Original Article

Defective Phosphatidylinositol 3-Kinase Signaling in Central Control of Cardiovascular Effects in the Nucleus Tractus Solitarii of Spontaneously Hypertensive Rats

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Recently we have shown functional involvement of the phosphatidylinositol 3-kinase (PI3K)–Akt–nitric oxide synthase (NOS) signaling pathway in central control of cardiovascular effects in the nucleus tractus solitarii (NTS) of normotensive Wistar-Kyoto (WKY) rats. In this study we determined whether PI3K/Akt signaling was defective in spontaneously hypertensive rats (SHR). WKY rats and SHR were anesthetized with urethane. Mean blood pressure (MBP) and heart rate (HR) were monitored intra-arterially. Unilateral microinjection (60 nL) of insulin (100 IU/mL) into the NTS produced prominent depressor and bradycardic effects in 8- and 16-week-old normotensive WKY and 8-week-old SHR. However, no significant cardiovascular effects were found in 16-week-old SHR after insulin injection. Furthermore, pretreatment with PI3K inhibitor LY294002 and NOS inhibitor L-NAME into the NTS attenuated the cardiovascular response evoked by insulin in WKY and 8-week-old SHR but not in 16-week-old SHR. Unilateral microinjection of 1 mmol/L of PI(3,4,5)P_3 (phosphatidylinositol 3,4,5-triphosphate), a phospholipids second messenger produced by PI3K, into the NTS produced prominent depressor and bradycardic effects in 8- or 16-week-old WKY rats as well as 8-week-old SHR but not in 16-week-old SHR. Western blot analysis showed no significant increase in Akt phosphorylation in 8-week-old pre-hypertensive SHR after insulin injection. Similar results were also found in hypertensive 16-week-old SHR. Our results indicate that the Akt-independent signaling pathway is involved in NOS activation to regulate cardiovascular effects in the NTS of 8-week-old pre-hypertensive SHR. Both Akt-dependent and Akt-independent signaling pathways are defective in hypertensive 16-week-old SHR. (*Hypertens Res* 2008; 31: 1209–1218)

***Key Words*: phosphatidylinositol 3-kinase, insulin, central cardiovascular regulation, spontaneously hypertensive rat, nucleus tractus solitarii**

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Introduction

Recent evidence has indicated that insulin function in the central nervous system may play a role in the development and establishment of hypertension (*1*). This action is initiated by the interaction of insulin with its receptors localized in brain areas such as the nucleus tractus solitarii (NTS) (*2*) and hippocampus. Physiological mechanisms associated with insulin have been shown to be involved in the stimulation of the phosphatidylinositol 3-kinase (PI3K)–Akt–nitric oxide synthase (NOS) pathway in the NTS of Wistar-Kyoto (WKY) rats (*3*). However, the significance of the insulin-mediated system in the control of blood pressure (BP) is not well understood in spontaneously hypertensive rats (SHR).

SHR display essential hypertension as well as insulin resistance and are generally considered to be a suitable model for studying genetic hypertension and related disorders commonly coexisting in patients, when compared with their normotensive WKY controls (*4*). Progressive hypertensive status is detected in SHR starting from 2 to 3 weeks after birth, with the animals becoming hypertensive 12 to 14 weeks later (*5*). Obesity and impaired glucose tolerance have recently been linked to a defect in the enzyme fatty acid translocase (*6*). However, the cause of hypertension remains elusive, although a previous study suggested that defects in the activity of endothelial insulin might contribute to the disease (*7*). For example, endothelial nitric oxide synthase (eNOS) null mice exhibited insulin resistance that was shown to be associated with hypertension (*8*) and coronary artery disease (*9*).

It is well known that the binding of insulin with its receptors results in activation of the insulin receptor (IR) tyrosine kinase. The activated kinase then phosphorylates tyrosine residues of IR substrates (IRSs). IRSs are adaptor proteins that transduce signals from IR to downstream signaling cascades, including phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. Interestingly, there is increasing evidence that impaired IRS-mediated signal transduction is a major contributor to insulin resistance in SHR (*10*). In addition, several studies have shown that enhancing insulin sensitivity with insulin sensitizers may improve insulin resistance and limit the development of adverse cardiovascular consequences (*11*). These observations suggest that signaling defects at the IR or post-receptor levels can lead to insulin resistance and may be associated with cardiovascular diseases, including hypertension.

Nitric oxide (NO) has important modulatory functions in the NTS, including the modulation of arterial BP and sympathetic nerve activity (*12*). NO is produced from the conversion of L-arginine to L-citrulline by a family of enzymes known as NOS. A previous study has shown that a depressed L-arginine/NO pathway is the primary cause of hypertension in SHR (*13*). Our previous study has also shown that increased productions of NO may be attributable to the regulation of central cardiovascular effects in the NTS of WKY rats (*3*). The aim of the present study is to determine whether PI3K-Akt-NOS signaling in the NTS of SHR plays a functional role in the central control of cardiovascular effects. Our results showed the presence of an additional signal transduction pathway involved in insulin-mediated cardiovascular effects in the NTS of SHR that is NOS-dependent but independent of the protein kinase Akt.

Methods

This study was reviewed and approved by the Research Animal Facility Committee and was conducted according to the Guidelines for Animal Experiments of Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan.

Animals and Hemodynamic Measurements

Animal studies were performed on male WKY rats and SHR at 8 and 16 weeks of age that weighed 250 to 300 g. Rats were anesthetized with urethane (1.0 g/kg i.p. and 300 mg/kg i.v. if necessary). The preparation of animals for intra-NTS microinjection and the methods used in localization of the NTS have been described previously (*12*). Briefly, A polyethylene cannula was placed in the femoral vein for drug administration. BP was measured directly through a cannula placed in the femoral artery and connected to a pressure transducer (Gould P23 ID; Gould Instruments, Cleveland, USA) and polygraph (Gould RS3800; Gould Instruments). HR was monitored continuously by a tachograph preamplifier (Gould 13-4615-65; Gould Instruments). Tracheostomy was performed to maintain airway patency during the experiment.

For brain stem nuclei microinjection, the rats were placed in a stereotaxic instrument (Kopf, Tujunga, USA), with the head flexed downward at a 45° angle. The dorsal surface of the medulla was exposed by limited craniotomy, and the rats were rested for at least 1 h before experiments. Single-barrel glass cannulas were prepared (0.031-inch o.d., 0.006-inch i.d.; Richland Glass Co., Vineland, USA) that had external tip diameters of 40 μm. The cannula was connected to a Hamilton microsyringe by polyvinyl tubing. The cannulas were filled with L-glutamate (0.154 nmol/60 nL) to functionally identify the NTS. A specific decrease in BP and heart rate (HR) (≥ -35 mmHg and -50 bpm) was demonstrated after microinjection of L-glutamate into the NTS (*14*). After that, BP and HR were observed through microinjection of insulin or inhibitors.

Central Administration of Insulin and Drugs

To investigate the effect of preadministration of the PI3K inhibitor LY294002 on cardiovascular responses to insulin in the NTS, different groups of animals were first injected with insulin (100 IU/mL; Novo Nordisk, Bagsværd, Denmark) into the unilateral NTS. The rats were then allowed to rest for at least 30 min until the mean BP and HR had returned to

basal levels. After this, the changes in mean BP and HR were observed by microinjection of the same dose of insulin 10 min after intra-NTS administration of the PI3K inhibitor LY294002 (10 μmol/L; Sigma, MI, USA) or vehicle. The cardiovascular action of the same dose of insulin was observed after 10 to 90 min. Similar experimental procedures were used to study the effects of pretreatment with N^G-nitro-L-arginine methyl ester (L-NAME, 33 nmol/60 nL; Sigma) on insulin in the NTS. During the study, a negative control experiment was performed.

Western Blot Analysis

The NTS tissues were separated carefully from individual rats under microscopic examination. Briefly, tissues on both sides of the dorsomedial part of the medulla oblongata at the level of the NTS (1 mm rostral or caudal from the obex) were collected by micropunches made with a stainless steel bore (1 mm i.d.). The individual NTS tissues with or without insulin and LY294002 injections were immediately excised for protein extraction. Total protein was prepared by homogenized NTS in lysis buffer containing 20 mmol/L imidazole-HCl (pH 6.8), 100 mmol/L KCl, 2 mmol/L $MgCl_2$, 20 mmol/L EGTA (pH 7.0), 300 mmol/L sucrose, 1 mmol/L NaF, 1 mmol/L sodium vanadate, 1 mmol/L sodium molybdate, 0.2% Triton X-100, and proteinase inhibitor cocktail (Roche, Mannheim, Germany) for 1 h at 4°C. Proteins extracted (30 μg per sample assessed by BCA protein assay; Pierce, Rockford, USA) were subjected to 7.5% to 10% SDS-Tris glycine gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, Buckinghamshire, UK). The membrane was blocked with 5% nonfat milk in PBST buffer (10 mmol/L Tris [pH 7.5], 100 mmol/L NaCl, 0.1% Tween 20), incubated with anti-IR antibody (Santa Cruz Biotechnology, Santa Cruz, USA), anti–p-Akt^{S473} and Akt antibody (Cell Signaling Technology, Beverly, USA) at 1:1,000, respectively, or anti–β-actin antibody (Sigma Chemical Co., St. Louis, USA) at 1:1,000 in PBST with bovine serum albumin, and incubated for 1 h at room temperature. Peroxidase-conjugated anti-mouse or anti-rabbit antibody (1:5,000) was used as a secondary antibody. The membrane was developed with an ECL-Plus protein detection kit (Amersham).

Immunohistochemistry Analysis

Immunohistochemical staining was performed according to procedures described previously (*3*). In brief, the rat brain was fixed with 4% formaldehyde. Paraffin-embedded serial sections were cut at 5-μm thickness. The sections were deparaffinized, biotin-blocked (biotin-blocking system; DakoCytomation Ltd., Cambridgeshire, UK), microwaved (0.01 mol/L citric buffer, pH 6.4), quenched (3% H_2O_2/methanol), blocked (3% goat serum), and incubated in anti-IR antibody (1:100; Santa Cruz Biotechnology), or anti–phospho-tyrosine antibody (1:100; Upstate, Charlottesville, USA), at 4°C overnight. Next, the sections were incubated with biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, USA) for 1 h and AB complex (1:100) for 30 min at room temperature. The sections were visualized using diaminobenzidine substrate for 5 min (Vector Laboratories) and counterstained with hematoxylin. The sections were then photographed with an Olympus microscope equipped with a CCD imaging system (Olympus, Tokyo, Japan).

Statistical Analysis

A paired *t*-test (before and after pretreatments) or Dunnett's test was applied to compare group differences when significant effects were noted by 1-way ANOVA. Differences with a probability value $p<0.05$ were considered statistically significant. All data are expressed as the mean±SEM.

Results

Comparison of Insulin-Mediated Cardiovascular Effects in WKY and SHR

Unilateral microinjection of insulin (60 nL, 100 IU/mL) into the NTS produced prominent depressor and bradycardic effects in 8-week and 16-week-old WKY rats (Fig. 1A and C). These results were consistent with our previous report (*3*). For the 8-week-old prehypertensive SHR, insulin microinjection into the NTS caused prominent depressor and bradycardic effects similar to those in WKY rats (Fig. 1C). Interestingly, no significant cardiovascular effects were observed in 16-week-old hypertensive SHR after insulin injection (Fig. 1B and C). The basal MBP and HR were 76±3 mmHg and 315±18 bpm (8-week-old WKY), 81±3 mmHg and 311±10 bpm (16-week-old WKY), 90±4 mmHg and 306±10 bpm (8-week-old SHR), and 109±2 mmHg and 297±11 bpm (16-week-old SHR).

IR and Its Phosphorylation in WKY and SHR

In order to clarify whether the IR expression level is suppressed in 16-week-old SHR, the NTS tissues from individual SHR and WKY rats were dissected and analyzed by an immunoblotting assay using anti-IR antibody. Figure 2A shows that the IR protein levels in the NTS of 16-week-old SHR were similar to those in the age-matched WKY. Interestingly, the ratio of IR to actin (internal control) was not significantly different in SHR and WKY rats (Fig. 2A, right). Similar results were also observed in the 8-week-old pre-hypertensive SHR and age-matched WKY (data not shown). We further used immunohistochemistry analysis to examine the IR expression in the NTS of SHR and WKY. No differences in IR-positive cells between SHR and WKY were found (the SHR results are shown in Fig. 2B; WKY, data not shown). This result was consistent with the findings from immunoblotting analysis (Fig. 2A).

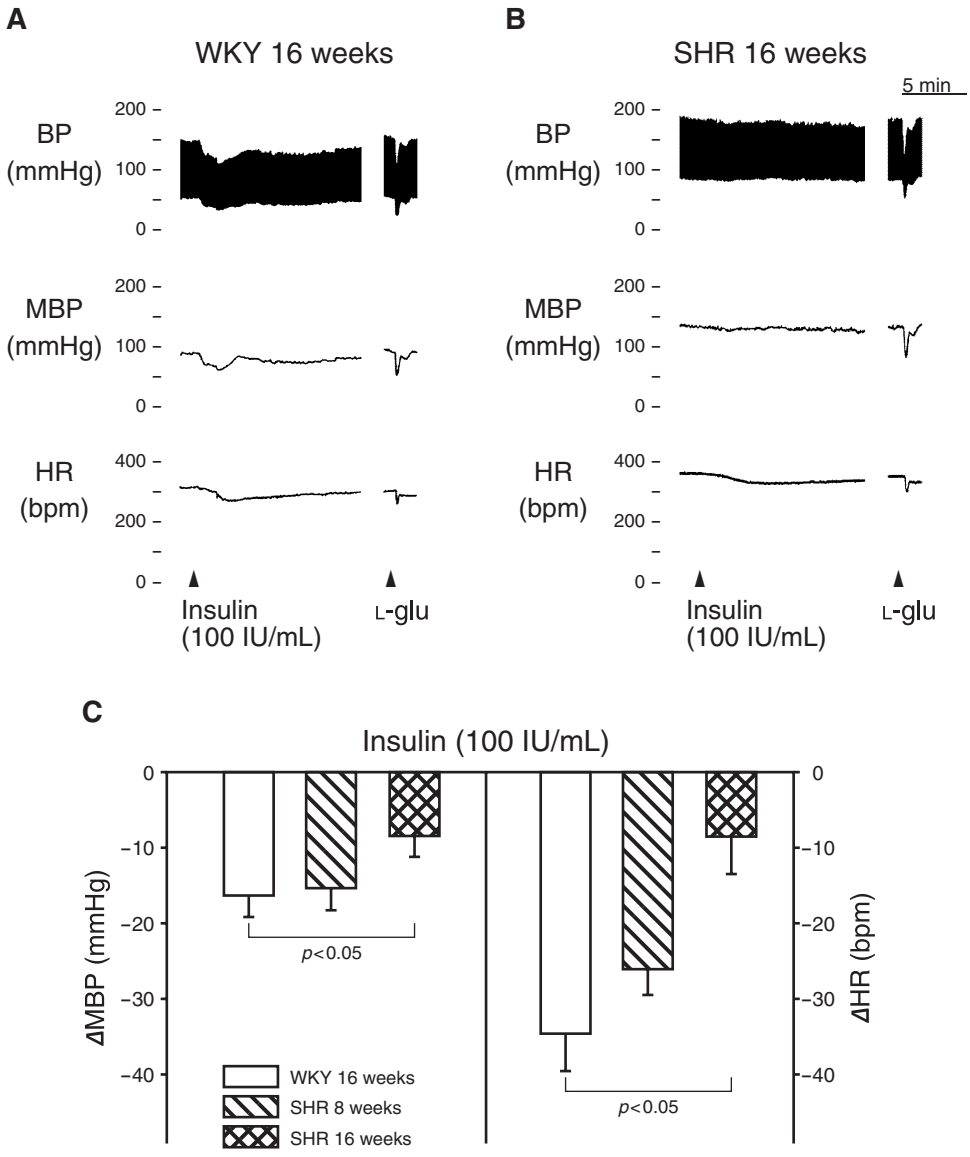

Fig. 1. *Cardiovascular effects of unilateral injection of insulin (100 IU/mL) into 16-week-old WKY (A) and SHR (B). Insulin was injected at the indicated time points. BP, blood pressure; MBP, mean blood pressure; HR, heart rate. HR was recorded at a paper speed of 3 mm/min. The horizontal bar represents recording during 5-min intervals. C: Effects of unilateral intra-NTS administration of insulin on MBP and HR. Vertical bars represent the SEM change from baseline values. Each bar represents the average data from 8 rats. $p < 0.05$, compared with the control value.*

We further investigated whether phospho-tyrosine immunoreactivity increases in the NTS after insulin microinjection. As shown in Fig. 2C, many neuronal-like cells in the NTS of SHR showed strong and specific phospho-tyrosine immunostaining on the cell membrane after insulin microinjection, whereas most of the cells in the control (without insulin microinjection) showed very weak or no immunostaining patterns. These two pieces of evidence suggested that neither the suppression of IR number nor a defect of IR activation caused the defect of the insulin-mediated cardiovascular effects in SHR.

Inhibitory Effects of LY294002 and L-NAME in Cardiovascular Regulation of SHR

We then investigated whether PI3K-Akt-NOS signaling was defective in the NTS of SHR. The specific PI3K inhibitor LY294002 was used to investigate the insulin-mediated cardiovascular effects in an insulin microinjection experiment. The results showed that pretreatment with LY294002 (10 μmol/L) attenuated insulin-induced cardiovascular effects in 8-week-old prehypertensive SHR (Fig. 3A). The cardiovascular responses to insulin were also attenuated by prior treat-

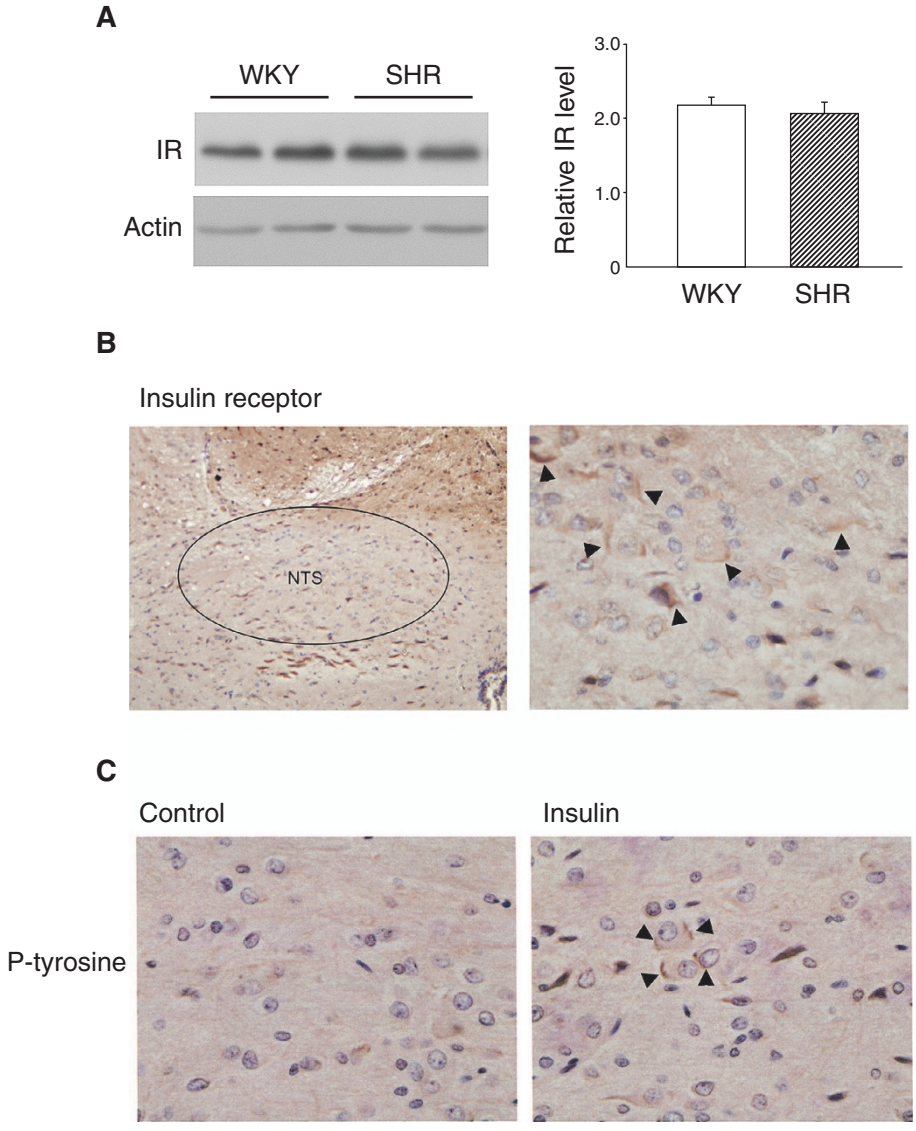

Fig. 2. *Insulin receptor protein expression and phosphorylation analysis in SHR. A: Western blot analysis of insulin receptor expression in WKY and SHR. Two representative cases are shown. Note that the insulin receptor expression was similar in SHR and WKY (n = 8). B: In-situ expression of insulin receptor in SHR by immunohistochemical analysis. Arrowheads show insulin receptor expression on the cell surface (400×, right). C: Phospho-tyrosine expression in the NTS of SHR after insulin injection by immunohistochemical analysis. Note the many phospho-tyrosine–positive cells (arrowheads) after insulin injection (400×, right) compared to the control (left).*

ment of LY294002 in both 8-week and 16-week-old WKY rats (Fig. 3A). However, no significant inhibitory effects were found in hypertensive 16-week-old SHR (Fig. 3A). In the control study, administration of Opti-MEM (the solvent of LY294002) in place of LY294002 did not modify the cardiovascular effects of insulin (data not shown).

In addition, the NOS inhibitor L-NAME was used to study the role of NOS in SHR. The results showed that the depressor and bradycardic responses to insulin were significantly attenuated in 8-week-old SHR after pretreatment with L-NAME (Fig. 3B). Similar results were also found in 8-week and 16-week-old WKY rats (Fig. 3B). Prior administration of L-NAME, however, did not attenuate the cardiovascular effects of insulin in 16-week-old hypertensive SHR (Fig. 3B). Taken together, these results suggested that the signaling pathway PI3K-Akt-NOS was defective in the insulin-mediated cardiovascular effects in the NTS of hypertensive SHR but not in the 8-week-old prehypertensive SHR or normotensive WKY rats.

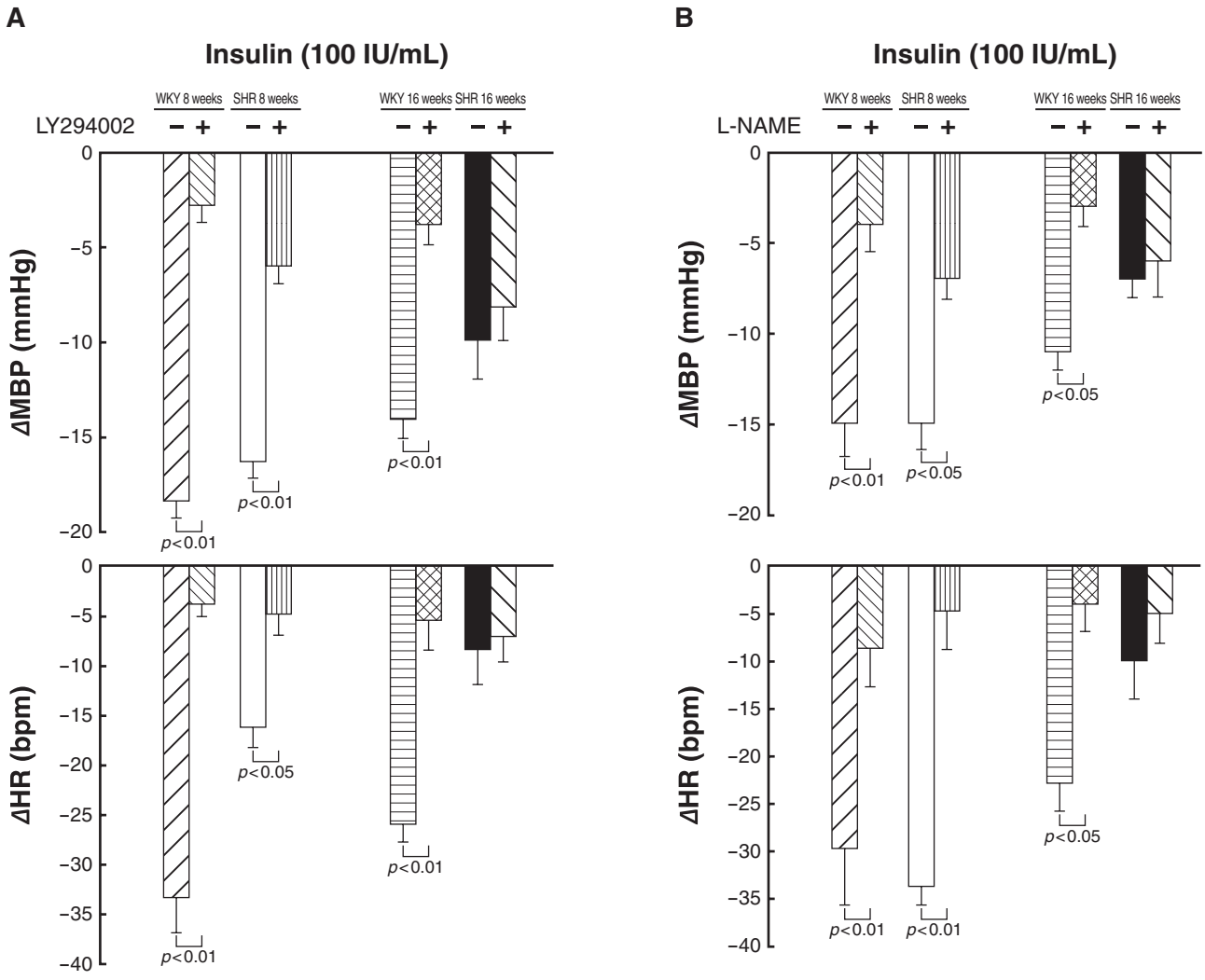

Fig. 3. *Comparative mean blood pressure (MBP) and heart rate (HR) effects of insulin (100 IU/mL) by the PI3K inhibitor LY294002, or NOS inhibitor L-NAME on unilateral intra-NTS administration of substances in WKY rats and SHR. Vertical bars represent the SEM change from baseline values. Each bar represents the average data from 8 rats. $p < 0.05$, compared with the control value.*

Involvement of PI(3,4,5)P_3 in Insulin-Mediated Cardiovascular Effects in the NTS of WKY Rats and Prehypertensive SHR

Synthetic phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P_3) was then used in the NTS microinjection experiment to test whether it elicited Akt and NOS signaling to produce cardiovascular effects in SHR. Unilateral microinjection of PI(3,4,5)P_3 (1 mmol/L) into the NTS produced prominent depressor and bradycardic effects in normotensive 8-week-old (not shown) or 16-week-old WKY rats (Fig. 4A and B). In contrast, weak depressor and minor bradycardic effects were shown when unilateral microinjection of PI(3,4,5)P_3 into the NTS in 8-week or 16-week-old SHR (Fig. 4A and B). Interestingly, there was a significant difference in the duration time of depressor effects of PI(3,4,5)P_3 between SHR and WKY rats (Fig. 4A). It is worth mentioning that administration of Opti-MEM (the solvent of PI(3,4,5)P_3) did not modify the cardiovascular response in the NTS. We then examined whether Akt phosphorylation levels were altered between WKY and SHR when PI(3,4,5)P_3 was microinjected into the NTS. Figure 4C shows that the phosphorylation level of Akt was elevated in normotensive WKY rats after PI(3,4,5)P_3 microinjection but not in SHR. These results further indicated that the defect of insulin-mediated cardiovascular effects in SHR was not due to the production of D-3 phosphoinositides by PI3K, but rather the D-3 phosphoinositide failed to act as a second messenger in the PI3K-signaling pathway in SHR.

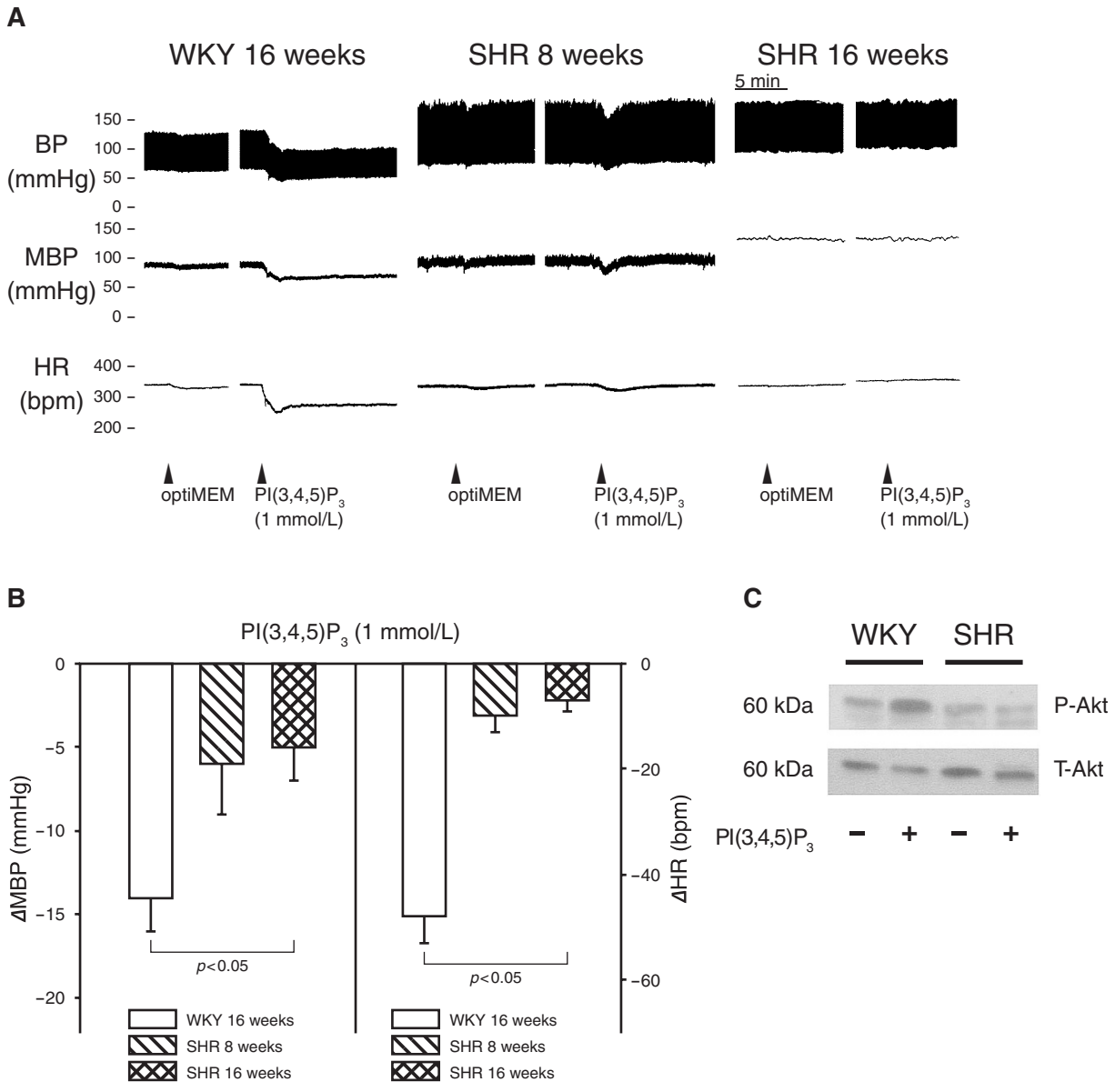

Fig. 4. *Cardiovascular effects and Akt phosphorylation of unilateral injection of PI(3,4,5)P_3 (1 mmol/L) into the NTS in 8-week-old SHR and 16-week-old WKY rats and SHR. A: BP, blood pressure; MBP, mean blood pressure; HR, heart rate. HR was recorded at a paper speed of 3 mm/min. The horizontal bar represents recording during 5-min intervals. B: Comparative MBP and HR effects of PI(3,4,5)P_3 on unilateral intra-NTS administration of substances in WKY rats and SHR. Vertical bars represent the SEM change from baseline values. Each bar represents the average data from 8 rats. $p < 0.05$, compared with the control value. C: Western blot analysis of Akt phosphorylation in NTS after PI(3,4,5)P_3 injections in WKY rats and SHR. Note the increased Akt phosphorylation in WKY rats after PI(3,4,5)P_3 injection (lane 2) but not in SHR (lane 4). T-Akt, total Akt; P-Akt, phosphorylated Akt.*

Induction of Akt Phosphorylation by Insulin Injection into the NTS of SHR

In order to clarify the role of the Akt in insulin-mediated depressor and bradycardic effects in SHR, Akt phosphorylation was determined by Western blot analysis in insulin microinjection experiments. Not surprisingly, there was no significant difference in Akt phosphorylation after insulin injection into the NTS of either 8-week-old (1.2±0.1-fold; Fig. 5A, lanes 7–9 and Fig. 5B) or 16-week-old SHR (1.1±0.2-fold; Fig. 5A, lanes 10–12 and Fig. 5B) when compared with that in WKY rats (Fig. 5A, lanes 1–6 and Fig. 5B).

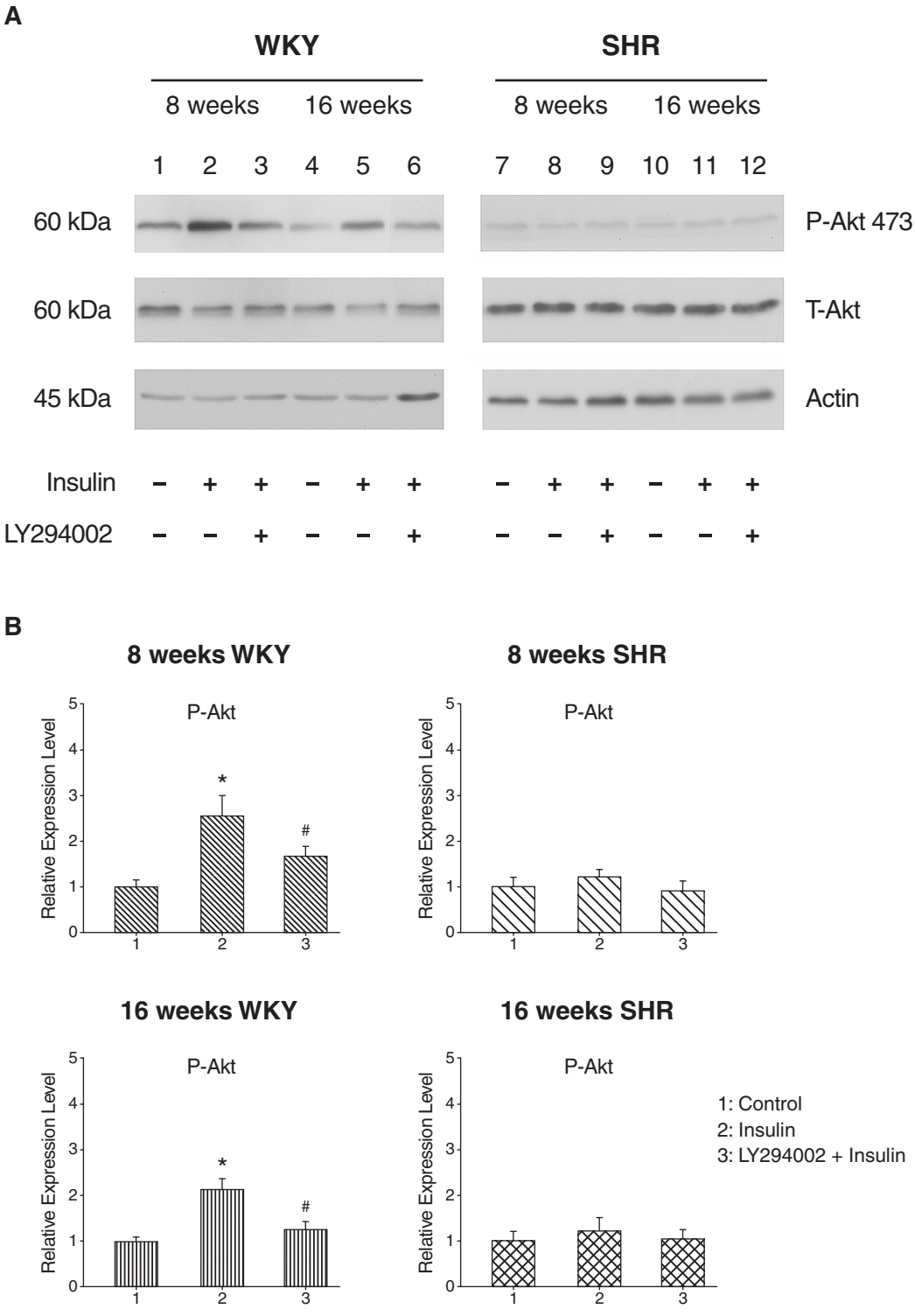

Fig. 5. *Western blot analysis of Akt phosphorylation in NTS after insulin and LY294002 injections in WKY rats and SHR. A: A Western blot depicting P-Akt protein in the NTS of 8-week-old and 16-week-old WKY rats and SHR after insulin microinjection (lane 2 vs. lane 5). Pretreatment with LY294002 after insulin-induced Akt phosphorylation in WKY rats and SHR (lane 3 vs. lane 6). B: Densitometric analysis of the P-Akt level before and after insulin and LY294002 injections in WKY rats and SHR. Bars are the mean ±SEM of 4 independent experiments. T-Akt, total Akt; P-Akt, phosphorylated Akt. *$p < 0.05$ vs. bar 1, $^{\#}p < 0.05$ vs. bar 2.*

These results were consistent with the data from $PI(3,4,5)P_3$ microinjection experiments.

Discussion

Our previous study demonstrated that insulin signaling plays an important role in regulating BP in the NTS (*3*). Recent reports have also shown that the impairment of insulin signaling in the brain has been linked to neurodegenerative diseases (*15*) and hypertension (*1*). For example, patients suffering from Alzheimer's and Parkinson's diseases exhibit reduced expression of IR in the brain (*16*). The course of genetic hypertension in SHR bears a resemblance to that of essential hypertension in humans. Thus, SHR have been the most extensively used animal model for genetic hypertension, increased stroke damage, and insulin resistance syndromes. These investigations have revealed several abnormalities of vasoregulatory factors, including the renin-angiotensin system, catecholamines, vasopressin, and vasoactive intestinal peptide in SHR (*17*).

Therefore, there are many possibilities concerning the insulin-resistant states in the insulin signaling pathway in SHR. For instance, recent studies have revealed a chromosomal deletion mutation in the SHR*Cd36* at the peak of linkage of these quantitative trait loci (QTLs) and concluded that *Cd36* deficiency gives rise to insulin resistance, defective fatty acid metabolism, and hypertriglyceridemia in this strain (*18*, *19*). Moreover, insulin-resistant IR substrate (IRS)-1 knockout mice are hypertensive (*20*). Some studies have also shown that reduced insulin signaling through the IRS-PI3K-Akt-eNOS pathway could contribute to the endothelial dysfunction of SHR (*21*). Furthermore, the Rho–Rho-kinase pathway in the NTS was suggested to contribute to BP regulation *via* the sympathetic nervous system *in vivo*, suggesting that activation of this pathway is involved in the central mechanisms of hypertension (*22*). The first genetic association with human essential hypertension involved the IR gene (*23*, *24*). However, our results showed that the defect of insulin-mediated cardiovascular effects in the NTS of SHR was due to neither the suppression of the IR number (Fig. 2A and B) nor the phosphorylation of the IR (Fig. 2C).

It is also notable that angiotensin II (Ang II) is reported to be involved in the development of both hypertension and insulin resistance. Recent evidence indicated that norepinephrine (NE) neuromodulation by Ang II was involved in the activation of the Ras-Raf-MAPK signal pathway in WKY rat brain neurons (*25*). However, this pathway is only partially responsible for this action of Ang II in SHR brain neurons. On the other hand, studies from several laboratories have shown that insulin produces non-uniform regional sympathetic nerve responses. Rahmouni *et al*. (*26*) indicated that PI3K and MAPK are major intracellular mediators in the regulation of sympathetic outflow by insulin.

Furthermore, microinjection of arginine, the substrate of NOS, into the NTS of either prehypertensive or hypertensive SHR induced effects very similar to those in WKY rats (Hsiao *et al*., unpublished results). These results indicated that NO production plays a cognitive role in regulating the cardiovascular function in the NTS of both SHR and WKY rats. The present study further suggested that the existence of a PI3K-dependent but not Akt-dependent signaling pathway in NTS may mediate the NO production and regulate the bradycardic effects in prehypertensive SHR. We believed that a progressive defect of this unknown mechanism together with the defect of the Akt-NOS signaling in SHR demonstrated in this study caused the prehypertensive SHR to develop hypertension. There are many other possibilities to cause the defect of NO regulation and hypertension in SHR during the development of hypertension. For example, the involvement of MAPK cannot be ruled out at the present time. A recent study showed that a MAPK-dependent insulin-signaling pathway was related to the production of NO in the vascular endothelium of mesenteric arteries from SHR (*7*). Thus, the MAPK-dependent insulin signaling pathway in NTS remains speculative and is currently under investigation.

The present study also showed that insulin injection failed to induce Akt phosphorylation in both 8-week and 16-week-old SHR. However, pretreatment with L-NAME or LY294002 significantly attenuated the bradycardiac and hypotensive responses to insulin injection into the NTS in 8-week-old SHR but not 16-week-old SHR. There are several possible explanations for these results. First, the failure of Akt phosphorylation in the SHR may have been the result of a time-dependent process in which the defect of Akt phosphorylation in the 8-week-old SHR was somewhere between that in WKY and 16-week-old SHR. Secondly, the results showed that the lack of a discrepancy in Akt phosphorylation between 8-week-old SHR and 16-week-old SHR may have been due to the sensitivity of the immunoblot experiment. Thirdly, pretreatment with the NOS inhibitor L-NAME can inhibit the bradycardiac and hypotensive responses to insulin in 16-week-old SHR but not in 8-week-old SHR, indicating that eNOS functions normally in 8-week-old SHR but not in 16-week-old SHR. Fourthly, MAPK signaling can be activated by PI3K, and our unpublished data show that MAPK signaling can modulate phosphorylation and activation of eNOS in NTS (Hsiao *et al*., unpublished results). Taken together, these results suggest that there may be phosphorylation systems other than Akt signaling that can modulate NO in the NTS.

In conclusion, our present study showed defective PI3K signaling in SHR. Our results support the idea that Akt downstream singling was intact; however, Akt upstream regulators such as IRS1–4, PDK-1, and other PI3K-related signaling pathways remain to be elucidated.

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