Increased astrocytic expression of mitochondrial antioxidant enzymes in active multiple sclerosis lesions

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Abstract

Axonal degeneration is widespread in multiple sclerosis (MS) lesions and is considered to be the main pathological correlate of permanent disability in patients. Recent evidence suggests that reactive oxygen species (ROS) derived from inflammatory cells drive axonal degeneration in active MS lesions by inducing intra-axonal mitochondrial dysfunction. Besides being a target of exogenous ROS, mitochondria are also capable of producing vast amounts of ROS themselves. To protect mitochondria from ROS-induced damage, an intricate system of mitochondrial-specific antioxidant enzymes exists that detoxifies the various ROS. Peroxiredoxin-3 (Prx3) and thioredoxin-2 (Trx2) are two key antioxidant enzymes which work in concert to detoxify peroxides in mitochondria. In this study, we analyzed expression of Prx3 and Trx2 in various MS white matter lesions and observed a striking upregulation of these mitochondrial antioxidants in reactive astrocytes in inflammatory MS lesions. To assess the functional role of this upregulation, we overexpressed Prx3 and Trx2 in an astrocytoma cell line. Astrocytoma cells overexpressing mitochondrial antioxidant enzymes were less susceptible to ROS-induced cell death compared to control cells. Moreover, we observed that neuroblastoma cells co-cultured with either Prx3 or Trx2 overexpressing astrocytoma cells were less vulnerable to exogenous ROS compared to neuroblastoma cells co-cultured with control astrocytoma cells. Taken together, we here provide strong evidence of increased expression of Prx3 and Trx2 in reactive astrocytes in inflammatory MS lesions. We speculate that upregulation of mitochondrial antioxidants preserves mitochondrial function and protects astrocytes from oxidative stress and subsequent cell death, and might even contribute to axonal survival.

Keywords
mitochondrial antioxidants; multiple sclerosis; reactive oxygen species; peroxiredoxin-3; thioredoxin-2
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Introduction

Multiple sclerosis (MS) is the leading cause of non-traumatic neurological disability among young adults in Europe and North-America.\(^1\) MS is generally characterized as an immune-mediated disease in which infiltrating macrophages and T-lymphocytes induce focal demyelination and associated neurodegeneration.\(^1,2,3\) Currently, it is widely accepted that neuro-axonal injury and loss represent the major pathological correlates of chronic disability in MS.\(^4\) Therefore, unravelling mechanisms that contribute to axonal degeneration and indentifying pathways that are involved in the protection of axons in MS are of utmost importance to bring disease progression to a halt.

Evidence is accumulating that reactive oxygen species (ROS) play an important role in axonal injury in MS.\(^5\) ROS are abundantly produced during the inflammatory phase of the disease by infiltrated macrophages and activated microglia. Besides their role in axonal degeneration ROS also induce myelin phagocytosis and oligodendrocyte cell death.\(^5\) Inflammation-derived ROS also contribute to mitochondrial dysfunction, which will consequently lead to increased mitochondrial ROS production.\(^6\) Importantly, ROS-mediated mitochondrial dysfunction will further induce axonal degeneration and neuronal loss.\(^7-10\)

As mitochondria produce large amounts of ROS, and mtDNA lacks protective histones, mitochondria are extremely vulnerable to ROS.\(^11\) Therefore, mitochondria are equipped with a specific antioxidant apparatus, which under physiological circumstances reduces virtually all mitochondrial ROS. The mitochondrial peroxiredoxin-thioredoxin antioxidant system is essential to detoxify various ROS in mitochondria.\(^12,13\) Peroxiredoxin-3 (Prx3) is the mitochondria-specific member of the peroxiredoxin family, which catalyzes the reduction of peroxides.\(^14\) It contains a redox-active cysteine residue that can be oxidized by peroxides to cysteine sulfenic acid (\(-\text{SOH}\)). To regain antioxidant function Prx3-SOH is reduced by thioredoxin-2 (Trx2), which in turn becomes oxidized. Trx2 is a mitochondria-localized member of the thioredoxin family, which are capable of removing disulfide bonds in proteins.\(^15\) Moreover, Trx2 is capable of directly reducing various ROS, and is involved in gene expression, cell growth and apoptosis by reducing proteins involved in these pathways.\(^15\)

Preservation of mitochondrial function is especially important in the central nervous system (CNS) as neurons are highly dependent on oxidative metabolism. This is further underscored by the neuroprotective properties of the mitochondrial antioxidants Prx3 and Trx2.\(^15-17\) These studies show that complete depletion of these antioxidants is lethal to neurons, while overexpression or intraventricular administration of Prx3 and Trx2 reduces neuronal cell death and improves neuronal function in various animal models for ischemia. Recently, we have showed that the expression of Prx3 and Trx2 is reduced in MS cortex compared to control grey matter, which was due to decreased expression of the transcriptional co-regulator PGC-1a. Immunohistochemistry, revealed a predominant neuronal localization of Prx3, Trx2 and PGC-1a indicating that the loss of expression reflects reduced neuronal expression. As ROS are abundantly produced during the inflammatory phase of the disease\(^18\) leading to mitochondrial dysfunction we set out to explore the cellular distribution of Prx3 and Trx2 in inflammatory MS white matter lesions.

We show that the expression of Prx3 and Trx2 is markedly increased in active white matter lesions, particularly in reactive astrocytes. To mimic this situation in vitro we generated astrocytic cell lines overexpressing Prx3 and/or Trx2, and subsequently demonstrated that overexpression of either Prx3 or Trx2 protects astrocytoma cells from ROS-induced cell death. Neurons co-cultured with mitochondrial antioxidant overexpressing astrocytes...
showed enhanced protection against exogenous ROS, indicating that increased astrocytic Prx3 and Trx2 in MS lesions may represent an intrinsic defence mechanism to promote axonal survival during an inflammatory-driven oxidative attack.

Material and Methods

Brain tissue

Formalin-fixed, paraffin-embedded brain sections were obtained from 11 patients and 6 matched non-neurological controls, in collaboration with the Netherlands Brain Bank, Amsterdam, The Netherlands. Detailed clinical data are summarized in table 1. The study was approved by the institutional ethics review board (VU University Medical Center, Amsterdam) and all donors or their next of kin provided written informed consent for brain autopsy, use of material and clinical information for research purposes.

Immunohistochemistry

Five um-thick paraffin sections were collected on SuperfrostPlus glass slides (VWR International; Leuven, Belgium) and dried overnight at at 37º C. Sections were deparaffinized in a series of xylene (3 x 5 min), 100% ethanol, 96% ethanol, 70% ethanol and water. Endogenous peroxidase was blocked by incubating the sectionse in methanol with 0.3% H₂O₂. Next sections were incubated with appropriate primary antibodies (see table 2) in phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA; Roche diagnostics GmbH, Mannheim, Germany) overnight at 4°C and stained with the EnVision horseradish peroxidase (DAKO, Glostrup, Denmark) kit followed by 3,3’diaminobenzidine-tetrahydrochloridedihydrate (DAB; DAKO). After a short rinse in tap water, sections were counterstained with haematoxylin for 1 min and intensely washed with tap water for 5 min. Images were taken on a Leica DM4000B microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). All primary antibodies were diluted in 0.01 mol/L phosphate buffered saline (PBS; pH 7.4) containing 1% BSA and 0.05% Tween-20 (SigmaAldrich, St Louis, MO, USA), which also served as a negative control.

Cell culture and lentiviral induced (over)expression

The astrocytoma cell line U373 and the co-cultures were cultured in DMEM/F12 (Life Technologies, Vienna, Austria) and the human neuroblastoma cell line SH-SY5Y was cultured in Opti-MEMα/HAM F-12 (1:1, Life Technologies, Vienna, Austria) both containing 10% foetal calf serum (FCS, Life Technologies), 2 mM L-glutamin (Life Technologies), and penicillin/streptomycin (50mg/ml; Life Technologies) in 24-well plates (Greiner Bio-One, Frickenhausen, Germany) at 5% CO₂ and 37º C. To overexpress Prx3, its coding sequence was amplified from primary human astrocyte cDNA and cloned into the lentiviral vector pRRL-cPPT-CMV-X2-PRE-SIN (kindly provided by Dr. J. Seppen, Academic Medical Center, Amsterdam, the Netherlands). Trx2 was kindly provided by Professor Jones (Emory University, Atlanta, GA, USA) in a pCDNA3.1-v5 vector which was subsequently cloned into the same lentiviral vector.19 Lentiviral vectors were produced by co-transfecting subconfluent human embryonic kidney (HEK) 293T cells with the MAZ expression plasmid and lentiviral packaging plasmids (pMDLg/pPRE and pRSV-Rev), using calcium phosphate as a transfection reagent. Lentiviral vectors were collected 24 h after transfection. The supernatant was centrifuged to remove cell debris and stored at -80ºC. U373 cells were
transduced with the lentivirus-containing Prx3 and Trx2. Forty-eight hours after infection of U373 cells with the lentiviruses, stable cell lines were selected by puromycin treatment (2 lg/mL; SigmaAldrich). The expression knockdown efficiency was determined by western blotting. Subsequently, stable cell lines were used to study the effects of increased Prx3 or Trx2 (from here on termed U373Prx3 and U373Trx2, respectively) expression on resistance against tert-butyl hydrogen peroxide (SigmaAldrich). U373 cells stably transduced with the empty pRRL vector served as control (U373mock). Finally, SH-SYSY neuroblastoma cells were stably transduced with the SHC003 turboGFP vector (SigmaAldrich), to generate a green fluorescent cell line.

**Western blot**

Protein isolation from U373 cells was performed using M-PER buffer supplemented with protease and phosphatase inhibitors according to manufacturer’s protocol (Thermo Scientific, Rockford, IL, USA). Protein concentrations were measured using BCA protein assay (Thermo Scientific). Western blot was performed as described previously. In short, equal amounts of protein (25–100µg) were separated on 10% SDS-PAGE gels and transferred to PVDF membranes (Bio-Rad Laboratories, Berkeley, CA, USA). After blocking in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, AK USA), membranes were incubated with appropriate primary antibodies (for details, see table 2) overnight in Odyssey blocking buffer at 4°C. Primary antibodies were detected by incubation with appropriate IRDye secondary antibodies (LI-COR Biosciences) for 1 hour at RT in Odyssey blocking buffer and quantified using the Odyssey infrared imaging system (LI-COR Biosciences). Actin quantification was used to correct for total protein loading variation.

**Functional analysis of cells overexpressing Prx3 and Trx2**

U373Prx3, U373Trx2 and U373mock were plated in 96-well plates (Greiner Bio-One, Frickenhausen, Germany) and grown until confluent. Hereafter, resistance against exogenous ROS was determined in shRNA transduced cells. Therefore, cells were treated with various concentrations of tert-butyl hydrogen peroxide for 4 hours. After which cell viability was assessed using the LIVE/DEAD Viability/Cytotoxicity kit (Invitrogen) according to manufacturer’s protocol. Fluorescent signals of alive and dead cells were measured with the Fluostar Galaxy (BMG Labtech, Ortenberg, Germany) and ratio between dead and live cells was calculated.

To assess neuroprotective properties of astrocytic overexpression of Tnx2 and Prx3, GFP-positive SH-SYSY cells were seeded on confluent monolayers of mock and overexpressing U373 cells. After 24h, cells were treated with tert-butyl hydrogen peroxide for 4 hours and washed twice. Then, remaining GFP signal was measured using the Fluostar Galaxy.

**Statistical analysis**

Protein expression levels of shRNA treated cells were compared using Student’s t-test. 2-Way ANOVA with Bonferroni post-hoc test was used to asses differences in dead : live cell ratios and neuroprotective properties of astrocyte cell lines.

**Results**

Mitochondrial antioxidant expression in MS white matter

Mitochondria are endowed with an intricate system of antioxidant enzymes. Here,
we analyzed the expression of peroxiredoxin-3 (Prx3) and thioredoxin-2 (Trx2), two key mitochondrial antioxidant enzymes that protect mitochondria against ROS-induced damage.

Inflammatory MS lesions are characterized by demyelination (Fig 1A) and abundant leukocyte infiltrates including foamy macrophages (Fig 1B). In active lesions the overall

**Figure 1. Increased mitochondrial antioxidant expression in astrocytes in inflammatory MS lesions.** Active demyelinating lesions are characterized by loss of proteolipid protein (PLP; A) and presence of foamy macrophages (B, inset). Immunostaining for peroxiredoxin 3 (Prx3) revealed similar Prx3 expression and distribution in normal-appearing white matter (NAWM; C) compared to control white matter (data not shown). Whereas Prx3 expression in inflammatory MS lesions was clearly increased in cells that morphologically resemble reactive astrocytes (D). Thioredoxin 2 (Trx2) was similarly expressed in MS brains, with Trx2 expression being comparable to control in NAWM (E) and clearly increased in certain cells in inflammatory MS lesions (F). Double immunofluorescent staining of Prx3 (G) and glial fibrillary acidic protein (GFAP; H) confirmed astrocyte specific upregulation of Prx3 in astrocytes (I). Likewise, double staining of Trx2 (J) with GFAP (K) revealed astrocytic localization of Trx2 in inflammatory MS lesions (L).
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intensity of Prx3 (Fig 1D) and Trx2 (Fig 1F) immunoreactivity was increased compared to normal appearing white matter (Fig 1C & E) and white matter from non-neurological controls (data not shown). We observed a marked cellular upregulation of Prx3 which appeared to be specific for cells resembling reactive astrocytes (Fig 1D, inset), whereas Trx2 immunostaining was enhanced throughout the brain parenchyma and in reactive astrocytes (Fig 1F, inset). Colocalization studies with glial fibrillary acidic protein (GFAP; Fig 1H & K) confirmed that both Prx3 (Fig 1G) and Trx2 (Fig 1J) were consistently upregulated in astrocytes in inflammatory MS lesions (Fig 1I and L). Thus far, we did not observe differences in Prx3 and Trx2 expression in axons or oligodendrocytes in MS lesions compared to axons in NAWM or control. However, immunofluorescent double stainings are warranted to exclude such a change.

Overexpression of Prx3 and Trx2 in astrocytoma cells improves mitochondrial function and reduces ROS-mediated cell death U373Prx3, U373Trx2 and U373mock

In order to investigate the protective effects of increased astrocytic Prx3 and Trx2 expression in active white matter MS lesions we stably transduced U373 astrocytoma cells with lentivirus containing human Prx3, Trx2 or empty vector. U373Prx3 cells exhibited a 6-fold increase in Prx3 protein expression compared to U373mock, while Trx2 expression was only slightly increased. Trx2 overexpression in U373 cells led to a 200% increase in Trx2 protein levels compared to U373mock, remarkably Prx3 protein expression was also 2-fold increased in these cells (Fig 2A-B). Since Prx3 and Trx2 catalyze the reduction of various peroxides, including hydrogen peroxide, we treated U373Prx3, U373Trx2 and U373mock cells with increasing concentrations of tert-butyl hydrogen peroxide. Overexpression of Prx3 and Trx2 in U373 cells markedly increased the resistance of U373 cells against an oxidative attack (Fig 2C). 4h treatment with 250 µM tert-butyl hydrogen peroxide led to a significant increase in cell death in U373mock cells, whereas U373Prx3 and U373Trx2 cells were completely protected against ROS mediated cell death at this concentration.

It has been described that overexpression of mitochondrial antioxidant enzymes can protect neurons under pathological conditions. Here we set out to investigate whether Prx3 and Trx2 overexpression in U373 cells are able to protect neurons from ROS-induced cell death. GFP expressing neuroblastoma cells were generated and cultured on a monolayer of U373Prx3, U373Trx2 and U373mock cells. Decreased death of neuronal cells was observed in co-cultures with U373Prx3, U373Trx2 and U373mock cells after treatment with different concentrations of tbH2O2, indicating the neuroprotective properties of astrocytes (Fig 2D). Moreover, 250 µM tert-butyl hydrogen peroxide led to less neuronal death in cocultures with either U373Prx3 or U373Trx2 when compared to U373mock.

Discussion

In the present study we show for the first time that the expression of peroxiredoxin-3 (Prx3) and thioredoxin-2 (Trx2), two key mitochondrial antioxidant enzymes, is markedly increased in astrocytes in active multiple sclerosis (MS) lesions. Our in vitro data show that overexpression of Prx3 and Trx2 protects astrocytoma cells against an oxidative attack. Moreover, co-culture experiments demonstrate that increased astrocytic expression of mitochondrial antioxidants decreased ROS-induced neuronal cell death in vitro. We speculate that upregulation of Prx3 and Trx2 in astrocytes in inflammatory MS lesions represent an endogenous defence mechanism to protect astrocytes and surrounding...
ROS unambiguously play a cardinal role in MS pathology and are strongly associated with inflammation and mitochondrial (dys)function. In most cells, mitochondria are the main site of ROS production and are therefore particularly susceptible to oxidative damage. To counteract the detrimental effects of ROS, mitochondria are endowed with their own efficient antioxidant apparatus, including the antioxidant enzymes Prx3 and Trx2. Although virtually all cells are equipped with various antioxidants, some cells are more effective in handling oxidative stress than others. Astrocytes, for instance, effectively react to increased ROS in an active MS lesion by inducing cytoplasmic antioxidant levels,
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whereas this upregulation is absent in oligodendrocytes and axons.\textsuperscript{21} This inability of axons and oligodendrocytes to increase their antioxidative capacity contributes to the extensive oxidative damage in axons and oligodendrocytes and subsequent cell death in MS lesions.\textsuperscript{18,21} Our observation that astrocytes markedly upregulate Prx3 and Trx2 in active MS lesions emphasizes the ability of astrocytes to robustly respond to an oxidative insult. At first sight, axons and oligodendrocytes, the main victims of the oxidative attack in inflammatory lesions, did not seem to enhance mitochondrial antioxidant expression in active MS lesions. However, further studies are warranted to exclude the upregulation of Prx3 and Trx2 in axons and oligodendrocytes in active MS lesions.

Production of most antioxidant enzymes, including those involved in glutathione metabolism and heme oxygenase-1 are coordinated by the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2).\textsuperscript{22} To date, efforts aimed at increasing the cells antioxidant capacity focussed mainly on activation of the Nrf2 pathway. Prx3 and Trx2, however, are not regulated by this transcription factor (unpublished data). Instead, Prx3 and Trx2 expression is under the control of PGC1-α, a mitochondrial transcriptional co-regulator which is responsible for the transcription of many nuclear-encoded mitochondrial molecules, including components of the oxidative phosphorylation complexes and various antioxidant enzymes (Witte, submitted).\textsuperscript{23} PGC1-α is known to be activated upon increased oxidative stress, indicating that the enhanced expression of mitochondrial antioxidant enzymes in inflammatory MS lesions is likely an astrocytic response to the oxidative environment.\textsuperscript{5} Future studies are needed to confirm astrocytic PGC1-α. In vitro, we have evidently shown that increased expression of Prx3 and/or Trx2 protects astrocytoma cells from ROS-induced cell death. This corroborates previous studies, that showed that overexpression of Trx2 in HeLa and COS7 cell lines protects cells against TNF-α- and hydrogen peroxide-induced cell death.\textsuperscript{11} Moreover, Prx3 overexpression in mice leads to decreased mitochondrial ROS production and increased resistance to oxidative stress-induced cell death and apoptosis.\textsuperscript{24} Lastly, stereotactic injection of Prx3 in combination with Trx2 is protective against ischemia and more effective than injection of Prx3 alone.\textsuperscript{17} In MS, inflammation-derived ROS can initiate cell death or tissue destruction through damaging mitochondria. This is supported by previous studies which show that exogenous ROS induces cell death through a caspase-3-independent apoptotic mechanism involving an increase in mitochondrial ROS, pro-apoptotic proteins, loss of mitochondrial membrane potential and release of cytochrome C.\textsuperscript{25, 26, 27} Taken together, our findings infer that increased mitochondrial antioxidant capacity protects astrocytes against inflammation-induced cell death.

Neurodegeneration is generally accepted as the main cause of irreversible neurological disability in MS patients.\textsuperscript{4} Recently, it has been shown that intra-axonal mitochondrial pathology precedes axonal degeneration in EAE spinal cord. This could be counteracted by the administration of exogenous antioxidants, indicating that inflammation-derived ROS are key in mitochondrial dysfunction and subsequent axonal degeneration.\textsuperscript{28} In contrast to the increased expression of Prx3 and Trx2 in astrocytes in active MS lesions, we previously showed that Prx3 and Trx2 levels are decreased in cortical neurons of MS patients. (Witte, submitted). In this study we show that enhanced astrocytic mitochondrial antioxidants are able to protect adjacent neurons from ROS-induced cell death \textit{in vitro}, accentuating the neuroprotective properties of astrocytic mitochondrial antioxidants. It has been described that neurons are more resistant to oxidative stress in the presence
of astrocytes. Moreover, overexpression of Nrf2 in astrocytes delays disease onset and increases the lifespan in mouse models for different neurodegenerative diseases, demonstrating the important role of astrogial antioxidant capacity in neuronal survival. However, the ongoing axonal damage in active MS lesion suggests that the increase in astrogial Prx3 and Trx2 is not sufficient to protect all neighboring axons.

(Neuro)protective properties of Prx3 or Trx2 overexpression were surprisingly similar, even though lentiviral transduction of astrocytoma cells with Prx3 was more successful. This could indicate that Trx2 is more efficient in detoxifying hydrogen peroxide. However, U373Trx2 cells also contained increased Prx3 levels, which could indicate that concurrent mild upregulation of both antioxidants is just as protective as a 6-fold induction in Prx3 levels. Thus far, it remains to be elucidated why Prx3 expression is increased after lentiviral transduction of astrocytes with Trx2.

Nowadays evidence is accumulating that antioxidant therapy represents an attractive strategy to reduce axonal and neural degeneration. In fact oral antioxidants treatment in a murine model of X-linked adrenoleukodystrophy, a rare disease characterized by inflammatory demyelination and axonal pathology halts axonal degeneration. Moreover, activation of the Nrf2 pathway by BG12, a dimethyl fumarate compound currently in phase III trials, has shown to improve the outcome of MS patients. However, the present study indicates that mitochondrial antioxidants, which are not regulated by Nrf2 (unpublished data), also represent an interesting therapeutic targets. We propose that a combined therapy targeting mitochondrial antioxidants and Nrf2-driven antioxidants may be of particular interest in combating neurodegeneration in MS.

In conclusion, we have shown increased mitochondrial antioxidant enzyme expression in astrocytes in active MS lesions. Our in vitro data indicate that overexpression of specific mitochondrial antioxidant enzymes preserves mitochondrial function during an oxidative insult and protects astrocytes from ROS-mediated cell death. Finally, using neuron-astrocyte co-cultures we demonstrate that Prx3 and Trx2 overexpressing astrocytes are able to reduce ROS-induced neuronal cell death.

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Chapter 4


Tables

Table 1 Clinical data of MS patients and non-neurological controls

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SP = secondary progressive MS; PP = primary progressive MS; ND = not determined; m = male; f = female; A = active lesion; CA = chronic active lesion; CIA = chronic inactive lesion

Table 2 Antibody details

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IHC, immunohistochemistry; WB, western blot; HLA-DR, human leukocyte antigen-DR; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1-α; GFAP, glial fibrillary acidic protein; NA, not applicable

* Sources: Serotec, Oxford, UK; eBioscience, San Diego, CA; Santa Cruz Biotechnology, Santa Cruz, CA; Abfrontier, Seoul, Korea; Sigma-Aldrich, St Louis, MO; Monosan, Uden, The Netherlands.