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Transient ischemia mediates dissimilarities in nitric oxide synthase activity in the spinal cord regions

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In the present study we investigated the effect of transient ischemia (13 and 17 min) followed by 1h and 3h of reperfusion on the Ca^{2+}-dependent nitric oxide synthase (NOS) activity. The enzyme activity was measured by the conversion of ^{14}[C]arginine to ^{14}[C]citrulline and determined in the dorsal horn, intermediate zone and ventral horn of the gray matter and in the dorsal, lateral and ventral columns of the white matter of the rabbit spinal cord. Transient ischemia induced by infrarenal balloon occlusion of the abdominal aorta for 13 min was not strong enough to cause significant changes in the catalytic NOS activity, although the experimental animals tended to show a mild deficit in hindlimb movement. The increase of enzyme activity was significant in the dorsal horn, dorsal and lateral column after 17 min ischemia. During subsequent reperfusions catalytic NOS activity undergoes a permanent increase in the dorsal horn and a transient increase in the intermediate zone. The neurological impairment of the hindlimbs, characterized by a partial or total paraplegia after 17 min ischemia and subsequent reperfusions may reflect differences in the integrity of spinal interneurons in the intermediate zone, i.e., the region being highly sensitive to transient spinal cord ischemia. Our results indicate that catalytic NOS activity may be regionally and temporally affected due to an ischemic insult and following subsequent reperfusions.

Key words: transient ischemia, nitric oxide synthase, rabbit, spinal cord regions.

Abbreviations: AA, arachidonic acid; DBP, distal blood pressure; DTT, dithiothreitol; H_4B, tetrahydrobiopterin; NADPHd, nicotinamide adenine dinucleotide phosphate diaphorase; NO, nitric oxide; NOS, nitric oxide synthase; PTFE, polytetrafluoroethylene.

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Introduction

Spinal cord ischemia followed by subsequent neurological deficit, i.e. paresis or paraplegia, represents a major complication after repair of the thoracoabdominal aorta. It has been reported previously that ischemia participates in the secondary cell death after a traumatic injury (Zhou et al., 1999) and during various pathological states (Kirsch et al., 1992; Linnik et al., 1993; Li et al., 1995; Sakurai et al., 1997). The secondary processes are seen to arise from the derangements of the normal metabolic and physiologic functions and thus contributing significantly to an overall neuronal injury. Although intense investigations have been undertaken identifying the mechanisms underlying neuronal degeneration after ischemic states, the basis for the neurological outcome, however, remains unclear.

Nitric oxide (NO), derived from constitutively expressed isoforms of nitric oxide synthase (NOS, endothelial and neuronal), is known to play an important role in the modulation of neuronal signals and regulation of the cerebral blood flow (Faraci & Brian, 1994). The release of this signaling molecule into the microenvironment can affect NO-mediated processes by two manners. The first, represented by the basal level of NO provides an anterograde and retrograde communication between neurons and/or the neurons and glia, and is essential maintaining the structural integrity of neurons (Blot et al., 1994; Yezierski et al., 1996). The second, increased in response to certain stimulation (Dawson et al., 1992) may play an important role in the pathogenesis of some destructive processes (Gross & Wolin, 1995). In the spinal cord, a high number of NOS-immunoreactive and/or NADPH-diaphorase-positive neurons was found in the superficial layers of the dorsal horn (laminae I-II) (Callsen-Cencic et al., 1999; Lukáčová et al., 1999; Maršala et al., 1999), and, as stated previously, this neuronal population can survive an ischemia/reperfusion insult (Maršala et al., 1997). Morphological data have shown that the neurons in the intermediate zone (lamina VII) and in the ventral horn (laminae VIII-IX), expressing under physiological conditions a low NOS activity (Lukáčová et al., 2002) are more vulnerable to ischemia than those in the dorsal horn and in the pericentral region (lamina X) (Hayashi et al., 1998; Sakurai et al., 1998).

The aim of the present study was to investigate the effect of 13 and 17 min ischemia followed by 1h and 3h of reperfusion on calcium-dependent NOS activity in regions of the lumbosacral spinal cord. We intended to find the correlation between catalytic NOS (cNOS) activity and the intensity of functional deficit.

Material and methods

Forty-nine adult Chinchilla rabbits of both sexes, weighing 2.5–3.5 kg were used in the experiment. They were divided into seven experimental groups: (1) control animals ($n = 7$); (2,3) animals subjected to 13 and 17 min ischemia without reperfusion, respectively (n = 14); (4,5,6,7) animals subjected to 13 and 17 min ischemia followed by 1 and 3h of reperfusion ($n = 28$).

Operative procedure

After being premedicated with ketamine (40 mg/kg) and xylazine (8 mg/kg, i.m.) the animals were anesthetized with 1.0–2.0% halothane in room air, delivered via face mask during operation. Under sterile conditions, femoral arteriotomy was performed 3–4 cm distal to the inguinal ligament. The left femoral artery was cannulated with an 18-gauge polytetrafluoroethylene (PTFE) catheter for monitoring distal blood pressure (DBP). Before catheter insertion, heparin 500 IU was administered intravenously. Aliquots of arterial bood samples (1 mL) taken shortly before ischemia, at ten minute of each ischemia and 5 minutes after aortic occlusion were drawn for measurement of blood gases and pH. No differences in physiological parameters were observed within the intraischemic periods, therefore the blood pressure, blood gases and pH data were averaged. A 5-Fogarty arterial embeloctomy catheter (120805F Baxter Healthcare Corporation, USA) was inserted 15 cm into the right femoral artery (de Haan et al., 2000). This resulted in a balloon location 0.5–1.5 cm distal to the left renal artery. The balloon was inflated for 13 and 17 min until the loss of pulsatile distal aortic pressure was observed. During the process of ischemia, the body temperature of the rabbits, monitored with a rectal thermometer was maintained at 37 °C with a heating pad. The backbone (segments L_4–S_4) of experimental animals was excised and the spinal cord was quickly extruded into an ice-cold isotonic solution. The spinal cord was carefully frozen and stored in liquid nitrogen. The L_4–S_4 segments were cut in a cryostat (−18 °C) into 600 μm slices. The tissue samples were punched by needles (id 0.6 or 0.8 mm) from the gray matter regions: laminae I-VI (dorsal horns), laminae VII and X (intermediate zone and pericentral region), laminae VIII-IX (ventral horns), and the white matter columns: dorsal, lateral and ventral on a plate cooled with the liquid nitrogen (−15 °C).

Nitric oxide synthase radioassay

Catalytic NOS activity was determined by the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline according to the method of Bredt & Snyder (1990). Frozen spinal cord samples were homogenized in 250–300 μL 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 papain). Aliquots of the homogenates (200 μg/mL) were incubated for 45 min (37 °C) with solution A (100 μL) consisting the 100 μM arginine, 1 mM NADPH, 1 mM dithiothreitol (DTT), 1.5 mM FAD, 1 mM EDTA, 20 mM $CaCl_2.6H_2O$, 10 mM tetrahydrobiopterin (H_4B), 50 mM HEPES, 1 mM calmodulin, 50 mM TRIS-HCl and solution B (100 μL) containing 2.5 μL L-[^{14}C]arginine (321nCi). The reaction was stopped by addition of 1 mL of ice-cold 100 mM HEPES buffer and 10 mM EDTA, pH = 5.5. Samples were applied to a Dowex AG 50 W-X8 cationic-exchange column (Na^+ form) to remove the L-[^{14}C]citrulline. The columns were washed with 2 mL of deionized water to elute the L-[^{14}C]citrulline. Then the aliquots of samples (0.5 mL) were mixed with 5 mL of Bray's fluid in scintillation vials and counted in the Beckman LS-3801 Liquid Scintillation System. Cpms were converted to dpms using [^{14}C] L-quenched standards. The levels of L-[^{14}C]citrulline were computed after substracting the blank that represented nonspecific radioactivity in the absence of enzyme activity. Protein determination was done using a BRADFORD (1976) assay. The results from analyses were expressed as pmol/mg protein.

Neurological evaluation

The neurologic status of each animal was evaluated at the end of respective reperfusion intervals and was graded according to Tarlov's criteria as: 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 inability to draw legs under body or hop; 3-ability to draw legs under body and hop but not normally; and 4 – normal motor function.

Statistical analysis

The results of NOS activity were statistically evaluated by ANOVA as well as by the Turkey-Kramer test and have been given as means ± SEM.

Results

Table 1 summarizes physiological parameters, i.e. the pH, pCO_2, pO_2 and DBP monitored in arterial blood before, during and after ischemia. The pH levels and pO_2 were not different in the control and experimental groups. However, pCO_2 decreased significantly in both, the ischemic and reperfusion periods. Within the intraischemic periods, there was an enormous decrease ($p < 0.05$) of the DBP. In the reperfusion period the mean of distal arterial blood pressure markedly increased ($p < 0.05$) and was comparable with the control values.

Table 1. Physiological variables.

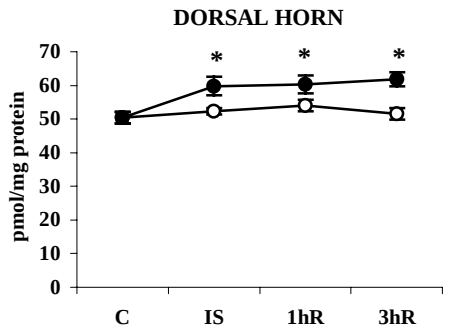
	Preischemia	Ischemia	Reperfusion
pH	7.35 ± 0.04	7.38 ± 0.05	7.33 ± 0.05
pCO2 [kPa]	7.47 ± 0.84	5.77 ± 0.92*	5.73 ± 0.51*
pO2 [kPa]	7.38 ± 1.12	8.44 ± 1.33	7.94 ± 0,86
DBP [Torr]	71.03 ± 11.18	18.56 ± 5.78*	63 ± 6.96

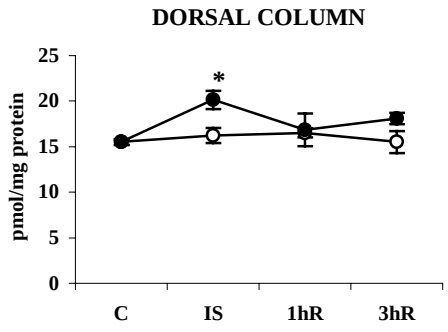
Physiological variables were measured before, during and after ischemia obtained from femoral arterial blood as described in experimental procedure. Data are given as means ± SEM. * $p < 0.05$ with respect to preischemia. DBP – distal blood pressure.

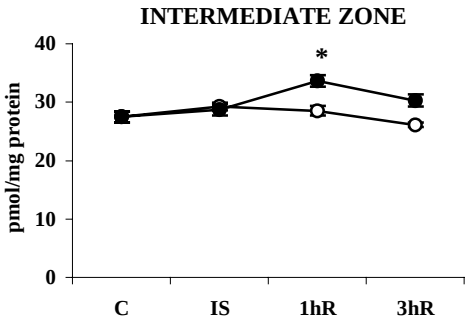
Table 2. Neurological evaluation.

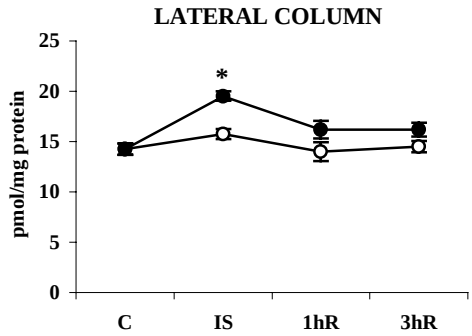
Degree of neurological injury	13'IS/1hR	13'IS/3hR	17'IS/1hR	17'IS/3hR
	number of animals			
0			4	4
1			2	3
2	3		1	
3	3	6		
4	1	1		

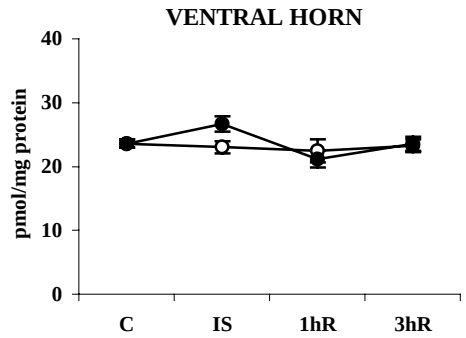
Neurologic evaluation of the function of the hindlimbs: 0, paraplegic with no lower-extremity function; 1, poor lower-extremity function, weak antigravity movement only; 2, some lower-extremity motor function with good antigravity strength but inability to draw legs under body or hop; 3, ability to draw legs under body and hop but not normally; and 4, normal motor function. Seven animals were evaluated in each experimental group. IS – ischemia; R – reperfusion.











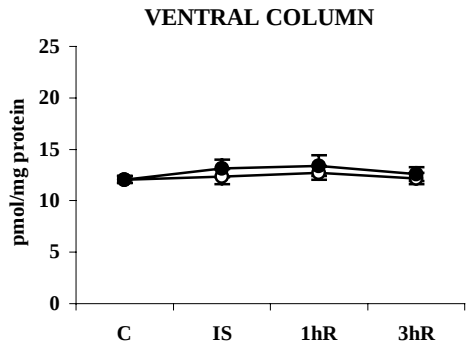


Fig. 1. The catalytic NOS activity in the gray matter regions – dorsal horn, intermediate zone and ventral horn of the lumbosacral spinal cord segments after 13 and 17 min ischemia followed by 1 h and 3 h of reperfusion. The results are expressed in pmol/mg protein. Data are given as means ± SEM. * $p < 0.05$ with the respect to control. C – control, IS – ischemia, R – reperfusion. ○ 13 min ischemia; ● 17 min ischemia.

Fig. 2. The catalytic NOS activity in the white matter regions – dorsal column, lateral column and ventral column of the lumbosacral spinal cord segments after 13 and 17 min ischemia followed by 1 h and 3 h reperfusion. The results are expressed in pmol/mg protein. Data are given as means ± SEM. * $p < 0.05$ with the respect to control. C – control, IS – ischemia, R – reperfusion. ○ 13 min ischemia; ● 17 min ischemia.

Data indicating the time course of the catalytic NOS activity in the ischemic spinal cord are illustrated in Figures 1 and 2. Ischemia performed for 13 min and that followed by 1 h and 3 h of reperfusion did not produce significant changes of the catalytic NOS activity in any region of the

gray and white matter. A statistically significant increase of the enzyme activity was noted after 17 min ischemia in the dorsal horn, dorsal column and lateral column. In the dorsal horn, catalytic NOS activity persisted elevated at 1 h and 3 h of reperfusion. Reestablishment of the blood supply to ischemic spinal cord caused the upregulation of the enzyme activity in the intermediate zone during first period of reperfusion. In the white matter columns the catalytic NOS activity was not significantly changed in any reperfusion period.

The neurological function of experimental animals was tested at the end of both survival intervals (Table 2). The recovery of motor function was not constant after 1 h of reperfusion. Ischemia induced for 13 min followed by 1 h and 3 h of reperfusions caused only mild neurological impairment, mostly characterized by the ability of experimental animals to draw their legs under the body and hop but not normally. Fully developed paraplegia was noted in most of animals subjected to 17 min ischemia and 1 h and 3 h of reperfusion. The rabbits suffered from partial or total paraplegia and maintained grade 1 or 0 deficit.

Discussion

This study demonstrates the changes of the catalytic NOS activity in regions of the spinal cord after transient ischemia (13 and 17 min) followed by two short reperfusion periods and compares them with neurological outcome of experimental animals. The catalytic NOS activity was modified differently in the gray and white matter regions, showing an apparent increase at 17 min ischemia in the dorsal horn of the gray matter and in the dorsal and lateral columns of the white matter. In a group of animals subjected to 13 min ischemia and subsequent reperfusions, the enzyme activity was not significantly changed, although, the rabbits tended to show a mild neurological deficit. The results indicate that re-supplying of oxygen to severely damaged spinal cord tissue became progressively worse, since NOS activity was significantly upregulated in affected regions of the spinal cord and, the incidence of full-developed paraplegia was found in most of animals subjected to 17 min ischemia and subsequent reperfusions.

The metabolic changes resulting from critical reduction of a substrate delivery and a release of active factors into the spinal extracellular space during ischemia strongly reflect metabolic demands of neuronal pools in the spinal cord. Regional differences in the catalytic NOS activity found in our study inside ischemia itself should be linked to a different regulation of ion channels affecting the excitatory or inhibitory response of spinal cord neurons (Pehl & Schmid, 1997), and a heterogenous release of free fatty acids (Halát et al., 1987). It was shown previously that arachidonic acid (AA) causes a strong enhancement of NOS activity in the cultured spinal cord neurons (Toborek et al., 2000). In the current study a significant increase of catalytic NOS activity was noted during 17 min ischemia in the dorsal horn, dorsal and lateral column. A high 9-fold elevation of AA from fasciculus gracilis forming a part of the dorsal column, and 8-fold increase in the dorsal part comprising the dorsal horn and dorsal part of the lateral column during ischemia itself (Halát et al., 1987) should be in part responsible for the observed phenomenon.

It is most likely that the mechanism of AA-induced upregulation of NOS relates to AA-induced alterations in the ion homeostasis and increased levels of the intracellular calcium (Toborek et al., 1999). Polyenoic FFA, notably arachidonate, are during the renewal of oxygen metabolized and provide a substrate for free radicals. Previous studies have reported that ischemia/reperfusion may lead to the neuronal NOS expression only in certain types of neurons (Kader et al., 1993; Pavel et al., 2001). In the current study, the most significant increase of catalytic NOS activity was limited only to the dorsal horn, where NOS activity was upregulated during 17 min ischemia and in subsequent reperfusion periods. In intact spinal cord, the most intensive neuronal NOS-IR is present in small neurons of superficial layers and in large neurons of deeper dorsal horn layers (Valtschanoff et al., 1992; Maršala et al., 1999). In this region of the spinal cord there is a high level of protein kinase C (Worley et al., 1987). Because under ischemia/reperfusion conditions the protein kinase phosphorylation system is impaired (Kochhar et al., 1989) and is not able to inhibit NOS activity completely, NOS-IR neurons may provide a main source for NO formation. In addition, NOS expression should be increased in neurons of the dorsal horn, that do not express NOS (Xu et al, 1998). The generation of an enormous amount of NO in this region of the spinal cord can be mediated through counterbalance between the neuronal NOS having a neurotoxic effect (Dawson et al., 1991) to spinal cord and endothelial NOS involved in vasodilation (Faraci, 1991).

In animals with fully developed spastic paraplegia, lateral part of the intermediate zone (laminae V–VII) is highly sensitive and typically af-

fected under conditions of partial ischemia (Jacobs et al., 1987; Maršala et al., 1989, 1994). The neurological impairment found in our study may partly reflect the changes of catalytic NOS activity in intermediate zone, i. e. in the region, having the highest baseline blood flow and the biggest reduction of blood flow during aortic occlusion. A significant increase of catalytic NOS activity was found in the intermediate zone (laminae VII) during 1 h of reperfusion. However, the enzyme activity did not gradually increase due to a longer reperfusion period. Under ischemia/reperfusion where NO has been speculated to be a toxic substance, other toxic agents, such as hydrogen peroxide and superoxide are also present. It is more likely that intermediate zone, the region with the highest metabolic rate, will display a high degree of sensitivity to ischemia/reperfusion and is adversely affected mainly due to the burst of free oxygen radicals in the early reperfusion period.

Our data demonstrate the changes in catalytic NOS activity, differing significantly during ischemia/reperfusion insults in the specific spinal cord regions.

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