Large neutral amino acids block phenylalanine transport into brain tissue in patients with phenylketonuria

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Large neutral amino acids (LNAAAs), including phenylalanine (Phe), compete for transport across the blood-brain barrier (BBB) via the t-type amino acid carrier. Accordingly, elevated plasma Phe impairs brain uptake of other LNAAAs in patients with phenylketonuria (PKU). Direct effects of elevated brain Phe and depleted LNAAAs are probably major causes for disturbed brain development and function in PKU. Competition for the carrier might conversely be put to use to lower Phe influx when the plasma concentrations of all other LNAAAs are increased. This hypothesis was tested by measuring brain Phe in patients with PKU by quantitative 1H magnetic resonance spectroscopy during an oral Phe challenge with and without additional supplementation with all other LNAAAs. Baseline plasma Phe was ~1,000 μmol/l and brain Phe was ~250 μmol/l in both series. Without LNAA supplementation, brain Phe increased to ~400 μmol/l after the oral Phe load. Electroencephalogram (EEG) spectral analysis revealed acutely disturbed brain activity. With concurrent LNAA supplementation, Phe influx was completely blocked and there was no slowing of EEG activity. These results are relevant for further characterization of the LNAA carrier and of the pathophysiology underlying brain dysfunction in PKU and for treatment of patients with PKU, as brain function might be improved by continued LNAA supplementation.


Introduction

In phenylketonuria (PKU), an inherited disorder of amino acid (AA) metabolism (McKusick 261600), blood levels of phenylalanine (Phe) are extremely elevated because of a deficiency of phenylalanine hydroxylase (PAH; Enzyme Commission 1.14.16.1). Untreated PKU leads to disturbed brain development with profound retardation, microcephaly, epilepsy, and other neurologic symptoms, which can be widely prevented by early institution of a Phe-restricted diet. Elevated Phe levels also acutely impair brain function in early treated, and thus normally developed, patients, which was proved using neuropsychologic tasks and electroencephalogram (EEG) spectral analysis (1–3).

Because some patients are not able to adhere rigorously to the Phe-restricted diet during early years of life, and given that most patients relax or stop the diet during adolescence, alternative treatment regimens have been developed. To overcome suspected cerebral dopamine and serotonin depletion, patients with PKU who were off diet were treated with the neurotransmitter precursors tyrosine (Tyr) and tryptophan (Trp) (4). To reduce Phe influx into the brain, Berry et al. (5) supplemented the branched-chain AAs valine, isoleucine, and leucine (VIL). However, widespread acceptance could not be obtained for either of these approaches.

Despite tremendous progress in the understanding of the molecular basis of PKU, the causes of brain damage in untreated patients and of the mechanisms underlying impaired brain function occurring with acutely elevated Phe levels are still unclear. Direct effects of Phe, as well as imbalances of the large neutral amino acids (LNAAAs) in brain, are thought to be major causes (6, 7).

In earlier studies, it was demonstrated that cerebral Phe can be readily detected noninvasively by proton magnetic resonance spectroscopy (1H-MRS) (8, 9) and that quantitative 1H-MRS can be applied to reliably determine its concentrations (9, 10). It was also shown that the influx of Phe through the blood-brain barrier (BBB) during an oral Phe challenge can be monitored by 1H-MRS (11). The aim of the present study was to use this approach to further investigate Phe transport through the BBB in patients with PKU by manipulating blood concentrations of Phe and the LNAAAs valine, methionine, isoleucine, leucine, Tyr, histidine, and Trp, while monitoring brain activity by EEG spectral analysis.

Methods

Human subjects. Six male patients with PKU were included in this study, which was approved by the Ethics Committee of the University of Heidelberg. Before the study, the experimental protocol was explained to all subjects, and their written informed consent was obtained.

The mean age of the patients was 27.6 years (range 26–30) (Table 1). None of the patients had any history of other factors disturbing brain development. Start of dietary treatment, defined by the first plasma level of Phe <600 μmol/l after diet onset, was at a mean age of 38 days (range 24–63) after birth.
Clinical phenotype was determined on the basis of Phe tolerance at five years of age (12), and standardized oral protein loading was performed mostly at the age of six years (13). Four patients (nos. 1, 2, 3, 6) still practiced a Phe-restricted diet. Long-term adult biochemical control was determined as the mean of six-month medians that were obtained from all Phe values in the last three years prior to the study.

Clinical data. Clinical evaluation (Table 1) included the identification of PAH mutations, which was based on a combination of denaturing gradient gel electrophoresis (DGGE), restriction enzyme analysis, and sequence analysis. Referring to in vitro expression of mutations and genotype-phenotype relations, mutations were classified as either severe, moderate, or mild (12). All patients were examined with a standardized neurologic investigation, routine EEG (10/20), standard magnetic resonance imaging (MRI) examination (grading of white matter abnormalities according to Priet et al. (14)), and intelligence testing (revised Wechsler Adult Intelligence Scale).

Ten healthy volunteers with a mean age of 25.3 years (range 20–46) were examined with 1H-MRS at a single point in time to obtain unimpaired normal 1H-MRS spectra. Their neurologic status and brain MRI scans were normal. Heterozygous status for known PAH mutations was excluded, and plasma Phe and LNAA concentrations (Table 2) were determined according to the procedures used in the patients.

Experimental protocol of Phe and LNAA intake. The study was constructed in a cross-over repeated measurement design to determine treatment effects in the six patients with PKU. Design and timing of all investigations are depicted in Figure 1. All patients were administered a single oral dose of purified l-Phe (100 mg/kg body weight; time 0 h) in each of two test series. In one series, termed Phe-LNAA (solid lines in all figures), Phe intake was supplemented for 12 h with an LNAA mixture containing 150 mg/kg body weight of each of the following AAs: valine, methionine, isoleucine, leucine, Tyr, histidine, and Trp. The LNAA mixture was divided into five equal portions and started two hours before the Phe load. The subsequent four portions were taken every three hours (one, four, seven, and 10 h postload). During the experiments, the usual diet was unchanged, except that the patients still on diet did not consume their regular AA mixture. To avoid hypoglycemia due to the intake of large amounts of free AAs, regular small meals rich in free carbohydrates and poor in protein were given during both series, always about two hours before blood drawings. The results of this Phe-LNAA series were compared with an otherwise identical baseline series without LNAA intake (termed Pheonly; dotted lines in all figures). To control for effects of order, in three patients, the Phe+LNAA series was performed first; in the others, the order was reversed. The interval between the two series was four to eight weeks. Ten days after the start of each series, plasma Phe and LNAA concentrations were monitored to ensure return to pretreatment levels.

Amino acid analysis. Plasma Phe levels during the study were determined by an enzymatic assay (Quantase Phe assay; Porton, Cambridge, United Kingdom). Valine, methionine, leucine, isoleucine, Tyr, and histidine were analyzed by automated ion-exchange chromatography with ninhydrin, using an AA analyser (Sykam, Munich, Germany) following standard procedures. Plasma Trp was determined by HPLC (Beckman System Gold, Palo Alto, California, USA). To compare AA plasma levels during the LNAA supplementation and the baseline period, the area under the curve was calculated for a time range from 0.5 to 11 h after Phe load. During the Pheonly series, plasma LNAA analysis was restricted to –0.5, 0.5, one, five, 11, and 24 h after Phe load. Blood samples for AA analysis in control subjects and for pretreatment values in the patients were taken in the morning two hours after a light standardized breakfast.

Effects of Phe loading and LNAA supplementation on carrier-mediated Phe transport at the BBB were estimated, based on the $K_m$-normalized Phe ratio (ratio of Phe to the other LNAAAs) regulating the influx through the BBB under AA competition for the same carrier (15):

$$K_m\text{-normalized PHE ratio} = \frac{[\text{PHE}]}{\sum K_m^{\text{PHE}} [\text{LNAA}] - [\text{PHE}] K_m^{\text{PHE}}}$$

In equation 1, $K_m^{\text{PHE}}$ and $K_m^{\text{LNAA}}$ are the absolute $K_m$ values of Phe and all other competing LNAAAs, respectively, and $[\text{PHE}]$ and $[\text{LNAA}]$ are their respective plasma concentrations. $K_m$ values (Table 2) determined in vivo in the conscious rat were used (16). Similarly, $K_m$-normalized Tyr and Trp ratios were derived.

Proton MRS. To determine brain tissue concentrations of Phe, localized 1H-MRS was performed on a routine 1.5 T MR scanner (General Electric Medical Systems, Milwaukee, Wisconsin, USA) preloaded (minus one hour) and six, 12, and 24 h postload. Spectra from a large volume placed above the ventricular system (white and gray matter of both hemispheres) were recorded using a quadrature head coil. Voxel volume was typically 60 × 50 × 20 mm. Axial $T_2$-weighted MR images were used for accurate (re-)positioning of the MRS voxel. Four spectra of 128 acquisitions were acquired in each session, using a PRESS sequence (Bottomley, Pennsylvania, USA) with outer volume suppression, water presaturation, and phase rotation (echo time 20 ms, repetition period three seconds, 2,048 data points) as described earlier (9). For the determination of different brain compartments and water referencing, a series of separate recordings with different echo times, but without water suppression, was acquired.

The data processing scheme used was similar to the one described in ref. 9. It is based on a three-compartment model (brain tissue, cerebrospinal fluid [CSF], blood) in which the visibility of plasma Phe was set to zero and the Phe content of CSF was assumed to be equal to that of brain tissue (rather than a steady-state relation with regard to plasma Phe, as assumed in ref. 9). Conversion to absolute concentration units was performed based on a proton signal density taken from the literature and on the unsuppressed water signal. The determination of the Phe peak area had been redesigned compared with ref. 9. It is now performed using a versatile time-frequency domain-fitting program (17) with the following steps. To parameterize normal background signals, the averaged spectrum of four normal subjects was fitted using 23 Voigt lines and a common nonanalytic line shape defined on the unsuppressed water peak (10 lines in the relevant downfield region). The spectrum of an aqueous 25 mM Phe model solution (pH 7.05) was similarly fitted and parameterized (11 Voigt lines to define the complicated strong coupling pattern). The combination of the two parameter sets (i.e., the models of Phe and the in vivo background signals each treated as nuclear magnetic resonance entities) was then used to determine the Phe peak areas in all in vivo spectra. Because the signal phase and line shapes were determined on the unsuppressed water signal, the peak area determination of the low signal-to-noise ratio peak of Phe was thus essentially reduced to amplitude fitting of background and Phe models in the real part of an absorption mode spectrum. This approach is illustrated in Figure 2 for a single spectrum (4 × 128 acquisitions) of a patient with PKU acquired before the Phe load (Figure 2a). The parameterized and line shape–adapted models of in vivo background signals and Phe are displayed in Figure 2, e and f, respectively, whereas the trace in Figure 2g contains the residual spectrum, i.e., original (Figure 2a) minus best fit (Figure 2d). Spectra from model solutions of the LNAA mixture as well as Tyr and Trp were recorded to rule out confounding signal contributions of the LNAAAs.
Brain tissue concentrations are given in units of micromole per kilogram of wet weight. Plasma concentrations are listed in units of micromole per liter. Concentration gradients of Phe between plasma and brain are reported as dimensionless values, obtained by a unit conversion using brain and plasma density values of 1.05 kg/l.

**EEG analysis.** To monitor brain activity during the two series in the six patients with PKU, EEGs in the relaxed awake state with eyes closed were derived -2.5 h preload (before first LNAA intake) and -0.5 h preload (after first LNAA intake), as well as 5.5, 11.5, and 23.5 h postload immediately before the 1H-MRS session. To control for diurnal variations of EEG spectra, Phe+LNAA and Pheonly series were parallel with respect to the time of Phe loading and subsequent EEG investigations, thereby ensuring that diurnal variations of EEG activity were taken into account. Electrode locations were according to the 10/20 system (monopolar, common reference, F = 128 Hz, time constant = 1.0). EEG activity was digitized with 256 Hz and stored on a personal computer. From each single recording of about 10 min duration, 16–20 artifact-free sections of 4.096 s were chosen visually. Spectral analysis was performed by fast Fourier transformation. Relative power (calculated as percentage of total power within the 1.5–25 Hz range) was computed for the 3.5–7.5 Hz, δ (1.5–3.5 Hz), θ (3.5–7.5 Hz), α1 (7.5–9.5 Hz), α2 (9.5–12.5 Hz), β1 (12.5–18.5 Hz), and β2 (18.5–25 Hz) bands from averaged spectra. In addition, the mean frequency of the power spectrum from 1.5 to 25 Hz (mean power frequency [MPF]), the frequency of maximum power within the α band (peak frequency [PF]), and the proportion of the power in the α band to the power in the δ band (δ/α ratio), were derived from power spectra. Statistical evaluation was restricted to lead O2. Comparability results were determined at other locations. All EEGs were also assessed by standard visual criteria.

**Statistical analysis.** Statistics were compiled by SAS, version 6.12 (SAS Inc., Cary, North Carolina, USA). Comparison of means within, as well as between, the two series (Phe+LNAA, Pheonly) were compared by Student’s paired t tests. Because of the small number of observations, correlational analysis was done by calculation of rank correlational coefficients (Kendall’s τ).

**Results**

**Clinical data.** On the basis of clinical data (Table 1), five patients corresponded to moderate PKU, and one patient had a severe type. Mutations were mostly typical for European patients with PKU, e.g., R408W, R261Q, G272X. In P225R, first described in a Sweden, and in Y166X, which was not described earlier, the severity of the mutations are unknown. In patient no. 5, one mutation could not be determined, despite repeated DGGE analysis. Assuming a large deletion or mutation of one of the large introns, a null mutation (residual activity <1%) is most probable. Regarding Phe tolerance at five years (12) and data from standardized protein loading tests (13), clinical phenotype and genotype corresponded. Neurologic status was normal except for mild tremor, which was present in two patients. Long-term baseline Phe values during adult age were lower in patients on diet compared with patients off diet.

**Baseline measurements.** Plasma Phe was normal in all control subjects (mean 66 ± 9 μmol/l). In the six patients with PKU, preload plasma Phe values (Table 2) ranged between 796 and 1,459 μmol/l and were thus in the typical long-term range of these patients. Mean preload plasma Phe concentrations before start of the Phe load and LNAA treatment did not differ between both series (t = 0.76, not significant [ns]). Mean pretreatment plasma concentrations of all other LNAA (Phe+LNAA –10 and –2.5 h; Pheonly –0.5 h) were also comparable for the two series (Table 2 and Figure 3). Before the Phe load, mean K_m-normalized Phe ratios of 5.7 and 5.6 resulted for the Pheonly and the Phe+LNAA series, respectively (Table 3), in comparison with a mean K_m-normalized Phe ratio of 0.33 ± 0.04 in the control subjects. Preload K_m-normalized Tyr and Trp ratios for the PKU patients were 0.015 and 0.026, respectively, in both series (Table 3), compared with 0.09 (Tyr ratio) and 0.19 (Trp ratio) in the control subjects.

In all 12 preload 1H-MR spectra of the patients, excess Phe peaks at 7.37 ppm were identified; these peaks are attributed to the phenyl protons of Phe. Figure 2 contains the spectrum for one patient before the Phe load in the Pheonly series (plasma Phe of 1,193 μM; brain Phe 265 μmol/kg) and demonstrates the signal-to-noise ratio achieved for single data points. Absolute brain tissue concentrations of Phe are listed in Table 3. Mean statistical uncertainty of a single brain Phe measurement (mean SEM of four spectra for each session) was 17 and 19 μmol/kg for the Pheonly and Phe+LNAA series, respectively.
Table 2

Plasma LNAA concentrations in patients with PKU

<table>
<thead>
<tr>
<th>LNAA</th>
<th>$K_m$</th>
<th>Control</th>
<th>Pretreatment</th>
<th>Postload</th>
<th>Postload</th>
<th>Treatment</th>
<th>Postload</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>values</td>
<td>n = 10</td>
<td>Phe –0.5 h</td>
<td>six hours</td>
<td>12 h</td>
<td>Mean</td>
<td>24 h</td>
</tr>
<tr>
<td></td>
<td>(μmol/l)</td>
<td></td>
<td>LNAAs –0.5</td>
<td></td>
<td></td>
<td>0.5–11 h</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>32 ± 9</td>
<td>66 ± 9</td>
<td>1,036 ± 199</td>
<td>1,890 ± 244***</td>
<td>1,838 ± 181***</td>
<td>1,893 ± 203***</td>
<td>1,693 ± 196***</td>
</tr>
<tr>
<td>Valine</td>
<td>168 ± 72</td>
<td>211 ± 33</td>
<td>218 ± 59</td>
<td>204 ± 48</td>
<td>237 ± 21</td>
<td>198 ± 34</td>
<td>215 ± 55</td>
</tr>
<tr>
<td>Methionine</td>
<td>83 ± 16</td>
<td>41 ± 6</td>
<td>33 ± 8</td>
<td>30 ± 16</td>
<td>32 ± 11</td>
<td>27 ± 8</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>145 ± 29</td>
<td>67 ± 12</td>
<td>71 ± 14</td>
<td>54 ± 17*</td>
<td>68 ± 135</td>
<td>53 ± 14*</td>
<td>57 ± 18**</td>
</tr>
<tr>
<td>Leucine</td>
<td>87 ± 11</td>
<td>128 ± 22</td>
<td>136 ± 21</td>
<td>120 ± 42</td>
<td>135 ± 32</td>
<td>115 ± 37</td>
<td>109 ± 36*</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>86 ± 17</td>
<td>59 ± 11</td>
<td>43 ± 7</td>
<td>40 ± 11</td>
<td>54 ± 13</td>
<td>39 ± 9</td>
<td>47 ± 13</td>
</tr>
<tr>
<td>Histidine</td>
<td>164 ± 28</td>
<td>82 ± 9</td>
<td>85 ± 14</td>
<td>74 ± 15</td>
<td>82 ± 8</td>
<td>73 ± 12</td>
<td>78 ± 10</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>52 ± 14</td>
<td>68 ± 13</td>
<td>49 ± 10</td>
<td>34 ± 9**</td>
<td>41 ± 9</td>
<td>35 ± 8**</td>
<td>34 ± 6**</td>
</tr>
<tr>
<td>Lysine</td>
<td>178 ± 33</td>
<td>161 ± 33</td>
<td>150 ± 48</td>
<td>170 ± 63</td>
<td>146 ± 41</td>
<td>155 ± 49</td>
<td></td>
</tr>
</tbody>
</table>

$K_m$ values of AA transport at the blood-brain barrier determined in the conscious rat, according to ref. 19; the $K_m$ of lysine, which does not belong to the group of LNAA, was not determined. Normal data were obtained from 10 healthy subjects who also served in the determination of normal reference $^1$H-MR spectra. Significance values are given for differences.

1. $K_m$ values of AA transport at the blood-brain barrier determined in the conscious rat, according to ref. 19; the $K_m$ of lysine, which does not belong to the group of LNAA, was not determined.
2. Normal data were obtained from 10 healthy subjects who also served in the determination of normal reference $^1$H-MR spectra. Significance values are given for differences.

...
Lysine 149 ± 24 103 ± 32* 105 ± 53 101 ± 34* 129
Tyrosine 53
Leucine 146
Isoleucine 82
Methionine 40
Phenylalanine 1,063
between preload and postload values within the two series: *

Table 2 (Continued)

<table>
<thead>
<tr>
<th>LNAAs</th>
<th>Pretreatment Phe –0.5</th>
<th>LNAAs –2.5 h</th>
<th>Postload six hours²</th>
<th>Postload 12 h²</th>
<th>Treatment Mean 0.5–11 h</th>
<th>Postload 24 h³</th>
</tr>
</thead>
</table>

Phenylalanine 1,063 ± 189 1,887 ± 235** 1,669 ± 168** 1,755 ± 80*** 1,495 ± 128**
Valine 258 ± 72 817 ± 250*** 816 ± 191*** 622 ± 117*** 311 ± 72²
Methionine 40 ± 8 404 ± 178** 551 ± 192** 334 ± 108*** 123 ± 80²
Isoleucine 82 ± 11 377 ± 118*** 332 ± 109** 218 ± 46** 69 ± 22²
Leucine 146 ± 45 398 ± 105*** 338 ± 88** 243 ± 42** 115 ± 32²
Tyrosine 53 ± 22 198 ± 65** 202 ± 64** 181 ± 41** 68 ± 14²
Histidine 88 ± 13 224 ± 71** 226 ± 64* 174 ± 38** 104 ± 19²
Tryptophan 54 ± 16 234 ± 38*** 212 ± 67** 205 ± 31*** 54 ± 16
Lysine 149 ± 24 103 ± 32* 105 ± 53 101 ± 34² 129 ± 16

Discussion

In this study, brain Phe concentrations were measured by means of in vivo ¹H-MRS in two series of AA loading experiments in patients with PKU. Our results prove that despite significantly increasing plasma Phe values, cerebral Phe concentrations remained unchanged, or were even lowered, during concomitant high-dose oral intake of an LNAAs mixture. Although a complete block of Phe influx seems to be the most plausible explanation, other mechanisms (e.g., influences on cerebral Phe metabolism as well as AA efflux from brain) have to be considered. It was shown that high brain Phe concentrations cause a slowing of electrical brain activity and that this effect can be avoided by LNAAs supplementation. These main results are relevant to basic research.

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addressing the properties of the LNAA carrier through the BBB and to understanding the biochemical mechanisms underlying brain dysfunction in PKU. They are also significant for treatment of patients with PKU.

**LNAAs system.** Normal brain development and function depend on the continuous influx of nutrients through the BBB. Therefore, the properties of specific transport systems have been an essential issue in the understanding of physiological as well as pathophysiological conditions. For LNAAs, net uptake through the BBB is determined by their ratio in plasma and their different affinity to the stereospecific L-type AA carrier system (18–21). Carrier saturation and competitive inhibition of the influx of other LNAAs can be expected at supra-physiological plasma Phe levels usually found in PKU and may be present already at concentrations in the range of 200–500 μmol/l (18). The Km-normalized Phe/LNAA was calculated using measured plasma concentrations and Km values from the literature. It predicted that in the Pheonly series, the increase of plasma Phe should lead to a further increase in brain Phe uptake, whereas in the PheLNAA series, brain Phe uptake should decrease despite doubled plasma Phe. The 1H-MRS and EEG measurements presented prove these predictions to be correct and the underlying model valid.

The applicability of such a simple model to the in vivo situation is surprising because the calculation of LNAAs transport on the basis of apparent Km values under non–steady state conditions obviously disregards some important aspects. First, it may appear questionable to use Km values obtained in an animal study, but there exists agreement in the literature that Km values can be transferred from animals to humans (16, 18, 19, 21). Second, Km values reported in the literature vary to some degree, even within one species, depending on the experimental approaches used. However, the main results from using the equation for competing enzyme kinetics are well reproduced, even when other sets of Km values are used (19, 20). Only the use of Km values determined in vitro in isolated human capillaries (21) produced somewhat different results, with smaller effects of LNAAs competition. This confirms that results from in vitro studies cannot directly be transferred to the in vivo situation. Third, AA uptake by the brain cannot be fully described by a simple two-compartment model. AA transport also occurs at cellular and intracellular membranes (22). Thus, the distribution of AAs into different compartments and fluid spaces, as well as efflux from brain, ought to be considered (23, 24). Furthermore, gli and neuronal cells seem to be different with regard to their free AA pools (24). However, it appears that LNAAs uptake via the high-affinity L-type carrier at the endothelial wall constitutes the rate-limiting step under physio-

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### Table 3

| Plasma and brain Phe concentrations, Km-normalized Phe/LNAA ratio, plasma/brain Phe ratio, and EEG spectral parameters in PKU patients as functions of time |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| **Pheonly series (baseline)** | **PheLNAA series 100 mg/kg Phe at time 0 h** |
| **Preload** | **Postload** | **Postload** | **Postload** | **Postload** | **Postload** | **Postload** | **Postload** | **Postload** |
| **Plasma Phe (μmol/l)** | 1.036 ± 1.99 | 1.890 ± 2.44 | 1.838 ± 1.81 | 1.963 ± 1.96 | 1.963 ± 1.96 | 1.963 ± 1.96 | 1.963 ± 1.96 | 1.963 ± 1.96 |
| **Km-normalized Phe ratio** | 5.7 ± 1.4 | 12.0 ± 1.7 | 10.3 ± 2.2 | 11.1 ± 2.4 | 11.1 ± 2.4 | 11.1 ± 2.4 | 11.1 ± 2.4 | 11.1 ± 2.4 |
| **Km-normalized Tyr ratio** | 0.015 ± 0.003 | 0.007 ± 0.002 | 0.011 ± 0.003 | 0.011 ± 0.003 | 0.011 ± 0.003 | 0.011 ± 0.003 | 0.011 ± 0.003 | 0.011 ± 0.003 |
| **Brain Phe (μmol/kg wet weight)** | 252 ± 73 | 344 ± 54 | 377 ± 80 | 397 ± 88 | 397 ± 88 | 397 ± 88 | 397 ± 88 | 397 ± 88 |
| **EEG: Gd band (μHz)** | 19.6 ± 11.5 | 24.1 ± 14.7 | 23.5 ± 14.8 | 24.8 ± 13.8 | 24.8 ± 13.8 | 24.8 ± 13.8 | 24.8 ± 13.8 | 24.8 ± 13.8 |
| **EEG: Gd band (%)** | 16.2 ± 10.2 | 13.2 ± 9.4 | 14.2 ± 9.1 | 11.3 ± 9.1 | 11.3 ± 9.1 | 11.3 ± 9.1 | 11.3 ± 9.1 | 11.3 ± 9.1 |
| **EEG: P30 band (%)** | 4.5 ± 4.2 | 3.8 ± 3.0 | 3.5 ± 2.7 | 3.0 ± 2.1 | 3.0 ± 2.1 | 3.0 ± 2.1 | 3.0 ± 2.1 | 3.0 ± 2.1 |
| **EEG: P20 band (%)** | 9.7 ± 0.5 | 9.3 ± 0.6 | 9.6 ± 0.9 | 9.2 ± 0.5 | 9.2 ± 0.5 | 9.2 ± 0.5 | 9.2 ± 0.5 | 9.2 ± 0.5 |
| **EEG: P50 band (%)** | 9.3 ± 0.6 | 9.1 ± 0.9 | 9.3 ± 1.1 | 8.8 ± 0.8 | 8.8 ± 0.8 | 8.8 ± 0.8 | 8.8 ± 0.8 | 8.8 ± 0.8 |

*Significance values are given for differences between preload and postload values within the two series: *P < 0.05, **P < 0.01, ***P < 0.001. Because of repeated use of the preload data, significance with P < 0.05 should be treated with caution. *For the calculation of ratios, brain tissue concentrations were transformed to μmol/l according to a brain tissue density of 1.05 g/l. **Because of diurnal EEG variations, significance values are given for differences between the Pheonly and PheLNAA series: IP < 0.10, ***P < 0.05, ****P < 0.01.
logical conditions. Finally, the role of adaptive processes is still quite unclear. Based on results obtained with the double-indicator method, Knudsen et al. (25) speculated that interindividual differences between patients with PKU with regard to $K_m$ and $V_{max}$ could be explained by an upregulation of the number of BBB carriers. The individual time courses of our measured brain versus plasma Phe levels (not shown) appear to confirm a large individual spread in kinetic parameters.

$^1$H-MRS. As in earlier studies (8, 9, 11), it was possible to quantitate by $^1$H-MRS cerebral Phe concentrations in patients with PKU, thereby confirming the usefulness of $^1$H-MRS for the quantitative study of inborn errors of metabolism in general and PKU in particular. The presented results for the steady-state plasma/brain ratio for Phe are in close accordance with our earlier values (average of 4.0 for preload values of the Phe-only series versus 4.1 in ref. 11).

The preload values of Phe were somewhat different between the two series. It seems quite possible that this small difference is caused by the first dose of LNAAs taken before the baseline measurement. This explanation is supported by the finding that plasma and brain values correlate significantly for the Phe-only series, but not for the Phe-LNAA series. It indicates that an increase in plasma LNAA concentration without Phe challenge might be used to lower steady-state brain Phe levels.

With the current setup of $^1$H-MRS data acquisition and processing, brain Phe concentrations down to 100 $\mu$mol/kg can be determined reproducibly. This corresponds to a detectable proton density of 500 $\mu$mol/kg. Given this detection threshold and the spectral pattern of valine (six protons in an upfield doublet), one can estimate that brain valine content remains below 500 $\mu$mol/kg when plasma valine is between 600 and 900 $\mu$m. Similarly, we did not detect any interfering signals from Trp, Tyr, or histidine in the downfield region during the LNAA series.

With the detection limit achieved, $^1$H-MRS is a suitable noninvasive method to characterize the LNAA uptake at the BBB even in healthy subjects. However, it has to be taken into account that cerebral Phe concentrations are determined not only by AA influx but also by efflux and metabolism. These factors, which contribute to cerebral AA concentrations, in principle can be deconvoluted either by dynamically fitting the time course of cerebral Phe elevations or by the use of isotopically labeled precursors. This approach is particularly promising, as alternatives (25, 26) are invasive, requiring puncture of the internal jugular vein or application of radioisotopes, and are based on various assumptions.

Möller et al. (27) tried to determine the kinetic characteristics of Phe transport at the BBB by use of steady-state $^1$H-MRS measurements and, analogous to glucose transport kinetics (28), application of symmetric Michaelis-Menten kinetics with an arbitrary constant
Figure 4
Averaged 1H-MRS difference spectra (patients minus averaged normal spectra), acquired in vivo before as well as 6, 12, and 24 h after the oral Phe load. The increase of the Phe peak at 7.37 ppm during the Phe+LNAA series (dotted line) contrasts with the unchanged Phe peak 6 and 12 h postload in the Phe+LNAA series (solid line). When LNAA treatment was stopped, brain Phe also increased in this series (measurement at 24 h).

Phe consumption rate. This method strongly relies on data obtained from patients with extremely high plasma Phe values and is also susceptible to nonlinearities in Phe quantitation. Their apparent $K_m$ and $T_{max}/V_{max}$ values do not concur well with the current data if the same simplified model is applied.

Pathophysiology of PKU. The biochemical mechanisms leading to impaired brain development and function in PKU are still the subject of research. Direct effects of elevated Phe concentrations on several enzyme systems, and consequences of a concomitant depletion of other LNAAs in the brain, are thought to be the most important factors for the disturbed brain development in untreated PKU (7). Research has focused on myelin synthesis and turnover, protein synthesis, and amine neurotransmitters. Significant effects were observed with regard to the initiation and further processing of protein synthesis (29). Hyperphenylalaninemia led to a loss of several AAs, especially LNAAs, in the brain of newborn mice, with a concomitant decrease in the incorporation of AAs into protein. Interestingly, the coadministration of LNAAs prevented this effect (30). However, the significance of animal models using experimentally induced hyperphenylalaninemia (29, 30) remains questionable because of uncontrolled side effects of the PAH inhibitors used, e.g., α-methylphenylalanine.

In addition to their long-term impact on brain structure, high Phe concentrations have acute effects on brain function. However, it is possible that thresholds for acute Phe effects and chronic Phe neurotoxicity in the developing brain are different. It has been discussed that impaired synthesis of serotonin and dopamine could underlie these effects. Krause and colleagues (1, 2) found correlations between high plasma Phe levels with decreased performance on neuropsychologic tests of higher integrative function and characteristic changes in the frequency spectrum of the EEG background activity with high brain Phe concentrations, which were seen earlier in children (1) and adults (3) with PKU.

Disturbances of the free AA pools of the brain may have further implications for brain function because, in addition to serotonin and dopamine, other AAs (e.g., glutamate, aspartate, glycine) serve as neurotransmitters. Significant effects were observed with regard to the initiation and further processing of protein synthesis (29). Hyperphenylalaninemia led to a loss of several AAs, especially LNAAs, in the brain of newborn mice, with a concomitant decrease in the incorporation of AAs into protein. Interestingly, the coadministration of LNAAs prevented this effect (30). However, the significance of animal models using experimentally induced hyperphenylalaninemia (29, 30) remains questionable because of uncontrolled side effects of the PAH inhibitors used, e.g., α-methylphenylalanine.

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isoleucine, and 200 mg/kg leucine) was designed to inhibit Phe influx into the brain. Although plasma concentrations of Phe remained unchanged, an ~20% decrease of Phe concentrations in CSF seemed to confirm the hypothesis. In addition, improvements on a neuropsychologic task were shown for VIL treatment (5, 39). The VIL approach was also used in an animal model of maternal PKU (40). Rats exposed to high phenylalanine levels in utero showed characteristic learning deficits, which could be prevented by VIL treatment. Long-term VIL treatment did not show any side effects. However, as criticized by Hommes (41), CSF Tyr concentrations were lowered further during VIL treatment. It was also noted that the rate at which protein synthesis may be reduced in the PKU brain will depend on the supply of all essential AAs. Thus, the increase of only one (Tyr) or three (VIL) AAs may even accentuate disturbances of neurotransmitter or protein synthesis (42). VIL therapy did not find acceptance in PKU treatment. In the present study, Km-corrected Tyr, and Trp ratios improved during LNAA supplementation, even under the condition of steeply increasing plasma Phe levels.

To date, there exists only one report on the use of LNAAAs in human PKU. In addition to a Phe-restricted diet, Dotremont et al. (43) used a supplement of 0.8 g/kg body weight LNAA mixture. This was well tolerated without adverse effects. Laboratory evaluations were all normal, except for extremely low lysine levels. Also, in our study, a reversible ~30% decrease of plasma lysine was noticed during LNAA treatment. The causes for this effect are unclear. One possible explanation for this phenomenon could be a stimulation of protein synthesis. However, this result highlights the necessity of careful AA monitoring during supplementation trials. Referring to animal studies that proved lysine deficiency in hyperphenylalaninemic rats, Huether et al. (44) noted that additional lysine supplementation should be considered in PKU treatment. Unfortunately, further data highlighting cerebral Phe concentrations or evaluations of brain function were not reported by Dotremont et al. (43). Before use in the present study, identical LNAA supplementation was studied in three other adult control subjects with normal Phe plasma levels. This trial showed no side effects, and EEG spectra were not influenced by LNAA intake (data not shown).

In standard dietary treatment of PKU, large amounts of free AAs are consumed to compensate for the Phe-restricted, and hence, also natural protein–restricted, diet. The present results now raise the question whether such a regular AA mixture may have the previously unexpected effect of lowering brain Phe in addition to replacing natural protein. Ris et al. (45) found negative correlations between verbal intelligence quotient (IQ) and age when formula use was discontinued in adult patients with PKU. This correlation was even stronger than the association of verbal IQ and age when Phe exceeded 1,200 µmol/l. Whether patients off diet might profit from the continuing intake of AA mixtures was considered. This speculation is supported by our results. In addition, the profiles of LNAA increase (Figure 3) after repeated oral intake elucidate intestinal resorption kinetics and elimination from plasma. These aspects as well as the specific Km values of the AAs used should be considered in further experiments to obtain a more balanced pattern of LNAA concentrations.

Recently, the metabolic effects resulting from large intakes of AA mixtures have been reported (46). The recommended daily amount of ~1.6 g/kg body weight AA mixture was given in a single dose to control subjects, leading to a substantial increase of AAs. For example, leucine increased from ~100 µmol/l preload to ~350 µmol/l. Thus, AA increases were nearly in the range of AA concentrations measured in our study. Side effects of AA intake included an increase of insulin in plasma, leading to significant decreases in blood glucose. Most of these side effects, however, could be prevented by dividing the single dose into three portions per day.

In conclusion, these results show that transport kinetics of carrier systems at the BBB can be quantified non-invasively by 1H-MRS. Evidence is provided that LNAA supplementation blocks Phe influx at the BBB and prevents disturbances of brain electrical activity caused by

**Figure 5**
Averaged EEG power spectra from patients with PKU. During the Phe-only series (dotted line), a shift of the dominant peak of EEG background activity to the lower-frequency spectrum is demonstrated, which was prevented by LNAA treatment (solid line).
high cerebral Phe concentration in an acute-phase study. Further studies in patients with PKU who are off diet are now warranted to determine whether brain Phe concentration can be lowered during steady state and whether brain function can be improved by long-term continued LNA supplementation.

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