Interleukin-10 inhibits angiotensin II-induced decrease in neuronal potassium current

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Jiang N, Shi P, Desland F, Kitchen-Pareja MC, Sumners C. Interleukin-10 inhibits angiotensin II-induced decrease in neuronal potassium current. Am J Physiol Cell Physiol 304: C801–C807, 2013. First published February 20, 2013; doi:10.1152/ajpcell.00398.2012.— Previously we demonstrated that viral-mediated increased expression of the anti-inflammatory cytokine interleukin-10 within the paraventricular nucleus of the hypothalamus significantly reduces blood pressure in normal rats made hypertensive by infusion of angiotensin II. However, the exact cellular locus of this interleukin-10 action within the paraventricular nucleus is unknown. In the present study we tested whether interleukin-10 exerts direct effects at its receptors located on hypothalamic neurons to offset the neuronal excitatory actions of angiotensin II via its type 1 receptors. The results indicated the presence of immunoreactive interleukin-10 receptors on neurons in normal rat paraventricular nucleus and that receptors for this cytokine were also expressed in neurons cultured from the hypothalamus. Patch-clamp electrophysiological recordings from these cultures revealed that extracellular application of interleukin-10 alone did not exert any alterations in neuronal membrane delayed rectifier or transient potassium currents. However, angiotensin II elicited a significant decrease in delayed rectifier potassium current, an effect that was abolished by interleukin-10 application. Since decreases in delayed rectifier potassium current contribute to increased neuronal excitability, this result is consistent with a direct inhibitory action of interleukin-10 on angiotensin-induced excitation of hypothalamic neurons. As such, these data are the first indication of a neuronal locus of action of interleukin-10 to temper the actions of angiotensin II in the hypothalamus.

angiotensin II; neuron; interleukin-10; cytokine; potassium current

IT IS WELL KNOWN THAT THE CENTRAL NERVOUS SYSTEM (CNS) HAS AN IMPORTANT ROLE IN THE REGULATION OF ARTERIAL BLOOD PRESSURE (14, 17). This regulation involves interplay between neural circuits that include connections among hypothalamic paraventricular nucleus (PVN), brain stem (rostral ventrolateral medulla), and spinal cord (intermediolateral column) sites to ultimately modulate sympathetic outflow and blood pressure (7, 17, 21). Critical to this regulation is the peptide angiotensin II (ANG II), which can elicit increases in sympathetic outflow and blood pressure in normotensive rats by acting via its type 1 receptors (AT1R) in the PVN (9, 11, 36). Further, in animal models of neurogenic hypertension such as the spontaneously hypertensive rat, it is clear that overactivity of ANG II/AT1R in the PVN makes a significant contribution to the raised blood pressure, possibly via a paracrine stimulatory action on nearby sympathetic efferent neurons (29, 30).

The actions of PIC are normally counterbalanced in normal animals by anti-inflammatory cytokines (AIC) such as interleukin-10 (IL-10), which are secreted concomitantly from microglia and astroglia (1, 24, 25). Indeed, increased expression of IL-10 in the brain produced by intracerebroventricular gene transfer reduced hypothalamic inflammation during heart failure (33). In addition, studies from our group indicated that increased expression of IL-10 within the PVN reduces blood pressure in normal rats made hypertensive by infusion of ANG II but did not alter the blood pressure of normotensive control rats (29). However, the exact locus of this IL-10 action, whether it is at microglia or astroglia to prevent PIC synthesis (1, 24, 25) or directly at PVN sympathetic neurons to reduce their discharge, is unknown. The results presented in the current study provide the first evidence to support the latter scenario, demonstrating that while IL-10 does not alter basal K+ current in isolated hypothalamic neurons, it blocks the ANG II-induced decrease in delayed rectifier K+ current (I_Kr). Since decreases in I_Kr contribute to increased neuronal excitability, this result is consistent with a direct inhibitory action of IL-10 on ANG II-induced neuronal excitation.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley (SD) rats or timed (E13–15) pregnant female SD rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were kept on a 12:12-h light/dark cycle in a climate-controlled room. Rat chow (Harlan Teklad) and water were provided ad libitum. All animal use protocols were approved by the Institutional Animal Care and Use Committee of the University of Florida.

Chemicals

IL-10 was purchased from ProSpec-Tany TechnoGene (Ness Ziona, Israel) and ANG II, PD123319, poly-L-lysine hydrobromide, plasma-derived horse serum, and other basic chemicals were obtained from Sigma-Aldrich Chemical (St. Louis, MO). DMEM was purchased from Invitrogen (Carlsbad, CA). Anti-IL-10 receptor (R) antibody (M-20) and the corresponding blocking peptide [IL-10R (M-20)] were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse HuC/D neuronal protein monoclonal antibody (clone 16A11) was from Life Technologies (Carlsbad, CA). Mouse anti-

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NeuN monoclonal antibody was from Chemicon International (Temecula, CA). Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR). Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) was from Vector Labs (Burlingame, CA).

**Preparation of Neuronal Cultures**

Neuronal cultures were prepared from the hypothalamus of newborn SD rats exactly as detailed previously (31). Cells were dissociated from this area, pooled, and resuspended in DMEM containing 10% plasma-derived horse serum and were cultured in poly-l-lysine-precoated plastic culture dishes for 10–17 days before use.

**Immunostaining**

PVN. Immunoreactivity for IL-10R and the neuronal specific protein HuC/D was assessed within the PVN in brain sections as follows. Rats were deeply anesthetized with isoflurane, brains were perfused transcardially in situ with 0.9% saline containing 4% paraformaldehyde and then cryoprotected in 30% sucrose for 1 wk. Coronal sections (30 μm) were cut through the PVN and washed with PBS. The sections were blocked in 10% goat serum for 15 min, washed in PBS for 30 min, and then incubated overnight at 4°C in either mouse monoclonal anti-HuC/D (1:20) or rabbit antibody to determine if this negated the observed IL-10R staining. Sections were mounted on glass slides with coverslips using Vectashield mounting medium with DAPI.

Cell cultures. Immunoreactivity for IL-10R and the neuronal specific protein NeuN was assessed within hypothalamic neurons in culture as follows. Neuronal cultures were washed briefly with ice cold PBS and then fixed for 10 min with ice-cold 4% paraformaldehyde. The fixed cells were incubated with PBS containing 0.3% Triton X-100 (Sigma-Aldrich) for 20 min to improve antibody penetration. Goat serum (5%) in PBS was added to the dish for 30 min at 37°C to reduce nonspecific binding, followed by an additional wash with PBS. Immunostaining using mouse monoclonal anti-NeuN (1:200) or rabbit polyclonal anti-HuC/D (1:100) antibodies was carried out as described above for brain tissue. Following the immunostaining procedures, a drop of Vectashield mounting medium with DAPI was placed on each dish, followed by a coverslip.

Brain sections containing the PVN and hypothalamic cultures that underwent immunostaining procedures were examined at room temperature with an Olympus BX41 fluorescence microscope with X10/0.3 UPlanFLN and X40/0.75 UPlanFLN objectives. Digital images were captured using an Olympus DP71 microscope digital camera and processed with DP Manager software.

**Real-Time RT-PCR**

IL-10R and GAPDH mRNA levels in the PVN or cerebral cortex were analyzed via quantitative real-time RT-PCR. The PVN was dissected as detailed previously (19). Total RNA was isolated from the PVN or cerebral cortex using RNAeasy kits (Qiagen, Valencia, CA). Isolated RNA underwent DNase I (Bio-Rad, Hercules, CA) treatment as above with the following exceptions: (i) inclusion of the blocking peptide IL-10Rα (M-20) P (30-fold higher concentration than the anti-IL-10Rα antibody) to test the specificity of the primary antibody; and 2) omission of the rabbit polyclonal anti-IL-10R primary antibody to determine if this negated the observed IL-10R staining. Sections were mounted on glass slides with coverslips using Vectashield mounting medium with DAPI.

Electrophysiological Recordings

Electrophysiological recordings of delayed rectifier K⁺ current (I_Kv). A-type K⁺ current (I_A), and total outward K⁺ current (I_out) were made using whole cell patch-clamp procedures in the voltage-clamp mode, as detailed previously (19, 32, 35). Experiments were performed at room temperature (23–24°C) with an Axopatch 200B amplifier and a Digidata 1200B interface (Axon Instruments, Burlingame, CA). Patch electrodes were pulled (Flaming/Brown P-97, Sutter Instrument, Novato, CA) from borosilicate glass (Friedrich & Dimmock). Data acquisition and analyses were performed using pClamp 8 software and ClampEx software (v 8.0) software utility. The average cell capacitance was compensated 70–80%; series resistance (Rs) was compensated applying negative pressure to the patch electrode. Whole cell capacitance was compensated 70–80%; series resistance (Rs) was compensated 40–60% with Axopatch 200B compensation circuitry. Leak current was subtracted online using the P/4 method. Cell capacitance was determined in voltage-clamp mode using the “Membrane Test” function in the ClampEx software (v 8.0) software utility. The average cell capacitance for neurons used in this study was 26 ± 1.74 pF (range from 15 to 40 pF). Standard recording conditions for I_Kv were elicited by stepping to a conditioning voltage of −40 mV from the holding potential of −60 mV for 300 ms; the membrane was then depolarized from −40 to +60 mV in increments of 10 mV for 200 ms. A voltage of +60 mV produced the largest peak current in each recording. Standard recording conditions for total outward K⁺ current were elicited by stepping from a holding potential of −120 to +60 mV in increments of 10 mV for 200 ms. I_A was derived by subtraction (total outward K⁺ current − I_Kv). Data are expressed as current density (current amplitude divided by its capacitance; pA/pF). ANG II and IL-10 were applied via a gravity fed pipette that was placed −100 μm from the cell. For the ANG II experiments, the angiotensin type 2 receptor (AT2R) blocker PD123319 (0.001 mM) was included in the extracellular perfusate.
Statistical Analyses

Results are expressed as means ± SE. Analyses were performed with Student’s t-test when comparing only two groups or with ANOVA followed by Bonferroni analysis for multiple comparisons. Differences were considered significant at P < 0.05.

RESULTS

Localization of IL-10R on Neurons in the PVN

Since our overall goal was to determine if IL-10 modulates the inhibitory effects of ANG II on neuronal \( I_{Kv,i} \) was important to demonstrate the presence of IL-10R on neurons in the PVN, and on hypothalamic neurons in culture. The representative low (×10) and high (×40) power fluorescence micrographs in Fig. 1, A–C and D–F, are taken from the PVN at approximately −1.8 mm from bregma. They show IL-10R immunoreactivity in the PVN (green) colocalized with immunoreactivity for the neuron-specific marker HuC/D (red). IL-10R immunoreactivity was observed to be colocalized with neurons throughout the PVN (−1.6 to −2.2 mm relative to the bregma; not shown). In addition, either omission of the IL-10R antibody or preabsorption using the blocking peptide IL-10Rα (M-20) P resulted in no specific IL-10R immunostaining (Fig. 1, G and H). Based on this qualitative view, it is apparent that neurons within the PVN express immunoreactive IL-10R. The presence of IL-10R in the PVN was confirmed by real-time RT-PCR. The results presented in Fig. 1I show the presence of IL-10R mRNA in the PVN compared with that present in the cerebral cortex.

Localization of IL-10R on Hypothalamic Neurons in Culture

Having demonstrated that IL-10R are present in PVN neurons it was important to demonstrate that this expression was maintained in hypothalamic neurons, in culture that were to be used for electrophysiological recordings. These cultured neurons have been used by us previously in multiple experiments,
and contain functional AT1R (33). The representative high-power (×40 magnification) fluorescence micrographs in Fig. 2 are taken from hypothalamic neuronal cultures and show IL-10R immunoreactivity (green) colocalized with the neuron-specific marker NeuN (red) and DAPI nuclear stain (blue). These micrographs indicate the presence of IL-10R on neurons within hypothalamic cultures, and this was confirmed by the single cell RT-PCR analyses that revealed that IL-10R transcripts are present in single neuron samples (Fig. 2E).

IL-10 Does Not Alter Basal Potassium Currents

Considering that hypothalamic neurons in culture contain IL-10R, we first tested whether IL-10 elicits any changes in baseline neuronal $I_{Kv}$ currents. This concentration of IL-10 (10 ng/ml) was chosen based on doses that were effective in studies from other neuronal systems (3, 34). The representative current tracings presented in Fig. 3A demonstrate that extracellular application of this AIC has little effect on baseline $I_{Kv}$, $I_A$, and total outward $K^+$ current ($I_{Kv} + I_A$). This was confirmed by analyses that demonstrated no significant effect of IL-10 on $I_{Kv}$ and $I_A$ current densities (pA/pF; Fig. 3, B and C). Furthermore, calculation of the $I$-$V$ relationship of neuronal $I_{Kv}$ before and after application of IL-10 revealed that this cytokine did not alter the threshold, density, or amplitude of this current (Fig. 3D). Similar preliminary $I$-$V$ analyses for $I_A$ from two neurons indicated that IL-10 did not alter the voltage properties of this current (not shown). In addition to these results using 10 ng/ml IL-10, we also determined in preliminary studies that a lower concentration of IL-10 (1 ng/ml) was also ineffective in altering $I_{Kv} + I_A$.

IL-10 Inhibits ANG II-Induced Decreases in Neuronal $I_{Kv}$

In numerous previous studies we demonstrated that application of ANG II, acting via AT1R, produced a decrease of neuronal $I_{Kv}$ (19, 35). Even though IL-10 did not alter basal $I_{Kv}$ in hypothalamic neurons in culture, we tested whether it would modulate the inhibitory actions of ANG II on this current. In agreement with previous studies, extracellular perfusion of ANG II (100 nM) made in the presence of the AT2R blocker PD123319 (0.001 mM) produced a significant decrease in neuronal $I_{Kv}$, with almost 50% of the neurons tested responding to ANG II (Fig. 4, A and B). After the inhibitory action of ANG II on $I_{Kv}$ was recorded neurons underwent washout with normal extracellular perfusate for 5–7 min. This was followed by extracellular application of IL-10 (10 ng/ml) onto the same neuron for 5 min, followed by a second application of ANG II in the presence of IL-10. In this way we were able to test any effect of IL-10 in ANG II-responsive neurons. The results shown in Fig. 4 demonstrate that ANG II alone produced a significant (∼30%) decrease in $I_{Kv}$ current density. The decrease in neuronal $I_{Kv}$ produced by ANG II was abolished by application of IL-10 (Fig. 4, B and C). Recordings of the effects of ANG II on $I_{Kv}$ in the absence or presence of IL-10 were made during different voltage steps and enabled us to assess the current-voltage relationship in each treatment condition. As shown in the $I$-$V$ curves in Fig. 4C, left, ANG II

![Fig. 2. Localization of IL-10R in hypothalamic neurons in culture. Fluorescence micrographs (×40 magnification) showing IL-10R (A) and NeuN (B) immunoreactivities in neuronal cultures prepared from the hypothalamus of SD rats. Also shown are overlays between IL-10R and NeuN (C) and among IL-10R, NeuN, and the nuclear stain DAPI (D). Arrows indicate examples of PVN neurons containing IL-10R immunoreactivity. E: presence of IL-10R in single hypothalamic neurons. Ethidium bromide-stained agarose gel shows RT-PCR products of the expected size for IL-10R (101 bp) from 4 hypothalamic neurons in culture (lanes 1–4). Substitution of RNase free water for the cell sample mRNA produced no such RT-PCR products (lane N). L, DNA ladder.](http://ajpcell.physiology.org/)

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produced a significant decrease in the amplitude of $I_{Kv}$ at every voltage step from 0 to $+60$ mV. No such decreases in $I_{Kv}$ amplitude were evident when ANG II was applied in the presence of IL-10 (Fig. 4C, right). Importantly, repeated application of ANG II (100 nM) using the above protocol in the absence of IL-10 perfusion produced no significant lessening of the ANG II-induced decrease in $I_{Kv}$. Without IL-10 perfusion, the reduction in $I_{Kv}$ current density produced by the first and second applications of ANG II (100 nM) was $9.7 \pm 1.25$ and $8.8 \pm 1.5$ pA/pF, respectively ($n = 5$ neurons).

DISCUSSION

While it is known that IL-10R are present on astroglia, microglia, and neurons in the CNS, the immunostaining shown in the current study is the first demonstration that receptors for this AIC are located on neurons in the PVN. Furthermore, our immunostaining and single cell RT-PCR data demonstrate that IL-10R are maintained on isolated hypothalamic neurons in culture and the electrophysiological studies presented here demonstrate that IL-10 can act at these receptors to inhibit the decreases in neuronal $I_{Kv}$ elicited by ANG II. Since a decrease in $I_{Kv}$ contributes to increased neuronal excitability, our studies provide the first demonstration that IL-10 can exert direct actions to inhibit ANG II-induced increases in neuronal excitation. Further, our results suggest that this is one mechanism by which increased IL-10 levels in the PVN act to decrease hypertension induced by elevated ANG II. These findings raise a number of questions that have yet to be answered.

First of all, the electrophysiological recordings performed here were performed on hypothalamic neurons in culture and as such are a first step. While the data clearly show that IL-10 can inhibit ANG II actions on neurons, the next step will be to determine if this AIC can inhibit ANG II/AT1R actions on defined preautonomic neurons in the PVN. Such information would allow us to further link antihypertensive action of increased IL-10 expression in the PVN to inhibition of the neuronal actions of ANG II.

A further question concerns the mechanism by which IL-10 exerts its inhibitory action over ANG II. The mechanism is unlikely to involve a direct action of IL-10 on the $K^+$ channels...
IL-10 decreases the inhibitory effect of ANG II on neuronal $I_{Kv}$. 

**A:** representative tracings of the changes in $I_{Kv}$ elicited by extracellular application of ANG II (100 nM), IL-10 (10 ng/ml), and ANG II + IL-10 in combination. **B:** bar graph shows the ANG II-induced decrease in $I_{Kv}$ current density before and after application of IL-10 (10 ng/ml). All recordings were made in the presence of the AT2R blocker PD123319 (0.001 mM). Data are means ± SE from 6 neurons. *$P < 0.02$ vs. control (pre-IL-10); there was no significant difference between control and ANG II groups post-IL-10. **C:** left: $I$-$V$ relationship of $I_{Kv}$ current before and after extracellular application of ANG II in the absence of IL-10 treatment. Data are means ± SE from 7 neurons. *$P < 0.05$. C, right: $I$-$V$ relationship of $I_{Kv}$ current before and after extracellular application of ANG II, in the presence of IL-10 treatment. Data are means ± SE from 7 neurons.

In conclusion, our results demonstrate that IL-10 has the ability to inhibit the actions of ANG II on one of the membrane currents ($I_{Kv}$) that underlies the neuronal excitatory actions of this peptide (32, 35). Whether IL-10 can alter the actions of ANG II on other membrane currents/channels such as calcium current remains to be investigated. Nonetheless, the results imply that IL-10, produced from immune cells in the CNS (microglia and astroglia), can temper the neuronal actions of ANG II to increase sympathetic outflow and blood pressure. By extrapolation, while this IL-10-mediated mechanism may be of importance to temper ANG II-induced increases in sympathetic outflow in normal rats, it is clearly inoperative in neurogenic hypertension, where our previous results indicate that ANG II inhibits IL-10 production by microglia (20), tipping the balance in favor of PIC in hypertension. Another interesting point concerns the stimulus for IL-10 secretion. One study has demonstrated that activation of AT3R on CD8 T-regulatory cells increases IL-10 production (8). Considering this and the evidence for AT3R-mediated inhibition of sympathetic activity via brain cardiovascular control centers (13), opposite to the stimulatory effects of AT1R, it is possible to speculate that a stimulus for IL-10 production in the PVN is AT3R. However, while we have preliminary evidence that the PVN contains AT3R mRNA (Shi P and Sumners C, unpublished observations), whether or not IL-10 producing glia at this site contain AT3R is not known.

From a global standpoint, the current work aids in our understanding of the regulatory mechanisms that are involved in controlling ANG II’s neuronal actions in the brain. Since these regulatory mechanisms may ultimately fail or not be enough to combat the prohypertensive stimuli, the current
findings may open up a new avenue for development of future antihypertensive therapeutics.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: N.J. and C.S. conception and design of research; N.J., P.S., F.D., and M.C.K.-P., and C.S. interpreted results of experiments; N.J., M.C.K.-P., and C.S. approved manuscript; N.J. and M.C.K.-P., and C.S. edited and revised manuscript; N.J., P.S., F.D., M.C.K.-P., and C.S. approved final version of manuscript.

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