The integrated stress response prevents demyelination by protecting oligodendrocytes against immune-mediated damage

Wensheng Lin, Samantha L. Bailey, Hanson Ho, Heather P. Harding, David Ron, Stephen D. Miller, and Brian Popko

Introduction
Phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α) on serine 51 is a highly conserved point of convergence among distinct signaling pathways that adapt eukaryotic cells to diverse stressful conditions. eIF2α phosphorylation promotes a stress-resistant state by global attenuation of protein biosynthesis and induction of numerous stress-induced cytoprotective genes. This eIF2α phosphorylation–dependent, stress-inducible pathway has been referred to as the integrated stress response (ISR; refs. 1–3). Four different kinases are known to phosphorylate eIF2α. Pancreatic endoplasmic reticulum kinase (PERK), an ER-localized eIF2α kinase, is activated by ER stress, the stress of accumulating unfolded or misfolded proteins in the ER (1–3). General control nonderepressible-2 (GCN2) kinase is activated by amino acid starvation (1, 2). Double-stranded RNA-dependent protein kinase (PKR) and heme-regulated inhibitor (HRI) are activated by viral infection and iron deficiency, respectively (2).

Demyelination and oligodendrocyte loss are hallmarks of the immune-mediated CNS disorder MS and its animal model, EAE (4, 5). The T cell–derived pleiotropic cytokine IFN-γ is regarded as a major proinflammatory cytokine that promotes myelin damage in MS and EAE (6, 7). Normal functions of IFN-γ include the promotion of Th1 T cell differentiation, activation of microglia/macrophages, induction of MHC class I and MHC class II expression, and upregulation of many inflammatory mediators such as TNF-α and iNOS. IFN-γ is undetectable in the normal CNS but becomes measurable during CNS inflammation, such as in the symptomatic phase of MS and EAE (6, 7). Administration of IFN-γ to MS patients and EAE mice enhances inflammation in the CNS and exacerbates clinical symptoms (8–10). Enforced expression of IFN-γ in the CNS of transgenic mice also results in CNS inflammation and myelin abnormalities (11, 12). Nevertheless, evidence is accumulating that IFN-γ is not exclusively detrimental in the pathogenesis of MS and EAE (13, 14). Mice with a mutation in either the gene encoding IFN-γ or that encoding its receptor remain susceptible to EAE and in fact develop EAE with higher morbidity and mortality (15, 16). Moreover, deletion of IFN-γ or its receptor makes resistant mouse strains susceptible to EAE (17). Importantly, it has also been shown that IFN-γ delivered intrathecally before disease onset protects C57BL/6 mice from EAE (18). Here we confirm the protective role of early CNS delivery of IFN-γ using transgenic mice that allow for temporally regulated delivery of IFN-γ to the CNS using a tetracycline controllable system (19–21) and demonstrate that these protective effects against EAE are mediated by activation of the PERK–phosphorylated eIF2α (PERK–p-eIF2α) ISR pathway in oligodendrocytes. Our observations suggest that therapeutic approaches to activate the ISR could prove beneficial in MS.

Results
IFN-γ protects against EAE-induced demyelination. To assess the role of IFN-γ in the pathogenesis of EAE, we used transgenic mice that allow for temporally regulated delivery of IFN-γ to the CNS using a tetracycline-controllable system (19–21). In these animals, expression of the tetracycline-controlled transactivator (tTA) is driven by the astrocyte-specific transcriptional regulatory region of the glial fibrillary acidic protein (GFAP) gene, and a mouse IFN-γ–expressing gene has been placed under the transcriptional control of the tetracycline response element (TRE). We refer to these mice as GFAP/tTA/TRE/Ifng. In this experimental system, 6-week-old tTA compound transgenic mice that had been maintained on doxycycline from conception (to suppress the expression of the TRE/Ifng transgene) were immunized with the myelin peptide.
Figure 1
CNS delivery of IFN-γ before EAE onset ameliorated the disease course dependent on the PERK pathway. (A) Expression pattern of total IFN-γ in the course of EAE (n = 3). (B) Expression pattern of transgenic IFN-γ in the course of EAE (n = 3). (C) Mean clinical score (n = 25). Error bars represent SD. *P < 0.05, **P < 0.01 versus IFN-γCNS−Perk+/. Student’s t-test.

significantly milder in the IFN-γCNS−Perk+/− mice (Figure 1C and Table 1). While all mice developed a typical EAE clinical phenotype, a significantly higher portion (20 of 25) of control IFN-γCNS−Perk+/− mice showed the severe EAE symptom of hind-limb paralysis compared with the IFN-γCNS−Perk+/+ mice (2 of 25; Table 1). Thus, these data confirm that CNS expression of IFN-γ before EAE onset attenuates disease severity.

Spinal cord tissue was prepared at the peak of disease and analyzed for tissue damage. Control mice exhibited typical EAE histopathology: destruction of myelin sheaths, axon damage, and oligodendrocyte loss (Figure 2, A–E) as well as perivascular inflammation (Figure 3, A–D). In contrast, myelin, axons, and oligodendrocytes remained largely intact in the lumbar spinal cords of IFN-γCNS−Perk+/+ mice (Figure 2, A–E, and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI29571DS1), and CD3-positive T cell infiltration was reduced (Figure 3, A, C, and D). Moreover, real-time PCR analyses revealed that the mRNA level of myelin basic protein (MBP) in the spinal cords of MOG35–55-immunized IFN-γCNS−Perk+/+ mice was not significantly decreased compared with age-matched nonimmunized, EAE-negative (i.e., healthy) mice. In contrast, the expression of MBP was significantly decreased in MOG35–55-immunized control IFN-γCNS−Perk+/+ mice, by approximately 30% and 22%, compared with naive and IFN-γCNS−Perk+/+ mice, respectively (Figure 2F). These data indicate that preemptive CNS delivery of IFN-γ protects against EAE-induced demyelination.

During EAE pathogenesis, T cells are primed in the peripheral immune system and enter the CNS well before the onset of clinical disease (23). Preemptive derepression of the TRE/ifng transgene to deliver the cytokine to the CNS before EAE onset did not affect T cell priming in the periphery (Supplemental Figure 2). Accordingly, we found that CNS delivery of IFN-γ did not significantly affect early infiltration by CD3-positive T cells at PID14 (Figure 3, E, F, I, and J) and had no significant effect on the number of CD11b-positive microglia/macrophages in the spinal cord at this time point (Figure 3, G–J). Importantly, real-time PCR analyses showed that CNS delivery of IFN-γ strongly enhanced the expression of iNos, TNF-α, IL-2, IL-12, and IL-10 in the spinal cord at EAE onset (Figure 3K); however, the derepression of the TRE/ifng transgene did not signifi-

Table 1
PERK-dependent attenuated disease severity induced by CNS delivery of IFN-γ before EAE onset

<table>
<thead>
<tr>
<th>Group</th>
<th>Time of EAE onset (PID)</th>
<th>Clinical score at disease peak</th>
<th>Incidence of hind-limb paralysis</th>
<th>Incidence of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γCNS−Perk+/+</td>
<td>13.6 ± 1.2</td>
<td>2.86 ± 0.80</td>
<td>10/25</td>
<td>0/25</td>
</tr>
<tr>
<td>IFN-γCNS−Perk+/-</td>
<td>14.0 ± 1.4</td>
<td>1.83 0.51</td>
<td>12/25</td>
<td>2/25</td>
</tr>
<tr>
<td>IFN-γCNS−Perk+/-</td>
<td>14.0 ± 0.9</td>
<td>2.67 ± 0.94</td>
<td>19/25</td>
<td>2/25</td>
</tr>
<tr>
<td>IFN-γCNS−Perk+/-</td>
<td>13.6 ± 0.6</td>
<td>2.71 ± 1.11</td>
<td>18/25</td>
<td>3/25</td>
</tr>
</tbody>
</table>

Data are mean ± SD (n = 25). *P < 0.001 versus IFN-γCNS−Perk+/.
There is accumulating evidence that IFN-γ protects against demyelination through a direct cytotoxic mechanism and contributes to the activation of most genes in the PERK-eIF2α pathway balances protein biosynthesis with ER-fold and can protect against subsequent exposure to more severe stresses. Several lines of evidence have suggested that modest ER stress protects cells from TNF-α exposure, oxidative/nitrative injury, and glutamate excitotoxicity. Importantly, these events are believed to play critical roles in oligodendrocyte death in MS and EAE. We have previously shown that the presence of IFN-γ in the CNS of adult mice induces a modest ER stress, but does not cause myelin abnormalities or oligodendrocyte loss (20). The PERK–p-eIF2α pathway balances protein biosynthesis with ER-folding capacity and contributes to the activation of most genes in the ER stress response (1, 3). It has been demonstrated that permissive conditional activation of the PERK–p-eIF2α pathway protects cells from the lethal effects of reactive oxidative/nitrative stress and ER stress (3). To determine whether the PERK pathway is involved in the protective effects of IFN-γ in EAE-induced demyelination, we monitored the level of p-PERK and p-eIF2α in oligodendrocytes during the course of EAE. Colocalization analysis with the CC1 antibody revealed that few oligodendrocytes were p-PERK–positive and p-eIF2α–positive in the spinal cords of IFN-γ-negative or transgenic mice at EAE onset, consistent with a previous report (35). In contrast, CNS delivery of IFN-γ markedly enhanced the activation of the PERK–p-eIF2α pathway in oligodendrocytes of IFN-γ-negative or transgenic mice (Figure 5). Double immunolabeling analyses showed that the immunoreactivity of p-PERK was undetectable in other types of cells such as CD3-positive T cells, CD11b-positive microglial cells, and GFAP-positive astrocytes in the lumbar spinal cords of mice on a Perk⁻⁻ background at PID17. In contrast, CNS delivery of IFN-γ did not prevent demyelination and axon damage in the lumbar spinal cord of mice on a Perk⁻⁻ background at PID17. Scale bar: 10 μm. (D and E) CC1 immunostaining showed that oligodendrocytes in the lumbar spinal cords of IFN-γ-negative or transgenic mice remained almost intact at PID17. In contrast, similar to control mice, IFN-γ-negative or transgenic mice lost the majority of oligodendrocytes in the demyelinated lesions of their lumbar spinal cords at PID17. Scale bar: 25 μm. (F) Real-time PCR analysis of the mRNA level of MBP in the indicated spinal cords at PID17 relative to that in the spinal cords of age-matched naive mice. Error bars represent SD. n = 3. *P < 0.05 versus IFN-γ-negative or transgenic mice (E and F).
IFN-γCNS−;Perk−/− mice at PID14 (Supplemental Figure 3). These data establish a correlation between the activation of the PERK-mediated ISR in oligodendrocytes and the protective role of IFN-γ against EAE-induced demyelination.

PERK is essential for the protective effects of IFN-γ in EAE. To explore a possible functional role of the ISR in protecting against EAE, we took a genetic approach. Mice that are heterozygous for a loss-of-function mutation in their Perk gene (also known as Eif2ak3) appear healthy but display evidence of haploid insufficiency (20, 21, 36). We crossed TRE/Ifng mice with Perk−/− mice (37) and then crossed the resulting progeny with GFAP/tTA mice to obtain compound GFAP/tTA;TRE/Ifng;Perk−/− mice and control GFAP/tTA;TRE/Ifng;Perk−/+ mice. Six-week-old GFAP/tTA;TRE/Ifng;Perk−/− mice that had been maintained under doxycycline repression from conception were immunized with the MOG35-55 peptide and simultaneously released from doxycycline repression at PID14 (n = 3). Real-time PCR analysis of the relative mRNA levels of CD3 and CD11b in the spinal cord at PID17 (n = 3). (E, F, and I) CD3 immunostaining showed that CNS delivery of IFN-γ did not significantly change the numbers of CD11b-positive microglia/macrophages in the lumbar spinal cord of mice on a Perk−/− background at PID17, but did not significantly affect T cell infiltration in mice on a Perk−/+ background. (B and C) CD11b immunostaining revealed that CNS delivery of IFN-γ did not significantly change the numbers of CD11b-positive microglia/macrophages in the lumbar spinal cord of mice on a Perk−/− or Perk−/+ background at PID14 (n = 3). (D) Real-time PCR analysis of the relative mRNA levels of CD3 and CD11b in the spinal cord at PID17 (n = 3). (G, H, and I) CD11b immunostaining showed that CNS delivery of IFN-γ did not significantly change the numbers of CD11b-positive microglia/macrophages in the lumbar spinal cord at PID14 (n = 3). (J) Real-time PCR analysis of the relative mRNA levels of CD3 and CD11b in the spinal cord at PID14 (n = 4). (K) Real-time PCR analysis for the expression pattern of cytokines in the spinal cord at PID14 (n = 4). Scale bars: 50 μm. Error bars represent SD. *P < 0.05 versus IFN-γCNS−;Perk−/− mice.

Neuropathologic analyses revealed that CNS delivery of IFN-γ before EAE onset did not prevent demyelination, axon damage, or oligodendrocyte loss (Figure 2) and did not change the immune response at the peak of disease in the spinal cords of Perk−/− mice (Figure 3, A–D, and Figure 4). In fact, MBP immunostaining, toluidine blue staining, and real-time PCR analysis for MBP consistently showed comparable demyelination at the peak of disease in the spinal cords of IFN-γCNS−;Perk−/− and control IFN-γCNS−;Perk−/+ or IFN-γCNS−;Perk−/+ mice (Figure 2). Importantly, the Perk−/− genotype did not significantly affect T cell priming in the peripheral immune system (Supplemental Figure 2), inflammatory infiltration, or the expression profile of cytokines in the course of EAE (Figure 3, A–D, and Figure 4), indicating that PERK does not modulate the primary inflammatory response in EAE. Thus, the activation of the PERK pathway by IFN-γ in oligodendrocytes is essential to the protection against EAE-induced demyelination.
Discussion
ISR activation by the ER stress response protects cells against subsequent cellular stresses. The ER stress response also controls an apoptotic program to eliminate cells whose folding problems in the ER can not be resolved by the adaptive coordinated responses (38, 39). While it has been shown that the apoptotic program controlled by the ER stress response plays an important role in the pathogenesis of various diseases such as diabetes mellitus and neurodegenerative diseases (40, 41), several lines of evidence suggest that a modest ER stress response that does not cause severe cell injury can protect against subsequent exposure to more severe stresses (32, 33, 38, 39). A recent study also shows that the ER stress response protects the heart from ischemia and reperfusion injury (42).

We have previously found that severe ER stress induced by IFN-γ in myelinating oligodendrocytes during development or in remyelinating oligodendrocytes in cuprizone-induced demyelinated lesions causes oligodendrocyte apoptosis and myelin abnormalities, and that PERK protects oligodendrocytes from these detrimental consequences of ER stress (20, 21). Nevertheless, the presence of comparable levels of IFN-γ in the CNS of adult mice induces only modest ER stress and does not diminish mature oligodendrocyte survival, even in heterozygous Perk⁻/⁺ mice (20). This presents a striking contrast to the deleterious effect of the mutation on survival of IFN-γ-exposed oligodendrocytes engaged in developmental myelination or pathologic remyelination (20, 21). Rather, as described here, modest ER stress induced by IFN-γ in mature oligodendrocytes of adult mice protects against EAE-induced demyelination, axonal damage, and oligodendrocyte loss.

Interestingly, the detrimental or beneficial effects of the ER stress response induced by IFN-γ do not correlate with the amount of IFN-γ present in the CNS. The ER stress response induced by a very low dose of IFN-γ (approximately 20 pg/mg) in the demyelinated lesions of cuprizone-treated mice, or a median dose (approximately 200 pg/mg) in the CNS of young, developing mice results in remyelinating oligodendrocyte or developing myelinating oligodendrocyte death, respectively (20, 21). In contrast, the ER stress response induced by a high dose of IFN-γ, approximately 500 pg/mg in the spinal cords of adult mice, protects oligoden-
Effects of IFN-γ in EAE are associated with activation of the PERK-eIF2α pathway in oligodendrocytes. The protective effects of IFN-γ in EAE are associated with activation of the PERK-eIF2α pathway in oligodendrocytes. (A and B) CC1 and p-PERK double labeling showed modest activation of PERK in a few oligodendrocytes in the lumbar spinal cords of control mice at PID14, and CNS delivery of IFN-γ strongly activated PERK in the majority of oligodendrocytes. (C and D) CC1 and p-eIF2α double labeling showed that modest activation of eIF2α in a few oligodendrocytes in the lumbar spinal cord of control mice at PID14, and CNS delivery of IFN-γ markedly activated eIF2α in the majority of oligodendrocytes. Scale bar: 10 μm. (E) Quantitative analysis showed CNS delivery of IFN-γ significantly increased the percentage of double-positive CC1/p-PERK and CC1/p-eIF2α cells in the spinal cords of IFN-γ−/− mice compared with IFN-γ−/− mice at PID14. Error bars represent SD. *P < 0.01 versus IFN-γ−/− mice.
ducng T cells in the peripheral immune system (49), whereas Wheeler et al. reported that the elevated level of IFN-γ in the CNS of TNF receptor 1-deficient mice protects against EAE but does not suppress the expression of IL-17 in the CNS (51). We show here that CNS delivery of IFN-γ before EAE onset did not significantly affect the expression of IL-17 in the spinal cord at PID14 (Figure 3K) or PID17 (Figure 4H), whereas we have previously shown that CNS delivery of IFN-γ at the recovery stage of EAE suppresses remyelination and significantly reduces the expression of IL-17 in the CNS (21). While an understanding of the effects of IFN-γ on the production of IL-17 in the peripheral immune system and the CNS at different stages of EAE requires further investigation, these data collectively indicate that the harmful and helpful actions of IFN-γ in EAE do not correlate with the level of IL-17 expression in the CNS.

Modulation of the microglia/macrophage response might also contribute to the observed effects of IFN-γ on the pathogenesis of EAE. The inflammatory roles reported for microglia/macrophages in the pathogenesis of MS and EAE include the presentation of antigens and the production of inflammatory mediators such as TNF-α and iNOS (52). Butovsky et al. reported that microglia activated by low doses of IFN-γ (20 ng/ml) support oligodendrogenesis, but microglia activated by higher doses of IFN-γ (100 ng/ml) inhibit oligodendrogenesis (53). We found that CNS delivery of IFN-γ did not significantly alter CD11b-positive microglia/macrophage infiltration or the induction of TNF-α and iNOS. MHC class II immunostaining also revealed that CNS delivery of IFN-γ did not significantly change the infiltration of MHC class II-positive antigen-presenting cells (Supplemental Figure 4). Furthermore, our previous and current data demonstrate that the beneficial and detrimental effects of IFN-γ on oligodendrocytes did not correlate with its dose (20, 21, 29). It thus appears unlikely that suppressing inflammatory function of microglia/macrophages contributes to the protective effects of high doses of IFN-γ observed in our model.

Currently approved disease-modifying treatments for MS that nonspecifically target immune responses have little impact on the progression of disability (54, 55), which may in part reflect the dual roles, beneficial and detrimental, of many inflammatory components in this disease (56, 57). The results of the present study may have important implications for the pathogenesis of MS and could lead to therapeutic strategies to specifically protect oligodendrocytes in individuals with MS. Thus, the ability of the PERK response described here to protect against immunological insult on oligodendrocytes suggests that protective therapies that specifically activate the ISR may reduce relapse rate and delay disability progression in MS patients.

Methods

**Mice and EAE immunization.** Line110 of GFAP/tTA mice on the C57BL/6 background were mated with line184 TRE/Ifng mice on the C57BL/6 background to produce GFAP/tTA;TRE/Ifng double transgenic mice (19). Moreover, TRE/Ifng mice were crossed with Perk−/− mice on the C57BL/6 background (37), and the resulting progeny were crossed to GFAP/tTA mice to obtain GFAP/tTA;TRE/Ifng double transgenic mice that were heterozygous for the Perk mutation. To prevent transcriptional activation of the TRE/Ifng transgene by the tTA, 0.05 mg/ml doxycycline was added to the drinking water and provided ad libitum from conception. EAE was induced as described previously (21) by subcutaneous injections at flanks and tail base of 200 μg MOG35-55 emulsified in complete Freund’s adjuvant (BD) supplemented with 600 μg *Mycobacterium tuberculosis* (strain H37Rv; BD). Two intraperitoneal injections of 400 ng pertussis toxin (List Biological Laboratories Inc.) were given 24 and 72 hours later. Clinical scores were recorded daily using a scale of 0–5 (0, healthy; 1, flaccid tail; 2, ataxia and/or paresis of hind limbs; 3, paralysis of hind limbs and/or paresis of fore limbs; 4, tetraparesis; 5, moribund or death).

All animal procedures were conducted in complete compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of The University of Chicago.

**T cell proliferation assay, cytokine assay, and ELISPOT assay.** Single-spleen cell suspensions were generated from mice at PID10. Red blood cells were lysed with buffered Tris ammonium chloride, washed, and plated at 5 x 10^6 splenocytes/well in serum-free media. Samples were plated in triplicate. OVA323-339 or MOG35-55 was added at various concentrations as indicated in Supplemental Figure 2. The cells were incubated at 37°C and 7.5% CO2. After 72 hours, cultures were pulsed with [3H]-thymidine (1 μCi/well; MP Biomedicals) and harvested 16 hours later. We determined cpm with a TopCount-NXT microplate scintillation and luminescence counter (Packard). We quantified cytokines from culture supernatants of triplicate wells using the 5 plex cytokine bead array kit (QIAGEN) according to the manufacturer’s instructions. ELISPOT assays were performed as previously described (58). We plated 2.5 x 10^6 splenocytes/well.

**Real-time PCR.** Anesthetized mice were perfused with PBS. RNA was isolated from the spinal cord using TRIzol reagent (Invitrogen) and treated with DNase I (Invitrogen) to eliminate genomic DNA. Reverse transcription was performed using the SuperScript First Strand Synthesis System for RT-PCR kit (Invitrogen). TaqMan real-time PCR was performed with iQ Supermix (Bio-Rad) on a Bio-Rad iQ Real-Time PCR detection system as previously described (21).

**ELISA.** Mice were perfused with ice-cold PBS. The spinal cords from 3 mice were removed, pooled, and immediately homogenized in 5 volumes of PBS with complete protease cocktail (Roche Diagnostics) using a motorized homogenizer. After incubation on ice for 5 minutes, the extracts were cleared by centrifugation at 10,000 g for 10 minutes. The protein content of each extract was determined by the DC protein assay (Bio-Rad). ELISA assays were performed using the Mouse IFN-γ Quantikine ELISA kit (R&D Systems) following the manufacturer’s instructions.

**Immunohistochemistry.** Anesthetized mice were perfused with 4% paraformaldehyde in 0.1 M PBS. Immunohistochemistry for CC1 (adenomatous polyposis coli [antibody 7], diluted 1:50; EMD Biosciences), MBP (diluted 1:1,000; Sternberg Monoclonals Inc.), p-eIF2α (diluted 1:50; Cell Signaling Technology), p-PERK (diluted 1:50; Cell Signaling Technology), CD3 (diluted 1:50; Santa Cruz Biotechnology Inc.), CD11b (diluted 1:50; Chemicon International), GFAP (diluted 1:1,000; Sternberg Monoclonals Inc.), and MHC class II (diluted 1:50; Chemicon International) was performed as previously described (20). We quantified the immunopositive cells by counting positive cells within the anteromedian white matter of the lumbar spinal cord, confined to an area of 0.1 mm². The percentage of double-positive cells was calculated by normalizing CC1/p-PERK or CC1/p-eIF2α double-positive cell numbers against CC1-positive cell numbers. The histological analysis was performed by a researcher blinded to experimental group.

**Toluidine blue staining.** For toluidine blue staining of 1-μm sections, we anesthetized and perfused mice with 0.1 M PBS containing 4% paraformaldehyde and 2.5% glutaraldehyde. The lumbar spinal cord were processed, embedded, sectioned, and analyzed as previously described (21). The total white matter area and the demyelinated area in the lumbar spinal cord were measured by ImageJ software (http://rsb.info.nih.gov/ij/). The percentage of demyelinated area was calculated by normalizing the demy-
eliminated area against the total white matter area. The histological analysis was performed by a researcher blinded to experimental group.

Statistics. Data are expressed as mean ± SD. Multiple comparisons were statistically evaluated by 1-way ANOVA followed by the Holm-Sidak test using SigmaStat 3.1 software (SAS Institute, Cary, NC). A P value less than 0.05 was considered significant.

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