Shift to an Involvement of Phosphatidylinositol 3-Kinase in Angiotensin II Actions on Nucleus Tractus Solitarii Neurons of the Spontaneously Hypertensive Rat

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Rationale: Central angiotensin (Ang) II inhibits baroreflex and plays an important role in the pathogenesis of hypertension. However, the underlying molecular mechanisms are still not fully understood.

Objective: Our objective in the present study was to characterize the signal transduction mechanism of phosphatidylinositol 3-kinase (PI3K) involvement in Ang II–induced stimulation of central neuronal activity in cultured neurons and Ang II–induced inhibition of baroreflex in spontaneously hypertensive rats (SHR) versus WKY rats.

Methods and Results: Application of Ang II to neurons produced a 42% greater increase in neuronal firing in cells from the SHR than the WKY rat. Although the Ang II–mediated increase in firing rate was abolished entirely by the protein kinase (PK)C inhibitor GF109230 in the WKY, blockade of both PKC and PI3K activity was necessary in the SHR. This was associated with an increased ability of Ang II to stimulate NADPH oxidase–reactive oxygen species (ROS)–mediated signaling involving phosphorylation of the p47phox subunit of the NADPH oxidase and was dependent on the activation of PI3K in the SHR. Inhibition of PI3K resulted in the reduction of levels of p47phox phosphorylation, NADPH oxidase activity, ROS levels, and ultimately neuronal activity in cells from the SHR but not the WKY rat. In addition, in working heart–brainstem preparations, inhibition of PKC activity in the nucleus of the solitary tract in situ abolished the Ang II–mediated depression of cardiac and sympathetic baroreceptor reflex gain in the WKY. In contrast, PKC inhibition in the nucleus of the solitary tract of SHR only partially reduced the effect of Ang II on the baroreceptor reflex gain.

Conclusions: These observations demonstrate that PI3K in the cardiovascular brainstem regions of the SHR may be selectively involved in Ang II–mediated signaling that includes a reduction in baroreceptor reflex function, presumably via a NADPH-ROS mediated pathway. (Circ Res. 2009;105:1248-1255.)

Key Words: hypertension • angiotensin II • phosphatidylinositol 3-kinase • reactive oxygen species • NADPH oxidase

It is well established that the brain angiotensin system plays a critical role in the control of cardiovascular functions, including blood pressure regulation. Physiological actions of central angiotensin (Ang) II include modulation of sympathetic pathways, increased secretion of vasoactive hormones such as vasopressin, and dampening of baroreceptor reflexes by stimulation of neurons in the cardioregulatory brain regions of the hypothalamus and the medulla oblongata.1,2 The importance of brain Ang II and the Ang II type 1 receptor (AT1,R) is further underscored by observations that hyperactivity of this hormone system is linked to numerous cardiovascular diseases such as hypertension and heart failure.3

The nucleus of the solitary tract (NTS), located in the dorsomedial medulla, is a central nervous region that is crucial for the effects of Ang II on arterial pressure and baroreceptor reflex function.4 For example, Ang II injected into the NTS depresses the arterial baroreflex in normotensive animals4,5 and baroreceptor activity is crucial in chronic hypertension.5,6 A number of studies have demonstrated that Ang II is selectively involved in Ang II–mediated signaling that includes a reduction in baroreceptor reflex function, presumably via a NADPH-ROS mediated pathway.6,7 Ang II–mediated Ang II–induced depression of cardiac and sympathetic baroreceptor reflex gain in the WKY. In contrast, PKC inhibition in the nucleus of the solitary tract of SHR only partially reduced the effect of Ang II on the baroreceptor reflex gain.

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regulation of blood pressure. In addition, evidence indicates that the sensitivity of NTS neurons to Ang II is altered, not much is known about the sequences for blood pressure homeostasis. These and other observations support the concept that a reduction in baroreceptor reflex sensitivity could produce long-lasting detrimental consequences for blood pressure homeostasis.

In spite of the evidence that the sensitivity of the NTS neurons to Ang II is altered, not much is known about the cellular/molecular basis of this alteration in the hypertensive condition, although this has been studied in normotensive rats. We have previously reported that Ang II produces a greater degree of activation of neuronal cells from a brainstem/hypothalamus coulture of spontaneously hypertensive rats (SHR) than the normotensive Wistar Kyoto (WKY) rats. This enhanced response of SHR neurons to Ang II appears to be the result of the presence of an additional signal transduction pathway for the AT₁R in the SHR that is linked to phosphatidylinositol 3-kinase (PI3K). Given these observations, coupled with the demonstrated sympathoactivation in the SHR and importance of the NTS in Ang II–mediated blood pressure regulation, we propose the following hypothesis: Ang II modulation of SHR dorsal medullary neurons involves activation of PI3K in a PI3K–NADPH oxidase–reactive oxygen species (ROS) signaling pathway and this has relevance to NTS control of baroreceptor reflex function. Thus, our objective in this study was to use a combination of neuronal cells in culture and a physiological in situ technique to provide evidence to support this hypothesis.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

In Vitro Studies
Neuronal cells in primary culture were established and electrophysiological recordings were made as described previously. Intra-cellular ROS levels in WKY rat and SHR neurons, as well as the NADPH oxidase activity, and phosphorylated levels of p47phox were measured. Please refer to the Online Data Supplement for details regarding these methods.

In Situ Studies
All animal procedures were carried out according to the United Kingdom Home Office Guidelines on Animals (Scientific Procedure) Act of 1986 and those approved by the Animal Care and Use Committees of the University of Bristol and the University of Florida.

In the in situ, arterially perfused working heart–brainstem preparation, as described previously, was used to assess the effect of Ang II in the NTS on cardiovascular autonomic functions before and after inhibition of protein kinase (PK)C in the NTS of the SHR and WKY. To examine the effect of Ang II in the NTS on cardiovascular autonomic functions before and after inhibition of PI3K in the NTS of the SHR, a dominant negative construct of the p85α subunit of the PI3K (DNp85α) was delivered in vivo in a lentiviral vector driven by EF1 promoter (LV-EF1-DNp85α-IRES-eGFP) (Online Data Supplement). The site of Ang II microinjection, the extent of the lentiviral transduction, and protein expression were all confirmed post hoc. Confirmation of the in vivo lentiviral-mediated inhibition of PI3K and NADPH signaling was performed using Western blotting. Cardiovascular parameters were analyzed using Spike2 software. For a detailed description of the methods, refer to the Online Data Supplement.

Results
Ang II–Induced Increase in Action Potential Firing Rate in the SHR Neuron Is Associated With Increased NADPH Oxidase–ROS Signaling
Our first objective was to determine whether a greater increase in Ang II stimulation of neuronal firing rates was associated with a greater response of Ang II on NADPH oxidase and ROS production in SHR versus WKY rat neurons by using the NADPH oxidase inhibitor, gp91ds-tat. Although there was no significant difference in the basal neuronal firing between the 2 rat strains, Ang II caused a 42±3% greater increase in this chronotropic effect in the SHR neuron than the WKY rat neuron (Figure 1). This increase was completely blocked by gp91ds-tat and not by scrambled gp91ds-tat. Comparison of ROS generation by dihydroethidium fluorogenic probe demonstrated insignificant difference in ethidium fluorescence at basal conditions between WKY rat and SHR neurons (see Online Figure I). However, treatment with 100nmol/L Ang II resulted in 61±5% greater ethidium fluorescence in the SHR neurons compared to WKY rat neurons (Online Figure I).

Involvement of PI3K in Ang II–Induced Signaling and Firing Responses of SHR but Not WKY Rat Neurons
Ang II treatment (100 nmol/L) resulted in increases in NADPH oxidase activity in both WKY rat and SHR neurons. However, this stimulation was 61±5% greater in the SHR neurons compared to WKY rat neurons (Figure 2). Our previous studies have shown that the firing response evoked by Ang II in neurons from WKY rats are mediated by NADPH-Ros signaling and completely blocked by inhibition of PKC. This was confirmed in the present study, ie, Ang II stimulation of NADPH oxidase activity was completely abolished by the PKC inhibitor, GF109230 (1 μmol/L) in neurons from WKY rats (Figure 2A). In contrast, this inhibitor only partially attenuated the effect of Ang II on NADPH oxidase in neurons from SHR (Figure 2A). GF109203 (1 μmol/L) did not alter basal NADPH oxidase activity in neurons from either strain of rat.

We next examined the role of PI3K. Treatment of WKY rat neuronal cells with 10 μmol/L LY294002, an antagonist of PI3K, did not influence Ang II–induced NADPH oxidase
activity (Figure 2A). In contrast, LY294002 (10 μmol/L) partially attenuated the stimulatory effect of Ang II on this enzyme in SHR neurons (Figure 2A). Finally, combined treatment of SHR neurons with 10 μmol/L LY294002 and 1 μmol/L GF109203 completely abolished Ang II–induced activation of NADPH oxidase activity (Figure 2A).

Neuronal cells were infected with LV-EF1α-DNp85α-eGFP to determine the role of a selective PI3K inhibition on ROS and NADPH oxidase. This treatment causes a 77% decrease in Akt phosphorylation, a measure of PI3K activity. This was associated with a ~60% inhibition of Ang II–induced increase in ethidium fluorescence (Online Figure I) and NADPH oxidase activity (Figure 2B).

Neurons from WKY rat and SHR were treated with GF109203 (1 μmol/L) and LY291002 (10 μmol/L) to evaluate the role of PKC and PI3K on neuronal firing. As expected, Ang II treatment caused a greater increase in neuronal firing rates in neurons from SHR compared to WKY rats. In neurons from WKY rat treatment with GF109203 completely blocked this increase in firing, whereas LY294002 had no effect (Figure 3A). In contrast, individual treatment with GF109203 or LY294002 only partially attenuated Ang II–induced firing response in neurons from SHR. However, a combination of GF109203 and LY294002 completely abolished Ang II action on neurons from the SHR (Figure 3B). Similar to the effects on NADPH oxidase, PKC inhibition completely abolished Ang II–induced increase in ROS in neurons from WKY rats and only partially inhibited ROS in neurons from SHR (Figure 4). Coincubation with GF109203 and LY294002 was required to completely abolish the effects of Ang II on ROS generation in neurons from SHR (Figure 4).

Phosphorylation of p47phox is critical in the activation of NADPH oxidase. Thus, our objective was to determine whether PI3K dependence of enhanced actions of Ang II in SHR neurons are reflected in the phosphorylation status of this NADPH oxidase subunit. Ang II treatment caused a 64% and 148% increase in phosphorylated p47phox in WKY rat and SHR neurons, respectively. PKC inhibitor GF109203 completely inhibited this phosphorylation in WKY rat neurons, whereas only 50% inhibition was observed in SHR neurons from SHR compared to WKY rats. In neurons from WKY rat treatment with GF109203 completely blocked this increase in firing, whereas LY294002 had no effect (Figure 3A). In contrast, combined treatment of SHR neurons with 10 μmol/L LY294002 and 1 μmol/L GF109203 completely abolished Ang II–induced increase in phospholipidosis and ROS (Figure 2B). Finally, combined treatment of SHR neurons with 10 μmol/L LY294002 and 1 μmol/L GF109203 was required to completely abolish the effects of Ang II on ROS generation in neurons from SHR (Figure 4).

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neurons (Figure 5). However, cotreatment with GF109203 and LY294002 completely blocked p47phox phosphorylation in SHR neurons (Figure 5).

In Situ Confirmation of an Additional Ang II Signaling Pathway Regulating Cardiovascular Functions in the NTS of the SHR

At a comparable rat age, pump flow rate, and perfusate vasopressin concentration, baseline perfusion pressure was higher in SHR (81±2 mm Hg) than WKY (71±2 mm Hg; P<0.01), consistent with previous reports.16 Additionally, we observed a significantly higher (integrated) sympathetic nerve activity (17±2) in the SHR than WKY rat (6±2; P<0.001) after correcting for electric noise. Heart rate was similar in both rat strains (WKY: 364±9 bpm; SHR: 334±11 bpm). Both cardiac and sympathetic baroreflex gains were not different in the SHR versus the WKY. In both strains of rat, microinjection of vehicle neither affected baseline parameters nor the baroreceptor reflex gain.

We tested the effect of PKC inhibition in the NTS of WKY and SHR on Ang II modulation of the baroreceptor reflex gain. In WKY, bilateral microinjection of Ang II into the NTS reduced cardiac baroreflex gain to 55±9% of its control value (P<0.05; Figure 6A and 6C). Following microinjection of GF109230, this Ang II–induced decrease in cardiac baroreflex gain was abolished, such that the gain was not different to control (Figure 6B and 6C). Similar results were observed for the sympathetic component of the baroreflex. Ang II in NTS of WKY rats reduced the baroreflex-mediated sympathetic inhibition to 47±5% of its control value (P<0.01; Figure 6A and 6D), yet this effect was abolished following pretreatment with GF109230 (Figure 6B and 6D). In contrast, in the SHR, pretreatment with GF109230 did not fully prevent the Ang II–mediated inhibition of baroreflex gain. In SHR, Ang II reduced cardiac baroreflex to 58±1% of control value in the vehicle-treated rats (P<0.05 versus control baroreflex gain, Figure 7A and 7C) and to 77±2% of control value after GF109230 treatment (P<0.05 versus control baroreflex gain; P<0.01 versus vehicle; Figure 7B and 7C). Sympathetic baroreflex gain in the vehicle pretreated SHR was reduced to 73±7% of the control value following the Ang II injection (P<0.05 versus control baroreflex gain; Figure 7A and 7D). Following the GF109230 treatment, the sympathetic baroreflex gain in the SHR was similar to its control baroreflex value (P=NS; Figure 7B and 7D). These observations suggest that the depressant actions of Ang II in the NTS on both the cardiac and sympathetic limbs of the baroreflex are mediated entirely by a PKC-dependent pathway in the WKY rat, whereas in the SHR, an additional cellular pathway exists.

In Vivo Validation of the Role of PI3K in the SHR

Considering our in situ results described above, and because our in vitro data implicating an additional role for PI3K in neurons...
from SHR but not in the WKY, we tested the functional implications of this on baroreflex gain in this rat strain only. We have used a combination of in vivo gene transfer and subsequently the in situ working heart-brainstem preparation to determine the cardiovascular implications of chronically blocking PI3K in the NTS on baroreceptor reflex gain of the SHR. At comparable perfusion rates and dose of vasopressin, there was no difference in cardio-respiratory parameters measured such as perfusion pressure, heart rate, and baroreceptor reflex gains (cardiac and sympathetic) in rats previously injected with LV-EF1α-DNp85α-eGFP compared to LV-EF1α-eGFP controls, which were also not different to nontransfected rats described above. Bilateral microinjection of Ang II in the NTS resulted in a decrease in cardiac baroreflex gain in the LV-EF1α-eGFP group to 50±3% of the control value (P<0.001; Figure 8A). In contrast, in LV-EF1α-DNp85α-eGFP–treated animals, this Ang II–induced decrease in cardiac baroreflex gain was reduced such that the gain was comparable to the control values (Figure 8A). Similarly, Ang II in NTS of the LV-EF1α–eGFP–treated group also reduced the baroreflex mediated sympathoinhibition to 62±10% of the control value (P<0.05; Figure 8B), an effect not seen in rats in which the NTS was pretransduced with LV-EF1α-DNp85α-eGFP and where the gain was similar to the value established pre–Ang II injection (Figure 8B). Finally, LV-EF1α-DNp85α-eGFP–treated NTS showed significant decreases in the PI3K and NADPH oxidase activities (Online Figure II). These observations suggest that in the SHR the depressant actions of Ang II in NTS on both the cardiac and sympathetic limbs of the baroreflex are mediated by a PI3K-dependent pathway. Post hoc histological analysis confirmed that expression of enhanced green fluorescent protein (eGFP) from both and LV-EF1α-DNp85α–eGFP– and LV-EF1α–eGFP–treated groups was comparable and restricted to the dorsal vagal complex (Online Figure III). Pontamine sky blue staining, used to indicate sites of microinjections of Ang II, was localized within areas expressing eGFP (Online Figure III).

Discussion
The present study examined the role of PI3K on Ang II regulation of neuronal activity in the SHR NTS. Major

![Figure 5. Blockade of PKC and PI3K on Ang II–induced P47phox phosphorylation in SHR and WKY rat neurons. Phosphorylation of p47phox was measured by using immunoprecipitation with anti-p47phox antibody followed by immunoblotting with anti-phosphoserine antibody in SHR and WKY rat neurons treated with the following conditions: control; Ang II (Ang) (100 nmol/L); Ang II plus GF109203 (Ang–GF) (1 μmol/L); and Ang II plus LY294002 (Ang+LY) (10 μmol/L). Bottom, Representative blot showing the levels of phosphorylated p47phox and total p47phox. Top, Bar graphs showing the mean level of phosphorylated p47phox in SHR and WKY neurons under each treatment condition. Data are means±SE (n=5 experiments). *P<0.01 vs respective control; #P<0.05 vs Ang II treatment.](http://circres.ahajournals.org/)

![Figure 6. Effect of Ang II in NTS on baroreflex of WKY rats pretreated with either vehicle or GF109203. A and B, Examples of recordings of integrated sympathetic nerve activity (μV), heart rate (HR) (bpm), and perfusion pressure (PP) (mm Hg) for vehicle-treated (A) or GF109203-treated (B) WKY rats. Baroreceptors were stimulated by pressor ramps (~2 to 5 seconds) before and after bilateral microinjections of Ang II (10 μmol/L; 50 to 60 nL/side). C and D, Group results for the cardiac (C) and sympathetic (D) baroreflex gain before and after Ang II injection in the NTS of WKY rats pretreated with either vehicle (black bars) or GF109203 (white bars), presented as percentage of control baroreflex gain (ie, the gain measured before any of the injections). *P<0.05, **P<0.01 vs control baroreflex gain; #P<0.05 vs vehicle.](http://circres.ahajournals.org/)
findings are as follows: (1) in dorsal medullary neurons from the WKY rat, Ang II–induced increase in firing is regulated by stimulation of PKC-NADPH oxidase-ROS mediated signaling pathway; and (2) a second Ang II–signaling pathway involving PI3K has been identified in neurons from the SHR. This signaling kinase is also linked to NADPH oxidase–ROS pathway and results in a greater Ang II stimulation of neuronal firing in neurons from the SHR. (3) Pharmacological inhibition of PKC in the NTS fully attenuates Ang II–induced decrease in cardiac and sympathetic baroreflex gain in the WKY, whereas this effect is only partial in the SHR. Collectively, these observations are consistent with our hypothesis that there is a greater sensitivity of NTS neurons to Ang II in the SHR, which may be a result of PI3K-mediated signaling transduction and has direct relevance to control of baroreflex function.

The actions of Ang II in NTS in normotensive rats are likely to be different to those in hypertensive rats. In normotensive rats, we have shown previously that Ang II–mediated depression of the baroreflex involves AT₁ receptor stimulation of endothelial NO synthase and NO generation.4,17 The latter pathway involved Gq protein–mediated activation of phospholipase C, which, through 1,4,5-inositol triphosphate, caused release of calcium from the inositol triphosphate–sensitive intracellular stores and calcium-calmodulin formation.11 Furthermore, we found that NO via cyclic adenosine diphosphate ribose/ryanodine–sensitive stores increased intracellular calcium levels in GABAergic interneurons in NTS of normotensive rats in vitro, which we speculated could increase GABA release.12 In addition, both Ang II and NO enhanced the magnitude of solitary tract evoked inhibitory postsynaptic potentials in NTS neurons in vitro.18 We have proposed that such a mechanism might cause a depression of the cardiac baroreflex in normotensive rats. Indeed, chronic depression of endothelial NO synthase activity increased baroreceptor reflex gain in both WKY rats and SHR.20,21 However, for the hypertensive rat, we have no proof that this was an NO-soluble guanylate cyclase mediated effect unlike that in normotensive rats.17 The present study compliments and advances our understanding of the mecha-
nism by which Ang II acts in the NTS of the SHR and, importantly, identifies a possible functional significance of the PI3K cellular pathway in this rat strain. We show, for the first time, that phosphorylation of the p47phox by a PKC-dependent mechanism that initiates this signaling cascade leads to increased neuronal activity in WKY rat neurons. In addition, we have identified coupling of PI3K with this cascade, which is responsible for enhanced phosphorylation of p47phox, leading to a greater stimulation of neuronal firing and specific to neurons from the SHR. The mechanism by which PKC and PI3K mediate p47phox phosphorylation, and whether both kinases phosphorylate the same serine residues in p47phox in these neurons, remains to be elucidated. However, our preliminary data with the use of decoy peptides based on the serine residues in the autoinhibitory region of the p47phox suggest that phosphorylation of serines 304 and 328 is important and that both PKC and PI3K inhibitors completely attenuate phosphorylation of these 2 serine residues. Finally, regarding the Ang II–PKC–NADPH oxidase–ROS–mediated signaling found in WKY rat neurons, we have not yet identified a functional role for this but, based on our previous data discussed above,4,12,17–21 do not believe it important for NTS baroreflex control.

Our previous studies have established that the expression of the AT1R is 2- to 4-fold higher in the neurons from SHR compared with those from WKY rat.1,22 This increase was consistent with elevated levels reported in the cardiovascular-relevant brain regions of the adult SHR.1 In addition, increased expression of the AT1R in the SHR neuron was associated with a greater firing rate response and neuromodulatory actions of Ang II in these strains of rat neurons.13 The present study shows that PI3K inhibition preferentially diminishes the firing responses evoked by Ang II in the SHR by inhibiting phosphorylation of p47phox, NADPH oxidase activity, and levels of ROS. This inhibition reduces the firing response to the level observed in neurons from WKY rats exposed to Ang II. Complete attenuation of Ang II response in neurons from SHR is only accomplished by addition of a PKC inhibitor. These observations support the hypothesis that PI3K-linked phosphorylation is consistent with previous observations,14 although its role in neurons of the SHR strain is novel.

Is this in vitro observation of any physiological relevance? Our in situ data suggest that the answer to this question is yes. Bilateral injection of a PKC inhibitor into the NTS of the WKY results in a complete attenuation of the Ang II–induced depressant effect on baroreflex gain (cardiac and sympathetic). However, this Ang II effect on both components of the baroreflex is only partially attenuated in the SHR. This suggests that an additional cellular pathway must exist in the NTS of SHR through which Ang II exerts its effects on the baroreflex. Based on our data present, herein we suggest that this may be PI3K-dependent. This would complement our previous studies showing an Ang II–dependent elevation in PI3K in the brainstem and hypothalamic cardiovascular regions of the SHR compared to the WKY,23 as well as our recent finding that chronic blockade of PI3K affects the spontaneous baroreflex gain and arterial pressure only in the adult SHR but not the WKY rat.15 Because PI3K blockade completely abolished the effect of exogenous Ang II in the NTS on the baroreceptor reflex in the SHR, we suggest that the Ang II–PI3K pathway may operate upstream of the PKC signaling in the same neurons. Furthermore, the enhanced Ang II–PI3K signaling in the NTS of SHR may cause other downstream effects which may also add to the baroreflex depression, such as elevating the levels of NO in the NTS as mentioned above.4,17,20,21 Recent studies report a defective PI3K-Akt-NOS signaling pathway in the NTS and inflammatory effects in the vessel walls of SHR.24,25 Our recent work has also shown that specific inflammatory condition may exist in the NTS of the SHR that may contribute to the neurogenic hypertension and dampening of the baroreflex gain.26–28 Furthermore, developmental differences in Ang II–PI3K signaling may exist between the P9 SHR used in vitro and the P21 SHR used in situ, as recently suggested16 which may also contribute to the perceived differences in the effects of the PI3K blockade. These apparent discrepancies may be related to the previously reported differences in AT1R signaling, because angiotensin II type 2 receptor signaling is predominant in the neurons derived from neonatal brainstem and shifts in favor of the predominantly AT1R-mediated signaling with age.29 However, the absence of any effects of LV-DNp85 in the NTS on the cardiovascular variables in the adult WKY15 indicates that the suggested developmental change in Ang II signaling, if indeed it exists, may be present only in the SHR.

The finding that Ang II in NTS depresses the cardiac and sympathetic component of the baroreflex in SHR is confirmatory of previous work using normotensive rats studied using the same in situ preparation,30 although baroreflex modulation of lumbar sympathetic activity by Ang II in NTS was less affected.31 The present study in the WKY and SHR demonstrates that the depressant action of Ang II in NTS affects both autonomic limbs of the baroreflex. We suggest that Ang II–driven PI3K activity in NTS neurons of SHR is a major modulator of one of the main homeostatic reflexes regulating arterial pressure. Interestingly, a prognostic indicator of morbidity and mortality for cardiovascular disease, including hypertension, is loss of heart rate variability and reduced baroreflex cardiac gain.32,33 Our data provide a potential explanation for this especially as Ang II activity appears elevated in SHR brainstem4 and that both exogenous (but presumably endogenous) Ang II and circulating Ang II (independent of the area postrema) can depress cardiac baroreflex gain via actions at the level of the NTS.4,34

In conclusion, our studies demonstrate the presence of an exclusive Ang II–AT1R signaling system in dorsal medullary neurons of the SHR involving PI3K kinase. This signaling is proposed to be involved in Ang II regulation of an impaired baroreflex in the SHR and thus may be a relevant target in the control of neurogenic hypertension.

Limitations
We acknowledge that usage of neuronal cultures in our in vitro experiments versus the P21 rats used in situ may pose certain questions regarding comparability of the data. However, the viability of the in situ method is highly dependent on the age of the animal which limited these experiments to P21. Nevertheless the P21 rats are still juvenile and in our opinion may have limited.
comparability to in vitro situation. Therefore, even though here we may allude to slight developmental differences in Ang II–PI3K signaling in the NTS in vitro versus the P21 SHR rats, we point out that the main difference is in the role of PI3K signaling in the NTS of WKY and SHR.

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Disclosures
None.

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Shift to an Involvement of PI3-Kinase in Angiotensin II Actions on Nucleus Tractus Solitarii Neurons of the Spontaneously Hypertensive Rat

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Short title: PI3K NADPH Oxidase-ROS Signaling in the NTS of the SHR
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Materials and Methods

**In vitro studies:**

**Preparation of neuronal cultures and electrophysiological recordings:**

Neuronal cells in primary culture obtained specifically from the dorsal brainstem (and included the NTS) were taken from 1-day-old WKY rats and SHR and were established for 14 days as described previously.¹ These cultures contain > 90% neurons and > 10% astrogial cells as determined by immunostaining with neuron- and glial-specific antibodies.²

Electrophysiological recordings were made in 14-day-old cultures of neuronal cells by recording spontaneous action potentials using the whole cell configuration in current-clamp mode as described previously¹, ³. The resting membrane potential was defined as the potential within a time period of 1 s during which there was no spontaneous action potential firing. The neuronal firing rate was measured as the number of fully developed action potentials (i.e. > 60 mV amplitude) per second (Hz).

**Biochemical measurements:**

Intracellular ROS levels in WKY rat and SHR neurons were measured with the oxidant-sensitive fluorogenic probe, Dihydroethidium (DHE) as described previously³. NADPH oxidase activity was determined using the lucigenin-derived chemiluminance method⁴. Phosphorylated levels of p47phox were measured by immunoprecipitation with anti-p47phox antibody (1:1500) followed by Western blotting using anti-phosphoserine antibody.³
In situ studies:

Measurement of cardiovascular autonomic functions:

Briefly, juvenile WKY and SHR (60-80 g, n=10 per strain) were deeply anaesthetized with halothane, until cessation of respiratory movements and absence of withdrawal reflex responses to noxious pinching of a paw. Rats were then bisected subdiaphragmatically, immersed in carbonated ice-cold Ringer’s solution, and decerebrated at the level of superior colliculus. A double lumen cannula was inserted retrogradely into the descending aorta, and the preparation was perfused with modified Ringer’s solution (32º C) using a peristaltic pump. Baseline flow rate was maintained at 22-24 ml/min. Arginine vasopressin (200-400 pM) was added to the perfusate to increase vascular resistance in order to optimize the baseline perfusion pressure (PP),\(^5\) which was monitored using the second lumen of the cannula, connected to a pressure transducer. The phrenic nerve activity was monitored to establish the viability of the preparation, and the electrocardiogram (ECG) was recorded to determine the heart rate (HR). Recordings of sympathetic nerve activity (SNA) were made using suction electrodes from the mid/lower thoracic sympathetic chain (T5-13). Arterial baroreceptors were stimulated by increasing the pump flow rate (~2-5 sec) to generate pressor ramps that were reproducible over the course of the experiment. The pressor ramps were timed to correspond with the phrenic burst activity.\(^6\) Cardiac and sympathetic baroreflex gains were measured before and after microinjection of Ang II (10 µM, 50-60nl per site) into the NTS, which was
visualized following resection of the cerebellum. Bilateral Ang II microinjections were made into comparable regions of the NTS that had received prior microinjections of either a PKC inhibitor GF109230 (10 nM, 50-60 nl per site) or vehicle. The working concentration of 10 nM of GF109230 was established in our preliminary experiments as the lowest dose that caused the maximum attenuation of Ang II-mediated baroreflex depression in the NTS of WKY rats. Microinjections were performed by applying pressure and observing the displacement of the meniscus using a binocular microscope fitted with a pre-calibrated eyepiece reticule. Microinjections were made over 1 minute to minimize tissue damage and performed at the level of calamus scriptorius, 350-500 µm from the midline and 500-600 µm ventral to the dorsal surface. At the end of each experiment, an injection of the dye (pontamine blue) was made into the same site to mark the injection sites. The SNA noise level was determined by application of lignocaine (2% w/v) directly on the sympathetic nerve at the end of each experiment.

**In vivo gene transfer into the NTS:**

To examine the effect of Ang II in the NTS on cardiovascular autonomic functions before and after inhibition of PI3K in the NTS of the SHR, a dominant negative construct of the p85α subunit of the PI3K (DNp85α) was delivered *in vivo* in a lentiviral vector driven by EF1 promoter (LV-EF1α-DNp85α-IRES-eGFP). With the IRES promoter eGFP was also driven by EF1 promoter and its visualization ensured expression of the DNp85α transgene. In addition, we injected LV-EF1α-
eGFP as a control. Lentiviral particles were prepared, titered and used for in vivo gene transfer as previously published by us. The viral titer was 1-2x10^{10} transfection units (TU)/ml.

Male SHR (n=10) were anaesthetized with a mixture of ketamine (60 mg/kg) and medetomidine (250 μg/kg), administered intramuscularly. The virus particles (either LV-EFα-DNp85α [LV-DNp85α] or LV-EFα-eGFP [LV-eGFP]) were microinjected bilaterally into the NTS at 3 separate sites spanning ±500 μm rostro-caudal to calamus scriptorius, 350-500 μm from the midline, and 500-600 μm ventral to the dorsal surface. The volume of each injection was approximately 100 nl containing 2-3x10^6 TU lentiviral particles, and was made over 1 minute to avoid tissue damage and allow good spread of the viral suspension. One week was allowed for recovery and gene expression prior to making measurements of cardiovascular autonomic function before and after NTS microinjections of Ang II, which were performed in situ as detailed above.

**Confirmation of in vivo lentiviral mediated protein expression by fluorescence microscopy:**

The site of Ang II microinjection, the extent of the lentiviral transduction, and protein expression were all confirmed post hoc. At the end of each experiment, the brainstem was removed and placed at 4°C in 10% formaldehyde/0.1 M PBS (pH 7.4) solution containing 10% sucrose. Transverse sections (40 μm) were cut on a freezing microtome throughout the caudal brainstem corresponding to the level of the NTS. The free-floating sections were collected in 0.1 M phosphate-
buffered saline (PBS) (pH 7.4), mounted onto glass microscope slides using 0.5% gelatin, cover-slipped using Vectashield Mounting Medium for Fluorescence (Vector Labs) and kept in the dark and at 4 °C to preserve the fluorescence. A Leica confocal fluorescence microscope equipped with the appropriate filters was used to detect green fluorescence resulting from lentiviral mediated expression.

**Confirmation of in vivo lentiviral-mediated inhibition of PI3K signaling by Western blotting:**

SHR (n=12) were microinjected with either LV- DNp85α or LV-eGFP in the NTS as described above. One week was allowed for recovery and gene expression. Following this, the rats were anaesthetized with halothane, and the NTS was bisected and samples were pooled between the rats within the same experimental group to yield sufficient protein. The protein levels of PI3-kinase p85α subunit, Akt, and p-Akt in the NTS were assessed by Western blot analysis, as described previously with primary antibodies purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA) with dilution 1:500 and a secondary antibody, anti-rabbit peroxidase-conjugated antibody (Bio-Rad, Hercules, CA; dilution 1:15,000). Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL Western blotting detection kit, Amersham Pharmacia Biotechnology), and films were analyzed using Quantity One Software (Bio-Rad).
Data Analysis:

Cardiovascular parameters were analyzed using Spike2 software. Baroreflex gains were measured on the linear part of the reflex function curve which was pre-determined for each rat. Baroreflex gain for HR was calculated as the maximum change in HR during a pressor ramp divided by the change in PP (ΔHR / ΔPP bpm/mmHg) before and after GF109230/vehicle or DNp85α/eGFP treatments and Ang II injections in the NTS, and presented as percentage of the control gain (i.e. the gain measured in the same rat prior to the injections of Ang II in the NTS). The sympathetic baroreflex gain was calculated as the ratio of the change in integrated SNA (0.1 sec time constant, noise level subtracted) during the pressor ramp (which was timed to coincide with the respiratory-related burst) against the average of two equivalent control periods of integrated SNA from the corresponding phase of preceding respiratory cycles. The SNA gain values were calculated before and after Ang II microinjections in all rat groups and expressed as percentage of the control gain. All data from in situ experiments were 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 and individual ‘p’ values are presented in figure legends.
Results

Confirmation of in vivo lentiviral-mediated inhibition of PI3K signaling

We studied the effect of lentiviral vector mediated overexpression of DNp85α on the PI3-kinase activity and NADPH oxidase activity in the NTS of SHR. One week transduction of LV-DNp85α resulted in about 10 fold increase in immunoreactive p85α protein. This was associated with a 78% decrease in phosphorylated Akt (Online figure IIA and IIB). In addition, transduction of LV-DNp85α decreased NADPH oxidase activity by 25% in the NTS of rats received LV-EF1α-DNp85α-eGFP (Online figure IIC) compared with LV-eGFP control. These results indicate that overexpression of dominant-negative p85α dramatically diminished PI3-kinase activity and significantly decreased NADPH oxidase activity in the NTS of SHR.

References


Figure Legends

Online figure I: Effect of Ang II on ROS production in WKY rat and SHR neurons.

ROS level was detected using the fluorogenic probe DHE.\textsuperscript{19} A through C, WKY rat neurons that were treated under the following conditions: A, Neurons in normal optical phase; B, Fluorescence micrograph of neurons loaded with DHE; C, Fluorescence micrograph of neurons treated with Ang II (100 nmol/L). D through F, SHR neurons that were treated under the same condition as WKY neurons described above. E: Bar graphs summarizing ethidium fluorescence intensity before and after treatment with Ang II (100 nmol/L) in both SHR and WKY rat neurons. Data are mean ± SE (n=19 to 22 fluorescent neurons). They were derived from three experiments and at least seven dishes in each experiment. *P<0.01 compared with their respective control (PBS). #P<0.05 compared between SHR and WKY rat neurons treated with Ang II (100 nmol/L). F: Bar graphs summarizing the effect of incubation of neurons with LV-eGFP or LV-DNp85α on Ang II-induced increases in ethidium fluorescence intensity of in both SHR and WKY rat neurons. Data are mean ± SE (n=21 to 23 fluorescent neurons). They were derived from three experiments and at least seven dishes in each experiment. *P<0.01 compared with their respective control (PBS). #P<0.05 compared LV-eGFP-transfected cells after Ang II (100 nmol/L) treatment.
Online figure II: Effects of LV-DNp85α on phosphorylation of Akt and NADPH oxidase activity in the NTS of SHR

SHR were injected with either LV-eGFP or LV-DNp85α into the NTS as described in the Methods. NTS were dissected, pooled and subjected to either Western blotting (A) or used for the determination of NADPH oxidase activity (C) as described in the Methods. A, representative blots showing DNp85α (Δp85), phosphorylated Akt, and Akt protein levels in the NTS of SHR after receiving microinjection of LV-eGFP or LV-NDp85α. B, Data are mean ±SE of pAkt. *p<0.05 vs. LV-eGFP (n=3 experiments from 6 rats). C, Data are mean ±SE of NADPH oxidase activity (n=3 experiments from 6 rats), *P<0.05 vs. LV-eGFP.

Online figure III. Confirmation of lentiviral-mediated transduction of NTS and summary of microinjection sites

A. A photomicrograph of a transverse section through the dorsomedial medulla showing an example of Ang II injection site into the NTS (arrow).

B. A diagram showing a summary of NTS injection sites. Open square and filled circles represent sites for vehicle and GF109203 experimental groups, respectively. (Bilateral microinjections were at -14.3 mm from Bregma).

C. Identification of transgene expression in the NTS following viral microinjection at the level of area postrema.

D, eGFP fluorescence at higher magnification (magnified from white dotted square in A, confirming neuronal transduction).
Abbreviations: AP-area postrema; CC-central canal; SOL-solitary tract, IV-fourth ventricle, DVN-dorsal vagal motor nucleus.

Online Figure I

![Experimental results showing Ethidium Fluorescence intensity in WKY and SHR models under different conditions.](image)

### E

**Ethyldium Fluorescence (Intensity unit)**

- **CON**
- **Ang II**

**WKY**

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<th>CON</th>
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**SHR**

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**#** indicates significant difference between groups.

### F

**Ethyldium Fluorescence (Intensity unit)**

- **SHR**
- **WKY**

**LV-eGFP**

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**LV-DNp85α**

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**#** indicates significant difference between groups.
Online Figure II

A

\[ \Delta p85 \]
\[ \beta\text{-actin} \]
\[ pA\text{kt} \]
\[ A\text{kt} \]

LV-eGFP  LV-DNp85α

B

\[ \text{pAkt/Akt protein (relative absorbance)} \]

LV-eGFP  LV-DNp85α

C

NADPH Oxidase Activity (counts/mg/min)

LV-eGFP  LV-DNp85α

*