EXPERIMENTAL STUDY

The effect of repeated sublethal ischemia on NO/cGMP signal transduction system in gray matter of the rabbit spinal cord

Pavel J, Lukacova N, Marsala J

*Institute of Neurobiology, Slovak Academy of Sciences, Košice, Slovakia.***pavel@saske.sk**

Abstract

Background: **The activation of the soluble guanylate cyclase and the production of cyclic 3',5'-guanosine monophosphate (cGMP) have been reported as the primary cellular response to nitric oxide (NO) in the nervous system. Previous results indicated, that three-fold sublethal ischemia repeated at 1-h intervals induces damage in gray matter of the spinal cord. However, little is known about the changes of NO/cGMP signal transduction system in gray matter of the spinal cord under conditions of repeated sublethal ischemia.**
M*ain purpose:* **The aim of this study was to compare the catalytic NOS activity and cGMP concentration in the gray matter regions of lumbosacral spinal cord segments (dorsal horn, zona intermedia, ventral horn) after three (8-, 8-, 9-min) sublethal occlusions repeated at 1-h intervals.**
Methods: **Twenty male rabbits, weighing 2.5—3.5 kg were used in the experiments. They were divided into two experimental groups: (1) control animals (n=10); (2) animals subjected to three brief (8-, 8-, 9-min) occlusions, each time repeated by reperfusion lasting for 1 h (n=10). Ischemia of lumbosacral segments was induced by ligation of the abdominal aorta just below the left renal artery (DeGirolami and Zivin, 1982). The catalytic NOS activity was determined by conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline according to the method of Bredt and Snyder (1990) slightly modified by Strosznajder and Chalimoniuk (1996). cGMP concentration was assessed by radioimmunoassay method (RIA).**
Results: **Repeated sublethal ischemia evoked a slight increase in catalytic NOS activity over control values in all gray matter regions. On the other hand, cGMP concentration in gray matter regions has a decreasing character, in a descending order: dorsal horn > zona intermedia > ventral horn. A significant impairment of NO-cGMP signal transduction was detected in the intermediate zone and ventral horns.**
Conclusions: **Our results indicate that threefold (8-, 8-, 9-min) sublethal ischemia repeated in 1 h intervals of reperfusion causes the impairment of NO/cGMP signal transduction system in gray matter of lumbosacral spinal cord segments and the extent of impairment is region-specific. This finding correlates with the neurological hindlimbs impairment in experimental animals. *(Tab. 1, Fig. 2, Ref. 39.)***
Key words: repeated sublethal ischemia, catalytic NOS activity, cyclic GMP, gray matter regions, spinal cord.

The primary cell response due to the nitric oxide (NO) stimulation known to occur in the central nervous system is the activation of soluble guanylate cyclase followed by cGMP formation. NO continuously released from endothelium of cerebral vasculature including capillary network (EDRF) is acting as a mediator allowing for a basilar vasodilatatory tone (Faraci, 1990). A prompt and precise control of the nitric oxide synthase (NOS) activity is one of the most significant components regulating the signal transduction by means of NO. It is well documented that the upregulation in NO synthesis may have an adverse effect and NO may become neurotoxic (Dawson et al., 1992), and, vice versa, a long-term inhibition of NOS activity at the spinal cord level can cause a time-dependent increase in blood pressure, a phenomenon adversely influencing the spinal arteriolar structure, vasoconstriction and permeability and, more dangerously, resulting in the formation of multilacunar infarcts of the gray and white matter (Blot et al., 1994). Several recent studies confirm the existence of a nitric oxide-cyclic guanosine monophosphate (NO-cGMP) signal transduction pathway in the central nervous system (Denninger and Marletta, 1999; Meller et al., 1992; Morris et al., 1994; Schmidt and Pehl, 1996). It should be noted that region-dependent diffe-

Address for correspondence: J. Pavel, RND, Institute of Neurobiology SAV, Soltesovej 4—6, SK-040 01 Košice, Slovakia.
Phone: +421.95.6785 064, Fax: +421.95.6785 074

Supported by VEGA grant No. 2/7222/20.

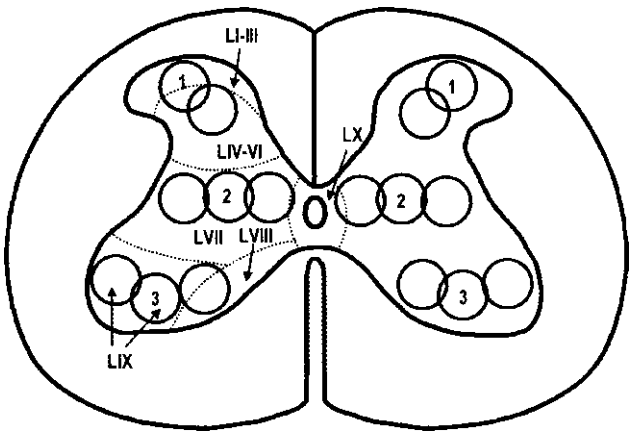

Fig. 1. Schematic drawing representing the regional and/or laminar division of gray matter.
No. 1 dorsal horns (laminae I-VI), No. 2 zona intermedia (lamina VII and lamina X), No. 3 ventral horns (laminae VIII-IX).

rences of the catalytic NOS activity and in the distribution of cGMP were found in the spinal cord (Lukáčová and Pavel, 2000; Pavel et al., 2000).

Some recent studies suggest that sublethal ischemia per se cannot cause a neuronal damage, but may induce so-called ischemic tolerance, i.e., a change influencing the regulation of the cell and tissue metabolic rate during a time-limited period during which the nervous tissue is getting more adaptive and more resistant against a secondarily applied lethal ischemia instituted shortly afterwards. However, if such sublethal ischemia is produced repeatedly, it can cause a damage ranging between a selective neuronal necrosis and gray matter infarction depending on severity and duration of ischemia (DeGirolami et al., 1984; Marcoux et al., 1982). On the other hand, some neurons can be damaged by sublethal ischemia if such ischemic intervention is repeated several times following short reperfusion periods (Araki et al., 1990; Kato and Kogure, 1990; Lukáčová et al., 1998). Some factors, e.g., the impairment of the proteosynthesis (Widmann et al., 1992), postischemic hypoperfusion, known to be maximally developed 1 h following a 2-min of ischemia (Kato et al., 1990), excitotoxic mechanisms (Kato and Kogure, 1990; Lin et al., 1992), the changes in the receptor sensitivity (Kato et al., 1991), regional changes in [^{3}H]inositol 1,4,5-triphosphate binding (Kato et al., 1994), the changes in the biogenesis of the membrane phospholipids (Lukáčová et al., 1998; Pavel and Lukáčová, 1999) and a high calcium accumulation (Araki et al., 1990) may cause the neuronal damage, a phenomenon seen most prominently after a sublethal ischemia repeated in short 1-h intervals. However, the influence of such sublethal ischemia repeated in 1 h intervals on NO-cGMP signal transduction system in the spinal cord remains unknown.

The aim of our experiments was i) to characterize the influence of sublethal ischemia repeated in short 1 h intervals on NO-cGMP signal transduction system in the spinal cord gray matter in the lumbosacral segments and, ii) to compare the changes in NO-cGMP pathway with functional disturbances of hindlimb motor activity.

Material and methods

The experiments were performed on twenty adult male rabbits, weighing 2.5—3.5 kg. The animals had unrestricted access to food and water. Ischemia of lumbosacral segments was induced under thiopental anesthesia (30 mg/kg, i.v.) by ligation of the abdominal aorta just below the left renal artery (DeGirolami and Zivin, 1982). The recirculation was started by simply removing the ligature. The experimental animals were divided into experimental groups as follows: 1) sham-operated control (n=10) and 2) animals subjected to three (8-, 8-, 9-min) ischemic insults repeated at 1h interval of reperfusion (n=10). At the end of the respective experimental intervals the backbone (segments L4-S1) was excised and the spinal cord was quickly extruded into an ice-cold isotonic saline. Spinal cord was cleaned from envelopes, carefully frozen and stored in liquid nitrogen and then cut in a cryostat (-15 °C) into 600 μm slices. The dorsal horns (No. 1), intermediate zone (No. 2) and ventral horns (No. 3) were punched by needles (id 0.6 or 0.8 mm) from the spinal cord slices on a plate cooled in liquid nitrogen (-15 °C) (Fig. 1).

NOS radioassay. Catalytic NOS activity was determined by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline according to the method of Bredt and Snyder (1990) with a slight modification by Strosznajder and Chalimoniuk (1996). Frozen spinal cord samples were homogenized in 100—150 μl of an ice-cold Tris-HCl buffer (10 mM, pH=7.4). Aliquots of the homogenates (200 μg/ml) were incubated for 45 min (37 °C) with 10 μM L-[^{3}H]arginine (1Ci), 1 mM NADPH, 1 μM calmodulin in HEPES buffer (50 mM, pH=7.4) containing 1 mM dithiothreitol, 1 mM EDTA, 100 μM FMN, 100 μM FAD, 2 mM $CaCl_2$, 15 μM tetrahydrobiopterin in a final volume of 300 l. The reaction was stopped by an addition of 1 ml of ice-cold HEPES buffer (100 mM, pH=5.5) containing 10 mM EDTA. Samples were applied to a Dowex AG 50 W-X8 cationic-exchange column (Na^+ form) to remove the L-[^{3}H]arginine. The columns were washed with 2 ml of deionized water to elute the L-[^{3}H]citrulline. The samples were centrifuged at 1000 g for 5 min and aliquots (0.5 ml) of supernatant fractions were mixed with 5 ml of Bray's fluid into scintillation vials and then counted in the Beckman LS-3801 spectrometer. Cpms were converted to dpms using [^{3}H]-quenched standards. Levels of L-[^{3}H]citrulline were computed after subtracting the blank, which represented nonspecific radioactivity in the absence of enzyme activity. Protein determination was done using a Bradford method (1976). The results of radioassay detection of catalytic NOS activity were expressed as dpm/g protein.

Cyclic Guanosine Monophosphate Radioimmunoassay. Before the assay, 1 volume of tissue samples, still frozen, was mixed with 10 volumes of perchloric acid 1.07 N. The cooled suspension of the spinal cord samples was sonicated and centrifuged for 1 min at 10,000 *g* and the supernatant was collected for the radioimmunoassay.

The Immunotech cGMP kit is based on the competition between the succinylated cGMP of the sample and a ^{125}I-labeled tracer for binding to the polyclonal antibody (Steiner et al., 1972). The amount of radioactivity was counted for 1 min in a gamma counter and the concentration of cGMP was calculated by interpolation from a standard curve. All analyses were carried out in duplicate. Protein determination was done using a Bradford assay

(1976). The results from the analyses were expressed as nmol cGMP/mg protein.

Neurological Evaluation. The degree of neurological impairment was evaluated at the end of reperfusion intervals and was graded according to Zivin et al. (1982): Grade 0: No neurologic impairment (freely movable and able to hop normally), Grade 1: Partial neurologic impairment (paretic hind limbs, usually abnormal sensation), Grade 2: Total neurologic impairment (full-developed flaccid paraplegia and absence of sensation).

Statistical Analysis The results of both the catalytic NOS activity and the cGMP level were statistically evaluated by ANOVA as well as by the Tukey-Kramer test and have been given as means±SEM.

Results

A radioassay (RIA) detection of the catalytic NOS activity and the assessment of cGMP concentration by RIA demonstrated prominent regional differences seen in the gray matter under physiologic conditions as well as under ischemia and reperfusion. Catalytic NOS activity detected in different regions of the gray matter under physiologic conditions was between 67.4 to 236.6 dpm/mg proteins and the concentration of cGMP ranged from 0.078 to 0.185 nmol/mg tissue wet weight (Tab. 1). Under physiologic conditions a correlation was noted between catalytic NOS activity and cGMP level in regions under study and, more interestingly, highest values of both enzymes could be found in the dorsal horn region. When compared with the control, a threefold sublethal ischemia caused a slight increase of catalytic NOS activity in all regions studied. An ascending order of this increase was noted as follows: dorsal horns<intermediate zone<ventral horns (Fig. 2). Contrary to this, the concentration of cGMP decreased below the control value in all regions as follows: in the dorsal horn about 16.2 %, in the intermediate zone 39.9 % and, more prominently, in the ventral horn about 59.8 %. A hardly discernible difference between catalytic NOS activity and cGMP synthesis was noted in the dorsal horns and, quite opposite, a significant difference was detected in the intermediate zone (55.8 %) and in the ventral horns.

A partial neurologic impairment was diagnosed after repeated sublethal ischemia in 9 of 10 experimental animals and classified as degree 1. A heavy neurologic impairment (degree 2) was noted in one animal.

Discussion

The results of the present study using a paradigm of the sublethal ischemia repeated at short ischemia and reperfusion intervals point to a region-dependent distribution of the catalytic NOS activity and cGMP in the gray matter of the lumbosacral segments both under physiologic or ischemia-reperfusion conditions. The correlation between the catalytic NOS activity and the level of cGMP in normal, control animals confirmed the presence of NO-cGMP signal transduction pathway in all regions of the spinal cord gray matter. A slight increase in the catalytic NOS activity thus pointing to an enhanced NO production known for its vasodilatatory effect seen after the application of a threefold repeated ischemia may reflect the changes in the activity of both constitutively expressed NOS isoforms, i.e., an endothelial and neuronal one. It

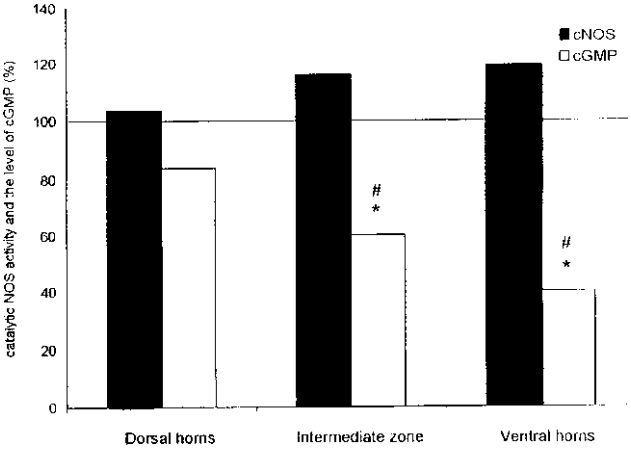

Fig. 2 Catalytic NOS activity and the level of cGMP in the dorsal horns, intermediate zone and ventral horns of the lumbosacral spinal cord of rabbit after three-fold (8-, 8-, 9-min) sublethal ischemia followed each time by 1h of reperfusion.
The result of cNOS activity expressed as dpm/μg protein and cGMP level expressed as nmol cGMP/mg wet. wt. were in the dorsal horns, intermediate zone and ventral horns of control taken as 100 %. The values were statistically evaluated by ANOVA as well as the Tuckey-Kramer test; #p<0.05 with respect to cNOS activity after repeated ischemia/reperfusions. Data are given as means (n=5) ± SEM; c NOS-catalytic nitric oxide synthase, cGMP-cyclic guanosine monophosphate.

is known that the expression of an inducible isoform of NOS, seen after occlusion of the middle cerebral artery (MCA) is, in comparison with the endothelial and neuronal NOS delayed and is seen 6—12 h postischemia (Iadecola et al., 1995).

It is known that the NO-cGMP is selectively localized in the superficial layers of the dorsal horns, i.e., in the loci playing a decisive role in the processing of the nociceptive stimuli and in the transmission of painful stimuli to second order neurons of the spinothalamic pathway. NOS synthesis along with cGMP formation may be the factor easing the transmission of the thermal nociceptive impulses in the corresponding microcircuits in the dorsal horn (Meller et al., 1992). It is well known that neurons synthesizing NO are anatomically positioned in those layers (laminae) of the dorsal horns, which are indispensable for the transmission of the nociceptive signals in the spinal cord (Valtschanoff et al., 1992; Dun et al., 1993). According to other sources the production of an endogenous NO is necessary for a tonic inhibition of the nociceptive processing of stimuli entering the dorsal horn via mechanoreceptive fibers of the dorsal roots (Zhuo et al., 1993). It seems likely, that NO produced

Tab. 1. Catalytic NOS activity and the level of cGMP in the spinal cord regions under physiological conditions.

Spinal cord region	Catalytic NOS activity (dpm/μg protein)	Concentration of cGMP (nmol/mg wet.wt.)
dorsal horns	236.6±8.4	0.185±0.003
intermediate zone	99.5±2.6	0.104±0.002
ventral horns	67.4±1.4	0.078±0.002

Data are given as means (n=5)±S.E.M.

during repeated sublethal ischemia may have an adverse effect on motor and sensitive activity of the spinal cord as well.

The superficial layers of the dorsal horns (laminae I-II) are known for a high number of NOS immunoreactive and NADPHd-exhibiting neurons (Maršala et al., 1999; Lukáčová et al., 1999), a neuronal population highly resistant against ischemia-reperfusion-induced damage (Maršala et al., 1997). Similar density of NOS immunoreactive neurons is not seen in the intermediate zone and in the ventral horn, a finding correlating with a comparatively low level of the catalytic NOS activity found in both regions. Previous results from our laboratory have shown, that NADPHd-exhibing neurons localized in the deep dorsal horn remained completely preserved after a three fold (8-, 8-, 9-min) ischemia followed by 1-h intervals of reperfusion (Maršala and Jalč, 2000). It is not surprising, therefore, that the region demonstrating a quite low damage is just the dorsal horn.

It is known that a three fold 5-min ischemia repeated at 1-h intervals may have a cumulative effect in the development of the brain edema in gerbils accompanied by a partial O_2 decrease in the cerebral cortex after each reperfusion period followed after a short-lasting ischemia (Nowak et al., 1990). These findings confirm that secondary hypoxia accompanying postischemic hypoperfusion may have a deleterious effect on the nervous tissue and, moreover, short postischemic reperfusion periods may aggravate the damage and the final neurological outcome. On the other hand, short several times performed ischemia repeated at 1-h intervals may gradually enhance the free radicals formation and diminish or depress the antioxidative protective activity of the nervous tissue. Since microvessels are considered to be the main target for free radicals destroying activity it seems possible that a three-times repeated sublethal ischemia may lead to a microvascular dysfunction and NO formation may act more adversely that protectively. Damaging effect of a threefold (8-, 8-, 9-min) sublethal ischemia repeated at 1-h intervals was fully confirmed in a study describing an enhanced membrane phospholipids degradation in all spinal cord regions (Lukáčová et al., 1998).

Prominent finding of the present study is the description of a laminar or region-dependent damage of NO-cGMP signal transduction system resulting after a threefold sublethal ischemia combined with a short 1-h intervals of reperfusion in the lumbosacral spinal cord segments. The intermediate zone and the ventral horns were the sites of a clearly expressed damage. Moreover, the damage of NO-cGMP pathway closely correlates with the degree of the neurologic impairment of animals subjected to the sublethal ischemia. It was clearly demonstrated that the avoidance reaction and the response to a painful stimulation were modified along with a deep motor disturbances affecting the hindlimbs. Simultaneously, the excretory functions were individually greatly disturbed. It is suggested that at least three factors may have an adverse effect on NO-cGMP signal transduction pathway after a threefold sublethal ischemia followed by 1-h reperfusion intervals: 1) the inhibition of sGC through cGMP-dependent protein kinase (PKG) (Ferrero et al., 2000). On the other hand, a cGMP-induced smooth muscle relaxation is mediated mainly by the activation of cGMP-dependent protein kinase (Cavajal et al., 2000); 2) an enhanced activity of cGMP-specific cyclic phosphodiesterases; 3) the low level of GTP as a specific substrate for sGC. According to Meller and Gebhart (1993) a continuous NO synthesis followed by a sGC activation in the lumbar spinal cord segments is necessary for maintaining the thermal hyperalgesia, ensuing in experimental models leading to a long-lasting pain NO may activate or inhibit the activity of the spinal cord neurons increasing the concentration of cGMP and, as was shown quite recently, these effects are lamina-dependent (Schmidt and Pehl, 1996). It should be admitted that astrocytes may have some influence on nociceptive signal transmission (Maihofner et al., 2000).

References

Araki T., Kato H., Kogure K.: Neuronal damage and calcium accumulation following repeated brief cerebral ischemia in the gerbil. Brain Res., 528, 1990, p. 114—122.

Blot S., Arnal J.F., Xu Y., Gray F., Michel J.B.: Spinal cord infarcts during long-term inhibition of nitric oxide synthase in rats. Stroke, 25, 1994, p. 1666—1673.

Bradford M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 1976, p. 248—254.

Bredt D.S., Snyder S.H.: Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. USA, 87, 1990, p. 682—685.

Carvajal J.A., Germain A.M., Huidobro-Toro J.P., Weiner C.P.: Molecular mechanism of cGMP-mediated smooth muscle relaxation. J. Cell Physiol., 184, 2000, p. 409—420.

Dawson T.M., Dawson V.L., Snyder S.H.: A novel neuronal messenger molecule in brain: the free radical, nitric oxide. Ann. Neurol., 32, 1992, p. 297—311.

DeGirolami U., Zivin J.A.: Neuropathology of experimental spinal cord ischemia in the rabbit. J. Neuropathol. Exp. Neurol., 41, 1982, p. 129—149.

DeGirolami U., Crowell R.M., Marcoux F.W.: Selective necrosis and total necrosis in focal cerebral ischemia. Neuropathologic observations on experimental middle cerebral artery occlusion in the macaque monkey. J. Neuropathol. Exp. Neurol., 43, 1984, p. 57—71.

Denninger J.W., Marletta M.A.: Guanylate cyclase and the NO/cGMP signaling pathway. Biochim. Biophys. Acta, 1411, 1999, p. 334—350.

Dun N.J., Dun S.L., Wu S.Y., Forstermann U., Schmidt, H.H.H.W., Tseng L.F.: Nitric oxide synthase immunoreactivity in the rat, mouse, cat and squirrel monkey spinal cord. Neuroscience. 54, 1993, p. 845—857.

Faraci F.M.: Role of nitric oxide in regulation of basilar artery tone in vivo. Amer. J. Physiol., 259, 1990, p. 1216—1221.

Ferrero R., Rodriguez-Pascual F., Miras-Portugal M.T., Torres M.: Nitric oxide-sensitive guanylyl cyclase activity inhibition trough cyclic GMP-dependent dephosphorylation. J. Neurochem., 75, 2000, p. 2029—2039.

Iadecola C., Zhang F., Xu S., Casey R., Ross M.E.: Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. J. Cereb. Blood Flow. Metab., 15, 1995, p. 378—384.

Kato H., Kogure K.: Neuronal damage following non-lethal but repeated cerebral ischemia in the gerbil. Acta Neuropathol., 79, 1990, p. 494—500.

Kato H., Araki T., Kogure K.: Role of the excitotoxic mechanism in the development of neuronal damage following repeated brief cerebral ische-

mia in the gerbil. Protective effects of MK-801 and pentobarbital. Brain Res., 516, 1990, p. 175—179.

Kato H., Araki T., Hara H., Kogure K.: Autoradiographic analysis of second-messenger systems in the gerbil hippocampus following repeated brief ischemic insults. Brain Res. Bull., 27, 1991, p. 759—765.

Kato H., Araki T., Hara H., Kogure K.: Regional changes in [3H]inositol 1,4,5-triphosphate binding in the gerbil brain following repeated sublethal ischemia. Brain Res. Bull., 33, 1994, p. 465—468.

Lin B., Globus M.I., Dietrich W.D., Busto R., Martinez E., Ginsberg M.D.: Differing neurochemical and morphological sequelae of global ischemia: comparison od single- and multiple-insult paradigms. J. Neurochem., 59, 1992, p. 2213—2223.

Lukáčová N., Čížková D., Maršala M., Jalč P., Maršala J.: Segmental and laminar distributions of nicotinamide adenine dinucleotide phosphate-diaphorase-expressing and neuronal nitric oxide synthase-immunoreactive neurons versus radioassay detection of catalytic nitric oxide synthase activity in the rabbit spinal cord. Neuroscience, 94, 1999, p. 229—237.

Lukáčová N., Jalč P., Maršala J.: Regional changes of membrane phospholipid concentrations in rabbit spinal cord following brief repeated ischemic insults. Mol. Chem. Neuropathol., 35, 1998, p. 61—76.

Lukáčová N., Pavel J.: Catalytic nitric oxide synthase activity in the white and gray matter regions of the spinal cord of rabbits. Physiol. Res., 49, 2000, p. 167—173.

Maihofner C., Euchenhofer C., Tegeder I., Beck K. F.,; Pfeilschifter J., Geisslinger G.: Regulation and immunhistochemical localization of nitric oxide synthases and soluble guanylyl cyclase in mouse spinal cord following nociceptive stimulation. Neurosci. Lett., 290, 2000, p. 71—75.

Marcoux F.W., Morawetz R.B., Crowell R.M., DeGirolami U., Halsey J.H., Jr.: Differential regional vulnerability in transient focal cerebral ischemia. Stroke, 13, 1982, p. 339—346.

Maršala J., Jalč P.: Short-term changes of NADPH diaphorase-exhibiting neuronal pools in the spinal cord of rabbit after repeated sublethal ischemia. Physiol. Res., 49, 2000, p. 157—165.

Maršala J., Kluchová D., Maršala M.: Spinal cord gray matter layers rich in NADPH diaphorase-positive neurons are refractory to ischemia-reperfusion-induced injury: a histochemical and silver impregnation study in rabbit. Exp. Neurol., 145, 1997, p. 165—179.

Maršala J., Maršala M., Vanický I., Taira Y.: Localization of NADPHd-exhibiting neurons in the spinal cord of the rabbit. J. Comp. Neurol., 406, 1999, p. 263—284.

Meller S.T., Dykstra C., Gebhart G.F.: Production of endogenous nitric oxide and activation of soluble guanylate cyclase are required for N-methyl-D-aspartate-produced facilitation of the nociceptive tail-flick reflex. Europ. J. Pharmacol., 214, 1992, p. 93—96.

Meller S.T., Gebhart G.F.: Nitric oxide (NO) and nociceptive processing in the spinal cord. Pain, 52, 1993, p. 127—136.

Morris R., Southam E., Gittins S.R., de Vente J., Garthwaite J.: The NO-cGMP pathways in neonatal rat dorsal horn. Europ. J. Neurosci., 6, 1994, p. 876—879.

Nowak T.S., Jr., Tomida S., Pluta R., Xu S., Kozuka M., Vass K., Wagner H.G., Klatzo I.: Cumulative effect of repeated ischemia on brain edema in the gerbil. Biochemical and physiological correlates of repeated ischemic insults. Adv. Neurol., 52, 1990, p. 1—9.

Pavel J., Lukáčová N.: Phospholipid composition in the spinal cord regions after sublethal ischaemia followed by short- and long-lasting reperfusions. Biologia, 6, 1999, p. 61—66.

Pavel J., Lukáčová N., Maršala J.: Regional changes of cyclic 3',5'-guanosine monophosphate in the spinal cord of the rabbit following brief repeated ischemic insults. Neurochem. Res., 25, 2000, p. 1131—1137.

Schmid H.A., Pehl U.: Regional specific effects of nitric oxide donors and cGMP on the electrical activity of neurons in the rat spinal cord. J. Chem. Neuroanat., 10, 1996, p. 197—201.

Steiner A.L., Ferrendelli J.A., Kipnis D.M.: Radioimmunoassay for cyclic nucleotides. 3. Effect of ischemia, changes during development and regional distribution of adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in mouse brain. J. Biol. Chem., 247, 1972, p. 1121—1124.

Strosznajder J., Chalimoniuk M.: Biphasic enhancement of nitric oxide synthase activity and cGMP level following brain ischemia in gerbils. Acta Neurobiol. Exp. (Warsz)., 56, 1996, p. 71—81.

Valtschanoff J.G., Weinberg R.J., Rustioni A.: NADPH diaphorase in the spinal cord of rats. J. Comp. Neurol., 321, 1992, p. 209—222.

Widmann R., Weber C., Bonnekoh P., Schlenker M., Hossmann K.-A.: Neuronal damage after repeated 5 minutes of ischemia in the gerbil is preceded by prolonged impairment of protein metabolism. J. Cereb. Blood Flow. Metab., 12, 1992, p. 425—433.

Zhuo M., Meller S.T., Gebhart G.F.: Endogenous nitric oxide is required for tonic cholinergic inhibition of spinal mechanical transmission. Pain, 54, 1993, p. 71—78.

Zivin J.A., DeGirolami U., Hurwitz E.L.: Spectrum of neurological deficits in experimental CNS ischemia. A quantitative study. Arch. Neurol., 39, 1982, p. 408—412.

Received March 28, 2001.
Accepted May 17, 2001.