Chronic Blockade of Phosphatidylinositol 3-Kinase in the Nucleus Tractus Solitarii Is Prohypertensive in the Spontaneously Hypertensive Rat

Jasenka Zubcevic, Hidefumi Waki, Carlos Diez-Freire, Alexandra Gampel, Mohan K. Raizada, Julian F.R. Paton

Abstract—Phosphatidylinositol 3-kinase (PI3K) within brain stem neurons has been implicated in hypertension in the spontaneously hypertensive rat (SHR). Previously, we demonstrated elevated expression of PI3K subunits in rostral ventrolateral medulla and paraventricular nucleus of SHRs compared with Wistar-Kyoto rats. Here, we considered expression levels of PI3K in the nucleus tractus solitarii, a pivotal region in reflex regulation of arterial pressure, and determined its functional role for arterial pressure homeostasis in SHRs and Wistar-Kyoto rats. We found elevated mRNA levels of p110β and p110δ catalytic PI3K subunits in the nucleus tractus solitarii of adult (12 to 14 weeks old) SHRs relative to the age-matched Wistar-Kyoto rats (fold differences relative to β-actin: 1.7±0.2 versus 1.01±0.08 for p110β, n=6, P<0.05; 1.62±0.15 versus 1.02±0.1 for p110δ, n=6, P<0.05). After chronic blockade of PI3K signaling in the nucleus tractus solitarii by lentiviral-mediated expression of a mutant form of p85α, systolic pressure increased from 175±3 mm Hg to 191±6 mm Hg (P<0.01) in SHRs but not in Wistar-Kyoto rats. In addition, heart rate increased (from 331±6 to 342±6 bpm; P<0.05) and spontaneous baroreflex gain decreased (from 0.7±0.07 to 0.5±0.04 ms/mm Hg; P<0.001) in the SHRs. Thus, PI3K signaling in the nucleus tractus solitarii of SHR restrains arterial pressure in this animal model of neurogenic hypertension. (Hypertension. 2009;53:97-103.)

Key Words: hypertension ■ brain stem ■ NTS ■ PI3K ■ baroreflex control

The pathogenesis of essential hypertension is multifactorial. In a majority of hypertensive cases, the underlying causes remain unknown and are most probably a product of complex interactions between susceptibility genes and environmental factors influencing neural, humoral, cellular, and subcellular mechanisms.1–3 Both animal4–7 and human studies8–11 suggest that overactive sympathetic nerve activity participates in the pathogenesis of hypertension. In a rat model of genetic hypertension (the spontaneously hypertensive rat [SHR]), sympathetic nerve activity is significantly higher in comparison with its normotensive control, the Wistar-Kyoto (WKY) rat.6 Interestingly, this raised level of activity precedes the onset of hypertension in this animal model, supporting a causative role in the development of this pathology.11

However, the roles of brain regions and intracellular signaling pathways in the pathogenesis of chronic sympathetic overactivity and hypertension are not fully understood. Our previous studies have demonstrated an elevated phosphatidylinositol 3-kinase (PI3K) signaling in presympathetic brain regions of the SHR12,13 We found that the mRNA levels of specific class I PI3K subunits (p85α, p110α, and p110β) were elevated within the rostral ventrolateral medulla (RVLM) and paraventricular nucleus of the SHRs compared with the WKY rats, which was accompanied by increased PI3K activity in brain stem/hypothalamic neuronal cultures made from SHRs.13 In addition, acute PI3K inhibition within the RVLM decreased blood pressure (BP) in the anesthetized SHRs to levels similar to those of the WKY rats,14 confirming a functional role for PI3K signaling pathway in this brain region unique to the SHR.

In this study, we quantified the level of gene expression of specific class I PI3K subunits (p85α, p85β, p110α, p110β, p110δ, and p110γ) in the nucleus tractus solitarii (NTS) of SHRs relative to WKY rats using real-time RT-PCR and assessed any functional role of PI3K signaling in this structure for chronic regulation of arterial pressure. The NTS is the principal termination site of baroreceptor afferents15,16 and, therefore, one of the key regulators of both baroreflex gain and the set point of arterial pressure.17–19 With accumulating evidence that the baroreceptor reflex plays a crucial role in the chronic regulation of arterial pressure,20 a reduction in its...
sensitivity may have long-lasting detrimental consequences for arterial pressure homeostasis. Here, we report differential expression of a subset of PI3K subunits in the NTS of SHRs versus WKY rats. Functionally, PI3K signaling in the NTS exerts a chronic restraining role on arterial pressure specific to the SHR.

Methods

Procedures were carried out according to the United Kingdom Home Office Guidelines on Animals (Scientific Procedure) Act of 1986. The animal were housed individually, allowed normal rat chow and drinking water ad libitum, and kept on a 12-hour light/12-hour dark cycle.

Differential Gene Expression in the SHRs and WKY Rats

Extraction and purification of RNA were conducted from 13- to 16-week-old SHRs and WKY rats (n=6 per strain), as described previously.21–23 Transcript abundance of β-actin and class I PI3K subunits (p85α, p85β, p110α, p110β, p110δ, and p110γ) were measured using quantitative RT-PCR (see the data supplement at http://hyper.ahajournals.org for details of the primers and the real-time RT-PCR procedures performed). Real time RT-PCRs were carried out using a DNA Engine Opticon 2 system (MJ Research) and the QuantiTect SYBR Green RT-PCR kit (Qiagen), as described previously.19 Expression of target genes in each sample was assessed in relation to a housekeeping gene (β-actin) using the comparative \((2^{-\Delta\DeltaCT})\) method.24 Fold differences against average values of WKY rats were calculated, as in Reference 19. Final products were confirmed to have correct sizes by gel electrophoresis.

Telemetric Recordings of Arterial Pressure

Male SHRs and WKY rats (13 to 16 weeks old; n=10 per strain) were anesthetized with ketamine (60 mg/kg) and medetomidine (250 μg/kg) intramuscularly. A radiotransmitter (TA11PAC40, Data Sciences International) was implanted to record arterial pressure (and from this, heart rate [HR] was derived) from the abdominal aorta, as described previously.25,26 Anesthesia was reversed with atipamezole (1 mg/kg). The rats were allowed to recover for 7 to 10 days before baseline telemetric measurements were taken. A full spectral analysis was performed on the BP signal to reveal potential mechanisms (see the data supplement for details and results of spectral analysis).

Cloning and Lentiviral Vector Production

DNp85 construct27 was cloned in a lentiviral (LV) vector driven by the human elongation factor 1 (EF1) promoter, as described previously28 (see the data supplement for details of our reasoning for the choice of the construct to reduce PI3K signaling). LV-expressing enhanced green fluorescent protein (LV-eGFP) driven by the same promoter was used as a control. The typical LV titer of the control LV-eGFP and LV-DNp85α vectors used were comparable, ranging from 1 to 2×10^6 transducing units per milliliter. This titer range was shown to be efficient in central neuronal transduction.29 Confirmation of the dominant-negative action of LV-EF1-α-DNp85α-IRES-eGFP (LV-DNp85α) in vitro is described in the online supplement (see the data supplement).

LV Microinjections into NTS

After control recordings of arterial pressure were taken for 24 hours, animals were reanesthetized. Three 200- to 300-nL volumes of either LV-DNp85α or LV-eGFP were microinjected bilaterally into the NTS at separate sites spanning ±500 μm rostrocaudal to calamus scriptorius, 350 to 500 μm from the midline, and 500 to 600 μm ventral to the dorsal surface. The animals were allowed to recover for 1 week before further arterial pressure measurements were taken. The site of the microinjection, the extent of the LV transduction, and protein expression were all confirmed by posthoc immunohistochemistry (see the data supplement for details of the immunohistochemical procedures).

Data and Statistical Analysis

Group data were expressed as mean±SEM 2-way repeated-measures ANOVAs, and the posthoc (Bonferroni) test was used to allow multiple comparisons of cardiovascular variables across time and between different groups. Paired/unpaired Student t test was used for further comparisons between 2 groups where applicable, with \(P<0.05\) considered significant.

Results

Quantitative Comparison of Transcript Abundances of Different PI3K Subunits in the NTS of Adult SHRs and WKY Rats

Using 40 cycles of real-time RT-PCR, we observed significantly higher transcript levels of both p110β and p110δ catalytic subunits of PI3K in the NTS of adult SHRs compared with the WKY rats (Figure 1). Specifically, the level of p110β mRNA transcript was significantly elevated in the SHRs compared with age-matched (adult) WKY rats (1.7±0.2 versus 1.01±0.08, respectively, normalized to β-actin; n=6 per strain; \(P<0.05\); Figure 1). Similarly, in SHRs, the level of p110δ mRNA transcript was significantly higher (1.62±0.15 versus 1.02±0.1, respectively; n=6 per strain; \(P<0.05\); Figure 1). In contrast, we failed to observe any differences in the NTS transcript abundances of p85α, p85β, p110α, and p110γ subunits of PI3K in the adult SHRs versus WKY rats relative to β-actin transcript levels (see Table S2 in the online data supplement). We confirmed the RNA quality by constructing melting curves for β-actin in the samples from both SHRs and WKY rats (see Figure S1).

Effects of DNp85α Expression in the NTS

Arterial BP

Considering that p110β and p110δ catalytic subunits of PI3K were upregulated in the NTS of SHRs (compared with WKY rats), we tested the functional impact of chronic PI3K blockade in the NTS on arterial pressure in both rat strains by LV-mediated expression of a dominant-negative for PI3K.

Because mature adult SHRs and WKY rats were used in the present study, with stable BP levels, we found no significant interaction between the time-related BP changes (ie, BP progression) and rat species (ie, WKY rats and SHRs). Over a period of 4 weeks postinjection, systolic BP (SBP) rose significantly in the SHRs injected with LV-DNp85α.

Figure 1. Endogenous expression of PI3K catalytic p110β and p110δ subunits in the NTS. A and B, Results of real-time RT PCR for p110β and p110δ, respectively. Data are expressed as the fold difference vs the WKY. \(\ast P<0.05\) vs WKY.
from a control of 175±3 mm Hg (day 0) to 191±6 mm Hg at day 28 compared with no change in SBP in SHRs injected with the control LV (LV-eGFP; n=5 per group; P<0.01, Figure 2A). In contrast, there were no significant differences in SBP between the WKY rats injected with either LV-DNp85α or LV-eGFP at any time point over the 4 weeks of monitoring (n=5 per group; Figure 2). Similarly, we observed significant increases in both the diastolic BP (DBP; from 130±2 to 157±5 mm Hg at day 28; P<0.05; Figure 2B) and the mean BP (MBP; from 145±2 to 165±5 mm Hg at day 28; P<0.05; Figure 2C) in the LV-DNp85α–treated SHRs. No significant changes were detected in DBP or MBP of WKY rats injected with either virus (Figure 2B and 2C). A significant rise in SBP, DBP, and MBP values in the LV-DNp85α SHR was first observed as early as 7 days after the viral injection (data not shown). Changes in night versus day BPs were also assessed in the LV-eGFP– and LV-DNp85α–treated SHRs (Figure S3). We found that the SBP, DBP, and MBP of SHRs significantly rose after the LV-DNp85α injection during both the light and dark phases compared with the LV-eGFP–treated SHRs, the values of which remained unchanged (Figure S3). These results suggest that increased PI3K signaling in the NTS of SHRs exerts a chronic restraining role on arterial pressure in the SHRs.

**HR and Spontaneous Baroreceptor Reflex Gain**

Over the period of 28 days of monitoring, the HR values significantly rose from 331±6 to 342±6 bpm (n=5 per group; P<0.05; Figure 2D) in the SHRs injected with LV-DNp85α compared with the LV-eGFP–treated SHRs, in which the HR values showed a significant decline over the time (from 346±7 to 322±6 bpm at day 28; P<0.05; Figure 2D). Similar to the LV-eGFP–treated SHRs, we found that the HR values in both the LV-DNp85α– and LV-eGFP–treated WKY groups also significantly decreased over time to a similar extent (Figure 2D). We attributed this to the age-related decreases in the HR values reported previously in mature rats.\(^{29–32}\) We also observed a significant decrease in values of the spontaneous baroreflex gain (sBRG; from 0.7±0.07 to 0.5±0.04 ms/mm Hg; n=5; P<0.001; Figure 2E). Changes in the night (dark phase) versus day (light phase) HR and sBRG values in LV-DNp85α– and LV-eGFP–treated SHRs followed a similar pattern in both phases (Figure S3). These results suggest a role for PI3K in the chronic modulation of the cardiac component of the baroreflex and regulation of HR in the NTS of the SHR.

**Confirmation of the LV-Mediated Gene Expression in NTS**

Posthoc examination of the sites of LV injections revealed abundant eGFP fluorescence in numerous cells of the NTS of all animal groups. Some eGFP-positive staining was also found in the dorsal vagal motor nucleus, because of the inevitable unsolicited viral spread in this area, which is in very close proximity to NTS and contains dendrites from dorsal vagal motor nucleus neurons. No eGFP-positive staining was found in any other medullary nuclei known to control.

---

**Figure 2.** DNp85α expression in the NTS of SHRs, but not WKY rats, raises arterial blood pressure (ABP). SBP (A), DBP (B), and MBP (C) are significantly elevated 14 days after the injection of LV-EF1α-DNp85α-IRES-eGFP (LV-DNp85α). They remained elevated for the 28-day observation period. No changes were seen in the control SHR, where LV-EF1α-eGFP (LV-eGFP) was injected into NTS or when LV-DNp85α or LV-eGFP was injected into the NTS of WKY rats. In D, NTS injection of LV-DNp85α significantly elevated HR in the SHR vs the LV-eGFP-treated SHR, which, however, showed a significant decrease in HR over time. In the WKY rat, there were no significant differences between the effects produced by either the LV-eGFP or LV-DNp85α on HR, with both groups showing a similar significant decrease in HR over time. In E, sBRG (PI) is significantly reduced after LV-DNp85α NTS injection in the SHR vs the effect of the LV-eGFP injection. There was no effect of either virus injection on sBRG (pulse interval) in the WKY rats, \(\dagger P<0.05, \dagger\dagger P<0.01, \) and \(\dagger\dagger\dagger P<0.001\) vs “Before.” \(\ast P<0.05, \ast\ast P<0.01, \) and \(\ast\ast\ast P<0.001 \) vs LV-eGFP transduction. N=5 per group.
the cardiovascular system, indicative of an absence of both retrograde and anterograde transport of this vector. Immunostaining for neuronal nuclei (marker) (NeuN) and double-fluorescence labeling revealed that a proportion (≈45%) of the eGFP-positive cells were double labeled and, therefore, neuronal (Figure 3). Comparable regions of the NTS were transduced with both viral vectors in all of the animal groups that spanned regions from −13.80 to −14.60 mm relative to bregma, 300 to 800 μm from midline, and 400 to 800 μm below the dorsal surface of the medulla (Figure 3).

**Discussion**

The most significant finding of the present study is that chronic and specific suppression of PI3K signaling in the NTS of the SHR causes arterial pressure to increase. This chronic effect, together with increases in HR and depression of the spontaneous baroreflex gain, are exclusive to the SHR and not seen in the WKY strain. These observations suggest that PI3K signaling is more active in the NTS of the SHR than the WKY rat and that this pathway could act to restrain the hypertension in the SHR.

**PI3K Expression in the NTS of the SHR Versus WKY Rat**

We provide the first direct evidence for increased PI3K transcript abundance within the NTS of the SHR relative to a normotensive rat strain. Specifically, we demonstrate increased gene transcript abundances of p110β and p110δ catalytic subunits of PI3K in the NTS of SHR compared with the WKY rat. This is consistent with our previous study, which also showed an elevated mRNA level of p110β in both the paraventricular nucleus and RVLM neurons of the SHR and was similarly accompanied by increased PI3K activity. These latter regions also showed alterations in p110α and p85α subunits. Therefore, the elevated mRNA levels of the catalytic p110β and δ PI3K subunits in the NTS of SHR seen in the present study may also be accompanied by elevated PI3K activity in these rats. We recognize that a caveat of the present study is that, for technical limitations (see below), we did not show directly increased PI3K activity in NTS of the SHR versus the WKY rat. However, an effective and robust way to test whether PI3K protein activity was elevated was to block the activity of p110β and p110δ catalytic subunits simultaneously by antagonizing the regulatory subunit p85α on which their catalytic activity depends. This would reveal whether the activity of PI3K was upregulated in the SHR and, importantly, whether this had any functional implications for arterial pressure control in the SHR.

We hypothesized that the elevated PI3K mRNA in the NTS may be of neuronal origin. This is supported by abundant transgene expression in NTS neurons, as revealed by the expression of eGFP in NeuN (a specific neuronal marker) immunopositive cells (Figure 3), consistent with the known neuronal tropism of the lentivirus in the NTS. However, a limitation of the present study is that it fails to pinpoint the exact neuronal phenotype in which this elevation of PI3K signaling is occurring. Considering that the differences in mRNA levels of p110β and p110δ are relatively small between SHRs and WKY rats (~1.7-fold and ~1.6-fold, respectively), this may reflect a highly selective and cell type-specific PI3K elevation in NTS neurons exerting a powerful effect on cardiovascular autonomic activity in the SHR. We discuss putative neuronal phenotypes below. Although we have not demonstrated reduced PI3K activity in vivo, which would be technically difficult, in our previous studies we have demonstrated the capability of the DNp85 construct to reduce the PI3K activity. Here we also demonstrate expression of the DNp85α protein in vivo, as evidenced from the expression of eGFP using the internal ribosome entry site construct (Figure 3). All told, our data support the presumption that our viral-induced intervention reduced PI3K activity in NTS in vivo.

*Figure 3. A, Schematic of the area transduced by LV-DNp85α spanning from −14.80 to −13.80 mm from bregma, with the greatest transfection observed at −14.30 to −14.08 mm from bregma. Red box in A indicates the region corresponding with images in B. B, Validation of transgene expression was visualized by eGFP fluorescence (left). Middle panel shows immunostaining for NeuN, and the right panel shows an overlay of eGFP and NeuN; the LV-DNp85α-transduced neurons are indicated by white arrows. The images were taken at approximately −14.30 mm from bregma. CC indicates central canal; Sol, solitary tract; DVN, dorsal vagal motonucleus; AP, area postrema. Scale bar=50 μm.*
Cardiovascular Functional Role of PI3K in the NTS of SHRs

Dysregulation within the NTS has been linked to the development of hypertension in animal models, including the SHR.\textsuperscript{17,19,21,26} To test for an overall functional role of PI3K in the NTS for regulating arterial pressure, we chronically blocked PI3K activity by expressing a dominant-negative of the regulatory p85a PI3K subunit using LV gene transfer (see online supplement and below for further discussion), because this provided the means to block the catalytic activity of the 2 subunits (p110\(\beta\) and p110\(\alpha\)) that we found to be overexpressed in the NTS of the SHR. We found a chronic and sustained elevation of arterial pressure (SBP, DBP, and MBP) in the SHR but no change in normotensive WKY rats. In the SHR, this was accompanied by an increase in very low frequency, very low frequency+low frequency (Figure S4), and HR and decreases in the sBRG and high frequency of HR variability (Figure S4). Increases in both very low frequency and very low frequency+low frequency could imply alterations in the sympathetic and hormonal controls of arterial pressure, whereas reductions in the high frequency of HR variability indicate loss of vagal tone.\textsuperscript{34–36} This suggests that PI3K activity in the NTS of the SHR controls autonomic activity destined for both the heart and vasculature. Moreover, suppression of the sBRG after PI3K blockade is consistent with the reduced cardiac vagal activity (high frequency of pulse interval).\textsuperscript{37} We recognize the limitations of our analysis in that we are unable to provide a full baroreceptor reflex function curve. However, the sBRG values obtained here are of physiological relevance, because they fall around the operating point of this reflex in an unrestrained conscious rat.\textsuperscript{38} Whether the gain of the sympathetic vasconstrictor component has also been modified remains unknown. Because both sBRG and SBP are affected by PI3K blockade in the SHR, it is unclear whether the increase in arterial pressure is caused by a depression of the baroreceptor reflex and/or direct action on neurons determining the set point of arterial pressure.

Others have shown that acute blockade of PI3K activity in other regions of the brain stem can affect arterial pressure. In the RVLM, wortmannin (a PI3K antagonist) decreased arterial pressure in anesthetized SHRs but not WKY rats.\textsuperscript{14} Our data are consistent with the holistic viewpoint that PI3K activity in the SHR is increased in key regions of the medulla and hypothalamus known to control vasomotor tone (eg, RVLM and paraventricular nucleus).\textsuperscript{12–14} However, the question of the signaling downstream of PI3K remains uncertain. It is generally believed that the main downstream effector of PI3K activation is the protein kinase B (PKB)/Akt pathway.\textsuperscript{39,40} We have confirmed in the present study in vitro that overexpression of DNp85a blocks PI3K activity\textsuperscript{13} to reduce activation of PKB/Akt signaling (see online supplement for details). However, this does not mean that PKB/Akt is involved in NTS signaling in the SHR in vivo. Indeed, others have shown in other animal models of hypertension that increased levels of PI3K subunits and PI3K activity did not necessarily lead to elevation of PKB/Akt signaling,\textsuperscript{41} suggesting the presence of a PI3K-dependent but a PKB/Akt-independent signaling pathway. A recent study also revealed that baseline phosphorylated (but not total) PKB/Akt levels are lower in the NTS of SHRs compared with the WKY rats.\textsuperscript{42} These authors also showed that injection of phosphoinositide(3,4,5)P\(_3\) (a phospholipids second messenger produced by PI3K, thereby mimicking activation of PI3K) in the NTS of the SHR failed to activate the downstream PKB/Akt but produced cardiovascular responses that are in full agreement with the findings of our present study (ie, depressor action). Therefore, although we are confident in the ability of our construct to reduce PI3K activity, the relevance of this to the downstream PKB/Akt in vivo remains uncertain.

Because angiotensin II potentiates PI3K signaling and release of catecholamines from brain stem SHR neurons,\textsuperscript{12,13} the A2 neurons in NTS may mediate the hypertensive effect after chronic PI3K blockade in the NTS of SHRs. Although we did not assess the proportion of A2 neurons that were transfected by LV-DNp85a in the present study, it is likely that many A2 neurons were transduced, because their location in NTS included neurons that had been transduced. The A2 noradrenergic cells are involved in BP homeostasis. Indeed, several studies reported that selective destruction\textsuperscript{43,44} or electric “silencing”\textsuperscript{26} of A2 neurons causes either lability\textsuperscript{43,44} or increased BP being more pronounced in the SHR.\textsuperscript{26} This last study also emphasized the putative homeostatic role of A2 neurons in SHRs in restraining BP in this model of hypertension. The contribution of A2 neurons to baroreflex regulation is not well established and remains controversial.\textsuperscript{45,46} However, cardiac baroreceptor reflex was attenuated after the A2 neurons were lesioned.\textsuperscript{44} In addition, vagal sensory afferents could be found in close proximity to the NTS catecholamine neurons.\textsuperscript{47} Therefore, the A2 neuronal population, or at least the portion that regulates cardiovascular homeostasis, seems to be sympathoinhibitory.

Finally, as the dendrites of dorsal vagal motor nucleus project up into the NTS,\textsuperscript{48} some of these were also transduced with the LV-DNp85a. Thus, there is a possibility that a component of the cardiovascular effects seen in the present study is because of the blockade of PI3K in this nucleus, especially because some of these motoneurons have chronotropic influences.\textsuperscript{49}

**Elevated PI3K in the NTS of SHR: Cause or Consequence of Hypertension?**

Because the present study compared the adult SHRs (with fully developed hypertension) with their age-matched WKY controls, we cannot conclude whether the elevated PI3K signaling in the NTS is a cause or consequence of the hypertensive phenotype. However, we showed previously that the PI3K signaling was enhanced in cultured brain stem/hypothalamic neurons from the neonatal SHR,\textsuperscript{12,13} indicating that the raised PI3K signaling in the brain stem is a congenital feature of the SHR. Future studies could assess whether chronic blockade of PI3K signaling in NTS in the developing SHR has any effect on the hypertension developed with maturation. Holistically, in the SHR, PI3K activity appears to be elevated in all of the cardiovascular control regions studied to date (RVLM, paraventricular nucleus, and now NTS, shown herein) and that PI3K activity is presumed to exert an excitatory effect on cardiovascular neurons di-
rectly or when driven by an endogenous ligand.50 Because we centered our LV injections to a sympathoinhibitory region of the NTS, its blockade resulted in a pressor effect. All told, it would appear that PI3K activity within the brain of the SHR plays a major role in the maintenance of the precise level of the hypertension by acting at regions exerting excitatory or inhibitory influences on sympathetic activity.

Future Perspectives

This study reveals a novel role for the PI3K signaling pathway in the NTS of SHRs in restraining BP in this animal model of human hypertension. Future studies should seek to determine the genomic and/or modulatory factors that cause this pathway to predominate in the NTS of the SHR, as well as to identify which specific neuronal types are involved. Such information might allow the development of new tools for specific activation of this signaling pathway in NTS, which may provide a powerful intervention for lowering arterial pressure chronically.

Sources of Funding

The financial support of the National Institutes of Health (HL033610) and the British Heart Foundation is acknowledged. J.F.R.P. was in receipt of a Royal Society Wolfson Research Merit Award.

Disclosures

None.

References


Downloaded from http://hyper.ahajournals.org/ by guest on May 3, 2016


Chronic Blockade of Phosphatidylinositol 3-Kinase in the Nucleus Tractus Solitarii Is Prohypertensive in the Spontaneously Hypertensive Rat

Jasenka Zubcevic, Hidefumi Waki, Carlos Diez-Freire, Alexandra Gampel, Mohan K. Raizada and Julian F.R. Paton

Hypertension. 2009;53:97-103; originally published online November 17, 2008; doi: 10.1161/HYPERTENSIONAHA.108.122341

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/53/1/97

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2008/11/17/HYPERTENSIONAHA.108.122341.DC1.html

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Chronic blockade of PI3K in the nucleus tractus solitarii is pro-hypertensive in the spontaneously hypertensive rat

Jasenka Zubcevic*, Hidefumi Waki§, Carlos Diez-Freire†, Alexandra Gampel, Mohan K Raizada‡, Julian FR Paton

Department of Physiology & Pharmacology, Bristol Heart Institute, School of Medical Sciences, University of Bristol, Bristol, BS8 1TD, UK

*Current address: Department of Integrative Physiology, Health Science Center, University of North Texas, Ft. Worth, TX 76107, USA

†Department of Physiology and Functional Genomics, University of Florida, PO Box 100274, Gainesville, FL 32610-0274, USA

§Department of Physiology, University of Wakayama, Wakayama, Japan

Corresponding author: Professor Julian F. R. Paton, Department of Physiology and Pharmacology, Bristol Heart Institute, School of Medical Sciences, University of Bristol, Bristol, BS8 1TD, UK

Phone: +44-(0)117-331-2275; fax: +44-(0)117-331-2288; email: Julian.F.R.Paton@bris.ac.uk

Word count: 2,686
Methods for real-time RT-PCR

Primer design

Oligonucleotide primers (forward and reverse) for p85α, p85β, p110β, p110δ, and β-actin were purchased ready-made (Qiagen; the approximate location of the selected primer products can be found on the company website www.qiagen.com) (Table S1). The oligonucleotide primers that were manually designed were p110α and p110γ. In case of p110α, the forward and reverse primers were designed based on the Rattus norvegicus GenBank sequence for p110α (GenBank entry AAK83379; http://www.ncbi.nlm.nih.gov/), using an online ‘Primer 3’ software for primer design (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers were designed to flank the intron-exon boundaries to avoid amplification of any remaining genomic DNA. In the case of p110γ, with no available Rattus norvegicus GenBank sequence at the time of our study, the oligonucleotide primers were designed based on predicted cDNA sequences, which were based on mouse sequences (Mus musculus; GenBank entry XM 001076206). Once chosen, the forward and reverse oligonucleotide primer sequences were ‘blasted’ (http://www.ncbi.nlm.nih.gov/BLAST/) to search for sequence similarities. This eliminated usage of primers that have a high sequence similarity to genes other than the gene of interest, and therefore maximized specific gene recognition and real-time RT-PCR amplification. The chosen oligonucleotide primers were purchased from MWG Biotech (http://www.mwg-biotech.com). The details of the manually designed primers are presented in Table S1.
**Methods for confirmation of dominant negative action of LV-EF1α-DNp85α-IRES-eGFP in vitro**

(i) **Utilizing the dominant negative action of DNp85α**

The DNp85α is a mutant version of the wild type p85α regulatory subunit constructed by specific deletion of 35 amino acids (from residues 479-513) from the wild type protein and insertion of two other amino acids (Ser-Arg) in the deleted position\(^3\). The resulting protein is 76.5 KD in size, and is devoid of the region necessary for its binding to the many catalytic PI3K subunits\(^3\). The overexpression results in the mutant protein (DNp85α; not bound to a catalytic subunit) competing with endogenous p85α (bound to a catalytic subunit), for binding to the tyrosine-phosphorylated adaptor proteins/receptors, thereby blocking endogenous PI3K activity, characterised by a decrease in the levels of phosphorylated PKB/Akt protein downstream in the PI3K cell signalling pathway\(^4\) (see also Figure S2 below). This is because the PI3K catalytic subunits rely on their regulatory subunits for recognition of the extra-cellular stimuli and binding of the activated phosphorylated receptors, which leads to subsequent activation of the PI3K heterodimer\(^5,6,7\). As p85α is the most abundant regulatory subunit\(^8\), interruption of its endogenous action by DNp85α has a powerful effect on the overall PI3K cellular signaling\(^4\) (see also Figure S2 below).

(ii) **Viral Transfection of PC12 Cells**

The ability of the DNp85α protein expression to block the PI3K activity was tested *in vitro* in a rat neuronal cell line (PC12 cells). The cells were plated at 50% confluence in full growth medium in a 6-well plate and incubated for 48 hours. This was followed by incubation with 2 μl
of either the ‘mock’ virus (i.e. cell medium only), the control LV-EF1α-eGFP (LV-eGFP) or the LV-EF1α-DNp85α-IRES-eGFP (LV-DNp85α virus) (>10^{10} TU/ml), for 48 hours at 37°C in the presence of 8 μg/ml polybrene (Chemicon, USA), a reagent that increases the efficiency of retroviral infection/transfection\(^9\). The cells were then placed on serum-free cell medium for 12 hours, and either stimulated with 100 ng/ml of neuronal growth factor (NGF) for 2 hours, or left untreated. The cells were then lysed in the lysis buffer (BioRad, USA) containing protease inhibitors and centrifuged at low speed at 4°C to pellet the cellular membrane debris. The cellular protein was stored at -80°C prior to use.

(iii) Western Blotting

Western blotting was preformed using the Novex NuPAGE gel system (Invitrogen) according to the manufacturer’s instructions. Briefly, an equal volume of 2X sample buffer (250 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue) was added to cell lysates. Samples were run on 4-12% Bis-Tris gels in MOPS running buffer. Gels were transferred to PVDF membrane using NuPAGE transfer buffer followed by blocking in 5% bovine serum albumin (BSA) in Tris-buffered saline+0.1% Tween (TBT) for 1 hour at room temperature (RT). Incubation with primary antibodies was overnight at 4°C: mouse monoclonal anti-β-actin antibody (Sigma-Aldrich), Akt antibody (Cell Signaling) and anti-phospho-Akt (pAkt) antibody (Cell Signaling). The anti-pAkt antibody detects protein levels of the PKB/Akt phosphorylated at Ser473, thereby indicating activation of the upstream PI3K. Therefore, a reduction in pAkt should indicate a reduced PI3K activity. Membranes were washed and incubated with secondary HRP-conjugated antibodies (Jackson Immunoresearch) in TBT for 1 hour at RT followed by washing in TBT.
HRP reaction product was visualized using the Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology) followed by exposure to Hyperfilm (Amersham Biosciences).

**Methods for telemetric measurements and data analysis**

Telemetric measurements were conducted at ‘day 0’ (i.e. baseline measurements one day before viral injections), and then at days 14 and 28 after the viral injections. On these days, the measurements were taken continuously for 5 minutes every hour over 24 hour period throughout the 12 hour light/dark cycle. Heart rate (HR) was derived from interpulse interval (PI). The spontaneous cardiac baroreflex gain (sBRG) was also determined from spontaneous changes in SBP and HR using methods originally described by Oosting et al.\textsuperscript{10}, and subsequently adopted by us\textsuperscript{11}. SBP and HR variability were determined using a fast Fourier transform (FFT) algorithm as previously described\textsuperscript{11} in order to derive low frequency (LF) band of SBP (0.27-0.75 Hz; an indicator of the level of vasomotor sympathetic activity\textsuperscript{12,13}), high frequency (HF) band of pulse interval (PI) (0.75-3.3 Hz, reflective of cardiac parasympathetic tone\textsuperscript{12}) and very low frequency (VLF) band of SBP (0-0.27 Hz, reflective of changes in sympathetic outflow related to thermoregulation, hormonal activity or changes in blood flow to meet local metabolic demands\textsuperscript{14,15}). VLF and VLF+LF (SBP) (an additional measure of sympathetic activity\textsuperscript{16}) and HF(PI) represent the mean values after viral gene delivery at the time of maximum change in SBP values for each individual rat (for SHR-DNP85α group only).
Methods for immunohistochemistry

At the end of each experiment, animals were anaesthetized terminally with sodium pentobarbitone (100 mg/kg i.p.) and trans-cardially perfused with 150 ml of phosphate-buffered saline (0.1 M PBS, pH 7.4) followed by 200 ml of 4% formaldehyde in 0.1 M PBS (pH 7.4). The brains were removed and placed overnight at 4°C in 2% formaldehyde/0.1 M PBS (pH 7.4) solution containing 10% sucrose as a cryoprotectant. Transverse sections (40 µm) were cut on a freezing microtome throughout the caudal brainstem (from approx. -14.60mm to -13.30mm from Bregma) to obtain a range of slices corresponding to the NTS region. The free-floating sections were collected in 0.1 M phosphate-buffered saline (PBS) (pH 7.4). Immunohistochemistry was performed for eGFP expressed by LV and a neuronal marker (neuronal nuclear antigen; NeuN). Free-floating sections were washed in PBS, followed by 5 minute incubation in the antigen unmasking solution (L.A.B. Solution, Polysciences, USA) at room temperature. The sections were then incubated with blocking solution (10% goat serum, 0.3% Triton X, 0.1 M PBS pH 7.4) for 15 minutes at room temperature, followed by an incubation with the primary antibody (1/2000 mouse anti-NeuN; Invitrogen) for 48 hours at 4 °C in a solution containing 1% goat serum and 0.3% Triton X in 0.1 M PBS pH 7.4. The sections were then exposed to AlexaFluor 594 conjugated goat anti-mouse IgG (1/500, Invitrogen) also containing 1% goat serum and 0.3% Triton X in PBS, for 1 hr at RT. The sections were then mounted onto glass microscope slides using 0.5% gelatin and coverslipped using Vectashield Mounting Medium for Fluorescence (Vector Labs). A Leica confocal fluorescence microscope was used to detect red and green fluorescence corresponding to AlexaFluor 594 (excitation/emission maxima of 590/617 nm) of the endogenous NeuN and lentiviral eGFP proteins, respectively. NeuN-positive
and eGFP-fluorescent cells were captured in the same focal planes using x63 objective to reveal the degree of double labeling.

Results

Lentiviral expression of DNp85α in PC12 cells In Vitro

When infected with either the LV-DNp85α, or the control LV-eGFP virus, PC12 cells showed robust expression of transgenes, as illustrated by the abundant eGFP expression 48 hours after viral infection (Figure S2). Western blotting performed with the anti-pAkt antibody revealed a decrease in protein levels of pAkt in the PC12 cells infected with the LV-DNp85α, compared to those in the cells infected with the LV-eGFP or the mock-infected cells (Figure S2). This was apparent in both the basal (without NGF) cell state as well as in the NGF-stimulated PC12 cells (Figure S2).

Variability in SBP and HR

Power spectral analysis of SBP variability in the SHR LV-DNp85α experimental group revealed a significant increase in the very low frequency (VLF) of SBP from 5.6±0.1 to 8.6±0.6 ms²/mmHg² (n=5, P<0.01, Figure S4) and the VLF+low frequency (LF) SBP values (from 9.3±0.1 to 12.3±0.7 ms²/mmHg², n=5, P<0.05, Figure S4), but no significant change in LF (data not shown). We also detected a significant decrease in high frequency (HF) variability of pulse interval (PI) in the LV-DNp85α–transduced SHR (from 17.7±0.7 to 15.4±0.6 ms²/Hz, n=5, P<0.05, Figure S4). No significant change in the HF/LF of PI was found. These data suggest
chronic and direct PI3K signaling in NTS is contributing to the hypertension in the SHR by regulation of both the parasympathetic (cardiac) and sympathetic (non-cardiac) limbs of the autonomic nervous system.
References


<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accretion number</th>
<th>Details</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>p85α</td>
<td>NM 013005</td>
<td>QT00184485, Qiagen</td>
<td>112 bp</td>
</tr>
<tr>
<td>p85β</td>
<td>NM 022185</td>
<td>QT00188335, Qiagen</td>
<td>130 bp</td>
</tr>
<tr>
<td>p110α</td>
<td>AAK83379</td>
<td>(forward) CATCAGTGCTCAAGGACAA (reverse) TCCAAAAAGTGCCCCAAAATC</td>
<td>180 bp</td>
</tr>
<tr>
<td>p110β</td>
<td>NM 053481</td>
<td>QT00190176, Qiagen</td>
<td>88 bp</td>
</tr>
<tr>
<td>p110δ</td>
<td>NM 001108978</td>
<td>QT00379456, Qiagen</td>
<td>73 bp</td>
</tr>
<tr>
<td>p110γ</td>
<td>XM 001076206</td>
<td>(forward) TCACCTCCCTGATTGGCTAC (reverse) TGGCATAGAGTTGGCATCC</td>
<td>132 bp</td>
</tr>
<tr>
<td>β-actin</td>
<td>NM 031144</td>
<td>QT00193473, Qiagen</td>
<td>145 bp</td>
</tr>
</tbody>
</table>

Primer details used for quantitative RT-PCR.
**Table S2**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>p85α</td>
<td>1.04±0.1</td>
<td>1.18±0.1</td>
</tr>
<tr>
<td>p85β</td>
<td>1.03±0.1</td>
<td>0.97±0.2</td>
</tr>
<tr>
<td>p110α</td>
<td>1.02±0.1</td>
<td>1.19±0.2</td>
</tr>
<tr>
<td>p110γ</td>
<td>1.02±0.1</td>
<td>1.16±0.1</td>
</tr>
</tbody>
</table>

PI3K subunit gene expression (fold difference) in the NTS of WKY and SHR. Expression of target genes relative to β-actin gene in each sample were derived using the comparative \(2^{-\Delta\Delta CT}\) method. Fold differences were calculated against the average of age-matched WKY. For p85α, p85β, p110α and p110γ gene expression, no significant differences were found between WKY and SHR.
Figure S1: Melting curves for β-actin, p110β and p110δ. At the end of the 40 cycle quantitative RT-PCR runs, melting curves were generated for every primer. The T(m) (i.e. peak of the melting curve) for each specific amplicon is calculated by the OpticonMONITOR™ 2.0 software and it is indicative of the RNA quality, as well as the product specificity and homogeneity. A: β-actin melting curve. B: p110β melting curve. C: p110δ melting curve.
Figure S2: Lentiviral over expression of DNp85α blocks the downstream PKB/Akt signalling. In A, the schematics of the lentiviral vector constructs used, driven by the elongation factor 1α (EF1α) promoter with the inserted transgene (DNp85α) and eGFP which was used as a reporter gene. Internal ribosomal entry site (IRES) allowed the expression of the second gene
(eGFP) under the same EF1α promoter. An additional vector was used as a control, containing only the reporter eGFP. In B, eGFP fluorescence corresponding to the level of transgene expression in PC12 cell culture 48 hours after viral transfection. In C, Western blot from PC12 cell homogenates at 48 hours after viral transfection. Cells were either ‘mock’-transfected, or transfected with the control (eGFP) vector or the DNp85α (DNp85α). Note a marked decrease in phosphorylated PKB/Akt (pAkt) protein level in both the basal (-neuronal growth factor, NGF) as well as in NGF-stimulated (+NGF) DNp85α –transfected when compared to both ‘mock’- and GFP-transfected cells. The decrease in pAkt is matched by a consequent increase in the PKB/Akt (Akt), and no change in the house-keeping β-actin protein levels.
Figure S3: **DNp85α expression in the NTS of SHR raises arterial pressure.** SBP (in A), DBP (in B) and MBP (in C) are significantly elevated 14 days after the injection of LV-EF1α-DNp85α-IRES-eGFP (LV-DNp85α) but not the control LV-EF1α-eGFP (LV-eGFP) in the NTS of SHR, during both the light and dark phases (12 hour light/dark cycle). They remained elevated for the twenty-eight day observation period. In D: NTS injection of LV-DNp85α significantly elevated HR in the SHR during both the light and dark phases compared to the LV-eGFP treated SHR. In E: sBRG (PI) is significantly reduced after LV-DNp85α NTS injection in the SHR compared to the effect of the LV-eGFP injection during both the light and dark phases. †P<0.05 and ††P<0.01 compared with ‘Before’. *P<0.05, **P<0.01 and ***P<0.001 compared with LV-eGFP transduction. *N=5 per group.*
Figure S4: DNp85α expression in the NTS of SHR raises the sympathetic and decreases vagal activity. In the SHR but not WKY rat, VLF(SBP) (A) and VLF+LF(SBP) (B) significantly increased, whilst HF(PI) (C) decreased significantly following NTS injection of LV-DNp85α. ‘Before’ indicates mean control values before viral injection, and ‘After viral
injection’ refers to mean VLF, VLF+LF and HF values taken at the time corresponding to a maximum SBP response of each SHR, irrespective of the day of post-viral transfection †P<0.05 and ††P<0.01 compared with ‘Before’. N=5 per group.