

Future Role of Large Neutral Amino Acids in Transport of Phenylalanine Into the Brain

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ABSTRACT. *Objective.* The treatment of phenylketonuria (PKU) in children and adults has been difficult because of erosion of dietary adherence, leading to poor school performance, impairment of executive functioning, loss of IQ, and deterioration of white matter in the brain. Mutant PKU mice produced by exposure to N-ethyl-N'-nitrosourea (ENU) were used to examine the effect of large neutral amino acid (LNAA) supplementation on brain and blood phenylalanine (Phe).

Methods. Mice with PKU, genotype ENU 2/2 with features of classical PKU, were supplemented with LNAA while on a normal diet. Two dosages of LNAA were given 0.5 g/kg and 1.0 g/kg by gavage. Blood Phe was determined in the experimental, control, and sham-treated mice. Brain Phe was determined by magnetic resonance spectroscopy after perchloric acid extraction. Branched-chain amino acid transferase (BCAT) was determined in brain as a marker for energy metabolism.

Results. Blood Phe was reduced in the LNAA-treated mice by an average of 15% (0.5 g/kg) and 50% (1.0 g/kg) in 48 hours. There was a sustained decrease in the blood Phe levels over a 6-week trial. The untreated mice and sham-treated mice maintained high blood Phe throughout the experiments. Brain Phe level determined by magnetic resonance spectroscopy showed a decline of 46% after the LNAA treatment. BCAT levels were lower (33%) in the ENU 2/2 mice compared with wild-type. The BCAT normalized in mice with PKU that were treated with LNAA.

Conclusion. The results suggest that giving LNAA lowered brain and blood Phe levels in mice with PKU. Energy metabolism generated from BCAT also improved in mice with PKU after treatment with LNAA. Data from the mice suggest that LNAA should be considered among the strategies to treat PKU in humans. *Pediatrics* 2003; 112:1570–1574; *phenylketonuria, PKU, large neutral amino acids, LNAA, blood-brain barrier.*

ABBREVIATIONS. PKU, phenylketonuria; Phe, phenylalanine; LNAA, large neutral amino acid; VIL, valine, isoleucine, and leucine; MRS, magnetic resonance spectroscopy; BCAT, branched-chain amino acid transferase; ENU, N-ethyl-N'-nitrosourea; NMR, nuclear magnetic resonance; GI, gastrointestinal.

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There has been considerable experience for the past 40 years in the treatment of phenylketonuria (PKU) with a phenylalanine (Phe)-restricted diet.¹ Restriction of Phe in the early years of life is successful; however, "diet for life" has been difficult, because the strict dietary regimen leads to erosion of dietary adherence as children get older.^{2–5} As a result, the desired blood Phe is not maintained. The National Institutes of Health Consensus Development Conference on Treatment of PKU recommends maintaining blood Phe levels at 2 to 6 mg/dL from ages birth to 12 years and 120 to 900 $\mu\text{mol/L}$ after 12 years, with levels 120 to 600 $\mu\text{mol/L}$ strongly encouraged.⁶ A majority of adolescents and adults have blood Phe levels $\geq 900 \mu\text{mol/L}$. There is ample documentation that the rise of blood Phe leads to poor school performance, impairment of executive functioning, loss of IQ, and deterioration of white matter in the brain.^{7–13} Therefore, alternative methods of treatment of PKU are being pursued.

The origin of the large neutral amino acid (LNAA) hypothesis to reduce brain Phe stems from the work of Olendorf et al¹⁴ on the transport of amino acids across the blood brain barrier. Phe and other LNAAs (tyrosine, tryptophan, threonine, isoleucine, leucine, valine, methionine, and histidine) share a common transporter to the brain and therefore compete with one another. As early as 1977, Kaufman¹⁵ suggested the use of LNAAs to compete with the entry of Phe into the brain as part of treatment for PKU. Pardridge¹⁶ used an experimental model to examine movement of amino acids across the blood brain barrier. He confirmed that a shared carrier was involved and that increasing other LNAAs competes with transport of Phe into the brain. Phe has the lowest Michaelis Constant (Km) and is preferentially transported by the LNAA carrier protein.

Clinical trials with partial LNAAs have been tried. Supplementation studies started in 1987 by Lou, who gave 160 mg/kg tyrosine to treated patients with PKU and found improved attention span and increased dopamine synthesis.¹⁷ Subsequent studies by Pietz et al¹⁸ showed no improvement when tyrosine was supplemented in patients with high blood Phe levels. Berry et al¹⁹ were the first to try to inhibit entry of Phe to the brain by increasing branch chain amino acids in the diet. They used valine 150 mg/kg, isoleucine 150 mg/kg, and leucine 200 mg/kg (VIL). The patients on VIL had substantial lowering of their

TABLE 1. PreKUnil (LNAA) Composition per Tablet

Amino Acid	mg
Tyrosine	256
Tryptophan	256
Arginine	35
Leucine	35
Isoleucine	35
Valine	35
Methionine	35
Threonine	35

cerebrospinal fluid Phe, but tyrosine levels also declined.²⁰ VIL is not a complete LNAA mixture and does not contain tyrosine or tryptophan, which are precursors for neurotransmitters.²¹

The first study of LNAA supplementation in the treatment of PKU was conducted by Dotremont et al²² on 4 patients using a low-protein diet 0.6 g/kg and 0.8 g/kg of LNAA. This treatment was problematic in that subjects developed a negative nitrogen balance as a result of lysine deficiency secondary to low-protein intake. The second study by Pietz et al²³ showed lowering of brain Phe in 6 male individuals with PKU who were given 100 mg/kg Phe load with and without LNAA supplementation. The group given LNAA had decreased entry of Phe to the brain as measured by magnetic resonance spectroscopy (MRS).

For clearly documenting the impact of LNAAs, direct examination of the influence on brain Phe is needed. Some investigators have developed techniques using MRS to evaluate the brain level of Phe in patients with PKU.^{24–26} The mouse model for PKU is used in this study to demonstrate the effect of LNAA supplementation on blood Phe levels and brain Phe levels and to find out whether energy substrates from branched-chain amino acids (eg, acetyl-CoA, succinyl-CoA) become available by following the activity of branched-chain amino acid transferase (BCAT), an enzyme that is dependent on substrate concentration.

METHODS

Mice with PKU, genotype N-ethyl-N'-nitrosourea (ENU) 2/2 (classical PKU), were purchased from Jackson Laboratories (Bar Harbor, ME). These mice were produced at the University of Wisconsin by exposure to ENU, which causes a homozygous F263S mutation, resulting in classical PKU. Mice were genotyped to verify that they were homozygous for the F263S mutation according to the method of McDonald and Charlton.²⁷ PreKUnil, a preformulated tablet containing LNAA, was obtained from NiLab (Korsøer, Denmark). The composition of PreKUnil is shown in Table 1. ENU 2/2 mice were given either 0.5 g/kg or 1.0 g/kg PreKUnil suspended in sesame oil by gavage. Sham-treated mice were given sesame oil with no PreKUnil by gavage. The control mice were untreated ENU 2/2. Blood Phe and tyrosine were determined from blood on filter paper using tandem mass spectroscopy (Neogen, Pittsburgh, PA) at 0 time and at 48 hours. In another trial, 0.5 g/kg PreKUnil was given for 6 weeks, and blood Phe and tyrosine were determined at 0 time and then at weekly intervals.

For the study of brain Phe, ENU 2/2 mice were anesthetized and the brain was removed, separated into cortex and subcortex regions, and cooled immediately in liquid nitrogen. The brains were homogenized by mechanical crushing, and perchloric acid (12%) was used for extraction. The supernatant was neutralized with the pH adjusted to 7.2. The samples were lyophilized and rehydrated with 100% D2O for ¹H nuclear magnetic resonance

TABLE 2. Blood Phe Levels in PKU Mice Treated With PreKUnil

LNAA	Blood Phe mg/dL	
	Zero Time	48 Hours
0.5 g/kg	37.8	32.0
1.0 g/kg	37.0	18.4

(NMR) measurement. High-resolution 750-MHz proton NMR spectra were run on the supernatants. NMR measurements were performed on a Varian Unity-plus spectrometer using water-suppressed proton NMR spectroscopy. The NMR parameters for the single-pulse experiments were as follows: TR = 10 seconds, acquisition time = 3 seconds, saturation delay = 2 seconds, signal averages = 128. The residual water peak was set to 4.70 ppm. Peak integrals for the 3 aromatic resonances of Phe (integral range: 7.29–7.43 ppm) were compared with the creatine methyl (singlet, 3.04 ppm) to calculate relative metabolite concentrations.

BCAT activity was assayed by the method of Yvon et al.²⁸ The reaction mixture contained 70 mM Tris-HCl (pH 8.0), 3 mM L-isoleucine, 10 mM α -ketoglutarate, 0.05 mM pyridoxal 5-phosphate, and brain extract in a total volume of 1 mL. The reaction was performed at 37°C for 15 minutes. The reaction mixture was mixed with the colorimetric L-glutamic acid assay (Boehringer Mannheim, Mannheim, Germany) to form the coupled reaction as described earlier.²⁸ The coupled reaction mixture was read in the absorbance at 492 nm.

RESULTS

The mean blood Phe for mice ($n = 2$) that were given 0.5 g/kg LNAAs and 1.0 g/kg per day for 48 hours is shown in Table 2. Mice that were given 0.5 g/kg LNAAs had a 15% decrease in blood Phe, whereas mice that were given 1.0 g/kg LNAA had a 50% decline in blood Phe. Table 3 shows the blood Phe levels of PKU mice that were treated with 0.5 g/kg/d LNAAs for 6 weeks. In this experiment, there were sham and untreated mice as controls, with 2 mice in each group. The mice that were treated with LNAAs had a sustained decrease in blood Phe over the 6 weeks of the study.

BCAT activity in brain extracts was determined in wild-type mice ($n = 4$), PKU mice ($n = 4$), and PKU mice that were treated with 1.0 g/kg/d PreKUnil ($n = 4$). Table 4 shows that BCAT activity in the PKU mice is one third that of the wild-type mice. The ENU mice that were treated with PreKUnil had enzyme activity close to that of the wild-type mice.

The perchloric acid extracts from the brain of wild-type mice ($n = 3$), untreated PKU mice ($n = 3$), and LNAA-treated PKU mice (0.5 g/kg; $n = 9$) were subjected to MRS determination using 750-MHz NMR spectra as shown in Fig 1. Representative spectra show that the expanded region with aromatic Phe

TABLE 3. Blood Phe Levels of PKU Mice Treated With PreKUnil (LNAA), 0.5 g/kg/d

	ENU2/2	Sham-Treated Blood Phe mg/dL	LNAA-Treated ($n = 2$)
Baseline	30.0 \pm 3.7	25.9 \pm 1.2	34.9 \pm 1.9
1 wk	31.3 \pm 0.7	28.2 \pm 0.7	23.1 \pm 0.8
2 wk	34.1*	30.1 \pm 0.6	23.9 \pm 1.9
3 wk	32.2 \pm 1.3	25.5 \pm 0.6	22.9 \pm 1.1
4 wk	32.5 \pm 1.1	27.5 \pm 0.5	23.6 \pm 1.2
6 wk	33.0 \pm 1.2	30.4 \pm 1.4	19.3 \pm 5.7

* $n = 1$.

TABLE 4. BCAT in Brain of PKU Mice With and Without LNAA

Mice (<i>n</i> = 4)	BCAT (mU/mg Protein)
Wild-type	0.13
Untreated ENU 2/2	0.04
Treated ENU 2/2	0.14

resonances are discernible and are higher in the untreated PKU mice and intermediate in the LNAA-treated mice.

Extracts of brain of mice were subjected to MRS for determining the levels of Phe in different regions of the brain (cortex and subcortex). Wild-type mice, untreated mice, and mice that were treated with 0.5 g/kg or 1.0 g/kg/d PreKUnil were examined. There were 3 mice in each group. Table 5 shows that the integration of the Phe peaks related to the integral of

TABLE 5. Whole-Brain, Cortex, and Subcortex Phe in Mice With PKU After PreKUnil (LNAA) Treatment

Mice (<i>n</i> = 3)	Creatine Integral	Phe Integral	Ratio (%) Phe/Creatinine
Wild-type			
Cortex	99.23	0.77	0.078
Untreated			
Cortex	91.28	8.72	9.55
Subcortex	90.62	9.38	10.35
Whole brain	83.81	16.19	19.32
Treated			
Cortex (0.5 g/kg)	95.58	4.42	4.62
Subcortex (0.5 g/kg)	94.23	5.77	6.12
Whole brain (1.0 g/kg)	93.75	6.25	6.67

creatine. In all cases, the brain Phe decreased in the treated group compared with the untreated animals.

Phe in whole-brain extracts in wild-type (*n* = 3), untreated PKU (*n* = 3), and LNAA-treated mice (*n* =

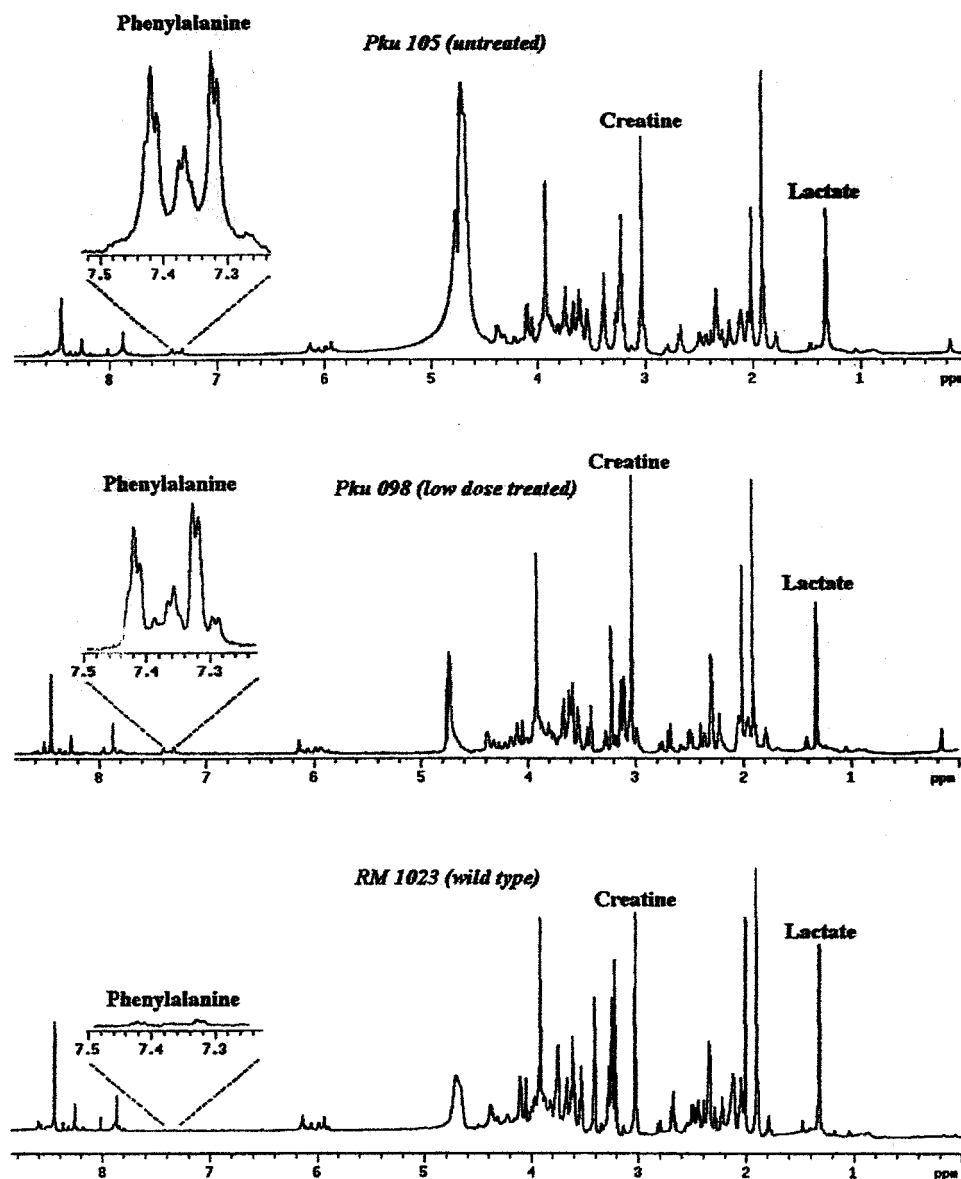


Fig 1. Representative 750-MHz NMR spectra of brain extracts from untreated (top) PKU mice, LNAA-treated PKU mice, and wild-type mice. The expanded regions show the aromatic Phe resonances, preserving the vertical scale across samples.

9) was determined. The ratio of Phe/creatinine was significantly increased in untreated PKU mice compared with wild-type mice ($P < .01$), and the Phe/creatinine ratio was significantly reduced in LNAA-treated mice compared with untreated PKU mice ($P = .01$) as shown in Fig 2.

DISCUSSION

The lack of adherence to diet in the treatment of PKU has resulted in neuropsychological deficits, even with early detection and treatment.²⁹ This has caused reassessment of treatment strategies and prompted the National Institutes of Health recommendation for treatment guidelines.⁶ The difficulty in attaining the goal of blood Phe of 120 to 600 $\mu\text{mol/L}$ in adolescents has been documented by the recent report from Walters et al.³ There has been an ongoing attempt to find other modalities for therapy. Experiments with the enzyme Phe ammonia lyase, which degrades Phe in the gastrointestinal (GI) tract, are now being pursued.^{30–32} The idea of using the competition of LNAAs on the blood-brain barrier to inhibit Phe entry to the brain has resurfaced. The blood brain barrier for LNAAs is mediated by a transporter with the lowest K_m for Phe. Therefore, this amino acid is favored to enter the brain compared with the other LNAAs.^{14,16} With high blood Phe levels, other LNAAs will be compromised in their ability to enter the brain. Lower levels of tyrosine, tryptophan, and branched-chain amino acids have been reported in patients with PKU and PKU mice.^{17,33} For overcoming these problems, short-term experiments using LNAAs in the treatment of PKU are being conducted.²⁴ It seems that MRS is an emerging strategy to measure brain Phe in humans.²⁶ We have used the mouse model of PKU to examine the possibility of reducing the influx of Phe to the brain of the mouse. The results of our experiments clearly show a significant reduction of brain Phe level in the dosages of LNAAs used. Furthermore, blood Phe levels in the mice that received LNAAs showed a significant decrease. Although brain Phe level decrease has been shown in humans,²⁴ the drop

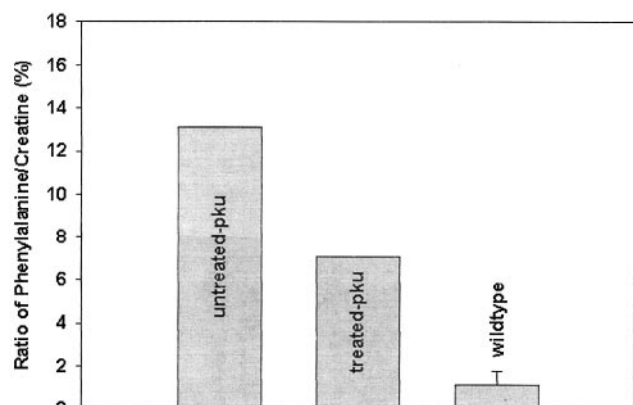


Fig 2. The ratio of Phe/creatinine in brain samples from mice with PKU, untreated mice ($n = 3$), 0.5 mg/kg treated mice ($n = 9$), and wild-type mice ($n = 3$). The untreated and wild-type mice were significantly different ($P < .01$), and the LNAA-treated and the untreated mice were significantly different ($P = .01$).

in blood Phe in patients with PKU has not been reported and needs to be investigated. Because the carrier for LNAAs is found in the GI tract with a higher K_m than the brain transporter, it may be possible to reduce the transport of Phe from the GI tract to the blood system by increasing the LNAAs in the diet.

For determining whether energy substrate acetyl-CoA and succinyl-CoA can improve in the brain after treatment with LNAAs, BCAT activity was examined in PKU mice and those that were treated with LNAAs. The data shown indicate a significant increase in BCAT activity in LNAA-treated mice, suggesting improved energy metabolism after treatment. Kang and Smith³³ showed a low level of leucine and other branched-chain amino acids in the PKU mouse brain and decreased protein synthesis. We do not know whether LNAA supplementation in mice with PKU would improve protein synthesis in the brain. Restoring the level of BCAT to normal with LNAAs may be a way that other metabolic pathways can be normalized in the brain of the PKU mouse. The mouse data presented are important and may have considerable effect on patients who are treated with LNAAs. The data from this study suggest that LNAAs can be used as a supplement to the conventional treatment of PKU and should allow for a more liberal diet and improved compliance.

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