) attenuated the increases in BP produced by Ang II without significant effect on HR (Figure 1A through 1C). This inhibition was evident at a dose of 200 ng (49%) and was maximal at 800 ng (70%) of gp91ds-tat. In contrast, scrambled-gp91ds-tat, which does not inhibit NADPH oxidase, showed no significant effects on the Ang II–induced pressor action in WKY rats (Ang II, 41±3 mm Hg and Ang II+Scrambled gp91ds-tat, 36±6 mm Hg, n=5). Next, we examined the effect of gp91ds-tat on the dipsogenic response produced by central administration of Ang II. ICV injection of 30 ng Ang II caused a significant increase in water intake by WKY rats (Figure 1D). This response was inhibited by 70% in gp91ds-tat pretreated WKY rats. However, scrambled gp91ds-tat had no inhibitory effect (Figure 1D). Collectively, these data establish that NAD(P)H oxidase–derived ROS is involved in the CNS-mediated cardiovascular and dipsogenic effects of Ang II, confirming previous observations.13,21
Ang II Stimulates NAD(P)H Oxidase in Neuronal Cultures

Treatment of neuronal cultures with Ang II resulted in a 2-fold increase in NAD(P)H oxidase activity (supplemental Figure IA, available online at http://circres.ahajournals.org). This increase was blocked by preincubation of neurons with losartan. In addition, preincubation of neurons with gp91ds-tat, but not with scrambled gp91ds-tat attenuated the Ang II–induced activation of NAD(P)H oxidase (supplemental Figure IB).

Ang II Increases ROS Production in Hypothalamus-Brain Stem Neuronal Cultures

The fluorogenic probe, DHE, was used to assess the effect of Ang II on ROS production. Insignificant ethidium fluorescence was seen in control untreated neurons (Figure 2A and 2B). However, treatment with 100 nmol/L Ang II resulted in a significant increase in the density of ethidium fluorescence within neurons (Figure 2C and 2G). Losartan (1 μmol/L) completely inhibited the Ang II–induced increase in fluorescence (Figure 2D through 2G). In addition, the Ang II–induced increase in ethidium fluorescence was blocked by gp91ds-tat and not by scrambled gp91ds-tat (Figure 3A through 3G). This indicated that Ang II, via activation of the AT1 receptor, increases superoxide production, which is blocked by inhibition of NAD(P)H oxidase.

Role of ROS in the Chronotropic Action of Ang II

Initially, we used Tempol, a superoxide scavenger, to determine the role of ROS in Ang II–induced increases in neuronal activity. Tempol (100 μmol/L) exerted no significant effect on the basal firing rate of WKY rat neurons (Figure 4A, 4C, and 4D). However, Tempol treatment resulted in a 70% attenuation of the chronotropic effect induced by Ang II. Neurons were then superfused with xanthine-xanthine oxidase (X-XO) to test whether intracellular accumulation of ROS increases neuronal firing rate and mimics the effects of Ang II. Superfusion with X (10 mmol/L)-XO (20 mU/mL) resulted in a 2-fold increase in neuronal firing (0.6 ± 0.0 Hz control versus 1.2 ± 0.2 Hz X-XO treatment Figure 4B and 4C). Finally, preincubation of neurons with Tempol, significantly blocked the X-XO–induced increases in neuronal firing rate (Figure 4C). Taken together, these data suggest that ROS are involved in the chronotropic action of Ang II in neurons.

Next, we used gp91ds-tat to confirm the involvement of ROS in this Ang II action. Figure 5 shows that Ang II (100 nmol/L), as expected, caused a 3-fold increase in neuronal firing rate. This effect was significantly attenuated by superfusion of neurons with gp91ds-tat (5 μmol/L; Figure 5A and 5C). In contrast, scrambled gp91ds-tat did not alter the Ang II–induced increases in firing rate of WKY rat neurons (Figure 5B and 5C).

ROS-Induced Increase in Neuronal Activity Involves $I_{\text{Kv}}$

Modulation of membrane K$^+$ and Ca$^{2+}$ currents, including inhibition of $I_{\text{Kv}}$, is responsible for the Ang II–induced increase in neuronal firing. Thus, we hypothesized that the ROS-mediated chronotropic action of Ang II may involve inhibition of $I_{\text{Kv}}$. We recorded $I_{\text{Kv}}$ by using voltage steps from −40 to +10 mV in our whole-cell patch clamp model. Figure 6A shows that the Ang
II–induced inhibition of $I_{Kv}$ was reversed by treatment with 5 μmol/L gp91ds-tat and not by its scrambled control. In addition, superfusion with 100 μmol/L Tempol completely blocked Ang II–induced inhibition of $I_{Kv}$ (Figure 6B). However, the hydrogen peroxide (H$_2$O$_2$) scavenger, PEG-Catalase (250 U/mL) did not affect the effect of Ang II on $I_{Kv}$ (Figure 6B), indicating that the inhibition of $I_{Kv}$ may be superoxide (O$^-$)-dependent. Superfusion with X (10 mmol/L)-XO (20 mU/mL) significantly decreased $I_{Kv}$, and PEG-catalase treatment failed to influence this inhibition (Figure 6C and 6D). Finally, treatment of neurons with 5 μmol/L gp91ds-tat did not influence the X-XO–induced inhibition of $I_{Kv}$. Collectively, these data indicate that ROS generation may mediate the inhibitory effects of Ang II on $I_{Kv}$.

Our next group of experiments was aimed at further examining the mechanisms by which ROS may mediate $I_{Kv}$ and alter neuronal firing. The data presented in Figure 7A indicate that the delayed rectifier $K^+$ (Kv) channel blocker 4-AP (5 mmol/L) significantly reduced $I_{Kv}$. Furthermore, 4-AP elicits a significant increase in neuronal firing (Figure 7A). Thus, we used the inside-out patch clamp recording technique to examine the effects of ROS on Kv channels. The data indicates that extracellular application of 5 mmol/L 4-AP via the recording pipette significantly blocked the single channel
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continue with the stimulation of BP and drinking responses of 
series of events that begin with the activation of AT1R and 
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pressor effects elicited by Ang II via the brain. The following 
neurons in an H2O2-independent mechanism.

### Discussion

This study provides the first evidence that the Ang II–induced increase in neuronal activity is mediated by ROS in a single neuron and that this effect involves direct inhibition of \( I_{Kv} \), and Kv channels. Based on these observations, we propose a series of events that begin with the activation of AT1R and continue with the stimulation of BP and drinking responses of Ang II (Figure 8). Activation of neuronal AT1R by Ang II stimulates two protein kinases, CaMKII and PKC\( \alpha \), that may be critical in the Ang II regulation of ROS generation. ROS then directly inhibits \( I_{Kv} \) via modulation of Kv channels and increases neuronal firing rate. Increasing the activity of CNS neurons is ultimately responsible for the dipsogenic and pressor effects elicited by Ang II via the brain. The following evidence supports this proposal. Studies from Davission and her colleagues12,14 have clearly established that Ang II stimulates ROS generation in the brain. In addition, interruption of ROS accumulation either by overexpression of SOD or the dominant-negative mutant of Rac1 abolished the Ang II–induced BP and drinking responses.13 Rac1 activation has been proven to be an obligatory step in the activation of NAD(P)H oxidase.22 Our present study provides further support for these observations. We demonstrate that Ang II–induced increases in NAD(P)H oxidase activity, neuronal ROS production, and dipsogenic and pressor responses are attenuated by inhibition of NAD(P)H oxidase by gp91ds-tat. These findings are consistent with recent reports implicating the involvement of the ROS system in the central effects of Ang II on sympathetic discharge, neural excitation, and neural regulation of hypertension.14,23–25

In previous studies, we had established that the Ang II stimulation of neuronal AT1R activates CaMKII and PKC\( \alpha \), two protein kinases that are critical in the regulation of neuronal activity and \( I_{Kv} \).8 Inhibition of both kinases is required for complete attenuation of the Ang II–mediated inhibition of \( I_{Kv} \), and increases in neuronal activity.8,9 This in vitro observation is validated by in vivo data. For example, our studies have shown that ICV administration of Ang II stimulates both CaMKII and PKC\( \alpha \) in the hypothalamus and that inhibition of these kinases attenuates the dipsogenic action of Ang II.16 The critical question that remains to be answered is whether CaMK II and PKC\( \alpha \) are involved in the generation of ROS. There is no direct evidence for this in our neuronal cells at the present time. However, ample evidence exists indicating that both CaMK II and PKC\( \alpha \), in addition to many other protein kinases, are involved in the generation of ROS in many cells including neurons.22,26
Finally, we propose that ROS generation by Ang II is proximal to the effect of this hormone on $I_{Kv}$ and neuronal firing. This conclusion is supported by the following: (1) inhibition of ROS accumulation by either gp91ds-tat or Tempol attenuates Ang II–induced inhibition of $I_{Kv}$ and increases of neuronal firing; (2) the ROS generator, xanthine-xanthine oxidase, mimics Ang II actions on $I_{Kv}$ and neuronal firing; and (3) xanthine-xanthine oxidase inhibits that Kv channel open-probability, an effect similar to that reported by Ang II on potassium channel activity.27–28 These effects of ROS on neuronal firing, $I_{Kv}$, and Kv channel opening seem to be mediated by generation of superoxides because they are not influenced by catalase. Thus, ROS influences $I_{Kv}$ and neuronal firing by directly affecting $K^+$ channel activity. Finally, Wang et al29 have reported that Ang II–induced regulation of the NTS neuronal activity is mediated by ROS and involves Ca$^{2+}$ channels. This, taken together with our present data, suggests that ROS may regulate neuronal activity by influencing both $K^+$ and Ca$^{2+}$ channels directly. Consistent with this view is our previous observations that Ang II activates Ca$^{2+}$ channels in brain neurons.8

In summary, we have identified the NAD(P)H oxidase as the major source of Ang II–induced ROS production in neurons. This NAD(P)H oxidase–derived ROS accumulation is critical in the neuronal chronotropic effect of Ang II and contributes to this hormone’s regulation of cardiovascular function and fluid balance. We speculate that NAD(P)H oxidase–derived ROS is important in the pathogenesis of central Ang II–dependent cardiovascular diseases. Further studies are necessary to evaluate the therapeutic potential of this observation on central-mediated cardiovascular diseases.

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**Figure 7.** Direct effect of ROS on single delayed rectifier $K^+$ channel in neurons. A, Left, Tracings of $I_{Kv}$, recorded in the presence or absence of 4-Aminopyridine (4-AP, 5 mmol/L). Data are representative of 4 neurons. Right, Representative tracings showing neuronal APs under the following treatment conditions: Basal (Con), followed by treatment with 4-AP (5mmol/L). Data are representative of 5 neurons. B, Representative single-channel current traces were recorded from inside-out excised patches under the following treatment conditions (from top to bottom): before (Con) and after extracellular application of 4-Aminopyridine (4-AP 5mmol/L); before (Con) and after bath perfusion of 10 mmol/L Xanthine – 20 μm/mL xanthine oxidase (X-XO); control (Con), superfusion of PEG-Catalase (Cat, 250 U/mL), superfusion of PEG-Catalase plus Xanthine-Xanthine oxidase (Cat+X-XO). C, Current-voltage relationship for the delayed rectifier $K^+$ channel activity in inside-out membrane patches. Each data point is mean±SE (n=4). Average slope conductance was $15.9±0.5$ pS. D, Bar graph showing the delayed rectifier $K^+$ channel open possibility (NPo) recorded from inside-out membrane patches under the treatment conditions described in B. Data are mean±SE from 4, 6, 6 patches of 4-AP, X-XO, Cat groups, respectively. $^*P<0.005$ compared with respective control.

**Figure 8.** Proposed mechanism of the involvement of ROS in Ang II actions in central neurons. Ang II binds to the AT1R, leading to G protein–dependent PKC/CaMKII activation, NAD(P)H oxidase assembly, and generation of $\cdot O_2^-\cdot$. ROS accumulation stimulates neuronal activity by modulation of potassium channel activity. This ultimately leads to increase in BP and dipsogenic actions of central Ang II. Ang II indicates angiotensin II; AT1R, angiotensin type 1 receptor; CaMKII, Ca$^{2+}$/calmodulin-dependent protein kinase II; X-XO, xanthine-xanthine oxidase; ROS, reactive oxygen species; $Kv$, delayed rectifier $K^+$ channel.
References

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Legend to Figure 1:

Effect of Ang II and gp91ds-tat on neuronal NAD(P)H oxidase activity

A: Effect of Ang II: neuronal cultures were pretreated without (open bars) or with (filled bars) 1µmol/L Losartan (Los) for 5min. This was followed by incubation with 100nmol/L Ang II for an additional 5min. Cells were collected and NAD(P)H activity was measured and expressed as mean light emission count/mg protein/min. Data are mean ± SE (n=9). *Significantly different from control PBS (Con) treatment (p<0.05).

B: Effect of gp91-ds-tat: Neuronal cultures were pretreated with 5µmol/L gp91-ds-tat (gp, filled bars) or scrambled gp91ds-tat control (Scra, open bars) for 10min prior to treatment with 100nmol/L Ang II for 5min as described under 2A. Data are mean ± SE (n=12). *Significantly different from PBS control (P<0.01).
On-line Supplement: Figure 1

A

NAD(P)H Oxidase activity (Counts/mg/min)

Con
Los

PBS Ang II

B

NAD(P)H Oxidase Activity

Scra
gp

PBS Ang II

*
On-Line Supplement

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