Nimodipine Attenuates Both Increase in Cytosolic Free Calcium and Histologic Damage Following Focal Cerebral Ischemia and Reperfusion in Cats

Daisuke Uematsu, MD, Joel H. Greenberg, PhD, William F. Hickey, MD, and Martin Reivich, MD

To clarify the mechanism of its effect on ischemic stroke, we investigated the effect of nimodipine, a dihydropyridine calcium antagonist, on changes in cytosolic free calcium, cortical blood flow, and histologic changes following focal cerebral ischemia and reperfusion in 14 cats. Using indo-1, a fluorescent intracellular Ca2+ indicator, we simultaneously measured changes in the Ca2+ signal ratio (400:506 nm), reduced nicotinamide adenine dinucleotide fluorescence (464 nm), and reflectance (340 nm) during an ultraviolet excitation (340 nm) directly from the cat cortex in vivo. In six cats treated with vehicle only, the calcium signal ratio increased from 5 minutes after middle cerebral artery occlusion to 30 minutes into reperfusion. The elevation of cytosolic free calcium was significantly attenuated by nimodipine, which was administered by intravenous infusion in eight cats starting 5 minutes after occlusion. Nimodipine had no effect on cortical blood flow during ischemia but induced a hyperperfused state following reperfusion. Nimodipine did not modify changes in the mitochondrial oxidation-reduction state. Nimodipine proved to have beneficial effects on recovery of the electroencephalogram following reperfusion as well as on the extent of focal histologic damage. Our results suggest that nimodipine, when administered during the early stage of focal ischemia, can favorably modify the outcome of stroke by reducing the Ca2+ entry during both the ischemic and reperfusion periods. (Stroke 1989;20:1531-1537)

In contrast to a relatively good understanding of the hemodynamic and metabolic aspects of stroke, a great deal remains to be clarified concerning the biochemical mechanisms underlying ischemia-induced cellular injury. In the past decade several mechanisms have been postulated to be involved in the production of ischemic and reperfusion injury: acidosis,1 accumulation of free fatty acids and prostanoids,2 free radical reactions,3,4 excitotoxins,5 and Ca2+-induced injury.6–9 An excessive entry of Ca2+ into ischemic cells seems to play a central role in all of these mechanisms. An increase in cytosolic free calcium ([Ca2+]i) triggers numerous Ca2+-dependent enzymatic processes as well as superoxide radical reactions, especially following reperfusion, leading to irreversible cellular injury. During severe ischemia, when the plasma membrane is depolarized due to energy depletion, a large amount of Ca2+ enters through the voltage-sensitive Ca2+ channels.10 Ca2+ can also enter the cells through receptor-operated ion channels that open during activation of N-methyl-D-aspartate (NMDA) receptors.11,12

We have recently demonstrated in a cat focal ischemia model using indo-1, a new fluorescent [Ca2+]i indicator, that the level of cortical [Ca2+]i increases in severe stroke and that the subsequent alterations of [Ca2+]i during reperfusion are closely related to the degree of electroencephalographic (EEG) recovery. This fluorometric technique offers a distinct advantage since the time course of changes in [Ca2+]i can be assessed in vivo along with changes in nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide (NAD/NADH) oxidation-reduction (redox) state and hemodynamics.13

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Nimodipine, a dihydropyridine derivative, has been considered to be a promising calcium antagonist for the treatment of cerebral ischemia because of its preferential vasodilatory effect on the cerebral vessels and its excellent permeability through the blood–brain barrier.14,15 Beneficial effects of nimodipine on ischemic stroke have been reported in recent clinical trials16,17 as well as in several experimental models of cerebral ischemia.18–23 It has not been determined, however, whether nimodipine owing its favorable effects to its vasodilatory action or to the direct blockade of Ca\(^{2+}\) entry into ischemic cells. To clarify the mechanism of its action, our present study focuses on the effects of nimodipine on alterations of [Ca\(^{2+}\)], local cortical blood flow (ICBF), NAD/NADH redox state, and focal histologic changes in a cat middle cerebral artery (MCA) occlusion and reperfusion model.

Materials and Methods

Details of the surgical preparation have been described.13,24 Twenty-one adult male cats weighing 2.3–3.3 kg were anesthetized by halothane inhalation, with induction and maintenance doses of 5.0% and 1.0%, respectively. Following tracheostomy and immobilization with 3 mg/kg i.v. gallamine triethiodide, the cats were artificially ventilated. Both femoral arteries and one femoral vein were catheterized for monitoring arterial blood pressure, for analyses of arterial blood gases, and for administration of drugs. The left lingual artery was also canulated so that a bolus of 0.3 ml saline could be intermittently injected to obtain hemodilution curves from the cortical vascular bed. A quartz cranial window equipped with silver EEG electrodes and inlet-outlet tubing was placed into a burr hole made in the skull over the exposed left middle ectosylvian cortex. The exposed cortex was continuously superfused with artificial cerebrospinal fluid (CSF) as described elsewhere.13 A main trunk of the left MCA was exposed microsurgically via a transorital approach using a modified O’Brien and Waltz technique.25

Artificial CSF containing 7 \( \mu \)M indo-1-acetoxymethyl ester (indo-1-AM, Molecular Probes Inc., Eugene, Oregon) was superfused for 2 hours at 37° C. The lipophilic membrane-permeant indo-1-AM can penetrate approximately 500 \( \mu \)m into the cortex and is converted by the action of intracellular esterase to the membrane-impermeant indo-1, which is trapped intracellularly as a specific Ca\(^{2+}\) indicator.13 The remaining extracellular dye was washed out by a subsequent 30-minute superfusion with artificial CSF. A small cortical area of interest was illuminated with ultraviolet light (340±12 nm), and optical signals including indo-1–Ca\(^{2+}\) fluorescence (400 and 506 nm), NADH fluorescence (464 nm), and reflectance (340 nm) were measured with four photomultiplier tubes with appropriate barrier filters. To minimize the effect of photobleaching on the dye, [Ca\(^{2+}\)], was measured intermittently for 2 hours after the indo-1 loading. NADH fluorescence was corrected for reflectance, and the indo-1–Ca\(^{2+}\) signals were corrected for changes in both reflectance and NADH fluorescence as described elsewhere.13 The corrected Ca\(^{2+}\) signal ratio (400:506 nm) was used as a measure of changes in [Ca\(^{2+}\)]. Changes in reflectance are inversely proportional to those of local cortical blood volume.26 Mean transit time of focal cortical circulation was assessed from the hemodilution curves based on an area-over-height analysis. Changes in ICBF were calculated by dividing changes in local cortical blood volume by mean transit time according to the Stewart-Hamilton equation.27

Following dye loading, 15 minutes were allowed for stabilization of the optical signals. The main trunk of the left MCA was occluded for 1 hour with a miniature aneurysm clip. To standardize the severity of ischemia, we used 14 cats in which the mean EEG amplitude decreased to <20% of control during the first 5 minutes of ischemia. In eight cats, 5 minutes after MCA occlusion, an intravenous infusion of nimodipine was started at a dose of 5 \( \mu \)g/kg/min for the first 3 minutes followed by 1 \( \mu \)g/kg/min for the remainder of the experiment. The rate of infusion was approximately 0.08 ml/min. In six cats, vehicle (polyethylene glycol and ethanol, 1:1 [vol:vol]) was infused in the same manner. After 3 hours of reperfusion, the brain was perfused with a buffered 3% glutaraldehyde solution from a catheter in the ascending aorta. The brain was then removed from the cranial cavity and postfixed in a 10% formaldehyde solution for 5 days. Two coronal sections were made at the same level as the optical measurements were stained with hematoxylin and eosin for light microscopy. Based on criteria described elsewhere,28 histologic evaluation was performed by a neuropathologist blinded to treatment. The outline of the region of histologic damage in the same cortical gyrus as the optical measurements was marked on the stained section by the neuropathologist, and the area of this region was determined using an image analyzer with a video camera. This permitted a direct correlation to be made between alterations of [Ca\(^{2+}\)], and histologic damage. The area of damage in the cortical gyrus from which the optical measurements were made divided by the total area of the left hemisphere in the section was used as a measure of the extent of focal histologic damage.

The vehicle-treated and nimodipine-treated groups were compared using multivariate analysis of variance. If significance was attained, probing tests at individual times were undertaken using Student’s two-tailed \( t \) test (or Wilcoxon’s signed rank or rank sum tests as appropriate). The results were considered statistically significant if the probability value was <0.05. All values are expressed as mean±SEM.

Results

Physiological parameters including mean arterial blood pressure (MABP), plasma glucose concentra-
TABLE 1. Physiological Data for Cats Subjected to Middle Cerebral Artery Occlusion and Reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean arterial blood pressure (mm Hg)</th>
<th>PaO2 (mm Hg)</th>
<th>PaCO2 (mm Hg)</th>
<th>Arterial pH</th>
<th>Plasma glucose concentration (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5 min before occlusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nimodipine</td>
<td>92±5</td>
<td>110±7</td>
<td>35±2</td>
<td>7.29±0.02</td>
<td>115±12</td>
</tr>
<tr>
<td>Vehicle</td>
<td>81±8</td>
<td>99±5</td>
<td>37±2</td>
<td>7.30±0.02</td>
<td>124±16</td>
</tr>
<tr>
<td><strong>After 1-hr occlusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nimodipine</td>
<td>83±4</td>
<td>119±5</td>
<td>36±1</td>
<td>7.26±0.02</td>
<td>146±15</td>
</tr>
<tr>
<td>Vehicle</td>
<td>77±4</td>
<td>106±5</td>
<td>38±3</td>
<td>7.27±0.02</td>
<td>135±18</td>
</tr>
<tr>
<td><strong>After 30-min reperfusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nimodipine</td>
<td>85±3</td>
<td>119±6</td>
<td>35±1</td>
<td>7.25±0.02</td>
<td>148±13</td>
</tr>
<tr>
<td>Vehicle</td>
<td>77±6</td>
<td>108±5</td>
<td>39±2</td>
<td>7.27±0.01</td>
<td>129±21</td>
</tr>
</tbody>
</table>

Values are mean±SEM, n=8 for nimodipine and n=6 for vehicle.

Ten minutes after MCA occlusion, ICBF decreased to 17±6% and 14±4% of control in the vehicle-treated and nimodipine-treated groups, respectively (Figure 2). Mean ICBF of the groups did not differ significantly during ischemia (<20% of control). During reperfusion, recovery of ICBF was significantly greater in the nimodipine-treated than in the vehicle-treated group: 172±28% vs. 80±14% of control (p<0.05) at 10 minutes, 151±22% vs. 68±14% of control (p<0.02) at 30 minutes, and 139±24% vs. 65±11% of control (p<0.05) at 60 minutes. There was an overall significant difference between groups (F1,12=5.64, p=0.035). The nimodipine-treated group exhibited postischemic hyperperfusion in contrast to the relative hypoperfusion seen in the vehicle-treated group.

In the vehicle-treated group, the Ca2+ signal ratio started to increase relative to control 5 minutes after the MCA occlusion (1.41±0.01 vs. 1.68±0.10, p<0.05) and remained elevated throughout ischemia and reperfusion (Figure 3). A steep increase in

![Graph of time course of mean±SEM changes in electroencephalogram (EEG) amplitude ipsilateral to ischemic hemisphere during middle cerebral artery occlusion (MCAO) and reperfusion (R) in cats. EEG amplitude of both vehicle-treated (○, n=6) and nimodipine-treated (●, n=8) groups was drastically depressed immediately after MCAO. Reduction of EEG amplitude during ischemia was similar in both groups, whereas recovery of EEG amplitude during reperfusion was significantly enhanced by treatment with nimodipine (*p<0.05 different from vehicle-treated group).](http://stroke.ahajournals.org/)

**FIGURE 1.** Graph of time course of mean±SEM changes in electroencephalogram (EEG) amplitude ipsilateral to ischemic hemisphere during middle cerebral artery occlusion (MCAO) and reperfusion (R) in cats. EEG amplitude of both vehicle-treated (○, n=6) and nimodipine-treated (●, n=8) groups was drastically depressed immediately after MCAO. Reduction of EEG amplitude during ischemia was similar in both groups, whereas recovery of EEG amplitude during reperfusion was significantly enhanced by treatment with nimodipine (*p<0.05 different from vehicle-treated group).
the Ca\textsuperscript{2+} signal ratio was observed between 10 and 20 minutes after MCA occlusion, presumably due to voltage-dependent Ca\textsuperscript{2+} entry during the neuronal depolarization that has been shown to occur during this period.\textsuperscript{10} In three of six vehicle-treated cats, [Ca\textsuperscript{2+}], showed a tendency to recover following reperfusion, whereas the other three cats exhibited a further increase in [Ca\textsuperscript{2+}], consistent with our previous results.\textsuperscript{13}

In the nimodipine-treated group, the Ca\textsuperscript{2+} signal ratio started to increase relative to control 5 minutes after MCA occlusion (1.39±0.01 vs. 1.52±0.05, p<0.05) and remained significantly elevated for the first 30 minutes of ischemia (Figure 3). A steep increase in [Ca\textsuperscript{2+}], was not seen in the nimodipine-treated group. There was an overall difference in the Ca\textsuperscript{2+} signal ratio between groups (F\textsubscript{12}=32.8, p=0.0001), the difference being significant from 20 minutes after MCA occlusion until the end of the [Ca\textsuperscript{2+}], measurement (30 minutes into reperfusion), indicating a beneficial effect of nimodipine on Ca\textsuperscript{2+} homeostasis throughout ischemia and reperfusion.

The NAD/NADH redox state became reduced after MCA occlusion in both groups, followed by a recovery during reperfusion (Figure 4). There was no significant difference in the time course of changes

**Figure 2.** Graph of mean±SEM changes in local cortical blood flow (ICBF) calculated from changes in local cortical blood volume and mean vascular transit time during middle cerebral artery occlusion (MCAO) and reperfusion (R) in cats. ICBF of both vehicle-treated (o, n=6) and nimodipine-treated (●, n=8) groups decreased to <20% of control 10 minutes after MCAO, with ICBF depression during ischemia being comparable. During reperfusion, nimodipine-treated cats showed marked hyperperfusion, whereas ICBF of vehicle-treated group remained slightly depressed compared with control. *p<0.05 different from vehicle-treated group.

**Figure 3.** Graph of time course of mean±SEM Ca signal ratio indicating changes in cytosolic free calcium during middle cerebral artery occlusion (MCAO) and reperfusion (R) in cats. Vehicle-treated group (o, n=6) exhibited marked elevation of Ca signal ratio compared with nimodipine-treated group (●, n=8) (*p<0.05, **p<0.01 different from vehicle-treated group).
in NAD/NADH redox state between groups at any time ($F_{1,13}=0.14$, $p=0.7150$).

The area of histologic damage in the cortical gyrus from which $[\text{Ca}^{2+}]$, was measured was 4.0±0.7% in the vehicle-treated group and 1.6±0.3% in the nimodipine-treated group (Figure 5). Thus, the extent of focal damage was significantly reduced by treatment with nimodipine ($p<0.05$).

**Discussion**

Our studies indicate that nimodipine can attenuate the increase in $[\text{Ca}^{2+}]$, that occurs in the cortex during focal cerebral ischemia and reperfusion.

Nimodipine was also shown to have favorable effects on the recoveries of EEG amplitude and ICBF during reperfusion as well as on histologic ischemic changes. Infusion of nimodipine was started 5 minutes after MCA occlusion since we have previously shown that $[\text{Ca}^{2+}]$, starts to increase between 5 and 10 minutes following severe ischemia. The degrees of reduction in ICBF as well as in EEG amplitude during ischemia were comparable between groups. Nimodipine did not have any effect on ICBF during ischemia, but it did produce hyperperfusion during the subsequent reperfusion. Our results are in good agreement with those of Gotoh and colleagues, 29 who showed that nimodipine administered 5 minutes after MCA occlusion did not modify the pattern of ICBF distribution during ischemia. Date and Hossmann30 also found that posttreatment with nimodipine reduced both intracortical and extracortical vascular resistance during ischemia without changing ICBF due to a decrease in systemic arterial blood pressure. Cerebral autoregulation has been shown to be disrupted by treatment with nimodipine.31 The marked increase in ICBF we observed in the nimodipine-treated group during reperfusion may be due to restored perfusion of dilated cortical vessels in the absence of autoregulation. In both vehicle-treated and nimodipine-treated groups, mean ICBF during ischemia was below the threshold (20% of control) at which extracellular $\text{Ca}^{2+}$ has been shown to enter ischemic cells.10,28 Consequently, attenuation of the ischemia-induced $[\text{Ca}^{2+}]$, increase by nimodipine cannot be due to its hemodynamic effect, but instead may presumably be attributed to its direct blocking effect on $\text{Ca}^{2+}$ entry. Hadani et al32 reported that nicardipine, another dihydropyridine derivative, reduced $\text{Ca}^{2+}$ accumulation and electrolyte derangements in
focal cerebral ischemia in rats, ascribing its protective effect against ischemic brain damage to a direct blockade of Ca\(^{2+}\) entry. A contradictory result was reported by Harris et al.,\(^{33}\) who described that pretreatment with nimodipine increased the ICBF threshold for disturbance of extracellular Ca\(^{2+}\) and K\(^+\) homeostasis in a baboon MCA occlusion model. Recently, Tsien and colleagues\(^{34,35}\) have characterized three subtypes of voltage-sensitive calcium channels (VSCCs) in neurons (L, T, and N) with different dihydropyridine sensitivities and electrophysiological properties. Nimodipine has been found to block only the L-type VSCCs in neuronal cell bodies.\(^{34}\) Accordingly, the small increase in [Ca\(^{2+}\)]\(_i\) we observed in the nimodipine-treated group during ischemia may be explained by a synaptic Ca\(^{2+}\) entry through the other subtypes of VSCC or by a receptor-operated Ca\(^{2+}\) entry during activation of NMDA receptors.\(^{11,12}\)

Following reperfusion, all eight nimodipine-treated cats exhibited consistent recovery of [Ca\(^{2+}\)]\(_i\), whereas half of the six vehicle-treated cats showed a further increase in [Ca\(^{2+}\)]\(_i\). There remained a significant difference in mean [Ca\(^{2+}\)]\(_i\) between the groups during the first 30 minutes of reperfusion. We speculate that in the vehicle-treated group integrity of the plasma membrane may have been disrupted due to abnormal lipolysis, protelysis, and protein phosphorylation that were induced by the high [Ca\(^{2+}\)].\(^9\) If ion homeostasis across the plasma membrane is severely impaired during ischemia, a further influx of Ca\(^{2+}\) could occur during reperfusion, triggering superoxide radical reactions\(^{4}\) as well as numerous Ca\(^{2+}\)-dependent enzymatic processes\(^{5,6}\); both events are deleterious to cellular viability. Nimodipine appears to protect against the irreversible membrane damage mediated by an increase in [Ca\(^{2+}\)].

Recovery of EEG amplitude during reperfusion was significantly enhanced by nimodipine treatment. Hoffmeister et al.\(^{18}\) and Mabe et al.\(^{22}\) reported a similar beneficial effect of nimodipine on the recovery of EEG. The recovery of EEG amplitude seen in the nimodipine-treated group cannot be explained solely by the hyperperfused state following reperfusion since ICBF in the vehicle-treated group also returned to 80% of control after 10 minutes of reperfusion. We have already shown that the recovery of EEG amplitude during reperfusion correlates well with [Ca\(^{2+}\)], but not with ICBF.\(^{28}\) Histologic damage in the same cortical gyrus from which [Ca\(^{2+}\)] was measured was significantly ameliorated by nimodipine treatment. Mohamed et al.\(^{21}\) and Steen et al.\(^{19}\) reported a similar histologic improvement with nimodipine treatment in different models of experimental ischemia, although negative results have been reported by other investigators.\(^{29,36-38}\) Mohamed et al.\(^{21}\) showed histologic improvement in the periphery of the ischemic area as well as in the cortex but not in the core of the lesion. We also found significant histologic improvement in the cortex. Cellular energy state assessed by the mitochondrial NAD/NADH redox state was not influenced by nimodipine treatment, supporting previous experimental evidence that nimodipine does not modify cerebral energy metabolism during ischemia.\(^{39,40}\)

Although the effect of nimodipine has been examined in a number of experimental models of ischemia, the results are somewhat conflicting. Possible factors contributing to the variable results include differences in species, in anesthesia, in doses and timing of nimodipine treatment, in type of ischemia (global vs. focal, complete vs. incomplete), in presence or absence of reperfusion, and in timing of evaluation. Timing of the treatment seems to be most crucial; nimodipine tends to be effective when administered before or immediately after the ischemic insult. We administered nimodipine early (5 minutes) after MCA occlusion. At that time nimodipine appears to attenuate an elevation of [Ca\(^{2+}\)], during ischemia independent of any hemodynamic effect. [Ca\(^{2+}\)], during reperfusion was also significantly lower in the nimodipine-treated group. Nimodipine led to a better recovery of EEG amplitude as well as to less histologic damage following the ischemic insult. These data suggest that the beneficial effects of nimodipine may be related to an attenuation of the increase in [Ca\(^{2+}\)], during ischemia and reperfusion rather than to a hyperperfused state following reperfusion.

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KEY WORDS • calcium • cerebral ischemia • nimodipine • cats
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