Protective Action by Methylprednisolone, Allopurinol and Indomethacin Against Stroke-Induced Damage to Adenylate Cyclase in Gerbil Cerebral Cortex

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SUMMARY Adenylate cyclase activity was investigated in either homogenate or particulate fractions from the frontal cerebral cortex of the gerbil following five experimental conditions of bilateral ischemia. After periods of 15 min ischemia, 15 min ischemia plus 15 min of recirculation or 60 min ischemia the enzyme generally displayed enhanced responses to GTP, norepinephrine (NE), dopamine (DA), NE + GTP and DA + GTP. Pretreatment of the gerbils with methylprednisolone, allopurinol or indomethacin did not significantly influence the outcome of these findings. When the animals were subjected to 60 min ischemia plus 15 min of reflow, enzyme responses to the stimulatory agents including forskolin and NaF were all reduced. Pretreatment with methylprednisolone, allopurinol or indomethacin prevented the damage to adenylate cyclase in the 60 min ischemia plus 15 min reflow animals. When animals were made ischemic for 15 min followed by one week of recovery, enzyme sensitivity to GTP, calmodulin-Ca++, NE, combinations thereof and forskolin were reduced in only the particulate fractions. Enzyme damage was reversed following methylprednisolone. Enzyme damage may result from generation of free radicals during reflow and drugs that either inhibit synthesis pathways generating free radicals, stabilize cell membranes or act as free radical scavengers may be therapeutically beneficial under specific conditions of stroke.

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depressed. Neither guanylate cyclase (EC 4.6.1.2) nor cyclic AMP phosphodiesterase (EC 3.1.4.17) activities were altered in these experiments.

The damage to central enzymes seen after a period of ischemia followed by recirculation has been termed "secondary ischemia" and has been attributed to various biochemical processes namely: release of cellular lysosomes, breakdown of the blood-brain barrier, Ca++ influx, formation of free radicals, rise in extracellular K+, prostaglandin formation, cellular acidosis and damage to cell membrane components resulting from activation of membrane phospholipases.

Therefore, any agent offering protection against one or more of these processes might afford a degree of therapeutic benefit toward stroke-induced damage. In the present investigation we report that the greatest damage to the adenylate cyclase system in the gerbil cerebral cortex was seen following reflow of blood subsequent to 60 min of bilateral ischemia. Injections of methylprednisolone, an agent that stabilizes cell membranes, prevents lysosomal breakdown and acts as a free radical scavenger prevented the damage to adenylate cyclase. In addition, allopurinol (inhibits xanthine oxidase) and indomethacin (inhibits cyclo-oxygenase and prostaglandin synthesis) two agents that inhibit enzymatic processes associated with free radical formation were likewise effective. Furthermore it is not known what influence the release of lysosomes, Ca++, free radicals, acidosis, etc. would have on central acenylate cyclase. In an attempt to circumvent some of these considerations a particulate form of the enzyme was evaluated in addition to cortical homogenates.

Methods

Female gerbils (Meriones unguiculatus, 35–50 gm) were purchased from Tumblebrook Farms, West Brookfield, Massachusetts. Bilateral ischemia was performed according to the procedure of Lust and Pas-
sonneau. The animals were fasted for 24 hours followed by anesthesia (thiopental 35 mg/kg). A midline cervical incision was made and both common carotid arteries were isolated and looped with silk thread (6-0). After a 3 hour recovery period, both carotid arteries were occluded using aneurysm clamps. Ischemia was allowed for either 15 min (reversible) or 60 min (irreversible). In designated experiments the clamps were removed from the 15 min ischemia animals and recirculation of blood was allowed for either 15 additional min or 7 days. With the 60 min ischemic period specified animals received a period of 15 min of reflow. 

Reflow was verified by visual inspection of blood flowing past the point of occlusion. Almost all animals (95%) survived the period of 15 min ischemia. With the use of thiopental as an anesthetic 80% survive the 60 min ischemia and associated reflow. At the termination of the ischemia experiments the animals were decapitated and the frontal cortex (rostral to the anterior commissure) from both sides was rapidly removed and homogenized in glycyl-glycine buffer (2 mM plus 1 mM MgSO₄ with 1.6 mM EGTA, pH 7.4). In one group of animals the cortical homogenates were centrifuged at 800 x g for 8 min. The resulting supernatant was recentrifuged at 27,000 x g for 20 min. This latter procedure was repeated and the final pellet (calmodulin-free) again was resuspended in glycylglycine buffer and used as a particulate form of adenylate cyclase (60 ug enzyme protein per sample). The method of Lowry and coworkers was used for determinations of sample proteins.

**Adenylate Cyclase Determination**

Our previous method was followed in order to assay adenylate cyclase. The enzyme preparations (100 ul) were added to a reaction mixture containing the following at final concentrations and volumes: (a) 40 ul solution containing isobutyl methylxanthine-papaverine (0.2 mM each), phosphoenol pyruvate (1 mM), pyruvate kinase (8 ug), Hepes buffer (40 mM, pH 7.4), and buffered mercaptoethanol (1 mM); (b) 20 ul of control solutions, drugs, GTP, or catecholamines. In addition, in designated particulate samples 3.42 ug of calmodulin plus Ca²⁺ (10⁻⁴ M) were added to the assay mixtures; (c) the reactions were initiated by adding 40 ul of ATP (2 mM) - MgSO₄ (4 mM), vortexing and incubating 8 min at 37° C. Enzyme reactions were terminated by capping the tubes and boiling in a water bath (4 min). After cooling and centrifugation a sample of the supernatant was taken for assay of cyclic AMP. Adenylate cyclase activity was expressed as pico moles of cyclic AMP formed per min per mg protein. All assays for individual samples were in duplicate.

**Drug Injections**

Methylprednisolone sodium succinate (Solu-Medrol, a gift from Upjohn Co) was injected (30 mg/kg) 30 min prior to clamping the carotid arteries. In the animals that were allowed to recover for one week, methylprednisolone was also injected at 8 and 16 hours post-ischemia. Indomethacin (Sigma Chem. Co.) was injected 2 mg/kg, 1 hour prior to bilateral ischemia while allopurinol (Sigma Chem. Co.) was injected 5 mg/kg 30 min before ischemia. Sham operated gerbils were injected with distilled water containing 0.9% benzylalcohol used to dissolve methylprednisolone, 30% ethanol used to solubilize indomethacin or 50% dimethylsulfoxide used to dissolve allopurinol. All drugs were injected ip. In designated samples these drugs or equivalent vehicle solutions were added to adenylate cyclase preparations in vitro. Furthermore the drugs were injected into selected unoperated gerbils and their lack of influence on the adenylate cyclase preparations was noted. We also evaluated the effects of in vivo injections of methylprednisolone on cyclic AMP dependent phosphodiesterase assayed in vitro using the cortical homogenates under conditions of high (0.4 mM) or low (1 uM) substrate (cyclic AMP). For this experiment previous methodology was followed.

The Student's paired t test was used to compare data between sham operated control and ischemic animals. Data between drug injected or ischemic animals and sham operated controls was compared using the Student's two-tailed t test. A 'P' value of less than 0.05 was considered to be a significant change. Either 4 or 5 animals were used for each experimental condition.

**Results**

**Normal Animals**

In homogenates of gerbil frontal cortex both NE and DA (10⁻⁴ M) or GTP (10⁻⁵ M) elevated adenylate cyclase by 30-40% over respective basal activity. In the presence of GTP the action of NE and DA was enhanced in an additive manner to a degree of stimulation of 65-85% over basal enzyme activity. Forskolin (10⁻³ M) and NaF (5 mM) stimulated adenylate cyclase to approximate values of 160 and 250% respectively (fig. 1, tables 1-4).

Adenylate cyclase in particulate preparations of frontal cortex was activated by 10⁻⁵ M GTP (37%), 3.4 ug calmodulin- 10⁻⁴ M Ca²⁺ (24%), 10⁻⁴ M NE (20%), NE + calmodulin-Ca²⁺ in the absence (33%) or presence of GTP (55%) and 10⁻⁵ M forskolin (233%) (fig. 2).

**a. Fifteen min Ischemia with No Recirculation**

Homogenates of frontal cortex displayed an increased adenylate cyclase sensitivity to DA, DA + GTP, forskolin and NE + GTP following a 15 min period of bilateral clamping of the carotid arteries. When methylprednisolone was preinjected 30 min prior to the ischemia the only observation that differed from sham-operated controls was that the enzyme was more sensitive to GTP alone (table 1). Neither allopurinol nor indomethacin when preinjected into the animals had any prominent effects on adenylate cyclase
when assessed after 15 min of ischemia (data not shown).

The observations for the particulate enzyme preparations were somewhat different to that of the homogenates. In this case sensitivities of adenylate cyclase were significantly augmented in response to GTP, NE, DA and forskolin following 15 min ischemia. Pretreatment of the gerbils with methylprednisolone apparently reduced to sham control values the adenylate cyclase (homogenate) elevation by NE and DA + GTP (table 2). Pretreatment with either allopurinol or indomethacin did not produce any change in the enhanced enzyme activation (both homogenate and particulate) as a consequence of 15 min ischemia followed by 15 min reflow (data not shown).

### b. Fifteen Min Ischemia Plus 15 Min Recirculation

In this situation the sensitivity of adenylate cyclase in the homogenate to GTP, DA, NE, DA + GTP and NE + GTP was enhanced in the ischemic animals. The particulate enzyme was more sensitive to NE and DA + GTP. Pretreatment of the gerbils with methylprednisolone apparently reduced to sham control values the adenylate cyclase (homogenate) elevation by NE and NE + GTP (table 2). Pretreatment with either allopurinol or indomethacin did not produce any change in the enhanced enzyme activation (both homogenate and particulate) as a consequence of 15 min ischemia followed by 15 min reflow.

### Table 1. Effect of 15 min Bilateral Ischemia (no reflow) on Adenylate Cyclase in Homogenates of Gerbil Frontal Cortex

<table>
<thead>
<tr>
<th>Additions</th>
<th>Sham</th>
<th>Control</th>
<th>Methylprednisolone</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP 10^-5 M</td>
<td>36 ± 2</td>
<td>33 ± 2</td>
<td>61 ± 6*</td>
</tr>
<tr>
<td>DA 10^-4 M</td>
<td>31 ± 2</td>
<td>41 ± 7*</td>
<td>42 ± 5*</td>
</tr>
<tr>
<td>NE 10^-4 M</td>
<td>37 ± 1</td>
<td>43 ± 7</td>
<td>53 ± 5*</td>
</tr>
<tr>
<td>DA + GTP</td>
<td>65 ± 3</td>
<td>78 ± 2*</td>
<td>85 ± 5*</td>
</tr>
<tr>
<td>NE + GTP</td>
<td>78 ± 3</td>
<td>98 ± 2*</td>
<td>110 ± 20</td>
</tr>
<tr>
<td>Forskolin 10^-5 M</td>
<td>149 ± 6</td>
<td>133 ± 15</td>
<td>175 ± 5</td>
</tr>
<tr>
<td>NaF 5 mM</td>
<td>180 ± 30</td>
<td>190 ± 25</td>
<td>255 ± 10</td>
</tr>
</tbody>
</table>

Values are the mean ± SE of 4 experiments.

*Significantly different (p < 0.05) than corresponding sham-operated control.

Basal enzyme activities (pmol cyclic AMP formed/min/mg protein) were: sham = 43 ± 4; ischemia control = 41 ± 4; and ischemia with methylprednisolone pretreatment = 39 ± 7.

### Table 2. Effect of 15 min Bilateral Ischemia Plus 15 min Reflow on Adenylate Cyclase in Homogenates of Gerbil Frontal Cortex

<table>
<thead>
<tr>
<th>Additions</th>
<th>Sham</th>
<th>Control</th>
<th>Methylprednisolone</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP 10^-5 M</td>
<td>33 ± 2</td>
<td>43 ± 4*</td>
<td>45 ± 3*</td>
</tr>
<tr>
<td>DA 10^-4 M</td>
<td>27 ± 2</td>
<td>35 ± 3*</td>
<td>36 ± 9</td>
</tr>
<tr>
<td>NE 10^-4 M</td>
<td>38 ± 2</td>
<td>50 ± 2*</td>
<td>39 ± 6</td>
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<tr>
<td>DA + GTP</td>
<td>68 ± 2</td>
<td>79 ± 4*</td>
<td>83 ± 3*</td>
</tr>
<tr>
<td>NE + GTP</td>
<td>77 ± 3</td>
<td>98 ± 7*</td>
<td>82 ± 6</td>
</tr>
<tr>
<td>Forskolin 10^-5 M</td>
<td>165 ± 16</td>
<td>152 ± 13</td>
<td>172 ± 6</td>
</tr>
<tr>
<td>NaF 5 mM</td>
<td>240 ± 15</td>
<td>300 ± 24</td>
<td>238 ± 22</td>
</tr>
</tbody>
</table>

Values are the mean ± SE of 4 experiments.

*Significantly different (p < 0.05) than corresponding sham-operated control.

Basal enzyme activities (pmol cyclic AMP formed/min/mg protein) were: sham = 42 ± 4; ischemia control = 44 ± 3; and ischemia with methylprednisolone pretreatment = 41 ± 6.
responses to GTP, DA, DA + GTP and NE + GTP.

Effects of 60 min ischemia plus 15 min reflow. In fact the clamping of the carotids prevented the damaging effects of 60 min ischemia plus 15 min reflow. Pretreatment of the animals with methylprednisolone (30 mg/kg, 30 min prior to bilateral clamping of the carotids) prevented the damage to adenylate cyclase elicited by the 60 min stroke plus 15 min reflow (table 4). Some cases pretreatment with allopurinol or indomethacin reversed the deficits in enzyme activity (data not shown).

d. Sixty Min of Ischemia Plus 15 Min of Recirculation

As noted in our previous work this experimental situation resulted in the greatest damage to the cerebral adenylyl cyclase system. In cortical homogenates enzyme responses to DA, NE, NE + GTP, NaF, and forskolin were attenuated. Pretreatment with methylprednisolone (30 mg/kg, 30 min prior to bilateral clamping of the carotids) prevented the damaging effects of 60 min ischemia plus 15 min reflow. In fact the responses to GTP, DA, DA + GTP and NE + GTP were significantly greater than the sham-operated controls (fig. 1). Furthermore, pretreatment of the gerbils with allopurinol (5 mg/kg 30 min prior to ischemia) or indomethacin (2 mg/kg 60 min before ischemia) also reversed the damage to adenylyl cyclase elicited by the 60 min stroke plus 15 min reflow (table 4). In some cases pretreatment with allopurinol or indomethacin reversed the damage to the enzyme above that of the sham controls. Specific situations when this happened were: with indomethacin — GTP, DA, NE, DA + GTP and NE + GTP; and with allopurinol — DA.

Adenylyl cyclase in the cortical particulate fraction was likewise less responsive to DA, DA + GTP and forskolin as a consequence of 60 min ischemia plus 15 min reflow. Pretreatment of the animals with methylprednisolone, allopurinol or indomethacin prevented the deficits in enzyme activity (data not shown).

e. Fifteen Min Ischemia Plus One Week of Recovery

Homogenates of the cortical enzyme from these experimental animals only revealed a tendency (nonsignificant) to be less sensitive to GTP, NE, DA and forskolin. These deleterious effects were however, not present when the gerbils were given 3 treatments with methylprednisolone (30 mg/kg, 30 min prior to ischemia and 8 and 16 hrs post ischemia) (data not shown). On the other hand, the particulate enzyme displayed deficits to all parameters of activation, namely GTP, calmodulin-Ca++, NE, NE+ calmod-
ulcin-Ca+++, NE + GTP + calmodulin-Ca+++, and forskolin. Treatment with the 3 injections of methylprednisolone prevented the damage to the particulate enzyme (fig. 2).

f. Lack of Drug Effects in Control Animals

When methylprednisolone, allopurinol or indo-
methacin were pre-injected into sham-operated gerbils under similar conditions as the stroke animals, they did not produce any changes in the sensitivity of either basal adenylate cyclase (either particulate or homogenate preparations) or to the degree of enzyme activation by GTP, catecholamines, forskolin or NaF (data not shown). The drugs at concentrations from 10^{-4} to 10^{-6} M when added in vitro to a control enzyme preparation (homogenates) neither influenced the basal activity of adenylate cyclase nor its activation by NE, GTP or forskolin. In this case control preparations of adenylate cyclase contained equal amounts of solvents used to dissolve the drugs.

In our earlier paper the activity of cyclic AMP phosphodiesterase was not altered by conditions of bilateral ischemia and reflow. Adrenal corticosteroids, at high concentrations have been found to inhibit phosphodiesterase. In the present experiments (15 and 60 min ischemia with or without 15 min recirculation) cyclic AMP phosphodiesterase was again measured in homogenates of cerebral tissue from the sham control, ischemic or ischemic plus methylprednisolone-treated animals. When assayed under conditions of high (4 mM) or low (1 uM) cyclic AMP concentrations, the activity of phosphodiesterase did not vary among the four experimental conditions (data not shown).

Discussion

The present experiments as well as earlier studies reveal the presence of a catecholamine, GTP, NaF and forskolin sensitivity of adenylate cyclase in homogenates of the gerbil frontal cortex. Likewise the action of catecholamines was additive in the presence of GTP. The particulate fraction presumably devoid of calmodulin-Ca++ was similarly sensitive to the above agents. Moreover, addition of calmodulin-Ca++ back to the particulate preparation gave progressively greater enzyme responses in the presence of GTP and/or NE. These latter findings suggest that calmodulin-Ca++ may play a minor role with regard to the stimulation of adenylate cyclase in the gerbil cortex. Earlier work had established a link between calmodulin-Ca++ and adenylate cyclase activation in specified brain regions from other species.

In our previous study we were unable to ascribe a role in the gerbil cerebral for calmodulin-Ca++ activation of cyclic AMP phosphodiesterase as previously described by Uzunov and Weiss for other species. The prominent stimulatory actions of forskolin on adenylate cyclase in the present study has been attributed to its effect on the catalytic site of the enzyme.

The slight elevation of cerebral adenylate cyclase in response to catecholamines and/or GTP as observed under conditions of 15 min ischemia with or without recirculation or 60 min of ischemia (no recirculation) is in accord with our earlier work. In other investigations using gerbils, mice or rats, ischemic or anoxic insults to the brain resulted in increased steady-state levels of cyclic AMP as well as elevated activity of Na+, K+ - ATPase at various time periods up to an hour. Perhaps the added sensitivity of the enzyme in our work is reflected in part to these elevated concentrations of cyclic AMP. However, ischemic conditions evoke marked releases of transmitters and adenosine which most certainly elevate the cyclic nucleotide. In addition, bilateral ischemia does result in varying degrees of seizure activity in gerbils. Seizures readily elevate cyclic AMP in the brain, however, it is not known under acute situations whether or not convulsant activity influences the sensitivity of adenylate cyclase to agonists. In the present study, seizures in gerbils preanesthetized with thiopental were not a major problem such as we have observed using other modes of anesthesia (unpublished findings). In most cases the animals rendered ischemic for 60 min with or without reflow were extremely lethargic.

Damage to the adenylate cyclase system only occurred when bilateral ischemia (60 min + 15 min reflow) caused reductions in the ability of DA, NE, NE + GTP, NaF and forskolin to activate the enzyme. Fifteen min of ischemia plus one week of reflow did reduce mainly the responses of the particulate enzyme to calmodulin-Ca+++, NE, GTP (and combinations thereof) and forskolin. This latter instance was the only situation in which particulate fractions showed a remarkable difference in neurohumoral sensitivity when compared with the homogenate enzyme. The basis for these variant findings is currently unknown. Interestingly, basal activity of adenylate cyclase was unaltered in these reflow experiments. Therefore damage to the enzyme must be directed toward its sensitivity to agonists acting at receptor (catecholamines), transducer (GTP) or catalytic (NaF and forskolin) sites. It is presently unknown as to which particular biochemical event produces this enzyme insensitivity. A likely candidate might be lactate accumulation. Hopefully this was minimized following fasting of the gerbils. Other possibilities are considered below. Adenylate cyclase damage following reflow from ischemia in gerbils was likewise reported in the initial study by Schwartz et al.

If methylprednisolone was preinjected before bilateral ischemia the above noted damage to adenylate cyclase as a consequence of recirculation was prevented. Pretreatment of dogs with methylprednisolone has likewise been shown to slow the rise in cardiac cyclic nucleotides, limit the breakdown of lysosomes and enhance hemodynamic and contractile indices of the heart following a period of ischemia plus recirculation. Corticosteroids have been used with varying degrees of success to treat brain edema. In addition these drugs prevent the release of arachidonic acid by inhibiting the action of phospholipases. Thus during reflow the metabolic cascade of events resulting in the breakdown of membrane phospholipids, the synthesis of vasoactive prostaglandins and the formation of free
radicals is inhibited. The stabilization of cell membranes and lysosomes plus the prevention of free radical production along with improved hemodynamic indices are the most likely sites of therapeutic action of glucocorticoids following an ischemic insult.11, 12, 32, 34

Pretreatment of gerbils with indomethacin also protected the adenylate cyclase system in the gerbil brain against the consequences of recirculation following 60 min of bilateral ischemia. Indomethacin has been shown in ischemic gerbil brain to block the formation of prostaglandins. Moreover, indomethacin-treated gerbils were observed to recover more rapidly.35, 36 In related studies involving experimental ischemia, pretreatment of different species with indomethacin afforded protection to cerebral blood vessels, improved vascular flow, prevented cerebral edema at low blood flow states and enhanced recovery.34, 37, 38 Indomethacin inhibits cyclo-oxygenase and hence the prostaglandin synthesis pathways are blocked. During the subsequent steps of prostaglandin synthesis, especially following ischemia, free radicals are generated which have been shown to damage the integrity of phospholipid components in cell membranes. This damage is especially evident during reflow following ischemia when the blood source of metabolites is available to the central tissue due to a breakdown of the blood-brain barrier. In addition, thromboxane A2 is synthesized from platelets in the microvasculature thereby promoting platelet aggregation and vasoconstriction of cerebral arteries. For some unexplained reason the proper balance between prostacyclin synthesis (disperses platelets and dilates vessels) is upset and the tissue damage is worsened.10-12

Other agents acting as either free radical scavengers of inhibitors of other enzymatic pathways that would form these highly reactive compounds might be expected to provide protection of cell membranes from the deleterious effects of recirculation following interruption of blood flow to the brain.39 In the present experiments pretreatment with allopurinol did provide such protection of adenylate cyclase. Since the enzyme exists as a complex embedded in the cell membrane it would be expected to suffer damage as a result of phospholipase action and free radical disruption of phospholipids.12, 13 The integrity of the phospholipid components of the cell membrane is necessary for full expression of receptor mediated adenylate cyclase activity.39 It is presently unknown whether free radicals actually produce direct damage to brain adenylate cyclase. However, when fat cell membranes were exposed to ionizing radiation at low temperatures subsequent damage to the enzyme was observed. Free radicals do damage Na+, K+-ATPase under in vitro conditions,41 however, whether such a mechanism occurs during cerebral ischemia is an equivocal topic.28

Since stroke is the major neurological problem in the United States, studies directed toward identification of altered biochemical marker sites within the brain would provide a rationale to evaluate therapeutic agents which could lead to either a prevention of stroke or aid in the recovery of such patients. Future questions to be asked might include: identification of other molecular markers; evaluation of other potentially therapeutic agents eg., barbiturates, naloxone, calcium blockers, etc; and a more precise determination of time and dose schedules for these drugs.

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