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N^G-Nitro-L-arginine methyl ester reduces necrotic but not apoptotic cell death induced by reversible focal ischemia in rat

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Abstract

During cerebral ischemia, nitric oxide (NO) production via stimulation of NO synthase, is likely one of the major events leading to neuronal death. Recently, we have demonstrated that after reversible focal ischemia, apoptosis was implicated in the penumbra whereas necrosis was prominent in the ischemic core. We have now examined the effect of a non-specific inhibitor of NO synthase, N^G-nitro-L-arginine methyl ester (L-NAME, 3 mg kg^{-1} i.p., 5 min and 3 h after the onset of ischemia), on the progress of apoptotic and necrotic nuclei following transient focal cerebral ischemia, using DNA electrophoresis and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL assay). Our results indicated that L-NAME prevented the loss of necrotic, but not apoptotic cells.

Keywords: Cerebral ischemia; Nitric oxide (NO); Reperfusion; DNA fragmentation; Chromatin condensation; Apoptotic body; TUNEL assay

1. Introduction

The neuroprotective effect of N^G-nitro-L-arginine-methyl ester (L-NAME), inhibitor of nitric oxide synthase (NOS), following focal cerebral ischemia (Nowicki et al., 1991; Buisson et al., 1992) indicates that NO contributes to neuronal death. Earlier studies have suggested that apoptosis participate in cell death following global and focal ischemia (for review see Charriaut-Marlangue et al., 1996a). Recent observations show that following focal ischemia, in the surrounds of the necrotic focus, degenerating neurons express apoptotic features. Presence of chromatin condensation and apoptotic bodies was demonstrated by hematoxylin, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL assay) of DNA fragments and Hoechst staining (Li et al., 1995a; Charriaut-Marlangue et al., 1996b) or by ultrastructure (Li et al., 1995b). Nitric oxide (NO) has been demonstrated to cause either apoptosis (Albina et al., 1993; Sarih et al., 1993) or necrosis (Mitrovic et al., 1995) in cell culture. In order to study, whether NO triggers neuronal apoptosis or necrosis following ischemia, we have investigated the effect of L-NAME, on DNA fragmentation damage produced by a transient middle cerebral artery (MCA) occlusion.

2. Materials and methods

Experiments were performed in strict accordance with the NIH guidelines and the French Department of Agriculture (Licence No. 01352).

2.1. Ischemia

Ischemia was performed on male Sprague-Dawley rats by occluding the left MCA and both common carotid arteries (CCAs) for 1 h as previously reported (Margaill et al., 1996). Rats were killed at 6 ($n = 7$, for TUNEL assay), 18 ($n = 8$, for DNA electrophoresis) and 24 ($n = 27$) h after the onset of ischemia. Three of the 7 rats killed at 6 h, 4 of the 8 rats killed at 18 h and 12 of the 27 rats killed at 24 h were treated with L-NAME (3 mg kg^{-1} i.p.) 5 min and 3 h after the onset of ischemia, whereas the others were treated with its vehicle (distilled water).

2.2. Histology

Whole brains of rats killed 24 h after the onset of ischemia were rapidly frozen in isopentane (−40°C), and

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subsequently stored at −40°C. Coronal cryostat sections (50 μm thick), at different levels, bregma 4.7 to −6.3 mm (Paxinos and Watson 1982), were prepared. Striatal and cortical areas of infarction were measured using an image analyzer (IMSTAR, Paris, France), after cresyl violet staining. Volumes of infarction, corrected for edema according to (Golanov and Reis, 1995) were calculated by integrating the necrotic areas.

2.3. DNA electrophoresis

Ipsilateral striatum and cortex were dissected out, then homogenized in 10 volumes of TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. The homogenates were incubated in 3 volumes of extraction buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 0.5% SDS, 100 μg/ml proteinase K) and genomic DNA was extracted as previously described (Charriaut-Marlangue et al., 1995). Naive rats were used as control.

2.4. TUNEL assay

The in situ labeling of fragmented DNA was performed on tissue sections (bregma −0.3 mm) from rats killed 6 h after the onset of ischemia, as previously reported (Charriaut-Marlangue et al., 1996b). Briefly, sections were incubated with terminal deoxynucleotidyl transferase (0.1 U/μl; Gibco) and biotin-16-dUTP (20 μM; Boehringer Mannhein), then visualized with streptavidin-biotin-peroxidase complex and diaminobenzidine. Apoptotic and necrotic nuclei were counted by the use of a 63× oil immersion objective as previously described (Charriaut-Marlangue et al., 1996b).

3. Results

1 h MCA and CCAs occlusion followed by 24 h reperfusion resulted in a delineated necrotic volume both in the cortex and striatum (respectively, 207 ± 46 and 45 ± 8 mm^3, $n = 15$). L-NAME (3 mg/kg), administered i.p. 5 min and 3 h after the onset of ischemia, significantly reduced the cortical infarcted volume by 24% (157 ± 38 mm^3, $n = 12$, $P < 0.01$) in rats killed 24 h after ischemia onset. In contrast, L-NAME did not alter the necrotic volume in the striatum (43 ± 7 mm^3).

The bulk of the genomic DNA extracted from naive rat brain demonstrated that its integrity was largely preserved (Fig. 1, lane C). 18 h following ischemia onset, DNA analysis demonstrated oligonucleosomal-sized DNA fragments of 180–200 bp and multiples thereof (ladder) associated with random DNA fragmentation (smear) in the striatum and cortex (Fig. 1). In contrast, in animals given L-NAME, DNA laddering was more conspicuous in the two regions. Furthermore, the quantity of uncleaved DNA present in the wells was more important compared to that extracted from untreated animals, indicating less DNA cleavage into low molecular weight fragments.

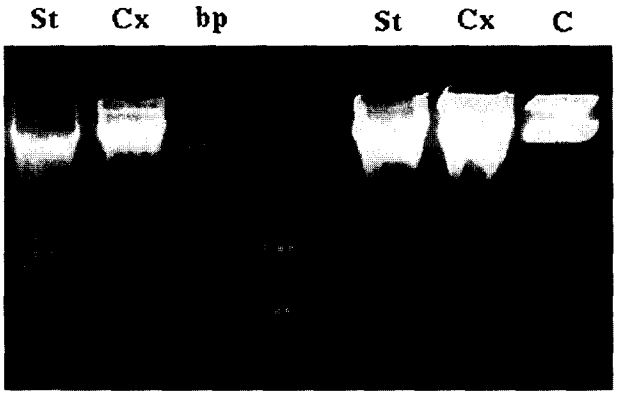

Fig. 1. Treatment with L-NAME did not reduce DNA fragmentation at 18 h after ischemic onset. Typical agarose gel electrophoresis of the genomic DNA (10 μg) from the striatum (St) and cortex (Cx) in untreated and L-NAME treated ischemic rats. Note that DNA laddering is more conspicuous in rats treated with L-NAME as compared to untreated ischemic rats. C represents DNA extracted from the brain of a control rat. bp, one-kilobase DNA ladder.

Analysis of chromatin condensation and fragmentation in cell nuclei was demonstrated with the TUNEL assay in rats killed 6 h following the onset of ischemia. As shown in Fig. 2, numerous TUNEL-positive nuclei were observed in the head of caudate putamen (CPu) showing chromatin condensation (arrowheads in A_1). In the striatal core as in the rostral cortex (not shown), several cells containing detectable concentrations of DNA ends, showed a more diffuse labeling not only in cell body but also in dendritic processes (open arrow in A_2), and were described as necrotic nuclei (Charriaut-Marlangue and Ben-Ari, 1995). In contrast, in the ischemic core of rats treated with L-NAME (Fig. 2B, B_1 and B_2), the number of necrotic nuclei was markedly reduced by 87% (see Fig. $2B_2$ and Table 1). Chromatin condensation around the margin of the nucleus and numerous apoptotic bodies were conspicuous (little arrows in Fig. 2 B_2). Apoptotic nuclei detected in the CPu were reduced (Table 1) whereas these found in horizontal diagonal band and islands of Calleja following

Table 1
Quantification of ipsilateral apoptotic (AC) and necrotic (NC) nuclei in coronal sections from rats ($n = 3$) subjected to 1 h MCA and CCAs occlusion, treated with L-NAME (3 mg kg^{-1}, i.p.) 5 min and 3 h after the onset of ischemia and followed by reperfusion, compared to untreated ischemic rats

	L-NAME	AC	NC
Head CPu	−	423 ± 92 [a]	63 ± 39 [a]
	+	136 ± 95	N.D. [b]
Striatal core	−	196 ± 53 [a]	192 ± 64 [a]
	+	75 ± 24	25 ± 4

[a] Reported in Charriaut-Marlangue et al. (1996b). [b] Not detectable.

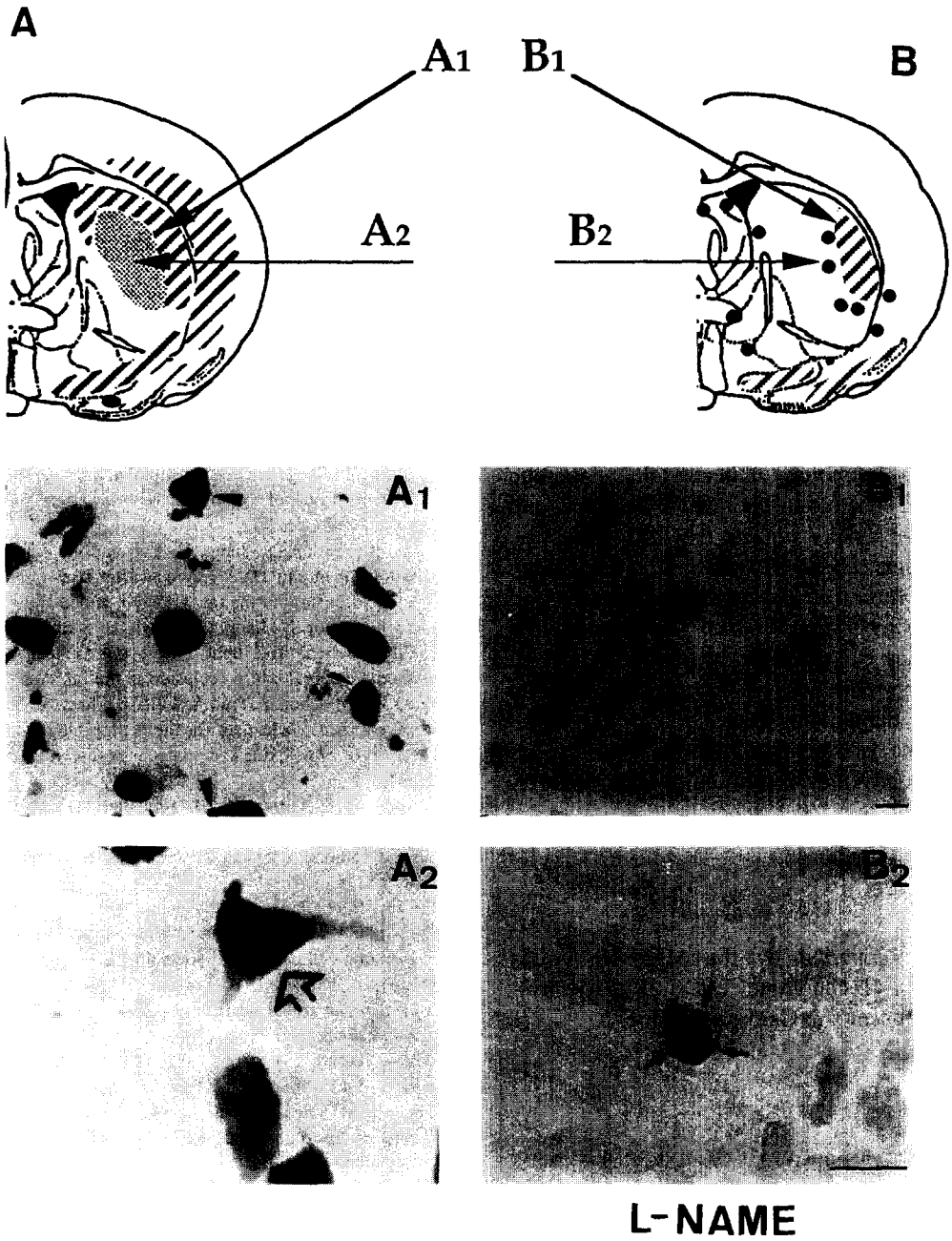

Fig. 2. Schematic diagrams and DNA nick-end labeling of apoptotic and necrotic neurons from ischemic (A, A_1, A_2) and L-NAME given (B, B_1, B_2) rats, 6 h after ischemic onset. (A) Distribution of high (grey area) and low (hatched area) ratio of apoptotic nuclei (described in detail in Charriaut-Marlangue et al., 1996b). (A_1) Presence of numerous apoptotic neurons (with chromatin condensation) in the head of the CPu. (A_2) Presence of numerous necrotic neurons (with a TUNEL labeling of nucleus and dendritic processes, open arrow) in the striatal ischemic core. (B) Distribution of apoptotic nuclei in L-NAME given rats. (B_1) Note that only a few apoptotic nuclei are present in the CPu. (B_2) Note the absence of necrotic nuclei and the presence of preapoptotic nuclei exhibiting chromatin buttoning.

ischemia (Charriaut-Marlangue et al., 1996b), were always detected in L-NAME given rats (Fig. 2A compared to Fig. 2B).

4. Discussion

This report demonstrates that a low dose of L-NAME (3 mg kg^{-1}), reduces the infarct volume measured at 24 h after transient focal ischemia, and decreases the necrotic but not the apoptotic component of cell death. In this model, chromatin condensation, nucleus segmentation and apoptotic bodies have been identified in penumbra whereas necrotic nuclei were mainly located in the ischemic core (Charriaut-Marlangue et al., 1996b). This assumption was strengthened by a high magnification in light microscopy analysis on TUNEL-positive nuclei showing chromatin

condensation in apoptotic nuclei and diffuse staining in necrotic nuclei (Charriaut-Marlangue and Ben-Ari, 1995).

Inhibition of NOS reduced DNA smears, most likely the result of single-stranded breaks from random DNA degradation, and necrotic nuclei in the striatum and cortex of the ipsilateral hemisphere. Our results may suggest that part of DNA fragmentation following ischemia can be induced by NO cytotoxicity, probably by its reaction with superoxide radicals ($O_2^{\cdot -}$) to form peroxynitrite ($OONO^-$), which decomposes to yield hydroxyl or hydroxyl-like free radicals ($^{\cdot}OH$) (Lipton et al., 1993). Oxygen radicals have toxic effects not only on proteins and lipid membranes but also on DNA, and may account for rapid or cumulative neuronal damage (Coyle and Puttfarcken, 1993; Slater et al., 1995). Furthermore, mapping of TUNEL-positive nuclei following NOS inhibition, indicates that apoptotic nuclei were mainly located in the MCA territory and adjacent areas. These results suggest that NO is not the sole inducer triggering cell death following reversible ischemia and its inhibition may develop damage to a lesser extent and with a slower time course. Inhibition of the synthesis of NO may be desirable in stroke models in which there is overproduction of NO, since apoptotic process leads to less damage for tissues.

In conclusion, the present study is the first one to indicate that NO toxicity may contribute to necrotic DNA damage following transient focal ischemia.

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