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Interleukin-1 β protects astrocytes against oxidant-induced injury via an NF κ B-dependent upregulation of glutathione synthesis

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Abstract

Astrocytes produce and export the antioxidant glutathione (GSH). Previously, we found that interleukin-1 β (IL-1 β) enhanced the expression of astrocyte system x_c⁻, the transporter that delivers the rate-limiting substrate for GSH synthesis —cyst(e)ine. Herein, we demonstrate directly that IL-1 β mediates a time-dependent increase in extracellular GSH levels in cortical astrocyte cultures, suggesting both enhanced synthesis and export. This increased GSH production was blocked by inhibition of nuclear factor κ B (NF κ B) activity but not by inhibition of p38 MAPK. To determine whether this increase could provide protection against oxidative stress, the oxidants tert-butyl hydroperoxide (tBOOH) and ferrous sulfate (FeSO₄) were employed. IL-1 β treatment prevented the increase in reactive oxygen species produced in astrocytes following tBOOH exposure. Additionally, the toxicity induced by tBOOH or FeSO₄ exposure was significantly attenuated following treatment with IL-1 β , an effect reversed by concomitant exposure to L-buthionine-S,R-sulfoximine (BSO), which prevented the IL-1 β -mediated rise in GSH production. IL-1 β failed to increase GSH or to provide protection against t-BOOH toxicity in astrocyte cultures derived from IL-1R1 null mutant mice. Overall, our data indicate that under certain conditions IL-1 β may be an important stimulus for increasing astrocyte GSH production, and potentially, total antioxidant capacity in brain, via an NF κ B-dependent process.

Keywords

oxidative stress; glioprotection; IL-1; central nervous system; neuroprotection

INTRODUCTION

Depending on the context, alterations in astrocyte physiology following neurological injury can promote neuronal survival or facilitate death (John et al. 2005; Liberto et al. 2004; Maki et al. 2013; Sofroniew and Vinters 2010). Astrocytes, contribute to the central nervous system (CNS) inflammatory response (Brambilla et al. 2005; Farina et al. 2007; Hewett 2009). Yet, they also play a key role in antioxidant protection and in glutathione (GSH)

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homeostasis (Dringen et al. 2000; Dringen et al. 1997; Raps et al. 1989; Sagara et al. 1993; Shih et al. 2003).

Prototypical inflammatory mediators, like the cytokine interleukin-1 β (IL-1 β), also produce dichotomous results within the CNS [dichotomy reviewed in (Hewett et al. 2012)]. IL-1 β is induced/upregulated in brain tissue following acute injury and is found to be elevated in more classical neurodegenerative diseases. The predominate viewpoint is that it contributes to and/or sustains pathophysiological processes (Allan and Rothwell 2001; Davies et al. 1999; Denes et al. 2011; Ferrari et al. 2006; Loddick and Rothwell 1996; Sheng et al. 1996; Sheng et al. 2000; Yan et al. 2014). However, other studies demonstrate that IL-1 β can play an important role in protection and repair (Albrecht et al. 2002; Carlson et al. 1999; John et al. 2005; Saavedra et al. 2007; Wang et al. 1994). It may do both by modifying astrocyte behavior (Nguyen et al. 2002; Zhao and Schwartz 1998).

Indeed, work from our laboratory would suggest the protective versus deleterious effects of IL-1 β on astrocytes could be context-dependent. Previously, we found that IL-1 β enhanced the expression and functional activity of the cystine/glutamate transporter (system x_c⁻) in astrocytes, producing excitotoxic neuronal cell death, but only in the context of energy deprivation (Fogal et al. 2007; Jackman et al. 2010). Interestingly, this same transporter is well characterized with respect to its participation in the uptake of cystine for the synthesis of the neuroprotective antioxidant molecule glutathione (GSH) (Bannai and Tateishi 1986). This raises the intriguing possibility that IL-1 β may upregulate processes that fundamentally protect against oxidative stress. As such, the ability of IL-1 β to regulate astrocytic GSH production and the susceptibility of cells to oxidative stress in the presence and absence of IL-1 β was evaluated herein. Overall, we found that interleukin-1 β protected astrocytes against oxidant-induced injury via an NF κ B-dependent upregulation of glutathione synthesis. Part of the work has been published in abstract form (He et al. 2013; Jackman et al. 2011).

MATERIALS AND METHODS

Animals

This study was conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals and was approved by the Institutional Animal Care and Use Committee of both the University of Connecticut Health Center and Syracuse University. CD1 mice were obtained from Charles River Laboratories (Wilmington, MA) and were used in all experiments unless indicated otherwise. IL-1RI null mutant animals (Stock # 003245) and its background strain (C57BL/6 Stock # 000664) were purchased from The Jackson Laboratory (Bar Harbor, ME). Because IL-1RI null mice were bred to homozygosity for the cell culture experiments, animals from the background strain were bred in parallel and their offspring used as wild-type controls.

Cell Culture Media

Media stock (MS): L-glutamine-free modified Eagle's medium (Earl's salt; MediaTech, Herndon, VA) supplemented with L-glutamine, glucose, and sodium bicarbonate to a final

concentration of 2.0, 25.7, and 28.2 mM, respectively; *Glial plating media*: MS containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) and 10% heat-inactivated calf serum (CS; Hyclone, Logan, UT), 10 ng/ml epidermal growth factor (Invitrogen, Carlsbad, CA), and 50 IU penicillin/50 µg/ml streptomycin (Gibco/BRL); *Maintenance media*: MS containing 10% CS and 50 IU penicillin/ 50 µg/ml streptomycin.

Primary Astrocyte Cultures

Cortices from postnatal day 1–3 CD1, C57BL/6 or *il1r1* null mutant mouse pups were dissected, pooled, and cells dissociated by exposure to trypsin (0.025%, 15 min, 37 °C). Cells, diluted 2 hemispheres/10 ml of glial plating medium/plate, were plated into multi-well culture plates (Falcon Primaria; BD Biosciences, Lincoln Park, NJ) as described (Hamby et al. 2006; Uliasz et al. 2012). DNA extracted from extraneous brain was used to confirm genotype. Once confluent, astrocyte monolayers were treated with 8 µM β-D-arabinofuranoside (Sigma, St. Louis, MO) once for 4–6 days to reduce the number of microglia. Cells were then placed in maintenance media that was replaced once per week until experimentation. Purified astrocyte cultures were generated by removing residual microglia by treating monolayers with 50–75 mM L-leucine methyl ester for 30–90 min, one day prior to experimentation (Hamby et al. 2006; Uliasz et al. 2012). Cultures were maintained at 37°C in a humidified 6.0% CO₂, 21% O₂ -containing incubator and were used for experimentation at 35 days *in vitro*.

IL-1β Treatment

Cells were treated with 3 or 10 ng/ml recombinant murine IL-1β (R&D Systems, Minneapolis, MN) in an incubation buffer composed of MS (*vide supra*) containing 0.1% fatty acid free BSA (Sigma, St. Louis, MO) and placed in a humidified 37°C normoxic (21% O₂) incubator containing 6% CO₂ for the times indicated in each figure legend.

Drug Exposure

SB203580 or TPCA-1—Exposure to the selective p38 MAP kinase inhibitor, SB203580 (Choi and Friedman 2009), or the IKKβ inhibitor, TPCA-1 (Podolin et al. 2005), was carried out in a humidified 37°C normoxic incubator containing 6.0% CO₂ and 21% O₂. Cells were treated with the drugs 30 min prior to addition of IL-1β. A 10 mM stock solution of SB203580 was prepared in Cellgro® water (Corning), while a 20 mM stock of TPCA-1 was made in DMSO; both were stored at –20°C until used. In the latter case, the experimental conditions contained identical concentrations of DMSO which never exceeded 0.1%.

L-buthionine-S,R-sulfoximine (BSO)—A 100 mM stock solution of BSO was prepared in Cellgro® water every three months and stored at 4°C. The stock was diluted to its final concentration in MS or MS containing 0.1% fatty acid free BSA and added to the cultures at the concentrations and durations detailed in each figure legend.

Tert-butyl hydroperoxide (tBOOH) treatment—A stock solution of tBOOH (1.5 M in H₂O; Acros Organics) was made and stored at 4°C. tBOOH was added to the cultures (final concentration: 0.1–1.5 mM) in MS. Experiments were terminated 2.5–3.5 hr later by addition of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to assess

for cell survival (*vida infra*). Tissue culture supernatant was removed for the measurement of lactate dehydrogenase (LDH) activity as an assessment of cell death (*vida infra*).

FeSO₄ Treatment—Stock solutions of FeSO₄ (10 mM in H₂O) and its carrier, sodium pyruvate (NaP; 20 mM in DMSO) (Kress et al. 2002), were made fresh daily. FeSO₄ and NaP were spiked into the cultures to achieve a final concentration of 1.5 or 2 μ M and 20 μ M, respectively. Because iron-containing compounds interfere with the LDH assay by directly oxidizing NADH, the MTT assay was utilized for the measurement of cell survival.

Measurement of GSH

Total extracellular and intracellular glutathione (GSH + GSSG) concentrations were determined using the GSH-Glo glutathione assay (Promega, Madison, WI) per manufacturer's instruction. Media was collected for analysis of the extracellular GSH ([GSH]_e) and GSH-Glo reaction buffer was added to the cells for analysis of the intracellular GSH ([GSH]_i). Additional reaction buffer was made per manufacturer's instructions and 200 μ L was added per well as a dilution factor to keep relative light units within the dynamic range of the standards. To determine total GSH levels, glutathione disulfide (GSSG) within the samples was converted to GSH with the reducing agent TCEP-HCl (final concentration = 1 mM; 10 min; 25°C; Thermo Scientific; Waltham, MA). Luciferase activity was measured using an Optocomp II luminometer (MGM Instruments) or a Synergy² microplate reader (BioTek, Winooski, VT). Total intracellular or extracellular GSH were normalized to standards prepared in GSH-Glo reaction buffer and MS containing 0.1% fatty acid free BSA, respectively. Extracellular GSSG was calculated based on the equation $GSSG = (\text{total GSH} - \text{reduced GSH}) / 2$. [GSH]_i were normalized to cellular protein. Standards were linear over the range of 0–5 μ M.

Immunoblotting

Total cell lysates—Purified astrocytes cultured in 6-well plates were washed once with ice-cold PBS, harvested by gentle scraping into one ml ice-cold PBS, and then pooled (two wells from two separate plates). Cells were spun (700 g; 5 min; 4°C) and the resulting pellet was suspended in lysis buffer containing: 20 mM HEPES (pH 7.4), 2 mM EGTA (pH 8.0), 50 mM β -glycerol phosphate (pH 7.2), 1 mM DTT, 1 mM Na₃VO₄, 5 mM NaF, 1% Triton X-100, 0.2 mM PMSF and 1 X Complete Protease Inhibitor (Roche). The resuspended pellet was then incubated on ice (30 min). Cellular debris was removed by centrifugation (12,000 g; 20 min; 4°C). Supernatants were stored at –80°C until used for detection of activated (phospho-) p38 MAP kinase.

Nuclear fractionation—Astrocytes cultured in 100 mm dishes were harvested by gentle scraping and spun (700 g; 5 min; 4°C). The resulting pellets were washed once with cold PBS and resuspended by gentle trituration in a cytosol extraction buffer [1 X Complete protease inhibitor, 10 mM HEPES (pH 7.4), 60 mM KCl, 1 mM EDTA, 1 mM DTT and 0.075% NP40]. After a three min incubation on ice, the suspensions were spun (210 g; 5 min; 4°C) and the supernatants removed. The resulting pellets were washed once with cytosol extraction buffer without NP40 and spun (210 g; 5 min; 4°C). These pellets were resuspended in a nuclear extraction buffer [1 x Complete protease inhibitor, 20 mM

Tris.HCl (pH 8.0), 420 mM NaCl, 1.5 mM MgCl₂, 25% glycerol, 0.2 mM EDTA and 0.1% NP40] and incubated on ice for 10 min. Cellular debris was removed by centrifugation (16,500 g; 10 min; 4°C). Fractions were stored at –80°C until used for detection of p65.

Gel Electrophoresis and Protein Detection—Thirty to 50 µg of protein (BCA assay; Pierce; Rockford, IL) was separated by 8% or 12% SDS-PAGE under reducing conditions and electrophoretically transferred to a PVDF membrane (Bio-Rad; Hercules, CA). Membranes were blocked (Odyssey® blocking buffer; at 25°C for 1 hr) and then probed (4°C overnight) with an anti-phospho-p38 MAPK rabbit polyclonal antibody (1:500 dilution; Cell Signaling) or an anti- NFκB p65 rabbit polyclonal antibody (1:200 dilution; Santa Cruz) and a mouse monoclonal antibody directed against β-actin (1:4000 dilution; Sigma; overnight). Species-specific secondary antibodies labeled with spectrally distinct IRDye® fluorescent dyes (LI-COR Biosciences; Lincoln, NE) were used to detect primary antibodies (1 hr at 25°C). Results were recorded on LI-COR ODYSSEY® Fc Imaging system (LI-COR Biosciences) and protein levels quantified using Image Studio 3.1 (LI-COR Biosciences; Lincoln, NE). p-p38 MAPK and p65 levels were normalized to their respective β-actin levels. Antibodies directed against GAPDH were used to check for cytosolic protein contamination within the nuclear fraction and none was found (data not shown).

Immunocytochemistry

Nuclear translocation of p65 in primary astrocytes was also assessed by immunocytochemistry. Cell staining was done essentially as described (Hamby et al. 2006). Briefly, cultures were fixed using 4% paraformaldehyde (30 min, 25°C), made permeable with 0.25% Triton X-100 in PBS (7 min, 25°C), and blocked with 10% normal goat serum (NGS) in PBS (1 hr, 25°C). Next, cultures were labeled with 2 µg/ml NFκB p65 antibody (rabbit polyclonal IgG, Santa Cruz Biotechnology, Dallas, TX) in PBS containing 2% NGS (overnight, 4°C). Then cells were incubated with 0.75 µg/ml Cy3-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) in PBS containing 2% NGS and 1 µg/ml DAPI (30 min, 25°C). Fluorescent photos were acquired by a DP73 digital color camera (Digital Video Camera Co) mounted on an Olympus IX50 inverted microscope outfitted with epifluorescence controlled by CellSens Standard (Olympus, Center Valley, PA) software. Brightness and contrast were standardized for each picture.

Detection of Reactive Oxygen Species by Dihydroethidium (DHE) Fluorescence

Astrocytes in 24 well plates were treated with IL-1β (3ng/ml) or its vehicle for 48 hr. Cells were then exposed to 0.7 mM tBOOH or its vehicle for 45 min following which cells were washed (2 × 400 µl) into MS made with phenol red free Eagle's medium. A stock solution of DHE (10 mM) was made in 100% DMSO and diluted into phenol red free MS to a final concentration of 20 µM (DMSO = 0.2%). DHE was added to the cells for 30 min (37°C). Cells were washed (2 × 400 µl) to remove any unincorporated DHE. Fluorescent images were randomly acquired from culture wells using a DP73 digital color camera (Digital Video Camera Co) mounted on an Olympus IX50 inverted microscope outfitted with epifluorescence and controlled by CellSens Standard (Olympus, Center Valley, PA) software. Exposure time, brightness and contrast were standardized for each picture. For

quantification, the total fluorescent intensity was normalized to the total cell number in each individual picture using ImageJ software (V1.49o, National Institute of Health).

Measurement of Cell Death and Viability

Cell death was quantitatively determined by spectrophotometric measurement of LDH activity as described previously (Uliasz and Hewett 2000). Data are expressed as a percentage of total astrocytic LDH activity (defined as 100% cell death) determined by exposing parallel cultures to 0.9 or 1.5 mM tBOOH for 20–24 hr.

Cell viability was quantified via colorimetric analysis of MTT (Sigma, St. Louis, MO) reduction as previously described (Lobner 2000). Following treatment, MTT was added to the cultures (final concentration = 300 µg/ml) for at least 3 hr at 37°C, after which the solution was carefully aspirated, and the resulting crystals solubilized in acidified isopropanol (90% isopropanol; 10% 1 N HCl; 400 µl/well). Two hundred µl was transferred to a 96-well plate and absorbance at 540 nm was measured against a 690 nm background subtraction (SpectraMax M2, Molecular Devices). Percent viable astrocytes was quantified by normalization of experimental MTT absorbance values to values obtained from untreated control cells (i.e., highest absorbance = 100%) as well as cells treated with 1.5 mM tBOOH or 125 µM FeSO₄/20 µM NaP, which results in complete loss of viability (defined as 0%).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism (Version 6.0.1, GraphPad Software, Inc.) as described in each figure legend. As percentage data and normalized data are by nature non-normally distributed, such data were first transformed via arcsin square root or $-1 \times \log(Y)$, respectively, before analysis. In all experiments, data are expressed as the mean + SEM. Significance was assessed at $p < 0.05$.

RESULTS

Treatment of purified cortical astrocytes with IL-1 β (3 ng/ml) resulted in a time-dependent increase in GSH that accumulated in the extracellular medium ([GSH]_e) at both 24 and 48 hr, while intracellular levels remained— for the most part— unchanged (Figure 1A). Basal [GSH]_e as well as the IL-1 β -mediated enhancement were concentration-dependently blocked by concomitant treatment with MK-571 (25–100 µM; Figure S1), suggesting that release occurred via the multidrug resistant protein, MRP-1, as has been reported previously (Hirrlinger et al. 2002). The fact that the extracellular reduced /oxidized glutathione (GSH:GSSG) ratio increased throughout the IL-1 β treatment indicated that IL-1 β did not *a priori* cause oxidative stress (Figure 1B); this result was confirmed via direct measurement of reactive oxygen species (ROS) as shown in Figure 9.

To understand the mechanism governing the increased GSH production, we investigated whether activation of the IL-1 β canonical signaling pathways, namely p38 MAPK and/or NF κ B, was responsible for the IL-1 β -mediated increase in [GSH]_e. Treatment of astrocyte cultures with IL-1 β resulted in a rapid activation of p38 MAPK as determined by increased detection of phospho-p38 in total cellular lysates (Figure 2A). Although the selective inhibitor, SB203580, significantly attenuated p38 MAPK activation (Figure 2A), it did not

reduce — but actually increased— [GSH]_e following IL-1 β treatment (Figure 2B). Next, we employed indirect immunofluorescence cytochemistry (ICC) to visualize whether changes in the subcellular localization of the p65 component of the NF κ B complex in astrocytes occurred following IL-1 β exposure. We found that under basal conditions (0 min), p65 is exclusively present within the cytoplasm of astrocytes, but translocates to and accumulates in the nucleus within 15 min of IL-1 β stimulation, largely returning to baseline levels by 2 hr (Figure 3). A 30 min pretreatment with the I κ B/IKK inhibitor, TPCA-1, reduced the IL-1 β -mediated nuclear translocation of p65 (Figures 4, 5A). Furthermore — at concentrations that suppressed p65 translocation (Figures 4, 5A) — this same inhibitor prevented the increase in [GSH]_e levels (Figure 5B), indicating that the regulation of GSH production by IL-1 β in astrocytes is dependent on NF κ B activation.

To determine whether IL-1 β could, *a priori*, confer selective resistance to oxidative insults via modulation of GSH synthesis, astrocyte cell death was compared between vehicle and IL-1 β -treated cultures following incubation with increasing concentrations of ferrous sulfate FeSO₄/Na pyruvate or tBOOH. These oxidants were chosen as detoxification has been demonstrated to be GSH-dependent (Dringen et al. 1998; Milchak and Douglas Bricker 2002), as confirmed herein (Figure 6). Specifically, in our model system reduction of GSH synthesis via incubation of cells with buthionine sulfoximine (BSO), a γ -glutamylcysteinyl ligase inhibitor (Griffith 1982), enhanced both astrocyte cell death induced by both FeSO₄ (1.5 and 2 μ M) and a sub-lethal concentration (0.3 mM) of tBOOH. BSO alone was not toxic (Figure 6).

Interestingly and importantly, the decrease in astrocyte viability that followed FeSO₄ exposure (1.5 μ M) was significantly attenuated by a 48 hr pretreatment with IL-1 β (Figure 7A). Likewise, exposure to tBOOH produced a concentration-dependent increase in astrocyte cell death that was attenuated at all concentrations by IL-1 β treatment (Figure 7B). This protection was occluded by simultaneous exposure to BSO (50 μ M), at a concentration that reduced IL-1 β -mediated increase in [GSH]_e to basal levels (Figure 8). Direct confirmation that IL-1 β protects astrocytes by reducing oxidative stress is shown in Figure 9. Exposure to tBOOH (0.7 mM) for 45 min resulted in an increase in ROS production that was completely abolished by prior treatment of the cultures with IL-1 β . IL-1 β exposure alone did not increase ROS generation (Figure 9). Finally, astrocyte cultures derived from *il1r1* null mutant mice, that is derived from mice lacking the IL-1 β signaling receptor, show no increase in [GSH]_e following stimulation with IL-1 β , nor do these cells enjoy any protection from IL-1 β treatment as compared to astrocytes derived from wild-type control mice (Figure 10). All together, these results indicate that the antioxidant properties of IL-1 β rest on its ability to increase GSH production in an NF κ B-dependent manner.

DISCUSSION

GSH is the predominate low molecular weight thiol in cells. As an essential antioxidant, GSH reacts non-enzymatically with free radicals, and also functions as a critical cofactor for the antioxidant enzyme glutathione peroxidase. GSH also plays important roles in cell metabolism and proliferation, gene expression, cytokine production, and signal transduction [for review see (Lu 2009; Wu et al. 2004)]. Regulation of GSH levels in brain, which has

high oxygen consumption, is extremely important for maintenance of its proper function. Since circulating GSH is unable to penetrate the blood brain barrier (Cornford et al. 1978), GSH within the CNS must be generated *in situ*. In brain, astrocytes play a prominent role in GSH metabolism (Dringen et al. 2000; Keelan et al. 2001; Sun et al. 2006). The fact that astrocytes synthesize and release GSH into the extracellular space is well known (Dringen et al. 1999; Hirrlinger et al. 2002; Sagara et al. 1996; Wang and Cynader 2000; Yudkoff et al. 1990) and represents an important method by which thiol levels in the CNS are maintained (Dringen et al. 1999; Keelan et al. 2001; Wang and Cynader 2000). Remarkably, it has been estimated that $\approx 10\%$ of astrocytic GSH is released per hour (Dringen et al. 1997), indicating that synthesis must match release in order to maintain a constant concentration of GSH. We previously demonstrated in a mixed cortical cell culture system that IL-1 β increased system x_c^- activity (Fogal et al. 2007), then localized the phenomenon to occur exclusively in astrocytes, not neurons or microglia (Jackman et al. 2010). Due to the well-characterized ability of system x_c^- to import cystine to support synthesis of the antioxidant molecule, glutathione (GSH) (Bassi et al. 2001; Bridges et al. 2001; Sato et al. 1999), herein, we assessed whether IL-1 β could enhance GSH production and if so, whether this increase could protect cells from oxidant injury.

In this study, we demonstrate that IL-1 β significantly increases $[GSH]_e$ (Figure 1A). Despite our ability to measure only a modest change $[GSH]_i$, it has been reported that the release of GSH by astrocytes depends strongly on the intracellular GSH concentration (Sagara et al. 1996). Hence, the elevated $[GSH]_e$ that follows IL-1 β exposure is most likely a direct consequence of increased synthesis followed by release. Moreover, the fact that MK-571 effectively blocked the release of GSH from astrocytes under both basal and IL-1 β -stimulated conditions (Figure S1) suggests that the transporter, multidrug resistant protein 1 (MRP-1), is involved in release, as has been demonstrated previously (Hirrlinger et al. 2002; Minich et al. 2006).

GSH levels in astrocytes can be experimentally regulated — either positively or negatively — using a variety of treatment strategies [for review see (Dringen 2000)]. Studies assessing the ability of IL-1 β , specifically, to affect GSH levels have produced dichotomous results. While we demonstrate that IL-1 β is a potent positive regulator of GSH synthesis/release in mouse astrocytes (Figure 1A), intracellular levels of reduced GSH were shown to be decreased following IL-1 β exposure in astrocytes derived from rat (Singh et al. 1998). Similar results were demonstrated in a human glioma cell line (Malaplate-Armand et al. 2000). The depletion of GSH in these studies was attributed to the production of oxidative stress occurring secondary to IL-1 β treatment (Malaplate-Armand et al. 2000; Singh et al. 1998). In contrast, using the GSH:GSSG ratio as a proxy for the cells redox potential/oxidant status (Schafer and Buettner 2001) as well as direct measurement of ROS, we find that IL-1 β does not contribute to oxidative stress in murine astrocytes (Figure 1B,9). Enhanced cellular synthesis of GSH has been demonstrated in human endometrial stromal cells and mouse endothelial cells following treatment with IL-1 β as well (Lee et al. 2009; Urata et al. 1996).

The absence of $[GSH]_e$ in astrocytes derived from cortical tissue of mice null for the IL-1 β signaling receptor, IL-1R1 (Figure 10A), not only confirms the purity of our recombinant

IL-1 β preparation, but presents clues as to the mechanism by which IL-1 β may regulate GSH synthesis. Since the canonical signaling pathways for IL-1R1 include NF κ B and p38 MAPK [for review see (Dinarello 2009)], we assessed their involvement. In agreement with previous studies (Dunn et al. 2002; Friedman et al. 1996; Guo et al. 2004; Molina-Holgado et al. 2000; Xia and Zhai 2010), we demonstrate that treatment of murine astrocytes with IL-1 β leads to the activation of both NF κ B and p38 MAPK (Figures 2, 3). Interestingly, the IL-1 β -mediated increase in [GSH]_e was found to be independent of p38 MAPK activation (Figure 2), with its inhibition actually resulting in an enhancement of GSH production. These results suggest strongly that p38 MAPK is a negative regulator of IL-1 β signaling under these conditions. This is in agreement with previous papers utilizing microglia-conditioned medium and TNF α (Correa et al. 2011; Correa et al. 2012) as stimuli.

In contrast, inhibition of NF κ B activation, did prevent the IL-1 β -mediated increase in [GSH]_e (Figure 5). Given its role as a transcription factor (O'Neill and Kaltschmidt 1997), it seems plausible that treatment with IL-1 β enhances the expression of proteins involved in GSH synthesis. In support of this contention, we previously demonstrated that IL-1 β transcriptionally regulates the substrate specific light chain for system x_c⁻ (xCT) leading to increased uptake of cystine in astrocytes (Jackman et al. 2010). Whether other components of the astrocyte GSH metabolism machinery are also regulated and contribute to the increased GSH production following IL-1 β exposure is a subject of investigation on-going in the laboratory.

GSH has been reported to protect astrocytes against various insults (Badawi et al. 2012; Kim et al. 2003; Scheiber and Dringen 2013). Herein, we demonstrate that IL-1 β exposure renders mouse astrocytes more resistant to toxic insult mediated by the organic hydroperoxide, tBOOH, and by FeSO₄ (Figure 7), most likely by eliminating the enhanced ROS production that follows their exposure (shown directly for tBOOH in Figure 9). We chose these two stressors specifically as it has been demonstrated previously and confirmed herein that their detoxification is GSH-dependent (Figure 6). Indeed, inhibition of GSH production by pretreatment with BSO eliminates the IL-1 β -mediated protective response (Figure 8). Likewise, astrocyte cultures derived from *il1r1* null mutant mice demonstrate no increase in GSH and enjoy none of the protective effects of IL-1 β (Figure 10). The less fulminate protection demonstrated against the toxicity of FeSO₄ as compared to tBOOH could be due to the fact that, along with GSH, catalase has been shown to be involved in the detoxification of iron (Liddell et al. 2004), whereas the elimination of tBOOH is solely GSH-dependent (Dringen et al. 1998).

This effect of IL-1 β likely has broader implications beyond its glioprotective benefit. Numerous studies demonstrate that GSH released by astrocytes, that is, the cycling of GSH and/or its metabolites between astrocytes and neurons, is necessary for the maintenance of neuronal GSH levels (Dringen et al. 2000; Dringen et al. 1999; Schulz et al. 2000). Additionally, depletion of astrocyte GSH renders neurons more susceptible to oxidative insults (Abramov et al. 2003; Drukarch et al. 1997; Gegg et al. 2005; Shih et al. 2003). Hence, this IL-1 β -mediated enhanced GSH production and release from astrocytes could potentially be neuroprotective as well.

The notion that protective effects could be mediated by an inflammatory mediator is not unprecedented. With respect to IL-1 β , its ability to mount protective responses in the CNS and in the periphery has previously been reported [for review see (Hewett et al. 2012)]. For instance, pretreatment with IL-1 β protected mice against lethal challenge via radiation or infectious agent (Neta et al. 1986; Schwartz et al. 1987; van der Meer et al. 1988) and ameliorated ischemic injury in rat hearts (Brown et al. 1990). Within the CNS, IL-1 β treatment protected rat organotypic hippocampal cultures from simulated ischemia (Pringle et al. 2001) and endogenously produced as well as exogenously administered IL-1 β has been demonstrated to be a mediator of ischemic tolerance in gerbil (Ohtsuki et al. 1996). Further, IL-1 β enhanced neuronal sprouting and/or regeneration *in vitro* and in the hippocampus and ventral tegmental nucleus *in vivo* (Fagan and Gage 1990; Temporin et al. 2008; Wang et al. 1994) and IL-1 β -deficient mice were worse than their WT counterparts at blood brain barrier repair following corticectomy as assessed via extravasation of IgG into brain parenchyma (Herx and Yong 2001). Finally, chronic overexpression of IL-1 β ameliorated amyloid load observed in the hippocampus in a mouse model of Alzheimer's disease (Shafteel et al. 2007), although control animals demonstrated impaired spatial and contextual memory development (Matousek et al. 2010; Moore et al. 2009).

Despite its ability to support normal CNS physiology and to participate in protective and/or reparative processes, IL-1 β has also been demonstrated to contribute to the pathology underlying a number of CNS disorders/diseases [see our current review for details (Hewett et al. 2012)]. Indeed, our own previous work indicated that IL-1 β potentiated neuronal injury under conditions of energy deprivation (Fogal et al. 2005; Fogal et al. 2007; Jackman et al. 2010; Uliasz et al. 2012). Given these pleiotrophic actions, whether IL-1 β initiates or results from damage and whether it promotes, halts or repairs injury, will have to be systematically evaluated in each experimental condition. The ultimate outcome may depend heavily on its local concentration, the environmental milieu, the cellular target, the presence or absence of negative feedback regulators, and the temporal characteristics of the response (Hewett et al. 2012). Understanding better the physiological and pathophysiological consequences of IL-1 β signaling in brain may help us devise strategies to harness its beneficial effects while employing strategies to reduce its ability to facilitate injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Main Points

1. IL-1 β increases astrocyte GSH production via an NF κ B-dependent, p38 MAPK-independent mechanism.
2. The glioprotective effect of IL-1 β against oxidant injury is mediated by enhanced GSH production.

Figure 1. IL-1 β increases astrocyte GSH levels

(A) Pure astrocytes in 24 well plates (400 μl well volume; $104.02 \pm 0.46 \mu\text{g protein/well}$) were incubated with IL-1 β (3 ng/ml) or vehicle for 24 or 48 hr, after which total intracellular and supernatant GSH levels were measured. Data are expressed as mean + SEM. An asterisk (*) denotes significant between-group (comparisons between vehicle and IL-1 β -treated cultures) differences as assessed by two-way ANOVA followed by Bonferroni's test for multiple comparisons. $n=6$ from three separate dissections. (B) Pure astrocytes were treated with IL-1 β (3 ng/ml) or vehicle (0 hr) for the times indicated after which GSH levels were

measured and GSSG levels calculated (see methods). Data are expressed as the ratio of GSH:GSSG (mean + SEM). n = 6 from two separate dissections.

Figure 2. IL-1β-mediated p38 MAPK activation does not underlie its ability to increase GSH production in cortical astrocytes

(A) Cells were incubated with or without the selective p38 MAPK inhibitor, SB203580, for 30 min in maintenance medium after which IL-1β (final concentration = 10 ng/ml) or vehicle was spiked in. Ten min later, protein was isolated and activated p38 MAPK (p-p38) was detected via western blot. Phosphorylation levels of p38 MAPK were quantified and normalized to their respective β-actin levels, and expressed as mean fold change over control (0 μM SB203580 without IL-1β). The graph represents the combined data of three

independent experiments. An asterisk (*) indicates values significantly different from IL-1 β alone as determined by two-way ANOVA followed by Sidak's multiple comparison's test. **(B)** Purified astrocyte cultures (n=16 from four separate dissections) were treated with SB203580 for 30 min, after which IL-1 β (final concentration = 3 ng/ml) or vehicle was spiked in. Forty-eight hr later, total extracellular GSH levels were measured. Data are expressed as mean + SEM. An asterisk (*) indicates values significantly different from their respective control value (-IL-1 β) whereas a pound sign (#) denotes values significantly greater than IL-1 β alone as determined by two-way ANOVA followed by Sidak's multiple comparisons test.

Figure 3. Astrocytic activation of transcription factor NF- κ B following IL-1 β stimulation
Purified astrocytes were incubated with IL-1 β (3 ng/ml) for the times indicated. Protein levels of p65 were examined by indirect immunofluorescence microscopy. Representative photos depict phase contrast (left panels), p65 nuclear translocation (red), DAPI (blue), and merged (magenta) images from three independent experiments.

Figure 4. The IKK inhibitor, TPCA-1, prevents IL-1 β -mediated nuclear translocation of p65 as assessed by indirect immunofluorescence

Purified astrocytes were treated with TPCA-1 (10 or 20 μ M; 30 min) after which vehicle (Ctrl) or IL-1 β (final concentration = 3 ng/ml) was added directly to the wells. After 15 min, cells were fixed and processed for immunocytochemistry (see methods). Representative photos depict phase contrast (left panels), p65 labeling (red), and their corresponding DAPI (blue) as well as the merged (magenta) images from three independent experiments.

Figure 5. TPCA-1 inhibits IL-1 β -mediated p65 protein nuclear translocation and GSH production

Purified astrocyte cultures were treated for 30 min with TPCA-1 at the indicated concentrations, after which IL-1 β (final concentration = 3 ng/ml) or vehicle (– IL-1 β) was added. (A) Twenty min later, cells were harvested, nuclei isolated and 15–30 μ g of nuclear protein was separated by SDS-PAGE (12% gel). Western blot analysis was performed using antibodies directed against p65 and β -actin (loading control). Lane 1; Vehicle; Lane 2, IL-1 β ; Lane 3, Vehicle + 10 μ M TPCA-1; Lane 4, IL-1 β + 10 μ M TPCA-1; Lane 5, Vehicle + 20 μ M TPCA-1; Lane 6, IL-1 β + 20 μ M TPCA-1. For quantification, p65 protein levels

were normalized to their respective β -actin levels and expressed as mean + SEM fold change over vehicle alone. The graph represents the combined data from four independent experiments. **(B)** Forty-eight hr after treatment, the extracellular GSH was measured. Data are expressed as mean + SEM; n=7 cultures from two separate dissections. Asterisks (*) denote significant within-group differences (+ or - IL-1 β treated cultures, each compared to their respective 0 μ M TCPA-1) as determined by two-way ANOVA followed by Sidak's multiple comparisons test.

Figure 6. GSH dependency of FeSO₄ and tBOOH detoxification

(A) Pure astrocyte cultures (n=12 from two separate dissections) were incubated with BSO (100 μM) or vehicle for 48 h, after which cultures were exposed to FeSO₄ (1.5 or 2.0 μM) for 2.5–4 hr. Cell viability is expressed as the mean + SEM MTT absorbance normalized to untreated cultures. An asterisk (*) denotes significant within-group differences (+ or - BSO treated cultures, each compared to their respective 0 μM FeSO₄) whereas a pound (#) indicate significant between-group (+ and - BSO at each FeSO₄ concentration) differences determined by two-way ANOVA followed by Bonferroni's test for multiple comparisons.

(B) Pure astrocytes were incubated with vehicle or BSO (100 μ M) for 24 hr, after which they were exposed to a normally innocuous concentration of tBOOH (0.3mM). Three and ½ hr later, the percentage of cells surviving was determined. Values are expressed as mean + SEM MTT absorbance normalized to the untreated group; n=6 cultures from three separate experiments. An asterisk (*) indicates a significant between-group difference (+ and - BSO) as assessed by two-way ANOVA followed by Bonferroni's test for multiple comparisons.

Figure 7. IL-1 β treatment renders astrocytes less susceptible to both FeSO₄- and tBOOH-mediated oxidative injury

(A) Pure astrocytes (n=6 from two separate dissections) were incubated with IL-1 β (3 ng/ml) or vehicle (-IL-1 β) for 48 h, after which FeSO₄ was spiked in for a final concentration 1.5 μ M. MTT was added 2.5–4 hr later to terminate the experiment. Data are expressed as the mean + SEM MTT absorbance normalized to untreated group. Asterisks (*) denote significant within-group differences (+ or - IL-1 β , each compared to their respective - FeSO₄) whereas pound signs (#) represent significant between-group differences (+ and - IL-1 β with or without 1.5 μ M FeSO₄) as determined by two-way ANOVA followed by

Bonferroni's test for multiple comparisons. **(B)** Pure astrocytes were incubated with IL-1 β (3 ng/ml) or vehicle (-IL-1 β) for 48 hr, after which cells were treated with increasing concentrations of tBOOH for 2.5 – 3.5 hr. Values are expressed as the mean LDH release + SEM normalized to the mean LDH released by cells treated with 0.9 mM tBOOH exposure, which results in complete astrocyte cell death; n=8 cultures from three separate dissections. An asterisk (*) indicates a significant between-group difference (+ and - IL-1 β at each tBOOH concentration) as assessed by two-way ANOVA followed by Bonferroni's test for multiple comparisons.

Figure 8. BSO occludes the protective effect of IL-1 β against oxidative injury

Pure astrocyte cultures ($n=7-8$ from three separate dissections) were incubated with BSO (+ BSO; 50 μM) or vehicle (- BSO) in the presence (+) or absence (-) of IL-1 β (3ng/ml). (A) After 48 hr, the total extracellular GSH levels were measured and values expressed as mean + SEM. (B) These same cells were then exposed to tBOOH (0.7mM) and the percent cell death measured 2.75 hr later. Values are expressed as the mean LDH release + SEM normalized to LDH levels released following exposure to 1.5 mM tBOOH for 24 h, which results in complete astrocyte cell death. (A, B) Asterisks (*) denote significant between-

group differences (+ and – IL-1 β with or without BSO) whereas a pound sign (#) represents significant within-group differences (+ or – IL-1 β treatment) determined by two-way ANOVA followed by Bonferroni's multiple comparisons test.

Figure 9. ROS generation in astrocytes detected by oxidation of DHE

Purified astrocyte cultures were treated with IL-1 β (3 ng/ml) or vehicle for 48 h, after which tBOOH (0.7 mM) was added for 45 min. (A) Representative photos depict phase contrast (left panel) as well as DHE fluorescence (right panel) from four independent dissections. (B) For quantification, total fluorescent intensity was normalized to total cell number in each image and expressed as mean + SEM fold change over control (– IL-1 β , – tBOOH); n = 4. Asterisks (*) denote significant within-group differences (+ or – IL-1 β treatment groups) and a pound sign (#) represents significant between-group differences (+ and – IL-1 β with or without tBOOH treatment) determined by two-way ANOVA followed by Bonferroni's multiple comparisons test.

Figure 10. IL-1 β does not facilitate GSH production or protection in astrocytes derived from IL1R1 null mutant mice

Pure astrocyte cultures derived from either wild-type (+/+) or *il1r1* knock-out mice (-/-) were treated with IL-1 β (3 ng/ml) or vehicle (- IL-1 β) for 48 hr, after which (A) supernatant GSH levels were measured (n=6 from two dissections) or (B) cells were exposed to 0.7mM tBOOH for 2–2.5 hr (n=11 from four dissections). Cell death, normalized to levels found following exposure to 1.5 mM tBOOH (= 100%), was assessed via the LDH assay. (A, B) Asterisks (*) denotes significant between-group differences (+ and - IL-1 β in same

genotype) whereas pound signs (#) represents significant within-group differences (+ or – IL-1 β between genotypes) as determined by two-way ANOVA followed by Bonferroni's test for multiple comparisons.