Hypoxic Tolerance Enhanced By \(\beta\)-Hydroxybutyrate-Glucagon in the Mouse

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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 increased 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. Increases in hypoxic tolerance have been observed in several studies 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 Wilheljm 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 after 30 min subjected to hypoxia (4–5% \(O_2\)). 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
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.

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-constantin) 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).

Pretreatment

In the first series of mice BHB was given IV at 12 mg/mouse. This dose was selected on the estimation that 12 mg of BHB distributed evenly and instantaneously throughout the extracellular fluid of a mouse would yield 10–15 mM BHB. G was given IV at 2.85 mg/mouse. In the IP series injections of 30, 60 and 120 mg/mouse were used to obtain as high as possible blood BHB with a single injection. In the IP series G was given IV at 4 mg/mouse. All IV and IP pretreatments were given in 0.25 ml volumes.

Blood BHB Analysis

Representative mice from each group were treated as described above. At 1, 2, 5, 10, 20 and 30 min after injection blood samples were taken by decapitating the mice and collecting the draining blood. The samples were rapidly centrifuged for 3 min and the plasma analyzed for BHB by spectrophotometry (Gilford 3500 computer-directed analyzer). 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 control group was done with a Student’s t-test or analysis of variance. Comparison of blood BHB levels throughout the groups was done using the Wilcoxon-Mann-Whitney Rank Sum test. Average values are expressed as means ± standard error of the mean (SEM); the sample size is designated (n).

Results

Intravenous BHB

The IV administration of BHB (12 mg/mouse) produced no statistically significant increase in hypoxic survival time. G, likewise, did not increase survival time. However, when these 2 agents (BHB + G) were combined, the mean hypoxic survival time increased from 185 ± 23 sec in saline injected control mice to 305 ± 64 sec (fig. 1). This represents an increase to 165% of control.

Following IV BHB (12 mg/kg) mean blood levels (fig. 2) of BHB fell from approximately 5 mM at 20 seconds to 0.7 mM 10 min later. At 30 min, the time which corresponds to the onset of hypoxia in the survival time studies, the levels of blood BHB are not significantly above saline injected animals. The mice given BHB and G had mean blood BHB levels not statistically different from mice receiving BHB alone. The mean blood BHB levels in saline injected mice at 30 min was 0.34 ± 0.07 mM. No statistically significant difference in Tb was detected either before or during hypoxia between control and experimental animals.

Intraperitoneal BHB

The time course of blood BHB after IP injection is quite different from that following IV injection (fig. 3). Blood levels rise sharply over the first few minutes and reach peak levels at about 10 min and gradually return toward control levels. At 30 min there is still a substantial elevation above control. The addition of G causes no further increase in blood BHB after 30, 60 or 120 mg/mouse BHB (fig. 4).

When BHB is given alone there is a trend toward increased survival time to hypoxia but in no group is the increase statistically significant (fig. 5). When G is given in addition to the BHB there is a statistically significant increase (p < 0.02 by Student’s t-test) in survival time at 30 and 60 mg/mouse BHB. At 120 mg/mouse BHB with G the average survival time was similar to untreated saline controls.
β-HYROXYBUTYRATE, GLUCAGON IN HYPOXIA/Eiger et al.

Discussion

In the present study, as well as work from other laboratories, the increased tolerance to hypoxia or ischemia that is coincident with documented or probable ketosis was, as well, coincident with conditions known to be characterized by elevated blood glucagon. In fasted and alloxan diabetic mice, in rats fasted overnight before exposure to altitude, in monkeys fasted overnight or mice on a ketogenic diet, both ketones and G would be elevated and the tolerance to hypoxia is increased. It is not surprising, therefore, that exogenous BHB, when given alone, failed to increase hypoxic tolerance, whereas when BHB was combined with G a significant increase in survival time was produced. This effect of the combined substances suggests a possible modulation of cerebral ketone uptake and/or cerebral metabolism by this pancreatic hormone. Direct measurement of tissue metabolites would be required to define such an effect.

It has been reported that brain uptake and utilization of BHB is directly related to blood BHB levels. We interpreted the observed trend toward increased hypoxic tolerance with increasing blood levels of BHB (see fig. 5) as indicative of increased brain utilization of ketones. G may have been acting simply by further elevating the blood BHB levels. The blood levels of BHB, however, were found not to be elevated in groups given G in addition to BHB (figs. 3, 4) and yet a significant increase was seen in hypoxic survival time. Therefore, G appears to be necessary for the BHB to exert its protective effect but at the same time does not appear to be working by altering blood BHB levels. At the highest dose of IP BHB (120 mg) with G, no protection was observed. This could be due to osmotic toxicity of this amount of BHB enhanced by G. In preliminary work we found that 240 mg BHB IP given alone killed mice, presumably due to osmotic shock.

In a previous study, 1, 3-butanediol elevated blood BHB and increased hypoxic tolerance. No exogenous G was given and yet significant protection from hypoxia was observed. Although not measured, it is possible that blood levels of G were elevated in that study, inasmuch as other alcohols have been reported to increase blood G levels.12

The primary explanation for this protective effect may be an alteration in cerebral function. Although G is not known to have direct effects on cerebral metabolism, it might alter cerebral ketone metabolism and/or the uptake of BHB. Either G or BHB might alter cerebral metabolism in a manner distinct from any effect related to the metabolism of ketones. An os-

![Figure 2](image-url)  
**Figure 2.** Blood levels of BHB as a function of time after IV injections of either 12 mg BHB (○) or 12 mg BHB with 4 μg glucagon (●). Bars are SEM. Bars smaller than the circles are not shown. N = 4 for all open circles. N = 3 for all closed circles except at 20 and 30 minutes where N = 2. Untreated controls had an average blood level of 0.35 ± 0.04 mM BHB.

![Figure 3](image-url)  
**Figure 3.** Blood levels of BHB as a function of time after IP injection of 60 mg BHB. Bars are SEM. N values at each point are given above the bars.
motic effect, alteration in blood gases, changes in blood flow, changes in glucose metabolism, or combinations of such changes could yield a protective effect. To separate out which if any of these effects plays a role in this protection from hypoxia would require measurements of blood and tissue metabolic substrates and intermediates which is beyond the scope of this study. Thus, the exact mechanism of action for G in this phenomenon is unknown. Our data only support a dependence upon G for exogenous BHB to be effective in increasing hypoxic survival time in mice.

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Transient Appearance of "No-Reflow" Phenomenon in Mongolian Gerbils

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SUMMARY We investigated the existence of the "no-reflow" phenomenon in focal cerebral ischemia. Regional cerebral blood flow was studied in Mongolian gerbils perfused with a carbon-black particle suspension after cerebral ischemia prior to decapitation and compared with $^{14}$C-antipyrine autoradiographic images. The correlation between the occurrence of the "no-reflow" phenomenon and systemic arterial blood pressure change was also examined. We found that the phenomenon was transient in character and that its manifestation was related to the transient fall in arterial blood pressure observed immediately after clip release and with stagnation of venous blood flow. The phenomenon disappeared in animals in which the arterial blood pressure was artificially increased after clip release.

FOLLOWING THE RELEASE of arterial obstruction, some brain areas of rabbits subjected to ischemia had incomplete filling of the vascular tree when perfused with carbon-black particle suspensions.\(^1\)\(^-\)\(^2\) This, the "no-reflow" phenomenon, was thought to occur because of ischemic swelling of endothelial and perivascular glial cells.\(^2\) The "no-reflow" phenomenon was later confirmed in cats by Olsson and Hossmann\(^1\) who induced total cerebral circulation arrest by clamping the ascending aorta and/or all 4 vessels ascending to the brain.\(^1\)\(^-\)\(^*\)

The present study was undertaken to examine the existence of this phenomenon following focal cerebral ischemia which is common in cerebral infarction. We perfused a carbon-black particle suspension through the femoral vein of living gerbils in order to avoid artificial perfusion and studied the regional cerebral blood flow (rCBF) on $^{14}$C-antipyrine autoradiographic images. In addition, we investigated the correlation between the occurrence of the "no-reflow" phenomenon and systemic arterial blood pressure changes and the effect of sympathomimetics on the phenomenon.

Materials and Methods

Two groups of adult Mongolian gerbils of either sex were used. In the first group, the common carotid artery was occluded bilaterally under light ether anesthesia, using Scovill's aneurysmal clips. The second group consisted of ischemia-sensitive gerbils which were selected as described previously;\(^6\) in this group unilateral occlusion of the left common carotid artery was performed.

1. Assessment of "No-Reflow" Phenomenon by Carbon-Black Perfusion

Cerebral blood flow was restored 30 sec to 15 min after 5, 15, 30, or 60 min bilateral, or 30 min, 1, 3 or 6 h unilateral occlusion. The animals were lightly anesthetized with ether and, 30 sec prior to decapitation, 1 ml of a carbon-black suspension for biological use (Pelikan Werke, W. Germany) was injected into the femoral vein of living gerbils in order to avoid artifactual perfusion and studied the regional cerebral blood flow (rCBF) on $^{14}$C-antipyrine autoradiographic images. In addition, we investigated the correlation between the occurrence of the "no-reflow" phenomenon and systemic arterial blood pressure changes and the effect of sympathomimetics on the phenomenon.

2. Morphological Assessment of rCBF by $^{14}$C-Antipyrine Autoradiography and Benzidine Preparations

Cerebral blood flow was restored 30 sec to 1 h after 1 h bilateral, or 6 h unilateral occlusion. The animals...
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