Drug-Induced Neuroprotection From Global Ischemia Is Associated With Prevention of Persistent but Not Transient Activation of Nuclear Factor-κB in Rats

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Background and Purpose—Nuclear factor-κB (NF-κB) is an oxidative stress responsive transcription factor that is transiently activated in most forebrain neurons in response to transient global ischemia. However, in hippocampal CA1 neurons destined to die, NF-κB remains persistently activated. The present study was performed to determine whether an antioxidant (LY231617) that afforded neuroprotection in previous studies had any effect on NF-κB activation in hippocampal CA1 neurons after global ischemia.

Methods—Rats were subjected to 30 minutes of forebrain ischemia by 4-vessel occlusion (4-VO) and killed at 24 and 72 hours after ischemia. LY231617 was administered orally at a dose of 50 mg/kg 30 minutes before 4-VO and again 4 hours after 4-VO. Neuronal damage was evaluated in sections stained with cresyl violet. Other sections were immunostained with antibodies to NF-κB p50 to assess nuclear localization. An electrophoretic mobility shift assay was performed on nuclear extracts from sham- and LY231617-treated rats at 24 and 72 hours after ischemia.

Results—The administration of LY231617 had a significant protective effect on hippocampal CA1 neurons at 72 hours after ischemia (control group, 16±7 neurons/mm; treated group, 294±35 neurons/mm, P<.02) and prevented nuclear translocation of activated NF-κB as normally seen at 72 hours after ischemia in untreated controls. In contrast, the untreated controls showed activated NF-κB at 72 hours after ischemia. At 24 hours after ischemia, both the control group and the LY231617 group showed intense nuclear localization of NF-κB.

Conclusions—Activation of NF-κB in vitro has been reported to promote proapoptotic as well as antiapoptotic mechanisms, depending on the cell type being investigated. In the present in vivo study, the role of the transient activation of NF-κB observed at 24 hours may be responsible for the induction of protective factors in neurons that survive the ischemic insult, whereas the persistent activation of NF-κB in hippocampal neurons could be responsible for the induction of proteins that result in CA1 neuronal death. (Stroke. 1998;29:677-682.)

Key Words: apoptosis ■ cerebral ischemia, global ■ nuclear factor-κB ■ rats

The large pyramidal neurons in the CA1 sector of the hippocampus are extremely vulnerable to short periods of global ischemia. Neurons in this brain area have a remarkable pattern of cell death because it takes approximately 72 hours for these neurons to die.1 Although perhaps still controversial, evidence has been recently published supporting the view that forebrain neurons exhibiting delayed cell death die by apoptosis or PCD.2,3 In fact, many of the factors that are known to be released after transient global ischemia (TNF-α, H2O2, glutamate, interleukin-1β) have been shown to induce apoptosis of cells in vitro as well as nuclear activation of NF-κB.4-16

The role of activation of NF-κB in events leading to cell death is unclear because NF-κB activation has been shown to be both proapoptotic12,17-19 and antiapoptotic20-24 depending on the conditions and the cell types under investigation. Cytokines such as TNF-α and interleukin-1β, which are released in response to cerebral ischemia, are known to be strong activators of NF-κB.5,8 Furthermore, TNF-α is well known for its ability to induce apoptosis; however, recent data suggest that it can also have a protective influence.23 Thus, production of cytokines and subsequent activation of NF-κB could have a biphasic role in mechanisms controlling cell survival. Production of moderate levels of cytokines and NF-κB may not be sufficient to cause neuronal death but may serve to “alert” the neuron to a potentially dangerous insult so that it can produce protective factors that enhance survival. This may explain why subthreshold ischemic insults or cytokine pretreatment can protect against subsequent larger ischemic insults.26,27

Several genes exist with NF-κB consensus sequences located in their promoter regions. Some of these downstream targets of

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NF-κB activation is known to be protective, such as Cu/Zn SOD and Mn SOD, whereas others are destructive, such as NO synthase. If NF-κB had an influence on neuronal cell survival, it would be expected that certain downstream targets of NF-κB would be transcriptionally regulated proteins that control cell survival. One example might be illustrated by recent observations on Bcl-x. We recently reported that after global ischemia, there was a significant increase in the proapoptotic short splice variant of Bcl-x (Bcl-xs) in the hippocampal area. Furthermore, NF-κB p50 and NF-κB p65 acted in synergy to transactivate the Bcl-x promoter in cotransfections in 293 cells, but in the hippocampus after global ischemia, the short splice variant was elevated. In support of this, it was recently reported that there was a decrease in the Bcl-x long-form/Bcl-x short-form transcript ratio that preceded DNA fragmentation after global ischemia. The antioxidant LY231617 has been shown to reduce neuronal injury after global ischemia in rats. Although LY231617 possesses antioxidant activity as one of its properties, the precise mechanism through which LY231617 prevents neuronal injury after global ischemia is not known. Various antioxidants and transition metal chelators have been shown in vitro to block the activation of NF-κB. It was the purpose of the present study to determine whether LY231617 administration affected the activation of NF-κB in response to global ischemia.

Materials and Methods

Global Forebrain Ischemia and Brain Tissues

Transient forebrain ischemia was induced by 4-VO as previously described. Briefly, Wistar rats (Hilltop Laboratories, Scottsdale, Pa) were prepared for forebrain ischemia under 2% halothane inhalation anesthesia by electrocauterization of the vertebral arteries bilaterally and placement of atrumatic clamps around the common carotid arteries without interruption of the arterial blood flow. On the following day, forebrain ischemia was induced by tightening of the clamps for 30 minutes. Body temperature was maintained at 37°C during and for 30 minutes after 4-VO by means of heat lamps. All animal procedures were performed in compliance with the institute's animal care and use committee. LY231617 was given orally at a dose of 50 mg/kg 30 minutes before 4-VO and again 4 hours after 4-VO. Controls received 2% acacia vehicle. After ischemia, the animals were perfused with a periodate-lysine-paraformaldehyde solution. The brains were postfixed by immersion for 24 hours, cryoprotected in 30% sucrose, and frozen in dry ice-cooled isopentane. Coronal sections (18 μm) were cut and mounted on slides, and alternate sections were stained with cresyl violet. Animals were killed at 24 (n=5) and 72 (n=5) hours after ischemia for evaluation of neuronal death, immunocytochemical analysis, and EMSA. An identical number of sham controls were evaluated. Hippocampal CA1 damage was determined by counting the live neurons. Level of significance was determined using the Mann–Whitney U test.

Antibodies

Polyclonal antisera generated to specific regions of NF-κB were used in the various assays. The antisera used for immunocytochemistry, Ab 293, was kindly supplied by Dr Warner Greene (Gladstone Institute of Virology and Immunology, University of California, San Francisco). Ab 392 was made against the N-terminal peptide of NF-κB. It was the purpose of the present study to determine whether LY231617 administration affected the activation of NF-κB in response to global ischemia.

Nuclear Protein Extracts and EMSA

The region of the dorsal hippocampus containing the CA1 layer from 6 sham or 6 ischemic/reperfused rat brains was homogenized in a microfuge tube with buffer A (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 0.5 mmol/L DTT, 1 mmol/L AEBSF, and 10 μg/mL leupeptin). The nuclei were collected by centrifugation at 500 g for 10 minutes and washed once with buffer A containing 0.2% NP-40. After centrifugation at 500 g for 10 minutes, the nuclear proteins were then extracted with buffer B (20 mmol/L HEPES, pH 7.9, 0.42 mol/L NaCl, 20% glycerol, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L AEBSF, and 10 μg/mL leupeptin) for 30 minutes at 4°C and microfuged for 30 minutes. The nuclear extracts were microdialyzed against buffer C (20 mmol/L HEPES, pH 7.9, 20% glycerol, 0.1 mol/L KCl, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L AEBSF) with an ultrafree centrifugal filter (Millipore) and frozen at −80°C.

For determination of protein–DNA interactions, the double-stranded oligonucleotide containing NF-κB consensus binding sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was purchased from Promega and end-labeled with γ[32P]-ATP according to manufacturer’s recommendation. The NF-κB binding reactions were performed in a final volume of 20-μL mixtures containing buffer (10 mmol/L HEPES, pH 7.5, 1 μg poly(dI-dC), 0.1 mmol/L NaCl, 0.8 mmol/L EDTA, 1 mmol/L DTT, 0.05% NP-40, 4% glycerol), 10 μg nuclear proteins, and 100,000 cpm of radiolabeled probe. The mixtures were incubated at room temperature for 20 minutes. For competition assay, 100-fold excess amount of cold NF-κB oligonucleotide was preincubated with nuclear proteins for 15 minutes before the addition of labeled probe. The DNA–protein complexes were resolved on a 5% nondenaturing polyacrylamide gel in 0.5X TBE buffer. The gel was dried and exposed to x-ray film overnight at −70°C. EMSA was performed on pooled nuclear extracts from 6 sham- and 6 LY231617-treated rats at 24 and 72 hours after ischemia. Semiquantitation of the EMSA was performed with densitometry.

Results

Oral administration of LY231617 almost totally prevented hippocampal CA1 neuronal damage at 72 hours after ischemia (Table 1). At 72 hours after ischemia, rats treated with the acacia vehicle showed extensive neuronal damage. At 24 hours, there was no histological evidence of CA1 neuronal damage (Fig 1A and Fig 1B). Also at 24 hours after 4-VO, intense nuclear localization of NF-κB p50 was present in both
the vehicle-treated control and the group treated with LY231617 (Fig 1C and 1D). At 72 hours after 4-VO, intense nuclear localization of NF-κB p50 was observed in the vehicle-treated control group (Fig 2C). However, in the animals treated with LY231617, nuclear localization of NF-κB p50 was absent (Fig 2D). Nissl-stained sections revealed that there was extensive neuronal degeneration of the CA1 layer in the control group (Fig 2A), whereas the CA1 neurons in the LY231617-treated group were protected (Fig 2B). The results clearly indicate that LY231617 treatment did not prevent nuclear localization of NF-κB at 24 hours; however, it totally blocked the persistent nuclear localization of NF-κB observed at 72 hours. When body temperature was monitored every 30 minutes for a period of 24 hours after oral administration of LY231617 or vehicle, no significant difference was observed between the LY231617-treated group and the acacia vehicle group.

The results of the EMSA (Fig 3) clearly demonstrate that at 24 and 72 hours after 4-VO, nuclear levels of activated NF-κB are considerably elevated above those of sham-operated controls. Treatment with LY231617 completely blocked the persistently elevated nuclear levels of NF-κB normally seen at 72 hours after 4-VO but failed completely to inhibit the activation of NF-κB at 24 hours after 4-VO. Semiquantitation of the EMSA by densitometric analysis (Table 2) supported these observations.

The pattern of increased nuclear levels of NF-κB at 24 and 72 hours as shown in Fig 3 was highly repeatable and was replicated in seven studies; the effect of LY231617 was replicated three times for the present study.

**Discussion**

The results of this study demonstrate that oral administration of LY231617 is highly effective in reducing neuronal damage in response to transient global forebrain ischemia. Although antioxidant activity is one of the properties of LY231617, it still may possess other activities that might account for its high degree of efficacy. In fact, a number of factors including free radicals, glutamate, calcium overload, NO, and various cytokines have been proposed to have an important role in causing neuronal death after short periods of global ischemia.

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**TABLE 1. Effect of Oral Administration of LY231617* on Hippocampal CA1 Neuronal Damage After 30 Minutes of Global Ischemia**

<table>
<thead>
<tr>
<th>Group</th>
<th>Time, h</th>
<th>No. of Rats</th>
<th>Viable CA1, neurons/mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acacia control</td>
<td>24</td>
<td>6</td>
<td>290±19</td>
</tr>
<tr>
<td>LY231617</td>
<td>24</td>
<td>5</td>
<td>310±29</td>
</tr>
<tr>
<td>Acacia control</td>
<td>72</td>
<td>5</td>
<td>16±7 (P&lt;.02)</td>
</tr>
<tr>
<td>LY231617</td>
<td>72</td>
<td>5</td>
<td>294±35</td>
</tr>
</tbody>
</table>

Values are mean±SE.

*LY231617 was administered orally at a dose of 50 mg/kg 30 minutes before ischemia and again 4 hours after ischemia.
TABLE 2. Densitometric Analysis of EMSA for NF-κB Shown in Fig 3

<table>
<thead>
<tr>
<th>Group (Nuclear Extracts)</th>
<th>Relative Density Compared to Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hela+ cold competitor</td>
<td>ND</td>
</tr>
<tr>
<td>Hela</td>
<td>ND</td>
</tr>
<tr>
<td>Sham</td>
<td>1</td>
</tr>
<tr>
<td>Sham+ cold competitor</td>
<td>0.002</td>
</tr>
<tr>
<td>4VO*</td>
<td>21</td>
</tr>
<tr>
<td>4VO+ cold competitor*</td>
<td>0.003</td>
</tr>
<tr>
<td>4VO+LY23167*</td>
<td>1.5</td>
</tr>
<tr>
<td>4VO+LY23167+ cold competitor*</td>
<td>0.001</td>
</tr>
<tr>
<td>4VO†</td>
<td>18</td>
</tr>
<tr>
<td>4VO+ competitor†</td>
<td>0.001</td>
</tr>
<tr>
<td>4VO+LY23167†</td>
<td>14</td>
</tr>
<tr>
<td>4VO+LY23167+ cold competitor†</td>
<td>0.005</td>
</tr>
</tbody>
</table>

ND indicates not determined.
*72 h after ischemia/reperfusion; †24 h after ischemia/reperfusion.

LY231617 may have acted to interfere with some downstream lethal event common to all the above agents.

Interestingly, all of these agents are known to activate NF-κB when exposed to cells in vitro. The activation of NF-κB by these agents appears to be via the generation of reactive oxygen species. It is unlikely that reactive oxygen species are directly responsible for neuronal death because they are present only for short periods of time after ischemia, and in the case of hippocampal CA1 pyramidal cells, it takes approximately 72 hours for neuronal cell death to occur. It is reasonable to postulate then that some form of PCD occurs in approximately 72 hours for neuronal cell death. It is reasonable to postulate then that some form of PCD occurs in these neurons, possibly as a result of the production of transcription factors such as NF-κB because of the length of time required for cell death to occur. Transcription factor activation could eventually lead to the production of proteins that result in neuronal death. Several recent studies have demonstrated in situ DNA fragmentation as well as biochemical evidence of DNA laddering and the production of proapoptotic proteins in the hippocampal CA1 sector after global ischemia. These findings add support for the view that at least a portion of the CA1 pyramidal neurons die by apoptosis or PCD. However, the production of proapoptotic proteins could lead to necrotic cell death in cells where protein synthesis is compromised.

In addition to the release of glutamate, calcium overload, and the production of NO and cytokines, which are known to cause apoptosis or delayed necrosis in vitro, it is well documented that reactive oxygen species are produced during the reperfusion phase subsequent to global ischemia. This has been confirmed in the same 4-VO model used in the present study. We previously demonstrated in this model the presence of activated NF-κB by EMSA, Western blots, and nuclear localization by immunocytochemistry, and we confirmed that the active complex consists of a heterodimer of NF-κB p50 and NF-κB p65. In the present study, the EMSA results confirm the immunocytochemical observation.

NF-κB was previously reported to be able to modulate apoptosis. All of those studies were performed in vitro, and the effect of NF-κB on apoptosis appeared to depend on the cell type and treatment conditions. Some in vitro studies have demonstrated that NF-κB is necessary for PCD, while others have demonstrated that NF-κB inhibited PCD. For example, one recent study demonstrated that blockade of NF-κB activation prevented the excitotoxicity exerted by glutamate on primary neuronal cultures and on brain slice cultures. In contrast, other recent in vitro studies have reported that NF-κB activation can prevent TNF-α-induced cell death. It appears then that NF-κB activation can steer a cell in either of two directions. One is to induce cellular defense mechanisms against PCD and the other is to induce the production of proteins that lead to cell death.

The transient elevation of NF-κB at 24 hours after ischemia in neurons that survive the ischemic insult may be responsible for the induction of protective factors. The 30-minute period of ischemia could be considered a subthreshold insult for most of the forebrain neurons except those in the hippocampal CA1 sector, the corpus striatum, certain layers of the cerebral cortex, and cerebellar Purkinje cells. The cytokines TNF-α and interleukin-1β, which are known to be powerful activators of NF-κB, have been shown to exert a neuroprotective effect in vitro when administered before an insult that normally causes neuronal cell death. In fact, the neuroprotective effects of TNF-α could be mimicked by exposure of cultures to an antisense construct to 1κB, which is thought to disinhibit NF-κB.

The possibility of a deleterious role for NF-κB must also be considered. NF-κB can cause transcription of the inducible NO synthase gene, and NF-κB consensus sequences are present in the promoter region of the neuronal NO synthase gene. Furthermore, NO is known to be a factor in neurotoxicity. The 5′-flanking region of the cytosolic phospholipase A2 (cPLA2) gene contains consensus NF-κB sequences. We have recently reported induction of cPLA2 after global ischemia. Transcription of this gene could result in the eventual production of eicosanoid products that are neurotoxic. Evidence that transcription factors of the NF-κB/Rel family are involved in PCD is continuously increasing. Potential target genes for NF-κB are among the genes induced on apoptosis. They include p53, c-myc, Fas/Apo-1 ligand, and interleukin-converting enzyme. Stimuli that activate NF-κB can transcriptionally activate these death genes, and where examined, their upstream promoter regions contain potential NF-κB–binding motifs. In contrast, the activity of NF-κB is downregulated by the antiapoptotic protein Bcl-2. Recently, it was shown that Bcl-2 and Bcl-x long-form mRNA were expressed after global ischemia in both surviving and dying neurons, but their proteins were expressed primarily in neurons destined to survive. Thus, the proapoptotic influence of NF-κB could continue unopposed.

It is unclear why LY231617 prevented the persistent activation of NF-κB observed at 72 hours but did not prevent the transient activation of NF-κB at 24 hours after ischemia. However, blockade by LY231617 of the late rise of NF-κB at 24 hours after ischemia could provide a way to selectively inhibit NF-κB–mediated PCD.
We also reported that after ischemia and NF-κB activation, Bcl-x messenger RNA levels increase in the CA1 hippocampal region, but as a result of this transcriptional increase, it is Bcl-xL, the proapoptotic form of Bcl-x, that is elevated. By preventing the persistent activation of NF-κB by LY23167 treatment, the production of this death protein would be blocked. These findings are in concert with those of Honkoneni et al who found that after global ischemia, there was a decrease in the Bcl-xL:Bcl-xS mRNA ratio that preceeded DNA fragmentation. Although it is not clear how LY23167 prevented the NF-κB activation seen at 72 hours, there is evidence that the prolonged activation of NF-κB shifts the balance of neuronal cell survival-related proteins in favor of those that induce cell death. Because the time course for cell death after global ischemia in other brain areas such as the corpus striatum differs from that observed in the hippocampus, the mechanisms that lead to neuronal cell death may differ. The potential factors leading to neuronal death reported in this study may be applicable only to the hippocampal CA1 sector. Additional studies are required to determine whether similar mechanisms are involved with ischemic neuronal death in other brain regions.

References

This study sought to determine whether the neuroprotective effects of the antioxidant LY231617 would influence patterns of NF-κB activation in the CA1 hippocampus after 30 minutes of global forebrain ischemia in rats. Pretreatment and posttreatment with LY231617 prevented hippocampal CA1 neuronal injury at 3 days after ischemia. Although at 24 hours intense nuclear NF-κB p50 immunoreactivity was present in vehicle-treated and LY231617-treated rats, NF-κB p50 immunoreactivity was observed only in the vehicle group at 3 days.

NF-κB has previously been shown to promote proapoptotic as well as antiapoptotic mechanisms. Thus, the ability of LY231617 to block NF-κB localization at 3 days is discussed in terms of apoptotic cell death. The role of programmed cell death in the pathophysiology of CA1 vulnerability after transient global ischemia remains controversial. This study adds evidence for the activation of a biochemical pathway believed to be important in cell apoptosis and other gene transcription events. Future studies are required to determine how LY231617 prevents NK-κB activation and to clarify how this agent affects cell-death programs. From a clinical standpoint, these studies are important because they demonstrate significant neuroprotection with oral administration of LY231617 following a relatively prolonged global ischemic insult.

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