The Effect of Diazepam on Neonatal Seizure: *In Vivo* ^{31}P and ^1H NMR Study¹

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ABSTRACT. It is assumed that when anticonvulsants arrest seizure, there is rapid return of brain high energy phosphates and brain lactate to control values. To test this hypothesis, diazepam was administered to neonatal dogs during flurothyl-induced seizure. *In vivo* ^{31}P nuclear magnetic resonance spectroscopy disclosed that diazepam quickly arrested electrographic seizure and restored brain phosphocreatine and inorganic phosphate to baseline values. In contrast, *in vivo* ^1H nuclear magnetic resonance spectroscopic measurements showed that arrest of seizure with diazepam did not return brain lactate to control values. The sustained increase in cerebral blood flow and prolonged elevation of brain lactate, acetate, valine, and succinate in the postictal period indicate that metabolic recovery of the brain occurs over an extended period of time after the normalization of EEG, phosphocreatine, and brain pH. (*Pediatr Res* 25:27-31, 1989)

Abbreviations

NMR, nuclear magnetic resonance
PCr, phosphocreatine
pH_i, intracellular pH
CBF, cerebral blood flow

Diazepam is widely used in the treatment of status epilepticus because it rapidly arrests electrographic seizure. Nonetheless, the potential beneficial effects of diazepam on brain metabolism during the postictal period have not been fully defined in the neonate. Previous studies of postictal brain metabolism in the adult experimental animal (1, 2) necessitated multiple groups of animals at different time points (1-4). Moreover, brain pH was measured by indirect methods or by invasive techniques which themselves could perturb brain metabolism.

The goal of these experiments was to use NMR spectroscopy to assess rate of normalization of brain metabolism after treatment of neonatal status epilepticus with diazepam. Seizure was induced with flurothyl (bistrifluorethyl ether; Flura Corp., Newport, TN), a convulsant gas which causes seizure by opening of sodium channels in the cell membrane (2). Previous studies have shown that flurothyl seizure retards brain growth in the neonatal rat (5) and produces neuronal necrosis in the adult rat (4). In

this study, four types of investigations were performed: *in vivo* ^{31}P NMR to follow brain high energy phosphate metabolism and brain pH; *in vivo* ^1H NMR to measure brain lactate; *in vitro* ^1H NMR spectroscopy to determine concentrations of brain excitatory and inhibitory amino acids; and iodoantipyrine autoradiography to assess regional cerebral blood flow.

MATERIALS AND METHODS

Animal preparation. The method of preparation has been recently described (6). Mongrel dogs (4-12 days old) were anesthetized with halothane, 1-4%, while undergoing tracheotomy and femoral arterial catheterization. Pancuronium bromide (0.5 ml subcutaneously) was then administered, halothane was withdrawn, and the animals were mechanically ventilated (PaO₂, 80-120 mm Hg; PCO₂, 30-40 mm Hg). Muscle paralysis was continued throughout the experiment. To minimize discomfort, animals were ventilated with 30% O₂ and 70% N₂O (1, 2), and topical anesthetic (lidocaine jelly, 1%) was applied to all incision sites. Anesthesia during the ictus was produced by the seizure itself which results in loss of awareness. The EEG, blood pressure, blood glucose, and blood lactate were monitored during the experiment (6). Experiments were approved by the Yale University Animal Care Committee and carried out with the adherence to "Guiding Principles for the Care of Animals," of the American Physiological Society, and in accordance with federal regulations.

Animals were randomly assigned either to a continuous seizure or to a seizure plus diazepam group. Animals in the continuous seizure group were subjected to 60 min of seizure which was induced by continuously vaporizing flurothyl (0.13 ml/min) within the ventilator tubing. Flurothyl was similarly administered to those animals randomized to the seizure plus diazepam group, but diazepam (0.67-1.0 mg/kg intravenously) was given 15 min after onset of seizure. An additional dose of diazepam was administered 15 min later (30 min after seizure onset) to assure suppression of electrographic seizure.

***In vivo* ^{31}P NMR studies.** *In vivo* ^{31}P data were collected using a 2.0 Tesla, 31-cm bore diameter superconducting magnet (General Electric CSI II, (CSI II; General Electric, Fremont, CA) and a 2-cm circular transmitter-receiver surface coil tuned for phosphorus (34.5 MHz, 512 acquisitions, repetition rate, 1.0 s). Reflection of cranial muscle was not done because previous imaging studies (6) show that most of the signal is derived from the relatively large brain of the neonatal dog. Moreover, the PCr/P_i ratio in the control state was near unity, indicating minimal contamination from surrounding muscle (7). Peak areas were not corrected for differential saturation because quantitative measurement of individual resonances was not attempted.

Sets of ^{31}P NMR spectra were acquired every 15 min throughout the experiment. The curve-fitting software "GEMCAP" (Nicolet Computer Graphics Div., Martinez, CA) was used to deter-

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mine areas of individual resonances where peak overlap occurred. Brain pH_i was determined from experimental spectra by noting the chemical shift of the P_i peak with respect to that of PCr, as previously described (8).

In vivo ¹H NMR studies. *In vivo* ¹H NMR spectra were obtained with a ¹³³T spin echo sequence (9) using the same spectrometer (¹H frequency, 85.6 MHz; 32 scans). A separate group of animals and a dedicated proton coil (General Electric, 2-cm circular) were used to maximize signal to noise and improve temporal resolution. Brain lactate was quantitated by killing the animal in

the spectrometer at the end of the experiment, acquiring a spectrum 20 min postmortem, and correlating the intensity of the lactate signal to the enzymatically derived lactate concentration in brain (9).

In vitro ¹H NMR studies. *In vitro* ¹H NMR investigations were performed in control animals, continuously seizing animals, and animals whose seizures were arrested with diazepam. Brains of these animals were frozen *in situ* with liquid nitrogen (10) and later dissected and extracted with HClO₄. Extracts were analyzed for metabolite concentrations by conventional spectrophotometric analysis (11) and with high resolution ¹H NMR spectroscopy (500 MHz Bruker WM-500 spectrometer; NMR parameters: 25°C, 30° pulse, 6-s repetition time, 160 scans). Resonances were assigned relative to sodium 3-trimethyl silyl propionate (6).

Regional CBF. Regional CBF was measured by quantitative autoradiography ([¹⁴C] iodoantipyrine) (12) in a parallel group of animals subjected to the same experimental conditions. CBF was determined at the end of the 60-min period of experimental observation.

Statistical analyses. The need for a control group is obviated in *in vivo* NMR studies because animals serve as their own controls. Sequential intragroup data obtained with *in vivo* NMR were therefore analyzed with repeated measures analysis of variance ANOVA and the Newman-Keuls *post hoc* test. Because the *in vitro* NMR and CBF studies necessitated multiple groups, statistically significant ($p < 0.05$) differences were analyzed with randomized ANOVA. All values are mean $\pm$ SE.

Fig. 1. Systemic changes during flurothyl seizure. Blood glucose rises to similar degree in animals subjected to continuous flurothyl seizure (right) and those treated with diazepam (left). However, administration of diazepam is associated with decrease in lactate. A similar degree of metabolic acidosis occurred in both groups of animals. Data from a total of 12 animals (continuous flurothyl seizure, seven; flurothyl plus diazepam, five) are depicted.

RESULTS

Systemic changes. Onset of seizure resulted in systemic arterial hypertension, whereas administration of diazepam caused a slight reduction in blood pressure (control, 93 ± 6 mm Hg; flurothyl, 108 ± 8 ; flurothyl and diazepam, 81 ± 3 ; $p = 0.012$). Flurothyl seizure caused significant increase in blood glucose, which was most prominent early in the course of seizure (Fig. 1). Blood lactate increased in response to flurothyl seizure, but then plateaued once seizure was treated with diazepam. Reduc-

Fig. 2. ³¹P NMR spectra and EEG during flurothyl seizure and after treatment with diazepam. The ratio of PCr to inorganic phosphate (dashed line between PCr and P_i peaks) abruptly decreases with onset of seizure and then gradually recovers. Note the rapid termination of electrographic seizure after treatment with diazepam. Abbreviations: PM, phosphomonoesters; PD, phosphodiester; γ , α , and β are resonances of ATP.

tion in arterial pH was similar in both the continuous flurothyl and the diazepam-treated animals.

Cerebral physiologic changes. The EEG (Fig. 2) showed low amplitude (1–2 μ V), 10–11 Hz activity as the dominant rhythm during the control period (Fig. 2). Flurothyl produced sharp waves, spikes, and spike/slow wave discharges (25–50 μ V, 1–2/s) whose amplitude and frequency gradually decreased approximately 10 min postadministration. Subsequent doses of flurothyl caused the discharges to return to the initial amplitude and frequency. Administration of diazepam promptly arrested electrographic seizure. The EEG reverted to low amplitude activ-

ity. Thereafter, further administration of flurothyl failed to induce paroxysmal activity. CBF increased significantly during flurothyl seizure (Table 1). CBF values in the diazepam-treated animals were intermediate between those of the control animals and the continuous flurothyl animals.

In vivo ³¹P NMR. Serial ³¹P NMR spectra from the continuous flurothyl group disclosed significant fall in PCr (0 min, $8.4 \pm 1.1\%$ total mobile phosphates; 15 min, $5.8 \pm 0.6\%$, $p < 0.05$; 30 min, 6.0 ± 0.9 , $p < 0.01$; 45 min, 5.1 ± 1 , $p < 0.01$; 60 min, 5.4 ± 1.2 , $p < 0.01$) (Fig. 3) and significant increase in levels of P_i (0 min, $4.3 \pm 1\%$ total mobile phosphates; 15 min, 7.6 ± 1.5 ; 30 min, 9.8 ± 2.5 ; 45 min, 8.7 ± 1.6 ; 60 min, 8.6 ± 2.0 ($p < 0.05$ for all values versus 0 min).

Table 1. CBF (ml/100 g/min) during flurothyl seizure and after treatment with diazepam*

Brain region	Control (n = 6)	Flurothyl (n = 4)	Flurothyl-diazepam (n = 5)
Frontal cortex	31 ± 2	$96 \pm 18^\dagger$	64 ± 14
Parietal cortex	34 ± 3	$79 \pm 16^\ddagger$	63 ± 14
Temporal cortex	29 ± 2	$109 \pm 25^\dagger$	$58 \pm 13^\S$
Occipital cortex	30 ± 4	$101 \pm 36^\ddagger$	63 ± 15
Caudate	31 ± 5	$86 \pm 22^\ddagger$	64 ± 11
Hippocampus	32 ± 5	$94 \pm 22^\dagger$	$50 \pm 10^\S$
Thalamus	39 ± 7	$125 \pm 25^\dagger$	$69 \pm 14^\S$
Hypothalamus	34 ± 8	$138 \pm 26^\dagger$	$65 \pm 18^\parallel$
Cerebellum	32 ± 8	73 ± 23	54 ± 13
Corpora quadrigemina	39 ± 8	$139 \pm 51^\ddagger$	75 ± 18
Pons	46 ± 9	$119 \pm 22^\dagger$	$87 \pm 10^\ddagger$
Medulla	55 ± 13	$124 \pm 22^\ddagger$	84 ± 11
Spinal cord	40 ± 6	$102 \pm 16^\dagger$	$75 \pm 12^\ddagger$
Corpus callosum	15 ± 3	40 ± 23	32 ± 11
Periventricular white	7 ± 3	$27 \pm 8^\ddagger$	16 ± 5

* Values are mean $\pm$ SE.

$^\dagger p < 0.01$ versus control.

$^\ddagger p < 0.05$ versus control.

$^\S p < 0.05$ versus flurothyl.

$^\parallel p < 0.01$ versus flurothyl.

Fig. 4. Brain and blood lactate during flurothyl seizure, after treatment with diazepam, and postmortem. Lactate rises rapidly during flurothyl seizure with peak value of 7 mmol/kg. Clearance of lactate occurs after treatment with diazepam but nonetheless has not reached baseline 45 min after the end of seizure. Data represent mean of four animals.

Fig. 3. High energy phosphates and brain pH during flurothyl seizure and after treatment with diazepam. PCr declines and then plateaus during continuous flurothyl seizure (left). There is little change in ATP. Administration of diazepam promptly restores PCr levels to their original values (right). pH_i also begins to rise after treatment with diazepam. Data (mean) from a total of 12 animals (continuous flurothyl seizure, seven; flurothyl plus diazepam, five) are shown.

Table 2. ^1H *in vitro* NMR measurement of brain metabolites*

Metabolite	Controls (n = 6)	Flurothyl (n = 11)	Flurothyl- diazepam (n = 7)
Blood glucose	8.6 $\pm$ 0.8	15.0 $\pm$ 1.7	13.2 $\pm$ 0.7
Blood lactate	1.7 $\pm$ 0.3	3.8 $\pm$ 0.3	1.8 $\pm$ 0.1
Brain glucose	2.6 $\pm$ 0.4	3.3 $\pm$ 0.5	5.3 $\pm$ 0.5
Brain lactate	1.75 $\pm$ 0.17	5.14 $\pm$ 0.55†	1.68 $\pm$ 0.23‡
Creatine	2.9 $\pm$ 0.2	3.2 $\pm$ 0.2	3.2 $\pm$ 0.2
PCr	2.2 $\pm$ 0.1	2.1 $\pm$ 0.1	2.1 $\pm$ 0.2
ATP	1.6 $\pm$ 0.1	1.7 $\pm$ 0.2	1.6 $\pm$ 0.2
Alanine	0.51 $\pm$ 0.05	1.07 $\pm$ 0.08†	0.49 $\pm$ 0.07‡
Aspartate	2.0 $\pm$ 0.2	1.5 $\pm$ 0.1	1.3 $\pm$ 0.1§
N-acetyl aspartate	2.2 $\pm$ 0.1	2.6 $\pm$ 0.2	2.7 $\pm$ 0.2
γ -aminobutyric acid	1.5 $\pm$ 0.1	1.6 $\pm$ 0.1	1.4 $\pm$ 0.1
Glutamate	5.9 $\pm$ 0.5	5.4 $\pm$ 0.4	5.2 $\pm$ 0.4
Glycine	1.8 $\pm$ 0.1	2.3 $\pm$ 0.2	3.3 $\pm$ 0.2†‡
Taurine	7.1 $\pm$ 0.4	8.0 $\pm$ 0.4	7.5 $\pm$ 0.7
Valine	0.06 $\pm$ 0.02	0.16 $\pm$ 0.03†	0.14 $\pm$ 0.02†
Acetate	0.35 $\pm$ 0.02	0.22 $\pm$ 0.01†	0.19 $\pm$ 0.01†
Inositol	8.5 $\pm$ 0.7	7.7 $\pm$ 0.4	7.7 $\pm$ 0.5
Succinate	0.22 $\pm$ 0.02	0.18 $\pm$ 0.01	0.36 $\pm$ 0.04†‡
Propylene glycol	ND	ND	0.67 $\pm$ 0.26

* Values are mean $\pm$ SE; ND = not detected; values adjusted to normalize total creatine.

† $p < 0.01$ versus control.

‡ $p < 0.01$ flurothyl versus flurothyl-diazepam.

§ $p < 0.05$ versus control.

|| $p < 0.05$ flurothyl versus flurothyl-diazepam.

Changes in high energy phosphates were similar in the flurothyl-diazepam group, with significant decline in PCr during the ictus. There was rapid restitution of PCr after treatment with diazepam. Intracellular pH declined during flurothyl seizure (control, 7.23; flurothyl, 6.98; $p = 0.041$) and then returned to control levels 30 min after administration of diazepam (15 min, 6.92; 30 min, 7.12).

In vivo ^1H NMR. *In vivo* ^1H NMR studies showed that the rise in cerebral lactate occurred soon after the onset of flurothyl seizure (Fig. 4). Diazepam caused marked decline in brain lactate, but it still had not returned to control values 45 min later. At all times, blood lactate was greater than brain lactate. Brain lactate fell more rapidly after seizure than did blood lactate.

In vitro ^1H NMR. *In vitro* ^1H NMR spectroscopic analyses revealed that acetate and aspartate declined during seizure and further decreased during diazepam-induced recovery (Table 2). The changes in alanine and taurine concentrations mirrored those of cerebral and blood lactate, *viz.* increase during seizure and decrease during recovery. Brain glucose and valine concentrations increased during both the seizure and the recovery. Succinate increased during the recovery period. The appearance of the vehicle, propylene glycol, marked the entry of diazepam into the brain. Brain lactate reflected blood lactate concentrations in the control group and diazepam-treated group. The brain: blood glucose ratio declined during flurothyl seizure, suggesting increased glucose utilization (control, 0.3; flurothyl, 0.22). After treatment with diazepam, the brain: blood glucose ratio rose (diazepam, 0.4).

DISCUSSION

Previous studies addressing the postictal brain metabolism were hampered by the inability to determine simultaneously brain pH and brain energy state (1). Indirect methods of measuring brain pH sometimes yielded conflicting results (13), while direct measurement with intracerebral electrodes traumatized the brain. In the present study, *in vivo* ^1H NMR spectroscopy permitted the simultaneous determination of blood and tissue lactate concentrations and led to several noteworthy findings.

First, there was a disparity between brain pH and brain lactate levels. Despite persistently elevated brain lactate levels after seizure, brain pH returned to normal. The phenomenon of nonacidotic elevation of brain lactate has also been documented with *in vivo* NMR in the rabbit (14).

A second conclusion is that the clearance of lactate was incomplete 45 min postictally. After an initial decline in the postictal period, lactate plateaued in the brain. This finding is juxtaposed to that in the adult rat in whom lactate returns to control values 45 min after seizure (2). A third finding was widening of the lactate gradient between blood and brain during diazepam-induced recovery. Initially, the cerebral and blood lactate decreased in parallel, but subsequently diverged, suggesting postictal systemic lactate production (14). Lactate flowing down the widened blood-brain gradient could be entering the brain as rapidly as the brain was able to metabolize it. Net cerebral uptake of lactate also occurs in neonatal hypoxia-ischemia when plasma levels exceed those of brain tissue (15).

Several hypotheses have been advanced to explain persistent lactate elevation under aerobic conditions. Lactate enters the neonatal brain and can serve as metabolizable fuel when glucose becomes relatively limited (6, 16, 17). Lactate may also be produced under aerobic conditions to share a carbon source for oxidation in tissues such as heart and brain that have large and critical energy requirements (18). Persistent increased sympathetic nervous system activity such as seizure may cause normoxic lactic acidemia (19). Brain lactate also remains elevated after brief electroshock seizure in the neonatal dog (20). Non-hypoxic lactate elevation during seizure may improve CBF and reduce excitability (15).

This study also demonstrates *in vivo* that PCr levels recovered within 15 min once seizure was arrested. The recovery of PCr preceded the recovery of pH_i. Assuming creatine kinase is at equilibrium, this implies that ADP decreased during this interval. This study corroborates previous studies in the adult rat (4), rabbit (21), and neonatal dog (6), which show only modest decrease (10–20% of control values) of brain ATP during seizure. Even in "hypermetabolic" structures such as substantia nigra, there is only slight decline in brain ATP levels and brain energy charge (25 and 10%, respectively) (4).

The ^1H *in vitro* NMR data similarly showed that restitution of other important amino acids and metabolites is incomplete by 45 min after seizure. Alanine returned toward normal in the diazepam-treated animals, but aspartate and acetate were reduced. The increase in succinate observed during recovery may be explained by the catabolism of γ -aminobutyric acid via the succinic semialdehyde pathway. Decrease in acetate may reflect increased acetylation of compounds such as N-acetyl aspartate, N-acetyl glutamate, acetylcholine, and others. N-acetyl aspartate may serve as a shuttle ferrying acetyl groups across the mitochondrial membrane (22). The exact pattern of change in putative amino acid neurotransmitters including aspartate, glutamate, γ -aminobutyric acid, glycine, and taurine during seizure activity may vary with the type of convulsant agent, duration of seizure activity, species of experimental animal, and region of brain assayed (2, 3, 13, 23–25).

The CBF studies showed that flurothyl seizure increased blood flow to a degree comparable to that seen in bicuculline-induced seizure (8). Moreover, there was persistent elevation of cerebral perfusion after seizure had been arrested. This was an unexpected finding because diazepam, particularly in combination with nitrous oxide (26), reduces CBF by as much as 60% of control values.

Would flurothyl seizure produce neuronal necrosis in the neonatal animal as it does in the adult rat (27)? Preliminary studies in this laboratory at light microscopic level showed no evidence of neuronal necrosis after 3 h of bicuculline seizure in the neonatal dog (R. S. K. Young, unpublished observations). Differences in receptor development in the developing animal may preclude neuronal necrosis (28). Further experiments are planned to explore these differences.

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