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Astrocytes, but Not Microglia, Rapidly Sense H$_2$O$_2$ via STAT6 Phosphorylation, Resulting in Cyclooxygenase-2 Expression and Prostaglandin Release

Soo Jung Park,*† Jee Hoon Lee,*† Hee Young Kim,*† Youn Hee Choi,†‡ Jung Sup Park,*† Young Ho Suh,* Sang Myun Park,*† Eun-hye Joe,*† and Ilo Jou*†

Emerging evidence has established that astrocytes, once considered passive supporting cells that maintained extracellular ion levels and served as a component of the blood–brain barrier, play active regulatory roles during neurogenesis and in brain pathology. In the current study, we demonstrated that astrocytes sense H$_2$O$_2$ by rapidly phosphorylating the transcription factor STAT6, a response not observed in microglia. STAT6 phosphorylation was induced by generators of other reactive oxygen species (ROS) and reactive nitrogen species, as well as in the reoxygenation phase of hypoxia/reoxygenation, during which ROS are generated. Src–JAK pathways mediated STAT6 phosphorylation upstream. Experiments using lipid raft disruptors and analyses of detergent-fractionated cells demonstrated that H$_2$O$_2$-induced STAT6 phosphorylation occurred in lipid rafts. Under experimental conditions in which H$_2$O$_2$ did not affect astrocyte viability, H$_2$O$_2$-induced STAT6 phosphorylation resulted in STAT6-dependent cyclooxygenase-2 expression and subsequent release of PGE$_2$ and prostacyclin, an effect also observed in hypoxia/reoxygenation. Finally, PGs released from H$_2$O$_2$-stimulated astrocytes inhibited microglial TNF-α expression. Accordingly, our results indicate that ROS-induced STAT6 phosphorylation in astrocytes can modulate the functions of neighboring cells, including microglia, through cyclooxygenase-2 induction and subsequent release of PGs. Differences in the sensitivity of STAT6 in astrocytes (highly sensitive) and microglia (insensitive) to phosphorylation following brief exposure to H$_2$O$_2$ suggest that astrocytes can act as sentinels for certain stimuli, including H$_2$O$_2$ and ROS, refining the canonical notion that microglia are the first line of defense against external stimuli. The Journal of Immunology, 2012, 188: 000–000.

Astrocytes and microglia are resident immune-competent cells in the CNS, which, until several decades ago, was considered an immune-privileged organ devoid of lymphatic drainage and protected by the presence of the blood–brain barrier (1, 2). The outdated notion that glia are simply supporting cells that hold neurons together has been displaced by subsequent findings that revealed the diverse functions of these cells. Recently, glial functions associated with neuronal synaptic plasticity and maintenance of brain homeostasis have attracted considerable research attention (2).

Astrocytes account for ∼50–90% of all brain cells, depending on the particular brain region. Their vast numbers are reflected in diverse functions, ranging from assisting in neurogenesis and determining the microarchitecture of the gray matter to defending the brain from various external and internal insults, functions that collectively serve to maintain the brain microenvironment (1–4).

Therefore, if astrocytes malfunction, the result can be disturbances in homeostasis that lead to neurodegenerative brain diseases, including Alzheimer’s disease, Parkinson’s disease, and various forms of dementia. Generally, microglia are characterized by a very low threshold of activation; thus, they respond to even minor pathological challenges or tissue alterations. Microglia continually survey their environment with highly motile extensions. Their activation occurs within minutes but can be long-lasting. Thus, microglia perform critical roles as a first line of defense against changes in the brain microenvironment in addition to their function in innate immunity (5, 6).

Many signaling pathways involved in immune and inflammatory responses are also associated with glial cell activation. Representative inflammatory signals, including NF-κB, STATs, and TLR signaling are also involved in glial activation induced by IFNs and LPS (7, 8). Originally identified as downstream targets of IFNs, members of the STAT family of transcription factors are now known to transduce signals generated by other cytokines and growth factors (9). They are also involved in glial activation induced by LPS and gangliosides, as well as IFNs (7, 10, 11). The analysis of STAT knockout mice revealed specific biological functions for individual STAT proteins (12). STAT family proteins respond to a variety of extracellular signaling molecules and mediate diverse cellular processes, including immune responses, differentiation, cell survival, proliferation, and oncogenesis. STATs also function as sensors of cellular stress, including oxidative stress (9, 13).

STAT6 is canonically activated by IL-4 and IL-13 and plays a key role in Th2 polarization of the immune system (14, 15). In
contrast to other STATs, which are activated by diverse stimuli, STAT6 is largely unresponsive to cytokines and growth factors other than IL-4 and IL-13. Although some reports suggested that STAT6 is involved in leukemia and lymphoma tumor progression (16, 17), the mechanism by which it is activated in such environments is largely unknown.

Reactive oxygen species (ROS) perform functions ranging from protecting against microbial infection to acting as signaling molecules (18, 19). When ROS accumulation exceeds cellular reducing capacity, the resulting oxidative stress may lead to neuronal death and, thereby, foster the progression of diverse neuronal pathologies, including stroke, ischemia/reperfusion injury, Parkinson’s disease, and Alzheimer’s disease (20–22). Although antioxidant enzyme systems in the brain are well developed, there is a tipping point in the redox balance beyond which it is virtually impossible for the brain to recover from oxidative injuries. In this context, it is critical for the brain to be able to detect ROS rapidly enough to mount an antioxidant response capable of maintaining redox homeostasis.

In this study, we present the novel finding that H2O2 rapidly induces the phosphorylation, nuclear translocation, and binding of STAT6 to the promoter of COX-2 gene, resulting in the expression and subsequent release of PGE2 and PGL2 in primary rat brain astrocytes. STAT6-dependent cyclooxygenase (COX)-2 expression was also observed in hypoxia/reoxygenation (H/R). Adding to the importance of these findings is the observation that STAT6 in astrocytes is much more sensitive to ROS-dependent phosphorylation than is microglial STAT6. Therefore, astrocytes, through this STAT6-activation mechanism, could function as ROS sensors in the brain.

Materials and Methods

Reagents and Abs

H2O2, N-acetylcysteine (NAC), catalase, PGE2, paracetamol (methyl viologen), FeSO4, sodium nitroprusside dihydrate, filamin III, methyl-β-cycloexodrin (MBcD), and Percoll were purchased from Sigma-Aldrich (St. Louis, MO). IFN-γ, pyrazolopyrimidine (PP)2 and PP3, JAK inhibitor (JAKi), and NS398 were purchased from Calbiochem (Butler Pike, PA). IL-4 was purchased from R&D Systems (Minneapolis, MN). Abs against JAK3, SP1, and COX-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). An Ab against Iba-1 was obtained from Wako (Osaka, Japan). All other Abs were obtained from Cell Signaling Technology (Beverly, MA). Enzyme immunoassay kits for PGE2 and 6-keto PGF1α were from GE Healthcare Biociences (Little Chalfont, U.K.) and Cayman Chemicals (Ann Arbor, MI), respectively.

Cell cultures from adult rat brain

Primary astrocytes and microglia were obtained from the cerebral cortices of 1-d-old Sprague Dawley rats (Samtako), as previously described (23). Briefly, cortices were triturated in MEM containing 10% FBS (Hyclone, Logan, UT) to yield a single-cell suspension and plated in 75-cm² T-flasks (0.5 hemisphere/flask). After culturing for 2–3 wk, microglia were detached from flask walls by shaking, and the remaining adherent astrocytes were detached with trypsin/EDTA, and recovered cells were plated on dishes. Microglia were harvested from flasks by mild shaking and filtered through a nylon mesh to remove astrocytes and clumped cells. The isolated cells were resuspended in MEM containing 10% FBS and seeded into six-well plates at a density of 1 × 10⁶ cells/well. Viable cell numbers were counted using a hemacytometer after staining with 0.2% trypan blue. One day later, the cells were washed and exposed to H2O2 or IL-4. The purity of astrocyte and microglial/macrophage cultures was confirmed using real-time PCR (Supplemental Fig. 1C).

Western blot analysis

Cells were washed with PBS and lysed in RIPA buffer (11) containing 1 μM protease inhibitor mixture set III (Calbiochem), 1 mM EDTA, 1 mM EGTA, and 0.5 mM Na3VO4. Cell lysates were microcentrifuged for 20 min at 13,000 rpm at 4°C. Supernatant proteins were separated by SDS-PAGE on 10% gels and transferred to nitrocellulose membranes (Whatman). After first blocking by incubating in TBST (0.1% Tween-20) containing 5% skim milk for 1 h, membranes were probed with primary Abs and HRP-conjugated secondary Abs (Invitrogen, Carlsbad, CA). Immunoblot proteins were visualized using an ECL system (AB Frontier). Quantification of band intensity was performed using Image J software (National Institutes of Health).

Measurement of ROS

For visualization of intracellular ROS levels, cells were incubated with 5 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA [DCF]; Invitrogen) for 30 min. The cells were then washed with Dulbecco’s PBS (2.68 mM KCl, 1.47 mM KH2PO4, 136.89 mM NaCl, 8.1 mM Na2HPO4), and fluorescence and phase-contrast images were collected using a fluorescence microscope (Zeiss Axiovert 200M; Carl Zeiss).

Immunofluorescence staining

Astrocytes cultured on coverslips were washed twice with ice-cold PBS and fixed with methanol at −20°C. Fixed cells were washed with PBS and blocked with 1% BSA for 30 min at room temperature. Cells were incubated overnight at 4°C with primary Abs against STAT6 (Chemicon, Temecula, CA; diluted 1:100) and GFAP (Sigma-Aldrich; diluted 1:300), followed by incubation for 1 h at room temperature with secondary Abs (Alexa Fluor 488-conjugated, diluted 1:600; Alexa Fluor 555-conjugated, diluted 1:500). Cells were then washed, mounted in mounting solution (Vector Laboratories, Burlingame, CA) containing DAPI, and observed under a confocal microscope (LSM 510; Carl Zeiss).

EMSA

EMSAs were performed as previously described (11). Briefly, cells were harvested and suspended in 900 μl hypotonic solution (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) containing 0.5% Nonidet P-40. Cells were centrifuged at 500 × g for 10 min at 4°C, and the pellet (nuclear fraction) was saved. The nuclear fractions were incubated with reaction buffer and γ32P-labeled probe, with or without a 50-fold excess of unlabeled probe. DNA–protein complexes were separated on 6% polyacrylamide gels in Tris/glycine buffer, and dried gels were exposed to X-ray film. A 20-bp oligonucleotide with the sequence 5′-GTA TTT CCC AGG AAA GGA AC-3′ was used as a consensus STAT6 binding site (Santa Cruz Biotechnology), and a 20-bp oligonucleotide with the sequence 5′-TAA CTT CCC TGA AGA ACA-3′ was used as a consensus STAT6 binding site (Santa Cruz Biotechnology), and a 20-bp oligonucleotide with the sequence 5′-TAA CTT CCC TGA AGA ACA-3′ was used as a probe for the STAT6 binding site in the rat COX-2 promoter (5′-TAA CTT CCC TGA AGA ACA-3′ was used as a probe for the STAT6 binding site in the rat COX-2 promoter (11)−1177 to 1158 (Bioneer). For supershift experiments, nuclear extracts were incubated with 2 μg anti-STAT6 Ab (Santa Cruz Biotechnology) for 1 h before addition of the γ32P-labeled probe.

Transfection of small interfering RNAs

SMART pools of small interfering RNAs (siRNAs) against c-Src and STAT6 and nontargeting control siRNAs were purchased from Dharmacon.
Hypoxic reoxygenation

Hypoxia was induced in cultured astrocytes by replacing media with serum-free MEM that had been saturated with N2 gas for 1 h. Dishes were transferred to an anaerobic chamber (Forma Scientific, Marietta, OH) maintained at 37°C under a humidified atmosphere of 5% CO2, 10% H2, and 85% N2. After culturing under hypoxic conditions for 3 h, cells were reoxygenated by incubating under normoxic conditions (95% air, 5% CO2) for the indicated periods.

Treatment with filipin III or MβCD

Filipin III, a polyene macrolide that binds cholesterol, and MβCD, a cholesterol depleter, were used to investigate the involvement of lipid rafts. After treating with filipin III or MβCD for 10 min or 1 h, respectively, cells were treated with H2O2 for 10 min and then harvested. For reassembly of lipid rafts, cells were recovered for 2 h after washout of the media and then treated with H2O2 for 10 min.

Isolation of detergent-insoluble fractions

Detergent-insoluble fractions were obtained as previously described (27). Briefly, cells were washed twice with ice-cold PBS and lysed with HEPES buffer containing 1% Triton X-100. Cells were then incubated for 30 min and centrifuged at 12,000 × g for 30 min at 4°C. The resulting supernatants (detergent-soluble fraction) and pellets (detergent-insoluble fraction) were washed with 1 ml cold HEPES buffer without detergent, sonicated with lysis buffer, and centrifuged at 12,000 × g for 30 min at 4°C. Each fraction was analyzed by Western blotting.

Assessment of cell viability

For MTT colorimetric assays, cells were incubated with 0.5 mg/ml MTT for 4 h at 37°C. The MTT solution was then removed, and formazan crystals generated by reduction of MTT by living cells were dissolved in dimethyl sulfoxide. Color formation was quantified by measuring OD at a wavelength of 540 nm using a microplate reader. For Alamar blue assays, cells were incubated for 3 h in one-tenth volume of Alamar blue (Biosource, Carlsbad, CA) and then fluorescence was measured in a spectrofluorometer (Molecular Devices, Sunnyvale, CA) at excitation and emission wavelengths of 560 and 590 nm, respectively.

RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen), and cDNA was synthesized from 1 µg total RNA in a total volume of 30 µl using avian myeloblastosis virus reverse transcriptase (GenDEPOT, Barker, TX), according to the manufacturers’ instructions.

For semiquantitative RT-PCR, 3 µl cDNA served as template; the final reaction volume was 25 µl. Each reaction contained the gene-specific primers listed in Table I (Bioneer), PCR reagents, and amfiEco Taq DNA Polymerase (GenDEPOT). PCR was performed over 20–35 cycles, and a cycle number in the exponential range was chosen for each individual PCR test. The cycle numbers were 23 for GAPDH and 25 and 28 for TGF-β and TNF-α, respectively. The PCR conditions were as follows: 2 min at 94°C; followed by 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and a final extension at 72°C for 10 min. Amplified products were separated by electrophoresis on 1% agarose gels and detected under UV light. Band intensities were quantified using Image J software.

For quantitative real-time PCR, 1 µl cDNA served as template; the final reaction volume was 20 µl. Each PCR reaction contained 2× KAPA SYBR Fast Master Mix (Kapa Biosystems, Woburn, MA) and 200 nM the primer sets listed in Table I (Bioneer). The PCR conditions were as follows: denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 3 s, 55°C for 15 s, and 72°C for 20 s. After PCR, melt curves (60–95°C) were constructed to ensure accurate product identification and to demonstrate product purity. Relative gene expression levels were calculated using the 2−ΔΔCt method (28). GAPDH housekeeping gene levels were used for normalization. The data are presented as fold changes relative to untreated controls.

PG assays

Astrocytes were exposed to 0.5 mM H2O2 for 10 min, after which H2O2 was removed by washout with fresh, serum-free medium. Cells were incubated for an additional 24 h, and cell culture media were collected and stored at −70°C until use. The levels of PGE2 and 6-keto PGF1α in culture media were assayed using an enzyme immunoassay kit, following the manufacturer’s protocol.

Preparation of astrocyte-conditioned media

Astrocytes grown to confluence were incubated with/without 0.5 mM H2O2 for 10 min, after which media were replaced with fresh, serum-free media with/without the COX-2 inhibitor NS398 (10 µM). Cells were then incubated for 24 h, at which time astrocyte-conditioned media (ACM) were collected and stored at −70°C until use.

Statistical analysis

Differences among groups were determined using one-way ANOVA. A p value ≤ 0.05 was considered significant. Values are presented as mean ± SEM.

Results

H2O2 induces phosphorylation, nuclear translocation, and binding of STAT6 to target gene promoters in brain astrocytes

Given the close relationship between STAT transcription factors and ROS (29), we screened the phosphorylation profiles of STATs in rat primary astrocytes following exposure to H2O2. Among STAT family proteins, STAT6 was prominently phosphorylated in H2O2-treated brain astrocytes (data not shown). A subsequent, detailed examination of changes in STAT6 phosphorylation in H2O2-treated primary astrocytes showed that STAT6 phosphorylation was dependent on H2O2 concentration (up to 0.5 mM) and time (up to 60 min) (Fig. 1Aa, 1Ab). On the basis of these results, we used a 10-min treatment with 0.5 mM H2O2 as a paradigm for inducing brief oxidative stress in subsequent experiments. To demonstrate that ROS are generated intracellularly by exogenous H2O2 treatment, we visualized intracellular ROS levels using a DCF-based analysis. In line with previous reports that H2O2 readily penetrates the cell membrane (30, 31), we found that H2O2 treatment induced a readily detectable increase in intracellular DCF fluorescence (Fig. 1Ba). In addition, H2O2-induced STAT6 phosphorylation was abrogated by inclusion of catalase, an H2O2 decomposer, or NAC, an intracellular ROS scavenger (Fig. 1Bb). After STAT is activated by phosphorylation, it is translocated to the nucleus and binds to the promoters of target genes. H2O2-induced phosphorylation of STAT6 was followed by STAT6 nuclear translocation, as shown in fractionation experiments (Fig. 1Ca) and immunohistochemical analyses (Fig. 1Cb). Moreover, as shown in EMSA analyses using a probe containing a consensus STAT6-binding sequence, STAT6 binding to target gene promoters increased following treatment with H2O2 (Fig. 1Cc). To determine whether this was unique to astrocytes, we examined ROS-induced STAT6 phosphorylation in microglia, representative early responders to external stimuli and damage. Unlike astrocytes, primary microglia exhibited negligible levels of phosphorylated STAT6 after treatment with H2O2, whereas STAT6 phosphorylation in microglia was readily induced by IL-4, used as a positive control (Fig. 1D). We confirmed the above findings using cells obtained from the adult rat brain. STAT6 was phosphorylated upon addition of H2O2 to 0.05 mM, and the extent of phosphorylation increased, in a dose-dependent manner, as the H2O2 level increased to 0.5 mM (Fig. 2A). Exposure to 100 µM H2O2 induced STAT6 phosphorylation in a manner similar to that noted in neonatal cells (Fig. 2B versus Supplemental Fig. 3A). In agreement with the results obtained using neonatal microglia, adult microglia were much less sensitive to STAT6 phosphorylation following exposure to H2O2 than were adult astrocytes (Fig. 2C).
STAT6 phosphorylation is induced by other ROS and reactive nitrogen species generators

Free radicals are generated from diverse sources in addition to H$_2$O$_2$. To examine whether other ROS and reactive nitrogen species (RNS) generators induce STAT6 phosphorylation, we treated cells with a mixture of H$_2$O$_2$ and FeSO$_4$ (to generate hydroxyl radicals), paraquat (ROS generator), or sodium nitroprusside dihydrate (RNS generator) and then examined STAT6 phosphorylation using Western blot analysis. STAT6 was rapidly (within 10 min) phosphorylated by all ROS and RNS generators tested (Fig. 3A), indicating that these effects were not limited to H$_2$O$_2$.

In contrast, Western blot analyses showed that, under these same experimental conditions, STAT6 in primary microglia was not phosphorylated by any of these free-radical generators (Fig. 3B).

STAT6 phosphorylation occurs in parallel with ROS generation under H/R conditions

To evaluate the physiological significance of STAT6 phosphorylation as an ROS sensor, we examined whether STAT6 was phosphorylated in response to H/R, a representative condition in which ROS are generated (32). STAT6 was promptly phosphorylated in astrocytes exposed to a 10-min reoxygenation period after 3 h of hypoxia (H3/R) and then returned to control levels after 1 h. However, with prolonged reoxygenation, STAT6 phosphorylation exhibited a biphasic pattern, reappearing at 2 h and then disappearing after 4 h (Fig. 4A). Intracellular ROS generation demonstrated using DCF staining paralleled STAT6 phosphorylation (Fig. 4B), indicating that ROS generated during reoxygenation induced STAT6 phosphorylation in astrocytes when the effects of hypoxic insults were experienced.

H$_2$O$_2$-induced STAT6 phosphorylation is mediated by Src and JAKs and does not involve IL-4/IL-13 receptors

Next, we examined the mechanism underlying H$_2$O$_2$-induced STAT6 phosphorylation. Because JAKs (JAK1, JAK2, JAK3, and Tyk2) and c-Src are well-known upstream signaling molecules for STATs (33, 34), we examined their activation in H$_2$O$_2$-treated astrocytes using the appropriate anti–p-JAK/c-Src Abs. H$_2$O$_2$ treatment led to a rapid increase in the phosphorylation of JAKs (JAK1, JAK2, JAK3, and Tyk2) and c-Src (Fig. 5A). To test the
involvement of these factors in mediating STAT6 phosphorylation, we used pharmacological inhibitors and siRNAs. Inhibition of c-Src by the specific inhibitor PP2 (Fig. 5B) or siRNA-mediated c-Src knockdown (Fig. 5C) suppressed the phosphorylation of JAKs, c-Src, and STAT6. Pretreatment with the pan-JAK inhibitor, JAKi, suppressed the phosphorylation of JAKs and STAT6 but not c-Src (Fig. 5B), indicating that H2O2 induced STAT6 phosphorylation via the c-Src–JAK pathway. Because STAT6 is a major downstream target of IL-4 and IL-13 signaling, we examined whether H2O2-induced STAT6 phosphorylation was achieved through either receptor. Using siRNA against IL-4Rα, a common receptor subunit for both IL-4 and IL-13 (14), we found that knockdown of IL-4Rα did not attenuate H2O2-induced STAT6 phosphorylation, but it did inhibit IL-4–induced STAT6 phosphorylation, indicating that H2O2-induced STAT6 phosphorylation is independent of IL-4 and IL-13 signaling (Fig. 5D).

H2O2-induced STAT6 phosphorylation is regulated through STAT6 translocation into lipid rafts

As shown in Fig. 5D, H2O2-induced STAT6 phosphorylation did not occur through IL-4R, raising the question: how does H2O2 induce STAT6 phosphorylation? H2O2 easily penetrates into membranes and regulates many cellular functions, such as inflammation and cell death, via lipid rafts (23, 35), which are known to serve as signaling platforms in which multiple signaling molecules aggregate and regulate cellular events in response to diverse stimuli. To investigate whether oxidative stress-induced STAT6 phosphorylation is dependent on lipid rafts in primary astrocytes, we disrupted rafts using filipin III or MβCD, as described in Materials and Methods. Our results showed that treatment with either filipin III (Fig. 6Aa) or MβCD (Fig. 6Ab) inhibited H2O2-induced STAT6 phosphorylation. Moreover, reassembly of lipid rafts by removing filipin restored the STAT6-phosphorylation response to added H2O2 (Fig. 6Aa). These results indicate that ROS-induced STAT6 phosphorylation is mediated through lipid rafts. Lipid rafts are known to be insoluble in nonionic detergents, such as Triton X-100, at 4˚C. Thus, to confirm that H2O2 recruits STAT6 into lipid rafts, we isolated detergent-resistant and -soluble microdomains and analyzed each fraction by Western blotting. STAT6 and p-STAT6 translocation into lipid rafts was induced by H2O2 within 5 min and was maintained for 60 min (Fig. 6B). Taken together, our results suggest that ROS induces the rapid translocation of STAT6 into lipid rafts.

H2O2 induces COX-2 expression in a STAT6-dependent manner

Next, to elucidate the cellular events that ensue following transient H2O2 treatment, we screened for several potentially H2O2-inducible molecules after treating with 0.5 mM H2O2 for 10 min, a treatment that did not affect cellular viability (Fig. 7Aa) but that induced prominent STAT6 phosphorylation (Fig. 7Ab). We next examined the expression levels of several downstream STAT6 effectors, including COX-2, heme oxygenase-1, nerve growth factor, Bcl-XL, and Bcl-2, using RT-PCR (Supplemental Fig. 2). COX-2 synthesis was dependent on the expression of STAT6; this was shown using both real-time PCR and Western blotting (Fig. 7B). EMSA experiments using a probe containing the STAT6 binding site of the COX-2 gene further showed that STAT6 binding to the COX-2 promoter was enhanced by H2O2 and reduced in a supershift assay using an anti-STAT6 Ab (Fig. 7C). Similarly, COX-2 expression, induced under 3H/R, was suppressed by STAT6 knockdown (Fig. 7D).

H2O2-induced COX-2 expression leads to the STAT6-dependent release of PGE2 and PGI2

COX-2 is a major enzyme in the synthesis of various PGs from membrane phospholipids (36). Therefore, we next examined which PGs were prominently synthesized in response to H2O2 treatment.
and assessed the STAT6 dependence of their synthesis. Real-time PCR (Fig. 8Aa), using the primer pairs listed in Table I, and Western blot analyses (Fig. 8Ab) demonstrated that transcript and protein levels of microsomal PGE synthase (mPGES) and PGI synthase (PGIS) were induced by transient H$_2$O$_2$ treatment, whereas those of hematopoietic PGD synthase were not induced under the same experimental conditions. To determine whether the increased expression of mPGES and PGIS resulted in the STAT6-dependent release of PGE$_2$ and PGI$_2$, we measured PGs in media...
Conditioned by normal and STAT6 siRNA-transfected astrocytes using ELISA. The release of PGE2 and 6-keto PGF1α, a stable metabolite of PGI2, into the media was increased by H2O2 and suppressed by STAT6 knockdown (Fig. 8B).

**Induction of the STAT6–COX-2 pathway by brief exposure to H2O2 in brain astrocytes modulates the inflammatory microenvironment**

COX-2 expression in the brain, as well as in peripheral tissues, is well known to play a central role during the inflammatory cascade, modulating the release of several inflammation-related cytokines under pathophysiological conditions (37). To test whether astroglial COX-2 expression and the subsequent release of PGE2 and PGI2 might directly or indirectly regulate neighboring cells, we incubated microglia with ACM obtained following the treatments indicated in Fig. 9Aa and examined microglial expression of pro- and anti-inflammatory cytokines. Expression of mRNA for the proinflammatory cytokine TNF-α in microglia was reduced following exposure to ACM and was further reduced by the addition of H2O2-preconditioned ACM (H-ACM) compared with control-preconditioned ACM (Fig. 9Ab). In contrast, mRNA expression of the potential anti-inflammatory modulator TGF-β was unchanged by ACM and H-ACM. To confirm that the reduction in TNF-α expression induced by H-ACM in microglia was dependent on COX-2, we tested ACM obtained by incubating with the selective COX-2 inhibitor NS398, with or without H2O2. The suppression of TNF-α mRNA expression in rat primary microglia by H-ACM was reversed by treatment with NS398 (Fig. 9Ab), whereas the extent of such suppression induced by ACM was not changed upon addition of NS398, showing that both COX-2–dependent and -independent regulatory signals were at play. Next, to assess the involvement of the STAT6 pathway, we measured TNF-α mRNA expression following siRNA-mediated STAT6 knockdown. H-ACM from STAT6-knockdown cells reversed the decrease in TNF-α expression induced by H-ACM from control siRNA-transfected cells (Fig. 9B). Finally, to test that these COX-2–dependent effects were mediated by PGE2 and PGI2 released by astrocytes, we directly treated microglia with PGE2 and carbaprostacyclin, a stable analog of PGI2. Both treatments induced a dose-dependent inhibition of microglial TNF-α mRNA expression (Fig. 9C). Collectively, these results show that astroglial COX-2 induced by transient exposure to H2O2 modulates microglial TNF-α release through COX-2–induced release of PGE2 and PGI2.

**Discussion**

In this study, we demonstrate that brief exposure of astrocytes to H2O2 rapidly induced STAT6 activation, resulting in COX-2 induction and release of PGE2 and PGI2, revealing that STAT6 functions as a downstream target of H2O2. Considering that STAT6 in astrocytes responds rapidly to ROS, including H2O2, the ROS–STAT6 activation circuit in astrocytes could function as a sentinel, sensing external injuries (including oxidative stress) and subsequently modulating the brain microenvironment.

Because the brain is a metabolically active and highly oxygen-demanding organ, it is imperative that it tightly regulates its oxidative status (38). Cellular responses to oxidative stress are accompanied by marked alterations in gene expression mediated by transcription factors (39, 40). The STAT proteins are well-known transcription factors involved in various cellular stresses, including oxidative stress (41). Our data demonstrated that STAT6 was
rapidly phosphorylated in the presence of H$_2$O$_2$ and returned to basal levels after H$_2$O$_2$ removal, revealing that STAT6 is very sensitive to H$_2$O$_2$. In cultured astrocytes, phosphorylation of STAT6 was observed after 10 min of H$_2$O$_2$ treatment with increasing concentrations starting at 50 μM and after 1 min of 0.5-mM H$_2$O$_2$ treatment (Fig. 1A). During global ischemia and reperfusion, the concentrations of striatal H$_2$O$_2$ increased from basal levels of 25–60 μM to 110 μM during ischemia and further to 160 μM during reperfusion using microdialysis (42). An H$_2$O$_2$ concentration <100 μM has been considered subtoxic in in vitro studies (42). Under our experimental conditions, 100 μM H$_2$O$_2$ induced STAT6 phosphorylation within 5 min. The level of phosphorylation increased for 30 min following H$_2$O$_2$ exposure and decreased thereafter. COX-2 expression commenced 0.5 h after 100 μM H$_2$O$_2$ exposure and peaked at 2 h (Supplemental Fig. 3). The decrease in the extent of phosphorylation evident at later times was attributable to destruction of H$_2$O$_2$ by astroglial cells. Exogenously added H$_2$O$_2$ is taken up by such cells via a process characterized by first-order kinetics, with the time taken to achieve 50% of the ultimate uptake depending on both cell type and cell numbers (43, 44). To minimize the impact of astroglial uptake and destruction of H$_2$O$_2$ on peroxide levels, as well as to exclude indirectly propagating signals, we chose to confine the H$_2$O$_2$ (0.5 mM) treatment time to only 10 min; the peroxide was then washed away.

Both astrocytes and microglia perform many functions both in the normal immune system and during inflammation, and they release cytokines, chemokines, and inflammatory mediators, including NOs (45, 46). Microglia are the resident macrophages of the brain, are readily activated in response to changes in the microenvironment, and also serve as a major source for ROS generation and reservoir of accumulating ROS during brain inflammation (47, 48). However, our results demonstrate that microglia were much less sensitive to STAT6 phosphorylation following exposure to oxidative stimuli than were astrocytes. This greater vulnerability of the astroglial STAT6 response to ROS compared with that of microglia is very interesting. Because the extent of oxidative stress depends on cellular antioxidant capacity, we first determined the variance resulting from differences in plated cell numbers and antioxidant enzyme distribution between astrocytes and microglia. In line with previous reports (43, 44), the total antioxidant capacities of the two cell types did not differ significantly (data not shown). Thus, difference in the cellular distribution of enzymes involved in phosphorylation/dephosphorylation and redox signaling between the two cell types might be responsible for this differential sensitivity. An example of this is provided by our previous study, which showed that H$_2$O$_2$ induced phosphorylation of the Src homology 2 domain-containing protein-tyrosine phosphatase-2 in astrocytes but not in microglia, an effect that was mediated via lipid rafts (23). The present results reveal

### Table I. PCR primers used in this study

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Sequence (5’→3’)</th>
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<tr>
<td>GAPDH</td>
<td>F: TCCCTCAAGATTGTGCAGCAA R: AGATCCCAACAGGATCATT</td>
</tr>
<tr>
<td>COX-2</td>
<td>F: CCT AGG GGT TAC CTC TCA CA R: TGA CCA AGG TGTT CCA AG</td>
</tr>
<tr>
<td>HO-1</td>
<td>F: ACT TTC AGA AGG GTC AGG TGT CC R: TTT AGG AGG AAG CCG GTC TTA G</td>
</tr>
<tr>
<td>NGF</td>
<td>F: GCC CAC TGG ACT AAA CTT CAG C R: CCC TGG CTT TGT TCT TCT</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>F: GTA CCT GGG CCA CAA CGT AG R: TGA TTT GAC CAT TTT CCT GA</td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>F: GCT GGG ACA CTT TGG TGG AT R: GAG CCC AGC AGA ACT ACA CC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: GAG GAC CCT GGA TAC CAA CTA CTG R: GGT TGG CCA GGC TCC AAA TGG AG</td>
</tr>
<tr>
<td>TGF-β</td>
<td>F: CTG GAC CTC AAG TCT CTA CTT CA R: TAG CCT CTC TGG GAG AT</td>
</tr>
<tr>
<td>IL-4Rα</td>
<td>F: GCC CAA AAG GGT CAT CAT C R: GTG ATG CCA TGG ACT GTC G</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: GGC CAA AAG GGT CAT CAT C R: GTG ATG CCA TGG ACT GTC G</td>
</tr>
<tr>
<td>COX-2</td>
<td>F: TGG GAA GCT TCC TCC AAT CAC CT R: GTG AAG TGG TGG CCA AGG AA</td>
</tr>
<tr>
<td>GEAP</td>
<td>F: TTC TCG TAC AGA CTT TCT CC R: CCC TCT AGG ACT GCC GTA CT</td>
</tr>
<tr>
<td>S100b</td>
<td>F: GAG GAG GAG ACT GGA GAG AG R: GCC ACC TTC AGA TGG GAG AC</td>
</tr>
<tr>
<td>Iba-1</td>
<td>F: TTT ATG TCG ATG AGG AGG AG R: CTT CCA CAT GGG AGG CAT CA</td>
</tr>
<tr>
<td>CD11b</td>
<td>F: CTT CTC CCT CAG GAA TGA CA R: CTG CCT CAG GGA TCC GTA AA</td>
</tr>
<tr>
<td>mPGES-1</td>
<td>F: ATG ACT TCC CTG GGT TGG ATG GAG R: ACA GAT GTG GGG CCA CTT CCC AGA</td>
</tr>
<tr>
<td>PGIS</td>
<td>F: TGG CCA AGG GGT CAT GCA AC R: CAG GTC GAA ATG ACT CAG CA</td>
</tr>
<tr>
<td>H-PGDS</td>
<td>F: ATC TGG TGT GAG CAA TCG AG R: CCC AGC CAA ATC TGG GTT TT</td>
</tr>
</tbody>
</table>

F, Forward; HO, heme oxygenase-1; H-PGDS, hematopoietic prostaglandin D synthase; NGF, nerve growth factor; R, reverse.
that H$_2$O$_2$-induced STAT6 phosphorylation was also regulated via lipid rafts. Lipid rafts are membrane microdomains enriched in cholesterol, sphingolipid, and phospholipids with long and saturated acyl chains that function as signaling platforms for the assembly of molecular-signaling modules during a number of cellular events (49). Several ROS-regulated cellular processes are also regulated through lipid rafts (50–52). Considering that phosphorylation status is the net result of the interactions of phosphatases and kinases, changes in the redox status and recruitment of these protein-modifying enzymes to lipid rafts by oxidative stimuli might underlie the differential sensitivity of the two cell types.

Under our experimental conditions, activation of STAT6 contributed to COX-2 expression, as evidenced by the results of experiments using siRNA in H$_2$O$_2$-treatment and H/R conditions (Fig. 7). In contrast to our results, previous reports indicated that IL-4–induced STAT6 represses COX-2 in human follicular dendritic cells (53), and unphosphorylated STAT6 contributes to constitutive COX-2 expression in lung cancer (54). STAT6 is overexpressed in several human malignancies, including prostate and colon cancer, lymphoma, leukemia, and glioma (16, 55, 56). In such cells, STAT6 induces resistance to apoptosis, resulting in cancer cell growth and increased tissue invasiveness. We also examined whether STAT6 activation was associated with astrocyte cell death or resistance to death; we used an oxidative preconditioning model to this end. In line with previous reports (57, 58), astrocytes pre-exposed to 0.5 mM H$_2$O$_2$ for 10 min showed resistance to prolonged lethal H$_2$O$_2$ exposure (Supplemental Fig. 4B, 4C). However, in astrocytes, H$_2$O$_2$-induced STAT6 activation was neither directly associated with cell death under conditions of oxidative stress nor exerted any salvage effect triggered by oxidative preconditioning (Supplemental Fig. 4D).

Our data demonstrate that the COX-2 products, PGE$_2$ and PGI$_2$, were released from ROS-induced astrocytes. Numerous studies in animal models of cerebral ischemia, neurodegeneration, and inflammation described both defensive and damaging effects of PGE$_2$ acting through different E-prostanoid (EP) receptors (59–61). Specifically, PGE$_2$, acting through the EP1 receptor, is reported to be responsible for excitatory neurotoxicity, as well as the neurotoxicity associated with oxygen/glucose deprivation and middle cerebral artery occlusion (59). PGE$_2$ was also reported to have the opposite effect, exerting neuroprotective functions through the EP2 receptor in cerebral ischemia (61). PGI$_2$ is well known for its vasodilating, antiplatelet, and anti-inflammatory actions (62). Thus, COX-2–derived PGs could contribute to brain injury or neuroprotection in excitotoxicity/hypoxia paradigms while opposing inflammation-mediated secondary neurotoxicity. In this study, we found that H$_2$O$_2$–STAT6–mediated release of PGE$_2$ and PGI$_2$ in astrocytes attenuated TNF-α mRNA expression in rat primary microglia (Fig. 9). PG-mediated regulation of myeloid TNF production was reported (63, 64). We presented only TNF-α release, but other inflammatory mediators might be modulated. PGE$_2$, acting via the EP4 receptor, exerts anti-inflammatory effects by reducing proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, in brain microglia (60). The roles of PGs and the subsequent defense-versus-damage outcomes are diverse and even contradictory, depending on specific injury context and cell type. Accordingly, if our in vitro data are to be transferred to therapeutic applications, further studies using animal models and corroboration of our findings in clinical samples are required.

Taken together, our results demonstrate that, through rapid phosphorylation in response to ROS, STAT6 in astrocytes can function as an oxidative stress sensor. In addition to the canonical notion that microglia are the first line of defense against external and internal changes and damages, our results indicate that, under certain circumstances, especially oxidative stress, astrocytes can serve a similar function.
References


10. ACTIVATED ASTROGLIAL STAT6 FUNCTIONS AS AN ROS SENSOR


Disclosures

The authors have no financial conflicts of interest.

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