

Region-specific sensitivity of the spinal cord to ischemia/reperfusion: the dynamic of changes in catalytic NOS activity

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Abstract This study was designed in order to consider whether the release of neuronally derived nitric oxide (NO) in the lumbosacral spinal cord during ischemia/reperfusion is region-specific and whether changes in Ca^{2+} -dependent NO synthase (cNOS) activity parallel with functional outcome. The cNOS activity was measured in the spinal cord regions after 13-, 15- and 17-min ischemia alone and that followed by 24 h of reperfusion. In addition, the Tarlov's criteria were applied to define the neurological consequences of ischemia/reperfusion in experimental animals. Based on the results, it is evident that only the 17-min ischemia alone was quite sufficient to cause changes in cNOS activity, however, without alterations in functional outcomes. On the other hand, the ischemic episodes followed by reperfusion caused dynamic, region-specific alterations in cNOS activity and consequently led to deterioration of motor function of hindlimbs in affected animals. Our results indicate that the motoneurons in the ventral horns respond more sensitively to ischemia/reperfusion than do neurons localized in the other spinal cord regions and that changes in cNOS activity may also influence the axonal conductance in the white matter and account for the impairment of motoneuronal activity in affected animals.

Keywords Calcium-dependent nitric oxide synthase activity · Transient ischemia · Spinal cord · Neurological outcome

Introduction

Paraplegia/paraesthesia represents a disastrous and unpredictable complication after successful surgery on the thoracoabdominal aorta. It is generally accepted that the most common mechanism participating in spinal cord injury during these surgical interventions is direct tissue ischemia. Ischemia is frequently caused by permanent exclusion of the essential intercostal arterial blood supply to the spinal cord or temporary interruption of blood flow in the spinal cord [1]. In the central nervous system (CNS), both neuronal cell bodies/dendrites located in the gray matter and myelinated axons in the white matter are reversibly and/or irreversibly damaged. Several laboratories have provided evidence that white matter is comparatively resilient to ischemia [2] because axons exhibit a relatively low metabolic rate and do not express glutamate receptors capable of mediating excitotoxic damage [3]. Other data have revealed that spinal cord ischemia caused by trauma or complications associated with aortic surgery affects the white matter in particular [4–6]. Several factors could account for ischemia-induced damage of gray and/or white matter spinal cord structures; however, the exact mechanism underlying delayed/secondary damage of the spinal cord is still unclarified [7, 8].

Nitric oxide (NO) is a pluripotent signaling molecule to which diverse physiological and pathophysiological neuronal functions have been attributed. Its production is catalyzed by nitric oxide synthase (NOS) during the conversion of L-arginine to L-citrulline. At least three different

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isoforms of this enzyme have been identified and characterized. Two of them, neuronal NOS (nNOS) and endothelial NOS (eNOS), are calcium dependent. They are constitutively expressed in neurons and endothelial cells, in which they produce nontoxic amounts of NO, which subsequently exerts its function in inter- and intra-cellular communication and in the regulation of cerebral blood flow [9, 10]. Under various ischemia/reperfusion conditions, NO may have either neuroprotective *or neurotoxic* effects [11–14]. It depends on the amount of NO released into the microenvironment, the cell type affected by ischemia, and the length and severity of the ischemic insult. Nelson et al. [15] have demonstrated that neuronal NOS knockout mice are resistant to neuronal damage following stroke resulting from middle cerebral artery (MCA) occlusion. There is also evidence that the pharmacologic inhibition of nNOS [16] or its gene deletion confers neuroprotection in animal models of transient focal cerebral ischemia [17]. In a model of transient spinal cord occlusion, preischemic administration of NG-nitro-L-arginine-methyl ester (L-NAME), a nonselective-inhibitor of NOS, significantly improved neurologic and histopathologic outcome [12]. Furthermore, the region-specific response of the spinal cord to ischemia has been previously reported in a model of transient ischemia followed by short periods of reperfusion (1 and 3 h) [18] and in a model of repeated short-lasting spinal cord ischemia [19]. The results provide evidence that regions showing no cNOS activity under physiological conditions are extremely susceptible to a reduction of oxygen supply and glucose and inevitably become damaged. The progression into irreversible damage of the spinal cord can nevertheless take several days and may include inducible NOS synthase (iNOS), which is expressed in glial cells and macrophages after exposure to cytokines and/or lipopolysaccharides [20].

Characterization of region-specific differences in NO production in the spinal cord after ischemia is of major clinical importance, since ischemic injury does not develop uniformly. It seems that specific spinal cord areas may be viable for longer times and could therefore be responsive to delayed therapeutic interventions. The main aim of the present study was to ascertain the dynamic of changes in cNOS activity in the gray and white matter spinal cord regions at the level of lumbosacral (L4–S4) segments after 13-, 15- and 17-min ischemia alone. Furthermore, we were also interested in the region-specific response of cNOS activity 1 day after postischemic injury, since this could provide information on the development of neurological deficit.

Materials and methods

We used 49 adult male Chinchilla rabbits weighing 2.5–3.5 kg in the experiment. Experimental protocols were

approved by the Institute of Neurobiology Animal Care Committee, with the aim of minimizing both the suffering and the number of animals used. All experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animals were divided into seven experimental groups: control animals ($n = 7$), animals subjected to 13-, 15- and 17-min ischemia alone ($n = 21$), and animals subjected to 13-, 15- and 17-min ischemia followed by 24 h of reperfusion ($n = 21$).

Surgical procedure

Before operation, the animals were premedicated with an intraperitoneal injection of ketamine (40 mg/kg) and xylazine (8 mg/kg) in the ratio 1:3, thus preventing them from being stressed. Afterwards, deep anesthesia was induced by continuous inhalation of 1.0–2.0% halothane via a face mask. Under sterile conditions, the left femoral artery was clipped with optical scissors 3–4 cm distal to the inguinal ligament, and an 18-gauge polytetrafluoroethylene (PTFE) catheter was inserted inside for monitoring distal blood pressure. Before catheter insertion, heparine 500 IU was administered intravenously. Subsequently, a 5-Fogarty arterial embolectomy catheter (120805F Baxter Healthcare Corporation, Deerfield, IL) was embedded approximately 15 cm into the right femoral artery. This resulted in a balloon location 0.5–1.5 cm distal to the left renal artery. The balloon was inflated for 13-, 15- and 17-min, respectively, until the loss of pulsatile distal blood pressure was observed. During the whole experimental procedure, the body temperature of animals monitored with a rectal thermometer was maintained at 37°C with a heating pad. The vertebral canal was opened by the total transection of the backbone at two sides, limiting the extent of lumbosacral segments, and the spinal cord was extruded as soon as possible into an ice-cold isotonic solution. The lumbosacral spinal cord was carefully frozen and stored in liquid nitrogen. Serial 600- μ m-thick slices were cut on a cryostat (Leica) at -18°C . The tissue samples were pricked out by needles (id 0.6 or 0.8 mm) from the gray matter regions: dorsal horns (laminae I–IV), intermediate zone and pericentral region (laminae VII and X), ventral horns (laminae VIII–IX), and the white matter regions: dorsal, lateral and ventral columns on a plate cooled with liquid nitrogen (-15°C).

Nitric oxide synthase radioassay

Calcium-dependent NOS activity was determined by the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline according to the method of Bredt and Snyder [21]. Frozen spinal cord samples were homogenized in 250–300 μ l of ice-cold Tris–HCl buffer (10 mM, pH = 7.4), containing 1 mM EDTA

and protease inhibitors (pancreas-extract, pronase, termolysin, chymotrypsin, trypsin and pappain). Aliquots of homogenates (200 µg/ml) were incubated for 45 min at 37°C with solution A (100 µl) containing 100 µM arginine, 1 mM NADPH, 1 mM dithiothreitol (DTT), 1.5 mM FAD, 1 mM EDTA, 20 mM CaCl₂ · 6H₂O, 10 mM tetrahydrobiopterin (H₄B), 50 mM HEPES, 1 mM calmoduline, 50 mM Tris-HCl and solution B (100 µl) containing 2.5 µl L-[¹⁴C]arginine (321nCi). The reaction was stopped by the addition of 1 ml ice-cold 100 mM Hepes buffer and 10 mM EDTA, pH = 5.5. Samples were applied into a Dowex AG 50 W-X8 cationic-exchange column (Na⁺ form) to remove L-[¹⁴C]citrulline. The columns were washed with 2 ml of deionized water to elute L-[¹⁴C]citrulline. Then aliquots of samples (0.5 ml) were mixed with 5 ml of Bray's fluid in scintillation vials and counted in a Beckman LS-3801 Liquid Scintillation System. Cpms were converted to dpms using [¹⁴C] L-quenched standards. The levels of L-[¹⁴C]citrulline were computed after subtracting the blank that represented nonspecific radioactivity in the absence of the enzyme activity. Protein determination was done using the method described by Bradford [22]. The results from analyses were expressed as pmol/mg protein.

Neurological evaluation

The motor function of hindlimbs in animals subjected to ischemia/reperfusion was evaluated and graded according to Tarlov's criteria as follows: 0, paraplegic with no lower extremity function; 1, poor lower extremity function, weak antigravity movement only; 2, some lower extremity motor function with good antigravity strength, but an inability to draw hindlimbs under the body or hop; 3, ability to draw hindlimbs under the body and hop, but not normally; 4, normal motor function of hindlimbs.

Statistical analyses

The results were statistically evaluated by ANOVA and Tukey-Kramer test and are expressed as means ± SEM.

Results

Neurologic assesment

The motor function of hindlimbs was tested at the end of each ischemia/reperfusion period (Table 1). In the control group, none of the animals exhibited malfunction in the movement of the lower extremities. Approximately half of the animals subjected to 17-min ischemia followed by 24 h of reperfusion displayed some motor function of the hindlimbs with good antigravity strength, but the inability to draw their legs under the body or to hop. The remainder were able to draw their legs under the body and to hop, but not normally. Only one experimental animal was found to be partially paraplegic with poor lower extremity function and weak antigravity movement of the hindlimbs. Similarly, 15-min ischemia followed by 24 h of reperfusion caused partial paraplegia only in one experimental animal. Some motor function of the hindlimbs with good antigravity movement was noticed in two animals, while three animals were not able to draw their legs under the body, although they hopped, though not normally. One animal was not affected by 15-min ischemia followed by 24 h of reperfusion with respect to neurological impairment of the hindlimbs. The 13-min ischemia followed by 24 h of reperfusion had no impact on lower extremity motor function in three animals. Four animals exhibited almost normal motor function of the hindlimbs, though they were not able to hop normally.

cNOS activity

Under physiological conditions cNOS activity varied in both the gray matter and white matter spinal cord regions (Tables 2, 3). The highest enzyme activity was detected in the dorsal horns (50.42 pmol/mg of protein) and in the dorsal columns (15.526 pmol/mg of protein). In comparison to the dorsal columns, cNOS activity in the lateral and ventral columns (14.265 and 12.072 pmol/mg of protein) was only slightly lowered, while in the intermediate zone

Table 1 The neurological evaluation of the lower extremity motor function

Degree of neurological impairment (grade 0–4)	Control (n = 7)	13'IS/24 h R (n = 7)	15'IS/24 h R (n = 7)	17'IS/24 h R (n = 7)
0, Paraplegic with no lower extremity motor function	–	–	–	–
1, Poor lower extremity motor function	–	–	1	1
2, Some lower extremity motor function	–	–	2	3
3, Ability to draw legs under body	–	4	3	3
4, Normal motor function	7	3	1	–

The degree of neurological impairment was evaluated at the end of the reperfusion period and graded according to Tarlov's criteria (grades 0–4). Seven animals (n = 7) were evaluated in each experimental group

IS ischemia, R reperfusion

Table 2 Catalytic NOS activity in the gray matter regions (dorsal horn, intermediate zone, ventral horn) of the lumbosacral spinal cord segments (L4–S4) in control, after partial ischemia (13, 15 and 17 min) and 24 h after ischemic insult

Experimental groups	Gray matter regions		
	Dorsal horn	Intermediate zone	Ventral horn
Control	50.42 ± 1.76	27.47 ± 0.90	23.58 ± 0.66
13'IS	52.25 ± 2.76	29.26 ± 1.03	23.01 ± 1.16
13'IS/24 h R	52.94 ± 1.22	24.01 ± 0.69	21.60 ± 0.90
15'IS	52.97 ± 0.81	27.80 ± 2.29	24.84 ± 1.48
15'IS/24 h R	54.35 ± 2.11	26.62 ± 0.86	27.90 ± 0.57*
17'IS	59.74 ± 0.98*	28.71 ± 0.66	26.66 ± 1.45
17'IS/24 h R	68.15 ± 2.03*	31.19 ± 0.69	27.20 ± 0.97*

The results from analyses were expressed as pmol/mg protein. The values were statistically evaluated by ANOVA and Tukey-Kramer test and have been given as means ± SEM. Data are the means of seven experiments ($n = 7$)

* $P < 0.05$ compared with control

IS ischemia, R reperfusion

Table 3 Catalytic NOS activity in the white matter regions (dorsal columns, lateral columns and ventral columns) of the lumbosacral spinal cord segments (L4–S4) in control, after partial ischemia (13, 15 and 17 min) and 24 h after ischemic insult

Experimental groups	White matter regions		
	Dorsal columns	Lateral columns	Ventral columns
Control	15.53 ± 0.30	14.27 ± 0.57	12.07 ± 0.35
13'IS	16.23 ± 1.01	15.77 ± 0.47	12.35 ± 0.85
13'IS/24 h R	15.06 ± 0.32	15.16 ± 0.34	10.04 ± 0.28
15'IS	15.63 ± 1.23	16.10 ± 1.08	12.79 ± 0.50
15'IS/24 h R	17.01 ± 0.55	11.86 ± 0.46**	9.27 ± 0.29
17'IS	20.14 ± 0.83*	19.53 ± 0.50*	13.15 ± 0.73
17'IS/24 h R	13.48 ± 0.85*,**	14.53 ± 0.46**	9.73 ± 0.77*,**

The results from analyses were expressed as pmol/mg protein. The values were statistically evaluated by ANOVA and Tukey-Kramer test and have been given as means ± SEM. Data are the means of seven experiments ($n = 7$)

* $P < 0.05$ compared with control; ** $P < 0.05$ compared with ischemia alone

IS ischemia, R reperfusion

and ventral horns (27.472 and 23.576 pmol/mg of protein) it reached only about the half of the enzyme activity measured in the dorsal horns.

The different vulnerability of the gray and white matter spinal cord regions to ischemia/reperfusion is shown in Tables 2 and 3. While 13- and 15-min ischemia alone had no effect on cNOS activity in any of the gray and white matter spinal cord regions, reestablishment of the blood

flow after 15-min ischemia caused a significant increase in cNOS activity in the ventral horns. In comparison to ischemic values, the enzyme activity decreased in the lateral columns.

Most striking were the alterations in cNOS activity resulting from 17-min ischemia alone and that followed by 24 h of reperfusion. A 17-min ischemia alone significantly increased cNOS activity in the dorsal horns, whereas the intermediate zone and ventral horns remained unaffected. On the other hand, a 17-min ischemia followed by 24 h of reperfusion caused a significant increase in cNOS activity in the dorsal horns (by 35%) as well as in the ventral horns (by 15%). With regard to the white matter regions, the dorsal and lateral columns exhibited an increase in cNOS activity during the ischemic episode by 30% and 37% in comparison to control values. After 1 day of recirculation, a significant decrease in cNOS activity occurred in all three white matter columns, but only when the results were compared to the 17-min ischemia alone. Among the most pronounced changes were those found in the dorsal columns, where they reached 33%. Enzyme activity had returned to control values in the lateral columns, but remained significantly reduced in the dorsal and ventral columns.

Discussion

In this study we clearly demonstrate the region-specific differences in cNOS activity in the spinal cord gray and white matter resulting from transient abdominal aorta occlusion lasting 13, 15 and 17 min ischemia, respectively, and each time followed by 24 h of reperfusion. In addition, we provide outcomes of neurological assessment, which can partially demonstrate the impact of individual ischemia/reperfusion periods on lower extremity motor disturbances resulting probably from damage to ventral horn motoneurons.

It is evident that neither 13-min ischemia alone nor that followed by 24 h of reperfusion is sufficient to induce changes in cNOS activity in any of the gray and white matter spinal cord regions investigated. Nevertheless, three of seven experimental animals subjected to 13-min ischemia followed by 24 h of reperfusion exhibited futile motor dysfunction with the ability to draw legs under the body and to hop, but not normally. In this group of animals, some nNOS-immunoreactive motoneurons were noted in the region of ischemic penumbra [14]. In necrotic as well as nonnecrotic regions, an increase of NADPHd staining was detected in vessels. No signs of necrosis were observed in the NADPHd-rich areas of the superficial dorsal horn or lamina X. In the ventral horn, a decreased number of neutral-red-stained motoneurons was noted after 13-min

ischemia followed by 1 day of reperfusion [14]. While 15-min ischemia alone did not alter cNOS activity in any of the gray and white matter spinal cord regions, 17-min ischemia produced a prominent increase in enzyme activity in the dorsal horns. During the following 24 h of reperfusion, enzyme activity significantly increased in both the dorsal and ventral horns. The response of cNOS activity to ischemia/reperfusion in the ventral horns seems to be accompanied by a striking deterioration in the neurological status of the affected animals. Approximately half of the experimental animals subjected to 15-min or 17-min ischemia followed each time by 24 h of reperfusion exhibited partial paraplegia with poor lower extremity motor function and weak antigravity movement of the hindlimbs (grade 2) or some lower extremity motor function with good antigravity strength, but an inability to draw the legs under the body or to hop (grade 3). The results are in good agreement with the data reported by other authors showing the effect of ischemia/reperfusion on changes in immunohistochemical and histochemical detection of cNOS and a direct relation of these differences with functional deficit [14, 23, 24]. The existence of nNOS immunopositive alpha motoneurons, even if mild in appearance, was seen after 17-min abdominal aorta occlusion and 1 week of reperfusion in the group of paraparetic animals [24]. The results show that the synthesis of nNOS in this group of animals is marginal, but still possible. In animals with fully developed paraplegia, the necrotic matrix emerged in the ventral horn of lower lumbar and sacral segments, leading to a total loss of motoneurons. Results reported by Schreiberova et al. [24] and those by Ashwal et al. [25] have shown that nNOS is more active in the necrotic matrix than in the penumbra region. Perturbations in lower extremity motor function combined with increased cNOS activity in the ventral horns, where the medial and lateral sets of alpha-motoneurons are located, suggest that NO produced by increased cNOS activity may be involved in motor neuronal damage following spinal cord ischemia and subsequent reoxygenation. It is very well known that during ischemia/hypoxia the endogenous excitatory amino acids glutamate and aspartate are continuously synthesized in deleterious concentrations [26, 27]. This phenomenon probably plays a crucial role in the NO-mediated neurotoxicity because sustained activation of the *N*-methyl-D-aspartate (NMDA) receptor leads to increased intracellular levels of Ca^{2+} [28], which in turn activate different Ca^{2+} -dependent enzymatic cascades, including NOS [29, 30]. Thus, activated NOS generates toxic amounts of NO, which can freely diffuse to adjacent target neurons, where it promotes oxidative damage by reacting with superoxide anion to form peroxynitrite and by perturbing iron metabolism [31, 32]. In addition, it should be highlighted that under ischemic

conditions NOS might generate peroxynitrite directly by producing both NO and superoxide, since substrates and co-factors might be rate limiting. Furthermore, energy failure, DNA damage and inhibition of DNA synthesis [33–35] resulting in programmed cell death may be the direct consequence of enhanced production of NO or its derived species [36]. The pronounced elevation of cNOS activity in the dorsal horns after 17-min ischemia and its further progression during reperfusion implies that neurons, mainly those associated with dorsal root afferents, are also vulnerable to ischemic insult. In the dorsal horns, especially in its superficial layers (laminae I–III), there is an abundance of NADPH diaphorase-positive and/or nNOS immunoreactive neurons [37]. This fact is now generally attributed to NO-neuroprotective effects, most probably to vasodilatory action of NO on microcirculation, and is confirmed by the evident resistance of dorsal horn neurons and lack of evident necrotic foci in the corresponding areas under ischemic conditions [11].

Recently, it has been reported that NOS is distributed not only in specific gray matter profiles, but is also present in the white matter columns containing different short- and long-projection axons [38, 39]. A 17-min ischemia alone caused a significant increase in cNOS activity, particularly in the dorsal and lateral columns. Based on the findings mentioned above, we tentatively assume that increased cNOS activity in both white matter columns may be the direct consequence of enhanced intra-axonal leakage of Ca^{2+} through reverse $\text{Na}^+-\text{Ca}^{2+}$ exchange observed in the previous experiments during axonal injury [40, 41]. Moreover, lethal axoplasmic Ca^{2+} overload may result in the activation of lipases and consecutive accumulation of heterogeneous free fatty acids (FFA), especially arachidonic acid (AA) [42], which has been reported to cause strong enhancement of cNOS activity in cultured spinal cord neurons [43]. Completely different results were noted in the white matter columns after 17-min ischemia followed by 1 day of reperfusion. A strong decrease of cNOS activity in these structures is probably coupled with axonal degeneration. It has been suggested that during reoxygenation re-energized mitochondria rapidly accumulate massive amounts of Ca^{2+} [44], possibly in an attempt to clear Ca^{2+} overloaded axoplasm [45]. Such high elevations of matrix Ca^{2+} are likely lethal and may underlie part of the reoxygenation injury known to be manifested by the segmental swelling of myelinated axons and the formation of spaces between myelin sheaths and axolemma [46].

In conclusion, we can state that our data clearly illustrate the considerably different responsiveness of gray and white matter spinal cord regions to transient ischemic insult alone and that followed by 24 h of reperfusion, demonstrated by quite distinctive alterations in cNOS activity and possible consequences resulting from such an elevation.

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