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ORIGINAL ARTICLE

The correlation between nitric oxide and vascular endothelial growth factor in spinal cord injury

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Study design: Prospective, randomized, placebo-controlled, experimental study.
Objectives: The issue of whether nitric oxide (NO) production is beneficial or deleterious on ischemic injuries of the central nervous system still remains doubtful. Vascular endothelial growth factor (VEGF) is known to induce the release of NO from endothelial cells. However, the effect of NO on VEGF synthesis is not clear. We aimed to determine the effects of L-arginine and NG-nitro-L-arginine methyl ester (L-NAME) on VEGF synthesis and free radicals in a rat model of spinal cord ischemia-reperfusion (IR) injury.
Setting: Surgical Research Laboratory of a Medical School.
Material and methods: Twenty-eight Wistar rats were divided into four groups as follows ($n=7$): Sham, IR injury, L-arginine, and L-NAME. Infrarenal abdominal aorta was occluded to induce spinal cord ischemia. L-Arginine (100 mg/kg) and L-NAME (10 mg/kg) were given before aortic occlusion. Biochemical assays of malondialdehyde (MDA), NO and VEGF were carried out in spinal cord specimens.
Results: L-Arginine treatment significantly increased MDA and NO, but decreased VEGF levels in spinal cord. However, nonselective inhibition of NOS with L-NAME significantly decreased MDA and NO, but increased VEGF levels. Besides, the positive linear correlation between MDA and NO, and negative linear correlations between MDA, NO and VEGF levels have also been demonstrated.
Conclusion: Nonselective inhibition of NO synthase activity with L-NAME attenuated free radical formation and increased VEGF level when compared with NO precursor L-arginine in a rat model of spinal cord ischemia. We suggest that inhibition of NO synthase, as well as induction of VEGF, may be a therapeutic option in spinal cord IR injury.
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Keywords: spinal cord injury; nitric oxide; vascular endothelial growth factor

Introduction

A dual role has been described for nitric oxide (NO) in ischemia-reperfusion (IR) injury depending on its concentration, source, redox state and concurrence with other molecules.[1] As a free radical itself, NO plays a major role in IR injury, because under certain conditions NO may act as a cytotoxic molecule.[2] On the other hand, the enhanced NO production in perivascular nerves and cerebrovascular endothelium may have a neuroprotective role by enhancing blood flow in the peri-infarct area.[3] Thus, NO may serve as both a mediator of neurotoxicity and as a neuroprotective agent during focal cerebral ischemia.

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Vascular endothelial growth factor (VEGF), a 45-kDa heparin-binding secreted homodimeric glycoprotein, is a potent mitogen of the vascular endothelium which stimulates angiogenesis and increases vessel permeability.[4,5] VEGF stimulates NO release from vascular endothelium and acts synergistically with NO in the cases of angiogenesis, permeability and anti-apoptotic effects on endothelial cells.[4,6] A trophic and protective role for VEGF in nervous system has also been described previously.[6,7] Moreover, Widenfalk *et al*.[5] showed that VEGF treatment improves functional outcome and decreases secondary degeneration in experimental spinal cord contusion injury.

As summarized above, the effect of NO modulation with L-arginine and NG-nitro-L-arginine methyl ester (L-NAME) on VEGF level in spinal cord IR injury is not defined. Accordingly, we aimed to determine the effects of NO donor 'L-arginine' and nonselective NO synthase (NOS) inhibitor

'L-NAME' on VEGF synthesis and free radicals in a rat model of spinal cord IR injury.

Materials and methods

The Institutional Animal Care and Use Committee approved this study. We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were strictly followed during the course of this research. This study was carried out on 28 adult male albino Wistar rats weighing between 200 and 250 g. The animals were acquired from the university vivarium sources and were housed in individual cages in a temperature and light–dark cycle-controlled environment with free access to food and water.

Experimental design
Animals were randomly assigned to experimental groups and were operated on in random order. Investigators blinded to the experimental groups made outcome assessments. Animals were divided into four groups as follows, where each was consisting of seven rats: Sham operation (Sham), spinal cord IR injury (IR injury), spinal cord IR injury and 'L-arginine' treatment (L-arginine), spinal cord IR injury and 'L-NAME' treatment (L-NAME).

Surgical procedure
Animals fasted for 12 h were anesthetized with a mixture of ketamine (20 mg/kg) and xylazine (9 mg/kg) injected intramuscularly, in an oxygen–room air mixture, and were allowed to breathe spontaneously. Experiments were performed at ambient room temperature (22–24°C). Rectal temperatures were maintained at 37–38°C using a heating lamp. A midline laparotomy was performed after skin shaving and preparation with 10% povidone–iodine solution. The intestines were reflected to the right and the infrarenal abdominal aorta was exposed and isolated. Abdominal aorta was occluded by placement of a microvascular clamp around the aorta at a level just distal to the left renal artery in order to induce ischemia in the lumbosacral segments of the spinal cord. Aortic clamps were removed after 30 min of warm ischemic insult and the lumbosacral segments of the spinal cord were reperfused for 120 min. Aortic occlusion and reperfusion were confirmed by the loss and reappearance of satisfactory pulsation in the distal aorta. In sham-operated animals (group 1), the same surgical procedure was applied without cross-clamping the abdominal aorta. An intraperitoneal dose of 100 mg/kg L-arginine (Sigma Chemical Co., St Louis, MO, USA) was given just before aortic occlusion in group 3. An intraperitoneal dose of 10 mg/kg L-NAME (Sigma Chemical Co.) was administered before aortic occlusion in group 4. All animals were exsanguinated through the right atrium at the end of the reperfusion. The whole lumbosacral segment of spinal cord was extracted and washed with saline solution, placed into glass bottles, labeled and stored in a deep freeze (−78°C) until the biochemical assays.

Biochemical procedures for malondialdehyde and NO assays
Briefly, the frozen sample of rat spinal cord was weighed and homogenized (Ultra Turrax T25, Staufen, Germany) (1:10, w/v) in 100 mmol/l phosphate buffer (pH 7.4) containing 0.05% sodium azide in an ice bath. The homogenate was sonicated (Bandelin, Berlin, Germany) for 30 s and centrifuged (5000 *g* for 10 min). The supernatant was frozen at −78°C in aliquots until used for biochemical assays. The protein content of the supernatant was determined using the Lowry method.[8]

Malondialdehyde assay Malondialdehyde (MDA) was estimated by the double-heating method of Draper and Hadley.[9] The principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA. For this purpose, 2.5 ml of 100 g/l trichloroacetic acid solution was added to 0.5 ml supernatant in each tube and the tubes placed in a boiling water bath for 15 min. After cooling in tap water, the tubes were centrifuged at 1000 *g* for 10 min, and 2 ml of the supernatant was added to 1 ml of 6.7 g/l TBA solution in a test tube and the tube placed in a boiling water bath for 15 min. The solution was then cooled in tap water and its absorbance was measured using a spectrophotometer (Shimadzu UV-1601, Kyoto, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of the MDA–TBA complex (absorbance coefficient $e = 1.56 \times 10^5\,\mathrm{cm}^{-1}\mathrm{M}^{-1}$) and is expressed as nanomoles per milligram of protein (nmol/mg protein).

NO assay. Because NO rapidly converts into nitrate and nitrite, sample reduction followed by measurement of nitrite by Griess reaction[10] has been used in determining total *in vivo* NO production. Reduction of nitrate to nitrite was accomplished by catalytic reaction using cadmium. The nitrite produced was determined by diazotization of sulfonilamide and coupling to naphthylethylene diamine. Absorbance of this complex was measured at 540 nm. Results were given as nmol/mg protein.

Biochemical procedure for VEGF assay
Tissue samples were homogenized in 400 μl PBS (pH 7.2). The homogenates were centrifuged at 15 000 r.p.m. for 30 min at 4°C. The supernatant was collected and stored at −78°C until analysis. VEGF concentration was determined using enzyme-linked immunosorbent assay kit for VEGF (Biosource, Camarillo, CA, USA) according to the manufacturer's specifications. The VEGF level of each sample was evaluated as the VEGF protein concentration (mg/l) divided by the total protein concentration (g/l) dissolved in sodium dodecyl sulfate solution. Results are expressed as picograms per milligram of tissue protein (pg/mg protein).

Statistical analysis
All statistical analyses were performed using SPSS 11.0. software (SPSS Inc., Chicago, IL, USA). Data were expressed

as mean plus or minus the standard error of the mean (s.e.m.). Differences among groups were analysed by one-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni test. Pearson correlation analysis was also carried out. A probability value less than 0.05 was considered significant ($P<0.05$).

Results

MDA levels of all experimental groups were shown in Figure 1. MDA was found 13.21 ± 0.70 in group 1. When spinal cord IR injury was induced, MDA level significantly rose to 19.60 ± 1.05 in group 2 ($P<0.05$). L-Arginine administration resulted in a further significant increase in MDA level to 25.29 ± 1.41 in group 3 ($P<0.05$). However, NOS inhibition with L-NAME administration significantly decreased the MDA level to 13.99 ± 0.79 in group 4 ($P<0.05$). MDA levels positively correlated with NO levels ($r^2=0.895$; $P<0.05$). A negative linear correlation was also noted between the levels of MDA and VEGF ($r^2=-0.427$; $P<0.05$).

NO levels of all experimental groups were shown in Figure 2. NO level was 36.91 ± 4.18 in group 1. A non-significant increase in NO level to 42.43 ± 4.72 was seen with the induction of spinal cord IR injury in group 2 ($P>0.05$). L-Arginine administration significantly increased the NO level to 118.99 ± 6.14 in group 3 ($P<0.05$). Inhibiting NOS with L-NAME significantly decreased the NO level to 33.94 ± 3.42 in group 4 ($P<0.05$). NO levels negatively correlated with the VEGF levels ($r^2=-0.427$; $P<0.05$).

VEGF levels of all experimental groups were shown in Figure 3. VEGF level was 753.14 ± 58.40 in group 1. Although it was not found to be significant when compared with group 1, spinal cord IR injury decreased VEGF level to 591.43 ± 43.64 in group 2 ($P<0.05$). L-Arginine administration decreased VEGF level significantly to 491.14 ± 27.56 in group 3 when compared with group 1 ($P<0.05$). When L-NAME administration inhibited NOS in group 4, VEGF level significantly increased to 1030.57 ± 66.38 ($P<0.05$). VEGF negatively correlated with both NO and MDA, as noted previously.

Discussion

In the present study, we found that L-arginine treatment significantly increased MDA and NO, but decreased VEGF levels in spinal cord. However, nonselective inhibition of NOS with L-NAME significantly decreased MDA and NO, but increased VEGF levels. Since VEGF is closely related with angiogenesis, its increase with L-NAME may play an important role in modulating spinal cord injury after an ischemic insult. To the best of our knowledge, the negative linear correlation between NO and VEGF levels has been demonstrated for the first time in spinal cord IR injury. This finding may induce the further investigation of VEGF in spinal cord IR injury, and may provide data for the consideration of VEGF as a therapeutic option.

It is increasingly apparent that VEGF may subserve multiple repair roles in the central and peripheral nervous systems that include angiogenic, blood–brain barrier permeabilizing, neurotrophic, gliotrophic and anti-apoptotic actions.[6] Expression of VEGF has been shown to be induced by several factors. One of the most important responses, in keeping with its role as an angiogenetic factor, is induction by hypoxia. VEGF transcription in hypoxic cells is upregulated by hypoxia inducible factor-1 alpha (HIF-1α).[7,11] In a previous study, it has been shown that NO plays an

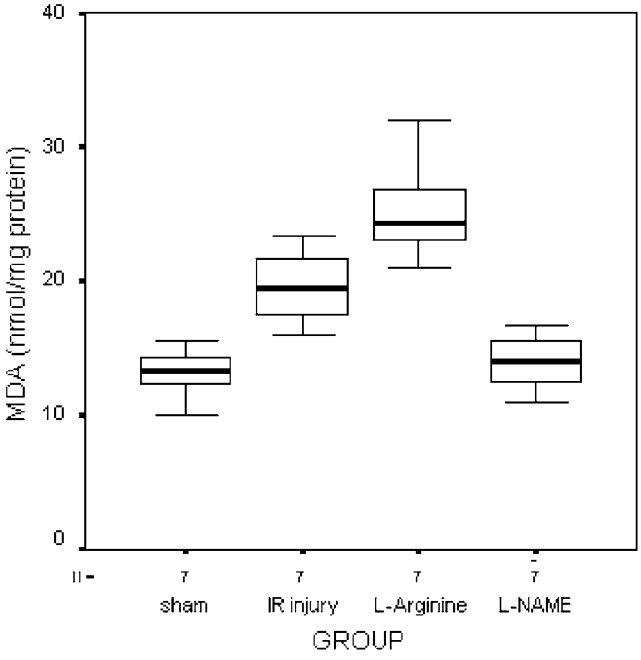

Figure 1 Boxplot of the MDA levels of groups. Horizontal bar represents the 90th, 75th, 50th (median), 25th and 10th percentiles.

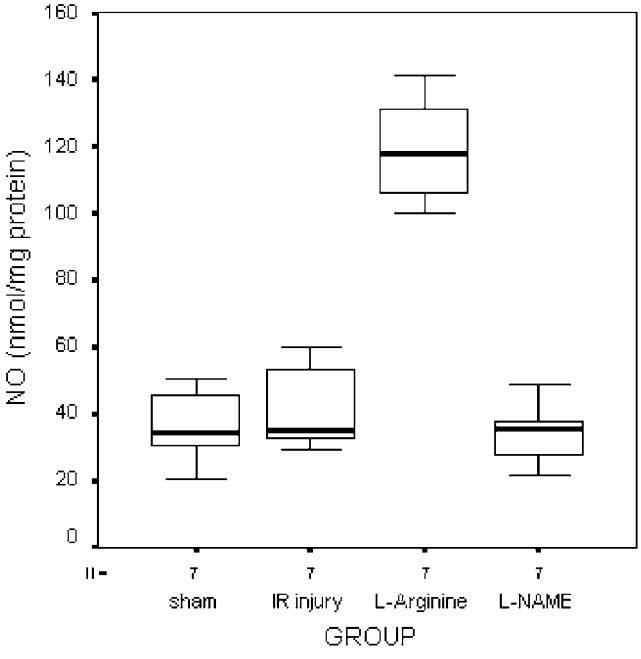

Figure 2 Boxplot of the NO levels of groups. Horizontal bar represents the 90th, 75th, 50th (median), 25th and 10th percentiles.

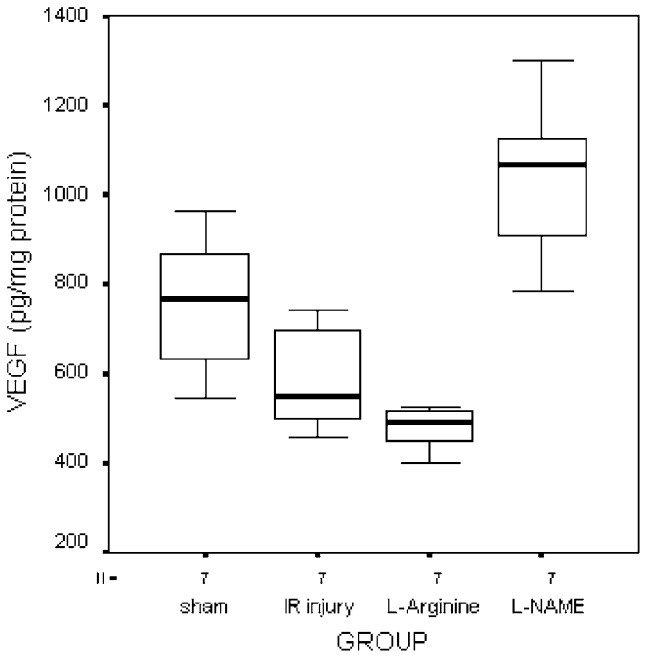

Figure 3 Boxplot of the VEGF levels of groups. Horizontal bar represents the 90th, 75th, 50th (median), 25th and 10th percentiles.

important role in the immunohistochemical expression of HIF-1α.[12] NO, in addition to mediating some of the VEGF effects, enhances its production through stabilization of HIF-1α.[7] VEGF is critical for angiogenesis but fails to induce neovascularization in ischemic tissue lesions in mice lacking endothelial NOS.[13] Interestingly, a negative linear correlation was found between the NO and VEGF in our study. VEGF is known to induce the release of NO from endothelial cells.[6,7] However, the effect of NO on VEGF synthesis is not clear. The results of Dulak *et al*.[14] indicate that endogenous NO enhances VEGF synthesis. In another study, inhibition of i-NOS resulted in reductions in wound VEGF expression. It has been concluded that VEGF production in granulation tissue is dependent on the presence of functionally active i-NOS and hence, the production of NO.[15] On the other hand, very similar results with ours have been achieved by Shihab *et al*.[16] In a rat model of chronic cyclosporine nephrotoxicity, VEGF expression is increased by NO blockade with L-NAME and decreased by NO enhancement with L-arginine.[16] In the present study, nonselective inhibition of NOS with L-NAME significantly decreased the free radical formation and NO production, and also significantly increased the VEGF level after spinal cord ischemia and reperfusion.

In a previous study, the use of NO donor nitroglycerine is associated with a high incidence of neurologic injury after thoracic aortic cross clamping in dogs.[17] To further evaluate the role of NO in spinal cord ischemia, Zhou *et al*.[18] sought to examine the expression of NOS in motoneurons of ventral horn of the lumbosacral spinal cord by using immunohistochemical methods in a rat model. Their work has shown that n-NOS and, i-NOS immunoreactivities can be induced in the ventral-horn motoneurons of lumbosacral spinal cord following transient ischemia. It is suggested that NO may be involved in the selective and delayed neuronal death in the spinal cord to the ischemic insult.[18] Beneficial effects of NOS inhibition on the neurological recovery after spinal cord injury has also been shown.[3,19] Nemoto *et al*.[3] has demonstrated that the inhibition of NO synthesis accelerates the recovery of reflex potential after transient spinal cord ischemia. Suzuki *et al*.[19] reported that NOS inhibition during the early stage of spinal cord injury has beneficial effects on the recovery of neurological function and the histopathological changes in the chronic stage. Recently, Genovese *et al*.[20] have demonstrated that inhibition of i-NOS resulted in a significant reduction in secondary damage, and this therapeutic efficacy was associated with the prevention of a spinal cord ischemia-induced decrease in n-NOS and e-NOS activities. Thus, one may speculate that the selective inhibition of i-NOS activity after spinal cord ischemia and reperfusion is expected to reduce the generation of NO-derived cytotoxic species, like peroxynitrites, which could render to be harmful for cell survival. This point represents one of the limitations of our study. Selective inhibition of NOS might have been preferred in this study instead of nonselective inhibition in order to see the isolated effects of all isoforms of NOS activities. However, in a previous work from Mexico, Diaz-Ruiz *et al*.[21] has documented that all isoforms of NOS seem to be involved in the tissue response to spinal cord injury by contributing to NO formation rate at the site of injury, at different times after insult, through diverse mechanisms and perhaps from varied cell sources. This finding may justify the usage of L-NAME for nonselective NOS inhibition in this study.

Although the previous studies discussed above support the aggrevating role of NO in spinal cord ischemia, the question whether NO production is beneficial or deleterious on ischemic injuries of the central nervous system still remains unanswered. For example, it has been suggested that nonselective inhibition of NOS activity has aggravating effects on the neurological and histopathological outcomes after transient spinal cord ischemia.[22] The very recent data from Chu and Wu[23] also adds a new line of evidence that expression of NOS is beneficial to the axonal regeneration of the injured spinal motoneurons. A very interesting data, opposite to ours, has been published previously.[11] The results of this above-mentioned study suggest the neuroprotective benefit of L-arginine in a rat model of spinal cord ischemia. In the above-mentioned study, L-arginine also reversed the increase in MDA levels to a considerable extent, and NO has been suggested to act as a free radical scavenger. As a free radical itself, it is hard to describe NO as a free radical scavenger. Although similar experimental models have been preferred in these two studies, achieving the inverse results with L-NAME when compared with L-arginine further supports the reliability of our data in this study.

The only limitation of this study may be that it focuses entirely on the lumbosacral segments of the spinal cord. MDA, NO and VEGF levels might also be measured in the adjacent spinal cord segments not effected by ischemia, and compared with the lumbosacral segments of the spinal cord. We believe that the sham-operated group better corresponds this necessity, because the consequences of IR injury might

also be seen in the adjacent segments of lumbosacral spinal cord.

Conclusion

Nonselective inhibition of nitric oxide synthase activity with L-NAME attenuated free radical formation and increased VEGF level when compared with NO precursor L-arginine in a rat model of spinal cord ischemia. To the best of our knowledge, it is the first evidence showing the negative linear correlation between NO and VEGF levels in a rat model of spinal cord ischemia and reperfusion. We suggest that inhibition of NOS, as well as induction of VEGF, may be a therapeutic option in IR-induced spinal cord injury. However, further data are needed in order to clarify the role of NO and VEGF in this model.

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