Chronic Hypertension Enhances Presynaptic Inhibition by Baclofen in the Nucleus of the Solitary Tract

Weirong Zhang, Steve Mifflin

Abstract—The selective γ-aminobutyric acid B-subtype receptor agonist baclofen activates both presynaptic and postsynaptic receptors in the brain. Microinjection of baclofen into the nucleus of the solitary tract increases arterial pressure, heart rate, and sympathetic nerve discharge consistent with inhibition of the arterial baroreflex. The magnitude of these responses is enhanced in hypertension and is associated with increased postsynaptic GABA\(_B\) receptor function. We tested whether a presynaptic mechanism contributes to the enhanced baclofen inhibition in hypertension. Whole-cell recordings of second-order baroreceptor neurons, identified by 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodide labeling of aortic nerve, were obtained in brainstem slices from normotensive control and renal-wrap hypertensive rats. After 4 weeks, arterial blood pressure was 162±9 mm Hg in hypertensive (n=6) and 107±3 mm Hg in control rats (n=6/11; P<0.001). Baclofen reduced the amplitude of excitatory postsynaptic currents evoked by solitary tract stimulation and the EC\(_{50}\) of this inhibition was greater in control (1.5±0.5 μmol/L; n=6) than in hypertensive cells (0.6±0.1 μmol/L; n=9; P<0.05). Baclofen (1 μmol/L) elicited greater inhibition on evoked response in hypertensive (58±6%; n=9) than in control cells (40±6%; n=8; P<0.05). Another index of presynaptic inhibition, the paired-pulse ratio (ratio of second to first evoked response amplitudes at stimulus intervals of 40 ms), was greater in hypertensive (0.60±0.08; n=8) than in control cells (0.48±0.06; n=5; P<0.05). The results suggest that in renal-wrap hypertensive rats, baclofen causes an enhanced presynaptic inhibition of glutamate release from baroreceptor afferent terminals to second-order neurons in the nucleus of the solitary tract. This enhanced presynaptic inhibition could contribute to altered baroreflex function in hypertension. (Hypertension. 2010;55[part 2]:481-486.)

Key Words: baroreceptor ■ baroreflex ■ blood pressure ■ cardiovascular regulation ■ hypertension

The nucleus of the solitary tract (NTS) is the first central integration site of arterial baroreceptor afferents.\(^1,2\) Baroreceptor afferent terminals release glutamate to activate second-order neurons in the NTS. This excitatory transmission is under dynamic modulation from various inhibitory neurotransmitters or neuromodulators, including the γ-aminobutyric acid (GABA) via both ionotropic GABA\(_A\) receptors and metabotropic GABA\(_B\) receptors.\(^3-5\)

GABA\(_B\) receptor-mediated modulation of excitatory baroreceptor inputs in the NTS has been demonstrated and this inhibition has presynaptic and postsynaptic components. Microinjection of selective GABA\(_B\) receptor agonist baclofen into the NTS results in an increase in arterial blood pressure, heart rate, and renal sympathetic nerve discharge.\(^6-8\) These baclofen-induced responses could be mediated by GABA\(_B\) receptor-mediated inhibition of presynaptic glutamate release or postsynaptic neuronal responses to glutamate in NTS neurons integrating baroreceptor afferent inputs.\(^5-9,11\) NTS microinjection of baclofen-induced cardiovascular responses are enhanced in several animal models of chronic hypertension, including spontaneously hypertensive rats,\(^12,13\) DOCA-salt hypertensive rats,\(^14\) and the 1-kidney, renal-wrap rat model of hypertension.\(^4-9,10,11,15\) However, the cellular mechanisms underlying the enhanced effects of baclofen in hypertension are still not well-understood.

Previous studies from this laboratory have demonstrated that 1-kidney, renal-wrap hypertension is associated with increased GABA\(_B\) receptor-mediated inhibition of baroreceptor-evoked discharge in NTS neurons\(^10\) and increased expression of GABA\(_B\) receptor mRNA in the NTS.\(^9\) These data support the concept that enhanced GABA\(_B\) receptor function may contribute to enhanced baclofen inhibition observed in chronic hypertension. We also demonstrated in a brain slice preparation from the same rat model of chronic arterial hypertension that the postsynaptic effect of baclofen (increased potassium conductance) was enhanced.\(^4\) However, it is not known whether presynaptic baclofen inhibition also contributes to the enhanced responses to baclofen observed in hypertension.

To further clarify GABA\(_B\) receptor-mediated cellular mechanisms in chronic hypertension, the present study investigated presynaptic inhibition by baclofen on synaptic transmission of baroreceptor afferents to NTS neurons using the

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same 1-kidney, renal-wrap hypertension rat model. An in vitro patch-clamp method was used to investigate the effect of baclofen on presynaptic release of glutamate to second-order baroreceptor neurons in the NTS. The results demonstrated that after chronic hypertension, there is enhanced baclofen-mediated presynaptic inhibition of glutamate release from baroreceptor afferents. This enhanced presynaptic baclofen inhibition could contribute to the enhanced baclofen-induced pressor response observed in chronic hypertension.9–11,15

Materials and Methods

All 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 and Renal-Wrap Chronic Hypertension Model

Male Sprague-Dawley rats (100 to 125 grams; Charles River, Wilmington, Mass) were anesthetized with a combination of ketamine (75 mg/kg intraperitoneal; Ft Dodge) and medetomidine (0.5 mg/kg intraperitoneal; Pfizer). Under aseptic conditions, crystals of anterograde fluorescent dye 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodide (DiA, D-3883; Molecular Probes) were gently applied unilaterally to the intact aortic nerve to visualize baroreceptor synaptic terminals and neurons receiving these synaptic contacts.3,4,16,17 The area was then embedded with silicone adhesive (Kwik-Sil; WPI).

Hypertension was induced using a 1-kidney, renal-wrap procedure. Immediately after these labeling procedures, a figure-8 renal wrap and contralateral nephrectomy were performed on these animals.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 (1 mg/kg intraperitoneal; Pfizer) at the conclusion of the surgical procedures. Postoperative analgesics (Nubain, intramuscular) 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. 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 Cambridge Electronics Design Axodigit (Cambridge Electronics Design). Blood pressure was measured for 3 hours in all hypertensive (HT) rats and in 6 of 11 normotensive (NT) rats, and measurements made during the last hour were used as an index of mean arterial pressure.

Brainstem Slice Preparation

The next day after blood pressure measurement, rats were anesthetized with isoflurane and the brain stem was rapidly removed and placed in ice-cold, high-sucrose, artificial cerebrospinal fluid that contained (in mmol/L): 125 NaCl, 1 MgCl2, 1 CaCl2, 2 MgSO4, 1.25 NaH2PO4, 26 NaHCO3, 10 glucose, and 206 sucrose, pH 7.4 when continuously bubbled with 95% O2/5% CO2, and superfused at a rate of ~2 mL/min. All images were captured with a charge-coupled device camera (IR-1000, charge-coupled device 100; Dage-MTI) and displayed on a television monitor. Patch pipettes were pulled from borosilicate glass capillaries with an inner filament (0.90 mm inner diameter, 1.2 mm outer diameter; WPI) and were filled with a solution of the following composition (in mmol/L): 125 CsCl, 1 MgCl2, 10 HEPES, 1.1 EGTA, 2 Mg2ATP, 0.3 Na3GTP, and 5 QX-314. The pH was adjusted to 7.3 with CsOH. The combination of CsCl and QX-314 in pipette solution reliably blocks the postsynaptic effect of baclofen, which is primarily an increase in potassium conductance.19–20 The pipette resistance ranged from 2 to 4 MΩ. A seal resistance of at least ≥1 MΩ and an access resistance <20 MΩ, which changed <15% during recording, were considered acceptable. Series resistance was optimally compensated. Cells were clamped at a membrane potential of ~60 nV. Recordings of postsynaptic currents began 5 minutes later, after whole-cell access was established and the current reached a steady state. Recordings were made with the AxoPatch 200B amplifier and pClamp software version 8 (Axon Instruments). Whole-cell currents were filtered at 2 kHz, digitized at 10 kHz with the DigiData 1200 interface (Axon Instruments), and stored in a PC computer for offline analysis. All experiments were performed at room temperature.

Whole-cell recording experiments were performed in DiA labeled second-order baroreceptor neurons in the NTS (Figure 1A, B). Evoked excitatory postsynaptic currents (eEPSC) were elicited by electric stimulation of the ipsilateral solitary tract using a concentric bipolar electrodes (FHC) with a tip diameter of 0.2 mm. Square electric pulses of 0.1 ms duration with a frequency of 0.2 Hz were delivered through a stimulus isolator A360 (WPI), in series with a programmable stimulator (Master8; AMPI). Recordings of eEPSC were performed in the presence of the GABA_A receptor antagonist picrotoxin (100 μmol/L). Bath application of drugs typically lasted ~3 to 5 minutes to achieve steady-state and begin drug effect tests. For establishing a dose–response curve of baclofen-induced inhibition of eEPSC, sequential bath application of baclofen was performed at concentrations of 0.03, 0.1, 0.3, 1, 3, and 10 μmol/L. Each concentration was applied for at least 3 minutes before tract stimulation. For paired-pulse stimulation, 2 synaptic responses (A1 and A2) were evoked by a pair of stimuli given at an interval ranging from 20 to 200 ms. Paired-pulse ratio (PPR) was calculated as the amplitude ratio of the second synaptic response to the first synaptic response (A2/A1).

Data Analysis

All data were presented as means±SEM. For baclofen-induced inhibition on eEPSC, dose–response curves were fitted by the Hill equation: I/I_{max} = 1/[1 + (EC_{50}/[ligand])^H], where I_{max} is the maximum response, EC_{50} is the concentration of ligand producing a half-maximal response, and H is the Hill coefficient. Differences in hypertensive effects were tested by an unpaired t test. For the comparison of hypertension or baclofen effect on PPR, a 2-way ANOVA (factors: hypertension and baclofen) was performed. For the comparison of PPR at different pulse intervals, a 1-way repeated-measure ANOVA was performed. Statistics were performed using SigmaStat (v2.03; SPSS software), and graphs were made with SigmaPlot (v8.0; SPSS software). Differences were considered statistically significant for P<0.05.

Results

Four weeks after renal-wrap/sham-operated procedures, renal-wrap HT rats had significantly higher mean arterial pressure (162±9 mm Hg; n=6) than control NT rats (107±3 mm Hg; n=6/11; P<0.05), indicating the successful results of renal-wrap surgery. This result is consistent with our previous studies with the same hypertension model.3,4,15,21–22
All in vitro whole-cell recording experiments were performed in second-order baroreceptor neurons in the NTS, identified by the presence of fluorescent dye DiA labeled boutons (Figure 1B). In our preparation of horizontal brainstem slices, fluorescent puncta were located medial to the solitary tract, usually in characteristic clusters. Clusters of dye puncta lay in close proximity to the soma membrane of individual neurons, usually forming a circle or near-circle, leaving the center of the soma void of labeling, and such positioned cells were considered to be anatomically identified second-order baroreceptive NTS neurons. Nonlabeled neurons might be second-order neurons contacted by nonbaroreceptor afferents; higher-order NTS neurons receiving baroreceptor afferent inputs or other afferent inputs; or neurons that do not receive any tractus input. Analysis of these types of neurons was beyond the scope of this study. Electric stimulation of ipsilateral tract was performed in voltage-clamp mode (Figure 1C). There is little variability of onset latencies of eEPSC (standard deviation of eEPSC onset latency in each cell ranging from 44 ± 187 µs with a mean variability of 121 ± 12 µs), further indicating a monosynaptic input from afferent terminals of baroreceptors.23

**Whole-Cell Recordings in Second-Order Baroreceptor Neurons in the NTS**

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**Baclofen Effect on eEPSC in the NTS**

Application of baclofen did not elicit a discernible change in holding current, indicating that our pipette solution successfully blocked postsynaptic effects of baclofen. We did not observe a difference in the amplitudes of eEPSC between NT and HT NTS neurons (211.0 ± 30.5 pA, n = 10 vs 172.5 ± 20.7 pA, n = 11; P > 0.05) and onset latency of eEPSC (4.9 ± 0.6 ms vs 4.2 ± 0.5 ms; P > 0.05). Baclofen inhibited eEPSC amplitude in both HT and NT NTS neurons (Figure 2A), but the
inhibition was significantly greater in HT neurons (155.4±20.1 pA–60.6±9.2 pA; 58±6% inhibition; n=9) than in NT neurons (226.9±29.1 pA–143.4±25.1 pA; 40±6% inhibition; n=8; P<0.05; Figure 2B). No significant effect of baclofen on onset latency was observed in both NT (4.5±0.7 ms vs 4.4±0.7 ms; P>0.05) and HT cells (4.8±0.5 ms vs 5.1±0.4 ms; P>0.05). Baclofen effects were blocked by the selective GABA\textsubscript{B} receptor antagonist CGP 35348 (200 μmol/L; n=3; data not shown), confirming that baclofen was acting on GABA\textsubscript{B} receptors. Dose–response curves revealed that the EC\textsubscript{50} of baclofen inhibition of eEPSC amplitude, calculated from the mean of the EC\textsubscript{50} of each single neuron exposed to whole-dose range of baclofen, was significantly smaller in HT neurons than in NT neurons (0.6±0.1 μmol/L, n=9 vs 1.5±0.5 μmol/L, n=6; P<0.05; Figure 2C).

**Baclofen Effect on Paired-Pulse Tract Stimulation eEPSC**

A paired-pulse stimulation protocol was used as one of the means to identify potential neural plasticity in synaptic transmission mediated by presynaptic mechanisms.\textsuperscript{24} Differences in PPR suggest alterations in the probability of release of available transmitter or the size of a release-ready pool of vesicles. At pulse intervals ranging from 20 to 200 ms, the amplitude of second eEPSC was consistently smaller than that of first one; therefore, the PPR value was <1 (Figure 3A, B). PPR of <1 were observed in all neurons tested. The PPR was attenuated as the paired-pulse interval was increased. PPR at a paired-pulse interval of 20 ms was significantly smaller than PPR at all other pulse intervals tested (P<0.001 in control and baclofen groups). When paired-pulse stimuli were applied in the presence of baclofen, the second eEPSC was minimally reduced by baclofen, although the first eEPSC was greatly attenuated, leading to increased PPR (Figure 3A). The baclofen effect on PPR was observed between pulse intervals from 20 to 200 ms (Figure 3B). At a paired-pulse interval of 40 ms, the PPR was significantly greater in HT neurons (0.60±0.08; n=8) than in NT neurons (0.48±0.06; n=5; P<0.05). Baclofen increased the PPR in both types of neurons; however, the baclofen effect was significantly greater in HT neurons (0.92±0.07; 172±22% increase; n=8) than in NT neurons (0.67±0.05; 148±16% increase; n=5; P<0.05; Figure 3C).

**Discussion**

These results demonstrate that activation of presynaptic GABA\textsubscript{B} receptors with baclofen inhibits glutamate release from baroreceptor afferent terminals to second-order baroreceptor neurons in the NTS and GABA\textsubscript{B} receptor-mediated presynaptic inhibition was enhanced in chronic hypertension. Considered along with our previous report of enhanced postsynaptic GABA\textsubscript{B} receptor function,\textsuperscript{4} enhanced presynaptic GABA\textsubscript{B} receptor function could contribute to the enhanced blood pressure and sympathetic responses to NTS microinjections of baclofen observed in various animal models of chronic hypertension. Our present study provides strong evidence that chronic arterial hypertension induces profound changes in the function of presynaptic and postsynaptic GABA\textsubscript{B} receptors.

The NTS is the first central integration site of arterial baroreceptor afferents.\textsuperscript{1,2} Glutamate is the primary neurotransmitter relaying information from baroreceptor afferent terminals
to second-order neurons within the NTS. This robust glutamatergic transmission is under constant modulation by various receptors and ion channels, including GABA receptors. Baclofen activates GABA receptors and inhibits NTS neurons receiving baroreceptor afferent inputs, which will blunt the baroreflex and induce a pressor response. GABA inhibition is tonically active because injections of GABA receptor antagonists into the NTS lower blood pressure and heart rate. Activation of GABA receptors can have dual effects to presynaptically inhibit excitatory neurotransmitter release and decrease postsynaptic neuronal excitability in the NTS, thus reducing the transmission of the integrated afferent input to other sites in central baroreflex pathways.

Enhanced arterial blood pressure, heart rate, and sympathetic nerve discharge induced by NTS injections of baclofen have been described in several animal models of chronic hypertension. Using in vitro whole-cell recordings, we previously demonstrated that chronic hypertension significantly enhanced baclofen-induced postsynaptic outward currents, as significantly lower EC50 for baclofen effect in HT than in NT cells. This is consistent with previous studies showing that chronic hypertension increases mRNA expression of GABA receptors and baclofen binding in the NTS. The current study extends these previous findings and demonstrates that hypertension also enhances baclofen inhibition of glutamate release from baroreceptor afferent terminals. We examined presynaptic inhibition using 2 complementary analyses: the amplitudes of eEPSC and PPR. Both approaches indicate that baclofen inhibition of glutamate release from baroreceptor afferent terminals is enhanced in hypertension. Insights into presynaptic transmitter release can also be obtained by analysis of spontaneous and miniature EPSC. The frequency of spontaneous and miniature EPSC represents the glutamate release probability from presynaptic terminals. However, our focus was on modulation of transmitter release from baroreceptor primary afferent fibers, and analyses of spontaneous and miniature EPSC cannot differentiate EPSC that originate from peripheral sources as opposed to central sources.

The mechanisms underlying the development of hypertension-enhanced function of GABA receptors in the NTS are unknown. Recent studies suggest that the availability of surface GABA receptor could be modulated by glutamate in central neurons, suggesting the alteration in GABA receptor could be the direct result of high blood pressure-induced increase in afferent inputs. In addition, elevated circulating angiotensin during hypertension may cause enhanced function of GABA receptor in the NTS. Obviously, these potential mechanisms will need further investigation.

The EC50 of presynaptic baclofen inhibition is noticeably smaller than the postsynaptic baclofen inhibition described in our previous study. This is consistent with an earlier in vitro current-clamp study that found the EC50 of presynaptic inhibition was an order of magnitude lower than that of postsynaptic inhibition. Although it is difficult to directly compare these 2 values, it is suggested that depending on GABA concentrations within the synaptic cleft and the precise location of GABAergic terminals, GABA receptors could preferentially mediate presynaptic inhibition. Enhanced presynaptic inhibition of glutamate release in hypertension could reduce information flow within central pathways of the arterial baroreflex. However, it is possible that at least some of the second-order baroreceptor neurons reported in this study are GABAergic neurons. Therefore, reduced excitation of GABAergic neurons could also determine the ultimate physiological significance of enhanced GABA receptor inhibition in hypertension.

Perspectives
The baroreflex serves to stabilize arterial blood pressure fluctuations under physiological normotensive conditions and in chronic hypertension. However, in chronic hypertension, baroreflex function is reset to higher levels of blood pressures, representing a new balance between increased excitatory afferent inputs attributable to increased blood pressure and GABA-mediated inhibition. Enhanced GABA receptor-mediated inhibition could be critical in baroreflex adaptation in chronic hypertension. Along with our previous study, we provided direct evidence of enhanced baclofen effect at presynaptic and postsynaptic sites of second-order baroreceptor neurons in the NTS. Altered function of GABA receptors could be crucial in determining baroreflex function in chronic hypertension.

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Disclosures
None.

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