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Lack of iNOS induction in a severe model of transient focal cerebral ischemia in rats

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Abstract

Calcium-independent nitric oxide synthase (NOS) activity has been reported in ischemic brains and usually attributed to the inducible isoform, iNOS. Because calcium-independent mechanisms have recently been shown to regulate the constitutive calcium-dependent NOS, we proposed to confirm the presence of iNOS activity in our model of transient focal cerebral ischemia in rats. Our initial results showed that, in our model, ischemia induced an important increase in brain calcium concentration. Consequently, the determination of calcium-independent NOS activity required a higher concentration of calcium chelator than classically used in the NOS assay. In these conditions, calcium-independent NOS activity was not observed after ischemia. Moreover, our ischemia was associated with neither iNOS protein expression, measured by Western blotting, nor increased NO production, evaluated by its metabolites (nitrate/nitrite). Our results demonstrate that iNOS activity may be overestimated due to increased brain calcium concentration in ischemic conditions and also that iNOS is not systematically induced after cerebral ischemia.
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Keywords: Calcium-independent nitric oxide synthase; Inducible nitric oxide synthase; Transient focal cerebral ischemia; 1400W; ARL 17 477

Introduction

Nitric oxide (NO) is a physiological messenger synthesized by a family of enzymes, the NO-synthases (NOS, EC 1.14.13.39), including neuronal nNOS, endothelial eNOS and an inducible isoform, iNOS, originally isolated from macrophages (Alderton et al., 2001; Förstermann et al., 1991). Both nNOS and eNOS are constitutively expressed and calcium-dependent. By contrast, iNOS is expressed in response to various inflammatory stimuli, and its activity is independent of intracellular calcium concentrations. Excess production of NO has been implicated in a variety of diseases, including cerebral ischemia (see for review Bolaños and Almeida, 1999; Iadecola, 1997; Moro et al., 2004; Samdani et al., 1997; Verrecchia et al., 1995). NO can be neuroprotective or neurotoxic during cerebral ischemia, depending on the NOS isoform involved. eNOS produces NO with beneficial effects (vasodilation, inhibition of platelet aggregation and polymorphonuclear neutrophil adhesion; Huang et al., 1996), whereas nNOS is deleterious (Escott et al., 1998; Hara et al., 1996; Huang et al., 1994; Margaill et al., 1997; Nagafuji et al., 1995; Yoshida et al., 1994; Zhang et al., 1996b). These effects occur in the early phase of ischemia, while iNOS activity increases later following cerebral ischemia. The deleterious effects of the NO produced by iNOS were demonstrated using selective iNOS inhibitors. Aminoguanidine, a partially selective iNOS inhibitor, reduced infarct volume in models of transient (Iadecola et al., 1996; Park et al., 2004; Spinnewyn et al., 1997; Zhang et al., 1996a; Zhu et al., 2002) and permanent

Abbreviations: CCA, common carotid artery; DMSO, dimethyl sulfoxyde; eNOS, endothelial NO-synthase; iNOS, inducible NO-synthase; LPS, lipopolysaccharide; MCA, middle cerebral artery; nNOS, neuronal NO-synthase; NO, nitric oxide; NOS, nitric oxide synthase; NOx, nitrites/nitrates; SEM, standard error of mean; TBS, Tris-buffered saline.

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ischemia (Cockroft et al., 1996; Iadecola et al., 1995b; Sugimoto and Iadecola, 2002). In our laboratory, we demonstrated that the highly selective iNOS inhibitor 1400W and an antisense directed against iNOS reduce post-ischemic damage induced by the occlusion of the left middle cerebral artery (MCA) and the ipsilateral common carotid artery (CCA) for 2 h (Parmentier et al., 1999; Parmentier-Batteur et al., 2001). The neuroprotective effect of 1400W was confirmed recently in transient and permanent models of cerebral ischemia (Armengou et al., 2003; Pérez-Arensio et al., 2005). In addition, ischemia produced a smaller infarct in iNOS knockout mice than it did in wild-type mice (Iadecola et al., 1997; Park et al., 2004). Thus, iNOS may be an attractive target with a wide therapeutic window for treating stroke.

The induction of iNOS in ischemic brain tissue has been demonstrated using several techniques. iNOS mRNA and protein have been detected either by RT-PCR (Galea et al., 1998; Iadecola et al., 1996; Park et al., 2004), Western blotting (Parmentier-Batteur et al., 2001; Pérez-Arensio et al., 2005) or immunohistochemistry (Galea et al., 1998; Iadecola et al., 1996). The activity of this ischemia-induced iNOS has been measured by omitting calcium from the medium in the radioenzymatic method of Bredt and Snyder (1989), modified by Iadecola et al. (1995a).

However, recent experimental results have shown that calcium-independent mechanisms also regulate the activity of nNOS (Okada, 1995; Wang et al., 1997) and eNOS (Butt et al., 2000; Caulin-Glaser et al., 1997; Corson et al., 1996; Dimmeler et al., 1999; Fleming et al., 1997, 1999; Igarashi et al., 1999; MacCabe et al., 2000; Montagnani et al., 2001; Tsukahara et al., 1994).

The present study was therefore carried out to confirm the presence of calcium-independent NOS activity due to iNOS after ischemia.

Materials and methods

All experiments were performed on male Sprague–Dawley rats (weighing 300 to 330 g, Iffa Credo, L'Arbresle, France), in compliance with French regulations on the protection of animals used for experimental and other scientific purposes (D2001-486), as well as with the EC regulations (OJ of EC L358 12/18/1986). The animals were housed at a controlled temperature, maintained on a 12 h light/12 h dark cycle throughout the experiments and allowed access to food and water ad libitum.

Chemicals were purchased from Sigma Chemical Corporation (Saint Quentin Fallavier, France) except those stated specifically.

Induction of transient focal cerebral ischemia

Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.; Prolabo, Fontenay s/Bois, France) and allowed to breathe spontaneously. The left MCA was exposed via a temporal craniotomy and occluded with a microclip (zen type temporary clip, 13 × 0.4 mm, Ohwa Tsusho, Tokyo, Japan). Both CCAs were concomitantly clamped. The microclip was placed on the MCA at a site proximal to the lenticulostriate arteries to produce cortical and striatal infarctions and kept in place for 20 min. The clip was then removed, and the CCA circulation restored. Reperfusion in the three arteries was checked under a microscope. The same surgery was performed in sham-operated rats, but the MCA and CCAs were not occluded. The body temperature was monitored throughout surgery by a rectal probe and maintained at 37 ± 0.5°C with a normothermic blanket control unit (Harvard apparatus, Edenbridge, Kent., UK). After surgery, rats were returned to their home cage and fed with mashed laboratory chow.

Experiment 1: Time course of calcium-independent NOS activity after ischemia

NOS activities were measured in the brains of non-operated rats (n = 8) and after 15, 24, 48 and 72 h of reperfusion in ischemic rats (n = 5–6 per time) and the corresponding sham-operated rats (n = 5 per time). Rats were killed with an overdose of sodium pentobarbitone (200 mg/kg; Sanofi, Libourne, France), and their brains were removed and sliced into six 2 mm thick coronal sections using a matrix. The cortex and striatum of the third section (9.7 mm to 7.7 mm anterior to the interaural line) were dissected out, immediately frozen and stored at −40°C for later determination of NOS activity.

Calcium-independent NOS activity was measured by the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline as described previously (Bredt and Snyder, 1989; Grandati et al., 1997; Iadecola et al., 1995a). Briefly, brain samples were homogenized with 5 volumes of ice-cold buffer (A) (20 mM HEPES, 0.32 M sucrose, 1 mM dithiothreitol, 10 mg/l leupeptin, 10 mg/l pepstatin A and 1 mM EGTA to chelate endogenous calcium; pH 7.4) and centrifuged for 15 min at 20,000 × g at 4°C. Supernatants (25 μl) were then incubated for 30 min at 37°C with 100 μl of reaction mixture (B) containing 200 μM NADPH, 50 μM tetrahydro-L-biopterin and 1 μCi/ml L-[^{14}C]arginine monohydrochloride (278 mCi/mmol; Amersham, Saclay, France) in buffer (A) and 75 μl of distilled water. The reaction was stopped by adding 1 ml of ice-cold buffer containing 30 mM HEPES and 3 mM EDTA (pH 5.5) and applied to Dowex AG50WX8-400 (Na^+ form) columns to remove L-[^{14}C]arginine. Columns were eluted, and L-[^{14}C]citrulline was measured by scintillation counting. Protein concentration was quantified by the Bradford (1976) method, using bovine serum albumin as a standard. Data are expressed as picomoles L-[^{14}C]citrulline formed per milligram protein per minute (pmol/mg protein/min).

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