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 than controls. Butanediol induced ketosis is also associated with a reduced neurologic deficit in the hypoxic rat. Furthermore, this enhanced hypoxic tolerance in mice was potentiated by exogenous glucagon (GG). In *in vitro* studies on rat brain slices we found that GG stimulates the incorporation of the ketone beta-hydroxybutyrate (BHB) into carbon dioxide both in the presence or absence of added glucose. In addition, Harris et al have reported a stimulatory effect of GG on liver mitochondrial respiratory rates in the presence of BHB as a substrate. These results suggest an hypothesis that GG, in addition to its systemic effects, may increase hypoxic tolerance by modifying brain metabolism of ketones.

The ability of glucagon (GG) to alter cell metabolism is widely recognized, but its mechanisms and sites of action remain to be fully elucidated. For example, acute GG treatment of intact rats increases hepatic mitochondrial respiration and calcium fluxes. Suzuki reported that intravenous injection of GG in rats in-
The tissue was transferred to a glass homogenizing vessel and homogenized with 10 upward and 10 downward strokes of a motor-driven Teflon pestle (1,450 rpm, no-load speed) having a wall clearance of 0.66 mm. The tissue was homogenized further with two passes of a tight-fitting pestle (wall clearance 0.20 mm). Approximately 4 ml of the crude homogenate was saved on ice for later use. The homogenizing vessel and pestles were washed with fresh medium, and the washings plus additional fresh medium were added to the homogenate to give a dilution of 1 gm tissue per 20 ml of homogenate.

The remainder of the homogenate was centrifuged at 3,200 x g (average) for 10 min. The resulting supernatant was strained through gauze and centrifuged at 10,000 x g for 15 min. Four milliliters of the resulting postmitochondrial supernatant were saved on ice for later use. The crude mitochondrial pellet was gently rinsed with fresh medium to remove fluffy, brown or loosely-adhering material. In our initial experiments the pellet was sequentially washed with and gently resuspended in fresh medium, and centrifuged again at 10,000 x g for 15 min. This procedure was repeated twice to obtain the final mitochondrial fraction. However, in the majority of experiments the first mitochondrial pellet was washed once with fresh medium as described above, then mixed with and resuspended in 30 ml (approximately 5 volumes) of medium containing 3% (w/v) Ficoll-400 (Pharmacia), and 10 ml aliquots were layered over 30 ml of medium containing 6% Ficoll. This was centrifuged at 10,000 x g for 30 min. Pellets obtained after centrifugation through Ficoll-containing media were rinsed with and resuspended gently in Ficoll-free medium. The suspensions were combined, gently homogenized by hand with two strokes of a tight-fitting pestle, and centrifuged at 10,000 x g for 15 min. The final pellet was rinsed once and gently resuspended in a small volume of Ficoll-free medium to give a protein concentration of approximately 30 mg/ml, based on a Biuret assay using bovine serum albumin as a standard. Protein yields are shown in table 1.

Most of the mitochondrial preparation was used immediately for polarographic determination of oxidative phosphorylating activity. A small aliquot was frozen and stored at —79°C for later determination of cytochrome content.

Mitochondrial Respiration

We used a Gilson Oxygraph equipped with a Clark-type electrode to study mitochondrial oxidative phosphorylation. The standard assay medium contained 0.25 M sucrose, 5 mM K2HPO4 and approximately 1.2 mg/ml of mitochondrial protein in 15 mM morpholino-propane-sulfonic acid (MOPS) buffer (pH 7.40 at 25°C). Respiratory substrates studied were Tris-glutamate, Tris-malate, sodium beta-hydroxybutyrate, Tris-succinate (plus 1 μg rotenone/mg protein), or combinations of these substrates as noted below. A 1 mg/ml glucagon stock solution was prepared by dissolving GG in equal volumes of brain incubation medium and sufficient 3.6 N HCl to reach pH 2.5 to 3.0, and was stored at 4°C as recommended by the supplier.

Materials and Methods

Animals

For each mitochondrial preparation 20–30 male Sprague-Dawley mice (strain HA-ICR) having an average body weight of approximately 25 g and free access to food and water were killed between 8:00 and 9:00 a.m. The animals were decapitated and the entire contents of the calvarium were removed and immediately placed in a beaker of cold isolation medium (0.3 M sucrose and 5 g/dl bovine serum albumin in 1 mM Tris-EDTA buffer, pH 7.40 at 4°C). This procedure took an average of 16 sec per mouse. Average brain wet weight was 360 ± 7 mg (mean ± 1 SEM). All remaining isolation steps were done at 4°C.

Mitochondrial Isolation

We used a modification of the protocol devised by Clark and Nicklas19 to obtain a preparation of primarily nonsynaptosomal ("free") mitochondria. The brains were washed free of extraneous blood with fresh isolation medium, then minced with scissors in a second aliquot of medium (4 ml per gram of brain wet weight). The tissue was transferred to a glass homogenizing vessel and homogenized with 10 upward and 10 downward passes of a motor-driven Teflon pestle (1,450 rpm, no-load speed) having a wall clearance of 0.66 mm. The tissue was homogenized further with two passes of a tight-fitting pestle (wall clearance 0.20 mm). Approximately 4 ml of the crude homogenate was saved on ice for later use. The homogenizing vessel and pestles were washed with fresh medium, and the washings plus additional fresh medium were added to the homogenate to give a dilution of 1 gm tissue per 20 ml of homogenate.
<table>
<thead>
<tr>
<th>Table 1</th>
<th>Protein Yields and Cytochrome Concentrations of Brain Mitochondria Isolated by Differential Centrifugation With or Without Ficoll 400*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifugation</td>
<td>Without Ficoll</td>
</tr>
<tr>
<td>Protein yield</td>
<td>15.52 ± 1.04</td>
</tr>
<tr>
<td>Cytochromes a + a3</td>
<td>0.189 ± 0.008</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>0.025 ± 0.001</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>0.116 ± 0.006</td>
</tr>
<tr>
<td>Cytochrome c1</td>
<td>0.042 ± 0.002</td>
</tr>
</tbody>
</table>

*Values reported are arithmetic means ± 1 SEM. Numbers in parentheses indicate the number of preparations from which reported data were obtained. Protein yield is expressed as mg protein recovered per gram of brain wet weight. Cytochrome content is expressed as nmols/mg of mitochondrial protein.

When GG was used, we added 20 ul of the stock solution immediately after adding 2 mM substrate, 2 min before inducing State 3 respiration by ADP addition. Total assay volume was 1.605 ml. Thus, final GG concentration was 3.6 μM. The respiratory substrates used and the inclusion of GG were varied randomly, and the assays were generally run at least twice for each condition studied.

The data reported were obtained from 4 to 12 mitochondrial preparations. State 4 respiratory rates were determined for approximately 2 min at which time a known amount of Na2-ADP (usually 500 nmoles; the exact concentration of the stock solution was determined spectrophotometrically) was added to induce State 3 respiration. When the mitochondria had returned to State 4 respiration a second aliquot of ADP was added so that respiratory rates and the ADP:O ratio could be measured in duplicate. Mitochondrial respiratory control ratios (RCR) were calculated as the quotient of the State 3 rate of oxygen consumption and the subsequent State 4 rate. When succinate was used as a substrate, mitochondrial respiratory control indices (RCI) were calculated as the quotient of the State 3 rate of oxygen consumption and the previous State 4 rate. The ADP:O ratio was calculated as the amount of oxygen consumed in the presence of a known amount of added ADP. The oxidative phosphorylation rate (OPR) was calculated as the product of the State 3 respiratory rate and the accompanying ADP:O ratio. The OPR estimates the rate of mitochondrial ATP synthesis, normalized per mg of mitochondrial protein or per mol cytochrome aa₃ per min. Maximal (uncoupled) rates were produced by addition of 2,4 dinitrophenol.

Oxygen consumption was computed according to the method of Chance and Williams. Oxygen solubility in the medium was first assumed to equal that for solubility in 0.155 N sodium chloride. To reduce data variability further, the oxygen solubility was also corrected to account for latitude, daily barometric pressure, ambient temperature, and the partial pressure of water at the cuvette temperature (28.5°C). Thus, the daily adjusted oxygen content ranged from 394 to 406 nanomols oxygen per ml of solution.

Cytochrome Content

Thawed mitochondria were diluted to a protein concentration of approximately 12 mg/ml with 0.1 M potassium phosphate buffer (pH 7.40). Cytochrome content was determined using an Amino-Chance DW-2 spectrophotometer according to the method described by Williams. Both reference and sample cuvettes contained approximately 6 mg of mitochondrial protein and 0.5% sodium deoxycholate in 0.1 M potassium-phosphate buffer (pH 7.40), with a total cuvette volume of 2.0 ml. After recording a stable baseline over the wavelength range of 500 to 650 nm, the reference was oxidized by the addition of 10 μl of 50 mM KFe(CN)₆ in phosphate buffer, and the sample was reduced by the addition of 10 μl of 50 mM L-ascorbic acid in phosphate buffer, plus a few grains of crystalline Na₂S₂O₄ were added and the scan repeated until there were no further changes in the spectra. Cuvette masks were used to minimize light scattering. Cytochrome content was calculated using equations and constants described by Azzone et al.

Chemicals and Reagents

Purified GG was a generous gift of Dr. M. A. Root of Lilly Research Laboratories (Indianapolis, IN). Fresh crystalline Na₂S₂O₄ was obtained from Fluka, AG. Ficoll 400 was purchased from Pharmacia. All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). All inorganic salts were reagent grade or better. All aqueous solutions were made with double-distilled water purified further by passing it through a Hydro Service 0A-18 organic adsorbent column and two D-18M mixed bed ion exchange columns (Hydro Services and Supplies Inc., Durham, NC).

Statistics

Experiments were designed so that the effects of GG addition on mitochondrial respiratory parameters could be evaluated using paired t-tests. Between-group comparisons of data obtained with different mitochondrial preparations under differing conditions were made using nonpaired (Student) t-tests. Exact probabilities were calculated using an Amdahl 470/v7 computer. Only differences achieving a "p" value less than 0.05 were considered statistically significant. Except where noted otherwise, data are reported as arithmetic means plus or minus one standard error of the mean (SEM).

Results

In order to assess the purity of the mitochondrial preparations, protein yields and cytochrome concentrations were determined for organelles isolated by protocols with or without centrifugation through Fi-
coll-containing isolation medium, as summarized in table 1. A typical cytochrome difference spectrum is shown in figure 1.

Figure 2 shows representative oxygen consumption records for two mitochondrial preparations studied in the absence and presence of GG with glutamate serving as the sole substrate. The major effects of GG in this tightly-coupled (mean RCR about 8) preparation were significant increases of both ADP-stimulated and basal oxygen consumption rates. Control respiratory data were comparable to those reported by others. Although slight differences may be explained in terms of isolation and assay protocols, animal species, and the use of whole brain rather than discrete brain regions.

Major functional indicators of mitochondria respiring with and without GG in the presence of glutamate and the other substrates or substrate combinations are summarized in table 2 and figure 3. Glucagon significantly increased both State 3 and State 4 respiratory rates (fig. 3), the ADP-O ratios, and the oxidative phosphorylation rate (table 2) of mitochondria utilizing glutamate as the sole substrate. Lack of an effect of the hormone on RCR was due to the fact that the significant increase of State 3 respiratory rates was accompanied by a slight but consistent and statistically significant ($p = 0.0002$) increase of State 4 respiration.

Combining glutamate with malate or with BHB increased both State 3 and State 4 respiratory rates (fig. 3) and OP rates were also increased significantly. However, based on nonpaired t-tests only the changes of State 3 respiratory rates were significantly greater with substrate combinations than with single substrates. Adding GG significantly ($p = 0.0172$) increased State 3 respiratory rates of mitochondria respire in the presence of glutamate plus BHB (fig. 3), but did not further increase ADP-stimulated respiration of organelles respiring with glutamate plus ma- late. Lack of further stimulation by GG in the latter instance may have been due to the fact that ADP-stimulated respiratory rates in the presence of glutamate plus malate were not appreciably different from maximal (uncoupled) rates of oxygen consumption measured in the presence of 2,4-dinitrophenol.

In an attempt to determine whether GG-induced stimulation of glutamate-supported respiration might be mediated by extramitochondrial contaminants, we evaluated the effects of the hormone in preparations to which aliquots of crude whole-brain homogenate or postmitochondrial supernatant were added. Also, since GG effects might be mediated by formation of cyclic-AMP due to plasmalemmal contamination, the effects of the hormone on oxygen consumption were evaluated in the presence of exogenous nucleotide.

Statistical analysis of the effects of crude brain homogenate, postmitochondrial supernatant, and 3',5'-cAMP on mitochondrial respiration are presented in table 3. Without GG, crude homogenate significantly increased both State 3 and State 4 respiratory rates and significantly decreased both RCR and the ADP-O ratio compared to homogenate-free controls. As shown in table 3, the stimulatory effect of GG on State 3 and State 4 respiratory rates was still apparent in the presence of crude homogenate, postmitochondrial supernatant, and 3',5'-cAMP; however, these changes do not reach the 0.05 level of statistical significance for State 3 respiration with crude homogenate and 3',5'-cAMP, or for State 4 respiration with 3',5'-cAMP.

Figure 4 illustrates the stimulatory effect of GG on mitochondrial oxidative phosphorylation rates under control conditions and in the presence of other additives. A statistically significant stimulation by GG was
Table 2 Effects of Various Respiratory Substrates and of Glucagon (GG) on in vitro Performance of Isolated Murine Brain Mitochondria*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>N</th>
<th>RCR -GG</th>
<th>+ GG</th>
<th>ADP-0 -GG</th>
<th>+ GG</th>
<th>OPR+ -GG</th>
<th>+ GG</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLU</td>
<td>12</td>
<td>8.0 ± 0.4</td>
<td>8.2 ± 0.4</td>
<td>2.39 ± 0.06</td>
<td>2.56 ± 0.05</td>
<td>434 ± 36</td>
<td>597 ± 54</td>
</tr>
<tr>
<td>MAL</td>
<td>6</td>
<td>2.6 ± 0.1</td>
<td>2.6 ± 0.2</td>
<td>2.42 ± 0.06</td>
<td>2.37 ± 0.10</td>
<td>210 ± 10</td>
<td>251 ± 40</td>
</tr>
<tr>
<td>SUC + ROT</td>
<td>6</td>
<td>2.8 ± 0.2</td>
<td>2.6 ± 0.2</td>
<td>1.70 ± 0.069</td>
<td>1.81 ± 0.02</td>
<td>338 ± 10</td>
<td>336 ± 40</td>
</tr>
<tr>
<td>BHB</td>
<td>11</td>
<td>3.1 ± 0.5</td>
<td>2.9 ± 0.3</td>
<td>2.06 ± 0.07</td>
<td>2.22 ± 0.15</td>
<td>268 ± 34</td>
<td>291 ± 35</td>
</tr>
<tr>
<td>GLU + MAL</td>
<td>4</td>
<td>8.5 ± 0.3</td>
<td>7.9 ± 0.2</td>
<td>2.42 ± 0.04</td>
<td>2.37 ± 0.09</td>
<td>897 ± 51</td>
<td>877 ± 49</td>
</tr>
<tr>
<td>Glu + BHB</td>
<td>7</td>
<td>5.9 ± 0.6</td>
<td>6.9 ± 0.7</td>
<td>2.44 ± 0.07</td>
<td>2.46 ± 0.04</td>
<td>744 ± 85</td>
<td>851 ± 63</td>
</tr>
</tbody>
</table>

Values reported are arithmetic means ± 1 SEM. Details of the experimental conditions are noted in the text.

Glucagon concentration was 3.6 μM.

OPR = oxidative phosphorylation rate expressed as mol ADP/mol aa3 per min.

*Values reported are arithmetic means ± 1 SEM. Details of the experimental conditions are noted in the text.

retained in the presence of postmitochondrial supernatant and 3',5'-cAMP. We were unable to identify hormone-stimulated inhibition of state 3 respiratory rates were already near-maximally stimulated in the absence of GG.

Discussion

The major points of the discussion focus on how our results could contribute to the understanding of the mechanism by which BHB plus GG prolong hypoxic survival time of the intact animal, and evidence for the mitochondrial oxidation phosphorylation.

Possible Relationships to Hypoxic Tolerance: Clinical experience and data obtained with different animal models led us to predict that hypoxia involves an initial compromise of brain function followed by respiratory then cardiovascular collapse. Lundy et al2 have shown that rats exposed to hypoxia first lose brain electrical activity, approximately 84 seconds later stop breathing, and then approximately 71 seconds later experience cardiovascular collapse. Additionally, the prolonged hypoxic survival associated with induced ketosis in rats can be attributed to a prolonged time to cessation of brain electrical activity.3 Likewise, Herin et al20 and McDonald et al21 have found that, in hypoxic dogs, the EEG goes flat before the animal experiences cardiovascular collapse. Previous data from our laboratory show that mice with elevated blood ketones, either due to fasting or administration of the ketone precursor 1,3-butanediol, have increased tolerance to hypoxia.4 Direct administration of the ketone beta-hydroxybutyrate itself, combined with GG, also prolongs hypoxic survival of mice.5 Collectively, the data suggest that interventions prolonging brain electrical activity by a process presumably involving ketone metabolism may be influenced directly or indirectly by elevated levels of GG.

In the intact animal or tissue, hypoxia causes accumulation of lactic acid intracellularly, with stimulation then inhibition of glycolysis and subsequent suppression of ATP production. During hypoxia, neither aerobic (mitochondrial) metabolism nor glycolysis can produce ATP at a rate sufficient to maintain brain function. Isolated mitochondria are distinct from the
by hypoxia. If this protection by stimulating mitochondrial substrate oxidation, in which tissue oxygen levels are reduced but all oxygen is not eliminated. Glucagon might confer antioxidative properties to mitochondria and specifically alter glutamate-supported oxidative metabolism.

A direct mitochondrial action would be independent of the membrane-associated 3',5'-cyclic AMP stimulatory effect. Glucagon's stimulatory effect on respiration is thought to be mediated by activation of adenylate cyclase, producing an increase in cytosolic 3',5'-cAMP, and ultimately acting to stimulate electron flow between cytochromes c and d. Such evidence does not exclude the possibility of a potential direct action of this peptide hormone on mitochondria. Our data support this hypothesis, suggesting that GG can act directly on isolated mitochondria and specifically alter glutamate-supported oxidative metabolism.

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glucagon consumption measured in the presence of 2,4- 

Dinitrophenol. However, the respiratory control was not changed by adding GG, indicating that the effect of GG is not an uncoupling of oxidative phosphorylation.

In conclusion, GG stimulated both ADP-dependent 

and -independent oxygen consumption by isolated 

murine brain mitochondria. This stimulation occurs 

with glutamate as a substrate but not with malate, 

succinate, or BHB. In addition to membrane-associated 

stimulation of cellular respiration reported by oth-

er's, these data support the hypothesis that in vivo GG 

can act directly and selectively on brain mitochondria 

to stimulate oxidative phosphorylation. These data 

also suggest a possible role for GG in the protection 

of the brain from hypoxic damage by sustaining mito-

chondrial oxidative phosphorylation necessary for 

brain cellular function.

Acknowledgement

We thank Dr. Leena Mela for her suggestions during the development of this study, Dr. Minor J. Coon for allowing us to use the Aminco- 

Chance DW-2 spectrophotometer and Dr. Dennis Koop for assistance with the cytochrome assay.

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cyclic AMP in glucagon-induced stimulation of ruthenium red 
in sensitive calcium transport in an endoplasmic reticulum-rich frac-
Presynaptic Inhibitory Action of Adenosine on Neuromuscular Transmission in the Canine Cavernous Carotid Artery

SHIGERU FUJIWARA, M.D., D.M.SC., NEAL F. KASSELL, M.D., TOMIO SASAKI, M.D., D.M.SC., MASANORI YAMASHITA, M.D., D.M.SC., MARIO ZUCCARELLO, M.D.

SUMMARY We investigated the effect of adenosine on neurogenic contraction of the canine cavernous carotid artery, using an isometric tension recording device and transmural nerve stimulation. Adenosine, in concentrations under $10^{-6}$M, had no relaxing effect on the contractions produced by high KCl solution or $10^{-5}$M norepinephrine. Transmural nerve stimulation (stimulus: 1 msec duration, 100V intensity) evoked a frequency-dependent contraction, which was abolished by $3 \times 10^{-4}$M tetrodotoxin. Adenosine in concentrations of $10^{-6}$M and $10^{-5}$M, inhibited the neurogenic contraction at each frequency, more so in the low frequency range. This inhibitory effect of adenosine was significantly antagonized by $10^{-5}$M theophylline.

Pretreatment with $2 \times 10^{-5}$M dipyridamole had no effect on neurogenic contractions, but augmented the inhibitory effect of adenosine. $10^{-5}$M theophylline did not augment the neurogenic contractions. The findings that both dipyridamole and theophylline failed to affect the neurogenic contractions in the absence of adenosine suggests that the presynaptic autoinhibition mechanism of adenosine may not be involved in neuromuscular transmission in this tissue. These results suggest that there is a presynaptic adenosine receptor in the nerve terminal which inhibits the release of neurotransmitter in canine cavernous carotid artery. It is also probable that the vasodilating effect of adenosine in the cavernous carotid artery is mainly due to its inhibitory effect on neurotransmission rather than to a direct relaxing effect on smooth muscle.

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ADENOSINE can markedly affect the blood flow in the cerebral vascular bed as well as in various peripheral vascular beds. It is well known that the vasodilating action of adenosine is due not only to a direct inhibitory effect on vascular smooth muscle, but also to presynaptic inhibition of adenergic transmission in peripheral vascular beds. However, there has been no extensive investigation into the vasodilating action of adenosine on the internal carotid artery system.

Recently, a great deal of evidence has been accumulated that the internal carotid artery system, as well as small vessels, play an active role in the regulation of cerebral blood flow, and an abundant effenter innervation by both adrenergic and cholinergic nerve fibers was demonstrated in the cavernous carotid artery (CCA). Furthermore, when we recorded the neurogenic contraction of canine CCA, the amplitude of these contractions was much larger than that of the carotid bifurcation, and we found that the neuromuscular transmission mechanism of canine CCA was different from that of peripheral arteries (Fujiwara, S, unpublished data).

In this experiment, we investigated the effect of adenosine on canine CCA, especially concerning its effect on neurogenic contraction, in order to clarify the presynaptic role of adenosine and to discern the precise role of cerebral blood flow regulation.
Substrate-specific stimulation by glucagon of isolated murine brain mitochondrial oxidative phosphorylation.
L G D'Alecy, C L Myers, M Brewer, C L Rising and M Shlafer

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