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The PGE₂ EP2 receptor accelerates disease progression and inflammation in a model of amyotrophic lateral sclerosis

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Abstract

Objective—Inflammation has emerged as an important factor in disease progression in human and transgenic models of amyotrophic lateral sclerosis (ALS). Recent studies demonstrate that the prostaglandin E₂ EP2 receptor is a major regulator of inflammatory oxidative injury in innate immunity. We tested whether EP2 signaling participated in disease pathogenesis in the G93A superoxide dismutase (SOD) model of familial ALS.

Methods—We examined the phenotype of G93A SOD mice lacking the EP2 receptor and performed immunocytochemistry, quantitative reverse transcriptase polymerase chain reaction, and Western analyses to determine the mechanism of EP2 toxicity in this model.

Results—EP2 receptor is significantly induced in G93A SOD mice in astrocytes and microglia in parallel with increases in expression of proinflammatory enzymes and lipid peroxidation. In human ALS, EP2 receptor immunoreactivity was upregulated in astrocytes in ventral spinal cord. In aging G93A SOD mice, genetic deletion of the prostaglandin E₂ EP2 receptor improved motor strength and extended survival. Deletion of the EP2 receptor in G93A SOD mice resulted in significant reductions in levels of proinflammatory effectors, including cyclooxygenase-1, cyclooxygenase-2, inducible nitric oxide synthase, and components of the NADPH oxidase complex. In alternate models of inflammation, including the lipopolysaccharide model of innate immunity and the APPSwe-PS1ΔE9 model of amyloidosis, deletion of EP2 also reduced expression of proinflammatory genes.

Interpretation—These data suggest that prostaglandin E₂ signaling via the EP2 receptor functions in the mutant SOD model and more broadly in inflammatory neurodegeneration to regulate expression of a cassette of proinflammatory genes. Inhibition of EP2 signaling may represent a novel strategy to downregulate the inflammatory response in neurodegenerative disease.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating and fatal neurodegenerative disease, characterized by the progressive loss of motor neurons in ventral spinal cord and motor cortex. Pathological findings in human ALS demonstrate marked inflammation with activation of astrocytes and microglia, induction of expression of pro-inflammatory enzymes and cytokines, and increased oxidative stress (reviewed in McGeer and McGeer¹). Although the precise

molecular trigger of ALS is unknown, this chronic inflammatory state is hypothesized to accelerate disease progression, leading to secondary neurotoxicity with sustained loss of motor neurons.

Genetic forms of ALS account for 5–10% of ALS cases, and mutations in the Cu, Zn-superoxide dismutase (SOD1) gene comprise a large subgroup of these hereditary forms. As in human ALS, transgenic mice expressing mutant SOD alleles experience development of age-dependent motor deficits, motor neuron loss, and increased oxidative and inflammatory changes (reviewed by Hensley and colleagues²). Recent findings demonstrate a critical role of inflammatory cells in disease progression. Genetic deletion of mutant SOD proteins in astrocytes and microglia^{3,4} results in a significant extension of survival, confirming the dominant role of glial activation and secondary neurotoxicity in the mutant SOD model. The importance of inflammatory oxidative injury has been further substantiated recently with the demonstration of a toxic gain of function of mutant SOD proteins that results in increased NADPH oxidase activity with reactive oxygen species (ROS) production.⁵

Significant interest has been generated regarding the function of the pro-inflammatory enzyme cyclooxygenase-2 (COX-2) and its downstream prostaglandin signaling pathways in mediating secondary neuronal injury in models of neurodegeneration, including ALS, Parkinson's disease, and Alzheimer's disease (reviewed by Minghetti⁶). COX-1 and the inducible COX-2 catalyze the first committed step in prostaglandin biosynthesis and are the pharmacological targets of non-steroidal anti-inflammatory drugs (NSAIDs). In ALS, COX-2 expression is increased,^{7–10} and increased levels of prostaglandin E₂ (PGE₂) in cerebrospinal fluid of ALS patients have been documented^{10,11} (however, see also Cudkowicz and colleagues¹²). In mutant SOD1 mice, levels of COX-2 messenger RNA (mRNA) and PGE₂ are increased,^{10,13} and administration of COX-2 inhibitors extends survival and reduces levels of cerebrospinal fluid PGE₂.^{13–15} These observations suggest that COX-2, via its downstream prostaglandin products, contributes to inflammatory toxicity in this model.

The prostaglandins PGE₂, PGD₂, PGF_{2α}, PGI₂, and TXA₂ are lipid messengers that bind to classes of G-protein-coupled receptors designated EP (for E-prostanoid receptor), FP, DP, IP, and TP receptors.¹⁶ Recent studies demonstrate that the PGE₂ EP2 receptor promotes inflammatory injury in models of innate immunity in Alzheimer's disease.^{17–19} The EP2 receptor mediates a dominant component of oxidative inflammatory injury in the lipopolysaccharide (LPS) model of innate immunity.^{18,19} In a transgenic model of familial Alzheimer's disease, genetic ablation of EP2 leads to significant decreases in lipid peroxidation associated with 50% decreases in Aβ peptide levels and amyloid deposition.¹⁷ These findings suggest that PGE₂ signaling through the EP2 receptor may function in regulating the inflammatory oxidative response in the setting of neurodegeneration. We examined whether EP2 signaling promoted disease progression in the G93A SOD model. We found that deletion of the PGE₂ EP2 receptor in G93A SOD mice improved motor strength and extended survival, and this was associated with reduced levels of proinflammatory effector proteins. Similar downregulation of proinflammatory effector proteins was also identified in the LPS and APPSwe-PS1ΔE9 models, suggesting that EP2 regulation of downstream proinflammatory effectors represents a common inflammatory pathway in neurodegenerative models.

MATERIALS AND METHODS

Animals

This study was conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals. Protocols were approved by the Institutional Animal Care and Use Committees at Johns Hopkins and Stanford Universities.

Three cohorts of mice were generated. (1) a cohort consisted 1-, 3.3-, and 4-month old male G93A SOD and wild-type littermates (B6Jl-TgN(SOD1-G93A)1Gur; Jackson Laboratories, Bar Harbor, ME) for expression and lipid peroxidation studies; (2) a cohort of G93A SOD mice bred serially to C57B6 EP2^{-/-} and EP2^{+/+} mice to generate SOD:EP2^{+/+}, SOD:EP2^{+/-}, and SOD:EP2^{-/-} mice (n=6–18 female mice per genotype) assessed for grip strength and survival; mice were killed if unable to right themselves within 30 seconds of being placed on their side, and this time point was recorded as the time of death; and (3) a cohort of SOD:EP2^{+/+} and SOD:EP2^{-/-} mice (n=7–9 female mice; age range, 150–159 days) for expression and lipid peroxidation studies. Transgene copy number was assayed using quantitative genomic polymerase chain reaction, and relative levels of mutant SOD did not vary between EP2^{+/+} and EP2^{-/-} backgrounds.

The intracerebroventricular injection of LPS (2µg/mouse; CalBiochem, San Diego, CA) in C57B6 EP2^{+/+} and EP2^{-/-} mice was performed as described previously,¹⁸ and brains were analyzed at 24 hours (n = 4–6 twelve-month old male mice per genotype). C57B6 APPSwe-PS1ΔE9 mice in EP2^{+/+} or EP2^{-/-} backgrounds were generated as described previously¹⁷ and tested at 12 months of age (n = 6–8 male mice per genotype).

Grip strength testing

Grip-strength testing was performed weekly by an examiner blinded to genotype by measuring peak force (Columbus Instruments, Columbus, OH). Four repetitions for each mouse were averaged.

Immunocytochemistry

Free-floating sections of lumbar spinal cord were generated and processed as described previously.²⁰ The following primary antibodies were used: anti-EP2 (1/1000; Cayman Chemicals, Ann Arbor, MI), NeuN (1/1000; Chemicon, Temecula, CA), anti-Iba1 (1/500; Wako, Richmond, VA), anti-glial fibrillary acidic protein (anti-GFAP; 1/2000; Dako, Carpinteria, CA), and anti-p67^{phox} (1:500; BD Biosciences, Franklin Lakes, NJ). Secondary antibodies and detection reagents included donkey anti-mouse Alexa 555, anti-rabbit Alexa 486, and Zenon 555 for detection of detection of Iba1 (Molecular Probes, Eugene, OR). Specific staining of the EP2 antibody was confirmed using blocking peptide, no primary antibody, and immunostaining of EP2^{-/-} brains. Images were acquired as a Z-series on an AxioImager microscope (Carl Zeiss, Thornwood, NY) and deconvolved using SlideBook4 (Intelligent Imaging Innovations, Denver, CO). The ratio of EP2-Iba1/Iba1 and EP2-GFAP/GFAP cells was calculated (n = 4 male 3-month-old mice per genotype, three sections 150 µm apart). Human paraffin sections from ALS and control spinal cords were generously provided by the University of Washington Alzheimer's Disease Research Center Neuropathology Core and Elizier Masliah at University of California at San Diego, and were stained for EP2 and GFAP, as previously described.¹⁷

Real Time Quantitative Polymerase Chain Reaction

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA), and integrity of RNA was confirmed by measuring A260/A280 and electrophoresis on agarose gels. Total RNA was treated with DNase (Invitrogen), and the reaction terminated by heating at 65°C for 10 minutes. First-strand cDNA synthesis was performed with 1.5 µg of total RNA, 4 units of Omniscript enzyme (Qiagen, Valencia, CA) and 0.25 µg of random primer in a reaction volume of 20 µl at 37°C for 1 hour. Reverse transcribed complementary DNA was diluted 1:20 in RNase free ddH₂O for subsequent reverse transcriptase polymerase chain reaction. Real-time polymerase chain reaction was performed by using 5µl of complementary DNA, 0.25–0.5 µM of primer, and 2X SYBR Green Super Mix (Qiagen) with final volume of 25 µL in the DNA Engine Opticon System (Bio Rad, Hercules, CA). Melting curve analysis confirmed the specificity in

each reaction. Primers specific for 18s ribosomal RNA were used as an internal control for relative quantification. Samples without reverse transcriptase served as the negative control. Primers are listed in the Table.

Western analysis

Twenty micrograms of cervical spinal cord protein was fractionated by SDS-PAGE. Blots were probed with antibodies against p67^{phox}, inducible nitric oxide synthase (iNOS), COX-2, FasL (n-20; Santa Cruz Biotechnology, Santa Cruz, CA), neuronal nitric oxide synthase (nNOS; Cell Signaling, Danvers, MA), and β -tubulin (Promega, Madison, WI). Immunoreactivity was detected and quantified as described previously.¹⁷ All experiments were confirmed in triplicate.

Measurements of lipid peroxidation

F₂-isoprostanes and F₄-neuroprostanes were measured by gas chromatography with negative ion chemical ionization mass spectrometry as described previously.¹⁷

Statistical analysis

Quantitative data are expressed as mean $\pm$ standard error of the mean and analyzed using analysis of variance or Student's *t*-test. Two-way analysis of variance with repeated measures (time in weeks and genotype) was used for grip strength, with genotype as the main effect between weeks 10 and 19. Differences in survival were assessed using Kaplan-Meier analysis with log-rank statistical analysis. *p* values less than 0.05 were considered significant.

Results

G93A Superoxide Dismutase Mice Demonstrate Increased Neuronal Lipid Peroxidation and Age-Associated Increases in Expression of Prooxidant Enzymes

Because of the significant lipid content of brain, a sensitive measure of oxidative damage is the level of lipid peroxidation. Evidence of protein modification by the lipid peroxide 4-hydroxynonenal has been found in sporadic ALS.²¹ We measured F₂-isoprostanes, which are free radical-generated isomers of prostaglandin PGF_{2 α} and F₄-neuroprostanes, which are oxidized products of neuronal docosahexanoic acid in cerebral cortex of 4-month-old male wild-type mice and G93A SOD littermates. There was a significant increase in levels of F₄-neuroprostanes in aged G93A SOD mice, indicating a selective increase in neuronal lipid peroxidation in this model (Fig 1A).

Proinflammatory proteins capable of generating ROS include iNOS, COX-2, and NADPH oxidase. NOS isoforms produce NO that can combine with superoxide to yield the highly reactive radical peroxynitrite. The NADPH oxidase complex is the principal source of superoxide and consists of the following: three cytoplasmic subunits, p40^{phox}, p47^{phox}, p67^{phox}; two membrane subunits, gp91^{phox} and p22^{phox}; and the guanosine triphosphate-binding protein Rac1. A third source of ROS is COX-1 and COX-2 that produce superoxide in the conversion of arachidonic acid to PGH₂ as a by-product of the peroxidase activity of the enzyme.²² We examined the regulation of expression of selected NADPH oxidase subunits, COX-1, the inducible COX-2, and iNOS in 1- and 3.3-month-old male wild-type and G93A SOD lumbar spinal cord. Reverse transcriptase polymerase chain reaction demonstrated no differences in expression between genotypes at 1 month of age, but did show a significant increase in iNOS and COX-2 mRNA, as well as NADPH oxidase subunits (gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox}), at 3.3 months of age in G93A SOD mice (see Figs 1B–D).

Localization of the Prostaglandin E₂ EP2 Receptor in Spinal Cord of G93A Superoxide Dismutase Mice

Precedent exists for dynamic upregulation of the EP2 receptor in a model of innate immunity.²³ Accordingly, we tested whether the EP2 receptor was induced in aged G93A SOD mice (Fig 2). In wild-type lumbar cord, EP2 colocalized with the neuronal marker NeuN (see Fig 2C), consistent with previous staining in rat spinal cord²⁴; there was limited expression in astrocytes or microglia (see Figs 2A, B). However, in G93A SOD littermates, EP2 was significantly induced in GFAP-positive astrocytes and Iba1-positive microglia (see Figs 2A, B, quantified in D). These data indicate that the EP2 receptor is dynamically induced in astrocytes and microglia in aging G93A SOD mice.

The EP2 Receptor Is Induced in Glial Cells in Human Amyotrophic Lateral Sclerosis Spinal Cord

Immunostaining for the EP2 receptor was performed in paraffin sections of human spinal cord of patients who had died of ALS and of causes not related to motor neuron disease. In control spinal cord, EP2 receptor was present only in motor neurons in ventral horn (Figs 3A, C). In ALS spinal cord, the EP2 receptor was also highly expressed in motor neurons (see Figs 3B, D), but in addition was abundantly expressed in glial cells that were morphologically consistent with astrocytes (see Fig 3D). Further examination of astrocytes using double labeling with anti-EP2 and anti-GFAP antibodies showed an increase in astrocytic EP2 expression in ALS spinal cord as compared with control tissue (see Figs 3E, F), indicating that EP2 is induced in astrocytes in spinal cord in ALS.

Phenotype of G93A Superoxide Dismutase Mice Lacking the EP2 Receptor

To test the function of the PGE₂ EP2 receptor, we compared cohorts of G93A SOD lacking the EP2 receptor (SOD:EP2^{-/-}) with littermate G93A SOD possessing one or both alleles of EP2 (SOD:EP2^{+/+} and SOD:EP2^{+/-}; Fig 4). A significant increase in survival was observed in SOD:EP2^{-/-} mice compared with SOD:EP2^{+/+} and SOD:EP2^{+/-} mice (see Fig 4A; $p < 0.02$). The mean survival was 148.3 ± 2.8 days for SOD:EP2^{-/-} mice compared with 138.7 ± 1.8 days for SOD:EP2^{+/+}, EP2^{+/-} control mice. Two-way analysis of variance with repeated measures demonstrated that deletion of the EP2 receptor resulted in better motor strength over time ($p < 0.05$; see Fig 4B); there were no significant differences in weight (see Fig 4C). EP2^{+/+} and EP2^{-/-} mice did not harbor differences in weight or grip strength (data not shown). Survival does not differ between EP2^{-/-} and wild-type mice.²⁵

The EP2 Receptor Regulates Messenger RNA and Protein Levels of Multiple Prooxidant Proteins

We investigated whether EP2 signaling altered FasL-Fas regulation of nNOS, a well-described mechanism of motor neuron apoptosis in mutant SOD models.²⁶ Deletion of EP2 did not alter nNOS or FasL expression (see Supplementary Fig 1), indicating that PGE₂ EP2 signaling accelerated disease progression by a mechanism distinct from the FasL-Fas-nNOS proapoptotic pathway.

We then tested whether the EP2 receptor regulated levels of proinflammatory enzymes that are typically upregulated in G93A SOD mice (see Fig 1). A significant reduction of expression in spinal cord lysates was observed for COX-1 and COX-2 mRNA and COX-2 protein in the EP2^{-/-} background (Figs 5A–C). Similar decreases were also observed for iNOS and endothelial nitric oxide synthase (eNOS) (see Figs 5D–F), and for components of the proinflammatory NADPH oxidase complex (see Figs 5G–I). Measurement of F₂-isoprostanes and F₄-neuroprostanes in G93A SOD:EP2^{-/-} mice demonstrated a trend toward decreased levels of F₄-neuroprostanes (see Fig 5J), partially reversing the selective increase in neuronal

lipid peroxidation seen in this model (see Fig 1A). No differences in levels of COX-1, COX-2, iNOS, eNOS, nNOS, or subunits of the NADPH oxidase complex were observed in EP2^{+/+} versus EP2^{-/-} mice (see Supplementary Fig 2). Taken together, these data indicate EP2 deletion abrogates the induction of a class of proinflammatory genes normally induced in this model of ALS that are implicated in secondary toxicity to motor neurons.

Deletion of EP2 Receptor in the Lipopolysaccharide and APPSwe-PS1ΔE9 Models Also Decreases Expression of Proinflammatory Genes

We tested whether EP2 signaling also regulated proinflammatory gene expression in two related models, the LPS model of innate immunity and the APPSwe-PS1ΔE9 model of amyloidosis, both of which exhibit a significant inflammatory oxidative response that is abrogated with deletion of EP2.^{17–19} Deletion of EP2 significantly blocked the increases in expression of COX-2, iNOS, and NADPH oxidase subunits p47^{phox}, p67^{phox}, and gp91^{phox} (Figs 6A, B) in hippocampi from EP2^{-/-} and EP2^{+/+} mice subjected to intracerebroventricular LPS. In aged APPSwe-PS1ΔE9 mice, there was a significant induction of EP2 expression in APPSwe-PS1ΔE9 compared with wild-type that was associated with increased COX-2 and p67^{phox} (see Figs 6C, D). This upregulation was abrogated with deletion of EP2 (see Fig 6E). This suggests the model (see Fig 6F) where PGE₂ signaling through the EP2 receptor on a glial cell (astrocyte and/or microglia) upregulates expression of proinflammatory genes and proteins including NADPH oxidase subunits, COX-1 and COX-2, and iNOS. Because COX-1 and COX-2 catalyze the first committed step in prostaglandin synthesis and further PGE₂ production, regulation of COX-1 and COX-2 expression by EP2 effectively creates a feed-forward cycle in which additional PGE₂ synthesis perpetuates and amplifies EP2 signaling and upregulation of proinflammatory proteins.

Discussion

In this study, we have tested whether PGE₂ signaling via the EP2 receptor impacts on the inflammatory response and course of disease in the G93A SOD model of ALS. EP2 signaling accelerates disease progression and acts upstream to regulate a cassette of proinflammatory genes known to induce inflammatory oxidative stress. This mechanism appears to be conserved in alternate models of neuroinflammation, including the LPS model of innate immunity and a model of amyloid deposition, suggesting a common proinflammatory response regulated by PGE₂ signaling via the EP2 receptor in models of neurodegeneration.

Deletion of the EP2 receptor improved survival and motor strength, and was associated with a broad downregulation of proinflammatory gene and protein expression. COX-1 and COX-2, multiple subunits of NADPH oxidase, and the iNOS and eNOS isoforms were significantly downregulated with deletion of EP2. The decrease in levels of COX-1 in the EP2^{-/-} background supports a proinflammatory function for COX-1.^{27,28} The induction of eNOS may reflect induction of this isoform in endothelial and astrocytic cells.²⁹ The effect of EP2 deletion on the NOS isoforms was specific to iNOS and eNOS, and did not impact on the FasL-nNOS pathway²⁶ in which NO-mediated increases in FasL-Fas signaling upregulate nNOS expression in motor neurons with subsequent NO neurotoxicity.

COX-2 and iNOS induction have been implicated as key effectors of neuronal damage from activated microglia. Coordinated regulation and synergistic action of both COX-2 and iNOS have been documented in settings of inflammation and cerebral ischemia.^{30,31} The mechanism of transcriptional regulation of these proinflammatory genes by EP2 was not explored in this study, and is likely to involve multiple and overlapping signal transduction pathways including the mitogen-activated protein kinase signaling cascades and their nuclear factor-κB and AP-1 targets. In addition, the downregulation of iNOS in the EP2^{-/-} background suggests the

possibility that COX-2 enzymatic activity may be secondarily attenuated, given recent findings demonstrating that iNOS S-nitrosylates COX-2 and can enhance its catalytic activity.³²

Increased expression of the gp91^{phox} subunit of NADPH oxidase has been demonstrated in mutant SOD mice and human ALS, and deletion of this subunit attenuates protein carbonylation and disease progression in G93A SOD mice.^{33,34} In this study, we find that the EP2 receptor regulates expression of multiple components of the NADPH oxidase complex that are induced in the G93A SOD model, including the membrane component p22^{phox} and the cytoplasmic subunits p40^{phox}, p47^{phox}, and p67^{phox}. NADPH oxidase is a major producer of superoxide anions in response to inflammatory stimuli. The neurotoxic effects of NO occur after its reaction with superoxide to form peroxynitrite, which oxidizes proteins, lipids, and DNA. Importantly, the superoxide with which the NO reacts is derived from microglial NADPH oxidase.³⁵ Thus, the full effect of microglial production of ROS is a combination of NADPH oxidase and iNOS activities, both of which are targets of EP2 receptor regulation.

In previous studies, we have demonstrated a protective effect of EP2 activation in hippocampal neurons and in organotypic spinal cord and hippocampal slices subjected to glutamate excitotoxicity, as well as in vivo in models of cerebral ischemia.^{20,24,36} The protective action of EP2 in vitro in glutamate excitotoxicity is protein kinase A dependent and is mediated by neuronal EP2 receptor signaling.²⁰ However, in models where inflammation and glial activation dominate, EP2 signaling in activated microglia is deleterious and causes secondary neuronal damage in vitro and in vivo.^{18,19,37,38} Thus, the toxic effects of EP2 signaling in this study would be consistent with the following: (1) microglial or astrocytic proinflammatory effects of EP2 signaling, or both; and (2) the possibility that motor neuron death in organotypic spinal cord slices subjected to glutamate toxicity does not model motor neuron injury in mutant SOD models.

Recent studies have highlighted the role of inflammatory cells and non-cell-autonomous motor neuron death in models of ALS (reviewed in Boillee and colleagues³⁹). This can now be better understood in light of recent findings indicating a potent toxic gain of function of mutant SOD protein. Harraz and colleagues⁵ have demonstrated that mutant SOD proteins bind the gp91^{phox} activator Rac1, resulting in overproduction of ROS by NADPH oxidase and secondary neurotoxicity. The significant role of the overactive NADPH oxidase in the mutant SOD model is evident in the marked prolongation of survival with genetic or early pharmacological inactivation of NADPH oxidase.^{5,34} In this regard, despite the marked overactivity of NADPH oxidase in G93A SOD mice, deletion of the EP2 receptor nevertheless led to downregulation of NADPH oxidase subunits, COX-1 and COX-2, iNOS, and eNOS, genes whose transcription is redox sensitive.⁴⁰ It is likely that sporadic ALS may also involve a selective vulnerability of motor neurons to inflammatory oxidative stress, and in the absence of a constitutively overactive NADPH oxidase, inhibition of EP2 signaling may exert even greater protective effects. Importantly, in models of neuroinflammation that do not involve altered endogenous NADPH oxidase activity, for example, the LPS model of innate immunity or the mutant amyloid precursor protein model of amyloidogenesis, the EP2 receptor appears to regulate the same class of proinflammatory effector genes, with a significant impact on levels of oxidative stress.^{17,18} The EP2 receptor may function as a transducer of the innate immune response in neurodegenerative models where inflammation is a central component. In the G93A SOD model, increased levels of innate immune response proteins, including TLR4 and CD14, have been demonstrated, and induction of these and other innate immune response proteins have been demonstrated in other neurodegenerative models such as Alzheimer's and Parkinson's diseases.⁴¹ The identification of pathways that regulate this neuroinflammatory response is therefore essential, not only to define mechanisms that propel disease progression, but also to identify appropriate therapeutic targets. The findings presented here suggest that PGE₂ EP2 signaling may be a commonly shared regulator of this response.

The non-cell-autonomous toxicity of EP2 is illustrated in the model (see Fig 6F) that places the PGE₂ EP2 receptor upstream of a cassette of proinflammatory genes. Because EP2 signaling upregulates COX-1 and COX-2 expression, this represents a feed-forward cycle leading to enhanced production of PGE₂ and EP2 signaling, which will, in turn, lead to increased COX-1 and COX-2 expression. Conversely, inhibition of COX activity with NSAIDs, selective COX-2 inhibitors, or selective blockade of the EP2 receptor would be expected to suppress this cycle and reduce injury. Regarding a potential preventive effect of NSAIDs against development of ALS, it is interesting to speculate whether early NSAID use might act preventatively to decrease the risk for development of ALS, as has been noted for Alzheimer's disease.^{42,43} Interruption of EP2 signaling may be a useful strategy to selectively downregulate expression of proinflammatory enzymes critical to perpetuating the neuroinflammatory cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Aging G93A superoxide dismutase (SOD) mice demonstrate increased oxidative stress in neurons and induced expression of proinflammatory enzymes. (A) Gas chromatography mass spectrometric quantification of lipid peroxidation in 4-month-old male G93A SOD and wild-type (WT) mice demonstrates a selective increase in *F4*-neuroprostanes (neuroPs) but not *F2*-isoprostanes (isoPs) in cerebral cortex (**p* < 0.05). (B–D) Lumbar spinal cord messenger RNA of 1- and 3.3-month-old male G93A SOD and WT mice was assayed for expression of inducible nitric oxide synthase (*iNOS*) (B), cyclooxygenase-1 (*COX-1*) and *COX-2* (C), and NADPH oxidase subunits *gp91^{phox}*, *p22^{phox}*, *p47^{phox}*, and *p67^{phox}*. (D) Significant increases occur in 3.3-month-old but not 1-month-old G93A SOD mice relative to WT age-matched littermates for *iNOS*, *COX-2*, and NADPH oxidase subunits (*n* = 6–10 per age per genotype; **p* < 0.05

in 3-month-old WT vs mutant SOD mice). There is a developmental downregulation of p67^{phox} between 1 and 3.3 months of age in WT spinal cord (*p < 0.01).

Figure 2.

EP2 is induced in astrocytes and microglia in G93A superoxide dismutase (SOD) mice. (A) EP2 receptor colocalizes with the astrocytic marker glial fibrillary acidic protein (GFAP) in 3.3-month-old male G93A SOD but not wild-type (WT) lumbar cord (vertical arrows). (B) EP2 receptor colocalizes with the microglial marker Iba1 in SOD but not WT lumbar cord (horizontal arrows). (C) EP2 colocalizes with NeuN in motor neurons in both SOD and WT lumbar cord. Scale bar = 10 μ m. (D) EP2 is induced in SOD as compared with WT lumbar ventral horn. Left graph represents percentage EP2-immunopositive Iba1 microglial cells; right graph represents percentage EP2-immunopositive GFAP astrocytes. There is a significant increase in ratio of EP2-Iba1/Iba1 cells and EP2-GFAP/GFAP cells in SOD versus WT spinal cord (n = 4 per genotype with three sections counted 150 μ m apart; ***p < 0.01).

Figure 3.

EP2 is induced in human amyotrophic lateral sclerosis (ALS) spinal cord in astrocytes. (A) Low magnification (40X) view of ventral horn (vh) and lateral corticospinal tract (cst) in control human thoracic cord demonstrates EP2 expression in large motor neurons. (B) Low-magnification view (original magnification X40) of same region in ALS thoracic cord demonstrates motor neuron expression of EP2, as well as EP2 staining of the hypercellular infiltrate in corticospinal tract and ventral horn. (C) Higher magnification (original magnification, X400) view shows perinuclear staining of EP2 in motor neurons in ventral horn. (D) Similar view of ALS ventral horn shows a degenerating EP2-positive motor neuron (asterisk) and also EP2-immunopositive cells (arrows) that are morphologically consistent with astrocytes. (E, F) EP2 is expressed in astrocytes (arrow); EP2 immunoreactivity (brown) is prominently expressed in a glial fibrillary acidic protein staining astrocyte (purple) in ALS (F) as compared with control ventral horn (E).

Figure 4.

Phenotypes of G93A superoxide dismutase (SOD) mice lacking the EP2 receptor. **(A)** Deletion of the EP2 receptor resulted in increased survival in G93A SOD mice ($p < 0.02$) ($n = 11$ and 22 female G93A SOD mice in EP2^{-/-} [green lines] and EP2^{+/+} and EP2^{+/-} [red lines] backgrounds). **(B)** Deletion of the EP2 receptor in aging G93A SOD mice resulted in improved grip strength. Two-way analysis of variance with repeated measures (time in weeks and genotype) between weeks 10 and 19 showed a significant effect of genotype [$F(1,19) = 5.32$; $p < 0.05$]. **(C)** No differences were observed in weight.

Figure 5.

Expression levels of proinflammatory enzymes are reduced in G93A superoxide dismutase (SOD) mice lacking the EP2 receptor. **(A)** Reverse transcriptase polymerase chain reaction (RT-PCR) quantification of cyclooxygenase-1 (COX-1) and COX-2 messenger RNA (mRNA) in lumbar spinal cord of female G93A SOD mice in EP2^{+/+} and EP2^{-/-} backgrounds. Significant decreases are observed in the EP2^{-/-} background for both COX-1 and COX-2 ($n = 5$ for each genotype; $*p < 0.05$). **(B)** Protein levels of COX-2 were assayed in cervical cord lysates of female G93A SOD mice in EP2^{+/+} and EP2^{-/-} backgrounds, and show a significant reduction by quantitative Western analysis in EP2^{-/-} background, quantified in **(C)** ($n = 4-5$ samples/genotype; $*p < 0.05$). **(D)** RT-PCR quantification shows significant decreases in expression for both inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) in the EP2^{-/-} background ($n = 5$ for each genotype; $p < 0.05$). **(E)** Protein levels of iNOS in cervical cord lysates are reduced by quantitative Western analysis in G93A SOD mice lacking the EP2 receptor and are quantified in **(F)** ($p < 0.05$). **(G)** Levels of six NADPH oxidase subunits were assayed by RT-PCR of lumbar spinal cord of G93A SOD mice in the EP2^{-/-} and EP2^{+/+} backgrounds. Significant decreases occur in the EP2^{-/-} background for the membrane-bound subunit p22^{phox}, and for the cytoplasmic subunits p40^{phox}, p47^{phox}, and p67^{phox} ($n = 5$ for each genotype; $p < 0.05$). **(H)** Protein levels of p67^{phox} in cervical spinal cord lysates are reduced by quantitative Western analysis in G93A SOD mice in the EP2^{-/-} background (first four lanes) but do not vary in wild-type versus EP2^{-/-} mice (last four lanes). **(I)** Quantification of p67^{phox} immunoreactivity ($**p < 0.01$; $n = 4-5$ samples/genotype). **(J)** Gas chromatography mass spectrometry quantification of lipid peroxidation in cerebral cortex shows a trend toward decreased levels of F₄-neuroprostanes (NeuroPs) in the EP2^{-/-} background ($n = 4-5$ per genotype). IsoPs = isoprostanes.

Figure 6.

EP2 deletion decreases expression of proinflammatory genes in the lipopolysaccharide (LPS) model of innate immunity and the APPSwe-PS1ΔE9 model of familial Alzheimer's disease (AD). (A) By quantitative reverse transcriptase polymerase chain reaction (RT-PCR), expression of cyclooxygenase-2 (COX-2), p47^{phox}, p67^{phox}, gp91^{phox}, and inducible nitric oxide synthase (iNOS) are induced 24 hours after intracerebroventricular (ICV) LPS in wild-type mice (*p < 0.05; vehicle vs LPS; n = 4–6 mice/genotype), but in EP2^{-/-} mice, this induction is blocked (*p < 0.05; EP2 wild type vs EP2^{-/-} with LPS). Gray bars represent EP2^{-/-}; black bars represent EP2^{+/+}. (B) Representative quantitative Western analysis demonstrates induction of p67^{phox} in cytosolic and membrane fractions with ICV LPS; the induction of p67^{phox} is inhibited with deletion of EP2. (C) Quantitative RT-PCR demonstrates a significant increase in EP2 receptor expression in 12-month-old APPSwe-PS1ΔE9 mice (n = 7–9 male twelve-month-old mice per genotype; **p < 0.01). (D) By RT-PCR, levels of COX-2 and p67^{phox} are increased in 12-month-old APPSwe-PS1ΔE9 mice, but (E) this increase is abrogated with deletion of the EP2 receptor (*p < 0.05; n = 4–9 twelve-month-old male mice per genotype). (D) White bars represent wild type; black bars represent APPS. (E) Black bars represent APPS:EP2^{+/+}; gray bars represent APPS:EP2^{-/-}. (F) Model of proinflammatory mechanism of prostaglandin E₂ (PGE₂) EP2 signaling: PGE₂ signaling through the EP2 receptor on a glial cell (astrocyte or microglia) upregulates expression of proinflammatory proteins including subunits of the NADPH oxidase complex, COX-1 and COX-2, and iNOS, which are capable of producing ROS that damage neurons (yellow arrow), as evidenced by increased neuronal F₄-neuroprostanes (see Fig 1). Because COX-1 and COX-2 catalyze the first committed step in prostaglandin synthesis leading to further PGE₂ production, regulation of COX-1/2 expression by EP2 creates a feed-forward cycle in which further PGE₂ synthesis perpetuates and amplifies EP2 upregulation of proinflammatory proteins. Inhibition of COX activity with NSAIDs would block this feed-forward pathway.

Table**Primers used for RT PCR**

	Forward	Reverse	Accession number
18s rRNA	5'-cggctaccacatccaaggaa-3'	5'-gctggaattaccgcggct-3'	AY248756
p67phox	5'-gccggagacgccagaagagcta-3'	5'-ggggctgcgactgagggtgaa-3'	NM010877
gp91 phox	5'-ccaactgggataacgagttca-3'	5'-gagagtttcagccaaggcttc-3'	NM007807
p47 phox	5'-tacagcaaaagacaggactgggtt-3'	5'-gaggcacttgctttctgcaaact-3'	NM010876
p22 phox	5'-tcccattgagcctaaacccaagga-3'	5'-tcttcaccctcactcggcttctt-3'	NM007806
p40 phox	5'-gagggttcaccagccactttgttt-3'	5'-ettgcagtgaaaaggctgttctt-3'	NM008677
rac-1	5'-aggaagagaaaatgcctg-3'	5'-agcaaaagcgtacaaaggt-3'	BC003828
iNOS	5'-tgacggcaaacatgacttcag-3'	5'-gccatcgggcactctgga-3'	MMU43428
COX-1	5'-tgggcttcaaccttctcaac-3'	5'-ggcacacggaaggaaacata-3'	BC023322
COX-2	5'-cctctgcgatgctcttcc-3'	5'-tcacacttatactgtcaaatcc-3'	BC052900
nNOS	5'-ttgtgggaatagcgtgacagcaga-3'	5'-acacacacacacacacacacac-3'	AF534821
eNOS	5'-atgcctacagcattggttgaagg-3'	5'-aagcatatgaagggcagcagga-3'	BC052636