SUMMARY In previous studies from our laboratory a positive correlation between elevated blood ketone levels and the survival time (ST) during hypoxia (4–5% oxygen) was observed in fasted and alloxan diabetic mice. To test the hypothesis that ketosis was somehow increasing the tolerance of mice to hypoxia, we induced ketosis by either oral (PO), intraperitoneal (IP), or intravenous (IV) 1,3-butanediol (BD). Blood beta-hydroxybutyrate increased from $0.83 \pm 0.15$ mM to $3.32 \pm 0.08$ mM for PO, $1.2 \pm 0.2$ mM for IV and $0.83 \pm 0.15$ mM for IP. BD was associated with an increase in ST to 458% (n = 19) when given PO, 217% (n = 12) by IP route, and 560% (n = 13) by the IV route. The effect of ambient temperature ($T_a$) on this phenomenon was evaluated at 12, 22, 32, and 34°C. At each $T_a$, IV BD at 1.4 mmole/mouse was associated with an increase in ST to 525, 559, 151, and 145% of control, respectively. The absolute ST of both control and treated mice was greater at $T_a$ of 12 and 22°C than at 32 and 34°C. Rectal temperature was continuously recorded and no differences were detected between control and treated groups just prior to hypoxia except for the group treated at $T_a$ of 12°C. Hypoxia, however, was associated with a decrease in body temperature in each group. It is concluded that the artificial induction of ketosis by BD is associated with an increase in ST of mice exposed to hypoxia.

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Butanediol Induced Ketosis Increases Tolerance to Hypoxia in the Mouse

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BRAIN SUBSTRATE availability* has been reported to modify survival time or brain damage in various animals exposed to brain hypoxia and ischemia. In a previous study from our laboratory, a correlation was noted between increased blood ketone levels and hypoxic tolerance. In other studies, in which elevated blood ketones could be predicted, an increased tolerance to hypoxia or ischemia has been noted. On the basis of this correlation, it was desirable to determine if an intentional elevation in blood ketone levels could produce an increased tolerance of the brain for hypoxia. A wide variety of procedures can elevate blood ketone levels (i.e., fasting, ketogenic diet, uncontrolled diabetes). For this study we have chosen the non-toxic alcohol 1,3-butanediol (BD). BD is converted in the body to beta-hydroxybutyrate (BHB) by alcohol dehydrogenase and aldehyde dehydrogenase. Although present in the brain, alcohol and
aldehyde dehydrogenases have their greatest activity in the liver. The indirect elevation of blood ketones by BD was chosen because the constant conversion of BD to BHB produced a relatively stable level of BHB in the blood. In a subsequent study, the somewhat more complex dynamics of BHB administration in combination with glucagon (G) are evaluated in terms of the direct ketogenic modulation of hypoxic tolerance.

In this study we tested the hypothesis that the administration of BD, which is coincident with the elevation of blood ketones, can induce an increased tolerance to hypoxia that cannot be explained by the hypothermic effects of the alcohol.

Materials and Methods

The animal model used has been previously described by Wilhelm and Arnfred, Steen and Michenfelder, and used by our laboratory. Adult male Sprague-Dawley albino mice (HA-ICR) weighing between 20 and 40 g were pretreated and then subjected to hypoxia (4–5% oxygen). The pretreatments involved the induction of ketosis by BD administered by one of 3 routes: IV, IP or PO. Body temperature, ambient temperature, survival time, or blood BHB levels were determined.

Experimental Hypoxia. For each trial, 2 control and 3 experimental animals were used. Each mouse was placed in an airtight, 110 ml flow through chamber. Five chambers were mounted in parallel and continuously flushed with a gas mixture. The composition of the gas mixture was continuously monitored with an oxygen analyzer (Beckman OM-14). The ambient temperature was maintained at one of 4 temperatures (13, 22, 32, or 34°C). Hypoxia was induced by first flushing the system at 1.7 l/min with an 8% oxygen, balance nitrogen, gas mixture. After 20–25 sec the chamber oxygen was reduced to between 4 and 5% at a flow rate of 1.3 l/min. The average oxygen content during the hypoxic period was 4.74 ± 0.04% (n = 92). Survival time, as used in this study and previous studies, refers to the time from the onset of hypoxia (4–5%) to the cessation of spontaneous ventilation.

Seven animals were exposed to hypoxia and, at the moment spontaneous ventilation stopped, were taken out of their chambers and immediately subjected to thoracotomy. In all 7 animals tested, the heart was still beating at thoracotomy. Thus, the cessation of respiration is probably not related to heart death and, likewise, contains no direct evidence of the exact time of brain death. Nonetheless, the procedure represents a simple, reproducible model of global hypoxia.

Temperature Measurements. Deep body temperature (Tb) was measured in each animal by inserting a thermocouple approximately 2 cm into the mouse’s rectum. The thermocouple wire (36 gauge copper-constantan) was encased in polyethylene tubing (PE 50) and secured by tape to the mouse’s tail. Body temperature was recorded at about 30 sec intervals using a temperature recorder (Honeywell Electronik 112). Cooling curves (fig. 1) were generated with rewarmed dead mice to determine the rate of decline in Tb from a nonmetabolizing body. Separate curves were plotted for each of the ambient temperatures (Ta). Selected carcasses were heated above Ta and then allowed to cool under the same conditions as the hypoxia experiments. The cooling curve data was taken to bracket the range of Tb observed for each Ta.

The individual curves were averaged by recording elapsed time and Tb from an arbitrary “zero” time. In order to test if the Tb effects were peculiar to BD treated mice, a separate series of mice were treated with diazepam (7.5 mg/kg, IV) and subjected to hypoxia at either 22 or 33°C.

1,3-Butanediol Administration. Five control studies were required to determine the route and amount of BD needed to induce ketosis as well as the time course of the induced elevation of BHB. In the first study, acute toxicity was tested in 68 mice given 0.25 ml injections containing from 1.1 to 7.7 mmol BD. All intravenous (IV) injections were given in the tail vein. These mice were maintained under normal housing conditions and those that were alive after 2 days were considered survivors. In the second group of 34 mice, the blood BHB levels were measured at 9 intervals from 5 to 70 min after 1.4 mmol BD, IV, per mouse. In the third study, 60 mice were given 0.25 ml IV injections containing from 0.50 to 1.75 mmol BD. After a 30 min wait, this group was tested for hypoxic tolerance. In the fourth group of 12 mice, 1.4 mmole BD was given IP and after 30 min hypoxic tolerance was tested. The fifth group of 19 mice received a
special diet* (United States Biochemical Corporation) containing 20% BD with no carbohydrate for 12 days prior to testing. Blood levels of BHB were measured in 4 mice receiving the special diet for 12 days and 4 mice 30 min after receiving IP BD.

Blood BHB Analysis. Representative mice from each group were treated as described above. Blood samples were taken by decapitating the mice and collecting the draining blood. One group of samples was deproteinized and neutralized for subsequent study by enzymatic analysis (beta-hydroxybutyrate dehydrogenase method) on a Farrand Ratio Fluorometer-2. A second group of samples was rapidly centrifuged for 3 min and the plasma analyzed for BHB by spectrophotometry (Gilford 3500 computer-directed analyzer.) These 2 procedures showed no significant differences when compared so the results of each assay were combined. Each of the values for blood BHB were obtained under normoxic conditions because we were unable to obtain blood samples during hypoxia or at the moment of "death."

Data Analysis. Statistical analysis was performed with the aid of Michigan Interactive Data Analysis System (MIDAS) on an Amdahl 470/v7 computer facility. Comparison of survival time for each experimental group with the control group was done with Student's t-test unless the experimental group had animals that lived past the end of the predefined end of the testing period. In this case, comparisons were done by nonparametric analysis using the Wilcoxon-Mann-Whitney (WMW) Rank Sum test. All groups, with the exception of those with survivors, are expressed as mean ± standard error of the mean (SEM); the sample size is designated (n).

Results

Butanediol Administration. The acute toxicity of BD occurs over a very narrow range increasing from zero to 100% mortality between 2.2 and 4.4 mmoles per mouse (fig. 2). At a dose of 1.4 mmoles per mouse no mortality is observed under normoxic conditions. After administration of 1.4 mmoles per mouse BD, the blood level of BHB increases rapidly from a control level (saline injected mice) of 0.33 ± 0.06 mM (n = 15) to 1.20 ± 0.25 mM (n = 7) at 30 min (fig. 3). The increase in blood BHB is statistically significant (p < 0.01 Student's t-test) from 5 to 70 min after injection. A statistically significant (p < 0.05 WMW Rank Sum test) increase in average hypoxic survival time was observed 30 min after 1.67 mmoles/mouse BD. At the highest dose given (1.67 mmoles/mouse) survival time was still almost twice that observed in controls. The major protective effect seen at lower doses (fig. 4) was no longer present, presumably due to combinations with some osmotic or CNS toxicity at this high dose.

Figure 5 shows the statistically significant (p < 0.0001 WMW Rank Sum test) increase in hypoxic survival time associated with PO, IV, and IP administration of BD. Mice alive after 3600 sec of hypoxia were considered "survivors" and included in the averages as if they had died at 3600 sec. Two of 19 in the PO group, 1 of 13 in the IV group, and none of 12 in the IP group were "survivors." If these 3 survivors are excluded completely, the average hypoxic survival times are 597.4 and 605.1 sec, respectively for PO and IV groups, each of which is statistically different from control (p < 0.001 Student's t-test). The filled dot over the control group represents a single aberrant mouse that is excluded from the average control because it had a survival time more than 5 standard deviations from the mean. The blood level of BHB was 3.32 ± 0.80 mM (n = 4) in the PO

*This diet contains 4% salt mixture USP XVII, 27% vitamin free casein, 20% 1,3-butanediol, 30% non-nutritive cellulose, 10% cottonseed oil, and a total vitamin supplement.
BUTANEDIOL INDUCED KETOSIS IN HYPOXIA/Kirsch et al.

1.6 r^1.2 CD

(3) (3) (3) (4) (4)

FIGURE 3. The closed circles (•) represent the average blood beta-hydroxybutyrate level at a given time after the injection of 1,3-BD. The line extending above and below the circle is the standard error of the mean. The number in parenthesis corresponds to the sample size for that particular circle. The open circle (○) on the vertical axis represents the blood beta-hydroxybutyrate level of animals given saline in place of BD.

II 20 40 60 Time after IV injection (minutes)

(13) **

0.50 1.00 150 2.00

1,3-butanediol (mmoles/mouse)

animals and 0.83 ± 0.15 mM (n = 4) in the IP animals.

Temperature Effects. A statistically significant increase in hypoxic survival time was observed at each of the T_a tested (fig. 6). At the 2 lower T_a the survival time was 550% of control and at the 2 higher T_a 150% of control. Both the control and experimental groups had lower survival times at the higher T_a. When polynomial regression of the cooling curve data (fig. 1) is plotted along with the time course of cooling during hypoxia, no apparent difference in the rate of temperature decrease was observed between control and treated animals (fig. 7). In the table it can be seen that no difference was detected in either initial T_b or body weight in any of the control or experimental groups. The T_b at the onset of hypoxia was lower at a T_b of 12°C only in the treated mice. When individual T_b at the onset of hypoxia is plotted against the corresponding survival time, there appears to be a negative correlation (fig. 8). However, when

FIGURE 4. Each dot represents the average hypoxic survival time of mice receiving intravenous BD 30 min prior to hypoxia. The dashes above and below the dots are the standard error of the mean. The number in parenthesis is the sample size for each group. The number of animals which survived hypoxia 900 sec or more are shown in the boxes above their respective dots. These five individuals were averaged in with their groups as if they had died at 900 sec (* = p < 0.05, ** = p < 0.001 Wilcoxon-Mann-Whitney Rank Sum test).
polynomial regression was done at the first through sixth levels, no $r^2$ greater than 0.5 was obtained.

Diazepam produced a statistically significant increase in hypoxic survival time from 121.5 ± 17.9 to 257.2 ± 40.7 sec at a T$_a$ of 22.5°C. At a T$_b$ of 33.4°C, no difference was detected between control and treated animals (fig. 9).

**Discussion**

In this study blood BHB was intentionally elevated by PO, IV and IP administration of BD. As in previous studies, ketosis was associated with an increased tolerance to hypoxia. The exact mechanism of this protection from hypoxia is obscure, but certain features of the phenomenon are beginning to emerge. There is no simple relationship between hypoxic tolerance and blood BHB. In fasted and experimental diabetic mice, as well as in those treated with BD, the level of BHB in the blood and the duration of survival is not linearly related. Among other things, differences in blood glucose levels between the ketotic groups may account for some of the nonlinearity. The evaluation of T$_a$ and T$_b$ in this response indicates that temperature can modify the protective effect of the ketosis induced by BD (figs. 6 & 8). Neither T$_a$ nor T$_b$, however, can account totally for this protection.

The mechanism we propose, whereby ketosis increases the brain's tolerance to hypoxia, involves the preferential metabolism of ketones, rather than glucose, as an energy substrate, thus minimizing the deleterious accumulation of brain lactic acid during hypoxia. The first requirement for this mechanism to operate is that BHB be available to the brain. The direct measurement of blood BHB indicates that at least the blood contains the necessary increase in ketones. An alternate source of BHB would be the conversion of BD in the brain to BHB. This is possible both because BD is a permeable alcohol and because the brain contains the appropriate enzymes for conversion of BD to BHB. Elevated BHB has been shown to be a negative modifier of brain phosphofructokinase thus establishing the necessary conditions for the preferential metabolism of ketones. This inhibition of glucose breakdown would also minimize the accumulation of lactic acid during hypoxia.

**Table 1.**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Control (n = 20)</th>
<th>Experimental (n = 10)</th>
<th>Control (n = 20)</th>
<th>Experimental (n = 10)</th>
<th>Control (n = 15)</th>
<th>Experimental (n = 15)</th>
<th>Control (n = 20)</th>
<th>Experimental (n = 10)</th>
<th>Control (n = 15)</th>
<th>Experimental (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19-21 °C</td>
<td>37.7 ± 0.2</td>
<td>37.7 ± 0.2</td>
<td>37.4 ± 0.5</td>
<td>37.2 ± 0.3</td>
<td>39.6 ± 0.2</td>
<td>40.2 ± 0.2</td>
<td>39.6 ± 0.2</td>
<td>39.4 ± 0.0</td>
<td>40.3 ± 0.2</td>
<td>40.4 ± 0.2</td>
</tr>
<tr>
<td>21-23 °C</td>
<td>37.9 ± 0.2</td>
<td>36.4 ± 0.3</td>
<td>37.2 ± 0.5</td>
<td>36.3 ± 0.3</td>
<td>39.8 ± 0.2</td>
<td>40.2 ± 0.2</td>
<td>39.7 ± 0.2</td>
<td>40.7 ± 0.2</td>
<td>40.3 ± 0.0</td>
<td>40.3 ± 0.0</td>
</tr>
<tr>
<td>23-25 °C</td>
<td>33.2 ± 1.8</td>
<td>30.9 ± 1.6</td>
<td>32.3 ± 1.4</td>
<td>30.4 ± 1.7</td>
<td>29.0 ± 1.4</td>
<td>29.2 ± 1.0</td>
<td>29.3 ± 1.2</td>
<td>30.0 ± 0.4</td>
<td>30.2 ± 0.4</td>
<td>30.3 ± 0.4</td>
</tr>
<tr>
<td>25-27 °C</td>
<td>765.6 ± 15.4</td>
<td>765.0 ± 26.4</td>
<td>835.4 ± 14.8</td>
<td>835.4 ± 17.1</td>
<td>100.1 ± 4.8</td>
<td>151.6 ± 7.3</td>
<td>151.6 ± 7.3</td>
<td>151.6 ± 7.3</td>
<td>139.8 ± 8.7</td>
<td>139.8 ± 8.7</td>
</tr>
<tr>
<td>27-29 °C</td>
<td>145.7 ± 525.4**</td>
<td>149.4 ± 559.2**</td>
<td>151.4 ± 48.8</td>
<td>159.8 ± 7.9</td>
<td>96.4 ± 7.3</td>
<td>145.0 ± 7.8</td>
<td>145.0 ± 7.8</td>
<td>145.0 ± 7.8</td>
<td>145.0 ± 7.8</td>
<td>145.0 ± 7.8</td>
</tr>
</tbody>
</table>

Values are grouped according to 4 ambient temperatures indicated at the top of each column.

* = p < 0.01 (Student's t-test)  ** = p < 0.001 (Wilcoxon-Mann-Whitney Rank Sum test).
Ketogenic diets have been reported to have an anti-convulsive effect. The results of the current study cannot rule out the possibility that anticonvulsive properties of ketones may likewise be responsible for the increased tolerance to hypoxia. Diazepam is well known for its anticonvulsive properties. The increased hypoxic tolerance seen with diazepam, in our study, was eliminated by the elevations of $T_a$. Unfortunately, BHB levels were not measured in these animals. A similar elevation in $T_a$ reduced, but did not eliminate, the protective effect of BD.

Another possible mechanism of protection might be attributable to a general reduction in cerebral function. The mice fed BD seemed behaviorally normal and those injected either IV or IP seemed more tranquil but still possessed motor capability and pain responses. There is little doubt that an alcohol such as BD could alter cerebral metabolism and/or function.
FIGURE 8. Individual hypoxic survival time is plotted against the corresponding body temperature at the onset of hypoxia. The open circles (○) represent mice treated with BD. The closed circles (●) represent saline control mice. Mice surviving more than 3600 sec of hypoxia are not shown on this graph.

The extent to which such an alteration contributes to the protective effect is currently obscure. It should be noted, however, that ketosis induced by fasting and experimental diabetes is also associated with an increase in hypoxic survival time.

It is concluded that elevated blood BHB is in part correlated with the increased tolerance to hypoxia seen in fasted, diabetic and butanediol-treated mice. Other apparently minor components of this response are the hypothermic effect and a possible anticonvulsive or metabolic effect. Perhaps some of the variability in the relationship between BHB levels and hypoxic survival time can be accounted for by differing blood levels of other substrates and metabolically active hormones. The interaction between BHB and glucagon is the subject of another study.

Acknowledgment

Metabolic substrate analyses were made possible by assistance from the Biochemistry Core Laboratory of the Michigan Diabetes Research and Training Center (USPHS AM20572). The heparin used in this study was graciously supplied by The Upjohn Company, Kalamazoo, MI. The temperature monitoring equipment and temperature control chamber were made available by Dr. M. J. Kluger. We thank Amy Goldfaden for her technical assistance. The secretarial assistance of Ms. Betty Martin and Susan Koch is appreciated.

References

Hypoxic Tolerance Enhanced By β-Hydroxybutyrate-Glucagon in the Mouse

Steven M. Eiger, C.C.S., Jeffrey R. Kirsch, B.S., and Louis G. D'Alecy, D.M.D., Ph.D.

SUMMARY A correlation has been observed between increased blood ketones and the tolerance of mice to hypoxia (4-5% oxygen). In previous studies fasted mice, alloxan diabetic mice and mice given 1,3-butanediol were found to be ketotic and to have increased tolerance to hypoxia. We attempted to induce a similar increase in hypoxic tolerance by direct elevation of blood ketones with IV and IP beta-hydroxybutyrate (BHB). No increase in hypoxic tolerance was observed with BHB alone. Inasmuch as fasting and alloxan diabetes are both associated with elevated blood glucagon (G), hypoxic tolerance tests were made 30 min after G alone or a combination of G plus BHB. The mice given G alone or BHB alone had hypoxic survival times not different from saline controls. The mice given G plus BHB had increased survival times that could not be explained on the basis of a G mediated alteration in blood BHB.

THE TOLERANCE OF MICE to hypoxia has been used by several laboratories as a model of cerebral hypoxia and ischemia.1-3 Increases in hypoxic tolerance have been observed in several studies4-6 when the systemic metabolism produced elevated blood beta-hydroxybutyrate (BHB). In many of the conditions used in these studies elevated blood glucagon (G) would be anticipated. The aim of this study was to determine if direct administration of BHB alone or in combination with G could modify survival time of mice which had been exposed to hypoxia (4-5% oxygen).

Materials and Methods

The animal model used has been previously described by Wilhelmin and Arnfred,2 Steen and Michenfelder4 and used by our laboratory.1 Adult male Sprague-Dawley albino mice (HA-ICR) weighing between 20 and 40 g were pretreated and after 30 min subjected to hypoxia (4-5% O2). The pretreatments involved IV or IP injections of BHB with or without IV G. Corresponding injections of saline were given to each group. Body temperature, ambient temperature, survival time, or blood BHB levels were determined.

Experimental Hypoxia

For each trial, 2 control and 3 experimental animals were tested simultaneously. Each mouse was placed in an airtight, 110 ml flow-through chamber. Five chambers were mounted in parallel and continuously flushed with a gas mixture. The composition of the gas mixture flushing the chambers was continuously monitored with an oxygen analyzer (Beckman OM-14). The ambient temperature was maintained at 22°C. Hypoxia was induced by first flushing the system at 1.7 l/min with an 8% oxygen, balance nitrogen gas mixture. After 20-25 sec the chamber oxygen was reduced to between 4 and 5%, at a flow rate of 1.3 l/min. The exact level of oxygen in the chambers was recorded for each trial. The survival...
Butanediol induced ketosis increases tolerance to hypoxia in the mouse.
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