RESEARCH ARTICLE

Evaluation of plasma GRP78 levels in patients with autism spectrum disorder

Selma Cirrik[1], Erman Esnafoglu[2], Oznur Adiguzel[2]

[1] Ordu University, Faculty of Medicine, Department of Physiology, Ordu - Turkey
[2] Ordu University, Faculty of Medicine, Research and Training Hospital, Department of Child and Adolescent Psychiatry, Ordu - Turkey

ABSTRACT

Objective: The role of endoplasmic reticulum (ER) stress in the pathogenesis of autism spectrum disorder (ASD) has been previously reported in both experimental and postmortem studies. However, no circulating marker of ER stress has been identified so far in ASD patients. In the present study, the plasma level of glucose-regulated protein 78 (GRP78) was investigated in ASD patients as a potential marker for ER stress.

Method: Plasma samples taken from healthy control subjects (n=26) and ASD patients (n=29) were used to evaluate circulating GRP78 levels. Plasma GRP78 concentrations were measured by ELISA. The severity of the disease in the ASD group was determined by the childhood autism rating scale.

Results: There was no significant difference between the median values of plasma GRP78 levels in ASD (12.40) and healthy control groups (11.11) (p<0.218). Plasma GRP78 concentration was 12.81±4.90 ng/mL in the ASD group and was not significantly different from the control values (11.12±3.83 ng/mL).

Conclusion: Although ER stress was associated with ASD, our results showed that plasma levels of GRP78 did not change in ASD patients. Our results suggest that GRP78 is not an appropriate circulatory marker to evaluate ER stress in ASD patients.

Keywords: Autism spectrum disorders, autism, endoplasmic reticulum stress, GRP78, pathogenesis

INTRODUCTION

The endoplasmic reticulum (ER) is a membranous organelle that plays a role in some essential cellular functions such as protein synthesis, calcium storage, and lipid biosynthesis. The newly synthesized transmembrane proteins and export proteins are post-translationally modified and properly folded with the assistance of chaperone proteins in the ER lumen. Various conditions, for instance excessive protein synthesis, oxidative stress, infection, toxins, energy depletion or the presence of mutant proteins, may impair protein processing and cause unfolded or misfolded proteins to accumulate in the ER lumen; this situation is defined as ER stress and activates the unfolded protein response (UPR) in the cell (1,2). UPR aims to clear misfolded/unfolded proteins from the ER lumen to ensure protein quality control and cell survival. To this end, it induces a global translation attenuation, except for some ER chaperone proteins such as glucose-regulated protein (GRP) 78, GRP94, and protein disulfide isomerase, which are upregulated by UPR-induced signaling pathways. Translocation of unfolded/misfolded proteins to the cytoplasm,

ubiquitination and further proteasomal degradation, which is defined as ER-associated degradation, is also upregulated during UPR. UPR-related changes are mediated by three cellular pathways; inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6), and protein kinase RNA (PKR)-like ER kinase (PERK). If the UPR induced adaptive changes are not sufficient to resolve ER stress, pro-apoptotic processes are initiated by the upregulation of various cellular death genes, including C/EBP homologous protein (CHOP) and caspase-12 (1-4).

There is accumulating evidence that reveals the role of prolonged ER stress or improper UPR in the development and progression of various diseases, including type 2 diabetes, liver diseases, atherosclerosis, and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease (4-7). Furthermore, recent reports also suggest the involvement of ER stress-induced neuronal apoptosis in some psychiatric diseases including schizophrenia, depression, bipolar disorder, and post-traumatic stress disorder (3,8-12). Interestingly, besides different neurodegenerative diseases, ER stress and related mechanisms are also charged with autism spectrum disorder (ASD) (13-16). ASD is caused by defective development and congenital dysfunction of the central nervous system (CNS) and is classified as a neurodevelopmental disorder. The main characteristics of ASD are impaired social interaction, irregular verbal and nonverbal communication, and restricted and repetitive behaviors. Different factors such as chromosomal abnormality, gene mutation and parental factors (i.e. alcohol and/or tobacco use, age) are held responsible for the ASD development and result in abnormal neuronal differentiation, neuronal maturation, and synaptogenesis (17,18). However, the exact molecular mechanisms in ASD are still unclear. Recently, ER stress, which contributes to the progression of neurodegenerative diseases, has been proposed to explain the pathogenic mechanism underlying ASD. For example, Crider et al (14) reported the dysregulation of ER stress genes in the brains of ASD patients. They showed that mRNA levels of ER stress markers such as ATF4, ATF6, PERK, XBP1, CHOP, and IRE1 increased in the postmortem middle frontal gyrus of ASD patients. They also reported a significant positive correlation between mRNA levels of ER stress genes and the diagnostic score for stereotyped behavior in subjects with ASD (14). In another study, Kawada et al. (16) showed an association between ASD and ER stress activation in the brain using a mouse

model established by injection with valproic acid (VPA). The researchers reported increased levels of GRP94, an ER stress marker, in the cerebral cortex and hippocampus of mice with ASD (16). Literature findings regarding the role of ER stress in neurodevelopmental diseases other than ASD are limited. However, oxidative stress and ER stress were identified as important mechanisms in neurodevelopmental impairments of the fetal brain caused by prenatal exposure to drugs such as cocaine, methamphetamines and nicotine (19).

To date, no markers for ER stress in the circulation of these patients have been reported. GRP78 is one of the ER-resident chaperone proteins and plays an important role in the activation of the main UPR pathways. As GRP78 is upregulated during ER stress, it is considered as an indicator for ER stress both in tissue and blood. Circulatory GRP78 level was measured in a limited number of studies and no study was related to neurodegenerative/psychiatric disease (20-22). Nevertheless, obesity, which is a hepatic ER stress condition, was reported to induce a GRP78 elevation in plasma as well as in the liver (20). Considering the studies reporting blood-brain barrier (BBB) disruption during ASD (23-25), we assumed that the increased GRP78 level in the brain tissue is reflected in blood, and we aimed to investigate the plasma GRP78 level in ASD patients as a potential marker for ER stress.

METHOD

Plasma samples from healthy control subjects (n=26) and ASD patients (n=29) were used to evaluate circulating GRP78 levels.

Participants and Ethical Conditions: Participants were recruited from the Child and Adolescent Psychiatry outpatient clinic of Ordu University Faculty of Medicine Training and Research Hospital. The study was approved by the Ordu University Faculty of Medicine Ethics Committee (2018/102). Children aged 3-15 years with a diagnosis of ASD (ASD group) and healthy children of the same age group (control group) were included in the study. The family of each participant was informed about this study and written consent was obtained. Patients with a history of any systemic disease, infection, drug use, neurological or psychiatric disease were excluded from the study.

The diagnosis of ASD was made according to DSM 5 criteria. To measure ASD severity, the Childhood Autism Rating Scale (CARS) was used. The healthy control group consisted of volunteers who came to the

outpatient clinic for simple reasons and were not diagnosed with DSM 5. Healthy subjects were given a sociodemographic form and completed the Schedule-Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version, DSM-5 (K-PADS-PL-DSM-5).

Samples were taken from all subjects just before breakfast between 08:00-11:00 in the morning under sterile conditions. Routine biochemistry, hemogram, and thyroid function tests were requested for routine evaluation of the patient's health status. Plasma was separated from blood samples and stored at -80 degrees until the ELISA experiments. In order to minimize the analytical variation, plasma samples from subjects meeting the criteria described above were collected for 6 months, rather than individual measurements. Then, on the same day, GRP78 was measured on plasma samples of 26 control and 29 patients using the same ELISA kit.

Sociodemographic Form: Designed by researchers this form includes different information about the subjects such as age, gender, birth history, birth weight, breastfeeding period, psychomotor development history, drug use, general medical condition, and psychiatric comorbidity, place of residence, body mass index (BMI), and parents' ages.

Childhood Autism Rating Scale (CARS): CARS consists of 15 items used to generate a total score that defines the severity of autism. A total score between 30 and 36.5 indicates mild to moderate autism, while the range between 37 and 60 denotes severe autism. CARS is scored by observing the child and interviews conducted with the family (26).

Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version, DSM-5 (K-SADS-PL): K-SADS-PL-DSM-5 is an effective measurement tool for screening childhood psychiatric disorders. Its Turkish validity and reliability were proven by Unal et al. (27). K-SADS-PL-DSM-5 is a semi-structured interview schedule and includes the newly-added DSM 5 diagnoses. In the present study, the age range of the participants was 3-15, and K-SADS-PL was used for both preschool and school-age children (28).

Measurement of Plasma GRP78 levels: Plasma GRP78 levels in control and ASD subjects were measured with a commercial ELISA kit using the Sandwich-ELISA working principle (Elabscience; E-EL-H5586). The sensitivity of this assay was 0.38 ng/mL and the detection range was 0.63-40 ng/mL. During the experiments, plasma samples and standards were added to the appropriate micro ELISA plate wells pre-coated with an antibody specific to human GRP78. After incubation at 37°C for 90 min, biotinylated detection antibody and avidin-horseradish peroxidase conjugate were added sequentially to each well. Following the incubation and washing of the free components, the substrate solution was added to each well and incubated. After the termination of the enzyme-substrate reaction, the optical density was measured at 450 nm wavelength. GRP78 levels in the samples were calculated by comparing the optical density of the samples with the standard curve.

Statistical Analysis
Statistical analysis was performed using SPSS 22.0 software. Categorical data comparisons between groups were made using the Chi-Square test. The normal distribution of numerical data was checked using the Shapiro-Wilk test. The Mann-Whitney-U test was used to compare numerical variables due to the small number of subjects and the lack of normal distribution. Correlation analysis was performed using the nonparametric Spearman test. Data are presented as mean±SD and median (min-max). $P < 0.05$ was considered statistically significant.

RESULTS

Our results showed that there were no significant differences between the groups in terms of age, gender, BMI, birth weight, and breastfeeding duration (p=0.189; p=0.696; p=0.099; p=0.890, p=0.358, respectively).

Compared as an independent variable, the GRP78 level did not differ significantly between the groups (p=0.218) (Table 1, Figure 1). Although there was no statistical difference, the GRP78 values were found to be higher in the patient group.
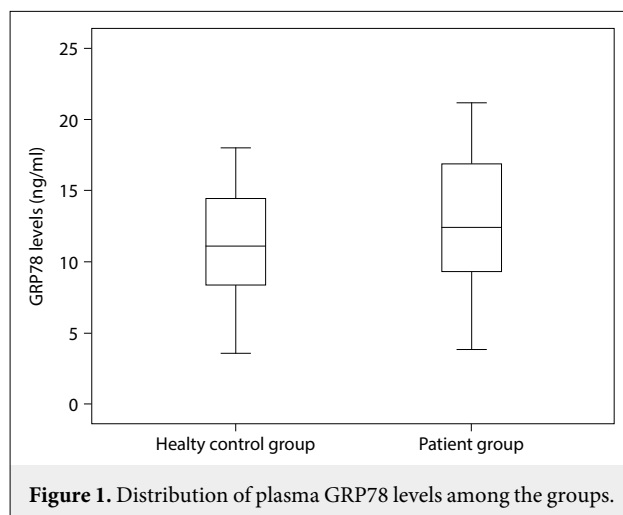

Figure 1. Distribution of plasma GRP78 levels among the groups.

Table 1: Group characteristics and plasma GRP78 levels

	Patient group (n=29)	Healthy control group (n=26)	p (df:1)
Gender (Female/Male)	3/26	4/22	0.696[1]
Age (mean±SD)	6.96±3.10	7.97±3.19	0.189[2]
Median (min-max)	6.5 (3.5-13)	6.5 (4-13)	
BMI (mean±SD)	18.11±3.08	16.69±2.24	0.099[2]
Median (min-max)	17.10 (14.34-24.99)	16.53 (13.51-22.77)	
CARS (mean±SD)	48.67±6.92	-	-
GRP78 levels (mean±SD)	12.81±4.90	11.12±3.83	0.218[2]
Median (min-max)	12.40 (3.79-21.18)	11.11 (3.54-18.00)	

BMI: Body Mass Index; CARS: Childhood Autism Rating Scale; df=degree of freedom
[1]Chi-square test [2]Mann Whitney-U test

Correlation analysis showed no significant relationship between GRP78 and age (r=-0.088 and p=0.534), BMI (r=0.149 and p=0.290) and CARS (r=0.092 and p=0.504).

DISCUSSION

The present study aimed to reveal whether the circulating GRP78 concentration changes in children with ASD and our results showed for the first time that plasma GRP78 is at similar levels in both ASD and control groups.

Studies in both animal models and patients with ASD reported that ER stress is one of the underlying mechanisms of ASD pathology. One of the experimental models used in these studies is the VPA model in which VPA administration to pregnant mice increases the ASD development risk in offspring by 3-5-times (29). The cerebral cortex and hippocampus of VPA-treated offspring showed an increased level of GRP94, an ER stress marker, compared to healthy control animals (16). Similarly, studies in autistic men showed that ER stress-related signals such as IRE1 alpha, ATF6, and PERK were activated in the cerebellum, hippocampus, and prefrontal cortex (13,14).

ER stress activation is implicated in brain overgrowth and synaptogenesis in ASD. Brain overgrowth in the early stages of development is a hallmark of ASD. In these patients, gray and white matter volumes, as well as brain weight are higher than healthy individuals. Neuronal differentiation and maturation were found to be increased in the early period of life in ASD cases. However, neurite growth and development of synaptic connections have deteriorated (15,30). ER stress induces neural stem cell differentiation, and this mechanism may play a role in

the brain overgrowth in ASD (15,16). Although ER stress accelerates neuronal differentiation, the expression level of MAP-2 (a dendrite marker) decreases during ER stress (31). Consistent with this finding, axon and dendritic lengths of cultured neurons were inhibited during ER stress (15,16). Consequently, ER stress may cause differentiation of neuronal stem cells, but delay maturation of neuronal extensions and synaptogenesis. On the other hand, mutation of the cell adhesion molecule-1 (CADM1) and neuroligin, which are involved in the pathogenesis of ASD, induces ER stress. Unfolded CADM1 accumulation in the ER lumen inhibits dendrite extension (32,33). Finally, the relationship between ER stress and neuronal differentiation/maturation seems to be one of the mechanisms underlying ASD.

As summarized above, the pathogenesis of ASD is somehow related to ER stress. So far, human studies have been conducted on post-mortem brain tissues. In this study, GRP78 concentration was examined in blood samples taken from children with ASD for the first time to monitor ER stress in the CNS. We think that such a marker might be important to evaluate the severity of ASD. Our results showed that there was no significant change in GRP78 level in the circulation of these patients. Since the presence of ER stress was detailed extensively, the unchanged GRP78 level in plasma might be explained by the fact that GRP78 is too large to cross the BBB even though this barrier is damaged, or that ER stress in the CNS is not high enough to increase circulating GRP78 level. However, GRP78 measurements in the cerebrospinal fluid would provide more accurate results.

The present study has some limitations. First of all, it was conducted with a small number of subjects and we think that insignificant changes may be due to Type

II error. The degradation of GRP78 during its storage period of up to 6 months, may be another limitation of this study. Moreover, this study is a cross-sectional study and it was carried out in a certain time period. We do not know how the GRP78 level changes in the years after birth. For this reason, it may be more appropriate to evaluate it within the first two years of life when CNS development is faster.

Although ER stress was associated with ASD, our preliminary results showed that plasma levels of GRP78 did not change in ASD patients. We suggest that GRP78 is not an appropriate circulatory marker to evaluate ER stress in ASD patients. However, this result does not mean that ER stress has no role in the ASD pathogenesis, when evaluated together with the other literature findings mentioned above. It is evident that further research on this subject conducted with a larger and younger sample group and testing different ER stress markers are needed.

Contribution Categories		Author Initials
Category 1	Concept/Design	S.C., E.E.
	Data acquisition	E.E.
	Data analysis/Interpretation	O.A.
Category 2	Drafting manuscript	S.C.
	Critical revision of manuscript	S.C., E.E.
Category 3	Final approval and accountability	S.C., E.E., O.A.
Other	Technical or material support	N/A
	Supervision	N/A

Ethics Committee Approval: The study was approved by the Ordu University Faculty of Medicine Ethics Committee (2018/102).

Informed Consent: Informed consent was obtained from all participants for this study.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors declare that there is no conflict of interest.

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