Chronic Valproate Treatment Increases Expression of Endoplasmic Reticulum Stress Proteins in the Rat Cerebral Cortex and Hippocampus

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Background: Sodium valproate is a highly effective treatment for bipolar disorder, but its mechanism of action remains poorly understood. We recently found with differential display polymerase chain reaction that valproate regulates the expression of the endoplasmic reticulum stress protein GRP78 in the rat cerebral cortex. In our study, we investigated the effect of this drug on the other members of the endoplasmic reticulum stress protein family, GRP94 and calreticulin, and we studied the brain regional distribution of GRP78, GRP94, and calreticulin.

Methods: Immunohistochemistry was used to measure protein levels of GRP78, GRP94, and calreticulin after treatment with sodium valproate (300 mg/kg, intraperitoneal) in specific rat brain regions.

Results: We report here that chronic treatment with valproate also increased expression of other members of the endoplasmic reticulum stress protein family, such as GRP94 and calreticulin. The brain regional distribution of these changes was similar for all three proteins, with marked increase detected in the frontal cortex, parietal cortex, and CA1 region of the hippocampus.

Conclusions: Because GRP78, GRP94, and calreticulin possess molecular chaperone activity and bind Ca\(^{2+}\) in the endoplasmic reticulum, the pharmacologic action of valproate may involve one or more of these processes. Biol Psychiatry 2000;48:658–664 © 2000 Society of Biological Psychiatry

Key Words: GRP78, GRP94, calreticulin, mood stabilizers, valproate, anticonvulsants

Introduction

Sodium valproate (VPA) is a first-line treatment for bipolar disorder (BD), with a broad spectrum of efficacy in this disorder (Davis et al 2000; Keck et al 1998; Tohen and Grundy 1999). Despite extensive investigation, however, the mechanism of its therapeutic action is still poorly understood. Earlier studies found that chronic treatment with VPA regulated the expression of several genes such as Na\(^+\) channel subunit (Yamamoto et al 1997) and myristoylated alanine-rich C kinase substrate (MARCKS; Lenox et al 1996). Using differential display polymerase chain reaction, we recently reported that chronic treatment with VPA increased mRNA and protein expression of 78-kilodalton glucose-regulated protein (GRP78) in rat brain (Wang et al 1999). This suggests a novel and potentially clinically relevant mechanism through which this drug may work.

GRP78, along with GRP94 and calreticulin, are gene products induced as part of the stress response in eukaryotic cells. They play an important role in the cellular response to stress and are involved in endoplasmic reticulum (ER) glycoprotein trafficking, molecular chaperone activity, and ER Ca\(^{2+}\) binding (Brostrom et al 1998; Kim et al 1987; Little et al 1994; Wooden et al 1991). Because these proteins act in concert, it was of interest to study the effects of VPA on other members of the ER protein family. Moreover, understanding the brain regional distribution of any changes in expression is critical to clarifying the functional significance of these drug effects. In the present study, we used immunohistochemistry to study GRP78, GRP94, and calreticulin expression in rat brain after chronic treatment with VPA and report here brain-specific changes in all three members of the ER stress protein family.

Methods and Materials

Animals and Drug Treatment

Male Sprague–Dawley rats (150–170 g) were housed two per cage and maintained on a 12-hour light/dark cycle with food and water freely available. Rats were divided into 2 groups: control animals (n = 6) and VPA-treated animals (1 day [1d], 7 days [7d], and 14 days [14d], n = 6 for each time point). Rats were injected intraperitoneally (IP) daily with saline or VPA (300 mg/kg).

After treatment, rats were anesthetized with sodium pentobar-
bital (60 mg/kg) and perfused transcardially with 250 mL of 100 mmol/L phosphate buffer (pH 7.5) at room temperature. Brains were removed, rapidly frozen in −80°C and serial 20 μm coronal sections (from Bregma −2.30 mm to Bregma −4.80 mm) prepared at −18°C. The sections were thaw-mounted on poly-L-lysine-coated glass slides. Each slide contained corresponding sections from one animal in each treatment groups (control, 1d, 7d, and 14d) to allow comparisons across groups and minimize differences in background.

**Immunohistochemical Staining**

Immunohistochemistry was carried out using the streptavidin-peroxidase method (Elias et al 1989; Ozaki et al 1997). Briefly, sections were treated with 0.3% Triton X-100/phosphate-buffered saline (PBS) and incubated in 3% H2O2/methanol to block endogenous peroxidase. The sections were preincubated with normal serum and then incubated at 4°C for 48 hours with specific primary antisera that recognize GRP78 (Stressgen Biotechnologies, Victoria, Canada), GRP94 (Stressgen Biotechnologies), and calreticulin (Upstate Biotechnology, Lake Placid, NY). These primary antisera were used at dilutions of 1:800, 1:800, and 1:1000 for GRP78, GRP94, and calreticulin, respectively. The sections were then incubated for 30 min with biotinylated secondary antibody (Zymed, South San Francisco, CA), and finally with streptavidin-horseradish peroxidase (HRP) conjugate for 30 min at room temperature. The peroxidase reaction was carried out with a DAB Kit (Zymed) according to the manufacturer’s protocol. The specificity of immunoreactivity was tested by incubating rat brain sections with no primary antibody or after the addition of nonimmune rabbit serum in which no immunostaining was observed.

**Quantitative Image Analysis**

Immunohistochemically stained sections were examined at 50× magnification by creating a digitized image with a microcomputer imaging device (MCID) image analysis system (Brock University, St. Catherines, Canada) attached to a light microscope (Zeiss Axioskop, Oberkochen, Germany) with a high-resolution charge-coupled device (CCD) camera (MTI CCD 72). The density of immunoreactive positive neurons in frontal cortex, parietal cortex, dentate gyrus, and CA1 and CA3 of hippocampus were measured using standard landmarks. Background values were obtained at a ten small boxes cursor in the molecular layer of cortex. The density of immunostaining in different regions was expressed as relative optical density (ROD), which was the density of each region divided by the background value of the same section. Six sections from each animal per each antibody through each region of interest were counted, and the same regions were compared as closely as possible between animals.

**Statistical Analysis**

Data were presented as the mean ± SEM. from six independent experiments. Statistical analysis of data was performed with one way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test.

**Results**

GRP78, GRP94, and calreticulin immunoreactivity were restricted to the cytoplasm of neurons with substantial overlap across the expression pattern for each protein (Figure 1). In saline-treated rats, immunoreactive positive neurons of GRP78, GRP94, and calreticulin were distributed throughout the entire brain. The pyramidal cells in the parietal cortex showed particularly strong immunoreactivity to GRP78, GRP94, and calreticulin when compared with other cells (Figures 2, 3, and 4). The immunoreactivity of GRP94 and calreticulin was relatively weaker in
dentate gyrus compared with CA1 and CA3 regions of hippocampus (Figures 3 and 4).

GRP78 immunoreactivity was significantly increased (Figure 5A) by intraperitoneal injection of VPA for 1, 7, and 14 days in both frontal and parietal cortex ($p < .01$). Sodium valproate increased GRP78 expression by 30%, 38%, and 28% in the frontal cortex and 21%, 21%, and 17% in the parietal cortex. Although VPA treatment for 1, 7, and 14 days had no effect on GRP78 expression in CA3 region of hippocampus, VPA significantly increased GRP78 expression in CA1 and dentate gyrus ($p < .05$). VPA increased GRP78 expression by 38%, 27%, and 20% at 1, 7, and 14 days in CA1; by 46% and 43% at 1 and 7 days in dentate gyrus ($p < .01$).

Figure 2. GRP78 immunoreactivity in different brain regions of the rat treated with sodium valproate (VPA). Rats were treated with saline (a, e, and i) and VPA (300 mg/kg, intraperitoneal) for 1 day (b, f, and j), 7 days (c, g, and k), and 14 days (d, h, and l). Arrowheads point to pyramidal cells. Magnification 100×. DG, dentate gyrus.
Similarly, calreticulin immunoreactivity was significantly increased (Figure 5B) after intraperitoneal injection of VPA for 1, 7, and 14 days in frontal cortex, parietal cortex, CA1, and dentate gyrus, but not in the CA3 region. VPA increased calreticulin expression by 22%, 38%, and 5% at 1, 7, and 14 days in frontal cortex ($p < .01$); by 18%, 29%, and 16% at 1, 7, and 14 days in parietal cortex ($p < .01$); by 39%, 38%, and 25% at 1, 7, and 14 days in CA1 ($p < .05$); and by 30% and 28% at 1 and 7 days in dentate gyrus ($p < .05$).

Finally, intraperitoneal injection of VPA for 1, 7, and 14 days significantly increased GRP94 immunoreactivity in all five measured regions including frontal cortex, parietal cortex, CA1, CA3, and dentate gyrus. VPA increased GRP94 expression by 38%, 29%, and 27% at 1 and 7 days, and 14 days in frontal cortex ($p < .05$); by 24%, 28%, and 35% in parietal cortex; by 39%, 38%, and 25% in CA1; and by 30% and 28% at 1 and 7 days in dentate gyrus.

Figure 3. GRP94 immunoreactivity in different brain regions of the rat treated with sodium valproate (VPA). Rats were treated with saline (a, e, and i) and VPA (300 mg/kg, intraperitoneal) for 1 day (b, f, and j), 7 days (c, g, and k), and 14 days (d, h, and l). Arrowheads point to pyramidal cells. Magnification 100×. DG, dentate gyrus.
at 1, 7, and 14 days in parietal cortex ($p < .05$); by 53%, 49%, and 55% at 1, 7, and 14 days in CA1 ($p < .05$); and 32%, 35%, and 41% at 1, 7, and 14 days in dentate gyrus.

**Discussion**

We previously demonstrated that VPA increased GRP78, one of the ER stress proteins in rat cerebral cortex (Wang et al 1999). To further study the effect of VPA on the closely related ER stress proteins, we used immunohistochemical staining with specific antisera to determine GRP78, GRP94, and calreticulin expression and report here that VPA increased GRP78, GRP94, and calreticulin expression in frontal cortex, parietal cortex, CA1, and dentate gyrus. Because this drug increases ER stress proteins, this suggest that it may
regulate the cellular response to stress to bring about its therapeutic effects.

ER stress proteins function in ER glycoprotein trafficking, have molecular chaperone activity, and protect cells from the deleterious effects of damaged proteins by binding and disposing of malfolded protein (Kim et al. 1987; Little et al. 1994; Wooden et al. 1991). ER stress proteins are also Ca\(^{2+}\) binding proteins and play an important role in maintaining ER Ca\(^{2+}\) homeostasis (Lièvremont et al. 1997). Because VPA appears to increase expression of ER stress proteins that perform these particular processes, our findings suggest that VPA treatment may target one or more of these processes. Although the mechanism of the regulation of ER stress proteins by VPA is not yet known, it is possible that this regulation is important in the pharmacologic action of VPA.

The effect of VPA on ER stress protein expression may also be relevant to the pathophysiology of BD. It has been known that intracellular free Ca\(^{2+}\) in peripheral blood cells was increased in BD subjects (Dubovsky et al. 1992). Increasing evidence suggests that altered polyphosphoinositide (PI)-generated second messenger signaling occurs in blood cells and brain tissue obtained from BD patients, which may relate to these increased intracellular Ca\(^{2+}\) levels (Jope et al. 1996; Wang et al. 1997). Under these conditions, an increase in ER stress proteins might be expected to bind ER Ca\(^{2+}\) and prevent depletion of ER stores due to increased PI signaling and in turn lower intracellular Ca\(^{2+}\) levels. Some recent evidence suggests that VPA inhibits 5-hydroxytryptamine-induced Ca\(^{2+}\) influx in C6 glioma cells (Yamaji et al. 1996), consistent with this hypothesis.

VPA-induced increases in ER stress proteins may have a neuroprotective role in specific brain regions in which cellular damage may occur in BD. Although there is no direct evidence to support this hypothesis, there is increasing interest in the role of ER stress proteins in central nervous system (CNS) disease and injury. GRP78 expression is induced after CNS injuries (Lowenstein et al. 1994), such as seizures, global ischemia, and acute trauma, and in neurodegenerative diseases like Alzheimer’s (Hamos et al. 1991). ER stress proteins induction by chronic VPA treatment may play a neuroprotective role by clearing malfolded proteins.

In conclusion, chronic treatment with the mood stabilizer VPA upregulates expression of ER stress proteins GRP78, GRP94, and calreticulin in rat cerebral cortex and hippocampus, which may have particular relevance for the prophylactic effect of VPA in the long-term management of patients with BD. The molecular chaperone and Ca\(^{2+}\) binding activities of ER stress proteins may be involved in the pharmacologic action of VPA.

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**References**


