Phenytoin Affects Metabolism of Free Fatty Acids and Nucleotides in Rat Cerebral Ischemia

Hiroyuki Kinouchi, MD, PhD, Shigeki Imaizumi, MD, PhD, Takashi Yoshimoto, MD, and Masakichi Motomiya, MD

We investigated the effects of phenytoin on the rate of enzymatic release of free fatty acids and on the levels of energy metabolites and nucleoside phosphates in ischemic brain. Phenytoin (10 mg/kg i.v.) was administered 30 minutes before the onset of ischemia induced in 30 male Wistar rats by occluding the basilar and both common carotid arteries. The rats' brains were frozen in situ after 0, 5, or 30 minutes of ischemia or 10, 30, or 60 minutes of recirculation following 30 minutes of ischemia (n=5 at each time). Nucleoside triphosphate levels were higher in the phenytoin-treated rats than in corresponding untreated rats at each time during and after ischemia. Phenytoin significantly attenuated the accumulation of lactate and free fatty acids (arachidonic acid and stearic acid) during ischemia and accelerated their recovery during recirculation. These results suggest that phenytoin has favorable protective effects on ischemic brain and that phenytoin may inhibit calcium-mediated phenomena, especially the inositol cycle, in cerebral ischemia. (Stroke 1990;21:1326-1332)

An ischemic energy crisis accelerates calcium influx into the cytosol from the extracellular space or subcellular organelles such as mitochondria or the endoplasmic reticulum. An increase in the concentration of free calcium in the cytosol activates phospholipase, which liberates free fatty acids (FFAs), especially arachidonic acid, and impairs the integrity of membranes. Increases in the concentrations of FFAs, mitochondrial uncouplers, disturb the respiratory chain and induce brain edema. The arachidonic acid cascade operates through auto-oxidation and peroxidative pathways (cyclooxygenase and lipoxygenase pathways). On the other hand, it has been stated that free radicals, prostaglandins, and leukotrienes are related to brain damage.

Phenytoin stabilizes membranes, mainly by changing the intracellular or extracellular cation levels. At therapeutic concentrations, phenytoin blocks several calcium-mediated neuronal functions, including protein phosphorylation, neurotransmitter release, and depolarization-dependent calcium accumulation. It has also been reported that phenytoin reduces ischemia-produced loss of hippocampal CA1 neurons in gerbils after forebrain ischemia. Delayed neuronal death is thought to be closely related to neurotransmitter release and calcium accumulation.

Previously we showed that the phenytoin protects the brain against hypoxia and ischemia. Our current study deals with the effects of phenytoin on the rate of enzymatic release of FFAs, on the concentration of energy metabolites, and on mononucleotide metabolism in ischemic brain.

Materials and Methods

The solvents used for high-performance liquid chromatography were of high-performance liquid chromatography grade; other solvents were of analytical grade. All solvents were purchased from Wako Chemicals, Tokyo, Japan. Standard nucleotides and lipids were purchased from Sigma Chemical Co., St. Louis, Mo. Wistar rats were purchased from Shizuoka Laboratory Animal Center, Shizuoka, Japan.

We used 60 male Wistar rats weighing 200–250 g. After induction of anesthesia with 2.5% halothane, the rats were given 0.8 mg/kg i.v. pancuronium bromide, were tracheotomized, and were mecha-
The brain was dissected under liquid nitrogen, and the frontoparietal cortex was chiseled out and stored at -80°C until use. The 200-250 mg frozen brain samples were weighed, pulverized under liquid nitrogen, and homogenized in 1 ml of 3 M HClO₄; 4 ml of ice-cold water was added, and the resulting mixture was centrifuged at 10,000g for 25 minutes at 0°C. The supernatant was neutralized with 2 M KHCO₃ and centrifuged at 10,000g for 20 minutes at 0°C. The supernatant was then separated and filtered through a 0.45 μm Millipore filter (Bedford, Mass.), and the filtrate was analyzed in a Hitachi high-performance liquid chromatograph with a Model 655 solvent delivery pump detector system (Tokyo, Japan). The mobile phase for the analysis of nucleoside monophosphates consisted of 6% CH₃CN, 0.067 M NH₄Cl, 0.01 M KH₂PO₄, and 0.01 M K₂HPO₄. The mobile phase for the analysis of nucleoside diphosphates and nucleotide triphosphates consisted of 6% CH₃CN, 0.2 M NH₄Cl, 0.03 M KH₂PO₄, and 0.03 M K₂HPO₄. Flow rate was 1 ml/min, and temperature was 70°C. Concentrations were quantified by using an 833-type data processor (Hitachi). Glucose and lactate were assayed by the method of Lowry and Passonneau. The sum of the adenine nucleotide concentrations and the energy charge (the adenosine triphosphate [ATP] concentration plus one-half the adenosine diphosphate [ADP] concentration divided by the sum of the ATP, ADP, and adenosine monophosphate [AMP] concentrations) were also calculated.

The FFAs were extracted from a 200 mg brain sample with chloroform:methanol (2:1) according to the methods of Folch et al. ²⁵ Heptadecanoic acid (Gaschrockyo, Tokyo, Japan) was used as an internal standard. The FFAs were converted into methyl esters with diazomethane and analyzed using gas-liquid chromatography in a Hitachi 263-70 FID gas chromatograph with a glass column (3 mm i.d. x 2 m) containing 15% diethylene glycol succinate (Gaschrome, Tokyo, Japan). The rate of recovery of the internal standard was 89.3%.

Values for the two treatment groups were compared using Student's t test. A probability value of <0.05 was considered to indicate a significant difference.

### Table 1. Effects of Phenytoin on Adenine Nucleotides and Intermediates of Glycolysis in Rat Model of Cerebral Ischemia

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Adenosine triphosphate</th>
<th>Adenosine diphosphate</th>
<th>Adenosine monophosphate</th>
<th>Sum of adenine nucleotides</th>
<th>Energy charge</th>
<th>Glucose</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min ischemia</td>
<td>5</td>
<td>2.77±0.09</td>
<td>0.250±0.022</td>
<td>0.054±0.002</td>
<td>3.05±0.08</td>
<td>0.951±0.005</td>
<td>3.51±0.30</td>
<td>1.65±0.32</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>5</td>
<td>2.67±0.11</td>
<td>0.231±0.010</td>
<td>0.058±0.010</td>
<td>2.96±0.12</td>
<td>0.952±0.003</td>
<td>3.19±0.19</td>
<td>2.03±0.06</td>
</tr>
<tr>
<td>5 min ischemia</td>
<td>5</td>
<td>0.32±0.04</td>
<td>0.305±0.002</td>
<td>1.50±0.05</td>
<td>2.16±0.10</td>
<td>0.228±0.021</td>
<td>0.67±0.06</td>
<td>12.60±0.93</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>5</td>
<td>0.76±0.12</td>
<td>0.310±0.020</td>
<td>1.35±0.07</td>
<td>2.42±0.17</td>
<td>0.363±0.027†</td>
<td>1.04±0.02†</td>
<td>9.84±0.20†</td>
</tr>
<tr>
<td>30 min ischemia</td>
<td>5</td>
<td>0.08±0.01</td>
<td>0.165±0.020</td>
<td>1.67±0.09</td>
<td>1.74±0.12</td>
<td>0.076±0.008</td>
<td>0.06±0.02</td>
<td>14.83±0.43</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>5</td>
<td>0.13±0.04*</td>
<td>0.179±0.0035</td>
<td>1.61±0.03</td>
<td>1.91±0.12</td>
<td>0.112±0.008*</td>
<td>0.12±0.01*</td>
<td>11.89±0.45*</td>
</tr>
<tr>
<td>10 min recirculation</td>
<td>None</td>
<td>0.94±0.02</td>
<td>0.280±0.016</td>
<td>0.71±0.06</td>
<td>1.93±0.09</td>
<td>0.506±0.045</td>
<td>3.88±0.13</td>
<td>10.51±0.24</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>5</td>
<td>1.43±0.05*</td>
<td>0.211±0.014</td>
<td>0.50±0.03*</td>
<td>2.14±0.13</td>
<td>0.728±0.022*</td>
<td>4.16±0.53∗</td>
<td>8.73±0.27†</td>
</tr>
<tr>
<td>30 min recirculation</td>
<td>None</td>
<td>1.50±0.05</td>
<td>0.202±0.010</td>
<td>0.023±0.001</td>
<td>1.72±0.05</td>
<td>0.831±0.005</td>
<td>4.05±0.20</td>
<td>7.94±0.60</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>5</td>
<td>1.60±0.06</td>
<td>0.223±0.010</td>
<td>0.016±0.002*</td>
<td>2.12±0.03*</td>
<td>0.870±0.010*</td>
<td>4.39±0.11</td>
<td>6.32±0.22</td>
</tr>
<tr>
<td>60 min recirculation</td>
<td>None</td>
<td>1.60±0.08</td>
<td>0.212±0.010</td>
<td>0.027±0.002</td>
<td>1.83±0.09</td>
<td>0.942±0.006</td>
<td>4.50±0.09</td>
<td>6.64±0.32</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>5</td>
<td>1.93±0.02*</td>
<td>0.203±0.001</td>
<td>0.021±0.001</td>
<td>2.17±0.01</td>
<td>0.946±0.001</td>
<td>4.47±0.13</td>
<td>4.54±0.40*</td>
</tr>
</tbody>
</table>

Values are mean±SEM μmol/g wet wt.

*p<0.05; †p<0.01, respectively, different from no treatment by Student's t test.
Results

In both untreated and phenytoin-treated groups, ATP concentration decreased markedly after the onset of ischemia (Table 1); however, phenytoin-treated rats showed significantly smaller decreases in ATP concentration than untreated rats. After 60 minutes of recirculation, ATP concentration returned to 58% and 72.3% of control (0 minutes of ischemia) in the untreated and phenytoin-treated groups, respectively. The phenytoin-treated groups exhibited higher energy phosphates than the untreated groups during ischemia after 60 minutes of recirculation. The phenytoin-treated groups were significantly lower than those of the untreated groups after 10 and 30 minutes of ischemia; however, no significant difference between groups was found after 60 minutes of recirculation. ADP concentration did not differ significantly between groups at any time. The phenytoin-treated groups exhibited higher energy charges than the untreated groups during ischemia and after 10 and 30 minutes of recirculation; however, no significant difference between groups was observed after 60 minutes of recirculation.

In the untreated groups, concentrations of cytidine triphosphate and uridine triphosphate decreased markedly during ischemia, but guanosine triphosphate (GTP) concentration remained at approximately 12% of control even after 30 minutes of ischemia (Table 2). GTP, cytidine triphosphate, and uridine triphosphate concentrations recovered to 73.2%, 77.1%, and 79.5% of control, respectively, after 60 minutes of recirculation. The phenytoin-treated groups exhibited higher GTP levels than the untreated groups at every time. Phenytoin-treated rats had a significantly higher cytidine triphosphate concentration than untreated rats after 5 minutes of ischemia; however, the cytidine triphosphate concentration was below the level of detection in both groups after 30 minutes of ischemia. The time course of the uridine triphosphate concentration was almost the same as that of cytidine triphosphate. The concentration of guanosine monophosphate increased after the induction of ischemia.

In untreated groups, the concentration of glucose decreased during ischemia, while that of lactate increased ninefold after 30 minutes of ischemia (Table 1). The lactate concentration decreased during recirculation but was still higher than control after 60 minutes of recirculation. On the other hand, phenytoin attenuated the rate of decrease of the glucose concentration and the rate of increase of the lactate concentration during ischemia and accelerated recovery of the lactate concentration toward control during recirculation.

In the untreated groups, the total amount of FFAs rapidly increased, to 5.7 times control after 5 minutes and to 11.8 times control after 30 minutes of ischemia (Table 3). The total amount of FFAs decreased during recirculation but did not return to control after 60 minutes. Of the FFAs, the concentration of arachidonic acid increased most remarkably after 5 minutes of ischemia (Figure 1). The concentrations of palmitic acid and stearic acid increased after 30 minutes of ischemia (Figure 2). The concentrations of individual FFAs tended to decrease during recirculation. The concentration of unsaturated fatty acids returned almost to control after 30 minutes of recirculation, though saturated FFAs such as stearic

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**Table 2. Effects of Phenytoin on Guanine Nucleotides, Cytidine Triphosphate, and Uridine Triphosphate in a Rat Model of Cerebral Ischemia**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Guanosine triphosphate</th>
<th>Guanosine diphosphate</th>
<th>Guanosine monophosphate</th>
<th>Cytidine triphosphate</th>
<th>Uridine triphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>0.500±0.015</td>
<td>0.175±0.007</td>
<td>&lt;0.002</td>
<td>0.048±0.004</td>
<td>0.215±0.008</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>5</td>
<td>0.526±0.017</td>
<td>0.168±0.005</td>
<td>&lt;0.002</td>
<td>0.045±0.002</td>
<td>0.222±0.008</td>
</tr>
<tr>
<td>5 min ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>0.098±0.008</td>
<td>0.189±0.010</td>
<td>0.028±0.002</td>
<td>&lt;0.002</td>
<td>0.023±0.003</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>5</td>
<td>0.195±0.009*</td>
<td>0.197±0.013</td>
<td>0.022±0.002</td>
<td>0.014±0.002†</td>
<td>0.048±0.002†</td>
</tr>
<tr>
<td>30 min ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>0.058±0.008</td>
<td>0.140±0.016</td>
<td>0.077±0.007</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>5</td>
<td>0.083±0.005†</td>
<td>0.165±0.007</td>
<td>0.059±0.003</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>10 min recirculation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>0.182±0.007</td>
<td>0.160±0.022</td>
<td>&lt;0.002</td>
<td>0.013±0.003</td>
<td>0.095±0.003</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>5</td>
<td>0.231±0.028†</td>
<td>0.161±0.005</td>
<td>&lt;0.002</td>
<td>0.018±0.004</td>
<td>0.115±0.009</td>
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<tr>
<td>30 min recirculation</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>0.318±0.010</td>
<td>0.130±0.002</td>
<td>&lt;0.002</td>
<td>0.027±0.002</td>
<td>0.146±0.006</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>5</td>
<td>0.378±0.006†</td>
<td>0.150±0.006*</td>
<td>&lt;0.002</td>
<td>0.034±0.002†</td>
<td>0.154±0.003</td>
</tr>
<tr>
<td>60 min recirculation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>0.366±0.007</td>
<td>0.125±0.021</td>
<td>&lt;0.002</td>
<td>0.037±0.005</td>
<td>0.171±0.008</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>5</td>
<td>0.407±0.010†</td>
<td>0.132±0.003</td>
<td>&lt;0.002</td>
<td>0.041±0.002</td>
<td>0.174±0.007</td>
</tr>
</tbody>
</table>

Values are mean±SEM μmol/g wet wt.
*p<0.01; †p<0.05, respectively, different from no treatment by Student's t test.
TABLE 3. Effects of Phenytoin on Total Free Fatty Acids in a Rat Model of Cerebral Ischemia

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 min ischemia</th>
<th>5 min ischemia</th>
<th>30 min ischemia</th>
<th>10 min Recirculation</th>
<th>30 min Recirculation</th>
<th>60 min Recirculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>182.0±15.0</td>
<td>1,035.0±61.8</td>
<td>2,146.2±130.9</td>
<td>1,043.2±76.2</td>
<td>769.0±63.0</td>
<td>397.2±16.8</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>164.2±13.0</td>
<td>745.8±35.9*</td>
<td>1,422.1±44.7*</td>
<td>967.5±64.7</td>
<td>356.3±77.8*</td>
<td>314.6±19.1*</td>
</tr>
</tbody>
</table>

Values are mean±SEM nmol/g wet wt (n=5). Free fatty acids were extracted by the methods of Folch et al.25 and analyzed by the method of Yoshida et al.26

*p<0.01; **p<0.05, respectively, different from no treatment by Student’s t test.

acid remained higher (Figure 2). Generally, the FFA levels in the phenytoin-treated groups were lower than those in the untreated groups at each time, but after 5 minutes of ischemia the difference between groups was significant only for arachidonic acid and stearic acid. After 5 minutes of ischemia much less arachidonic acid was liberated in the phenytoin-treated rats than in the untreated rats (Figure 1).

Discussion

We evaluated the effects of phenytoin during and after transient cerebral ischemia on the levels of mononucleotides and FFAs; the former are indicators of energy metabolism and the latter are indicators of membrane disintegration via mechanisms such as deacylation, peroxidation, or free radical reaction.

It is evident from this experiment that phenytoin contributes to saving energy. Phenytoin-treated rats had significantly higher ATP concentrations and lower lactate concentrations not only during ischemia but also after 10 and 60 minutes of recirculation. Moreover, the levels of GTP, cytidine triphosphate, and uridine triphosphate were significantly higher in phenytoin-treated rats. The effects of ischemia on cerebral adenine nucleotide metabolism have been studied. However, only a few reports have dealt with nucleotides other than the adenosine phosphates.27,28

Guanine nucleotides participate in protein synthesis. Dwyer and Wasterlain29 reported that ternary complexes were strongly inhibited by a decreased GTP/guanosine diphosphate (GDP) ratio. After 60 minutes of recirculation, the GTP/GDP ratio in our phenytoin-treated rats was higher than that in our untreated rats. Thus, it is possible that phenytoin exerts its effects on protein synthesis. On the other hand, Nowak et al.30 concluded that the regulation of protein synthesis during recirculation was independent of the GTP/GDP ratio. Further investigations are needed to clarify the relation between the GTP/GDP ratio and protein synthesis during recirculation.

Cytidine triphosphate is closely related to de novo synthesis of phospholipid, and uridine triphosphate to glycogenesis. We found no significant differences in uridine triphosphate levels between groups at any time.

With regard to the mechanisms of phenytoin’s protection of the brain from ischemia, several possibilities can be considered: 1) preserving intracellular/extracellular ion homeostasis through maintenance of Na,K-ATPase, reduction of sodium-dependent action potentials,32,33 or inhibition of sodium conduc-

Oleic acid (C18:1) Arachidonic acid (C20:4) Docosahexaenoic acid (C22:6)

![Graph of mean±SEM concentrations of free fatty acids in brain samples from phenytoin-treated (*) or untreated (○) rats, five for each determination. *p<0.01, **p<0.05 different from untreated by Student’s t test.](http://stroke.ahajournals.org/DownloadedFrom)
In recent studies, at the onset of ischemia the degradation of polyphosphoinositides by phospholipase C is primarily responsible for the increase in the levels of FFAs (particularly stearic acid and arachidonic acid), and during the late stage of ischemia phospholipase A₂ mainly causes the increase in the concentrations of other FFAs such as palmitic acid, oleic acid, or docosahexaenoic acid. This degradation of polyphosphoinositides, which is well known as the inositol cycle, has been observed during stimulation of brain slices by neurotransmitters and receptor agonists. This reaction is initiated by the activation of phospholipase C, which is stimulated by calcium ions. Whether the action of phenytoin is related to inhibition of the calcium system or to other actions is still unclear, and further investigations will be needed. However, our data (in which phenytoin attenuated not only the increases in the concentrations of arachidonic acid and stearic acid during the early stage of ischemia but also the increases in the concentrations of other FFAs after 30 minutes of ischemia) suggest that phenytoin inhibits presynaptic and postsynaptic calcium-mediated phenomena, especially the inositol cycle, in cerebral ischemia. Taft et al. reported that phenytoin reduced the ischemia-produced loss of hippocampal CA1 neurons in gerbils after forebrain ischemia. These authors also mentioned that phenytoin effects produced by inhibition of calcium uptake is a particularly attractive hypothesis in regard to the possible involvement of calcium ions in models of selective neuronal vulnerability.

FFAs are considered to be uncouplers of mitochondrial oxidative phosphorylation. Hence, an increase in the concentrations of FFAs in the brain suggests that mitochondrial function is disturbed. Furthermore, it has been reported that FFAs, especially polyenoic fatty acids, bring about brain edema in brain slices or in vivo experiments. Also, arachidonic acid accumulated during temporary ischemia is converted into biologically active substances such as prostaglandins, leukotrienes, or free radicals after ischemia. These substances are considered to be important factors in the development of posts ischemic brain edema. From the therapeutic point of view, it is obvious that attenuating the increases in FFA levels during ischemia has a favorable effect on recovery from ischemic brain damage. However,
References


32. Hershkowitz N, Ayala GF: Effect of phenytoin on pyramidal neurons of the rat hippocampus. *Brain Res* 1981;208:475-492


**KEY WORDS** • cerebral ischemia • fatty acids, nonesterified • phenytoin • rats
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Stroke. 1990;21:1326-1332
doi: 10.1161/01.STR.21.9.1326

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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