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Mechanism of AVP release and synthesis in chronic salt-loaded rats

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Abstract

Arginine vasopressin (AVP) is involved in osmotic regulation in the brain and peripheral tissues. To elucidate the regulatory mechanism that involves AVP release in hyperosmolality, we investigated the regulation of the synthesis and release of AVP in chronic salt-loaded rats. In chronic salt-loaded rats, which were generated by free access to water containing 2% NaCl for 7 days, plasma osmolality was significantly increased compared with control value. When tested, the AVP content was significantly higher in plasma but lower in the pituitary and whole brain (hypothalamus, cortex and striatum) than in control rats. The expression of AVP mRNA in the brain was significantly up-regulated compared with that in control rats. These data lead to the suggestion that hyperosmolality stimulates AVP release from the brain and subsequently induces AVP synthesis in the brain. On the other hand, mRNA levels of vasopressin V_{1a} receptor ($V_{1a}R$), whose down-regulation is known to be a counteraction to the $V_{1a}R$ activation, was not changed in the brain, suggesting that the AVP seems not to interact with the $V_{1a}R$ in the brain. These results suggest that hyperosmosis promotes the release of AVP into plasma, the subsequent induction of AVP mRNA in the brain and its action on the peripheral tissues.

Introduction

Arginine vasopressin (AVP) is a peptide hormone which plays an important role in cardiovascular homeostasis through multiple actions including vasoconstriction, baroreceptor modulation, antidiuresis, platelet aggregation and adrenocorticotrophin release from the anterior pituitary (Jackson 1996). AVP is synthesized in the supra-optic and paraventricular nuclei of the hypothalamus and transported down axons to the sites of hormone storage and release in the pituitary. AVP release is subject to wide and rapid fluctuations in demand from the pituitary, in which there is a large storage pool of hormone available for immediate release (Johnston 1985).

Plasma levels of AVP are elevated in a number of disease states including diabetes (Brooks et al 1989), dehydration (Arnauld et al 1993) and hypertension (Trinder et al 1992). AVP actions are mediated via G-protein-coupled receptors classified into V_{1a}, V_{1b} and V_2. The V_{1a} receptor ($V_{1a}R$), one of the V_1 subtype, is found on platelets, and in the brain, liver and kidney (Morel & O'Carrol 1992). $V_{1a}R$ is widely distributed in the brain and has been proposed to mediate the effects of AVP on memory and learning, antipyresis, brain development, blood flow to the choroid plexus, cerebrospinal fluid production and regulation of smooth muscle tone in superficial brain vasculature. The plasma level of AVP is elevated in patients with major depression, which influences the activity of the hypothalamic–

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pituitary–adrenal axis (van Londen et al 1997). In gerbils, transient cerebral ischaemia leads to the increase of AVP content in brain and the down-regulation of V_1 receptors in the cerebral cortex (Liu et al 1991). Recently, our data suggest that hepatic $V_{1a}R$ in db/db mice is down-regulated accompanied by an increase in AVP mRNA level (Morita et al 2001).

In this paper, we examined regulation of AVP release and synthesis, particularly in the brain, and the involvement of $V_{1a}R$ in brain with chronic salt-loaded rats.

Materials and Methods

Animals

Male Sprague-Dawley rats (aged 7 weeks), weighing 176–191 g, were given free access to food and water. Each four rats were equally divided into a non salt-loaded group (control rats) or a salt-loaded group in which rats were given 2% NaCl in their water for 7 days.

Control and salt-loaded rats were weighed and killed by decapitation. Mixed arterial-venous blood from the trunk was collected in heparinized test tubes. The brain was immediately removed from the cranium. Hypothalamic, cortex and striatum regions were separately excised after removal of the pituitary stalk, cerebellum and brain stem (Jacobowitz 1974). In this study, whole brain is defined as total brain tissue removal of pituitary stalk, cerebellum and brain stem. Tissues were frozen in liquid nitrogen and kept frozen at −80°C until extraction. Brain tissues were homogenized in 1.0 mL of 0.1 M HCl using a Polytron (Kinematica GmbH, Westburg, NY). Homogenates were stored at −20°C until the AVP content was measured. A sample of the trunk blood was used for the determination of haematocrit, and the remainder was centrifuged at 3000 *g* for 5 min and the supernatant (plasma) was frozen at −20°C. The haematocrits were measured with Celltac A (Nihon Kohden, Tokyo, Japan). Plasma osmolalities were measured with Osmostat (Kyoto Dai-ichi Kagaku, Kyoto, Japan) on the day of experiment.

Northern hybridization

The cDNA clones coding for rat AVP and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were obtained by polymerase chain reaction (PCR) performed on rat brain cDNA. A set of primers were designed from the published sequence of the AVP mRNA supplemented with Apa I and Nsi I digestion site (Sense-Apa I: 5′-ATT GGG CCC TGC AGC GAT GAG AGC TGC GTG G-3′, bases 325–376; Antisense-Nsi I: 5′-GCT ATG CAT TTA TTT TCC ATG CTG TA-3′, bases 562–588) (Schmale et al 1983; Rehbein et al 1986). Primers of G3PDH (sense; 5′-TGA AGG TCG GTG TCA ACG GAT TTG GC-3′; antisense: 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′) were purchased from the Clontech (Palo Alto, CA) (Tso et al 1985). The PCR products of AVP (281 bp) and G3PDH (1053 bp) were subcloned into pGEM-T Easy Vector (Promega Co., Madison, WI) (pGEM-AVP) and TA Cloning vector (Invitrogen, NV Leek, The Netherlands) (pTA-G3PDH), respectively. The identities of the obtained clones were confirmed by nucleotide sequence analysis.

The probes of AVP and G3PDH for Northern hybridization were prepared by digesting the vectors with Nsi I and Apa I and with EcoR I, respectively. After purification by agarose gel electrophoresis, probes were labelled using DNA labelling kit (Takara, Kyoto, Japan) with [α-^{32}P]dCTP (Amersham, Buckinghamshire, UK). cDNA probes were purified by extraction with Nuctrap push columns (Stratagene, La Jolla, CA).

Northern hybridization procedures were carried out as described in Maniatis et al (Sambrook et al 1989). Total RNA was extracted from whole brain (except for pituitary stalk, cerebellum and brain stem) using TRIzol (Gibco BRL, Gaithersburg, MD). RNA samples (25 μg) were electrophoretically separated in denaturing 1% agarose gels and transferred to nylon membranes. Blots were prehybridized for 6 h at 42°C in hybridization buffer (0.75 M NaCl, 1% SDS (sodium dodecyl sulfate), 20 mM Tris-HCl (pH 7.5), 2.5 mM EDTA, 50% formamide and 2% Denhardt's solution (1 × Denhardt's solution: 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficol)) and subsequently hybridized with the ^{32}P-labelled AVP probe (3×10^5 count min^{-1} μL^{-1}) for 20 h at 42°C. Following hybridization, the blots were washed twice in 2 × SSC (saline sodium citrate) for 10 min at 25°C, twice in 0.5 × SSC plus 0.1% SDS for 30 min at 65°C and twice in 0.5 × SSC at room temperature. The washed nylon membrane was exposed in an Imaging Screen Cassette BI (Bio-Rad, Hercules, CA) overnight for AVP and for 2 h for G3PDH. The extent of mRNA levels was determined by measuring the pixel density unit (PDU) of autoradiographic images on the screen as determined by Molecular Imager System (Bio-Rad). Data was presented as the ratio of AVP extent to G3PDH extent. The blots were stripped for reprobing in boiling 0.1% SDS for 30 min for hybridization of another probe.

Measurement of vasopressin

Plasma AVP concentration and AVP contents in brain tissues were measured by radioimmunoassay as previously reported (Nagai et al 1996). AVP was extracted from samples with Sep-Pak C18 (Waters, Millipore Corp., Bedford, MA) and eluted with 50% acetonitrile in 0.1% trifluoroacetic acid. The eluates were evaporated, the residues were dissolved in 0.05 M phosphate buffer (pH 7.4). After tissue samples from the brain tissues were diluted with assay buffer (whole brain; 1:1071, pituitary; 1:102900, striatum and cortex; 1:10, hypothalamus; 1:10), the AVP levels were determined with a radioimmunoassay kit (AVP-RIA, Mitsubishi Kagaku, Tokyo, Japan).

Quantitative RT-PCR

For AVP, the sense primer (5′-TGCTTCGGGCCG-AGCATCTGC-3′) and antisense primer (5′-TGGCCC-GTCCAGCTGCGTGGCGTTGCTC-3′) correspond to bases 166–186 and 426–399 from ATG, respectively (261 bp) (Schmale et al 1983). For $V_{1a}R$, the sense primer (5′-TCATGATCGCCACCTCTTGGGTGCTGA-3′) and antisense primer (5′-TCATGCTATCGGAGTC-ATCCTTGGCGAA-3′) correspond to bases 506–532 and 1162–1135 from ATG, respectively (657 bp) (Morel & O'Carroll 1992). For G3PDH, the sense primer (5′-GCCTCGTCTCATAGACAAGATGGTGA-3′) and antisense primer (5′-CTCAGT ATCCTTGCTGGGC-TGGGTGGTCCA-3′) correspond to bases −19–+7 and 1040–1011 from ATG, respectively (1059 bp) (Nagai et al 1996). Each PCR product is detected as the anticipated single band in the agarose gel electrophoresis.

Synthesis of cDNA was carried out using reverse transcriptase (RT; Gibco BRL, Rockville, MD). Total RNA (5 μg) of an individual sample was mixed with oligo dT primers and incubated at 70°C for 10 min. After cooling for 1 min, samples were incubated with Superscript II enzyme for 1 h at 42°C in 20 μL assay buffer (40 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM Tris-HCl (pH 8.4), 500 μM dNTPs (deoxyribonucleoside 5′-triphosphates), 0.1 mg mL^{-1} bovine serum albumin). The reactions were stopped by heating at 72°C for 10 min, cooled down and incubated with RNase H (2 IU) at 37°C for 20 min. cDNA was diluted with 18.1 μL water (0.25 μg μL^{-1} total RNA) and stored at −20°C.

For PCR, PCR master mixes (20 μL) containing 500 μM dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.125 mM $MgCl_2$, 3.0 IU Taq DNA polymerase (Takara) were added to 2 μM of both primers, and 2 μL of the diluted RT reaction. After the initial melt step (94°C for 3 min), the cycles were carried out with PCR Thermal Cycler MP (Takara) as follows: melt step, 94°C for 1 min; annealing for 1 min at 60°C; extension for 2 min at 72°C. A final extension at 72°C was carried out for 7 min. Control without the addition of RT was run in parallel to rule out genomic amplification. A water blank instead of sample cDNA was included in each assay to detect any contamination.

Accurate quantitation by RT-PCR requires demonstration of an exponential range which varies among samples (Sanna et al 1993; Yokoi et al 1993). The exponential curve of PCR was generated with a varying number of cycles on a fixed amount of cDNA. The cycle number for quantitative RT-PCR in each gene was determined within exponential range of the PCR amplification (data not shown). The cycle numbers determined in AVP, $V_{1a}R$ and G3PDH were 20, 24 and 14, respectively. For the quantification of PCR product, 10 μL of each PCR product was electrophoresed on 2% agarose gel. The PCR products separated on the gels were visualized by staining with 2% SYBR Green (Molecular Probes, Leiden, The Netherlands). The extent of mRNA level was determined by measuring the optical density unit (OPU) of the SYBR green-staining band on the agarose gel as defined by a fluorescence imaging analyzer (Takara). The expression levels of AVP or $V_{1a}R$ mRNA are expressed as the ratio of G3PDH expression level.

Statistics

Data are expressed by means ± s.e.m. for four rats. Values were analysed with Student's *t*-test ($*P < 0.05$, $**P < 0.01$) compared with control rat group.

Table 1 Biological characterization of chronic salt-loaded rats.

	Control rats	Salt-loading for 7 days
Body weight (g)		
pre	244 ± 4	247 ± 6
post	311 ± 5	212 ± 11**
Haematocrit (%)	40.5 ± 0.9	48.3 ± 1.1*
Plasma osmolality (mPosm)	280 ± 1	344 ± 7**

Values are means ± s.e.m., n = 4; $*P < 0.05$, $**P < 0.01$ vs control group (Student's *t*-test).

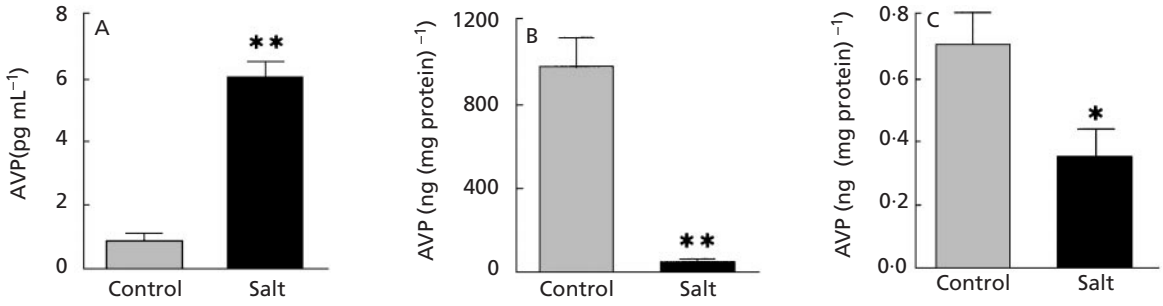

Figure 1 AVP levels in plasma (A), pituitary (B) and whole brain (except pituitary stalk, cerebellum and brain stem) (C) of chronic salt-loaded rats (Salt) and control rats. $*P < 0.05$, $**P < 0.01$ versus control group, with Student's *t*-test.

Table 2 Distribution of AVP in normal rat brain.

	AVP content (ng (mg protein)$^{-1}$)	AVP total content (ng (total tissue)$^{-1}$)
Whole brain	0.74	56.00
Cortex	0.0013	0.2000
Striatum	0.055	6.600
Hypothalamus	15	47
Pituitary	850	1100

Results

The state of salt-loading of rats was evaluated by comparing body weight, plasma osmolality and the haematocrit. A significant rise in plasma osmolality of chronic salt-loaded rats was observed compared with that of control rats (Table 1). The haematocrit was significantly elevated (Table 1). These data indicate that drinking 2% NaCl solution for 7 days resulted in significant weight loss, plasma hyperosmolality and hypovolaemia.

We compared the AVP contents of plasma, pituitary and brain between salt-loaded rats and control rats. The plasma AVP level was significantly higher in salt-loaded rats (Figure 1A) but the AVP content in the pituitary and whole brain (including striatum, hypothalamus and pituitary) was significantly lower (Figures 1B and 1C).

To estimate AVP distribution in whole brain of normal rats, the AVP content in the cortex, striatum, hypothalamus and pituitary was measured. As shown in Table 2, the AVP content in the pituitary was higher than in other brain sections. The AVP content in cortex and striatum was much lower than in pituitary and hypothalamus (Table 2). Whole brain section contains parts of cortex, striatum and hypothalamus, but not of pituitary. The total AVP content in whole brain and hypothalamus was 56 ng and 47 ng, respectively. These data suggested that a large part of AVP content in whole brain (except for pituitary) is derived from that in the hypothalamus.

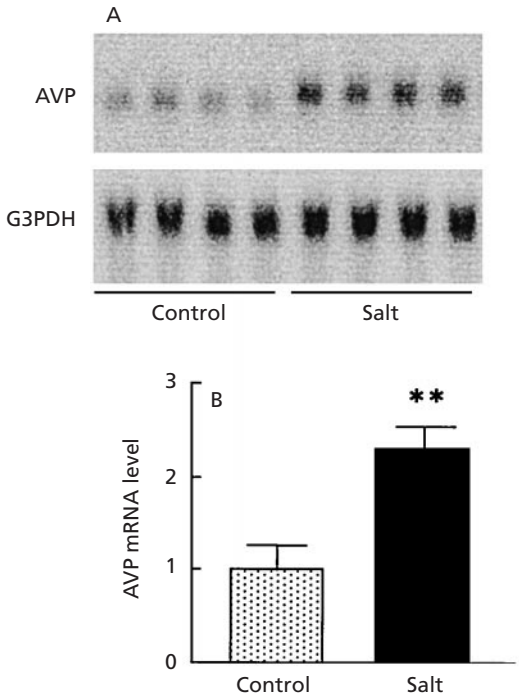

Figure 2 Northern blot analysis of mRNA levels of AVP in whole brain from total RNA (25 μg) of chronic salt-loaded rats (Salt) and control rats. A. Northern blotting pattern of AVP and G3PDH in each rat. B. Data quantified with Northern blotting image. The mRNA level was quantified as pixel density units (PDU) of AVP and G3PDH bands on Northern hybridization image. Data was presented as the ratio of AVP PDU to G3PDH PDU. The ratios were normalized with the value of control group. $*P < 0.05$, $**P < 0.01$ versus control group, with Student's *t*-test.

To evaluate the AVP mRNA level in salt-loaded rats, we performed Northern hybridization analysis. Bands of whole brain AVP mRNA were found at approxi-

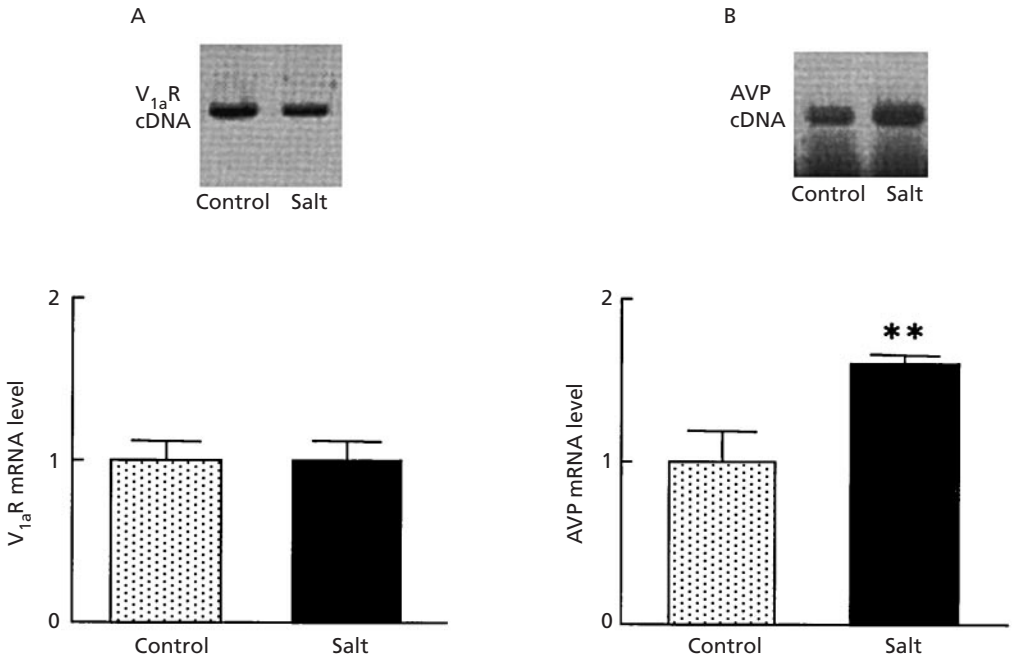

Figure 3 Quantitative RT-PCR analysis of mRNA levels of $V_{1a}R$ (A) and AVP (B) in chronic salt-loaded rats (Salt) and control rats. Upper panel shows representative pattern of agarose gel electrophoresis with SYBR green-stained PCR product from total RNA (0.25 μg) of rat whole brain. Lower panel shows comparison of mRNA expression level between control group and chronic salt-loaded rats (Salt). The normalized data is expressed as the ratio of mRNA level to G3PDH as described in Material and Methods. $^{**}P < 0.01$ versus control group, with Student's *t*-test.

mately 680 bp in the Northern hybridization pattern (Figure 2A). Northern hybridization image analysis revealed that whole brain AVP mRNA level was significantly high in salt-loaded rats (Figure 2B). AVP mRNA size in the whole brains from salt-loaded rats was increased compared with that in control rats (Figure 2A; upper panel). On the other hand, there was no difference in the size of whole brain G3PDH mRNA between two groups (Figure 2A; lower panel). The size changes in AVP mRNA bands were not due to gel or transfer artifacts.

The amount of $V_{1a}R$ mRNA in brain is so small that its level was detected only for a long exposure by Northern hybridization (Morel & O'Carroll 1992). RT-PCR is so sensitive that it can discriminate between very low abundance mRNA species. To estimate $V_{1a}R$ mRNA level in brain of salt-loaded rats and control rats, we performed quantitative RT-PCR. An insignificant difference between the two groups was seen in the whole brain $V_{1a}R$ mRNA level (Figure 3A). A single band of 657 bp was identified as the expected product size for $V_{1a}R$. On the other hand, the AVP mRNA level in the whole brain of salt-loaded rats was significantly higher than that of control rats (Figure 3B). These data were consistent with those of Northern hybridization (Figure 2A). The single PCR product of AVP gave the predicted 261 bp in length (Figure 4B). No genomic contamination was observed in any total RNA preparation as confirmed through the use of PCR primer pairs that were localized to different exons of the AVP and $V_{1a}R$ genes (data not shown). Rat G3PDH was used as the housekeeping gene for analysing relative differences in mRNA levels between two groups.

Discussion

The present data demonstrate that in rats freely drinking 2% NaCl solution for 7 days, AVP contents in both the whole brain and the pituitary were significantly decreased and the plasma AVP level was significantly increased compared with the control value. George (1976) reported that the AVP level of both neurohypophysial hormones in the neural lobe and hypothalamic nuclei was decreased in rats drinking hypertonic saline for 7 days. In contrast, Ciosek et al (1993) reported that in rats freely drinking 2% NaCl solution for 3 days, the hypothalamic AVP content was significantly raised

compared with the control value, but the neurohypophysial AVP content was somewhat decreased, although the decrease was not significant. Thus, there is no consistent finding in AVP content in the brain regarding rats given sodium chloride, possibly due to differences in experimental conditions such as the state of hyperosmolality. Our data were close to those of George et al (1976).

To examine the distribution of AVP in the brain of normal rats, we quantified the amount of AVP in brain. Pituitary, used for storage of AVP in the brain (Jackson 1996), preserved 95% of brain AVP. Our data also showed that, except for pituitary, the amount of AVP in the hypothalamus was the largest in whole brain. Thus, these data suggested that changes in AVP content in whole brain may be mainly reflected by those in hypothalamus. The actual AVP content in the hypothalamus is a result of two variables: neurohormonal synthesis rate and the rate of neurohormonal transport towards pituitary. Similarly, the actual AVP amount in the neurohypophysis (pituitary) results from both supply from the hypothalamus and release into the blood. In rats drinking hypertonic saline in our experiments, the decrease of neurohypophysial AVP content is due to enhanced constant hormonal release into the blood, sequentially leading to the decrease of AVP content in the hypothalamus by transportation to the pituitary.

Our data demonstrated that expression level and size of AVP mRNA in whole brain of the chronic salt-loaded rats were increased compared with those of control rats. The observations are in agreement with other Northern hybridization data (van Tol et al 1987; Pu et al 1995) and solution hybridization data (Burbach et al 1986). We carried out in-situ hybridization analysis, indicating that AVP mRNA was predominantly expressed in the supraoptic and paraventricular nuclei of the hypothalamus in normal rats (data not shown). Therefore, the augmentation of AVP mRNA level in the whole brain (Figures 2 and 3) may be mainly derived from that in hypothalamic neurons. These data suggest that AVP mRNA level was increased in the supraoptic and paraventricular nuclei of the hypothalamus in chronic salt-loaded rats. It is plausible that hypovolaemic and hypertensive stimulation gives rise to AVP release, and changes the length of the brain AVP mRNA response in order to complement amount of stored AVP. This means an increase in the poly (A) tail of AVP mRNA after osmic stimulation in rats. The elongation of the poly (A) tail is considered to be involved in the stability of mRNA (Carrazana et al 1988; Carter & Murphy 1991). In this study, bands of AVP mRNA in the chronic salt-loaded rat migrated closer to an origin, suggesting an elongation of the poly (A) tail of AVP mRNA. Therefore, it is suggested that elongation of the poly (A) tail occurs under conditions of chronic salt-load, leading to more stable mRNA.

The mechanism whereby AVP gene expression in chronic salt-loaded status is enhanced is not well understood. However, the following explanation may be possible. Decreased AVP content in the pituitary gland due to an intensive secretory stimulus may facilitate its biosynthesis. A stimulation, which excites magnocellular neurons in the hypothalamus to secrete AVP, may also simultaneously enhance the process for AVP mRNA transcription as a feed-back mechanism. On the other hand, our chronic osmotic stimulation with oral NaCl load for 7 days continuously increased plasma osmolality and decreased plasma volume. In rats, chronic hyperosmotic and hypovolaemic stimulation is thought to induce constant release of AVP from the pituitary into blood, transfer of AVP from the hypothalamus to pituitary and synthesis of AVP mRNA and AVP peptide in the hypothalamus.

It is known that the regulation of mRNA level of receptors is induced by receptor–ligand interactions (Trinder et al 1992). The level of receptor mRNA would give us the information on receptor–ligand interaction. $V_{1a}R$ is the major vasopressin receptor in brain. In adrenalectomized rats given corticosteroid, the plasma AVP level is increased and $V_{1a}R$ is decreased (Patchev & Almeida 1995). We are interested in the fluctuation of $V_{1a}R$ mRNA expression in the brain of chronic salt-loaded rats. Our present finding, that the level of brain $V_{1a}R$ mRNA was not changed in chronic salt-loaded rats, suggests that AVP would not mainly act on the brain in chronic salt-loaded rats.

In conclusion, hyperosmolality by chronic salt-loading results in the involvement of the cascade of release and synthesis of AVP (i.e., the increased level of plasma AVP and the decreased contents of hypothalamus and pituitary AVP) and the up-regulation of brain AVP mRNA level. The discrepancy of change of AVP levels in plasma and in brain tissue suggests that the rate of AVP released from the brain is greater than the rate of AVP synthesis in the brain. The $V_{1a}R$ expression data suggest that AVP released from the brain seems to act on peripheral tissues via the circulation in blood but not in brain tissue.

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