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Proteinase-activated receptors (PARs), a newly discovered subgroup of G-protein coupled receptors, are widely expressed by neural cells, but their roles in the nervous system remain uncertain. In this study, we report that PAR-2 was up-regulated on neurons in conjunction with neuroinflammation in brain tissue from patients with HIV-1-associated dementia. The inflammatory cytokines TNF-α and IL-1β were also increased in HIV-1-associated dementia brains compared with patients without dementia (p < 0.05), but these same cytokines induced PAR-2 expression on neurons. Enhanced PAR-2 expression and subsequent activation prevented neuronal cell death and induction of the tumor suppressor, p53, caused by the HIV-encoded protein, Tat (p < 0.01). Intrasratial implantation of a PAR-2 peptide agonist also inhibited Tat-induced neurotoxicity in a mouse model of HIV neuropathogenesis (p < 0.05). Moreover, PAR-2 null animals showed more severe neuroinflammation and neuronal loss caused by Tat neurotoxicity (p < 0.05). TNF-α protected wild-type neurons from Tat-related neurotoxicity, but in PAR-2-deficient neurons, the same concentrations of TNF-α were cytotoxic (p < 0.001). Thus, neuroinflammation can exert protective effects by which it induces PAR-2 expression with the ensuing abrogation of neuronal death. The Journal of Immunology, 2005, 174: 7320–7329.

Proteinase-activated receptors (PARs) are a novel family of G-protein coupled receptors that are activated by the proteolytic unmasking of a tethered ligand at their N terminus. Four members of this family have been cloned and identified; among them PAR-1, -3, and -4 are cleaved by thrombin, whereas PAR-2 is activated by trypsin and mast cell tryptase (1, 2). In the absence of proteolytic cleavage, PARs can also be activated by short peptides resembling the tethered ligand in sequence (e.g., TFFLRR for PAR-1 and SLIGRL for PAR-2). These peptides have been used as selective agonists for studying the function of PARs in vivo and in vitro. Although PARs were first discovered in a search for the receptors mediating the hormone-like effects of thrombin on platelets, subsequent studies showed their widespread expression in different cell types in the gastrointestinal, cardiovascular, respiratory, genitourinary, and central, and peripheral nervous systems (3–9). All four PARs have been shown to be expressed in the CNS, while their activating proteases can either be produced within the brain or originate from extravasated plasma during inflammatory processes (10).

Most studies of PARs expressed in the brain have focused on the role of PAR-1 in neuropathogenesis. Through a variety of signal transduction mechanisms, PAR-1 has been shown to mediate effects of thrombin on astrocytic proliferation, process retraction, growth factor production, and also neuronal morphology and survival (11, 12). PAR-1 has also been implicated in the pathogenesis of neuroinflammatory/degenerative disorders (13, 14). Previous studies from our group demonstrated an important role for astrocytic PAR-1 up-regulation and activation in the neuropathogenesis of HIV-1 infection (15), characterized by neuroinflammation and neuronal injury. Indeed, neuronal dysfunction and death likely underlie the motor, behavioral, and cognitive impairments that are present among patients with HIV-associated dementia (HAD), which usually becomes manifest during AIDS. Neuropathologically, brains of patients with HAD frequently exhibit neuroinflammation, including astrogliosis, multinucleated giant cells, and neuronal apoptosis (16). Despite the severe neuronal loss observed in HIV-1 infection, minimal infection of neurons has been reported (17–19). This consistent observation has led to the assumption that indirect pathways, including the release of host inflammatory molecules such as cytokines, chemokines, and matrix metalloproteinases, in addition to other potential host neurotoxins and also HIV-encoded proteins, Tat and gp120, play central roles in HIV-induced neuronal death (20–24).

Although not as extensively studied as PAR-1, PAR-2 has also been shown to be involved in the pathogenesis of systemic inflammatory disorders (25–29). Nonetheless, PAR-2 is expressed on hippocampal, cortical, thalamic, hypothalamic, and striatal neurons in the brain and on dorsal root ganglia, myenteric, and submucosal neurons.
in the peripheral nervous system (7–9, 30, 31). Brain-expressed trypsinogen IV (32), a splice variant of pancreatic trypsinogen III, and mast cell-derived tryptase are considered potential PAR-2-activating proteinases in the brain (10). Despite the widespread expression of PAR-2 in the CNS, its role in neurodegenerative/inflammatory disorders is unknown. To explore the role of the PAR-2 axis in the context of HIV-induced brain disease, we investigated the expression of the receptor and its putative activating proteinase in brain samples from patients with HIV infection. Experiments were conducted to assess the effect of HIV-induced inflammatory stimuli on neuronal PAR-2 expression and to determine the effects of receptor activation on neuronal survival in vitro and in vivo. In addition, experiments were performed to explore the downstream mechanisms of PAR-2 effects on neuronal survival and their interplay with TNF-α during HIV infection.

**Materials and Methods**

**Human brain tissue samples**

Brain tissue (frontal lobe) was collected at autopsy from patients seropositive for HIV-1 and stored at −80°C, as described previously (35–38), from which RNA was extracted. Patients were stratified to demented (HAD) and nondemented (ND) HIV/AIDS groups based on standard neurological and neuropsychological testing (39, 40). The HAD group consisted of 8 male patients (age, 39.4 ± 4.3 years; CD4 count, 47.6 ± 28.3/mm³), whereas the ND group consisted of 10 males and 2 females (age, 37.6 ± 2.5 years; CD4 count, 88.25 ± 47.5/mm³) (38). In addition, tissue sections from the frontal lobe from HAD and ND patients were prepared.

**Real-time RT-PCR**

Cultured cells and brain tissue were homogenized and lysed in TRIzol (Invitrogen Life Technologies) according to the manufacturer’s guidelines. Total RNA was isolated and dissolved in diethylpyrocarbonate-treated water, and 1 μg of RNA was used for the synthesis of complementary DNA and PCRs as described previously (41). Primers were as follows: GAPDH, 5′ primer: 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′, 3′ primer: 5′-CGG AGT CAA CGG ATT TGG TCG-3′; PAR-2, 5′ primer: 5′-CTG GCC ATT GGG GTC TTT CTG TTC-3′, 3′ primer: 5′-GGC CCT CTT CCT TTT CTT CTC TGA-3′; trypsinogen, 5′ primer: 5′-TCA GCG AAC AGT GGG TGG TAT CAG-3′, 3′ primer: 5′-GAG GGG CGG TGG GCA CAG-3′; TNF-α, 5′ primer: 5′-ATT CAG GAA TGT GTG GCC TGC-3′, 3′ primer: 5′-GTT TGA ATT CTT AGT GGT TGC-3′; IL-1β, 5′ primer: CCA AAG AAG AAG ATG GAA AAG CG-3′, 3′ primer: GGT GCT GAT GTA CCA GTT GCC-3′. Semiquantitative analysis was performed by monitoring in real time the increase of fluorescence of the SYBR Green dye on a Bio-Rad i-Cycler, as previously reported (41). All

**FIGURE 1.** PAR-2 up-regulation on neurons in brains of HAD patients. TNF-α (A) and IL-1β (B) mRNA levels were up-regulated in brain tissue of HAD patients, as assessed by real-time RT-PCR, compared with ND HIV/AIDS patients. PAR-2 mRNA levels (C) are increased in the brains of HAD patients, but (D) trypsinogen levels are not significantly different compared with ND patients. mRNA levels are shown as relative fold change (RFC) ± SEM compared with ND patients. PAR-2 immunoreactivity in ND (E, inset shows the staining with Ab absorbed with immunogen peptide) and HAD brain sections (F, inset shows the colocalization of PAR-2 with NeuN neuronal marker), showing that PAR-2 immunoreactivity was up-regulated on neurons (arrows) in sections from HAD patients. Trypsinogen immunoreactivity in ND (G) and HAD brain section (H, inset shows the colocalization of trypsinogen with NeuN neuronal marker) was also localized on neurons. (Original magnification: ×400; Student’s t test; *, p < 0.05).
data were normalized against the GAPDH mRNA levels and expressed relative to controls.

Cell lines, transfections, and treatments
As previously described (42), mouse primary neuronal cultures were established from 18-day-old PAR-2 null (knockout (KO)) (43) and wild-type embryos. Primary human fetal neurons (a gift from Dr. V. W. Yong, University of Calgary, Calgary, Alberta, Canada) were prepared and cultured as described (42). LAN-2 human cholinergic neuroblastoma cells were grown in MEM containing 10% FBS and 1% N2 supplement and then differentiated for 2 days in L-15 medium, containing 10% FBS and 1 mM dibutyryl-cAMP (44). All of the steps were done without trypsin. LAN-2 cells were transfected with a pcDNA3.1 vector containing a human PAR-2 cDNA (45) using FuGene6 Transfection Reagent (Roche Applied Sciences) and then selected with Geneticin for 2 mo (0.7 mg/ml). Mouse and human recombinant TNF-α and IL-1β were applied to neuronal cultures for 24 h (R&D Systems). Neurotoxic supernatants (Tat S/N) from monocytoid (U937) cells transfected with an HIV-1 expressing vector, derived from a patient with HAD, were used in accordance with previous studies (44, 46).

PAR-2 agonists
The activating peptide (AP) sequence (SLIGRL-NH2), corresponding to the tethered ligand domain of PAR-2, as well as a mutated inactive peptide (AP) sequence (LSIGRL-NH2), synthesized by the Peptide Synthesis Facility (University of Calgary) were prepared in 25 mM HEPES buffer, pH 7.4, and standardized by quantitative amino acid analysis and mass spectrometry to confirm peptide concentration and purity (>95%) for in vitro and in vivo application (47).

Calcium-signaling assay
The calcium-signaling assay was performed as described previously (48). Briefly, cells at 90% confluence in 75-cm2 flasks lifted with calcium-free isotonic PBS containing 0.2 mM EDTA and pelleted before resuspension in 1 ml of AIM-V medium, and 0.25 mM sulfinpyrazone. A total of 10 μl of 2.5 mg/ml Flu-3 acetoxyethyl ester (Molecular Probes) was added to the cells followed by gentle shaking for 20 min at room temperature. Cells were then washed in PBS and resuspended in calcium assay buffer (150 mM NaCl, 3 mM KCl, 1.5 mM CaCl2, 10 mM glucose, 20 mM HEPES, 0.25 mM sulfinpyrazone, pH 7.4). Fluorescence measurements were performed on a PerkinElmer fluorescence spectrometer 650-10S, with an excitation wavelength of 480 nm and emission recorded at 530 nm.

Immunohistochemistry
Paraffin-embedded sections (5 μm) of human or mouse brain tissue were deparaffinized and hydrated using decreasing concentrations of ethanol. Sections were boiled in 0.01 M citrate buffer, pH 6.0, for 10 min for PAR-2, neuronal nuclei Ag (NeuN), and glial fibrillary acidic protein (GFAP) staining. Endogenous peroxidases were blocked by incubating sections in 3% hydrogen peroxide for 20 min. To prevent nonspecific binding, sections were preincubated with 1% normal goat serum for 1 h at room temperature. Primary Abs including polyclonal antiserum (B5) raised in rabbits to a peptide corresponding to rat PAR-2 (GPNSKGRSLIGRLDT46P-YGGC; 1/1000) (49, 50), rabbit anti-human pancreatic trypsin antisera (Advent Research and Technology; 1/1000), mouse anti-NeuN (Chemicon International; 1/200) and anti-GFAP (Dako; 1/200), were diluted in PBS/5% serum and incubated overnight at 4 degrees, followed by washing. For double labeling with anti-NeuN Ab, an alkaline phosphatase-conjugated goat anti-mouse Ab (Jackson ImmunoResearch Laboratories) was used. Alkaline phosphatase activity was detected with 3,3‘-diaminobenzidine tetrachloride (Vector Laboratories). For double labeling with anti-NeuN Ab, an alkaline phosphatase-conjugated goat anti-mouse Ab (Jackson ImmunoResearch Laboratories) was used. Alkaline phosphatase activity was detected by an NBT/BCIP substrate (Vector Laboratories). For double labeling with anti-neuron marker, Cy3-conjugated goat anti-mouse or Alexa 488-conjugated goat anti-rabbit Abs (Molecular Probes) were used for NeuN and GFAP detection, respectively.

**FIGURE 2.** Inflammatory cytokines induce neuronal PAR-2 expression. PAR-2 mRNA levels are increased in human neuronal (LAN-2) cells after treatment with TNF-α (10 ng/ml) (A) or IL-1β (20 ng/ml) (B), mRNA levels are shown as relative fold change (RFC) ± SEM compared with controls. PAR-2 immunoreactivity (C) is enhanced in neuronal (LAN-2) cells after treatment with TNF-α or IL-1β. Quantification of PAR-2 immunoreactivity in neuronal (LAN-2) cells shows a significant increase after treatment with TNF-α (D) and IL-1β (E). (Original magnification, ×400; Student’s t test and Tukey-Kramer multiple comparisons test; *, p < 0.05; **, p < 0.01).

**FIGURE 3.** PAR-2 mediates calcium signaling in neurons. A, Neurons transfected with a PAR-2-transferring vector show increased PAR-2 immunoreactivity in cytoplasm and plasma membrane. B, PAR-2 agonist peptide (SLIGRL-NH2) induces a robust calcium signal in transfected neurons, but not in mock-transfected neurons. PAR-1 activation by PAR-1 AP (TFFLR) was maintained in both transfected and mock-transfected neurons. (Original magnification, ×400).
Quantitative immunodetection assay

Differentiated LAN-2 cells, plated in 96-well plates, were used for in-cell quantitative immunocytochemistry, according to the manufacturer instructions (LI-COR Biosciences). Briefly, cells with or without pretreatment with PAR-2 agonist peptide (2 h) were exposed to Tat S/N for 12 h before fixation with 4% paraformaldehyde. Cells were then washed and permeabilized with PBS/0.1% Triton X-100, blocked with Odyssey Blocking Buffer followed by immunostaining using a primary polyclonal rabbit anti-cleaved-caspase-3 (1/100 dilution; Cell Signaling) or a primary polyclonal rabbit anti-p53 (1/100 dilution; Santa Cruz Biotechnology) and a goat anti-rabbit IR Dye800 secondary Ab (1/800 dilution; Rockland Immunochemicals). Immunoreactivity was measured and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences). For fluorescent labeling, the same anti-p53 Ab plus anti-nonphosphoNeurofilament (SMI-32) mouse monoclonal (Sternberger Monoclonals) were used, followed by Alexa 488-conjugated goat anti-rabbit or Cy3-conjugated goat-anti-mouse secondary Abs (Molecular Probes), respectively.

Neuronal survival assays

Lactate dehydrogenase (LDH) assays were performed on the supernatants from LAN-2 neuroblastoma and mouse primary neurons based on the manufacturer’s instructions (Cytotoxicity Detection kit; Roche Applied Science). Trypan blue dye exclusion was performed by incubating LAN-2 cells with 0.4% paraformaldehyde. Cells were then washed and permeabilized with PBS/0.1% Triton X-100, blocked with Odyssey Blocking Buffer followed by immunostaining using a primary polyclonal rabbit anti-cleaved-caspase-3 (1/100 dilution; Cell Signaling) or a primary polyclonal rabbit anti-p53 (1/100 dilution; Santa Cruz Biotechnology) and a goat anti-rabbit IR Dye800 secondary Ab (1/800 dilution; Rockland Immunochemicals). Immunoreactivity was measured and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences). For fluorescent labeling, the same anti-p53 Ab plus anti-nonphosphoNeurofilament (SMI-32) mouse monoclonal (Sternberger Monoclonals) were used, followed by Alexa 488-conjugated goat anti-rabbit or Cy3-conjugated goat anti-mouse secondary Abs (Molecular Probes), respectively.

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Lactate dehydrogenase (LDH) assays were performed on the supernatants from LAN-2 neuroblastoma and mouse primary neurons based on the manufacturer’s instructions (Cytotoxicity Detection kit; Roche Applied Science). Trypan blue dye exclusion was performed by incubating LAN-2 cells with 0.4% paraformaldehyde (Invitrogen Life Technologies) for 4 min, before washing with PBS and counting. Primary mouse neurons grown on eight-well chamber slides were fixed and stained with mouse anti-microtubule-associated protein-2 (MAP-2) mAb (Sigma-Aldrich) followed by Cy3-conjugated goat anti-mouse secondary Ab (Molecular Probes). Scion Image software was used to quantify MAP-2 reactivity after cytokine/Tat S/N treatment (Scion).

Neurobehavioral studies

Male PAR-2-null (KO) and wild-type (WT) littermate C57BL/6 mice between 8 and 10 wk of age (43) and 3-wk-old CD-1 mice (Charles River Laboratories) were used in the present studies. Animals were housed in a biocontainment facility according to the guidelines of the Canadian Animal Care Committee. Animals were placed in a stereotaxic frame under Ketamine/Xylazine anesthesia. Peptides and/or Tat S/N were delivered into the right striatum. In vivo neurological injury was assessed according to the Ungerstedt model (44, 46, 51). In short, ipsiversive rotations as well as total number of rotations were monitored over 10 min after i.p. injection of amphetamine (1 mg/kg) on days 3 and 7 following striatal injection. Increased ipsiversive rotary behavior is indicative of neurological injury. Animals were sacrificed after 7 days, and brain sections were prepared for immunohistochemical analysis. All experiments were approved by the University of Calgary Animal Care Committee.

Results

PAR-2 expression is increased on neurons in HIV-induced neuroinflammation

HIV-1 infection of brain induces neuroinflammatory pathways associated with infiltrating macrophages and microglial activation, and up-regulation of inflammatory mediators (52). We examined mRNA levels of two major proinflammatory cytokines, TNF-α...
Pyramidal neurons in the frontal and parietal cortices (Fig. 1) was markedly enhanced in demented cases, especially in demented brains (Fig. 1), but its expression on ND (Fig. 1E) groups. PAR-2 immunoreactivity was localized in neurons for both PAR-2 mRNA was up-regulated in the brain tissue of HAD patients with and without HAD. The mRNA of both cytokines showed significant up-regulation in HAD brains compared with ND HIV/AIDS patients, similar to previous studies (53, 54). Although PAR-2 may modulate inflammation in certain circumstances (6, 55–57), little is known about its role in neuroinflammation, particularly in the CNS. To investigate PAR-2 expression in the brain, PAR-2 mRNA levels were evaluated, in addition to its activating protease, trypsinogen IV (32) in the same brain samples. PAR-2 mRNA was up-regulated in the brain tissue of HAD patients compared with the ND group (Fig. 1C), whereas trypsinogen mRNA levels did not differ between groups (Fig. 1D). Interestingly, we found that the mRNA levels of PAR-2 were highly correlated with both IL-1β (Pearson r = 0.82; p < 0.0001) and TNF-α (Pearson r = 0.93; p < 0.0001) mRNA levels in both groups. PAR-2 immunoreactivity was localized in neurons for both ND (Fig. 1E) and demented brains (Fig. 1F), but its expression on neurons was markedly enhanced in demented cases, especially in pyramidal neurons in the frontal and parietal cortices (Fig. 1F, arrows), regions that are particularly susceptible to HIV-mediated injury (58). Indeed, PAR-2 immunoreactivity was colocalized with neuronal nuclear marker, NeuN (Fig. 1F, inset). The immunoreactivity of trypsinogen was also localized to neurons with no evident difference between the ND (Fig. 1G) and the demented patients (Fig. 1H). Like PAR-2, trypsinogen was also colocalized with NeuN immunoreactivity (Fig. 1H, inset). These observations indicated that PAR-2, but not trypsinogen, was up-regulated on neurons in conjunction with neuroinflammation and HIV infection. 

**Inflammatory cytokines induce PAR-2 expression on neurons**

Given the induction of proinflammatory cytokines TNF-α and IL-1β in the brain upon HIV infection, we next studied the effect of these cytokines on PAR-2 expression in cultured human neuronal (LAN-2) cells. Treatment of neuronal cells with TNF-α (10 ng/ml) (Fig. 2A) or IL-1β (20 ng/ml) (Fig. 2B) for 24 h caused significant up-regulation of PAR-2 mRNA levels for both cytokines, which was associated with a corresponding increase in PAR-2 immunoreactivity on cultured neurons (Fig. 2C). Moreover, PAR-2 protein expression on cultured neurons showed significant up-regulation with TNF-α (Fig. 2D) and IL-1β (Fig. 2E) treatments, indicating that proinflammatory cytokines induced PAR-2 expression in neurons.

**PAR-2 expression and activation on neurons reduces Tat-induced neurotoxicity in vitro and in vivo**

To investigate whether PAR-2 up-regulation on neurons might affect neuronal survival, we transfected neuronal (LAN-2) cells with a PAR-2-expressing vector (45). Stable transfection resulted in a substantial increase in PAR-2 immunoreactivity compared with mock-transfected cells (Fig. 3A). Mock-transfected neurons do not express PAR-2 at sufficient levels to induce calcium mobilization in response to a PAR-2 AP (SLIGRL-NH2), despite being sensitive to a PAR-1 AP (TFLLR) (Fig. 3B). However, PAR-2-transfected neurons treated with different concentrations of SLIGRL-NH2, PAR-2 AP showed robust calcium responses, with concurrent preservation of PAR-1 AP effects (Fig. 3B). These findings emphasized the importance of increased PAR-2 expression levels to activate a response involving an increase in intracellular calcium in neurons. Previous studies indicate that HIV Tat is capable of mediating neuronal apoptosis through activation of proximal monoyctoid cells and secretion of neurotoxins (59, 60). Exposure of human fetal neurons to neurotoxic supernatants from HIV tat-transfected monoyctoid cells (Tat S/N) led to a marked reduction in MAP-2 reactivity (Fig. 4A), compared with untreated cultures. Supernatants from monoyctoid cells transfected with an empty vector or a tat sequence from an ND patient did not cause neuronal injury (data not shown), similar to previous studies (44, 46). A 75% reduction of MAP-2 immunoreactivity in human fetal neurons caused by Tat S/N treatment (Fig. 4B) was associated with neuronal death. Tat S/N treatment of neuronal (LAN-2) cells was also associated with increased immunoreactivity of the cleaved form of caspase-3 (Fig. 4C) and a 4-fold increase in neuronal death, measured by trypan blue exclusion, compared with mock-treated cultures (Fig. 4D). Pretreatment with the PAR-2 AP (SLIGRL-NH2), but not the mAP, significantly reduced the level of Tat S/N-induced cell death in PAR-2-transfected neurons (Fig. 4E). Conversely, mock-transfected neurons were not protected by the PAR-2 AP (SLIGRL-NH2; Fig. 4E), underscoring the importance of increased receptor levels in terms of cell responsiveness (e.g., as with calcium signaling).

To explore whether PAR-2 activation may also play a protective role in vivo, we simultaneously implanted Tat S/N with or without the PAR-2 APs or mAPs (inactive) into the right striatum of 3-wk-old CD-1 mice. Seven days after implantation, neurobehavioral analysis showed a significantly lower number of ipsiversive rotations, indicative of neuronal injury (46, 51), in animals receiving 100 μg/kg of the PAR-2 AP together with Tat S/N (Fig. 4F), compared with animals receiving only Tat S/N or Tat S/N with the mAP. A nonsignificant dose-dependent trend was observed with 10 and 100 μg/kg of the PAR-2 AP. Thus, these findings revealed that PAR-2 activation exerted a neuroprotective effect, countering the neurotoxic effects of Tat S/N.

**PAR-2 activation diminishes Tat-induced p53 induction in neurons**

Previous studies have reported that p53 is up-regulated in neurons in vivo by HIV infection (61) and in vitro by Tat S/N (44). To explore
the downstream mechanisms for the neuroprotective effects of PAR-2, p53 levels in PAR-2-transfected neurons were investigated. Tat S/N treatment of PAR-2-transfected neurons resulted in increased p53 expression compared with untreated PAR-2-transfected neurons (Fig. 5A). Similarly, quantification of p53 expression following Tat S/N application revealed enhanced p53 expression in both PAR-2-transfected (Fig. 5B) and mock-transfected neurons (data not shown). In PAR-2-transfected cells, pretreatment with the PAR-2 AP significantly decreased Tat S/N-induced p53 expression (Fig. 5B). A similar trend that did not reach statistical significance was also observed in mock-transfected cells (data not shown). This lack of a clear-cut neuroprotective action in the mock-transfected cells can be correlated with diminished PAR-2 expression. Notwithstanding, our results imply that in principle, p53-mediated neuronal apoptosis caused by Tat S/N can be mitigated by PAR-2 activation.

TNF-α protects neurons against Tat S/N

Previous studies indicate that TNF-α signaling in the nervous system can have both neurodegenerative and neuroprotective effects (62–65). Although it can induce or promote neuronal death in

FIGURE 6. TNF-α is neuroprotective in the presence of PAR-2. A, TNF-α pretreatment decreases Tat S/N toxicity in WT primary mouse neuronal cultures (WT), while TNF-α treatment (up to 100 ng/ml) per se does not affect neuronal survival. In contrast, TNF-α-treated neurons from PAR-2 null (KO) animals showed reduced viability that was further exacerbated by treatment with Tat S/N. B, Quantification of MAP-2 reactivity showed that TNF-α does not affect WT neuron survival. C, TNF-α treatment protected PAR-2 WT neurons from Tat S/N neurotoxicity. D, Conversely, TNF-α decreased PAR-2 KO neuronal viability. E, In contrast to WT neurons, TNF-α pretreatment exacerbated Tat S/N-mediated neuronal injury in PAR-2 KO neurons. Each culture was done in triplicate from several different animals. Neuronal survival has been assessed by measuring MAP-2 reactivity and expressed as a relative fluorescence unit (RFU) ± SEM compared with control. (Original magnification: ×400; Tukey-Kramer multiple comparisons test; *, p < 0.05; **, p < 0.01; ***, p < 0.001).
FIGURE 7. PAR-2 deficiency exacerbates Tat S/N neuropathological and behavioral outcomes. A. Neuropathological analysis of striatum from PAR-2 WT and KO animals, implanted with Tat S/N. Labeling for the neuronal, NeuN, and astrocytic, and GFAP markers shows more severe neuronal loss and astrocytosis in PAR-2 KO animal brains in the vicinity of the implantation site. B. Quantitative studies showed a marked decrease in NeuN in KO animals, whereas GFAP reactivity is significantly higher in KO animals compared with WT littermates following Tat S/N implantation. C. Neurological damage, assessed at days 3 and 7 in animals receiving intrastriatal implants of Tat S/N, revealed that PAR-2 KO animals show a higher percent of ipsiversive rotations at day 7 after Tat S/N implantation, indicative of more severe neurobehavioral deficit (Student’s t test; *p < 0.05).

some model systems, pretreatment of neurons with TNF-α has also been shown to decrease neuronal vulnerability to such injurious stimuli as glucose deprivation, excitotoxicity, and β-amyloid toxicity (64–66). To investigate whether TNF-α might affect neuronal survival in the context of HIV-induced neuroinflammation, primary neuronal cultures from 18-day-old mouse embryos that were PAR-2 WT or null (KO) were pretreated with 10–100 ng/ml murine TNF-α for 24 h and then exposed to HIV Tat S/N for 48 h (Fig. 6A). TNF-α treatment alone did not affect the viability of WT neurons, as assessed by MAP-2 reactivity (Fig. 6, A and B). In contrast, Tat S/N treatment led to a marked reduction of MAP-2 reactivity in WT neurons (Fig. 6A). Interestingly, pretreatment with TNF-α, rescued WT neurons from Tat S/N-induced neurotoxicity (Fig. 6A) in a concentration-dependent manner (Fig. 6C). To explore the potential role of PAR-2 in this TNF-α-mediated protective effect, primary neurons established from PAR-2 null (KO) embryos were used (right micrographs in Fig. 6A). In contrast to the WT neurons, TNF-α treatment decreased the survival of PAR-2 null (KO) neurons (Fig. 6, A and D). Indeed, in the PAR-2 KO cells, TNF-α treatment appeared to enhance Tat S/N-induced neurotoxicity (two bottom right photomicrographs in Fig. 6A) in a concentration-dependent manner (Fig. 6E). Similar results were obtained using LDH release as an index of cytotoxicity in TNF-α and Tat S/N-treated neurons (data not shown). Thus, our findings suggested that TNF-α was protective in PAR-2-expressing (WT) neurons, but cytotoxic without or in combination with Tat S/N in PAR-2 null (KO) neurons.

PAR-2 deficiency exacerbates HIV Tat-induced neuropathological and neurobehavioral deficits in mice

To examine the PAR-2-mediated neuroprotective effects in vivo, Tat S/N was implanted into the right striatum of littermate PAR-2-expressing (WT) or null (KO) mice, followed by neurobehavioral and neuropathologic analyses. NeuN immunoreactivity was reduced in PAR-2 KO animals in the vicinity of the implantation site compared with PAR-2 WT animals (Fig. 7A). Similarly, neuronal counts of NeuN-positive cells were significantly reduced in PAR-2 KO animals (Fig. 7B). Astrogliaisis was increased in the PAR-2 KO animals relative to PAR-2 WT animals (Fig. 7A), which was also confirmed by GFAP-positive astrocyte counts (Fig. 7B). Indeed, neuronal injury and loss was closely associated with enhanced astrogliosis in PAR-2 KO animals, as indicated by the merged NeuN-GFAP immunoreactivity (Fig. 7A). Neurobehavioral analyses revealed that 7 days after implantation, significantly higher ipsiversive rotations were observed in PAR-2-deficient animals, indicative of more severe neurological injury (Fig. 7C). Comparison of baseline rotary behavior in PAR-2 KO and WT animals before implantation did not reveal significant differences between groups (data not shown). Thus, these observations confirm our in vitro data (Fig. 4), suggesting that PAR-2 exerts a neuroprotective effect in the context of HIV-1 infection.

Discussion

Despite their widespread expression in brain, the role of PARs in neurodegenerative disorders remains largely undefined. In this study, we show that PAR-2 is up-regulated at both the mRNA and protein levels in brains of patients with HAD. In addition, neuronal PAR-2 was inducible in cultured human neuronal (LAN-2) cells by the proinflammatory cytokines, IL-1β and TNF-α, whose expression was also increased in HAD brains. In vitro experiments with a differentiated human neuronal cell line showed that PAR-2 activation can decrease the susceptibility of these cells to HIV-Tat-induced neurotoxins, involving a p53-mediated pathway. In
vivo implantation of a PAR-2 AP also reduced Tat-S/N-induced neurobehavioral deficits in PAR-2-expressing animals. Conversely, intrastratal implantation of Tat S/N led to a more prominent neuronal loss, associated with enhanced astrocytosis and neurodegenerative deficits in PAR-2 null (KO) animals, leading us to conclude that PAR-2 regulation and expression in the context of neuroinflammation assumes a neuroprotective function.

TNF-α is one of the key regulators of inflammation in different neuroinflammatory disorders including stroke, multiple sclerosis, Alzheimer’s disease, and HAD (67–70). Although TNF-α is chiefly known for its neurotoxic effects (62, 63), several reports have also shown a neuroprotective role for this cytokine, including protection against excitotoxicity, glucose deprivation, and β-amyloid toxicity (64–66). In this study, we show that pretreatment of primary neurons with TNF-α renders them more resistant to HIV Tat-induced neurotoxicity. The ability of TNF-α to cause an up-regulation of PAR-2 may explain in part its neuroprotective activity. Interestingly, we found that TNF-α exerts a toxic action in PAR-2-deficient neurons, at the same concentrations that enhance WT neuronal viability, thereby potentiating Tat neurotoxic activity. There is substantial evidence to suggest that the signaling mechanisms leading to the protective vs degenerative effects of TNF-α, involves NFκB vs death domain-mediated signaling (65, 71). Whether activation of PAR-2, as a potential NF-κB activator (55, 72), may tip the balance of TNF-α signaling from death domain-mediated signaling toward NF-κB-mediated signaling, is an interesting question to explore in future.

Molecular mechanisms of HIV-induced neuronal death have been under intense investigation. The tumor suppressor transcription factor, p53, has been recently shown to be a mediator of neuronal apoptosis following a variety of neurodegenerative stimuli (73). Studies done in vitro have shown neuronal p53 up-regulation caused by excitotoxicity, DNA damage, hypoxia, and growth factor withdrawal (74). Elevated p53 immunoreactivity has been detected in CNS tissue of patients diagnosed with other neurodegenerative disorders including Alzheimer’s disease, Parkinson’s disease, Down’s syndrome, Huntington’s disease, and amyotrophic lateral sclerosis (73, 74). Interestingly, absence or down-regulation of p53 protects neurons against some juvenile stimuli (74). It has been shown that p53 is up-regulated on cortical neurons of patients with HIV-1 infection and its induction has been reported to play a role in the neuronal death caused by HIV Tat and gp120 molecules (44, 61). In this study, we show that PAR-2 activation on neurons prevents p53 up-regulation by Tat-induced neurotoxins, a phenomenon that could mediate the neuroprotective effect of PAR-2. Stress-activated protein kinases, particularly p38 MAPK and JNK are among the principal p53 phosphorylating and stabilizing agents (73, 74). ERK is the other MAPK pathway that acts in direct opposition to JNK and p38 MAPK pathways; and its activation has been shown to protect against p53-mediated neuronal apoptosis (74). PAR-2 activation leads to activation of the ERK1/2 pathway, whereas it weakly stimulates p38 MAPK and does not activate JNK (2). Indeed, a very recent study of experimental stroke shows that PAR-2 null mice exhibit a more severe phenotype that is accompanied by reduced phosphorylated-ERK expression (75). Thus, it is conceivable that preferential ERK pathway activation may be responsible for PAR-2-mediated p53 suppression and a neuroprotective effect.

Although most studies of HIV-1 neuropathogenesis have focused on the pathogenic mechanisms of neuronal cell death, we found that PAR-2 expression may play a protective role in the context of HIV-induced neuroinflammation. Although neuroinflammation can cause neuronal death, there is also existing evidence showing that neuroinflammation can also confer neuroprotection, depending on the profile of inflammatory molecules activated (76–78). Indeed, the protective role of neuroinflammation-induced PAR-2 is likely an early event in the disease course, which is eventually overwhelmed by progressive disruption of innate immunity and neuronal loss in conjunction with immunosuppression. Nonetheless, identifying the mechanisms by which the beneficial effects of inflammation are exerted in the nervous system will facilitate the development of therapeutic strategies for neuroinflammatory/degenerative disorders where inflammation itself is difficult to control.

Disclosures

The authors have no financial conflict of interest.

References


