Protein disulfide isomerase (PDI) is an oxidoreductase assisting oxidative protein folding in the endoplasmic reticulum of all types of cells, including neurons and glia. In neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS), up-regulation of PDI is an important part of unfolded protein response (UPR) that is thought to represent an adaption reaction and thereby protect the neurons. Importantly, studies on animal models of familial ALS with mutant Cu/Zn superoxide dismutase 1 (SOD1) have shown that the mutant SOD1 in astrocytes or microglia strongly regulates the progression of the disease. Here, we found an early up-regulation of PDI in microglia of transgenic (tg) mutant SOD1 mice, indicating that in addition to neurons, UPR takes place in glial cells in ALS. The observation was supported by the finding that also the expression of a UPR marker GADD34 (growth arrest and DNA damage-inducible protein) was induced in the spinal cord glia of tg mutant SOD1 mice. Because mutant SOD1 can cause sustained activation of NADPH oxidase (NOX), we investigated the role of PDI in UPR-induced NOX activation in microglia. In BV-2 microglia, UPR resulted in NOX activation with increased production of superoxide and increased release of tumor necrosis factor-α. The phenomenon was recapitulated in primary rat microglia, murine macrophages and human monocytes. Importantly, pharmacological inhibition of PDI or its down-regulation by short interfering RNAs prevented NOX activation in microglia and subsequent production of superoxide. Thus, results strongly demonstrate that UPR, caused by protein misfolding, may lead to PDI-dependent NOX activation and contribute to neurotoxicity in neurodegenerative diseases including ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by a loss of motoneurons in the motor cortex, brainstem and spinal cord (SC). About 90% of all ALS cases are sporadic. The second most frequent cause of familial ALS is attributed to dominantly inherited mutations in Cu/Zn superoxide dismutase 1 (SOD1) (1).

Gliarial cells play a major role in neurodegenerative disorders, including ALS. Both in ALS patients and the transgenic (tg) mice expressing mutant SOD1, astrogial dysfunction and microgliosis precede the clinical manifestations (2). Although the expression of mutant SOD1 in motoneurons has an impact on disease onset, the presence of mutant protein in surrounding glia is thought to strongly influence the disease progression (3,4).

The mechanism of how mutant SOD1 causes direct and glia-mediated neurotoxicity is not fully understood. In vivo ALS studies indicate that NADPH oxidase (NOX) activation and superoxide production are elevated in microglia and may contribute to motoneuron death (5). Importantly, it has been demonstrated that mutant SOD1 can bind Rac1, a small G protein that activates NOX complex (6), and cause sustained NOX activation, leading to increased superoxide production (7). Mutant SOD1 may also increase the secretion...
of tumor necrosis factor-α (TNFα) in a NOX-dependent manner (8).

Protein disulfide isomerase (PDI) is an enzyme in the endoplasmic reticulum (ER) catalyzing the formation and breakage of protein disulfide bonds. Under ER stress, PDI is thought to protect cells from apoptosis by re-arranging disulfides in misfolded proteins (9). In a number of neurodegenerative diseases, including ALS, misfolding and aggregation of proteins overwhelms the protein-folding capacity of the ER and induces excessive unfolded protein response (UPR) (10), which subsequently leads to apoptosis or autophagy (11). Considering that ER stress and UPR have been implicated in ALS, it is not surprising that PDI is up-regulated in the SC of G93A-SOD1 rat and mouse (12–14). In fact, PDI may have a central role in ALS pathogenesis, as it can directly interact with mutant SOD1 and prevent SOD1 aggregation both in vivo and in vitro (13,14). Interestingly, it has been reported that in the brains of Parkinson’s and Alzheimer’s disease patients as well as in the SCs of ALS patients and G93A-SOD1 mice, PDI is S-nitrosylated and therefore inactive (15,16). S-nitrosylation in turn leads to the accumulation of polyubiquitinated proteins and the activation of UPR. In non-neuronal cells, such as vascular cells and macrophages, PDI has been found to be associated with NOX and to act as a redox-sensitive regulatory protein of several NOX isoforms (17).

Altogether, recent investigations have demonstrated that PDI can interact with mutant SOD1 in neurons as well as with NOX in macrophages, and that mutant SOD1 regulates NOX activation in microglia. In light of the previous findings, our aim here was to explore whether PDI expression is up-regulated in glial cells of mutant SOD1 mice as a part of UPR, and whether UPR results in PDI activity-dependent NOX activation in microglia and other cells of monocytic lineage.

RESULTS

Prominent induction of PDI in microglia in the SC at the early symptomatic phase of ALS

Previous studies have shown that PDI, a protein associated with UPR, is up-regulated in the SCs in animal models of ALS, and strong expression of PDI was observed in motoneurons at the end-stage of disease (13). To more closely investigate the PDI expression in motoneurons at the time of disease onset, SC sections of 20-week-old tg and wild-type (wt) mice were stained and quantified for co-localization of PDI and SMI-32 in the SC ventral horns. PDI co-localized with SMI-32 staining both in wt and tg animals (Supplementary Material, Fig. S1A and B), and there was no difference in the PDI-positive area co-localizing with SMI-32 between the two mouse groups (Supplementary Material, Fig. S1C). However, the intensity of PDI immunoreactivity co-localizing with SMI-32 was modestly yet still significantly increased in tg animals (P < 0.05) (Supplementary Material, Fig. S1D), suggesting an increased PDI expression in motoneurons.

To assess the gliosis, an early event in the course of ALS, SC sections were first stained for astrocyte and microglia markers [GFAP (glial fibrillary acidic protein) and CD45, respectively, Supplementary Material, Fig. S2A and B]. In the ventral horn, GFAP immunoreactivity was up-regulated 3-fold and CD45 immunoreactivity even 7-fold in tg mice when compared with wt mice (Supplementary Material, Fig. S2C and D). Importantly, in the white matter, the increase of CD45 immunoreactivity was even more dominating, showing 20-fold increase (P < 0.01) in tg mice compared with the wt mice (Supplementary Material, Fig. S2F), whereas GFAP immunoreactivity in this lumbar SC region was increased only 2.5-fold (P < 0.05) (Supplementary Material, Fig. S2E). These results confirm the previous findings that microgliosis is more prominent than astrogliosis as an early hallmark of the degeneration of SC motoneurons (18,19). In addition, our data and the work done by Alexianu et al. (20) suggest that microglial activation is even more dramatic in the white matter than the ventral horn early in the course of motoneuron degeneration of tg mice.

To address the question whether cell types other than motoneurons, especially microglia, show altered expression of PDI at the time of the disease onset, the SC sections stained for microglia and astrocyte markers were double-labeled with PDI antibody, and the possible co-localization of these markers was quantitatively analyzed. When the ventral horn was analyzed, a prominent PDI immunoreactivity was detected in astrocytes (Fig. 1A and B) and microglia (Fig. 2A and B), but their contribution to the total PDI immunoreactive area was minor compared with the predominant and large neuronal structures. However, the PDI-positive area in the astrocytes was increased by 10% (P < 0.05) (Fig. 1C) and their PDI expression level (Fig. 1D) was increased even 8-fold (P < 0.001) in the tg SC ventral horn when compared with corresponding wt tissue. CD45-positive cells showed similar PDI immunoreactivity as GFAP-positive cells in the ventral horn. However, owing to unspecific binding of the secondary antibody to lipofuscin present in neuronal cell bodies, which caused a problem while imaging CD45 immunoreactivity at high magnification, no quantifications were done. Considerably less PDI immunoreactivity was detected in the SC white matter than in the gray matter (Fig. 2A–C). In the white matter, PDI immunoreactivity localized to structures of small cell bodies and long cellular projections. Confocal microscopy revealed that the PDI immunoreactivity co-localized with both astrocytes (Supplementary Material, Fig. S3A and B) and microglia (Fig. 2C and D) in the white matter. Importantly, although the PDI-positive area in the astrocytes was the same for tg and wt mice in the white matter (Supplementary Material, Fig. S3C), this value was 27% higher (P < 0.01) for PDI-positive area in microglia of tg mice than wt mice (Fig. 2E). Moreover, although weighted co-localization coefficient did not show changes in PDI expression levels in astrocytes between wt and tg mice (Supplementary Material, Fig. S3D), it revealed >3.5-fold increase in microglial PDI expression in the SC white matter of tg mice (Fig. 2F). Taken together, our findings indicate that PDI-immunoreactive populations of microglia and astrocytes as well as PDI expression level per cell in these cell populations increase around the cell bodies of degenerating motorneurons in the tg mice. Importantly, at the same time, in the anterior column of the white matter where the axons of the degenerating motoneurons emerge, it is only proliferating...
microglia that increase the population of PDI-immunoreactive cells as well as the level of PDI expression per cell. Moreover, as the up-regulation of PDI is a substantial part of UPR, these results suggest that UPR is induced especially in a considerable portion of microglia in the lumbar SC early in the course of motoneuron degeneration of tg mice.

UPR is induced in astrocytes and microglia at the early symptomatic phase of ALS

UPR and ER stress of neurons are involved in the ALS pathology (reviewed in 21). According to the previous studies, ER stress has been sometimes observed upon CNS insults also in astrocytes (22). However, although macrophages are well-known to induce ER stress in atherosclerosis (23), not much is known about ER stress in microglia. As the induction of PDI in SC astrocytes and microglia of the tg mice may indicate ER stress in glia during early ALS pathogenesis, we next assessed whether UPR takes place in the tg mouse glia by staining SC sections with an antibody against GADD34, (growth arrest and DNA damage-inducible protein) a widely used UPR marker (24). As a cell cycle protein, GADD34 is up-regulated in response to DNA damage, cell cycle arrest and ER dysfunction (25). In addition to strong GADD34 immunoreactivity in motoneurons, a faint and very sparse GADD34 immunoreactivity was seen in other cells co-localizing with GFAP- and CD45-immunoreactive glia in both the ventral horn and the white matter of wt mice (Fig. 3 and Supplementary Material, Fig. S4). Interestingly, quantitative confocal microscopy revealed that although the weighted co-localization coefficient for astrocytes was significantly increased in the ventral horn but not in the white matter (Fig. 3E and Supplementary Material, Fig. S4E) of the tg mice when compared with wt mice, the same co-efficient for microglia was significantly increased in the white matter (Fig. 3F) of the tg mice when compared with wt mice. Thus, the expression of both PDI and GADD34 is increased in ventral horn astrocytes and in white matter microglia in tg mice, suggesting segregation of these two glial populations regarding their ER stress and UPR in ALS.

UPR results in increased NOX activation coupled with enhanced superoxide production in microglia and other cells of monocytic lineage

ER stress capable of inducing UPR has been previously shown to result in the activation of NOX in peripheral macrophages (26). To explore whether UPR increases NOX activation in a PDI-dependent manner in microglia, we utilized murine BV-2 microglial cells treated with 2 mM dithiothreitol (DTT), a well-described and extensively used inducer of UPR (27). For the verification of the model, proteolytic activation of ATF6, a key molecule in the initiation phase of UPR (28), was measured. Proteolysis of ATF6 was strongly induced upon DTT treatment, as ATF6 was almost completely undetectable already at 0.5 h time point as illustrated by western blotting in Figure 4A (lanes 3 and 4).

There are various forms of NOX in monocytic cells and they all generate superoxide by transferring electrons from NADPH across the membrane inside the cell and couple them to molecular oxygen, resulting in superoxide release. In addition, UPR-induced NOX activation results in the generation of reactive oxygen species (ROS) in peripheral macrophages (17). Thus, we used the NADPH-dependent superoxide production as a measure of NOX activation and monitored the rate of superoxide generation using a lucigenin-based assay. As expected, DTT treatment significantly increased NOX activation (P < 0.05, Fig. 4B) in BV-2...
To confirm that UPR does indeed result in increased NOX activation in these cells, we also tested two other well-characterized UPR inducers: heat shock and sodium meta-arsenite (NaAsO₂). Both treatments resulted in a strong and significant increase in NOX activation (Fig. 4B). To exclude the possibility that UPR-induced NOX activation coupled with increased superoxide production is a feature specific only to BV-2 microglia cell line, we also exposed RAW 264.7 macrophages (Fig. 4C), primary rat microglia (Fig. 4D) and human bone marrow-derived monocytes (Fig. 4E) to DTT and measured the superoxide production. Analysis of all these different cells of monocytic lineage demonstrated a significant increase in superoxide production in RAW 264.7 macrophages (170%, \( P < 0.05 \)) and in primary microglia (493%, \( P < 0.001 \)) as well as a strong trend toward increased superoxide production in monocytes (130%), providing evidence that UPR-induced NOX activation associated with increased superoxide production is a common feature of all cells of monocytic lineage. In addition, the results suggest that among different cell types of monocytic lineage, UPR-induced NOX activation is especially robust in differentiated microglia.

Previously, overexpression of G93A-SOD1 has been reported to increase NOX activation in MO59J cells (7) and to enhance oxidative stress in microglial BV-2 cells (8). To verify these findings in our model, we transiently transfected BV-2 cells with wt and G93A-SOD1 constructs (Fig. 4F) and measured the NOX activation 24 h after the transfection. Although the expression levels of G93A-SOD1 was rather modest, the expression of this mutant SOD1 gene resulted in significantly increased, NADPH-dependent NOX activity compared with the effect of wtSOD1 expression (Fig. 4G). This effect was significantly inhibited by 300 \( \mu \text{M} \) bacitracin (Supplementary Material, Fig. S5).

**Figure 2.** PDI co-localizes with CD45 in both the ventral horn and the white matter of SC, and the co-localization is induced at the early symptomatic phase of ALS. (A–D) SC sections of wt (A and C) and tg (B and D) mice were stained with 4',6-diamidino-2-phenylindole (DAPI), anti-CD45 and anti-PDI, and confocal images of ventral horns (A and B) and the white matter (C and D) were taken. Immunoreactivity of CD45 and PDI was modest in wt SC tissues, but co-localization could be observed. In tg tissues, the immunoreactivity of CD45 and PDI was stronger and prominent co-localization could be observed. (E) Overlap coefficient of CD45 and PDI was significantly higher in the tg SC white matter compared with wt, indicating increased PDI-positive area in tg microglia (\( P < 0.001 \)). (F) In addition, tg mice had significantly increased weighted co-localization coefficient in the white matter of SC than wt mice, indicating significantly increased PDI expression in CD45-positive cells (\( P < 0.001 \)). Scale bar 20 \( \mu \text{m} \).

**UPR enhances TNFα secretion in microglia**

In addition to increased superoxide production, the release of TNFα is another potential toxic consequence of increased NOX activation in BV-2 cells (8). Thus, we wanted to evaluate whether the secretion of TNFα by BV-2 cells was also enhanced during UPR. The cells were exposed to DTT as described and the amount of TNFα released to the cell culture medium was determined by ELISA. Indeed, UPR resulted in about 5-fold increase in the amount of TNFα secreted by BV-2 cells (Fig. 4H).
PDI inhibition decreases UPR-induced NOX activation

PDI has been reported to regulate NOX activation in macrophages (29). Because we detected a clear up-regulation of PDI protein expression in tg mouse microglia in vivo, indicating that UPR takes place in these cells, and we also found that UPR increases NOX activation in cultured microglia, we next investigated whether microglial NOX activation is regulated by PDI. When UPR was induced by DTT or NaAsO₂ in the presence of bacitracin, a well-described PDI inhibitor (30), the NOX-mediated superoxide production was significantly reduced (Fig. 4I). To further verify that pharmacological inhibition of PDI inhibits the UPR-induced NOX activation, we also tested the effect of thiomuscimol, a novel PDI inhibitor (31) on DTT- and NaAsO₂-induced NOX activation.

Thiomuscimol inhibited the NOX activation with the same potency as bacitracin (Fig. 4I). Importantly, bacitracin significantly reduced the NOX activation also in DTT-treated RAW 264.7 macrophages (Fig. 4C) and human monocytes (Fig. 4E) and had a strong trend toward decreased superoxide production in rat primary microglia (Fig. 4D), suggesting that PDI activity is required for UPR-induced increase in NOX activation in cells of monocytic lineage.

Short interfering RNA-mediated down-regulation of PDI decreases NOX activation

Short interfering RNAs (siRNAs) targeted against PDI significantly down-regulated PDI expression in BV-2 cells (Fig. 5A).
Figure 4. Induction of UPR increases NOX activation in a PDI-dependent manner. (A) Induction of UPR by DTT results in proteolytic processing of ATF6. BV-2 cells were treated with 2 mM DTT for the times indicated, after which the cells were lysed and proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting. Proteolytic processing was detected after 0.5 h DTT treatment as indicated by disappearance of a 90 kDa band. β-Actin was used as loading control. (B) Effect of different UPR inducers on NOX activation in BV-2 cells. UPR was induced as described in supplement data. All UPR inducers caused significantly increased $\frac{O_2}{O_2^+}$ generation, DTT ($P < 0.05$), NaAsO$_2$ ($P < 0.05$) and heat shock ($P < 0.01$). (C–E) Effect of UPR induction and PDI inhibition in RAW 264.7 cell line, rat primary microglia and human primary monocytes. UPR was induced with DTT as in Figure 4B, and PDI activity was inhibited by bacitracin (300 μM). NOX activation was measured as described in Figure 4B. (C) Significantly higher NOX activation was detected in RAW 264.7 cells exposed to DTT than in control cells ($P < 0.05$), and the increase could be abolished with bacitracin ($P < 0.05$). (D) In rat primary microglia, UPR induction also increased NOX activation significantly ($P < 0.01$) and there was a trend toward decreased NOX activation in cells treated with bacitracin. (E) A trend toward increased NOX activation upon UPR induction by DTT was observed in human primary monocytes. NOX activation was significantly decreased by bacitracin ($P < 0.01$). (F and G) Effect of wt and tg SOD1 on NOX activation. (F) BV-2 cells were transiently transfected with wt- and G93A-SOD1 to induce the expression of human SOD1. After 24 h of transfection, cells were collected and proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting. Expression level of wt SOD1 was very prominent, whereas the protein levels of
After 48 h of transient down-regulation of PDI, UPR was induced with DTT, the remaining PDI was inhibited with bacitracin and NOX activation was measured. Although the NOX activation profile was similar to mock-transfected cells, PDI-siRNA-transfected cells had significantly lower NOX activation even at the control level (Fig. 5B). Most importantly, PDI-siRNA-transfected cells showed significantly lower NOX activation than mock-transfected cells upon UPR induction with DTT (Fig. 5B). In addition, PDI inhibition by bacitracin resulted insignificantly lower NOX activation in PDI-siRNA-treated cells compared to mock-transfected cells (Fig. 5B).

**DISCUSSION**

In this study, we demonstrated for the first time segregated increase of PDI expression in microglia of the SC white matter and in the astrocytes of the ventral horn of tg mutant G93A-SOD1 mice. As the increase of PDI expression was associated with an increased expression of GADD34 in these cells, the results strongly suggest that astrocytes and microglia sustain ER stress-related UPR response early in the course of motoneuron degeneration in tg ALS mice. Importantly, by *in vitro* studies, we were able to show that PDI acts as an important regulator of NOX activation and subsequent ROS production in microglial cells, suggesting that the up-regulated PDI might not have only protective role in neurodegenerative diseases associated with protein aggregation, but it may also contribute to the oxidative stress in a cell-type-specific manner. In addition to bringing up a novel role for PDI, current data emphasize the role of glial cells in ALS and provide further evidence for ALS as a non-cell autonomous disease, advancing the understanding of disease pathology.

**PDI co-localizes with CD45-positive microglia and is induced at the early symptomatic phase of ALS**

PDI has been shown to be up-regulated in the SC of ALS mice and rats, where it is thought to have a beneficial role by countering misfolding and aggregation of mutant SOD1 (13). Moreover, PDI has been detected also in the swollen neurites and neuronal cytoplasmic inclusions of ALS patients (32). In agreement with earlier findings, we detected PDI expression in the SC motoneurons and this expression was significantly (by 40%) increased in tg mice compared with controls at the disease onset.

White matter damage has been reported in several neurological disorders, including multiple sclerosis, stroke and traumatic injury (33). Furthermore, analyses of post-mortem tissues have revealed that, within the white matter of the SC of ALS patients, there is a loss of large myelinated fibers in the corticospinal tracts and ventral roots (34). On the other hand, microglia have been proposed to mediate the white matter damage (35). In harmony with earlier results (36), we detected increased CD45 immunoreactivity in the ventral horn and white matter of tg animals indicative of microgliosis.

Importantly, we demonstrated for the first time a significantly increased co-localization of PDI with a microglial marker CD45 in the SC white matter of ALS tg mice compared with the wt mice, indicative of higher number of PDI-positive microglia in the tg than wt tissue. In addition, we showed 384% up-regulation in the microglial PDI expression in the ALS SC. In contrast, there was no significant difference in the co-localization of astrocyte marker GFAP and PDI between tg and wt white matter, indicating that the phenomenon was cell-type-specific in this tissue. These findings suggest an important role for microglia in the white matter damage during ALS progression and indicate that ER stress-mediated mechanisms take place in microglia early in the course of ALS pathogenesis.

**PDI co-localizes with GFAP-positive astrocytes and is induced at the early symptomatic phase of ALS in the ventral horn**

Activation of astrocytes is one of the most prominent features of ALS pathology. Consistent with earlier reports (37), we detected significant astrogliosis in the tg SC compared with wt SC, in both the ventral horn and white matter. Strong immunoreactivity of PDI was observed in both wt and tg tissues. Interestingly, we made a novel finding that the number of PDI-positive astrocytes was increased in the ventral horn upon the disease pathogenesis, as the co-expression of PDI and astrocytic marker GFAP was significantly higher in the tg ventral horn than in wt. Altogether, the *in vivo* data suggest that PDI activity in glial cells may play a prominent role in ALS pathogenesis.

**UPR is induced in astrocytes and microglia at the early symptomatic phase of ALS**

UPR has been implicated in SC motoneurons both in G93A-SOD1 mice and in ALS patients (13,14). This is also evidenced by a finding that G85R-SOD1 ALS mice deficient for pancreatic ER kinase, a signal transduction protein that contributes to UPR by regulating protein translation, show dramatically accelerated disease onset as well as shortened lifespan (38). Moreover, a motoneuron subtype that selectively develops ER stress response coupled with microglial activation and consequent axonal degeneration has been identified in G93A SOD1 mice (39). Our data demonstrate up-regulation...
of PDI and GADD34 in astrocytes and microglia in the lumbar SC of tg mice early after detectable onset of the disease and suggest that ER stress and UPR are also taking place not only in the motoneurons but in glial cells as well in ALS. Importantly, our results also suggest a segregation of UPR into ventral horn astrocytes and predominance of microglial UPR into white matter microglia. This segregation is likely to reflect differential role of UPR in astrocytes around degenerating motoneuron cell bodies and microglia around both motoneuron cell bodies and neurites. Considering the early degenerative changes in motoneuron axons and the role of microglia in front-line defense, it is not surprising that microglial UPR precedes and/or dominates over astrocytic UPR during early motoneuron degeneration.

**UPR induces PDI-dependent NOX activation in microglia**

After establishing the presence of two major phenomena, PDI expression and UPR, in the glial cells of tg animals SCs, we assessed UPR and PDI activity in cell models.

Since PDI has been found in association with NOX (17) and might therefore cause imbalance in the redox environment, we hypothesized that PDI activity could be coupled to NOX-mediated ROS production during UPR in microglial cells. Our experimental model where murine microglial BV-2 cells were treated with DTT recapitulated UPR as demonstrated by ATF-6 processing and TNF-α release. Indeed, the NOX activation was increased upon UPR induction and it was clearly PDI-dependent as assessed by PDI inhibitors. In concert with pharmacological inhibition, down-regulation of PDI expression with siRNAs led to decreased NOX activation. Moreover, the phenomenon was also applicable to human primary monocytes, rat primary microglia and murine macrophage type of cells.

The current data suggest that PDI is an important regulator of UPR-triggered NOX activation in many cell types of hematopoietic origin. In agreement with recent studies (7), the expression of G93A-SOD1 resulted in increased NOX activation in microglial cells, indicating that mutant SOD1 is capable of triggering the UPR and consequential superoxide production.

Protein folding in ER is a highly redox-dependent pathway, associated with the formation of disulfide bonds and their further processing (40). The ER-resident oxidase Ero1, a flavoenzyme, which uses molecular oxygen as an electron acceptor, transfers oxidizing equivalents mainly to its substrate PDI (41). Oxidized glutathione and hydroperoxide produced by Ero1 have been implicated as potentially dangerous byproducts of oxidative protein folding in the ER (42,43). For the time being, there is no clear consensus whether Ero1-mediated extensive oxidation in the ER leads to augmented oxidative stress or acts as a part of homeostatic redox control mechanisms (44). Our results suggest that the cellular response triggered by ongoing protein aggregation and UPR may be coupled with misbalance in protein folding pathway resulting in increased ROS production. In this context, increased oxidative stress upon UPR can be regarded as a convergence of a number of both proapoptotic and proadaptive mechanisms (17).

In microglia cells, ROS production may depend on PDI, which associates with NOX and regulates its function. In agreement with our results, it was recently shown that the overexpression of PDI promoted NOX activation in vascular smooth muscle cells (45). Our notion is also supported by findings that PDI closely associates with p22phox subunit of phagocyte NOX, and that NOX activation directly correlates with PDI expression levels (29).

The question of how exactly PDI activates NOX remains open. Several models have been proposed where PDI interacts with catalytical or regulatory subunits of NOX (46). The current evidence suggests that PDI reductase activity may be required. The crucial role of PDI reductase in PDI-mediated NOX activation is further supported by the observation that...
bacitracin, an inhibitor of PDI reductase activity (47), is able to suppress superoxide production in several cell types.

Altogether, our study sheds light on the deleterious role of PDI in NOX-expressing cells upon protein aggregation, where the attempted protein refolding in ER results in increased PDI activity, eventually leading to NOX activation and oxidative stress (Fig. 6). This role of PDI is essentially different from the one so far described in ALS motoneurons, and may give a basis for the exploration of non-cell autonomous neurodegeneration also in other diseases linked to protein aggregation. Our work also provides a rationale for paradoxical strategy to inhibit one of the key players in protein folding—PDI, as a therapeutic approach in neurodegenerative diseases where protein aggregation takes place.

MATERIALS AND METHODS

Tg mouse model of ALS

Tg (B6.Cg-Tg-(SOD1-G93A)1Gur/J) mice carrying a high copy number of human mutant G93A-SOD1 were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and maintained in the C57BL/6j strain. Wt littermates were used as control animals. Early symptomatic, 20-week-old, male mice were used for histological studies (wt n = 6, tg n = 7 and 8 sections/animal). Animal experiments were conducted according to the national regulations and the Council of Europe (Directive 86/609). All data are presented with SD error bars.

Cell cultures

The mouse BV-2 microglia cell line was kindly provided by Professor Rosario Donato (University of Perugia, Italy). Cells were routinely grown in RPMI medium (Sigma, St Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA), 4 mM l-glutamine (Gibco) and 5 μg/ml gentamycin (Biochrome AG, Germany). RAW 264.7 cells (ATCC laboratories cat TIB-71™) were grown according the manufacturer’s protocol. Rat primary microglia were isolated from neonatal rats according to the method modified from Giulian and Baker (48). Mononuclear cells were isolated from human blood (see Supplementary Material for details). The study was approved by the Board of Research Ethics, Hospital District of Northern Savo, Finland, carried out according to the World Medical Association Declaration of Helsinki, and informed consent was obtained from all subjects.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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