Research Article

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Endoplasmic reticulum stress in leukocytes from phenylketonuric patients

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Abstract

Objectives: Phenylketonuria (PKU) is a proteinopathy due to the deficiency of phenylalanine hydroxylase (PAH) enzyme. The pathological elevation of phenylalanine (Phe) and its metabolites in PKU is linked to neurological hallmarks and mental disabilities. The aim of this study was to examine the hypothesis that high levels of Phe caused endoplasmic reticulum (ER) stress in PKU patients.

Methods: We primarily evaluated ER stress markers glucose-regulated protein78 (GRP78) and C/-EBP homologous protein (CHOP), and thiobarbituric acid-reactive substances (TBARS) as a biomarker of oxidative stress in leukocytes and correlated it with blood Phe values from patients with PKU. Patients in this study were selected from individuals who were diagnosed with PKU as a result of the national neonatal screening program and undergone treatment at our university hospital. The subjects were divided into four groups: healthy controls, patients with hyperphenylalaninemia (HPA), BH_4-responsive patients with PKU and patients with classic PKU. GRP78, CHOP and TBARS levels were estimated in leukocytes isolated from whole blood of subjects, Phe and tyrosine levels were determined in plasma.

Results: The levels of Phe in BH_4-responsive PKU and classic PKU groups were statistically higher as compared to healthy controls, and Phe levels were higher in classic PKU compared to HPA group. CHOP levels were elevated by 35.3% in BH_4-responsive group compared to control. GRP78, CHOP and TBARS showed no statistical differences between control and patient groups. GRP78 was also negatively correlated with Phe levels.

Conclusions: These results suggested that blood Phe concentrations might not be associated to ER stress in white blood cells obtained from the PKU patient groups under treatment.

Keywords: endoplasmic reticulum stress; leukocytes; phenylalanine; phenylketonuria.

Introduction

Phenylketonuria (PKU), a well-known misfolding disease, is an inborn error of L-phenylalanine (Phe) metabolism characterized by mutations in the Phe hydroxylase (PAH) gene. PAH enzyme catalyzes the conversion of Phe to tyrosine in the presence of a cofactor tetrahydrobiop terin (BH_4), Fe^{+2} and O_2 [1]. Defaults in either PAH or the generation or recycling of BH_4 can result in hyperphenylalaninemia (HPA) leading to mental disabilities. Untreated PKU patients exhibit high levels of Phe in their bloodstream and tissues [2, 3]. Normal blood Phe concentration is 50–110 µmol/L. The untreated PKU patients who depict a level of 120–600 µmol/L of Phe prior to starting therapy are classified as having mild HPA (sometimes those with plasma Phe concentration 150–360 µmol/L on newborn screening called as HPA). Individuals with Phe concentrations ranging from 600–1,200 µmol/L are classified as mild PKU (occasionally those with values of 900–1,200 µmol/L are termed as a moderate classification). Phe levels ranging over 1,200 µmol/L is called classic PKU [1, 4, 5]. Patients with Phe levels >360 µmol/L should be checked for responsiveness to BH_4. A decline in blood Phe value of 30% or more than baseline shows response to BH_4 therapy [6, 7].

Accumulation of unfolded/misfolded proteins or pathological stresses such as the existence of mutated proteins that cannot fold correctly in the ER during protein maturation, metabolic disturbances and other perturbations affecting ER homeostasis can lead to ER stress. In such situation, cells make a respond to ER stress by the activation of the unfolded protein response (UPR). The UPR is a complex signaling

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program mediated by three ER transmembrane receptors in mammalian cells. They include activating transcription factor 6 (ATF6), inositol requiring kinase 1 (IRE1) and RNA-dependent protein kinase (PKR)-like ER kinase (PERK). ER stress triggers the UPR. The misfolded/unfolded proteins are recognized by GRP78, which subsequently releases itself from PERK, IRE1, and ATF6, resulting in their activation. The activated GRP78 and CHOP constitute bio-markers for ER stress. The balance between the prosurvival chaperone GRP78 and CHOP dictates the cell destiny upon ER stress [8]. The failure in the UPR program to re-establish regular ER functions and attenuate the stress state, triggers cellular inflammation and/or apoptotic signals mechanisms. As a result of these signals mechanisms, severe or prolonged ER stress results in cell death if the stress condition cannot be resolved [8–11]. For instance, the presence of ER stress in patients-derived fibroblasts with homocystinuria showed increased levels of GRP78, PERK and CHOP proteins involved in the ER stress response [12]. Fumarylacetate produced by tyrosine degradation induces an ER stress response that causes the induction of GRP78 and CHOP expression, suggesting that ER stress is interested in hereditary tyrosinemia type I [13]. GRP78 responds quickly to ER stress to improve cell survival. Besides, ER stress-induced activation of CHOP has been shown to have a role in the pathogenesis of inflammation and a pro-apoptotic transcription factor activated during UPR [14–16]. However, there is still scanty information about the physiopathology related of the ER stress in patients with PKU in association with misfolding disease. Theoretically, due to the presence of misfolded PAH enzymes; ER stress is expected to increase in patients with BH_4-responsive PKU. In late-diagnosed classical PKU, failure of PAH activity results in elevated levels of Phe and its metabolites phenylpyruvate, phenylacetate, phenyllactate, and phenylethylamine in blood and tissues of effected patients. These elevated organic compounds may also be the cause of ER stress. Main

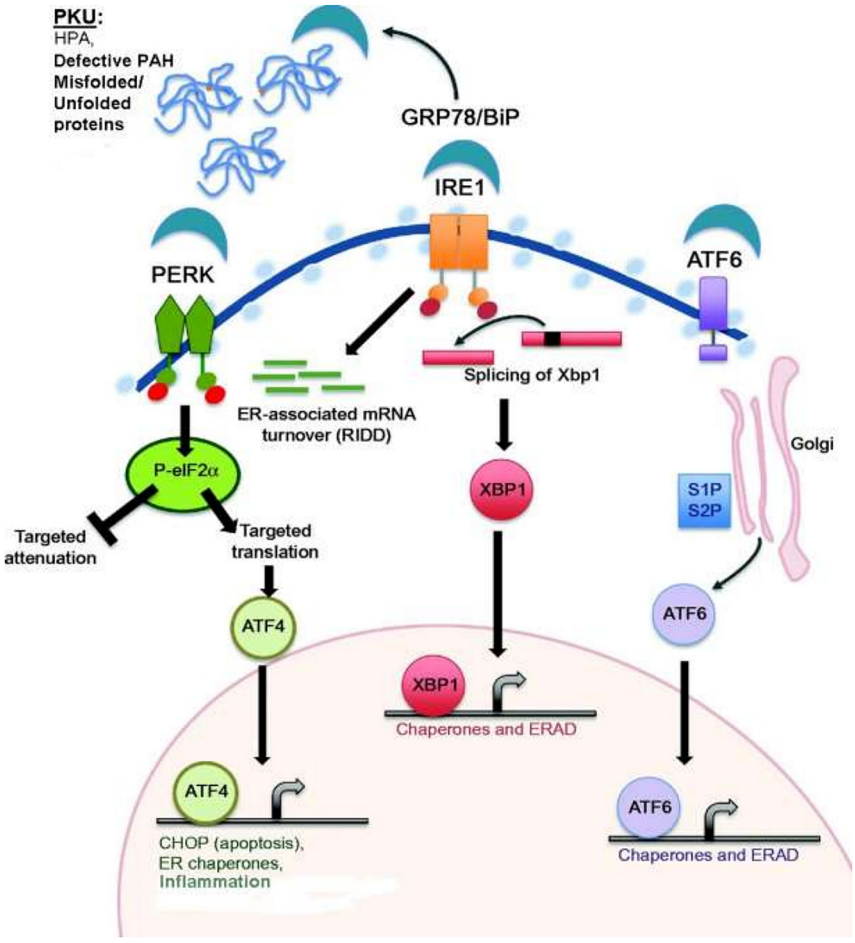

Figure 1: The basic components of the unfolded protein response (UPR). ER stress occurs when protein misfolding/unfolding or defective proteins augment over threshold levels, activating a signaling mechanism and transcriptional events known as the UPR to re-establish ER homeostasis. Though GRP78 represents an indicator marker of for the pro-survival (or a potent anti-apoptotic factor) efforts of the UPR, ER stress-induced expression of CHOP has been associated with cytokine-induced inflammatory responses, its high-level expression is an indicative marker of a switch to a pro-apoptotic function. Figurative knowledge and explications modified from cited references [14–17]. PKU, phenylketonuria; HPA, hyperphenylalaninemia; PAH, phenylalanine hydroxylase; PERK, RNA-dependent protein kinase (PKR)-like ER kinase; IRE1α, inositol-requiring enzyme 1α; ATF6, activating transcription factor 6.

components of the UPR related to PKU may be depicted hypothetically in Figure 1.

Some authors reported some forms of alterations in the pathophysiology of PKU disease. These authors observed the deleterious effects of Phe and its metabolites, the presence of oxidative stress in PKU and DNA injury in leukocytes from PKU patients [18–20]. Nevertheless, the relation between ER stress and the pathogenesis of PKU has not been exactly investigated up to date. In this work, we made an attempt to address this relation and examined various parameters of oxidative and ER stress such as GRP78, CHOP, and TBARS in leukocytes from untreated HPA and treated PKU patients and evaluated the relationship between oxidative and ER stress markers in relation to plasma Phe levels in PKU.

Materials and methods

The current study was approved by the Ethical Committee of Hospital of Ondokuz Mayıs University (KAEK 2018/250), in Samsun/Turkey. The parents of the individuals participated in the current study let an informed consent. Treatment is composed of protein-restricted diet supplemented with a phe-free combination fortified with micronutrients like trace elements, vitamins in different compositions. The patient group with HPA and BH_4-responsive PKU was not under dietary treatment. Diet-compromised PKU patient group was within the acceptable limits for treatment compliance by age group. Of these patients, nine HPA patients (5 girls and 4 boys, age: 1.80 ± 2.18 years), eight PKU patients (3 girls and 5 boys, age: 3.14 ± 4.33 years) with BH_4-responsive and fourteen classic PKU patients (9 girls and 5 boys, age: 12.29 ± 10.07 years) admitted at the Department of Pediatric Nutrition and Metabolism, ten children (6 girls and 4 boys, age: 10.01 ± 3.81 years) served as healthy control subjects who were selected randomly from patients came to the healthy child outpatient clinic for routine follow-up and agreed to participate in the study were included in this study. Exclusion criteria included a variety of conditions related to the ER stress such as any infection, acute or chronic inflammatory disease, obesity, cardiovascular, renal or hepatic dysfunction and the other metabolic diseases especially MTHFR polymorphisms with elevated homocysteinemia.

Blood sampling: Venous blood samples after 2-h fasting were drawn into a tube containing an anticoagulant (EDTA) and plasma separated after centrifugation (1,500 g 10 min) at 4 °C. Then, 4 mL of whole blood was gently pipetted into a falcon tube containing Ficoll-Histopaque (density 1.077 g/mL) solution (4 mL). Later, this falcon tube was centrifuged at 400 g for 20 min over Ficoll-Histopaque, and then the interface with leukocytes was transferred to a sterile falcon tube. Leukocytes transferred into a sterile falcon were washed with 4 mL of cold PBS, and centrifuged at 250 g for 10 min. After removing the supernatant, cell pellets were suspended in 0.5 mL of PBS solution. In order to remove erythrocytes (RBCs) from suspended pellets, 0.5 mL of RBC lysis buffer was added to the tubes, gently mixed, and sits for 10 min in cold room at 4 °C and later centrifuged at 250 g for 10 min. The supernatant was discharged and the leukocytes pellets were re-suspended in 350 µL of leukocyte lysis buffer, vortexed and submitted to ultrasonication for 15 min. The leukocytes lysate thus recovered in eppendorf tubes were frozen to −80 °C for subsequent analyses.

Biochemical analyses: Phe and tyrosine concentrations were determined in fresh plasma samples by HPLC using the inherent fluorescence of aromatic amino acids. Plasma precipitated with 6% perchloric acid was separated using a base-deactivated C_{18} column with 5% acetonitrile in water as the mobile phase. Fluorescent measurements at an excitation wavelength of 215 nm and emis sion 283 nm showed only three peaks for the internal standard, tyrosine and Phe eluting within 9 min. The levels of GRP78 and CHOP were estimated in leukocyte cell lysates by using ELISA kits (USCNK-SEC343HU, USCNK-SEJ282HU). TBARS formed as a byproduct of lipid peroxidation was detected in leukocyte lysates by the TBARS assay using thiobarbituric acid as a reagent at 532 nm spectrophotometer (UV-160A Shimadzu), measuring malondialdehyde (MDA) present in the sample with assay of TBARS [21]. Protein concentration in leukocyte lysates was also determined by using the method of Lowry [22]. The levels of measured parameters are represented as µmol/L for Phe and tyrosine, ng/mg of protein for GRP78 and CHOP, and µmol/mg of protein for TBARS.

Statistical analysis: SPSS Statistics 22.0 software was used for statistical analyses. Data were initially examined for the normality assumption analyzed by Shapiro–Wilk test. Accordingly, GRP78 and tyrosine data were analyzed by parametric one-way ANOVA test, whereas non-parametric statistical analyses were used for Phe, CHOP and TBARS data, and then Kruskal–Wallis test used to estimate the

Table 1: Phenylalanine and tyrosine concentrations in plasma, GRP 78, CHOP and TBARS levels in leukocyte lysates of the study groups.

Parameters	Groups			
	Healthy control (n=10)	HPA (n=9)	BH_4-Responsive PKU (n=8)	Classic PKU (n=14)
Phenylalanine, µmol/L	[a]68.85 (25.66)	[b]136.56 (93.73)	[c]192.27 (341.69)	[d]473.45 (478.37)
Tyrosine, µmol/L	72.25 (19.45)	68.95 (35.80)	71.00 (42.86)	73.09 (60.81)
GRP78 (ng/mg of protein)	5.15 (2.70)	4.29 (3.70)	5.29 (2.78)	3.94 (1.60)
CHOP (ng/mg of protein)	0.22 (0.16)	0.22 (0.07)	0.34 (0.22)	0.21 (0.25)
TBARS (µmol/mg of protein)	29.94 (9.17)	35.56 (17.13)	34.79 (12.34)	39.65 (25.39)

Data present as median and interquartile range (IQR). a–c: $p<0.006$; a–d: $p<0.001$; b–d: $p<0.026$; no significant difference was observed for the other parameters between the groups, $p>0.05$.

statistical significance of the differences in the groups. Correlation analysis was performed using the Spearman Correlation method. The results are represented as median and interquartile range (IQR). p-Values below 0.05 were considered statistically significant.

Results

Table 1 presents Phe and tyrosine concentrations in plasma, GRP78, CHOP and TBARS levels in leukocyte cell lysates for healthy controls, HPA, BH_4-responsive and classic PKU patients. Phe concentrations were statistically higher in BH_4-responsive PKU and classic PKU groups compared to healthy controls (p=0.006, p=0.001), and also in classic PKU group according to HPA group (p=0.026). No significant difference was observed for tyrosine levels between the groups (p>0.05).

To evaluate whether the blood high levels of Phe cause oxidative and ER stress in leukocytes, we have estimated GRP78, CHOP and TBARS in patients and

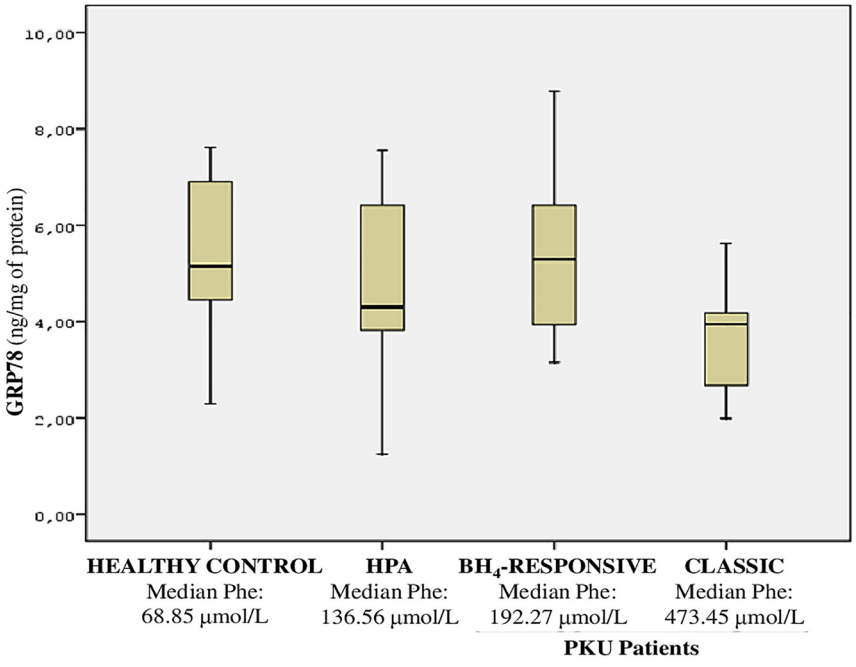

Figure 2: GRP78 values of peripheral white blood cells from healthy controls, HPA and two groups of PKU patients, one with median phe: 192.27 µmol/L (n=8) and the other with median phe: 473.45 µmol/L (n=14). The levels of GRP78 in leukocyte lysates were slightly heightened by 2.64% in BH_4-responsive PKU compared to the control.

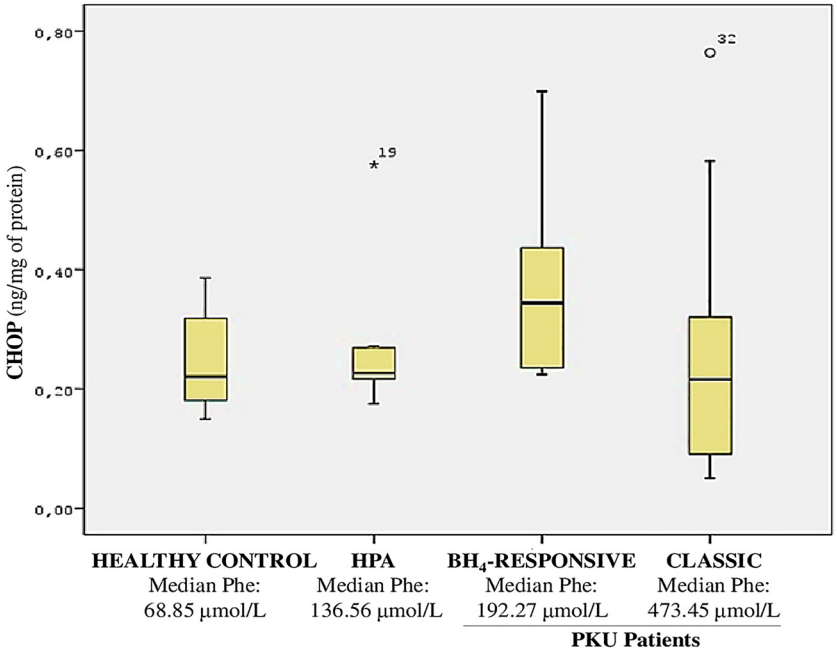

Figure 3: CHOP values of peripheral white blood cells from healthy controls, HPA and two groups of PKU patients, one with median phe: 192.27 µmol/L (n=8) and the other with median phe: 473.45 µmol/L (n=14) and healthy controls (n=10). The levels of CHOP in leukocyte lysates were mildly increased from 0.22 to 0.34 ng/mg protein (35.3%) in BH_4-responsive PKU compared to the control.

controls. In this manner, Figures 2 and 3 depicted GRP78 and CHOP levels in association with median values of Phe levels in two groups of PKU; CHOP levels in leukocyte lysates were determined higher by 35.3% in BH_4-responsive PKU group according to the control. The results demonstrated that, there was no significant difference between control subjects and patient groups in terms of GRP78 and CHOP levels related to the ER stress in leukocytes ($p>0.05$). GRP78 was also negatively correlated with Phe concentrations ($r=-0.368$, $p=0.02$), and CHOP was not correlated with Phe levels ($r=-0.038$, $p=0.812$).

In the view of a biomarker of oxidative stress, TBARS levels are presented in Figure 4 in relation to median Phe levels in the study groups. TBARS levels of leuko cyte lysates were higher by 15.80% in HPA, 13.94% in BH_4-responsive and 24.48% in classic PKU groups with respect to controls. But, TBARS levels in leukocytes of HPA, BH_4-responsive and classic PKU patients showed no significant differences from control subjects ($p>0.05$).

Discussion

This study is the first to report two ER stress related markers GRP78 and CHOP in leukocytes from HPA and PKU patients in relation to higher plasma Phe levels. Patients were exposed for long periods to high levels of Phe and its catabolic metabolites. CHOP level was higher by 35.3% in BH_4-responsive PKU as compared to healthy controls. GRP78 was also negatively correlated with Phe concentrations in this study.

Deficiency of PAH activity results in an accumulation of Phe and decreased levels of tyrosine in the body fluids in untreated PKU patients. The level of PAH activity is used to make the difference between classic PKU (hepatic PAH residual activity <1%, Phe levels above 1,200 mmol/L) from other milder forms (PAH residual activity 1–5%, Phe levels 600–1,200 mmol/L; and also permanent mild HPA patients: PAH residual activity >5%, Phe levels <600 mmol/L). Patients suffering from permanent HPA will have a normal life without treatment, whereas those with the other forms of the disease require a lifelong dietary Phe restriction. Accordingly, regular monitoring of blood Phe and tyrosine levels is necessary [23]. The ratio of Phe/tyrosine is also helpful in monitoring appropriate dietary intake. In the current study, tyrosine levels appear to be normal compared to the reference value (between 35 and 102 μmol/L) in HPA and treated PKU patients, suggesting that these individuals are not in situation of tyrosine deficiency.

The pathological elevation of Phe has been associated with neurological hallmarks, intellectual disability and behavioral disorders in PKU patients if left untreated [1, 2, 24]. Sirtori et al. [19] have shown that at least 600 μmol/L with a mean value (1,160 μmol/L) of Phe can come about oxidative stress in patients with PKU. In addition, DNA

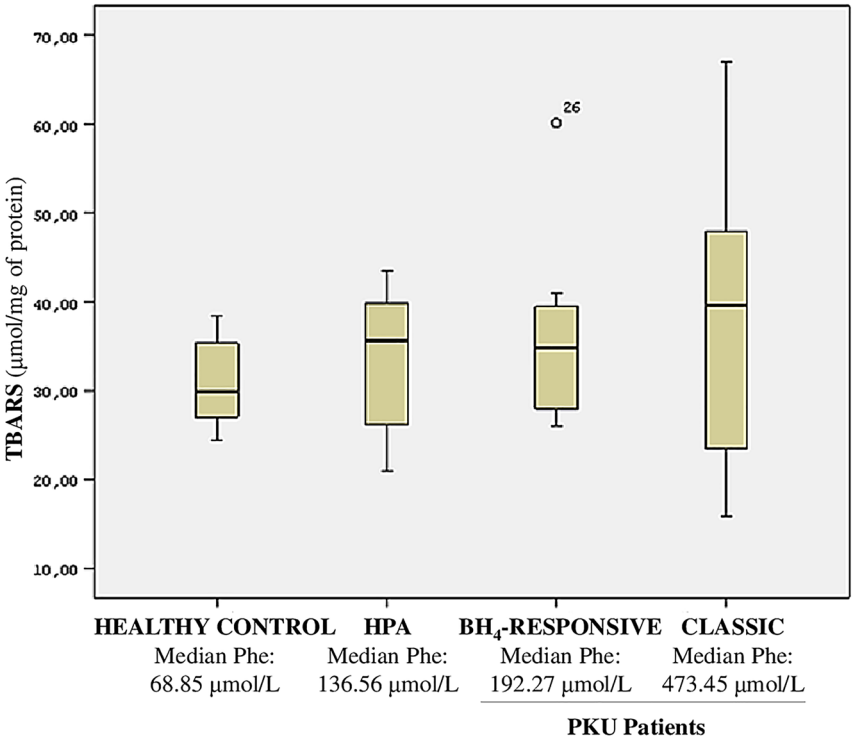

Figure 4: TBARS levels of peripheral white blood cells from healthy controls, HPA and two groups of PKU patients.

damage appeared in peripheral blood leucocytes from eight PKU patients who had well compliance with the proper diet (mean Phe: 396.4 μmol/L) and ten PKU patients who did not exactly adhere to the recommended diet (mean Phe: 848.8 μmol/L) [20].

The importance of oxidative stress in the pathogenesis of HPA and PKU is evolving continuously [18]. In this regard, researchers set up a correlation between high blood Phe concentrations, brain injury, neuropsychiatric disorders, and elevated lipid peroxidation markers MDA, TBARS and the others [18, 25, 26]. In this study, we noticed that TBARS was higher by a median of 15.8, 13.9, and 24.4% in leukocytes from HPA, BH_4-responsive and classic PKU groups in parallel with high plasma levels of Phe in HPA (median Phe: 136.56 μmol/L), BH_4-responsive (median Phe: 192.27 μmol/L) and classic PKU patients (median Phe: 473.45 μmol/L), respectively, compared with control subjects. The explanation of this correlation maybe due to minimum level of lipids peroxidation in peripheral white blood cells in relation with oxidative stress related to the high Phe levels in PKU patients, considering that oxidative stress might occur minimally in the patients studied.

Previous studies have reported that mutations in the PAH gene can lead to PAH protein misfolding, aggregation and earlier degradation [27–29]. In addition, patients with a mild to moderate HPA phenotype are very likely to respond to BH_4 (a pharmacological chaperone) therapy than those with classical PKU [30], also included in Turkish PKU patients [31], considering that PKU is a protein misfolding disorder with loss of PAH function. Now, researchers and clinicians assume that the rehabilitation of PAH function with the drug doses of BH_4 happens through correction of PAH protein misfolding. In the light of current scientific observations, we have hypothesized that misfolded/unfolded/defective proteins in the ER lumen of white blood cells from BH_4-responsive and classic PKU patients accumulate, ER stress can trigger in the cell, and then initiates the UPR. In the ER, misfolded proteins activate GRP78 (an ER chaperone) and induce the expression of ER-resident chaperons during the UPR [32]. The ATF4/PERK/CHOP signal pathway is a pro-apoptotic mechanism provoked by persistent ER stress [32]. CHOP is an essential pro-apoptotic factor in cells' ER stress [33]. In our study, GRP78 and CHOP levels were elevated by 2.64 and 35.3% in BH_4-responsive group according to controls as shown in Figures 2 and 3. It is probably, at least, that 35.3% increase in CHOP value might point out the rise of ER stress in PKU patients with BH_4-responsive. But, we observed that there were no significant changes of ER stress markers GRP78 and CHOP in leukocytes from treated patient groups in concentrations of blood Phe below ~300–500 μmol/L. GRP78 levels were statistically unaltered among the groups and also negatively correlated with patients' Phe values. This finding could be explained by the fact that the implicated Phe levels might not be associated with ER stress in PKU patients under therapy. In contrast to these findings, GRP78 and CHOP gene expression were found to be higher in leukocytes of metabolically unhealthy diabetic obese subjects compared to metabolically healthy obese [16]. In addition, 0.9 mM concentration of Phe leads to Phe-induced neuronal apoptosis measured by TUNEL assay in cultured neurons [34].

In conclusion, the relations between ER stress markers and high Phe levels showed that GRP78 was negatively correlated with Phe values and that CHOP was not correlated with Phe levels in HPA and treated PKU subjects. These results suggest that high Phe concentrations in the bloodstream may be not related to ER stress in the peripheral blood cells of PKU patients under treatment. Inability in unexplained advanced mental skills, even in ideally treated patients, might be associated with ER stress. ER stress and apoptosis in PKU subjects not receiving treatment need to be examined in further studies.

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