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Experimental evidence that phenylalanine is strongly associated to oxidative stress in adolescents and adults with phenylketonuria

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ABSTRACT

Few studies have looked at optimal or acceptable serum phenylalanine levels in later life in patients with phenylketonuria (PKU). This study examined the oxidative stress status of adolescents and adults with PKU. Forty PKU patients aged over fifteen years were enrolled, and were compared with thirty age-matched controls. Oxidative stress markers, anti-oxidant enzyme activities in erythrocytes, and blood anti-oxidant levels were examined. Nitric oxide (NO) production was also examined as a measure of oxidative stress. Plasma thiobarbituric acid reactive species and serum malondialdehyde-modified LDL levels were significantly higher in PKU patients than control subjects, and correlated significantly with serum phenylalanine level ($P<0.01$). Plasma total anti-oxidant reactivity levels were significantly lower in the patient group, and correlated negatively with phenylalanine level ($P<0.001$). Erythrocyte superoxide dismutase and catalase activities were higher and correlated significantly with phenylalanine level ($P<0.01$). Glutathione peroxidase activity was lower and correlated negatively with phenylalanine level ($P<0.001$). The oxidative stress score calculated from these six parameters was significantly higher in patients with serum phenylalanine of 700–800 μmol/l. Plasma anti-oxidant substances, beta-carotene, and coenzyme Q_{10} were also lower ($P<0.001$), although the decreases did not correlate significantly with the phenylalanine level. Serum nitrite/nitrate levels, as stable NO products, were higher together with low serum asymmetric dimethylarginine, as an endogenous NO inhibitor. Oxidative stress status is closely linked with serum phenylalanine levels. Phenylalanine level in should be maintained PKU below 700–800 μmol/l even in adult patients.

1. Introduction

Phenylketonuria (PKU) is an autosomal recessive disorder caused by a deficiency in hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1) and is usually diagnosed early in life. Unless the affected child is maintained on a strict low-phenylalanine diet, PKU leads to mental retardation, seizures, behavioral difficulties, and other neurological symptoms [1]. After the introduction of newborn mass screening for this disorder in the 1960s–70s, affected infants generally achieved normal development through early-intervention dietary treatment. Initially, the low-phenylalanine diet prescribed for classical

Abbreviations: ADMA, asymmetric dimethylarginine; CoQ_{10}, coenzyme Q_{10}; GPx, glutathione peroxidase; MDA-LDL, malondialdehyde-modified LDL; NO, nitric oxide; NOx, nitrite and nitrate; PKU, phenylketonuria; SOD, superoxide dismutase; TAR, total anti-oxidant reactivity; TBARS, thiobarbituric acid reactive species.

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PKU was discontinued by the second decade of life. However, growing evidence suggests that a high serum phenylalanine level can cause neuropsychological and psychosocial problems in diet-off adult patients [2], and that those symptoms improved after resumption of a phenylalanine-restricted diet [3]. Current recommendations are therefore that PKU patients continue the phenylalanine-restricted diet therapy for life, although the optimal serum phenylalanine levels in later life have yet to be established.

High phenylalanine concentrations in the brain correlate with the neurological signs and brain dysfunction characteristic of PKU, although the exact mechanisms and pathophysiology underlying these symptoms remain unclear. The possible culprits include changes in protein synthesis, transport of large neutral amino acids across the blood-brain barrier, synthesis of monoamine neurotransmitters, activity of glutamate receptors, and energy metabolism in the brain [4,5].

Oxidative stress influences many biological functions and long-term prolongation of oxidative stress contributes to the development and progression of various diseases, including neurological and cardiovascular disease. Brain has a high content of lipids that are vulnerable to oxidation, such as unsaturated fatty acids, yet the neural antioxidant defense system is relatively weak [6–8].

Recent studies demonstrated that oxidative stress status is pronounced in PKU patients [9–13]. In addition, it has been shown that, at least in animal models, the administration of anti-oxidants can improve the evolution of PKU [14,15]. However, the available data is limited, and more information is required before any clinically relevant conclusions are derived. The present study examined surrogate markers for oxidative stress and erythrocyte anti-oxidant enzymes in a group of PKU patients aged over fifteen years that showed widely differing dietary patterns and serum phenylalanine levels among individuals. We also investigated nitric oxide (NO) production because it is sensitive to changes in oxidative stress status and is associated with vascular tone, neurological function, apoptosis, and anti-inflammatory responses [16–18].

The purpose of this study was to evaluate oxidative stress status in PKU patients with respect to their serum phenylalanine levels, with the view to establish an optimal or acceptable serum phenylalanine level in later life.

2. Materials and methods

2.1. Subjects

We enrolled 40 PKU patients (28 females and 12 males) ranging in age from 15 to 50 years (mean ± SD, 28.4 ± 11.3 years). Phenylalanine hydroxylase (PAH) deficiency was diagnosed at the participating institutions, based on the absence of neurological deterioration on a low phenylalanine diet, analysis of dihydropteridine reductase activity in erythrocytes, biopterin loading test, and/or pteridine analysis in urine. Patients younger than 33 years were found to have hyperphenylalaninemia by mass screening at around five days of age. The remaining patients (>34 years) presented at hospital at the ages of 1–6 years for precise assessment of mental retardation and delayed motor development, and were found then to have hyperphenylalaninemia. After diagnoses, all patients were placed on a phenylalanine-restricted diet. As adults, these patients received a various range of dietary restrictions, from the maintenance of strict phenylalanine restriction to mildly restricted diets. Consequently, the plasma phenylalanine levels among the patients enrolled in this study ranged from 180 to 1800 μmol/l.

We also enrolled 30 healthy subjects as the control subjects (20 females and 10 males; range, 17–49 years; mean, 29.5 ± 7.5 years), who were matched to the patients in age, body mass index, and biological data.

2.2. Study design

The following blood markers were measured as indicators of oxidative stress: plasma levels of thiobarbituric acid-reactive species (TBARS), representing fatty acid peroxidation; total antioxidant reactivity (TAR), reflecting the ability to attenuate reactive species in the early phase; and, serum levels of malondialdehyde-modified low-density lipoprotein (MDA-LDL), representing oxidized LDL (Ox LDL) [19]. Urinary markers of oxidative stress also measured were acrolein–lysine to reflect the level of lipid peroxidation products in plasma and urine, and urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) to reflect oxidative DNA damage [19].

Beta-carotene and alpha-tocopherol in plasma were examined as anti-oxidant substances [19]. Plasma coenzyme Q_{10} (CoQ_{10}), an electron carrier from mitochondrial respiratory chain complexes I and II to complex III, was also examined because CoQ_{10} serves as an anti-oxidant and its production is closely linked with phenylalanine metabolism [12,20,21]. Catalase, superoxide-dismutase (SOD), and glutathione peroxidase (GPx) were measured as anti-oxidative enzymes in erythrocytes.

To estimate NO metabolism, serum levels of nitrite/nitrate (NOx) were assayed as stable metabolites of NO, along with asymmetric dimethylarginine (ADMA), a competitor of arginine for NO synthase (NOS; EC 1. 14. 13. 39), and plasma arginine and citrulline, constituting the NO-citrulline cycle.

Blood was drawn from a peripheral vein in the morning after overnight fasting. Serum and plasma were obtained for the determinations of TBARS, TAR, MDA-LDL, alpha-tocopherol, beta-carotene, CoQ10, NOx, ADMA, and amino acids. Erythrocytes were washed in cold 0.9% NaCl solution. Lysates for the determinations of anti-oxidative enzyme activities were prepared by adding 100 μl of washed erythrocytes to 1 ml of distilled water, and then freezing at −80 °C until further analysis. Urine samples (5–15 ml) for oxidative stress markers were collected 0.5–2 h before blood sample collection.

This study protocol was approved by the relevant institutional review boards. All patients or their parents provided written informed consent before the start of the study.

2.3. Assays for blood and urinary oxidative stress markers

Plasma TBARS levels were determined using a fluorometric assay as described previously [22]. Plasma TAR levels, which represent the capacity to attenuate oxidants, was determined based on the luminol chemiluminescence intensity induced by 2, 2′-azo-bis-2-amidinopropane, according to Lissi et al. [23]. Serum MDA-LDL levels were determined using a sensitive enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody reactive to MDA-apo B as described previously [24]. Plasma beta-carotene, alpha-tocopherol, and CoQ_{10} (ubiquinol-10 plus ubiquinone-10) levels were measured using high-performance liquid chromatography [25].

Urinary acrolein–lysine and 8-OHdG levels were determined using competitive ELISA kits: ACR-Lysine Adduct ELISA (NOF Corp, Tokyo, Japan) and 8-OHdG Check (Institute for the Control of Aging, Shizuoka, Japan), respectively. These values were presented as acrolein–lysine and 8-OHdG to creatinine ratios.

The intra- and inter-assay coefficients of variation were less than 10% for all measurements.

2.4. Assays for anti-oxidative enzyme activities in erythrocytes

The SOD activity was determined by spectrophotometry at 505 nm (RANSOD kit; Randox Laboratories Ltd; Antrim, UK). Catalase activity was determined as described previously [24]. In brief, we monitored the decrease in absorbance at 240 nm in a reaction medium containing 20 mM H_2O_2, 10 M potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/ml. The GPx activity was determined by the method of Wendel

[26]; this involved monitoring the disappearance of NADPH at 340 nm in a medium containing 2 mM glutathione, 0.15 U/ml glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxidase, and 0.1 mM NADPH. The intra- and inter-assay coefficients of variation were less than 10% for the respective enzyme assays.

2.5. Assays for NOx and ADMA

Serum NOx levels were measured using the Griess method, with a nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). Serum ADMA levels were determined using an ELISA kit (DLD Diagnostika GmbH, Hamburg, Germany).

2.6. Statistical analyses

Differences between patients and controls were analyzed using Student's *t*-test. The relation between each pair of parameters was estimated using Pearson's correlation test. *P* values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Urinary and blood oxidative stress markers in PKU patients

The data in Table 1 clearly indicate enhanced oxidative stress in PKU patients. Three oxidative stress markers, TBARS, MDA-LDL, and TAR, were significantly different in PKU patients than the control. The mean level of urinary oxidative marker, acrolein–lysine, was significantly higher than in controls, while the 8-OHdG level was similar in the two groups. These data provided strong evidence for increased lipid peroxidation and enhanced oxidative stress in the affected patients.

Erythrocyte SOD and catalase activities were significantly higher in PKU patients, indicating heightened responsiveness to the enhancement of oxidative stress, supporting the above notion. On contrast, GPx activities were significantly lower. Among anti-oxidative substances, beta-carotene and CoQ_{10} levels were significantly lower in PKU patients than those in the controls, while alpha-tocopherol levels were similar in the two groups.

Table 1
Blood and urinary oxidative stress markers and anti-oxidant enzyme activities in erythrocytes.

	PKU patients (n = 42)	Healthy controls (n = 30)
Blood TBARS (nmol/mg protein)	5.05 ± 1.16[¶]	3.79 ± 0.46
Blood TAR (nmol/mg protein)	1.36 ± 0.40[§]	2.15 ± 0.38
Blood MDA-LDL (IU/ml)	61 ± 18[§]	39 ± 11
Blood alpha-tocopherol (mg/dl)	0.74 ± 0.13	0.85 ± 0.14
Blood beta-carotene (mg/dl)	28.2 ± 15.0[§]	46.5 ± 13.5
Coenzyme Q_{10} (ng/ml)	482 ± 102[§]	970 ± 237
Urinary 8-OHdG/Cr (ng/mg Cr)	7.68 ± 1.65	8.23 ± 1.49
Urinary acrolein–lysine/Cr (mmol/mg Cr)	279 ± 142[¶]	199 ± 99
SOD activity in erythrocytes (unit/mg protein)	1.54 ± 0.25[¶]	1.12 ± 0.21
Cat activity in erythrocytes (pmol/mg protein)	3.49 ± 0.47[¶]	2.79 ± 0.34
GPx activity in erythrocytes (mU/mg protein)	0.511 ± 0.168[§]	0.756 ± 0.122
Blood NOx (μmol/l)	47.2 ± 17.2[¶]	30.9 ± 10.7
Blood ADMA (μmol/l)	0.44 ± 0.10[*]	0.61 ± 0.10
ADMA/NOx	0.014 ± 0.008[¶]	0.020 ± 0.007
Blood arginine (μmol/l)	66 ± 22[¶]	95 ± 22
Blood citrulline (μmol/l)	32 ± 9[*]	26 ± 6

[*] $p<0.05$, compared with the control (Student's *t*-test).
[¶] $p<0.01$, compared with the control (Student's *t*-test).
[§] $p<0.001$, compared with the control (Student's *t*-test).

3.2. Correlations between serum phenylalanine and parameters of oxidative stress in PKU patients

In PKU patients, blood TBARS, TAR, and MDA-LDL levels correlated significantly with serum phenylalanine levels: TBARS, r = 0.709; TAR, r = −0.871; MDA-LDL, r = 0.663 (Fig. 1), whereas urinary acrolein levels did not correlate with those of 8-OHdG (acrolein, r = 0.159; 8-OHdG, r = 0.012). With respect to anti-oxidative substances that were significantly different between PKU patients and controls, beta-carotene but not CoQ_{10} showed a significant negative correlation with serum phenylalanine levels in PKU patients (r = −0.421; Fig. 2). Erythrocyte SOD and catalase in PKU patients correlated significantly with serum phenylalanine levels (catalase, r = 0.672; SOD, r = 0.647). In contrast, GPx activity correlated negatively with phenylalanine level (r = −0.877; Fig. 3).

3.3. NO metabolism

Serum NOx levels were significantly higher in PKU patients than in the controls, whereas serum ADMA levels and the ADMA: NOx ratios (ADMA/NOx) were significantly lower than the respective control levels (Table 1). Serum arginine levels were also significantly lower in the patient group compared to the control, while serum citrulline

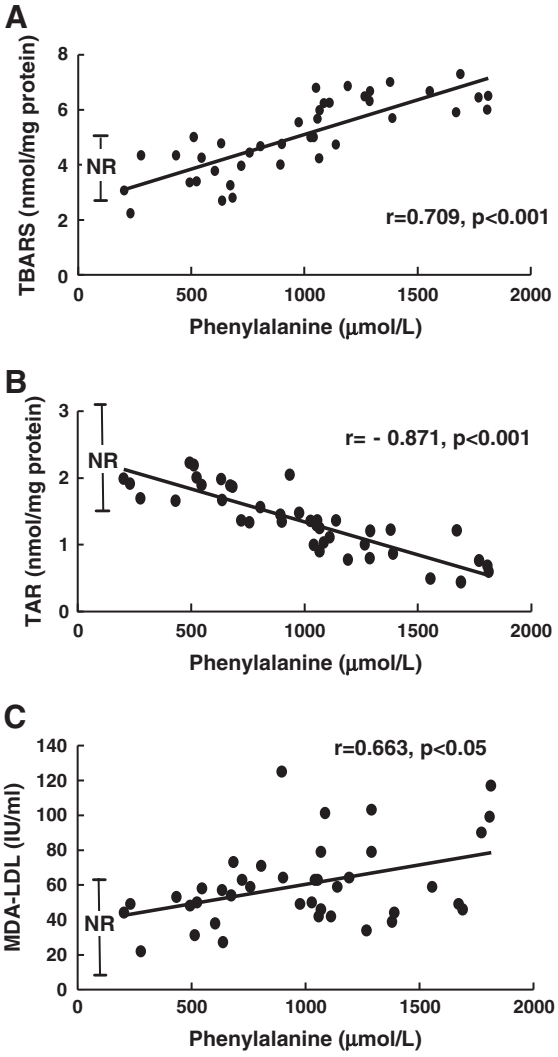

Fig. 1. Scatter graphs of thiobarbituric acid reactive substance (A), total anti-oxidant reactivity level (B), and malondialdehyde-modified LDL level (C) against plasma phenylalanine level. NR, normal range (mean ± 2 SD) obtained from 30 age-matched healthy controls.

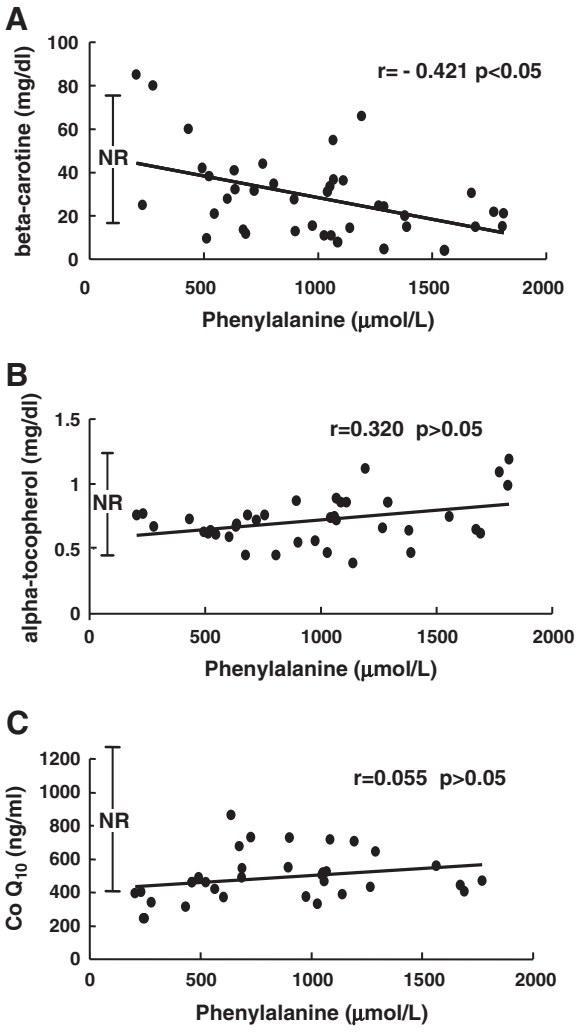

Fig. 2. Scatter graphs of beta-carotene (A), alpha-tocopherol (B), and coenzyme Q_{10} (C) against plasma phenylalanine level. NR, normal range (mean ± 2 SD) obtained from 30 age-matched healthy controls.

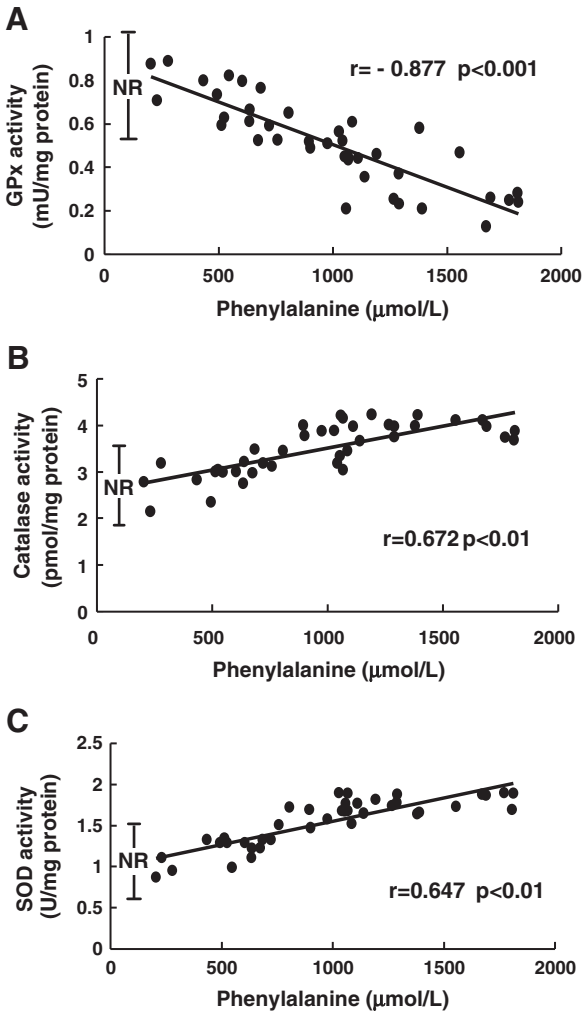

Fig. 3. Scatter graphs of GPx (A), catalase (B), and SOD (C) activities in erythrocytes against plasma phenylalanine level. NR, normal range (mean ± 2 SD) obtained from 30 age-matched healthy controls.

levels were significantly higher in the patient group. (Table 1). In PKU patients, the NOx, ADMA, and ADMA/NOx values did not correlate significantly with serum phenylalanine levels, although NOx tended to be lower in patients with phenylalanine levels of >900 μmol/l (Fig. 4).

3.4. Oxidative stress score

Of note, the values of several parameters of oxidative stress were beyond the range of the respective control values (mean ± 2 SD) in patients with serum phenylalanine levels of >600–800 μmol/l (Figs. 1–3). To evaluate the relationship between serum phenylalanine level and oxidative stress more comprehensively, we scored (0, 1, or 2 points) three blood oxidative stress markers (TBARS, TAR, and MDA-LDL) and three erythrocyte anti-oxidative enzymes (SOD, catalase, and GPx), which exhibited significant correlations with serum phenylalanine levels. For TBARS, MDA-LDL, catalase, and SOD, values ranging mean + 1 SD of healthy controls were scored as 0 point, between mean + 1 SD and + 2 SD were scored as 1 point, and more than mean + 2 SD were scored as 2 points; for TAR and GPx, values between mean − 1 SD of normal control were scored as 0 point, between mean − 1 SD and − 2 SD were scored as 1 point, and those less than mean − 2 SD were scored as 2 points. We calculated the total score as the oxidative stress score (0–12 points). We were able to divide PKU patients into two groups at six points of oxidative stress score as shown in Fig. 5. All patients with less than six points showed less than 800 μmol/l phenylalanine, and all patients with more than six points showed more than 800 μmol/l. In other words, the oxidative stress in the PKU patients changed greatly at the border of 700–800 μmol/l.

4. Discussion

Oxidative stress and its implications have not been studied adequately in PKU, particularly in adult patients. According to earlier reports, irrespective of the blood phenylalanine level, plasma TBARS and TAR levels were increased and decreased, respectively, in PKU children [9], while GPx but not SOD and catalase activities were decreased [9,11]. On the other hand, Artuch et al. [10] reported that anti-oxidant enzyme activities were not changed in PKU patient groups consisting of children, adolescents, and young adults.

We often find it difficult to evaluate the oxidative stress status in children because of the changing anti-oxidative system during growth and development [19]. The present study thus targeted subjects aged over fifteen years, whose oxidative stress status might not be influenced by such factors [19]. Our findings confirmed that oxidative stress was enhanced in the PKU patient group compared to age-matched healthy controls. In addition, there was a significant correlation between serum phenylalanine levels in PKU patients and the magnitude of oxidative stress. In other words, high oxidative stress was noted in patients with

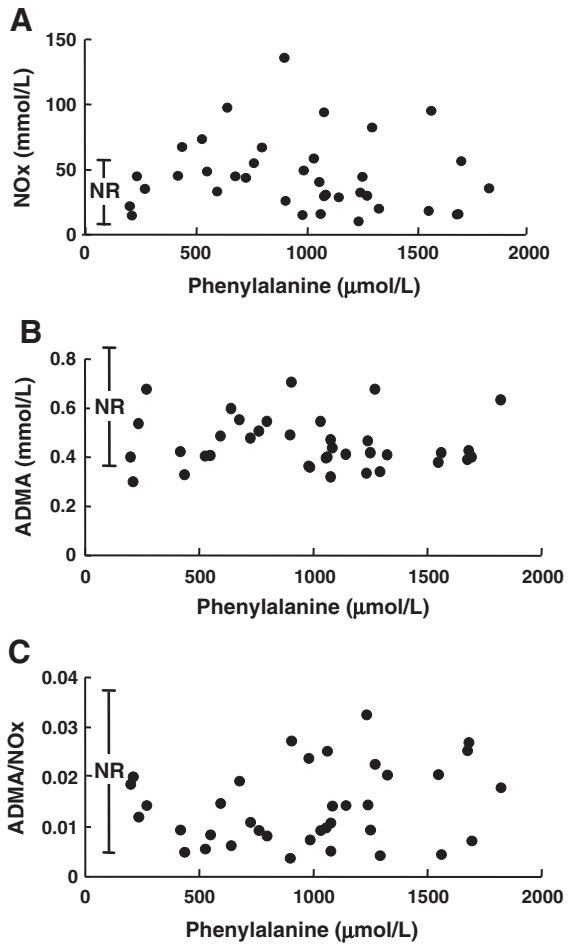

Fig. 4. Scatter graphs of NOx (A), ADMA (B), and NOx/ADMA (C) in erythrocytes against plasma phenylalanine level. NR, normal range (mean ± 2 SD) obtained from 30 age-matched healthy controls.

high serum phenylalanine levels based on the measurements of selected stress status markers (TBARS, TAR, and MDL-LDL), anti-oxidative stress enzymes (SOD and catalase), and anti-oxidants (beta-carotene). These results were supportive of a similar study in animals in which oxidative stress was closely linked with serum phenylalanine level [27].

Of note, CoQ_{10} was considerably reduced in the plasma of PKU patients. CoQ_{10} plays pivotal roles in the mitochondrial respiratory chain, cell signaling, and gene expression, and the pathogenic effects of CoQ_{10} depletion in PKU warrants further investigation. High phenylalanine inhibits mevalonic acid production via the suppression of

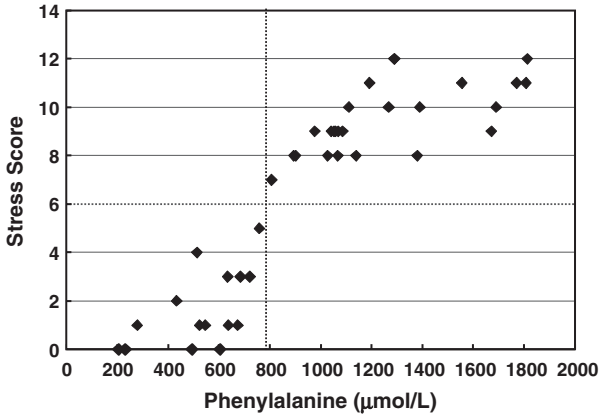

Fig. 5. Scoring of oxidative stress status according to serum phenylalanine level.

3-hydroxy-3-methylglutaryl-CoA reductase, leading to lower plasma CoQ_{10} levels [10,12,21]. In addition, strictly limited dietary intake including phenylalanine has resulted in CoQ_{10} deficiency in some cases, because phenylalanine and mevalonic acid are substrates for CoQ_{10} production [12]. We postulate that these factors also contributed to the low plasma CoQ_{10} level in our patients.

The low GPx activity could be also related to neuropsychiatric disturbances in PKU patients because GPx is highly expressed in brain and plays a pivotal role in the anti-oxidant defense system of the brain [8,28,29]. The mechanisms underlying the inhibition of GPx activity in PKU patients remain to be clarified. It is known that GPx activity is decreased with deficiencies in selenium, an essential cofactor for this enzyme [30]. Selenium levels were measured in approximately 50% of our patient group (19/40), but were normal. Hagen et al. [14] showed that phenylalanine directly suppresses GPx production or promotes GPx degradation in a rat model of hyperphenylalaninemia. In our patients, the GPx activity level exhibited a strong negative correlation with the serum phenylalanine level, suggesting that phenylalanine *per se* inhibits GPx activity.

PKU patients examined in our study also showed changes in parameters of NO metabolism. Considering that NO has a regulatory effect on neurological function, it is plausible that such abnormalities in the NO metabolism could mediate, at least in part, the neurological signs and symptoms in PKU patients [16–18]. In this study, serum NOx levels were increased, but serum ADMA levels and ADMA to NOx ratios were decreased compared to controls, and plasma arginine, an amino acid substrate for NO production, was decreased. A scatter graph of NOx against serum phenylalanine levels displayed a tendency for the NOx level to increase until a serum phenylalanine level of 900 μmol/l and subsequently decrease (Fig. 4). In contrast, ADMA/NOx tended to decrease until 900 μmol/l phenylalanine, and thereafter increase (Fig. 4). Irrespective of NOx level, ADMA levels remained fairly constant. Together, these findings suggested strongly that the regulation of NO production, in particular the inhibition by ADMA, is disturbed in PKU patients. We inferred the increase of NOx in the first phase as a consequence of the pronounced oxidative stress. As a possible mechanism for the decrease in NOx trend in the second phase at higher phenylalanine levels, we speculate that transcriptional suppression of the NOS gene or structural changes in NOS are induced with markedly enhanced oxidative stress [16,18].

Even if the adult brain in PKU develops resistance to high phenylalanine concentrations, such increased levels could still contribute to the development of neuropsychological and psychosocial problems in diet-off adult patients. Therefore, PKU patients are recommended to continue diet therapy for life. On the other hand, the appropriate or ideal serum phenylalanine levels in later life remain to be established. Diet therapy is difficult and therefore it is particularly important to patient quality of life to recommend an optimal blood phenylalanine level. Current recommended levels for adults on dietary treatment vary greatly from 600 to 1300 μmol/l in Europe and the USA. The present study now raises the possibility that increased oxidative stress in PKU patients >15 years of age could be avoided by keeping their serum phenylalanine level below 700–800 μmol/l. This relatively low phenylalanine levels to be maintained have been supported by the results of MR imaging [31–33]. Kono et al. [33] reported that PKU patients with serum phenylalanine levels beyond 550 μmol/l exhibited abnormal findings in posterior cerebral deep white matter on magnetic resonance imaging, irrespective of age.

5. Conclusion

Oxidative stress status is closely linked with serum phenylalanine levels. Based on our findings, we propose that serum phenylalanine level should be controlled below 700–800 μmol/l by dietary treatment even in later life.

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