The Role of Ethanol in Diluents of Drugs that Protect Mice from Hypoxia*

M.M. Moursi, B.A. Luyckx, and L.G. D’Alecy, Ph.D.

SUMMARY This study evaluates the hypothesis that ethanol alone, or in diluents for drugs used to protect hypoxic mice, is responsible in part for an increased tolerance to hypoxia (4-5% oxygen). The change in hypoxic tolerance following i.v. or i.p. administration of ethanol, diazepam, nimodipine and various diluent components was measured. Diazepam (50 mg/kg i.v.) increased hypoxic tolerance to 700 ± 47% (n = 11) of saline control, its diluent increased hypoxic tolerance to 468 ± 60% (n = 10) of saline control but the ethanol component of the diluent accounted for almost half of this diluent effect. Nimodipine (2 mg/kg i.p.), a calcium antagonist, increased tolerance to 180 ± 18% of control (n = 19) and nimodipine diluent showed an even greater increase to 226 ± 25% of control (n = 15). In this case essentially all of the protective effect of nimodipine diluent (81.3%) is accounted for by ethanol. Dose response curves indicate the maximum ethanol induced increase in hypoxic tolerance was approximately 335% of control at a dose of 2.4 g/kg. Buffers, etc. in the diluents evidently add to the protective effect of ethanol. Our data clearly indicate ethanol is the important component of some treatments which protect mice from hypoxia. The pharmacological activity of ethanol, even when used in a diluent, should not be ignored in evaluating therapeutic intervention for protection from hypoxia.

THE INCREASED SURVIVAL TIME in mice subjected to hypoxia has been investigated in several laboratories to identify procedures which may be of therapeutic value in cerebral hypoxia. The importance of these experiments stems from the high morbidity and mortality associated with cerebral hypoxia. The brain appears to be particularly sensitive to hypoxia presumably because of its high metabolic rate, relatively small stores of high energy phosphates and glucose as well as a relatively low capillary density. Although the cause of death or sequence of events leading to death in the hypoxic mouse model have not been established there is evidence to suggest that during hypoxia the cessation of spontaneous ventilation results from reduced brain activity. Recent work in our laboratory using a Levine rat preparation, which includes on-line monitoring of blood pressure, heart rate, respiratory rate, central venous pressure and electroencephalogram, indicates that loss of cerebral electrical activity invariably precedes loss of spontaneous respiration and ultimate loss of cardiac activity when an animal is exposed to hypoxia. In contrast it has been shown that cardiac function is maintained during hypoxia because coronary blood flow is increased by local mechanisms in order to maintain a relatively constant myocardial oxygen tension. Clinically, hypoxia may be encountered at high altitudes, during childbirth, deep-sea diving, as a complication during surgery, with a sedative overdose, or because of carbon monoxide poisoning. Furthermore, hypoxia has been identified as an avoidable extracranial factor associated with apnea due to head injury which, when controlled, could prevent severe hypoxic brain damage and ultimately minimize mortality. In each of these conditions the cerebral hypoxic damage is probably caused by lactic acid accumulation in the brain tissue which can lead to altered membrane structure and function, breakdown of blood brain barrier and to brain edema.

A variety of compounds are being evaluated as possible therapeutic agents for hypoxia. Our laboratory, for example, has shown that pretreatment with 1,3-butanediol (BD), a non-toxic ethanol dimer, significantly increases the tolerance of mice to hypoxia. Diazepam, presently administered as a therapeutic intervention in treating ischemia or hypoxia, reportedly provides this effect through its anticonvulsant properties and thereby presumably increases hypoxic tolerance by decreasing the brain’s oxygen requirements. It has been hypothesized that nimodipine, a calcium antagonist, may protect the brain from hypoxic damage because of its ability to increase cerebral blood flow. In evaluating the diluents of these potentially therapeutic agents (nimodipine and diazepam) it became apparent that ethanol was a common component to each. The present study, therefore was designed to test the hypothesis that ethanol alone, or in diluents of these drugs plays an important role in increasing the tolerance of the brain to hypoxia.

Materials and Methods

The animal model used has been previously described by Wilhjelm and Arnfred, Steen and Michenfelder, and used by our laboratory. White, male, albino, Sprague-Dawley, mice (HA-ICR) weighing between 20 and 40 g (free access to food and water) were exposed to premixed gases (4.62 and 8.61% oxygen) with varying intervals between pretreatment and hypoxia. The pretreatment involved i.v. or i.p. injection of saline; ethanol; nimodipine; diazepam; complete diluents of diazepam and nimodipine; as well as incomplete diluent formulations without the citrate, propylene glycol 400 or ethanol.
Table 1 Components of Drug Diluents

<table>
<thead>
<tr>
<th></th>
<th>Diazepam</th>
<th>Nimodipine</th>
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</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>10.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.5</td>
<td>—</td>
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<tr>
<td>Nimodipine</td>
<td>—</td>
<td>0.01</td>
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<tr>
<td>Water</td>
<td>38.0</td>
<td>69.8</td>
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<tr>
<td>Propylene glycol 400</td>
<td>40.0</td>
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<tr>
<td>Sodium benzoate</td>
<td>5.0</td>
<td>—</td>
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<tr>
<td>Benzoic acid</td>
<td>5.0</td>
<td>—</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>1.5</td>
<td>—</td>
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<tr>
<td>Polyethylene</td>
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<td>15.0</td>
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<tr>
<td>Sodium citrate</td>
<td>—</td>
<td>0.20</td>
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<tr>
<td>Citric acid</td>
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<td>0.03</td>
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Diazepam and nimodipine are both dissolved in diluents which contain ethanol as well as buffers. Components are given as percent weight to volume.

Pretreatment

Each injection was either i.p. (0.50 ml) or i.v. (0.25 ml via a 26 gauge needle into the lateral tail vein) and administered prior to the onset of hypoxia. The compounds studied were ethanol, diazepam (Valium-Roche Laboratories), nimodipine (Bay e 9736), diazepam diluent, nimodipine diluent (the diluent compositions are given in table 1) and incomplete diluent mixtures. Ethanol was diluted by volume with either normal saline or distilled water to obtain dose ranges of 0.2–2.1 mmol/mouse i.v. and 0.2–4.2 mmol/mouse i.p. Diazepam, which has been shown to provide protection in this model, was administered i.v. at a dose of 50 mg/kg. Tests on various diluent combinations of ethanol, water, citrate and propylene glycol 400 were made in an attempt to determine which of these components of the nimodipine diluent could account for the protective effect observed (see table 2). Incomplete diluents were prepared on the basis of concentrations given on the manufacturer information sheets; nimodipine diluent was supplied by the manufacturer. All injections were given 30 minutes prior to hypoxia. In a separate series of experiments nimodipine was injected i.p. at a dose of 2 mg/kg 0, 5, 15, 22.5, 30, and 45 minutes before hypoxia to determine which time interval between injection and hypoxia produced the maximum protective effect.

Experimental Hypoxia

For each trial, 2 saline injected and 3 drug pretreated 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 compressed air or premixed gas. The importance of simultaneously testing control and treated mice was recently restated in a critical assessment of this animal model by Artru and Michenfelder. The diurnal variation in hypoxic tolerance reported by Stufel et al. also focuses on the need for simultaneous testing of control and treated animals. Further, to assure the comparability of separate runs the composition of the gas mixture flushing the test chambers was continuously monitored with an oxygen analyzer (Beckman OM-14). The ambient temperature remained relatively constant at 21–23°C. Initially the mice were placed in air flushed chambers (20.95% oxygen). Hypoxia was induced by flushing the system for 20–25 seconds at 3.6 l/min with premixed 8.6% oxygen followed by 4.62% oxygen at 3.6 l/min. Survival time, as used in this and previous studies, refers to the time from onset of 4.62% oxygen exposure to the cessation of spontaneous ventilation. Individuals alive after 15 minutes were considered to be survivors and were averaged with the group using a hypoxic survival time value of 15 minutes. It should be pointed out that death by hypoxia is distinct from asphyxia in that the individual loses consciousness having shown no signs of discomfort. Once unconscious, a brief convulsion precedes cessation of spontaneous ventilation and ultimately death.

Data Analysis

Statistical analysis was performed with the aid of Michigan Interactive Data Analysis System on an Amdahl 470V/8 computer. Comparison of survival time of each experimental group with a control group was done by Student's t-test. All values are expressed as means ± one standard error of the mean; the sample size is designated within parentheses.

Table 2 Effect of Nimodipine and Diluent Components on Hypoxic Tolerance

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<tr>
<td>Nimodipine</td>
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<td>Citrate</td>
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<td>Saline</td>
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<td>Water</td>
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<td>Ethanol</td>
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<tr>
<td>Hypoxic survival</td>
<td>129.9</td>
<td>93.4</td>
<td>194.2</td>
<td>223.0</td>
<td>402.7</td>
<td>316.9</td>
<td>253.2</td>
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<td>±22.4</td>
<td>±22.9</td>
<td>±29.5</td>
<td>±20.1</td>
<td>±29.8</td>
<td>±34.5</td>
<td>±25.7</td>
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<td>(16)</td>
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Plus symbols (+ + +) indicate the presence of components in the test diluents or drug. Hypoxic survival time is reported as seconds of survival ± one standard error of the mean. Numbers in parentheses indicate sample size.
Drugs and their diluents were given either i.v. or i.p. both modes of administration of ethanol had to be tested. In figure 5, a dose-response relationship for i.v. versus i.p. injections of ethanol in

are compared to ethanol alone at a concentration (see table 1) equivalent to that in the diluents. In the case of diazepam the ethanol in the diluent, when given alone, can account for 28.6% of the protective effect and the complete diluent can account for 67.0% of the total effect. The situation is very different with Nimodipine, its diluent or simply the ethanol in the diluent produce increased survival times which were not significantly different from one another yet each was different from saline control. This suggests that nimodipine does not add to the protective effects of ethanol.

In an attempt to further clarify which components of these diluents, other than ethanol, were important in hypoxic protection, citrate, water and saline combinations were tested. The data in table 2 indicates that there is a protective effect of ethanol and citrate but propylene glycol 400 does not appear to be protective. In figure 4, dose-response relationships for ethanol in citrate, water, or saline indicate, in each case, a dose-dependant increase in survival time reaching a maximum at a dose of approximately 1.6 mmol/mouse. The maximum protection by ethanol in water (308% of control) was greater than the maximum for ethanol in saline (211% of control). At 1.3 to 1.7 mmol/mouse the citrate and water solutions both showed a statistically significant (p < 0.01) increase over ethanol in saline solutions.

Because the drugs and their diluents were given either i.v. or i.p. both modes of administration of ethanol had to be tested. In figure 5, a dose-response relationship for i.v. versus i.p. injections of ethanol in

Results

The major result of this study is that ethanol prolongs hypoxic survival in mice. This potentially therapeutic effect was identified when testing the diluents of diazepam and nimodipine. The principle active component of the diluents appears to be ethanol which when given alone, either i.v. or i.p., offers significant protection from hypoxia.

Figure 1 shows a statistically significant (p < 0.01) increase in hypoxic survival time from 110 ± 11 (saline treated) to 773 ± 52 seconds (6 mice surviving past 15 minutes) when diazepam (50 mg/kg) is given 30 minutes prior to hypoxia. The ethanol-containing diluent for diazepam, the composition of which is given in table 1, shows a statistically significant (p < 0.01) increase in survival time to 518 ± 66 seconds (1 mouse surviving past 15 minutes).

In figure 2 an increase in survival time following the injection of 2 mg/kg nimodipine is presented at 5, 15, 22.5, 30, and 45 minutes after injection. This figure shows that nimodipine produced a significant increase from control at 3 of the 6 times tested and waiting 30 minutes to the onset of hypoxia results in the maximum protective effect (180% of saline controls). The ethanol-containing diluent for nimodipine produced a statistically significant (p < 0.01) increase from saline control at each time point reaching a maximum at 5 minutes post-injection. In addition, the nimodipine diluent increased (p < 0.05) hypoxic tolerance over nimodipine, as supplied by the manufacturer, in four of the five time points tested.

In figure 3 hypoxic survival time is given as percent of saline control for diazepam, nimodipine, their diluents and ethanol. The diluents of these two compounds
DILUENTS AND ETHANOL vs. HYPOXIC SURVIVAL TIME

FIGURE 3. This bar graph shows the relative contribution of ethanol to the change in hypoxic survival time seen with diazepam (given i.v.) and nimodipine (given i.p.) [E on the ethanol bars indicates that the value was extrapolated from the ethanol dose response curves]. Data are normalized to percent of corresponding saline controls. Two stars indicate $p < 0.01$. Numbers in parentheses indicate sample size.

water are compared. The maximum effects (385 sec i.v.; 362 sec i.p.) achieved with each route of administration are similar; however the i.p. dose (3.14 mmol/mouse) needed to reach this maximum survival time is more than twice that of the i.v. dose (1.56 mmol/mouse).

Discussion

Our laboratory has demonstrated that an ethanol dimer, 1,3-butanediol is more effective in protecting mice from hypoxia than diazepam and more effective than the protection reported for barbiturates. Hypoxic survival time in mice is used by numerous investigators as a model for cerebral hypoxia to evaluate the efficacy of a variety of compounds in anticipation of identifying materials that could be considered clinically useful in protecting the brain from hypoxic damage. The assumption in all studies using this model is that the hypoxic insult involves the brain first and only secondarily cardiac function. Clinically, hypoxia is associated with drowsiness, disorientation and loss of consciousness, respiratory stimulation followed by respiratory depression and apnea. If the respiratory depression or apnea is not counteracted by artificial ventilation then the hypoxia will progress to anoxia and cardiovascular collapse. The precipitating factor in either event is the cessation of ventilation due to inadequate oxygen supply and loss of brain function. The protection offered by these compounds must in part counteract this loss of brain function due to hypoxia. This does not exclude the possibility that these same agents might also be of potential benefit to the myocardium under similar conditions.

The new information in this study is not the identification of yet another compound to be considered, but drawing attention to a material so commonly used that its pharmacological activity is often ignored. Emerson in 1942 was apparently the first to describe the protection from hypoxia provided by ethanol. We have confirmed this and have shown that two unrelated compounds, diazepam and nimodipine, both of which protect the hypoxic brain do so in part because of the ethanol in their diluents. Indeed, in the case of nimodipine, the compound actually appears to have no effect on survival time since nimodipine treated mice had survival times not different from those of mice treated with ethanol alone.

The major weakness in this study is that no mechanistic explanation for the increase in hypoxic survival time is identified. It is, however, the descriptive nature of the study that is of particular importance. Clearly any attempt to identify a mechanism of action for diazepam or nimodipine, in protecting the brain from hypoxia, would be misguided if the pharmacological activity of ethanol were to be ignored. Thus, although we do not understand the mechanism or mechanisms re-

DOSE RESPONSE OF ETHANOL vs. HYPOXIC SURVIVAL TIME

FIGURE 4. These curves show an increased tolerance to hypoxia with treatment by ethanol diluted in citrate, water or saline. Ethanol dissolved in saline is less protective than either the citrate or water solutions. All points without NS or stars are different than saline control groups ($p < 0.01$) by Student-t test. Numbers in parentheses indicate sample size.
sponsible for the increase in tolerance to hypoxia during diazepam, nimodipine or ethanol administration, the increases in survival time is evidently heavily dependent on the alcohol. In that 1,3-butanediol is a dimer of ethanol and is also effective in protecting the brain we offer some speculation as to possible common modes of action of these two alcohols.

With ethanol, as with 1,3-butanediol, a clear dose response relationship of increasing hypoxic tolerance with increasing dose is obtained. Both produce peripheral vasodilation and hypothermia which could lower whole body metabolism and produce a generalized hypothermia which could lower metabolic rate. However, the hypothermic effect of butanediol accounts for only part of the protective effect of this alcohol. Alternatively, the generalized central nervous system anticonvulsant and depressant effects of alcohols could contribute to the protective effect by a direct reduction of brain oxygen demand and function. This mechanism was explored in preliminary studies which indicate that the effect of 1,3-butanediol on brain tissue metabolites is not similar to that seen with other central nervous system depressants like barbiturates or ethanol.

Another potentially significant similarity in the biological effects of ethanol and its dimer, 1,3-butanediol is that both compounds have been reported to produce a systemic ketosis. Based on studies in our and other laboratories we have developed a hypothesis which contends that ketosis increases the brain’s tolerance to hypoxia, by shifting the energy producing substrate metabolism towards ketone utilization thus minimizing the deleterious accumulation of brain lactic acid during hypoxia. The current data does not test this hypothesis but is consistent with previous data which led to its development.

The effects of the citrate buffer were not as dramatic as the ethanol effects however it is interesting to note that some protection was offered. With the present data there is no way to distinguish blood buffering, tissue buffering or osmotic effects from some more complex involvement of citrate in brain metabolism. Under conditions of head or spinal cord injury the use of ethanol or related alcohols to protect from hypoxic injury may be contraindicated in that more extensive damage has been reported in animals pretreated with these compounds. Our results might therefore be more relevant to hypoxia uncomplicated by central nervous system trauma.

In conclusion our data confirm that of earlier studies by indicating that ethanol offers significant protection from hypoxia. By contributing to the apparent protection offered by currently used therapeutic agents, ethanol has undoubtedly confounded the interpretation of their mechanism of action. Although the specific mechanism of action has not been fully established the similarities between ethanol and BD suggest that this family of compounds may protect the hypoxic brain through a common mode of action.

Acknowledgment
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