

Reactive Oxygen Species from Human Astrocytes Induced Functional Impairment and Oxidative Damage

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Abstract Reactive oxygen species (ROS) have been shown to be a contributor to aging and disease. ROS also serve as a trigger switch for signaling cascades leading to corresponding cellular and molecular events. In the central nervous system (CNS), microglial cells are likely the main source of ROS production. However, activated astrocytes also appear to be capable of generating ROS. In this study we investigated ROS production in human astrocytes stimulated with interleukin (IL)-1 β and interferon (IFN)- γ and its potential harmful effects. Although IFN- γ alone had no effect, it potentiated IL-1 β -induced ROS production in a time-dependent manner. One of the sources of ROS in IL-1 β -activated astrocytes was from increased superoxide production in mitochondria accompanied by enhanced manganese superoxide dismutase and inhibited catalase expression. NADPH oxidase (NOX) may also contribute to ROS production as astrocytes express NOX isoforms. Glutamate uptake, which represents one of the most important methods of astrocytes to prevent excitotoxicity, was down-regulated in IL-1 β -activated astrocytes, and was further suppressed in the presence of IFN- γ ; IFN- γ itself exerted minimal effect. Elevated levels of 8-isoprostane in IL-1 β \pm IFN- γ -activated human astrocytes indicate downstream lipid peroxidation. Pretreatment with diphenylethylidene ammonium abolished the IL-1 β \pm IFN- γ -induced ROS production, restored glutamate uptake function and reduced 8-isoprostane to near control levels suggesting that ROS

contributes to the dysfunction of activated astrocytes. These results support the notion that dampening activated human astrocytes to maintain the redox homeostasis is vital to preserve their neuroprotective potential in the CNS.

Keywords Astrocytes · Interleukin 1 beta · Reactive oxygen species · Mitochondria · 8-Isoprostane · Lipid peroxidation

Background

Free radicals, naturally produced by aerobic organisms, are highly reactive with many cellular components, leading to potential damage. Besides normal aerobic respiration, high level of reactive oxygen species (ROS) can also be generated under various conditions, including inflammation. It has been shown that ROS contribute to several neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, HIV-1-associated dementia (HAD) and amyotrophic lateral sclerosis (ALS) [1–4]. ROS were also found to limit the lifespan of hematopoietic stem cells [5]. On the other hand, ROS also serve as important immune defense weapons and as trigger switches for signaling cascade pathways and gene expression. Studies have also shown that ROS production is essential for downstream signaling pathways [6, 7] and perhaps foster communication among various cellular processes to maintain homeostasis [8]. A shortage of ROS, termed reductive stress, can also disrupt many physiological signaling processes [9]. Reaction of ROS with target molecules can induce further ROS production in a chain reaction [10] and this can present dire consequences unless the ROS are removed in a timely fashion. Hence, the antioxidant system, including both enzymes and specific molecules, acts as scavengers of

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ROS and its downstream products to minimize the buildup of ROS and lessen the potential inflicted damage. As such, the balance between ROS and the antioxidant system plays a critical role in determining the fate of cells.

ROS can be generated from many sources, involving enzymatic and non-enzymatic processes [11]; mitochondria are considered to be one of the main contributors to ROS production [12]. ROS production has been demonstrated in macrophages and microglial cells through the activity of NADPH oxidase (NOX) [13–15], as well as in human astrocytic cell lines U-87 [16, 17], CRT-MG [18], astrocytes differentiated from human brain-derived progenitor cells [19] and in primary rat astrocytes [20]. However, in primary human astrocyte cultures, expression of NOX isoform NOX4, but not NOX2, has been reported [21] and NOX4 is the most widely distributed isoform [9]. Diphenyleneiodonium (DPI) is often used as an inhibitor of NADPH oxidase [13, 22–24], which catalyzes superoxide anion (O_2^-) production, although it is not specific for NOX.

Astrocytes are the most abundant glial cells in the central nervous system (CNS) and they play a major role in cross-talk with neurons, provide physical and functional support of neurons, and ultimately afford a level of protection for neurons, such as preventing glutamate excitotoxicity [25]. Damage to astrocytes could therefore be deleterious to neuronal function, resulting in apoptosis and neurotoxicity. The pro-inflammatory cytokine interleukin 1 (IL)-1 β , which is primarily released from activated microglial cells during inflammation, injury or other pathological conditions, is a robust activator of human astrocytes leading to inflammatory mediator production such as cytokines, chemokines, nitric oxide (NO) and ROS [19, 26–31] and to down-regulation of glutamate uptake [32]. Some of the downstream effects of ROS accumulation, including lipid peroxidation of membrane arachidonic acid leading to isoprostane formation in astrocytes, has also been demonstrated [33]. Interferon (IFN)- γ , which is largely secreted by lymphocytes trafficking into the brain during CNS infection and inflammation, potentiates IL-1 β -induced production of inflammatory mediators derived from astrocytes [27, 34], which could trigger unfavorable and injurious autocrine and paracrine conditions for the surrounding neuronal and glial cells. Therefore, dampening astrocytes activated by IL-1 β could be important in minimizing neurotoxicity. Because glutamate uptake has been shown to be inhibited by ROS in rodent astrocytes [35–37] and we have reported down-regulation of glutamate uptake by IL-1 β in human astrocytes [32], in this study we sought to investigate the production of ROS in IL-1 β \pm IFN- γ -activated human astrocytes and characterize the damage inflicted by ROS.

Materials and Methods

Reagents

The following reagents were purchased from the indicated sources: Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin (BSA), Hanks' balanced salts (HBSS), penicillin, streptomycin, trypsin, Tween 20, phosphate buffered saline (PBS), 3,3'-diaminobenzidine, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), diphenyleneiodonium (DPI) (Sigma-Aldrich, St. Louis, MO); heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT); 3H -glutamate (GE Healthcare, formerly Amersham Biosciences, Piscataway, NJ); acrylamide/bis-acrylamide gel and protein assay (Bio-Rad, Hercules, CA); gentamicin, Fungizone[®], RNase inhibitor, SuperScript[™] III reverse transcriptase, Hoechst 33342, MitoSOX Red mitochondrial superoxide indicator (Invitrogen, Carlsbad, CA); RNeasy mini kit (Qiagen, Valencia, CA); DNase (Ambion, Austin, TX); random hexamer and oligo (dT)_{12–18} (Gene Link, Hawthorne, NY); SYBR[®] Advantage[®] qPCR premix (Clontech, Mountain View, CA); dNTPs (GE Healthcare, Piscataway, NJ); CDP-Star substrate (Applied Biosystems, Foster City, CA); anti-rabbit IgG-alkaline phosphatase (AP) conjugate (Promega, Madison, WI); human recombinant IL-1 β and IFN- γ (R&D Systems, Minneapolis, MN); anti-p38, anti-extracellular signal-regulated kinase 1 and 2 (ERK1/2 or p44/42) MAPK, anti-Stat1(-Tyr701) and (Ser727) and anti- β -actin antibodies (Cell Signaling, Beverly, MA); anti-SOD2 and anti-catalase antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); H₂DCFDA, SB203580 (an inhibitor of p38 MAPK), SB202474 (negative control of SB203580), U0126 (an inhibitor of MAP kinase [MEK]1/2, upstream of ERK1/2), U0124 (negative control of U0126) and N^G-monomethyl-L-arginine (N^GMA, inhibitor of nitric oxide synthase) (EMD Biosciences, La Jolla, CA); 8-isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI).

Astrocyte Cultures

Astrocytes were prepared from 16- to 22-week-old aborted human fetal brain tissues obtained under a protocol approved by the Human Subjects Research Committee at our institution. Brain tissues were dissociated and resuspended in DMEM containing FBS (6 %), penicillin (100 U/ml), streptomycin (100 μ g/ml), gentamicin (50 μ g/ml) and Fungizone[®] (250 pg/ml) and plated onto poly-L-lysine (20 μ g/ml)-coated 75-cm² flasks at a density of 80–100 \times 10⁶ cells/flask and incubated in a 6 % CO₂ incubator. Culture medium was changed after 24 h and at a weekly interval. On day 21, flasks

were shaken at 180–200 rpm for 16 h followed by trypsinization with 0.25 % trypsin in HBSS for 30 min. After adding FBS (final concentration 10 %), centrifugation and washing, cells were seeded into new flasks with DMEM, as shown above, followed by media change after 24 h. The subculture procedure was repeated four times at a weekly interval to achieve highly purified astrocyte cultures (99 % of cells stained positive with anti-GFAP antibody) which were plated onto 4-well chamber slide (2×10^4 cells/well) for staining or MitoSOX Red mitochondrial superoxide indicator, 12-well (10^6 cells/well) for RNA extraction or Western Blot, 24-well (1×10^5 cells/well) for ^3H -glutamate uptake assay or 96-well (10^4 cells/well) plates for ROS production or 8-isoprostane assay. The number of cells plated for each culture vessel achieves 100 % confluence. Two days after cells plating, experiments were initiated.

^3H -Glutamate Uptake

Previously, we have determined the ^3H -glutamate concentration and optimal incubation time in astrocyte cultures [32] and adopted the procedure [38] to determine total ^3H -glutamate uptake versus nonspecific value. In this study, ^3H -glutamate (specific activity: 50 Ci/mmol) 10 nM in DMEM was added to cell cultures in 24-well plates (1×10^5 cells/well, 300 μl DMEM) for 10 min at 37 °C after treatment followed by washing with media and cell lysis with 2 N NaOH. Cell lysates were added to scintillation cocktail to measure ^3H -glutamate uptake activity. Values were adjusted to protein content.

ROS Assay

After treatment, cell culture media were removed and cultures in 96-well plates (10^4 cells/well, 100 μl DMEM) were washed with HBSS (with Ca^{2+}) once followed by incubation in H_2DCFDA (20 μM in HBSS with Ca^{2+}) for 45 min in the dark before the culture plate being read in a fluorescence plate reader (Molecular Devices, Sunnyvale, CA) at $\text{Ex}_{485\text{nm}}$ and $\text{Em}_{538\text{nm}}$.

MitoSOX Red Mitochondrial Superoxide Indicator

After treatment, MitoSox reagent was applied to astrocyte cultures on chamber slide (2×10^4 cells/well, 500 μl DMEM) and incubated for 10 min according to the manufacturer's protocol. After washing cells were counterstained with Hoechst 33342, washed and mounted in warm buffer to be microphotographed under fluorescent microscope.

NO Assay

After treatment, astrocyte culture supernatants from 96-well plates (10^4 cells/well, 100 μl DMEM) were collected to measure nitrite (NO_2^-) release using Griess reagent, which reflects NO production in cultures as previously described [34]. In brief, Griess reagent (freshly mixed before use) consisting of equal volumes of 0.1 % naphthylendiamine dihydrochloride in distilled H_2O and 1 % sulfanilamide and 6 % H_3PO_4 in distilled H_2O , was added in equal volume to astrocyte culture supernatants. After 10 min incubation at room temperature, the mixtures were read with a microplate reader at 550 nm and NO_2^- level was extrapolated from a standard curve generated with a series of concentrations of sodium nitrite (NaNO_2 , 1–62.5 μM). The detection limit for NO_2^- was 0.5 μM .

Cell Viability Assay

To determine the effect of treatment on astrocyte viability a MTT assay, which provides quantitative assessment of mitochondrial integrity [transformation of tetrazolium salt via mitochondrial succinic dehydrogenases to formazan crystals (purple)], was used. After treatment of astrocytes MTT (final concentration of 1 mg/ml) was added to cell cultures for 4 h followed by addition of lysis buffer [20 % SDS (w/v) in 50 % N,N-dimethyl formamide, pH 4.7, adjusted with 2.5 % acetic acid and 1 N HCl (32:1)] for 16 h. Cell lysate was collected and absorbance was read at 600 nm (Molecular Devices, Sunnyvale, CA) to reflect possible cytotoxicity caused by treatment. Another assay using trypan blue exclusion test, in which live cells with intact cell membranes exclude certain dyes like trypan blue while dead cells do not, was used to verify cell viability. Cell suspension was mixed with 0.4 % trypan blue and examined under light microscope to visualize if blue cells (i.e. dead cells) were present.

Real-Time Polymerase Chain Reaction

Total RNA extracted from astrocytes in 12-well plates (10^6 cells/well, 600 μl DMEM) with RNeasy mini kit after treatment was treated with DNase and reverse transcribed to cDNA with oligo (dT)_{12–18}, random hexmer, dNTPs, RNase inhibitor and SuperScriptTM III reverse transcriptase. Mixtures of diluted cDNA, primers and SYBR[®] Advantage[®] qPCR premix were subjected to real-time PCR (Stratagene, La Jolla, CA) according to manufacturer's protocol. Primer sequences were sense 5'-TGGCACCCTTTTACACTGACATCC-3' and antisense 5'-CTCCCACTAACATCACCACCTCATAG-3' for NOX2 (gp⁹¹phox, 217 bp), sense 5'-ACCC TGTTGGATGACTGGAAAC-3' and antisense 5'-CCCAT CTGTTTACTGAGGTACAG-3' for NOX4 (180 bp),

sense 5'-GAGCCTGCTGACTAAACTGGAGAT-3' and anti sense 5'-CCCCTGATGGAGTCTTCTTCT-3' for NOX5 (174 bp), sense 5'-TTCTGGACAAACCTCAGCCCTAAC-3' and antisense 5'-CCTTATTGAAACCAAGCCAACC-3' for SOD2 (MnSOD, 163 bp), sense 5'-CCTTTTTGCCTATCCTGACTC-3' and antisense 5'-GTTGAATCTCCGCACTTC TCCAG-3' for catalase (238 bp), sense 5'-GACCTGCTGGA TTACATCAAAGCACT-3' and antisense 5'-CTTTG GATTATACTGCCTGACCAAGG-3' for HPRT (hypoxanthine phosphoribosyltransferase, 240 bp).

Western Blot

Cell lysates collected from 12-well plates (10^6 cells/well, 600 μ l DMEM) after treatment were electrophoresed in 12 % acrylamide/bis-acrylamide, electrotransferred onto nitrocellulose membrane and probed with antibodies for SOD2, catalase, MAPKs (p38 or p44/42), Stat1 and β -actin followed by AP-conjugated secondary antibodies with chemiluminescence detection using image station (Carestream Health (formerly Kodak), New Haven, CT).

8-Isoprostane Assay

After treatment, astrocyte culture supernatants from 96-well plates (10^4 cells/well, 100 μ l DMEM) were collected for 8-isoprostane detection according to manufacturer's protocol. Elevated level of 8-isoprostane was projected as oxidative stress marker.

Statistical Analysis

Data are expressed as mean \pm MSE as indicated. For comparison of means of multiple groups, analysis of variance (ANOVA) was used, followed by Tukey's test.

Results

^3H -Glutamate Uptake Activity and ROS

Reports of inhibition of glutamate uptake by ROS in rodent astrocytes [35–37] led us to conduct experiments using DPI, a classic but non-specific NOX inhibitor, prior to cytokine exposure to verify if ROS was involved in our human astrocyte cultures. We confirm that exposure to IL-1 β \pm IFN- γ exerts significant suppression of ^3H -glutamate uptake activity in human astrocytes [32], while IFN- γ alone shows no effect (Fig. 1). Pretreatment with DPI produces a remarkable blockade of the suppression of ^3H -glutamate uptake activity (Fig. 1) suggesting the involvement of ROS in IL-1 β \pm IFN- γ 's inhibitory effect. This observation led us to investigate ROS production by human astrocytes

using the same treatment paradigm. In our astrocyte cultures, IL-1 β \pm IFN- γ treatment at 24 h did not induce cell loss, reduced viability or apoptosis verified by trypan dye exclusion and MTT assay (data not shown).

ROS Production in Human Astrocytes

Human astrocytes treated with IL-1 β alone (0.01–10 ng/ml) induce peak levels of ROS starting at 0.1 ng/ml (Fig. 2a) while minimal ROS induction is observed by IFN- γ treatment alone (Fig. 2a, b) at 24 h. However, the combination of IL-1 β and IFN- γ robustly potentiates ROS production (Fig. 2a, b). It appears that IFN- γ at 200 U/ml maximizes the potentiation of IL-1 β -induced ROS at 0.1 ng/ml, while IL-1 β at 10 ng/ml potentiates IFN- γ -induced ROS in a concentration-dependent manner (Fig. 2b). By the same token, levels of ROS production were undetectable for all treatments at 3 h and 8 h (Fig. 2c). By 24 h, only IL-1 β or IL-1 β + IFN- γ -treated astrocytes show a marked increase of ROS (Fig. 2c). Therefore, the 24 h treatment time was selected for the subsequent experiments.

To microphotograph ROS production, astrocytes untreated or treated with IFN- γ , IL-1 β or IL-1 β + IFN- γ for 24 h were washed and incubated with H₂DCFDA (20 μ M) followed by

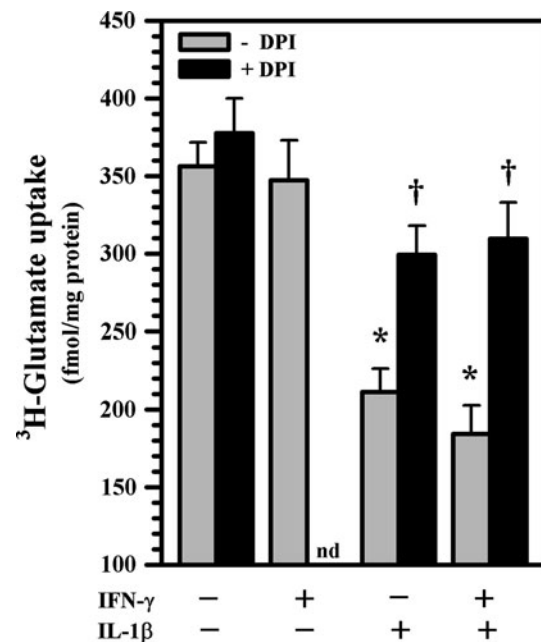


Fig. 1 ^3H -glutamate uptake activity in human astrocytes. Human astrocyte cultures in 24-well plates (1×10^5 cells/well, 300 μ l DMEM) were untreated or pretreated with DPI (300 nM) for 3 h prior to IL-1 β \pm IFN- γ exposure for 24 h followed by addition of ^3H -glutamate (10 nM) for 10 min before harvesting for quantification. Data presented are mean \pm MSE of triplicates from four separate experiments using astrocytes derived from different brain tissue specimens. nd: not done; * p < 0.05 versus untreated control, † p < 0.05 vs. IL-1 β or IL-1 β + IFN- γ correspondingly

visualization under fluorescent microscope. Minimal ROS fluorescence is observed in untreated control or IFN- γ -treated cells, while IL-1 β exposure induces enhanced ROS fluorescence (Fig. 2d). In IL-1 β + IFN- γ -activated astrocytes, ROS production is dramatically increased, as demonstrated by enhanced fluorescence (Fig. 2d).

NADPH Oxidase (NOXs) in Astrocytes

The plasma membrane- and membrane of phagosome-associated NOXs are the main sources of O₂⁻. To verify whether NOXs play a role in ROS production, we first pretreated

astrocytes with DPI overnight, followed by IL-1 β + IFN- γ exposure for 24 h. A robust blockade of ROS production by DPI is evident in a concentration-dependent fashion (Fig. 3). Cytotoxicity by DPI was not found at the concentrations used in these experiments as measured by trypan blue exclusion assay (data not shown). We then used real-time RT-PCR to assess expression of different NOX isoforms in human astrocytes and found medium NOX4 (Ct 24–26) to moderate NOX5 (Ct 28–30) to low NOX2 (Ct 30–33) mRNA expression in astrocytes (Fig. 4). These results suggest that NOXs may contribute to ROS production in IL-1 β \pm IFN- γ -activated astrocytes.

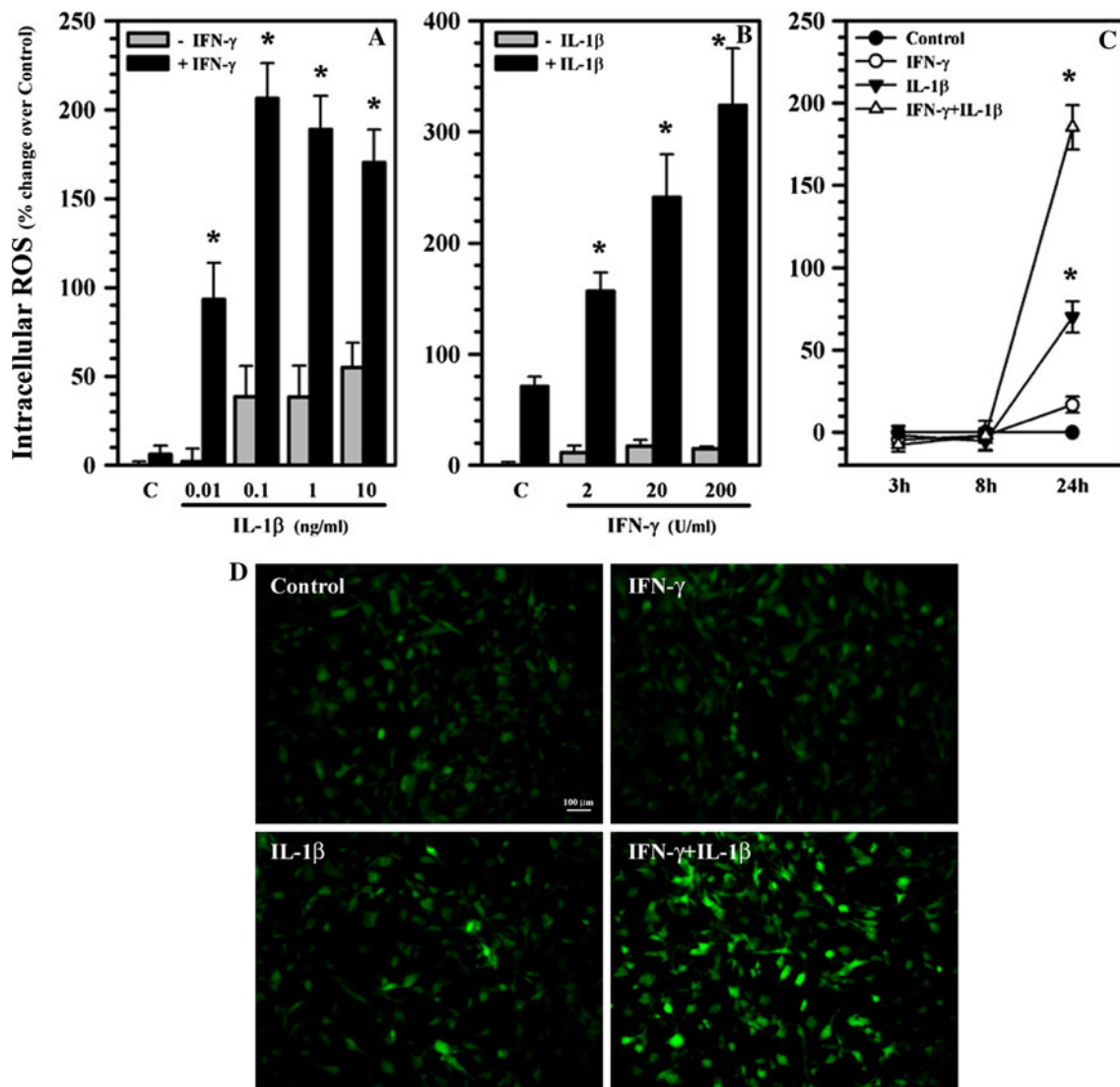


Fig. 2 ROS production in human astrocytes. Human astrocyte cultures in 96-well plates (10^4 cells/well, 100 μ l DMEM) were untreated (c) or treated with either **a** IL-1 β (0.01–10 ng/ml) \pm IFN- γ (200 U/ml); **b** IFN- γ (2–200 U/ml) \pm IL-1 β (10 ng/ml) for 24 h; **c** IL-1 β (10 ng/ml) alone or in combination with IFN- γ (200 U/ml) for 3, 8 and 24 h; or **d** IFN- γ (200 U/ml), IL-1 β (10 ng/ml) or IL-1 β + IFN- γ for 24 h. After washing, cultures were incubated with

H₂DCFDA (20 μ M) for 45 min and read at EX_{485nm} and Em_{538nm} (a–c) or microphotographed under fluorescent microscope (d). Data presented are mean \pm MSE of triplicates of three separate experiments using astrocytes derived from different brain tissue specimens. * p < 0.05 versus respective **a** IL-1 β , **b** IFN- γ alone or **c** untreated control

Mitochondria and ROS Production

Mitochondria are the primary sources of energy and ATP for any cell via the electron transport chain, which generates ROS as a natural byproduct. We used the MitoSOX™ Red mitochondrial superoxide indicator to stain and image untreated (control) or DPI pretreated astrocyte cultures prior to IL-1β exposure for 24 h. Treatment with IL-1β dramatically increases superoxide production (substantial red stain) (Fig. 5) while the untreated control or IFN-γ alone (data not shown) show minimal superoxide production (no positive red stain). Pretreatment with DPI appears to abolish the superoxide production (minimized to almost no red stain) suggesting the inhibitory action on flavoproteins in mitochondria. Similar findings in IL-1β + IFN-γ-activated astrocytes were also observed (data not shown). These results suggest that mitochondria also contribute to ROS release in IL-1β ± IFN-γ-activated astrocytes.

Effects of IL-1β and IFN-γ on Antioxidant Enzyme Expression

ROS production by activated human astrocytes led us to investigate whether antioxidant enzymes were affected by

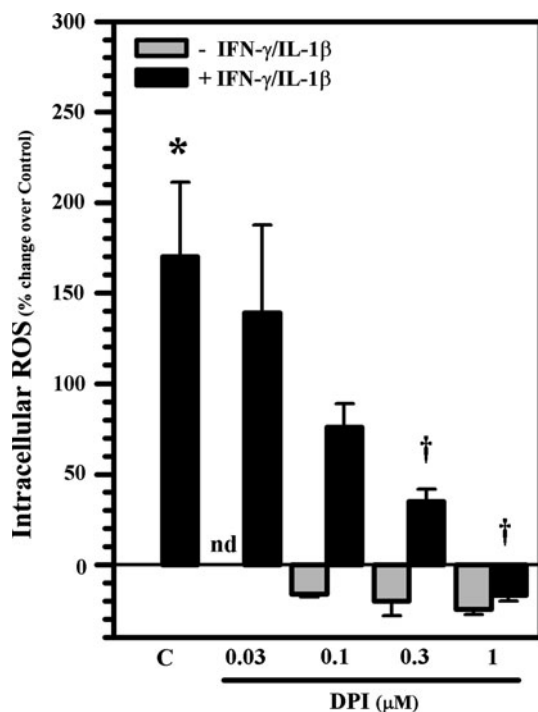


Fig. 3 Inhibition of ROS production in human astrocytes. Human astrocyte cultures in 96-well plates (10⁴ cells/well, 100 μl DMEM) were untreated (c) or pretreated with DPI (0.03–1 μM) overnight prior to IL-1β (10 ng/ml) + IFN-γ (200 U/ml) exposure for 24 h before being washed and incubated with H₂DCFDA (20 μM) for ROS measurement. Data presented are mean ± MSE of triplicates of three separate experiments using astrocytes derived from different brain tissue specimens. nd: not done; **p* < 0.05 versus untreated control, †*p* < 0.05 versus IL-1β + IFN-γ

IL-1β ± IFN-γ treatment. We found that expression of superoxide dismutase 2 (SOD2 or MnSOD) is up-regulated and catalase (CAT) is down-regulated by IL-1β ± IFN-γ treatment, while there is no effect by IFN-γ alone (Fig. 6a, b). The result of increased MnSOD (SOD2) expression could be a response to increased superoxide production in mitochondria. With suppressed CAT expression, we speculate that H₂O₂ generated from MnSOD reaction may not be removed efficiently and its accumulation could impose further deleterious effects on astrocytes already under oxidative stress.

IL-1β and IFN-γ Induced Signaling Pathways in Human Astrocytes

Many reports have demonstrated the involvement of ROS in MAPK pathways [5, 6, 15, 39]. In this study, we first investigated several signaling pathways in human astrocytes exposed to IL-1β ± IFN-γ. We found that within 15 min of IL-1β treatment, phosphorylation of p38, p44/42 MAPK and Stat1(Ser727) is induced and the activation continues for at least 1 h (Fig. 7). There is no activation of these signaling pathways by IFN-γ exposure alone, except that of pStat1(Tyr701), nor is there any potentiation of IL-1β-induced signaling by IFN-γ (Fig. 7). Quantitation and normalization of band density was performed for each target for visual assessment (Fig. 7).

P38 MAPK in ROS Production and Glutamate Uptake

Since IFN-γ did not potentiate IL-1β-induced signaling, we then used inhibitors to analyze the signaling pathways

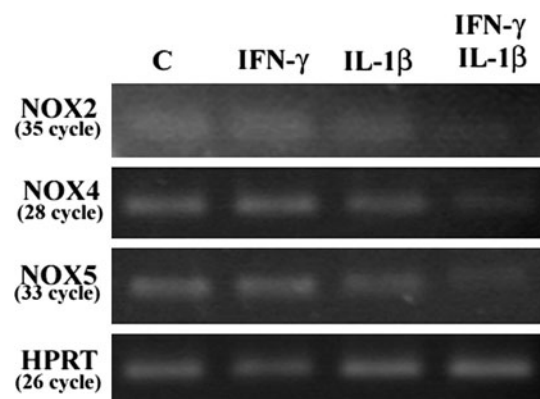


Fig. 4 Expression of NOX isoforms in human astrocytes. After treatment total RNA (1 μg) collected from astrocyte cultures in 12-well plates (10⁶ cells/well, 600 μl DMEM) was treated with DNase and reverse transcribed to cDNA followed by qPCR for NOX isoforms and housekeeping gene HPRT. PCR products were electrophoresed in 4 % NuSieve 3:1 agarose gel and stained with ethidium bromide. Data presented are representative of three separated experiments using astrocytes derived from different brain tissue specimens

Fig. 5 Mitochondrial ROS production in human astrocytes. Human astrocyte cultures in 4-well chamber slide (2×10^4 cells/well, 500 μ l DMEM) were untreated (Control) or pretreated with DPI (1 μ M) overnight prior to IL-1 β (10 ng/ml) exposure for 24 h before being stained with MitoSOXTM Red mitochondrial superoxide indicator. After washing, cells were counterstained with Hoechst 33342, washed and mounted in warm buffer to be microphotographed under a fluorescent microscope

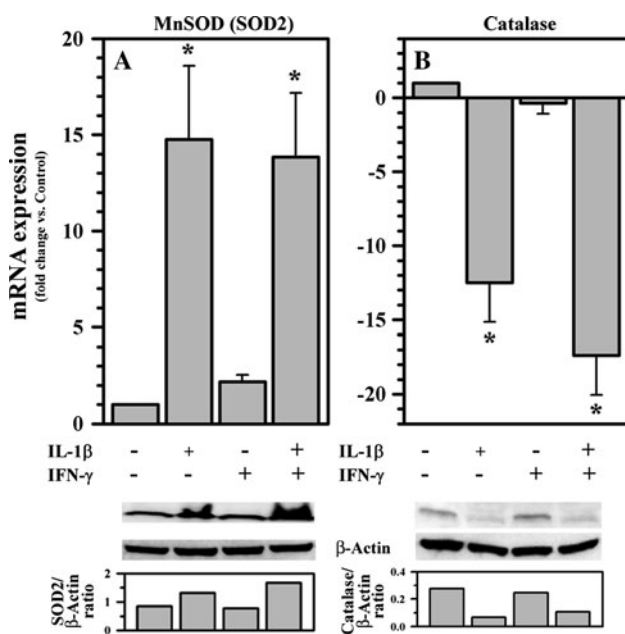
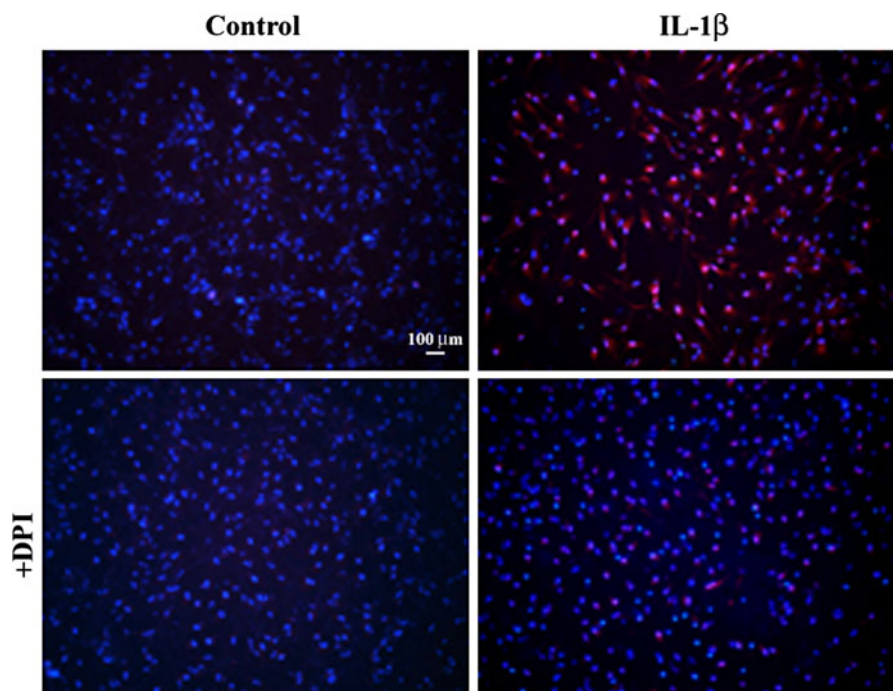


Fig. 6 Effects of IL-1 β and IFN- γ on antioxidant enzymes in human astrocytes. Total RNA (1 μ g) of cell culture lysates collected from human astrocyte cultures in 12-well plates (10^6 cells/well, 600 μ l DMEM) untreated or treated with IL-1 β (10 ng/ml) \pm IFN- γ (200 U/ml) for 24 h were DNase treated and reverse transcribed to cDNA followed by qPCR or electrophoresed, transblotted and probed, respectively, to assess **a** SOD2 and **b** catalase expression. Densitometric measurement (normalized to β -actin) of the bands is shown. Data presented are mean \pm MSE of three separate experiments using astrocytes derived from different brain tissue specimens. * $p < 0.05$ versus untreated control

involved in IL-1 β + IFN- γ -induced ROS production. After pretreatment with SB203580 (p38 MAPK inhibitor), SB202474 (negative control of SB203580), U0126 (p44/42 MAPK inhibitor) and U0124 (negative control of U0126) prior to IL-1 β + IFN- γ exposure, ROS production is suppressed only by the p38 MAPK inhibitor SB 203580 in a concentration-dependent fashion (Fig. 8a), while the p44/42 MAPK inhibitor U0126 shows no effect (Fig. 8b). This finding suggests that p38 MAPK is required for IL-1 β + IFN- γ -induced ROS production in astrocytes. Using a similar pretreatment paradigm, SB203580 but not SB202474 reverses the inhibition on 3 H-glutamate uptake activity (Fig. 8c) indicating that p38 MAPK mediates this inhibitory effect of IL-1 β + IFN- γ .

Effects of IL-1 β and IFN- γ on Lipid Peroxidation

Lipid peroxidation is one of the end results of free-radical-induced injury and 8-isoprostane, a prostaglandin (PG)-F₂-like compound (8-*epi* PGF_{2 α}) produced by peroxidation of arachidonic acid, has been shown to have biological activity [40]. Although 8-isoprostane is present in normal healthy subjects, an elevated 8-isoprostane level is a signal of oxidative stress. After exposure for 24 h, 8-isoprostane levels in culture supernatants are markedly enhanced in IL-1 β \pm IFN- γ -treated human astrocytes and are significantly suppressed by pretreatment with DPI (Fig. 9), suggesting the contribution of ROS to the lipid peroxidation. Although no detectable NO was present at 24 h by Griess reagent

Fig. 7 IL-1 β - and IFN- γ -induced signaling pathways in human astrocytes. Human astrocyte cultures in 12-well plates (10⁶ cells/well, 600 μ l DMEM) were untreated (as control) or treated with IL-1 β (10 ng/ml) \pm IFN- γ (200 U/ml) for 15, 30 and 60 min before collecting cell lysates to be electrophoresed, transblotted to nitrocellulose membrane and probed for p38 or p44/42 MAPK, Stat1 or β -actin (as internal control). Densitometric measurement (normalized to total p38 or p44/42 or β -actin) of the bands is shown. Data presented are representative of three to five separate experiments using astrocytes derived from different brain tissue specimens. All targets of the same experiment were electrophoresed and analyzed in individual blot without re-probing

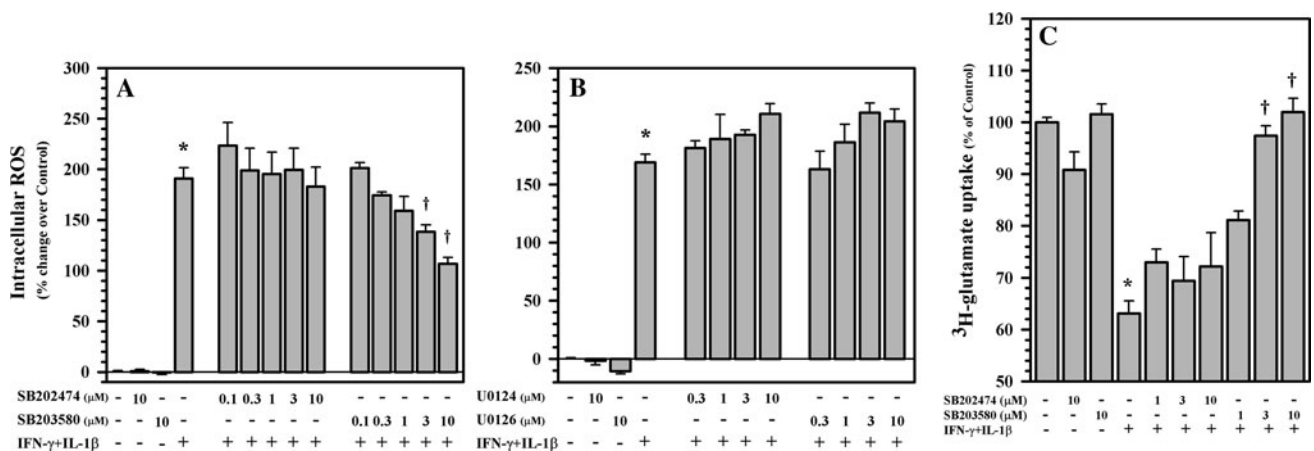
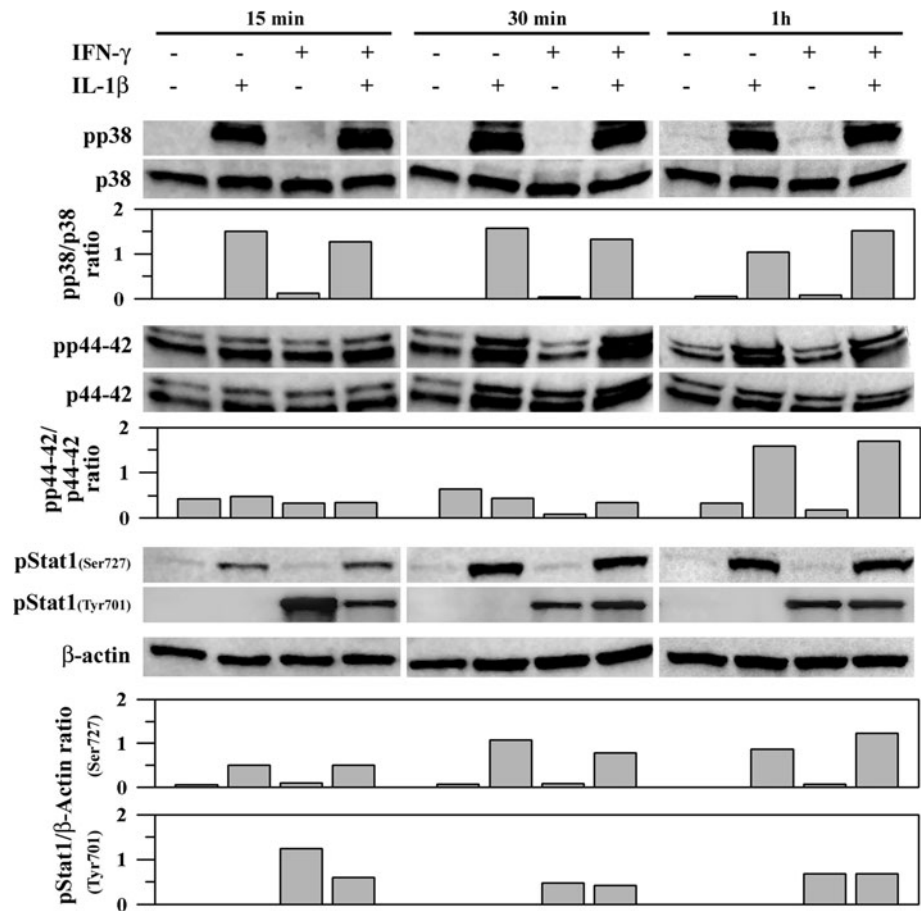


Fig. 8 Involvement of p38 MAPK in ROS production and glutamate uptake in human astrocytes. Human astrocyte cultures in 96-well plates (10⁴ cells/well, 100 μ l DMEM) were untreated (as control) or pretreated with **a** SB203580 (p38 MAPK inhibitor) and SB202474 (as negative control) or **b** U0126 (ERK1/2 MAPK inhibitor) and U0124 (as negative control) for 1 h prior to IL-1 β (10 ng/ml) + IFN- γ (200 U/ml) exposure for 24 h before being washed and incubated with H₂DCFDA (20 μ M) for ROS measurement. **c** Human astrocyte cultures in 24-well plates (1 \times 10⁵ cells/well, 300 μ l DMEM) were

untreated (as control) or pretreated with SB203580 (p38 MAPK inhibitor) and SB202474 (as negative control) for 1 h prior to IL-1 β (10 ng/ml) + IFN- γ (200 U/ml) exposure for 24 h followed by addition of ³H-glutamate (10 nM) for 10 min before harvesting for quantification. Data presented are mean \pm MSE of triplicates of three to five (**a**, **b**) and three (**c**) separate experiments using astrocytes derived from different brain tissue specimens. **p* < 0.05 versus untreated control; †*p* < 0.05 versus IL-1 β + IFN- γ

(detecting presence of nitrite indicative of NO production), we used the NOS inhibitor N^GMA as pretreatment to see if NO might also play a role in 8-isoprostane production. No significant reduction of 8-isoprostane level was found (data not show), suggesting that NO is not involved in IL-1 β + IFN- γ -induced 8-isoprostane release in human astrocytes at 24 h.

Discussion

In coordination with antioxidant enzymes and molecules, ROS produced under normal condition can be managed and homeostasis can be maintained without triggering injury to the host. However, ROS can damage cells under conditions such as aging, inflammation, or other pathological circumstances. In the CNS, activated microglial cells, the resident macrophages of the brain, are the main source of phagocytic ROS production, primarily through NADPH-oxidase activity as demonstrated by studies using DPI or siRNA treatment and knock-out animal models [41–43]. Human astrocyte cell lines have also been shown to produce ROS through NADPH-oxidase activity

[17, 18] while no ROS source was identified in astrocytes differentiated from human neural progenitor cells [19]. Recently, we reported induced heme oxygenase 1 expression in IL-1 β -activated astrocytes is likely due to oxidative stress [44]. In this study IL-1 β + IFN- γ are able to induce ROS in astrocytes but not in primary microglial cells (unpublished observation) suggesting different regulatory mechanisms between these two types of glial cells. In IL-1 β -activated astrocytes some IFN-stimulated genes (ISG) were induced and the phosphorylation and nuclear translocation of IFN regulatory factor 3 (IRF3) and the expression of IFN- β and chemokines (CXCL10 and CCL5) were demonstrated in IL-1 β signaling [31]. Also, IFN- γ might prime astrocytes for heightened responses to IL-1 β as it was reported in macrophages exposed to LPS [45]. These effects might reflect on the exacerbated ROS production in IL-1 β + IFN- γ compared to IL-1 β exposure alone in this study.

Previously, mRNA expression of NOX4 was demonstrated in primary human astrocytes [21] and NOX4 was also found to localize to mitochondria [46]. Here we found medium to moderate to low expression of NOX isoforms NOX4, NOX5 and NOX2, respectively, in human astrocytes (NOX1 and NOX3 were undetectable) suggesting that NADPH-oxidase may contribute to ROS production in IL-1 β + IFN- γ -activated astrocytes.

Mitochondrial ROS formation is a natural product and can be potentiated by exogenous factors such as oxidants [47]. Our results show enhanced superoxide production in mitochondria by IL-1 β + IFN- γ activation, which is abated by DPI, since flavoproteins are present in mitochondria. Involvement of other processes in ROS production, such as free iron, cyclooxygenase and monoamine oxidase, however, cannot be ruled out. Future experiments using specific inhibitors may clarify the contribution of these processes to ROS generation in activated human astrocytes. With enhanced superoxide production, increased MnSOD expression and suppressed catalase expression found in this study, it is logical to speculate that H₂O₂ formed may not be removed promptly and its accumulation could be damaging; an example of this includes inhibition of glutamate uptake in rodent astrocytes [35–37]. Others have shown increased H₂O₂ production with enhanced MnSOD activity [48–50]. However, one must bear in mind that catalase is not the only antioxidant molecule capable of removing H₂O₂, as glutathione peroxidases and peroxiredoxins are also able to convert H₂O₂ to water [8]. Although we did not find glutathione levels to be affected until 72 h, we did observe that mRNA expression of enzymes involved in glutathione synthesis and recycling were suppressed to an extent in IL-1 β + IFN- γ -activated astrocytes (unpublished observation).

Reports have shown that there is an interaction between MAPK signaling and ROS [5, 15, 39, 51] and both are required in normal physiological and biological processes.

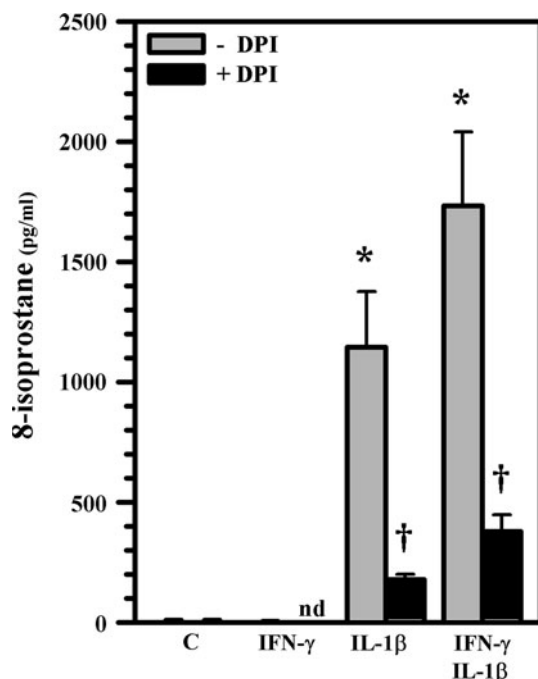


Fig. 9 ROS-induced 8-isoprostane production in human astrocytes. Human astrocyte cultures in 96-well plates (10^4 cells/well, 100 μ l DMEM) were untreated (c) or pretreated with DPI (1 μ M) overnight prior to IL-1 β + IFN- γ exposure for 24 h before harvesting culture supernatants for 8-isoprostane measurement. Data presented are mean \pm MSE of triplicates of three separate experiments using astrocytes derived from different brain tissue specimens. nd: not done; * p < 0.05 versus untreated control; † p < 0.05 versus IL-1 β or IL-1 β + IFN- γ correspondingly

Phosphorylation of p38 and p44/42 MAPK within 15 min seems to occur much earlier than the H₂DCFDA-detectable ROS at 24 h and the ROS production was dampened only by the p38 inhibitor SB203580, but not by the p44/42 MAPK inhibitor U0126 in IL-1 β + IFN- γ -activated astrocytes. Therefore, activation of p38 MAPK appears to be required for ROS production in IL-1 β + IFN- γ -activated astrocytes. Results showing the reversal of glutamate uptake suppression by p38 inhibitor SB203580 further supported the belief of p38 MAPK activation playing a role in ROS production leading to the inhibitory effect of IL-1 β + IFN- γ on ³H-glutamate uptake of astrocytes.

Elevated 8-isoprostane levels from lipid peroxidation is considered an indicator of ROS damage [52] and increased 8-isoprostane levels in serum correlate negatively with pulmonary function in patients with systemic sclerosis [53]. In rat astrocyte cultures, isoprostane levels increase by many fold after mechanical trauma or injury by free radicals [33]. Because 8-isoprostane exerts biological functions such as vasoconstriction and reducing blood flow [40], the increased isoprostane levels in injured astrocytes might be deleterious to the nearby cerebral vessels [33]. Stimulation of 8-isoprostane production in IL-1 β \pm IFN- γ -activated astrocytes and its suppression by DPI, but not by the NOS inhibitor N^GMA, further implicates ROS production in activated human astrocytes as a source of lipid peroxidation.

Previously, we have shown induction of apoptosis and inhibition of glutamate transporter expression/activity and glutamine synthetase activity in human astrocytes exposed to IL-1 β [28, 32, 54]. It was also reported that increased mitochondrial ROS in CA1 astrocytes contributed to the loss of glutamate transporter activity leading to CA1 neuronal injury [55]. These results, together with the finding of increased lipid peroxidation in this study, suggest that ROS production in activated astrocytes may either set the stage for functional impairment of astrocytes indirectly leading to neuronal injury, or possibly lead to direct damage to the surrounding neurons.

In summary, we have demonstrated that the inflammatory cytokines IL-1 β and IL-1 β + IFN- γ induce ROS in human astrocytes and that NADPH oxidase and mitochondria may contribute to ROS production. A hallmark of ROS damage in IL-1 β \pm IFN- γ -activated astrocytes is the significantly suppressed ³H-glutamate uptake and elevated 8-isoprostane level indicative of oxidative stress-induced lipid peroxidation, which may have deleterious consequences for the surrounding cells and tissues. With ROS being shown to contribute to many neurodegenerative diseases and astrocytes being capable of ROS production it will be vital to maintain astrocyte function and integrity at a homeostasis state to be neuroprotective. Further detailed studies are needed to study the downstream effects of ROS

generated by IL-1 β \pm IFN- γ -activated human astrocytes on neurotoxicity.

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