Effect of Calcium Antagonists on Postischemic Protein Biosynthesis in Gerbil Brain

Y. Xie, MD; K. Seo, MD; K. Ishimaru, MD; and K.-A. Hossmann, MD, PhD

Background and Purpose: Prolonged inhibition of protein synthesis precedes delayed neuronal death in the CA1 sector of the hippocampus after transient cerebral ischemia. Organic calcium antagonists have been recommended for alleviation of ischemic neuronal damage. The present study was undertaken to investigate whether these drugs improve the recovery of protein biosynthesis after interruption of cerebral blood flow.

Methods: Cerebral protein synthesis was measured biochemically and autoradiographically in gerbils submitted to 5 minutes of bilateral occlusion of the common carotid arteries followed by 2 hours or 2 days of recirculation. Flunarizine (25 mg/kg) or nimodipine (1.5 mg/kg) were applied intraperitoneally shortly after ischemia.

Results: Treatment with either calcium antagonist did not markedly influence postischemic recovery of protein synthesis in the resistant regions of the brain and did not prevent the persisting inhibition in the vulnerable stratum pyramidale of the CA1 sector of the hippocampus.

Conclusions: The postischemic application of the organic calcium antagonists nimodipine and flunarizine does not promote postischemic recovery of protein synthesis. The beneficial effects of these drugs must, therefore, be based on other mechanisms. (Stroke 1992;23:87-92)

The phenomenon of selective injury of hippocampal CA1 pyramidal neurons after a brief period of global cerebral ischemia is widely used for studying the molecular events associated with ischemic cell death. Apart from acute processes such as disturbances of energy metabolism, accumulation of acidic metabolites, release of excitatory amino acids, or cytosolic calcium overload, several long-lasting biochemical alterations seem to be of particular pathogenetic importance. One of these is the postischemic inhibition of cerebral protein biosynthesis. Transient interruption of cerebral blood flow in gerbils for as little as 5 minutes causes a severe depression of protein synthesis lasting for several hours. In the CA1 region protein synthesis does not recover at all, which may be the cause of the delayed structural disintegration of pyramidal neurons in this region.

In a previous study we reported that administration of a low dose of pentobarbital shortly after the beginning of recirculation prevents selective neuronal damage in the CA1 sector of the hippocampus in Mongolian gerbils. This treatment did not reverse the initial postischemic inhibition of protein synthesis, but it promoted subsequent recovery. We therefore concluded that it is not the initial depression of the protein-synthesizing machinery, but the ability of the neurons to reactivate this machinery during the postischemic recovery process, that determines the degree of ischemic injury.

In the present study we investigate the effects of the organic calcium entry blockers flunarizine and nimodipine on this process. We considered these drugs of particular interest because of their beneficial effects in different ischemia/hypoxia animal models and because of the widely accepted hypothesis that postischemic intracellular calcium overload initiates the pathological cascade leading to ischemic cell death (for review see References 19 and 20).

Materials and Methods

L-(4.5-3H)Isoleucine, L-(3.4.5-3H)leucine, L-(ring-2.3.5.6-3H)tyrosine, L-(ring-2.3.4.5.6-3H)phenylalanine, and L-(methyl-3H)methionine were purchased from New England Nuclear, Dreieich, FRG. Prior to injection, the tritium-labeled amino acids were mixed and diluted with unlabeled L-amino acids and physiological saline to an equimolar solution of 0.538 mM for each.
amino acid and a radioactivity of 0.2 mCi/ml (specific radioactivities 75.1, 77.3, 79.6, 79.6, and 74.3 Ci/mmole, respectively).

Unlabeled l-aminocids, N,N-dimethyl-5-amino-naphthalene-1-sulfonic acid chloride (DNS-Cl), and DNS-aminocids were obtained from Sigma, Heidelberg, FRG. Acetonitrile, methanol, and water used for high-performance liquid chromatography (HPLC) were from Baker Chemical, Groß-Gerau, FRG; all other reagents were obtained from Merck, Darmstadt, FRG.

Adult male Mongolian gerbils (Meriones unguiculatus, 50–80 g body weight) were anesthetized with 2% halothane in 30% oxygen/70% nitrous oxide. Both common carotid arteries were exposed and cross-clamped with atraumatic aneurysm clips for 5 minutes. Sham-operated gerbils served as controls. During ischemia halothane anesthesia was discontinued. Rectal temperature was maintained at 37°C. Arterial blood pressure and the electroencephalogram were monitored continuously. After removing the clips proper reestablishment of blood flow through the carotid arteries was confirmed by microscopic inspection.

For the measurement of protein synthesis, catheters were inserted into a femoral artery and a femoral vein. A tracer quantity of the five tritium-labeled neutral amino acids was injected intravenously over 30 seconds (1 mCi/kg, 13.4 nmol/kg). During the incorporation time blood was withdrawn continuously from the femoral artery (0.27 ml/hr) using a constant-speed withdrawal pump (Harvard Apparatus, Millis, Mass.). Forty-five minutes after the injection of tracer the gerbils were decapitated. The brains were removed rapidly from the skulls and frozen at -50°C. Plasma was prepared from collected arterial blood samples. The plasma and brain tissue were stored at -80°C until further processing.

The following seven groups consisting of six gerbils each were investigated: group I, sham-operated controls; group II, 5 minutes of bilateral carotid artery occlusion followed by 2 hours of recirculation (tracer injection after 75 minutes of recirculation); group III, same as group II but with intraperitoneal injection of 25 mg/kg flunarizine immediately after ischemia; group IV, same as group II but with intraperitoneal injection of 1.5 mg/kg nimodipine; and groups V, VI, and VII, same as groups II, III, and IV but with recirculation period of 2 days.

For the measurement of protein synthesis, tissue samples were taken from the front pole of frozen brains and sonicated in 0.5 ml ice-cold water. Homogenates were precipitated with an equal volume of 10% trichloroacetic acid (TCA) and washed twice with 0.5 ml of 5% TCA. For determination of the specific radioactivity of the tracer in the tissue free amino acid pool, supernatants were extracted five times with ether to remove TCA and evaporated to dryness. The dried samples were redissolved in 0.2 ml of 40 mM lithium carbonate buffer (pH 9.5) and dansylated according to the method of Badoud and Pratz. Amino acid analysis was performed by HPLC on a reversed-phase column (Bio-Sil ODS-5S, 250x4 mm, Bio-Rad Instruments, Munich, FRG) using a linear gradient of 14–55% acetonitrile in phosphate buffer. The radioactivities of collected fractions were measured in a liquid scintillation counter (LS 7000, Beckman Instruments, Inc., Fullerton, Calif.). Plasma samples were deproteinized with 5% TCA and processed in the same way as the tissue samples.

For the determination of radioactivity incorporated into brain proteins, pellets after TCA washing were redissolved in 1 ml of 0.2% sodium carbonate in 0.1N NaOH (pH 10.2). Aliquots were taken for the measurement of radioactivity. Amino acid incorporation into proteins was estimated by calculating the ratio of TCA-precipitable radioactivity to total tissue radioactivity (fractional protein radioactivity).

The regional pattern of protein synthesis was assessed by quantitative autoradiography. Twenty-micrometer coronal cryostat sections passing through the dorsal hippocampus were prepared at -20°C. Selected sections were wash-incubated in 10% TCA overnight to remove free amino acids. Autoradiographs were prepared by exposing the TCA-eluted sections side by side with their uneluted neighboring sections to beta-sensitive film (Ultratm, LKB Produkter AB, Bromma, Sweden). After exposure for 3–12 weeks, the autoradiograms were analyzed with a computer-controlled image processing system (ID 2000, DeAnza Systems, Fremont, Calif.). For quantification, tritium polymer standards (Amersham, Braunschweig, FRG) were calibrated with cryostat sections of gray matter tissue homogenates. Tissue radioactivity was expressed as nanocuries per milligram wet weight.

The fractional protein radioactivity was determined by digitizing the autoradiograms obtained from neighboring sections with and without TCA wash-incubation and dividing the corresponding radioactivities pixel by pixel using the image processing system. All values are expressed as mean±SD. Differences between controls and ischemic gerbils and between drug-treated and untreated animals were tested by nonparametric analysis of variance (Kruskal-Wallis test) followed by a simultaneous multiple comparison procedure according to Nemenyi.

**Results**

Mean arterial blood pressure (MABP) in the control gerbils was 81±13 mm Hg. Treatment with nimodipine transiently decreased MABP to 55±4 mm Hg. After 2 days MABP returned to 71±8 mm Hg. This change had no effect on the brain amino acid pool (see below). Flunarizine treatment did not affect MABP (76±13 mm Hg).

The content and specific radioactivity of amino acids in the precursor pools are summarized in Table 1. In control animals the combined plasma concentration of the five amino acids amounted to 0.218 nmol x μl⁻¹. The specific radioactivity of these amino acids in plasma samples collected continuously during the 45-
minute incorporation period was 0.58 nCi×nmol⁻¹. After 2 hours or 2 days of recirculation following 5 minutes of forebrain ischemia, no consistent changes occurred. Treatment with flunarizine or nimodipine induced a persistent increase in the plasma concentration of the amino acids up to 2 days without significantly altering their specific radioactivity (Table 1).

The free amino acid content in the brain was of the same order of magnitude as in plasma (Table 1). The increased plasma amino acid content induced by the calcium antagonists was associated with an elevated amino acid level in brain tissue. During early recirculation (2 hours) there was a twofold to threefold increase in the specific activity of the brain amino acid pool despite a constant specific activity in plasma. Treatment with a calcium entry blocker attenuated but did not prevent the changes in precursor specific activity in the brain (Table 1).

The total radioactivity measured in tissue samples from the frontal pole amounted to about 1 nCi×mg⁻¹ wet weight and differed little among groups (Table 2). Radioactivity incorporated into the TCA-precipitable fraction of tissue samples from the frontal pole, in contrast, exhibited substantial alterations. After 2 hours of recirculation following 5 minutes of ischemia, the fractional protein radioactivity declined from 0.54 to 0.27 in the untreated gerbils. Treatment with flunarizine or nimodipine did not attenuate this reduction (fractional protein radioactivities of 0.28 and 0.26, respectively). After 2 days of recirculation amino acid incorporation improved substantially to 0.42 in the untreated animals and to 0.42 and 0.51 in the flunarizine- and nimodipine-treated gerbils, respectively.

Fractional protein radioactivity of the control gerbils measured by quantitative autoradiography was between 0.56 and 0.72, that is, higher than in tissue

| TABLE 1. Tritium-Labeled Amino Acids in Plasma and Brain Tissue Precursor Pools Before and After 5 Minutes of Bilateral Carotid Artery Occlusion in Gerbils |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Group           | Plasma (nmol×μl⁻¹) | Brain (nmol×mg⁻¹) | Plasma (nCi×nmol⁻¹) | Brain (nCi×nmol⁻¹) |
| Controls        | 0.218±0.02       | 0.237±0.03       | 0.58±0.18         | 0.10±0.04        |
| Ischemic        |                  |                  |                  |                  |
| 2 hours of recirculation |         |                  |                  |                  |
| Without treatment | 0.234±0.04     | 0.275±0.06       | 0.57±0.26         | 0.37±0.20*       |
| Flunarizine     | 0.446±0.09†     | 0.334±0.10       | 0.51±0.23         | 0.20±0.09*       |
| Nimodipine      | 0.400±0.09      | 0.347±0.11       | 0.37±0.05         | 0.24±0.12*       |
| 2 days of recirculation |         |                  |                  |                  |
| Without treatment | 0.250±0.09     | 0.261±0.08       | 0.49±0.15         | 0.13±0.04        |
| Flunarizine     | 0.381±0.14††    | 0.314±0.09       | 0.68±0.39         | 0.18±0.08*       |
| Nimodipine      | 0.402±0.14††    | 0.275±0.07       | 0.55±0.22         | 0.14±0.05        |

Labeled amino acids were injected intravenously 45 minutes before decapitation. Values are mean±SD, n=6 in each group.

* p<0.05 different from sham-operated control animals.
† p<0.05 different from untreated animals (Kruskal-Wallis test followed by multiple comparisons according to Nemenyi²²).

| TABLE 2. Tritium-Labeled Amino Acid Incorporation Into Brain Proteins of Gerbils After 5 Minutes of Bilateral Carotid Artery Occlusion |
|-----------------|-----------------|-----------------|-----------------|
| Group           | Total (nCi×mg⁻¹) | TCA-precipitable (nCi×mg⁻¹) | Fractional protein radioactivity |
| Controls        | 0.946±0.11      | 0.506±0.05      | 0.54±0.03       |
| Ischemic        |                  |                  |                  |
| 2 hours of recirculation |         |                  |                  |
| Without treatment | 1.014±0.22     | 0.232±0.07*     | 0.27±0.11*       |
| Flunarizine     | 0.97±0.22       | 0.272±0.07†     | 0.28±0.04†       |
| Nimodipine      | 1.14±0.15†      | 0.293±0.10†     | 0.26±0.11†       |
| 2 days of recirculation |         |                  |                  |
| Without treatment | 0.917±0.18     | 0.392±0.16†     | 0.42±0.11†       |
| Flunarizine     | 1.04±0.20       | 0.435±0.12      | 0.42±0.11†       |
| Nimodipine      | 1.21±0.13†      | 0.612±0.10†     | 0.51±0.04        |

Labeled amino acids were injected intravenously 45 minutes before decapitation. Fractional protein radioactivity is ratio of TCA-precipitable radioactivity to total tissue radioactivity. Values are mean±SD. TCA, trichloroacetic acid. *t p<0.001 and p<0.05, respectively, different from controls.
Control

2h Recirculation

Untreated

Nimodipine

Flunarizine

2 days

FIGURE 1. Autoradiograms of tritium-labeled amino acids incorporated into brain proteins of control gerbil and gerbils submitted to 5 minutes of ischemia followed by 2 hours or 2 days of recirculation. Compare untreated animals and animals receiving either 1.5 mg/kg i.p. nimodipine or 25 mg/kg i.p. flunarizine immediately after ischemia. Note persisting inhibition of protein synthesis in CA1 sector of hippocampus (arrows) of both untreated and treated gerbils.

samples. This is attributed to a loss of tritium-labeled water in the dried cryostat sections due to metabolism of the tritium-labeled amino acids. Two hours after 5 minutes of ischemia fractional protein radioactivity in the untreated animals was severely depressed throughout the brain with the exception of the dentate gyrus, which at this time was only slightly affected (Figure 1). After 2 days of recirculation untreated gerbils exhibited substantial recovery in the parietooccipital cortex and CA3 sector (90% of control), partial recovery in the thalamus (78% of control), and only minor recovery in the selectively vulnerable CA1 sector of the hippocampus (45% of control).

In drug-treated gerbils amino acid incorporation returned to about 75% of control in the CA1 sector and to or above control in all other brain regions. Inspection of the autoradiograms revealed that recovery in the CA1 subfield was not equivalent to the normal laminar pattern; only two of the six flunarizine-treated and one of the six nimodipine-treated gerbils exhibited the typical pattern of high radioactivity in the pyramidal layer. In the other animals incorporated radioactivity was distinctly suppressed. This is not different from the case in the untreated gerbils, of which two of six exhibited normal and the other four exhibited severely suppressed amino acid incorporation into the CA1 pyramidal neurons (Figure 1).

Discussion

After transient cerebral ischemia, protein synthesis recovers more slowly than energy metabolism. This has been attributed to the disaggregation of ribosomes that occurs shortly after the restoration of blood flow and is only slowly reversed. This disturbance, however, does not lead to irreversible injury. Only in regions with persisting inhibition of protein synthesis, such as the CA1 sector, does neuronal death occur after a delay of several days.

The present study was undertaken to investigate whether calcium antagonists may interfere with this process. It is widely held that delayed neuronal death in the CA1 sector of the hippocampus is caused by calcium-mediated processes. It is, therefore, conceivable that posts ischemic depression of protein synthesis is a consequence of this disturbance because several steps of protein synthesis are directly or indirectly modulated by the calcium/calmodulin system. The beneficial effect of calcium antagonists on selective vulnerability reported by several au-
Theors15-18 may, therefore, be related to a restitution of normal postischemic protein synthesis.

The results of our present study do not support this hypothesis. Neither flunarizine nor nimodipine had a major effect on postischemic suppression or the subsequent reversal of neuronal protein synthesis in the vulnerable or resistant parts of the brain. The slight improvement of protein synthesis in the CA1 sector after 2 days of recirculation was not associated with the normal laminar organization. In treated as in untreated gerbils, four or five of six animals exhibited a distinct reduction of incorporated radioactive activity in the pyramidal layer. The improvement of amino acid incorporation in the CA1 sector of treated gerbils, in consequence, seems to be due to glial rather than neuronal metabolism.

The absence of a therapeutic effect of the calcium antagonists is in contrast to a previous study of postischemic barbiturate application that led to the full recovery of protein synthesis in the CA1 sector after 2 days of recirculation.13 This difference may be due to the different pharmacological profiles of these drugs. The organic calcium antagonists nimodipine and flunarizine act primarily on voltage-sensitive channels, that is, nimodipine acts on the L-type31,32 and flunarizine on the T-type33 channel. Barbiturates, in contrast, also inhibit excitatory amino acid receptors, particularly of the non-N-methyl-D-aspartate type.34,35 There is increasing experimental evidence that activation of these receptors and the resulting massive cation fluxes are critical steps in the cascade of pathological events leading to neuronal injury (for review see Reference 36). It is, therefore, conceivable that inhibition of the voltage-dependent calcium channels alone does not lower the intracellular calcium activity below the threshold for restoration of protein synthesis.

In conclusion, the present study does not provide evidence for the reversal by organic calcium antagonists of the postischemic inhibition of protein synthesis. A possible therapeutic effect, therefore, must be related to other mechanisms.

References


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**KEY WORDS** • cerebral ischemia • flunarizine • nimodipine • gerbils
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