Focal Ischemia Causes an Extensive Induction of Immediate Early Genes That Are Sensitive to MK-801

Yolanda Collaco-Moraes, BSc; Benjamin S. Aspey, MPhil; J.S. de Belleruche, PhD; Michael J.G. Harrison, FRCP

Background and Purpose. There is strong evidence to implicate glutamate in the cerebral damage caused by ischemia. In this study we investigated the role of glutamate receptors in mediating effects of middle cerebral artery occlusion (MCAO) on immediate early gene expression in the rat by quantitation of mRNA levels.

Methods. The effect of MCAO on the induction of immediate early genes was studied in five regions, both ipsilateral and contralateral to the occlusion: the core ischemic area of the cortex in the central region of the middle cerebral artery territory, the surrounding area, frontal cortex, occipital cortex, and hippocampus. Levels of c-fos, c-jun, zif-268, and krox-20 mRNA were measured by Northern and slot blot analysis.

Results. A large induction of c-fos mRNA was obtained in all four cortical regions ipsilateral to the occlusion, with the greatest effect detected in the core area. Little effect was detected in the ipsilateral hippocampus and in all contralateral regions. Pretreatment with MK-801 (3 mg/kg) largely inhibited the induction of c-fos mRNA, indicating that the induction was mediated through an N-methyl-D-aspartate subtype of glutamate receptor. MCAO also produced a significant induction of c-jun and zif-268 mRNA in ipsilateral cortical regions.

Conclusions. These results indicate that MCAO causes a profound modulation of the expression of multiple genes in an extensive area of cerebral cortex extending beyond the immediate area supplied by the middle cerebral artery. The marked effect of MK-801 indicates the potential importance of glutamate antagonists in restricting the widespread deleterious effects of glutamate.

Key Words: cerebral arteries • cerebral ischemia, focal • gene expression • N-methyl-D-aspartate • rats

The sequence of events through which ischemia leads to cell death is poorly understood. A number of endogenous components have been implicated in the subsequent neurodegenerative processes, such as glutamate, free radicals, calcium, and polyamines, all of which have been shown to be elevated in association with the ischemic insult. Agents such as glutamate and calcium have established rapid and potent physiological actions that are exaggerated during ischemia, leading to a massive and uncontrolled amplification of their normal effects. An important component of the physiological action of glutamate that is likely to underlie synaptic potentiation is the regulation of transcription. However, the persistent activation that results from elevated glutamate release after tissue damage in ischemia greatly augments this response, with potentially harmful consequences. The immediate early genes are the most rapidly induced by neuronal injury; they encode for transcription factors, which in turn regulate the expression of a number of target genes or late genes, some of which may contribute to neuronal death or survival. An extensive induction of immediate early genes can be observed in the brain following ischemic insult, and many of these genes have been studied in detail. In this study we characterized the extent of induction of three immediate early genes in response to focal ischemia produced by middle cerebral artery occlusion (MCAO). The immediate early genes that were investigated were c-fos, c-jun, and zif-268. We know that c-fos and c-jun proteins work in a coordinated action through the formation of both heterodimers and homodimers in the regulation of other genes. Their induction in response to a number of physiological and experimental stimuli has been characterized, eg, peripheral nerve stimulation, intracerebral electric stimulation, kindling, trauma, seizure activity, pentylene tetrazol-induced seizures, and excitotoxin injection. The induction of c-fos mRNA has previously been demonstrated in hippocampus in models of global ischemia in which transient occlusion of carotid arteries is followed by various periods of reperfusion. In these studies transient c-fos and c-jun mRNA changes are most marked in granule cells of the dentate gyrus and occur after 30 minutes, with maximal changes at 1 hour. A later peak of induction was seen in CA1 cells of the hippocampus at 24 to 48 hours.

In the present study we concentrated on the use of a focal ischemia model that is more consistently sensitive to glutamate antagonists selective for both N-methyl-D-aspartate (NMDA) and non-NMDA receptors.
The following five regions were dissected from each side of the brain, as indicated in Fig 1. The “core” region coincided with the main area of cortical ischemia, as indicated after intracranial ink perfusion in preliminary experiments, and corresponded to the central territory of the middle cerebral artery. Although histological signs of cortical infarction during the first 4 hours after MCAO are still evolving and variable in size and location, our ink perfusion studies (Y.C.-M., B.S.A., J.S. de B., M.J.G.H., unpublished data, 1993) showed that cortical blood flow was impaired, with vasodilation of penetrating vessels and patchy capillary filling within the core region. The second region was that medially adjacent to the core region, representing an ischemic “penumbra” where anterior cerebral artery and middle cerebral artery territories overlapped. The third, frontal region consisted of principally anterior cerebral artery territory with some middle cerebral border zone, and the occipital region was served mainly by the posterior artery. In addition, the hippocampus was removed. All tissue samples were rapidly frozen in liquid nitrogen and stored at ~70°C before extraction.

### Extraction and Analysis of mRNA

Total RNA was isolated from tissue samples by acid guanidinium thiocyanate-phenol-chloroform extraction.20 The resulting RNA was analyzed by Northern blotting and slot blotting, as previously described.21 Detection of c-fos mRNA on Northern and slot blots was carried out by means of a 1-kb Pst I fragment of p-fos 1 derived from a cDNA sequence coding for v-fos,22 kindly donated by Dr N. Wilkie. The proto-oncogene c-jun was detected with JACI, a mouse cDNA (supplied by ATCC). The zif-268 probe was derived from a 900-bp insert in pGEM4 coding for murine zif-268, and the krox-20 probe was derived from a 500-bp insert in pBluescript M13+ coding for murine krox-20.22 An 800-bp cDNA fragment of β-tubulin derived from a human fetal brain library24 was used as a reference probe.

Northern blot filters were preincubated in hybridization buffer (50% formamide, 5x SSPE [0.09 mol/L NaCl, 0.05 mol/L sodium phosphate, pH 7.4, 5 mmol/L EDTA], 5x Denhardt’s solution [0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone], 0.5% [wt/vol] sodium dodecyl sulfate [SDS], and 100 μg/mL salmon sperm DNA) for 2 hours at 65°C, and the filters were exposed to preflashed Hyperfilm-MP (Amersham International PLC) with intensifying screens at ~70°C.

Quantitation of c-fos mRNA, c-jun, zif-268, and krox-20 mRNA was carried out relative to β-tubulin mRNA levels by slot blot analysis as follows. Slot blot filters were hybridized with [32P]cDNA and then washed and autoradiographed as described above. Hybridization was carried out at three dilutions as previously described22 to confirm the linear relationship between densitometric signal and concentration necessary for accurate quantitation. Each slot blot filter was then stripped of labeled probe by washing in 5 mmol/L tris(hydroxymethyl)-aminomethane HCl, pH 8.0, 2 mmol/L EDTA, 0.1x Den-
TABLE 2. Physiological Parameters Immediately Before Euthanasia for Control and MK-801–Treated Animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Arterial Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P02, mm Hg</td>
</tr>
<tr>
<td>Control</td>
<td>105.9±36.0</td>
</tr>
<tr>
<td>MK-801</td>
<td>153.9±30.0</td>
</tr>
</tbody>
</table>

BG indicates blood glucose; MABP, mean arterial blood pressure; and Temp, rectal temperature. Values are mean±SD.

Results

Physiological Parameters

Preocclusion and postocclusion values for arterial parameters and rectal temperature were maintained within physiological limits (Tables 1 and 2). There were no significant differences in preocclusion values between control and MK-801–treated animals before treatment. During the period after MCAO, PO2 and PCO2 in controls decreased, whereas pH and glucose increased. This may have resulted from a small degree of hyperventilation due to the light levels of pentobarbital anesthesia maintained after surgery. Arterial blood pressure was relatively high in all animals during this time. Pentobarbital has been shown to have partial neuroprotective effects at the doses used in this study, and it was important to reduce such effects as much as possible. The reduction in PCO2 and increase in pH did not occur in MK-801–treated animals after MCAO, and PO2 increased. This probably resulted from a small anesthetic effect of MK-801 at 3 mg/kg, which prevented some of the hyperventilation seen in controls. Except for a transient fall in blood pressure immediately after MK-801 infusion in the four treated animals, ranging from 11% to 62% of the predosed value and lasting less than 3 minutes, blood pressure and blood glucose were not significantly different from those of controls.

Effect of MCAO on c-fos mRNA in Cortical Regions Ipsilateral and Contralateral to the Occlusion

Levels of c-fos mRNA are normally low or absent in brain tissue. Permanent unilateral MCAO caused a substantial induction of c-fos mRNA ipsilateral to the occlusion most markedly in the core and penumbra regions, as shown on the Northern blot (Fig 2). The contralateral hemisphere and hippocampus, both ipsilateral and contralateral to the occlusion, showed little induction of c-fos mRNA.

Quantitation of c-fos mRNA relative to β-tubulin mRNA was carried out by slot blot hybridization (Fig 3) and showed that a significant increase was obtained in core, penumbra, and occipital regions ipsilateral to the occlusion compared with the contralateral side and with unoperated control levels; the magnitude of the increase in ipsilateral cortex relative to contralateral cortex was 5.5- and 7.7-fold in core and penumbra regions, respectively, and 3-fold in occipital cortex. Changes in the frontal cortex were similar to those obtained in the occipital cortex (data not shown). No change in the hippocampus was detected when comparing the ipsilateral to the contralateral side (Fig 3).

Effect of MK-801 on c-fos mRNA Induction by MCAO

Pretreatment of animals 5 minutes before occlusion with 3 mg/kg MK-801 caused a substantial attenuation of the level of c-fos mRNA induction in the ipsilateral core, penumbra, and occipital cortex by 73%, 54%, and 84%, respectively, reaching the level of c-fos mRNA in the contralateral cortex, as shown by slot blot hybridization of mRNA (Fig 4) and when quantitated relative to β-tubulin mRNA (Fig 4). The level of c-fos mRNA in the contralateral cortex and hippocampus was unaffected by treatment with MK-801.

Induction of c-jun and zif-268 mRNA by MCAO

Induction of c-fos mRNA was also accompanied by a significant induction in two other immediate early genes, c-jun and zif-268. The effect on c-jun was mainly restricted to the core area (Fig 5), where a significant increase of 86% was detected. More marked increases in zif-268 of twofold to fourfold were seen throughout the ipsilateral cerebral cortex. Significant increases in zif-268 mRNA were detected in the core, penumbra, and occipital cortex of 132%, 285%, and 107%, respectively. Levels of zif-268 mRNA in the hippocampus were unaffected. The widespread effect on zif-268 induction in all ipsilateral cortical regions compared with β-tubulin mRNA is shown in Fig 6. No significant increase was detected in krox-20, another member of the zinc finger family.

TABLE 3. Levels of zif-268 mRNA in the Core, Penumbra, and Occipital Cortex

<table>
<thead>
<tr>
<th>Region</th>
<th>Control</th>
<th>MK-801</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core</td>
<td>1.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Penumbra</td>
<td>1.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Occipital</td>
<td>0.8</td>
<td>1.2</td>
</tr>
</tbody>
</table>

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Fig 2. Northern blot analysis of c-fos mRNA in brain regions ipsilateral and contralateral to middle cerebral artery occlusion. Total RNA (20 μg) was loaded for each region, separated by electrophoresis, and hybridized as described in "Materials and Methods." Note that c-fos mRNA is most evident in the core (C) and penumbra (P) regions ipsilateral to middle cerebral artery occlusion. L indicates left; H, hippocampus; and R, right.
family of immediate early genes, in the core region, but a significant increase of 135% was detected in the penumbra (Fig 5).

**Discussion**

In this study we have shown that focal ischemia due to permanent MCAO causes a selective ipsilateral induction of immediate early genes, which are dependent on the presence of glutamate receptors, in that this effect can be largely inhibited by pretreatment with the glutamate antagonist MK-801. It is particularly noteworthy that the extent of the induction was greater than that immediately surrounding the ischemic area and extended beyond the middle cerebral artery territory. The surgical procedure to occlude the middle cerebral artery by the method of Tamura et al is quite invasive, and although not reported by previous studies, some induction of c-fos mRNA in the ischemic core area may have resulted from the trauma of craniotomy, exposure of the cortex, and electrocauterization. Immunocytochemical studies have demonstrated the extensive induction of c-fos protein after MCAO, photochemically induced infarction, mechanical trauma, and localized cerebral devascularization, which is widespread and extends throughout the injured hemisphere.

As in the present study, this induction has been shown to be sensitive to MK-801, and it has been postulated that the widespread response is due to cortical spreading depression, which also induces c-fos and is sensitive to MK-801. Furthermore, the incidence of spreading depression after focal ischemia in the rat is also reduced by MK-801. Support for this proposed mechanism comes from the observation that c-fos mRNA induction does not occur in the hippocampus (Fig 2). As with MK-801, treatment with antisense oligodeoxynucleotides to the NMDA-R, receptor using this MCAO model is also known to significantly reduce the size of the area of infarction. Treatment with MK-801 alone has also been shown to induce c-fos immunoreactivity in neurons in the deep cortical layers and thalamus and also to induce heat-shock protein (HSP) 72 in the posterior cingulate and retrosplenial cortex. However, in this study no clear evidence of such an effect with drug treatment was evident. Although levels of c-fos mRNA in the contralateral core region (nonischemic) were higher in MK-801–treated animals than vehicle-treated animals (Fig 4), this effect did not reach significance and was not evident in other cortical regions. This indicates that the predominant effect of MK-801 in this model is to prevent the induction of c-fos mRNA initiated by ischemia.

Induction of c-fos mRNA has been recorded previously with both temporary and permanent MCAO, but in this study we focused on extensive regional quantitation of mRNA to present data on this and other immediate early genes and the effect of MK-801 in permanent MCAO. Confirmation that elevated levels of c-fos mRNA rise from increased rates of transcription rather than a reduced turnover of mRNA comes from recent studies of focal ischemia with reperfusion, in which nuclear run-on assays also demonstrate significant c-fos mRNA induction. The involvement of c-fos and c-jun in the regulation of gene expression in ischemia is also supported from mobility shift assays of immediate early genes.
It is important to establish whether the changes in gene expression contribute to the toxic effects of the lesion and hence are of a harmful nature or whether they are a parallel mechanism that may be involved in mediating a potentially neuroprotective effect. In this respect Combs et al. were able to show that the potentiating effect of hypoglycemia on cell death after transient ischemia in gerbils was associated with a suppression of c-fos mRNA induction, indicating the potential involvement of c-fos in a protective mechanism.

Focal ischemia has also been shown to be associated with an induction of HSP70 mRNA, which, like the induction shown by c-fos mRNA, is quite extensive ipsilateral to the lesion. However, an absolute quantitation of the response was not carried out in this study; rather, semiquantitative analysis was performed from in situ hybridization studies. Immunochemical localization of HSP70 during rat focal ischemia has been shown to occur initially in neurons, followed by microglia and endothelial cells. Induction of HSP70 mRNA has also been demonstrated after global ischemia in gerbil hippocampus, which is sensitive to MK-801.

Models of global ischemia followed by reperfusion have also been shown to cause an induction of c-fos mRNA. In addition, a delayed induction of ornithine decarboxylase mRNA has also been shown in these studies, which is maximal at 4 to 8 hours after reperfusion. Effects of cell loss produced by global ischemia have not been consistently shown to be dependent on glutamate-mediated neurotoxicity because they are not abolished by treatment with MK-801. Similarly, the effect of global ischemia on ornithine decarboxylase mRNA induction was not prevented by MK-801 treatment.

Therefore, two mechanisms are likely to be involved in the injury responses to focal and global ischemia. In the case of focal ischemia, a more localized glutamate-mediated neurotoxicity is likely to predominate, whereas global ischemia brought about, for example, by bilateral carotid occlusion followed by reperfusion is likely to be dominated by a number of vascular factors, perhaps mediating alterations in free radical release.

After an ischemic insult, the process of neuronal cell death may extend for several hours, and several factors may contribute to this process. During this time, it may be possible to rescue some cells, whereas others may be irreversibly damaged. However, key trigger factors that initiate either a neurodegenerative cascade or potentially neuroprotective mechanisms are likely to rise from the elevated levels of extracellular glutamate and its effect on gene expression described here. The elucidation of these processes is an important goal.

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**References**


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