Nitric Oxide Regulation System in Degenerative Lumbar Disease

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Summary: Elevated nitric oxide (NO) production derived from NO synthase (NOS) activity has been shown in cerebrospinal fluid (CSF) in patients with degenerative lumbar disease (DLD). However, the regulatory mechanism of NO and the relationship of NO to clinical manifestations are unclear. In the present study, concentrations of NOx (nitrate NO\textsubscript{3} and nitrite, NO\textsubscript{2}\textsuperscript{-}) L-arginine (a substrate for NOS), and asymmetric dimethyl arginine (ADMA, an endogenous NOS inhibitor) in CSF were measured in two major DLDs: lumbar intervertebral disc herniation (LDH; 13 cases) and lumbar spinal canal stenosis (LSCS; 28 cases), and were compared with the levels in control patients with traumatic diseases. The levels of NO regulatory markers were also assessed according to Japanese Orthopedic Association (JOA) scores for the management of low back pain. NOx levels in LSCS patients (9.65±0.74 μM) were significantly higher than those in controls (5.26±0.27 μM) and in LDH patients (4.27±0.35 μM) (p<0.01). ADMA levels were lower in LDH patients (0.016±0.008 pM) than in controls (0.045±0.011 pM) (p<0.05), whereas those in LSCS patients (0.05±0.006 pM) were the same as in the controls. There was no significant difference in L-arginine levels among the groups. Nitrate or nitrite levels in these DLDs correlated with some of the JOA scores. The NO\textsubscript{2}\textsuperscript{-} concentration was positively correlated with both JOA15 and JOA6 scores in LDH cases (p<0.01). In LSCS cases, a positive correlation was found between NO\textsubscript{2}\textsuperscript{-} concentration and JOA6 score, and between NO\textsubscript{2}\textsuperscript{-} concentration and JOA9 (p<0.01). In immunohistochemical analysis, inducible NOS and dimethylarginine dimethylaminohydrolase were expressed in mononuclear cells in tissues obtained from LSCS patients along with nitrotyrosine deposition, a footprint of NO radical formation. Our data suggested that the NO regulatory mechanism controlling the pathogenesis and progression of LDH might differ from that of LSCS.

Key words nitric oxide (NO), inducible nitric oxide synthase (iNOS), asymmetric dimethyl arginine (ADMA), dimethylarginine dimethylaminohydrolase (DDAH), lumbar intervertebral disc herniation, lumbar spinal canal stenosis, degenerative lumbar disease

INTRODUCTION

Nitric oxide (NO) was initially discovered as an endothelium-derived relaxing factor, and is an important mediator in the regulation of vascular tone; it is formed from the amino acid precursor L-arginine by NO synthase (NOS) [1-4]. At least three isozymes of NOS exist: endothelial (constitutive) NOS (eNOS),...
cytokine-inducible NOS (iNOS), and neural (constitutive) NOS (nNOS). Accumulative evidence suggests that NO plays important roles in various disease processes [5,6]. In the field of orthopaedic surgery, Farrell et al. [7] first demonstrated a high concentration of NO in the synovial fluid of patients with rheumatoid arthritis and osteoarthritis, suggesting a close association between NO production and the pathogenesis of arthritis. Excess NO production has been shown to be involved in the pathogenesis of neural degenerative diseases such as Alzheimer’s disease [8-12]. Elevated NO production has also been shown in cerebrospinal fluid (CSF) in patients with degenerative lumbar disease (DLD) as compared with pain-free control patients [13,14]. Insights into the mechanisms by which NO production or NO activity is altered at various stages of DLD may lead to new therapeutic strategies. However, the regulatory mechanisms of NO and the relation of these mechanisms to clinical manifestations in DLD are unclear.

N⁶,N⁶-Dimethyl-L-arginine (ADMA) is an endogenous NOS inhibitor of eNOS and iNOS activities [15,16]. It has been demonstrated that increased plasma levels of ADMA are associated with hypertension [17], hypercholesterolemia [18], and atherosclerotic diseases in humans [19,20]. ADMA is metabolized by a key enzyme, N⁶,N⁶-dimethylarginine dimethylaminohydrolase (DDAH) [21-23]. DDAH has been identified in human aortic and brain tissues [24-28]. It has been reported that oxidized low-density lipoprotein upregulates the synthesis of ADMA in cultured human endothelial cells in association with decreased DDAH activity [27]. In vascular smooth muscle cells, cytokine-induced nitric oxide production is regulated not only by iNOS activity but also by DDAH [29]. Collectively, the ADMA-DDAH system may be involved in the regulation of endogenous NO synthesis and in the pathogenesis of human diseases [29-33] (Fig. 1). However, the potential role of this system in DLD has not been fully explored.

In the present study, concentrations of NOx (nitrate and nitrite), L-arginine, and ADMA were measured in patients who were diagnosed with one of two major DLDs: lumbar intervertebral disc herniation (LDH) or lumbar spinal canal stenosis (LSCS). The levels of NO regulatory markers were also assessed according to Japanese Orthopedic Association (JOA) scores for the management of low back pain [34]. A possible difference in NO regulatory mechanisms in the pathogenesis and progression of these DLDs is discussed.

**MATERIALS AND METHODS**

**Subjects and collection of CSF**

CSF was obtained from patients with LDH (n=13, 9 males and 4 females; mean age, 44 years; range, 18-73 years) and from patients with LSCS (n=28, 23 males and 5 females; mean age, 69 years; range, 50-87 years). The clinical status of DLD was evaluated according to JOA scores for the management of low back pain. A full score was 29 points: three subjective symptoms (JOA9; 9 points), three clinical signs (JOA6; 6 points), and seven activities of daily living (14 points). Lower leg pain (1B) and gaiting ability (1C) were also evaluated [34]. Hospitalized patients with traumatic disease who were free from chronic lumbar pain and free from specific neuromuscular diseases were used as controls (n=14, 6 males and 8 females; mean age, 57 years; range, 17-90 years). Control cases included fractures of the tibia (n=6), femur (n=4), patella (n=1), and calcaneum (n=1) and injury to ankle tendons (n=1). Informed consent for this study was obtained from all subjects. Collection of 1.5 ml of CSF was performed at the lumbar puncture during myelography in the DLD cases or during lumbar anesthesia in the control cases. The CSF was
collected mainly from the L4/5 level. The samples were collected in microcentrifuge tubes and centrifuged at 14,000 rpm for 10 min. The supernatant was stored at −80°C until use.

**Measurement of NO, ADMA, and L-arginine**

The production of nitric oxide (nitrite, NO$_2^-$ and nitrate, NO$_3^-$) in CSF was measured by the Griess method [35] using an ENO-10 NOX analyzer (Eicom, Kyoto, Japan), and the data were analyzed with a Chromatocorder 21 (System Instruments, Tokyo, Japan). The analyzing system was calibrated by a NOx standard solution (Tokyo Kasei Kogyo, Tokyo, Japan) before measurement. The total nitrite reacted with the Griess reagent to form a purple azo compound. The absorbance at 540 nm was measured spectrophotometrically. Nitrate was reduced to nitrite using a Cd-Cu reduction column (Tokyo Kasei Kogyo). Each sample was measured three times, and the average was used for the data analysis.

ADMA was analyzed by high-performance liquid chromatography (HPLC) employing a column switching method [29] consisting of two separation steps: a precolumn isolated the crude fraction containing dimethylarginines and then an analytical column separated ADMA. Samples were deproteinized in the manner described above, and the supernatants obtained were incubated for 3 min with OPA (o-phthalaldehyde) reagent (2.3 mg/ml OPA in borate buffer, pH 9.7, containing 2.3 mg/ml N-acetyllysine) before automatic injection (injection volume, 15 μl) into the HPLC column (temperature, 49°C). HPLC was performed on a Hitachi L-6300 system equipped with a F-1080 fluorescence detector for excitation at 348 nm and emission at 450 nm. The precolumn, an anion-exchange column (Shodex NH2P50, 4.6 mm×30 mm), was run with a mobile phase of 75 mM sodium acetate buffer (pH 6.0) containing 3% acetonitrile at a flow rate of 0.8 ml/min. The analytical column, an ODS column (L-column ODS, 5 μm, 4.6 mm×250 mm, Chemical Inspection and Testing Institute, Tokyo, Japan), was run with a mobile phase of 75 mM sodium acetate buffer (pH 6.0) containing 2.5% tetrahydrofuran at a flow rate of 0.8 ml/min. The intra- and inter-assay variences were 2.8 and 2.4%, respectively, and the detection limit of the assay was 0.02 nmol/ml. L-arginine was also analyzed by HPLC at the Special Reference Laboratory (SRL; Tokyo, Japan).

**Immunohistochemical analysis**

Resected yellow ligament tissues were obtained from surgical cases of LSCS. The tissues were fixed in 10% formalin for 24 hrs. After dehydration with 70-100% ethanol, the tissues were embedded in paraffin wax. Sections (2 μm) were stained with hematoxylin and eosin for conventional histological characterization of the lesions. Another series of sections was used for immunohistochemical detection with an LSAB staining kit (DAKO Japan, Kyoto, Japan), and counterstaining was performed with Mayer’s hematoxylin. To detect iNOS protein, the sections were incubated with a 1:200 dilution of rabbit polyclonal anti-mouse macrophase NOS antibody (Affinity Bioreagents, Golden, CO, USA). To detect DDAH protein, the sections were incubated with a 1:400 dilution of a polyclonal rabbit anti-human DDAH antibody (Kato S. et al., manuscript in preparation). Sections were also tested with a 1:200 dilution of rabbit polyclonal anti-nitrotyrosine antibody for detecting the footprint of peroxynitrite production (Upstate Biotechnology, Lake Placid, NY, USA). All primary antibodies were incubated overnight at 4°C. For the negative control, primary antibodies were omitted or were replaced by nonspecific serum. For cell identification, antibodies against vimentin (for myofibroblasts), CD34 (for endothelial cells), CD68 (for macrophages), or S-100 (histiocytes) (Dako Japan) were also tested.

**Statistical analysis**

The experimental groups were compared by analysis of variance (ANOVA), and, when appropriate, by Scheffe's test for multiple comparisons. All data were expressed as means ± S.E. A level of p<0.05 was accepted as statistically significant. Common logarithms were used for the analysis of the NOx data. The correlations between each of the NO regulatory factors (log [NO$_2^-$], log [NO$_3^-$], L-arginine concentration, ADMA concentration) and patient age, and the correlations between each factor and JOA scores were analyzed by Pearson correlation coefficients.

**RESULTS**

**Levels of NO regulatory factors in CSF of DLD patients**

The levels of nitrite (1.71±0.18 μM), nitrate (7.94±0.56 μM), and total NOx (9.65±0.74 μM) in LSCS patients were significantly higher than those in controls (NO$_2^-$, 1.13±0.07 μM, p<0.05; NO$_3^-$, 4.13±0.20 μM, p<0.01; total NOx, 5.26±0.27 μM,
p<0.01, respectively). On the other hand, levels of nitrite (1.15±0.02 μM), nitrate (3.12±0.33 μM), and total NOx (4.27±0.35 μM) in LDH patients did not differ significantly from the control values (Fig. 2). There was no significant difference in the L-arginine level among groups (17.06±0.86 pM in control; 17.23±0.78 pM in LDH; 18.9±0.52 pM in LSCS) (Fig. 3A). The ADMA level was lower in LDH patients (0.016±0.008 pM) than in controls (0.045±0.011 pM) (p<0.05), whereas the ADMA level in LSCS patients (0.05±0.006 pM) was similar to that in controls (Fig. 3B).

**Correlation coefficients between NO regulatory factors and patient age**

To investigate whether aging affects the concentrations of NO regulatory factors in CSF, correlation coefficients were analyzed in each study group (controls, LHD cases, and LSCS cases). No age-related change in the concentration of nitrite, nitrate, L-arginine, or ADMA was observed in either of the groups (Table 1).

**Correlation coefficients between NOx and JOA score in LDH and LSCS**

The data on the correlation coefficients between NOx and JOA scores in DLD cases are summarized in Table 2. There was no significant correlation between total NOx and JOA scores. However, the NOx concentration in CSF was positively correlated with both JOA15 and JOA6 in LDH cases (p<0.01, respectively). In LSCS cases, a positive correlation was found in the relationship between NOx concentration and JOA6 score, and between NOx concentration and JOA9 (p<0.01).

**Immunohistochemical detection of iNOS, DDAH, and nitrotyrosine in LSCS**

Immunohistochemical analysis was performed

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**Fig. 2.** NOx concentrations measured in the CSF of DLD patients. Concentrations of nitrite (NO2⁻) and nitrate (NO3⁻) were separately measured by the Griess method as described in Materials and Methods. Open bars, control; Hatched bars, LDH; Solid bars, LSCS cases. The values shown are means±SE.

**Fig. 3.** L-arginine (A) and ADMA (B) concentrations in CSF of DLD patients. Both concentrations were measured by the HPLC method as described in Materials and Methods. Open bars, control; Hatched bars, LDH cases; Solid bars, LSCS cases. The values shown are means±SE.
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<tr>
<th>TABLE 1.</th>
<th>The relationship between NO regulatory markers (nitrite, NO\textsubscript{2}⁻ ; nitrate, NO\textsubscript{3}⁻ ; L-arginine, L-Arg; and ADMA) in CSF and age in the control, LDH, and LSCS cases.</th>
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<th>TABLE 2.</th>
<th>The relationships between NOx (nitrite, NO\textsubscript{2}⁻ ; nitrate, NO\textsubscript{3}⁻ ) in CSF and JOA score in the LDH and LSCS cases.</th>
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*P; statistically significant.
using yellow ligament tissues which were surgically resected in the LSCS cases that showed relatively high levels of NOx in CSF (n=2). Conventional histological examination revealed hyaline and myxomatous degeneration in the fibrotic yellow ligament tissue of these LSCS cases, in association with decreased numbers of viable mesenchymal cells. iNOS and DDAH were expressed in some of the mononuclear cells in the lesion, which were thought to be S-100-positive histiocytes. Nitrotyrosine deposition was observed in the cells as well as in the matrix (Fig. 4).

**DISCUSSION**

NO regulatory factors in CSF and aging

Reports on age-related changes in NOx concentration in CSF have been controversial. It has been reported that the NOx concentration in CSF decreases with age in association with the age-dependent decrease in NADPH-diaphorase activity in the brain,

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a marker for neurons containing NOS [36,37]. On the other hand, another study reported no age-related change in NOx concentration in CSF [38]. In the present study, the NOx concentration in CSF did not show any correlation with aging in either of the study groups (control, LDH, and LSCS groups) (Table 1). Thus we concluded that age-matching analysis was not necessary for the interpretation of the present data. L-arginine is a substrate for NOS [1-4]; age-related changes in the level of L-arginine in CSF were not seen, which was consistent with previous reports [38,39] (Table 1). The present data did not suggest age-related change in ADMA, an endogenous NOS inhibitor (Table 1). The reduction of ADMA in the CSF during aging and in patients with Alzheimer’s disease has been reported [39], although another report found no specific change in the ADMA concentration in the CSF of Alzheimer’s disease patients [40]. Thus a larger number of materials will need to be analyzed by a standardized method in order to reach a conclusion about the relationship between these NO regulatory factors and aging or age-related diseases.

**NOx levels in CSF of DLD**

NOx (NO\(^2\)- and NO\(^3\)-) levels in LSCS cases were significantly higher than those in the controls and in LDH cases. NOx levels were comparable between LDH cases and control cases (Fig. 2). It has been reported that NOx was significantly higher in patients with DLD (including both LDH and LSCS) than in an age-matched control group, and that the duration of pain was much longer in the DLD group than in the control group [36]. Although this report may be partially consistent with our data, the difference in NOx levels between LDH and LSCS cases should be discussed. It has been reported that, in various DLD cases, the disc level at the compressed spinal nerve is positively correlated with elevated NO levels in CSF [41]. Decreased NOx levels in CSF of DLD cases have been shown to be associated with post-operative pain relief [42]. NOx activity in the spinal cord is mainly localized in the cornu posterior and substantia intermedia, but not in the cornu anterior [43]. In a rat model, electrical stimulation of the dorsomedial white matter adjacent to the substantia gelatinosa in the spinal cord elicited the release of NO [44]. Collectively, a close association between the NOx level in CSF and the regulatory mechanism of pain or the sensory nerve system may be suggested. However, in the present study there was no statistical difference in the average JOA9 score (subjective symptoms including pain) between LDH cases (3.8±0.47 points) and LSCS cases (3.4±0.35 points). The only correlation recognized was between JOA9 score and NOx level in LSCS cases (Table 2). Thus, the mechanism of pain and the involvement of the sensory nerve system may not fully account for the difference in NO regulation between LDH and LSCS cases.

Since we did not find elevated NOx levels in CSF of LDH cases, we further examined individual cases. Two LDH cases (n=13) showed relatively high levels of NOx, and magnetic resonance imaging revealed stenotic change in the spinal canal caused by disc herniation (data not shown). It is possible that the mechanism associated with spinal canal stenosis is closely related to the elevation of NOx level in CSF even in the LDH cases. Moreover, it has been reported that microcirculatory disturbance of vessels in the cauda equina plays an important role in the development of intermittent claudication and multiple-level cauda equina compression in LSCS [45,46]. These reports suggest a close relationship between circulatory disturbance and mal-regulation of NOx level in LSCS [5,6]. Taken together, these findings suggested that the elevation of NOx levels in LSCS but not in LDH may be based on various pathophysiological factors such as the mechanism of pain, stimulation of the sensory nerve system, mechanical spinal stenosis, and microcirculation. Further study should be conducted to elucidate the NO regulatory mechanism in DLD.

**ADMA-DDAH system in DLD**

As mentioned in the introduction section, NOS activity is potentially regulated by the ADMA-DDAH system in addition to the post-transcriptional regulation of the NOS gene [29]. As the NOx level in CSF was significantly elevated in LSCS cases, immunohistochemical analysis was performed using resected yellow ligament tissue to test the possible involvement of the ADMA-DDAH system. In the degenerative ligament tissue, mononuclear cells (histiocytes) expressed both iNOS and DDAH in association with cellular and stromal deposition of nitrotyrosine, a footprint of peroxynitrite (Fig. 4). Thus, cytokine-induced NO synthesis may take place in the lesion, together with involvement of the ADMA-DDAH system, which we can assume is the cause of NO radical formation leading to tissue destruction.

In the present study, ADMA levels were lower in the CSF of LDH cases than in control cases, whereas
the level of NOxs was unchanged. Although ADMA levels in the CSF of LSCS cases were comparable with those in control cases, the levels of NOxs were elevated (Fig. 3 and Fig. 2). These data suggest that ADMA-mediated regulation of NOS activity cannot completely explain total NOx production in CSF. NOx in CSF may be differentially (or more systematically) regulated in the injured tissue.

CONCLUSION

In the present study, we measured the concentrations of NOx, L-arginine, and ADMA in the CSF of LDH and LSCS cases. NOx levels were significantly higher in LSCS cases than in control or LDH cases. Thus the NO regulatory mechanism controlling the pathogenesis and progression of LDH may differ from that of LSCS. Although the ADMA-DDAAH system may be partly involved in the NO regulatory system in DLD, the possibility that the regulation of ADMA level in CSF is independent from that of local DDAAH activity cannot be denied. Understanding the NO regulatory mechanism in DLD may lead to the development of new therapeutic approaches.

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REFERENCES


