EFFECT OF NITRIC OXIDE METABOLISM UPON PERIPHERAL BLOOD CHEMILUMINISCENCE AND LEUKOCYTE AND ERYTHROCYTE ADHERENCE IN MULTIPLE SCLEROSIS

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VLIV METABOLISMU OXIDU DUSNATÉHO NA CHEMILUMINISCENCI OBVODOVÉ KRVE A ADHERENCI LEUKOCYTŮ A ERYTROCYTŮ U NEMOCNÝCH ROZTROUŠENOU SKLERÓZOU MOZKOMÍŠNÍ

Abstract

Mayer M, Hermanova Z:
Effect of nitric oxide metabolism upon peripheral blood chemiluminiscence and leukocyte and erythrocyte adherence in multiple sclerosis
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Background: **It has become increasingly apparent that nitric oxide (NO) and related metabolic and effector systems play an important role in immune regulations, in inflammatory response and in cellular and tissue damage in multiple sclerosis (MS) and in its experimental models.**
Objectives: **Various adherence interactions and metabolism of reactive oxygen species belong to the cellular functions associated with NO metabolism. The aim of the present study was to evaluate the effect of chemical compounds affecting NO metabolism on adhesive properties of leukocytes and erythrocytes and on the blood chemiluminiscence in MS patients in comparison with controls. The effects of N^G-Methyl-L-Arginine (N^GMLA), a specific competitive NO antagonist, L-arginin, NO precursor, and sodium nitroprusside, an NO-releasing agent were tested.**
Me*thods:* **Luminol dependent chemiluminiscence was measured with and without respective drug administration, the results were expressed as chemiluminiscence index. Adherence of peripheral blood cells to nylon wool was used for the adherence studies, the results were expressed in a form of adhesivity index. Studies were performed in a group of patients with active multiple sclerosis, in a group of neurological controls and in a group of healthy controls. The differences between MS patients and control groups were statistically evaluated using Mann and Whitney U-test.**
Main results: **Sodium nitroprusside stimulated the luminol-dependent chemiluminiscence in MS patients and inhibited it in**

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Abstrakt

Mayer M., Heřmanová Z.:
Vliv metabolismu oxidu dusnatého na chemiluminiscenci obvodové krve a adherenci leukocytů a erytrocytů u nemocných roztroušenou sklerózou mozkomíšní
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Pozadí problému: V posledních letech přibývá informací o důležité roli oxidu dusnatého (NO) v imunitních regulacích, zánětlivých procesech a v mechanismech buněčného a tkáňového postižení u roztroušené sklerózy mozkomíšní (RS) a u jejích experimentálních modelů.
Cíl a východiska studie: Adherenční interakce a metabolismus reaktivních sloučenin kyslíku patří k buněčným funkcím úzce s metabolismem NO souvisejícím. Cílem předkládané studie bylo zjistit účinek látek ovlivňujících metabolismus NO a na adhezivní vlastnosti leukocytů a erytrocytů na chemiluminiscenci obvodové krve u nemocných s aktivní RS a u kontrolních skupin zdravých kontrol a kontrol neurologických. Testovali jsme účinek N^G-methyl-L-argininu (N^GMLA), specifického kompetitivního NO antagonisty, L-argininu, metabolického prekursoru NO, a nitroprussidu sodného, látky uvolňující NO.
Metody: Luminol-dependentní chemiluminiscence byla měřena bez přítomnosti a s přidáním příslušné látky a adherence buněk obvodové krve k nylonové vatě byla použita pro studie adherence. Neparametrický Mannův—Whitneyho U-test by použit pro statistické vyhodnocení.
Hlavní výsledky: Nitroprusid sodný statisticky významně stimuloval luminol-dependentní chemiluminiscenci u nemocných RS a inhiboval ji v obou kontrolních skupinách. Statisticky signifikantní inhibice leukocytární adherence za přítomnosti argininu byla zjištěna u nemocných RS ve srovnání se zdravými kontrolami, u kterých byla pozorována stimulace adheren-

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both control groups. The difference between MS patients and the control patient subgroup was proved to be statistically significant. Statistically significant inhibition of leukocyte adherence by arginine was found in MS patients in comparison to healthy controls, in whom stimulation of adherence was observed. When differentiating the effect of the agents upon particular leukocyte subpopulations the only statistically significant difference was found with arginine and granulocytes, the trend being similar as in the total leukocyte adherence. Sodium nitroprusside further stimulated the adherence of erythrocytes in MS patients and inhibited it in controls.
Conclusions: **Results indicate nitric oxide-dependent alterations of oxidative metabolic burst and of cellular adherence properties in patients with active multiple sclerosis. Our findings also question possible participation of granulocytes and erythrocytes in multiple sclerosis pathogenetic cascade.** ***(Tab. 3, Ref. 49.)***
Key words: adherence, erythrocytes, granulocytes, chemiluminiscence, leukocytes, multiple sclerosis, nitric oxide.

ce. Při diferenciaci efektu zkoumaných látek na hlavní leukocytární subpopulace byl statisticky průkazný rozdíl zjištěn s neutrofilními granulocyty a argininem. Nitroprusid sodný dále stimuloval adherenci erytrocytů u nemocných RS a inhiboval ji u kontrol.
Závěry: Výsledky ukazují u nemocných s aktivní RS na alterace metabolického oxidativního metabolismu a adherenčních interakcí závislé na metabolismu NO. Dále z výsledků vyplývá otázka možné participace neutrofilních granulocytů a erytrocytů obvodové krve v některých imunopatologických interakcích v patogenetické kaskádě u RS. *(Tab. 3, lit. 49.)*
Klíčová slova: adherence, erytrocyty, granulocyty, chemiluminiscence, leukocyty, roztroušená skleróza, oxid dusnatý.

Introduction

It has become increasingly apparent that nitric oxide (NO) and related metabolic and effector systems play an important role in immune regulations, in inflammatory response and in cellular and tissue damage in multiple sclerosis (MS) and in its experimental models (8, 11, 31, 33, 44). Various adherence interactions and metabolism of reactive oxygen species and associated redox-based cascades belong to the cellular functions tightly linked to NO metabolism (1, 2, 6, 8, 12, 20, 22, 33, 35, 44). In our previous studies we found abnormalities of leukocyte adherence behaviour of MS leukocytes after antigenic and ionophoretic stimulation, apparently dependent on the arachidonic acid metabolism and probably other oxidative processes (25, 26, 27). Further, some authors (34, 15, 16) including our group (28, 30) described in MS patients also alterations of oxidative metabolic burst of peripheral blood and leukocytes measured by luminol-dependent chemiluminiscence.

The aim of the present study was to evaluate the effect of chemical compounds affecting NO metabolism on the adhesive properties of leukocytes and erythrocytes and on the blood chemiluminiscence in patients with active MS in comparison with controls. The effects of N^G-methyl-L-arginine (N^GMLA), a specific competitive NO antagonist, L-arginine, NO precursor, and sodium nitroprusside (SNP), a NO-releasing agent were tested.

Materials and methods

Chemicals

N^GMLA was used in the final concentration of 0.25 μM/L, L-arginine in the final concentration of 0.5 μM/L, sodium nitroprusside in the final concentration of 0.25 μM. N^GMLA and L-arginine was made by Sigma Chemicals, USA, sodium nitroprusside by Lachema, Czech Republic. Luminol (5 amino-2,3-dihydro-1,4-phtalayinedione) was by Lumac Medical Products, USA.

Chemiluminiscence

150 μL of peripheral heparinized (10 i.u./mL) blood were mixed with 75 μL of physiological saline alone (in the control) or with the respective compound to achieve the desired final concentration. Then 15 μL of Luminol were added and chemiluminiscence was measured with Lumac/3M Biocounter Model 2010A six times in 15 minute intervals. The maximum and mean chemiluminiscence counts were evaluated. All the tests were performed in duplicate and mean values were used. The results were expressed as chemiluminiscence index (CLI):

$$\frac{\text{counts with the respective compound}}{\text{counts in the control group}}$$

The values exceeding 1.0 thus reflect inhibition of chemiluminiscence in the presence of the respective compound and *vice versa*.

Blood cell adhesivity

Initially, the blood was processed as described above. 150 μL of peripheral heparinized blood were mixed with 75 μL of physiological saline alone (in the control group) or with the respective compound. The mixture was then aspirated in triplicate (in 50 μL volume) into the pipette tips filled with 25 μg of nylon wool and then incubation for 45 minutes at 37 ˚C followed. The content was expelled by a pipette piston and total count of the non-adhering red and white blood cells was determined by a routine manual technique (Bürker's chamber). Leukocytes were further differentiated by a standard manual technique (Giemsa—Romanowski stain) and absolute numbers were calculated for the particular leukocyte populations (lymphocytes, monocytes, neutrophilic granulocytes). Mean values from three parallel tests were taken for further processing. The results were expressed for erythrocytes, neutrophilic granulocytes, monocytes and total lymphocytes as adhesivity index (AI):

$$\frac{\text{cell number with the respective drug}}{\text{cell number in the control}}$$

The values exceeding 1,0 thus reflect the adherence inhibition in the presence of the respective compound and *vice versa*.

Patients and controls

The MS group consisted of 21 patients (16 females, 5 males, aged 25—57 years) with clinically definite disease, in relapse or with secondary progressive course. The control group (total of 23

persons) consisted of two subgroups. In the subgroup of healthy volunteers were 7 females and 4 males, aged 20—52 years. The subgroup of neurological controls consisted of patients with intracranial tumours (7 males, 5 females) — 3 with astrocytoma, 2 with glioblastoma, 3 with CNS metastases, 3 with meningioma, 1 with acustic neurinoma. The age ranged from 32—59 years. The blood was sampled after informed consent was obtained.

Statistics

Statistical differences between the results obtained in the MS and the control groups were evaluated using the nonparametrical Mann and Whitney U-test. The MS group was compared with the control group as a whole and with the particular subgroups separately. Values of two-tailed probability less than or equal to Z=0.05 were considered statistically significant.

Results

Maximum (peak) chemiluminiscence

Sodium nitroprusside stimulated the luminol-dependent chemiluminiscence in MS patients (CLI 1.185±0.151) and inhibited it in both control groups (CLI being 0.630±0.110 in the patient sub-group, 0.960±0.167 in healthy controls). The difference between MS patients and the control patient sub-group was statistically significant (Z=0.0247). Using N^GMLA and arginine, no apparent differences between the MS patients and the control groups were found. The results are summed up in table 1.

Mean chemiluminiscence

Using this type of chemiluminiscence evaluation, analogical trends as with the peak chemiluminiscence were observed. The results however did not reached statistically significant level (data not shown).

Leukocyte adherence

Statistically significant (Z=0.026) inhibition of leukocyte adherence by arginine was found in MS patients (AI 1.233±0.133) in comparison to healthy controls, where stimulation of adherence was observed (AI 0.868±0.169). Results are summed up in table 2. When differentiating the effect of the agents upon the particular leukocyte subpopulations the only statistically significant difference was found with arginine and neutrophilic granulocytes, the trend being similar as in the total leukocyte adherence. (AI 1.112±0.098 in MS group, 0.752±0.220 in the healthy control group, 1.099±0.324 in the patient control group). The difference between the MS and the healthy control groups is statistically significant (Z=0.046).

Erythrocyte adherence

Sodium nitroprusside stimulated adherence of erythrocytes in MS patients (AI 0.940±0.065) and inhibited it in controls (AI 1.168±0.104 in the healthy controls, 1.227±0.154 in the patient controls). There is a statistically significant difference between MS patients and the control group as a whole (Z=0.0248). Results are summed up in table 3.

Discussion

The role of nitric oxide in the MS pathogenesis has recently become a subject of intensive interest and there is an increasing

Tab. 1. Effect of sodium nitroprusside, N^G-methyl-L-arginine (N^GMLA), and L-arginine upon the peak luminol-dependent chemiluminiscence of peripheral blood in multiple sclerosis patients (MS), healthy controls and neurological controls. Expressed as chemiluminiscence index — see materials and methods. Mean values ± standard error are given.
Tab. 1. Vliv nitroprusidu sodného N^G-methyl-L-argininu (N^GMLA) a L-argininu na maximální luminol-dependentní chemiluminiscenci obvodové krve u pacientů s roztroušenou sklerózou mozkomíšní (MS), u zdravých kontrol a u neurologických kontrol. Vyjádřeno jako chemiluminiscenční index. Udány střední hodnoty ve skupinách ± střední chyba.

Compound	MS patients	Healthy controls	Neurological controls
N^GMLA	1.615±0.374	1.492±0.248	1.04±0.213
Arginine	1.186±0.342	1.103±0.161	1.270±0.359
Sodium nitroprusside	1.185±0.151*	0.960±0.167	0.630±0.110*

* – statistically significant difference, Mann and Whitney U-test, Z = 0.0247

Tab. 2. Effect of sodium nitroprusside, N^G-methyl-L-arginine (N^GMLA), and L-arginine upon the adherence of leukocytes in multiple sclerosis patients (MS), healthy controls and neurological controls. Expressed as adhesivity index – see materials and methods. Mean values ± standard error are given.
Tab. 2. Vliv nitroprusidu sodného, N^G-methyl-L-argininu (N^GMLA) a L-argininu na adherenci leukocytů u pacientů s roztroušenou sklerózou mozkomíšní (MS), u zdravých kontrol a u neurologických kontrol. Vyjádřeno jako adherenční index. Udány střední hodnoty ve skupinách ± střední chyba.

Compound	MS patients	Healthy controls	Neurological controls
N^GMLA	1.188±0.109	1.189±0.133	1.154±0.148
Arginine	1.233±0.133*	0.868±0.169*	1.059±0.169
Sodium nitroprusside	1.023±0.094	1.088±0.179	1.369±0.171

* – statistically significant difference, Mann and Whitney U-test, Z = 0.026

Tab. 3. Effect of sodium nitroprusside, N^G-methyl-L-arginine (N^GMLA), and L-arginine upon the adherence of erythrocytes in multiple sclerosis patients (MS), healthy controls and neurological controls. Expressed as adhesivity index – see materials and methods. Mean values ± standard error are given.
Tab. 3. Vliv nitroprusidu sodného, N^G-methyl-L-argininu (N^GMLA) a L-argininu na adherenci erytrocytů u pacientů s roztroušenou sklerózou mozkomíšní, u zdravých kontrol a u neurologických kontrol. Vyjádřeno jako adherenční index. Udány střední hodnoty ve skupinách ± střední chyba.

Compound	MS patients	Healthy controls	Neurological controls
N^GMLA	1.020±0.073	1.076±0.089	1.138±0.158
Arginine	1.046±0.077	1.073±0.083	1.128±0.117
Sodium nitroprusside	0.940±0.065*	1.168±0.104*	1.227±0.154*

* – statistically significant difference (MS versus total controls), Mann and Whitney U-Test, Z = 0.248

evidence of enormous complexity of the effects of this molecule. NO may act as an important neuroprotective agent but also, in a form of peroxynitrite anion ($ONOO^-$), as one of the most toxic compounds produced in mammal organisms (1, 8, 9, 19, 22, 31, 47). This molecule may have both immunostimulating and immunosuppressive properties (1, 37). In experimental models of demyelination, the NO effects may substantially differ in dependence to gender of the experimental animals, to the stage of the disease and to a number of other factors (5, 8, 9, 13, 37, 41).

Luminol-dependent chemiluminiscence is a sensitive tool for evaluating the oxidative metabolism of activated leukocytes and of some other types of cells and NO release is a part of this integrated metabolic response (2, 20, 48). Luminol-dependent chemiluminiscence reflects predominantly the production of H_2O_2 together with the NO/peroxynitrite formation (2, 20). In our experiments, sodium nitroprusside, NO-releasing agent**,** stimulated the luminol-dependent chemiluminiscence in MS patients and inhibited it in both control groups. L-arginine, NO precursor and N^GMLA, a competitive NO antagonist had no apparent influence in the experimental system used. These findings point to the fact that not only up-regulation of the L-arginine/NO pathway, but also increased sensitivity to NO effects upon the metabolism of reactive oxygen species (metabolic oxidative burst) in patients with active MS must be taken into consideration. Cellular adherence represents another important cellular function associated with NO (6, 12, 35). In our previous studies, we have found abnormities of leukocyte adherence after antigenic and ionophoretic stimulation in MS patients (25, 26, 27). In our present experiments, excess of L-arginine, a precursor of L-arginine/NO pathway, significantly inhibited the leukocyte adherence to nylon wool in MS patients in comparison to healthy controls, where stimulation of adherence was observed. First, these results indirectly confirm the role of NO metabolism in leukocyte adherence and, further, they indicate a NO-dependent adherence alteration in MS. When differentiating the effect of the agents upon the particular leukocyte subpopulations the only statistically significant difference was found with neutrophilic granulocytes, the trend being similar as in the total leukocyte adherence.

Granulocyte involvement does not belong to generally accepted parts of MS pathogenesis despite various granulocyte functions have been reported to be altered in MS (39, 27, 28, 32) and some recent investigations suggest more significant and specific role of neutrophilic granulocytes in experimental demyelination and in MS (24, 49). Neutrophils have been proved to posses important immunoregulatory functions and they reveal an interesting ability to undergo a complete cycle of priming/depriming and they can thus flexibly control inflammatory interactions (4).

In our experiments, sodium nitroprusside stimulated adherence of erythrocytes in MS patients and inhibited it in controls. Erythrocyte adhesive abnormity associated with NO in MS might seem to be a rather unusual finding. Nevertheless, the functional, biochemical and morphological alterations of MS erythrocytes have been repeatedly reported. These alterations include increased osmotic and mechanical fragility of the erythrocyte membrane (21), increased lability of the erythrocyte membrane at higher temperatures (7) and changes in electrophoretic mobility and in sedimentation of erythrocytes in the presence of polyunsaturated fatty acids (10, 29). This should not necessarily mean a direct involvement of erythrocytes in MS pathogenesis. On the other hand, erythrocytes could enter the inflammatory cascade by affecting the metabolism of active lipidic compounds, especially eicosanoids, and they could directly influence the intravascular pathology by their interactions with leukocytes and endothelial cells (36, 43, 45).

According to a broad spectrum of investigations ranging from classical histopathological works to recent immunological and magnetic resonance imaging studies (14, 17, 18, 23, 33, 46, 40, 49), the endothelial and vascular component of MS pathogenesis deserves greater attention and NO metabolism play here an important part. Interactions between leukocytes and endothelial cells together with thrombocytes (3, 38, 42) and erythrocytes (36, 43, 45) emerge as an extremely complex spatiotemporal effector and regulatory network. Taken together, our results indicating NO-dependent alterations of metabolism of oxygen reactive species and of the cellular adhesivity in MS patients should be seen in this context.*

List of abbreviations

AI — adhesivity index
CLI — chemiluminiscence index
CNS — central nervous system
MS — multiple sclerosis
N^GMLA — N^G-methyl-L-arginine
NO — nitric oxide
$ONOO^-$ — peroxynitrite anion

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ODBORNÉ PODUJATIA SLOVENSKEJ INTERNISTICKEJ SPOLOČNOSTI ROKU 2000

1. Spolok bratislavských lekárov *Večer I. internej kliniky*
   Termín konania: 31.1.2000 o 17. h
   Téma: Zaujímavé kazuistiky
   Miesto konania: poslucháreň NTÚ, LFUK, Sasinkova 4, Bratislava

2. *Dérerov memoriál — 40. ročník*
   Termín konania: 31.3.2000
   Téma: Manažment pri akútnom infarkte myokardu
   Miesto konania: poslucháreň NsP Kramáre, Bratislava
   Kontaktná osoba: doc. MUDr. M. Pavlovič, CSc., I. interná klinika Dérerovej nemocnice, Bratislava

3. *Prvá poľsko-slovenská konferencia v internej medicíne*
   Termín konania: 26.—27.5.2000
   Miesto konania: Katowice, Poľsko

4. *The 5th Mitteleurope-Countries Meeting in Internal Medicine*
   Termín konania: 29.6.—1.7.2000
   Téma: Terapia sepsy, nové liečebné postupy v gastroenterológii, kardiológii, endokrinológii, reumatológii a onkológii a pri metabolických ochoreniach. Nežiaduce účinky liekov. Edukačné a koncepčné problémy internej medicíny.
   Miesto konania: SÚZA, Drotárska 46, Bratislava
   Kontaktná osoba: MUDr. S. Kiňová, I. interná klinika FN a LFUK, Mickiewiczova 13, Bratislava. Fax: 07.5292 5875, E-mail: kinova@faneba.sk

5. *Dni mladých internistov*
   Termín konania: 25.—26.5.2000
   Miesto konania: LF Martin
   Kontaktná osoba: prof. MUDr. M. Mokáň, CSc., I. interná klinika FN