Increased PI3-Kinase in Presympathetic Brain Areas of the Spontaneously Hypertensive Rat
Shereeni J. Veerasingham, Masanobu Yamazato, Kathleen H. Berecek, J. Michael Wyss, Mohan K. Raizada

Existing evidence led us to hypothesize that increases in p85α, a regulatory subunit of PI3-kinase, in presympathetic brain areas contribute to hypertension. PI3-kinase p85α, p110α, and p110δ mRNA was 1.5- to 2-fold higher in the paraventricular nucleus (PVN) of spontaneously hypertensive rats (SHR) compared with their controls, Wistar Kyoto rats (WKY). The increase in p85α/p110δ was attenuated in SHR treated with captopril, an angiotensin (Ang)-converting enzyme inhibitor, from in utero to 6 months of age. In the rostral ventrolateral medulla (RVLM), p110δ mRNA was 2-fold higher in SHR than in WKY. Moreover, the increases in mRNA were associated with higher PI3-kinase activity in both nuclei. The functional relevance was studied in neuronal cultures because SHR neurons reflect the augmented p85α mRNA and PI3-kinase activity. Expression of a p85 dominant-negative mutant decreased norepinephrine (NE) transporter mRNA and [3H]NE uptake by ~60% selectively in SHR neurons. In summary, increased p85α/p110δ expression in the PVN and RVLM is associated with increased PI3-kinase activity in the SHR. Furthermore, normalized PI3-kinase p85α/p110δ expression within the PVN might contribute to the overall effect of captopril, perhaps attributable to a consequent decrease in NE availability.

PI3-kinase signaling mediates augmented Ang II-mediated norepinephrine (NE) neuromodulation in hypothalamus/brainstem neuronal cultures. In addition, PI3-kinase inhibition within the rostral ventrolateral medulla (RVLM), a major presympathetic area, decreases blood pressure (BP) of the SHR to levels similar to that of their normotensive controls, Wistar Kyoto rats (WKY). Thus, it appears that a central PI3-kinase signaling pathway is critical for hypertension, possibly as a mediator of Ang II-induced NE neuromodulation.

Class I PI3-kinases consist of a catalytic subunit (p110α, β, δ, and γ) and a regulatory subunit (p85α and its alternatively spliced forms, p85β, p55γ, and p101).1 Ang II stimulation results in prolonged increases in p85α-associated PI3-kinase activity in SHR neurons,7 indicating that the p110 isoforms that dimerize with p85α mediate this effect. Based on these findings, we hypothesized that increases in expression of p85α in presympathetic brain areas contribute to hypertension. Thus, our aims were to determine whether p85α expression is increased in presympathetic brain areas of SHR and to assess if this increase is normalized after angiotensin-converting enzyme inhibition. We focused on the RVLM and the paraventricular nucleus (PVN), a nucleus with substantial noradrenergic input, because altered Ang and NE mechanisms in these nuclei have been linked to hypertension.4–6,8

Materials and Methods
PI3-kinase mRNA was assessed in PVN and RVLM micropunches of WKY, SHR, and SHR that received an angiotensin-converting enzyme inhibitor (captopril, in utero to 6 months, 40 mg/L;8). BP and cardiac and coronary artery hypertrophy were evaluated.9 Primary neuronal cultures were derived from the hypothalamus/brainstem of newborn SHR and WKY.7 PI3-kinase activity10 was assessed in micropunches and neuronal cultures. The cultures were infected with an adenoviral vector that expresses a p85 dominant-negative mutant10 (AdΔp85) or enhanced green fluorescent protein (Ad.eGFP). PI3-kinase protein, Δp85 expression, and phosphorylation (Ser 473) of Akt, a downstream effector of PI3-kinase, were determined. NET mRNA and maprotiline-sensitive [3H]NE uptake2 were measured 48 hours after adenoviral infection. An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results
Early, long-term captopril treatment prevented hypertension and cardiac hypertrophy in SHR (supplemental Figure I and Table in the online data supplement), consistent with a previous report.10

Increased PI3-Kinase Expression and Activity in Presympathetic Areas Is Associated With Hypertension
In the PVN, p85α mRNA was ~60% higher in SHR than in WKY. This increase was prevented by captopril treatment (Figure 1A). However, p85α mRNA in the RVLM did not vary significantly between groups. Next, we assessed mRNA levels of PI3-kinase catalytic subunits that dimerize with p85α. PI3-kinase p110δ mRNA was increased in the PVN and RVLM of SHR compared with WKY, but only the increase in the PVN was prevented in the SHR group that received an angiotensin-converting enzyme inhibitor captopril (Figure 1C). Whereas an increase in PI3-kinase p110α mRNA
was noted in the PVN of SHR, it was not normalized after captopril treatment (Figure 1B and 1C). PI3-kinase p110β expression did not differ between groups (data not shown) and subunit expression did not vary between groups in the anterior hypothalamic area (Figure II in the online data supplement). In SHR, p85α-associated PI3-kinase activity was increased ∼2.5-fold in the PVN (Figure 1F), but not in the RVLM (data not shown). However, p110δ-associated PI3-kinase activity was increased ∼1.5-fold in the SHR RVLM (Figure 1F). A significant increase in p85α mRNA and protein (∼40%), but not PI3-kinase p110 subunits, was detected in neurons derived from prehypertensive SHR and was associated with increased PI3-kinase activity (Figure 1D to 1F). This suggests that the increased hypothalamic p85α expression and PI3-kinase activity in SHR is not secondary to hypertension.

Expression of Δp85 Decreases NE Uptake Selectively in SHR Neurons

We studied the effect of decreased p85/p110 association in neuronal cultures to determine the effect of decreased p85α on NE neuromodulation. Transduction with Ad.Δp85 resulted in ∼10-fold increase in immunoreactive p85α protein and decreased Akt phosphorylation in WKY and SHR neurons compared with Ad.eGFP-infected controls (Figure 2B), establishing that Δp85 functioned as dominant-negative. [3H]NE uptake was 3-fold higher in Ad.eGFP-infected SHR than WKY, consistent with increased NE neurotransmission in SHR neurons.3 In addition, transduction with Ad.Δp85 decreased NET mRNA and [3H]NE uptake by 50% to 60% exclusively in SHR neurons (Figure 2C and 2D). These results indicate that p85 expression is critical for NE uptake in SHR, with decreased p85/p110 association resulting in decreased NE neuromodulation.

Discussion

The present study provides the first direct evidence for increased PI3-kinase expression and activity within presympathetic brain areas of the SHR. Our findings suggest that normalized PI3-kinase p85α/p110δ expression within the PVN might contribute to the overall effect of captopril, possibly via modulation of NE availability.

We demonstrate increased gene expression of p85α, p110α, and p110δ in the PVN of SHR compared with WKY. In contrast, only p110δ mRNA was increased in the SHR RVLM. Increased p85α/spliced variant-associated PI3-kinase activity was detected in SHR PVN, but not in the RVLM. Because wortmannin inhibition of the RVLM decreases BP exclusively in SHR,2 it would suggest that regulatory subunits other than p85α that associate with p110δ are involved. We detected increased p110δ-associated PI3-kinase activity in the RVLM of SHR. Thus, our study indicates that increased p85α/p110α and p85α/p110δ association in the PVN, and p110δ association with p85α/p55γ in the RVLM, likely account for the increased PI3-kinase activity in SHR. In addition, p85α expression and its associated activity were increased in neuronal cultures derived from prehypertensive SHR. Furthermore, the increase in p85α appears to precede the increases in p110 expression as only p85α expression was increased in SHR cultures. Moreover, the increases in PI3-kinase mRNA appear to be specific for presympathetic areas, because subunit expression was comparable between strains in the anterior hypothalamic area. Finally, a transient Ad.Δp85 gene transfer in the PVN resulted in sustained...
decreases in heart rate in SHR, but not WKY, in preliminary experiments. Whereas a decrease in BP was not evident, it is likely that long-term expression of Δp85 may be required to reflect changes in BP. Thus, these data provide compelling evidence in support of our hypothesis that p85α expression, at least in the PVN, contributes to hypertension in the SHR.

Enhanced angiotensinergic mechanisms in the RVLM and PVN contribute to hypertension. The enhanced tonic sympatho-excitatory activity of RVLM vasomotor neurons in SHR is largely mediated by Ang type I (AT₁) receptor activation. This is mainly attributable to an exaggerated tonic excitatory input to RVLM AT₁ receptors from the PVN. Augmented noradrenergic mechanisms also contribute to hypertension. In the SHR RVLM, increases in tyrosine hydroxylase, NET, and adrenergic α1A receptors parallel the development of hypertension. In addition, NE synthesis appears to be enhanced in the PVN of SHR. In cultured hypothalamus/brainstem SHR neurons, Ang II stimulation results in augmented NE synthesis and uptake via PI3-kinase signaling. Our findings of increased PI3-kinase subunit expression in the PVN and RVLM of SHR are consistent with increased NET expression in the SHR.

Figure 2. A, Representative Western blots of p85α, phosphorylated Akt (pAkt) and Akt in neurons expressing enhanced green fluorescent protein or Δp85. The p85α antibody also recognizes Δp85. B, Densitometry of pAkt normalized to Akt. C, NET mRNA. D, Maprotiline-sensitive [³H]NE uptake in WKY and SHR neurons infected with Ad.eGFP (white bars) or Ad.Δp85 (black bars). Values are mean±SE; n=3; experiments of pooled triplicate treatment/group. * P<0.05 vs SHR Ad.eGFP; #P<0.05 vs other groups. AU indicates arbitrary units (mRNA quantity).

In conclusion, this study indicates that increased PI3-kinase subunit expression in the PVN and RVLM is associated with hypertension in the SHR. Furthermore, the effect of captopril treatment may be mediated by normalization of p85α/p110β expression in the PVN and consequent NE neuromodulation.

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References
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Expanded Materials and Methods

Chemicals and antibodies were purchased from Sigma (St. Louis, MO) except where otherwise noted.

Animal Protocols

SHR and WKY rats (Harlan Sprague Dawley, Indianapolis, IN) were fed regular rat chow and allowed tap water ad libitum. A second group of SHR received an ACE inhibitor, captopril, in their drinking water (40 mg/L), from in utero to 6 months of age (SHR-CAP). Early, long-term ACE inhibition prevents the development of hypertension in SHR via alterations in central angiotensinergic mechanisms. Systolic BP was measured by tail-cuff plethysmography. The rats were euthanized at 6 months of age and 1 mm diameter, 500 µm thick punches of the PVN and RVLM were rapidly frozen in liquid nitrogen. Cardiac and coronary artery hypertrophy were evaluated by histological and morphometric analysis as previously described. All animal protocols complied with the guidelines of the Institutional Animal Care and Use Committees of the participating institutions.

Neuronal Cell Cultures and Gene Transfer

Primary neuronal cell cultures were derived from the hypothalamus and brainstem of newborn SHR and WKY rats (Charles River, Wilmington, MA) and established as previously described. These cultures consisted of 85-90% neurons and 10-15% glia. Purified high-titer stocks of adenoviral vectors that expresses a p85 dominant negative mutant (Ad.Δp85, kindly provided by Dr. David Murphy, University of Bristol, UK) or enhanced green fluorescent protein (Ad.eGFP, Clontech, Palo Alto, CA) were generated by double CsCl banding. Adenoviral titers were evaluated in HEK 293 cells by spot assays using an Adeno-X rapid titer kit (Clontech, Palo Alto, CA). Neuronal cultures were infected with either Ad.eGFP or Ad.Δp85 on day 5 of culture (MOI of 2) and used 48 hours after infection for in vitro assays.
**Real-Time RT-PCR**

Total RNA was isolated from micropunches or neuronal cultures using a RNaqueous-4-PCR kit (Ambion, Austin, TX). Two-step real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed according to the manufacturer's protocols on an ABI Prism 7000 HT Detection System (Applied Biosystems, Foster City, CA). Primers and probes were as follows: NET: 5’-TCACCGGACTGGCTGATGA-3’ (forward), 5’-CCGAGGGTGACACACATG-3’ (reverse) and 5’-6FAM-TCCAGGTCTGAAGCGACACAGA-MGBNFQ-3’ (probe); PI3-kinase catalytic α polypeptide (Pik3ca): 5’- TCGAGGTGGTGAGAAACTCTCA-3’ (forward), 5’-CCTTCAGGCCTCTTTTGCA-3’ (reverse), and 5’-6FAM-CCATCATGCAGATTC-MGBNFQ-3’ (probe); PI3-kinase catalytic δ polypeptide (Pik3cd): 5’-CAGTGTGGAGCAGTGCACCTT-3’ (forward), 5’-CTGCTGTACATGATCCACAATGG-3’ (reverse) and 5’-6FAM-ATGGACTCCAAAATGAA-MGBNFQ-3’ (probe). Assays-on-demand kits were used for PI3-kinase regulatory subunit, polypeptide 1 (Pik3r1) and PI3-kinase catalytic β polypeptide (Pik3cb), and ribosomal RNA (18S) was used as an endogenous control (all: Applied Biosystems, Foster City, CA). The Pik3r1 probe spans exon 6 to 7 and therefore detects p85α, but not its splice variants.

**Western Blot Analysis**

Neuronal cultures were solubilized in RIPA buffer containing protease inhibitors and centrifuged at 15,000g. Equivalent amounts of the supernatant were separated by SDS-PAGE and transferred to nitrocellulose membranes for western analysis using p85α (1:200; Upstate Biotechnology, Lake Placid, NY), p110α (1:250, BD Biosciences, San Diego, CA), p110β, p110δ (both: 1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA), phosphorylated Akt (Ser 473) and Akt (both: 1:1000, Cell Signaling Technology Inc., Beverly, MA) antibodies. The p85α antibody also detects Δp85 expression. PI3-kinase subunit densities were normalized to endogenous α-tubulin.
**PI3-kinase Activity Assay**

Neuronal cultures or micropunches were solubilized in PI3-kinase lysis buffer containing protease inhibitors and centrifuged at 15,000g. Equal protein amounts of the supernatant were incubated with either p85\(\alpha\) or p110\(\delta\) antibody and protein A agarose beads (Upstate Biotechnology, Lake Placid, NY). PI3-kinase activity was determined as previously described.\(^6\) The p85\(\alpha\) antibody recognizes an epitope common to p85\(\alpha\) and its spliced variants. Hence, PI3-kinase activity measurements of p85\(\alpha\) immunoprecipitates assessed the activity of p110\(\alpha\), \(\beta\) and \(\gamma\) catalytic subunits associated with p85\(\alpha\) or its spliced variants. Activity measurements of p110\(\delta\) immunoprecipitates assessed PI3-kinase activity of the p110\(\delta\) subunit associated with the regulatory subunits it dimerizes with (p85\(\alpha\), its spliced variants, p85\(\beta\) or p55\(\gamma\)).

**NE Uptake Assay**

Maprotiline-sensitive neuronal \(^{3}\text{H}\)NE uptake was determined as previously described.\(^7\) Incubations with \(^{3}\text{H}\)NE were at 37°C for 30 minutes.

**Experimental Groups and Data Analysis**

Eight-10 rats per group were used. For mRNA quantification, micropunches from 2 rats were pooled for each measurement. In vitro assays were performed in triplicate. RNA quantity was normalized to 18S ribosomal RNA and expressed relative to a calibrator sample using the comparative CT method (User Bulletin #2, Applied Biosystems, Foster City CA). Western blots and PI3-kinase activity autoradiograms were analyzed by densitometry (GS700, Biorad Laboratories, Hercules, CA). All results are expressed as mean ± SE. Data was analyzed by ANOVA followed by the Student Newman-Keuls correction for multiple pair-wise comparisons. Unpaired t-tests were used for comparisons between two groups. Values of \(P < 0.05\) were considered statistically significant.
Legends

Table 1: Effect of early, long-term captopril treatment on BP, cardiac and arterial hypertrophy in SHR. Values are Mean ± SE. n = 8-10 rats/group. HW/BW, heart weight/body weight ratio, * p < 0.05 versus other groups, # p < 0.05 versus SHR.

Figure 1: Effect of early, long-term captopril treatment on cardiac and perivascular fibrosis in SHR. A, B, and C, Syrius red stained left ventricular myocardial sections from WKY rats, SHR and SHR-CAP (captopril 40mg/L drinking water, in utero to 6 months), respectively. D, E and F, mid-myocardial sections showing small arteries from WKY rats, SHR and SHR-CAP, respectively. SHR exhibit multiple focal areas of cardiac fibrosis (B) and dense perivascular fibrosis (E) which was prevented by captopril treatment (C and F). Scale bars in C and F represent 1 mm in A, B, C and D, E, F respectively.

Figure 2: PI3-kinase subunit mRNA expression in the AHA. PI3-kinase subunit mRNA did not vary significantly between WKY (white bars) and SHR (black bars). Values are mean ± SE. n = 4-5/group, each sample consisted of micropunches from 2 rats. AU: Arbitrary units.

References


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<thead>
<tr>
<th>Group</th>
<th>Systolic BP (mmHg)</th>
<th>HW/BW Ratio</th>
<th>Interstitial Collagen (%)</th>
<th>Wall/Lumen Ratio</th>
<th>Perivascular Collagen (%)</th>
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<tr>
<td>WKY</td>
<td>112 ± 3</td>
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<td>4.26 ± 0.19 *</td>
<td>3.99 ± 0.13 *</td>
<td>0.67 ± 0.01 *</td>
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<td>SHR-CAP</td>
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<td>3.22 ± 0.09</td>
<td>1.76 ± 0.11 #</td>
<td>0.39 ± 0.02</td>
<td>0.23 ± 0.02 #</td>
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Figure 2, Online Supplement

![Bar chart showing protein levels with error bars.](image)