Adenylate Cyclase and Histopathological Changes in the Gerbil Brain Following Prolonged Unilateral Ischemia and Recirculation

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SUMMARY This study was designed to correlate histopathological changes in gerbil brain following unilateral primary and secondary ischemia to enzymatic-adenylate cyclase damage. At three hrs permanent occlusion of the right common carotid artery only minimal histological changes were evident in cerebrum, hippocampus, striatum and olfactory tubercle while the enzyme responses were unremarkable. Severe histological and enzymatic alterations were present at one hour of recirculation subsequent to 3 hrs of unilateral occlusion. Similar damage was evident at 6 and 24 hrs permanent occlusion. Principal enzyme damage was directed toward basal activity, as well as stimulation of the catalytic (forskolin-sensitive) sites on the enzyme complex. For the most part the transducer (GTP-sensitive) site was unaffected by ischemia until 24 hr ligation. These changes were observed in only those gerbils developing severe symptoms of stroke.

DUE TO AN INCOMPLETE CIRCLE OF WILLIS (lack of posterior communicating arteries) perfusion of the gerbil forebrain is dependent upon carotid blood flow. In addition, each cerebral hemisphere is isolated in 20 to 50% of gerbils because of a lack of anastomosis between the anterior cerebral arteries. Thus occlusion of one carotid artery produces a model of ipsilateral cerebral ischemia and edema in these animals. Therefore this model allows for study of not only ischemic regions of the brain, but for concurrent effects on areas remote from the focal injury as well.

The consequences of both unilateral primary ischemia and secondary ischemia involving reflow of blood to damaged brain tissue have been examined in metabolic, enzymatic and morphological studies. One such metabolic system investigated is that of the cyclic nucleotides. Subsequent to an ischemic insult levels of adenosine 3',5' monophosphate (cyclic AMP) are elevated in the cerebrospinal fluid of humans, as well as in the cerebral cortex of rapidly fixed gerbil brain. During reinstitution of blood flow guanosine 3',5'-monophosphate (cyclic GMP) levels in the gerbil brain decrease while cyclic AMP concentrations remain elevated. Investigations using a model of bilateral ischemia reveal that regional damage to adenylate cyclase in the gerbil brain requires a prolonged period of ischemia followed by extended periods of reflow. Only preliminary investigations have evaluated the effects of unilateral ischemia on this enzyme system. It was the intent of the present study to examine the temporal influence of unilateral ischemia with or without reflow on regional activity of adenylate cyclase using this gerbil model. Responses of the enzyme were measured under basal conditions, as well as with applied stimuli to the catalytic (forskolin-sensitive), transducer (GTP-sensitive) and receptor (catecholamine-sensitive) sites on the enzyme. An attempt is made to correlate these biochemical parameters to histopathological alterations within the ischemic brain.

Materials and Methods

Animals

The right common carotid in anesthetized (ketamine 80 mg/kg) female gerbils (25—45 gms, Tumblebrook Farms, West Brookfield, MA) was isolated and clamped with an aneurysm clip. Symptomatic and asymptomatic animals were sacrificed at 3, 6 and 24 hrs after occlusion. The clamp was removed in 5 animals at 3 hrs occlusion to allow recirculation of 1 hr. Resumption of blood flow through the artery was visually ascertained. The right common carotid was exposed and isolated from the adjacent nerve and vein but was not clamped in sham-operated gerbils. Sham-operated gerbils were sacrificed 90 min post-manipulation of the vessel. Symptomatic (ULS) gerbils were distinguished from asymptomatic (ULA) by the presence of neurological signs of stroke and by the central distribution patterns of systemically injected Evan’s Blue dye.

Histology

Thirty min prior to sacrifice the animal was reanesthetized with ketamine and Evan’s Blue dye (10%, 0.5 ml) was injected into the femoral artery. Selected animals from each time frame (3, 6 and 24 hrs permanent occlusion and 3 hr occlusion with 60 min reflow) were reserved for histological examination. The brains were quickly removed and emersion-fixed in 4% paraformaldehyde and 5% sucrose. Fixed brains were rapidly frozen in isopentane (prechilled in liquid N2) and sectioned serially into 16-20 μm sections on a cryostat. Sections were stained with Cajal’s reduced silver stain for frozen sections. Unstained sections were scanned using a Leitz MPV-3 microscopefluorrometer to quantitate...
the intensity and location of Evan’s Blue dye within the parenchyma.

Adenylate Cyclase Assay

Enzyme activity from all animals was evaluated with the exception of gerbils chosen at random for histological examination. Selected areas of the brains were rapidly dissected out and homogenized in glycyglycine buffer (2 mM plus 1 mM MgSO4 and 1.2 mM EGTA, pH 7.4). An aliquot was taken for protein determination by the method of Lowry et al.15 Adenylate cyclase was determined as previously described.11,12 The assay consisted of: a) 60 μg (olfactory tubercle and striatum) and 90 μg (frontal cortex and hippocampus) of homogenate protein in 100 μl buffer; b) a 40 μl solution containing isobutylxanthine-papaverine (both at 0.3 mM), pyruvate kinase (8 μg/mL), Hepes buffer (40 mM, pH 7.4), S-adenosylmethionine (10 μM) and buffered mercaptoethanol (1 mM); c) 20 μl of either control solutions, (0.5% ethanol, distilled water or bovine serum albumin, 40 μg/ml), GTP, catecholamines or forskolin. The assay was maintained at 4°C until reactions were initiated by adding 40 μl of ATP (2 mM)-MgSO4 (4 mM), vortexing and incubating at 37°C for 8 min. The reaction was terminated by boiling for 4 min in a water bath. This was followed by cooling and centrifugation. A 40 μl sample of the supernatant was removed for assay of cyclic AMP.16 Adenylate cyclase activity was expressed as picomoles (pmol) of cyclic AMP formed per min per mg of sample protein. All assays for individual samples were conducted in duplicate. All aspects of adenylate cyclase activation were examined. Basal activity is that level of cyclic AMP generated at 8 min in the absence of stimulatory agents. The stimulatory agents were selected to activate the various components of the adenylate cyclase complex. The receptor site herein responds to NE and dopamine. Receptor activation is magnified via GTP interacting through the transducer site of the enzyme. The catalytic component of adenylate cyclase is presumed to face the interior of the cell and synthesizes cyclic AMP from ATP in the presence of Mg++. Forskolin is postulated to enhance this catalytic activity.11,12

Materials

Ketamine-HCL (Ketalar, injectable 10 mg/ml) was purchased from Parke-Davis. Forskolin (purchased from Calbiochem-Behring) was initially dissolved in 50% ethanol and stored frozen as a 1 mM stock solution. Catecholamines were solubilized in bovine serum albumin (40 μg/ml). Norepinephrine (NE) was used as the "d" form.

Statistics

Statistical evaluations were carried out using a one-way analysis of variance and significance was assessed by the Student-Newman Keuls a posteriori test. The paired, Student's t test was used to examine possible differences between the right and left halves of the same brain. In all cases the difference was considered significant if the probability of the null hypothesis was equal to or lesser than 0.05.

Results

Distinction Between Unilateral Asymptomatic (ULA) and Unilateral Symptomatic (ULS)

The unilateral model of cerebral ischemia in the gerbil allows comparison between the control (non-occluded) and ischemic (occluded) halves of the brain. However, the anomalous cerebral vascular patterns of this animal necessitate distinguishing asymptomatic (those animals with varying degrees of communication between the anterior cerebral artery)17 from symptomatic (those animals with homolateral stroke from occlusion of one carotid artery). Although observation of behavior is a reliable indicator of the presence or absence of a stroke, we were also interested in the extent of the ischemic area visualized with Evan’s Blue dye.

Evan’s Blue dye was used as a marker of vascular distribution and also as an indicator of vascular permeability in those animals in which blood flow through the carotid was resumed after 3 hrs of occlusion. The dye was homogeneously distributed throughout both halves of the brain in sham and ULA animals. One animal that appeared asymptomatic for 24 hrs had a small focus without dye in the region of the right, dorsolateral parietal cortex indicative of a lack of flow within that region. The dye was confined to mainly the left half of the brain in ULS. Occasionally at midline there was an extension of either the ischemic or normoxic demarcation. Mayevsky et al.,18 have recently described an interhemispheric blood supply from the contralateral carotid in the gerbil. There was an increased width and 'sponginess' (indicative of microscopic vacuolization) in the right hemisphere of ULS sacrificed at 6 and 24 hrs after occlusion. The right half of the brains of those animals in which blood flow was reestablished also displayed a certain degree of 'sponginess' however, less marked. The hippocampus of one of these animals had striae of pinpoint hemorrhages throughout.

Thirty-seven percent of animals with unilateral occlusion of the right common carotid were designated as ULS. Symptoms included circling movements, neck and trunk torsion, hemiparesis (usually ipsilateral) and sometimes, seizures. One or more of these symptoms became apparent 30 to 60 min after occlusion in one group of animals while another group did not manifest symptoms until 2–3 hrs after occlusion. The former group, classified as rapidly progressive stroke as described by Yanagihara19 was used for biochemical determinations at 3 (with and without recirculation) and 6 hrs. Animals with delayed onset of symptoms survived to 24 hrs and were regarded as moderately symptomatic. However, these animals eventually displayed severe neurological symptoms by the time of sacrifice.

Effect of Unilateral Carotid Occlusion on Adenylate Cyclase Activity

When homogenates from the 4 brain regions namely, frontal cortex, hippocampus, striatum and olfactory tubercle, were evaluated for adenylate cyclase...
activity in ULA or animals displaying mild symptoms, there were no differences in enzyme responses between left and right halves of the brain. This observation occurred under conditions of either basal activity, or GTP, forskolin and NE stimulation.

Basal adenylate cyclase activities within the 4 brain regions of sham-operated and ULS are presented in figure 1. The frontal cortex, hippocampus, striatum and olfactory tubercle were chosen as representative of the blood supply of the internal carotid and show enzymatic liability to bilateral ischemia. Basal activity significantly decreased in areas of the right (ischemic) half of the brain compared to the left (normoxic) at 6, 24 and 3 hrs (one hr reflow) postocclusion. Additional basal activity depression was seen in the right olfactory tubercle when compared to the left at 3 hrs (permanent ligation), but not when compared to sham-operated controls (Newman-Keuls test).

The ability of GTP (10^{-5} M) to activate adenylate cyclase was uniformly depressed at 24 hrs in all areas of the right hemisphere in comparison with the left side. Similar depression of GTP action was seen in only the frontal cortex and striatum after 3 hrs ligation plus one hr of reflow (fig. 2). There was a consistent trend (nonsignificant, analysis of variance) towards an increase in activation of the enzyme in both hemispheres after 3 hrs of ischemia. Those 3 hr animals in which blood flow was resumed displayed no such trend.

Forskolin (10^{-5}M) stimulation of adenylate cyclase was attenuated in each right brain area as compared to the left at 6 and 24 hrs. No changes were seen between the right and left hemispheres at 3 hrs of ischemia unless reflow occurred in which case the cortical, hippocampal and striatal enzymes displayed insult (fig. 3).

The ability of NE (10^{-4} M) to stimulate adenylate cyclase within the cortex and hippocampus is depicted in figure 4. Great disparities were noted in the NE-stimulation of the enzyme within the right cortex and hippocampus as compared to the left. The cortical and hippocampal enzyme displayed a decreased responsiveness to NE at 6 hrs and 24 hrs of ischemia and with 3 hrs plus 1 hr recirculation. In addition, enzyme activation in the right hippocampus appeared depressed at 3 hrs (no reflow).

An attempt was made to evaluate dopamine (DA) elicited responses in the striatum and olfactory tubercle. However percent stimulation of the enzyme over basal activity ranged from 0 to 126% in sham-operated and ULA. In view of the lack of consistent controls it was difficult to analyze any DA-activated changes oc-
curing within the striatum and olfactory tubercle of ULS.

Histopathological Changes

The right and left frontal cortex, hippocampus, striatum and olfactory tubercle were examined with light microscopy at each time frame. Since each area displayed a similar injury profile, the frontal cortex was chosen as representative. On the normoxic left side no neuropathology was evident at 3 hrs ligation (with or without reflow). With prolonged occlusion (6 and 24 hrs) edema consisting of sparse vacuolization with few pyknotic neurons was seen on the left side. With respect to the experimental right side of the brain moderate damage was seen at 3 hrs of occlusion as evidenced by an increase in the perineuronal space and heterogeneous neuronal changes (fig. 5). When reflow was reinstated after 3 hrs of occlusion, cellular disruption was evident throughout the right cortex. Thus there was a virtual lack of normal appearing neurons (fig. 6).

Progression of neuropathological changes occurred in the right side of the frontal cortex at 6 to 24 hrs permanent occlusion (data not shown). There was almost a total loss of neuronal structure (especially at 24 hrs) and an increase in background due to degenerating fibers and terminals. At 24 hrs there was a confluent necrosis in all areas examined of the right half of the brain.

The olfactory tubercle was utilized to conduct a microspectrofluorometric scan of the frequency distribution of Evan’s Blue dye. These scans evaluated the intensity and site of Evan’s Blue fluorescence within an arbitrary scale of 0 to 200 units. Evan’s Blue fluorescence has been used previously to quantify vascular protein leakage. No dye was present in the occluded (right) side of the brain indicating a complete cessation of blood flow. Dye was present in only the vasculature of the control (left) half of the brain (data not shown). The right hemisphere of those gerbils in which blood flow was resumed after 3 hrs of occlusion displayed a lack of vascular pattern with dye dispersed throughout the parenchyma (See fig. 7 for representative olfactory tubercle). Twenty-seven percent of the total area scanned was labelled on the left half of the brain while 98% was labelled on the right half. These results indicate a total disruption of the vascular endothelium coincident with recirculation.

In some preliminary work with Evan’s Blue perfusion at 3 hrs plus 1 hr reflow we evaluated adenylate cyclase responses. The presence of both anesthesia and Evan’s Blue dye gave enzymatic damage profiles identical to those seen in figures 1–4.

Discussion

Previous investigations have noted the ambiguity of neurological symptoms of the gerbil model of cerebral ischemia. Using the classification of Yanagihara we evaluated at 3 and 6 hrs only those animals displaying severe neurological symptoms by 30 min of occlu-

Figure 3. Regional forskolin (10^{-5} M) stimulation of adenylate cyclase during various periods of occlusion and with 1 hr reflow. Values are the mean percentage stimulation over basal activity ± SE of 4–6 experiments per time period. *p < 0.05, paired-t test compared to corresponding side. Hatched bars = sham-operated gerbils, open bars = control left side and shaded bars = ischemic right side.

Figure 4. Norepinephrine (NE) (10^{-5} M) activation of adenylate cyclase within the left (open circles) and right (closed circles) cortex and hippocampus. Enzyme responses in sham-operated animals are denoted as open squares. Values are the mean percentage stimulation over basal activity ± SE of 4–6 separate experiments per time period. *p < 0.05, paired-t test compared to corresponding side.
FIGURE 5. Photomicrograph (silver stain) of the left (A, 40×) and right (B, 64×) frontal cortex of a gerbil 3 hrs after occlusion of the right common carotid.

sion. Although we found no apparent differences in basal or agonist-activation of adenylate cyclase in either hemisphere of asymptomatic (ULA) gerbils, changes have been observed in blood flow, glycolytic intermediates, P-creatine and lactate levels of the occluded hemisphere of these animals.13 Previous investigations have also compared the contralateral (non-ischemic) hemisphere to the ipsilateral (ischemic) half of the brain in symptomatic animals with regard to other metabolic profiles namely, protein synthesis, cyclic nucleotide content and cyclic nucleotide related enzymes.4,5,9,10,19 However, biochemical alterations have been noted to occur concurrently during ischemia in the control-contralateral hemisphere of symptomatic gerbils. Therefore we felt that any investigation utilizing this unilateral model of ischemia in the gerbil should include comparisons between the ipsilateral (ischemic) hemisphere and both the contralateral hemisphere and sham-operated controls. On the other hand, we could detect no significant differences between the enzyme parameters within brain regions of sham-operated gerbils as compared to contralateral (control) brain regions of symptomatic animals.

This enzyme investigation dealt with three phenomena: 1) the temporal changes during periods of prolonged ischemia upon components of the adenylate cyclase system; 2) the effect of recirculation upon the enzyme during the postictal period; and 3) the selective vulnerability to ischemia of different brain regions with regard to the adenylate cyclase system as previously shown during bilateral ischemia.12 No qualitative histopathological differences were noted between the four regions examined during prolonged ischemia i.e., each region was severely compromised. In addition, adenylate cyclase activity from the 4 areas displayed similar damage profiles at each time frame. Shorter primary and secondary ischemic time periods have indicated selective damage to limbic and striatal subregions with regard to deoxyglucose uptake.21 Previous work has shown that cortical adenylate cyclase is more resistant to short-term ischemia.11,12 Regional enzyme damage was, however, found after bilateral ischemia of 60 min plus reflow.12

Ischemia for a period of time longer than 3 hrs is required before both overt histological and enzymatic (adenylate cyclase) injury becomes manifest in the gerbil forebrain. Raichle22 has recently reviewed the literature and reported that in several instances there is partial recovery of cerebral tissue after prolonged ischemia. At 3 hrs of ischemia only the ipsilateral olfactory tubercle displayed a significant decrease in basal adenylate cyclase activity but only when compared to the corresponding contralateral tuberculum. However, at 6 and 24 hrs basal activity of all areas examined was...
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Depressed with the exception of the frontal cortex. Schwartz, et al, likewise found no differences in cortical basal activity at 3 or 6 hrs of ischemia.

Agonist stimulation of the enzyme was evaluated using GTP, forskolin and DA or NE as respective activators of the transducer, catalytic and receptor components of the enzyme complex. The GTP-responsiveness of adenylate cyclase was unaffected by ischemia of even 6 hrs duration. Stimulation of the enzyme by GTP was significantly decreased only at 24 hrs within all areas of the ipsilateral hemisphere and within the cortex and striatum after reflow. The nonsignificant trend towards an increase in GTP activation at 3 hrs in both hemispheres during ischemic conditions has been observed in two other experimental paradigms, that of 15 mins bilateral ischemia plus 15 mins reflow and 60 mins of ischemia without reflow. This broad fluctuation in GTP-stimulated activity of adenylate cyclase is indicative of an increased lability, or perhaps, flexibility of the guanine nucleotide regulatory protein(s) to ischemia.

On the contrary, alterations in forskolin-activation of the enzyme appear to be analogous to changes in basal activity of the enzyme in that stimulated activity is depressed by 6 hrs of ischemia. Forskolin is thought to stimulate primarily the catalytic site of adenylate cyclase. However, recently the specificity of forskolin's action on the adenylate cyclase complex has been questioned and may require GTP for full expression of activity. A plausible explanation for attenuated basal and forskolin stimulation of adenylate cyclase could be roughly drawn between our findings and those of Yanagihara. Yanagihara found a marked depression of protein synthesis in cerebral tissue at 3 and 6 hrs of unilateral ischemia in gerbils. This may be reflected in either diminished synthesis of adenylate cyclase or an enhanced breakdown of vulnerable enzyme proteins. Since GTP coupled enzyme responses were still present at 6 hrs of ischemia, forskolin activation of adenylate cyclase must occur via a coupling of additional labile proteins as alluded to by a number of recent studies. In addition, Brooker, et al, reported that when protein synthesis inhibitors were present in cultured astrocytoma cells, forskolin activation of adenylate cyclase was selectively diminished while that of GTP, as assessed by cholera toxin stimulation, was spared.

Mrusulja and coworkers found that if cerebral blood flow was re-established (secondary ischemia) even after prolonged ischemia, energy metabolites return to approximate pre-ischemic values. The paradox of normalization of energy state despite increased cellular
damage due to reflow has been attributed to acidosis, an increased intracellular calcium accumulation and/or the generation of free radicals. In preliminary work, we have indeed observed a direct free radical-induced damage to adenylate cyclase. In the present study at 3 hrs of occlusion of the common carotid artery little cellular or enzyme damage was observed unless reflow was permitted. Schwartz et al. also found changes in adenylate cyclase activity only after reperfusion following 1 hr of unilateral carotid ligation in the gerbil cerebral cortex. Moreover, microspectrofluorometric analysis of the brains of gerbils undergoing 3 hrs of ischemia revealed a total disruption of the vasculature during recirculation. Using a variety of experimental techniques and different species, post-ischemic morphological damage has likewise been demonstrated following primary ischemia.

Only slight histopathological damage was seen at 3 hrs in gerbils with permanent unilateral carotid occlusion. Severe damage with progressing cellular death was evident from 6 to 24 hrs. These findings are in accordance with a similar study by Yanagihara.

The present findings show that unilateral carotid occlusion in the gerbil forebrain requires a time period of over 3 hrs unless reflow is permitted before prominent enzyme and cellular damage is demonstrated. However, no attempt was made either histologically or biochemically to ascertain whether or not there was a specific vulnerability of certain cell types within each region of ischemic insult. In addition, enzyme alterations could have been present in small focal areas, but were not demonstrable within the region as a whole.

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