Chronic Hypertension Enhances the Postsynaptic Effect of Baclofen in the Nucleus Tractus Solitarius

Weirong Zhang, Myrna Herrera-Rosales, Steve Mifflin

Abstract—Microinjection of the inhibitory neurotransmitter γ-aminobutyric acid B-subtype receptor agonist baclofen into the nucleus tractus solitarius increases arterial blood pressure and sympathetic nerve discharge. The baclofen-induced pressor response is enhanced in chronic hypertension. We hypothesized that a postsynaptic mechanism contributes to the enhanced responses to baclofen in hypertension. We investigated the postsynaptic effect of baclofen on second-order baroreceptor neurons, identified by 1,1'-dilinoleyl-3,3',3'-tetra-methylindocarbocyanine, 4-chlorobenzenesulphonate labeling of the aortic nerve, in nucleus tractus solitarius slices from sham-operated normotensive and unilateral nephrectomized, renal-wrap hypertensive rats. After 4 weeks, arterial blood pressure was 153±7 mm Hg in hypertensive rats (n = 9) and 93±3 mm Hg in normotensive rats (n = 8; P < 0.05). There was no difference in resting membrane potential (54.5±0.7 versus 53.3±0.6 mV) or input resistance (1.07±0.11 versus 1.03±0.11 GΩ) between hypertensive and normotensive neurons (both n = 18). Baclofen induced a net outward current in nucleus tractus solitarius neurons in the presence of 1 μmol/L tetrodotoxin. The EC₅₀ of the baclofen effect was greater in normotensive cells (9.1±3.2 μmol/L; n = 5) than hypertensive cells (3.0±0.5 μmol/L; n = 7; P < 0.05), and baclofen (10 μmol/L) induced a greater decrease in input resistance in hypertensive cells (61±2%; n = 6) than in normotensive cells (45±4%; n = 9; P < 0.05). Both potassium and calcium channels were involved in the baclofen-evoked whole-cell current. The results suggest an enhanced postsynaptic response to activation of inhibitory neurotransmitter γ-aminobutyric acid B-subtype receptors in second-order baroreceptor neurons in the nucleus tractus solitarius in renal-wrap hypertensive rats. This enhanced inhibition could alter baroreflex function in chronic hypertension. (Hypertension. 2007;49[part 2]:659-663.)

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The nucleus tractus solitarius (NTS) is the first site of baroreceptor afferent integration within the central nervous system.1,2 The synaptic transmission of baroreceptor afferents within the NTS is constantly modulated by both excitatory and inhibitory inputs mediated by many neurotransmitters, including the inhibitory neurotransmitter γ-aminobutyric acid (GABA). Microinjection of baclofen, a selective GABA B-subtype (GABAB) receptor agonist, into the NTS results in an increase in arterial pressure, heart rate, and renal sympathetic nerve discharge,3–5 which are expected, because baclofen inhibits NTS neurons that integrate baroreceptor afferent inputs.6–8 This baclofen-induced pressor response is enhanced in several animal models of chronic hypertension, including the spontaneously hypertensive rat,9,10 deoxycorticosterone salt–hypertensive rats,11 and 1-kidney, renal wrap models of hypertension.6–8,12 Baclofen can presynaptically inhibit glutamate release from afferent terminals and postsynaptically induce outward current to reduce neuronal excitability in the NTS.13 However, it is not known to what extent postsynaptic GABAB receptor-mediated inhibition contributes to the enhanced baclofen-induced pressor response in chronic hypertension.14

Previous studies from this laboratory have demonstrated that renal-wrap hypertension is associated with increased GABAB receptor–mediated inhibition of baroreceptor-evoked discharge in NTS neurons8 and increased expression of GABAB receptor mRNA in the NTS.7 To clarify GABAB receptor–mediated cellular mechanisms in the neuronal adaptations to chronic hypertension, the present study investigated the postsynaptic effect of baclofen on NTS neurons receiving monosynaptic afferent inputs from baroreceptors and the influence of chronic hypertension on the postsynaptic response to baclofen. We addressed these questions using an in vitro patch-clamp method to directly investigate the postsynaptic effect of baclofen on second-order baroreceptor neurons in the NTS. The results demonstrated that, after chronic hypertension, second-order neurons showed enhanced postsynaptic responses to baclofen. This enhanced postsynaptic baclofen effect could contribute to the enhanced baclofen-induced pressor response observed in chronic hypertension.14

Methods
All of the experimental protocols in this work were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio.
Surgical Preparation for Labeling Aortic Nerve
Male Sprague-Dawley rats (100 to 125 g, Charles River, Wilmington, MA) were anesthetized with a combination of ketamine (75 mg/kg IP; Ft Dodge) and medetomidine (0.5 mg/kg IP; Pfizer). Under aseptic conditions, crystals of anterograde fluorescent dye 1,1'-dilinoleyl-3,3',3'-tetra-methylindocarbocyanine, 4-chlorobenzensulfonate ([DiA] D-3883, Molecular Probes) were gently applied unilaterally to the aortic nerve to visualize baroreceptor synaptic terminals and neurons receiving these synaptic contacts.15-17 The area was then embedded with silicone adhesive (Kwik-Sil, WPI). Anesthesia was terminated at tipanezole (1 mg/kg IP, Pfizer) at the conclusion of the surgical procedures. Postoperative analgesics (Nubaine, IM) were available as needed. The rats were allowed to recover for ≥1 week before performing renal wrap/sham surgery.

Chronic Hypertensive Model
Rats were anesthetized with the ketamine/medetomidine described above. Hypertension was induced using a figure-8 renal wrap of 1 kidney, and contralateral nephrectomy were performed.18 Control animals were sham-operated rats that only received a unilateral nephrectomy but no wrap of the contralateral kidney. Anesthesia was terminated by atipamezole, and postoperative analgesics (Nubaine, IM) were available as needed.

Four weeks after renal wrap or sham surgery, an arterial catheter was placed in the femoral artery while the animal was under ketamine/medetomidine anesthesia as described above. After a 2-day recovery period, the blood pressure of the conscious animal was measured by connecting the arterial catheter to a pressure transducer (Kobe) and displayed by means of a Cambridge Electronics Design A/D converter (CED). Blood pressure was measured for 3 hours, and measurements made during the last hour were used as an index of mean arterial pressure.

Brain Slice Preparation
After the day following the blood pressure measurement, the rat was anesthetized with isoflurane, and the brain stem was rapidly removed. The brain stem horizontal slices (250-µm thick) were cut with a sapphire knife (Delaware Diamond Knives) and mounted in a vibrating microtome (VT 1000E, Leica Microsystems). Then the slices were incubated for 95% O2/5% CO2 and perfused at a rate of 2 mL/min. All of the experiments were performed at room temperature.

Whole-cell recording experiments were performed in DiA-labeled second-order baroreceptor neurons in medial NTS. All of the experiments were performed in the presence of 1 µmol/L of tetrodotoxin (TTX) to block sodium channels and action potential-dependent neurotransmitter release. All of the drugs were dissolved in normal aCSF unless otherwise stated. For recordings of baclofen-induced currents, sequential bath application of baclofen was performed at concentrations of 0.3, 1, 3, 10, 30, and 100 µmol/L. Each concentration was applied for 2 min with an interval of ≥15 minutes, during which normal aCSF was applied. To investigate current–voltage relationships of the baclofen effect, a voltage step protocol was performed in control condition and after holding the current stabilized during application of 10 µmol/L of baclofen. Clamping membrane potentials were changed from −130 mV to −30 mV in 10-mV steps. The duration of each step was 500 ms, and voltage steps were applied every 2 s.

For recording calcium channel currents, barium was used as the current carrier. The bath solution contained (in mmol/L): 100 NaCl, 40 tetraethylammonium hydrochloride, 2.5 KCl, 5 BaCl2, 10 HEPES, and 10 glucose (pH adjusted to 7.3 with NOH). The electrode solution contained (in mmol/L): 140 CsCl, 1 MgCl2, 10 HEPES, 10 EGTA, 2 MgATP, and 0.3 Na3GTP (the pH was adjusted to 7.3 with CsOH). Barium currents were evoked every 5 s by a 200-ms voltage step from −60 mV to 40 mV from a holding potential of −80 mV.

Data Analysis
All of the data were presented as mean±SEM. For baclofen-induced outward currents, dose-response curves were fitted by the Hill equation: \( I = I_{\text{max}} \times \left[1 + \left(\frac{[EC_{50}]/[Ligand]\times P}{\text{n}_I}\right)^{n_H}\right] \), where \( I_{\text{max}} \) is the maximum response, \( EC_{50} \) is the concentration of ligand producing a half-maximal response, and \( n_I \) is the Hill coefficient. A t test was used to test differences between sham and hypertensive groups. Statistics were performed using SigmaStat (version 2.03, SPSS Software), and graphs were made with SigmaPlot (version 8.0, SPSS Software). Differences were considered statistically significant for \( P<0.05 \).

Results
Baclofen Effect in Hypertensive Rats
Four weeks after renal wrap or sham surgery, renal wrap hypertensive (HT) rats had significantly higher mean arterial pressure (153±7 mm Hg; \( n = 9 \)) than control normotensive (NT) rats (93±3 mm Hg; \( n = 9 \), \( P<0.05 \)). All of the in vitro whole-cell recording experiments were performed in medial NTS slices collected the day after blood pressure measurement, and second-order baroreceptor neurons were identified by the presence of DiA-labeled attached boutons (Figure 1A). Chronic hypertension did not significantly alter resting membrane characteristics of the second-order NTS neurons. There was no difference in resting membrane potential (54.5±0.7 mV versus 53.3±0.6 mV; both \( n = 18 \)) and input resistance (1.07±0.11 GΩ versus 1.03±0.11 GΩ; both \( n = 18 \)) of second-order neurons between HT and NT rats.

As reported previously,13 bath application of baclofen induced outward currents in second-order NTS neurons at a holding potential of −60 mV and in the presence of 1 µmol/L TTX, indicating a direct postsynaptic effect (Figure 1B). There was a dose-dependent increase in outward current with baclofen application on both NT and HT cells (Figure 1C). The EC50 of baclofen effect was significantly greater in NT
rats than in HT rats (9.1 ± 3.2 µmol/L, n = 5 versus 3.0 ± 0.5 µmol/L, n = 7; P < 0.05). During application of 10 µmol/L baclofen (Figure 1D), HT cells responded with a greater outward current than NT cells (32.6 ± 4.2 pA, n = 10 versus 16.3 ± 2.3 pA, n = 12; P < 0.01). This concentration of baclofen elicited a greater reduction in input resistance in HT cells than in NT cells (39 ± 2% of control, n = 6 versus 55 ± 4% of control, n = 9; P < 0.05).

Current–voltage relationships were investigated during application of 10 µmol/L of baclofen (Figure 2A). Application of 10 µmol/L of baclofen increased the current response to each voltage step. The baclofen equilibrium potential (E_b) was similar between HT and NT cells (Figure 2B; 77 ± 3 mV, n = 7 versus 74 ± 2 mV, n = 6; P > 0.05). There was a significant increase in slope conductance in all of the second-order neurons during the 10 µmol/L of baclofen application, as calculated from the slope of current–voltage curve between −130 mV and 40 mV. However, HT cells had greater increase in conductance than NT cells (254 ± 31% versus 184 ± 13%, n = 7; P < 0.05).

GABA_B Receptors Mediate Baclofen Effect
In 2 NT cells, application of 20 µmol/L of selective GABA_B receptor antagonist SCH 50911 did not induce discernible change on holding currents, indicating no tonic activation of postsynaptic GABA_B receptors in the brain slice from NT rats. To confirm the selective effect of baclofen on GABA_B receptors, coapplication of 10 µmol/L of baclofen with 20 µmol/L of SCH 50911 did not significantly alter holding current currents, suggesting that baclofen-evoked outward current was because of activation of GABA_B receptors. After washout, baclofen application induced outward currents of 13.9 ± 1.4 pA.

Ionic Mechanisms of Baclofen Effect
Under our experimental conditions, the calculated potassium equilibrium potential was −98 mV, which was more negative than E_b presented above (74 ± 2 mV; n = 6). Thus, a separate group of 16 control NT rats was used to identify the ionic mechanisms of the baclofen-induced current. Application of 1 mmol/L of barium, a nonselective potassium channel blocker, increased the current to approximately 40 pA in both HT and NT cells (Figure 2C). This increase was significantly greater in HT cells (204 ± 31% versus 132 ± 13% in NT cells; n = 10; P < 0.05).
blocker, induced a mild, apparently inward current of 6.9±2.1 pA (n=6) coupled with an increase in membrane resistance (136±9% of control; P<0.05). Coapplication of 10 μmol/L of baclofen with barium significantly reduced the evoked outward current in NTS neurons (8.0±1.2 pA versus 16.3±2.3 pA; P<0.05) and attenuated the baclofen-evoked reduction in input resistance (65±3% of control versus 55±4% of control; P<0.05). Furthermore, after application of 1 mmol/L barium, the \( E_b \) shifted to more positive potentials (−63±8 mV; n=5; Figure 3A) than the \( E_b \) under control condition (74±2 mV; n=6). These data suggest that the baclofen effect on second-order baroreceptor neurons in the NTS was largely mediated by potassium channels.

Baclofen has been reported to alter calcium currents.19–21 Therefore, we investigated the role of calcium channels in a baclofen-induced current. In the presence of 200 μmol/L of cadmium, a nonselective calcium channel blocker, the reversal potential of the baclofen-induced current shifted to the more negative value of −100±15 mV (n=5), that is, close to the calculated potassium equilibrium potential of −98 mV under our experimental conditions (Figure 3B). We also investigated baclofen-mediated inhibition of calcium channels by measuring the baclofen effect on barium currents in 7 NTS cells. Baclofen (10 μmol/L) was applied after stable barium currents were obtained. The voltage-activated barium currents were detectable at −40 mV and peaked at −10 mV. The inverted bell shape of the current–voltage curve was not changed by baclofen. We observed an inhibition of barium currents during baclofen application. The inhibition by 10 μmol/L of baclofen was 18.9±5.8% at 0 mV and 21.4±5.1% at −10 mV (all P<0.05; Figure 3C). Cadmium (200 μmol/L) abolished barium currents, confirming that the currents were carried through calcium channels.

**Discussion**

These results demonstrate that activation of postsynaptic GABA<sub>B</sub> receptors with baclofen induces outward currents in second-order baroreceptor neurons in the NTS, which will decrease neuronal excitability. This GABA<sub>B</sub> receptor–mediated inhibitory effect was enhanced after chronic hypertension. Both potassium and calcium channels were involved in the baclofen-evoked currents in the NTS. Enhanced postsynaptic GABA<sub>B</sub> receptor function could contribute to the enhanced baclofen-induced pressor response observed in vivo in various animal models of chronic hypertension. Our present study did not examine presynaptic inhibition by baclofen. This component of baclofen-mediated inhibition may or may not be altered in chronic hypertension.

An initial key component of the baroreflex arc is the synaptic integration of baroreceptor afferent inputs within the NTS. Baclofen decreases the action potential discharge of NTS neurons receiving baroreceptor afferent inputs6,8 and induces a pressor response.3 Activation of GABA<sub>B</sub> receptors can presynaptically inhibit excitatory neurotransmitter release and decrease postsynaptic neuronal excitability via activation of a potassium conductance,13 thus reducing the transmission of baroreceptor afferent inputs to other sites in baroreflex pathways.2 An enhanced baclofen-induced pressor response is a well-described phenomenon after chronic hypertension, suggesting an enhanced inhibitory effect of baclofen. However, it is difficult to achieve conclusive evidence on the role of presynaptic and/or postsynaptic mechanisms underlying the baclofen-induced pressor response and its alterations in hypertension using an in vivo approach. The current study used in vitro whole-cell recording methods to directly investigate the postsynaptic responses to baclofen after chronic hypertension. We observed that chronic hypertension significantly enhanced baclofen-induced postsynaptic outward currents. There is significantly lower EC<sub>50</sub> for baclofen-induced outward current comparing HT with NT cells, suggesting that chronic hypertension enhanced neuronal sensitivity to baclofen. The results of the current study are consistent with previous biochemical studies on NTS GABA<sub>B</sub> receptors. A previous study from our laboratory has found that in the NTS of renal-wrap HT rats there is increased expression of GABA<sub>B</sub>.
receptor mRNA. A similar study reported that baclofen binding in the NTS was increased in spontaneously hypertensive rats when compared with control rats. The enhanced baclofen effect could be mediated by an increased number of GABA<sub>B</sub> receptors and/or increased flow through individual channels on second-order neurons after chronic hypertension.

The changes that we report here are considered adaptations in response to a chronically elevated blood pressure. The exact stimulus, or stimuli, that induces an increase of postsynaptic response to activation of GABA<sub>B</sub> receptors is unknown. The stimulus could be increased excitatory baroreceptor afferent inputs to NTS neurons because of the elevated blood pressure. The stimulus could be systemic and/or local tissue-generated neurohormonal alterations in chronic hypertension (eg, angiotensin).

In the present study, the baclofen equilibrium potential was more positive than the potassium reversal potential. Our result is similar to the baclofen equilibrium potential of −73 mV reported in a previous study in medial NTS under a similar experimental condition. Postsynaptic baclofen effects are primarily mediated by activation of potassium channels. In addition, baclofen also inhibits voltage-dependent calcium channels. Our results confirmed these findings and showed that blocking voltage-dependent calcium channels shifted E<sub>K</sub> closer to potassium equilibrium potential, whereas blocking potassium channels shifted E<sub>K</sub> further away from potassium equilibrium potential, suggesting that both channels are involved in the baclofen effect on second-order baroreceptor neurons in the NTS. Future studies will be needed to explore the role of different types of calcium and potassium channels and their relative contributions to the enhanced baclofen-induced inhibition observed in chronic hypertension.

**Perspectives**

The baroreflex serves to minimize arterial blood pressure fluctuation under physiological conditions and remains functional in chronic hypertension. However, baroreflex regulation of sympathetic discharge is reset to higher pressures in chronic hypertension. It has been proposed that this resetting reflects a new balance between increased excitatory afferent inputs because of increased blood pressure and GABA-mediated inhibition. Such balance would maintain regulatory function of baroreflex during chronic hypertension. Enhanced GABA<sub>B</sub> receptor–mediated inhibition could be a crucial factor in baroreflex adaptation in chronic hypertension. The current study provides direct evidence of an enhanced postsynaptic baclofen effect on second-order baroreceptor neurons in the NTS. This enhanced postsynaptic GABA<sub>B</sub> receptor function could be crucial in modulating baroreflex function in chronic hypertension. Future studies will investigate the effect of chronic hypertension on presynaptic GABA<sub>B</sub> receptor function, including both excitatory and inhibitory inputs to second-order NTS neurons. Furthermore, the impact of chronic hypertension on different ion channels mediating postsynaptic baclofen effects will be investigated.
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