Not in the mind but in the cell: increased production of cyclo-oxygenase-2 and inducible NO synthase in chronic fatigue syndrome

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Submitted: June 9, 2007 *Accepted: July 12, 2007*

Key words: **chronic fatigue syndrome; inflammation; oxidative stress; inducible NO synthase; cyclooxygenase 2; immunity; depression; cytokines**

Neuroendocrinol Lett 2007; **28**(4):463–469 **PMID:** 17693978 NEL280407A16

Abstract

Chronic fatigue syndrome (CFS) is a medically unexplained disorder, characterized by profound fatigue, infectious, rheumatological and neuropsychiatric symptoms. There is, however, some evidence that CFS is accompanied by signs of increased oxidative stress and inflammation in the peripheral blood. This paper examines the role of the inducible enzymes cyclo-oxygenase (COX-2) and inducible NO synthase (iNOS) in the pathophysiology of CFS. Toward this end we examined the production of COX-2 and iNOS by peripheral blood lymphocytes (PBMC) in 18 CFS patients and 18 normal volunteers and examined the relationships between those inflammatory markers and the severity of illness as measured by means of the FibroFatigue scale and the production of the transcription factor nuclear factor kappa beta (NFκβ).

We found that the production of COX-2 and iNOS was significantly higher in CFS patients than in normal controls. There were significant and positive intercorrelations between COX-2, iNOS and NFκβ and between COX-2 and iNOS, on the one hand, and the severity of illness, on the other. The production of COX-2 and iNOS by PBMCs was significantly related to aches and pain, muscular tension, fatigue, concentration difficulties, failing memory, sadness and a subjective experience of infection.

The results suggest that a) an intracellular inflammatory response in the white blood cells plays an important role in the pathophysiology of CFS; b) the inflammatory response in CFS is driven by the transcription factor NFκβ; c) symptoms, such as fatigue, pain, cognitive defects and the subjective feeling of infection, indicates the presence of a genuine inflammatory response in CFS patients; and d) CFS patients may be treated with substances that inhibit the production of COX-2 and iNOS.

ORIGINAL ARTICLE

INTRODUCTION

There is now evidence that chronic fatigue syndrome (CFS) is accompanied by signs of severe oxidative and nitrosative stress and activation of the inflammatory response system (IRS).

There are reports showing that CFS is accompanied by a decreased antioxidant status in the blood. Thus, Maes *et al.* [1,2] found significantly lower serum levels of zinc, a strong antioxidant, and dehydroepiendrosterone-sulfate, a hormone with strong antioxidant properties, in patients with CFS as compared with normal controls. Other findings point toward increased oxidative and nitrosative stress in CFS. The findings include: increased isoprostane levels and oxidized low density lipoproteins (LDL) [3]; higher LDL thiobarbituric acid reactive substances (TBARS) [3]; c) elevated protein carbonyl levels [4]; and d) an increased response to incremental exercise, which is associated with a lengthened and accentuated oxidative stress [5]. Also, in animal models for CFS an increased oxidative stress had been described [6]. We found that CFS is accompanied by increased levels of IgM antibodies directed against fatty acids (oleic acid), by-products of lipid peroxidation (MDA and azelaic acid), and anti-S-farnesyl-L-cysteine, and NO derivates, such as nitro-tyrosine, nitro-phenylalanine, nitro-arginine, nitro-tryptophan and nitro-cysteine [7]. This shows that CFS is characterized by an IgM-mediated immune response directed against autoepitopes, which are normally hidden from the immune system, but which have become immunogenic through different mechanisms, such as a) oxidative damage to lipid membranes and synthesis of by-products of lipid peroxidation; and b) modification of endogenous proteins by nitrosative stress (NO and peroxynitrite). In conclusion, CFS is accompanied by decreased antioxidative defences and by increased oxidative and nitrosative stress, which has generated a variety of oxidatively modified neoepitopes, which have acquired immunogenicity and thus may serve as a trigger to impair or bypass immunological tolerance [7].

Besides increased oxidative stress there is also an activation of the IRS in CFS. Thus, CFS is accompanied by a) immune activation, characterized by an increased expression of T cell activation markers, such as CD26 and CD38 [8] and alterations in cytokine production [9–11]; b) increased plasma concentrations of the alpha2 globulin fraction obtained by electrophoresis and decreased serum zinc levels [1]; and c) signs of decreased cellular immune responses (another hallmark of an IRS response), such as decreased mitogen–induced lymphocyte responses and specific defects in early T cell activation, i.e. a diminished mitogen-induced expression of the early activation marker CD69 [8,12–14].

Probably, activation of the IRS and the increased oxidative and nitrosative stress are intertwined phenomena in CFS [7]. Indeed, activation of the IRS is accompanied by an increased production of oxygen radicals, e.g. H_2O_2 (peroxides) and $2O^{2-}$ (superoxide), which may damage lipid membranes in the brain, muscle, and nerve cells; and by nitrosative stress formed by activated neutrophils and monocytes, e.g. nitrogen monoxide (NO) or peroxynitrite ($ONOO^-$).

One mechanism which may explain both activation of the IRS and increased oxidative/nitrosative stress in CFS is an increased intracellular production of the important inflammatory and oxidative mediators, i.e. cyclo-oxygenase-2 (COX-2) and inducible NO synthase (iNOS). COX-2 is a key enzyme which catalyzes the transformation of arachidonic acid to prostaglandins and prostacyclins. COX-2 is undetectable in most normal tissues and is an oxidatant-inducible gene, which expression is upregulated during inflammation. It becomes abundant in activated macrophages and other cells at sites of inflammation [15]. iNOS is preferentially localized in macrophages and upon stimulation by specific cytokines can generate nitric oxide (NO). NO synthesis by macrophages and neutrophils has multiple roles in the development of inflammation and in oxidative and nitrosative stress and it plays an important role in the cytotoxic activity and in the pain syndrome associated with inflammation [16,17]. Since CFS is accompanied by oxidative stress and by activation of the IRS we expect to find an increased production of COX-2 and iNOS in those patients.

Recently, we have shown that nuclear factor kappa beta (NFκβ), the major upstream, molecular mechanism which regulates many inflammatory and oxidative stress mediators, is highly significantly increased in CFS patients as compared to normal volunteers [18]. Upon activation NFκβ is translocated from the cytoplasma to the nucleus where NFκβ binds specific promoter sequences of DNA and induces transcriptional activation of amongst other things iNOS and COX-2 [19,20]. Based on the above, we expect to find significant positive correlations between increased COX-2 and iNOS production and the increased production of NFκβ in CFS.

The aims of the present study were to examine 1) whether CFS is accompanied by an increased production of COX-2 and iNOS; 2) whether the increased COX-2 and iNOS production are driven by an increased production of NFκβ; and 3) the symptom profiles of increased COX-2 and iNOS production in CFS.

SUBJECTS AND METHODS

Subjects

Thirty-six subjects participated in the present study, 18 patients with CFS and 18 age-sex matched and unrelated controls. The patients were admitted to the M-Care4U Outpatient Clinics, Belgium. The diagnosis of CFS was made by means of the Centers for Disease Control and Prevention (CDC) criteria [21]. These criteria include: the patient must have a severe chronic fatigue of six months or longer, while there is no other known medical condition which can explain the fatigue; and the patient

must have four or more of the following symptoms: substantial impairment in short-term memory or concentration, sore throat, tender lymph nodes, muscle pain, multi-joint pain without swelling or redness, headache of a new type, pattern or severity, unrefreshing sleep, and post-exertional malaise lasting more than 24 hours. The total sum on the FibroFatigue scale, i.e. the Fibromyalgia and Chronic Fatigue Syndrome Rating Scale [22] was used to compute the severity of illness. This scale measures 12 CFS (and fibromyalgia) symptoms, i.e. pain, muscular tension, fatigue, concentration difficulties, failing memory, irritability, sadness, sleep disturbances, autonomic disturbances, irritable bowel, headache, and subjective experience of infection.

Subjects with a life-time diagnosis of psychiatric DSM-IV disorders, such as bipolar disorder, depression, anxiety disorders, schizophrenia, substance use disorders and organic mental disorders were excluded to participate in the present study. Also, subjects with medical illnesses, e.g. diabetes type 1 or type 2, inflammatory bowel disease, essential hypertension, and arteriosclerosis; and subjects who ever had been treated with anti-psychotic drugs, anticonvulsants or mood stabilizers and subjects who had been taking other psychotropic drugs during the last year prior to the studies were omitted. None of the subjects suffered from acute inflammatory and allergic reactions for at least 2 months prior to the study. All subjects had normal values for routine blood tests, such as alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), calcium, creatinine, electrolytes, thyroid stimulating hormone (TSH), total protein, and iron or transferrin saturation. No one had positive IgM antibody titers for EBV or CMV. Patients and controls gave written informed consent after the study protocol was fully explained. The study has been approved by the local ethical committee.

Methods

COX-2, iNOS and NFκβ concentrations were determined on the cell lysates of unstimulated mononuclear cell isolates. PBMCs were obtained through histopaque - 1077 density gradient centrifugation of peripheral blood samples of patients and controls. Isolated cells were resuspended in RPMI 1640 medium (Sigma R-0278) supplemented with 10% foetal calf serum and cultured overnight (18 hours). After harvesting the cells and washing with PBS/PIB (phosphate inhibitor buffer) cells were lysed with Nonidet P-40 and a nuclear cell extract was obtained after resuspension of the pellet in complete lysis buffer according to the protocol of the TransAM™ NFκβ p50 Transcription Factor Assay kit (Cat 41`096 & 41596; Active Motif, Carlsbad, California). Lysates of the unstimulated PBMCs were tested in batch for their iNOS concentrations through the Quantikine Human iNos immunoassay of R&D Systems (Minneapolis) and for their COX-2 content through the TiterZyme® EIA for human cyclo-oxygenase-II (catalog no 900-094, Assay Designs, Inc, Ann Harbor). Concentrations are expressed in ng/ml for the COX-2 and iNOS concentrations and in ng/well for NFκβ p50. All lysates were stored frozen at –80 °C until the measurements were performed simultaneously on the complete batch of samples. The intraassay coefficients of variation were less than 6%.

Statistics

Group mean differences were assessed by means of analysis of variance (ANOVA) and covariance (ANCOVA). Relationships between variables were ascertained by means of Spearman's rank order correlation coefficients, and by means of multiple regression analyses and canonical correlation analysis. The diagnostic performance of increased iNOS and COX-2 was checked by means of ROC (receiver operating characteristics) analysis with computation of the area under the ROC curve, sensitivity, specificity and predictive value of a positive test result (PV+) and with kappa statistics. The significance was set at $\alpha = 0.05$ (two tailed).

RESULTS

There were no significant differences in age ($F = 0.01$, $df = 1/34$, $p=0.9$) between normal controls (mean age $\pm$ SD = 42.7 $\pm$ 8.9 years) and CFS patients (43.0 $\pm$ 9.2 years). There were no significant correlations between age and either COX-2 ($r=0.02$, NS) and iNOS ($r=0.06$, NS).

Figure 1 shows the production of COX-2 and iNOS in both study groups. ANOVA shows significantly higher COX-2 production in CFS patients than in normal controls ($F = 86.2$, $df = 1/34$, $p=0.0000004$). ANOVA showed significantly higher iNOS production in CFS patients than in normal volunteers ($F = 28.1$, $df = 1/34$, $p=0.00005$). Covarying for age in ANCOVAs did not change any of these results.

ROC analysis performed on the COX-2 and iNOS values showed that the area under the ROC curve (AUC) was highly significant for COX-2 (AUC = 99.4%) and iNOS (AUC = 90.7%). Using a cut off value for COX-2 >4.6 ng/mL, we found a significant discrimination of CFS patients from normal controls with a sensitivity of 100%, specificity = 94.4 % and a PV+ = 94.5% ($\kappa=0.94$, $t=17.22$, $p<10^{-4}$). Using a cut off value for iNOS >14.2 ng/mL, we found a significant discrimination of CFS patients from normal controls with a sensitivity of 77.8%, specificity = 94.9% and a PV+ = 93.3% ($\kappa=0.72$, $t=6.26$, $p<10^{-4}$).

Spearman's rank order correlation analyses showed significant relationships between COX-2 production and the total score on the FibroFatigue scale ($r=0.65$, $p=0.004$). COX-2 production was significantly related to aches and pain ($r=0.51$, $p=0.03$), fatigue ($r=0.82$, $p=0.0001$), concentration difficulties ($r=0.52$, $p=0.02$), failing memory ($r=0.77$, $p=0.0004$), sadness ($r=0.47$, $p=0.04$), and subjective experience of infection ($r=0.68$, $p=0.002$).

Spearman's rank order correlation analyses showed significant relationships between iNOS production and

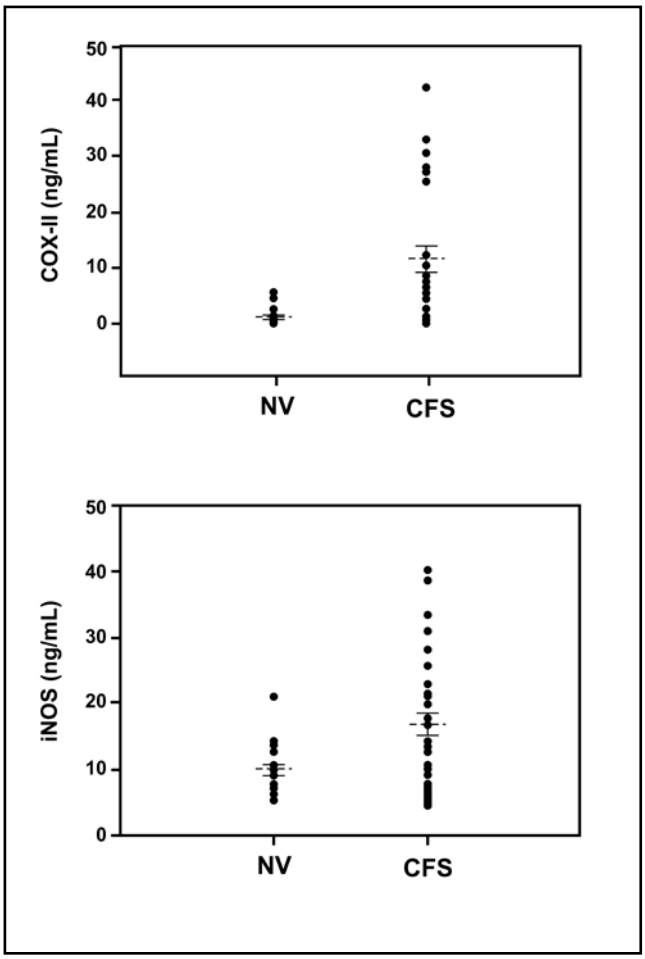

Figure 1. Scatter plot of the measurements of cyclo-oxygenase-2 (COX-2) and inducible NO synthase (iNOS) in 18 patients with chronic fatigue syndrome (CFS) and 18 age-sex matched normal volunteers (NV).

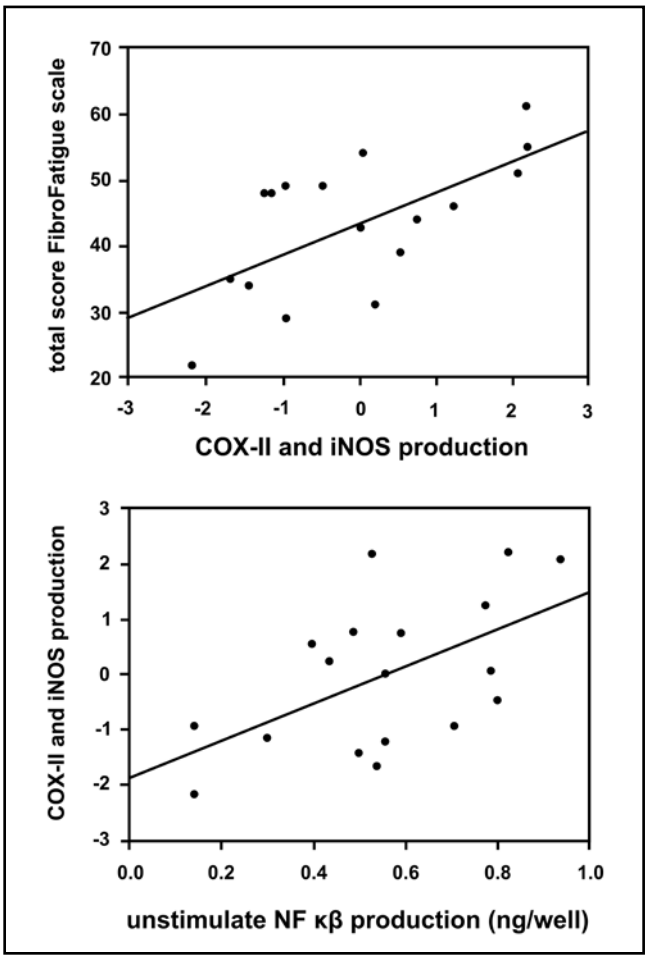

Figure 2. Regressions of the total score on the FibroFatigue scale on the integrated index of COX-2 and iNOS production (the first PC subtracted from both data) and of the integrated index of COX-2 and iNOS production on the unstimulated production of nuclear factor kappa beta (Nfκβ) in 18 subjects with chronic fatigue syndrome.

the total score on the FibroFatigue scale (r=0.51, p=0.03). Spearman's rank order correlation analyses showed that iNOS production was significantly correlated to fatigue (r=0.60, p=0.008), concentration difficulties (r=0.61, p=0.007), failing memory (r=0.71, p=0.001), and subjective experience of infection (r=0.61, p=0.008).

Table 1 shows the results of canonical correlation analyses with the symptoms of the FibroFatigue scale as dependent variables and the production of COX-2 and iNOS as independent variables. Inspecting the loadings on the first component shows that aches and pain, fatigue, concentration difficulties, failing memory, sadness and a subjective feeling of infection are significantly related to the production of COX-2 and iNOS.

As an integrative index for the intracellular inflammation in CFS, we computed the first principal component (PC) extracted from both the COX-2 and iNOS values. The first PC explained 95.5% of the variance in the COX-2 and iNOS data. Figure 2 shows that there was a significant and positive correlation (r=0.55, p=0.01) between the total score on the FibroFatigue scale and the first PC.

We also computed the intercorrelations between iNOS, COX2 and the production of NFκβ. The production of NFκβ was significantly higher (F = 20.1, df = 1/34, p=0.0002) in the CFS patients (0.357 ± 0.244 ng/well) than in the normal volunteers (0.087 ± 0.092 ng/well). In the total study group, Spearman's rank order correlation analyses showed significant and positive intercorrelations between the production of COX-2 and iNOS (r=0.89, p=0.0000). The unstimulated production of NFκβ was significantly correlated to the production of iNOS (r=0.76, p=0.0000) and COX-2 (r=0.78, p=0.0000). Also, in the subgroups of patients with CFS and in normal controls, the above relationships remained significant. Figure 2 shows that the first PC subtracted from the COX-2 and iNOS values was significantly and positively correlated (r=0.78, $p<10^{-4}$) to the production of NFκβ.

Table 1. Results of the canonical correlation analysis with the symptoms of the Fibrofatigue scale as dependent variables and the production of cyclo-oxygenase (COX-2) and inducible NO synthase (iNOS) as explanatory variables.

Dependent variables (FibroFatigue scale)	Loadings on the first eigenvector
aches and pain	**0.57**
muscular tension	0.28
fatigue	**0.78**
concentration difficulties	**0.43**
failing memory	**0.71**
irritability	0.09
sadness	**0.43**
sleep disturbances	–0.31
autonomic disturbances	0.15
irritable bowel	–0.14
headache	0.24
subjective experience of infection	**0.50**
Independent variables	
COX-2	**0.95**
iNOS	**0.58**

The significant loadings (>0.40) are in bold and underlined.

DISCUSSION

This is a first study which shows that CFS is accompanied by a significantly increased production of COX-2 and iNOS. Thus, the results of the present study confirm those of previous studies (see Introduction) that CFS is accompanied by inflammation and increased oxidative stress. 1) Indeed, COX-2 is a key regulatory enzyme in the synthesis pathway of eicosanoids, which are responsible for multiple inflammatory actions in tissues [23]. COX-2, which is located in the endoplasmatic reticulum and in the perinuclear envelope, extracts arachidonic acid from the membrane and decores it with peroxides. The resulting molecule is modified by peroxides and other enzymes yielding mature prostaglandins. The latter modulate pain signaling, inflammation and smooth muscle contraction [24]. 2) iNOS is preferentially localized in macrophages and upon stimulation by specific cytokines can generate nitric oxide (NO). NO is a major signalling molecule in neurons and in the immune system, either acting within the cell in which it is produced or by penetrating cell membranes to affect adjacent cells. NO is central to the early macrophage immune responses to invading microorganisms [25]. NO toxicity is linked to its ability to combine with superoxide anions (O^{2-}) to form peroxynitrite ($ONOO^-$), an oxidizing free radical that can cause DNA fragmentation, lipid oxidation and tyrosine nitration [16,17,26]. Thus, increased iNOS production in CFS may – in part – explain the signs of oxidative and nitrosative stress in CFS (for review: see Introduction).

Another major finding of the present study is that the increased COX-2 and iNOS production are significantly related to core symptoms of CFS, such as fatigue, pain symptoms, cognitive symptoms (concentration difficulties and failing memory), sadness, and the subjective experience of infection. These findings extent those of earlier results showing that signs of inflammation and oxidative or nitrosative stress are related to those symptoms profiles. Indeed, there is now a vast literature that inflammation may induce fatigue and depression [27–29]. Also, in CFS significant correlations are found between 1) the severity of fatigue and increased oxidative stress and decreased antioxidant defences [30]; 2) fatigue and pain symptoms and the IgM-related immune responses mounted to neoepitopes formed by oxidative and nitrosative damage to lipid and protein structures [7]; and 3) isoprostane levels and joint pain and postexertional malaise [3]. Moreover, a causal role for oxidative stress in muscle fatigue and pain of CFS patients is provided by research showing that the response of those patients to incremental exercise associates a lengthened and accentuated oxidative stress response with marked alterations of the muscle membrane excitability [5] and that administration of NAC, a strong antioxidant, may delay muscle fatigue during repetitive handgrip exercise, supporting the hypothesis that oxidative stress is a causal factor in human muscle fatigue [31].

It is well-known that COX-2 is implicated in the production of musceloskeletal pain and that COX-2 inhibitors may alleviate these symptoms [32]. COX-2-selective inhibition has also a beneficial effect on muscle fatigue resistance in elderly patients with inflammation of infectious origin [33]. iNOS has been shown to play a role in fibromyalgia, a disorder which is highly comorbid with CFS and which shows a highly significant symptomatic overlap with CFS. Thus, iNOS protein content in muscle biopsy is increased in fibromyalgia and is negatively correlated with total exercise time. Moreover, the higher iNOS contents are related to reduced responses to aerobic exercise and reduced maximal exercise time. The increased dialysate lactate in response to stimulation of iNOS suggests that fibromyalgia patients may be more sensitive to the suppressive effects of nitric oxide on oxidative phosphorylation [34]. COX-2 overexpression may induce specific cognitive deficits and COX-2 inhibition may alleviate cognitive defects [35,36]. The

correlation between the FibroFatigue scale item the subjective experience of infection and COX-2 and iNOS production shows that this symptom reflects genuine inflammatory responses in patients with CFS . Previously, we have reported that the subjective experience of infection is related to another marker of IRS activation in CFS, i.e. lowered serum zinc [1].

The third major finding of this study is that both the production of iNOS and COX-2 are significantly related to the production of NFκβ in CFS patients and normal volunteers. It is known that NFκβ plays a critical role mediating COX-2 [19] and iNOS production [37,38]. Thus, our findings indicate that the increased production rates of both iNOS and COX-2 in CFS are at least partly driven by increased NFκβ production.

In summary, CFS is accompanied by an increased production of COX-2 and iNOS, which is driven by an increased production of NFκβ and which is related to the severity of key symptoms, such as fatigue, pain, sadness, cognitive disorders and a subjective feeling of infection. Therefore, it may be hypothesized that an intracellular inflammatory response in the white blood cells plays an important role in the inflammatory pathophysiology of CFS, which points towards IRS activation and increased oxidative stress; and that the symptoms expressed by CFS reflect a genuine inflammatory response. The results also suggest that CFS patients should be treated with substances that inhibit COX-2 and iNOS.

ACKNOWLEDGEMENTS

The research reported was supported by a NARSAD Distinguished researcher award to M.Maes and by M-CARE4U and CRC-MH, Antwerp, Belgium.

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