Thromboxane Synthesis Inhibitor in a Beagle Pup Model of Perinatal Asphyxia

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During perinatal asphyxia, cerebral blood flow is markedly reduced in the gray and white matter of the telencephalon. Since previous work has implicated prostaglandins in the control of blood flow, we tested the hypothesis that a thromboxane synthesis inhibitor would improve cerebral blood flow and blunt the metabolic alterations that accompany asphyxia. Forty-three newborn beagles 2–7 days old were anesthetized, ventilated, and randomized to insult (5 minutes of asphyxia) or no insult and received treatment with either the thromboxane synthesis inhibitor CGS 13080 (CIBA-GEIGY Corp.) (0.06 mg/kg/hr i.v. infusion) or saline. Cerebral blood flow was measured in 25 pups. Pups received treatment 30 minutes before insult or no insult. In pups randomized to insult and receiving saline, cerebral blood flow increased during insult in the medulla but decreased elsewhere. Pups randomized to insult and treated with thromboxane synthesis inhibitor had increased cerebral blood flow during insult in all cerebral regions studied. In addition, these pups experienced a significantly higher incidence of intraventricular hemorrhage than did pups randomized to insult and receiving saline. In other experiments with 18 pups, brain extracts were prepared for proton nuclear magnetic resonance spectral analysis of high-energy phosphorylated compounds and lactate levels. In pups exposed to insult and receiving saline, mean±SD phosphocreatine concentration fell from 1.9±0.1 to 0.4±0.1 mmol/kg, lactate concentration increased from 2.0±0.5 to 3.3±0.4 mmol/kg, and the calculated pH fell 0.8 units. There were no differences between groups. Our findings suggest that therapeutic strategies for perinatal asphyxia must extend beyond blood flow maintenance to methods that may reduce cerebral metabolic needs. We can then hope to prevent neurologic changes. (Stroke 1989;20:809–814)
6-keto-PGF\textsubscript{\textalpha}, the stable metabolite of PGI\textsubscript{2} in humans,\textsuperscript{21} and therefore may alter CBF under physiologic and/or pathologic conditions. The newborn beagle pup provides an excellent model for study of the developing nervous system.\textsuperscript{12,22} We employed the thromboxane synthesis inhibitor CGS 13080, imidazo(1,5-\textalpha)-pyridine-5-hexanoic acid (CIBA-GEIGY Corp., Summit, New Jersey), to test the hypothesis that a thromboxane synthesis inhibitor would improve CBF and thus blunt the metabolic alterations that accompany insult in the newborn beagle pup model of perinatal asphyxia.

**Materials and Methods**

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

Newborn beagle pups (24–72 hr) were rapidly anesthetized with 20 mg/kg i.p. pentobarbital. They were then tracheostomized, paralyzed with 1 mg/kg s.c. gallamine triethiodide, and ventilated with a mixture of 30% oxygen and 70% nitrous oxide for analgesia. With pups under local anesthesia with 1% lidocaine hydrochloride, bilateral femoral venous and arterial catheter were inserted by cutdown procedures. For the radioactive microsphere determination of CBF, pups had a left carotid artery line implanted by cutdown under local anesthesia. This line was advanced to the left ventricle. Systemic arterial and left ventricular pressure were continuously monitored. Body temperature was maintained at 36.5–37.5° C. Ventilatory rate and total volume were adjusted to maintain arterial normoxia (40–60 mm Hg) and normocapnia (30–40 mm Hg).

CGS 13080 was prepared daily by dissolving CGS 13080 in freshly prepared normal saline, heating to 50° C, cooling, and filtering before use. Solution was prepared to permit a 0.06-mg/kg/hr dose in a 2-ml/hr volume. We chose this dose to correspond with previously reported neonatal pig studies.\textsuperscript{23} CGS 13080 was administered via a femoral venous catheter connected to a continuous infusion pump.

Two parallel sets of experiments were performed, one set for determination of CBF using microspheres and another set for proton nuclear magnetic resonance (NMR) spectral analyses. The experimental design for each set consisted of 1) stabilization, 2) randomization to one of four groups, 3) ventilation and administration of 0.06 mg/kg/hr i.v. CGS 13080 by continuous infusion or an equal volume of saline during 30 minutes of observation, 4) insult (systemic asphyxia by the discontinuation of mechanical ventilation and simultaneous cross-clamping of the respiratory lines) or routine ventilation for 5 minutes, 5) reinstitution of ventilation for insulted pups, and 6) observation for 60 minutes.

CBF was measured at 0, 30, 35, and 95 minutes using known amounts of radioactivity as chromium-51, cerium-141, strontium-85, or scandium-46 in 15-μm microspheres (300,000–800,000 microspheres in 2.0 ml saline, New England Nuclear, Boston, Massachusetts) as previously described.\textsuperscript{24} For the NMR spectral analyses of cerebral metabolic state, the pups also underwent craniotomies before stabilization. Brains were prepared for NMR studies at 35 minutes by freezing in situ with liquid N\textsubscript{2} and storing at −60° C. Extraction procedures and NMR spectroscopy were performed as previously reported.\textsuperscript{25,26} The change in intracellular pH (∆pH\textsubscript{ic}) was calculated as ∆log (PCr/Cr),\textsuperscript{27} where CPK is creatine phosphokinase, PCr is phosphocreatine concentration, and Cr is creatine concentration. There are several sources of possible error in the NMR measurement, including variations in signal intensity and in the signal-to-noise ratio. In our studies, successive spectra obtained on a single sample were extremely reproducible, and the variation in signal intensity as reflected by the signal height revealed the variation to be <0.1%. The signal-to-noise ratio for the concentration standard 3-(trimethylsilyl)propionate (TSP) at 1.4 mmol/l was at least 500:1. Assuming that a signal three times the height of the peak-to-peak noise may be observed, the threshold of detection for a singlet such as TSP was 0.076 mmol proton nuclei/l (12 mmol 1\textsuperscript{H} nuclei/l/free induction decay). Although a number of factors (including acquisition and processing parameters, field homogeneity [shim], and receiver characteristics) can affect this figure, these sources of error would primarily affect signal with a low intensity, such as the methyl doublets of valine (1.05 ppm) and β-hydroxybutyrate (1.21 ppm). In addition to the factors intrinsic to the signal (e.g., three proton nuclei split into a doublet), the threshold value was strongly influenced by dilution, the ratio of the amount of buffered D\textsubscript{2}O used to redissolve the lyophilized extract to the initial weight of the brain extracted. These considerations yield a mean threshold of detection of 0.04 mmol/kg frozen weight of sample for a doublet. Dividing the threshold of detection concentration by 3 yields an estimate of the error based on signal-to-noise ratio considerations.

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

**Results**

CBF was determined in 25 pups. Eight were randomized to treatment with insult, seven to treatment without insult, five to saline with insult, and five to saline without insult. At 30 minutes, mean arterial blood pressure (MABP) was 71.2±3.6, 71.6±5.9, 74.0±6.2, and 75.0±3.2 mm Hg, respectively; no significant difference among groups was noted. MABP of both groups without insult remained stable during the experiment. In contrast, MABP of both groups with insult decreased significantly during insult (to 36.2±3.1 and 35.0±3.2 mm Hg for the treatment and the saline groups, respectively) followed by rapid improvement to baseline (t=0) val-
ues after re instituted of ventilatory support. MABP of both groups then remained stable. Three-way ANOVA demonstrated a significant effect of time (p<0.001) and a significant insult×time interaction (p<0.001) but no effects of treatment or insult. No other interactions were significant.

Before randomization, PO₂, PCO₂, and pH in the treatment with insult, treatment without insult, saline with insult, and saline without insult groups were 105.6±9.9, 100.7±13.1, 94.2±15.2, and 91.0±14.2 mm Hg; 34.0±1.2, 33.7±1.1, 33.8±1.5, and 32.9±0.7 mm Hg; and 7.35±0.01, 7.37±0.02, 7.29±0.03, and 7.33±0.03, respectively. There were no differences among groups in any parameter. In response to discontinuation of ventilatory support, both groups with insult developed significant hypoxemia (12.0±1.0 and 14.3±2.9 mm Hg for the treated and the saline groups, respectively), hypercarbia (66.2±2.0 and 6014±3.8 mm Hg, respectively), and acidosis (7.09±0.01 and 7.02±0.05, respectively). Ventilatory support was re instituted at preinsult ventilator settings, and 60 minutes later (at 95 minutes) PO₂ and PCO₂ values had returned to preinsult levels although both groups with insult remained acidotic. ANOVA for PO₂, PCO₂, and pH demonstrated significant (p<0.001) effects of time and significant (p<0.001) insult×time interactions but no effect of treatment vs. saline.

CBF values are found in Table 1. At 0 minutes, there were no differences among groups. CBF in brainstem structures and the thalamus was increased significantly at 35 minutes and that in the hemispheric gray and white matter was significantly decreased in the saline with insult group. CBF values for all regions studied had returned to baseline or somewhat below baseline by 95 minutes. CBF in brainstem structures and the thalamus was significantly increased at 35 minutes in the treated with insult group. CBF was, however, significantly higher than that for the saline with insult group, except for the midbrain. In addition, CBF in the hemispheric gray and white matter group, in contrast to the saline with insult group, in which CBF decreased in response to insult.
No pup in the treated without insult group had a germinal matrix hemorrhage or an intraventricular hemorrhage. One pup in the saline without insult group had a germinal matrix hemorrhage. Five of eight pups in the treated with insult group had neuropathologic evidence of a germinal matrix hemorrhage and/or an intraventricular hemorrhage, compared with one of seven in the saline with insult group (p<0.05).

NMR spectroscopic analysis of high-energy cerebral metabolites were performed on 18 pups; five were randomized to treatment with insult, four to treatment without insult, four to saline with insult, and five to saline without insult. MABP and arterial blood gas and values were similar to those for the CBF set of experiments. Three-way ANOVA demonstrated similar changes for these parameters.

Figure 1 shows a representative spectrum of a perchloric acid extract of neonatal dog cerebrum frozen in situ during normoxic, normocarbic conditions. Signals from a wide variety of metabolites have been identified. Quantitative analysis was performed on the 13 metabolites listed in Figure 1.

Table 2 lists the metabolite concentrations observed by NMR spectra expressed in mmol/kg wet tissue wt. Although an effect of insult was seen for lactate (p<0.01), creatine (p<0.001), phosphocreatine (p<0.001), inositol (p<0.001), and calculated pH (p<0.001), there were no other differences between the groups with insult by ANOVA. Unpaired t tests revealed a significant difference between the glutamine values at 35 minutes (p<0.05).

Discussion

The cerebrovascular role of endogenously synthesized PGs has yet to be fully explained, and these compounds may be of most importance in the injured brain. Studies of neonatal beagles and guinea pigs have demonstrated the release of PGE₂ during asphyxia, and the same prostanoid has been shown to increase CBF and produce intraventricular hemorrhage in newborn beagles. Similarly, several studies suggest that cyclooxygenase inhibitors decrease PG-mediated CBF in neonatal animals. Leffler et al reported increased cortical subarachnoid fluid concentrations of 6-keto-PGF₁α, thromboxane B₂, and PGE₂ in piglets exposed to a model for perinatal asphyxia. Systemic administration of indomethacin decreased these findings. Similarly, using indomethacin blockade of PG synthesis, Wagerle et al demonstrated that metabolites of the cyclooxygenase pathway may play a role in the regulation of CBF in newborn piglets during hypoxia and hypercapnia.

Intraventricular hemorrhage, or hemorrhage into the germinal matrix tissues of the developing brain with extension into the ventricular system and cerebral parenchyma, occurs in 40–50% of infants of ≤34 weeks’ gestational age. Infants with intraventricular hemorrhage are at increased risk for neonatal seizures, hydrocephalus, and death as well as subsequent neurodevelopmental handicap. Intraventricular hemorrhage is believed secondary to CBF changes. Rennie et al has demonstrated elevated levels of PGI₂ in newborn preterm infants who subsequently develop intraventricular hemorrhage, and studies in both experimental beagle pups and preterm infants have demonstrated that indomethacin prevents neonatal intraventricular cerebral hemorrhage and decreases this vasodilating prostaglandin.

Thromboxane synthesis inhibitors such as CGS 13080 have been reported to prevent sudden coronary death in experimental rabbits and to improve septic pulmonary hypertension in newborn piglets. Such agents have recently been tested in humans, and in addition to inhibiting the production of

![Figure 1. Representative proton nuclear magnetic resonance spectrum of perchloric acid extract of neonatal beagle cerebrum frozen in situ under normoxic, normocarbic conditions. Labeled signals indicate chemical shifts in parts per million (ppm) of some resonances of metabolites listed in Table II. Spectra of extracts were obtained on Bruker AM 500 spectrometer at 500 MHz at 25°C using 30° pulse width (4 μsec) and 6-second repetition rate. Metabolite concentrations were measured by comparing intensity of identified resonances to intensity of 3-(trimethylsilyl)propionate methyl resonance. ATP, adenosine 5'-triphosphate; GLC, glucose; NAA, N-acetyl aspartate; LAC, lactate; INO, inositol; PCr, phosphocreatine; CR, creatine; TAU, taurine; GB, γ-aminobutyric acid; ASP, aspartate; GLN, glutamine; GLT, glutamate; and ALN, alanine.](http://stroke.ahajournals.org/lookup/figure/78764)
of thromboxane A₂, they also lead to increased synthesis of PGI₂ and PGE₂. Studies of the thromboxane synthesis inhibitor UK 38,485 (dazmagrel) revealed that, although producing the expected changes in PG levels, dazmagrel failed to improve cerebral perfusion in adult dogs and cats exposed to models of cerebral ischemia.

We investigated the influence of the thromboxane synthesis inhibitor CGS 13080 on CBF and metabolism in newborn beagle pups exposed to perinatal asphyxia. Based on the predictive value of a low 5-minute Apgar score and the practical time of instituting life-saving support measures, 5 minutes of asphyxia was chosen for these experiments. For saline-treated pups, insult produced marked increases in CBF to the medulla, midbrain, and pons; CBF fell in cortical gray structures. In contrast to saline-treated pups, pretreatment with CGS 13080 increased CBF in cortical gray and white matter structures and significantly increased CBF in brainstem structures.

Of interest, there was no significant difference in the severity of intracellular acidosis between the groups with insult despite improved CBF in the treated group. For both groups, ΔpH CK of −0.8 during asphyxia suggested significant acidosis in excess of the change predicted by PaCO₂ and cerebral lactate concentration.

Although the neurochemical changes observed between the groups receiving saline were similar to those reported in adult rabbits, our data indicated that asphyxia was better tolerated by neonatal pups. The amino acid concentrations determined in our study were in agreement with those reported in the neonatal dog. Concentrations of alanine, glutamate, and glutamine increased minimally, indicating small perturbations of cytosolic redox state and ammonia metabolism. ATP concentrations were unchanged. The marked drop in concentrations of phosphocreatine, produced by acidosis, suggested a significant increase in energy utilization since the rate of ATP hydrolysis is a major contributor to intracellular acid production. Changes in glucose, lactate, and phosphocreatine concentrations observed between the treated groups were similar to those measured in the saline with insult group. Little change in concentration was seen for alanine, glutamate, and glutamine. Thus, the effect of this thromboxane synthesis inhibitor on the neurochemical changes accompanying asphyxia were rather modest; no obvious benefit could be demonstrated.

Finally, our experiments demonstrated a high incidence of germinal matrix/intraventricular hemorrhage in the treated pups with insult. This lesion is associated with marked increases in CBF in newborn beagle pups, and the elevations in CBF associated with the treatment and asphyxial insult most likely produced this lesion. Treated pups without insult did not experience a high rate of hemorrhage. This is consistent with the finding that the CGS 13080 did not increase CBF in this group.

Our data demonstrate that CGS 13080 increases CBF during an insult designed to mimic perinatal asphyxia to regions of the developing brain known to be at risk for ischemic damage. However, maintenance of CBF did not prevent those cerebral metabolic changes believed to lead to neurologic changes. Indeed, the exuberant perfusion of damaged tissue that CGS 13080 produced may have led to germinal matrix/intraventricular hemorrhage in this model of developing brain.

Acknowledgments

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### Table 2. Metabolite Concentrations in Brains of Newborn Beagle Pups by Nuclear Magnetic Resonance

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CGS 13080 Without insult</th>
<th>CGS 13080 With insult</th>
<th>Saline Without insult</th>
<th>Saline With insult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>2.0±0.5</td>
<td>3.3±0.4*</td>
<td>1.2±0.2</td>
<td>3.4±0.6*</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.9±0.5</td>
<td>2.1±0.2</td>
<td>2.1±0.3</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>Total creatine</td>
<td>4.1±0.3</td>
<td>3.6±0.2</td>
<td>3.5±0.2</td>
<td>3.8±0.2</td>
</tr>
<tr>
<td>Creatine</td>
<td>2.2±0.1</td>
<td>3.2±0.2*</td>
<td>1.9±0.1</td>
<td>3.2±0.3*</td>
</tr>
<tr>
<td>Phosphocreatine</td>
<td>1.9±0.1</td>
<td>0.4±0.1*</td>
<td>1.6±0.1</td>
<td>0.5±0.2*</td>
</tr>
<tr>
<td>ATP</td>
<td>1.3±0.1</td>
<td>0.8±0.1</td>
<td>0.9±0.2</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>N-acetyl aspartate</td>
<td>1.4±0.1</td>
<td>1.6±0.1</td>
<td>1.6±0.2</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>Glutamate</td>
<td>3.4±0.4</td>
<td>3.4±0.2</td>
<td>3.5±0.2</td>
<td>3.9±0.2</td>
</tr>
<tr>
<td>Aspartate</td>
<td>1.1±0.1</td>
<td>1.1±0.1</td>
<td>1.1±0.1</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>γ-Aminobutyric acid</td>
<td>1.0±0.1</td>
<td>1.0±0.1</td>
<td>0.9±0.1</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Taurine</td>
<td>3.3±0.1</td>
<td>2.1±0.5</td>
<td>3.4±0.6</td>
<td>2.4±0.9</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.52±0.07</td>
<td>0.4±0.02</td>
<td>0.34±0.04</td>
<td>0.40±0.01</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1.8±0.3</td>
<td>1.2±0.1†</td>
<td>1.6±0.2</td>
<td>1.8±0.2†</td>
</tr>
<tr>
<td>Inositol</td>
<td>5.2±0.5</td>
<td>3.9±0.2*</td>
<td>3.9±0.2</td>
<td>4.2±0.2</td>
</tr>
<tr>
<td>ΔpHCK</td>
<td>0</td>
<td>−0.8±0.1*</td>
<td>0</td>
<td>−0.8±0.1*</td>
</tr>
</tbody>
</table>

Data are mean±SEM mmol/kg except for ΔpH CK.

* p<0.05 different from without insult.
† p<0.05 different from saline.
Fisk and the secretarial assistance of Mrs. Marie Campbell.

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KEY WORDS • asphyxia • cerebral blood flow • dogs
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