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SPECIAL REPORT

Loss of NADPH-diaphorase containing neurones after reversible focal ischaemia in rats delayed by L-NAME

[1]Isabelle Margaill, Monique Allix, *Christiane Charriaut-Marlangue, Roger G. Boulu & Michel Plotkine

Laboratoire de Pharmacologie, Université René Descartes, 4 avenue de l'observatoire, 75006 Paris, France and *INSERM U29, 123 Bld de Port-Royal, 75014 Paris, France

In the present study, NADPH-diaphorase histochemistry was used to assess the temporal evolution of the number of nitric oxide (NO)-synthase containing neurones after reversible focal cerebral ischaemia in rats. The number of NADPH-diaphorase containing neurones was reduced by 50% and 90% respectively 6 and 24 h after ischaemia. L-NAME, a NO-synthase inhibitor, prevented the loss of NADPH-diaphorase containing neurones observed 6 h after ischaemia but not 24 h after ischaemia, suggesting that in the early phase, nitric oxide is involved in this phenomenon.

Keywords: Nitric oxide; NADPH-diaphorase; focal ischaemia; reperfusion; L-NAME

Introduction Increasing evidence demonstrates a deleterious role for neuronal nitric oxide synthase (NOS) in focal cerebral ischaemia (Yoshida *et al.*, 1994; Huang *et al.*, 1994). This raises the question of NOS containing neurone vulnerability to NO. In the present study, the temporal evolution of the number of NOS containing neurones after transient cerebral focal ischaemia in rats was assessed by use of NADPH-diaphorase histochemistry (Bredt *et al.*, 1991; Hope *et al.*, 1991). The effect of N^G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, on the vulnerability of NOS-containing neurones to ischaemia was studied.

Methods The following experiments were performed in strict accordance with NIH guidelines and French Department of Agriculture (Licence No. 01352).

Male Sprague-Dawley rats (300-350 g) were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and allowed to breathe spontaneously. The left middle cerebral artery (MCA) was exposed *via* a temporal craniotomy and occluded in a proximal site with a Zen type clip (13 mm × 0.4 mm, Ohwa Tsusho Co., Ltd Tokyo, Japan). Simultaneously both CCAs were occluded. After 1 h of ischaemia, the microclip occluding the MCA was removed and recirculation within the CCAs was allowed. Normothermia (37-38°C) was maintained until recovery from anaesthesia by means of a heating blanket.

Rats were reanaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.) at various times after the onset of ischaemia: 3 h ($n=6$), 6 h ($n=13$) and 24 h ($n=12$). Six of the 13 rats killed 6 h after ischaemia and 6 of those killed 24 h after ischaemia were treated with L-NAME (3 mg kg^{-1}, i.p., 5 min and 3 h after the onset of ischaemia). Nine unoperated rats were used as controls. Anaesthetized rats were then perfused transcardially with saline containing heparin 500000 iu l^{-1} followed by 4% paraformaldehyde. Brains were removed, and after postfixation and cryoprotection, coronal sections (30 μm thick) were cut in a cryostat at −20°C. Sections were treated for NADPH-diaphorase staining according to the technique described by Hope *et al.* (1991). Cortical and striatal neurones of the ischaemic hemisphere showing NADPH staining were counted under the microscope on coronal sections located at interaural 8.7 mm. Indeed, preliminary histological studies showed that this level exhibited the largest infarcted area 24 h after ischaemia (70% of the total area section).

Results are expressed as mean ± s.e.mean. Statistical analysis was performed by ANOVA followed by PLSD Fisher test.

Results The number of NADPH-diaphorase containing (NADPH-d(+)) neurones progressively decreased after reversible focal ischaemia in both the infarcted cortex and striatum (Table 1). At the cortical level, this decrease was significant in rats killed 6 h (44%, $P<0.01$) and 24 h (88%, $P<0.001$) after ischaemia. At the striatal level, the number of NADPH-d(+) neurones of the infarcted hemisphere was significantly reduced by 35% ($P<0.01$), 52% ($P<0.001$) and 97% ($P<0.001$) respectively in animals killed 3, 6 and 24 h after MCA occlusion.

In animals treated with L-NAME and killed 6 h after ischaemia, the number of NADPH-d(+) cortical and striatal neurones (83 ± 5 and 149 ± 14 respectively) were significantly higher than in the untreated ischaemic rats (49 ± 10, $P<0.05$ and 92 ± 17, $P<0.05$) and was not significantly different from the control group (88 ± 8 and 190 ± 13) (Figure 1). On the contrary, in rats killed 24 h after ischaemia, the number of cortical and striatal NADPH-d(+) neurones of L-NAME-treated (20 ± 2 and 27 ± 10) and untreated rats (11 ± 2 and 6 ± 2) did not differ significantly.

Table 1 Temporal evolution of NADPH-diaphorase containing neurones in rats submitted to reversible focal ischaemia

Time after ischaemia onset	*Number of NADPH-d(+) neurones* (coronal section: 8.7 mm anterior to interaural line)	
	Cortex	*Striatum*
Control ($n=9$)	88 ± 8	190 ± 13
3 h ($n=6$)	72 ± 10 (NS)	124 ± 19 (**)
6 h ($n=7$)	49 ± 10 (**)	92 ± 17 (***)
24 h ($n=6$)	11 ± 2 (***)	6 ± 2 (***)

Comparison versus control: NS, non significant; **$P<0.01$; ***$P<0.001$.

[1] Author for correspondence.

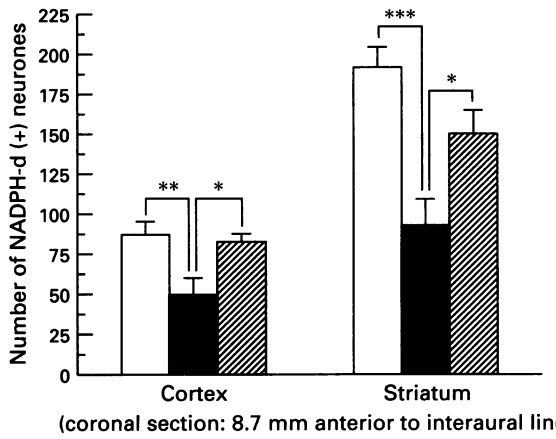

Figure 1 Effect of L-NAME ($3\,mg\,kg^{-1}$, i.p.) on the number of NADPH-diaphorase containing neurones in cortex and striatum of rats killed 6 h after the onset of ischemia. Open column: control non-ischaemic group ($n=9$), solid column: control ischaemic group ($n=7$), hatched column: L-NAME-treated ischaemic group ($n=6$). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Discussion NADPH-d(+) neurones were shown to be selectively spared in human neurodegenerative diseases such as Alzheimer's and Huntington's diseases (Ferrante *et al.*, 1985; Hyman *et al.*, 1992) and in an experimental model of ischaemia (Uemura *et al.*, 1990). The situation appears different in reversible focal ischaemia in rats. Indeed, the present study clearly demonstrates that 24 h after transient MCA occlusion, neuronal NADPH-d staining disappeared in the cortex as well as in the striatum. This result is in accordance with the data of Iadecola *et al.* (1995) showing the loss of NADPH-d(+) neurones 24 h after permanent focal ischaemia. This loss is very rapid since as by little as 6 h after ischaemia, about 50% of NADPH-d(+) neurones had already disappeared in both structures studied. NO appears to be implicated in this phenomenon since L-NAME, a NO-synthase inhibitor, reduced the loss of NADPH-d(+) neurones observed 6 h after ischaemia. However, L-NAME was ineffective in the loss of NADPH-d(+) neurones observed 24 h after ischaemia. It is unlikely that this lack of effect is due to a decline in L-NAME activity since this inhibitor induces prolonged NOS inhibition (Iadecola *et al.*, 1994). Thus, it appears that NO exerts its deleterious effect only in the early phase after ischaemia and that other mechanisms occurring in a later phase induce neuronal loss.

In conclusion, this study, the first one to examine the temporal evolution of NADPH-d(+) neurones in a model of reversible focal ischaemia, indicates that these neurones are highly sensitive to ischaemia. Moreover, our results suggest that during the early phase of reperfusion, NOS containing neurones are submitted to a self-destruction phenomenon.

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