Basal and angiotensin II-inhibited neuronal delayed-rectifier \( K^+ \) current are regulated by thioredoxin

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Matsura T, Harrison RA, Westwell AD, Nakamura H, Martynyk AE, Sumners C. Basal and angiotensin II-inhibited neuronal delayed-rectifier \( K^+ \) current are regulated by thioredoxin. Am J Physiol Cell Physiol 293: C211–C217, 2007. First published March 14, 2007; doi:10.1152/ajpcell.00615.2006.—In previous studies, we determined that macrophage migration inhibitory factor (MIF), acting intracellularly via its intrinsic thiol-protein oxidoreductase (TPOR) activity, stimulates basal neuronal delayed-rectifier \( K^+ \) current \( (I_{Kv}) \) and inhibits basal and angiotensin (ANG) II-induced increases in neuronal activity. These findings are the basis for our hypothesis that MIF is a negative regulator of ANG II actions in neurons. MIF has recently been reclassified as a member of the thioredoxin (Trx) superfamily of small proteins. In the present study, we examined whether Trx influences basal and ANG II-modulated \( I_{Kv} \) in an effort to determine whether the Trx superfamily can exert a general regulatory influence over neuronal activity and the actions of ANG II. Intracellular application of Trx (0.8–80 nM) into rat hypothalamic/brain stem neurons in culture increased neuronal \( I_{Kv} \), as measured by voltage-clamp recordings. This effect of Trx was abolished in the presence of the TPOR inhibitor PMX 464 (800 nM). Furthermore, the mutant protein recombinant human C32S/C35S-Trx, which lacks TPOR activity, failed to alter neuronal \( I_{Kv} \). Trx applied at a concentration (0.08 nM) that does not alter basal \( I_{Kv} \) abolished the inhibition of neuronal \( I_{Kv} \) produced by ANG II (100 nM). Given our observation that ANG II increases Trx levels in neuronal cultures, it is possible that Trx (like MIF) has a negative regulatory role over basal and ANG II-stimulated neuronal activity via modulation of \( I_{Kv} \). Moreover, these data suggest that TPOR may be a general mechanism for negatively regulating neuronal activity.

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small proteins (including Trx) may serve as a regulator of both basal and stimulated neuronal activity.

MATERIALS AND METHODS

Animals. For the experiments described here, we utilized adult Sprague-Dawley (SD) rats as breeders to produce rat pups that were used for the production of neuronal cultures. The adult breeder rats were purchased from Charles River Farms (Wilmington, MA). All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Invitrogen (Grand Island, NY). Rabbit anti-Trx1 polyclonal antibody was purchased from Chemicon International (Temecula, CA). Plasma-derived horse serum (PDHS), 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron), ANG II, PD-123319, goat anti-rabbit IgG, NADPH, insulin, 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB), and all other chemicals were obtained from Sigma (St. Louis, MO). Mouse Trx and rat Trx reductase were purchased from American Diagnostica (Stamford, CT). PMX 464 [formerly AW464; (4-oxo-4-benzo[b][1,4]thiazinyl-2-yl)-4-hydroxy-1,3-benzenedisulfonic acid (Tiron)] was obtained from Applied Biosystems (Foster City, CA). Mouse Trx and rat Trx reductase were kindly provided by the Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital. Mouse Trx (C32S/C35S-Trx) were kindly provided by the Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital. Cells-to-cDNA II kits were purchased from Ambion (Austin, TX). Primers for Trx and 18S for real-time RT-PCR were obtained from Applied Biosystems (Foster City, CA).

Preparation of neuronal cultures. Neuronal cultures were prepared from the brain stem and a hypothalamic block of newborn SD rats as described previously (25). Cultures were grown in DMEM containing 10% PDHS for a further 12–15 days before use.

Electrophysiological recordings. Electrophysiological recordings of K+ currents were carried out in cultured neurons as detailed by us recently with the whole cell configuration of the patch-clamp technique (17). Total K+ current was recorded by stepping from a holding potential of −80 mV to +10 mV for 100 ms every minute. Ik, was measured directly by stepping from −40 mV to +10 mV for 100 ms. To inactivate I=k, we applied depolarizing pulses to −40 mV from a holding potential of −70 mV for 50 ms (37). In most cases, this 50-ms prepulse to −40 mV was able to inactivate this fast K+ current, although in some neurons a remnant component of outward inactivating current was present. For total K+ current and Ik, current amplitudes were measured at 50 ms from the onset of the test pulse. Current density was derived by dividing current amplitude (pA) by membrane capacitance (pF), which was measured with the Membrane test of pCLAMP 8.0. The average cell capacitance for neurons used in this study was 33.7 ± 1.3 pF (n = 137), ranging from 12 to 74 pF. Ik, was elicted by depolarization of the membrane potential to +42.5 mV from a holding potential of −110 mV for 100 ms every minute (17, 34). Ik, amplitude was measured as the peak current during the depolarizing pulse.

Analysis of Trx mRNA. cDNA was produced from control or ANG II-treated neuronal cultures with the Cells-to-cDNA II kit. Levels of Trx mRNA were quantified by real-time RT-PCR, essentially as described previously for MIF (28). Data were normalized to 18S RNA.

Analysis of Trx protein. Proteins were isolated from control or ANG II-treated neuronal cultures, and the expression of Trx was assessed via Western immunoblots. This was achieved with rabbit anti-Trx1 (1:2,500; primary antibody), peroxidase-conjugated goat anti-rabbit IgG (1:10,000), and a chemiluminescence method using ECL Western Blotting reagents (Amersham Biosciences, Piscataway, NJ). All procedures were as detailed by us previously for the analysis of MIF (4). Trx protein levels were quantified by densitometry using a calibrated imaging densitometer (model GS710, Bio-Rad Laboratories, Hercules, CA) and were expressed as a ratio of Trx to β-actin levels in the same samples. Quantity One software (Bio-Rad Laboratories) was used to validate the linearity of the obtained signals.

Data analyses. Results are expressed as means ± SE. Statistical significance was evaluated with ANOVA and Student’s t-test. Differences were considered significant at P < 0.05; n refers to the number of cells examined or the number of experiments.

RESULTS

Trx increases basal neuronal I_Kv, but not neuronal I_A. In the first set of experiments, we examined whether Trx, which exhibits TPOR activity via its -Cys-X-X-Cys- motif, can modulate basal neuronal K+ current. In ∼80–90% of the neurons tested, Trx (8 nM), applied via intracellular perfusion, produced a significant increase in I_Kv, measured at +10 mV. This effect became apparent by 10 min, increased slowly, and reached a maximum at ∼15–20 min after the start of Trx administration (Fig. 1). The current-voltage (I-V) relationships of I_Kv, recorded before and after treatment of neurons with the same concentration of Trx, are shown in Fig. 2. Trx increased the amplitude and density (pA/pF) of I_Kv at all tested potentials without a significant change in the threshold of I_Kv activation. The effect of Trx on I_Kv was concentration dependent, with the increase in current density (pA/pF) from 31.70 ± 2.51 (mean ± SE) in controls to 43.71 ± 3.53 (Fig. 3, A and B). The same concentration of Trx substantially increased total K+ current, activated by a depolarizing pulse to +10 mV from the holding membrane potential of −60 mV (Fig. 4C, top). Subtraction of the control recording from the Trx data revealed that the Trx-sensitive K+ current is a slow-activating current that does not exhibit any obvious
inactivation during a 100-ms depolarizing pulse (Fig. 4C, bottom). The total K⁺ current density, measured at 50 ms from the onset of the test pulse and therefore reflecting predominantly \( I_{Kv} \) amplitude, revealed that Trx (8 nM) produced a significant increase in \( I_{Kv} \) (Fig. 4D).

**Mechanism of Trx-induced increases in neuronal \( I_{Kv} \).** Considering that MIF alters neuronal \( I_{Kv} \) via its TPOR activity, our next objective was to investigate whether the stimulatory action of Trx on neuronal \( I_{Kv} \) was mediated via a similar mechanism. The first strategy we used was to test the effects of PMX 464, which inhibits the TPOR activity of Trx by cross-linking irreversibly to cysteine residues 32 and 35 at the Trx active site via its two \( \beta \)-carbon atoms (3, 19, 20, 35), on Trx-stimulated neuronal \( I_{Kv} \). First we tested the effects of 800 nM PMX 464, injected intracellularly as described in MATERIALS AND METHODS, on basal neuronal \( I_{Kv} \). As shown in Fig. 5A, PMX 464 alone at this concentration elicited a minor decrease in basal neuronal \( I_{Kv} \) by 10.220.33.5 on June 21, 2017 http://ajpcell.physiology.org/ Downloaded from

Fig. 1. Thioredoxin (Trx) increases neuronal delayed-rectifier K⁺ current (\( I_{Kv} \)). A: depolarizing voltage command protocol used to elicit \( I_{Kv} \), and representative current tracings of neuronal \( I_{Kv} \), which were recorded under control conditions and after intracellular application of Trx (8 nM). Recordings were made during 100-ms voltage steps from −40 mV to +10 mV. B: time course of changes of neuronal \( I_{Kv} \), elicited by intracellular administration of Trx (8 nM). * \( P < 0.05 \) compared with pre-Trx recordings. Data are means ± SE from 8 neurons. Waveforms at bottom are representative recordings from 3, 13, 23, and 33 min.

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Fig. 2. Effects of Trx on neuronal \( I_{Kv} \): current-voltage (I-V) relationship. A: top: depolarizing voltage command protocol used to elicit \( I_{Kv} \). \( I_{Kv} \) was elicited by 100-ms depolarizing pulse from −40 to +40 mV in 10-mV steps (see MATERIALS AND METHODS for details). Bottom: representative superimposed current traces before (Control) and after application of Trx (8 nM). B: I-V relationship of \( I_{Kv} \), before and after intracellular application of Trx (8 nM). Data are means ± SE from 4 neurons.
neuronal $I_{Kv}$, produced by 8 nM Trx. Trx and PMX 464 were premixed in pipette solution, in order to decrease the TPOR activity of Trx. Intracellular application of this mixture did not alter neuronal $I_{Kv}$, in contrast to the stimulatory action of Trx alone in a separate group of neurons (Fig. 5B). Inclusion of the PMX 464 solvent (0.005% DMSO) within the pipette solution did not alter the effects of Trx on neuronal $I_{Kv}$ (30.95 ± 1.30 to 38.59 ± 1.72 pA/pF; $n = 6$, $P < 0.01$). Thus the data shown in Fig. 5B provide further support for the idea that the effects of Trx on $I_{Kv}$ are mediated via its TPOR activity.

This idea was strengthened by data from experiments in which we tested the effects of rhC32S/C35S-Trx, a Trx mutant protein that lacks TPOR activity, on basal neuronal $I_{Kv}$. The data presented in Fig. 5C show that intracellular application of 8 nM rhTrx produced a similar significant increase in neuronal $I_{Kv}$ as observed with mouse Trx (Fig. 3). However, rhC32S/C35S-Trx (8 nM) did not alter neuronal $I_{Kv}$ after intracellular application (Fig. 5C). It should be noted that the lack of TPOR activity of rhC32S/C35S-Trx was confirmed by insulin reduction assays. rhC32S/C35S-Trx displayed only 11.59% of the insulin-reducing activity of wild-type rhTrx.

Having demonstrated that the stimulatory action of Trx on neuronal $I_{Kv}$ appears to involve a TPOR mechanism, we next tested whether this action of Trx involves scavenging of ROS, analogous to the actions of MIF on this $K^+$ current (17). The strategy used here was to test the effect of Tiron, a cell-permeant low-molecular-weight phenolic compound and scavenger of intracellular superoxide anions (11, 36), on the Trx-induced increase in neuronal $I_{Kv}$. Neuronal cultures were pretreated with Tiron (1 mM) for 30 min, conditions that have been used previously to scavenge superoxides in neurons in culture (23). The Tiron pretreatment was followed by record-

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ings of $I_{K_v}$ before and after intracellular application of Trx (8 nM). The data presented in Fig. 5D demonstrate that under these experimental conditions Trx failed to produce any changes in $I_{K_v}$ in the Tiron-pretreated neurons. Thus the data demonstrate that a superoxide scavenger can prevent the stimulatory action of Trx on neuronal $I_{K_v}$.

**ANG II increases expression of Trx in neuronal cultures.**

ANG II elicits an increase in the expression of MIF mRNA and protein in hypothalamus-brain stem neuronal cultures from SD rats (4, 28). Using similar neuronal cultures, we examined whether ANG II (1 μM) increases the expression of Trx protein and mRNA. As demonstrated in Fig. 6A, ANG II increased Trx mRNA expression within 0.5 h. In addition, ANG II produced a time-dependent increase in Trx protein expression, with a maximal effect at 5 h after incubation (Fig. 6B).

**Intracellular application of Trx blunts decrease in neuronal $I_{K_v}$ produced by ANG II.** In previous studies, we demonstrated (28) that concentrations of MIF that do not alter basal neuronal firing are able to significantly attenuate the chronotropic action of ANG II. In addition, we have shown that ANG II decreases $I_{K_v}$ via ANG II type 1 (AT1) receptors (26), and that this is one of the effects underlying the neuronal chronotropic action of ANG II (33). Here we tested whether a concentration of Trx (0.08 nM) that does not alter neuronal $I_{K_v}$ can affect the ANG II-induced inhibition of this current. Since ANG II elicits an increase in neuronal $I_{K_v}$ via the ANG II type 2 (AT2) receptor (37), these recordings were made in the presence of the AT2 receptor blocker PD-123319 to ensure that the ANG II-induced responses recorded here were AT1 receptor mediated. Bath application of ANG II (100 nM) in the presence of PD-123319 (1 μM) elicited a significant decrease in $I_{K_v}$ within 2–3 min (Fig. 7, left). Intracellular application of Trx (0.08 nM) alone did not elicit any change in the $I_{K_v}$ current density (31.50 ± 1.03 to 31.50 ± 1.15 pA/pF; n = 12). However, intracellular application of Trx (0.08 nM) abolished the decrease in neuronal $I_{K_v}$ induced by ANG II in the presence of PD-123319 (Fig. 7, right).

**DISCUSSION**

The present study provides the first demonstration that Trx can influence neuronal $K^+$ current. We have demonstrated here that intracellular application of Trx increases basal $I_{K_v}$ in hypothalamus/brain stem neurons in culture but does not alter neuronal $I_A$. Furthermore, low concentrations of Trx that do not influence basal neuronal $I_{K_v}$ abolish the inhibition of this $K^+$ current that is induced by the peptide ANG II. In light of the fact that $I_{K_v}$ is one of the currents that are important for the maintenance of neuronal action potentials, it is likely that Trx can modulate both the basal neuronal firing and the stimulatory actions of ANG II on neurons within the hypothalamus and brain stem. Considering our results that show that ANG II increases the intracellular expression of Trx in hypothalamus-brain stem neuronal cultures, it is possible that Trx serves as a negative feedback regulator of ANG II actions in neurons. This is significant, since it is well known that ANG II is an important CNS regulator of blood pressure and fluid balance (5, 18).

The rationales for focusing on Trx in the present study were as follows. First, Trx exhibits both TPOR and antioxidant activity similar to MIF, and may exhibit the same effect on neuronal $I_{K_v}$ as MIF. Second, both Trx and Trx reductase are
located in many tissues including the brain, and in situ hybridization studies have revealed that Trx mRNA is highly expressed in certain regions that are involved in neuroendocrine and/or cardiovascular control, such as the PVN of the hypothalamus and the nucleus of the solitary tract (14). Thus if Trx serves as a negative regulator of ANG II, it may play a pivotal role in controlling sympathetic outflow and cardiovascular control. Third, in general, intracellular concentrations of Trx in several tissues are 100- to 1,000-fold less than those of glutathione (GSH), another common antioxidant defense (24). However, it is likely that the antioxidant role of Trx is more significant in neurons because the level of GSH is relatively low in these cells (21).

The mechanism of Trx action in modulating basal neuronal $I_{Kv}$, appears to involve the intrinsic TPOR activity of the Trx molecule, which is mediated via its -Cys-X-X-Cys- motif at residues 32–35. We conclude this because the effect of Trx on basal neuronal $I_{Kv}$, was abolished by simultaneous application of PMX 464, which attaches to the aforementioned cysteine residues and specifically inhibits the TPOR activity of Trx (3, 19, 20). The effect of Trx on $I_{Kv}$, was also abolished by pretreatment with Tiron, which scavenges superoxide anions, indicating that scavenging or sensing of ROS may be critical to this Trx action. In addition, the rhC32S/C35S-Trx TPOR-negative mutant did not alter neuronal $I_{Kv}$. The data from the insulin reduction assay support the results of the patch-clamp experiments, strongly suggesting that Trx is stimulating $I_{Kv}$, via its intrinsic TPOR activity.

On the basis of these data we speculate that Trx scavenges ROS via its TPOR activity and that this may be a significant mechanism for increasing $I_{Kv}$, since ROS can modulate the activity of membrane ion channels. For example, it has been demonstrated that the fast activation of certain $I_{Kv}$, is mediated by oxidative processes (1), and also that ROS can alter K$^+$ channel activity (10). Interestingly, this proposed mechanism of action of Trx is similar to that described for the stimulatory action of MIF on neuronal $I_{Kv}$, (17). Despite the above-described findings, other possible mechanisms of action of Trx should also be considered. For example, Trx may reduce another protein disulfide, and that protein may directly modulate K$^+$ channels and thus influence K$^+$ current. A further possibility is that Prxs may serve as an effector of Trx to scavenge ROS instead of Trx itself. The expression of Prxs II-V has been observed in the cytoplasm of mouse neurons (7). If rat neurons also contain Prxs, there is a possibility that intracellular application of Trx may reduce the intracellular Prxs, and that the reduced Prxs in turn may scavenge ROS. Future electrophysiological recordings will assess the role of Prxs in the actions of Trx on basal neuronal $I_{Kv}$.

The mechanisms by which Trx at low concentrations inhibits ANG II-induced increases in neuronal $I_{Kv}$, are not known but may also involve the above-described proposed TPOR/ROS mechanisms. We are proposing that this is the case because low levels of MIF, which do not alter basal neuronal firing, abolish the neuronal chronotropic actions of ANG II via a TPOR/ROS-scavenging mechanism (28). Thus the same may be true for Trx; at the low concentrations used it does not alter basal $I_{Kv}$, but still may retain a sufficient level of TPOR activity and ROS-scavenging capability to inhibit the actions of ANG II, which depend on ROS generation (29).

Even though it has been shown that ANG II increases the expression of Trx protein in peripheral blood mononuclear cells from Wistar-Kyoto rats (31), the present studies are the first to demonstrate that ANG II increases Trx expression in neurons. The promoter region of the Trx gene contains many possible regulatory binding motifs compatible with inducible expression, including AP-1, AP-2, and NF-κB, etc. (8), and also has an antioxidant response element (16, 30). Activation of neuronal ANG II receptors results in the modulation of multiple intracellular signaling pathways (27), including the induction of AP-1 (6). Thus AP-1 will be a primary target in our future studies on the mechanisms of ANG II-induced Trx production.

In this study, 0.08–80 nM of Trx had concentration-dependent stimulatory effects on neuronal $I_{Kv}$. These levels of Trx may, at first glance, appear insignificant since the intracellular concentration of endogenous Trx is roughly in the range of 1–15 μM (24) and the intracellular content of another major antioxidant, GSH, is in the millimolar range (1–11 mM) in many other tissues (24). It is imperative, however, to recall that physiological redox status is a very delicate balance between the formation and the elimination of ROS. Many enzyme systems, including NAD(P)H oxidase, xanthine oxidase, and myeloperoxidase, produce ROS under normal conditions. On the other hand, many enzyme systems, including superoxide dismutase, the GSH system, the Trx system, and MIF scavenge ROS. The plethora of recent literature demonstrating that low amounts of various ROS have physiologically relevant functions, rather than pathological consequences, in cellular signaling indicates that even a small perturbation of the redox balance in the intracellular environment can be meaningful. Therefore, we speculate that, even at this small concentration, Trx may serve to produce enough increase in the total amount of ROS scavenging systems to tip the redox balance of the local intracellular environment.

To summarize, the present data provide in vitro evidence that Trx acts intracellularly to produce a specific modulatory action on one of the K$^+$ currents that is the basis of the neuronal action potential and thus neuronal firing. Furthermore, this action of Trx likely involves a TPOR/ROS mechanism. However, this work raises some broader issues. The first is the issue of whether such actions of Trx on basal and ANG II-modulated K$^+$ current occur in vivo in rat brain and contribute to regulation of the physiological actions of ANG II, similar to MIF (13). The second issue is whether TPOR may be a general mechanism for regulating neuronal activity and the actions of ANG II. The third issue is whether Trx and MIF, acting via similar TPOR mechanisms, are members of a novel class of intracellular inhibitors of ANG II action in neurons.

GRANTS

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REFERENCES


