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BiP mRNA expression is upregulated by dehydration in vasopressin neurons in the hypothalamus in mice

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ABSTRACT

The immunoglobulin heavy chain binding protein (BiP) is an endoplasmic reticulum (ER) chaperone that facilitates the proper folding of newly synthesized secretory and transmembrane proteins. Here we report that BiP mRNA was expressed in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus in wild-type mice under basal conditions. Dual *in situ* hybridization in the SON and PVN demonstrated that BiP mRNA was expressed in almost all the neurons of arginine vasopressin (AVP), an antidiuretic hormone. BiP mRNA expression levels were increased in proportion to AVP mRNA expression in the SON and PVN under dehydration. These data suggest that BiP is involved in the homeostasis of ER function in the AVP neurons in the SON and PVN.

1. Introduction

The immunoglobulin heavy chain binding protein (BiP), also referred to as the 78-kDa glucose regulated protein (GRP78), is one of the most abundant endoplasmic reticulum (ER) chaperones [6,12,23]. BiP binds to newly synthesized secretory and transmembrane proteins, and facilitates protein folding by recognizing unfolded polypeptides [11]. BiP also binds to misfolded proteins with transport from the ER blocked, maintains them in a state to be refolded correctly, and prevents their aggregation [11]. The perturbation of the homeostasis of the ER results in the accumulation of unfolded proteins in the ER lumen (*i.e.* ER stress), and consequently triggers responses known as the unfolded protein response (UPR) [28]. While BiP is bound to the ER stress transducers such as inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6), and protein kinase RNA-like ER kinase (PERK) under nonstress conditions, it preferentially binds to unfolded proteins and dissociates from the transducers under ER stress conditions, leading to the activation of UPR [4,31]. Since the expression of BiP is reportedly increased under ER stress [11,17,18], the induction can serve as a general indicator of ER stress as well as UPR being triggered [19].

Arginine vasopressin (AVP), an antidiuretic hormone that promotes the reabsorption of water from the kidney, is synthesized in the magnocellular neurons of the supraoptic nucleus (SON) and paraventricular nucleus (PVN) in the hypothalamus [5]. The synthesis and release of AVP are regulated physiologically by plasma osmolality and blood volume or pressure [2,10,15,16,32]. The process from synthesis to release includes transcription, translation of mRNA, folding of the precursor within the ER, cleavage of the precursor during axonal transport, and release of AVP into the systemic circulation [7,8]. The failure of the process could cause a depletion of the hormone, a disorder called diabetes insipidus, in which the urine volume could increase as much as 10 L/day in humans; ER stress has been implicated in the pathogenesis of some genetic types of the disease such as familial neurohypophysial diabetes insipidus (FNDI) [3,9,13,14,22] and Wolfram syndrome [27]. Apart from these genetic diseases, however, it remains unclear whether the AVP neurons might be subjected to ER stress in any physiological conditions, and if so, how the neurons cope with it.

In the present study, we examined the expression of BiP mRNA in the AVP neurons in the SON and PVN in wild-type mice, and

Abbreviations: BiP, immunoglobulin heavy chain binding protein; GRP78, 78-kDa glucose regulated protein; ER, endoplasmic reticulum; UPR, unfolded protein response; AVP, arginine vasopressin; SON, supraoptic nucleus; PVN, paraventricular nucleus; OT, oxytocin; CRH, corticotropin-releasing hormone.

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also evaluated the changes in BiP mRNA expression in comparison with AVP mRNA expression under water-deprivation conditions. Since a previous study demonstrated that increased AVP mRNA levels caused by dehydration remained elevated for a relatively long period [36], we employed repeated water-deprivation in the present study in order to further increase AVP mRNA levels.

2. Materials and methods

2.1. Animals

C57BL/6J male mice (Chubu Science Materials, Nagoya, Japan) were maintained under controlled conditions (23.0 ± 0.5 °C, lights on 9:00 to 21:00). All procedures were approved by the Animal Experimentation Committee of the Nagoya University Graduate School of Medicine, and performed in accordance with the institutional guidelines for animal care and use.

2.2. Water deprivation

Mice were divided into 4 groups: (1) those with access to water *ad libitum* throughout the experiments (control group), (2) those subjected to water deprivation for 48 h, (3) those subjected to water deprivation for 48 h once a week for 2 weeks, and (4) those subjected to water deprivation once a week for 4 weeks. Mice in dehydrated groups were sacrificed immediately after the last 48-h water deprivation. All mice were sacrificed at the age of 12 weeks.

2.3. Brain collection for in situ hybridization and immunohistochemistry

Mice were anesthetized with diethyl ether and then by intraperitoneal injection of pentobarbital sodium (50 μg/g, Abbott Laboratories, Abbott Park, IL) and transcardially perfused with a cold fixative containing 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4. After fixation, brains were removed and immersed in the same fixative for 2 h at 4 °C. The brains were kept in phosphate-buffered saline (PBS) containing 20% sucrose at 4 °C for cryoprotection. They were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical, Tokyo, Japan) and stored at −80 °C until sectioning. Brains were cut into 16 μm sections on a cryostat at −20 °C; then, according to the brain atlas [25], slices at −0.7 mm caudal from the bregma were thaw-mounted on Superfrost Plus microscope slides (Matsunami, Tokyo, Japan), and stored at −80 °C until either *in situ* hybridization or immunohistochemistry was performed.

2.4. Probes for BiP, AVP, oxytocin (OT), and corticotropin-releasing hormone (CRH) mRNA for in situ hybridization

The BiP exonic probe was constructed from a 922-bp fragment containing bases 852–1773 of the mouse BiP cDNA subcloned into pCRII TOPO (Invitrogen, San Diego, CA). The antisense probe was linearized by XhoI and the sense probe was linearized by HindIII. The AVP exonic probe constructed from a 200-bp fragment of the rat AVP cDNA was kindly provided by Dr. Harold Gainer [National Institutes of Health (NIH), Bethesda, MD]. A pGEM-3Z plasmid containing a 476-bp SstI fragment of rat OT gene was kindly provided by Dr. W. Scott Young (NIH). The CRH exonic probe constructed from a 408-bp fragment of the rat CRH cDNA was the kind gift of Dr. Kelly E. Mayo (Northwestern University, Evanston, IL). Highly specific antisense probes of BiP, AVP, OT, and CRH mRNA and the sense probe of BiP mRNA were synthesized as described previously [14]. SP6 RNA polymerase was used for antisense probes of BiP, AVP, and OT mRNA, and T7 RNA polymerase was used for antisense probe of CRH mRNA and sense probe of BiP mRNA. The digoxigenin-labeled antisense RNA probe from AVP cDNA templates was prepared by using digoxigenin-UTP (Roche Diagnostics, Indianapolis, IN).

2.5. In situ hybridization and quantification

Prehybridization, hybridization, and posthybridization procedures were performed as described previously [30]. To visualize digoxigenin staining, sections were rinsed twice in buffer 1 (100 mM Tris–HCl and 150 mM NaCl, pH 7.5) for 5 min, followed by 30 min in buffer 1 containing 3% normal goat serum and 0.3% Triton X-100, before overnight incubation with alkaline phosphatase-conjugated antidigoxigenin antiserum (Roche Diagnostics, 1:1500 in 3% normal goat serum/0.3% Triton X-100 in buffer 1). Sections were consecutively rinsed in buffer 1 for 10 min, in buffer 2 (100 mM Tris–HCl, 100 mM NaCl, and 50 mM $MgCl_2$, pH 9.5) for 5 min, and incubated for 75 min at 37 °C in the dark in buffer 2 containing 0.34 mg/ml nitroblue toluidinium salt (Roche Diagnostics). Sections were rinsed four times in buffer 2, dipped briefly in distilled water and 70% ethanol, and air-dried.

Sections were exposed to Kodak BioMax MR film (Kodak, Rochester, NY) for various periods yielding appropriate signal intensities. The expression levels of BiP and AVP mRNA in the SON and PVN were quantified by measurements of the integrated optimal densities (optic densities × area) of the film images using ImageJ software (http://rsb.info.nih.gov/ij/).

Adjacent sections hybridized with antisense probes for BiP, AVP, OT and CRH mRNA, or sense probe for BiP mRNA were dipped in Kodak Autoradiography Emulsion Type NTB (Kodak) and developed after the exposure for 14 h (AVP mRNA in SON and PVN), 17 days [AVP mRNA in suprachiasmatic nucleus (SCN)], 24 days (OT and CRH mRNA), or 33 days (BiP mRNA antisense and sense probes). The sections hybridized with digoxigenin-labeled RNA probe for AVP mRNA and ^{35}S-labeled RNA probe for BiP mRNA were dipped in Ilford Nuclear Research Emulsion K5D (Polysciences, Warrenton, PA) and developed after the exposure for 7 days.

The number of cells stained with digoxigenin for AVP mRNA was counted on both sides of SON and PVN in each mouse, and cells were considered to express BiP mRNA when the grains of BiP mRNA were more than three-fold the background density.

2.6. Immunohistochemistry

Slides were washed in 0.1 mol/l PBS, followed by incubation in blocking solution (20% normal goat serum in PBS) for 30 min. The tissues were then incubated with rabbit anti-BiP antibody (Ab21685; Abcam, Cambridge, MA) at 1:100 solution in PBS with 0.3% Triton X-100 and 1% normal goat serum overnight at 4 °C. The sections were rinsed with PBS for 15 min and incubated with biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) at 1:200 in PBS for 3 h at room temperature (RT). After being rinsed, tissues were incubated in avidin–biotin complex solution (1:100; Vector Laboratories) for 90 min at RT, and then immersed in PBS containing 0.1% 3,3′-diaminobenzidine dihydrochloride (Sigma–Aldrich, St. Louis, MO). Antibody-binding sites were made visible by adding 0.004% hydrogen peroxide.

2.7. Statistical analysis

The statistical analyses were performed with one-way ANOVA, followed by Fisher's protected least significant difference test. Results are expressed as means ± SE, and differences were considered statistically significant at $p < 0.05$.