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# Expression of NOS-2, COX-2 and Th1/Th2 cytokines in migraine

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**Abstract** Nitric oxide (NO) probably plays an important role in the pathogenesis of migraine without aura (MWA). As the activation of NO-ergic cascade has been shown to be closely linked to cyclooxygenase pathway and to cause some differences in peripheral blood lymphocyte populations, we investigated if the Th1/Th2 balance in peripheral blood of MWA patients was affected in comparison to controls. Twenty-six MWA patients and 10 healthy controls (C) were enrolled in this study. Blood samples were taken at baseline (T0) and during an induced migraine attack (T1). Total RNA from human peripheral blood lymphocytes (PBLs) was isolated and reverse-transcribed to prepare complementary DNA. COX-2, NOS-2 and β-actin were amplified using PCR. PBLs from patients and controls were stimulated with phorbol 12-myristate 13-acetate plus ionomycin in the presence of brefeldin A. Cells were surface-stained with fluorochrome-conjugated anti-CD3 and anti-CD8 monoclonal antibodies (mAbs) and intracellularly stained with fluorochrome-conjugated anti-IFN-γ or anti-IL-4 mAbs. The level of cytokine expression was analyzed by gating on the $CD4^+$ lymphocyte subset. Th1 and Th2 type cytokines (IFN-γ, IL-2, IL-4) were directly assayed in serum by ELISA. Preliminary results indicate that NOS-2 was upregulated in MWA patients at basal time if compared to controls, whereas after NOD administration NOS-2 was significantly decreased. COX-2 was upregulated in MWA patients at basal time and it had an opposite trend after NOD administration. The homeostatic Th1/Th2 balance defined by the IFN-γ or IL-4 cytokine expression was unchanged in MWA patients in comparison to controls, and NOD administration did not affect that pattern. The cell activation machinery was not altered after mitogenic activation, as shown by CD69 expression level. Cytokine serum levels showed no significant changes in all studied situations. This study confirms the relevance of the NOS/COX system in MWA but, in contrast with previous studies, excludes their effect and activation on peripheral cytokine production. More sophisticated experimental models are needed to investigate the ability of NOS/COX to activate migraine pain.

**Key words** Migraine • Nitric oxide • Cycloxygenase • Cytokines • T-helper lymphocytes

## Introduction

Inducible and constitutive nitric oxide synthases (NOS) have been detected in all areas of human brain, suggesting that nitric oxide (NO) plays a role in central nervous system (CNS) neurotransmission and in the regulation of cerebral circulation [1, 2]. Nitroglycerin derivatives – as NO donors (NOD) – have been widely used in the past to provoke experimental attacks of both migraine and cluster headache [3, 4]. In the last decade the scientific relevance of the NOD experimental model turned on NO ability to activate the endothelial pathways and/or the CNS nuclei by stimulating and/or controlling the following networks: (i) the trigeminovascular system in migraine [5–7]; (ii) the hypothalamic area and peripheral endothelial function in cluster headache [8, 9]; (iii) the central pain sensitization pathway in chronic tension headache [10]; and (iv) the local release of proinflammatory cytokines in cervicogenic headache, a still debated headache syndrome [11]. Today the molecular role of NO – at both central and peripheral levels – is mainly recognized in migraine [12].

Inducible NOS (iNOS or NOS-2) expression is activated by several cytokines, LPS and prostaglandin $E_2$ ($PGE_2$). In several physiological and pathological conditions, nitric oxide and prostanoids work cooperatively and synergistically. The inducible forms of NOS and cyclooxygenase (COX) (COX-2) are concurrently induced in inflammatory tissues in an experimental model. Prolonged, excessive induction of COX-2 and NOS-2 may have several deleterious effects on tissues. COX-2 catalyzes the synthesis of inflammatory prostaglandins, notably $PGE_2$, which may cause matrix deposition and suppresses the immune response [3]. The small amount of NO produced continuously by neuronal and endothelial NOS is involved in retrograde neurotransmission and vasodilatation, respectively. In contrast, a massive amount of NO produced by inducible NOS may be involved in anti-bacterial, -parasital and -viral action and, under particular conditions, has cytotoxic activity and damages several host tissues. Furthermore, both NOS-2 and COX-2 may generate a large amount of oxygen radicals that combine with NO to form $NO_3\cdot$, extremely toxic for cells.

NOS and COX are two enzymes that interact with one another, mutually stimulating or inhibiting their activities, but at the moment the modality of interaction is unknown. Modulation of the COX pathway by NO and vice versa has been studied in several systems with no uniform results.

NO plays a relevant role in the peripheral induction of immune responses and is a potent modulator of the balance between functionally distinct Th1 and Th2 cell subsets. Th1/Th2 cytokines may influence the spreading of pain-producing processes in migraine.

Considerable evidence suggests the existence of functionally polarized responses by $CD4^+$ T helper cell subsets that depend on the cytokines they produce. Based on these observations, in 1986 Mosmann and colleagues [13] divided helper cells into two populations secreting distinct and cross-regulating cytokine patterns. Th1 cells are responsible for chronic inflammation, cytotoxic T cell activation and protection against intracellular pathogens; they secrete interferon (IFN)-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-β. Th2 cells have a role in driving the antibody response, thereby protecting against extracellular pathogens, and secrete IL-4, IL-5, IL-6, and IL-10 [13]. Different types of immune responses evoke different types of cytokine production and variable proportions of Th1 and Th2 subsets. In normal individuals, the Th1/Th2 balance is constant and it is perturbated in immune responses and immunological diseases. Thus, cytokine expression patterns have been used to distinguish normal and altered cell functions in a variety of clinical conditions. In the past, conflicting results have been reported regarding the peripheral cytokine secretion pattern in migraine [14–16], suggesting that different routes are involved in the generation of migraine pain. In the present study, we investigated the NOS-2/COX-2 network and its possible influence on Th1/Th2 balance and cytokine production.

## Materials and methods

### Subjects

We studied 26 patients with migraine without aura (IHS code 1.1) [17] (21 women, 5 men; mean age, 35.7 years; range, 19–53 years) and 10 age-matched healthy controls (8 women, 2 men; mean age, 31.7 years; range, 23–44 years). There was no coexistence of tension-type headache in the patients. The study protocol was approved by the Institutional Ethics Board and informed written consent was obtained from all participants of the clinical study.

Whole blood samples (23 ml) were taken at baseline (T0) for both groups, and during a migraine attack (T1) induced with 5 mg isosorbide dinitrate for patients only. All samples were processed immediately after collecting.

### RT-PCR analysis of NOS-2 and COX-2

Total RNA from human peripheral blood lymphocytes was isolated using the Trizol reagent (Life Technologies) and RNA was quantified by UV absorption at 260 nm. The RNA (2 μg) was reverse-transcribed into complementary DNA with superscript reverse transcriptase (RT) (Gibco), following the manufacturer's instructions. A mixture of 2 μg RNA, 1 μl 10 mM dNTP, 1 μl random primers and sterile distilled water to 12 μl was heated to 65° C for 5 min and then quickly chilled on ice. Then 4 μl 5x first strand buffer, 2 μl 0.1 M DTT, and 1 μl RNase-OUT were added and after an incubation at room temperature for 10 min and at 42°

C for 2 min, 200 U Superscript II was added. The reaction was run at 42° C for 50 min and inactivated for 10 min at 72° C. The oligonucleotide primers used to amplify sequences of NOS-2, COX-2 and β-actin are listed in Fig. 1. RT-generated fragments corresponding to human NOS-2, COX-2 and β-actin were amplified using polymerase chain reaction (PCR).

PCR was performed in a 100-µl reaction containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 U Taq polymerase, 20 pmol oligonucleotide primers and RT products (1/10 of the RT reaction). After an initial denaturation step (5 min at 95° C), NOS-2 was amplified by 35 cycles (1 min at 94° C, 1 min at 65° C, 2 min at 72° C), COX-2 by 35 cycles (1 min at 94° C, 1 min at 60° C, 1 min at 72° C), and β-actin by 35 cycles (30 s at 94° C, 30 s at 62° C, 30 s at 72° C). The final extension period was 3.5 min at 72° C. PCR products were analyzed in a 1.2% Sepharide gel containing 0.5 µg/ml ethidium bromide in tris-EDTA buffer. RT-PCR data are expressed as percent values compared to reference gene expression (β-actin).

| | | |
|---|---|---|
| NOS-2 | Forward | 5′ -TCCGAGGCAAACAGCACATTCA-3′ |
| | Reverse | 5′ -GGGTTGGGGGTGTGGTGATGT-3′ |
| COX-2 | Forward | 5′ -TTCAAATGAGATTGTGGGAAAATTGCT-3′ |
| | Reverse | 5′ -AGATCATCTCTGCCTGAGTATCTT-3′ |
| β-actin | Forward | 5′ -ACCAACTGGGACGACATGGAG-3′ |
| | Reverse | 5′ -CGTGAGGATCTTCATGAGGTACTC-3′ |

**Fig. 1** Sequences of primers used to amplify NOS-2, COX-2 and β-actin cDNAs

### Intracellular detection of Th1/Th2 cytokines

Peripheral blood from migraine patients and healthy donors was collected in heparinized tubes. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (Lympholyte, Cedarlane Laboratories, Hornby, Canada) density-gradient centrifugation, resuspended in RPMI-1640 medium (Gibco, Grand Island, New York) containing 10% heat-inactivated fetal calf serum (Hyclone, Logan, Utah), 100 U/ml penicillin and 100 µg/ml streptomycin. PBMC were stimulated for 18 h at 37° C, in 5% $CO_2$ with 25 ng/ml PMA (phorbol-12-myristate-13-acetate) in combination with 1 µg/ml ionomycin (Sigma). Brefeldin-A (Sigma) was added to the cells at a concentration of 10 µg/ml to prevent cytokine release by disrupting intracelluar Golgi-mediated transport and allowing cytokines to accumulate. Cultures without exogenous stimuli were included to record the spontaneous activation and secretion of cytokines by $CD4^+$ T cells. After incubation, cells were stained for flow cytometry according to the method described by Becton Dickinson Immunocytometry Systems (BD Biosciences, Erembodegem, Belgium). Surface phenotyping was performed using a combination of anti-CD3-PerCP and anti-CD8-FITC or anti-CD8-PE monoclonal. For activation detection, the anti-CD69-FITC-labelled antibody was used. All antibodies were from Becton Dickinson. The cells were incubated in 2 ml lysing solution for 10 min at room temperature, pelleted and incubated in 500 µl FACS permeabilizing solution for 10 min at room temperature in the dark, then washed and pelleted. The following cytokine-specific mAbs were used: anti-IFN-γ-FITC and anti-IL-4-PE (Becton Dickinson). Cells were washed and pellets were fixed in 1% paraformaldehyde. The immunofluorescence analysis was performed on a FACScalibur flow cytometer equipped with the CellQuest software (Becton Dickinson). A total of 15 000 events was collected and the level of cytokine expression on activated cells was analyzed by gating on the $CD3^+CD8^-$ population, which is equivalent to the $CD4^+$ subset, and compared with the level of unstimulated T cells.

### Serum cytokine assay

Th1 and Th2 type cytokines (IFN-γ, IL-2, IL-4) were directly assayed in serum using commercial ELISA kits (R&D Systems) following the manufacturers instructions.

### Statistical analysis

Calculations were done blindly by two different operators using the SAS System for statistical analysis. The data were reported as means ± SEM. Paired comparison *t* test procedure was used to reveal differences between the obtained data. Values of *p* (for a confidence interval of 95%) <0.05 were set as the significant level for hypothesis testing.

## Results

Preliminary results of this ongoing study are reported.

NOS-2 was upregulated in peripheral blood lymphocytes from patients with migraine without aura compared to controls (T0 vs. controls 47.2%±3.1% vs. 34.0%±0.9%; $p<0.001$). After NOD administration, NOS-2 expression decreased significantly (T0 vs. T1, 47.2%±3.1% vs. 37.1%±1.8%; $p<0.01$) while it was lower compared with controls (T1 vs. controls, 36.1%±2.5% vs. 34.0%±0.9%; $p<0.01$) (Fig. 2).

COX-2 was upregulated in migraine patients at baseline (T0 vs. controls, 49.2%±3.1% vs. 38.3%±2.5%; $p<0.02$). It was significantly decreased after NOD administration (T1 vs. T0, 37.1%±3.4% vs. 49.2%±3.1%; $p<0.01$) (Fig. 2).

The homeostatic Th1/Th2 balance defined by the IFN-γ or IL-4 cytokine expression was unchanged in migraine patients in comparison to controls (Fig. 3). NOD administration did not affect this pattern. Moreover, the cell activation machinery was not altered, as demonstrated by CD69 expression observed after mitogenic activation (Fig. 4).

Cytokine (IL-2, IFN-γ, IL-4) serum levels showed no significant changes in control subjects and in patients, even during a NOD-induced migraine attack (Table 1).

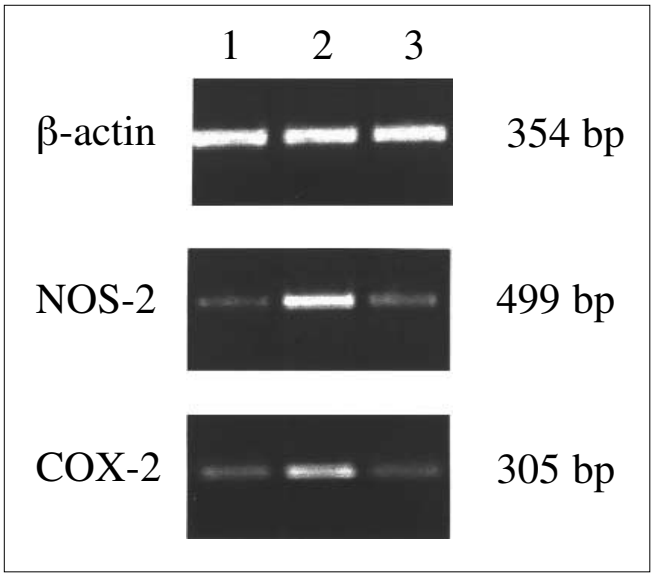


**Fig. 2** RT-PCR assessment of the β-actin, NOS-2 and COX-2 mRNA expression in peripheral blood lymphocytes in controls (n=10) and patients with migraine without aura (n=26). *Lane 1*, healthy subjects; *Lane 2*, migraine patients at baseline; *Lane 3*, migraine patients after NOD administration. The samples in the figure are representative of one patient (#15) and one control (#9)

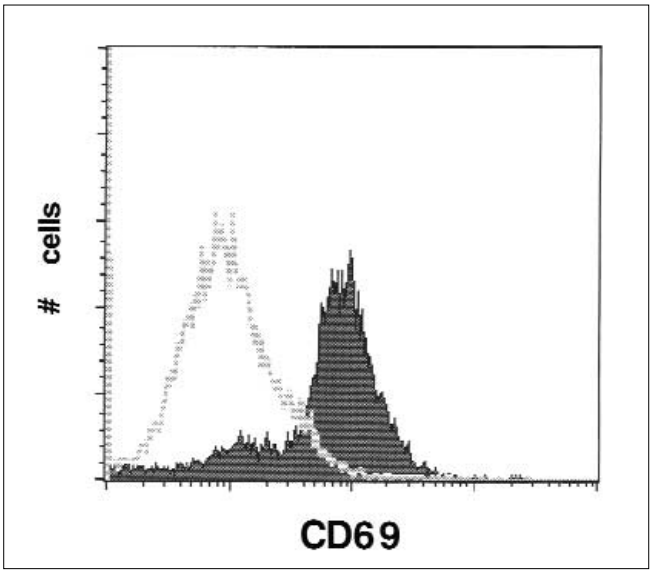


**Fig. 3** Expression of the lymphocyte activation marker CD69 on a representative sample of unstimulated (*grey line*) or activated (*shaded area*) peripheral blood mononuclear cells from a migraine patient. Surface staining with anti-CD3-PerCP and anti-CD69-FITC. Flow cytometric analysis of CD69 stained cells was performed on $CD3^+$ gated events

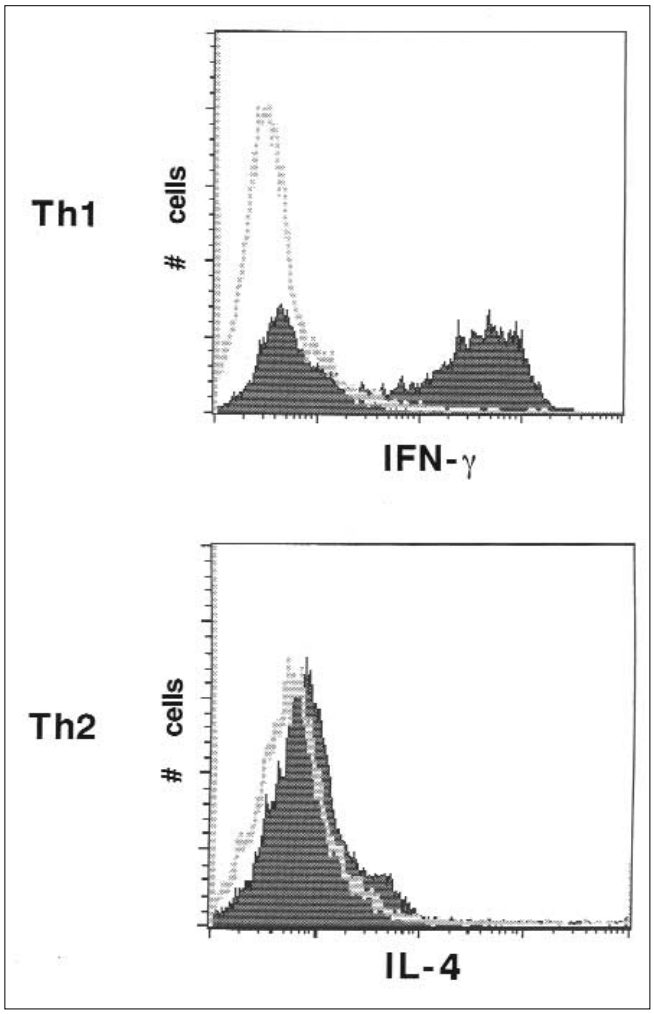


**Fig. 4** Th1 and Th2 cytokine expression in representative non-activated (*grey line*) or activated (*shaded area*) peripheral blood mononuclear cells from a patient with migraine. Surface staining with anti-CD3-PerCP and anti-CD8-FITC or PE. After permeabilization, intracellular staining with anti-IFN-γ-FITC or anti-IL-4-PE. Flow cytometric analysis of Th1 or Th2 cytokine expression was performed on $CD3^+/CD8^-$ gated cells

**Table 1** Serum cytokines in migraine without aura patients (n=26) and in healthy subjects (n=10)

| | | Migraine without aura patients | |
|---|---|---|---|
| Cytokine | Controls | Baseline | NOD-induced migraine |
| IFN-γ (UI/ml) | 10.0±1.3 | 10.9±1.5 | 10.3±1.8 |
| IL-2 (pg/ml) | ND | 34.5±1.5[a] | 34.5±1.1[a] |
| Il-4 (pg/ml) | 1.37±0.3 | 1.27±0.4 | 1.84±0.6 |

[a] Values represent levels in just 2 patients, while they were not detectable in the other 24 patients
*ND*, not detectable

## Discussion

The outcomes from the present study indicate that the peripheral model for studying the NO effects in migraine is still not adequately proved. Nevertheless our data further support the evidence regarding the key role played by NO in migraine.

In fact, in our previous work [18] we demonstrated high serum level of nitrites in patients with migraine without aura and, in the present paper, we report an up-regulation of NOS-2 in peripheral blood lymphocytes of migraine patients even in between attacks. After administration of an NO donor, we detected a decrease in NOS-2 expression, thus suggesting a negative regulatory feedback played by NO itself on NOS activity. On the other hand, because of the deleterious effects of high amounts of NO on host tissues, it is necessary that the NOS activity be strictly regulated. Our data [19] and that of others [20, 21] demonstrated that even COX-2 is overexpressed in migraine patients in between attacks when the NO serum level is quite high.

Interestingly, when serum NO levels became very high, as happens after NOD administration, NO itself could play an inhibitory role on COX-2 activation: in fact COX-2 expression was decreased with respect to the baseline value. These data are not surprising. In fact, the reciprocal modulation between NOS-2 and COX-2 and their metabolites is, in other experimental systems, well known. Moreover, the NO level in migraine patients seems not to be sufficient for inducing significant modifications of the Th1/Th2 balance. The patients' lymphocytes, in fact, did not show any significant difference in their subsets and cytokine pattern release.

We investigated a possible modulation of Th1/Th2 balance in MWA patients and controls since the imbalance of these T cell subsets in clinical medicine may be a consequence of a disease or may itself be a cause of it [22]. NO is believed to incluce migraine and also to influence peripherally and centrally both endothelial and nociceptive regulatory pain pathways [23, 24]. Our data indicate that although the activation mechanism of Th cells is functioning, as demonstrated by increased CD69 expression, the expression of Th1-type cytokines such as IFN-γ, or Th2-type cytokine such as IL-4, did not change in migraine patients compared to controls. It is conceivable that non-infectious inflammatory processes in the CNS do not directly involve peripheral blood lymphocytes. Furthermore, the timing of NO-inducer exposure with respect to the activation status of $CD4^+$ T cells may be critical for determining the effect of migraine attack on cellular function.

The present study showed that the level of IFN-γ- or IL-4-producing cells in peripheral blood of either untreated or NOD-treated migraine patients did not differ from those of healthy controls. This observation has been confirmed by the lack of change of cytokine serum levels. However, during our study, we revealed high inter- and intra-individual variability of immunological functionality, so that it is necessary to extend the present study to a larger group of patients.

In conclusion, the present study confirms the relevance of the NOS/COX system in migraine without aura but it seems to be unable to cause a shift of Th1/Th2 ratio and the intra- and extracellular levels of peripheral cytokines. More sophisticated experimental models are needed to investigate the ability of the NOS/COX network to activate migraine pain.

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