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Effects of nitric oxide on expressions of nitrosocysteine and calcium-activated potassium channels in the supraoptic nuclei and neural lobe of dehydrated rats

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Abstract

Nitric oxide (NO) is an important gas mediator in the signal transduction cascade regulating osmotic function in the hypothalamo-neurohypophysial system. We previously found that increased nitric oxide synthase (NOS) activity in the supraoptic nuclei (SON) and neural lobe following osmotic stimulation and NO could regulate the expression of Ca²⁺-activated K⁺ channel (BK channels) protein in the magnocellular system during dehydration. The aim of the current study is to examine the role of NO in the regulation of nitrosocysteine and BK channel protein in the magnocellular system in dehydrated animals. Using Western blot analysis and quantitative immunofluorescent staining study, we found that water deprivation in rats significantly enhanced the expression of nitrosocysteine protein in SON and neural lobes. Immunohistochemistry study indicated that dehydration significantly increased the profiles of SON neurons co-expressing nitrosocysteine with BK-channel protein. Intracerebroventricular administration of L-NAME (an inhibitor of NO synthase) significantly reduced the neuronal profiles of nitrosocysteine, as well as their co-expression with BK-channel in SON of dehydrated rats. However, treatment of sodium nitroprusside (a donor of NO) increased this co-expression. Our results indicate that NO signaling cascade may control the expression of BK channels through the regulation of nitrosocysteine in SON and neural lobe of rats during osmotic regulation.

Nitric oxide (NO), a gas molecule generated endogenously from the amino acid L-arginine by nitric oxide synthase (NOS), is a freely diffusible intercellular messenger that functions in various cells in the nervous system. In the hypothalamo-neurohypophysial system, NO mediates neuronal synaptic transmission and plasticity in the regulation of vasopressin and oxytocin secretion (16,17). As the important neuroendocrine cells, the supraoptic nuclei (SON) and paraventricular nuclei (PVN), as well as in their axon terminals in the neural lobe, synthesizes vasopressin and oxytocin in response to osmotic alternations in physiological and pathophysiological states (2,16). The expression of NOS protein and mRNA was widely

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reported in the entire hypothalamo-neurohypophyseal system (18,30). As a marker of NOS activity, NADPH-diaphorase was reported to reside in this system (2,23). The activated NO system was reported to involve the response of magnocellular neurons to acute and chronic osmotic insults, such as dehydration and hypovolemia (17,18,30). These studies indicate that disturbances of fluid balance triggered the system to produce NO to meet the increasing demand for NO modulation in the magnocellular system.

We previously reported that osmotic stimulation significantly increased the NOS activity in the SON and neural lobe in rats (17,18). In several studies, NO was reportedly generated dynamically during conditions of normal hydration to inhibit the secretion of both vasopressin and oxytocin in the neuroendocrine system (5,11,16,21,26). Inhibition of oxytocin secretion by NO was found in experimental animals when the intracellular and extracellular volumes was reduced or the plasma levels of angiotensin II elevated (16). These results suggest a preferential role of NO in the hypothalamo-neurohypophyseal system to control fluid balance physiologically. In a previous study, we found that the effect of NO on the hypothalamo-neurohypophyseal system could be independent from the activation of soluble guanylyl cyclase and cGMP production (28). Recently, accumulated evidence suggest that the highly labile NO reacts with cysteine thiol groups of cell membrane proteins to affect NO-related bioactivity in NOS-expressing cells (3,4,8). Ahern et al. reported that in the posterior pituitary, the large conductance Ca^{2+} -activated K^{+} channels (BK channels) were activated significantly by NO in a cGMP-independent mechanism in the axon terminals (1). BK channels induced the neuronal excitability significantly enhancing the regulation of neurotransmitter release since it was involved in the repolarization of action potentials (15). In the magnocellular neurons, NO activates BK channels in the posterior pituitary and depresses the excitability of the terminals. This reduced impulse activity could lead to the inhibitory action of NO on hormone secretion (16,17). Recent findings from our lab indicated that water deprivation significantly upregulated BK channel protein in magnocellular neurons and that NO levels affected this regulation (17). Since NO-mediated nitrosylation of receptor proteins could serve as a ubiquitous post-translational modification that dynamically regulates a wide functional spectrum of neurotransmission receptors, the NO regulated-BK channel protein expression in magnocellular neurons may use this mechanism in response to osmotic stimulation (9,10,19). The aim of this investigation was to use immunoblot analysis and quantitative immunofluorescent staining approaches to detect whether the NO levels regulate the expression of nitrosocysteine and BK channel proteins in hypothalamo-neurohypophyseal system in dehydrated rats.

The animal protocol was approved by University of Texas Medical Branch Institutional Animal Care and Use Committee. Male Sprague Dawley animals (8–10 weeks old) were anesthetized with sodium pentobarbital (40 mg/kg, ip) and an icv guide cannula was implanted into one lateral cerebroventricle stereotactically (22). We chose pentobarbital as the anesthetic because it has no effect on NOS activity (17,29).

We first detected the changes in the expression of BK-channel protein in the SON and neural lobe by using Western blot analysis. Groups of rats ($n=20$ in each group) were either deprived of water for 24 hr or given water *ad libitum*. After the anesthesia procedure with sodium pentobarbital, the brains of animals were quickly removed. The collected SON and neural lobe tissues were homogenized with an ultrasonic cell disruptor and the concentration of protein solutions were measured using a BCA kit (Pierce, Rockford, IL) on a microplate reader. Equal amounts of protein sample (30 μg) were size fractionated by 10–20% (w/v) gel electrophoresis (SDS-PAGE) and were transferred onto a PVDF membrane. The blots were incubated in blocking buffer for 1 hr at room temperature and then incubated with a specific primary monoclonal antibody to nitrosocysteine (1:500, A. G. Scientific, CA) overnight at 4°C. The blots were washed extensively with washing buffer and incubated with horseradish peroxidase-

conjugated anti-mouse IgG (Santa Cruz, CA) in 5% (w/v) milk in incubation buffer for 2 hr. The membranes were washed with buffer three times for 30 min and enhanced with the chemiluminescence reagent (ECL Kit, Amersham, IL). The expression of β -actin was also determined at the same time by re-blotting the membranes with the antibody against β -actin (Sigma Company). The blots were apposed to autoradiographic films, and the intensity of specific immunoreactive bands was acquired and quantified using a densitometric scanning analyses software (AlphaEase software). The results were expressed as a ratio of density of the detected band over that of β -actin (17).

For immunostaining studies, we tested the changes in the expression of nitrosocysteine and BK channels in magnocellular neurons of the SON in dehydrated and effects of NOS inhibitors were detected. To manipulate NO level, we treated the animals with NO donor, SNP and NOS inhibitor, L-NAME and the control solution, artificial cerebrospinal fluid (aCSF). Three groups of hydrated rats (n=6 each) received an icv injection of either the vehicle aCSF (5 μ l), sodium nitroprusside (SNP, 0.68 μ g; Sigma Chemical Company, St. Louis, MO), or N^G nitro-L-arginine methyl ester (L-NAME, 200 μ g; Sigma Chemical Company, St. Louis, MO).

To perform the immunocytochemistry study, systematic random sampling was collected and three pairs of sections were chosen for the staining by using antibody against nitrosocysteine protein (17). Following incubation with 10% normal goat serum in PBS, the sections were incubated for 48 hr with the primary monoclonal antibodies against nitrosocysteine and the CyTM 3-conjugated goat anti-mouse antibody was added and incubated with the sections (Jackson ImmunoResearch Labs, West Grove, PA). The sections were rinsed and viewed with an Olympus microscope. Five sections were recorded and the number of outlined neurons on each section was accumulated to represent the total number of positive neuronal profiles (6). We further detected the changes in the co-expression profiles of nitrosocysteine with BK channels and the effect of NO. Briefly, in another parallel immunostaining experiment, after the completion of nitrosocysteine staining, the slides were re-incubated with a polyclonal anti-BK channel antibody (1: 200; Alomone Labs, Jerusalem, Israel) for another 24 hr. The slides were then rinsed in 0.1M PBS and incubated for 2 hr in CyTM 5-conjugated goat anti-rabbit IgG (Jackson Labs). After another washing with PBS, the slides were covered with the medium and the signals were visualized by a two-channel microscope. Data were analyzed for significance using a *t*-test or one-way analysis of variance (17).

The Western blot study showed nitrosocysteine protein expression in the hypothalamic nuclei. The molecular weight of the band corresponding to this protein was at approximately 52 kDa. Nitrosocysteine was located in the SON (left panel in Figure 1) and axon terminals in the neural lobe (right panel in Figure 1) in normal hydrated conditions (marked as Con. in Figure 1). However, following water dehydration in rats, the expression of nitrosocysteine protein was enhanced significantly (marked as Exp. in Figure 1) in the SON (27 ± 4.4 vs 40 ± 7) and the neural lobes (46.2 ± 6.9 vs 88 ± 9.4). The β -actin expression did not show any changes between the two groups examined (Figure 1).

The immunostaining study indicated that approximately 70% of neurons express nitrosocysteine and BK channels (a neuronal marker, NeuN was tested with co-expression of Bk channel in preliminary study, data not shown). Quantitative analysis showed that following dehydration, the profiles of SON neurons expressing nitrosocysteine increased significantly (Figure 2, Panel A1, A2). The positive neuronal profiles for BK channels in SON neurons also increased significantly (Figure 2, Panel B1, B2). We also have found that the profile number of examined SON neurons expressing nitrosocysteine significantly increased in dehydrated rats, when compared to that of water-sated rats (Figure 2D, 185 ± 17 vs 132 ± 10 , $p < 0.05$). The profile number of SON neurons expressing BK channel also significantly increased (Figure

2D, 133 ± 13 vs 89 ± 8). We also performed the double-staining experiment to localize the co-expression of nitrosocysteine and BK-channel proteins in SON neurons (Figure 2C).

Finally, we tested the effects of NO levels on the expression of nitrosocysteine in the SON neurons. We manipulated the NO level by the administration of SNP or L-NAME respectively, into the cerebroventricular system. This treatment changed the level of NO and produced proportional changes in the profiles of neurons expressing nitrosocysteine and BK channels (Figure 3). The increased number of neuronal profile for nitrosocysteine in SNP-treated rats was not statistically significant when compared to that control group, aCSF (Figure 4A). However, when compared to animals treated with SNP, the L-NAME treatment-induced reduction in the profiles of neurons expressing nitrosocysteine was observed. (Figure 3, 4A). The effect of NO levels on the co-expression of nitrosocysteine and BK channel in SON neurons (Figure 2C) in dehydrated rats was indicated in Figure 4B and 4C. Dehydration produced an increase in the co-expression of nitrosocysteine and BK-channel proteins in SON (109 ± 17 vs 57 ± 12 ; Figure 4B). SNP significantly increased the co-expressing profile number of nitrosocysteine and BK channels by 34% (Figure 4C) while inhibition of NO levels with L-NAME reduced this co-expressing significantly (by 56%) in dehydrated animals (Figure 4C, #, $p < 0.05$).

The results of this study demonstrated that in the hypothalamo-neurohypophyseal system, an osmotic insult could enhance the nitrosylation of membrane protein such as BK channels since the data showed that the number of neurons expressing nitrosocysteine and BK channels were significantly elevated during dehydration. By using Western blot analysis, we found that the enhanced nitrosocysteine and BK channels occurred in parallel with upregulation in the amount of proteins in the SON, the anatomic site of the cell bodies of the magnocellular neurons, as well as in their axon terminals in the neural lobe. Also, the neuronal profile of SON cells with co-expressing nitrosocysteine and BK-channel proteins increased in response to water deprivation, and the changed NO levels, by manipulating NOS activity, produced an effect on their expression. The number of neuronal profiles with nitrosocysteine was increased following treatment of an NO donor, SNP, but this regulation is not significantly (Figs. 3, 4A). But, compared to the treatment of L-NAME, the result showed a significant difference as well as for controlling co-expression with BK-channel protein (Fig. 4A, B, C). Effects of NO on glial and/or cerebral vascular system could be impacted and resulted in the phenomena. This result revealed that, as one of the important membrane receptor proteins, the BK channels could be regulated by NO in the hypothalamic magnocellular system. The nitrosylation of BK-channel protein on the critical cysteine residue in magnocellular neurons may be involved in the NO-derived intracellular signal transduction mechanism of dehydration. This mechanism could be very critical in the central mechanism of osmotic regulation since the activation of BK channels reduces the excitability of terminals of the hypothalamo-neurohypophyseal system in animals which was consistent with one of our previous findings (1,16).

In one previous study, we used the antibody directed against the α subunit, whose specificity has been assessed for both mouse and rat BK channel α subunit. We found that water deprivation produced more expression of BK-channel protein (17). An immunostaining study on the sections of SON neurons also revealed both enhanced expression of BK channel and nitrosocysteine proteins in water-deprived rats. Thus, our results suggest that NO may modify the thiol groups of the α subunit of the BK channel to modulate the activity of magnocellular neurons. In accordance with this concept in previous studies, we also reported that dithiothreitol, a thiol-reducing agent, treated icv to water-sated rats increased the plasma levels of vasopressin and oxytocin as well as their localized enhancement of the expression in these neuronal populations (16,17). However, by reducing NO synthesis with L-NAME, this effect was also observed when NO levels were decreased. In this study, the profile number of SON neurons co-expressing nitrosocysteine and BK proteins responded to the levels of NO

following the manipulated procedure of NO system by NO donor or NOS inhibitors. It reflects that the BK-channel receptor protein may be one of the active targets of nitrosylated action by NO (12,19). The increased BK channel activity or protein expression in the neural lobe and the SON during dehydration could be part of the cellular regulatory mechanisms in response to osmotic stress. BK proteins are susceptible to nitrosylation by the action of NO since nitrosylation-mediated regulation on a single critical cysteine residue may also be subject to oxygen- or glutathione-dependent modification. This mechanism in the SON-involved osmotic regulation system suggested that nitrosylation of BK-channel protein could be a prototypic redox signal in response to water deprivation (17). It also reported that a number of cysteine residues in a protein could determine the susceptibility to S-nitrosylation (3,13). Of the seven cysteines in the Dexras 1 protein, at least one residue is nitrosylated (14). Several residues containing 84 cysteines with free-sulfhydryl groups in the ryanodine receptor were found to be very susceptible to nitrosylation (16). It has been postulated that residues adjacent to cysteine in receptor proteins enhance the nucleophilic properties of cysteine, and putative consensus sequences adjacent to the cysteine residue increase the susceptibility to S-nitrosylation (24, 25,27). The proximity of the NO generator and acceptor could be considered as another biochemical mechanism since it provide the selective targeting of NO to specific protein thiol groups *in vivo* (7). Thus, the NO production in the subcellular location and local chemical environment may to some extent indicate which proteins, such as BK channel protein, become nitrosylated at the susceptible cysteine residue (20). We found that there are certain co-localized neuronal profiles of nitrosylated protein with those expressing BK channels, and the NO-mediated changes in BK channel profiles happened shortly after the manipulation of NO levels. This indicates that the substantial cellular events in response to this situation might remain at the post-translational level. Furthermore, the detailed cellular mechanism of the transcriptional expression in this signal transduction cascade needs further intensive studies.

In summary, the results of our investigation suggest that NO plays an important role in the regulation of nitrosylation of BK channels in the hypothalamic magnocellular system in response to osmotic stimulation.

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Figure 1.

Western blot analysis of nitrosocysteine (52 kDa) in supraoptic nuclei (Supraoptic N.) and neural lobe following dehydration. Ratio of density of the immunoblotting bands of nitrosocysteine (B) relative to β -actin (loading control, 42 kDa) in the supraoptical nucleus (left panel, labeled as Superoptic N.) and neural lobe (right panel) of water-sated (labeled as Con., control in Figure 1) and water-deprived rats (labeled as Exp., experimental group in Figure 1). The bottom bar graphs (G1 and G2) indicated the statistical analysis (clear bars: water sated rats; hatched bars: water deprived animals). *, $p < 0.05$, water-deprived rats vs. water-sated rats.

Figure 2.

Effects of dehydration on the profiles of magnocellular neurons of the SON expressing nitrosocysteine and BK channels. Pictures of immunocytochemical staining of magnocellular neurons of the SON expressing nitrosocysteine (red color, pictures A1 and A2) and BK channels (green color, pictures B1 and B2). Panels A1 and B1 present as the tissue sections from the water-sated rats and panels A2 and B2 represent the water-deprived rats (Scale bar: 30 μ M). Panel C shows that the co-expression (the yellow color labeled with merge in the far right) of nitrosocysteine (left, red color; labeled as Nitro.) and BK channels (middle, green color, marked as BK) in one representative neuron from a rat with water-derived treatment (Scale bar in panel C: 5 μ M). Values represent means $\pm$ SEM, $n = 6$ per group. Statistics: Wilcoxon test, *, $p < 0.05$, water-deprived rats vs. water-sated rats (Panel D, clear bar, nitrosocysteine; hatched bar, BK channels).

Figure 3.

Effect of NO levels on the profiles of magnocellular neurons of the SON expressing BK channels. Representative pictures of immunocytochemical staining of magnocellular neurons of the SON expressing nitrosocysteine (upper panels, red color) and BK channels (down panels, green color) in rats treated with aCSF (left panels); SNP, a donor of NO (middle panels), and L-NAME, an inhibitor of NOS (right panels, scale bar; 20 μ M). Rats received an icv injection (5 μ l) of the vehicle aCSF, sodium nitroprusside (SNP, 0.68 μ g), or L-NAME (200 μ g).

Figure 4.

Bar graphs show the quantitative analysis of the neuronal profiles of nitrosocysteine (Panel A) and co-expression of nitrosocysteine and BK channels (Panel B and C) in rats with different treatments. There is no significant change in neuronal profiles of nitrosocysteine following SNP treatment (NS, not significant). However, L-NAME treatment reduces the numbers (Panel A, $p < 0.05$). Dehydration increased the co-expression profile number of nitrosocysteine and BK channel proteins (Panel B, $*p < 0.05$, vs water-sated group). SNP treatment enhanced the profiles of the co-expression of nitrosocysteine and BK channels, while L-NAME significantly decreases the numbers (Panel C). Values represent means $\pm$ SEM, $n = 6$ per group. Statistics:

one-way ANOVA (Panel A) and χ^2 (Panel B), NS: not significant; * $p < 0.05$, SNP vs L-NAME (Panel C).