ANTIOXIDANTS & REDOX SIGNALING
Volume 6, Number 3, 2004

Forum Original Research Communication

Nitric Oxide as a Prognostic Marker for Neurological Diseases

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ABSTRACT

The potential value of the nitric oxide (NO) level as a prognostic marker in human brain diseases is investigated. Cerebrospinal fluid (CSF) collected from neurological patients was examined for NO content using electron paramagnetic resonance (EPR) spectroscopy. In adult patients with meningitis, the level of NO was higher than that in other groups of brain disorders, such as brain traumas and brain tumors. Very high levels of NO in the CSF appeared to be correlated with a high incidence of fatal outcomes. In children with meningitis, it was possible to differentiate between viral and bacterial origin of the disease as evidenced by the EPR analysis of the CSF. The results indicated that NO levels in the CSF can be a useful prognostic marker in neurological diseases. *Antioxid. Redox Signal.* 6, 613–617.

INTRODUCTION

MANY TISSUES CONTAIN intracellular nitric oxide (NO) synthases (NOSs), a family of NO-generating enzymes that generate NO by converting L-arginine to L-citrulline in the cells of many tissues (2). NO interacts with various biomolecules as a biological mediator, modulator, and effector. Biosynthesis of NO in various tissues is due to the activity of the three isoenzymes of NOS (16). Among them, neuronal NOS and endothelial NOS are constitutive enzymes. NO produced by the above constitutive NOSs is involved in various physiological activities. The inducible NOS (NOS2) normally is not expressed in the majority of the cells, but may be induced by certain proinflammatory cytokines (16).

The decrease in basal NO release may favor the development of hypertension, thrombosis, vasospasm, and atherosclerosis (11). On the other hand, a high level of NO, which may develop as a result of excessive NOS2 expression, is toxic (3, 16). This condition is usually connected with a nonspecific immunological response, with the symptoms of various pathologies, and in the rejection of organ graft, as well as with a septic shock (15) and certain neurological dysfunctions (14, 17). Expression of NOS2 and production of NO are possible in the astrocytes, microglia, and macrophages of the central nervous system (4, 5). Oligodendrocytes were reported to express NOS2 in the rat (6), but no data pertaining to patients are available.

NO can be produced in animal and human body in a wide range of concentration. Sometimes, the level of NO in the organism may be very low and is attributed to an underproduction of NO. On the other hand, the accumulation of NO in excessive amounts due to its overproduction can also be observed. Between these two extremes, a level of NO production that could be defined as normal can be expected. For example, in the case of healthy individuals the concentrations between 4 and 10 μmol/L of NO present in the cerebrospinal fluid (CSF) can be considered normal (10, 13). Similar amounts can be found in patients with headache, vertigo, or hypertension or who are human immunodeficiency virus (HIV)-positive who did not reach an advanced stage of the disorder or disease (13). All of them can be classified to be neurologically healthy. In terminal HIV patients, the level of NO may reach 4 μmol/L or even drop to zero. In endothelial cells, a 1,000 times lower amounts (4 nmol/L) can be found in

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human umbilical veins. The levels of NO around 5–8 μmol/L are detectable in the islets of Langerhans, while they are slightly lower in peripheral blood, reaching a concentration around 3–4 μmol/L. The production of NO may reach a very high level under various circumstances. These are in particular: (a) infections, *i.e.,* 70 μmol/L of NO in blood during septic shock (14); (b) transplant rejection; (c) tumor defense, *e.g.,* human carcinoma growing in nude mice (9); and (d) autoimmune diseases, *e.g.,* multiple sclerosis (6).

Cytotoxicity of NO brings about cell death due to necrosis or apoptosis (12). The latter effect in particular manifests in various types of cells such as thymocytes, T cells, myeloid cells, or neurons. NO causes permeability of mitochondrial membranes, leading to the production of reactive oxygen species (14). NO and peroxynitrite lead to lipid peroxidation, consumption of intracellular antioxidants (1), DNA damage (7), and inhibition of enzymes, *e.g.,* aconitase, glyceraldehyde-3-phosphate dehydrogenase, and ribonucleotide reductase, and ultimately cause cytotoxicity. This article is mainly based on our clinical observations of neurological patients, both adult and children. The site where brain dysfunctions may be reflected is certainly the CSF. Samples of CSF are frequently examined for diagnosis of brain diseases. The concentration of NO in CSF can be conveniently and precisely determined by electron paramagnetic resonance (EPR) spectroscopy. We measured the level of NO in the CSF of several neurological patients, including adults and children. The results showed a high level of NO in patients with meningitis as compared with the patients with other brain diseases.

MATERIALS AND METHODS

CSF

CSF was obtained by lumbar puncture. This is usually done during routine diagnostic examinations. A very small quantity of the fluid is sufficient for the determination of NO by EPR spectroscopy. The CSF examined in the present study originated from two sources: 129 samples from the adult patients from the Clinics of Neurotraumatology of the Jagiellonian University and 72 samples from pediatric patients from the Department of Neuroinfections of Cracow City Hospital. The age of adult patients varied between 16 and 69 years (mean 42.3 ± 1.42 years), and the age of pediatric patients varied between 1 month and 14.5 years. Usually more than one determination of the NO content was done in the same patient during the treatment. This only seldom happened in the case of children because the examination was performed only for diagnostic purposes. All patients survived, and there was no need to monitor the progress following treatment and draw CSF more than once. All pediatric cases were limited to only two diseases: viral and bacterial meningitis. In contrast, the number of conditions that were treated in adult patients was much higher and included various types of hematomas, cerebral contusions, brainstem damages, brain abscesses, skull bone fractures, and others, as listed in Table 1. The other two brain diseases that were also covered by the protocol of observations included brain tumors and meningitis.

TABLE 1. CLINICAL DIAGNOSIS, NUMBER OF PATIENTS, AND NUMBER OF SAMPLES

Clinical diagnosis	*Number of*	
	Patients	*Samples*
Trauma: hematomas: subdural, epidural, intracerebral; cerebral contusions; brainstem damages; dura-plastics; brain abscesses; skull bone fractures	30	58
Brain neoplasms: astrocytoma; glioma malignum; oligodendroglioma; blastoma	10	41
Meningitis	21	30

Preparation of CSF for EPR analysis

The samples of CSF were drawn by lumbar puncture and stored for 1 h at 4°C before further handling. Seventeen milligrams of hemoglobin (Hb) (Sigma-Aldrich, Milwaukee, WI, U.S.A.) was mixed with 9 mg of sodium dithionite ($Na_2S_2O_4$) (Sigma-Aldrich) and dissolved in 0.1 ml of phosphate-buffered saline (PBS). Then 0.5 ml of CSF was added to this solution. This mixture was frozen after 7 min in liquid nitrogen and stored at this temperature until EPR analysis. The Hb was added as a spin trap for NO, while sodium dithionite was expected to reduce ferric iron and nitrite to NO. Samples were kept under an argon atmosphere to avoid the reaction of NO in the air with the sample. The control samples were treated exactly the same way as described above.

EPR measurements

EPR measurements were carried out at 77 K using a Bruker 300E EPR spectrometer (Bruker Instruments, Karlsruhe, Germany). The measurements were done at 20 mW microwave power, 5 G modulation amplitude, 100 kHz modulation frequency, 10 ms time constant, 500 G scan range, 9.48 GHz microwave frequency, and 4E5 receiver gain, using 10 scans at a 21-s scan time. The amplitude of the second line of the triplet hyperfine structure of the Hb-NO EPR signal (Fig. 1) was used for quantitation. 1,1-Diphenyl-2-picrylhydrazyl was used as a reference.

Statistical analysis

The mean and SEM of the data were calculated and presented. All data were subjected to one-way analysis of variance for statistical significance.

RESULTS

The patients included in the present study were diagnosed with many types of neurological diseases. The adult patients were divided into three groups (Table 1): (1) patients with craniocerebral traumas (58 samples from 30 patients), (2) patients with brain tumors (41 samples from 10 patients), and (3) patients with meningitis that was sometimes connected

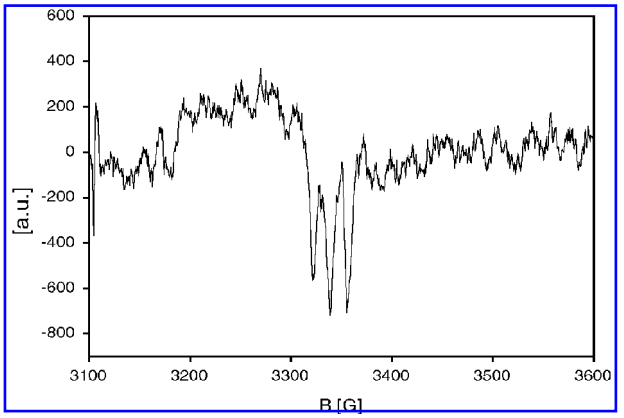

FIG. 1. A typical EPR spectrum of Hb-NO complex measured in the CSF of a patient with brain disease.

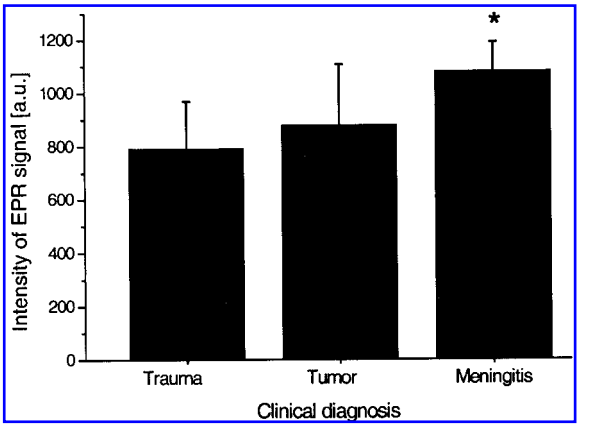

FIG. 2. EPR signal intensity due to NO in CSF from patients with various types of brain diseases. *$p < 0.05$, statistically significant compared with that of trauma.

with other diseases (30 samples from 21 patients). Glasgow Coma Score (GSC) (18) and Glasgow Outcome Scale (GOS) (8) were estimated as a neurological condition and the outcome of treatment, respectively. In the second group of patients only children were included. Their ages ranged between 1 month and 14.5 years, and they suffered from bacterial or viral meningitis (Table 2). In all cases, the level of NO in the CSF was determined by EPR spectroscopy.

Level of NO in the CSF of patients with various types of brain diseases

The pathological conditions included in the present study are listed in Table 1. The concentrations of NO in the CSF under these disease states are shown in Fig. 2. The data show that the amount of NO is the highest in the patients with meningitis [1,080 ± 110 arbitrary units (a.u.)], and it significantly exceeds the concentrations observed in the patients with brain trauma (791 ± 177 a.u.), but not in the patients with brain tumors (878 ± 229 a.u.).

Mortality in patients with various brain diseases

The percentage of mortality in the patients with brain disease is shown in Fig. 3. The mortality is the highest in the adult patients with meningitis, whereas it does not differ distinctly for the other two groups of diseases (brain tumors and brain lesions). The mortality goes hand in hand with the elevated concentrations of NO in the CSF of these patients, and its highest levels seem to be associated with the poorest chance of survival (Fig. 4). In addition, it may also be connected with appropriate values of GCS and GOS parameters (Fig. 5).

Concentration of NO in CSF of children with meningitis

Five types of bacteria and four types of viruses were diagnosed to be responsible for meningitis in children as shown in Table 2. The amount of NO was found to be markedly different in the meningitis of bacterial origin compared with that in the meningitis of viral origins (Fig. 6). The CSF contained a 10-fold higher concentration of NO in the cases of meningitis of bacterial origin (1,452 ± 172 a.u.) compared with the content found in the cases of meningitis of viral origin (144 ± 53 a.u.).

TABLE 2. CLINICAL DIAGNOSIS AND NUMBER OF SAMPLES FOR PEDIATRIC PATIENTS

Clinical diagnosis	*Number of samples*
Bacterial (*Neisseria meningitidis, Haemophilus influenzae, Escherichia coli, Enterobacter, Diplococcus pneumoniae*)	43
Viral (*Paramyxovirus paratides,* Coxsackie virus, varicella-zoster, ECHO group viruses)	29

ECHO virus, enteric cytopathic human orphan virus.

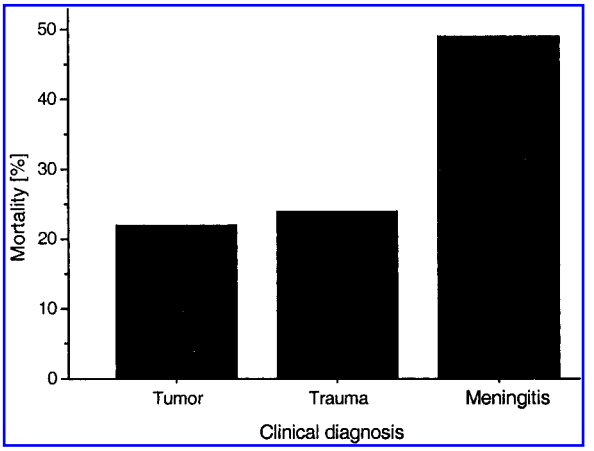

FIG. 3. Mortality in various types of brain diseases.

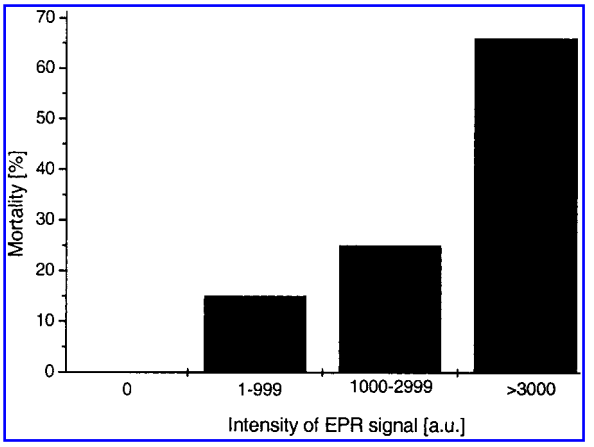

FIG. 4. Probability of mortality and EPR signal intensity of NO in CSF.

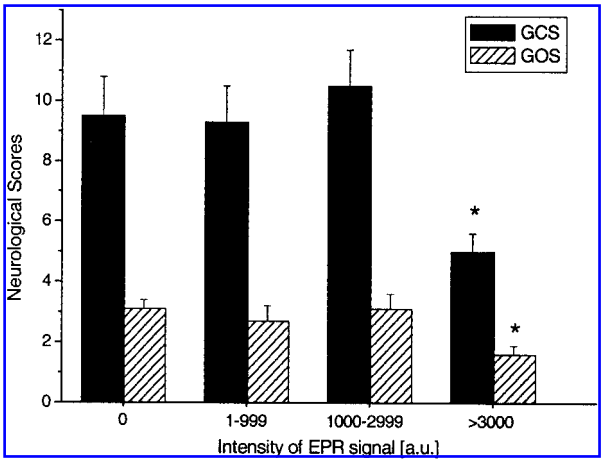

FIG. 5. Correlation of GCS and GOS neurological scores with EPR signal intensity of NO in the CSF of patients with brain diseases. Data are mean and SEM values. $*p < 0.05$, statistically significant compared with the respective scores with lower EPR intensities.

DISCUSSION

Cell communication and in particular neuronal transmission are crucial and vital processes that involve moderate amounts of NO. High concentrations of NO generated within the brain have been reported to cause damage (14, 17). Thus, it is important to determine the concentration of NO during brain diseases. This also explains the rationale for initiating the measurements of NO in our laboratory both in adult patients suffering from certain brain dysfunctions and in children with meningitis. Extensive differences in the amounts of NO were noticed between various individuals under examination. Therefore, it was justified to seek a correlation between the level of NO in the CSF of patients with neurological problems or those with bacterial or viral meningitis who have the best chances of survival.

On the contrary, it was observed that the decrease in the levels of NO, as a rule, was followed by an improvement in the patients. In light of these facts, it is likely that the level of NO may have the value of a useful prognostic marker, at least in certain types of brain diseases. The first question that may deserve consideration in this context is whether the excessive levels of NO in the body indicate deterioration in the condition of patients. In oncology, the measurement of the content of NO in body fluids is not clinically used. The only exceptions are the tumors of the central nervous system. Here the analysis of the NO content in CSF of patients has been conducted for the first time by our group and will perhaps become a useful prognostic marker. The amount of NO in the ascites fluid in the body cavity of some terminal patients is also under investigation for some time in our laboratory as well. The concentration of NO is also under investigation in the synovial fluid of patients with arthritis. The prognostic value of this parameter will be evaluated in the near future (19).

Quantitative EPR studies on the CSF appear to lead to a number of questions. First of all, one should ask whether the type of brain disease has any connection with the amount of NO that is found in the sample. As one can see from Fig. 2 such a relation can be found. The adult patients with meningitis seem to have the highest level of NO in the CSF, whereas those who suffer from brain trauma and tumors possess somewhat lower amounts (Fig. 2). This indicates that brain infections are associated with a more intense reaction of inducible NOSs than brain traumas and brain tumors. Quite interesting are the data obtained from children with meningitis. It can be concluded from the EPR results that the amount of NO markedly varies between bacterial and viral origin of the disease (Fig. 6). It is many times higher in bacterial meningitis as compared with the viral meningitis. It is very important to note that the EPR determination of the level of NO is much faster as compared with the time required for the bacteriological assay. So, it is convincing that the EPR measurement of NO in the CSF may very likely emerge as a useful prognostic marker in the future.

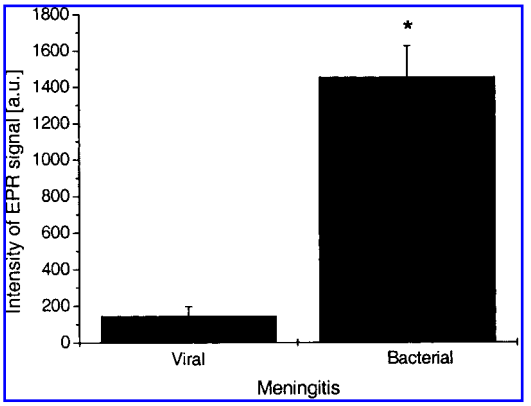

FIG. 6. Intensity of EPR signal of NO in CSF of children with viral and bacterial meningitis. Data are mean and SEM values. $*p < 0.05$, data statistically significant.

CONCLUSIONS

There is no general rule that would predict that high levels of NO should be deleterious or beneficial for an organism. Both options are possible in different animals and in humans. From the clinical observations based on the EPR data presented in this study it is concluded that: (1) The type of brain disease may determine an average content of NO in the CSF of patients; (2) a very high amount of NO in the CSF is associated with a poor prognosis, including a high probability of fatal outcome; (3) EPR measurement of NO concentration in CSF of children with meningitis makes it possible to differentiate between viral and bacterial background of this disease quickly and easily; and (4) all these facts seem to justify an expectation that the level of NO in the CSF of neurological patients is a likely useful indicator of prognostic value.

ACKNOWLEDGMENTS

The authors thank Dr. Periannan Kuppusamy for his suggestions in preparation and revising this manuscript.

ABBREVIATIONS

CSF, cerebrospinal fluid; EPR, electron paramagnetic resonance; GOS, Glasgow Outcome Scale; GSC, Glasgow Coma Score; Hb, hemoglobin; Hb-NO, nitrosylhemoglobin; HIV, human immunodeficiency virus; NO, nitric oxide; NOS, nitric oxide synthase; NOS2, inducible nitric oxide synthase; PBS, phosphate-buffered saline.

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Received for publication January 19, 2004; accepted February 19, 2004.

This article has been cited by:

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