Original Article

Valproic Acid Inhibits NA-K-2CL Cotransporter RNA Expression in Male But Not in Female Rat Thymocytes

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Abstract

Objective: The NKCC1 is a recognized tumorigenesis marker as it is important for tumor cell proliferation, differentiation, apoptosis, and tumor progression. The study aim was to investigate the effect of sodium valproate (VPA) on thymus NKCC1 RNA expression.

Material and Methods: Wistar rats, age 4 to 5 weeks, were investigated in the control and VPA-treated male and female gonad-intact and castrated groups. The treatment duration with VPA 300 mg/kg/d was 4 weeks. Rat thymus was weighted; its lobe was taken for the expression of NKCC1 RNA determined by the real-time polymerase chain reaction method.

Results: The RNA expression of the *Slc12a2* gene was found to be significantly higher in the gonad-intact male control compared with the gonad-intact female control ($P = .04$). There was a gender-related VPA treatment effect on NKCC1 RNA expression in thymus: The *Slc12a2* gene RNA expression level was found to be decreased in VPA-treated gonad-intact males ($P = .015$), and no significant VPA effects were found in the castrated males and in the gonad-intact and castrated females compared with the respective controls ($P > .05$).

Conclusions: The study showed a gender-related difference in the NKCC1 RNA expression in rat thymus. The VPA decreases the NKCC1 expression in the thymus only in gonad-intact male rats. The NKCC1 RNA expression downregulation by VPA could be important for further VPA pharmacological studies in oncology.

Keywords

valproic acid, thymus, NKCC1, gender, rat

Introduction

The valproic acid (VPA) is a histone deacetylase inhibitor.[1] The VPA alters the expression of genes, suppresses cell proliferation, inhibits cell growth through cell-cycle arrest, and increases apoptosis.[2-4] It belongs to a class of potent epigenetic modulators, acts as an immunomodulator, and is a potential investigational product for cancer treatment: The nonclinical data suggest that the VPA could be used in combination with several hormonal, cytotoxic and immunotherapeutic agents, and radiation therapy as an adjunctive product and with the existing therapies to increase the treatment effectiveness, disease-free survival, and to decrease resistance emergence in the cancer therapy.[5-8] The VPA was approved by the Competent Authorities as an investigational medicinal product for 84 clinical trials treating different cancers.[9]

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The valproic acid suppresses proliferation in vitro of T cells,[10] reduces lymphoproliferation,[11] and activates the lymphocyte apoptosis.[12] The VPA treatment for 8 weeks significantly reduces the spleen and lymph node weight and cellularity compared to control in MRL/lpr(−/−) mice females.[11] Thymus weight reduction was induced after VPA prenatal exposure in male but not in female newborn rats.[13] Age-related thymus involution depends on sex hormones.[14] The VPA decreases the rat thymus weight concomitantly with increasing in thymus the number of Hassall's corpuscles, which is gonad hormone dependent.[15] Hassall's corpuscles represent the terminal stage of thymic medullary epithelial cell differentiation[16,17] and participate in the removal of apoptotic or the maturation of thymocytes.[18,19] Thymocytes and thymic epithelial cells possess functional androgen receptors.[20] Many aspects of the VPA treatment significance in gender-related pathophysiological processes have not been elucidated.

The experimental data indicate that VPA has aquaretic and saluretic effects in rats: Alongside its diuretic effect, VPA enhances Na^+, Cl^-, and K^+ excretion with 24-hour urine.[21,22] Also, VPA significantly increases the urinary excretion of magnesium ions.[23] The abovedescribed saluretic effects of VPA on urinary ion excretion could be characteristic for the Na-K-2Cl (NKCC2) inhibition in rat kidney because NKCC2 inhibitors increase urinary monovalent as well as divalent cation excretion.[24] The NKCC1 is responsible for sodium and chloride (Cl^-) influx into thymocytes.[25] The intracellular Cl^- concentration would be one of the critical messengers in cell proliferation and differentiation processes.[26,27]

The abovedescribed facts induce the aim to test the VPA pharmacological effect on NKCC1 RNA expression in the rat thymus. The article presents data on the pharmacological VPA effect as the inhibition of NKCC1 RNA expression in rat thymocytes after the repeated VPA dosage treatment that is gender related.

Materials and Methods

Study Design

The effect of the VPA treatment on the thymus was investigated in the following 8 groups of age-matched Wistar rats of both genders: gonad-intact and castrated male and female controls and in respective male and female VPA-treated groups. The permission was obtained from the State Food and Veterinary Service of Lithuania to use experimental animals for research (2015-05-18 No. G2-28). The animals were purchased from the Animals Facility of the Veterinary Academy at the Lithuanian University of Health Sciences (Kaunas, Lithuania). The experiment was carried out at the Animal Research Center at the Lithuanian University of Health Sciences (Kaunas, Lithuania). The animals were housed in standard colony cages with free access to food, in the conditions of constant temperature (21°C [1°C]), humidity, and the light/dark cycle (12-hour/12-hour). A commercial pellet diet was provided ad libitum. The experiments were performed in compliance with the relevant laws and institutional guidelines for animal care in order to avoid any unnecessary animal distress.

For the experiment, 4 to 5 weeks aged Wistar rats were selected with the same animal number ($n = 6$) in the groups; there was no difference in rat weight among the formed groups. In the animal groups selected for castration, the male orchidectomy and female ovariectomy operations were performed. The castration was performed at the age of 28 (2) days (in the peripubertal period of animals). The accommodation period after the castration was 1 week. After the accommodation period, the treatment of gonad-intact and castrated animals was started. At the end of the experiment, one castrated VPA-treated female was eliminated from the study due to a fistula formed after the operation and significant weight loss.

Treatment with VPA aqueous solutions (300 mg/kg/d) in drinking water was used. The only source of drinking was the VPA solution for treated groups, and fresh tap water was provided for the control groups; VPA solution and water were offered to animals ad libitum. The treatment duration was 4 weeks.

Thymus Preparation

Completing the experiment, the animals were killed in a 70% CO_2 camera. To minimize the thymus contamination with red blood cells, the *carotid* arteries and the aorta were cut, and the animals exsanguinated. Upon killing the animals, their thymus was harvested and the contaminating blood was removed by rinsing with RPMI-1640 (Biological Industries, Israel). The weight of the thymus was evaluated, and the left rat thymus lobe samples of the study groups after thymus surrounding connective tissue were removed and the thymus lobe was stored in the RNA*later*RNA stabilization reagent (Qiagen, Germany) at −80°C until further RNA extraction and analysis.

Extraction of RNA From the Thymus

Rat thymus samples of all study groups were stored in RNA-*later*RNA stabilization reagent (Qiagen) at −80°C until further RNA extraction. The frozen tissue was ground in liquid nitrogen. Total RNA was extracted using the TRIzol Plus RNA purification kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instruction. The integrity of the total RNA was analyzed using the Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA) with an Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA). All RNA samples had the RNA integrity number (RIN) higher than 5. The quantity and quality of RNA samples were measured with a NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, MA). Only the RNA samples with the 260:280 ratio between 1.9 and 2.1 and the 260:230 ratio greater than 2.0 were used for the downstream analysis. The extracted RNA samples were stored at −80°C until further analysis.

Determination of the NKCC1 Expression in Thymus

RNA expression assay was performed for *Slc12a2* (Rn00582505_m1) and *Glpdh* (Rn01775763_g1) genes. High-capacity complementary DNA (cDNA) reverse transcription kit with RNase inhibitor (Applied Biosystems, Carlsbad, CA) was used for reverse transcription reaction in 20 µL reaction volume containing 50 ng of total RNA incubated at 25°C for 10 minutes, transcripted at 37°C for 120 minutes, and terminated by heating at 85°C for 5 minutes using Biometra TAdvanced thermocycler (Analytik Jena AG, Germany). The synthesized cDNA was stored at 4°C until use or at −20°C for a longer time. The real-time polymerase chain reaction (PCR) was run in triplicate with 4 µL of cDNA template in a 20 µL reaction volume (10 µL of TaqMan Universal Master Mix II, no UNG (Applied Biosystems, Carlsbad, CA), 1 µL of TaqMan gene expression assay 20× (Applied Biosystems, Carlsbad, CA), 5 µL of nuclease-free water (Invitrogen, Carlsbad, CA) with the program running at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The reaction was performed using an Applied Biosystems 7900 Fast real-time PCR system (Applied Biosystems, Carlsbad, CA).

Statistical Analysis

The statistical analysis was performed using the Statistical Package (IBM SPSS Statistics v22.0) for Windows. The normality assumption was verified by the Kolmogorov-Smirnov test. The animal weight data are expressed as the mean (standard deviation) values. The thymus weight data are presented as the median and the range (minimum and maximum values). Differences between 2 independent groups were evaluated using the nonparametric the Mann-Whitney *U* test. The 1e-way analysis of variance was used to determine significance among the groups, and post hoc tests with Fisher least significant difference were used for comparison among the individual groups. To investigate the *NKCC1* (*Slc12a2*) RNA expression changes in the VPA-treated group, the threshold cycle (CT) values were normalized with the control *Glpdh* gene; for the gene expression study, the delta delta threshold cycle ($2^{-\Delta\Delta CT}$) method was used to calculate the expression ratio between the VPA-treated (test) and control conditions of the target gene compared with the reference gene. Spearman rank correlation coefficient (*r*) was used to assess the relationship between thymus weight and ΔCT value. Differences at the value of $P < .05$ were considered significant.

Results

VPA Impact on Rat Thymus Weight

No statistically significant difference was found in thymus weight between the male and female control groups and the castrated male and female rat controls ($P > .05$). Comparing gonad-intact and castrated rats, the thymus weight in castrated rats of both genders was found increased: The gonad-intact and the castrated controls of both gender groups indicated a significant thymus weight increase in castrated males ($P = .02$) and females ($P = .001$). The thymus weight of gonad-intact control male and female rat groups was higher than in respective male and female rats treated for 4 weeks with 300 mg/kg VPA, although the difference was not significant ($P > .05$); a comparison of the thymus weight of castrated rats and castrated VPA-treated rats of both genders showed a thymus weight loss after VPA treatment, although no statistically significant difference was found ($P > .05$; Table 1).

Table 1. Rat Thymus Weight Data in Male and Female Study Groups.

Study Group	Thymus Weight (g), Median (Min-Max)	
	Males	Females
Gonad-intact rats		
Control	0.639 (0.483-0.823)	0.496 (0.495-0.508)
VPA treated	0.596 (0.551-0.624)	0.447 (0.3760.468)
Castrated rats		
Control	0.793 (0.682-0.982)[a]	0.808 (0.625-1.172)[b]
VPA treated	0.775 (0.669-0.887)	0.857 (0.364-1.185)

Abbreviation: VPA, valproic acid.
[a]*P* – significant compared with the gonad-intact male control.
[b]*P* – significant compared with the gonad-intact female control.

The VPA Impact on the NKCC1 RNA Expression in the Rat Thymus

The expression difference in the *Slc12a2* and *Glpdh* genes comparing the VPA-treated and the control groups as well as the data of male and female controls is considered as the ΔCT value. The *Slc12a2* RNA expression in the thymus after normalization with the *Glpdh* gene in analyzed rats groups is shown in Figure 1.

The significant difference was found between the ΔCT values of the gonad-intact male control and gonad-intact female control groups ($P = .04$). In the gonad-intact male group, the *Slc12a2* gene expression after normalization with *Glpdh* gene was found a significant difference between the VPA-treated and the control groups ($P = .015$). The difference between the ΔCT of a target and reference genes as expressed by the ΔΔCT is shown in Table 2. The RNA expression level ($2^{-\Delta\Delta CT}$) in the gonad-intact VPA-treated males was 0.116-fold lower compared with the control. This means the 88% downregulation of expression as the expression level is decreased by 88% to the level of 12% under control conditions. The significant difference between the ΔCT values of gonad-intact VPA-treated female and male groups was found ($P = .032$). Also, there was a nonsignificant *Slc12a2* gene expression change in the castrated male VPA-treated group compared with its control: Its expression level was found to be decreased by 70% in VPA-treated animals ($P = .471$). There was no statistical significance in the RNA expression analysis when comparing the control and the VPA-treated groups in both gonad-intact and castrated female rats ($P > .05$; Table 2 and Figure 1).

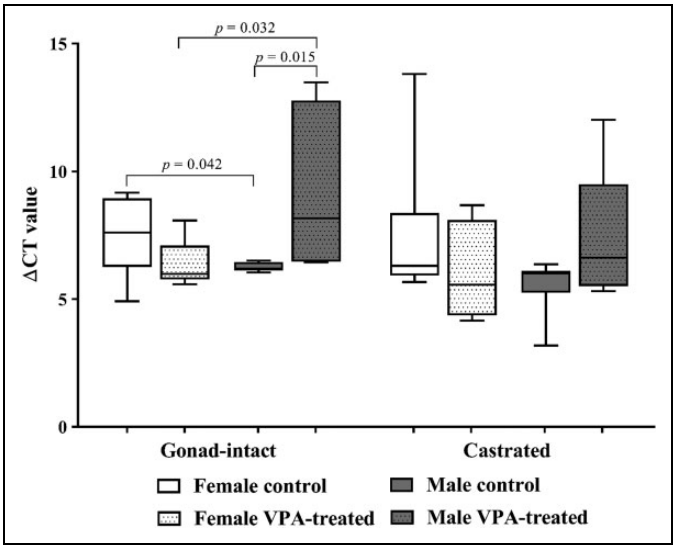

Figure 1. *Slc12a2* RNA levels in the rats after normalization with *Glpdh* gene. Delta threshold cycle (ΔCT) method was used for this analysis (the horizontal bars represent the mean, the minimal and maximal values are shown with short horizontal lines).

Table 2. RNA Expression of NKCC1 in Thymus of the Study Groups.

	CT Mean				
Study Group	*Glpdh*	*Slc12a2*	ΔCT	ΔΔCT	$2^{-\Delta\Delta CT}$
Gonad-intact female					
Control	23.530	31.130	7.600	−1.256	2.389
VPA treated	23.083	29.427	6.344[a]		
Gonad-intact male					
Control	22.905	29.134	6.229[b]	3.103	0.116
VPA treated	22.982	32.314	9.332[c]		
Castrated female					
Control	22.675	30.115	7.440	−1.340	2.531
VPA treated	24.831	30.931	6.100		
Castrated male					
Control	24.712	30.305	5.593	1.734	0.301
VPA treated	22.319	29.646	7.327		

Abbreviations: CT, threshold cycle; VPA, valproic acid.
[a]*P* – significant compared with the gonad-intact VPA-treated male.
[b]*P* – significant compared with the gonad-intact female control.
[c]*P* – significant compared with the gonad-intact male control.

No significant correlations between thymus weight and ΔCT values were found in the study groups (data are not shown).

Discussion

Three decades ago, researchers suggested that the anticonvulsant medicine VPA induces anticancer effect, inhibiting proliferation, inducing the differentiation, and immunogenicity of glioblastoma cells in vitro.[28] In 2001, VPA was reported to be the histone deacetylase (HDAC) inhibitor, and by this mechanism, it induces cancer cell proliferation, inhibition, and differentiation[29]; cell-cycle arrest; and apoptosis.[5] The VPA is a selective inhibitor of class I and IIa HDAC isoforms, with the highest selectivity for HDAC2.[30,31] The VPA increases the turnover of δ-aminobutyric acid (GABA) in neurons.[32] The GABA A receptor subunits form a functional chloride channel,[33] which is expressed in rat kidney and other tissue cells.[34-36]

The new VPA effect was observed: The VPA diuretic effect and its relation to Na^+, K^+, Cl^- and Mg^{2+} 24-hour urinary excretion; the total 24-hour diuresis and the 24-hour diuresis per 100 g of body weight were found to be significantly higher in VPA-treated rats of both genders than in the control groups with gender-related differences.[21-23] These data support the possible NKCC2 inhibition by VPA, as the increased diuresis and increased saluretic effect accompanied by an increase in divalent ions in the urine is characteristic of the NKCC2 inhibition in kidneys.[24,37] A gender difference in the NKCC2 in rat kidneys is known: The lower abundance of NKCC2 was observed in females compared with males.[38] It was supposed that the gender-related diuretic effect of medicines in rats could be due to males' larger body water content than in females.[39]

NKCC belongs to the Cl^- cotransporter family; there are 2 NKCC isoforms: NKCC1 and NKCC2. NKCC1 is distributed in various tissue types, and NKCC2 is expressed in the kidney.[40] The NKCC activity is controlled by phosphorylation; the phosphoacceptor sites are highly preserved in both NKCC1 and NKCC2, and these isoforms are activated in a similar phosphorylation manner of the N-terminus.[40,41] NKCC1 is expressed in most tissues, it transports 1 Na^+, 1 K^+ and 2 Cl^- ions into a cell and plays a major role in Cl^- accumulation.[40,42] By accumulating Cl^-, migrating cancer cells can utilize the electrochemical driving force for Cl^- efflux to osmotically release cytoplasmic water, thus modulating the cellular volume and cell migration: Pharmacological inhibition of NKCC1 with bumetanide reduces glioma cell migration and invasion.[43]

The NKCC1 activity in rat thymocytes was found to be related with the Cl^- influx, which is sensitive to the NKCC inhibitor furosemide.[25,44] NKCC1 is an important biomarker of the cell ion homeostasis regulation; it participates in the cell regulatory volume increase and can be activated in chloride depleted cells.[45] NKCC1 stimulation in the G_1 phase is essential for the proliferation of certain cell types via cell-cycle progression by modulating cell volume.[40]

The thymus is a valuable model in experimental research for evaluating the impact of medicinal products on thymocyte proliferation. The study results show that the NKCC1 RNR expression in the gonad-intact rat thymocytes is gender dependent: It was significantly more expressed in male rats compared with females. We did not find respective data concerning thymocytes in the literature, but there are data about gender-related differences in the NKCC function activity or its protein expression. On the day of birth, the NKCC1 messenger RNA (mRNA) level was higher in male than in female rat hypothalamus, and the total NKCC1 protein level was higher in the male than in female hypothalamus of rat fetuses.[46] The mean value of the $Na^+/K^+/2Cl^-$ cotransport protein in red blood cells was by 26% to 46% higher in men than that in women.[47,48] The

NKCC1 cotransport activity was found to be lower in women erythrocytes during the follicular phase[49]: The lowest in ovulatory women and the highest in men.[50] Other authors have shown that NKCC1 activity is unchanged during the menstrual cycle in female erythrocytes.[51] There is a difference in the activity of NKCC1 comparing human and rat cells: In human neurons, the rate of the Cl^- transport via NKCC1 was smaller than that in rat neurons by 61.7%.[52]

The study results show a gender-related repeated-dosage VPA treatment effect on the expression of NKCC1 in the rat thymus. The VPA was found as a significantly downregulated NKCC1 RNA expression in gonad-intact male thymocytes (expression level is decreased by 88% to the level of 12%), and nonsignificantly (decreased by 70% to the level of 30%) in the castrated VPA-treated male rats was observed. The tendency for VPA effect is clear and the lack of confidence possibly is related only with a small sample size. No significant VPA effect on the NKCC1 RNA expression in thymocytes was found in the gonad-intact and castrated female rats.

The study limitation is that the mRNA expression of NKCC1 is necessary to complete represents the transporter per se. Thus, further investigation of VPA effect on the transporter activity in relationship with gender and gonad hormone impact on the transporter protein level and NKCC1 function is necessary.

The NKCC activity in different cell types is regulated by oxidation and nitration; the oxidative/nitrosative stress effects on the NKCC activity may depend on the levels of free radicals or nitric oxide donors. Free radicals and protein tyrosine nitration can affect the NKCC structure and result in changes in its function.[53,54] The NKCC1 activity inhibition was found in endothelial cells exposed to the oxidant tertbutylhydroperoxide.[54] Oxidative stress parameters were elevated in VPA-treated patients with epilepsy as compared with the nontreated control.[55] The reactive oxygen species (ROS) production can cause direct damage to cellular DNA, protein, lipids, and can alter normal cell signaling pathways.[56] The VPA has been shown to increase ROS production and induce apoptosis in several cancer cell lines.[57] There are data on gender-related difference in ROS production. Female mice have a lower oxidative stress activity compared with males, so females may be better protected against the ROS damage. Ovariectomy enhanced the ROS production parameters, whereas an orchidectomy did not modify the oxidative stress in mice.[58] The male rats showed higher production of hydrogen peroxide in cardiac mitochondria compared to females.[59] The rat thymocytes proliferation was inhibited by the increased formation of ROS production.[60]

The study results show that castration significantly increased the thymus weight of both genders, and this was accompanied by the tendency to increase NKCC1 RNA. Castration induces an increased rat thymocyte proliferation with thymus hyperplasia.[13] The VPA treatment effect of decreased NKCC1 RNA expression in the castrated male rats was lower compared with the gonad-intact males, indicating a possible synergistic VPA effect with testosterone. Thymus cells have functional androgen receptors[20]; androgens induce a decline of thymus weight in NZB mice[61]; the surgical and chemical castration of male rats caused regeneration of thymus,[62] and castration of Sprague-Dawley rat enhanced thymic weight while gender hormones reduced the castration-induced thymus hypertrophy.[63] The study results show that the testosterone level declined after castration is related to vanishing the NKCC1 RNA expression gender difference noted among gonad-intact rats of both genders.

The study presents the gender-related VPA effect on the NKCC1 RNA expression in rat thymocytes. This imposes the necessity to evaluate the gender differences of VPA pharmacological efficacy in preclinical studies. The regulatory guideline for the study and evaluation of gender differences in the research of pharmaceuticals stresses the growing concerns that the investigational medicinal product development should provide adequate information about the effects of medicinal products in both genders.[64]

The researchers reviewed the importance of Na^+ and Cl^- ions in a tumor microenvironment as they are involved in cancer progression mechanisms.[65] NKCC regulating the urine excretion of Na^+ and Cl^-, as well as their intracellular and extracellular levels, increases its importance in cancerogenesis. NKCC1 plays an important role in cancer cell proliferation, apoptosis, and tumor progression,[45,26] especially in cancer cells with a high NKCC1 expression.[66,67] The tumors' intracellular Cl^- concentration could be one of the keys for anticancer therapy targets, and the regulation of the intracellular Cl^- concentration by NKCC1 activity would enhance the antitumor effect of anticancer medicines.[26,68]

Conclusions

The study showed a gender-related difference in the NKCC1 RNA expression in rat thymus, which is gonad hormone dependent. The VPA decreases the NKCC1 expression in the thymus of gonad-intact males, but it has no significant effect in gonad-intact female rats. The NKCC1 RNA expression downregulation by VPA as the antitumor effect could be one of the keys for anticancer therapy targets, especially in the personalized treatment of cancer with NKCC1 overexpression in cells.

Declaration of Conflicting Interests

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