Chronic AT₁ receptor blockade normalizes NMDA-mediated changes in renal sympathetic nerve activity and NR₁ expression within the PVN in rats with heart failure

Allison C. Kleiber, Hong Zheng, Neeru M. Sharma, and Kaushik P. Patel

Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, Nebraska

Submitted 23 October 2009; accepted in final form 17 February 2010

Kleiber AC, Zheng H, Sharma NM, Patel KP. Chronic AT₁ receptor blockade normalizes NMDA-mediated changes in renal sympathetic nerve activity and NR₁ expression within the PVN in rats with heart failure. Am J Physiol Heart Circ Physiol 298: H1546–H1555, 2010. First published February 19, 2010; doi:10.1152/ajpheart.01006.2009.—Exercise training normalizes enhanced glutamatergic mechanisms within the paraventricular nucleus (PVN) concomitant with the normalization of increased plasma ANG II levels in rats with heart failure (HF). We tested whether ANG II type 1 (AT₁) receptors are involved in the normalization of PVN glutamatergic mechanisms using chronic AT₁ receptor blockade with losartan (Los; 50 mg·kg⁻¹·day⁻¹ in drinking water for 3 wk). Left ventricular end-diastolic pressure was increased in both HF + vehicle (Veh) and HF + Los groups compared with sham-operated animals (Sham group), although it was significantly attenuated in the HF + Los group compared with the HF + Veh group. The effect of Los on cardiac function was similar to exercise training. At the highest dose of N-methyl-D-aspartate (NMDA; 200 pmol) injected into the PVN, the increase in renal sympathetic nerve activity was 93 ± 13% in the HF + Veh group, which was significantly higher (P < 0.05) than the increase in the Sham + Veh (45 ± 2%) and HF + Los (47 ± 2%) groups. Relative NMDA receptor subunit NR₁ mRNA expression within the PVN was increased 120% in the HF + Veh group compared with the Sham + Veh group (P < 0.05) but was significantly attenuated in the HF + Los group compared with the HF + Veh group (P < 0.05). NR₁ protein expression increased 87% in the HF + Veh group compared with the Sham + Veh group but was significantly attenuated in the HF + Los group compared with the HF + Veh group (P < 0.05). Furthermore, in vitro experiments using neuronal NG-108 cells, we found that ANG II treatment stimulated NR₁ protein expression and that Los significantly ameliorated the NR₁ expression induced by ANG II. These data are consistent with our hypothesis that chronic AT₁ receptor blockade normalizes glutamatergic mechanisms within the PVN in rats with HF.

In the present study, we hypothesized that chronic ANG II type 1 (AT₁) receptor blockade with losartan (Los) in rats with HF would normalize PVN NMDA-mediated RSNA responses as well as the increased NR₁ expression within the PVN. A portion of this data has been presented in abstract form (14).

METHODS

**Animals.** Male Sprague-Dawley rats weighing 220–280 g (Sasco Breeding Laboratories, Omaha, NE) were fed and housed according to institutional guidelines. Protocols were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). Rats were given rat chow and water ad libitum and were housed in a room with a 12:12-h light-dark cycle. Rats were allowed to acclimate for 1 wk before cardiac surgery.

**Induction of HF.** Rats were randomly assigned to either the sham-operated control (Sham) group or the HF group. HF was induced by ligation of the left coronary artery, which has been previously described (24, 26, 43). Left ventricular (LV) dysfunction was assessed using hemodynamic and anatomic criteria. After 3 wk of Los administration, echocardiograms were performed to measure LV end-sys-
tolic diameter (LVESD) and LV end-diastolic diameter (LVEDD); ejection fraction (EF) and LV end-diastolic pressures (LVEDP) were also measured. LVEDP was measured using a Mikro-Tip catheter (Millar Instruments, Houston, TX) inserted into the LV via the right carotid artery. To measure infarct size, the heart was dissected free of adjacent tissues, and the atria were removed. The right ventricle was opened with a lengthwise incision such that the heart was flattened with the LV lying in the middle with the right ventricle on either side of it. The right ventricle was removed, and the remaining LV was laid flat. A digital image of the LV was captured using a Kodak DC290 digital camera (Kodak, Rochester, NY), and the infarcted area and total LV area were quantified using SigmaScan Pro. Infarct size (in %) was determined by dividing the size of the infarcted area by the total size of the LV. Rats with elevated LVEDP (after the rat had been euthanized) and was determined by nerve activity recorded at the end of the experiment (after the rat had been euthanized). Nerve activity during the experiment was determined by nerve activity recorded at the end of the experiment (after the rat had been euthanized). The left kidney was exposed through a retroperitoneal flank incision. A branch of the renal nerve was isolated from fat and connective tissue. The central end of the nerve was placed on thin bipolar platinum electrodes. The nerve-electrode junction was fixed and electrically insulated from surrounding tissues with a Wacker Silgel platinum electrodes. The nerve-electrode junction was fixed and electrically insulated from surrounding tissues with a Wacker Silgel electrically insulated from surrounding tissues with a Wacker Silgel electrically insulated from surrounding tissues with a Wacker Silgel electrically insulated from surrounding tissues with a Wacker Silgel electrically insulated from surrounding tissues with a Wacker Silgel electrically insulated from surrounding tissues with a Wacker Silgel electrically insulated from surrounding tissues with a Wacker Silgel
**RESULTS**

**General data.** Hemodynamic and LV pressure data as well as morphological characteristics before the acute experiments are shown in Table 1. Data represent mean values from animals used for RSNA experiments in addition to animals in which NR1 mRNA and protein from the PVN were measured. Only rats with ≥30% infarct of the LV wall were included in the analysis for the HF groups. Hearts from rats in the Sham groups had no visible myocardial damage. LVEDSD and LVEDDD were significantly increased in the HF + Veh group compared with the Sham + Veh group. Los treatment in the HF + Los group had no effect on LVEDSD and attenuated LVEDDD compared with the HF + Veh group, although LVEDDD was still significantly elevated compared with both Sham groups. EF was decreased in the HF + Veh (50 ± 4%) and HF + Los (53 ± 14%) groups compared with the Sham + Veh (81 ± 2%) and Sham + Los (80 ± 8%) groups. LVEDP was significantly increased in the HF + Veh group (25.1 ± 2.0 mmHg) compared with the Sham + Veh group (4.7 ± 0.3 mmHg). LVEDP in the HF + Los group (14.4 ± 1.9 mmHg) was significantly attenuated compared with the HF + Veh group but was still significantly higher than that in the Sham + Veh and Sham + Los (6.9 ± 1.6 mmHg) groups. dP/dt was significantly decreased in both the HF + Veh (5.196 ± 413 mmHg/s) and HF + Los (5.754 ± 441 mmHg/s) groups.
compared with both the Sham + Veh (8,869 ± 363 mmHg/s) and Sham + Los (8,606 ± 566 mmHg/s) groups. Taken together, the ≥30% infarct size, increased LVEDP, LVESD, and LVEDD, and decreased dp/dt and EF indicate that rats in both HF groups were experiencing cardiac dysfunction.

Plasma ANG II data are also shown in Table 1. Plasma ANG II was increased in the HF + Veh group (207 ± 40 pg/ml) compared with the Sham + Veh group (68 ± 8 pg/ml). Rats treated with Los had plasma ANG II levels significantly elevated above that of the HF + Veh group. In the Sham + Veh group, the plasma ANG II concentration was 363 ± 54 pg/ml.

Microinjection of NMDA into the PVN. Figure 1 shows a schematic representation of sites of termination of NMDA injections into the PVN. Injections made outside of the PVN were not included in the analyzed data. Of 25 injections, 23 injections were found to be located within the PVN.

Figure 2 shows examples of responses of RSNA to microinjections of 200 pmol NMDA into the PVN of each of the experimental groups. Mean data demonstrating the increase in RSNA in response to the three doses of NMDA (50, 100, and 200 pmol) microinjected into the PVN are shown in Fig. 3A. At all three doses of NMDA, the increase in RSNA was greater in the HF + Veh group compared with the other groups. At 50 pmol, the response in the HF + Veh group was 35 ± 3% (n = 6), significantly higher (P < 0.05) than in the Sham + Veh (27 ± 2%, n = 6), Sham + Los (25 ± 2%, n = 5), and HF + Los (27 ± 2%, n = 6) groups. Similarly, the increase in RSNA in the HF + Veh group (60 ± 5%) in response to 100 pmol microinjected into the PVN was significantly higher (P < 0.05) than in the Sham + Veh (35 ± 3%), Sham + Los (29 ± 3%), and HF + Los (34 ± 2%) groups. At the highest dose of NMDA (200 pmol) microinjected into the PVN, the RSNA response was also significantly (P < 0.05) higher in the HF + Veh group (93 ± 13%) compared with the Sham + Veh (45 ± 2%), Sham + Los (40 ± 2%), and HF + Los (47 ± 2%) groups. There were no significant differences at any dose between the Sham + Veh, Sham + Los, and HF + Los groups. These data indicate that chronic AT1 receptor blockade by Los treatment normalizes the enhanced RSNA response to NMDA microinjected into the PVN in rats with HF.

Figure 3B shows the increase in MAP in response to NMDA injected into the PVN. At 50 pmol NMDA, the increase in MAP in the HF + Los group (5 ± 1 mmHg) was significantly lower
than in the HF + Veh group (11 ± 2 mmHg), although the response in the HF + Veh group was not significantly different from the Sham + Veh group (7 ± 1 mmHg). At 100 pmol NMDA, the increase in MAP in the HF + Veh group (15 ± 3 mmHg) was significantly (P < 0.05) higher than that in the Sham + Veh (7 ± 1 mmHg), Sham + Los (8 ± 2 mmHg), and HF + Los (7 ± 1 mmHg) groups. Similarly, at 200 pmol NMDA, the increase in MAP in the HF + Veh group (23 ± 5 mmHg) was significantly (P < 0.05) higher compared with the Sham + Veh (10 ± 2 mmHg), Sham + Los (11 ± 1 mmHg), and HF + Los (11 ± 1 mmHg) groups. Furthermore, the increase in MAP in the HF + Los group was not different from either of the Sham groups at any of the three doses of NMDA.

Figure 3C shows the increase in HR in response to three doses of NMDA injected into the PVN. While HR responses in the HF + Veh group tended to be higher than in the other groups at the two lower doses of NMDA (50 and 100 pmol), the differences were not statistically significant. At the highest dose of NMDA (200 pmol), the increase in HR in the HF + Veh group (31 ± 5 beats/min) was significantly (P < 0.05) higher than in the Sham + Veh (18 ± 2 beats/min), Sham + Los (18 ± 4 beats/min), and HF + Los (19 ± 3 beats/min) groups, and the HF + Los group was not different from the Sham + Veh or Sham + Los groups.

Expression of NR1 subunit mRNA and protein within the PVN. NR1 mRNA expression, as measured by real-time RT-PCR, is shown in Figs. 4 and 5. Examples of original real-time traces are shown in Fig. 4. Figure 5 shows the composite real-time RT-PCR data for the four experimental groups (n = 10 rats/group). Relative NR1 mRNA expression was significantly (P < 0.05) higher in the HF + Veh group (2.2 ± 0.3 relative units) compared with the Sham + Veh group (1.0 relative units). NR1 mRNA expression in the HF + Los group (1.0 ± 0.2 relative units), however, was significantly (P < 0.05) attenuated compared with the HF + Veh group and was not different from the Sham + Veh or Sham + Los groups (0.9 ± 0.2 relative units). These data indicate that chronic AT1 receptor blockade by Los normalizes the increased NR1 mRNA expression within the PVN in rats with HF.

Figure 6 shows NR1 protein expression as measured by Western blot and calculated as the ratio of the density of the NR1 band to the density of the β-tubulin band. Figure 6A shows a sample gel with NR1 bands in the four experimental groups. Composite data for NR1 protein expression are shown in Fig. 6B (n = 10 rats/group). The level of relative NR1 protein expression in the HF + Veh group (0.86 ± 0.11) was significantly (P < 0.05) higher than that in the Sham + Veh group (0.46 ± 0.06). NR1 protein expression was significantly (P < 0.05) attenuated in the HF + Los group (0.59 ± 0.07) compared with the HF + Veh group and was not different from the Sham + Veh or Sham + Los (0.51 ± 0.04) groups.

Figure 7 shows NR1 receptor protein expression within the SON, LH, and cortex and NR2B protein expression within the PVN, SON, LH, and cortex samples were obtained from the same coronal sections from which the PVN was punched. As shown in Fig. 7A, there were no significant differences in NR1 protein expression within the SON, LH, or cortex, suggesting that changes in NR1 expression are limited to the PVN. Additionally, as shown in Fig. 7B, there were no differences between groups in the expression of the NR2B subunit of the NMDA receptor within the PVN. Overall, these data show that chronic AT1 receptor blockade by Los normalizes the increased NR1 protein expression within the PVN in rats with HF.

Protein expression of NR1 subunit in the NG-108 cell line. NG-108 cells were treated with different concentrations of ANG II, and changes in NR1 subunit expression were analyzed by Western blot after 24 h (Fig. 8A). NR1 subunit expression,
as calculated by the ratio of the density of NR1 band to the density of GAPDH band, showed an increase with increasing concentrations of ANG II compared with the control. There was no significant change at lower doses; however, the effect was more pronounced at higher concentrations. At 50 μM ANG II, NR1 expression was increased by ~32% compared with the control. To ascertain whether this increase in the expression of NR1 subunit was mediated by an AT1 receptor-mediated mechanism, NG-108 cells were incubated with different concentrations of Los (10–50 μM). Treatment with Los (50 μM) for 24 h significantly ameliorates the NR1 expression in NG-108 cells (Fig. 8B).

**DISCUSSION**

In the present study, we showed that, in rats with HF, 3-wk Los treatment normalized the potentiated RSNA increase in response to NMDA microinjected into the PVN. We also showed that Los treatment in rats with HF normalized the increased mRNA and protein expression of the NMDA receptor subunit NR1. Taken together, these data support our hypothesis that chronic AT1 receptor blockade by Los treatment normalizes enhanced glutamatergic mechanisms within the PVN in rats with HF.

The coronary artery ligation model used to produce HF has been used extensively by this laboratory and others (26, 45). It has the advantage over other models of HF, such as ventricular...
pacing, in that ligation of the artery mimics blockade of the artery, a common observation in patients with HF. LVEDP and dP/dt are increased and EF is decreased in rats 6–8 wk after ligation of the coronary artery. In addition, this model produces an infarct that is \( \frac{1}{3} \) of the LV area. Taken together, these characteristics indicate that coronary artery ligation is an effective model of HF in rats.

According to histological data, the dye was distributed within the PVN, suggesting that the effects of the drug administrations were due to actions within the PVN. It is recognized that, although the spread of the dye is within the boundaries of the PVN, the spread of NMDA may not coincide perfectly with distribution of the dye. Our previous work (41–43) and work by others (33) have validated this technique of microinjection and appropriate controls in terms of, for example, adjacent nonactive sites, Veh controls, and leakage into the peripheral circulation. The Veh controls in this study and data with glass micropipette injections (data not shown) also suggest that any response or the absence of a response to microinjection of drugs into the PVN was not due to mechanical damage of the PVN by cannula placement or microinjection volumes.

The PVN, which is located within the forebrain, is involved in fluid balance and vasopressin release (39), receives afferent input from cardiac vagal neurons (20), and is directly involved in the modulation of sympathetic outflow (39). Preautonomic neurons of the dorsal and lateral parvocellular divisions of the PVN project to sympathetic preganglionic neurons within the intermediolateral cell column of the spinal cord (34) as well as to preautonomic neurons located within the rostral ventrolateral medulla (RVLM) (34). Given these characteristics of the PVN, in addition to the increased activity of PVN neurons in HF (9, 24), there is good evidence to indicate that the PVN is involved in the increased sympathoexcitation associated with HF. The results of the present experiments are in agreement with this idea. The excitatory glutamatergic mechanisms of the PVN are increased in HF, including increased responsiveness to NMDA microinjected into the PVN and increased expression of the NR1 subunit of the NMDA receptor.

While the PVN cannot receive direct input from plasma ANG II due to the presence of the blood-brain barrier, there are a number of circumventricular organs that project directly to the PVN, including the subfornical organ (SFO) (21) and organum vasculosum of the lamina terminalis (35). Of these, neurons from the SFO that project to the PVN have been shown to respond to bath-applied ANG II (2). SFO efferents to the PVN use ANG II as a neurotransmitter (18). An intravenous ANG II infusion increases Fos immunoreactivity in neurons within the SFO as well as the PVN (30). Thus, it is possible that the increased plasma ANG II in the HF state chronically activates PVN-projecting SFO neurons to alter the expression of the NMDA receptor via increased NR1 subunits, leading to the increased activation of preautonomic PVN neurons and subsequent augmented RSNA. The mechanism(s) by which NR1 gene expression is altered, however, is not clear.
Another explanation for the effect of Los on the increased glutamatergic mechanism in the PVN in HF may be through modulation of the brain renin-angiotensin system (RAS) in regions of the brain involved in neurohumoral control. RAS components, including renin, angiotensinogen, angiotensin-converting enzyme (ACE), ANG II, and ANG II receptors are found within the brain (4, 13, 27–29). ANG II acts as an excitatory neurotransmitter in the central nervous system (18). AT1 receptors have been immunohistochemically identified in sympathetic control regions, including the PVN (4, 27, 28), and injection of ANG II into these sites or intracerebroventricular ANG II injection elicits an increase in MAP (1, 5, 36). Additionally, bath-applied ANG II increases the discharge of PVN neurons retrogradely labeled from the RVLM, illustrating the PVN sympathoexcitatory response to ANG II. Furthermore, coincubation of AT1 receptors have been immunohistochemically identified in excitatory neurotransmitter in the central nervous system (18). ANG II acts as an excitatory neurotransmitter in the central nervous system (18). AT1 receptors have been immunohistochemically identified in sympathetic control regions, including the PVN (4, 27, 28), and injection of ANG II into these sites or intracerebroventricular ANG II injection elicits an increase in MAP (1, 5, 36). Additionally, bath-applied ANG II increases the discharge of PVN neurons retrogradely labeled from the RVLM, illustrating the PVN sympathoexcitatory response to ANG II. ANG II converts to Ang II, and ANG II receptors are expressed at high levels in brain regions involved in neurohumoral control. RAS mechanisms of action in the brain, including the PVN, have been extensively studied, and RAS mechanisms have been implicated in the regulation of sympathetic activity in HF. RAS activation in the brain has been linked to increased sympathetic activity in HF, and RAS inhibition in the brain has been shown to decrease sympathetic activity in HF. RAS mechanisms have also been implicated in the regulation of sympathetic activity in HF, and RAS inhibition in the brain has been shown to decrease sympathetic activity in HF. RAS mechanisms have also been implicated in the regulation of sympathetic activity in HF, and RAS inhibition in the brain has been shown to decrease sympathetic activity in HF.

In HF, the brain RAS is activated, particularly in the areas involved in neurohumoral control (10, 40). ACE and AT1 receptor binding density, as measured by autoradiography, are increased in the SFO, organum vasculosum of the lamina terminalis, and PVN 4 and 8 wk after coronary artery ligation in rats (40). AT1 receptor mRNA and protein expression are increased in RVLM punches from rabbits with HF (10). An acute intracerebroventricular ANG II infusion increases RSNA to a greater extent in rabbits with HF than in Sham rabbits, whereas intracerebroventricular Los infusion decreases RSNA in HF but not Sham rabbits (10), suggesting that ANG II, through AT1 receptors, contributes to increased sympathetic outflow (7, 44). Similarly, DiBona et al. (7) demonstrated that an acute intracerebroventricular or intravenous infusion of Los decreased the elevated RSNA in rats with HF. Zhang et al. (44) used a chronic intracerebroventricular infusion of Los to attenuate the potentiated increase in RSNA after air jet stress in rats with HF. The results of these studies suggest molecular and functional upregulation of the brain RAS in HF may be involved in the elevated sympathetic activity observed in HF (7, 10, 40, 44).

In an isolated cell culture system, incubation of ANG II with NG-108 cells, which have been known to have both AT1 and NR1 receptors (32), causes a dose-dependent increase in the expression of NR1 receptor protein. Furthermore, coincubation with Los prevents the upregulation of the ANG II-induced increase in NR1 protein. These data are consistent with the idea that increased ANG II in rats with HF may have caused the increase in NR1 protein expression within the PVN of rats with HF.

A neuronal intracellular signaling pathway has been proposed (38) in which activation of the AT1 receptor activates the Ras/Raf/MEK/Erk pathway, increasing the transcription of c-fos and c-jun, and the resulting proteins Fos and Jun, which combine to form the activating protein (AP)-1 transcription factor (38). The gene encoding the NR1 subunit, Grin1, contains an AP-1 consensus element (22), but, to our knowledge, the effect of AT1 receptor activation on AP-1-mediated Grin1 transcription and subsequent NR1 expression has not been examined. Thus, elements of an AT1 receptor-mediated pathway involving a transcription factor that has a binding element on the gene encoding NR1 have not been proposed, but a direct effect of AT1 activation on NR1 expression has not been studied until our in vitro data using NG-108 cell cultures. Again, it is important to note that, in our experiments, for a mechanism such as a direct effect of ANG II or Los on PVN neurons to occur, Los must be capable of crossing the blood-brain barrier, which is a matter that remains disputed (3, 17).

Los is an AT1 receptor antagonist that is commonly used in the treatment of patients with HF. In the present study, Los was administered to rats in drinking water, which mimics the method of delivery in HF patients. We chose this method over others, such as chronic infusion, since it was easy to ensure each rat received the correct dose and did not require surgical implantation of an intracerebroventricular catheter or an osmotic minipump. Furthermore, Los in the water did not affect the volume of water consumed per rat.

We found that NR1 protein expression was increased within the PVN. A functional NMDA receptor, however, is composed of both an NR1 and NR2 subunit (8, 37). The NR2B subunit is expressed at a higher level than NR2A, NR2C, and NR2D subunits within the parvocellular subdivisions of the PVN (12). In the present study, however, we found that NR2B expression was not altered by HF or by Los treatment. It is possible, however, that HF and/or Los treatment altered the expression of one or more of the remaining subunits within the PVN.
We also examined NR1 protein expression in three control areas taken from the same coronal section as the PVN punches. The SON, like the PVN, is located within the hypothalamus and is involved in cardiovascular control and fluid balance (31). However, HF and Los treatment did not alter NR1 expression within the SON. Anatomic control samples obtained from the LH, which is adjacent to the PVN (25), and the cortex showed no change in NR1 expression by HF or by Los treatment. Additionally, NMDA injected into sites outside of the PVN caused little or no change in RSNA. Taken together, these data indicate that the alterations in glutamatergic mechanisms associated with HF are limited to the NR1 subunit within the PVN.

In conclusion, the results of the present study indicate that chronic AT1 receptor blockade normalizes the augmented RSNA response to NMDA microinjected into the PVN and the enhanced expression of the NR1 subunit of the NMDA receptor. It is therefore possible that normalization of plasma ANG II levels is one mechanism by which exercise training normalizes enhanced glutamatergic mechanisms associated with HF.

ACKNOWLEDGMENTS

The technical assistance of Dr. Kurtis Cornish, Dr. Xuefei Liu, Dr. Lirong Xu, and Phyllis Anding is greatly appreciated.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant HL-62222 (to K. P. Patel) and American Heart Association Predoctoral Fellowship 05515518Z (to A. C. Kleiber).

DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES