Nitric oxide up-regulates the expression of calcium-dependent potassium channels in the supraoptic nuclei and neural lobe of rats following dehydration

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Abstract

Nitric oxide (NO) is a gas molecule to signal neurotransmission in the hypothalamo-neurohypophysial system during osmotic regulation. We previously reported that osmotic stimulation increased nitric oxide synthase (NOS) activity in the supraoptic nuclei (SON) and neural lobe. The aim of this study is to define the role of NO in the regulation of Ca\textsuperscript{2+}-activated KC channels (BK channels) expression in the magnocellular system following dehydration. We used Western blot analysis and quantitative immunocytochemistry to conduct the experiment in rats. In the immunoblot study, we found that water deprivation significantly increased the expression of BK channels in the SON and neural lobes. Dehydration also enhanced the profiles of neurons expressing vasopressin and oxytocin significantly. In about 70% of these neurons, BK channels were co-localized in the same neuron, and their expression increased significantly during dehydration. We further examined the effects of intracerebroventricular administration of sodium nitroprusside (a donor of NO) and l-NAME (an inhibitor of NO synthase) on expression of BK channels in the SON. We found that compared to animals treated with the donor of NO, there were significant decreases in the expression of BK proteins in animals receiving l-NAME. These results suggest that NO may enhance the expression of BK channels in the supraoptic nuclei and neural lobe of rats following dehydration.

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Nitric oxide (NO), a free gas produced endogenously from the amino acid \textit{l}-arginine by nitric oxide synthase (NOS), is an important modulator of vasopressin and oxytocin secretion from the hypothalamo-neurohypophysial system [13]. The neuronal NOS is expressed in the supraoptic nuclei (SON) and paraventricular nuclei (PVN), where vasopressin and oxytocin are synthesized in their axon terminals in neural lobe [2]. The gene expression of NOS [14,24,25], as well as NADPH-diaphorase activity, a marker of NOS [2,18], increase throughout the hypothalamo-neurohypophysial system when the activity of magnocellular neurons is enhanced during osmotic stimulation [22]. Previous studies indicate that NO is produced tonically during conditions of normal hydration to inhibit secretion of both vasopressin and oxytocin [4,8,13,16,21]. When intracellular and extracellular volumes decrease or plasma levels of angiotensin II elevate [13], NO inhibition of oxytocin secretion increases while that of vasopressin is removed. This results in a preferential release of vasopressin, a phenomenon physiologically significant for fluid balance.

We have shown that the action of NO on the hypothalamo-neurohypophysial system is independent from activation of soluble guanylyl cyclase and cGMP [22]. Recently, evidence has accumulated to demonstrate that NO reacts with thiol groups of cell membrane proteins to exert its biologic action [3,7]. Accordingly, we have shown that the activity of NOS increases in the SON and neural lobe during osmotic stimulation [22]. Previous studies indicate that NO production is elevated during disturbances of fluid balance, presumably to meet the increasing demand for NO modulation of the magnocellular system. Accordingly, we have shown that the activity of NOS increases in the SON and neural lobe during osmotic stimulation [22].
et al. demonstrated that NO activates large conductance Ca^{2+}-
activated K\(^+\) channels (BK channels) of the axon terminals in
the posterior pituitary by a cGMP-independent mechanism \[1\].

In neurons, BK channels participate in repolarization of action
potentials and fast after-hyperpolarization, affecting the release
of neurotransmitter \[12\], and therefore, the excitability of neu-
rons. Activation of BK channels by NO in the terminals of
magnocellular neurons in the posterior pituitary depresses the
excitability of the terminals. This is likely to inhibit impulse
activity that could explain the inhibitory action of NO on hor-
mone secretion. The objective of our studies was to test the
hypothesis that NO regulates the protein expression of BK chan-
nels of the hypothalamo-neurohypophysial system in rats.

The protocol was approved by the Institutional Animal Care
and Use Committee of The University of Texas Medical Branch.
The male SD animals (8–10-weeks-old) were housed under nor-
mal conditions for 1 week. Animals were anesthetized with
sodium pentobarbital (40 mg/kg, i.p.) and a chronic icv guide
cannula was stereotactically implanted into one lateral cere-
broventricle \[17\]. Pentobarbital has no effect on brain NOS
activity \[23\]. The animals were housed singly and allowed to
recover from surgery for 5–7 days before experimental manip-
ulation.

We first determined changes in the BK channels protein
expression in the SON and neural lobe using Western blot anal-
ysis. Three groups of rats \((n=20\) in each group) were either
deprived of water for 24 h or given water ad libitum. They were
anesthetized with sodium pentobarbital and the brains were
quickly removed. Using a stereomicroscope, the neural lobe was
carefully separated from the intermediate lobe and transferred
to a plastic tube maintained on dry ice. The SON was localized
laterally to the optic tract and removed with scissors. Both nuclei
were immediately transferred to a plastic tube maintained on dry
ice. Pools of neural lobes and SON from 20 rats in each group
were used for the Western blot analysis and were kept in the
freezer \((−70 \, ^\circ \text{C})\) until analyzed. The remainder of the brain was
frozen on dry ice, embedded with Tissue Tek OCT medium and
kept at \(−70 \, ^\circ \text{C}\). The sections were stained with thionin for ver-
fication of completeness of SON removal. Then, we examined
the changes in the expression of vasopressin, oxytocin, and BK
channels in magnocellular neurons of the SON using immuno-
chemistry. Two groups of rats \((n=6\) each) were either deprived
of water for 24 h or given water ad libitum. They were then anes-
ethesized for perfusion of the brain. Finally, we tested the changes
in the expression of BK channels in the magnocellular neurons of
the SON during manipulation of NO levels in the cerebroven-
tricular system. Three groups of normally hydrated rats \((n=6\)
each) received an icv injection \((5 \mu l)\) of either the vehicle arti-
ficial cerebrospinal fluid \((\mu lCSF)\), sodium nitroprusside \((SNP, 0.68 \mu g;\) Sigma Company, St. Louis, MO, USA), a donor of NO,
or N\(_{G}\)-nitro-l-arginine methyl ester \((l-NAME, 200 \mu g;\) Sigma
Company), an inhibitor of NOS. Five minutes later, the animals
were anesthetized and the brains were perfused. The pools of
SON and neural lobes were homogenized with an ultrasonic
cell disruptor \((Microson, USA)\), and the concentration of pro-
tein was measured using the BCA kit \((Pierce, Rockford, IL, USA)\)
on a microplate reader. Equal amounts of protein \((30 \mu g)\)
were size fractionated by 10–20% \((w/v)\) gel electrophoresis
(SDS-PAGE) and transferred onto a PVDF membrane. The
blots were placed in blocking buffer for 1 h at room temper-
ature and then incubated with a specific primary polyclonal
antibody to BK channels \((1:300;\) Alomone Labs, Jerusalem,
Israel) overnight at 4 \(^\circ\) C. The blots were washed three times
for 30 min each with washing buffer and then incubated with
horse-radish peroxidase-conjugated anti-rabbit IgG \((Santa Cruz,
San Francisco, CA, USA)\) in 5% \((w/v)\) nonfat milk in washing
buffer for 2 h. The membranes were washed with buffer three
times for 30 min and enhanced with chemiluminescence reagent
\((ECL\, kit;\) Amersham, Arlington Heights, IL, USA). The expres-
sion of \(\beta\text{-actin}\) was also determined as an internal control.
The blots were apposed to autoradiographic films and the intensity
of specific immuno-reactive bands was quantified using densit-
ometric scanning analyses software \((AlphaEase\, software)\). The
results were expressed as a ratio of density of the band of inter-
est over that of \(\beta\text{-actin}\). In immunocytochemistry, the rats
were perfused with 4% paraformaldehyde in 0.1 M PBS.
The brain was removed, blocked and post-fixed for 3 h. The block
was embedded in medium and kept in the ultra-cold freezer
at \(−70 \, ^\circ\) C. The block was serially sectioned \(10 \mu m\) thickness
in a cryostat \((−20 \, ^\circ\) C). Systematic random sampling was done
\[5\], and three pairs of sections chosen were taken and stained
for vasopressin, oxytocin, and BK channels. Following staining,
digitized images of each section were obtained with an Olym-
pus microscope. To count neurons, the image of each section
was displayed on a computer screen and a transparent \(\text{(plastic)}\)
sheet was placed over the image, and all labeled cell profiles
were outlined and counted. Five sections were counted, and the
number of neurons on each section was added to represent the
total number. Sections were incubated for 1 h with 10% normal
goose serum in PBS containing 0.3% Triton. Following a rinse
of PBS, the sections were incubated for 48 h with the primary anti-
bodies against vasopressin \((monoclonal\, anti-vasopressin\, PS41
antibody \((gift\, from\, Dr.\, Gainer,\, without\, cross-reaction\, with\, other
related peptide, such as oxytocin)\) \[26\] and a specific mon-
oclonal anti-oxytocin antibody \((Chemicon\, International, \,CA,
USA; \, without\, cross-reaction\, with\, vasopressin)\) at a dilution of
1:200. The sections were rinsed in PBS and placed in Cy\(\text{TM}
3\)-or Cy\(\text{TM}\, S\)-conjugated in goat anti-mouse \((Jackson\, Immuno-
search\, Labs,\, West\, Grove,\, PA,\, USA)\) at a dilution of 1:200
for 1 h. The slides were rinsed in 0.1 M PBS\(\star\) for 30 min and
incubated for 2 h in biotinylated goat anti-rabbit IgG \((Vector\, Labs\, Inc.,
Burlingame, CA, USA)\). After another 30-min wash, the bind-
ing of BK channels was visualized by incubating the sections
in streptavidin Alexa Fluor\(\text{TM}\, 488\) conjugate \((1:200;\) Molecular
Probes, Eugene, OR, USA) for 1 h. The sections were rinsed
again with PBS\(\star\) and coveredslipped with mounting medium and
observed under fluorescence microscope. Controls for the stain-
ing consisted of the same procedure, with the omission of the
antibodies. Data were analyzed for significance using a \(t\)-test
\((studies\, 1\) and 2) and one-way analysis of variance \((study\, 3)\).

The result in first study to show the changes in the expres-
sion of BK channels in the SON and neural lobe during water
Fig. 1. Western blot study of BK channel expression (120 kDa) in supraoptic nuclei (S-optic N.) and neural lobe during dehydration. Ratio of density of the immunoblots of BK channels (B) relative to β-actin (loading control, 42 kDa) in the supraoptical nucleus (left panel, labeled as S-optic N.) and neural lobe (right panel) of water-sated (labeled as Con., control, clear bars in Panel G1 and G2) and water-deprived rats (labeled as Exp., experimental group; hatched bars in G1 and G2). *p < 0.05, water-deprived rats vs. water-sated rats.

depression. Western blot analysis showed BK channel protein expression in the hypothalamic nuclei. The molecular weight of the band corresponding to the BK channels was 120 kDa. BK channels were expressed in the SON (left panel in Fig. 1) and axon terminals in the neural lobe (right panel in Fig. 1) in conditions of normal hydration (labeled as Con. in Fig. 1). Their expression enhanced significantly following water dehydration (labeled as Exp. in Fig. 1). The expression of β-actin did not show any changes between the two groups. Next, the changes in the expression of vasopressin, oxytocin, and BK channels in the SON during water deprivation were investigated by using immunostaining method. The immunostaining study indicated that approximately 70% of neurons expressing vasopressin co-expressed BK channels (data not shown). Fig. 2C showed one representative neuron with their co-localization (yellow labeled merge). Quantitative study showed that follow-

Fig. 2. Effects of dehydration on the profiles of magnocellular neurons of the SON expressing vasopressin and BK channels. Photomicrographs of immunocytochemical staining of magnocellular neurons of the SON expressing vasopressin (red color, pictures A1 and A2) and BK channels (green color, pictures B1 and B2). Panels A1 and B1 present as the sections from the water-sated rats and panels A2 and B2 represent as that from the water-deprived rats (scale bar: 30 μM). Panel C show that the co-expression (the yellow color labeled with merge in the far right one) of vasopressin (left, red color) and BK channels (middle, green color) in one representative neuron from a rat with water-derived treatment (scale bar in panel C: 5 μM). Rates were water-sated or deprived of water for 24 h before the brains were fixed for immunocytochemical staining. Values represent means ± S.E.M., n = 6 per group. Statistics, t-test; *p < 0.05, water-deprived rats vs. water-sated rats (Panel D, clear bar, vasopressin; hatched bar, BK channels).
Water deprivation (Figs. 2B1, B2, 3B1 and B2, * neurons expressing BK channels enhanced significantly after In agreement with the Western blot analysis, the profiles of oxyrons with positive staining of vasopressin also increased sig-
ificantly (Fig. 2D). To study another peptide hormone, oxy-
tocin in this system by using the same approach, we found that about 68% of the oxytoxin-positive neurons express BK chan-
nels protein (data not shown). The representative picture of the co-expression of oxytocin and BK channels was presented as the yellow color labeled with merge in Fig. 3C. Furthermore, after 24 h of water deprivation, both of the neuronal profiles of oxytocin and BK channels increased significantly when com-
pared to that of the water-sated group (Fig. 3A, B and D).

In agreement with the Western blot analysis, the profiles of neurons expressing BK channels enhanced significantly after water deprivation (Figs. 2B1, B2, 3B1 and B2, * p < 0.05, water deprived rats versus water-sated rats). In the group of control staining, the respective expression was absent when the antibod-
ies against peptides and proteins were omitted (data not shown).

Finally, the effects of nitric oxide levels on the expression of BK channels in the SON were tested. Changes in the availability of NO with administration of SNP, a NO donor or l-NAME, an NO synthase inhibitor, respectively, into the cerebroventricular system produced proportional changes in the profiles of neu-
rons expressing BK channels (Fig. 4). The increases in neuronal profile of positive BK channels were statistically significant compared to that of animals receiving the vehicle aCSF (p < 0.05, Fig. 4A, B and D). However, when compared to animals treated with the donor of NO, treatment with an NO synthase inhibitor, l-NAME significantly reduced (p < 0.05) the profiles of neurons expressing both proteins (Fig. 4B–D).

The results of these studies demonstrated that when the activ-
ity of the hypothalamo-neurohypophysial system increases dur-
ing dehydration, the number of neurons expressing vasopressin
and oxytocin, as well as BK channels, becomes elevated. These increases occurred in parallel with increases in the concentra-
tion of the proteins in the SON, the anatomic site of the cell bodies of the magnocellular neurons, and in their axon termi-

nals in the neural lobe. This indicates that as one of the important membrane proteins, the BK channels may be regulated by NO’s actions on the hypothalamic magnocellular system in the signal-
transduction mechanism of dehydration. This is significant in as much as activation of BK channels decreases the excitability of terminals of the hypothalamo-neurohypophysial system [1], a finding that is consistent with our hormone data [13]. BK channels consist of α and β subunits [9]. The β subunit contains the Ca2+-sensitive domain. The α subunit, which contains the pore-lining domain, has a molecular mass of 125 kDa. In our studies, we used the antibodies directed against the α subunit, whose specificity has been assessed for both mouse and rat BK channel α subunit. The molecular weight of the protein observed in our Western blot studies was 120 kDa, a value very close to that expected for BK channel. Thus, our results suggest that NO modifies thiol groups of the α subunit of BK channel to modu-
late the activity of magnocellular neurons. In accordance with this postulate in previous studies, we have shown that dithiothre-
itol, a thiol-reducing agent, administered icv to water-sated rats increased plasma levels of vasopressin and oxytocin [13]. This effect is similar to that when NO levels are decreased by dimin-
ishing its synthesis with l-NAME administered icv. We found that the changes in BK channels profiles happened at 5 min fol-
lowing the manipulation of NO levels. It suggests that the cellular changes in this situation might remain at the post-translational
Fig. 4. Effect of NO levels on the profiles of magnocellular neurons of the SON expressing BK channels. Photomicrographs of immunocytochemical staining of magnocellular neurons of the SON expressing BK channels in rats treated with aCSF (Panel A), SNP, a donor of NO (Panel B), and L-NAME, an inhibitor of NOS (Panel C, scale bar, 20 μM). Rats received an icv injection (5 μl) of the vehicle aCSF, sodium nitroprusside (SNP, 0.68 μg), a donor of NO, or L-NAME (200 μg), an inhibitor of NOS. Five minutes later, the brains were fixed for immunocytochemical staining. Panel D, Bar graph shows the quantitative analysis of the neuronal profiles in different groups. Values represent means ± S.E.M., n = 6 per group. Statistics, one-way ANOVA, *p < 0.05 aCSF vs. SNP; #p < 0.05, SNP vs. L-NAME.

The number of neurons expressing BK proteins may be one of the targets of nitrosylated action by NO. We found that there are certain co-localized neuronal profiles of nitrosylated protein with those expressing BK channels (unpublished communication). The mechanism appears to mediate NO’s action on several proteins susceptible to nitrosylation. According to Jaffrey et al. [10], there are a number of cysteine residues in a protein that determine the susceptibility to S-nitrosylation. For example, of the seven cysteines in the Deltas 1 protein, at least one is nitrosylated [11], and of the 84 cysteines with free sulphydryl groups in the ryanodine receptor the number of proteins expressing BK channels in this regimen needs further investigation.

level, but not at the transcriptional level. However, the detailed mechanism of the mRNA expression in this regimen needs further investigation.

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