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Fos proteins are not prerequisite for osmotic induction of vasopressin transcription in supraoptic nucleus of rats

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Abstract

While it is well known that osmotic stimulation induces the expression of Fos family members in the supraoptic nucleus (SON), it is unclear whether the induced protein products are involved in the regulation of the gene transcription of arginine vasopressin (AVP). In the present study, we examined the *in vivo* correlation between changes in AVP gene transcription and expression of the various Fos family members in the SON after acute osmotic stimuli. The data demonstrated that the peak of AVP transcription (measured by intronic *in situ* hybridization) observed 15 min after an injection of hypertonic saline preceded the expression of Fos proteins, which became detectable at 30 min and peaked at 120 min. Electrophoretic mobility shift assay showed that the expressed Fos proteins bound to the composite AP-1/CRE-like site in the AVP promoter. These data suggest that Fos proteins in the SON induced by acute osmotic stimuli could affect AVP gene transcription by binding to the AVP promoter, but that they are not prerequisite for the induction of AVP gene transcription.

Arginine vasopressin (AVP), or antidiuretic hormone, is synthesized in magnocellular neurons of the supraoptic (SON) and paraventricular nuclei (PVN), and transported through axons to the posterior pituitary from which it is released into the systemic circulation [22]. AVP plays a critical role in water and electrolyte balance by promoting water absorption through V2 receptors in the kidney [22]. The synthesis and release of AVP are tightly regulated by plasma osmolality, an activation of the system correlates well with activation of AVP gene transcription in the SON and PVN [1].

The isolation and characterization of the structure of the AVP gene from the genome of different species [10,20,24,25] have lead to a growing understanding of the mechanism regulating AVP transcription. Cyclic AMP increases in the SON of osmotically challenged rats [6,35], and *in vitro* studies have shown that the cyclic nucleotide potently induces the AVP gene [3,4,8,21,29]. While this suggest that cAMP plays a primary role, many reports have described the induction the expression of Fos family members in the SON after osmotic stimuli [7,11,13,15,19,26,27,31]. Proteins of Fos family bind to a specific site of gene promoter known as AP-1, and the binding of tissue extracts from the SON to the AP-1 consensus sequence are reportedly increased by osmotic stimuli [18,33]. Previous studies in cell lines also showed that Fos proteins could bind to the putative AP-1/CRE binding sites in the AVP promoter [9,21,34] and possibly affect the transcriptional activities [34]. However, it is still unclear whether AP-1 complex induced in the SON binds to the AVP promoter to

play any physiological role in the regulation of AVP gene transcription. Actually, a recent in vivo study [14] demonstrated a discrepancy in expression between *c-fos* mRNA and AVP heteronuclear (hn) RNA (or primary transcript which is commonly used as a sensitive indicator for gene transcription) [1,3,4]. In order to better understand the possible role of Fos proteins in the regulation of AVP gene transcription in the SON, we examined time course changes in expression of Fos members as well as their binding to the consensus AP-1 or the putative AP-1/CRE binding sites of the AVP promoter in relation to the changes in AVP gene transcription in osmotically stimulated rats.

Adult male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, USA), weighing 250-270 g, were entrained to a 12:12 light/dark schedule (lights on at 7:00 am) 2 weeks before experiments were carried out. Groups of 6 rats were subjected to the following experimental treatments: 1) undisturbed controls, 2) controls receiving an intraperitoneal (ip) injection of 2% body weight (BW) of isotonic (0.15 M) saline (IS), 3) an acute osmotic stimulus by an ip injection of 2% BW of hypertonic (1.5 M) saline (HS). The rats were decapitated 15, 30, 60, 120, 240, 360 and 720 min after the HS injection, and 120 min after the IS injection. All injections were performed so that rats were decapitated between 9:00 and 12:00 am. Brains were immediately removed, frozen on dry ice and stored at -70 C until sectioning. Experiments were done in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the *Guide for the Care and Use of laboratory Animals*, and the *Animal Welfare Act Regulations*, following experimental protocols that were approved by the NICHD *Animal Care and Use Committee*.

The rat AVP intronic probe (kindly provided by Dr. Thomas Sherman, Georgetown University, Washington DC) was a 735 bp fragment of intron 1 of the rat *AVP* gene subcloned into pGEM-3 and linearized with Hind III. The *c-fos* (-21 to +387), *fra-1* (+1 to +827), *fra-2* (+495 to +945) and *fosB* (+1693 to +2136) fragments were subcloned into pcRII (Invitrogen Carlsbad, CA), and linearized with XhoI (*c-fos*, *fra-1* and *fosB*) or Bam HI (*fra-2*). Synthesis of radiolabeled RNA probes, prehybridization and hybridization procedures were performed as previously described [2]. Sections were air-dried and exposed to x-ray films together with ¹⁴C standards. The exposure time yielding signal intensities within the linear range of the standards (one day for *AVP*hnRNA, 2 days for *c-fos* and *fra-2*, 7 days for *fosB*, 14 days for *fra-1*) was determined after exposing the sections to films for various periods. Optic densities (OD) were measured with the public domain NIH Image developed at the US National Institutes of Health and freely available at <http://rsb.info.nih.gov/nih-image>. Changes in mRNA or hnRNA levels after HS injection were quantified by the measurements of the integrated OD (OD x area) of the film images. Some sections were dipped in NTB 2 nuclear emulsion (Kodak, Rochester, NY) and developed after 5 (*c-fos* and *fra-2*), 14 (*fosB*) and 30 (*fra-1*) days.

The SON of 6 rats in each group was punched out and quick-frozen in dry ice. The punched-out SON was combined in 2 rats and total protein lysates were prepared using the same volume of lysis buffer as previously described (Baler and Klein, 1995). The same volume of protein solution was then subjected to Western blot and gel shift assay. The ³²P-radiolabeled probe was either an AP-1 consensus element (Promega, Madison, WI), or a synthetic 37-base pairs (bp) long, double stranded (ds) oligonucleotide probe of the rat AVP promoter containing an AP-1 like region shown underlined as below: 5- GTGTCCCCAGATGCCTGAATCACTGCT **GACAGCTTGG**-3. The probe also contains a CRE-like site (in bold). The following affinity-purified rabbit polyclonal IgG (Santa Cruz Biotechnologies, Santa Cruz, CA) were used: SC253 (anti-pan-Fos), SC7202 (anti-c-Fos), SC605 (Fra-1), SC48 (anti-FosB), and SC197 (anti-Max). The Fra-2-specific antisera used is described in a previous study [28]. Either SC197 or a preimmune rabbit serum was used as control. Binding reactions were performed by incubating 2 µl (Hiroshi; indicate µg of

protein) SON protein solution with 1 μ l 32 P-radiolabeled probe (it would be better to indicate molar concentration of dpm), 20 mM HEPES, 0.05 mg/ml poly(dI-dC), 1 mM EDTA, 0.5 mM MgCl₂, 10% glycerol and 1mM DTT with or without 1 μ l each antibody (20 μ l total volume). For western blot analysis SON total protein extracts were separated on a 10% SDS-PAGE, transferred onto PVDF (Schleicher & Schuell Bioscience, Inc., Keene, NH) and exposed to a pan-Fos antibody as previously described [5].

Statistical significance of differences between groups was calculated by a one-way ANOVA followed by Fisher's PLSD test. Results are expressed as the mean $\pm$ SE (arbitrary units) of the fold changes over basal expression. Differences were considered statistically significant at $p < 0.05$.

We first monitored the levels of mRNA for the Fos family members *c-fos*, *fra-1*, *fra-2* and *fosB* after ip injection of HS, using *in situ* hybridization (Fig. 1). Levels of *c-fos* mRNA were undetectable under basal conditions, started to increase by 15 min, reached almost peak levels at 30-60 min, started to decline at 120 min and returned to basal at 240 min after HS injection. On the other hand, *fra-1* and *fra-2* mRNAs were detectable before stimulation, raised more slowly reaching peak levels about 120 min and returned to basal levels by 720 and 360 min, respectively. Levels of *fosB* mRNA rose from undetectable levels to a peak at 120 min, decreasing thereafter but remaining detectable for up to 720 min after HS injection. In contrast to the effect of HS, an ip injection of IS used as control treatment had no significant effect on the expression of any member of the Fos family, as measured 120 min after injection. Specificity of the probes was confirmed by the lack of detectable signals using sense probes (data not shown).

As shown in Fig. 2A, western blot analysis of SON proteins using an anti-pan-Fos antibody revealed three different bands with relative mobilities of ~55, 46 and 42 kDa. All three bands started to increase by 30 min and reached a maximum by 120 min after HS injection, with the 46 kDa species displayed the highest increment. While the upper and middle bands became undetectable by 360 min and 720 min respectively, the lower band remained higher than basal levels by 720 min. On the other hand, AVP hnRNA levels in the SON increased rapidly following HS injection, showing a peak between 15 and 60 min and started a slow decline by 120 min, but remaining elevated for at least 720 min after HS injection (Fig. 2B). To examine the properties of the AP1 complex induced by hypertonic saline injection, gel shift assays were performed using SON protein extracts and either a consensus AP1 oligonucleotide (Fig. 3A), or one containing the AP1 sequence present in the rat AVP promoter (Fig. 3B). In the absence of antibodies (control), shifted bands were low in basal conditions as shown by arrows in Fig. 3A and B, control lanes. These bands increased in intensity markedly by 120 and 240 min and declined to levels still above basal by 360 min. Addition of pan-fos antibody almost completely suppressed the AP1 band and generated a supershifted band at all time points, indicating that the antibody recognized all components of the AP1 complex or a component which is required for the formation of the complex. While there were no apparent supershifted bands with *c-Fos* antibody, the intensity of AP-1 bands was substantially decreased, in particular in the reaction with the AVP promoter oligonucleotides (Fig. 3B). In contrast, the *Fra-2*- antibody caused a marked supershift of AP-1 band at all time points, particularly at 120 and 240 min post injection. *FosB* antibodies decreased slightly the intensity of AP-1 bands at 240 and 360 min without apparent supershifted bands. On the other hand, *Fra-1* antibodies failed to modify the AP-1 band, having no effect on the AP1 band and yielding no supershifted band.

In the present study, we first investigated the *in vivo* correlation between the changes in AVP gene transcription and expression of the various Fos family members in the SON by *in situ* hybridization and western blot analysis. Second, we monitored specific interactions of

these endogenous complexes with consensus AP-1 or the non-canonical, putative AP-1/CRE binding sites in the AVP promoter, using an electromobility gel shift assay. The present demonstration that AVP transcription had reached a peak before any production of Fos proteins suggests that while the endogenous AP-1 complex produced following osmotic stimulation can bind to the AVP promoter to possibly affect AVP gene transcription, it was not prerequisite for the induction of AVP gene transcription in the SON.

It is well recognized that osmotic stimulation induces *c-fos* mRNA and its protein c-Fos in the magnocellular neurons after osmotic stimuli [7,11,13,15,19,26,27,31]. It has also been suggested that Fos-related antigens could be induced in the SON in the chronically hyperosmotic states [19,26]. Our results using *in situ* hybridization are consistent with these studies and clearly showed that the mRNA levels of all members of Fos family are increased after an acute osmotic stimulus, with different time courses. The western blot with anti pan-Fos antibody showed at least three different immunoreactive species being induced in the SON after osmotic stimulus. Based on previous studies [5], the three bands revealed by the western blot are likely to correspond to c-Fos protein (56 kDA) and Fra-2 isoforms (42 and 46 kDA). Consistent with the rapid and transient induction of *c-fos* mRNA shown by the present study and previous reports [7,13,15,26,27], the upper band probably corresponding to c-Fos protein in the western blot was already apparent at 30 min after ip injection of HS, and almost disappeared by 360 min. While the exact identity of the western blot band is unclear, it is evident from the data that activation of AVP transcription occurs much earlier than the appearance of AP1 proteins. Although it is not possible to rule out that the sensitivity of the western blot was not sufficient to detect small amounts of c-Fos, the data suggest that c-Fos is not essential for the induction of AVP transcription.

Previous studies in cell lines demonstrated that Fos proteins could bind to the AP-1-like and CRE-like sites in the AVP promoter [9,21,34]. We have extended these findings by showing that the endogenous AP1 complex has the capacity to bind to the putative AP-1/CRE sequence of the AVP promoter. Furthermore, the EMSA using an AP-1 consensus sequence as well as the AVP promoter showed clearly supershifted bands with Fra-2 antibodies, suggesting that Fra-2 is a dominant Fos member in the SON in response to an osmotic stimulus. While AP-1 complex expressed in cell lines reportedly increased AVP gene expression, our time course study with western blot and intronic *in situ* hybridization demonstrated that AVP gene transcription increased substantially in the absence of apparent induction of Fos proteins. Rather, Fra-2 proteins were most abundant at a point when AVP gene transcription showed the first signs of deceleration (Fig. 1). Thus, it is possible that Fra-2 might play a role in not inducing but suppressing AVP gene transcription after osmotic stimuli. While this is consistent with a previous study showing that Fra-2 is a potential negative regulator of gene transcription [29], further studies are warranted to prove this hypothesis.

In contrast to the clear induction of *fosB* and *fra-1* mRNA in the SON following ip hypertonic saline, the gel shift assay showed little or no supershift or decrease in the AP1 band. While, this suggests a lower expression of FosB and especially of Fra-1, from the present data it is not possible to rule out that these factors are present in the AP1 complex following ip hypertonic saline and that they may have a role in regulating AVP gene expression. It should be also pointed out that this study has only focused on Fos proteins. Given that the number of proteins that can potentially act at AP-1 sites is much larger [17,23,32], the system regulating AVP expression via AP-1/CRE sites will be, in all likelihood, more complex. However, the almost total obliteration of the AP1 band with the pan-Fos antibody suggests that the Fos family is essential for the formation of the complex, and that any participation of Jun or other AP1 members must be in conjunction with Fos.

In addition of being an osmotic stimulus, ip injection of hypertonic saline is a painful stress and increases expression of AVP in hypophyseotropic neurons (16). While noxious stimuli markedly affect AVP neurons in the dorsomedial region of the PVN, they do not usually vasopressinergic neuron activity in the SON. Thus, it is unlikely the induction of Fos family members shown in this study in the SON reflect responses to the painful component of the stimulus.

In conclusion, we demonstrated that the induction of expression of Fos family by osmotic stimulation does not precede but follows the induction of AVP transcription. Thus, the induction of AP1 peaks at a time when AVP gene transcription starts to decline in the SON. Although it is not possible to rule out that a small induction of Fos proteins could be involved in the activation of AVP transcription by osmotic stimulation in rats, the data suggest that members of Fos family have alternative roles in the magnocellular neuron function.

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Figure 1.

Time course of expression of Fos family mRNA in the SON after hypertonic saline (HS) injection. Rats were decapitated at 15, 30, 60, 120, 240, 360 and 720 min after an ip injection of HS, or 120 min after isotonic saline (IS) injection. Bars represent the mean $\pm$ S.E. (arbitrary units) of the values obtained from 6 rats in each experimental group. Representative images of SON in rats injected with IS and HS (60 min after injection for c-fos, 120 min after injection for fra-1, fra-2 and fosB) are also shown. *, p<0.05 versus control.

Figure 2.

Time course of expression of Fos family proteins relative to AVP gene transcription in the SON after HS injection. **A.** Western blot analysis performed with pan-Fos antibody on whole cell protein extracts from pools of SON punches collected at the indicated times from rats without treatment or injected with either isotonic saline (IS) or hypertonic saline (HS). **B.** Quantitative *in situ* analysis of AVP hnRNA levels in the SON of rats without treatment or injected with either IS or HS. Bars represent the mean $\pm$ S.E. arbitrary units of the values obtained from 6 rats in each experimental group. Representative images of AVP hnRNA expression at each time point are also shown. *, $p < 0.05$ versus values at 60 min.

Figure 3.

AP-1 electrophoretic mobility gel shift assay (EMSA) with antibodies for Fos members. EMSA was performed in the presence of either a pan-Fos antibody or specific antibodies raised against the c-Fos, Fra-1, Fra-2 or FosB, and using proteins extracted from the SON of rats injected with either isotonic saline (control) or hypertonic saline (120, 240 or 360 min) and a ^{32}P -radiolabeled probe containing either a classical AP-1 consensus element (**A**) or the AVP derived, 37-bp long sequence containing both the AP-1 and CRE-like sites (**B**). The figure is representative of 3 experiments. The arrow indicates specific AP-1 binding, of which intensity was quantified and shown as bar graphs. The levels of AP1 intensity in control at each time point are shown as dotted lines. *, supershifted bands in pan-Fos and Fra-2 lanes. ns: non-specific bands shown in only lanes where serum was applied instead of IgG.