Effect of Altered Availability of Energy-Yielding Substrates Upon Survival from Hypoxia in Mice

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SUMMARY The duration of survival during a hypoxic or ischemic incident can be altered by barbiturate anesthesia. If this effect on the brain results from a reduction in lactic acid production by hypoxia, then a similar protective effect may be produced by altering substrate availability. Six groups of mice were subjected to hypoxia (4 to 5% O₂, balance N₂) at 30 to 35°C: 1. Hypoglycemia was induced by 2 U insulin injected ip 30 min prior to hypoxia. 2. Ketotic hypoglycemia was induced by fasting 85 to 90 hours prior to hypoxia. 3. Hyperglycemia was induced by iv dextrose. 4. Diabetic-ketotic-hyperglycemia was induced by iv alloxan 5 days prior to hypoxia. 5. One group was given both the insulin and dextrose in the above sequence. 6. In controls, saline was given iv or ip when appropriate. The mean survival time for ketotic-hypoglycemic mice was significantly higher than control. The mean survival time for the insulin-hypoglycemic mice was significantly lower than control. The remaining groups showed no difference from control. The observed improvement in survival time from hypoxia seen in the ketotic animals suggests that during hypoxia, the brain metabolizes ketones selectively and minimizes the production of lactic acid to maintain neuronal viability.

BARBITURATE ANESTHESIA and alterations in blood glucose have been reported to modify survival time or brain damage in various animal models of brain hypoxia and ischemia. If the increase in survival time and the decrease in brain injury are related to a reduction in hypoxia-induced production of lactic acid, then supplying the brain with a substrate other than glucose may afford protection from hypoxia. Under normal circumstances, the mammalian brain is an obligatory glucose user. During a prolonged fast, or in the uncontrolled diabetic state, ketosis develops and the brain adapts by metabolizing acetocetate and beta-hydroxybutyrate. The metabolism of these ketones requires the consumption of oxygen, but does not involve the production of lactic acid. Lactic acid, itself, or the hydrogen ion changes associated with the production of lactic acid have been suggested as a possible mediator of the neuronal damage associated with ischemia or hypoxia. It is assumed in the present study and previous studies using this animal model that neuronal metabolic derangements are responsible for the death of the animal during hypoxia. We tested the hypothesis that an alteration in the type or amount of substrate available for energy production could increase survival time during hypoxia.

Materials and Methods

The animal model used has been previously described by Wilhelm and Arnfred and Steen and Michenfelder. Adult, male Sprague-Dawley albino mice (ICR-ARS-HA) weighing between 15 and 30 g were pretreated and then subjected to hypoxia (4 to 5% oxygen). The pretreatment involved a modification of the availability of substrate for the metabolism of brain. This modification was induced by combination of dextrose infusion, insulin injection, fasting, or experimental alloxan-diabetes. For each group the survival time, and blood glucose and ketone levels were determined.

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Experimental Hypoxia

For each trial 2 control and 3 experimental animals were used. Each mouse was placed in an air-tight, 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 between 30 to 35°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 to 5%, at a flow rate of 1.3 l/min.

Survival time, as used in this study and previous studies,4,5 refers to the time from the onset of hypoxia (4–5%) to the cessation of spontaneous ventilation. This measurement contains no direct evidence of the exact time of brain or heart death. Thus the interpretation from “survival time” should be restricted to comparative studies. Nonetheless, the procedure represents a simple, reproducible model of global hypoxia. Total ischemia8 and relative ischemia11 may involve different metabolic consequences. The observer who monitored the survival was unaware of the classification of each test animal. In addition to the untreated controls, iv or ip injections of sterile saline were given to correspond with the insulin or dextrose infusion procedures. Seven control mice were used for determinations of blood glucose levels and 7 for blood ketone levels.

Hypoglycemia-Hypoxia

Hypoglycemia was induced either by insulin infusion or by fasting. Fifteen mice were injected ip with 2 units of soluble beef-pork-zinc-insulin (0.5 ml of 4 U/ml Iletin, R Lilly). After approximately 30 min, the survival time of each mouse was tested during hypoxia. Five additional animals were given insulin and sacrificed after 30 min for determinations of blood glucose. To test for the effect on survival of insulin alone, 5 animals were given insulin and not subjected to hypoxia. Seventeen animals were fasted for 85 to 95 hours. The animals were individually caged and allowed free access to water during the fasting period. At the end of the fasting period, each animal was exposed to hypoxia. Seven additional animals were fasted and then sacrificed for determinations of blood glucose and 5 for determination of blood ketone levels.

Hyperglycemia-Hypoxia

Hyperglycemia was induced by one of 3 methods: 1. Fifteen mice received an injection of 125 mg dextrose in 0.25 ml of water into the tail vein and within 15 min were exposed to hypoxia. Five additional mice were injected similarly with dextrose and sacrificed for determination of blood glucose. 2. Fifteen mice received 2 U of insulin ip 30 min prior to hypoxia and 125 mg of dextrose in 0.25 ml of water 15 min prior to hypoxia. Five additional mice given both insulin and glucose were sacrificed for blood glucose determinations. 3. Experimental diabetes was induced in 15 mice under general ether anesthesia by the intravenous injection of approximately 75 mg/kg alloxan. This dose of alloxan has been shown to be 88% effective in inducing experimental diabetes in mice.13 The mice were maintained for 5 days and allowed free access to food and water, after which they were subjected to hypoxia. Eight additional alloxan diabetic mice were sacrificed for determination of blood glucose levels and 5 for determination of blood ketone levels.

Blood Glucose and Ketone Analysis

Representative mice from each group were heparinized (750 U/kg iv) and ether-anesthetized. The brachial artery and vein were sectioned for the collection of at least 600 µl of mixed arterial and venous blood. Each sample was deproteinized and neutralized for subsequent enzymatic analysis (hexokinase method)14 of blood glucose levels using the reagents of Worthington Biochemicals and a Gilford 3500 Computer-Directed Analyzer. Blood ketones (beta-hydroxybutyrate and acetoacetate) were measured on deproteinized and neutralized blood samples using beta-hydroxybutyrate dehydrogenase enzymatic procedure15,16 modified for fluorimetry. Each of the values for blood glucose and ketones were obtained under normoxic conditions because we were unable to obtain blood samples during hypoxia or at the moment of “death” (cessation of spontaneous ventilation).

Data Analysis

Statistical analysis was performed with the aid of Michigan Interactive Data Analysis System (MIDAS) on an Amdahl 470/v6 computer facility. Comparison of survival time for each experimental group with control group was done with a Student’s t-test and the comparisons for blood glucose and blood ketones were done with the Wilcoxon-Mann-Whitney Rank Sum test. All values were expressed as means ± standard error of the mean (SEM); the sample size is designated (N).

Results

Normoglycemia

The average composition of the gas initially flushing the flow-through chambers was 8.15 ± 0.06% oxygen. Within 25 sec hypoxia was induced at an oxygen composition of 4.34 ± 0.04%. For all experiments the ambient temperature was 32.89 ± 0.19°C. The mean survival times for the saline (iv or ip) control groups were pooled (table). The mean survival time for all the normal glycemic control animals was 125.7 ± 4.8 sec. The mean blood glucose level in these normal glycemic control animals was 11.6 ± 0.7
mM (N = 7). The mean blood ketone (combined beta-hydroxybutyrate and acetoacetate) level was 0.413 ± 0.020 mM, (N = 7).

**Hypoglycemia**

The insulin pretreatment resulted in a blood glucose level of 6.4 ± 1.3 mM (N = 5). There was a statistically significant (p < 0.0001) reduction in mean survival time of this group to 72.5 ± 3.6 sec (N = 15). When hypoglycemia was induced by fasting, blood glucose level averaged 4.9 ± 0.8 mM (N = 7). In this group blood ketone levels showed a statistically significant (p < 0.005) increase to 2.29 ± 0.36 mM (N = 5). In these fasted hypoglycemic mice, 5 of the 17 mice exposed to hypoxia survived longer than 900 sec (15 min), after which the experiment was terminated. For purposes of analysis, these results were averaged by assuming only a 15-min survival. Thus, the mean survival time for this group was at least 526 sec.

**Hyperglycemia**

When hyperglycemia was induced by dextrose infusion, the blood glucose level averaged 41.5 ± 3.7 mM (N = 7). The mean survival time of 136.0 ± 9.2 sec (N = 15) was not statistically different from controls. Likewise, when insulin and dextrose were used, the survival time 112.5 ± 8.9 sec (N = 15) did not differ statistically from control. The blood glucose level in this group was 24.8 ± 3.5 mM (N = 5). In the diabetic hyperglycemic mice there was a statistically significant (p < 0.0001) increase in the survival time to 185.6 ± 16.8 sec (N = 15). The blood glucose levels in these experimental diabetic mice averaged 28.4 ± 2.1 mM (N = 8). The blood ketone levels (2.50 ± 0.63 mM) were significantly higher than control (p < 0.01) (N = 5).

**Discussion**

Our results indicate that, upon exposure to hypoxia (4-5% oxygen), the alloxan-diabetic and the fasted mice survive longer than normoglycemic mice. Inasmuch as the alloxan-diabetic mice are hyperglycemic, and the fasted mice are hypoglycemic, the increase in survival time cannot be accounted for by a simple modification in blood glucose level. The factor which is common to these two groups is the presence of ketosis. We hypothesize that this ketosis is causally related to the increased survival time in both these groups during the period of hypoxia. The fasted animals demonstrated an increase in survival time by over 300%. In fact, one-third of the fasted animals survived for the entire duration of the experiment (15 min). This increased survival time greatly exceeds that observed in the diabetic group. Several explanations are possible. First, in the fasted group the mean blood glucose level was less than half that observed in the control groups. The lower blood level of glucose is likely to reduce the amount of glucose available for brain metabolism, and hence, the potential for producing lactic acid during the period of hypoxia. Second, the elevated blood glucose level of diabetic mice is such that there may be a competition between glucose metabolism and ketone metabolism during hypoxia. The ketone metabolism would compete for coenzyme A, thus shifting the glucose metabolism toward the production of lactic acid. A third possible component in explaining the difference in survival time between these 2 ketotic groups is the difference in the level of ketosis in the individual animals. The fasted animals, however, had approximately the same level of ketones available for brain metabolism as the diabetic animals.

A statistically significant decrease in survival time was noted in the animals treated with insulin 30 min prior to hypoxia. The level of blood glucose in these animals was very similar to that observed in the fasted animals that had a prolonged survival. Therefore, it is again unlikely that blood glucose level accounts for the difference in survival between the fasted and the insulin group, or between the controls and the insulin group. Two possible hypotheses can be put forth. The first and most traditional explanation of this observation would be that insulin decreases the glucose availability and, combined with the hypoxia, decreases survival time. An alternative hypothesis can be suggested on the basis of observations made in cat, man, and rat that insulin facilitates the uptake of glucose by the brain. If insulin facilitates the uptake of glucose by the brain, this decrease in survival time could be attributed to an augmented glucose metabolism under hypoxic conditions resulting in an
accelerated production of lactic acid and its subsequent production of neuronal damage. This is clearly speculative in that lactic acid levels in the brain were not measured in these studies. Furthermore, it is generally accepted that insulin has no action at all on glucose transport in the brain. If this is the case then the explanation for the decreased survival time based upon augmented glucose uptake would be invalid.

In a recent study using the same animal model, mephobarbital was shown to produce a 300% increase in survival time at a dose level of 100 mg/kg iv of a racemic mixture or the anesthetically active isomer. This increase in survival time was attributed to the binding of anesthetic to a stereospecific receptor which was common for both the protective effect and the anesthetic effect. In our studies a similar level of protection was observed, and yet no anesthetic was used. Clearly, an alteration in cerebral metabolism is present in both the studies using mephobarbital and in our fasted or diabetic group of animals. Presumably, a simple reduction in the cerebral metabolic rate for glucose is present in the barbiturate-anesthetized animals. We are hypothesizing, however, that in our fasted animals and our alloxan-diabetic animals a shift toward ketone metabolism with its subsequent reduction in lactic acid production can afford a similar level of protection from hypoxia without a generalized reduction in cerebral energy metabolism.

Other investigators have shown that food-deprived monkeys recovered fully from 10 min of cardiac arrest (total ischemia), whereas, fed or glucose pretreated animals showed marked cerebral dysfunction. Myles has shown that fasting increases survival time in rats exposed to a 33,500 ft simulated altitude. This is consistent with our hypothesized reduction in brain glucose availability away from glucose and toward ketones. If it were possible to induce cerebral ketone metabolism some protective effect may be produced for the brain during hypoxia.

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