Summary and discussion
Monocyte-derived macrophages are considered the major cell type causing demyelination and axonal damage in MS lesions. During inflammation, macrophages produce large amounts of inflammatory mediators, including cytokines, chemokines, and reactive oxygen species (ROS), which play a role in the development of the disease. 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 (BBB) disruption and enhance leukocyte migration. In the brain parenchyma, ROS contribute to lesion persistence by mediating oligodendroglial damage and axonal injury. The studies described in this thesis aimed to elucidate how the BBB and the central nervous system (CNS) parenchyma react to an oxidative attack. Below, the results of these studies are summarized and discussed.

1. ROS play a role during monocyte migration into the CNS

1.1 ROS are produced upon the interaction of monocytes with brain ECs

An early step in MS lesion formation is the migration of monocytes from the circulation into the CNS. To enter the CNS, monocytes have to cross the blood-brain barrier (BBB), which mainly consists of highly specialized brain endothelial cells (ECs) and their tight junction (TJ) complexes. In general, transendothelial migration of leukocytes occurs in several consecutive steps, involving various sets of adhesion molecules like selectins and inflammatory mediators like chemokines. Previous studies from our group showed that ROS are of importance during the migration process, since ROS enhance monocyte adhesion and migration, whereas antioxidants reduce monocyte migration. In chapter 2, these studies were extended and we demonstrated that the interaction of monocytes with brain ECs induces the production of ROS by monocytes. Monocytes can produce ROS via the membrane associated NADPH-oxidase complex and via the enzyme xanthine oxidase. This may be triggered via integrin engagement during monocyte adhesion to brain endothelium. In addition, other monocytic cell surface proteins may be involved. For instance, the interaction of SIRPα with its ligand on brain ECs, CD47, is required for the transmigration of monocytes across brain endothelium. It has been described that ligation of signal regulatory protein α (SIRPα) on macrophages induces NADPH-oxidase activation, which may account for increased release of ROS that influences the BBB.

Brain ECs may also produce ROS upon the interaction with monocytes, which can contribute to BBB permeability and monocyte adhesion and migration. The adhesion molecule VCAM-1 is the major adhesion molecule on brain ECs involved in monocyte adhesion and migration during inflammation. Previous studies described that engagement of VCAM-1 on peripheral ECs induces ROS production by the NADPH oxidase complex. However, quantitative PCR experiments suggest that, in contrast to human umbilical vein endothelial cells that express abundant levels of NADPH oxidase 2 (NOX2) and NOX4, rat brain ECs express very low levels of NADPH oxidase molecules (unpublished results). These low levels may, however, be sufficient to induce
intracellular effects in brain ECs. It has been described that brain ECs can produce ROS via xanthine oxidase\textsuperscript{13}. Our group previously showed that inhibitors of xanthine oxidase decrease monocyte migration, suggesting that xanthine oxidase is the major source of ROS during transendothelial migration of monocytes\textsuperscript{4}. Future studies on the cellular source and molecular interactions involved in ROS production during adhesion of monocytes to brain ECs may lead to the selective inhibition of adhesion-induced ROS production and subsequent BBB-damage.

1.2 ROS affect BBB integrity and facilitate monocyte migration
The migration of leukocytes across the BBB requires the active participation of brain ECs to rearrange their cytoskeleton and TJs, thus allowing leukocytes to enter the CNS. TJ molecules, like occludin and claudin-5, are of importance for BBB integrity to keep immune cells out of the brain. Through its interaction with TJ molecules, the actin cytoskeleton plays an active role in maintaining TJ integrity and BBB function\textsuperscript{14}. In chapter 2, we demonstrate that adhesion of monocytes to an \textit{in vitro} model of the BBB induces permeability of a brain endothelial monolayer. The induced leakage is prevented by the presence of the ROS scavenger α-lipoic acid, suggesting that ROS are directly involved in monocyte-induced BBB permeability. In addition, extracellular ROS directly affect the integrity of the brain endothelial monolayer, by reducing transendothelial electrical resistance and enhancing paracellular permeability (chapter 3). Altered BBB integrity induced by ROS is paralleled by cytoskeleton rearrangements and redistribution and disappearance of TJ proteins claudin-5 and occludin from the intercellular junction.

Rearrangement of the brain endothelial actin cytoskeleton and TJ complexes involves intracellular signaling events\textsuperscript{15-19}. In chapter 3 we demonstrate that the effect of ROS on the actin cytoskeleton and TJs is mediated by signal transduction via RhoA, phosphatidylinositol 3-kinase (PI3 kinase) and protein kinase B (PKB) (figure 3, insert A). Therefore, agents that inhibit ROS-induced activation of these signal transduction pathways, such as antioxidants or signaling inhibitors for RhoA, PI3 kinase or PKB, may be tools to prevent transendothelial migration of monocytes. Indeed, the antioxidants α-lipoic acid (chapter 2) and luteolin\textsuperscript{20} reduce monocyte migration across the BBB \textit{in vitro}. In addition, antioxidants like α-lipoic acid (chapter 2)\textsuperscript{21,22}, luteolin\textsuperscript{20}, N-acetyl-L-cysteine\textsuperscript{23}, and N-acetylcysteine amide (AD4)\textsuperscript{24} prevent cellular infiltration into the CNS and reduce the development of clinical signs in EAE. Furthermore, overexpression of the endogenous antioxidant enzyme peroxiredoxin 1 (Prx1) in brain ECs increases BBB integrity and reduces transendothelial migration of monocytes (chapter 4), indicating that antioxidants may be a powerful tool to prevent cellular infiltration into the CNS. It has been shown that inhibition of the activation of small GTPases, especially RhoA, in the brain endothelium by various agents also results in a decreased cellular migration both \textit{in vitro} and \textit{in vivo} (unpublished results obtained in our group)\textsuperscript{15,16,25,26}. In addition, our group previously showed that inhibitors of the PI3 kinase pathway prevent superoxide-induced monocyte migration\textsuperscript{4}, suggesting that signaling pathways that are involved in ROS-induced BBB permeability indeed play a role in transendothelial migration of monocytes.
Besides RhoA, PI3 kinase and PKB, other signaling molecules may be involved in ROS-induced BBB permeability. It has been described that the phosphorylation state of occludin is important in TJ assembly and disassembly\textsuperscript{27}. A number of kinases have been associated with occludin-phosphorylation, including extracellular signal related kinase (ERK) and mitogen-activated protein (MAP) kinase, which have been linked to ROS-induced occludin disruption at epithelial junctions\textsuperscript{28-30}. These molecules may also be involved in BBB dysfunction and may be targets to inhibit transendothelial migration of monocytes. However, it should be taken into account that the above mentioned signal transduction pathways may play a role in many physiological processes. In addition, inhibitors of these pathways may affect leukocyte migration into other tissues, which is of importance for immune surveillance and removal of pathogens. Therefore, to prevent undesirable side effects, it is important to find signaling pathways that are specific for BBB dysfunction.

1.3 Other players during transendothelial migration of monocytes

Besides a direct effect on BBB integrity, ROS may also affect transendothelial migration of monocytes via the regulation of other molecules involved in cellular migration, such as adhesion molecules or matrix metalloproteinases (MMPs). Transendothelial migration of monocytes is mediated by various sets of adhesion molecules, including integrins expressed on monocytes and members of the immunoglobulin superfamily expressed on brain endothelium. Short-term incubation with superoxide or \(\alpha\)-lipoic acid did not alter adhesion molecule expression on monocytes or brain ECs (chapter 2). However, in acute EAE, treatment with \(\alpha\)-lipoic acid may have affected adhesion molecule activity on monocytes or brain ECs. Integrins are expressed on monocytes in their inactive form, but they are rapidly activated by intracellular stimuli via a process called inside-out signaling\textsuperscript{31}. ROS may induce integrin activation, thereby enhancing the adhesive and migratory capacity of monocytes\textsuperscript{5}. It has recently been reported that the platelet integrin \(\alpha_2\beta_3\) contains a redox site within its extracellular domain that is constituted of several unpaired cysteine residues\textsuperscript{32}. Integrins involved in monocyte migration across the BBB may also contain such redox sites, which may be susceptible to activation by ROS. Future studies are needed to demonstrate whether ROS affect the affinity of integrins expressed on monocytes or influences downstream signaling pathways of endothelial adhesion molecules, such as members of the immunoglobulin superfamily.

Infiltrating leukocytes and brain ECs secrete MMPs that degrade and remodel extracellular matrix components, such as fibronectin, collagen, and laminin\textsuperscript{33} and play a critical role during leukocyte migration across the BBB. The TJ molecule occludin contains a putative MMP cleavage site in its first extracellular loop\textsuperscript{34}, suggesting that MMPs may be involved in transmigration via cleavage of TJ molecules. This is supported by \textit{in vitro} observations that, upon the interaction of monocytes with brain ECs, occludin disappears from the junctions and is degraded, which could be prevented by MMP inhibitors\textsuperscript{35}. For peripheral endothelium, it has been described that both VCAM-1 crosslinking and extracellular addition of ROS activate endothelial cell-associated MMPs, which is dependent on the presence of ROS and facilitates transendothelial migration of
leukocytes\textsuperscript{36}. In addition, α-lipoic acid dose-dependently decreases the activity of MMP-9, thus affecting leukocyte migration into the CNS\textsuperscript{37}. These data suggest that ROS-induced MMP-activation may be of importance during monocyte migration across the BBB. Preliminary data indicate that ROS also induce activation of MMPs in brain ECs. Future studies should specify whether ROS-induced MMP-production in brain ECs plays a role during migration of monocytes into the CNS and whether this is a target for therapeutic intervention in neurological diseases associated with BBB dysfunction.

1.4 How do extracellular ROS activate intracellular signaling pathways in brain ECs?
It is not exactly clear how extracellular ROS, e.g. excreted by monocytes, induce activation of intracellular signal transduction pathways in brain ECs. Superoxide is highly reactive and it is assumed that superoxide cannot diffuse across the plasmamembrane. Hydrogen peroxide can diffuse across lipid bilayers, suggesting that hydrogen peroxide is the pertinent ROS inducing signal transduction. However, the observation that SOD can inhibit superoxide-induced PKB phosphorylation (chapter 3) and monocyte migration\textsuperscript{4} implies that the effect is mediated by superoxide and not by hydrogen peroxide or hydroxyl radicals. Another mediator of monocyte/ROS-induced BBB opening may be a nitric oxide (NO) derivative. Superoxide can react with nitric oxide to form highly reactive peroxynitrite. In vitro, increased NO-levels are detectable after 24 hours coculture of monocytes and brain ECs and not within the time frame of our experiments (unpublished results). Inhibition of NO production by N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) or induction of NO production by the NO donor S-nitroso-N-acetylpenicillamine (SNAP) had no significant effect on monocyte migration across brain ECs (data not shown), suggesting that NO is not involved in ROS-induced BBB dysfunction upon monocyte migration. Possibly, superoxide oxidizes transmembrane receptors at the extracellular domain, thereby inducing an intracellular signal that activates pathways involved in BBB dysfunction. Alternatively, ROS can react with cellular lipids, generating a spectrum of products, many of which contain functional groups capable of modifying proteins. These reactive lipid products may induce the activation of intracellular signal transduction pathways\textsuperscript{38}. Oxidized lipids can disrupt the organization of the cell membrane and mediate permeability changes through TJ desintegration\textsuperscript{39,40}. In addition, damage to mitochondria induced by lipid peroxidation can lead to further intracellular ROS generation. Subsequently, reactive lipid products may transducer ROS into cellular signals through post-translational modification of critical cellular proteins\textsuperscript{38,41-44}. Factors that protect cells from oxidative attacks may lead to the maintenance of BBB integrity under oxidative conditions and may thus prevent cellular migration into the CNS.

In conclusion, the effect of ROS, produced upon the interaction of monocytes with brain ECs, on BBB integrity and transendothelial migration of monocytes, may be impeded at multiple levels. First, the production of ROS upon adhesion of monocytes to brain ECs can be prevented by the inhibition of the cellular source or molecular interactions involved in ROS-production. Secondly, knowledge on the targets of ROS can lead to the development of compounds that contribute to the maintenance of BBB integrity under
oxidative conditions. And thirdly, ROS-induced BBB dysfunction can be prevented by antioxidants or specific inhibitors of pathways involved in ROS-induced BBB opening, such as RhoA, PI3 kinase and PKB. Agents that selectively affect these mechanisms may be used therapeutically to modulate neurological diseases complicated by ROS-induced BBB dysfunction (Figure 3, insert A).

2 Cellular adaptations to ROS-induced damage

ROS play a physiological role in numerous cellular regulatory processes. However, a disturbed balance between the rate of ROS production and the capacity of antioxidant defense may lead to oxidative stress and subsequent tissue damage. Besides their role in transendothelial migration of monocytes, ROS contribute to MS lesion persistence by mediating oligodendroglial damage and axonal injury. Macrophages produce large amounts of ROS during myelin phagocytosis. Subsequently, ROS induce damage to myelin lipids, oligodendrocytes and axons. Long-term exposure to ROS may provoke adaptive responses to counteract the oxidative attack by the induction of protective proteins. Redox sensitive transcription factors such as nuclear factor E2-related factor 2 (Nrf2) can contribute to adaptive responses to oxidative stress through the induction of endogenous antioxidant enzymes (reviewed in chapter 5). Under physiological conditions, Nrf2 is bound to Keap1 and located in the cytoplasm. However, upon oxidative stress, Nrf2 is released from Keap1 and translocates to the nucleus, where it activates ARE-mediated gene transcription and induces the coordinate transcription of ARE-regulated genes.

2.1 Expression of Nrf2/ARE regulated enzymes in MS lesions: indication of ongoing oxidative stress

In this thesis we demonstrate that protein expression of Nrf2/ARE regulated antioxidant enzymes, including NAD(P)H:quinone oxidoreductase (NQO1), catalase, superoxide dismutase 1 (SOD1), SOD2, peroxiredoxin 1 (Prx1), heme oxygenase 1 (HO-1), and glutathione peroxidase (GPx) is highly increased in both MS (chapter 7 and 8) and EAE lesions (chapter 6). In EAE, enhanced antioxidant enzyme expression correlated with disease severity and infiltration of monocyte-derived macrophages. Since transcription of endogenous antioxidant enzymes is triggered by ROS, enhanced expression of these enzymes suggests the presence of ongoing oxidative stress within active MS and EAE lesions. This is supported by the observation of enhanced levels of nitrotyrosine, an end product of peroxynitrite and biochemical marker for oxidative stress, in EAE lesions and MS lesions (chapter 6). Expression of endogenous antioxidant enzymes was mainly found in active lesions, which are marked by the presence of activated macrophages, suggesting that macrophages are the major source of ROS in MS lesions.

In MS lesions, high expression of endogenous antioxidant enzymes was found in macrophages containing myelin degradation products. During phagocytosis activated macrophages produce large amounts of ROS, which play a physiological role in bacterial
killing\textsuperscript{60}. During myelin phagocytosis, ROS are produced as well\textsuperscript{46}. To resist those ROS and subsequent reactive by-products, macrophages need defense mechanisms. It has been described that the Nrf2/ARE pathway plays an important role in antioxidant defense in macrophages\textsuperscript{61} and our data show that the Nrf2/ARE pathway is activated in infiltrated macrophages in MS lesions.

In addition to macrophages, enhanced expression of endogenous antioxidant enzymes was observed in astrocytes within MS lesions (Figure 3, insert B). In the CNS, expression of Nrf2/ARE driven enzymes preferentially occurs in astrocytes and much less in neurons, microglia or oligodendrocytes\textsuperscript{62-65}. Enhanced expression of Nrf2/ARE regulated genes in astrocytes in MS lesions suggests that endogenous antioxidants are protective against ROS-mediated toxicity in those cells, but other cell types, such as neurons and oligodendrocytes, may be overwhelmed by an oxidative attack, since they have lower antioxidant capacity\textsuperscript{48,66}. Therefore these cells may be more vulnerable to oxidative damage in MS.

2.2 Adaptations to oxidative stress in brain endothelium
Since ROS are produced during monocyte migration across the BBB, brain ECs were expected to express elevated levels of endogenous antioxidant enzymes in MS lesions. We detected weak to moderate expression of antioxidant enzymes in brain endothelium, but no differences were observed between MS lesions and normal appearing white matter (chapter 7 and 8). In vitro, we demonstrated that long-term exposure to superoxide induces the transcriptional activation of various endogenous antioxidant enzymes, such as SOD2, NQO1, and HO-1. In addition, superoxide induces alterations in protein expression, including the antioxidant enzyme Prx1 (chapter 4). This suggests that brain ECs have the capacity to express endogenous antioxidant enzymes when exposed to ROS. However, levels of ROS produced during migration may be sufficient to induce rearrangements of the actin cytoskeleton and TJs that are necessary for transendothelial migration of monocytes, but may be too low to activate the Nrf2/ARE pathway in brain ECs.

2.3 Therapeutic potential of endogenous antioxidant enzymes
Increased expression of endogenous antioxidant enzymes in MS lesions may function as a protective mechanism against ROS-mediated cellular toxicity. However, various studies showed that ROS-induced damage is present in MS lesions\textsuperscript{49,55,56,67-69}, suggesting that this response may not be sufficient or may be induced too late to be protective against ROS-induced injury. Therefore, targeting the Nrf2/ARE pathway in an earlier phase may represent a novel therapeutic approach for the treatment of oxidative stress-related neurological diseases, such as MS, stroke, or HIV-associated encephalitis.

In chapter 4, we studied the effect of enhanced antioxidant enzyme expression in brain ECs on transendothelial migration of monocytes. Overexpression of the antioxidant enzyme Prx1 enhanced BBB integrity and reduced cellular migration across brain endothelium. However, cellular adhesion and migration were not completely blocked by
Prx1 overexpression. In our cell line, Prx1 expression was approximately threefold increased. For Prx2, it has been described that thirty fold enhanced expression in peripheral ECs completely blocked inflammation-induced monocyte adhesion\textsuperscript{70}. Likely, higher expression of Prx1 or the induction of multiple antioxidant enzymes more potently reduces transendothelial migration of monocytes. Currently, a number of compounds are known that promote the transcriptional activation of the Nrf2/ARE pathway and induce the expression of multiple endogenous antioxidant enzymes, including tert-butylhydroquinone (tBHQ), dimethylfumarate, sulforaphane and 3-hydroxycoumarin\textsuperscript{71,72}. Preliminary studies from our group show that treatment of brain ECs with tBHQ dose-dependently reduces transendothelial migration of monocytes in vitro (Figure 1), suggesting that such compounds have potential for the treatment of neuroinflammation. Here, monocyte migration was also not completely inhibited, but optimization of the experimental conditions may increase the inhibitory effect of tBHQ on monocyte migration. Furthermore, other Nrf2/ARE inducing factors may more potently inhibit transendothelial migration of monocytes, which remains to be established.

![Figure 1. tBHQ dose-dependently reduces monocyte migration across brain endothelium.](image)

We also studied the effect of tBHQ treatment on neuroinflammation in vivo. Animals suffering from acute EAE (as described in chapter 6) were injected intraperitonally twice a day with tBHQ (15 mg/kg), starting from day 6 after immunization. At day 10 after immunization, vehicle treated animals started to lose tail tonus and at day 13 all animals were clinically ill (mean clinical score of 2.6 ± 0.12 at day 13, Figure 2). Animals treated with tBHQ revealed slightly reduced clinical signs (mean clinical score of 2.0 ± 1.18 at day 13). However, variation of clinical scores in this group was high and the reduction in clinical scores was not significant. Interestingly, upregulation of HO-1, SOD and catalase via specific enzyme inducers or viral vectors has been shown to ameliorate EAE\textsuperscript{73-75}, suggesting that enhanced expression of endogenous antioxidant enzymes may be valuable for the treatment of EAE. In addition, the beneficial effects of Nrf2/ARE activating compounds tBHQ and sulforaphane were demonstrated in animal models for other oxidative stress related neurological diseases, such as traumatic brain injury and stroke\textsuperscript{76-79}. Possibly, the dose of tBHQ we used was too low to induce functional levels of
endogenous antioxidant enzymes. In addition, induction of endogenous antioxidant enzymes earlier after immunization may be more effective in the treatment of acute EAE. Furthermore, other Nrf2/ARE inducing compounds, such as dimethylfumarate, sulforaphane and 3-hydroxycoumarin, may have more potency to induce the expression of antioxidant enzymes in the rat. Future studies should address these questions to provide insight into the value of Nrf2/ARE enzyme inducers for the treatment of neuroinflammatory diseases, such as MS, and other oxidative stress-related neurological disorders.

Figure 2. The effect of tBHQ treatment on the clinical course of acute EAE. Acute EAE was induced in 8- to 11-week-old male Lewis rat according to the protocol described in chapter 2. Animals were treated twice a day with 15 mg/kg tBHQ, starting from day 7 after immunization, by intraperitoneal injections. Squares represent vehicle treated EAE animals (n=8). Triangles represent animals treated with tBHQ (n=8). Data are expressed as mean clinical score ± SEM.

3. Concluding remarks

In this thesis we showed that ROS, which are produced upon the interaction of monocytes with brain ECs, play an important role during monocyte migration into the CNS by the induction of BBB dysfunction. As summarized in figure 3 (insert A), ROS activate RhoA, PI3 kinase and PKB, which induce rearrangements of the actin cytoskeleton and TJs, thus enhancing BBB permeability and facilitating monocyte migration into the CNS. In the brain parenchyma, ROS contribute to lesion persistence by mediating oligodendroglial damage and axonal injury. Data described in this thesis suggest that infiltrated macrophages are the major source of oxidative stress in MS lesions. In addition, we demonstrated that besides causing damage to the BBB, ROS induce adaptive responses in brain endothelial cells, astrocytes and infiltrated macrophages by the induction of endogenous antioxidant enzymes (Figure 3, insert A and B). However, in MS lesions, this response may not be sufficient or may be induced too late to be protective against ROS-induced damage. Therefore, antioxidant therapy, either via supplementation with exogenous antioxidants, or by the induction of endogenous antioxidant enzymes in an early phase of lesion formation, may be beneficial for the treatment of MS. At present, antioxidants like α-lipoic acid are used in
the clinic for the treatment of various diseases, such as diabetic polyneuropathy, diabetic nephropathy and burning mouth syndrome\textsuperscript{80-83}, without causing serious side effects.

Since ROS contribute to various pathological processes underlying MS lesion formation, scavengers of ROS may interfere at multiple levels (Figure 3). In the initial phase of MS lesion formation, ROS scavengers may prevent the migration of monocytes into the CNS, whereas in the brain parenchyma, antioxidants may prevent ROS-induced damage to oligodendrocytes and axons. Our findings have contributed to gaining insights into BBB dysfunction and may lead to new strategies for the treatment of MS to prevent lesion formation and progression.
Figure 3. Overview of pathological processes involved in MS lesion formation and the role of ROS herein.
1. Monocytes adhere to activated endothelium, which triggers ROS productions (Figure A at the opposite page). Next, ROS activate signal transduction pathways in brain ECs, such as RhoA and PI3 kinase, that induce cytoskeleton alterations and tight junction rearrangements, thus facilitating monocyte transendothelial migration. However, ROS also induce adaptive responses to oxidative stress in brain ECs. Within hours, ROS induce gene transcription of endogenous antioxidant enzymes, which leads to alterations in protein expression in the long term.
2. Monocytes accumulate in the perivascular space from where they migrate further into the brain parenchyma. In the brain parenchyma monocyte-derived macrophages phagocytose myelin, thus causing demyelination and axonal damage. During this process ROS are produced, which activate the cellular stress response and induce gene expression of endogenous antioxidant enzymes in macrophages themselves and in astrocytes (Figure B at the opposite page). Since ROS contribute to various pathological processes underlying MS lesion formation, scavengers of ROS may interfere at multiple levels. In the initial phase of MS lesion formation, ROS scavengers may prevent the migration of monocytes into the CNS. In the brain parenchyma, antioxidants may prevent ROS-induced damage to oligodendrocytes and neurons.
Summary and discussion
References

1. Butcher EC. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell 1991;67:1033-1036.


