Enhanced antioxidant enzyme expression in inflammatory multiple sclerosis lesions

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Abstract

Reactive oxygen species (ROS) contribute to the formation and persistence of multiple sclerosis (MS) lesions by acting on distinct pathological processes. In the initial phase of MS lesion development, locally produced ROS may induce blood-brain barrier disruption and enhance leukocyte migration and myelin phagocytosis. In advanced stages, ROS may contribute to lesion persistence by mediating oligodendroglial damage and axonal injury. Generally, high levels of ROS cause oxidative stress, which induces transcription of antioxidant response element (ARE)-regulated genes. These genes encode various endogenous antioxidant enzymes that confer protection against oxidative damage. Here, we studied the expression of endogenous antioxidant enzymes in control brain tissue and various stages of MS lesions. ARE-regulated antioxidant enzymes, including superoxide dismutase-1 and -2, catalase, peroxiredoxin-1 and heme oxygenase-1 are expressed by glial cells in white matter from control brains and normal appearing white matter. Interestingly, these cytoprotective antioxidant enzymes are markedly upregulated in active demyelinating MS lesions. Particularly, foamy macrophages containing myelin and cellular debris and hypertrophic astrocytes abundantly expressed endogenous antioxidant enzymes. We speculate that enhanced antioxidant enzyme production in inflammatory MS lesions indicates the occurrence of oxidative stress and may reflect an adaptive defense mechanism to counteract ROS-induced cellular damage.
Antioxidant enzyme expression in MS lesions

Introduction

Multiple sclerosis (MS) is neuropathologically characterized by infiltration of inflammatory cells, particularly T cells and monocyte-derived macrophages, into the central nervous system (CNS)\(^1\)-\(^3\). Once infiltrated into the brain parenchyma, leukocytes evoke a cascade of pathological processes that are characteristic for MS, including demyelination, axonal damage and loss of oligodendrocytes\(^4\). Last years, evidence is emerging that reactive oxygen species (ROS) contribute to several mechanisms underlying the pathogenesis of MS lesions. We previously showed that ROS are produced within minutes upon interaction of monocytes with brain endothelium\(^5\). Local formation of ROS causes opening of intercellular tight junction complexes and cytoskeleton rearrangements ultimately leading to impaired blood-brain barrier functioning and subsequent extravasation of leukocytes into the CNS\(^6;7\). In addition, oxygen radicals enhance myelin phagocytosis and breakdown by macrophages\(^8\), oligodendrogial damage\(^9;10\) and neuronal and axonal injury\(^11;12\). Together, there is ample evidence that ROS contribute to the formation and progression of MS lesions.

Generally, exposure of cells to high levels of ROS induces oxidative stress, a term generally defined as an imbalance between the formation pro-oxidants and the antioxidant capacity, ultimately resulting in ROS-induced damage to macromolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA. The CNS is especially sensitive to oxidative damage due to its high oxygen consumption and is therefore equipped with a battery of antioxidant enzymes regulating the cellular redox status\(^13;14\). Expression of these cytoprotective enzymes is coordinated by the transcription factor nuclear factor-E2-related factor (Nrf2), which upon oxidative stress translocates to the nucleus, where it activates antioxidant response element (ARE)-mediated gene transcription\(^15;17\). So far, over 200 Nrf2-ARE-driven genes, involved in detoxification and antioxidant defense, have been identified, including superoxide dismutase-1 and -2\(^18\), peroxiredoxin-1\(^19;20\), catalase\(^19\) and heme oxygenase-1\(^20\).

Superoxide dismutase generally exists in two forms inside the eukaryotic cell, superoxide dismutase-1 in the cytoplasm and outer mitochondrial space and superoxide dismutase-2 exclusively in the inner mitochondrial space\(^21;22\). Superoxide dismutase-1 and -2 catalyze the conversion of superoxide anion to hydrogen peroxide. The removal of hydrogen peroxide in cells is predominantly mediated by catalase\(^23\), glutathione peroxidase\(^24\) and peroxiredoxin-1\(^25\).

Catalase has one of the highest turnover numbers for all known enzymes and exists as a tetramer of four identical subunits. Each monomer contains a heme prosthetic group at the catalytic center that reacts with hydrogen peroxide\(^23\). Although catalase is very efficient in the breakdown of hydrogen peroxide it has become clear that peroxiredoxins may constitute the major hydrogen peroxide removing enzymes\(^25\). The family of Prxs consists of six distinct groups of thio-specific antioxidant proteins that are involved in the enzymatic degradation of hydrogen peroxide, organic hydroperoxides and peroxynitrite. Prxs also play a role in the modulation of cytokine-induced hydrogen peroxide levels, redox signaling, cell proliferation, differentiation and gene expression\(^25\).
Heme oxygenase-1 is the rate-limiting enzyme in the catabolism of heme and a crucial mediator of antioxidant and tissue-protective actions. Heme oxygenase-1 breaks down the porphyrin ring of heme into biliverdin, free iron and carbon monoxide. The formed biliverdin is rapidly converted by biliverdin reductase into billirubin. This biliverdin-billirubin system exerts potent antioxidant activity, whereas the pro-oxidant iron is directly sequestered and inactivated by co-induced ferritin^26^.

Antioxidant enzymes have been implicated in various neurodegenerative disorders and their expression is increased in brains of Alzheimer’s disease and Parkinson’s disease patients^27^-^31^. In active MS lesions superoxide dismutase-1 gene expression was significantly increased^32^, however, the distribution and cellular localization of antioxidant enzymes in MS brain tissue remains elusive. Interestingly, upregulation of heme oxygenase-1, superoxide dismutase and catalase via specific enzyme inducers or viral vectors have been shown to ameliorate experimental allergic encephalomyelitis, an animal model for MS.

Given that ROS are intimately involved in various processes underlying the formation and persistence of MS lesions, we propose that antioxidant enzyme expression may be altered in MS brains. Hence, we examined the distribution pattern of various endogenous antioxidant enzymes in well-characterized MS lesions. Our data reveal that antioxidant enzyme expression is strikingly enhanced in active demyelinating MS lesions. Activation of the Nrf2-ARE pathway under neuroinflammatory conditions may function as a compensatory mechanism to counteract the harmful effects of ROS and concomitant oxidative stress and damage.
Material and methods

Autopsy Material
Brain tissue from 21 patients with clinically diagnosed and neuropathologically confirmed MS was obtained at rapid autopsy and immediately frozen in liquid nitrogen (in collaboration with The Netherlands Brain Bank, coordinator Dr. Ravid). The Netherlands Brain Bank received permission to perform autopsies, for the use of tissue and for access to medical records for research purposes from the Ethical Committee of the VU University Medical Center, Amsterdam, The Netherlands. Three cases without neurological disease were selected as controls. Tissue samples from 4 control cases were taken from the subcortical white matter or corpus callosum. MS tissue samples were selected on the basis of post-mortem MRI and lesions were classified according to standard histopathological criteria as previously published\textsuperscript{33}. Relevant clinical information was retrieved from the medical records and is summarized in Table 1. All patients and controls, or their next of kin, had given informed consent for autopsy and use of their brain tissue for research purposes.

Table 1. Summary of patient details.

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**Immunohistochemistry**

For immunohistochemical staining, 5 µm cryosections were air-dried and fixed in acetone for 10 minutes. Sections were preincubated for 30 minutes with 20% animal serum, the source of which was determined by the specific secondary antibody used. Afterwards, sections were incubated overnight at 4°C with primary antibodies directed against CD68 (DAKO, Glostrup, Denmark, 1:1000), proteolipid protein (PLP, clone plpc1, Serotec Ltd, Oxford, UK, 1:500), major histocompatibility complex (MHC) class II (DAKO, Glostrup, Denmark, 1:100), glial fibrillary acidic protein (GFAP, DAKO, Glostrup, Denmark, 1:20), peroxiredoxin-1 (Alexis Biochemical, Lausen, Switzerland, 1:200), catalase (Abcam, Cambridge, UK, 1:500), heme oxygenase-1 (Stressgen, Victoria, Canada, 1:700), superoxide dismutase-1 (The Binding Site, Birmingham, UK, 1:100) and superoxide dismutase-2 (The Binding Site, Birmingham, UK, 1:300). Subsequently, sections were incubated with appropriate secondary biotin-labeled antibodies for one hour at room temperature and with ABC (DAKO, Glostrup, Denmark) according to the manufacturer's description. Diaminobenzidine (DAB) was used as chromogen. Between the incubation steps, sections were thoroughly washed with PBS. After a short rinse in tap water preparations were incubated with hematoxylin for 1 minute and extensively washed in tap water for 10 minutes. Finally, sections were dehydrated with ethanol followed by xylol. All antibodies were diluted in PBS containing 0.1% bovine serum albumin (BSA, Boehringer-Mannheim, Germany), which also served as a negative control.

For colocalization studies, sections were preincubated for 30 minutes with 20% animal serum, the source of which was determined by the specific secondary antibody used. Then, sections were incubated with antibodies directed against the various antioxidant enzymes. To detect peroxiredoxin-1, catalase and heme oxygenase-1 we used biotinylated swine anti-rabbit (DAKO, Glostrup, Denmark, 1:500) and for superoxide dismutase-1 and -2 sections were incubated with biotinylated donkey anti-sheep (Jackson ImmunoResearch Laboratories Inc. Westgrove, USA, 1:300) as secondary antibody. Streptavidin-labeled Alexa-488 (Molecular Probes, Leiden, The Netherlands, 1:400) was used as fluorochrome. To distinguish different cell types, sections were then incubated with mouse monoclonal antibodies directed against human MHC class II, CD68 (macrophages and microglia) and GFAP (astrocytes). Biotin-labeled rabbit anti-mouse antibody was used as secondary antibody and streptavidin-labeled Alexa-594 (Molecular Probes, Leiden, The Netherlands, 1:400) was used as fluorescent probe. Basement membranes were detected with a monoclonal antibody directed against collagen type IV (DAKO, Glostrup, Denmark, 1:100). Fluorescence analysis was performed with a Leica TCS SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany).
Results

Lesion classification
Classification of the lesions was based on standard immunohistochemical stainings for inflammatory cells (anti-CD68 and anti-MHC class II) and myelin (PLP) as described previously\textsuperscript{33-35}. Based on these findings 13 lesions sampled in this study were classified as active with abundant phagocytic perivascular and parenchymal macrophages containing myelin degradation products, 9 lesions as chronic active with a hypocellular demyelinated center and a hypercellular rim containing numerous macrophages and 8 lesions as chronic inactive with only a few leukocytes and extensive GFAP-immunoreactivity throughout the lesion, indicating astrogliosis.

Antioxidant enzyme expression in control brain tissue and normal appearing white matter
In normal appearing white matter we observed weak expression of superoxide dismutase-1 in microglia (Figure 1A) and occasionally in astroglial cells, while superoxide dismutase-2 immunostaining was predominantly found in astrocytes (Figure 1B). Catalase, peroxiredoxin-1 and heme oxygenase-1 immunoreactivity was confined to microglia (Figure 1C-E). The expression pattern of antioxidant enzymes in control brain tissue was similar to that observed in the normal appearing white matter. Omission of primary antibodies against distinct antioxidant enzymes completely blocked immunopositivity (data not shown).

Cellular localization of antioxidant enzyme expression in MS brain tissue
Superoxide dismutase-1 and -2
Enhanced superoxide dismutase-1 staining was observed in foamy macrophages and astrocytes throughout active demyelinating MS lesions containing large numbers of inflammatory cells (Figure 2A, B, D). In contrast, superoxide dismutase-2 immunoreactivity was less prominent in foamy macrophages than superoxide dismutase-1, but more pronounced in large hypertrophic astrocytes, particularly at the rim of the lesion area (Figure 2C, E). In both control white matter and MS tissue brain endothelial cells and oligodendrocytes displayed very weak superoxide dismutase-1 and -2 staining. Using double immunofluorescence stainings we confirmed that superoxide dismutase-1 immunoreactivity was observed in MHC class II-positive macrophages (Figure 2F-H) and GFAP-positive astrocytes (not shown). Likewise, colocalization of superoxide dismutase-2 with macrophages (MHC class II, not shown) and the astrocyte marker GFAP (Figure 2I) was detected. Chronic inactive lesions contained dispersed weakly superoxide dismutase-1 and -2 positive astrocytes (data not shown).
Figure 1. Antioxidant enzyme expression in normal appearing white matter. Superoxide dismutase-1 (A) and superoxide dismutase-2 (B) expression was mainly observed in astrocytes and microglial cells in the normal appearing white matter. Antibodies directed against catalase (C), peroxiredoxin-1 (D) and heme oxygenase-1 (E) stained microglial cells in normal appearing white matter.
Antioxidant enzyme expression in MS lesions

Figure 2. Superoxide dismutase-1 and -2 expression in active MS lesions. Active MS lesions are characterized by high numbers of MHC class II-positive macrophages (A). Figure B and C represents high power images of the area designated by the dashed red line in figure A. Superoxide dismutase-1 immunoreactivity was predominantly expressed in foamy macrophages (B, D, arrows) and to a lesser extent in astrocytes (B, D, arrowhead), whereas superoxide dismutase-2 was mainly expressed by astroglial cells (C, E, arrowheads) and macrophages (C, E, arrows). Double immunofluorescence stainings confirmed that superoxide dismutase-1 immunoreactivity colocalized with MCH class II-positive foamy macrophages (F-H, superoxide dismutase-1 in green and MHC class II in red) and superoxide dismutase-2 staining with GFAP-immunopositive astrocytes (I, superoxide dismutase-2 in green and GFAP in red).
Catalase and heme oxygenase-1

Catalase is expressed by CD68-positive macrophages (Figure 3C) and GFAP-positive astrocytes (Figure 3D), in particular reactive astrocytes at the rim of active lesions. Similarly, both macrophages and hypertrophic astrocytes (Figure 3E) displayed heme oxygenase-1 immunoreactivity in active plaques, whereas astrocytes in inactive lesions showed weak catalase (data not shown) and heme oxygenase-1 immunostaining (Figure 3F). Catalase and heme oxygenase-1 expression was weak or even absent in vascular cells and oligodendrocytes and no differences were observed comparing control white matter with MS tissue.

Peroxiredoxin-1

Peroxiredoxin-1 was abundantly expressed in macrophages with a foamy appearance (Figure 4A, arrows) and large reactive astrocytes (Figure 4B) in active demyelinating MS plaques. Astrocytes in chronic inactive MS lesions displayed peroxiredoxin-1 immunoreactivity, albeit less intense than in active lesions (data not shown). Using anti-peroxiredoxin-1 we observed a weak staining of blood vessels in control brain tissue and normal appearing white matter (Figure 4C). However in active inflammatory lesions we noticed a striking upregulation of peroxiredoxin-1 associated with the vasculature (Figure 4A, arrowhead, Figure D). Colocalization studies with collagen IV, a major basement membrane constituent, demonstrated that peroxiredoxin-1 was expressed at the abluminal site of the vascular basement membrane (Figure 4E).

Figure 3 (opposite page). Catalase and heme oxygenase-1 expression in active MS lesions. Lack of PLP immunoreactivity in an active demyelinating MS lesion (A) containing numerous MCH class II-positive macrophages and activated microglia (B). Figure C-E represent high power images of the area designated by the dashed red line in figure B. Catalase is expressed by macrophages throughout the active lesion area (figure C, arrows). Astrocytes at the edge of the lesion displayed strong catalase immunoreactivity (D, arrows). Catalase staining colocalized with CD68-positive macrophages (C, insert, catalase in green and CD68 in red) and GFAP-immunopositive astrocytes (D, insert, catalase in green and GFAP in red). Foamy macrophages (E, arrows) and astrocytes (E, arrowhead) are intensely stained for heme oxygenase-1. Chronic inactive lesions contained weak heme oxygenase-1 immunoreactive astrocytes (F, arrows) and intensely heme oxygenase-1 immunopositive corpora amylacea (F, arrowhead). Heme oxygenase-1 staining colocalized with CD68-positive macrophages (E, insert, heme oxygenase-1 in green and CD68 in red) and GFAP-immunopositive astrocytes (E, insert, heme oxygenase-1 in green and GFAP in red).
Antioxidant enzyme expression in MS lesions

A

B

C

D

E

F
Figure 4. Peroxiredoxin-1 expression in active MS lesions. Macrophages (A, arrows), blood vessels (A, arrowhead) and hypertrophic astrocytes (B, arrows) displayed peroxiredoxin-1 immunoreactivity in active MS lesions. Peroxiredoxin-1 staining was weakly associated with the vasculature in normal appearing white matter (C, arrow) and markedly enhanced in active lesions (D, CD68 in green and peroxiredoxin-1 in red, arrows). Peroxiredoxin-1 immunoreactivity was observed at the abluminal side of the vascular basement membrane (E, collagen type IV in green and peroxiredoxin-1 in red).
Discussion

In the last decade there has been a major interest in the role of ROS during neuroinflammation. ROS are produced by activated macrophages and microglial cells and contribute to the formation and progression of MS lesions by acting on distinct pathological processes including transendothelial monocyte migration and myelin phagocytosis. In addition, at high concentrations, ROS are important mediators of oxidative damage to cell structures, including lipids, proteins and nucleic acids. Markers of free radical-mediated damage are increased in both serum and CNS tissue of MS patients.

To reduce cellular damage induced by oxidative stress, brain tissue is endowed with various defense systems, including a vast array of antioxidant enzymes. Our data show that antioxidant enzymes are highly upregulated in active demyelinating MS lesions. We propose that enhanced redox enzyme production is an adaptive compensatory mechanism to counteract the increased formation of ROS and subsequent oxidative damage in MS brains. Antioxidant enzyme gene expression is increased in active MS plaques, however, this is the first immunohistological survey demonstrating that antioxidant enzymes are markedly upregulated in inflammatory demyelinating MS lesions. Particularly, foamy macrophages and hypertrophic astrocytes expressed considerable levels of antioxidant enzymes. In contrast, weak expression of antioxidant enzymes was observed in glial cells in control brain tissue, normal appearing white matter and chronic inactive MS lesions, suggesting that antioxidant enzyme production is particularly increased during neuroinflammation.

Here, we show that myelin-laden macrophages produce high amounts of antioxidant enzymes. Recently, Boven and colleagues demonstrated that myelin-containing macrophages express various anti-inflammatory proteins and lack pro-inflammatory cytokines. Our data suggests that, in addition to their potential anti-inflammatory phenotype, myelin-containing macrophages exhibit an adaptive protective mechanism to detoxify ROS. Interestingly, oxidized-low density lipid-laden macrophages, like myelin-containing macrophages, expressed high levels of the antioxidant enzyme peroxiredoxin.

Astrocytes in active MS lesions and at the edges of chronic active MS lesions expressed high levels of antioxidant enzymes, which correlated with the presence and extent of inflammation, and associated oxidative stress. In the CNS, expression of Nrf2-ARE enzymes preferentially occurs in astrocytes and we previously showed that the antioxidant protein NAD(P)H:quinone oxidoreductase 1 is mainly localized in astrocytes in active MS lesions. Furthermore, it has been demonstrated that astrocytes in spinal cord lesions from MS patients expressed enhanced levels of heme oxygenase-1 compared to astrocytes in spinal white matter of normal subjects. In line with this report, we did observe heme oxygenase-1-immunoreactive astrocytes, however most of the heme oxygenase-1 immunostaining was found in foamy macrophages in demyelinating MS lesions.
In contrast to macrophages and astrocytes, oligodendrocytes lacked antioxidant enzyme expression, suggesting that they are less capable of detoxifying ROS. In fact, several studies have shown that oligodendrocytes are extremely sensitive to oxidative stress \textit{in vitro} due to an impaired antioxidant defense mechanism\textsuperscript{10,48}. Lack of an adaptive compensatory mechanism to eliminate ROS make oligodendrocytes more vulnerable and prone to ROS-induced cell death in MS lesions and thus contribute to oligodendrocyte cell loss and concomitant demyelination.

Surprisingly, brain endothelial cells expressed relatively low amounts of antioxidant enzymes in MS brain tissue and no striking differences were observed between demyelinating MS lesions and normal appearing white matter. This observation may indicate that brain endothelial cells are less capable to enhance the production of endogenous antioxidants in response to oxidative stress. In addition to specialized brain endothelial cells the blood-brain barrier is composed of astrocyte endfeet, which surround brain endothelial cells and contribute to the brain endothelial phenotype\textsuperscript{49}. Surprisingly, we observed enhanced peroxiredoxin-1 expression, which was associated with the cerebrovasculature in areas of leukocyte infiltration. Double-immunofluorescence stainings revealed increased peroxiredoxin-1 immunostaining at the abluminal site of the vascular basement membrane suggesting that peroxiredoxin-1 expression is selectively upregulated in astrocyte endfeet.

In conclusion, we showed for the first time that superoxide dismutase-1 and -2, catalase, peroxiredoxin-1 and heme oxygenase-1 are highly upregulated in active demyelinating MS lesions, particularly in myelin-laden macrophages and hypertrophic astrocytes. Enhanced expression of Nrf2-ARE-regulated antioxidant enzymes may function as an adaptive protective mechanism against ROS-mediated cellular toxicity. In future, antioxidant enzymes or compounds that specifically activate the Nrf2-ARE pathway and thereby induce the production and activity of endogenous antioxidant enzymes may represent an attractive therapeutic target for future treatment strategies in MS and other neurodegenerative disorders.

\section*{Acknowledgements}

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References