In Situ Akt Phosphorylation in the Nucleus Tractus Solitarii Is Involved in Central Control of Blood Pressure and Heart Rate

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Background—Previously, we have shown that nitric oxide (NO) plays a significant role in central cardiovascular regulation and modulates the baroreflex in the nucleus tractus solitarii (NTS) of rats. NO production is mediated by activation of NO synthase (NOS). Insulin signaling was involved in controlling peripheral blood pressure via the activation of endothelial NOS. Here, we investigated whether the insulin signal transduction pathway is involved in controlling central cardiovascular effects.

Methods and Results—Insulin was injected into NTS of urethane-anesthetized male Wistar-Kyoto (WKY) rats. Unilateral microinjection (60 nL) of insulin (100 IU/mL) into the NTS produced prominent depressor and bradycardic effects in 8- and 16-week-old WKY rats. In addition, pretreatment with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 and the NOS inhibitor L-NAME into the NTS caused attenuation of the cardiovascular response evoked by insulin in either 8- or 16-week-old WKY rats. Western blot analysis showed a significant increase (2.6 ±0.4-fold; P < 0.05) in Akt phosphorylation after insulin injection, whereas LY294002 abolished the insulin-induced effects. In situ Akt phosphorylation was found in NTS by immunohistochemistry analysis after injection of insulin. This in situ Akt phosphorylation was abolished significantly after injection of LY294002.

Conclusions—Take together, these results suggest that the insulin-PI3K-Akt-NOS signaling pathway may play a significant role in central cardiovascular regulation. (Circulation. 2004;110:2476-2483.)

Key Words: insulin enzymes nitric oxide synthase

Nitric oxide (NO) has been indicated to affect sympathetic nerve activity, and it modulates blood pressure and heart rate (HR) in the nucleus tractus solitarii (NTS). However, the mechanisms for these actions have not been clarified. NO is produced by NO synthase (NOS). Previously, adenovirus-mediated gene delivery of endothelial NOS (eNOS) into NTS caused hypotension and bradycardia. The results suggested that genes involved in the eNOS upstream signal transduction pathway might control central cardiovascular effects.

Studies have shown that insulin receptors are present in the central nervous system, which indicates the significant role of insulin in the central nervous system. Havrankova et al have demonstrated that insulin has an effect on cardiovascular function. Other reports also showed the effects of insulin on arterial pressure and HR after peripheral injection and suggested a function of insulin in central cardiovascular control. Recent evidence has demonstrated that the peripheral and central influence by insulin on cardiovascular regulation is due to an influence it exerts in the sympathetic nervous system. Moreover, insulin receptors are shown to be immunochemically positive in the NTS. These data suggested that insulin-mediated signaling in the NTS may play a significant role in the regulation of cardiovascular activity.

It then becomes important to study insulin-mediated signaling in the NTS to understand how insulin regulates cardiovascular function. Previous research has shown that insulin can affect vasodilator actions in vivo that depend on endothelium-derived NO. NO is produced from the conversion of L-arginine to L-citrulline by a family of enzymes known as NOS. Immunohistochemical studies have demonstrated the existence of NOS in the NTS. Microinjections of the NOS inhibitor Nω-monomethyl-L-arginine (L-NMMA) into the NTS increase both arterial pressure and renal sympathetic nerve activity in anesthetized animals. These results indicate that the NOS/NO system is involved in central cardiovascular regulation. Observations from previous studies have indicated that insulin can be considered a factor potentially involved in cardiovascular regulation not only through its influence in the sympathetic nervous system (baroreceptor reflex) but also through its effects on vascular tone. Nevertheless, the actions of insulin and its downstream regulatory genes are still unexplored in the NTS.
Furthermore, recent studies reported that the insulin receptor tyrosine kinase, phosphatidylinositol 3-kinase (PI3K), and Akt may play roles in insulin-stimulated production of NO in endothelial cells. Insulin induces activation of PI3K to generate 3-phosphoinositide lipids. These lipid second messengers, together with phosphoinositide-dependent kinase 1, can lead to activation of protein kinase Akt and other kinases. Activated Akt has been shown to regulate cell growth, proliferation, and survival by phosphorylating a subset of Akt-targeting proteins. Interestingly, insulin-stimulated production of NO is inhibited by wortmannin (a PI3K inhibitor) in human umbilical vein endothelial cells (HUVECs), which suggests that NO is one of the downstream modulators of signaling molecules in insulin-signaling pathways. We have recently demonstrated that NO can cause regulation of blood pressure in the central nervous system. These data strongly suggest that the insulin-PI3K-Akt-NOS signaling pathway in the NTS is involved in central cardiovascular regulation.

In the present study, we investigated the cardiovascular effects of insulin in the NTS of Wistar-Kyoto (WKY) rats of different ages. Our results showed that insulin and its downstream molecules, such as PI3K, Akt, and NOS, are involved in the regulation of central cardiovascular effects in the NTS of WKY rats.

**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.

Figure 1. Cardiovascular effects of unilateral injection of insulin (100 IU/mL) into NTS before and after LY294002 (10 μmol/L) injection into 8-week-old (A) and 16-week-old (B) WKY rats. C, Repeat injections of insulin (100 IU/mL) into NTS to show reproducibility of insulin effects in 8-week-old WKY rats. Insulin and LY294002 were injected at indicated time points. BP indicates blood pressure; MBP, mean blood pressure; HR, HR recorded at paper speed of 3 mm/min. Horizontal bar represents recording during 5-minute intervals.
Animals and Hemodynamic Measurements
Animal studies were performed on normotensive male WKY rats at 8 and 16 weeks of age that weighed 250 to 300 g. Rats were anesthetized with urethane (1.0 g/kg IP and 300 mg/kg IV if necessary). The preparation of animals for intra-NTS microinjection and the methods used in localization of the NTS have been described previously. A polyethylene cannula was inserted into the femoral vein for administration of drugs, and blood pressure was measured directly via a cannula placed into the femoral artery and connected to a pressure transducer (P23 ID) and a polygraph (ATS5000). HR was monitored continuously with a tachograph preamplifier (13-4615-65; Gould). Tracheostomy was performed to keep the airway patent during the experiment.

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) into the unilateral NTS. The rats were then allowed to rest for at least 30 minutes until the mean blood pressure and HR had returned to basal levels. After this, the changes in mean blood pressure and HR were observed by microinjection of the same dose of insulin 10 minutes after intra-NTS administration of the PI3K inhibitor LY294002 (10 μmol/L, Sigma) or vehicle. The cardiovascular action of the same dose of insulin was observed after 10 to 90 minutes.

Similar experiment procedures were used to study the effects of pretreatment with N\textsuperscript{-}nitro-L-arginine methyl ester (L-NAME, 33 mmol/60 nL; Sigma) and L-NMMA (33 mmol/60 nL; Sigma) on insulin in the NTS. During the study, a negative control experiment was performed.

Western Blot Analysis
NTS with or without insulin and LY294002 injections were immediately excised to extract protein. 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\textsubscript{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 protease inhibitor cocktail (Roche) for 1 hour at 4°C. Proteins extracted (30 μg per sample assessed by BCA protein assay, Pierce) were subjected to 7.5% to 10% SDS-Tris glycine gel electrophoresis and transferred to a nitrocellulose membrane (Amersham). The membrane was blocked with 5% nonfat milk in TBST buffer (10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20), incubated with anti-p-Akt or anti-Akt antibody (Cell Signaling Technology) at 1:500 or 1:2000, respectively, in TBST with bovine serum albumin, and incubated for 1 hour at room temperature. Peroxidase-conjugated anti-mouse or anti-rabbit antibody (1:5000) was used as secondary antibody. The membrane was developed with the ECL-Plus detection kit (Amersham).

Immunohistochemistry Analysis
Immunohistochemical staining was performed to determine whether Akt phosphorylation occurred in situ after insulin injections in the NTS of WKY rats. The rat brain was fixed with 4% formaldehyde. Paraffin-embedded serial sections were cut at 4-μm thickness. The sections were dewaxed, quenched in H\textsubscript{2}O\textsubscript{2}/methanol, microwaved in citric buffer (pH 6.0), blocked in 5% goat serum, and incubated in immunohistochemistry-specific anti-phospho-Akt antibody (1:50) at 4°C overnight. Afterward, sections were incubated in biotinylated secondary antibody (1:100) for 1 hour and in AB complex (1:50) for 30 minutes in room temperature. The sections were visualized with the DAB substrate kit (Vector Laboratories) and counterstained with hematoxylin. The sections were then photographed with an Olympus microscope equipped with a Nikon Cool Scan 995 digital camera.

Results
Cardiovascular Effects of Insulin in the NTS
To examine the plausible connection from insulin to its downstream molecules, PI3K, Akt, and NOS, in the NTS, we investigated the cardiovascular effects of insulin in the NTS of urethane-anesthetized male WKY rats. As expected, unilateral microinjection (60 nL) of insulin (100 IU/mL) into the NTS produced prominent depressor and bradycardic effects in 8-week-old WKY rats (−18±1 mm Hg and −33±4 bpm, respectively; Figures 1A and 2). Similarly, significant cardiovascular effects were found in 16-week-old WKY rats after insulin injection (−14±1 mm Hg and −26±2 bpm, respectively; n=8; Figures 1B and 2). Administration of Opti-MEM (the solvent of LY294002) as a sham control in place of LY294002 did not modify the cardiovascular effects of insulin (data not shown). Furthermore, the reproducibility...
of insulin-induced responses was also investigated, and the results showed that insulin produced depressor and bradycardic effects after repeated injection in the NTS (Figure 1C). Pharmacological doses of insulin were used throughout this study to ensure the proper downstream response of insulin could be achieved. Pretreatment with the vehicle of insulin did not attenuate the cardiovascular effects in the NTS (data not shown).

Inhibitory Effects of LY294002 and L-NAME in Cardiovascular Regulation

To test whether the D-3 phosphoinositides and NO were involved in the insulin-mediated signaling to regulate cardiovascular function, we used the PI3K inhibitor LY294002 and the NOS inhibitor L-NAME, respectively, to examine their effects on blood pressure and HR. Interestingly, pretreatment with LY294002 (10 μmol/L) for 10 minutes elicited slight cardiovascular effects; however, the depressor and bradycardic responses to insulin were attenuated significantly in 8-week-old WKY rats, as shown in Figure 2 (from 18±1 mm Hg and 33±4 bpm to 2±1 mm Hg and 2±2 bpm, respectively; P<0.05, paired t test; n=8). For 16-week-old WKY rats, pretreatment with LY294002 also attenuated the cardiovascular response to insulin (from 14±1 mm Hg and 26±2 bpm to 2±1 mm Hg and 2±2 bpm, respectively; P<0.05, paired t test; n=8; Figure 2).

Prior treatment with L-NAME (33 nmol) also significantly attenuated the cardiovascular effects of insulin in 8-week-old WKY rats (from 15±3 mm Hg and 30±6 bpm to 4±2 mm Hg and 9±4 bpm, respectively; P<0.05, paired t test; n=8; Figures 3A and 4). Similar results were found in 16-week-old WKY rats, in which pretreatment with L-NAME also attenuated the cardiovascular response to insulin (from 11±1 mm Hg and 23±3 bpm to 2±2 mm Hg and 4±3 bpm, respectively; P<0.05, paired t test; n=8; Figures 3B and 4). Moreover, pretreatment with L-NAME (33 nmol) did not attenuate the cardiovascular effects of (R,S)-3,5-dihydroxyphenylglycine (DHPG), a specific agonist for group I metabotropic glutamate receptors. Thus, NOS blockade did not affect glutamate or a group I metabotropic glutamate receptors.

In addition, we used another nonspecific NOS inhibitor, L-NMMA, to confirm the findings with L-NAME. Figure 5 demonstrates that prior administration of L-NMMA (33 nmol) also significantly attenuated the cardiovascular effects of insulin in 8-week-old WKY rats (from 13±2 mm Hg and 37±11 bpm to 3±3 mm Hg and 7±3 bpm, respectively; P<0.05, paired t test; n=8). The attenuated effects caused by both LY294002 and L-NAME on insulin-mediated cardiovascular response reached a plateau 10 minutes after the injection and lasted for at least 60 or 90 minutes (Figures 1 and 3). In comparison, prior administration of vehicle did not modify the effects of insulin on mean blood pressure and HR (data not shown).

Interestingly, insulin did elicit a prominent pressor and
tachycardic response after L-NAME injection (Figure 3A). However, we observed variations of such responses in all animals studied (n=8). Less prominent pressor and tachycardic responses of insulin were also found in other animals (data not shown). We attributed the discrepancy to individual differences in our animal model during the study. Further studies are required to clarify the nature of this insulin-induced pressor and tachycardic response after injection of L-NAME.

**Akt Phosphorylation Induced by Insulin Injection in the NTS**

Insulin has been shown to stimulate the protein kinase Akt via activation of PI3K. To elucidate the effects of insulin on Akt in NTS, Akt phosphorylation was determined by Western blot analysis with a phospho-specific Akt antibody that has been shown to correlate with enzyme activity. Western blot analysis (Figure 6A) revealed a significant increase (2.6±0.4-fold, P<0.05) in Akt phosphorylation after insulin injection into NTS compared with control (Figure 6B, lane 2 versus lane 1). The increase in Akt phosphorylation was not due to the increase in total Akt protein, because there was no significant difference in total Akt protein among the groups studied (Figure 6C). Furthermore, LY294002 significantly blocked the insulin-induced increase in Akt phosphorylation (Figure 6A, lane 3, and Figure 6B; n=6).

To determine whether Akt phosphorylation occurred in situ after insulin injection, paraffin sections of the NTS were subjected to immunohistochemical staining analysis with an immunohistochemistry-specific phospho-Akt antibody. Figure 6D showed strong Akt phosphorylation in NTS, whereas LY294002 abolished the in situ Akt phosphorylation.

**Discussion**

The present study provides evidence that microinjection of insulin into the NTS induced depressor and bradycardic effects in normotensive WKY rats. In addition, the results also suggest that activation of PI3K-Akt-NOS signaling is involved, at least in part, in the regulation of various neural and cardiovascular functions of WKY rats.

A number of studies have shown that the biological actions of insulin in vascular cells include increases in amino acid transport, glycogen synthesis, and induction of eNOS. Although insulin receptors are widely dispersed throughout tissues of the periphery, and their function is well known, the existence and function of insulin receptors within the brain are somewhat of an enigma. Many studies including the present one, however, have suggested that insulin/NO signaling may exist in the NTS and may play a significant role in regulating cardiovascular activity. First, the NTS plays a vital role in baroreceptor, chemoreceptor, and cardiopulmonary afferent-mediated regulation of cardiovascular function. Second, the effect of insulin on the baroreceptor reflex in NTS is probably mediated by a change in sympathetic nervous activity. Third, insulin receptors are shown to be immunochemically positive in the NTS. Fourth, insulin infusion was found to stimulate local vasodilation by enhancing the action of NO. Fifth, our previous data have suggested that NO is involved in central cardiovascular regulation, and the depressor effect of NO in the NTS might be through inhibition of renal sympathetic nerve activity. Sixth, a NOS inhibitor can cause attenuation of baroreflex activation in the NTS (Figure 4). These results suggest that the cardiovascular function of insulin might be mediated through the NOS signaling pathway in the NTS.

The finding in the present study demonstrate for the first time that the insulin-PI3K-Akt-NOS signaling pathway is linked to cardiovascular effects in the NTS. In endothelial cells, it has been reported that impairment of the IRS-1/PI3K/phosphoinositide-dependent kinase 1/Akt insulin-signaling cascade determines impaired insulin-stimulated NO release. The present data suggest, by Western blot and immunohistochemistry analysis (Figure 6), that insulin can induce Akt phosphorylation in the NTS. Importantly, insulin-induced Akt phosphorylation in the NTS was significantly reduced by pretreatment with LY294002, which strongly suggests specific suppression of Akt activ-
ity in vivo through PI3K inhibition (Figure 6). Similar results have been demonstrated in HUVECs, in which the insulin-mediated activation of Akt in HUVECs was PI3K dependent, as confirmed by use of the pharmacological PI3K inhibitors wortmannin and LY294002. Although the downstream signaling mediated by Akt phosphorylation in the NTS remains unclear, recent reports indicate eNOS activation by Akt phosphorylation, in part, plays a role in regulating cardiovascular function. Moreover, both pharmacological and in vivo gene transfer experiments support a major role for eNOS in the NTS in the regulation of baroreceptor reflex gain. In addition, overexpression of eNOS in the NTS causes a greater depressor response in SHR (spontaneously hypertensive rats), which suggests that an abnormality of the NO pathway in the NTS may be related to the hypertensive mechanisms of SHR. This was also supported by a study that demonstrated that insulin-stimulated production of NO in HUVECs is completely blocked by L-NAME, which is consistent with the possibility that eNOS may be acutely regulated through ligand-activated tyrosine kinase receptors, such as the insulin receptor and insulin-like growth factor-I receptor. On the basis of those findings, we question whether eNOS activation is needed in the insulin-signaling pathway to regulate cardiovascular effects in the NTS.

Other mechanisms may also exist and cannot be ruled out at the present time. For example, a recent study provides another plausible connection between the PI3K/protein kinase B pathway and WNK1, which may influence blood pressure. Furthermore, the increase in Akt phosphorylation induced by insulin may be mediated in part by activation of insulin receptor. Further studies are needed to elucidate the mechanism.

In conclusion, we present a novel PI3K-Akt-NOS signaling in the NTS that allows us to dissect the insulin-signaling pathway related to production of NO in the regulation of blood pressure and HR. The present data demonstrate that the insulin-signaling pathway might couple to the activation of NOS via liberation of NO to participate in central cardiovascular regulation. They also suggest a possible interaction between insulin and PI3K-Akt-NOS-NO–mediated signaling in the NTS of normotensive WKY rats.

**Acknowledgments**

This work was supported by the National Science Council (NSC91-2320-B-075B-003), Kaohsiung Veterans General Hospi-
tal (VGHKS92-48), and the Ministry of Education Academic Excellence Program (91-B-FA08-1-4) to Dr Tseng.

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Figure 6. Western blot and immunohistochemical analysis of Akt phosphorylation in NTS after insulin and LY294002 injections. A, Western blot depicts abundant p-Akt protein in NTS of WKY rat after insulin microinjection (lane 2). LY294002 blocks insulin-induced expression of p-Akt in WKY rats (lane 3). B and C, Densitometric analysis of p-Akt and Akt level before and after insulin and LY294002 injections. Bars are mean±SE of 4 experiments. *P<0.05 vs lane 1 and #P<0.05 vs lane 2. D, In situ Akt phosphorylation after insulin injection into NTS of WKY rats revealed by immunohistochemical staining. a, H&E staining showing NTS and injection sites. Arrowheads indicate sites of injections in NTS. b and c, p-Akt immunostaining in NTS with phospho-Akt specific antibody. b, Insulin injection only. c, Insulin and LY294002 injections. Arrowheads indicate cells with Akt phosphorylation after insulin injection. Note Akt phosphorylation was decreased after LY294002 injection. Scale bar represents 50 μm.


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*Circulation*. 2004;110:2476-2483; originally published online October 4, 2004;
doi: 10.1161/01.CIR.0000145116.75657.2D

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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