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Inflammation Research

# The activation of protein kinase C induces higher production of reactive oxygen species by mononuclear cells in patients with multiple sclerosis than in controls

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**Abstract.** *Objective:* Recent findings have increasingly shown the importance of reactive oxygen species (ROS) in causing oxidative damage to macromolecules and in contributing to tissue degeneration in target organs of autoimmune diseases. This study was aimed at comparing the base line and induced production of ROS by peripheral blood mononuclear cells (PB MNCs) of patients with multiple sclerosis (MS) in remission and relapse, of patients with other neurological diseases (OND) and of healthy controls. In addition, we analyzed the underlying mechanism of ROS production.
*Methods:* PB MNCs were separated from 28 MS patients in remission and 13 in relapse, and from 29 healthy controls and 10 OND. ROS was measured by spectrofluorometry. Expression of proinflammatory cytokines was assessed by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Mitochondrial (mt) DNA haplotypes were determined by using restriction site polymorphism analysis.
*Results:* The base line and tumor necrosis factor (TNF)-$\alpha$ or interferon (IFN)-$\gamma$ induced ROS values were similar in the four groups, and the individual measures did not show a correlation with MS associated mtDNA haplotypes. Phorbol ester activation of protein kinase C (PKC) induced higher ROS production in all groups, however, with significantly greater values in the MS remission group. Calphostine C, a PKC inhibitor decreased or eliminated ROS production in a dose-dependent manner, suggesting further that it was predominantly or exclusively generated by PKC activated NADPH oxidase. A trend of increased TNF-$\alpha$ and IFN-$\gamma$ expression was noted in the MS relapse group, in contrast to the high ROS release in the MS remission group.
*Conclusion:* The detected phase difference between the highest ROS production vs TNF-$\alpha$ expression is compatible with the hypothesis that different subpopulations of monocytes/macrophages are involved. We suggest that the ROS producing subpopulation preferentially migrates into the central nervous system (CNS) during a relapse. The present study together with our previous observation on oxidative damage to DNA in active plaques delineates a molecular pathway likely involved in the histologic evolution of inflammatory demyelination.

**Key words:** Reactive oxygen species – Protein kinase C – Multiple sclerosis

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## Introduction

Several forms of molecular alterations secondary to chronic inflammation have been recognized including phosphorylation, nitration and oxidation of macromolecules. One of the best characterized molecular alterations includes oxidative damage to DNA, proteins and lipids caused by ROS [1–5]. ROS ($O_2^-$; $H_2O_2$; $OH^-$) are produced during the oxidative burst of activated macrophages and neutrophils involved in antimicrobial defense. The two major sites of ROS formation are the plasma membrane bound NADPH oxidase complex and the respiratory chain in mitochondria (6). NADPH catalyzes the production of $O_2^-$ from $O_2$ [6]. Superoxide dismutase (SOD) converts $O_2^-$ into $H_2O_2$, which in the presence of iron, undergoes a Fenton reaction to produce $OH^-$, a very damaging species reacting with macromolecules at a high rate [6]. In various autoimmune conditions (e.g. diabetes and asthma) a chronic increase in ROS production by monocytes/macrophages has been associated with oxidative damage to macromolecules and degeneration of affected tissues (e.g. diabetic vasculopathy) [1, 4, 5]. Increased production of ROS by activated monocytic, polymorphonuclear and microglial cells, and associated oxidative damage to proteins and lipids in the CNS, have been shown in late acute, hyperacute and early relapsing phases of EAE [7–9]. ROS degrade myelin lipids and proteins in vitro [10], and cause degeneration of cultured oligodendrocytes [11]. The iron-chelating agent desferrioxiamine prevents the tissue damaging effects of ROS and inhibits the development of EAE [12]. Increased

ROS production by monocytes and polymorphonuclear cells has been noted previously in small numbers of MS patients [13–15].

These studies collectively establish that autoimmune conditions are associated with increased production of ROS, which contribute to molecular damage in the target organs of immune attacks. Our previous study added to these observations that oxidative damage to DNA develops in association with inflammation in active MS plaques [16]. Histologic studies reveal that this oxidative damage is likely related to the presence of ROS and NO producing macrophages and glial cells in active lesions [2, 17, 18]. In T2-weighted MRI of early active lesions, a ring-like hypointense signal within the hyperintense region of demyelination is described [19]. Histologic correlation studies suggest that this ring-like appearance of a paramagnetic MRI signal is probably related to the presence of ROS produced in excess by infiltrating macrophages [17]. Despite the high potential of ROS for causing ultrastructural changes and degeneration through oxidative damage in plaques, they have not been thoroughly investigated in MS. In this preliminary study, we asked if ROS production correlates with disease activity, mtDNA haplotypes associated with MS, or with production of cytokines (TNF-$\alpha$, IFN-$\gamma$) known to parallel disease activity [20–24]. We also addressed what subcellular pathway is involved in ROS production, and how it relates to the immunopathology of MS.

## Patients and controls

Patients with clinically definite and laboratory supported relapsing-remitting (RR) MS [25] were recruited from the MS Clinic of MCP-HU. Heparinized blood was obtained from 28 patients during a remission phase of the disease and from 13 patients during a relapse phase of the disease (prior to methylprednisolone therapy). In addition, 29 healthy controls volunteered to participate in the study in the Blood Donor Center, MCP-HU. Ten patients with OND (having seizures, headache, stroke or polyneuropathy) were collected in the Hospitals of MCP-HU. Because of mtDNA haplotype analysis, only Caucasian males and females participated in the study with an age range of 18–54 years.

## Methods

MNCs were separated from PB by Ficoll-Paque density gradient centrifugation, and washed 6 times with phosphate buffered saline (PBS). MNCs were used freshly for ROS measurement and were frozen at –70 °C until used for DNA and RNA extraction.

### *Spectrofluorometric analysis*

A fluorometric microplate assay was used to measure oxidative products of PB MNC in the presence of 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) (Sigma) [26]. Extracellular ROS production was measured after incubation of cells at 37 °C for 30 minutes with (phorbol myristate acetate [PMA]; n-formyl-methionyl-leucyl-phenylalanine [fMLP]; TNF-$\alpha$; IFN-$\gamma$) (Sigma, Pharmingen) or without stimuli in the presence of DCFH-DA. In the presence of peroxides released by stimulated MNCs, non-fluorescent DCFH-DA is oxidized and converted into a fluorescent counterpart in the supernatant. This product was measured by a spectrofluorometer (CytoFluor Series 4000 Fluorescence Multi-well Plate Reader, PerSeptive BioSystems, Framingham, MA, USA) at an excitation wavelength of 485 nm and at an emission wavelength of 535 nm. The fluorescent microplate assay allowed us to run up to 96 samples simultaneously. The optimal cell number per well ($10^5$) and the concentration of DCFH-DA (10 ug/ml), IFN-$\gamma$ (100 U), TNF-$\alpha$ (100 U), PMA ($10^{-7}$ M) and fMLP ($10^{-7}$ M) were determined in preliminary kinetic studies, and were consistently used in each experiment. We also performed a kinetic study by adding increasing amounts ($10^{-15}$–$10^{-3}$ M) of $H_2O_2$ to wells containing DCFH-DA in the absence of cells. With logarithmic increments of $H_2O_2$ an exponential dose response curve was obtained, which was shifted to the right over time. The minimum detection level of $H_2O_2$ was $10^{-9}$ M. ROS production by $10^5$ MNC from MS patients and controls varied in a range equivalent to $10^{-8}$–$10^{-5}$ M $H_2O_2$, in which range the dose response curve was virtually linear. Calphostine C (10 uM) (Sigma, St. Louis, MO, USA), a non-isoform specific PKC inhibitor was used to confirm the involvement of this enzyme in ROS production (27). In preliminary studies with 1-10-50-100 uM calphostine C a dose-dependent reduction of the PMA induced ROS release was seen by MNCs. A dose of 10 uM was chosen for the study, because calphostine C at this concentration eliminated 80–100% of the PMA induced ROS production without decreasing the viability of MNCs. $Ca^{2+}$-influx dependence of the reaction was tested by using BAPTA-AM (1,2 bis-(2-aminophenoxy)ethane-NNN′N′-tetra-acetic acid) (100 uM) (Sigma) an intracellular $Ca^{2+}$-chelator, and by performing the assay in $Ca^{2+}$-free buffers (27). Catalase (Sigma) (100 U/ml), an $H_2O_2$ scavenger, and superoxide dismutase (SOD) (Sigma) (400 U/ml), an $O_2^-$-scavenger, were applied to analyze components of ROS.

mtDNA haplotypes of interest (J* and K*, shown to be associated with MS in Caucasians) were determined by restriction site polymorphism analysis of selected regions amplified by polymerase chain reaction (PCR), as described [20, 21, 28]. J* was defined as –13,704/*Bst*NI;–16,065/*Hinf* I; +14,798/*Dde*I, and K* was defined as –9052/*Hae*II; –9053/*Hha*I; +14,798/*Dde*I, where "+" indicates the presence and "–" indicates the absence of a (polymorphic) restriction site (20).

Cytokine expression of non-stimulated MNCs was determined by semiquantitative RT-PCR using specific primers for TNF-$\alpha$ and IFN-$\gamma$. For reverse transcription 1 ug RNA was used, and 2 ul of each cDNA sample was amplified. A cycle number of 27 was chosen (linear phase of amplification) in preliminary experiments. Twenty ul of PCR mixtures were separated by agarose gel electrophoresis, blotted onto a nylon membrane, and hybridized with a biotinylated PCR generated probe of both cytokines (Gibco-BRL, Gaithersburg, MD, USA). The expression of cytokines was normalized for the expression of $\beta$-actin. Bands were quantitated by densitometric analysis (Multiscan 17Sf II, Molecular Dynamics, Sunnyvale, CA, USA). Cytokine expression was correlated with ROS production by MNCs from patients and controls.

| | HC | OND | MS-remission | MS-relapse |
|---|---|---|---|---|
| DCFH-DA | 1790 ± 1349 | 1017 ± 311 | 2252 ± 1189 | 1414 ± 1093 |
| IFN-$\gamma$ | 2030 ± 1511 | 995 ± 286 | 2445 ± 1703 | 1524 ± 1111 |
| TNF-$\alpha$ | 1651 ± 1180 | 918 ± 203 | 2171 ± 1702 | 1388 ± 1028 |
| PMA | 3021 ± 2350 | 2508 ± 2753 | 5196 ± 3471 | 2683 ± 2490 |
| fMLP | 1835 ± 1311 | 1043 ± 302 | 2476 ± 1809 | 1450 ± 863 |

**Table 1.** Baseline and stimulated ROS production by PB MNCs of MS patients and controls.

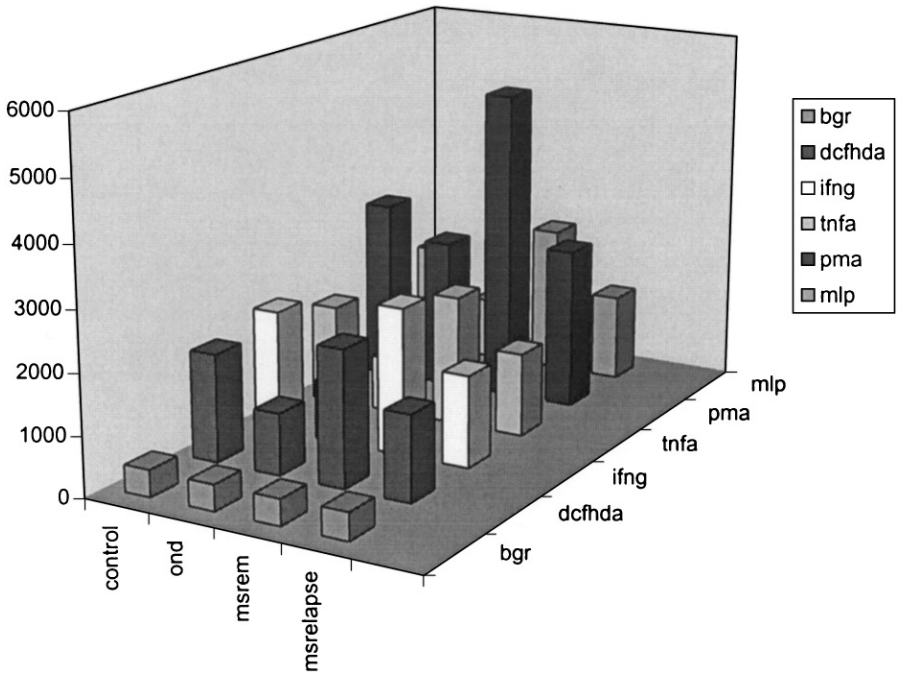


**Fig. 1.** Base line and INF-$\gamma$, TNF-$\alpha$, PMA and fMLP induced production of ROS by MNCs from MS patients and controls was measured by spectrofluorometric method. Results are expressed as mean ± SE of fluorescence units of ROS/$10^5$ MNCs. PMA induced ROS production by MNCs was significantly higher at p = 0.007 when the MS remission group was compared to the MS relapse group, at p = 0.004 when the MS remission group was compared to MS relapse and control groups, and at p = 0.0003 when the MS remission group was compared to all three other groups (Kruskal-Wallis non-parametric test).

*Statistical analysis*

The Kruskal-Wallis test (non-parametric), t-test and regression analysis were performed using the SAS version 6.12 computer program.

## Results

*ROS production by MNCs from MS patients and controls*

Unstimulated MNCs of MS patients during remission produced moderately higher amounts of ROS than normal and OND controls (p-value non-significant) (Table 1, Fig. 1). IFN-$\gamma$ , TNF-$\alpha$ or fMLP had no or small effect on ROS production in all three groups without statistical difference among groups. PMA induced higher ROS production in each group. The PMA induced ROS production was significant at p = 0.007 when the MS remission group was compared to the MS relapse group, at p = 0.004 when the MS remission group was compared to MS relapse and control groups, and at p = 0.0003 when the MS remission group was compared to all three other groups (Kruskal-Wallis non-parametric test) (Table 1, Fig. 1). When ROS production from patients treated and untreated with Betaseron or Avonex was compared within the MS remission group, no difference in mean values was seen.

Further functional analysis of ROS production was performed in MNCs from 4 MS patients in remission. Catalase (an $H_2O_2$ scavenger) in 100 U/ml did not reduce ROS values. SOD (an $O_2^-$ scavenger) in 400 U/ml dosage reduced the PMA-induced ROS values by 50%. The combination of catalase (100 U/ml) and SOD (400 U/ml) acted in a synergistic manner and resulted in a 2/3 reduction of ROS values. In addition, 1000 and 2000 U/ml concentrations of SOD completely eliminated PMA-induced ROS production, but no effect was seen with similar dosages of catalase alone. The PMA-induced increase in ROS production was not or only minimally inhibited by a Ca-influx inhibitor (15% reduction in the presence of 100 uM BAPTA AM). Ten uM calphostine C, a PKC inhibitor, reduced PMA-induced ROS production by 92%. These data suggest that increased ROS production in MS is PKC-dependent, and is predominantly composed of $O_2^-$ and $H_2O_2$.

*Correlation of ROS production with mtDNA haplotype*

Individuals in the study were screened for mtDNA haplotype K* or J* known to be associated with MS in Caucasians [20, 21]. The t-test revealed no difference in the base line or PMA induced ROS production when individuals positive or negative for mtDNA K* and J* haplotypes were compared (Table 2).

*Correlation of ROS production with the expression of INF-$\gamma$ and TNF-$\alpha$*

mRNA expression of IFN-$\gamma$ and TNF-$\alpha$ normalized to $\beta$-actin expression did not correlate with ROS production. However, a trend was seen for higher expression of both cytokines in the MS relapse group (Fig. 2).

**Table 2.** Spontanous and PMA induced ROS production by individuals positive or negative for the MS associated mtDNA haplotypes.

| | K* or J* mtDNA haplotype | No. of indivuduals | Mean values | |
|---|---|---|---|---|
| | | | DCFH-DA | DCFH-DA+PMA |
| MS relapse | + | 1 | 1499 | 2527 |
| | – | 8 | 1659 | 3365 |
| | | p-value | 0.91 | 0.81 |
| MS remission | + | 5 | 2202 | 5229 |
| | – | 19 | 2471 | 5433 |
| | | p-value | 0.73 | 0.91 |
| Control | + | 2 | 2259 | 3050 |
| | – | 23 | 1912 | 3078 |
| | | p-value | 0.73 | 0.99 |

The t-test reveals that MNCs of individuals positive or negative for the MS associated mtDNA haplotypes K* and J* [20, 21] produce similar amounts of ROS when the baseline and PMA induced values are compared. (Some individuals did not have sufficient number of cells to perform all the DNA and RNA studies).

## Discussion

In this study, we describe characteristic features of ROS production in MS patients. ROS production by PB MNCs was found to be independent of mtDNA haplotypes associated with MS [20, 21]. We observed small or no effect of pro-inflammatory cytokines on ROS production and no difference among groups under the condition tested. However, the PKC activator PMA induced a notable increase in ROS production in each group with significantly higher values in the MS remission group.

PKC is a phospholipid dependent serine/threonine kinase family with at least 11 isoenzymes involved in signal transduction in various cell types. Recent studies classify PKC isoenzymes into three groups according to their $Ca^{2+}$ dependence and substrate preference [29]. Based on this classification, the PMA responsive and $Ca^{2+}$ independent isoenzyme we identified in ROS production belongs to PKC group II. Another PKC-dependent mechanism, an IFN-$\gamma$ mediated $Ca^{2+}$-influx in T lymphocytes has been associated with MS (30) which, however, is likely related to a different ($Ca^{2+}$-dependent) PKC isoform.

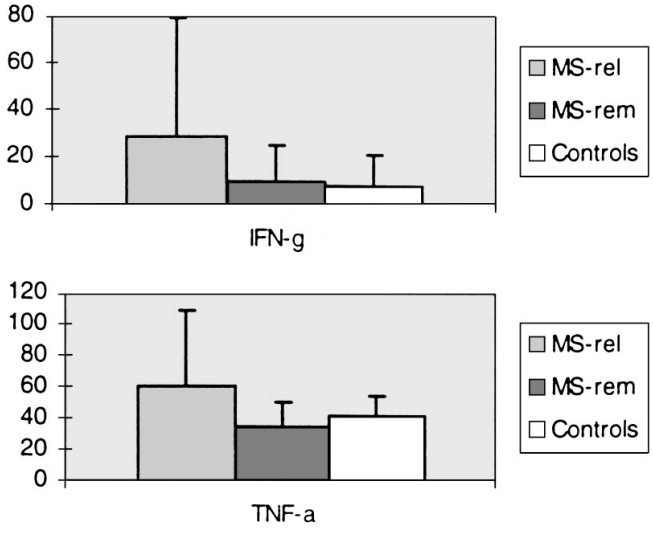


**Fig. 2.** IFN-$\gamma$ and TNF-$\alpha$ production by MNCs of MS patients and controls. mRNA expression of IFN-$\gamma$ and TNF-$\alpha$ was determined by Northern blot analysis. Densitometric values of cytokines normalized to those of $\beta$-actin are expressed as arbitrary units (mean ± SE). The Kruskal-Wallis test reveals no significant differences between the MS and control groups, but a trend for higher cytokine expression is seen in the MS relapse group.

The PKC isoenzyme involved in ROS production is thought to act through the phosphorylation and consequent membrane translocation of cytoplasmic NADPH oxidase [7, 31]. The present study suggests that PKC, in the presence of appropriate stimuli such as PMA, induces increased production of ROS by MNCs of MS patients in remission. The increased ROS production can be blocked by a PKC inhibitor, calphostine C. Although we did not separate adherent and non-adherent MNCs, the higher PMA induced ROS values point to monocytes, since lymphocytes show no detectable oxidative burst activity [13–15, 27, 32]. Since a trend was seen for increased expression of pro-inflammatory cytokines by MNCs, different subpopulations of monocytes/ macrophages may be involved in ROS and TNF-$\alpha$ production at the same time. Subpopulations of macrophages with high ROS release may preferentially migrate into the CNS and escape from ROS detection during a relapse. MRI detection of a paramagnetic signal (a potential ROS indicator) around the edges of acute demyelinating lesions [17, 19], and detection of oxidative damage to DNA in active plaques [16], supports this hypothesis. The question remains as to the cause and the cellular distribution of the PKC mediated increased ROS production in MS. It may be part of an inherent molecular abnormality responsible for a diminished threshold of several different activation pathways in immune cells, or it may be a consequence of increased levels of immune mediators. Activated PKC isoforms are likely involved not only in immune activation but also in other stages of pathogenesis such as the break-down of blood-brain barrier and dysfunction of oligodendrocytes. In cell cultures, PMA activation of a PKC isoform has been associated with increased endothelial permeability [33], or with down-regulation of myelin basic protein and 2′3′-cyclic nucleotide 3′-phosphohydrolase accompanied by demyelination and partial loss of differentiated oligodendrocytes [34].

In sum, the finding of increased PMA induced ROS production by PB MNCs of MS patients in remission seems

to be mediated by a PKC isoenzyme acting through the phosphorylation of NADPH oxidase. The phase difference between the highest ROS production and TNF-$\alpha$ expression suggests that distinct monocyte subpopulations are involved. A preferential transendothelial migration and pathogenic effect of ROS producing monocyte/macrophage subpopulation in the CNS is supported by previous observations. In vivo activators of PKC may include cytokines, phospholipids or small molecules released from CNS tissues damaged by inflammation. It remains to be investigated in what ways PKC relates to other immune activation pathways, and how the delineated *circulus vitiosus* (CNS tissue damage, release of potential ligands for PKC, immune activation, ROS production, CNS tissue damage) could be interrupted.

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