Beagle Pup Model of Perinatal Asphyxia: Nimodipine Studies

Laura R. Ment, William B. Stewart, Charles C. Duncan, and Bruce R. Pitt

Calcium antagonists may be of significant benefit in the pharmacotherapy of cerebral ischemia, possibly by improving postischemic cerebral blood flow (CBF). This study evaluated the effects of the calcium antagonist nimodipine on CBF in a newborn beagle pup model of perinatal asphyxia lasting 5 minutes. Immediately after the asphyxial episode, nimodipine (2 μg/kg/min) or saline was infused for 10 minutes, following which [14C]iodoantipyrine determinations of CBF were performed. In nonsuited pups, nimodipine caused both significant decreases in CBF to cortical and deep gray structures as well as a decrease in mean arterial blood pressure (MABP) (p < 0.05). In insulted pups, nimodipine similarly decreased MABP (p < 0.001) and CBF to cortical and deep gray matter regions. Nimodipine appeared to have no effect on arterial blood gases and EEG tracings in either insulted or nonsuited pups. Although nimodipine may be shown to improve neurologic outcome in asphyxiated newborn infants, the limits of this study do not show the mechanism to be that of improving CBF. (Stroke 1987;18:599-605)

During the past decade numerous investigators have hypothesized that the 1,4-dihydropyridine calcium antagonists may be of significant benefit in the pharmacotherapy of cerebral ischemia. Ischemia-induced alterations in calcium homeostasis are believed to be responsible for the initiation of the cascade of events leading to irreversible neuronal damage. Hypoxic/ischemic insults are known to result in cerebral metabolic disturbances characterized by decreased concentrations of high-energy phosphate compounds and by increased cerebral lactate. Cerebral blood flow (CBF) falls, and secondary failure of the ion pump results in neuronal depolarization and calcium entry. This imbalance in intracellular calcium provokes the accumulation of free fatty acids, which in turn uncouples oxidative metabolism and produces additional neuronal permeability changes. In response to ischemic insult, calcium channel blockers have been reported to both act on vascular smooth muscle to increase CBF and to block calcium influx at voltage-gated calcium channels to preserve neuronal integrity.

While much has been written about the efficacy of calcium antagonists in adult animal models of hypoxic/ischemic insult, little is known about the role of these agents in the response of the developing nervous system to such problems. Severe perinatal asphyxia, the classic hypoxic/ischemic insult of the newborn period, has been reported to occur in between 1 in 50 and 1 in 500 live births and the survivors of such insult demonstrate an incidence of 20–30% moderate to severe neurodevelopmental difficulties including cerebral palsy, mental retardation, and persistent seizure disorders. Studies of experimental neonatal animals and newborn infants with perinatal asphyxia have demonstrated alterations in CBF and neuropathologic changes throughout the developing brain.

Newborn beagle pups provide a good model for the study of the developing brain. Neuropathologic insults such as perinatal cerebral infarction and intraventricular hemorrhage may be produced by clinically relevant models and CBF may be determined. The purpose of this study was to evaluate the effects of the calcium antagonist nimodipine on CBF in newborn beagle pups exposed to a clinically relevant model of perinatal asphyxia. To approximate clinical events, nimodipine was administered as an i.v. infusion after asphyxial insult.

Materials and Methods

The following studies were performed with the approval of the Yale University Committee on Animal Care.

Newborn beagle pups (24–96 hours old) were lightly anesthetized with 20 mg/kg i.p. pentobarbital, tracheotomized after local 1% xylocaine infiltration, paralyzed with 1 mg/kg subcutaneous gallamine triethiodide, and ventilated with a mixture of 30% oxygen and 70% nitrous oxide for analgesia. Bilateral femoral venous and arterial lines were inserted, and arterial blood pressure was monitored by a pressure transducer and polygraph recorder. Body temperature was recorded by a thermal probe and maintained at 36.5–37.5°C using a warming light. Ventilatory rate and tidal volume were adjusted to maintain arterial normoxia (> 40 mm Hg) and normocapnia (30–40 mm Hg). Scalp EEG electrodes were placed, and EEG was continuously monitored using a Grass Model 7 EEG machine. The experiment was designed to correlate with clinical events in newborn infants. Animal preparation and

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stroke did not vary between pups, and when the pups were physiologically stable, they were randomly assigned to 1 of 4 treatment and drug groups: 1) insult and saline infusion, 2) insult and nimodipine infusion, 3) no insult and saline infusion, and 4) no insult and nimodipine infusion. The 20-minute experiment consisted of insult or no insult for 5 minutes followed by a resuscitation phase for 5 minutes and then saline or nimodipine infusion for 10 minutes; CBF was then determined immediately. There were no deaths and no episodes of pulselessness associated with this protocol. Insult consisted of temporarily withdrawing ventilatory support for 5 minutes. No-insult animals were ventilated and observed for a comparable time. The resuscitation phase consisted of re-instituting ventilatory support; no additional medications or fluids were administered during the resuscitation phase. Nimodipine was administered at 2 μg/kg/min in approximately 4–5 ml of saline for 10 minutes; control animals received an equal volume of saline diluent. Arterial blood gases were monitored throughout.

Nimodipine is light-sensitive; therefore, all syringes and catheters used for mixing and infusion of this medication were covered with aluminum foil. Nimodipine (Miles Laboratories, New Haven, Conn.) was supplied at a concentration of 1 mg/5 ml. Aliquots of this solution were diluted with 0.9% NaCl.

CBF was determined using a 5-second bolus venous infusion of 50 μCi [14C]iodoantipyrine (IAP) simultaneously with the withdrawal of arterial blood into an artificial organ system (approximately 80 cm of PE-60 polyethylene tubing) attached to a Harvard infusion/withdrawal pump calibrated to withdraw blood at a constant rate of 2.7 ml/min. The pups were then rapidly decapitated, and the brains were removed and placed in isopentane chilled to -60°C. Brain sections 32 μm thick were prepared with a cryostat maintained at -15 to -10°C. Every 25th section was placed on a glass slide, dried on a hot plate at 60–70°C, and placed in an x-ray cassette loaded with Kodak SB-5 film for 7 days. Calibrated plastic standards (Amersham) were placed adjacent to the tissue sections. Using a Leitz microdensitometer, local tissue concentrations were determined.

Arterial blood withdrawn during the [14C]IAP venous injection was placed in preweighed scintillation vials, and triplicate aliquots were treated with an equal volume of hydrogen peroxide. 14C was determined in brain/g) x (2.22 x 10^3 dpm/μCi) x (2.72 ml/min) (1)

All values are presented as means ± SEM. Two means were compared using t tests for paired observations, as appropriate, and 3 or more means were compared using analysis of variance (ANOVA). Significance was assumed at p < 0.05.

Results

Physiologic Data

[14C]IAP determinations of CBF were performed on 24 pups. Six pups were randomized to each of the 4 experimental groups. Arterial blood gas values and mean arterial blood pressure (MABP) are found in Table 1; no significant differences in any of these parameters were noted before the insult. MABP is also shown in Figure 1.

MABP of control pups was stable before drug administration. Insulted pups experienced a significant decrease in MABP followed by improvement in blood pressure prior to drug administration. A three-way ANOVA was used on the MABP data. There was a significant effect of time [F(3,60) = 18.89, p < 0.001] and of the time x drug interaction [F(3,60) = 6.89, p < 0.001] and time x group interaction [F(3,60) = 14.91, p < 0.001]. For the non-insulted animals, there was essentially no change in MABP over time before the initiation of drug infusion. For the saline-treated pups, MABP rose slightly, and for the nimodipine-treated pups, MABP decreased from 62.5 ± 7.4 to 53.8 ± 6.9 mm Hg over the insult [F(3,30) = 12.31, p < 0.05]. For the insulted pups, MABP dropped significantly during the insult and then rose sharply following resuscitation. During the infusion MABP remained stable for the saline-treated pups but dropped sharply for the nimodipine-treated pups (p < 0.001).

Analysis of the blood gas data demonstrated that insulted pups developed significant hypoxemia, hypercarbia, and acidosis (p < 0.001) in response to discontinuation of ventilatory support. Five minutes after reinstitution of ventilatory support at the ventilatory requirements of prior to insult, Po2 had returned to preinsult levels although the pups remained somewhat acidic and hypercarbic (p < 0.05 compared with preinsult values).

With respect to the entire experimental time interval, both Po2 and Pco2 changed over time for the insulted pups (p < 0.001) but not for the noninsulted pups, irrespective of drug. Insulted pups showed a decrease followed by a rise in pH [F(3,54) = 39.53, p < 0.001]; noninsulted pups had a very slight rise in pH over time (p < 0.01). Changes in pH occurred irrespective of drug.

EEG Data

The baseline EEG of the puppy cortex consists of a 4–10 Hz rhythm. In the insulted pups, asphyxia resulted in a gradual loss of amplitude and frequency over the first 2–3 minutes of the 5-minute insult; by 3 minutes all insulted pups had EEG evidence of electrocerebral silence. Within 30 seconds of initiation of the resuscitation phase, EEG activity returned to preinsult baseline and remained unchanged throughout the post-insult drug administration phase. No differences were noted in the EEGs of the nimodipine-treated pups compared to their saline counterparts.
pared with the saline-treated pups for either the insulted or noninsulted groups.

Cerebral Blood Flow Determinations

CBF values are found in Table 2. Two-way ANOVA with treatment and drug as the main factors were used to evaluate these data. The $F$ ratios and $p$ values are also indicated in Table 2. For all of the gray matter regions examined, there were significant effects of drug and treatment, but no significant interaction. In the frontal and temporal white matter regions, there were no significant effects of drug, treatment, or the interaction. For the parietal white matter, there was a significant effect of drug and a marginal effect of treatment, but no significant interaction. For the caudate nucleus, there was a significant effect of drug, treatment, and their interaction. Thus, the lowest caudate nucleus CBF values were found in insulted pups treated with nimodipine. For the germinal matrix, there was a marginal effect of drug and a significant effect of treatment, but no significant interaction.

Discussion

The study of CBF and metabolism has markedly improved the understanding of neonatal neurologic insults. Despite the development of sophisticated perinatal care, the survivors of serious perinatal asphyxia still experience a 10–20% incidence of serious neurodevelopmental handicaps. At delivery those infants may be profoundly hypoxic and hypotensive. When Nelson and Ellenberg reviewed the data of the Collaborative Perinatal Project of the National Institute of Neurologic and Communicative Disorders and Stroke of approximately 40,000 live births, an Apgar score of 3 or less at 5 minutes after birth was significantly correlated with adverse neurologic outcome. Forty-four percent of infants with such scores died within the first year, and 1 in 20 of the surviving infants developed major motor handicaps. The relative risk of these infants for cerebral palsy was 21 times that of infants with 5-minute Apgar scores of 7–10. Similarly, other studies have demonstrated abnormalities of technetium brain scans and computed tomography (CT) scans consis-

### Table 1. Physiologic Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before insult $t = 0$</th>
<th>After insult $t = 5$</th>
<th>Before infusion $t = 10$</th>
<th>After infusion $t = 20$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP (mm Hg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline No insult</td>
<td>69.8 ± 1.0</td>
<td>70.0 ± 1.0</td>
<td>69.5 ± 1.0</td>
<td>72.5 ± 1.8</td>
</tr>
<tr>
<td>Insult</td>
<td>68.9 ± 7.2</td>
<td>44.5 ± 6.1</td>
<td>56.9 ± 4.4</td>
<td>55.4 ± 5.4</td>
</tr>
<tr>
<td>Nimodipine No insult</td>
<td>62.0 ± 7.4</td>
<td>63.0 ± 6.0</td>
<td>62.5 ± 7.4</td>
<td>53.8 ± 6.9</td>
</tr>
<tr>
<td>Insult</td>
<td>71.2 ± 3.1</td>
<td>51.3 ± 4.9</td>
<td>61.8 ± 5.6</td>
<td>45.8 ± 5.5</td>
</tr>
<tr>
<td>$P_O_2$ (mm Hg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline No insult</td>
<td>90.7 ± 6.3</td>
<td>91.0 ± 7.0</td>
<td>96.3 ± 8.1</td>
<td>93.0 ± 5.1</td>
</tr>
<tr>
<td>Insult</td>
<td>82.4 ± 9.6</td>
<td>12.0 ± 3.8</td>
<td>85.9 ± 10.5</td>
<td>79.8 ± 8.9</td>
</tr>
<tr>
<td>Nimodipine No insult</td>
<td>96.0 ± 11.5</td>
<td>97.5 ± 10.0</td>
<td>67.0 ± 10.9</td>
<td>101.5 ± 10.9</td>
</tr>
<tr>
<td>Insult</td>
<td>86.1 ± 7.1</td>
<td>11.1 ± 4.6</td>
<td>93.5 ± 7.9</td>
<td>87.6 ± 8.3</td>
</tr>
<tr>
<td>$P_Co_2$ (mm Hg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline No insult</td>
<td>36.2 ± 0.9</td>
<td>37.0 ± 1.2</td>
<td>36.0 ± 1.7</td>
<td>38.2 ± 0.8</td>
</tr>
<tr>
<td>Insult</td>
<td>34.9 ± 1.2</td>
<td>67.8 ± 4.9</td>
<td>42.6 ± 5.0</td>
<td>38.8 ± 3.6</td>
</tr>
<tr>
<td>Nimodipine No insult</td>
<td>34.6 ± 1.4</td>
<td>35.0 ± 1.5</td>
<td>34.8 ± 1.2</td>
<td>33.5 ± 2.6</td>
</tr>
<tr>
<td>Insult</td>
<td>33.0 ± 1.3</td>
<td>66.5 ± 7.6</td>
<td>43.0 ± 4.3</td>
<td>36.8 ± 3.0</td>
</tr>
<tr>
<td>$pH$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline No insult</td>
<td>7.30 ± 0.03</td>
<td>7.30 ± 0.03</td>
<td>7.32 ± 0.02</td>
<td>7.32 ± 0.02</td>
</tr>
<tr>
<td>Insult</td>
<td>7.30 ± 0.03</td>
<td>7.06 ± 0.04</td>
<td>7.16 ± 0.03</td>
<td>7.22 ± 0.03</td>
</tr>
<tr>
<td>Nimodipine No insult</td>
<td>7.29 ± 0.03</td>
<td>7.30 ± 0.02</td>
<td>7.29 ± 0.02</td>
<td>7.29 ± 0.02</td>
</tr>
<tr>
<td>Insult</td>
<td>7.30 ± 0.01</td>
<td>7.04 ± 0.04</td>
<td>7.19 ± 0.03</td>
<td>7.27 ± 0.03</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.
tent with disruption of the normal neonatal blood–brain barrier and with edema formation throughout the hemispheres. 37,42 In addition, a significant depression of CBF may occur for many days following such insult. 31,33 Finally, follow-up CT studies have demonstrated both central and cortical tissue loss, 25,42 and postmortem examinations have documented neuropathologic changes throughout the developing brain. 35–37

Neonatal animal studies of perinatal asphyxia have confirmed the loss of the normal blood–brain barrier and noted the development of pressure-passive CBF. 28,43–45 Studies of fetal sheep, newborn piglet, and newborn beagle pup models of perinatal asphyxia have all demonstrated bradycardia and hypotension. 28,32,43–45 With severe insult, extremely low values of hemispheric blood flow have been reported. 28,32,43

Numerous recent investigations of the pathophysiology of irreversible neuronal damage following ischemic insult appear to indicate that disturbances in calcium homeostasis may be in part responsible for this process. 9,10 The low CBF and the decline in high energy phosphates evoked by hypoxic/ischemic insult may result in failure of the membrane ion pump and intraneuronal accumulation of both calcium and free fatty acids, 11–14 which have detrimental effects on other cellular systems, resulting in an uncoupling of oxidative phosphorylation and additional membrane permeability changes. In addition, transient increases in intracellular calcium may stimulate the release of excitotoxic neurotransmitters. 46,47

Treatment with calcium antagonists in the early phase after cerebral ischemia has been hypothesized to prevent intracellular calcium ion accumulation and thus prevent the cascade of events leading to ultimate neuronal destruction. 1–3 Furthermore, these drugs have

Table 2. Cerebral Blood Flow Values

<table>
<thead>
<tr>
<th></th>
<th>No insult</th>
<th>Insult</th>
<th>Drug</th>
<th>Insult</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Nimodipine</td>
<td>Saline</td>
<td>Nimodipine</td>
<td></td>
</tr>
<tr>
<td><strong>Gray matter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>F Ratios</strong></td>
</tr>
<tr>
<td>Frontal</td>
<td>24.8±3.2</td>
<td>10.32±2.9</td>
<td>11.2±4.4</td>
<td>5.71±1.0</td>
<td>9.32*</td>
</tr>
<tr>
<td>Temporal</td>
<td>31.9±3.5</td>
<td>17.1±6.1</td>
<td>15.2±4.4</td>
<td>8.1±0.8</td>
<td>6.26†</td>
</tr>
<tr>
<td>Parietal</td>
<td>26.7±3.7</td>
<td>17.7±5.9</td>
<td>17.4±5.1</td>
<td>7.9±0.9</td>
<td>4.13†</td>
</tr>
<tr>
<td><strong>White matter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal</td>
<td>5.0±0.6</td>
<td>3.4±0.9</td>
<td>3.2±1.0</td>
<td>2.3±0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Temporal</td>
<td>7.3±1.8</td>
<td>3.9±1.2</td>
<td>3.7±1.0</td>
<td>3.0±0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Parietal</td>
<td>5.2±0.6</td>
<td>3.4±1.0</td>
<td>3.7±0.9</td>
<td>2.2±0.4</td>
<td>4.77†</td>
</tr>
<tr>
<td><strong>Caudate nucleus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>26.8±1.8</td>
<td>7.5±2.7</td>
<td>9.6±3.2</td>
<td>4.7±0.4</td>
<td>18.82*</td>
</tr>
<tr>
<td><strong>Germinal matrix</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.6±0.7</td>
<td>3.6±1.4</td>
<td>3.0±1.0</td>
<td>1.9±0.1</td>
<td>3.63‡</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, ml/100 g/min. NS, not significant (p > 0.10).

*p < 0.005.

†p < 0.05.

‡Marginal p < 0.10.
been reported to increase CBF in nonischemic animal models by dilatation of the cerebral microvasculature\(^48,49\) and might therefore be expected to improve postischemic hypoperfusion. Finally, recent data suggest that calcium channel blockers prevent the release of excitatory neurotransmitters\(^46,47\) and in this fashion act as anticonvulsant agents.\(^50\)

Unfortunately, experimental studies from the past several years have resulted in conflicting results. Although Steen et al\(^51\) initially reported that pretreatment with nimodipine improved CBF and neurologic recovery after complete cerebral ischemia in dogs, subsequent studies by Steen et al\(^52\) and others\(^53,54\) demonstrated no improvement in neurologic outcome, CBF and cerebral metabolism, or postischemic cerebral calcium, free fatty acids, and ATP stores.

In contrast, reports\(^55,56\) of calcium entry blockers administered after complete cerebral ischemia have documented more favorable outcomes for treated animals. Vaagenes et al\(^57\) treated dogs that suffered 10 minutes of complete cardiac arrest with lidoflazine within 10 minutes of the insult and documented improved neurologic outcome; in this study there was no histopathologic difference between treated and untreated dogs. Steen et al\(^58\) similarly exposed pigtailed monkeys to 17 minutes of complete cerebral ischemia followed by resuscitation and supportive care. Those monkeys treated with nimodipine beginning 5 minutes after the insult had better neurologic and neuropathologic outcomes than their untreated peers.

Recent experimental animal and human data have failed to demonstrate any beneficial effect of treatment with calcium antagonists in pathologic circumstances. Vorstrup et al\(^59\) administered the calcium antagonist PY 108-068 to adult stroke patients and demonstrated a slight increase in CBF in the nonaffected hemisphere but diminished CBF in the ischemic areas of 3 of 5 patients. MABP decreased by 13% in patients treated with this agent, and their clinical symptoms were unchanged. Similarly, Phillis et al\(^60\) demonstrated that the calcium antagonists nifedipine and felodipine had no effect on the basal CBF of adult rats and depressed the increase in CBF elicited by anoxia in this model.

Studies of calcium antagonists in newborn animals are limited, although these agents have been shown to be clinically effective for the treatment of a variety of pediatric cardiovascular disorders. Silverstein et al\(^61\) examined the impact of pretreatment with flunarizine on the development of hypoxic/ischemic brain injury in immature rats. Although this group was unable to demonstrate any neuroprotective effect from verapamil, diltiazem, nicardipine, or nifedipine, flunarizine appeared to limit morphologic injury and this effect was reported to be independent of dopamine release. When Mogilner et al\(^62\) administered nimodipine to newborn lambs, this agent significantly increased both MABP and CBF. In response to moderate hypotension that did not appear to alter cerebrovascular autoregulation, nimodipine continued to increase CBF.

The current study investigated the possible role of nimodipine in the pharmacotherapy of perinatal asphyxia. The authors' previous study\(^60\) of CBF following asphyxial insult of 15 minutes demonstrated profound decreases in flow to all cerebral regions examined. Based on the predictive value of a low 5-minute Apgar score\(^23\) and the practical time of instituting life-saving support measures,\(^60\) 5 minutes of asphyxia followed by a 5-minute resuscitation phase were chosen for this experiment. Drug (i.e., nimodipine) was administered as soon as might be practically possible. In nonsaluted pups, nimodipine caused significant decreases both in CBF to cortical and deep gray structures as well as in MABP. Hernandez et al\(^61\) and Young et al\(^62\) investigated the autoregulation of CBF in newborn beagle pups in response to alterations in MABP, and the nimodipine-induced changes in MABP in our pups are not below the reported autoregulatory range in normal pups. Thus, although the changes in CBF in the nonsaluted pups should not be directly attributable to the failure of autoregulation, it may be that nimodipine in some fashion improves CBF response to changes in MABP, as noted by Harris et al.\(^60\) In insulted pups, nimodipine similarly decreased MABP and CBF to both cortical and deep gray matter regions. Nimodipine appeared to have no effect on arterial blood gases in either insulted or nonsaluted pups. Similarly, no differences were noted in the EEGs of nimodipine-treated compared with saline-treated pups. Although nimodipine may improve the neurologic outcome in asphyxiated newborns, it appears to be by some mechanism other than improving blood flow.

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References


**KEY WORDS**

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- asphyxia
- nimodipine
- beagle pup
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