Glucagon Stimulates Ketone Utilization by Rat Brain Slices

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SUMMARY Glucagon has been shown previously to increase further the enhanced tolerance for hypoxia observed in mice with elevated blood ketones. Glucagon is also known to increase blood glucose and to alter directly the metabolism of some (liver) cells. Both the increase in blood glucose and altered cellular metabolism could contribute to the increase in tolerance for hypoxia observed in mice given glucagon in combination with the ketone, beta-hydroxybutyrate. To evaluate the systemic component of this hypothesis, blood glucose, beta-hydroxybutyrate, and glucagon were elevated alone or simultaneously and hypoxic tolerance of mice was measured. To identify possible cellular effects of glucagon on glucose or ketone metabolism, we measured the incorporation of radiolabeled glucose or beta-hydroxybutyrate into CO₂ or total lipid in isolated rat brain slices. Both glucagon and glucose increased hypoxic tolerance of ketotic mice but our data do not support the hypothesis that glucagon's action was only through an elevation of blood glucose. In brain slices glucagon stimulated the incorporation of beta-hydroxybutyrate into CO₂ both in the presence or absence of additional glucose. These results demonstrate that glucagon has a direct effect on brain metabolism which may contribute to the increased tolerance for hypoxia. They, however, do not exclude the possibility that glucagon is working in addition to increase hypoxic survival in ketotic mice by increasing the availability of glucose to the brain.

CONTEMPORARY THERAPEUTIC MEASURES for hypoxic-ischemic brain injury involve anticoagulation, antiplatelet agents, volume expansion, hemodilution, induced hypertension, pharmacological vasodilators, barbiturate coma, profound cooling, steroids, diuretics and a variety of other miscellaneous treatments. None of these procedures have met with resounding success. We believe our work on induced ketosis represents a fundamentally different approach to the problem of cerebral protection.

Previous studies from our laboratory demonstrated that, when mice were placed in an hypoxic environment, those with elevated blood ketones survived up to five times longer. Furthermore, this enhanced hypoxic tolerance was potentiated by exogenous glucagon (GG). Before such manipulations of blood glucose, ketones or GG can be considered for clinical applicability, some basic understanding of the mechanism by which these procedures increase tolerance for hypoxia would be desirable.

In the intact animal GG is known not only to elevate blood glucose but to alter cellular metabolism. For example, it has been reported that the intravenous injection of GG increases oxygen consumption of subsequently prepared liver slices. In order to discriminate between a direct cellular effect and an indirect systemic effect of GG, Suzuki first isolated hepatocytes and then treated them with GG. In the presence of GG there was a 50% increase of ADP-dependent respiration. We have recently shown that GG stimulates oxidative phosphorylation of isolated brain mitochondria. Thus there is evidence for both a systemic effect of GG to elevate blood glucose and a possible cellular or subcellular effect of GG to stimulate metabolism.

Jowett and Quastel first acknowledged, and Owen et al subsequently defined the magnitude of the brain's ability to use a substrate other than glucose. Hawkins has described the regional specificity of cerebral ketone metabolism and Ruderman et al have demonstrated that the brain uses ketones as an energy source whenever they are available. Until recently, however, this growing body of knowledge about brain ketone metabolism had not been exploited as a possible approach to the prevention or treatment of brain damage due to hypoxic-hypoxia or ischemic-hypoxia.

In contrast to our initial descriptive experiments on hypoxic tolerance, the experiments presented here were designed to test the hypothesis that the increase in tolerance for hypoxia observed in the presence of both elevated ketones and GG is due to both a direct and indirect effect. The direct effect of GG would be the stimulation of brain oxidative phosphorylation, tested in a brain slice preparation, and the indirect effect would be through an increased substrate (glucose or ketones) supply to the brain, tested in both the brain slice preparation and the intact hypoxic mouse model. The slice preparation avoids the confounding influences of anesthesia on cerebral metabolism, the necessity for blood flow measurements to calculate cerebral metabolic rates for different substrates, and possible systemic effects of ketones, by determining the level of incorporation of radiolabeled glucose or beta-hydroxybutyrate (BHB) into carbon dioxide or total lipid. The hypoxic mouse model has been used as a reliable and reproducible method of testing the efficacy of a variety of different compounds for the protection of the hypoxic animal from death. The results support the hypothesis that GG, in addition to possible systemic
effects, may increase hypoxic tolerance by stimulating brain metabolism of ketones. This basic understanding of the mechanism by which induced ketosis may offer the brain significant protection from hypoxia now dictates serious consideration of the possible therapeutic usefulness of this new approach to cerebral hypoxic-ischemic injury.

**Materials and Methods**

**Hypoxic Survival Time Experiments**

The protocol for hypoxic gas exposure has been described previously. Briefly, mice weighing 20–40 g received both an intravenous (0.25 ml in lateral tail vein) and intraperitoneal (0.5 ml) injection 30 minutes prior to the onset of hypoxic gas exposure. Seven groups of mice were tested: (1) normal saline iv and ip; (2) 62.5 mg glucose iv and saline ip; (3) normal saline iv and 60 mg BHB ip; (4) 62.5 mg glucose iv and 60 mg BHB ip; (5) 125 mg glucose iv and 60 mg BHB ip; (6) 4.0 ug glucagon iv and 60 mg BHB ip; (7) 62.5 mg glucose and 4.0 ug glucagon iv and 60 mg BHB ip.

The BHB was a racemic sodium salt (Sigma Chemical Co.) and the glucagon (Lilly Research Laboratories) dissolved in sterile saline and contained 1.88 × 10^-6 % insulin.

For each experimental trial five mice, including 2 or 3 individuals from the control group and 2 or 3 from one of the treated groups, were tested. The importance of this 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 Stupfel et al also focuses on the need for simultaneous testing of control and treated animals. Premixed tanks of gas were used to assure the comparability of separate runs, and 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.

Hypoxic survival time (HST) as used in this and previous studies 13 refers to the time from onset of hypoxic gas exposure to the cessation of spontaneous ventilation. Any individual alive after 15 minutes was removed from the test chamber and a HST value of 15 minutes was averaged in with the appropriate group. It should be pointed out that hypoxia has been found to be an effective, humane, and safe means of animal euthanasia.17

**Blood Glucose and BHB Analysis**

In order to determine the blood glucose and BHB levels at the onset of hypoxia, mice were decapitated 30 minutes after the injection (iv and ip as in preceding paragraph) instead of being exposed to hypoxia. Blood was collected in a heparinized tube and immediately centrifuged to obtain plasma which was deproteinized and neutralized before enzymatic analysis. Glucose was measured with the hexokinase method 19 on a Gilford 3500 Computer-Directed Analyzer and BHB was measured with the beta-hydroxybutyrate dehydrogenase method 19 on a Farrand Ratio Fluorometer 2.

**Brain Slice Preparation**

Unmedicated, adult male Sprague-Dawley rats (457 ± 19 g, n = 54) fed ad libitum were decapitated and the brain quickly removed into iced incubation medium which was a modified Krebs-Ringer bicarbonate buffer solution described by Ide et al (20) containing Na, 146; K, 5; Ca, 2; Mg, 1; Cl, 113; HPO₄, 1 and HCO₃, 40 mM. The dura and arachnoid were removed and the brain was sliced with an automatic tissue slicer (Mcllwain, Beckman) to obtain, from each hemisphere, four 0.3 mm thick cortical slices (total average wet weight 37.4 ± 0.7 mg, n = 108) which were placed in 2.2 ml of medium in a 25 ml incubation flask (Kontos Glass Company). One or more of the following substrates were then added to the flasks prior to bubbling the medium with selected gas mixtures to obtain the following: 10 mM beta-hydroxy[3-14C]butyrate (1 uCi/flask) (New England Nuclear), 10 mM [U-14C]glucose (1 uCi/flask) (New England Nuclear), 10 mM unlabeled glucose, 10 mM unlabeled beta-hydroxybutyrate (Sigma Chemical Company), and 3.8 mM insulin free glucagon (Eli Lilly Co.). Each flask was bubbled for 5 minutes with 5, 40, or 95% oxygen combined with 5% carbon dioxide and enough nitrogen to complete the mixture. Capped flasks were incubated in a shaker bath for 2 hours at 37°C and 72 cycles/minute after which 0.2 ml of 10 N H₂SO₄ and 0.3 ml of 1 M methylbenzethonium hydroxide in methanol were injected through the stopper into the medium and center well respectively. The collection of CO₂ was considered complete after 90 minutes of additional incubation at room temperature and 36 cycles/minute (20). The well containing the trapped CO₂ was placed in 10 ml of ACS liquid scintillation fluid (Amer sham Inc.), capped, mixed by vortex and placed in a beta counter (Searle Isocap/300) for 30 minutes prior to counting to allow for loss of chemiluminescence. The slices were removed from the medium, blotted, weighed, and extracted with 20 ml of chloroform-methanol (2:1) according to Folch et al. 21 A 5 ml aliquot of the lipid phase was evaporated, redissolved in 10 ml of ACS liquid scintillation fluid, mixed and counted.

**Statistical Analysis**

Statistical analysis was done with the aid of the Michigan Interactive Data Analysis System (MIDAS) on an Amdahl 470/v7 computer facility. Analysis of variance and the two tailed Student's t test with Bonferroni weighting when appropriate were used to test for differences between groups. Each value is reported as the mean ± one standard error of the mean (SEM); the sample size is designated (n). All values from the brain slice experiments are expressed as nanomoles of substrate (either glucose or BHB) per 100 mg wet brain weight incorporated into either CO₂ or total lipid during the two hour incubation period to permit direct
comparison to published values. The HST values in all groups are expressed in seconds.

Results

Hypoxic Survival Time

For all hypoxic survival time trials the average oxygen concentration of the hypoxic gas mixture was 4.60 ± 0.02%. The mice used had an average of 43 ± 2 days and an average weight of 27.6 ± 0.3 g, n = 195. As presented in table 1, animals given BHB by itself (Group #3) or BHB with glucagon (group #6) had a significantly increased HST and blood BHB and a significantly lower blood glucose than saline pretreated control animals (Group #1). The combination of glucose and BHB (Groups #4 and #5) significantly increases HST, blood BHB, and blood glucose (Group #5 only). Glucagon significantly increased the HST of animals given both BHB and glucose but did not further increase the blood glucose level (Group #4 vs Group #7).

Brain Slices CO₂ Production

At each oxygen level the addition of glucose to the media stimulated (p < 0.05, Bonferroni) the production of CO₂ from BHB. With 95% O₂ the CO₂ production from BHB was further stimulated by the addition of GG both in the presence and absence of added glucose (fig. 1, left side). This effect, however, was not evident at 40% or 5% O₂. In the absence of GG (with or without added glucose) the reduction of oxygen to 40% stimulated the production of CO₂ from BHB over that at 95% oxygen. At 5% O₂ both groups with added GG had significantly less CO₂ production from BHB than at 95% O₂.

In contrast, in brain slices gassed with 95% O₂ the CO₂ production from glucose alone was 2102 nm/100 mg/2 hr and dropped to 1723 nm/100 mg/2 hr (p = 0.004, Bonferroni) from glucose alone but it was not different than the CO₂ production from glucose in the presence of added glucagon.

Total Lipid Production

As illustrated on the right of figure 1, in all groups tested total lipid production only accounts for a small fraction of the BHB or glucose which is metabolized by the brain slices. GG produced no statistically significant change in lipid production from BHB nor did reduction in the oxygen concentrations. The lipid production from glucose (42.3 nm/100 mg/2 hr) in brain slices gassed with 95% O₂, was not significantly altered by the addition of either BHB (38.8 nm/100 mg/2 hr) or GG (42.7 nm/100 mg/2 hr).

Discussion

This discussion is divided into two sections. The first section, Methodological Considerations, deals with the significance of the HST model as an indicator of brain failure; the adequacy of our use of the brain slice preparation including the levels of oxygen used; and the rationale for the dose of GG used in these studies. The second section, Physiological Considerations, deals with the interpretation of the results as far as they reflect on the question of substrate availability versus altered brain metabolism as a mechanism for protection of the brain from hypoxia. Possible clinical implications are discussed as long range implications of these studies.

Methodological Considerations

The measurement of mouse HST is a crude measurement and does not necessarily indicate the exact time of brain death. There is, however, evidence to suggest that the mouse hypoxic survival time model is an appropriate tool for the evaluation of interventions for the protection of the brain from hypoxia. Lundy et al.²² have shown that rats exposed to hypoxia first lose brain electrical activity, a few seconds later stop breathing and then experience cardiovascular collapse. Furthermore, as predicted by our mouse hypoxia studies,¹,³ he showed that in the hypoxic rat, induced ketosis prolonged the time to cessation of brain electrical activity. Likewise, Herin et al.¹⁹ have found in hypoxic dogs the EEG goes flat before the animal experiences cardiovascular collapse. Thus from studies with different animal models including the rat and dog we are led to predict that hypoxia in the mouse involves an initial

### Table 1: The Hypoxic Survival Time, the Blood Beta-hydroxybutyrate Concentration in mM and the Blood Glucose in mg/dl for Each of Seven Different Groups of Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>HST (seconds)</th>
<th>Blood BHB (mM)</th>
<th>Blood glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Saline</td>
<td>118 ± 4.3 (94)</td>
<td>0.28 ± 0.05 (8)</td>
<td>220.0 ± 9.9 (14)</td>
</tr>
<tr>
<td>2. GLU (62.5)</td>
<td>126 ± 10.5 (9)</td>
<td>0.13 ± 0.01 (10)</td>
<td>249.9 ± 4.7 (10)</td>
</tr>
<tr>
<td>3. BHB</td>
<td>*153 ± 9.8 (17)</td>
<td>*3.5 ± 0.33 (9)</td>
<td>*186.3 ± 11.9 (13)</td>
</tr>
<tr>
<td>4. GLU (62.5) + BHB</td>
<td>*189 ± 10.6 (22)</td>
<td>*3.2 ± 0.41 (9)</td>
<td>254.8 ± 16.2 (14)</td>
</tr>
<tr>
<td>5. GLU (125) + BHB</td>
<td>*207 ± 14.8 (21)</td>
<td>*3.8 ± 0.61 (5)</td>
<td>*404.8 ± 35.2 (12)</td>
</tr>
<tr>
<td>6. GG + BHB</td>
<td>*187 ± 14.4 (18)</td>
<td>*3.3 ± 0.23 (9)</td>
<td>*150.5 ± 7.5 (9)</td>
</tr>
<tr>
<td>7. BHB + GLU (62.5) + GG</td>
<td>*310 ± 47.7 (14)</td>
<td>*3.1 ± 0.19 (10)</td>
<td>*258.8 ± 30.0 (10)</td>
</tr>
</tbody>
</table>

The first column indicates the treatment given each group and the number in parentheses indicates the amount in mg given to each mouse prior to hypoxia. The stars (*) indicate p < 0.05 by the Student t test when compared to saline pretreated controls.
levels of in vitro oxygenation chosen likewise do not necessarily reflect a particular in vivo circumstance. The 95% oxygen level was chosen so that we could compare our values of CO₂ production to those in the literature. 21 Since the CO₂ production from brain slices utilizing glucose was the same at 95% oxygen and 40% oxygen but reduced when at 5% oxygen we predict that the 95% oxygen was not damaging or limiting to the slice but the 5% oxygen became a limiting factor. Thus in both the slice preparation and the mouse model there is a transition from adequate oxygen for function to hypoxic dysfunction.

The dose of glucagon used in the HST experiments was that which proved effective in our earlier study. 3 The concentration of glucagon in the brain slice medium was chosen on the basis of our experiments demonstrating the stimulatory effects of glucagon on isolated brain mitochondria 4 and from experiments of isolated hepatocytes showing similar stimulation of subsequently isolated mitochondria. 22

**Physiological Considerations**

In our previous studies we found that GG potentiated the beneficial effects of systemic ketosis by further increasing hypoxic tolerance in mice. In this study we tested two of the possible explanations for the observed potentiation, one dealing with the systemic effects of GG and the other focusing on a possible direct cellular effect of GG on brain metabolism. GG is known to increase blood glucose levels by systemically stimulating gluconeogenesis, and glycogenolysis and by inhibiting glycogen synthesis. Therefore, by administering both BHB and GG to mice an increase in HST could have been produced by simply increasing the availability of glucose in addition to BHB for brain metabolism.

Systemic administration of ketones is known to decrease blood glucose levels as was observed in this study, and previously has been shown to be due to inhibition of hepatic glucose output. 23 We observed that giving BHB-ketotic mice glucose not only increased the animals’ blood glucose levels (Group #5), it elevated their HST (Group #4 and #5) as compared to ketotic animals who did not receive glucose (Group #3). Our data, therefore, are consistent with the idea that increased substrate availability enhances hypoxic tolerance. In contrast, however, BHB-ketotic animals given GG instead of glucose (Group #6) did not have elevated levels of blood glucose and yet had elevated HSTs. Likewise, in one group of mice given both glucose and BHB (Group #4) the blood glucose and blood BHB levels were not different than a group of mice given GG in addition to the glucose and BHB (Group #7), yet HST in these animals receiving GG (Group #7) was much higher than the animals who did not receive GG (Group #4). This finding led us to conclude that the GG must be working by some mechanism other than by simply increasing brain substrate availability. This of course does not exclude the possibility that GG is increasing substrate uptake without increasing systemic levels of the substrates.
and Luyckx, however, have stated that GG has no direct effect on the peripheral glucose utilization.

A second explanation for GG's potentiation of increased hypoxic tolerance observed in ketogenic mice is that GG is altering cerebral metabolism. We have evaluated this possibility with the brain slice preparation and found that GG selectively stimulates the incorporation of BHB, rather than glucose, into CO$_2$ and, indeed, there is some evidence to suggest that GG may inhibit the incorporation of glucose into CO$_2$. One explanation for the proposed inhibition of glucose metabolism may be that GG has recently been shown to inhibit the activity of phosphofructokinase by decreasing the level of fructose 2,6-bisphosphate in intact cells. This inhibition would then suppress glucose utilization but not adversely effect BHB utilization since BHB does not utilize the glycolytic pathway. This contrasts with earlier studies of Lefebvre and Luyckx who reported that GG had no direct effect on the peripheral glucose utilization.

The GG stimulation of brain slice BHB metabolism which occurred in flasks gassed with 95% O$_2$ did not occur with slices gassed with mixtures of 40 and 5% oxygen; thus it is unclear at what level of reduced oxygen the stimulating effect of GG no longer increases ketone incorporation into slices. The reduced oxygen level alone did, however, increase the incorporation of BHB into CO$_2$ above the levels observed at 95% oxygen. This may suggest that hypoxia and GG might be stimulating BHB incorporation into CO$_2$ by similar intracellular pathways.

In conclusion the in vitro brain slices studies indicate that GG or 40% oxygen (relative hypoxia) stimulates CO$_2$ production from ketones. The in vivo studies suggest that some mechanism other than an elevation of blood glucose is responsible for the augmented survival of ketogenic mice exposed to GG. It is still, however, speculative that the stimulation of ketone metabolism observed in vitro is causally related to the augmented survival of ketogenic mice. The utilization of induced ketosis and GG in a more clinically relevant setting has yet to be pursued.

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