Endoplasmic reticulum stress in leukocytes from phenylketonuric patients

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), BH4-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 BH4-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 BH4-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 tetrahydrobiopterin (BH4), Fe²⁺ and O₂ [1]. Defaults in either PAH or the generation or recycling of BH4 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 BH4. A decline in blood Phe value of 30% or more than baseline shows response to BH4 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
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 BH4-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 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 Mayas University (KAES 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 BH₄-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 BH₄-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”的内容大体如下：

一些作者报告了PKU疾病病理生理学中的一些形式的改变。这些作者观察到Phe及其代谢物的有害影响，氧化应激在PKU和来自PKU患者的DNA损伤在白细胞中。然而，ER应力与PKU病理生理学的关系尚未被确切地调查。在这项工作中，我们试图解决这个问题，并检查各种氧化和ER应力参数，特别是在HPA和治疗过的PKU患者中，并评估ER应力和氧化应激起泡的关系与血浆Phe水平的关系。

**Materials and methods**

这项研究得到了Ondokuz Mayas University（KAES 2018/250）的医疗伦理委员会的批准，在Samsun/Turkey。参与该项研究的父母同意签署知情同意书。治疗由蛋白质限制饮食补充一个Phe免费组合，与微量营养素如微量元素，维生素组成。患者组包括HPA和BH₄应答性PKU，未接受饮食治疗。饮食不完全的PKU患者组是接受可接受的限制性摄入的。其中，九名HPA患者（5名女孩和4名男孩，年龄：1.80 ± 2.18岁），八名PKU患者（3名女孩和5名男孩，年龄：3.14 ± 4.33岁）伴有BH₄应答性，以及十四名经典PKU患者（9名女孩和5名男孩，年龄：12.29 ± 10.07岁）被送往儿童营养和代谢科。十名儿童（6名女孩和4名男孩，年龄：10.01 ± 3.81岁）作为健康对照组，入选条件包括各种与ER应力相关的条件，如感染、急性或慢性炎症性疾病、肥胖、心血管、肾脏或肝功能障碍和其他代谢疾病，尤其是MTHFR突变与高半胱氨酸血症。

**Blood sampling:** 静脉血样在2-h空腹后采取，放入含有抗凝剂（EDTA）的管中，分离血浆并经离心（1,500 g 10 min）于4 °C。然后，4 mL全血轻轻吸出放入一个含有Ficoll-Histopaque（密度1.077 g/mL）的溶液（4 mL）的管中。之后，这个管在400 g离心20 min后，从Ficoll-Histopaque上层中吸出，白细胞转移到一个无菌管中。白细胞转移入一个无菌管中后，洗涤并用0.5 mL PBS悬浮。为了去除红细胞（RBCs），从悬浮的细胞中添加0.5 mL RBC裂解缓冲液，轻轻混匀，置10 min于冷室，4 °C后，离心250 g 10 min。上清液弃去，细胞沉淀用PBS悬浮，然后，细胞沉淀与红细胞分层转移至一个无菌管中。用350 μL的血细胞裂解缓冲液，旋涡混匀并超声15 min。白细胞亚基由此恢复。

**Table 1:** 苯丙氨酸和酪氨酸浓度在血浆中的变化，GRP78、CHOP和TBARS水平在白细胞裂解液中的变化

| Parameters                  | Groups                        | Healthy control (n=10) | HPA (n=9) | BH₄-Responsive PKU (n=8) | Classic PKU (n=14) |
|-----------------------------|-------------------------------|------------------------|-----------|--------------------------|-------------------|
| Phenylalanine, μmol/L       |                               | 68.85 (25.66)          | 136.56 (93.73) | 192.27 (341.69)        | 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)     |

数据以中位数和四分位数范围表示（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, BH₄-responsive and classic PKU patients. Phe concentrations were statistically higher in BH₄-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 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 BH₄-responsive PKU compared to the control.

**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 BH₄-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 BH₄-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 BH₄-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 leukocyte lysates were higher by 15.80% in HPA, 13.94% in BH₄-responsive and 24.48% in classic PKU groups with respect to controls. But, TBARS levels in leukocytes of HPA, BH₄-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 BH₄-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...
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, BH4-responsive and classic PKU groups in parallel with high plasma levels of Phe in HPA (median Phe: 136.56 μmol/L), BH4-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 BH4 (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 BH4 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 BH4-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 BH4-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 BH4-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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