Hypoxia-Induced Increases in Hypoxic Tolerance Augmented by \(\beta\)-Hydroxybutyrate in Mice

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A standard murine model was used to determine whether acute pretreatment exposures to hypoxia could alter ultimate hypoxic survival time. Adult male albino mice (Mus musculus) weighing 25–30 g were subjected to three pretreatment hypoxic exposures (4.5% O\(_2\), balance N\(_2\)) of increasing duration (90, 120, and 150 seconds) with 300 seconds of normoxia between each pretreatment exposure and before testing of hypoxic survival time. Acute pretreatment exposures to hypoxia significantly increased mean±SEM hypoxic survival time from 108 ±4 to 403±42 seconds. Mean±SEM blood glucose concentrations increased significantly from 201 ±19 to 397 ±10 mg/dl immediately after hypoxic pretreatment. A significant increase in mean±SEM blood ketone concentrations, from 0.15±0.01 to 0.40±0.08 mM, was detected in the blood 1,800 seconds but not 300 seconds after hypoxic pretreatment. However, pretreatment with exogenous glucose or ketones alone, to mimic the blood levels seen after hypoxic pretreatment, failed to increase hypoxic survival time. In contrast, mice pretreated with hypoxic exposures plus the exogenous substrate \(\beta\)-hydroxybutyrate had an increased mean±SEM hypoxic survival time of 749±48 seconds and a decreased body temperature. Stepwise Cox regression analyses with body temperature as a fixed covariate suggest that this decrease in body temperature has a partial role in, but can not fully account for, the increased hypoxic survival time. These data suggest that sequential exposures to hypoxia induce metabolic changes that protect against the lethal effects of hypoxia, perhaps by altering substrate mobilization and utilization and/or by inducing a hypometabolic hypothermia. (Stroke 1989;20:1219–1225)

Hypoxia can produce significant clinical morbidity and mortality; however, naturally occurring hypoxia (e.g., in diving mammals and during hibernation) is often well tolerated. The mechanism of such natural adaptations to hypoxia are still poorly understood. The survival time of mice subjected to hypoxia is used by several laboratories to identify procedures that may be of therapeutic value in protection from hypoxia. Our study was designed to determine if acute exposure to hypoxia might produce a rapid biologic adaptation that subsequently would improve hypoxic tolerance. We hypothesized that, as a naturally occurring adaptive response to hypoxia, ketones would be mobilized and the blood ketone concentration would increase when ketones were made available in excess of utilization. In addition, administration of supplemental substrate, specifically \(\beta\)-hydroxybutyrate or glucose, might further extend hypoxic survival time (HST). Our laboratory has made some progress in evaluating ketone utilization by the brain as a protective and/or therapeutic intervention to minimize brain damage due to hypoxia or ischemia.\(^1\)-\(^6\) It is now well established that the brain uses ketones when they are available.\(^7\)-\(^8\) Preliminary studies,\(^5\) in which ground squirrels showed elevated blood ketone concentration and prolonged survival times when exposed to hypoxia, likewise suggest a biologic adaptation in which ketone mobilization contributes to enhanced tolerance of hypoxia. Understanding the acute response to hypoxia that prolongs survival in a hypoxic environment will help identify means of protection against hypoxia, which in turn may have therapeutic significance.

Materials and Methods

The hypoxia model has been previously described\(^5\),\(^10\) and is used by our laboratory.\(^1\)-\(^3\),\(^11\)
Adult male albino mice (Mus musculus, CD-1) weighing 25–30 g were pretreated and tested for HST or decapitated for blood analysis.

For each trial, groups of five (two control and three experimental or three control and two experimental) mice were tested simultaneously in individual airtight 110-ml flow-through chambers. Body temperature was continuously monitored with copper constantan thermocouples inserted 2 cm into the rectum, and ambient temperature was 22–24°C. Initially, the chambers were flushed with 20.95% O₂ (OM-14, Beckman Instruments, Inc., Fullerton, California). Hypoxia was induced by flushing the system for 20 seconds at 1.7 l/min with premixed 8.6% O₂, balance N₂ followed by 4.6% O₂, balance N₂ at 1.7 l/min. HST, as used in this and all previous studies,1–3,7,11 is the time from onset of 4.6% O₂ exposure to the cessation of spontaneous ventilation. The trial was concluded after 900 seconds, and at that time mice still alive were considered to have survived and were later decapitated. It should be noted that death due to hypoxia is distinct from death due to asphyxia in that with hypoxia, the individual becomes euphoric and then loses consciousness; after loss of consciousness, a brief convulsion precedes cessation of spontaneous ventilation and ultimate death. Parallel groups of mice were decapitated for blood analyses. Concentrations of D-/β-hydroxybutyrate in plasma were determined by a fluorometric modification of the method of Williamson and Mellanby.12 Glucose concentrations were spectrophotometrically determined on a Gilford 3500 Computer-Directed Analyzer (Oberlin, Ohio) using the procedure of Cooper Biomedical Co. (Statzyme Glucose Reagent; Malvern, Pennsylvania), based on the coupled enzyme method of Slein,13 using hexokinase and glucose-6-phosphate dehydrogenase, with modifications by Bondar and Mead.14

In the initial adaptation series, pretreatments consisted of one (I), two (II), or three (III) sequential exposures to hypoxia of increasing duration. Groups of two control and three experimental or three control and two experimental mice were placed in a 5-liter flow-through chamber flushed with either room air or 4.6% O₂, balance N₂ gas for successive intervals: I, 90 seconds; II, 90 then 120 seconds; or III, 90, then 120, and then 150 seconds; 300 seconds of normoxia was allowed both between each sequential exposure and before HST testing. A fourth group of mice (III+30) was exposed to hypoxia three times as described above and allowed 1,800 rather than 300 seconds of normoxia before HST testing or decapitation for blood sampling.

In the substrate addition series, 1,800 seconds before HST testing separate groups of mice were pretreated with either substrate alone 0.25 ml i.p. 62.5 μg dextrose in water (GLU), 0.25 ml i.p. 62.5 μg sodium salt of D-β-hydroxybutyrate in water (BHB), or 0.25 ml i.p. isotonic saline control (SAL) or substrate plus three hypoxic exposures (III+GLU, III+BHB, III+SAL) to assess whether additional substrate could further increase HST. Doses of substrate were chosen to mimic blood glucose concentration measured after three exposures to hypoxia and to reproduce the blood ketone concentration that we had previously found to be protective against hypoxia in this mouse model.2

In the modified series, 30 mice were uniformly sprayed with approximately 0.5 ml tap water 300 seconds before HST testing to mimic the reduction in body temperature seen after hypoxic pretreatments.

All values are expressed as mean±SEM. In the initial adaptation series, HST of control and experimental mice was compared by Student’s t test with Bonferroni’s correction across groups when the number of comparisons approximated the number of groups. When the number of comparisons exceeded the number of groups (as in body temperature analysis), Scheffe’s test was done at an allowance level of 0.95. No differences in HST were detected between mice receiving saline and those not receiving saline, so for clarity these groups were combined.

In the substrate addition series, survival curves for each group were generated using the computer program BMDP P2L,15 and were compared for two groups at a time. The generalized Wilcoxon test is analogous to the Kruskal-Wallis nonparametric ranking test. Breslow’s version of the generalized Wilcoxon statistic16 was computed to test the equality of any two survival curves. Results are reported as probability values.

The Cox proportional hazards regression model (BMDP P2L)17 was used to predict survival curves at high and low body temperatures. BMDP P2L analyzes survival data for which the time-to-time response is influenced by other measured variables, or covariates (e.g., body temperature), and quantifies the relation between survival and that covariate. The Cox model presumes that death rates are log-linear functions of the covariates. A set of regression coefficients is estimated that relates the effect of the chosen covariate to the survival function, and estimated survival is then plotted as a standard percent survival curve. Statistical analysis was performed with the aid of Michigan Interactive Data Analysis System (MIDAS) on an Amdahl 470V8 computer (Sunnyvale, California).

Results

In the initial adaptation series, the marked increase in HST following pretreatment is illustrated in Figure 1. Groups I and II had HSTs of 200% and 300% of their respective controls. Group III HST was 450% of control, with 18.6% of the experimental mice surviving the entire 900 seconds. There was a trend toward increased blood ketone concentration 300 seconds after hypoxia in Group III (Figure 2); however, when time in normoxia was increased to 1,800 seconds, a significant increase (0.40±0.07 mM) was observed compared with control concen-
PRETREATMENTS

Figure 1. Bar graph of mean ± SEM hypoxic survival time after hypoxic pretreatment in mice. Shaded bars, control mice receiving normoxia or no pretreatment (N). Open bars, mice receiving one (I), two (II), and three (III) pretreatment exposures to hypoxia (90, 120, and 150 seconds, respectively). Group III corresponds to mice designated "prehypoxia" in other figures; dashed bar represents group mean excluding eight mice that survived 900 seconds. Hypoxic survival time increased with number and duration of hypoxic pretreatments. n, number of mice; NS, no significant difference; *p<0.05, **p<0.01 different from N by Student's t test with Bonferroni's correction for three comparisons.

For the substrate addition series, the actual percent survival for each group over time is depicted in a standard survival curve15 in Figure 3. III+GLU appeared to offer some additional protection over III alone, but these survival curves do not differ significantly. However, III+BHB offered dramatic and significant protection (p=0.01, generalized Wilcoxon test), with nearly 50% of the experimental mice surviving the entire 900 seconds. These data indicate that pretreatment with ketones, but not with glucose, can further increase HST after hypoxic pretreatment.

Blood glucose concentration in III increased significantly compared with SAL (Table 1). This increase in blood concentration was mimicked by GLU, but the intervention did not correspondingly increase HST. III+GLU did not further increase...
FIGURE 3. Percent survival during hypoxia for mice. Generalized Wilcoxon test (analogous to Kruskal-Wallis nonparametric ranking test) was computed to test equality of survival curves between groups. All treatments significantly increased survival compared with saline control. d-3-Hydroxybutyrate (β-HB) Pre-Hypoxia provided most protection. All curves differ significantly at p<0.01 except Pre-Hypoxia vs. Glucose Pre-Hypoxia.

HST over III alone. The data suggest that an increase in blood glucose concentration cannot account for the increase in HST observed after hypoxic pretreatment.

Blood ketone concentrations were not significantly elevated by 300 seconds but by 1,800 seconds after hypoxic pretreatment were significantly higher than control (Figure 2). BHB alone to increase blood concentration to levels that were protective in previous studies did not increase HST as much as III alone. However, III+BHB increased blood ketone concentration over III alone but did not increase the blood concentration of glucose (Table 1). III+BHB significantly increased HST compared with SAL or III alone (Sheffe’s allowance of 0.95 and generalized Wilcoxon test at p<0.01).

Body temperature was reduced after hypoxic pretreatment, which may have contributed to the increased tolerance to hypoxia. Mean body temperatures at the onset of hypoxia were plotted against mean HSTs for each group (Figure 4). While BHB and GLU did not alter body temperature compared with SAL (mean of approximately 38°C), all hypoxia pretreatments depressed body temperature. However, III alone resulted in a mean body temperature that did not differ significantly (Sheffe’s allowance at 0.90 and 0.95) from that of III+GLU or III+BHB. Thus, while III+BHB significantly increased HST, it did not correspondingly decrease body temperature significantly compared with III alone or III+GLU. Published data indicate that mice treated with the β-hydroxybutyrate precursor 1,3-butenediol to elevate concentrations of circulating ketones have a HST greater than that of any group in the current study and a body temperature no cooler than that of any hypoxia-pretreated group. Likewise, water-cooled mice showed a significantly decreased body temperature compared with the non-hypoxia pretreated mice but did not show a significant increase in HST (Figure 4). These data suggest that a lowered body temperature may contribute to increased HST in all hypoxia-pretreated groups; however, lowered body temperature can not fully account for the increased HST seen in the III+BHB group.

Because body temperature was a confounding variable in comparing HST among groups, the step-wise Cox regression model was used to predict survival curves with the covariate body temperature fixed at 34°C, that of the coolest pretreatment group (Figure 5, top) and at 38.2°C, that of the SAL group (Figure 5, bottom). A general shift toward an increased predicted compared with actual survival curve (Figure 3) is obtained if the mice were to have entered the hypoxic challenge at 34°C. A general shift toward a decreased predicted compared with

FIGURE 4. Graph of mean±SEM body temperature at onset of hypoxia vs. mean±SEM hypoxic survival time for mice. n, number of mice for which temperature was obtained and number of mice tested for survival, respectively. Lower body temperature is associated with increased hypoxic survival time; however, body temperatures of saline, glucose (Glut), and β-hydroxybutyrate (β-HB) groups do not differ significantly by Scheffé’s test at 0.90 or 0.95. Body temperatures of three prehypoxia groups do not differ significantly by Scheffé’s test at 0.90 or 0.95. Data from mice given butanediol are from a previous study.
FIGURE 5. Estimated percent survival for mice as generated by stepwise Cox proportional hazards regression model17 using body temperature (Tb) as fixed covariate at (top) 34°C, mean Tb of coolest pretreatment group, and at (bottom) 38.2°C, mean Tb of saline control group. β-HB, D-β-hydroxybutyrate.

actual survival curve is obtained if the mice were to have entered the hypoxic challenge at the normal body temperature of 38.2°C. This modeling illustrates the effect of a narrow range of body temperatures on hypoxic tolerance. Further, it suggests that factoring out body temperature effects does not eliminate the protection from hypoxia that was observed with III alone or with III+BHB.

Discussion

Hypoxia is associated with significant morbidity and mortality in a number of clinical settings,18,19 necessitating an understanding of the mechanisms that may prolong or prevent hypoxic tissue damage and hypoxic death. It has been demonstrated in unventilated animal models of hypoxia that the brain is the organ most sensitive to hypoxia, and brain electrical activity is reduced in response to hypoxia and ultimately ceases prior to respiratory and cardiovascular collapse.6 The loss of cerebral function in hypoxia may be due to intracellular lactate accumulation, leading to altered membrane structure and function, breakdown of the blood–brain barrier, and development of brain edema,20 with subsequent loss of tissue viability due to energy insufficiency.21 Accordingly, to prolong tissue survival during hypoxia it is necessary to depress metabolic demand, to increase the capacity for substrate supply and utilization, and/or to increase end-product removal. Such changes might occur naturally (adaptive responses) or be induced as potential therapeutic manipulations.

Our three sequential exposures to hypoxia were associated with elevated blood ketone levels and increased tolerance to hypoxia. This increased tolerance was augmented by the addition of exogenous β-hydroxybutyrate. Could an alteration in substrate supply account for the hypoxia-induced increase in HST? Hochachka and Dunn21 suggest that in regard to hypoxic stress and glucose stores, glucose stores in the liver may not be adequate if the hypoxic stress is of rapid onset and if there is not enough time for glucose mobilization. In this case, the authors21 suggest that the sensitive tissue itself must have stored glycogen or an alternate substrate to use until oxygen is again abundant or until liver glucose is available. In our study, the provision of exogenous glucose or ketones to compensate for such a proposed lack of energy reserve did not significantly prolong HST. This suggests that either the substrate still could not get to the brain fast enough to be utilized or that the capacity of the brain must be modulated to utilize substrate. After hypoxic pretreatment, the addition of β-hydroxybutyrate but not glucose further increased HST, suggesting that ketones (but not glucose) augment hypoxia-induced protection.

It is well established that the brain can utilize ketones.7,8,22 Kirsch and D’Alecy2 originally proposed the concept of altered substrate availability, that the brain metabolizes ketones selectively in hypoxia, possibly to minimize the production of lactate and to maintain neuronal viability. When mice are made ketotic by fasting, by 1,3-butanediol administration,1,3 or by β-hydroxybutyrate plus glucagon administration,11 they survive a hypoxic environment longer than control mice, suggesting a protection of ketotic mice that may be due to the utilization of ketones. In no hypoxic survival study has the model allowed for examination of radiolabeled ketone utilization; thus, actual utilization by ketotic-hypoxic mice can only be inferred. However, Hawkins et al8 have demonstrated that the capacity of the brain to utilize β-hydroxybutyrate increases in starvation and in diabetes by 50–60%. It is conceivable that hypoxia increases the capacity of the brain to utilize and/or transport ketones by similar mechanisms. Flux across the blood–brain barrier or enzymatic activity could be acutely modified by pH or other changes in the cytosolic environment induced by hypoxia and/or ketosis. In our current study, BHB augmented the hypoxia-induced increase in hypoxic tolerance by an undefined mech-
anism. In other studies of rat brain slices, hypoxic brain tissue preferentially metabolized β-hydroxybutyrate instead of glucose. Whether a similar (relative or absolute) preference for ketones of the brain is induced after hypoxic pretreatment and whether this in turn could account for increased HST is only suggested by our current data and must be the subject of further studies.

If ketone utilization were protective in hypoxia, it might be expected that an animal would increase mobilization of ketones from fatty stores and/or increase the brain’s ability to transport/utilize ketones, similar to the increase in utilization capacity demonstrated in starvation and in diabetes. In our study, hypoxic pretreatment elevated blood ketone concentration, perhaps through mobilization of ketones from fatty stores and/or ketone concentration, which may be involved in hypoxic tolerance induced by hypoxic pretreatment. However, substrate manipulation appeared to act independently of body temperature. When hypoxic pretreatment was supplemented with substrate (III+BHB, III+GLU), there was no significant difference in body temperature among groups. Despite the similarity of body temperatures, III+BHB increased survival to beyond that accounted for by hypothermia alone. In addition, in previous studies with the ketogenic alcohol 1,3-butanediol, which is dehydrogenated to β-hydroxybutyrate, mice with body temperatures no lower than that of the hypoxia-pretreated group had an average HST markedly greater than that of any pretreatment group in our study. Thus, we conclude that β-hydroxybutyrate provides a protection independent of the hypothermia that is supplementary or synergistic with the hypoxic pretreatment-induced protection. If body temperature alone were responsible for the increased HST observed in hypoxic pretreatment (and hypoxia plus substrate pretreatment) groups, then eliminating this confounding variable by Cox modeling would have produced a parallel utilization (as indicated by reduced body temperature). Again, the direct demonstration of brain ketone oxidation is beyond the scope of our study.

In response to three sequential hypoxic exposures, mice demonstrated both increased hypoxic tolerance and decreased body temperature. Decreased body temperature may be an adaptive response to hypoxia that contributes to increased hypoxic tolerance. Adaptive hypothermia has been described recently by Hicks and Wood in lizards, and it is well established in diving turtles. Ground squirrels have recently been observed to alter their metabolism in response to hypoxia and to survive a hypoxic environment much longer than similarly sized rats. The idea that hypothermia is a protective response to hypoxia is not new. Hochachka and Dunn argue that hypoxia produces a metabolic depression and hypothermia, which is protective under hypoxic conditions.

Our current data are consistent with previous observations that hypoxia decreases body temperature. Body temperatures of 38°–34°C were observed in our hypoxia-pretreated mice, with or without additional substrate. Indeed, predicted survival curves are shifted when using both low and high body temperatures as fixed covariates, illustrating that body temperature modifies survival of all groups. It is possible that an adaptive hypothermia or metabolic depression induced by hypoxia may be a dominant contributor to the increased hypoxic tolerance induced by hypoxic pretreatment. However, substrate manipulation appeared to act independently of body temperature. When hypoxic pretreatment was supplemented with substrate (III+BHB, III+GLU), there was no significant difference in body temperature among groups. Despite the similarity of body temperatures, III+BHB increased survival to beyond that accounted for by hypothermia alone. In addition, in previous studies with the ketogenic alcohol 1,3-butanediol, which is dehydrogenated to β-hydroxybutyrate, mice with body temperatures no lower than that of the hypoxia-pretreated group had an average HST markedly greater than that of any pretreatment group in our study. Thus, we conclude that β-hydroxybutyrate provides a protection independent of the hypothermia that is supplementary or synergistic with the hypoxic pretreatment-induced protection. If body temperature alone were responsible for the increased HST observed in hypoxic pretreatment (and hypoxia plus substrate pretreatment) groups, then eliminating this confounding variable by Cox modeling would have produced a parallel utilization (as indicated by reduced body temperature). Again, the direct demonstration of brain ketone oxidation is beyond the scope of our study.

In summary, our study demonstrates an increase in hypoxic tolerance in mice induced by sequential hypoxic exposures of increasing duration. The mechanism of protection is not clearly defined; however, hypoxic pretreatment induces a metabolic depression (as indicated by reduced body temperature) that contributes to but does not account for increased HST. Hypoxic pretreatment potentially induces a mobilization of ketones, which may be involved in the increased HST. We mimicked the increased blood concentrations of glucose and β-hydroxybutyrate seen after hypoxic pretreatment; however, this failed to mimic the prolonged HST observed with hypoxic pretreatment alone. This suggests that simply elevating blood substrate concentrations is not sufficient to improve survival. With no hypoxic pretreatment, the supplemental substrate may not be utilized because the capacity of the brain has not been modulated. In hypoxia-pretreated mice, supplementation with the ketone β-hydroxybutyrate but not with glucose augments the hypoxia-induced
increase in hypoxic tolerance. Thus, hypoxic pre-treatment not only protects against the lethal effects of hypoxia, it also permits further protection afforded by supplemental ketones, perhaps by inducing hypometabolic hypothermia and/or by altering substrate mobilization and utilization.

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References


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