Effect of Phenylalanine and Its Metabolites on the Proliferation and Viability of Neuronal and Astroglial Cells: Possible Relevance in Maternal Phenylketonuria

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ABSTRACT

Phenylketonuria is a genetic defect that, without strict dietary control, results in the accumulation of phenylalanine (Phe) in body fluids. If a low-Phe diet is not maintained during pregnancy, the offspring of phenylketonuric women are born with mental retardation and microcephaly. Primary cultures of rat cerebellar granule cells, rat cortical astrocytes, human fetal astrocytes, and human neuroblastoma (SY5Y) cells and human astrocytoma (1321N1) cells were used to test the hypothesis that the microencephaly may be a result of neuronal cell death and reduced astrocyte proliferation. Exposure to Phe or to six Phe metabolites [phenylacetic acid (PAA), phenyllactic acid, hydroxyphenylacetic acid, phenylpyruvic acid, phenylethylamine (PEA), and mandelic acid] did not result in astroglial or neuronal cell cytotoxicity. Treatment of 1321N1 cells, human fetal astrocytes, or rat astrocytes with 5 mM Phe for 24 h decreased DNA synthesis 19 ± 4%, 30 ± 4%, and 60 ± 6%, respectively. This effect was concentration dependent, and flow cytometry revealed that Phe treatment resulted in the accumulation of cells in the G0/G1 phase of the cell cycle. In addition, in 1321N1 cells, exposure to 5 mM PAA, and in rat astrocytes, exposure to 0.5 mM PEA inhibited cell proliferation 42 ± 4% and 55 ± 4%, respectively. These metabolites also resulted in the accumulation of cells in the G0/G1 phase of the cell cycle. In human fetal astrocytes, 0.5 mM PEA and 0.5 mM PAA resulted in a 41 ± 12% and 52 ± 11% reduction proliferation, respectively.

Phenylketonuria (PKU) is a genetic defect in phenylalanine hydroxylase, an enzyme that converts phenylalanine (Phe) to tyrosine. In humans, PKU is characterized by a plasma Phe level above 1.2 mM (Rezvani, 1996), however, plasma Phe levels as high as 6 mM have been reported (Swaiman and Wu, 1984). At birth, an individual with classic PKU (cPKU) is clinically normal and, as a result of early diagnosis and treatment with a diet low in Phe, 97% of all diagnosed phenylketonuric children are intellectually normal (Williamson et al., 1981). If left untreated, however, cPKU results in mental retardation and seizures (Menkes, 1995; Rezvani, 1996). Discontinuation of dietary therapy after adolescence has essentially no adverse effect on the nervous system and, as a result, strict dietary control is often not maintained after 17 years of age (Potocnik and Widhalm, 1994; Wilkinson and Holbrook, 1998). Thus, although in the past individuals with cPKU did not reproduce due to their severe mental retardation, phenylketonuric women, having normal fertility, are having high-risk pregnancies: offspring of phenylketonuric mothers not under dietary control are born with maternal PKU (mPKU) (Levy and Ghavami, 1996). These individuals, although genotypically normal, have severe central nervous system (CNS) dysfunctions, including mental retardation, microcephaly, and seizures. For example, 73 to 100% of offspring from mothers with untreated cPKU were reported to have microcephaly, and 92 to 94% were classified as being mentally retarded (Lenke and Levy, 1980; Lipson et al., 1984).

Phe is primarily metabolized to tyrosine; however, in hyperphenylalaninemic individuals, the conversion of Phe to nontyrosine derivatives becomes significant. Thus, Phe is decarboxylated to phenylethylamine (PEA), 90% of which is oxidized to phenylacetic acid (PAA), and the remainder to mandelic acid (MA). In addition, Phe also can be transaminated to phenylpyruvic acid (PPA), which is converted to phenyllactic acid (PLA) and hydroxyphenylacetic acid (HPA). These Phe metabolites have all been shown to be signifi-

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ABBREVIATIONS: PKU, phenylketonuria; Phe, phenylalanine; cPKU, classic PKU; mPKU, maternal PKU; CNS, central nervous system; PEA, phenylethylamine; PAA, phenylactic acid; MA, mandelic acid; PPA, phenylpyruvic acid; PLA, phenyllactic acid; HPA, hydroxyphenylacetic acid; FBS, fetal bovine serum; MTt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; BrdU, 5-bromodeoxyuridine; DMEM, Dulbecco’s modified Eagle’s medium; MPPD, mevalonate pyrophosphate decarboxylase.
cantly elevated in hyperphenylalaninemic individuals and urinary excretion has been reported to be increased 6- to 16-fold (Michals et al., 1985; Tuchman et al., 1985; Kaufman, 1989; Clemens et al., 1990; Langenberg et al., 1992). Furthermore, in phenylketonuric individuals plasma levels of PLA, PPA, MA, and HPA in the range of 3 to 84 μM have been reported (Clemens et al., 1990; Langenberg et al., 1992). Whether Phe itself, or one of its metabolites, is responsible for the CNS dysfunctions associated with PKU is unknown. Limited evidence in humans seems to suggest that Phe, most likely in combination with one or more of its metabolites, may be the agent responsible for the CNS anomalies associated with PKU (Levy and Ghavami, 1996). Wen et al. (1980) estimated that levels of PAA in the brain can reach 0.4 to 3 mM, and animal studies suggest that PAA is the toxic metabolite (Loo et al., 1980, 1983; Manabe and Ohsawa, 1993). For example, Wen et al. (1980) reported that treatment of neonatal rats with PAA resulted in reduced size of the cerebellum and in a reduction in thickness of the molecular layer. Furthermore, in mouse embryos exposed to PAA in vitro, PAA resulted in a concentration-dependent increase in neural tube closure defects and in an incomplete expansion of the forebrain (Denno and Sadler, 1990). Similarly, in vitro exposure of mouse embryos to PEA resulted in a concentration-dependent increase in neural tube closure defects (Denno and Sadler, 1990). These investigators also reported that developmental exposure to PLA and PPA resulted in CNS anomalies, including neural tube closure defects (Denno and Sadler, 1990).

The mechanism(s) of the CNS dysfunctions associated with mPKU is unknown. It is evident, however, that exposure to high levels of Phe, or one of its metabolites, is toxic to the developing brain, whereas the fully developed brain is essentially unaffected (Potocnik and Widhalm, 1994). Thus, there appears to be a developmentally restricted window of vulnerability to hyperphenylalaninemia. Exposure to high levels of Phe during the brain growth spurt, the transient period of rapid brain growth that occurs primarily late in the third trimester of pregnancy and that is characterized by rapid glial cell proliferation, results in mental retardation and reduced brain size (Lipson et al., 1984; Menkes, 1995; Levy et al., 1996; Levy and Ghavami, 1996; Rezvani, 1996; Roricht et al., 1999). In addition, autopsies and magnetic resonance imaging of children with mPKU have revealed a loss of neurons (Lacey and Terplan, 1987; Levy et al., 1996) and animal studies have suggested a hyperphenylalaninemia-mediated increase in cell death in the developing brain (Reynolds et al., 1993). Thus, the current study was undertaken to determine whether the microencephaly and neuronal loss associated with developmental hyperphenylalaninemia may be due, at least in part, to enhanced neuronal cell death and decreased astrocyte proliferation.

**Experimental Procedures**

**Materials.** Pregnant Sprague-Dawley rats were obtained from B & K Universal (Kent, WA). Cell culture media, fetal bovine serum (FBS), trypsin, penicillin, and streptomycin were purchased from Life Technologies, Inc. (Grand Island, NY). Astrocyte basal medium and the human astrocyte growth medium kit were purchased from BioWhittaker (Walkersville, MD). The Hoechst 33258 and 3-14,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Molecular Probes (Eugene, OR). The lactate dehydrogenase (LDH) test kit, BSA, 5-bromodeoxyuridine (BrdU), t-Phe, PAA, PLA, HPA, PPA, MA, and PEA were purchased from Sigma (St. Louis, MO) and the protease inhibitor cocktail from Boehringer Mannheim (Indianapolis, IN). All other chemicals were obtained from common commercial suppliers.

**Neuronal Cell Tissue Culture.** Human SY5Y neuroblastoma cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) containing 5 mM glucose, 4 mM glutamine, and supplemented with 5% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. SY5Y cells plated at a density of 5 x 10^4 cells/cm^2 for experiments. Primary cultures of rat cerebellar granule cells were established as described previously (Oberdoerster and Rabin, 1999). Briefly, the cerebella from 7-day-old rats were removed and minced in DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin. The cerebella then were incubated in DMEM containing 0.1% trypsin and 0.2% deoxyribonuclease for 10 min at 37°C. The tissue was suspended in DMEM supplemented with 10% heat-inactivated FBS, 50 mM glucose, and 2.5 mM KCl, and then filtered through a 100-μm nylon mesh. Cells were plated at a density of 2 x 10^6/cm^2 onto tissue culture dishes coated with 500 μg/ml poly(t-lysine) for 15 min at 37°C to remove astrocytes. After shaking the flask to detach the neurons, the medium was filtered sequentially through 70- and 40-μm nylon mesh, and the cells plated at a density of 2 x 10^6/cm^2 onto brain tissue dishes coated with 500 μg/ml poly(t-lysine). On day 1 and day 5 after isolation, the cells were treated with 10 μM cytosine arabinoside for 24 h to remove any proliferating cells. Subsequently, medium was changed every 3 days and the cells used for experiments 8 days after isolation.

**Gliaal Cell Tissue Culture.** Human 1321N1 astrocytoma cells were maintained at 37°C in DMEM containing 5 mM glucose, 4 mM glutamine, and supplemented with 5% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. 1321N1 cells were plated at a density of 5 x 10^5 cells/cm^2 for experiments. Primary cultures of cortical astrocytes were prepared as described previously (Guizzetti et al., 1996). Briefly, the cerebral cortex from 21-day-old rat fetuses was removed and minced in DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin. The tissue was incubated in PBS containing 0.1% trypsin for 10 min at 37°C. The tissue was then suspended in DMEM supplemented with 10% FBS and filtered through a 100-μm nylon mesh. Cells were plated at a density of 1.4 x 10^5 cells/cm^2 in a T-flask coated with 20 μg/ml poly(t-lysine) and maintained in DMEM containing 5 mM glucose, 4 mM glutamine, and 10% heat-inactivated FBS at 37°C in a humidified atmosphere containing 95% air, 5% CO₂. The medium was changed every 3 to 4 days. On day 9 after isolation, oligodendrocytes and microglia were removed from the cultures by vigorously shaking the flasks overnight. The astrocytes were then removed in 0.125% trypsin and seeded into poly(t-lysine)-coated tissue culture plates at a density of 5 x 10^5 cells/cm^2 for experiments. Primary cultures of human fetal astrocytes were purchased from BioWhittaker and maintained on poly(t-lysine)-coated tissue culture plates according to the suppliers directions in astrocyte growth medium (BioWhittaker) containing 5% FBS. Human astrocytes were plated at a density of 4 to 5 x 10^4 cells/cm^2 for experiments.

**Evaluation of Cell Viability.** Cell viability was determined as described previously by measuring either the leakage of LDH from dead or dying cells into the culture medium (Guizzetti and Costa, 1996) or the reduction of MTT by viable cells (Oberdoerster and Rabin, 1999). The LDH activity (i.e., the reduction of pyruvate and the oxidation of NADH) was evaluated by using a commercially available kit (Sigma) according to the manufacturer’s directions. For determination of cell viability with MTT, cells, which were plated onto 24-well plates, were incubated with PBS containing 150 μg/ml MTT after the experimental treatment. After the incubation with MTT at 37°C, the medium was removed from the wells and the reduced MTT extracted in dimethyl sulfoxide. The optical density of the MTT was then determined (590 nm minus 650 nm).
Cell Proliferation. Proliferation assays were carried out as described previously (Guizzetti and Costa, 1996) by measuring the incorporation of \(^{3}H\)thymidine into cellular DNA. In brief, 1321N1 cells were plated into 24-well plates in DMEM containing 5% FBS. Rat cortical astrocytes were seeded into 24-well plates in DMEM containing 10% FBS. After 3 days, the cells were washed twice with PBS and incubated for 48 h in DMEM containing 0.1% BSA to halt cell proliferation. The cells were then treated with the various Phe metabolites for 24 h. \(^{3}H\)Thymidine (1 \muCi) was added to each well after 18 h and the cells fixed in methanol 6 h later. The DNA was precipitated in 10% trichloroacetic acid, dissolved in 1 N NaOH, and the amount of incorporated \(^{3}H\)thymidine measured by using a Beckman LS 5000CE scintillation counter.

Flow Cytometric Analysis. Flow cytometric analysis was carried out by using the BrdU/Hoechst 33258 method as described previously (Guizzetti and Costa, 1996). In brief, before the experiment, 1321N1 cells or rat cortical astrocytes were incubated in 0.1% BSA for 48 h as described above and then exposed to the various Phe metabolites in the presence of 150 \muM BrdU. After 48 h, the cells were removed from the tissue culture plate in 0.05% trypsin. The cells were collected by centrifugation at 1000g for 5 min and resuspended in Hoescht buffer (154 mM NaCl, 100 mM Tris, 0.5 mM MgCl\(_2\), 0.2% BSA, 0.1% IGEPA CA-630, 5.9 \mug/ml Hoechst 33258, pH 7.4) containing 10% dimethyl sulfoxide. Ethidium bromide (1.5 \mug/ml) was then added and the stained cells (10,000 cells/sample) analyzed 15 min later by using a Coulter EPICS Elite ESP flow cytometer (Hoechst 33258: \(\lambda_{\text{excitation}} = 350 \pm 20\) nm, \(\lambda_{\text{emission}} = 450 \pm 18\) nm; ethidium bromide: \(\lambda_{\text{excitation}} = 485 \pm 10\) nm, \(\lambda_{\text{emission}} > 590\) nm). The data were collected and analyzed using the MPLUS AV software program (Phoenix Flow Systems, San Diego, CA).

Total Cell Protein Determination. For the determination of total cellular protein, cells were incubated in 0.1 N NaOH and protein content measured by using the colorimetric Bio-Rad protein dye-binding procedure with BSA (fraction V) as a standard.

Statistical Analysis. The data were analyzed with a one-way ANOVA and the post hoc Fisher’s least-significant difference comparison test by using the StatView 512+ software program for the Macintosh computer.

Results

In a first series of experiments, primary cultures of rat cerebellar granule cells were used to determine whether exposure to Phe or to one of its metabolites results in neuronal cytotoxicity. In hyperphenylalaninemic individuals, brain PAA levels as high as 3 mM have been estimated (Wen et al., 1980) and plasma levels of PLA, PPA, MA, and HPA in the range of 3 to 84 \muM have been reported (Clemens et al., 1990; Langenbeck et al., 1992). The mitochondrial reduction of MTT and the release of LDH into the culture medium was unchanged by a 48-h exposure of the granule cells to PPA, HPA, MA, PEA, PLA, PAA, or Phe (Table 1A). Human SY5Y neuroblastoma cells also were used to determine the effect of Phe and its metabolites on neuronal cells of human origin. Similar to the effects in the granule cells, the reduction of MTT and the release of LDH into the culture medium was unchanged by a 48-h exposure of SY5Y cells to PPA, HPA, MA, PEA, PLA, PAA, or Phe (Table 1B). Proliferation of the SY5Y cells (measured by the incorporation of \(^{3}H\)thymidine into DNA) also was unchanged by a 24-h exposure to Phe or its six metabolites (data not shown).

Primary cultures of rat cortical astrocytes were used to determine whether exposure to Phe or to one of its metabolites results in decreased astrocyte proliferation. A 24-h exposure to PPA, HPA, MA, PLA, and PAA did not alter the

| TABLE 1 | Phenylalanine and its metabolites do not alter the viability of rat cerebellar granule cells or human SY5Y neuroblasto
cells
<p>| Cerebellar Granule Cell Cytotoxicity | MTT Reduction | LDH Release |</p>
<table>
<thead>
<tr>
<th>A. Treatment</th>
<th>% untreated cells</th>
<th>% untreated cells</th>
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<tbody>
<tr>
<td>0.5 mM PPA</td>
<td>95 ± 6</td>
<td>98 ± 7</td>
</tr>
<tr>
<td>0.5 mM HPA</td>
<td>102 ± 1</td>
<td>97 ± 6</td>
</tr>
<tr>
<td>0.5 mM MA</td>
<td>100 ± 2</td>
<td>113 ± 10</td>
</tr>
<tr>
<td>0.5 mM PEA</td>
<td>102 ± 6</td>
<td>122 ± 5</td>
</tr>
<tr>
<td>0.5 mM PLA</td>
<td>96 ± 2</td>
<td>105 ± 4</td>
</tr>
<tr>
<td>5 mM PAA</td>
<td>93 ± 3</td>
<td>121 ± 18</td>
</tr>
<tr>
<td>5 mM Phe</td>
<td>96 ± 6</td>
<td>106 ± 4</td>
</tr>
</tbody>
</table>

<p>| SY5Y Neuroblastoma Cytotoxicity | MTT Reduction | LDH Release |</p>
<table>
<thead>
<tr>
<th>A. Treatment</th>
<th>% untreated cells</th>
<th>% untreated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mM PPA</td>
<td>92 ± 2</td>
<td>93 ± 14</td>
</tr>
<tr>
<td>0.5 mM HPA</td>
<td>93 ± 3</td>
<td>104 ± 19</td>
</tr>
<tr>
<td>0.5 mM MA</td>
<td>104 ± 5</td>
<td>113 ± 17</td>
</tr>
<tr>
<td>0.5 mM PEA</td>
<td>102 ± 3</td>
<td>123 ± 10</td>
</tr>
<tr>
<td>0.5 mM PLA</td>
<td>99 ± 6</td>
<td>123 ± 15</td>
</tr>
<tr>
<td>5 mM PAA</td>
<td>100 ± 5</td>
<td>123 ± 17</td>
</tr>
<tr>
<td>5 mM Phe</td>
<td>101 ± 7</td>
<td>112 ± 9</td>
</tr>
</tbody>
</table>

Fig. 1. Phe and PEA inhibit serum-induced cortical astrocyte proliferation. Nonproliferating rat cortical astrocytes were incubated for 24 h with DMEM containing 10% FBS in the presence of 1 mM PPA, 1 mM HPA, 1 mM MA, 0.5 mM PEA, 1 mM PLA, 5 mM PAA, or 5 mM Phe. Cell proliferation was measured by the incorporation of \(^{3}H\)thymidine into cellular DNA. A 24-h exposure to 10% FBS resulted in a 6.22 ± 1.23-fold increase in \(^{3}H\)thymidine incorporation. Data are expressed as percentage of serum-stimulated cells and are plotted as mean ± S.E. (n = 3–6, **P < .01).
in \[^{3}H\]thymidine incorporation. In nonproliferating astrocytes none of the treatments had any effect on the basal amount of \[^{3}H\]thymidine incorporated into cellular DNA or on the mitochondrial reduction of MTT (data not shown), indicating a lack of cytotoxicity of these compounds.

The effect of Phe and PEA on serum-induced rat cortical astrocyte proliferation was concentration dependent. A 24-h exposure to 1 or 2.5 mM Phe resulted in a significant 17 and 44% decrease in \[^{3}H\]thymidine incorporation, respectively, with higher concentrations causing further inhibition (Fig. 2A). Similarly, exposure to 100 or 250 \(\mu\)M PEA resulted in a significant 18 and 28% decrease in serum-induced \[^{3}H\]thymidine incorporation, respectively (Fig. 2B). To determine whether the PEA- and Phe-induced inhibition of DNA synthesis was the result of cell cycle arrest, flow cytometric experiments evaluating the different stages of the cell cycle were carried out. After a 96-h exposure to DMEM containing 0.1% BSA, 77% of the cells were in the G0/G1 phase of the cell cycle (Table 2). A 48-h stimulation of the cells with serum resulted in an increase in the number of cells in the S1, the G2, and the new G0/G1 phase of the cell cycle. Treatment of serum-stimulated rat astrocytes with 1 mM PEA for 48 h resulted in a significant reduction in cells entering the S/G2 phase of the cell cycle. Similarly, exposure to 5 mM Phe resulted in the accumulation of cells in the G0/G1 phase of the cell cycle (Table 2).

Human 1321N1 astrocytoma cells were used to determine the effect of Phe and its metabolites on glial cells of human origin. Exposure to PPA, HPA, MA, PEA, or PLA did not alter serum-induced 1321N1 cell proliferation; however, PAA and Phe (both at 5 mM) significantly decreased 1321N1 cell proliferation by 42 and 19%, respectively (Fig. 3). The leakage of cellular LDH into the culture medium was used as a measure of cell viability to determine whether PAA and Phe are toxic to proliferating cells. Neither compound had any effect on LDH release (data not shown). In nonproliferating astrocytoma cells none of the treatments had any effect on the basal amount of \[^{3}H\]thymidine incorporated into cellular DNA or on the mitochondrial reduction of MTT (data not shown).

A 24-h exposure to Phe resulted in a concentration-dependent decrease in 1321N1 astrocytoma cell proliferation. Treatment with 5 and 10 mM Phe resulted in a 19 and 31% decrease in serum-induced \[^{3}H\]thymidine incorporation, respectively (Fig. 4A). The effect of PAA on 1321N1 cell prolif-

### Table 2

Phenylalanine and phenylethylamine inhibit cell cycle progression of rat cortical astrocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of Cells in Cell Cycle</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>G2/G1</td>
</tr>
<tr>
<td>0.1% BSA</td>
<td>76 ± 6</td>
</tr>
<tr>
<td>Serum</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>+0.1 mM PEA</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>+1 mM PEA</td>
<td>51 ± 2*</td>
</tr>
<tr>
<td>+1 mM Phe</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>+5 mM Phe</td>
<td>44 ± 6*</td>
</tr>
</tbody>
</table>

*P < .05.
eration was also concentration dependent. A 24-h exposure to 2.5 or 5 mM PAA resulted in a significant 27 and 42% decrease in serum-induced proliferation, respectively (Fig. 4A).

To determine whether the PAA- and Phe-induced inhibition of DNA synthesis was the result of cell cycle arrest, flow cytometric experiments evaluating the different stages of the cell cycle were carried out. After a 96-h exposure to DMEM containing 0.1% BSA, 98% of the cells were in the G0/G1 phase of the cell cycle (Table 3). A 48-h stimulation of the cells with serum resulted in an increase in the number of cells in the S1, G2, and new G0/G1 phase of the cell cycle. Treatment of serum-stimulated 1321N1 cells with 5 mM PAA for 48 h resulted in a significant reduction in cells entering the S/G2 phase of the cell cycle. Similarly, exposure to 10 mM Phe resulted in the accumulation of cells in the G0/G1 phase of the cell cycle (Table 3).

Because rat cortical astrocytes and human 1321N1 astrocytoma cells were differentially sensitive to PEA and PAA, we also used primary cultures of human fetal astrocytes to determine the effect of Phe and its metabolites on the proliferation of nontransformed human astrocytes. Exposure to PPA, HPA, MA, or PLA, all at 0.5 mM, did not alter serum-induced human astrocyte proliferation. A 24-h treatment with 0.5 mM PEA, 5 mM PAA, or 5 mM Phe, however, significantly decreased human astrocyte proliferation by 41, 52, and 30%, respectively (Fig. 5). In nonproliferating astrocytes none of the treatments had any effect on the basal amount of [3H]thymidine incorporated into cellular DNA or on the mitochondrial reduction of MTT (data not shown).

**Discussion**

Hyperphenylalaninemia during the brain growth spurt results in mental retardation and microencephaly (Lipson et al., 1984; Menkes, 1995; Levy and Ghavami, 1996; Rezvani, 1996). It is unclear whether Phe itself or one of its metabolites is involved in the CNS dysfunctions associated with hyperphenylalaninemia because in hyperphenylalaninemic individuals, alternate Phe conversion pathways, which are...
normally unimportant, become significant. Thus, the CNS may be sensitive not only to high concentrations of Phe but also to nontyrosine metabolites of Phe such as PEA, PAA, MA, PPA, PLA, and HPA. Denno and Sadler (1990), for example, have shown that exposure of whole mouse embryo cultures to PAA, PLA, PEA, or Phe results in neural tube closure defects. Furthermore, administration of PAA to neonatal rats has been reported to result in cerebellar atrophy (Wen et al., 1980). The reason for the CNS dysfunctions associated with developmental hyperphenylalaninemia are unclear; however, autopsies and magnetic resonance imaging of children with mPKU have revealed, in addition to microcephaly, a loss of neurons (Lacey and Terplan, 1987; Levy et al., 1996). It does not appear, however, that neurons are directly sensitive to hyperphenylalaninemia because we did not detect any cytotoxicity associated with a 48-h exposure of rat cerebellar granule cells or human SY5Y neuroblastoma cells to Phe or its metabolites. Silberberg (1967) similarly reported that a 10-day exposure to 9 mM Phe or 1.1 mM PAA was not toxic to mixed rat cerebellar cultures. A 10-day exposure of these cultures to 6.6 mM PAA, however, resulted in changes in cell morphology (e.g., intracellular vacuoles) that suggested a loss of cell viability. In addition, Silberberg (1967) noted that a 10- to 14-day exposure to Phe resulted in vacuole formation in glial cell populations. Thus, Phe and its metabolites appear not to be directly toxic to neurons. Rather, the metabolites may have an adverse effect on glial cells during development.

A reduction in the number of glial cells during development would not only result in microencephaly but also may contribute to a corresponding loss of neurons. Indeed, in the present study we demonstrate that exposure of human or rat astroglial cells to Phe results in decreased cell proliferation and cell cycle arrest. In addition, we show that primary cultures of human and rat fetal astrocytes are differentially sensitive to PAA. Although PEA treatment results in decreased proliferation of both cell types, exposure to PAA reduces human but not rat fetal astrocyte proliferation. Similarly, Pahan et al. (1997) reported that the viability of rat astrocytes was unchanged by an in vitro exposure to 5 mM PAA. The reason for this difference is unclear; however, a species difference cannot be ruled out because proliferating human 1321N1 astrocytoma cells also are sensitive to PAA. Furthermore, PAA has previously been shown to inhibit the growth of several other human cells (e.g., human pancreatic carcinoma cells, LNCaP prostate cancer cells, and human glioblastoma cells) (Samid et al., 1994; Danesi et al., 1996; Premakala et al., 1996; Harrison et al., 1998).

Phe, PAA, and PEA have all been shown to inhibit mevalonate pyrophosphate decarboxylase (MPPD), a key enzyme involved in both protein prenylation and cholesterol biosynthesis (Castillo et al., 1991; Harrison et al., 1998). In fact, exposure of cells to PAA has been reported to result in a decrease in protein isoprenylation. Thus, PAA may inhibit astrocyte proliferation by blocking the isoprenylation of key proteins that are required for proliferation (e.g., the Ras and Rho family of GTPases, nuclear lamins) (Samid et al., 1994; Danesi et al., 1996; Harrison et al., 1998). As mentioned above, inhibition of MPPD may result in the decreased availability of cholesterol and cholesterol has been shown to be critical for the formation of cell membranes in proliferating cells (Goldstein and Brown, 1990; Grunler et al., 1994). It is unlikely, however, that reduced cholesterol biosynthesis is responsible for the antiproliferative effects of PAA and Phe because the serum present in the culture medium contains cholesterol. Interestingly, Cuthbert and Lipsky (1991, 1995) report that inhibition of MPPD in 12 transformed cells lines induced the activation of an unidentified inhibitor of proliferation. This endogenous inhibitor appears to be synthesized from either mevalonate or mevalonate phosphates (Cuthbert and Lipsky, 1997). Thus, although speculative, this endogenous inhibitor also may be activated in proliferating astroglial cells exposed to Phe, PAA, or PEA.

It is evident that the developing nervous system but not the fully developed brain is sensitive to hyperphenylalaninemia (Potocnik and Widhalm, 1994). Thus, both mPKU and cPKU result in mental retardation. The mechanism(s) of mPKU and cPKU appears to be distinct because exposure to high levels of Phe during the brain growth spurt (i.e., mPKU) results in microencephaly, whereas postnatal hyperphenylalaninemia (i.e., cPKU) does not. The results from the present study may help to explain these differences because perturbations of glial cell proliferation during the brain growth spurt would be expected to result in microencephaly. Thus, although the effects of cPKU appear to be mediated by reduced glial cell function (e.g., hypomyelination) (Huether et al., 1982; Burri et al., 1990; Reynolds et al., 1993), the microencephaly and mental retardation associated with mPKU may be due, at least in part, to the reduced proliferation of astrocytes.

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References


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