Role of Tissue Lactate and Substrate Availability in 1,3-Butanediol-Enhanced Hypoxic Survival in the Mouse*

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SUMMARY Previously we found that 1,3-butanediol-treated mice live longer during hypoxia. We hypothesized that 1,3-butanediol could reduce the brain's accumulation of potentially cytotoxic lactate and/or elevate brain substrate availability (ketones or glucose) and thus maintain the brain's energy producing capability even during reduced oxygen availability. To test these hypotheses, whole brain metabolites from normoxic and hypoxic mice, pretreated with 1,3-butanediol or insulin, were compared to saline controls. During hypoxia both pretreated groups had lower brain lactate than controls. If lactate accumulation was the sole factor responsible for hypoxic tolerance, insulin should have increased brain lactate since insulin has been shown previously to reduce hypoxic tolerance. In normoxic mice the ratio of lactate to pyruvate and the level of malate and fumarate were not changed by 1,3-butanediol as is found with other agents known to protect the hypoxic animal. When substrate availability was directly elevated by beta-hydroxybutyrate and glucose administration hypoxic survival time increased thus implicating substrate availability as an important factor in hypoxic tolerance. We conclude that reduced brain lactate and augmented substrate availability both contribute to 1,3-butanediol-enhanced hypoxic tolerance in this animal model.

IN PREVIOUS STUDIES from our laboratory ketosis induced by 1,3-butanediol (BD) pretreatment was shown to be coincident with a dramatic increase in the tolerance of mice to hypoxia. Myles reports increased hypoxic tolerance in fasted, and presumably ketogenic, animals, exposed to simulated high altitude. Others have shown that a fasting individual's brain uses ketones for as much as 60% of their energy requirement. In conditions other than fasting, e.g. in newborn animals ketones are normally used for brain metabolism and newborn animals also have increased tolerance to hypoxia. Such changes in hypoxic tolerance during these ketotic conditions could, of course, be produced by some mechanism totally distant from ketosis.

We have identified two general hypotheses that initially offered plausible explanations for the increased hypoxic tolerance observed in animals rendered ketogenic by BD. The first hypothesis contends that tissue lactate is the key cytotoxic factor in that lactic acid accumulation has been correlated with autolysis of neuronal tissue. An alternative and less traditional hypothesis is that ketosis enhances hypoxic tolerance by directly or indirectly maintaining the brain's energy producing capability. Directly the ketones could enter the tricarboxylic acid cycle through conversion to acetyl CoA and indirectly they could, in addition, have a permissive effect on glycolytic flux by, for example, consuming reducing equivalents during the formation of fatty acids. It should be stressed that either of these ketone based mechanisms require oxygen and hence would only be functional during hypoxia and not during anoxia.

To test the hypothesis which is concerned with the role of tissue lactate we pretreated mice with either BD or insulin and measured brain metabolites after various times of exposure to hypoxia. To determine if elevated substrate availability would enhance hypoxic tolerance, the survival time of mice pretreated with either saline, BHB and dextrose, BHB alone or dextrose alone was compared. The results suggest that lactate accumulation and continued substrate supply are important factors in determining the outcome of a hypoxic challenge in BD pretreated animals.

Methods

General Procedure

The animal model used was described by Wilhelmi and Arnfred and Steen and Michenfelder and used in our laboratory. Adult male Sprague-Dawley albino mice (HA-ICR) weighing approximately 30 g were pretreated and then thirty minutes later exposed to either a normoxic gas mixture (room air) or a hypoxic gas mixture (approximately 4.5% O2, 95.5% N2). All IV injections were made with a 26 gauge needle into a lateral tail vein.

Brain Metabolite Experiments

For each trial two control (saline IV or IP) and three treated animals were used. The group of five mice was placed in an airtight 5.5 liter flow-through Dewar flask which was continuously flushed with the gas mixture at room temperature (22-24°C). The importance of simultaneously testing control and treated mice was recently restated in a critical assessment of this animal model. The diurnal variation in hypoxic tolerance reported by Stupfel et al also focuses on the need for simultaneous testing of control and treated animals. Further, to assure the comparability of separate runs

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the composition of the gas was continuously monitored with an oxygen analyzer (Beckman OM-14). Thirty minutes prior to hypoxia animals were pretreated by one of the following: 1.4 mmoles of BD in 0.25 ml sterile water IV; 0.25 ml of sterile saline (0.9% sodium chloride) IV; 2U of regular insulin in 0.5 ml of sterile saline IP; 0.5 ml of sterile saline IP. Hypoxia was induced by first flushing the flask at about 10 liters/min with an 8% O₂ and 92% N₂ gas mixture. After 20–25 seconds the chamber oxygen was reduced to about 4.5% at a rate of 10 liters/min. In the normoxic group the chamber was flushed at a rate of 10 liters/min with compressed room air (20.95% O₂). These procedures were used to mimic, as closely as possible, the timing, pretreatments, and gas exposures used in our previous studies thus permitting direct comparisons with previous data. After 30, 60, 90, or 120 sec of exposure to hypoxia the mice were submerged in approximately 4 liters of liquid N₂.

**Brain Metabolite Analysis**

The brains were dissected out of the skulls, ground in liquid N₂, with a mortar and pestle and poured into a preweighed vial containing 1 ml of 3M HClO₄. The dissection, grinding and deproteinization were done in a cold room at -20°C. After each vial was reweighed, 2 ml of deionized distilled water was added to the HClO₄-brain mixture. Each sample was homogenized for 30 seconds at maximum speed (Brinkman Polytron) at 4°C and centrifuged at 7710g for 20 minutes. The supernatant (2.7 ml) was withdrawn and neutralized with an 8% 0₂ and 92% N₂ gas mixture. After 20-25 sec the chamber oxygen was reduced to about 4.5% at a rate of 10 liters/min with compressed room air (20.95% O₂). These procedures were used to mimic, as closely as possible, the timing, pretreatments, and gas exposures used in our previous studies thus permitting direct comparisons with previous data. After 30, 60, 90, or 120 sec of exposure to hypoxia the mice were submerged in approximately 4 liters of liquid N₂.

**Hypoxic Survival Time Experiments**

For the reasons described above, 2 control and 3 experimental animals were placed in five individual airtight, 110 ml flow-through chambers mounted in parallel and continuously flushed with a monitored (Beckman OM-14) gas mixture at room temperature. When the animals were first placed in the chambers compressed room air was delivered to the chambers. Thirty minutes prior to the initiation of hypoxia animals were given an IV and IP injection. The IV injection was of either 62.5 mg dextrose in 0.25 ml sterile water or 0.25 ml of sterile normal saline. IP injections included 60 mg DL-BHB delivered in 0.5 ml sterile water or 0.5 ml of sterile normal saline. Hypoxia was induced by first flushing the system at 2 l/min with an 8% oxygen, balance nitrogen, gas mixture. After 20–25 sec the chamber oxygen was reduced to about 4.5% without changing the flow rate. Hypoxic survival time (HST) as described by Wilhjelm and Arnfred and Steen and Michenfelder and currently used in our laboratory is the time from the onset of hypoxia to the cessation of spontaneous ventilation. Although the exact relationship between HST and brain dysfunction has not been defined in this mouse model it is more clearly described in dogs. When hypoxia is used as euthanasia in dogs the EEG becomes isoelectric, with a normal or elevated blood pressure, then respiration is stimulated before being depressed and finally there is cardiovascular collapse. A similar sequence of isoelectric EEG, respiratory stimulation, then depression and final cardiovascular collapse was recently observed in rats exposed to unilateral carotid ligation and hypoxia. These patterns are consistent with the brain being the initial tissue compromised by hypoxia followed by respiratory failure and ultimately cardiovascular decompensation.

**Blood Glucose and BHB Analysis**

An additional group of mice was treated with both an IV and IP injection as described in the previous section. In order to obtain an assessment of the blood glucose and BHB levels at the onset of hypoxia these animals were decapitated 30 minutes after the injection instead of being exposed to hypoxia. Because decapitation was the method used to obtain the blood samples we were only able to obtain one sample per animal at any time. The blood was collected in a heparinized tube and immediately centrifuged to obtain the plasma which was then deproteinized and neutralized before analysis. Plasma glucose was measured enzymatically with the hexokinase method using reagents of Worthington Biochemicals on a Gilford 3500 Computer-Directed Analyzer. BHB was detected enzymatically (beta-hydroxybutyrate dehydrogenase method) on a Farrand Ratio Fluorometer 2.

**Data Analysis**

Statistical analysis was performed with the aid of the Michigan Interactive Data Analysis System (MIDAS) on an Amdahl 470/7 computer facility. Comparison of each experimental group with the control group was done with a two-tailed Student’s t-test using the Bonferroni correction for multiple comparisons where appropriate. All results are expressed as mean ± one standard error of the mean (SEM); the sample size is designated (n). Brain metabolites are all reported in µmoles/g wet weight and are not corrected for blood or cerebrospinal fluid metabolite content. Entrapped blood in brain tissue represents approximately 3% of the brain weight and is of unknown composition, thus making precise correction untenable.

**Results**

**Brain Metabolite Experiments**

**General**

The average age for 118 mice used in this protocol was 53 ± 2 days and average brain weight used for the analysis of metabolites was 377 ± 5 mg and included both fore- and hindbrain. Food and water was available to all animals up to the time of the experiment. The average oxygen content flushing the Dewar flask dur-
ing the hypoxic period was 4.72 ± 0.03%. With this low an inspired oxygen level the arterial oxygen tension although not measured, could not exceed 20 mmHg at sea level regardless of respiratory rate and cardiac output.

Glycolytic Intermediates

During normoxia the brain tissue lactate was 2.5 μmoles/g and was not changed significantly by pretreatment with BD or insulin. Each group of animals subjected to hypoxia showed an increasing tissue lactate level with increasing duration of hypoxic exposure (fig. 1). The level of lactate accumulation during hypoxia was significantly (by Bonferroni weighting of Student t) lower in both BD (p = 0.02 at 90 sec and p = 0.001 at 120 sec) and insulin (p = 0.002 at 60 sec) pretreated animals as compared to saline pretreated control animals. The difference between lactate in the saline and BD pretreated animals increases with the increase in duration of hypoxic exposure (fig. 1). During normoxia brain pyruvate was 0.26 ± 0.02 μmoles/g (n = 10) and was decreased significantly (P < 0.01) (34%) with BD pretreatment to 0.17 ± 0.02 (n = 10) and with insulin (P < 0.05) pretreatment to 0.17 ± 0.01 (n = 4). During hypoxia the saline treated animals had a brain pyruvate level of 0.27 ± 0.02) (n = 14) which was not statistically different from levels in BD (0.23 ± 0.01) (n = 16) or insulin (0.24 ± 0.01) (n = 8) treated animals. In one series of animals both lactate and pyruvate were measured simultaneously (table 1). In normoxic animals the lactate-pyruvate ratio was unaffected by BD pretreatment but increased by insulin pretreatment. With the exception of insulin pretreated animals, the lactate-pyruvate ratio increased after exposure to hypoxia. Whereas pretreatment with BD did not significantly alter the hypoxic lactate-pyruvate ratio, insulin pretreated animals had a ratio which was 37% lower than controls (p < 0.05).

Kreb Cycle Intermediates

Neither brain malate or fumarate levels were significantly different from saline controls in the normoxic BD pretreated animals (fig. 2). With exposure to hypoxia both the saline and BD pretreated animals showed marked increases in the accumulation of these metabolites. The hypoxic accumulation of malate however was slightly but significantly inhibited by BD when compared to the saline pretreated control group.

HST Experiments

The average age and body weight of mice used in these experiments was 39 ± 2 days and 27.3 ± 0.3 g respectively, n = 142. The average oxygen content of the gas flushing the test chambers was 4.62 ± 0.01% during the hypoxic period. Animals given saline IV and IP had average HST, plasma glucose and plasma BHB levels (table 2) comparable to those we have published previously. As expected animals receiving IP injections of BHB had HST and plasma BHB levels significantly higher than control. In animals receiving only IP BHB, plasma glucose levels were slightly lower than control. Elevation of substrate availability by pretreatment with dextrose IV produced an increase in plasma glucose, a decrease in plasma BHB and no increase in HST. In contrast animals pretreated with both dextrose and BHB had HST values greater than would be expected for a simple additive effect of the two substrates. The plasma BHB level was significantly greater than the saline IV, saline IP control and the plasma glucose was significantly (p = 0.05) greater than the saline IV, BHB IP group.
Table 1  Lactate-Pyruvate Ratio

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<tr>
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<th>Normoxia</th>
<th>Hypoxia</th>
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<tbody>
<tr>
<td>Saline</td>
<td>9.0 ± 1.0c</td>
<td>30.1 ± 2.6c,b</td>
</tr>
<tr>
<td>BD</td>
<td>11.2 ± 1.1c</td>
<td>29.1 ± 1.9c,b</td>
</tr>
<tr>
<td>Insulin</td>
<td>14.7 ± 1.8a,c</td>
<td>19.3 ± 2.3a,c</td>
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In this table the lactate-pyruvate ratios are given for each pretreatment group sacrificed in a normoxic environment or a hypoxic environment. The numbers in parentheses are the sample size for each bar. The line which extends above each bar represents one standard error of the mean. The dashed lines represent literature values of malate and fumarate expressed as percent of control for animals treated with 225 mg/kg sodium phenobarbital IP (line a) or 135 mg/kg sodium amyatal IP (line b) (Goldberg et al, 1966). * = p < 0.01 as compared to saline pretreated hypoxic animals.

Discussion

Methodological Considerations

Although many methods have been described for rapid fixation of brain metabolites we will discuss only those reported for the unanesthetized mouse. Three currently used fixation methods are freeze blowing,26 microwave irradiation (6 KW),27 and immersion in liquid N2.28-30 With these three methods, values of brain metabolites are generally comparable. It was necessary that we use immersion in liquid N2 because using either freeze blowing or microwave irradiation would require changes from the test conditions we had used previously1, 14, 15 and hence would make the current data not applicable as an explanation for the increased survival time observed in these studies.

Whole brain lactate measured in our study is within the normal range reported for normoxic mice using liquid N2 fixation.28, 29 Unaware of the local or global site of action of BD in the brain, we included both fore- and hindbrain for tissue analysis. Duffy et al30 have shown marked regional differences in brain lactate levels ranging from 1.32 mmole/kg wet weight in the parietal cortex to 3.10 mmole/kg wet weight in the medulla of a mouse. Therefore, compared to the literature values some of the absolute differences in metabolite levels presented in the current study may be attributed to the fact that others restrict their tissue samples to brain from the anterior cranial fossa. Since the comparisons were made between control and treated mice, that had been handled identically, such differences in absolute values should have little effect on the interpretation of the data.

BD was chosen for the brain metabolite experiments because we had previously shown1 that 1.4 mmoles BD given 30 minutes prior to hypoxia increased blood BHB concentration 364% and HST 560%. BD is a dimer of ethanol which is converted to BHB by alcohol dehydrogenase and aldehyde dehydrogenase, both of which are present in the liver and to a lesser extent in the brain.32 Thus BD is reliable and effective for elevating blood ketones and consistently increasing HST. Because BD has other systemic effects which may alter HST’s direct administration of BHB was chosen to focus in on the substrate altering effects related to the increased HST. BHB rather than acetoacetate was chosen for measurement since preliminary studies indicated that the contribution of acetoacetate to the total ketone pool was small. BHB and acetoacetate are in equilibrium and therefore an elevation of one of these substrates would tend to implicate an elevation in the other.

Evaluation of the Hypotheses

We have proposed two possible hypotheses to explain the increased tolerance to hypoxia seen with the elevation of blood ketones by BD. The first is a decrease in lactate accumulation, and the second involves the maintenance of the cerebral energy state.
either directly or indirectly by modification of substrate availability. Although neither of these explanations exclude one another, or other totally different hypotheses, we will try to focus on each theory as it is supported or refuted by the current data.

If it were assumed that death from hypoxia is linked to the accumulation of lactic acid then one would predict that BD-pretreated animals, with their augmented hypoxic tolerance, would have less brain lactic acid accumulation than control animals. Conversely in an animal with reduced hypoxic tolerance an increase in lactic acid accumulation would be predicted if tissue lactate were the sole determinant of hypoxic tolerance. Mice pretreated with insulin have been shown to have a decreased tolerance in hypoxia. 3, 14 This reduced hypoxic tolerance could of course be explained by a close association between substrate availability and hypoxic tolerance in which case either a reduction or no change in brain lactate would be predicted. On the other hand, if insulin pretreatment proved to exacerbate lactate accumulation this would support the hypothesis that lactate was the key cytotoxic factor in this animal model.

Recently, Rehncrona11 has reaffirmed lactate as an important factor which contributes to the neurolysis associated with ischemic hypoxia. Furthermore, Barlet13 has shown that animals treated with 6-aminonicotinamide, which is known to increase hypoxic survival time, have substantially less lactate accumulation during hypoxia than saline controls. Our data are consistent with that of Rehncrona and Barlet in implicating tissue lactate as an important factor in hypoxic damage. In light of the insulin data, however, it is unlikely that lactate is the sole determinant of neuronal failure. We showed that insulin pretreatment decreased HST14 and in the current study the same insulin pretreatment was associated with a reduction rather than an increase in brain tissue lactate as would be predicted if lactate was the sole determinant of neuronal damage.

The decrease in brain lactate observed in animals pretreated with BD could be a direct or indirect effect. It is possible for example, that BD is acting directly to inhibit glycolysis or indirectly through its ketone metabolite, BHB, to inhibit glycolysis. Ketone metabolism has been shown to elicit potent inhibition of glycolysis via negative modulation of phosphofructokinase and hexokinase probably because of elevated brain levels of citrate and ATP. 26 It is well documented in the literature34 and confirmed here that the brain level of lactate increases during hypoxia. The increase in the lactate-pyruvate ratio during hypoxia observed in BD and saline pretreated animals indicates that the brain metabolites are in a more reduced state than during normoxia. Since, however, pretreatment with BD did not alter the lactate-pyruvate ratio from control values during either normoxia or hypoxia it may be concluded that the increase in HST associated with BD is probably not due to a change in the brain's oxidative state. These are static measurements and thus do not exclude an effect due to dynamic changes which could not be detected with these methods. Regardless of how BD reduces brain lactate, we are led to conclude that a reduction in brain lactate contributes to the protective effects of BD.

Barbiturates as well as induced ketosis afford increased tolerance to hypoxia and ischemia. The exact mechanism of barbiturate protection has yet to be identified, but barbiturates are known to alter brain metabolism. 13 Specifically, phenobarbital and amytal cause a decrease or no change in brain citrate and isocitrate and a decrease in brain malate and fumarate. 35, 36 A decrease in lactate, pyruvate and the lactate-pyruvate ratio has also been reported with general anesthetics. 37 Thus one is led to examine patterns of change in brain tissue metabolites that could suggest a possible mode of action for potentially therapeutic agents like barbiturates and BD. Nevertheless, despite marked changes in cerebral metabolites, it can only be inferred that such changes have any bearing on hypoxic tolerance. In our study, pretreatment of normoxic mice with BD produced no statistically significant change in the brain levels of malate, fumarate, lactate or the lactate-pyruvate ratio. Furthermore, Veech et al26 showed that rats given BD have increases in brain citrate and isocitrate rather than a decrease as anticipated with a general anesthetic effect. Steen and Michenfelder19 have shown that barbiturates' protective effect is dependent on the anesthetic effect and distinct from the anticonvulsant effect. They have also shown that even slight modifications in barbiturate structure may greatly reduce its protective effect. Neither BD or BHB have structural characteristics resembling those of any of the barbiturates which have been shown to increase HST. Mice treated with BD parenterally were not anesthetized although they at times appeared intoxicated by the alcohol. Furthermore BD taken orally for 12 days protected from hypoxia without any clinical signs of intoxication or anesthesia. 1 Since the exact mechanisms by which barbiturates and BD afford protection from hypoxia have not been identified, one can not exclude the possibility that they are the same. The clinical picture, the molecular structure and the changes in tissue metabolites all suggest a distinct mechanism of action for BD and barbiturates despite the fact that both offer protection from hypoxia. The data do not exclude the possibility that some alcohol-like effect34, 39 contributes to the overall protection afforded by BD. We, however, believe that the alcohol-like effects will prove to be additive rather than alternative to the dramatic changes associated with altered substrate availability.

The second hypothesis attributes the enhanced tolerance to hypoxia observed with BD pretreatment to an increased and altered substrate availability. The reduced tolerance for hypoxia observed with the insulin-induced hypoglycemia may be explained by reduced substrate availability. Elevation of blood ketones whether by BD pretreatment or by BHB loading in the presence of hyperglycemia is associated with increased hypoxic tolerance. We have concluded that the protection from hypoxia associated with BD pretreatment may be attributed in part to a specific reduction in
brain lactate content. The decrease in brain lactate with a decrease in hypoxic survival time in response to insulin, however, casts some doubt on lactate accumulation as the only factor responsible for neuronal failure during hypoxia in this model. This disassociation between tissue lactate and HST does not eliminate the possibility that a reduction in brain lactate is the sole mechanism for the protection from hypoxia seen with BD. The data, however, implicates some additional mechanism, possibly elevated substrate availability, that allows for maintenance of spontaneous ventilation during hypoxia and thus contributes to the protective effect associated with BD administration.

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