Neuroscience 118 (2003) 107–113

COMBINED NIMODIPINE AND CITICOLINE REDUCE INFARCT SIZE, ATTENUATE APOPTOSIS AND INCREASE BCL-2 EXPRESSION AFTER FOCAL CEREBRAL ISCHEMIA

M. SOBRADO,[a]* M. G. LÓPEZ,[a] F. CARCELLER,[b] A. G. GARCÍA[a] AND J. M. RODA[b]

[a]*Instituto Teófilo Hernando, Departamento Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, Servicio de Farmacología Clínica e Instituto de Gerontología, Hospital de La Princesa, Madrid, Spain*

[b]*Unidad de Investigación Cerebrovascular, Cirugía Experimental, Hospital Universitario La Paz, Madrid, Spain*

Abstract—Cerebral ischemia triggers a multitude of pathophysiological and biochemical events that separately affect the evolution of focal ischemia and, therefore, stroke treatment should logically employ all known neuroprotective agents. We hypothesized that a treatment combining nimodipine and citicoline might have a potential neuroprotective effect. To assess this idea, Sprague–Dawley rats underwent transient bilateral common carotid artery ligation with simultaneous middle cerebral artery occlusion for 60 min. Four treatment groups were established. Animals received either: a) saline (control group); b) intracarotid nimodipine infusion during 30 min in the ischemia-reperfusion (nimodipine group); c) i.p. postischemic citicoline injections once daily for 7 days (citicoline group); or d) intracarotid nimodipine bolus during ischemia-reperfusion plus i.p. postichemic citicoline injections (combination group). They were killed after either 7 or 3 days after reperfusion. In the first case, the volume of the infarcted tissue was studied by a stereological procedure and in the second case, *in situ* end-labeling of nuclear DNA fragmentation (TUNEL) and Bcl-2 expression were employed to determine the level of apoptosis. The infarct volume was significantly reduced in both the nimodipine and the citicoline treatment groups after 7 days of reperfusion; combination of both drugs produced an additive effect. After 3 days of reperfusion, the number of Bcl-2-positive neurons was significantly increased while that of TUNEL-positive cells significantly decreased at the infarct border in the combined-treatment animals. Our findings demonstrate a neuroprotective effect from an acute single dose of nimodipine during ischemia-reperfusion and prolonged post-ischemic treatment with citicoline in a model of focal cerebral ischemia. These results suggest that a possible mechanism of neuroprotective action would be mediated by increased Bcl-2 expression and decreased apoptosis within the boundary zone of the infarct together with neutralization of the ischemia-reperfusion injury. © 2003 Published by Elsevier Science Ltd on behalf of IBRO.

*Corresponding author. Tel: +34-91-727-7128; fax: +34-91-727-7128.
E-mail address: monica.sobrado@uam.es (M. Sobrado).

Abbreviations: H&E, hematoxylin and eosin; %I, percent of neocortex that was infarcted; L, whole cortex of the contralateral hemisphere; MK801, (+)-10,11-dihydro-5-methyl-5H-dibenzo[*a,b*]cyclopentene-5,10-imine; R_N, spared cortex in the damaged hemisphere; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling.

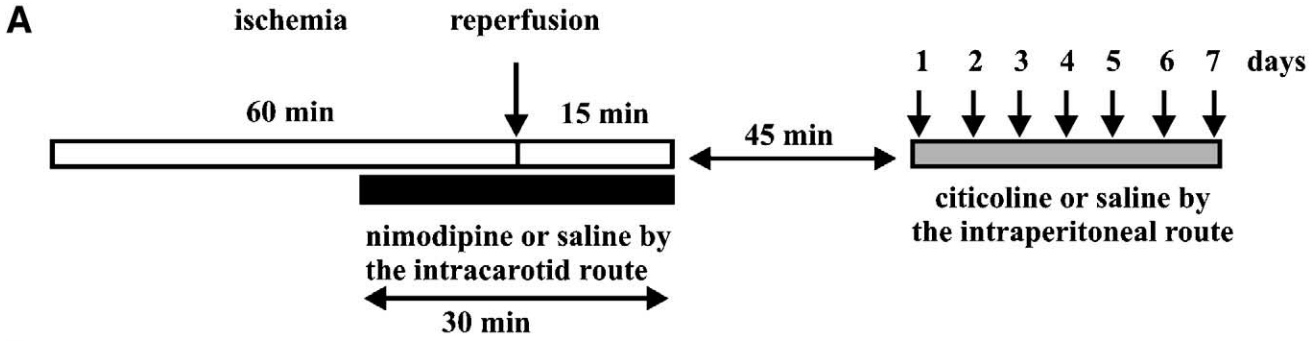
Key words: neuroprotection, ischemia-reperfusion, TUNEL, rats.

In a stroke, neurons die by necrotic mechanisms, mainly in the core zone, as well as by apoptotic mechanisms, particularly in the inner borders of the infarct (Benchoua et al., 2001). The presence of apoptotic cells in the penumbra suggests that apoptosis may contribute to the final infarct size (Guégan et al., 1996; Li et al., 1995a,b), and that this might be reduced with an appropriate pharmacological treatment. In many experimental situations, apoptosis is regulated by proteins of the Bcl-2 family. The antiapoptotic protein Bcl-2 has been implicated in the molecular pathology after brain injury, and is thought to provide protection from neuronal loss after transient focal ischemia (Chen et al., 1995). This protein effectively protects against apoptotic oxidative stress in PC12 cells (Kubo et al., 1996), reduces the rise of mitochondrial Ca^{2+}, presumably by preventing opening of the mitochondrial transition pore (Zamzami et al., 1996) and, finally, inhibits cytochrome c release, consequently blocking caspase activation and the apoptotic process (Kluck et al., 1997).

Many researchers have tested a variety of compounds with different mechanisms of action in the hope of reducing neuronal damage and rescuing neurons from the penumbra area. However, although some substances produced neuroprotection in experimental studies, they showed little benefit in clinical trials. The reason for this may be the multitude of mechanisms in cerebral ischemia that act in different ways to result in nervous tissue damage. Up to now, single drugs have failed to demonstrate clinical efficacy in stroke patients. Therefore, the search for a combination of drugs with complementary effects that could act at different steps in the ischemic cascade is attracting attention. In fact, some studies with combined treatments in experimental focal cerebral ischemia, i.e. the association of citicoline with either recombinant tissue plasminogen activator (Andersen et al., 1999) or MK-801 (Onal et al., 1997), have shown synergistic neuroprotective effects.

Our objective in this study was to test the effects of the combination of these two drugs: nimodipine, a dihydropyridine that blocks L-type Ca^{2+} channels, and citicoline, an endogenous compound that stabilizes membrane function and reduces free radical generation (Rao et al., 1999, 2000), in a rat model of reversible focal cerebral ischemia. The model we have used permits intracarotid injection of the potentially therapeutic compound directly to the ischemic cerebral tissue, before blood recirculation (Roda et

0306-4522/03$30.00+0.00 © 2003 Published by Elsevier Science Ltd on behalf of IBRO.
doi:10.1016/S0306-4522(02)00912-0



B

groups	intracarotid infusion (30 min)	intraperitoneal bolus (7 days)
control	saline	saline
nimodipine	nimodipine 40 μg/Kg/30min	saline
citicoline	saline	citicoline 250 mg/Kg/day
combination	nimodipine 40 μg/Kg/30min	citicoline 250 mg/Kg/day

Fig. 1. (A) Schematic representation showing the protocol used for the intracarotid and i.p. injections of the different pharmacological substances employed in rats subjected to transient focal cerebral ischemia. (B) Treatment protocol in the four groups.

al., 1995). The effectiveness of drug therapy is known to be limited by poor perfusion in the ischemic area when an intravenous route is employed. However, when an arterial route is used, the pharmacological substance arrives at the ischemic zone in a more rapid, concentrated and efficient way. This can help open the therapeutic window for delivering neuroprotective substances to the ischemic tissue (Clemens, 1995). Nimodipine was acutely injected by an intracarotid route in order to impregnate the ischemic cerebral tissue just before recirculation; on its part, citicoline was regularly administered by an i.p. route in order to alleviate the deleterious effects resulting from cell membrane damage, during either ischemia or reperfusion (Schabitz et al., 1996). In order to investigate the neuroprotective mechanism of the combination treatment against cortical infarction, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) histochemical and morphological examinations were used to detect apoptotic cells and antiapoptotic protein Bcl-2.

EXPERIMENTAL PROCEDURES

Subjects

Male Sprague–Dawley rats were obtained from our breeding colony derived from a stock originally purchased from Janvier (Legenes-St-Isle, France). Animals, weighing 275±25 g, were subjected to transient focal cerebral ischemia and assigned to one of the following treatment groups: a) nine animals received injections of intracarotid isotonic saline followed by i.p. isotonic saline (control group); b) nine animals received intracarotid nimodipine followed by i.p. isotonic saline (nimodipine group); c) nine animals received intracarotid isotonic saline followed by i.p. citicoline (citicoline group); and d) nine animals received intracarotid nimodipine followed by i.p. citicoline (combination group) (See Fig. 1C, for the experimental protocol followed in each group of animals).

Another 12 rats were used for histology and immunohistochemistry and the details are explained below.

This study was approved by the Animal Care and Use Committee of the Facultad de Medicina, Universidad Autónoma de Madrid, and conforms to the national and international guidelines on the ethical use of animals. Each experimental protocol was statistically designed to use the minimal number of animals.

Induction of transient focal ischemia

Surgery leading to focal cerebral ischemia was performed in all groups and was conducted under anesthesia with 400 mg/kg bodyweight of chloral hydrate injected intraperitoneally, and supplemented as necessary during the procedure. The femoral artery was cannulated with polyethylene tubing for continuous monitoring of arterial blood pressure and blood sampling for analysis of blood gases, pH and glucose. Measurements were performed before occlusion, 15 min after occlusion and 10 min after reperfusion. During surgery, body temperature was maintained at 37±0.5 °C using a servo-controlled rectal probe–heating pad, until recovery from anesthesia. The surgical procedure was a variant of that described by Chen et al. (1986) and Liu et al. (1989). A small craniectomy was made over the trunk of the right middle cerebral artery and above the rhinal fissure. The middle cerebral artery trunk was transitorily ligated just before its bifurcation between the frontal and parietal branches with a 9-0 suture. Complete interruption of blood flow was confirmed under an operating microscope. A thermistor probe was placed under the temporal muscle and over the cerebral artery region to measure brain temperature. This was maintained at 37±0.5 °C by means of a lamp located over the head. Both common carotid arteries were then occluded for 60 min. At the end of this time, blood flow was directly restored under the operating microscope in all three arteries (He et al., 1993; Lin et al., 1993). During the occlusion period, the right external carotid artery was retrogradely cannulated with the catheter tip placed near the origin of the internal carotid artery, as previously described (Carceller et al., 1993; Roda et al., 1991).

Protocol of drug administration

Intracarotid infusion of nimodipine (40 μg/kg/30 min; Nimotop solution, Bayer; Leverkusen, Germany) in physiological saline (nimodipine group and combination group) or the saline solution

alone (control group and citicoline group) was started 15 min before recirculation and maintained until 15 min after recirculation (Fig. 1). Nimodipine or the saline solution alone was supplied continuously for 30 min through an electronic pump (Harvard Apparatus, Millis, MA, USA). Animals were treated with an i.p. bolus of either citicoline (250 mg/kg/day; Ferrer Internacional, Barcelona, Spain), dissolved in physiological saline at a concentration of 75 mg/ml (citicoline and combination groups), or of vehicle alone (nimodipine and control groups) at 1 h after reperfusion and once a day for 6 days thereafter. Animals that died within 12 h of vessel occlusion were not included in the study. On the seventh day the animals were killed with an i.p. overdose of chloral hydrate and decapitated. The brains were quickly removed and coronally sectioned into six 2-mm-thick slices. For delineation of infarct area, the brain slices were incubated in a 2% solution of triphenyltetrazolium chloride and then fixed in a buffered formalin solution. The unstained area was defined as infarcted tissue.

Measurement of infarct volume

Morphometric determination of cortical infarct volume was obtained by means of an unbiased estimator of volume based on Cavalieri's principle (Avendaño et al., 1995). The ratio between the volume of the spared cortex in the damaged hemisphere (R_N) and that in the whole neocortex of the contralateral hemisphere (L) was calculated, and employed to detect differences in the amount of cortex that was damaged by the infarct in each animal. When expressed as a percentage this ratio indicates the percentage fraction of neocortex which was spared by the ischemia; therefore, the percent of neocortex that was infarcted (%I) is readily obtained by the formula: $\%I=[1-(R_N/L)]\times 100$.

Histology and immunohistochemistry

For morphological examinations on paraffin sections, 12 other rats ($n=3$ each group) were subjected to reversible focal cerebral ischemia by the same method described above. They were killed 72 h after middle cerebral artery occlusion and fixed by transcardial perfusion with 4% paraformaldehyde in 10% buffered formalin phosphate. The brains were removed from the skull and postfixed overnight in the same buffer at 4 °C. Each tissue block was dehydrated, embedded in paraffin, and cut into four coronal slabs (two anterior and two posterior to the anterior commissure) using brain matrix. The blocks were embedded in paraffin and 5-|μm sections were prepared for neuropathological studies.

Three sections from each rat ($n=9$) were stained with hematoxylin and eosin (H&E) and used to locate the infarct border after middle cerebral artery occlusion. Adjacent sections were stained with Bcl-2 immunostaining. A standard H&E-stained section through the anterior commissure, where the lesion was always detected in all animals, was used to observe the TUNEL staining (see below).

TUNEL

The detection of DNA fragmentation and apoptotic bodies in cells were assessed with a terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labeling assay using a kit (TdT mediated biotin-dUTP nick-end labeling; TdT-FragEL DNA fragmentation detection kit, Oncogene Research Products). Deparaffined and rehydrated sections were pretreated with proteinase K for 30 min at room temperature, then, after endogenous peroxidase activity was quenched with 2% H_2O_2 in methanol, slices were placed in equilibration buffer and then in working TdT enzyme for 1 h at 37° followed by stop and blocking buffer. Finally, the reaction was visualized by streptavidin–biotin–peroxidase complex and diaminobenzidine. Normal nuclei that had a relatively insignificant number of DNA 3'OH ends did not stain with this technique. For the negative controls the sections were incubated without the enzyme. Coronal sections stained with the TUNEL method were also counterstained with green methyl. Positive cells were counted under eight microscopic fields (250×) in the dorsal and ventrolateral cortical infarct border. Adjacent sections of tissue were stained with H&E to identify the infarct border in rat brain subjected to middle cerebral artery occlusion. Since TUNEL staining may detect cells undergoing necrotic as well as apoptotic cell death, we considered the presence of various types of chromatin condensation (peri-nuclear ring formation, patches or apoptotic bodies) as TUNEL-apoptotic neurons, and cells showing weak diffuse cytoplasmic staining or light nuclear labeling without nuclear condensation or apoptotic bodies as TUNEL-positive necrotic neurons (Ay et al., 2001; Elibol et al., 2001).

Detection of Bcl-2-positive neurons

After deparaffination and rehydration, sections were immersed in 3% H_2O_2 in methanol to quench endogenous peroxidase activity and then in 1% bovine serum albumin for 1 h. Sections were incubated overnight at 4°C in monoclonal rabbit antimouse *Bcl-2* (sc-7382; Santa Cruz Biotechnology; 1:100). Immunoreactivity was visualized using a goat-antimouse antibody (1:50) for 1 h at room temperature, followed by incubation with a peroxidase–antiperoxidase mouse complex (1:300). Sections were processed with 0.06% diaminobenzidine and 0.2% H_2O_2. Negative control sections were incubated without the primary antibody. Sections were counterstained with green methyl. For histopathological assessment, the adjacent sections from each brain stained with H&E were used to identify the infarct border.

Cortical areas in close proximity to the infarct border zone, dorsally and ventrally, were used for quantitative analysis of immunoreactivity in the peri-infarct tissue. Positive cells were counted under eight microscopic fields (250×) in the dorsal and ventrolateral cortical infarct border. Three sections per brain of three animals of each one of the four treatment groups were analyzed.

Statistics

Data are presented as mean±S.D. Differences in infarct volume, number of TUNEL- and Bcl-2-labeled cells were assessed using an analysis of variance followed by Fisher's test. $P<0.05$ was considered significant.

RESULTS

Measurement of infarct size

Physiological variables, including arterial blood pressure, blood gases, and glucose concentration, were not significantly different between any of the experimental groups before, during, or after middle cerebral artery occlusion (data not shown). The infarcted areas within the territory of the occluded middle cerebral artery were easily identifiable and had sharp, clearly discernable borders. The index used to detect differences in the volume of infarcted cortex is given by the ratio between the spared cortex of the damaged right hemisphere and the entire cortex of the left L. %I (see Experimental Procedures), was 26.7%±8.0 in the control group, 17.5%±5.7 in the nimodipine group, 15.5%±5.3 in the citicoline group, and 10.8%±4.5 in the combined treatment group ($n=9$ per group; Fig. 2). The mean value for infarct volume percentage was significantly reduced in the nimodipine, the citicoline and the combination groups compared with the control ($P<0.05$), with the largest reduction in the combination group. This reduction

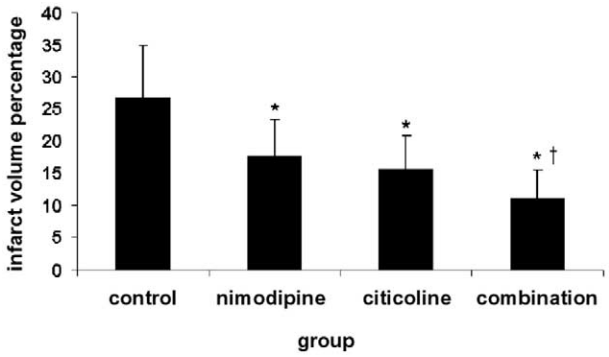


Fig. 2. Percentage of reduction in infarct volume after transient focal cerebral ischemia in rats treated with saline (control), nimodipine, citicoline or nimodipine plus citicoline (combination) (see protocols in Fig. 1). Data are means±S.D. of nine animals for each group. *$P<0.05$, significantly different from control. †$P<0.05$, versus nimodipine group.

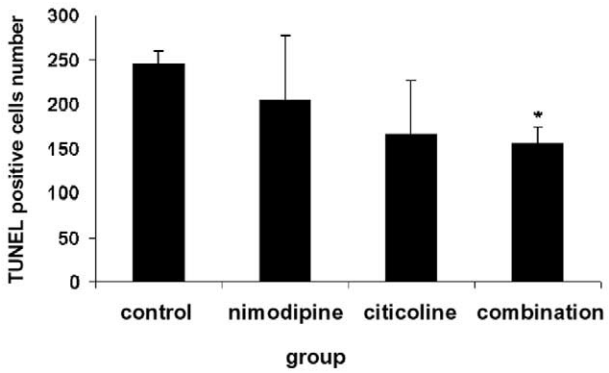


Fig. 4. Number of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL)-positive neurons corresponding to the four groups of treatment in the inner boundary zone of rats subjected to transient focal cerebral ischemia and 72 h of reperfusion. There was a significant reduction in the number of cells in the combination group when compared with the other three groups ($P<0.05$). TUNEL-positive cells were quantified on eight microscopic fields (×250) on coronal sections at the level of the anterior commissure in the inner boundary zone of the infarction. Bars=S.D.

was also significant when compared with the nimodipine treatment group ($P<0.05$). Taking the control group as 100% infarction, the reduction of cerebral infarction was 34% in the nimodipine group, 42% in the citicoline group and 59% in the combination group.

Detection of apoptotic cells with TUNEL staining

The border of the infarct was identified with H&E staining and TUNEL-positive neurons were counted in adjacent sections stained with the TUNEL method. No TUNEL labeling was observed in the contralateral non-ischemic hemisphere. At the inner border zone of infarction, few TUNEL-labeled cells displayed the diffuse staining throughout their cytoplasm characteristic of necrotic cell morphology, whereas many cells showed the characteristic appearance of apoptosis with intense staining, chromatin condensation and apoptotic bodies (Fig. 3). There were significantly fewer TUNEL-positive apoptotic cells in the combination treatment group than in the control group (154±19 versus 245±13 cells per section respectively, $P<0.05$) (Fig. 4).

Bcl-2 immunostaining

Bcl-2 immunoreactivity was not detected in the L after middle cerebral artery occlusion or in sections incubated without primary antibody. After 60 min of ischemia and 72 h of reperfusion, most of the Bcl-2-immunostained cells in the infarct border had morphological features that were characteristic of neurons (large cell bodies, axonal and dendritic processes, etc.) (Fig. 5). In the core region of the infarction, Bcl-2-positive staining was mainly limited to vessel walls (Chen et al., 1995).

The number of Bcl-2-positive cells was significantly higher in the rats treated with nimodipine plus citicoline than in the rats treated with isotonic saline (97±47 versus 62±35 cells respectively, $P<0.05$) (Fig. 6).

DISCUSSION

This study demonstrates that both an acute intracarotid injection of nimodipine or on repeated i.p. administration of

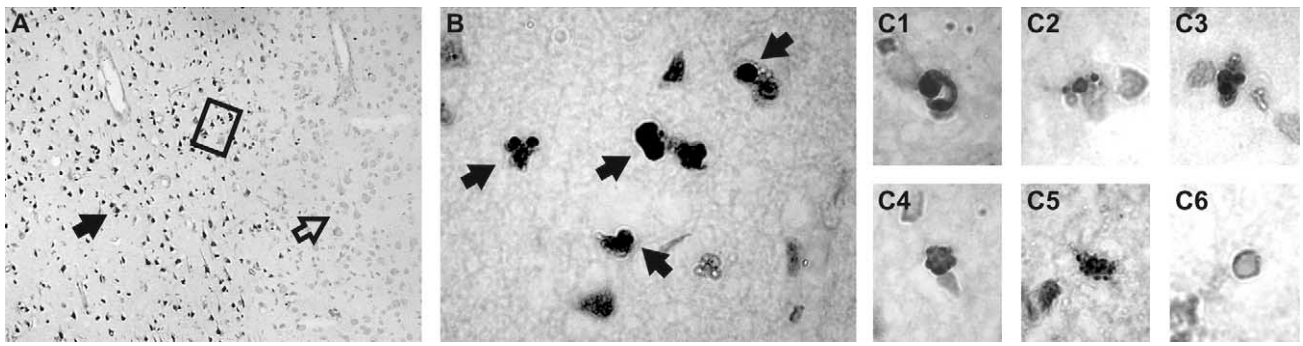


Fig. 3. *In situ* end-labeling of nuclear DNA fragmentation (TUNEL) staining (dark) of neurons presenting DNA degradation in the cerebral cortex corresponding to the occluded middle cerebral artery. (A) A part of the upper parietal cortex located between normal (open arrow) and ischemic (close arrow) tissue (100× magnification); (B) Magnification of a group of apoptotic cells framed in A (some arrowed), primarily located at the inner boundary zone of the ischemic core. (C) Morphologic features of apoptotic neurons showing apoptotic bodies (C1–C5) and perinuclear, ring-shaped chromatin condensation (C6) present in the inner boundary zone of the infarct with a 1000× magnification (methyl green was used as counterstain).

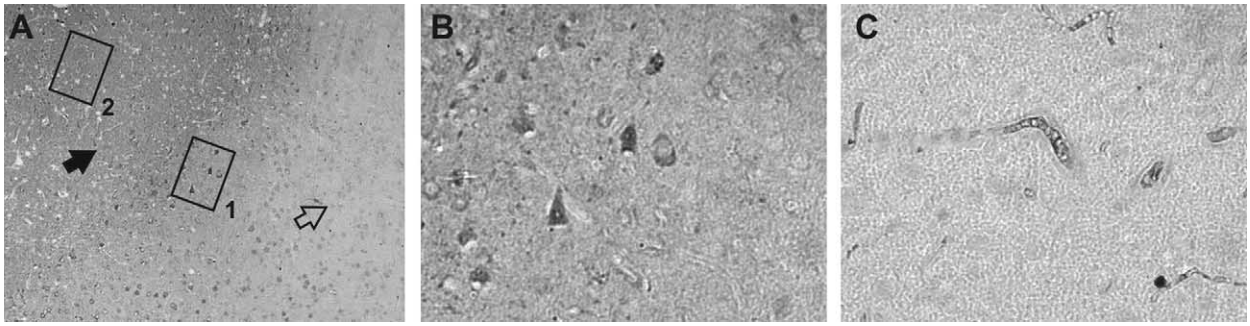


Fig. 5. Bcl-2 immunoreactivity in coronal brain sections from rats subjected to transient focal cerebral ischemia and 72 h of reperfusion. (A) Part of the upper parietal cortex between normal (open arrow) and ischemic (solid arrow) tissue with a 200× magnification. (B, C) Magnification of a group of Bcl-2 cells in the inner border of the infarction (frame one of A) or in a vessel wall in the core of the infarction (frame two of A), respectively, with an 800× magnification.

citicoline are effective neuroprotective measures that reduce infarct volume in an animal model of temporary focal cerebral ischemia with complete reperfusion and 7 days of survival. Furthermore, the combination of both drugs produced an even larger reduction, compared with when either drug was given separately. Likewise, this combined treatment decreased apoptotic cell death and increased Bcl-2 expression in the inner boundary zone of the infarction 3 days after reperfusion.

The main aim of any therapeutic intervention in brain ischemia is to reduce the size of the infarct. The study of the mechanism of action of pharmacological molecules is essential to elucidate our knowledge of their potential neuroprotective effect on focal ischemia brain injury. With the aim of advancing in this field, we conducted the second part of this work. Three days of survival were chosen in order to be able to administer, in addition to acute nimodipine, citicoline as a prolonged treatment. TUNEL staining and Bcl-2 expression were used to assess effectiveness.

After ischemic insult, apoptosis develops in the neurons at the inner border of the infarct (Choi, 1996; Linnik et al., 1993). During expansion of infarction, apoptotic neurons preferentially develop at the inner boundary of the infarct (Guégan et al., 1996; Li et al., 1995a; Li et al., 1995b). As demonstrated in the present study, TUNEL-positive cells were only located in the cerebral cortex of the regions corresponding to the occluded middle cerebral artery in control animals. The staining was significantly reduced in the rats subjected to the combination treatment.

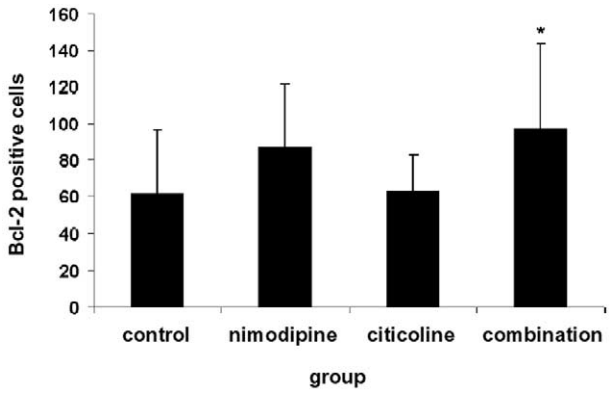


Fig. 6. Number of Bcl-2-positive neurons per section and three sections per brain in the inner boundary zone in control, nimodipine, citicoline and combination groups of rats subjected to transient focal cerebral ischemia and 72 h of reperfusion. There was a significant increase in the number of cells in the combination group when compared with the other three groups ($P<0.05$). Bars=S.D.

The neuroprotective mechanism of nimodipine could be attributed to the prevention of both necrotic and apoptotic death (Korenkov et al., 2000), since disturbances in cytosolic Ca^{2+} concentrations and intracellular Ca^{2+} compartmentalization are involved in the apoptotic mechanism (Krajewski et al., 1999; McConkey and Orrenius, 1995, 1997), and mitochondrial lesions or DNA injury have been ameliorated by Ca^{2+} channel blockers (Buttke and Sandstrom, 1994; Richter, 1993). Also, the protective effect of nimodipine could be directly related to the neutralization of the ischemic reperfusion injury (Roda et al., 1995).

For its part, citicoline, in addition to its well-known action of stabilizing both the cell membrane (Rao et al., 1999, 2000) and the glucose metabolism (Kakihana et al., 1988) and reducing lipid peroxidation in an ischemia reperfusion model (Fresta et al., 1994), has been proven to reduce the expression of procaspases-1,-2,-3,-6 and -8 and cleaved caspase-3 in the penumbra area cells, as well as reduce the number of cells bearing nuclear DNA fragmentation after focal cerebral ischemia (Krupinski et al., 2002). Furthermore, studies *in vitro* have shown that a lack of choline, a citicoline synthesis precursor, resulted in the loss of membrane phosphatidylcholine and sphingomyelin, with the consequent induction of apoptosis (Yen et al., 1999).

Bcl-2 was significantly increased in animals treated with the combination modality in the present study. It has been shown that Bcl-2 overexpression protects brain tissue from the ischemic injury caused by permanent middle cerebral artery occlusion (Linnik et al., 1995; Martinou et al., 1994). In addition, Bcl-2 is expressed in cortical neurons that survive focal cerebral ischemia (Chen et al., 1995) and endogenous levels of Bcl-2 can promote survival in ischemic neurons after transient focal cerebral ischemia (Chen et al., 2000). In a similar way, Bcl-2 is suggested to have prevented neuron death and activated microglia through an antioxidative mechanism after tran-

sient focal cerebral ischemia (Urabe et al., 1998). On the contrary, suppression of Bcl-2 is known to worsen ischemic damage after temporary middle cerebral artery occlusion (Chen et al., 2000) and in neuronal cultures subjected to ischemia-like conditions (White et al., 1997).

Due to the multiple mechanisms involved in ischemic injury, no single drug alone is capable of providing complete neuroprotection following transient focal cerebral ischemia. It is therefore useful to employ compounds with different mechanisms of action. By acting at different sites on the multifactorial ischemic brain injury cascade, the treatment combining nimodipine plus citicoline may help limit neuronal cell death. There is controversy about the effects of nimodipine: both experimental models and clinical trials have found that nimodipine is effective in some situations and ineffective in others (Gotoh et al., 1986; Horn et al., 2001; Kawaguchi et al., 1999; Mohr et al., 1994; Roda et al., 1995; Snape et al., 1993; Wahlgren et al., 1994). The same is true for citicoline (Clark et al., 1997, 1999, 2001; Schabitz et al., 1996; Tazaki et al., 1988). Nimodipine and citicoline are commonly used in clinical practice and, hence, are easily available for the exploration of whether a combination therapy with both agents would have cumulative neuroprotective effects in human stroke patients. The positive results obtained in this study in rats show that a combination of nimodipine plus citicoline is more effective than either of them alone, and suggest that this combined treatment could help in the management of cerebral ischemia.

In conclusion, our data prove that a combination of nimodipine plus citicoline ameliorates cerebral damage after transient focal cerebral ischemia, suggesting that the progression of the neuronal injury could have been limited either by reducing apoptotic processes or by neutralizing the ischemia-reperfusion damage, or both. This finding would widen the therapeutic window with the consequent benefit in clinical practice.

Acknowledgements—This study was supported by grants from Ferrer Internacional and from Spanish Government, DGICYT, No PM99-0004 to A.G.G. and PM99-0006 to M.G.L., Programa de Grupos Estratégicos del III PRICIT CAM, Madrid, to A.G.G. and CAM No. Ref.08.5/0042/2001 to M.G.L. Mónica Sobrado is a fellow of Fundación Teófilo Hernando, Madrid, Spain. Ferrer Internacional Company, Barcelona, Spain, provided Citicoline. To C. F. Warren for linguistic assistance.

REFERENCES

Andersen M, Overgaard K, Meden P, Boysen G, Choi SC (1999) Effects of citicoline combined with thrombolytic therapy in a rat embolic stroke model. Stroke 30:1464–1471.

Avendaño C, Roda JM, Carceller F, Díez-Tejedor E (1995) Morphometric study of focal cerebral ischemia in rats: a stereological evaluation. Brain Res 673:83–92.

Ay I, Sugimori H, Finklestein SP (2001) Intravenous basic fibroblast growth factor (bFGF) decreases DNA fragmentation and prevents downregulation of Bcl-2 expression in the ischemic brain following middle cerebral artery occlusion in rats. Brain Res Mol Brain Res 87:71–80.

Benchoua A, Guégan C, Couriaud C, Hosseini H, Sampaio N, Morin D, Onteniente B (2001) Specific caspase pathways are activated in two stages of cerebral infarction. J Neurosci 21:7127–7134.

Buttke TM, Sandstrom PA (1994) Oxidative stress as a mediator of apoptosis. Immunol Today 15:7–10.

Carceller F, Roda JM, Gutierrez-Molina M, Lopez-Pajares R, Albarino AR, Díez-Tejedor E (1993) Can nimodipine prevent ischaemic reperfusion injury in the rat brain? Neurol Res 15:395–400.

Chen J, Graham SH, Chan PH, Lan J, Zhou RL, Simon RP (1995) Bcl-2 is expressed in neurons that survive focal ischemia in the rat. Neuroreport 6:394–398.

Chen J, Simon RP, Nagayama T, Zhu R, Loeffert JE, Watkins SC, Graham SH (2000) Suppression of endogenous bcl-2 expression by antisense treatment exacerbates ischemic neuronal death. J Cereb Blood Flow Metab 20:1033–1039.

Chen ST, Hsu CY, Hogan EL, Maricq H, Balentine JD (1986) A model of focal ischemic stroke in the rat: reproducible extensive cortical infarction. Stroke 17:738–743.

Choi DW (1996) Ischemia-induced neuronal apoptosis. Curr Opin Neurobiol 6:667–672.

Clark WM, Warach SJ, Pettigrew LC, Gammans RE, Sabounjian LA (1997) A randomized dose-response trial of citicoline in acute ischemic stroke patients: Citicoline Stroke Study Group. Neurology 49:671–678.

Clark WM, Wechsler LR, Sabounjian LA, Schwiderski UE (2001) A phase III randomized efficacy trial of 2000 mg citicoline in acute ischemic stroke patients. Neurology 57:1595–1602.

Clark WM, Williams BJ, Selzer KA, Zweifler RM, Sabounjian LA, Gammans RE (1999) A randomized efficacy trial of citicoline in patients with acute ischemic stroke. Stroke 30:2592–2597.

Clemens JA (1995) Editorial comment. Stroke 26:1892.

Elibol B, Soylemezoglu F, Unal I, Fujii M, Hirt L, Huang PL, Moskowitz MA, Dalkara T (2001) Nitric oxide is involved in ischemia-induced apoptosis in brain: a study in neuronal nitric oxide synthase null mice. Neuroscience 105:79–86.

Fresta M, Puglisi G, Di Giacomo C, Russo A (1994) Liposomes as in-vivo carriers for citicoline: effects on rat cerebral post-ischaemic reperfusion. J Pharm Pharmacol 46:974–981.

Gotoh O, Mohamed AA, McCulloch J, Graham DI, Harper AM, Teasdale GM (1986) Nimodipine and the haemodynamic and histopathological consequences of middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab 6:321–331.

Guégan C, Boutin H, Boudry C, MacKenzie ET, Sola B (1996) Apoptotic death in cortical neurons of mice subjected to focal ischemia. C R Acad Sci III 319:879–885.

He YY, Hsu CY, Ezrin AM, Miller MS (1993) Polyethylene glycol-conjugated superoxide dismutase in focal cerebral ischemia-reperfusion. Am J Physiol 265:H252–H256.

Horn J, de Haan RJ, Vermeulen M, Limburg M (2001) Very early nimodipine use in stroke (VENUS): a randomized, double-blind, placebo-controlled trial. Stroke 32:461–465.

Kakihana M, Fukuda N, Suno M, Nagaoka A (1988) Effects of CDP-choline on neurologic deficits and cerebral glucose metabolism in a rat model of cerebral ischemia. Stroke 19:217–222.

Kawaguchi K, Henshall DC, Simon RP (1999) Parallel dose-response studies of the voltage-dependent Na+ channel antagonist BW619C89, and the voltage-dependent Ca2+ channel antagonist nimodipine, in rat transient focal cerebral ischaemia. Eur J Pharmacol 364:99–105.

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275:1132–1136.

Korenkov AI, Pahnke J, Frei K, Warzok R, Schroeder HW, Frick R, Muljana L, Piek J, Yonekawa Y, Gaab MR (2000) Treatment with nimodipine or mannitol reduces programmed cell death and infarct size following focal cerebral ischemia. Neurosurg Rev 23:145–150.

Krajewski S, Krajewska M, Ellerby LM, Welsh K, Xie Z, Deveraux QL, Salvesen GS, Bredesen DE, Rosenthal RE, Fiskum G, Reed JC (1999) Release of caspase-9 from mitochondria during neuronal

apoptosis and cerebral ischemia. Proc Natl Acad Sci USA 96: 5752–5757.

Krupinski J, Ferrer I, Barrachina M, Secades JJ, Mercadal J, Lozano R (2002) CDP-choline reduces pro-caspase and cleaved caspase-3 expression, nuclear DNA fragmentation, and specific PARP-cleaved products of caspase activation following middle cerebral artery occlusion in the rat. Neuropharmacology 42:846–854.

Kubo T, Enokido Y, Yamada M, Oka T, Uchiyama Y, Hatanaka H (1996) Oxygen-induced apoptosis in PC12 cells with special reference to the role of Bcl-2. Brain Res 733:175–183.

Li Y, Chopp M, Jiang N, Yao F, Zaloga C (1995a) Temporal profile of in situ DNA fragmentation after transient middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab 15:389–397.

Li Y, Sharov VG, Jiang N, Zaloga C, Sabbah HN, Chopp M (1995b) Ultrastructural and light microscopic evidence of apoptosis after middle cerebral artery occlusion in the rat. Am J Pathol 146:1045–1051.

Lin TN, He YY, Wu G, Khan M, Hsu CY (1993) Effect of brain edema on infarct volume in a focal cerebral ischemia model in rats. Stroke 24:117–121.

Linnik MD, Zahos P, Geschwind MD, Federoff HJ (1995) Expression of bcl-2 from a defective herpes simplex virus-1 vector limits neuronal death in focal cerebral ischemia. Stroke 26:1670–1674.

Linnik MD, Zobrist RH, Hatfield MD (1993) Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Stroke 24:2002–2008.

Liu TH, Beckman JS, Freeman BA, Hogan EL, Hsu CY (1989) Polyethylene glycol-conjugated superoxide dismutase and catalase reduce ischemic brain injury. Am J Physiol 256:H589–593.

Martinou JC, Dubois-Dauphin M, Staple JK, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D, Catsicas S, Pietra C (1994) Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron 13:1017–1030.

McConkey DJ, Orrenius S (1995) Calcium and cyclosporin A in the regulation of apoptosis. Curr Top Microbiol Immunol 200:95–105.

McConkey DJ, Orrenius S (1997) The role of calcium in the regulation of apoptosis. Biochem Biophys Res Commun 239:357–366.

Mohr JP, Orgogozo JM, Harrison MJG, Hennerici M, Wahlgren NG, Gelmers JH, Martinez-Vila E, Dycka J, Tettenborn D (1994) Meta-analysis of oral nimodipine trials in acute ischemic stroke. Cerebrovasc Dis 4:197–203.

Onal MZ, Li F, Tatlisumak T, Locke KW, Sandage BWJ, Fisher M (1997) Synergistic effects of citicoline and MK-801 in temporary experimental focal ischemia in rats. Stroke 28:1060–1065.

Rao AM, Hatcher JF, Dempsey RJ (1999) CDP-choline: neuroprotection in transient forebrain ischemia of gerbils. J Neurosci Res 58:697–705.

Rao AM, Hatcher JF, Dempsey RJ (2000) Lipid alterations in transient forebrain ischemia: possible new mechanisms of CDP-choline neuroprotection. J Neurochem 75:2528–2535.

Richter C (1993) Pro-oxidants and mitochondrial Ca2+: their relationship to apoptosis and oncogenesis. FEBS Lett 325:104–107.

Roda JM, Carceller F, Díez-Tejedor E, Avendaño C (1995) Reduction of infarct size by intra-arterial nimodipine administered at reperfusion in a rat model of partially reversible brain focal ischemia. Stroke 26:1888–1892.

Roda JM, Carceller F, Pajares R, Díez-Tejedor E (1991) Prevention of cerebral ischaemic reperfusion injury by intra-arterial administration of superoxide dismutase in the rat. Neurol Res 13:160–163.

Schabitz WR, Weber J, Takano K, Sandage BW, Locke KW, Fisher M (1996) The effects of prolonged treatment with citicoline in temporary experimental focal ischemia. J Neurol Sci 138:21–25.

Snape MF, Baldwin HA, Cross AJ, Green AR (1993) The effects of chlormethiazole and nimodipine on cortical infarct area after focal cerebral ischaemia in the rat. Neuroscience 53:837–844.

Tazaki Y, Sakai F, Otomo E, Kutsuzawa T, Kameyama M, Omae T, Fujishima M, Sakuma A (1988) Treatment of acute cerebral infarction with a choline precursor in a multicenter double-blind placebo-controlled study. Stroke 19:211–216.

Urabe T, Hattori N, Yoshikawa M, Yoshino H, Uchida K, Mizuno Y (1998) Colocalization of Bcl-2 and 4-hydroxynonenal modified proteins in microglial cells and neurons of rat brain following transient focal ischemia. Neurosci Lett 247:159–162.

Wahlgren NG, MacMahon DG, De Keyser J, Indredavik B, Ryman T (1994) Intravenous Nimodipine West European Stroke Trial (INWEST) of nimodipine in the treatment of acute ischaemic stroke. 4:204–210.

White MJ, Chen J, Zhu L, Irvin S, Sinor A, DiCaprio MJ, Jin K, Greenberg DA (1997) A Bcl-2 antisense oligonucleotide increases alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) toxicity in cortical cultures. Ann Neurol 42:580–587.

Yen CL, Mar MH, Zeisel SH (1999) Choline deficiency-induced apoptosis in PC12 cells is associated with diminished membrane phosphatidylcholine and sphingomyelin, accumulation of ceramide and diacylglycerol, and activation of a caspase. FASEB J 13:135–142.

Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, Kroemer G (1996) Mitochondrial control of nuclear apoptosis. J Exp Med 183:1533–1544.

(Accepted 1 November 2002)