Reduction of Neurologic Deficit by 1,3-Butanediol Induced Ketosis in Levine Rats

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SUMMARY The objective of this study was to determine if 1,3-butanediol would reduce a neurologic deficit in rats exposed to ischemic-hypoxia (Levine rats). Age and weight matched male Sprague-Dawley rats were anesthetized with 2% halothane. The right common carotid and external jugular vein were ligated and cannulated and EEG screws were implanted followed by a 2 hour recovery period. Thirty minutes prior to exposure the rats received either 1,3-butanediol (47 mmole/kg i.v.; n = 11) or an equal volume of saline (n = 10). The rats were then exposed to 4.5% O2 until mean arterial blood pressure fell to 70 mm Hg. The oxygen level was then increased to 8% for 30 minutes, after which the rats were returned to room air.

Posture, hemiparesis, circling, shuffling, activity, and ability to hang on to a vertical screen were scored 1 (normal) to 5 (severe deficit) at 2 and 20 hours after insult. The time to 70 mm Hg was extended from 7.9 ± 0.9 min for saline treated rats to 19.0 ± 2.3 min for the 1,3-butanediol treated rats (p < 0.001). All eleven 1,3-butanediol treated rats survived the hypoxic insult; 90% (9/10) saline treated rats died. In an attempt to reduce the insult, six additional saline treated rats were switched to 8% O2 at 75 mm Hg and still 4/6 died.

The mean score at 20 hours for three surviving saline treated rats was 3.4. A significantly better (p < 0.002) mean 20 hour score for the surviving 8/11 1,3-butanediol treated rats was 1.2. 1,3-butanediol increases survival and decreases the neurologic deficits associated with this ischemic-hypoxic insult.

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INTERVENTIONS IN CEREBRAL ISCHEMIA produce clinical benefit by reducing mortality and/or by lessening neurologic sequelae of a cerebral ischemic event. The mortality associated with spontaneous cerebrovascular events is well established.1,2 Equally well established, but less widely appreciated, is the magnitude of the residual disability that accompanies cerebral ischemia.3,4 This differs from ischemic events in other vascular beds in that the morbidity that accompanies myocardial infarction or peripheral vascular occlusive disease, while potentially disabling, pales in comparison to the morbidity of a cerebral infarction. Blindness, paralysis, aphasia, and loss of higher cortical function make cerebral infarctions more visibly disabling than comparable tissue insults in other vascular beds.

Previous studies from this laboratory have demonstrated enhanced survival following 1,3-butanediol administration in two animal models: mice exposed to a
hypoxic environment and rats heavily instrumented for a modified Levine (ischemic hypoxia) preparation. We have proposed that the increased survival in those studies was accomplished through a primary cerebral protective effect mediated by brain ketone metabolism. This general mechanistic hypothesis was supported by parallel studies of brain tissue metabolites and in vitro rat brain slices that indicated preferential brain utilization of ketones during hypoxia.

Of necessity these previous studies were terminal protocols. In this study, however, we used a sublethal, but significant, ischemic-hypoxic insult that produces a functional neurologic deficit. The specific aim was to assess the efficacy of 1,3-butanediol in lessening ischemic-hypoxic brain damage. Functional neurologic deficits as a measure of brain damage and protection is of particular clinical relevance because the ultimate test of a cerebral protective agent will be its ability to preserve cerebral function. In addition to obvious clinical relevance, such a model also allows consideration of mechanisms not possible with terminal protocols. In this study demonstration of an attenuation of the anticipated neurologic deficit would further support our hypothesis that 1,3-butanediol is an effective cerebral protective agent.

Materials and Methods

Adult male Sprague-Dawley rats, matched for age and weight, were used. Halothane (2%) anesthesia (chamber induction, mask maintenance) was used for the surgical preparation. Right carotid arterial and right jugular venous cannulations were accomplished using saline-flushed polyethylene (PE 50) tubing. The catheters were inserted 2-2.5 cm, secured to the cervical musculature, and passed through a subcutaneous tunnel to the nape of the neck.

Five electroencephalographic leads were placed after first shaving and incising the skin over the dorsal skull. The overlying connective tissue was cleared and two small burr holes were drilled 2 mm cephalad to the anterior suture and 2 mm lateral to the sagittal suture, and one drilled 3 mm cephalad to the posterior suture and 2 mm to the right of the sagittal suture. Self-tapping $\frac{3}{16}$ inch stainless steel screws, silver-soldered to 30 gauge multistranded stainless steel wire, were inserted into the burr holes. These EEG screws were isolated from surrounding tissues with dental acrylic. The wires were then passed through a subcutaneous tunnel to exit at the nape of the neck adjacent to the vascular cannulae. The incisions were closed with wound clips and a two-hour recovery period was allowed. Total time under halothane anesthesia was recorded.

Experimental Environment

The experimental environment was a cylindrical clear plastic chamber 30 cm long and 10 cm in diameter with large rubber stoppers at each end. The chamber was connected by polyethylene tubing to a source of premixed gas containing nitrogen and either 20.9% O$_2$ (room air), 4.5% O$_2$ or 8.0% O$_2$. There were two exhaust ports in the posterior stopper through which EEG leads, vascular cannulae, and the rat tail were passed. The latter was secured with tape. The rat’s body temperature was monitored with a rectal probe. The chamber environment was continuously monitored using an oxygen/temperature analyzer (YSI 2600) and chamber temperature was maintained at 30°C with a heat lamp. The vascular cannulae and EEG leads were connected by appropriate transducers to a multichannel oscillograph (Grass Model 7D Polygraph) to continuously record mean and pulsatile blood pressure, heart rate, respiratory rate, and EEG.

Protocol

All rats received either 1,3-butanediol or saline thirty minutes prior to hypoxic exposure. In the first experiment, thirty minutes prior to hypoxia, 11 rats received 1,3-butanediol (47 mmol/kg, i.v.) and 10 rats received an equivalent volume of saline. The animals were then exposed to 4.5% O$_2$ until their mean blood pressure fell to 70 mm Hg at which time the chamber oxygen tension was increased to 8.0% O$_2$ for thirty minutes. After the thirty minutes of 8.0% O$_2$ they were brought up to 20.9% O$_2$ (room air) and left in the chamber for an additional 15 minutes. In an attempt to reduce the mortality in the saline treated rats a second group of saline-treated rats was tested by changing from 4.5% to 8.0% O$_2$ at mean blood pressures of 75 mm Hg (n = 6).

Neurologic Assessment

We selected neurologic examinations which identify unilateral motor deficits in rats (table 1). These were performed at 2 and 20 hours by trained observers who were not aware of the treatment given to each rat. The individual rat neurologic score was the mean of the scores of each behavior listed in table 1.

Data Analysis

Statistical analysis was performed with the aid of the Michigan Interactive Data Analysis System (MIDAS) on an Amdahl 5860 computer. The two-tailed Student t test was used to compare the overall survival time, blood pressure, and neurologic score of the 1,3-butanediol treated group with the control group. All values are expressed as mean ± one standard error of the mean (SEM); sample size is designated (n). Survival rates were compared using the nonparametric Fisher’s Exact Test.

Results

No statistical difference was detected between the 1,3-butanediol and saline-treated groups in relation to age, weight, surgical time, blood loss, prehypoxia body temperatures, blood pressure, and respiratory rate (table 2). We were unable to detect any statistical differences between the saline treated rats that were changed from 4.5% O$_2$ to 8.0% O$_2$ at a mean arterial blood pressure of 70 and those changed at 75 mm Hg; therefore these two subgroups were combined and con-
rats was significantly lower (1.1 ± 0.1) than the score for the saline treated rats (3.40 ± 0.87) (p < 0.002) (fig. 1). Survival to the 20 hour neurologic exam was significantly increased for the 1,3-butanediol rats to 72.7% (8/11) as compared to only 18.8% (3/16) for the saline treated rats (p < 0.004). 1,3-butanediol treated rats had a significantly longer exposure to 4.5% O2 environment, 1143.6 ± 141 sec vs. 506.3 ± 43 sec for the saline rats (p < 0.001).

There were significant differences in the EEG patterns between the two groups. Only 2/11 1,3-butanediol treated rats ever achieved isoelectric EEG; however, 13/15 saline rats developed an isoelectric EEG (one rat had defective EEG lead wires). For the 2/11 1,3-butanediol rats that did achieve an isoelectric EEG the average time to isoelectric EEG was significantly increased from 445.71 ± 46 sec for the saline treated rats to 1556 ± 213.6 sec for the 1,3-butanediol rats (p < 0.001).

Discussion

Enhanced survival and a reduction of the functional neurologic deficits that accompany cerebral ischemic events are the ultimate goals of all proposed therapy for cerebral vascular disease. Previous work in this laboratory has demonstrated an increased survival after 1,3-butanediol pretreatment in experimental hypoxia and ischemic-hypoxia models. By design such studies are terminal protocols and do not allow assessment of residual neurologic deficits. This study, using a sublethal but, damaging, ischemic-hypoxic insult, demonstrates that pretreatment with 1,3-butanediol not only increases survival but protects the brain from the anticipated neurologic deficit.

In previous studies from our laboratory, ketosis induced by a variety of means, but in particular the administration of 1,3-butanediol, was shown to prolong survival in mice and Levine rats. In the mouse studies, protection of brain function could only be inferred by the prolonged hypoxic survival times (cessation of spontaneous ventilation). In our initial Levine rat study a clear progression from loss of brain electrical activity to respiratory dysfunction and finally to complete cardiovascular collapse was noted in both the control and 1,3-butanediol treated rats. This consistent sequence strengthened the inference of a primary cerebral protective effect because the prolonged survival of the 1,3-butanediol treated rats could be completely accounted for by the increase in the interval from the onset of hypoxia to loss of spontaneous cortical electrical activity. Nevertheless, for each of these studies, protection of brain function could only be inferred.

In this study pretreatment of rats with 1,3-butanediol significantly improved survival and neurologic score. This is even more remarkable in that total hypoxic exposure in the 1,3-butanediol treated rats was more than twice that (1144 vs. 506 sec) of the saline control rats. This increased hypoxic exposure resulted from the experimental design, which used biologic rather than chronologic endpoints. That is, it took longer for the 1,3-butanediol treated rats to lower their mean arterial pressure to 70 mm Hg. The additional
exposure to hypoxia introduced a strong bias against the neurologic score of the 1,3-butanediol treated group. The 1,3-butanediol pretreated rats should have, but did not, show an increase in the severity of their motor deficit. This would suggest that if the 1,3-butanediol pretreated rats had been exposed to hypoxia for only 306 sec, then they would have had an even lower morbidity and mortality.

In the rat, 1,3-butanediol is rapidly converted to the ketone beta-hydroxybutyrate by the enzyme alcohol dehydrogenase. With the dose of 47 mM/kg i.v., blood ketone (beta-hydroxybutyrate) levels are significantly elevated within minutes and remain elevated for hours. It is also well established that ketones are readily utilized as an alternate substrate for brain metabolism. Previous mouse studies have shown that ketosis induced by a variety of methods, such as fasting, diabetes, and ketogenic diet, also significantly increases hypoxic survival times. Biological studies of tissue metabolites and brain slice preparations from our laboratory have identified an increased and preferential utilization of ketones under hypoxic conditions. This preferential utilization of the ketogenic effect of 1,3-butanediol is linked to its ketogenic effect.

Our previous survival and biochemical studies utilized terminal preparations and thus were unable to address the issue of possible amelioration of neurologic deficit or the preservation of neurologic function after a sublethal insult. Obtaining a consistent model of lateralized neurologic damage is a major problem for investigators attempting to evaluate potential treatments for stroke. In this study, right carotid ligation coupled with exposure to 4.5% and 8% oxygen proved effective and reliable. Two levels of oxygen were necessary because exposure to 4.5% O2 rapidly produces an arterial pO2 of less than 20 mm Hg, which invariably proves fatal before a functional deficit can evolve. Exposure to a single gas with less severe hypoxia (8%) can be tolerated for prolonged periods and does not reliably produce lateralized deficits. Right carotid ligation combined with an initially severe hypoxia (4.5%) that is changed to a mild hypoxia (8.0%) at a specific biologic endpoint (mean systemic blood pressure 70–75 mm Hg) reliably produces a lateralized deficit. A biologic endpoint was purposely chosen as opposed to a simple elapsed time or chronologic endpoint because it was found empirically to increase the reproducibility of the preparation and reduce cardiovascular variation between the groups.

The neurologic scoring system used in our laboratory focuses on variables targeted primarily at motor

| TABLE 2. Numerical Values of the Measured Parameters and the Results of Statistical Analysis Between the 1,3-Butanediol and Saline Control Groups |
|-------------------------------------------------|-----------------|-----------------|
| Age (days)                                      | 61.1 ± 0.5      | 61.2 ± 0.6      | NS               |
| Weight (grams)                                  | 277.7 ± 4.5     | 278.1 ± 3.9     | NS               |
| Surgical time (min)                             | 41.6 ± 1.0      | 42.3 ± 2.2      | NS               |
| Blood loss (0–4)                                | 0.4 ± 0.2       | 0.5 ± 0.3       | NS               |
| Prehypoxia temp °C                              | 36.1 ± 0.2      | 37.0 ± 0.4      | NS               |
| Prehypoxia BP mm Hg                            | 125.2 ± 6.4     | 123.1 ± 1.5     | NS               |
| Prehypoxia respiratory rate                     | 105.3 ± 9.1     | 103.0 ± 6.8     | NS               |
| Mean 20 hr neuro score (1–5)                    | 1.1 ± 0.1       | 3.4 ± 0.87      | p < 0.002        |
| Percent survival (20 hrs)                       | 72.7% (8/11)    | 18.7% (3/16)    | p < 0.004*       |
| Hypoxic time to switch (sec)                    | 1143.6 ± 140.58 | 506.3 ± 43.0    | p < 0.001        |
| Percent who developed Iso-EEG                    | 18.2% (2/11)    | 86.7% (13/15)   | p < 0.001*       |
| Time to Iso-EEG (sec)                           | 1556.3 ± 213.6  | 445.7 ± 46.0    | p < 0.001        |
| Respiratory rate at switch                      | 73.5 ± 10.5     | 20.9 ± 7.1      | p < 0.002        |

NS = not statistically different to a p < 0.05 level when compared by the Student t test. P values were determined by the Student t test except for * which indicate values compared by the nonparametric Fisher Exact Test.

Figure 1. Mean 20 hour neurologic score of the 1,3-butanediol (open bar) vs. saline control group (hatched bar). * = statistical significance by the Student t test.
function: posture, hemiparesis, circling, shuffle, spontaneous activity, and the ability to hang on a vertical screen (table 1). All such neurologic examinations, particularly on small animals, can be quite subjective. To obviate this concern to the greatest degree possible we utilized experienced, but blinded examiners for scoring. Similarly, the heavy emphasis on motor function in this work was intentional, as the assessment of sensory and behavioral deficits is more subtle and requires more sophisticated and often more subjective techniques.

Loss of sensory, memory, and learning functions as well as impaired nocturnal and diurnal patterns are not currently detectable with the neurologic scoring used in this study. This restricted sensitivity should, however, be viewed in a positive light, in that, despite this restricted sensitivity, our neurologic scoring system graphically demonstrated the efficacy of 1,3-butanediol. Thus recognizing its limits, we have found the neurologic score to be reliable and consistent when performed by independent examiners.

Several other investigators have used the Levine preparations as a model for stroke research. Most have concentrated on blood flow, histologic or biochemical aberrations. Few studies have concentrated on neurologic deficit using this model. These assessed neurologic deficit in relation to the degree of necrosis, edema formation, or the specific neuroanatomic loci involved. We focused on functional neurologic deficits as an additional means of evaluating the protective effect of 1,3-butanediol. Neurologic assessment has obvious clinical relevance but also provides a means of approaching the question of the mechanism of action of 1,3-butanediol. That is, by demonstrating not only increased survival but also demonstrating reduced neurologic impairment the inference of a primary cerebral protective effect is further strengthened.

Of the 400,000 people who develop spontaneous cerebral vascular accidents each year, approximately 30–50% die during their initial hospitalization. If the patient survives, the functional deficits that accompany even small cerebral infarcts are often quite profound. In the Framingham Study of stroke survivors, because of the functional sequelae of a stroke, 71% of the afflicted were not able to return to their previous level of activity. Fully 16% of the stroke survivors were institutionalized, 31% were dependent on others for self care, 20% had dependent mobility, and 62% were less active socially. Motor and sensory deficits and visual impairment obviously restrict all activity; loss of higher cortical functions and communicative skills, however, are particularly devastating and may contribute directly and indirectly to the significant psychological changes noted in stroke survivors. The human suffering and socioeconomic cost are enormous. By concentrating on survival and neurologic deficit this study attempts to assess the ability of 1,3-butanediol to alter the two devastating sequelae of stroke; death and functional neurologic deficit.

Safe, effective, and readily available means of affording cerebral protection from ischemic hypoxia do not yet exist. This study indicates that 1,3-butanediol has the ability to reduce both mortality and morbidity in this hypoxic-ischemic rat model. If 1,3-butanediol continues to demonstrate efficacy when tested in other animal models, then it may have important clinical implications. Protection may be afforded in such clinically common acute ischemic events as stroke, stroke in evolution, and transient ischemic attacks, and perhaps as a protective agent during the temporary vulnerable periods associated with cardiac resuscitation, carotid clamping, and cardiopulmonary bypass.

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References

Brain Metabolic Changes in Young vs Aged Rats During Hypoxia

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SUMMARY Brain energy state and glycolytic metabolites were measured in young (6 month) and aged (28 month) male rats under normoxic (70% nitrous oxide, 30% oxygen) or hypoxic (PaO₂ = 25 mm Hg) test conditions. Hypoxic ischemia was induced in one cerebral hemisphere by ligation of one carotid artery. Under normoxic test conditions brain energy metabolite concentrations were similar between young and aged rats. Brain tissue glucose, glycogen, glucose-6-phosphate and citric acid cycle intermediate concentrations were decreased in aged rats during normoxia while fructose-6-phosphate and pyruvate were increased. Decreases in brain energy state and increases in lactate/pyruvate ratios were significant in both young and aged rats during hypoxia and were greater in aged animals in hypoxic-ischemic tissues. These results indicate that brain energy state is normal in aged rats under normoxic conditions but that hypoxic-ischemia produces a greater degree of brain energy failure compared to younger animals.

SEVERAL CHANGES HAVE BEEN REPORTED in brain energy metabolism with aging. Both cerebral blood flow (CBF) and cerebral oxygen metabolism (CMRO₂) have been reported to decrease in old subjects.¹⁻³ Other reports indicate no significant change in CMRO₂ in the aged but instead have shown a decrease in cerebral glucose metabolism (CMRgl).⁴⁻⁵ Changes in brain metabolism with aging may not only affect energy utilization under resting conditions but may also alter the subject’s ability to respond to hypoxic-ischemic challenges which may occur during stroke. Reports indicate that aged subjects have less capacity to respond to these challenges and have a higher incidence of mortality.⁶⁻⁷ The increased susceptibility of aged subjects to stroke may be due to a decrease in brain energy reserve. Ulfert, et al⁸ have reported that brain ATP and phosphocreatine concentrations are decreased as a function of aging. Such a change would decrease the amount of energy reserve available during hypoxic or ischemic challenges and increase the risk of brain damage during stroke. Since brain glucose oxidation is the primary mechanism to maintain the brain energy state, it is likely that changes in brain glucose metabolism may also play a role in the increased susceptibility of aged subjects to stroke. In order to evaluate brain metabolic changes which may occur during aging we have measured brain energy states and metabolites of glycolysis and the citric acid cycle in young and old rats under control and hypoxic-ischemia test conditions.

Methods

Male F-344 Rats, aged 6 months and 28 months were used in these experiments. These age groups correspond to a young adult and a senescent aged rat respectively. Rats were anesthetized with halothane, tracheostomized and artificially ventilated with 1% halothane in 69.5% nitrous oxide, 29.5% oxygen for surgical procedures. Heparinized saline filled catheters were inserted into the right femoral artery for measurement of arterial blood pressure and to obtain arterial blood samples. A catheter was also inserted into the right femoral vein for infusion of fluids. The right common carotid was dissected free from the cervical sympathetic and vagus nerves and ligated. The skull was exposed and a funnel was fixed over it, to be used later for freezing the brain. Following the completion of all surgery, the rat was allowed to stabilize while being ventilated with 70% nitrous oxide, 30% oxygen. Blood gas tensions were measured using an IL 1303 blood gas analyzer. Arterial PCO₂ was adjusted to 35–40 mm Hg. Body temperature was measured with a Yellow Springs thermistor probe and maintained at 37° C using overhead heat lamps.

At the end of the 45 minute stabilization period, young and aged rats were subjected to either normoxic
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