2-(1-Hydroxypentyl)-benzoate Increases Cerebral Blood Flow and Reduces Infarct Volume in Rats Model of Transient Focal Cerebral Ischemia

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ABSTRACT

2-(1-Hydroxypentyl)-benzoate (dl-PHPB), a derivate of 3-n-butyolphthalide (dl-NBP), is a novel drug candidate used for treatment of cerebral ischemia. The goal of the present study was to investigate the effects of dl-PHPB on infarct volume, neurological function, and cerebral blood flow (CBF) in transient focal cerebral ischemia. Therefore, an animal model of 2-h middle cerebral artery occlusion (MCAO) followed by 24-h reperfusion was used. Rats received dl-PHPB (1.3, 3.9, or 12.9 mg/kg) intravenously 10 min after the onset of MCAO. Compared with the vehicle control group (37.4%), infarct volume in dl-PHPB-treated groups was reduced significantly and dose-dependently to 25.4, 17.4, and 13.7%, respectively. The changes in neurological deficient were also observed in neurobehavioral test in a dose-dependent manner, and the neuronal score was improved significantly from the vehicle control of 3.2 to 2.7, 2.1, and 1.8, respectively. At the highest dose, the potency of dl-PHPB was similar to those of dl-NBP. CBF was quantified by using laser-Doppler flowmetry. During the ischemia, the regional CBF values of dl-PHPB groups were significantly higher than that of vehicle group. In addition, our study showed that dl-PHPB converted into dl-NBP very quickly in blood in vitro. Approximately 70% of dl-PHPB converted into dl-NBP in 5 min when dl-PHPB was added into plasma at final concentrations of 6, 30, and 60 μg/ml. This result demonstrated that the neuronal protection effects of dl-PHPB were mainly induced by dl-NBP, an active compound converted from its precursor, dl-PHPB. In conclusion, dl-PHPB can reduce infarct volume and improve neurobehavioral deficits in a rat model of transient MCAO. Those effects may partially be due to an increase in CBF by the active metabolite (dl-NBP) of dl-PHPB. Therefore, our results suggest that dl-PHPB may be useful for treatment of ischemia stroke.

Stroke is the third leading cause of death and one of the leading causes of adult disability in North America, Europe, and Asia (Higashida et al., 2003). Ischemic stroke occurs in 60 to 70% of all forms of stroke patients (Feng, 1999; Silvestrelli et al., 2002; Mahajan et al., 2004).

Ischemic stroke is initiated by a transient or permanent reduction in cerebral blood flow (CBF) that is restricted to the territory of major brain artery, such as the occlusion of middle cerebral artery (MCAO). MCAO impairs irrigation of focal brain areas, which show gradual reduction of CBF from the periphery to the core of the MCA territory (Bolander et al., 1989). The development of ischemic brain damage depends on the reduction of CBF below critical threshold level (Memezawa et al., 1992; Takagi et al., 1995). Then, the decrease of CBF in ischemic regions may result in an energy failure and further lead to an activation of the toxic intracellular pathway (Jorgensen and Diemer, 1982; Dirnagl et al., 1999; Hou and MacManus, 2002). The infarct volume is also dependent on the duration of ischemia (Soriano et al., 1997). Therefore, the severity of ischemia has two components: degree of CBF reduction and duration of the ischemic episode.

Potassium 2-(1-hydroxypentyl)-benzoate (dl-PHPB), derived from 3-n-butyolphthalide (dl-NBP), is a newly synthesized compound that is under development as a therapeutic drug for cerebral ischemia (Yang et al., 2002). As reported, dl-NBP is a primary naphtha component from seeds of Apium graveolens Linn. The phase 3 clinical trial of dl-NBP has been completed, and it was approved by the State Food
and Drug Administration of China at the end of 2002 as a new drug for treatment of ischemic stroke in clinic. Many basic and clinic studies have proved that dl-NBP is a potentially beneficial and promising drug for treatment of ischemic stroke with multiple actions that affect some pathophysiological processes, such as improving microcirculation of rat brain, inhibition of platelet aggregation, regulation of energy metabolism, inhibition of ischemia-induced oxidative damage, and neuron apoptosis (Chong and Feng, 1997; Yan et al., 1998; Xu and Feng, 2000, 2001; Dong and Feng, 2002). However, dl-NBP is difficult to use intravenously because of its hydrophobicity. Thus, dl-NBP is limited to use in the clinic for serious patients of ischemic stroke. Therefore, dl-PHPB was designed as a predrug of dl-NBP. The properties of dl-PHPB have been greatly improved. In addition, in our previous research, it was found that dl-PHPB could quickly convert to dl-NBP in vitro and in vivo (given to rat orally or intravenously). Recently, it was also found that dl-PHPB could inhibit platelet aggregation ex vivo and reduce thrombus formation in the arteriovenous shunt model in vivo (Zhang et al., 2004). In the present study, the effects of dl-PHPB on CBF, infarct volume, and neurological function were investigated in a rat model of transient focal cerebral ischemia.

Materials and Methods

Animals and Drugs. For all experiments, male Sprague-Dawley rats (weight, 270–330 g) were used. Animals were allowed free access to food and water in a temperature-controlled environment at 25°C before and after surgery. The experiments were performed in accordance with the guidelines for the care and use of laboratory animals and were approved by the Animal Care Committee of the Peking Union Medical College and the Chinese Academy of Medical Sciences.

dl-PHPB and dl-NBP were offered by the Department of Synthetic Pharmaceutical Chemistry of our institute with a purity of 99.9%. The chemical structures of these compounds are shown in Fig. 1. According to the molecular weight of two compounds, doses of dl-PHPB (1.3, 3.9, and 12.9 mg/kg) used to treat intravenously were equimolar to the doses of dl-NBP (1, 3, and 10 mg/kg), respectively. dl-PHPB was dissolved in 0.9% saline. dl-NBP was dissolved in component solvent (PEG-400/H2O, 1/3) (i.v.).

Animal Preparation and Experimental Model. After administration of atropine sulfate (0.5 mg/kg i.p.) to minimize bronchial secretions, the rats were anesthetized with 3% isoflurane in 40% oxygen and 60% nitrogen, orally intubated, and mechanically ventilated. Temperature probe was inserted into the rectum, and a separate heating lamp was used to maintain rectal temperature at normothermic level. The right femoral artery and vein were cannulated for monitoring before, during, and after reperfusion. In addition, the right common carotid artery and bifurcation were dissected free from surrounding nerves and fascia. The ECA was ligated distally, and a 4-0 nylon suture (its tip rounded by heating and coated with poly-L-lysine) was introduced into the lumen of the ICA through a small incision on the stump of the ECA. The suture was gently advanced into the ICA for 19 to 20 mm from the bifurcation of common carotid artery to block the origin of MCA, and the ipsilateral laser-Doppler signal decreased to approximately 20 to 30% of baseline. After 2 h of MCAO, reperfusion was achieved by withdrawal of the intraluminal suture from ECA. At 50 min of reperfusion, the laser-Doppler flow probe and catheter were removed. The trachea was extubated, and the neck incision was closed with silk sutures. The animal was allowed to awaken from anesthesia and survive for 24 h after ischemia.

Treatment Groups and Drug Administration. Rats were divided into six experimental groups randomly: three dl-PHPB-treated groups (1.3, 3.9, and 12.9 mg/kg), one dl-NBP-treated groups (10 mg/kg), one vehicle control group, and one sham-operated group. At 10 min after the onset of MCAO, drugs or vehicle were administered intravenously to rats in a volume of 1 ml/kg body weight.

Regional CBF Measurement. rCBF was measured continuously before and during the acute phase of ischemia and after reperfusion by means of a LDF. The rCBF value was calculated as the average value during a period of every 10 min. First, the rCBF was monitored for 10 min before MCAO to establish the baseline value (100%). Then, MCAO was induced by advancing the filament. After 2 h of occlusion, the filament was withdrawn. The changes in rCBF were expressed as a percentage of the baseline value.

Neurobehavioral Testing. After surgery, each animal’s neurological function was evaluated at 24 h of reperfusion. Neurological deficit was graded on a score of 0 to 4 as described previously (Menzies et al., 1992; Belavie et al., 1996; Mukuda et al., 2000): 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion and decreased resistance to lateral push; 3, forelimb flexion, decreased resistance to lateral push and unilateral circling; and 4, forelimb flexion and being unable or difficult to ambulate.

Infarct Assessment. At 24 h of reperfusion, the animals were anesthetized with trichloroacetaldehyde monohydrate (350 mg/kg i.p.) and decapitated. The brain was then rapidly removed, cut into six 2-mm-thick coronal sections (the last section was 4-mm-thick) by use of a rat brain matrix (RB4000C; ASI Instruments, Warren, MI), and stained with 4% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Co., St. Louis, MO) at 37°C for 30 min. Infarct area on each slice was determined by using digital imaging with a computerized image analyzer (SPOT 3.5 Biometrics software; Silicon Graphics, Inc., Mountain View, CA), and the infarct areas were calculated to obtain the infarct volumes per brain (in millimeters3). Infarct volumes were expressed as a percentage of the contralateral hemisphere volume by using an “indirect method” (area of intact contralateral [left] hemisphere minus area of intact regions of the ipsilateral [right] hemisphere) to compensate for edema formation in the ipsilateral hemisphere (Swanson et al., 1990; Mukuda et al., 2000).

\[ I\% = \left( V_r - V_c \right) / V_c \times 100\% \]

Where: 
- \( I\% \) = percentage of infarct volume
- \( V_r \) = total brain volume
- \( V_c \) = volume of intact contralateral (left) hemisphere
Conversion of dl-PHPB in Vitro. dl-PHPB was added into rat plasma at the final concentration of 6, 30, and 60 µg/ml and incubated for different periods (2, 5, 10, and 20 min). After the incubation, 200 µl of methanol was added into 100 µl of the plasma sample. Then, the samples were mixed by vortex for 20 min and centrifuged for 10 min at 8000 rpm. The supernatant was collected and directly injected into the high-performance liquid chromatography (Agilent 1100; Agilent, Palo Alto, CA) system. For quantification of dl-PHPB and dl-NBP, an Aichrom Bond-1 ODS column (particle size, 5 µm; 4.6 × 250 mm, pH 2–10; Abel Industries, Dumfries, VA) and the mobile phase comprised of methanol-0.1% phosphate buffer (50:50) were used. The flow rate was 1 ml/min. The wavelength for UV detection was 210 nm. The column temperature was kept at 25 ± 0.5°C. 4-Biphenylacetic acid (10 µl, 0.5 mg/ml) and diazepam (10 µl, 0.5 mg/ml) were used as the internal standard for quantification of dl-PHPB and dl-NBP, respectively.

Statistical Analysis. The results are expressed as mean ± S.E.M. Physiological data, neurological score, and infarct volume were statistically analyzed using one-way ANOVA followed by Dunnett’s test for comparing the treatment groups and vehicle control group. The data from rCBF were analyzed by two-way ANOVA to detect differences between groups and over time. Results were considered to show a significant difference when the P value was less than 0.05.

Results

Physiological Parameters before and after Brain Ischemia. The physiological parameters for all groups are provided in Table 1. All data were kept within normal physiological limits before, during, and after ischemia. There were no significant differences in arterial blood gases, glucose, pH, hematocrit, and MABP between the vehicle and dl-PHPB-treated groups.

The rectal temperature in all groups was regularly maintained at a normal level (37°C) during surgical procedures (before, during ischemia, and 30 min of postischemia) under conditions of anesthesia and temperature-controlled system as shown in Table 1. At later reperfusion time points (12 and 24 h of postischemia) after rats recovered from anesthesia, the temperature definitely increased and reached approximately 39°C in the vehicle group as well as in drug-treated groups. However, there were no significant differences between them.

Neurological Assessment. Before MCAO, neurological score was zero in all animals. After MCAO, high-grade neurological deficits were present. The results are shown in Table 2. Compared with vehicle-treated rats (score, 3.2 ± 0.2), treatment with dl-PHPB significantly and dose-dependently improved the neurological score at 24 h after ischemia. At the highest dose, the efficacy of dl-PHPB (score, 1.8 ± 0.2) was similar to dl-NBP (score, 2.0 ± 0.2).

Infarct Volume And Protective Effect of dl-PHPB. Coronal sections of rat brain stained with TTC are shown in Fig. 2. Infarct volume was measured in animals after transient MCAO after treatment with dl-PHPB, dl-NBP, or vehicle (Fig. 3). At doses of 1.3, 3.9, and 12.9 mg/kg, dl-PHPB could dose-dependently reduce the infarct volume significantly from a vehicle control of 37.4 to 25.4, 17.4, and 13.7%, respectively (P < 0.01 versus vehicle control). The infarct volume in dl-NBP group (10 mg/kg) was 16.3%. This indicates that dl-PHPB shows the similar efficacy with dl-NBP in the reduction of infarct volume at the corresponding dose.

TABLE 1

Physiological parameters in rats subjected to 2-h intraluminal suture occlusion of MCA and 24-h reperfusion

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle Control</th>
<th>dl-PHPB 1.3</th>
<th>dl-PHPB 3.9</th>
<th>dl-PHPB 12.9</th>
<th>dl-NBP (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MABP (mm Hg)</td>
<td>99 ± 6</td>
<td>100 ± 5</td>
<td>104 ± 6</td>
<td>98 ± 7</td>
<td>91 ± 12</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>7.27 ± 0.05</td>
<td>7.27 ± 0.03</td>
<td>7.30 ± 0.02</td>
<td>7.26 ± 0.02</td>
<td>7.29 ± 0.01</td>
</tr>
<tr>
<td>PaO2 (mm Hg)</td>
<td>94.5 ± 7.9</td>
<td>92.3 ± 7.2</td>
<td>97.9 ± 3.0</td>
<td>97.3 ± 12.9</td>
<td>95.6 ± 1.3</td>
</tr>
<tr>
<td>PaCO2 (mm Hg)</td>
<td>53.3 ± 4.8</td>
<td>52.1 ± 3.9</td>
<td>51.1 ± 0.9</td>
<td>50.7 ± 8.9</td>
<td>53.7 ± 0.3</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>48.1 ± 1.4</td>
<td>48.5 ± 1.0</td>
<td>47.3 ± 2.3</td>
<td>48.8 ± 0.6</td>
<td>49.2 ± 0.6</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>166.1 ± 10.5</td>
<td>166.9 ± 9.4</td>
<td>167.2 ± 14.4</td>
<td>174.3 ± 15.0</td>
<td>170.7 ± 11.4</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>37.1 ± 0.39</td>
<td>37.0 ± 0.28</td>
<td>37.2 ± 0.30</td>
<td>37.0 ± 0.19</td>
<td>37.1 ± 0.27</td>
</tr>
<tr>
<td>During ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MABP (mm Hg)</td>
<td>101 ± 11</td>
<td>99 ± 8</td>
<td>105 ± 12</td>
<td>102 ± 11</td>
<td>109 ± 8</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>7.24 ± 0.03</td>
<td>7.23 ± 0.04</td>
<td>7.27 ± 0.09</td>
<td>7.23 ± 0.04</td>
<td>7.25 ± 0.02</td>
</tr>
<tr>
<td>PaO2 (mm Hg)</td>
<td>101.2 ± 12.2</td>
<td>103 ± 5.8</td>
<td>99.8 ± 8.5</td>
<td>100.8 ± 6.4</td>
<td>97.4 ± 2.8</td>
</tr>
<tr>
<td>PaCO2 (mm Hg)</td>
<td>55.3 ± 4.0</td>
<td>53.6 ± 8.3</td>
<td>54.0 ± 9.0</td>
<td>54.3 ± 6.2</td>
<td>54.9 ± 4.1</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>47.6 ± 1.8</td>
<td>46.5 ± 4.0</td>
<td>47.6 ± 0.8</td>
<td>45.9 ± 4.3</td>
<td>48.0 ± 0.9</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>168.7 ± 8.9</td>
<td>163.8 ± 14.5</td>
<td>165.2 ± 8.9</td>
<td>170.9 ± 12.6</td>
<td>168.3 ± 10.5</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>36.8 ± 0.34</td>
<td>36.7 ± 0.21</td>
<td>36.8 ± 0.28</td>
<td>36.7 ± 0.42</td>
<td>36.9 ± 0.31</td>
</tr>
<tr>
<td>After ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MABP (mm Hg)</td>
<td>98 ± 9</td>
<td>103 ± 6</td>
<td>100 ± 7</td>
<td>96 ± 12</td>
<td>93 ± 9</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>7.25 ± 0.10</td>
<td>7.24 ± 0.02</td>
<td>7.22 ± 0.07</td>
<td>7.22 ± 0.05</td>
<td>7.25 ± 0.01</td>
</tr>
<tr>
<td>PaO2 (mm Hg)</td>
<td>106.2 ± 17.0</td>
<td>102.5 ± 7.4</td>
<td>103.1 ± 6.7</td>
<td>106.2 ± 13.3</td>
<td>103.4 ± 8.8</td>
</tr>
<tr>
<td>PaCO2 (mm Hg)</td>
<td>56.1 ± 3.7</td>
<td>52.1 ± 11.3</td>
<td>57.7 ± 8.7</td>
<td>54.8 ± 4.4</td>
<td>56.6 ± 4.9</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>48.0 ± 1.4</td>
<td>49.1 ± 3.4</td>
<td>47.3 ± 4.7</td>
<td>48.8 ± 0.6</td>
<td>49.2 ± 0.6</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>170.1 ± 11.9</td>
<td>169.2 ± 15.1</td>
<td>168.6 ± 9.5</td>
<td>178.1 ± 11.8</td>
<td>169.2 ± 9.8</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>36.9 ± 0.20</td>
<td>37.0 ± 0.27</td>
<td>37.1 ± 0.27</td>
<td>36.9 ± 0.15</td>
<td>37.0 ± 0.21</td>
</tr>
<tr>
<td>Temperature at later reperfusion (°C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 h after MCAO</td>
<td>38.3 ± 0.41</td>
<td>38.2 ± 0.33</td>
<td>38.4 ± 0.21</td>
<td>38.1 ± 0.26</td>
<td>38.0 ± 0.22</td>
</tr>
<tr>
<td>24 h after MCAO</td>
<td>38.9 ± 0.37</td>
<td>38.7 ± 0.29</td>
<td>39.1 ± 0.30</td>
<td>38.8 ± 0.33</td>
<td>38.6 ± 0.28</td>
</tr>
</tbody>
</table>

PaO2, arterial oxygen tension; PaCO2, arterial carbon dioxide tension.
farcted tissue is white, whereas live tissue is darkly stained by TTC. Intraluminal suture occlusion of MCA and 24-h reperfusion. The TTC-stained coronal brain sections are from representative animals of vehicle control group (A), dl-PHPB (1.3, 3.9 and 12.9 mg/kg)-treated group (B, C, and D), and dl-NBP (10 mg/kg)-treated group (E), respectively. Drugs were intravenously administered 10 min after the onset of MCAO. Infarcted tissue is white, whereas live tissue is darkly stained by TTC.

**Table 2**

Improvement in neurological behavior of dl-PHPB in rats after 2-h intraluminal suture occlusion of MCA and 24-h reperfusion. Values are mean ± S.E.M. (n = 10). dl-PHPB and dl-NBP were intravenously administered 10 min after the onset of MCAO.

<table>
<thead>
<tr>
<th>Group</th>
<th>Neurological Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>3.2 ± 0.2**</td>
</tr>
<tr>
<td>dl-PHPB 1.3 mg/kg</td>
<td>2.7 ± 0.1*</td>
</tr>
<tr>
<td>dl-PHPB 3.9 mg/kg</td>
<td>2.1 ± 0.2**</td>
</tr>
<tr>
<td>dl-PHPB 12.9 mg/kg</td>
<td>1.8 ± 0.2**</td>
</tr>
<tr>
<td>dl-NBP 10 mg/kg</td>
<td>2.0 ± 0.2**</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

**p < 0.01 vs. sham-operated group.
* p < 0.05. ** p < 0.01 vs. vehicle control group.

**Fig. 2.** Effect of dl-PHPB (i.v.) on infarct volume in rat brain after 2-h intraluminal suture occlusion of MCA and 24-h reperfusion. The TTC-stained coronal brain sections are from representative animals of vehicle control group (A), dl-PHPB (1.3, 3.9 and 12.9 mg/kg)-treated group (B, C, and D), and dl-NBP (10 mg/kg)-treated group (E), respectively. Drugs were intravenously administered 10 min after the onset of MCAO. Infarcted tissue is white, whereas live tissue is darkly stained by TTC.

**Fig. 3.** Improvement in infarct volume of dl-PHPB in rats after 2-h intraluminal suture occlusion of MCA and 24-h reperfusion. Values are mean ± S.E.M. (n = 10). dl-PHPB (1.3, 3.9, and 12.9 mg/kg) and dl-NBP (dose displayed represents the actual doses of 10 mg/kg) were intravenously administered 10 min after the onset of MCAO. **, P < 0.01; ***, P < 0.001 versus vehicle control group.

**Effects of dl-PHPB on Regional CBF.** Figure 4 shows the relative changes of rCBF with LDF during and after 2 h of MCAO. In all groups, MCA occlusion induced a similar immediate reduction of rCBF value to approximately 20 to 30% of the baseline level. During the ischemic period, the rCBF values in dl-PHPB groups and dl-NBP groups were significantly higher than that in vehicle group. Especially at high doses, dl-PHPB increased rCBF significantly from 20 min after administration and throughout the ischemic period. The potency of dl-PHPB was a little bit stronger than that of dl-NBP, but there was no significant difference between them at the corresponding dose. After recirculation, rCBF gradually recovered to approximately 70 to 80% of the baseline level. At the 50-min reperfusion, there were no significant differences between the vehicle and dl-PHPB groups. Two-way ANOVA of data, obtained after injection of dl-PHPB and dl-NBP, showed a significant effect of treatment (P < 0.05 versus vehicle control), but no effect of time during the ischemia.

**Conversion of dl-PHPB to dl-NBP in Vitro.** Using our analysis system, the rate of recovery from rat plasma was 98.3% for dl-PHPB and 84.7% for dl-NBP, respectively. It is shown in Fig. 5. After adding into plasma, the levels of dl-PHPB decreased very fast, and the levels of dl-NBP in plasma simultaneously increased. After 5 min of incubation of dl-PHPB with plasma, more than 70% of dl-PHPB was converted into dl-NBP. This indicated that dl-PHPB could convert into dl-NBP quickly and completely at concentrations of 6, 30, and 60 µg/ml.

**Discussion**

The ischemia-induced changes and functional impairments after permanent or transient occlusion of the MCA in rats closely resemble those observed after focal occlusion of the MCA in humans (Yamori et al., 1976), resulting in the frequent use of this model for evaluating neuroprotective agents (Muller et al., 1995; Engelhorn et al., 2004). Therefore, in present study, we used a rat model of 2-h intraluminal suture occlusion of MCA and 24-h reperfusion to produce transient focal cerebral ischemia.

As described above, the severity of damage after transient cerebral ischemia depends mainly on the duration of the ischemia and the degree of CBF reduction. For a given period of ischemia (2-h MCAO in our study), the infarct volume will be a function of CBF reduction. However, CBF in the ischemic core is usually strongly affected for quite a wide range of infarct sizes. Therefore, CBF in the periphery of the affected area (penumbra) can potentially indicate the extent of CBF alterations and, therefore, might give an estimation of the severity of ischemia (Menegazza et al., 1992; Soriano et al., 1997). Many kinds of methods are widely used for measuring CBF. All methods have strengths and weaknesses (Kramer et al., 1996). Compared with other methods, LDF provides a noninvasive and continuous measure of local CBF, increasing the ability to observe instantaneous changes in cerebral microcirculation. The changes of focal CBF after MCAO can be monitored by project zones of MCA in the surface of cerebra with LDF, which seems to give good estimations of CBF reductions. However, LDF did not record absolute blood flow, and it was very sensitive to an altered position of the LDF probe. In addition, under conditions of changed blood hematocrit or fluctuations of blood gases, LDF did not accurately measure CBF values (Kramer et al., 1996; Gu et al., 2003). Therefore, physiology parameters should be monitored at the same time.

dl-NBP was demonstrated as a useful drug for treatment of ischemic stroke in clinic studies. However, it was limited to use intravenously because of its property of hydrophobicity,
and it was difficult in the treatment of serious patients. Therefore, \(dl\)-PHPB was designed as a prodrug of \(dl\)-NBP, and its properties have been greatly improved. In this study, it was shown that approximately 70\% of \(dl\)-PHPB could convert into \(dl\)-NBP in 5 min when \(dl\)-PHPB was added into the plasma in vitro. A pharmacokinetic study in vivo also showed that \(dl\)-PHPB intravenously to rat, it was difficult to determine in the plasma. However, after \(dl\)-PHPB injection for 1 min, \(dl\)-NBP was clearly found in rat plasma with quite high levels (J. Li, L. He, and X. Wang, unpublished data). The present results showed that intravenous treatment of \(dl\)-PHPB (1.3, 3.9, and 12.9 mg/kg) could dose-dependently reduce the infarct volume significantly. In addition, at the highest dose, \(dl\)-PHPB showed slightly higher efficacy in reducing infarct volume than \(dl\)-NBP, although there was no significant difference between the two compounds.

Neurobehavioral deficits were also observed 24 h after MCAO. It showed that \(dl\)-PHPB could also improve the behavioral deficits in a dose-dependent manner. The improvement effects of \(dl\)-PHPB observed were highly correlated with the reduction of infarct volumes at the same doses.

To determine whether the infarct-reducing effects of \(dl\)-PHPB are due to an increase in blood flow during ischemia, we measured rCBF in the penumbral region of cortex (2 mm posterior and 3 mm lateral to the bregma), which is also the project zone of MCA in the surface of cerebral. Our data

**Fig. 4.** Relative rCBF measured with LDF in the MCA-supplied cortex of all groups over a period of 120 min after MCAO and then recorded for 50 min after reperfusion. Values are mean \(\pm\) S.E.M. (\(n = 9\)). \(dl\)-PHPB (1.3, 3.9, and 12.9 mg/kg) was intravenously administered 10 min after the onset of MCAO. A, rCBF over the entire period of measurement. B, rCBF from 10 to 120 min after MCAO shown on an expanded scale. \(*, P < 0.05; ***, P < 0.01\) versus vehicle control group.

**Fig. 5.** The conversion curve of \(dl\)-PHPB to \(dl\)-NBP in plasma. Values are mean \(\pm\) S.E.M. (\(n = 3\)). The concentrations of \(dl\)-PHPB and \(dl\)-NBP were determined by high-performance liquid chromatography method. After \(dl\)-PHPB was added into the plasma after 2, 5, 10, and 20 min, the plasma (100 \(\mu\)l) was deproteinated by 200 \(\mu\)l of methanol, and they were centrifuged. The supernatant was used for analyzing the drug level.
showed that treatment with dl-PHPB could significantly improve rCBF during the period of occlusion, whereas the improving effect at the highest dose was far more potent than that at the middle or lowest dose. At the highest dose of dl-PHPB injection, CBF values increased from approximately 27 to 40% of the preischemia values (CBF values in the vehicle-treated group decreased from 25% to 15%). Whereas CBF values in the middle-dose group only increased slightly from approximately 23% to 26%, similar to the effect in the lowest dose (from approximately 29% to 24%). Compared with the dose-dependent results from the infarct and neurobehavioral assessments, such results indicate that the neuroprotective effect of higher doses of dl-PHPB observed in infarct and in the neurobehavioral test was mainly due to an improvement of blood flow during ischemia. However, at lower doses, the neuroprotection of dl-PHPB seemed to be a result of multiple mechanisms such as antioxidative and antiapoptotic effects that have been observed in our research, same as in dl-NBP (Dong and Feng, 2002; Chang and Wang, 2003). After the withdrawal of the intraluminal suture, rCBF in all groups gradually recovered. There was also a trend that the CBF values displayed after reperfusion in the treated groups was shown to be obviously higher than that in vehicle groups. Especially at high doses, treatment with dl-PHPB increased the CBF level by approximately 51.6% than the vehicle group at 30 min after reperfusion, although there were no significant differences between the vehicle and the treated group at the same individual time points.

Various physiological variables influence CBF, including arterial blood gases, cerebral autoregulation, metabolic rate through vasoconstraining and functional balance between the endothelium-dependent vasodilators and the endothelium-derived constrictors. One important mediator controlling basal CBF is nitric oxide (NO). Endothelium-derived NO plays a crucial role in maintenance of blood vessel caliber and, therefore, blood flow throughout the vasculature. It is also important in preventing thrombosis through inhibition of platelet adhesion, activation, and aggregation (Markus, 2004). Arachidonate metabolism also plays an important role in modulating CBF. Thromboxane A2 (TXA2) and prostaglandin L2 (PGL2) are major metabolites of cyclooxygenase activation in platelets and in endothelial cells, respectively. TXA2 is a potent platelet aggregating and vasoconstricting substance, whereas PGL2 is a powerful antiplatelet and vasodilator agent (Peng et al., 2004). Previous studies showed that dl-NBP could induce the activation of endothelial NO synthase and thus increase the production of NO (Yan and Feng, 1998). Furthermore, dl-NBP could decrease the production of TXA2 and increase the production of PGL2 in cerebral cortex cells and decrease the ratio of TXA2/PGL2 in brain tissue after MCAO in rat (Chong and Feng, 1997). Therefore, such activities might also be involved in the rCBF improvement effects of dl-PHPB.

In conclusion, dl-PHPB significantly reduces infarct volume and improves neurobehavioural deficits in rat model of transient focal cerebral ischemia. Such effects may be partly due to the increase of rCBF. In addition, multiple mechanisms for neuroprotection might be involved in the effects of dl-PHPB. Therefore, further studies are needed to completely elucidate the mechanism accounting for the protective effects of dl-PHPB on cerebral ischemia.

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References


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