The NADPH Oxidase Pathway Is Dysregulated by the P2X7 Receptor in the SOD1-G93A Microglia Model of Amyotrophic Lateral Sclerosis

Savina Apolloni, Chiara Parisi, Maria Grazia Pesaresi, Simona Rossi, Maria Teresa Carri, Mauro Cozzolino, Cinzia Volonté and Nadia D’Ambrosi

J Immunol 2013; 190:5187-5195; Prepublished online 15 April 2013;
doi: 10.4049/jimmunol.1203262
http://www.jimmunol.org/content/190/10/5187

References
This article cites 43 articles, 11 of which you can access for free at:
http://www.jimmunol.org/content/190/10/5187.full#ref-list-1

Subscriptions
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscriptions

Permissions
Submit copyright permission requests at:
http://www.aai.org/ji/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/cgi/alerts/etoc
Inflammation and oxidative stress are thought to play determinant roles in the pathogenesis of amyotrophic lateral sclerosis (ALS). Degenerating motor neurons produce signals that activate microglia to release reactive oxygen species (ROS) and proinflammatory cytokines, resulting in a vicious cycle of neurodegeneration. The ALS-causing mutant protein Cu+/Zn+ superoxide dismutase SOD1-G93A directly enhances the activity of the main ROS-producing enzyme in microglia, NADPH oxidase 2 (NOX2), a well-known player in the pathogenesis of ALS. Considering that extracellular ATP through P2X7 receptor constitutes a neuron-to-microglia alarm signal implicated in ALS pathology, we used primary microglial cells derived from transgenic SOD1-G93A mice and SOD1-G93A mice lacking the P2X7 receptor to investigate the effects of both pharmacological induction and genetic ablation of receptor activity on the NOX2 pathway. We observed that, in SOD1-G93A microglia, the stimulation of P2X7 receptor by 2'-3'O-(benzoyl-benzoyl) ATP enhanced NOX2 activity in terms of translocation of p67phox to the membrane and ROS production; this effect was totally dependent on Rac1. We also found that, following P2X7 receptor stimulation, the phosphorylation of ERK1/2 was augmented in ALS microglia, and there was a mutual dependency between the NOX2 and ERK1/2 pathways. All of these findings suggest a noxious mechanism by which P2X7 receptor leads to enhanced oxidative stress in ALS microglia and identify the P2X7 receptor as a promising target for the development of therapeutic strategies to slow down the progression of ALS. The Journal of Immunology, 2013, 190: 5187–5195.

The latter function as signals to amplify the production of several proinflammatory and neurotoxic cytokines through the activation of downstream molecules, including MAPK (i.e., p38 and ERK1/2) (5).

A central activator of NOX2 is the Rho-GTPase family member Rac1, acting as a molecular switch cycling between its inactive GDP-bound state and active GTP-bound state (6). GTP hydrolysis by Rac1 is controlled through a redox-dependent interaction with the ubiquitous enzyme Cu+/Zn+ superoxide dismutase (SOD1), which is found mutated in ALS patients. When SOD1 is bound to Rac1 under reducing conditions, GTP hydrolysis is inhibited; in oxidizing conditions, SOD1 dissociates from Rac1, no longer inhibiting GTP hydrolysis. The ALS-causing glycine-to-alanine substitution at position 93 (G93A) on SOD1 protein renders the enzyme less sensitive to redox uncoupling, leading to enhanced Rac1/NOX2 activation (7) and resulting in overproduction of damaging ROS (8). Consistently, NOX2 is upregulated in activated ALS microglia, and the deletion of NOX2 can improve disease progression and survival of SOD1-G93A mice (9–11). Furthermore, uncontrolled ROS, together with inflammatory cytokines produced by microglia, mediate disease progression, causing direct neurotoxicity and increasing motor neuron susceptibility to ALS-triggering factors (2, 12). Among these, extracellular ATP was recently suggested as a possible candidate. The role of extracellular nucleotides is well recognized in proliferation, chemotaxis, phagocytosis, and the release of proinflammatory molecules, namely in all aspects of microglia functioning (13). A major player in the response of microglia to extracellular ATP is the ionotropic purinergic P2X7 receptor, which is responsible for the release of cytokines and induction of proinflammatory factors, such as TNF-α, IL-6, IL-1β, plasminogen, and cyclooxygenase-2 (COX-2) (14). Indeed, the proinflammatory function of P2X7 receptor has...
been involved in different neuropathologies with an inflammatory component, such as Alzheimer’s and Huntington’s diseases, multiple sclerosis, spinal cord injury, and neuropathic pain (15, 16). Recent articles reported that P2X7 receptor expression and modulation are linked to neuroinflammation in human ALS and in different animal models for the disease (17, 18). In particular, human postmortem ALS spinal cord displays a greater density of P2X7 receptor immunoreactivity in microglial cells, together with an increased production of COX-2 (19). A conspicuous P2X7 receptor immunolabeling clearly delineating microglial cells was also present at advanced stages of disease in spinal cord sections of transgenic rats expressing mutant SOD1 (20). Thus, this distribution of P2X7 receptor suggests its involvement in active microglia and neuronal cell loss in different animal models for the disease (17, 18). In particular, the European Guidelines for the use of animals in research (86/609/CEE) requires that all experiments should be designed to ensure that the number of animals used is as small as necessary to produce reliable results.

**Reagents**

BzATP, BBG, and all other reagents, unless otherwise stated, were obtained from Sigma-Aldrich (Milan, Italy). PD98059 and catalase (bovine liver) were purchased from Calbiochem (San Diego, CA). A-839977 and A-438079 were from Tocris Bioscience (Bristol, U.K.).

**Abs**

P2X7, rabbit polyclonal Ab (1:500) was purchased from Alomone Labs (Jerusalem, Israel); Rac1 mouse Ab (1:1000) was obtained from Millipore (Merck Millipore, Merck KGaA, Darmstadt, Germany); p67phox (1:200 for immunofluorescence and 1:500 for Western blot) and gp91phox (1:1000–1:2000) mouse mAbs were from BD Transduction Laboratories (Lexington, KY); p44/p42 MAPK (ERK1/2; 1:341FL; 1:2000) mouse Ab and phospho-p44/p42 MAPK (ERK1/2; Thr202/Tyr204; 1:1000) and phospho-p38 MAPK (Thr180/Tyr182; 1:28F; 1:700) rabbit Abs were from Cell Signaling Technology (Beverly, MA). LRp-linked anti-rabbit and anti-mouse Abs were from Cell Signaling Technology.

**Primary microglia cell cultures**

Mixed glial cultures from brain cortex were prepared as we described previously (21). Briefly, neonatal SOD1-G93A, P2X7−/−SOD1-G93A, and nt mice were sacrificed; after removing the meninges, cortices were minced and digested with 0.01% trypsin and 10 μg/ml DNase I. After dissociation, single cell suspensions were obtained by passing the cell suspension through 70-μm filters. Cells were resuspended in DMEM/F-12 media with glutamine (Life Technologies, Invitrogen, Paisley, U.K.) plus 10% FBS, 100 U/ml gentamicin, and 100 μg/ml streptomycin/penicillin at a density of 62,500 cells/cm². After ~15 doubling time, the cultures were split 1:5. A more refined pure microglial cell population was achieved by using 0.05% trypsin in DMEM/F-12 without FBS, for 10 min at 37°C to remove nonmicroglial cells (22). The resultant adherent microglial (≥95%) pure cultures were treated twice with DMEM/F-12 and kept in 50% fetal calf–conditioned medium at 37°C in a 5% CO₂ and 95% air atmosphere for 48 h until use.

**Protein extraction, SDS-PAGE, and Western blotting**

To isolate total-protein extracts, cells in serum-free medium were harvested in ice-cold RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) and treated with proteinase inhibitor mixture (Sigma-Aldrich). Lysates were kept on ice for 30 min and then centrifuged for 10 min at 14,000 x g at 4°C. Supernatants were collected and assayed for protein concentration using the Bradford assay (Thermo Scientific, Rockford, IL). Analysis of protein components was performed using Mini-PROTEAN TGX Gels (Bio-Rad Laboratories, Milan, Italy) and transferred onto nitrocellulose membranes (Amersham Biosciences, Colombo Monzese, Italy). After saturation with ECL Advance Blocking Agent (Amersham Biosciences), blots were probed overnight at 4°C with the specified Ab, incubated in 1:1 HRp-conjugated secondary Ab, and detected on x-ray film (Aurogene, Rome, Italy), using an ECL Advance Western Blotting Detection Kit (Amersham Biosciences). Quantifications were performed using a Kodak Image Station.

**Membrane translocation of p67phox**

After drug treatment, primary microglia were lysed in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 1.25 mM EGTA, and 10 mM PIPES [pH 7.3]) added to protease inhibitor mixture (Sigma-Aldrich) and sonicated (3 × 10 s, at 4°C using an ultrasonic processor [Hielscher, Teltow, Germany]). Cell lysates were centrifuged at 500 × g to remove mitochondria and nuclei, generating a postnuclear supernatant that was subsequently ultracentrifuged at 100,000 × g for 1 h at 4°C to spin down total membranes. The membrane fraction was resuspended in 100 μl relaxation buffer added to 1% Triton X-100, protein concentration was estimated using BCA assay, and 5 μg protein was analyzed using Western blot with anti-p67phox Ab. To normalize the total amount of cell membranes, Western blot with an Ab recognizing the mouse NOX2 membrane–resident gp91phox subunit was performed.

**Rac1 constructs, transfection, and virus transduction**

Human cDNA coding for Rac1 (GenBank accession number NM_006908) was cloned by RT-PCR from human SH-SY5Y neuroblastoma cells using...
the forward primer 5′-AAA GTC CAT GCA GCC ATC AAG TGT G-3′ and the reverse primer 5′-AAA TCT AGA TTA CAA CAG CAG GTA TTC C-3′. The resulting PCR fragment was inserted into BamHI/XbaI restriction sites of prRLSIN.cPT.PGK-eGFP lentiviral vector. Rac1-V12 and Rac1-N17 were produced by PCR site-directed mutagenesis using wild-type Rac1 plasmid as a template, followed by digestion with DpnI. All of the prRLSIN.cPT.PGK-eGFP constructs were verified by automated sequencing and then purified and co-transfected together with packaging vectors into HEK-293FT cells. Supernatants were collected after 48 and 72 h, and viral particles were concentrated by ultracentrifugation for 2 h at 26,000 rpm (Ultraloc Tubes, SW28 rotor, and Optima L-100 XP Ultracentrifuge; Beckman Coulter, Milan, Italy) and recovered by suspension in HBBS (Sigma-Aldrich). Titer of viral particles ranged between 106 and 107 TCID50. Viral particles and polybrene (8 μg/ml final concentration) were then added to isolated primary microglia. Lentiviral particles, at a multiplicity of infection (MOI) of 30, and 8 μg/ml polybrene (Sigma-Aldrich) were added to the culture. Supernatant was removed 5 h postinfection and replaced with DMEM-F12 medium containing 10% FBS. In all of the experiments, the efficiency of microglia transduction was ≥90%, as determined by counting the number of microglia expressing the GFP molecule and counterstaining nuclei with Hoechst 33258 (1 μg/ml for 5 min) by means of a fluorescence microscope. Cell morphology was visualized by Cy3-conjugated phalloidin staining (5 μg/ml). GFP–adenovirus construction was described previously (24) and used at an MOI of 30–50. All experiments were performed at 72 h postinfection.

Rac1 pull-down assay

The Rac-GTP pull-down assay was performed as previously described (24). Briefly, cells were lysed in a buffer containing 50 mM Tris [pH 7.2], 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, 10% glycerol, and 1% Nonidet P40 plus protease inhibitors. One fiftieth of the cell lysate was subjected to Western blotting. Cell lysates were mixed with 10 mg bacterially expressed GST-p21 activated kinase 1 protein (PAK) (rat PAK aa 1–252) bound to glutathione–Sepharose and incubated at 4°C with tumbling for 30 min. Beads were collected by centrifugation and washed twice in lysis buffer prior to the addition of Laemmli buffer and analysis with anti-Rac1 Ab by Western blot.

Intracellular ROS measurement

Because of the many limitations of standard fluorescent probes when measuring intracellular ROS by fluorescence microscope analysis, intracellular production of ROS was measured by monitoring the oxidation of the cell-permeable nonfluorescent reagent CellROX Deep Red Reagent (Molecular Probes, Carlsbad, CA), which, upon oxidation, exhibits a strong fluorogenic signal that is stable after fixation. Briefly, 2 × 105 cells were starved with serum-free medium and then exposed to treatments. Subsequently, cells were incubated with 5 μM CellROX Deep Red Reagent and 1 μg/ml Hoechst 33258 (for nuclei) for 30 min at 37°C and, after three washes with PBS, were fixed for 10 min in 4% paraformaldehyde. Cells were mounted and cover-slipped with Gel/Mount antifading (BioMeda, Burlingame, CA). Fluorescence was analyzed using a confocal laser scanning microscope (LSM700; Zeiss, Oberkochen, Germany) equipped with four laser lines: 405, 488, 561, and 639 nm. Images were analyzed with ImageJ software.

H2O2-production assay

Microglial cells were grown in 96-well fluorescence tissue culture plates at a density of 104 cells/well. After treatment, an Amplex Red Hydrogen Peroxide/Peroxidase Assay (Molecular Probes) was performed to detect H2O2 released from cells. In the presence of HRP, the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H2O2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product resorufin. Fluorescence was recorded from the blank, control, and treated cells, together with a positive control, in a multilabel counter (Victor3-V; PerkinElmer) by measuring (530–560Ex/590Em) fluorescence with a positive control, in a multilabel counter (Victor3-V; PerkinElmer). Following 5 h postinfection, in the presence of HRP, the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H2O2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product resorufin. Fluorescence was recorded from the blank, control, and treated cells, together with a positive control, in a multilabel counter (Victor3-V; PerkinElmer). Following 5 h postinfection, the addition of BzATP increases p67phox subunit translocation ~3.5-fold (with respect to nt cells) only in SOD1-G93A microglia, whereas a higher BzATP concentration (100 μM) results in only a 2-fold increase in the p67phox subunit in membranes of nt cells and a 4-fold increase in SOD1-G93A cells. The translocation of p67phox in SOD1-G93A membranes by BzATP was confirmed by immunofluorescence (Fig. 1B), because the generally diffused cytosolic staining of anti-p67phox Ab becomes enriched on cell membranes upon 100 μM agonist stimulation. This effect is completely prevented by the addition of BBG, A-839977, or A-438079 (all at 1 μM), as shown by immunofluorescence and Western blotting (Fig. 1B, 1C). Thus, the effectiveness of BzATP and the inhibition by specific antagonists indicate that the P2X7 receptor mediates the translocation of p67phox. Indeed, in SOD1-G93A microglia in which the P2X7 receptor is knocked out (Fig. 1D, left panel), stimulation with 100 μM BzATP fails to increase p67phox subunit in cell membranes, as demonstrated by immunofluorescence and Western blotting (Fig. 1D). These data reinforce the P2X7 receptor as a likely candidate to modulate the NOX2 pathway in ALS microglia (Fig. 8).

P2X7 receptor influences GTP-bound Rac1 level

The Rho-GTPase family member Rac1 is a central activator of NOX2 via a multistep mechanism involving binding to GTP, translocation to cell membrane and direct interaction with gp91phox (26). Thus, we analyzed the effect of BzATP on Rac1 activation in nt and SOD1-G93A microglia by measuring GTP-bound active Rac1 with a GST-PAK1 pull-down assay (Fig. 2). Western blot analysis of pulled-down Rac1 shows that, already under unstimulated conditions, SOD1-G93A microglia display a 3-fold higher amount of total Rac1. Despite this elevated Rac1 activity under basal conditions, SOD1-G93A microglia display a 3-fold higher amount of p67phox (1:200; BD Biosciences) in 1% BSA in PBS. The cultures were stained for 1 h with Cy2-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA). Nuclei were stained with 1 μg/ml Hoechst 33258 for 5 min, and the cells were mounted and cover-slipped with Gel/Mount antifading (BioMeda). Immunofluorescence was analyzed using a confocal microscope, as described above.

Data analysis

Data are presented as mean ± SEM. Statistical differences were verified by the Student t test using MedCalc (MedCalc Software, Mariakerke, Belgium), followed by individual post hoc comparisons (Fisher protected least significant difference). The p values < 0.05 were considered significant.

Results

Modulation of P2X7 receptor affects p67phox subunit translocation

To investigate the modulation of the NOX2 pathway by P2X7 receptor activation in SOD1-G93A microglia, we first examined the translocation of the NOX2 activator p67phox to cell membranes. As shown in Fig. 1A, under basal conditions, SOD1-G93A microglia display a higher amount of membrane-associated p67phox with respect to cells derived from nt animals (normalized to the membrane-resident gp91phox subunit). Activation of the P2X7 receptor by addition of BzATP increases the presence of p67phox in membranes of both nt and SOD1-G93A cells. However, 10 μM BzATP increases p67phox subunit translocation ~3.5-fold (with respect to nt cells) only in SOD1-G93A microglia, whereas a higher BzATP concentration (100 μM) results in only a 2-fold increase in the p67phox subunit in membranes of nt cells and a 4-fold increase in SOD1-G93A cells. The translocation of p67phox in SOD1-G93A membranes by BzATP was confirmed by immunofluorescence (Fig. 1B), because the generally diffused cytosolic staining of anti-p67phox Ab becomes enriched on cell membranes upon 100 μM agonist stimulation. This effect is completely prevented by the addition of BBG, A-839977, or A-438079 (all at 1 μM), as shown by immunofluorescence and Western blotting (Fig. 1B, 1C). Thus, the effectiveness of BzATP and the inhibition by specific antagonists indicate that the P2X7 receptor mediates the translocation of p67phox. Indeed, in SOD1-G93A microglia in which the P2X7 receptor is knocked out (Fig. 1D, left panel), stimulation with 100 μM BzATP fails to increase p67phox subunit in cell membranes, as demonstrated by immunofluorescence and Western blotting (Fig. 1D). These data reinforce the P2X7 receptor as a likely candidate to modulate the NOX2 pathway in ALS microglia (Fig. 8).
tion by 100 μM BzATP in SOD1-G93A microglia. Most importantly, active Rac1 is significantly decreased by BBG in SOD1-G93A microglia, even in the absence of P2X7 receptor exogenous stimulation (Fig. 2A, left panel, 2B), suggesting, overall, that basal P2X7 receptor activity contributes to the Rac1-NOX2 detrimental pathway in ALS microglia.

**NOX2 activation by P2X7 receptor is Rac1 dependent**

To determine the dependency of the P2X7 receptor/NOX2 pathway on Rac1 recruitment in SOD1-G93A microglia, we made use of SOD1-G93A microglia transduced with two Rac1 mutants. Microglia are very sensitive to activation as a consequence of experimental manipulations. To preserve their resting state, despite high-efficiency transduction of Rac1 mutants, we first compared lentiviral and adenoviral infections of microglia. As shown by green fluorescence (Fig. 3A), infection with lenti-GFP results in ∼80% efficiency, whereas the adeno-GFP yield is ∼60%.

Moreover, as indicated by Western blot analysis, infection with lenti-GFP does not modify the expression of CD68, a marker for active microglia, whereas adeno-GFP results in a 2-fold increase in CD68 expression (Fig. 3A). Thus, we used lentiviral infection to transduce a constitutively active (V12) and a dominant inactive (N17) form of Rac1 and measured the translocation of p67phox in membrane. As expected, the infections with mutated Rac1 constructs give rise to modifications of cellular morphology (Fig. 3B), because Rac1 is a regulator of actin cytoskeleton organization (27). In the presence of V12 Rac1, microglia show smaller cell bodies becoming characterized amoeboid, whereas the expression of N17 Rac1 mutant reduces the cell bodies and transforms the long cellular protrusions of control cells into a more spiny phenotype (Fig. 3B). The membrane levels of p67phox are increased in the presence of V12 Rac1, where cells show an active phenotype, whereas in the presence of N17 Rac1, the amount of membrane-bound p67phox is comparable to GFP-infected cells. Differently from GFP-infected control cells, the addition of 100 μM BzATP does not increase NOX2 activity when cells are transduced with any exogenous constitutively active or dominant-
negative form of Rac1 (Fig. 3B), demonstrating that NOX2 activation by the P2X7 receptor, in terms of p67 phox membrane translocation, is totally dependent on Rac1 recruitment and not on alternative pathways (Fig. 8).

Modulation of P2X7 receptor affects ROS production

Because the activity of NOX2 results in the generation of ROS, we investigated the effect of P2X7 receptor modulation on the production of ROS in nt and SOD1-G93A microglia. As shown in the time-course experiments in Fig. 4A, ROS production (measured as H2O2 content) stimulated by BzATP peaks at 10 min and then reaches a plateau. Consistent with activation of the NOX2 complex, 10 μM BzATP fails to stimulate ROS production in nt cells, but it results in a clear increase in ROS in SOD1-G93A microglia; when used at 100 μM, BzATP enhances the production of ROS to a significantly higher extent in SOD1-G93A microglia than in nt cells (2- and 1.3-fold, respectively, over untreated cells) (Fig. 4A, 4B). To ascertain whether ROS were generated by NOX2 activity, we stimulated microglial cells in the presence of apocynin, an inhibitor of the translocation of NOX2 cytoplasmic subunits (Fig. 4B). The addition of 250 μM apocynin prior to 100 μM BzATP results in 70% inhibition of ROS produced by SOD1-G93A microglia, suggesting NOX2 as a major source of ROS induced by BzATP. We also investigated ROS production by fluorescence analysis of the oxidative dye CellROX Deep Red Reagent in SOD1-G93A microglia stimulated with 100 μM BzATP, in the absence or presence of 50 U/ml catalase (to neutralize H2O2 possibly released in the medium), or of the antagonists BBG, A-839977, or A-438079 (all at 1 μM). As shown in Fig. 4C, although almost all of the cells (∼90%) are stained after treatment with BzATP, even in the presence of catalase, preincubation with the antagonists reduces CellROX Deep Red Reagent intensity down to control levels. These data indicate that BzATP stimulates ROS production via the P2X7 receptor and primarily via NOX2 activity (Fig. 8).

ERK1/2, but not p38 MAPKs, are differently recruited by P2X7 receptor stimulation in SOD1-G93A microglia compared with nt microglia

It is well established that intracellular ROS function as signaling molecules in microglia to amplify the production of several proinflammatory and neurotoxic cytokines. This is achieved through several downstream-signaling molecules, such as MAPK (i.e., p38 and ERK1/2) (5). Moreover, it was reported that, upon proper stimuli, these same kinases can be recruited upstream of NOX2 in the phosphorylation of p47phox and p67phox, a necessary for the translocation of these subunits to membranes. To dissect the signaling pathway leading to the increased response of SOD1-G93A microglial cells to BzATP, we investigated the phosphorylation of p38 and ERK1/2. Fig. 5A shows that 100 μM BzATP activates p38 with comparable kinetics in both nt and SOD1-G93A microglia: the expression of p-p38, normalized to total ERK1/2, peaks at 2.5 min and starts decreasing thereafter. Again, this effect is mediated specifically by the P2X7 receptor, because the addition of 1 μM BBG or ablation of the P2X7 receptor inhibits the upregulation of p-p38 in SOD1-G93A microglia (Fig. 5B) and nt cells (data not shown). Thus, P2X7 receptor stimulation induces a similar time- and dose-dependent p38 activation in nt and SOD1-G93A microglia. With the aim to ascertain whether this event is upstream of NOX2, as suggested by its early
activation, we measured the phosphorylation of p38 in the presence of 250 μM apocynin. As shown in Fig. 5B, 100 μM BzATP is still effective in stimulating p38 activity, even upon pretreatment with the NOX2 inhibitor, indicating that this event is upstream or independent from NOX2 activity.

Differently from what we observed with p38, the phosphorylation of ERK1/2 induced by 100 μM BzATP is augmented in SOD1-G93A microglia with respect to nt cells. As shown in the time-course experiments in Fig. 6, ERK1/2 start to be significantly phosphorylated after 10 min of treatment, but the amount of p-ERK1/2 (normalized to total ERK1/2) is higher in SOD1-G93A cells than in nt cells (5- and 3-fold increase over control, respectively) (Fig. 6A); ERK1/2 phosphorylation peaks at 60 min, exhibiting a 6- and 4.5-fold increase in SOD1-G93A and nt cells, respectively, over control and then decreases nearly to control levels after 120 min (Fig. 6B). The addition of 1 μM BBG returns ERK1/2 phosphorylation to basal levels at 30 min of stimulation and inhibits the phosphorylation in SOD1-G93A microglia by ∼70% at 60 min (Fig. 7A). In these cells, the addition of 1 μM A839977 or A438079 returns p-ERK1/2 to control levels after 60 min of BzATP stimulation (Fig. 7A). Moreover, in P2X7−/−/SOD1-G93A microglia, the addition of 100 μM BzATP for up to 60 min does not modify the levels of p-ERK1/2 (Fig. 7A). These data strongly indicate that the P2X7 receptor mediates the phosphorylation of ERK1/2 induced by BzATP. Because ERK1/2 activation by BzATP seems to be a late event, peaking at 60 min, we analyzed whether it could rely on NOX2 activation (Fig. 7B). In cells pretreated with 250 μM apocynin, ERK1/2 phosphorylation is reduced by 30% at both 30 and 60 min of stimulation with BzATP. This result suggests that, at least in part, ERK1/2 phosphorylation by BzATP lies downstream of NOX2 activation.

Conversely, we also tested whether inhibition of ERK1/2 phosphorylation could affect ROS production (Fig. 7C). As shown by fluorescence staining with CellROX Deep Red Reagent, ROS induction by 100 μM BzATP is clearly reduced by preincubation of SOD1-G93A microglia with the MEK pathway inhibitor PD98059, indicating that ERK1/2 activation is responsible, at least in part, for ROS production by BzATP. Overall, these data reveal a mutual dependency of NOX2 and ERK1/2 upon P2X7 receptor stimulation in SOD1-G93A microglia (Fig. 8).

**Discussion**

The aim of this work was to decode the main molecular pathways modulated by the P2X7 receptor that could account for neuro-
inflammation during ALS pathogenesis and to identify early signaling targets and effectors that might be useful in intercepting the progression of the pathology. To this end, we made use of primary microglial cells derived from transgenic SOD1-G93A mice and SOD1-G93A mice lacking the P2X7 receptor as models to investigate the effects of pharmacological induction or genetic ablation of the receptor activity. We found that P2X7 receptor stimulation in SOD1-G93A microglia increases NOX2 activity, ROS production, and GTP-Rac1 and p-ERK1/2 levels. Based on our results, we propose a novel mechanism by which the P2X7 receptor may lead to enhanced oxidative stress in ALS microglia. P2X7 receptor stimulation by extracellular ATP directly activates ERK1/2 and NOX2, and both of these pathways converge in ROS generation; the induction of NOX2 relies on GTP-Rac1 and p-ERK1/2 phosphorylation decreases ROS production, suggesting a mutual dependency between ERK1/2 and NOX2 upon P2X7 receptor stimulation. In these same experimental conditions, activation of p38 by BzATP is comparable in SOD1-G93A and healthy microglia. This may be explained by the fact that several kinases, in addition to MAPKs, such as protein kinase C, PAK, Akt and P38K, may be involved in NOX2 activation (28). This complexity in the phosphorylation of p47phox and p67phox suggests that the intracellular signaling pathways responsible for this event may be cell type and stimulus specific. Taken together, all of these data led us to hypothesize that, in SOD1-G93A mice, ATP released in vivo in the surrounding extracellular space of damaged motor neurons and slowly hydrolyzed in the proximity of microglia overactivates the P2X7 receptor, which is highly expressed in motor neurons and slowly hydrolyzed in the proximity of microglia.

Our findings contribute to improving the understanding of the biological processes that cause neuroinflammation in ALS and identify the P2X7 receptor as a promising target to halt the vicious cycle between uncontrolled neuroinflammation and neuron degeneration, because its inactivation affects the earliest steps of the proinflammatory action exerted by ALS microglia. The important role of microglia in the progression of ALS has been widely recognized (36); indeed, a substantial slowing of disease progression was obtained by excision of mutant SOD1 in the myeloid lineage of SOD1-G93A mice (37). However, treatment with traditional nonsteroidal anti-inflammatory drugs, with minocycline and apocynin, or the knocking down of specific components of the proinflammatory factors and neurotoxicity induced by microglia upon P2X7 receptor stimulation (21). In addition, several reports describe NOX2 as a source of damaging ROS in ALS (7, 9, 10, 12). Consistent with this, in the present study we found a basal upregulation of the Rac1-NOX2 pathway in the model of SOD1-G93A primary microglia, in line with previous results obtained in cell lines transiently infected with ALS-related genes (7, 12), and we also demonstrated that activation of the P2X7 receptor further enhances this pathway, thus producing additional oxidative stress. Furthermore, by overexpression of a dominant-negative or constitutively active form of Rac1 in ALS microglia, we established that p67phox subunit translocation on membranes is totally dependent on Rac1 recruitment and not on alternative pathways; thus, Rac1 activation is a prerequisite for NOX2 activation by the P2X7 receptor.

Because NOX2-derived ROS may activate MAPKs, which are known to be the core of the cell stress–response signaling network and, conversely, that oxidative stress also can occur through activation of MAPKs (28), we investigated the role of these relevant kinases, in particular p38 and ERK1/2, which are known to be activated by the P2X7 receptor in microglial cells (29) and to be implicated in ALS pathology (30, 31). Although the role for ERK1/2 in ALS is controversial, being overphosphorylated or dephosphorylated in different cells and conditions (32–34), persistent activation of p38 signaling was suggested to mediate neuronal apoptosis in ALS. Indeed, increased levels of p-p38 MAPKs are present in the motor neurons and microglia of the ventral spinal cord of mutant SOD1 mice, and p38 inhibition largely protects motor neurons and prevents proximal axon degeneration (35). In this study, we found that, following P2X7 receptor stimulation, the phosphorylation of ERK1/2 is augmented particularly in ALS microglia, and this effect is inhibited by the ROS inhibitor apocynin. We also demonstrated that inhibition of ERK1/2 phosphorylation decreases ROS production, suggesting a mutual dependency between ERK1/2 and NOX2 upon P2X7 receptor stimulation. In these same experimental conditions, activation of p38 by BzATP is comparable in SOD1-G93A and healthy microglia. This may be explained by the fact that several kinases, in addition to MAPKs, such as protein kinase C, PAK, Akt and P38K, may be involved in NOX2 activation (28).
inflammatory pathway did not yield satisfactory results (38–40), suggesting that a successful inhibition of microglia-mediated toxicity might require the attenuation of a broad spectrum of different pathways. In this study, we show that inhibition of the P2X7 receptor by genetic ablation or through the antagonists BBG, A-839977, and A-438079 suppress both early and late proinflammatory responses activated by BzATP in SOD1-G93A microglia (i.e., p67phox translocation, p-ERK1/2 levels, and overall ROS production). Moreover, we showed that BBG significantly inhibits GTP-Rac1 activation and, to a lesser extent, the translocation of p67phox, also in unstimulated conditions, suggesting that endogenous release of ATP might contribute to SOD1-G93A microglia basal activation (21) through stimulation of the P2X7 receptor. Altogether, these results could be the rationale for a preclinical study using A-839977 or BBG in ALS mice, because they can cross the blood–brain barrier (41, 42). Moreover, it is known that BBG has no toxicity and exhibits therapeutic effects in other animal models of neurodegenerative diseases (42). However, one complicating factor in inhibiting the action of the P2X7 receptor in vivo might result from the dichotomous role exerted by microglia in neurodegenerative diseases, such as ALS: initially, microglia have a neuroprotective action, clearing the source of the inflammatory stimuli (a condition known as M2 phenotype), whereas they transform into a toxic M1 phenotype that contributes to the acceleration of motor neuron degeneration (2, 43). Therefore, the challenge will be to inhibit the P2X7 receptor only when microglia convert to the dangerous M1 phenotype and have a detrimental role, and to preserve the neuroprotective aspect. In summary, we confirmed the P2X7 receptor as a promising target...
for the development of therapeutic strategies to slow down the progression of ALS.

Disclosures

The authors have no financial conflicts of interest.

References


