Elevated Blood Ketone and Glucagon Levels Cannot Account for 1,3-Butanediol Induced Cerebral Protection in the Levine Rat

Edward F. Lundy, Lawrence D. Klima, Thomas S. Huber, Gerald B. Zelenock, and Louis G. D’Alecy

1,3-Butanediol is an ethanol dimer that induces systemic ketosis. It has previously been shown to increase hypoxic survival time and reduce neurologic deficit in several experimental preparations. The aim of this study was to determine if the mechanism of 1,3-butanediol-induced cerebral protection was elevation of blood ketone levels, blood glucagon levels, or both. Blood β-hydroxybutyrate levels, glucagon levels, or both produced by a previously reported protective dose of 1,3-butanediol (47 mmol/kg) were simulated by direct i.v. infusion of the ketone β-hydroxybutyrate and glucagon separately and in combination, and the effect on hypoxic survival time in instrumented Levine rats (unilateral carotid ligation and hypoxic exposure) was determined. To test if the mechanism was a direct or osmotic effect of the alcohol, an equimolar dose of ethanol (47 mmol/kg) was administered and the effect on hypoxic survival time was compared with that produced by 1,3-butanediol. As in previous studies, 1,3-butanediol significantly increased hypoxic survival time (241% of control, Scheffe p<0.05). Various doses of β-hydroxybutyrate and glucagon were infused to approximate the blood levels of β-hydroxybutyrate and glucagon produced by a protective dose of 1,3-butanediol. Although β-hydroxybutyrate or glucagon infusions produced blood levels of these substances that were comparable with those produced by administering butanediol, they failed to prolong hypoxic survival time as long as 1,3-butanediol. No correlation was detected between hypoxic survival time and blood levels of β-hydroxybutyrate, glucagon, insulin, or glucose. An equimolar dose of ethanol did not significantly increase hypoxic survival time. Neither simple elevation of blood ketones or glucagon nor a direct or osmotic effect of the alcohol can explain the protective effects of 1,3-butanediol. (Stroke 1987;18:217–222)
Briefly, with rats under halothane anesthesia (2%, chamber induction, mask maintenance) the right carotid artery and right jugular vein were cannulated with saline-flushed polyethylene (PE-50) tubing. Catheters were inserted 2–2.5 cm, secured to the cervical musculature, and passed through a subcutaneous tunnel to the nape of the neck.

Three electroencephalographic leads were placed after shaving and incising the skin over the dorsal skull. Overlying connective tissue was then cleared and small burr holes were drilled, one 2 mm caudal to the anterior suture, another 2 mm lateral to the sagittal suture, and a third 2 mm cephalad to the posterior suture and 2 mm lateral to the sagittal suture on the right dorsal skull. Self-tapping 3/16-in. stainless steel screws, silver soldered to 30-gauge multistranded stainless steel wire, were inserted into the burr holes and 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 2-hour recovery period was allowed.

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% O₂ (room air) or 4.5% O₂. There were two exhaust ports in the posterior stopper through which EEG leads, vascular cannulas, and the rat’s tail were passed. The tail was secured with tape. The rat’s body temperature was monitored with a rectal probe. The chamber environment was continuously monitored using a oxygen/temperature analyzer (YSI 2600), and chamber temperature was maintained at 30°C with a heat lamp. Vascular cannulas 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. At the end of each experiment the animal was quickly removed from the chamber and decapitated to obtain blood for β-hydroxybutyrate, glucagon, insulin, and glucose determinations.

To establish baseline survival times and blood levels, 6 rats received 1,3-butanediol (47 mmol/kg i.v., total volume = 0.54 ml) 30 minutes before hypoxic exposure and 4 rats received an equivalent volume of saline 30 minutes before hypoxic exposure. In 4 previous studies this dose of 1,3-butanediol was found to be protective. In both groups survival time and blood β-hydroxybutyrate, glucagon, insulin, and glucose levels were determined. Various doses of β-hydroxybutyrate, glucagon, or both were then administered to test groups in an attempt to reproduce those blood levels produced by 1,3-butanediol. Because of the short half-life of β-hydroxybutyrate and glucagon and to maintain a constant elevation of β-hydroxybutyrate, we began an infusion (Harvard Infusion Pump Model 945) 15 minutes prior to the onset of hypoxia and continued it throughout the hypoxic exposure. The β-hydroxybutyrate and glucagon were diluted in distilled water. Seven dosage combinations were administered (Table 1). To control volume, flow was limited to either 0.077 or 0.3 ml/min. For each group the concentration of β-hydroxybutyrate or glucagon was adjusted to deliver the approximate desired dose. Because of slight variations in animal weights the dose per kilogram varied slightly (Table 1). Volume controls were produced by infusion of normal saline at either 0.077 ml/min (n = 6) or 0.3 ml/min (n = 19). To test whether the mechanism of protection was a direct effect of the alcohol or an osmotic effect, an equal molar dose per kilogram of ethanol (47 mmol/kg; total volume 0.54 ml) was administered intravenously 30 minutes before hypoxia and survival time was measured.

Statistical analysis was performed using the Michigan Interactive Data Analysis System (MIDAS) on an Amdahl 470/V6 computer. All values are expressed as mean ± SEM; sample size is designated n. One-way analysis of variance (ANOVA) was used to compare survival time, temperature, mean arterial blood pressure, heart rate, to isoelectric EEG, and ketone, glucagon, insulin, and glucose blood levels. Temperature, blood pressure, and heart rate were compared before and at onset of hypoxia, 5, 10, 15, 20, and 25 minutes into the experiment, at isoelectric EEG, and at the end of the experiment. Where appropriate, groups were compared using Scheffe 95% confidence intervals; if the difference was significant it was reported at the 0.05 level. Linear regression analyses were performed by the method of least squares.

### Results

Because no significant differences were detected in temperature, blood pressure, heart rate, time to isoelectric EEG, and hypoxic survival time among the 3 control groups receiving saline (0.3 ml/min, 0.077 ml/min, and bolus), they were combined and considered a single control group for subsequent analysis. Hypoxic survival time was significantly increased from 945 ± 76 seconds for the control group to

### Table 1. Actual Infusion Doses (mean ± SEM) of β-Hydroxybutyrate (BHB) and Glucagon, Separately or in Combination, and the Flow Rate for the 7 Different Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Flow (cc/min)</th>
<th>Dose BHB (mg/kg/min)</th>
<th>Dose Glucagon (mg/kg/min)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHB 5</td>
<td>0.077</td>
<td>4.6 ± 0.1</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>BHB 15</td>
<td>0.3</td>
<td>15.4 ± 1.9</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td>Glucagon 2</td>
<td>0.077</td>
<td>—</td>
<td>2.1 ± 0.1</td>
<td>6</td>
</tr>
<tr>
<td>Glucagon 8</td>
<td>0.3</td>
<td>—</td>
<td>8.4 ± 0.7</td>
<td>17</td>
</tr>
<tr>
<td>BHB 5 and glucagon 2</td>
<td>0.077</td>
<td>4.6 ± 0.6</td>
<td>2.4 ± 0.3</td>
<td>9</td>
</tr>
<tr>
<td>BHB 4 and glucagon 8</td>
<td>0.077</td>
<td>4.1 ± 0.1</td>
<td>8.4 ± 0.2</td>
<td>7</td>
</tr>
<tr>
<td>BHB 15 and glucagon 8</td>
<td>0.3</td>
<td>16.8 ± 3.3</td>
<td>8.6 ± 0.3</td>
<td>13</td>
</tr>
</tbody>
</table>
Table 2. Hypoxic Survival Time and Blood Levels of β-Hydroxybutyrate, Glucagon, Insulin, and Glucose

<table>
<thead>
<tr>
<th>Group</th>
<th>Hypoxic survival time (sec)</th>
<th>Blood BHB level (mM)</th>
<th>Blood glucagon level (pg/ml)</th>
<th>Blood insulin level (μU/ml)</th>
<th>Blood glucose level (mg%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>945 ± 76</td>
<td>0.25 ± 0.17</td>
<td>464.6 ± 53.6</td>
<td>59.9 ± 19.0</td>
<td>170.3 ± 8.1</td>
</tr>
<tr>
<td>BD 47</td>
<td>2280 ± 235</td>
<td>0.91 ± 0.08</td>
<td>800.3 ± 141.5</td>
<td>103.6 ± 28.9</td>
<td>178.0 ± 28.6</td>
</tr>
<tr>
<td>BD 20</td>
<td>1464 ± 234</td>
<td>0.59 ± 0.06</td>
<td>1403.8 ± 603.1</td>
<td>239.0 ± 61.2</td>
<td>144.5 ± 13.3</td>
</tr>
<tr>
<td>BHB 5</td>
<td>943 ± 254</td>
<td>1.96 ± 0.36</td>
<td>441.8 ± 181.1</td>
<td>118.7 ± 43.4</td>
<td>147.3 ± 10.5</td>
</tr>
<tr>
<td>BHB 15</td>
<td>772 ± 88</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Gluc 2</td>
<td>845 ± 193</td>
<td>0.25 ± 0.34</td>
<td>474.4 ± 162.7</td>
<td>95.5 ± 36.6</td>
<td>159.8 ± 11.8</td>
</tr>
<tr>
<td>Gluc 8</td>
<td>934 ± 96</td>
<td>0.26 ± 0.21</td>
<td>1601.8 ± 631.2</td>
<td>7.3 ± 1.7</td>
<td>199.0 ± 13.2</td>
</tr>
<tr>
<td>BHB 5 and gluc 2</td>
<td>1291 ± 224</td>
<td>1.52 ± 0.18</td>
<td>491.6 ± 106.8</td>
<td>82.0 ± 21.9</td>
<td>139.8 ± 18.5</td>
</tr>
<tr>
<td>BHB 4 and gluc 8</td>
<td>711 ± 79</td>
<td>1.63 ± 0.10</td>
<td>377.7 ± 88.7</td>
<td>16.9 ± 4.3</td>
<td>152.7 ± 16.1</td>
</tr>
<tr>
<td>BHB 15 and gluc 8</td>
<td>947 ± 87</td>
<td>6.27 ± 0.40</td>
<td>1120.9 ± 229.9</td>
<td>6.4 ± 1.0</td>
<td>199.4 ± 25.1</td>
</tr>
<tr>
<td>ETOH 47</td>
<td>1313 ± 200</td>
<td>0.41 ± 0.05</td>
<td>—</td>
<td>—</td>
<td>94.7 ± 15.1</td>
</tr>
</tbody>
</table>

BD 47 = 1,3-butanediol bolus at 47 mmol/kg; BD 20 = 1,3-butanediol bolus at 20 mmol/kg; BHB 5 = β-hydroxybutyrate infusion at 4.6 mg/kg/min; Gluc 2 = glucagon infusion at 2 ng/kg/min; Gluc 8 = glucagon infusion at 8 ng/kg/min; BHB 5 and gluc 2 = β-hydroxybutyrate infusion at 5 mg/kg/min and glucagon infusion at 2 ng/kg/min; BHB 4 and gluc 8 = β-hydroxybutyrate infusion at 4 mg/kg/min and glucagon infusion at 8 ng/kg/min; BHB 15 and gluc 8 = β-hydroxybutyrate infusion at 15 mg/kg/min and glucagon infusion at 8 ng/kg/min; ETOH 47 = ethanol bolus at 47 mmol/kg.

2280 ± 235 seconds for the 1,3-butanediol-treated animals (Scheffe p < 0.05).

Blood β-hydroxybutyrate, glucagon, insulin, and glucose levels associated with this protective dose were determined (Table 2). The various doses of β-hydroxybutyrate, or glucagon, or both produced blood levels comparable to those associated with 1,3-butanediol-induced protection (Figure 1). However, none of the groups receiving these infusions had a significant increase in hypoxic survival time (Figure 2). When hypoxic survival time is related to blood levels of β-hydroxybutyrate (Figure 3), glucagon (Figure 4), insulin (Figure 5), and glucose (Figure 6), it is clear that blood levels approximately those produced by 1,3-butanediol did not significantly increase hypoxic survival time like 1,3-butanediol.

We were unable to detect any significant correlation between hypoxic survival time and temperature, arterial blood pressure, and heart rate. The rats given 1,3-butanediol had increased hypoxic survival time and elevated blood ketones; however, regression analysis indicated that there was no significant correlation between hypoxic survival time and blood levels of the substrates β-hydroxybutyrate and glucose or the hormones glucagon and insulin.

An equal molar dose of ethanol (47 mmol/kg) did not significantly increase hypoxic survival time when compared with that of control animals (Scheffe or Student's t test with Bonferroni correction). The 1,3-butanediol-treated animals had a significantly increased (Scheffe p < 0.05) hypoxic survival time compared with those receiving ethanol (47 mmol/kg).

Discussion

As in previous mouse and rat studies, 1,3-butanediol significantly increased hypoxic survival time. Multiple doses of β-hydroxybutyrate, glucagon, and β-hydroxybutyrate plus glucagon were infused and the blood levels of β-hydroxybutyrate and glucagon produced by a protective dose of 1,3-butanediol were mimicked. Despite effectively bracketing the ketone or glucagon levels, no group exhibited a similar rise in hypoxic survival time compared with the group receiving 1,3-butanediol (Figures 2, 3, and 4). In addition, no significant correlation was detected between blood levels of β-hydroxybutyrate or glucagon and hypoxic survival time. The data therefore refute our original hypothesis that 1,3-butanediol provides cerebral pro-
Figure 2. The effects of various treatments on hypoxic survival time (seconds). *p = 0.05. BD47 = 1,3-butandiol 47 mmol/kg; BD20 = 1,3-butandiol 20 mmol/kg; BHB5 = β-hydroxybutyrate 5 mg/kg/min; BHB15 = β-hydroxybutyrate 15 mg/kg/min; G2 = glucagon 2 ng/kg/min; G8 = glucagon 8 ng/kg/min; BHB5 G2 = β-hydroxybutyrate 5 mg/kg/min and glucagon 2 ng/kg/min; BHB4 G8 = β-hydroxybutyrate 4 mg/kg/min and glucagon 8 ng/kg/min; BHB15 G8 = β-hydroxybutyrate 15 mg/kg/min and glucagon 8 ng/kg/min; ETOH47 = ethanol 47 mmol/kg.

Figure 3. β-Hydroxybutyrate blood levels (mM) are plotted against hypoxic survival time. Regardless of blood β-hydroxybutyrate levels no group exhibited hypoxic survival times similar to butandiol at 47 mmol/kg. 1 = saline; 2 = BD 20 mmol/kg; 3 = BHB 5 mg/kg/min; 4 = glucagon 2 ng/kg/min; 5 = glucagon 8 ng/kg/min; 6 = BHB 5 mg/kg/min and glucagon 2 ng/kg/min; 7 = BHB 4 mg/kg/min and glucagon 8 ng/kg/min; 8 = BHB 15 mg/kg/min and glucagon 8 ng/kg/min; 9 = ethanol 47 mmol/kg.

Figure 4. Glucagon blood levels (pg/ml) are plotted against hypoxic survival time. Regardless of blood glucagon levels no group exhibited hypoxic survival times comparable to butandiol at 47 mmol/kg. 1 = saline; 2 = BD 20 mmol/kg; 3 = BHB 5 mg/kg/min; 4 = glucagon 2 ng/kg/min; 5 = glucagon 8 ng/kg/min; 6 = BHB 5 mg/kg/min and glucagon 2 ng/kg/min; 7 = BHB 4 mg/kg/min and glucagon 8 ng/kg/min; 8 = BHB 15 mg/kg/min and glucagon 8 ng/kg/min.

Protection solely by elevating blood β-hydroxybutyrate and/or glucagon levels.

Alcohols, specifically ethanol, have been reported to have cerebral protective actions. However, ethanol did not significantly increase hypoxic survival time although the average survival time of the ethanol group tended to be higher than that of the saline controls. Butanediol-treated animals had a significantly longer hypoxic survival time compared with either the ethanol group or saline control group. Equimolar doses of butanediol and ethanol were chosen to match osmotic effects and because other studies have shown that, at equivalent doses, the 2 alcohols produce similar neurologic effects. Data from this study are consistent with data from other studies that indicate some cerebral protective effect with ethanol; however, our data also indicate that the protective effects of 1,3-butandiol cannot be completely explained by direct effects of the alcohol.

Hypothermia has long been known to provide cen-
nal nervous system protection.11,12 A suggested mechanism of 1,3-butanediol-induced cerebral protection is a possible alcohol-induced hypothermia. To minimize hypothermia as a contributing factor we controlled body temperature by maintaining chamber temperature at 30°C. Thirty degrees was chosen to reduce heat loss due to possible alcohol-related vasodilation. The chamber temperature was also well within the thermoneutral zone of the rat.13 We found no significant differences in the deep rectal temperature among the groups before the onset or 5, 10, 20, or 25 minutes after the onset of hypoxia. Also no significant correlation could be detected between temperature and survival time at any of the above times. These data agree with those of previous studies using mice and rats that indicated that the protective effects of 1,3-butanediol could not be fully explained by changes in body temperature.13

To minimize the volume infused, the time required to achieve constant blood β-hydroxybutyrate levels was determined. It required approximately 10 minutes to achieve stable blood levels. Therefore, we began infusions 15 minutes before hypoxic exposure.

To account for possible volume effects, 3 control groups receiving saline were used. No significant difference in hypoxic survival time among the volume controls was detected. This agrees with previous work1 and indicates that volume effects cannot explain the observed protection.

The association between increased blood levels of β-hydroxybutyrate and increased hypoxia–ischemia tolerance led us to hypothesize a causal link between these correlated variables. While this study indicates that an acute elevation (minutes before the insult) of blood ketone or glucagon levels is not sufficient to provide cerebral protection, it does not eliminate enhanced brain ketone metabolism as a component of the protective mechanism of 1,3-butanediol.

An induction period may be required before the brain can maximally transport and metabolize ketones. Several studies have indicated that β-hydroxybutyrate crosses the blood–brain barrier by carrier-mediated facilitated diffusion14,15 and that this transport mechanism is induced by starvation.16,17 After 5 days of starvation the transport rate of β-hydroxybutyrate was twice the rate expected if no induction had occurred.14 It has also been shown that the activity of β-hydroxybutyrate dehydrogenase, an enzyme required for ketone utilization, is increased eightfold after 72 hours of starvation.17 Such studies may explain why fasted or diabetic mice or mice fed a ketogenic diet, all of which have chronically elevated blood ketones, demonstrated increased tolerance to hypoxia.13

In contrast to acute ketone infusions, the ketone precursor 1,3-butanediol may be effective without an induction period. Like other alcohols, butanediol is lipophilic, and passively diffuses across the blood–brain barrier.18 Therefore, unlike β-hydroxybutyrate, which requires time for induction of blood–brain barrier transport mechanisms, transport requirements may be eliminated with 1,3-butanediol. Once across the blood–brain barrier, butanediol can be rapidly converted to β-hydroxybutyrate by alcohol and aldehyde de-
hydrogenases in the brain. In a brain slice study where absence of the blood–brain barrier would permit free ketone access to neurons, it has been reported that hypoxia induces preferential use of $\beta$-hydroxybutyrate.\textsuperscript{7}

The potential therapeutic efficacy of 1,3-butanediol has been repeatedly demonstrated in a variety of experimental models. It increased the hypoxic survival time of mice\textsuperscript{1,2} and rats\textsuperscript{3} and decreased neurologic deficits of Levine rats.\textsuperscript{4} The precise mechanisms of 1,3-butanediol protective action remains to be delineated.

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Key Words • stroke • ketosis • brain protection • butanediol • $\beta$-hydroxybutyrate • glucagon
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