The Differential Effects of Erythropoietin Exposure to Oxidative Stress on Microglia and Astrocytes in vitro

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Keywords

Glia · Hydrogen peroxide · Injury · Neuroinflammation

Abstract

The neonatal brain is especially susceptible to oxidative stress owing to its reduced antioxidant capacity. Following hypoxic-ischemic (HI) injury, for example, there is a prolonged elevation in levels of hydrogen peroxide (H_2O_2) in the immature brain compared to the adult brain, resulting in lasting injury that can lead to life-long disability or morbidity. Erythropoietin (Epo) is one of few multifaceted treatment options that have been promising enough to trial in the clinic for both term and preterm brain injury. Despite this, there is a lack of clear understanding of how Epo modulates glial cell activity following oxidative injury, specifically, whether it affects microglia (Mg) and astrocytes (Ast) differently. Using an in vitro approach using primary murine Mg and Ast subjected to H_2O_2 injury, we studied the oxidative and inflammatory responses of Mg and Ast to recombinant murine (rm)Epo treatment. We found that Epo protects Ast from H_2O_2 injury ($p < 0.05$) and increases secreted nitric oxide levels in these cells ($p < 0.05$) while suppressing intracellular reactive oxygen species ($p < 0.05$) and superoxide ion ($p < 0.05$) levels only in Mg. Using a multiplex analysis, we noted

that although H_2O_2 induced the levels of several chemokines, rmEpo did not have any significant specific effects on their levels, either with or without the presence of conditioned medium from injured neurons (NCM). Ultimately, it appears that rmEpo has pleiotropic effects based on the cell type; it has a protective effect on Ast but an antioxidative effect only on Mg without any significant modulation of chemokine and cytokine levels in either cell type. These findings highlight the importance of considering all cell types when assessing the benefits and pitfalls of Epo use.

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Injury to the developing brain is a leading cause of neurodevelopmental disabilities, resulting in life-long morbidity in survivors, with hypoxic-ischemic (HI) damage being the most common cause of brain injury among newborns [1, 2]. We previously reported that the developing brain is particularly sensitive to oxidative injury [3] and experiences a greater accumulation of hydrogen peroxide (H_2O_2) following HI injury compared to the adult brain [4]. This H_2O_2 subsequently combines with the free iron in the immature brain, producing damaging free radicals that disrupt key cellular processes that lead to cell death. While several antioxidative treatments have shown

promise over the years, none of them is yet clinically acceptable due to the uncertainty about their beneficial effects [5]. Consequently, there is an unmet need for treatment options for HI brain injury that address oxidative-stress-induced HI damage along with other effects such as inflammation, cell death, and repair.

Erythropoietin (Epo) is a hematopoietic cytokine with an established role in neuroprotection. Although it is primarily produced in the kidneys and liver, studies also show the expression of Epo receptor and Epo production in the brain early in development [6, 7]. Epo production is evident primarily in astrocytes (Ast) [8], and to a lesser extent in neurons [9] and oligodendrocytes [8, 10], where it occurs in a regulated manner. All these cells, along with microglia (Mg) [11], also express Epo receptor and see an upregulation after injury [12], suggesting that Epo has a role in postinjury processes in the brain. Indeed, several lines of evidence, both in vivo and in vitro, have now established that Epo has important neuroprotective, neurogenic, angiogenic, and oligodendroglial properties after HI [10, 13, 14]. Promising clinical trials with Epo as a potential treatment for preterm and term brain injury are currently underway [15].

Despite its promise as a potential treatment for HI injury, there are currently limited data on the specific effects of Epo on oxidative and inflammatory responses after HI injury, especially in glial cells. While some evidence suggests that Epo has antiapoptotic, antioxidative, and anti-inflammatory effects on Ast and Mg [16], few reports exist of posttreatment with Epo, and even fewer report Epo effects after a direct oxidative insult on these cells. Moreover, closer analysis of the literature suggests that Epo can have differential effects on different cell types [16], highlighting the importance of understanding the timing and pattern of these responses in determining effective dosing strategies for clinical trials.

The aim of this study was to characterize the oxidative and inflammatory responses of Ast and Mg to exogenous Epo following an oxidative insult utilizing H_2O_2 exposure as the model of oxidative stress injury in vitro. We have previously shown that H_2O_2 levels in the immature (but not adult) brain are sustained at high levels after injury for at least 5 days [17], rendering it highly susceptible to oxidative injury over a prolonged period. In order to mimic this in vitro, we utilized an enzymatic system where we used glucose oxidase and catalase to generate and break down (respectively) H_2O_2 and maintain it at a constant level [18, 19]. We have previously shown that this system can be successfully used in vitro on Mg to induce oxidative and inflammatory responses [18]. Here,

we extend this analysis to cultured Ast, examine the responses after treatment with Epo, and show that Epo has differential, pleiotropic effects on Mg and Ast.

Experimental Procedures

Materials

The following reagents were all sourced from the Cell Culture Facility at the University of California San Francisco, CA, USA: Hanks' balanced salt solution, Dulbecco's modified Eagle's medium (DMEM; phenol red-free, 4.5 g/L glucose, with no pyruvate), penicillin-streptomycin (P/S), fetal bovine serum (FBS; Axenia Biologix), and 0.05% trypsin. Glucose oxidase (GOX, G0543), catalase (CAT, C3155), H_2O_2 (H1009), and H2DCFDA (#D6883) were from Sigma (St. Louis, MO, USA). Neurobasal (NB) medium (12348-017), B27 (17504044), B27 without antioxidants (10889038), GlutaMax I (35050), Griess reagent (G7921), Live-Dead Assay (L3224), and the LDH cytotoxicity kit (11644793001) were from Roche. Recombinant murine (rm)Epo (959-ME-010) was from R&D Systems (reconstituted in 0.1% BSA PBS).

Mg and Ast Cultures and H_2O_2 Treatment

Animals were sacrificed in accordance with guidelines from the Institutional Animal Care and Use Committee at the University of California San Francisco, CA, USA. Mixed glial cultures were prepared from P1 C57BL/6 mice (Simonsen Laboratories, Gilroy, CA, USA). Their brains were isolated, stripped of meninges, mechanically dissociated, and trypsinized for 15 min at 37°C. Following resuspension in DMEM supplemented with 1% P/S and 10% FBS, cells were seeded (2 brains/T-75 flask) and then incubated at 37°C/5% CO_2 . Medium was changed the next day and then every 3 days subsequently. After 10–14 days, the flasks containing mixed glial cultures were shaken for 1–2 h at 200 rpm and 37°C to lift off the microglial cells, which were then plated into 24-well plates in DMEM supplemented with 1% P/S and 2% FBS overnight and used the next day. Cells from a 75-cm² flask were seeded into a 24-well plate. Ast contamination in Mg cultures was confirmed to be <5% using immunofluorescence (Iba-1 for Mg, glial fibrillary acid protein for Ast, and O_4 for oligodendrocyte labeling; data not shown).

Following Mg isolation, the flasks were replenished with fresh DMEM supplemented with 1% P/S and 10% FBS, sealed with Parafilm, and shaken for 24 h. The medium was replaced again, and the flasks resealed and shaken for another 24 h until no phase-dark cells (oligo-

dendroglia) were visible. Ast were then trypsinized with 0.05% trypsin, pelleted, and plated into 24-well plates at a density of 250,000 cells/well overnight and used the next day. The Mg contamination of Ast cultures was established to be <5% as confirmed by immunocytochemistry (Iba-1 for Mg, glial fibrillary acid protein for Ast, and O₄ for oligodendrocyte labeling; data not shown).

Mg and Ast Stimulation with H₂O₂ and Treatment with rmEPO

The GOX-CAT method is an enzymatic method that can be used to generate low, continuous H₂O₂ for over 24 h [18, 19]. GOX is a stable enzyme that remains fully active over 24 h at 37°C and generates H₂O₂ by consuming O₂, while CAT degrades H₂O₂ back to H₂O and O₂ and is not saturable, even at molar concentrations of H₂O₂. This means that while GOX generates H₂O₂ over 24 h, CAT continuously breaks it down in a typical exponential kinetic fashion that is directly dependent on the H₂O₂ concentration. Accordingly, stable H₂O₂ concentrations are generated that only depend on the ratio of enzyme activities. Thus, by regulating the specific dilutions of CAT (and thus the rate of H₂O₂ breakdown) in the medium, it is possible to produce controlled amounts of H₂O₂ continuously for at least 24 h within a variability of 20%. No significant hypoxia is induced for 24-well plates using a GOX activity of 1:100,000 and a medium volume of 0.5 mL [19].

The aims of the experiments were to study the responses of these cell types to low and high continuous exposures of H₂O₂. The low and high doses for each of the cell types was determined from titration studies where the dose eliciting minimal, i.e., ≤2-fold of control cell death, was considered a low dose, and that 2- to 4-fold of control cell death was equated as a high dose, as measured at 24 h after exposure. In all experiments, GOX was kept at a constant dilution of 1:100,000, whereas CAT dilution varied (1:20,000 [low], 1:960,000 [high for Mg]/1:1,280,000 [high for Ast]) resulting in H₂O₂ levels of around 1–3 μM. Since serum can contain CAT, all H₂O₂ treatments were carried out in serum-free DMEM (DMEM SF) with 1% P/S. DMEM SF served as a control for the H₂O₂ treatments. Following overnight plating in 2% FBS DMEM, the medium in the plates was replaced with prewarmed test medium (containing GOX-CAT/control) for 4 h. At 4 h, rmEpo was added into the medium at a final concentration of 10 ng/mL in DMEM SF (a volume of 10 μL), with the dose chosen based on previously published data showing this to be an effective dose for eliciting cellular effects [20, 21]; 10 μL DMEM SF was added into the non-Epo control wells at the same time.

All assays were run in duplicate and repeated independently 6 times. Experiments were ended 20 h later (24 h from the start of H₂O₂ treatment). For pretreatment studies, cells were exposed to 10 ng/mL Epo for 24 h prior to H₂O₂ exposure for 2 h.

In vitro Assays

All in vitro assays were carried out at the end of the study (24 h from the start of H₂O₂ treatment and 20 h from Epo addition), unless otherwise stated.

Viability Assay

Mg and Ast viability in response to H₂O₂ treatment were assessed using the LDH assay according to the manufacturer's instructions. LDH release into the medium was compared to total LDH in the medium following cell lysis. For pretreatment studies, LDH release was measured at the end of 2 h of H₂O₂ exposure.

Nitrite Assay

Nitrite levels in medium were used as a surrogate measure of nitric oxide (NO) using the Griess reagent according to the manufacturer's protocols. Briefly, at the end of the designated time period, 150 μL of medium from each well was mixed with 20 μL of Griess reagent and 130 μL of deionized water for 30 min at room temperature. Absorbance was measured and expressed relative to the reference (sodium nitrite) sample at 548 nm after normalizing to the amount of protein in the well.

Reactive Oxygen Species Assay

Cellular reactive oxygen species (ROS) content was measured using the H2DCFDA reagent. At the end of the designated time period, cells were loaded with 20 μM H2DCFDA for 45 min at 37°C. Following 2 washes with prewarmed Hanks' balanced salt solution, fluorescence absorption was measured at 485^{ex}/535^{em} nm. Results were normalized to the amount of protein in the well.

Mitochondrial Superoxide Assay

Mitochondrial superoxide levels were measured using MitoSOX Red indicator dye. As it is readily oxidized by superoxide but not by other ROS-/reactive nitrogen species-generating systems, it would give an accurate measure of superoxide levels in response to H₂O₂ treatment. At the end of the time period, cells were loaded with 5 μM MitoSOX dye in prewarmed DMEM SF for 10 min at 37°C. Following 2 washes in the same medium, fluorescence absorption was measured at 485^{ex}/590^{em} nm, and results were normalized to the amount of protein in the well.

Fig. 1. H₂O₂-induced LDH release is not attenuated by treatment with rmEpo at 4 h. Cultured primary murine astrocytes (Ast) and microglia (Mg) were exposed to continuous H₂O₂ for 4 h before treatment with 10 ng/mL rmEpo. LDH release into the medium was measured after 20 h of Epo treatment (24 h after the start of H₂O₂ injury). Mg (**a**) and Ast (**b**) LDH release. Pretreatment (1 h) with 10 ng/mL Epo before administering H₂O₂ for 2 h elicited protection in Ast (**d**) but not in Mg (**c**). Results are a composite of at least 6 (posttreatment) and 3 (pretreatment) independent assays. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ versus control.

Multiplex Assay for Cytokine Measurements

Cytokine protein expression in Mg-/Ast-conditioned medium was assessed according to the manufacturer's instructions on a custom mouse 15-plex cytokine MicroBead assay kit which allows for the simultaneous detection of 15 inflammatory molecules in a single 75-μL sample including KC, MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES, G-CSF, IL-1a, IL-1b, IL-4, IL-6, IL-9, IL-10, IL-13, and TNFα. Results were analyzed using a Bio-Plex workstation (Bio-Rad), and then levels were normalized to the total amount of protein in the appropriate well and subsequently expressed as fold of control. For discussion purposes, KC, MCP-1, MIP-1α, MIP-1β, MIP-2, and RANTES were loosely categorized as chemokines, with the remainder classified as cytokines. Protein concentrations were determined using the Bio-Rad DC protein assay. Data are expressed as picograms per microgram of protein. The level of sensitivity for each MicroBead cytokine standard curve ranged from 1 to 35 pg/mL. Two 96-well assays were performed in duplicate, with each plate containing Mg-/Ast-conditioned medium from 6 independent experiments. For neuron-conditioned medium

(NCM) studies, medium from 3 independent experiments was assessed.

Primary Cortical Neuronal Culture and Generation of NCM

Cultures were prepared from the cortices of embryonic day 15 C57BL/6 mice as previously described [22]. Briefly, embryos were harvested from the dam under terminal anesthesia, and the cortices were isolated, papain digested, and plated onto PDL-pre-coated 24-well plates in NB medium. On day 3 in vitro (DIV3), AraC was added to a final concentration of 5 μM, and on DIV4, half of the medium was exchanged for fresh NB medium with B27-antioxidants. Experiments were initiated on DIV6 when medium was replaced with fresh NB medium with B27-antioxidants containing 50 μM H₂O₂ (or just NB-antioxidants for control) for 30 min. At the end of 20 min, the NCM was collected and centrifuged at 5,000 rpm for 5 min at 4°C before storage at -80°C until use. On the day of use, NCM was thawed on ice and concentrated using 3,000-Da molecular-weight cut-off filters (UFC800324, Millipore). 50 μL of concentrated

NCM was added per well to the Ast or Mg culture, at the same time as H₂O₂.

Statistics

All statistical analyses were performed using Graph-Pad Prism v5.0 (La Jolla, CA, USA). Wilcoxon signed-rank tests were used to compare treatments with and without rmEpo and to control on data normalized to the protein content for each respective treatment well. All results are expressed as means $\pm$ SEM and significance was accepted at $p \leq 0.05$.

Results

Viability

Epo has been shown to be protective in a range of experimental paradigms. Here, we tested the neuroprotective capacity of rmEpo following H₂O₂ injury in primary Mg and Ast using LDH release as a measure.

As expected, H₂O₂ exposure induced significant cell death at the higher concentrations for both cell types ($p < 0.0001$; Fig. 1a–d). The addition of 10 ng/mL rmEpo following a 4-h H₂O₂-induced injury did not appear to be protective in either cell type (Fig. 1a–d), but when pretreated for 24 h with rmEpo, protection was seen in Ast ($p < 0.05$; Fig. 1f) but not in Mg (Fig. 1e).

Oxidative Stress

Nitrites

The effects of Epo on NO production/nitrite levels in cultured cells vary and appear to be largely due to factors like which type of cell or stimulus is involved [16]. We tested whether rmEpo would alter nitrite levels in cultured Mg and Ast stimulated with H₂O₂.

There were no significant changes in nitrite levels in Mg with either H₂O₂ or rmEpo treatment (Fig. 2a). In Ast, however, nitrite levels were considerably suppressed after H₂O₂ (Fig. 2b), and following rmEpo treatment, they were significantly increased (Fig. 2b, $p < 0.05$ and $p < 0.01$, respectively, for low and high continuous H₂O₂).

Reactive Oxidative Species

Epo has been widely reported to have antioxidative properties. However, most in vitro studies utilize a pretreatment paradigm. The aim of our study was to evaluate the posttreatment effects of rmEpo on the intracellular levels of ROS.

In Mg, low-dose H₂O₂ significantly increased ROS levels, and rmEpo treatment significantly suppressed the flu-

orescence of high continuous H₂O₂ cultures (Fig. 2c; $p < 0.05$) but had no effect on low H₂O₂ cultures. In Ast, there were no significant changes in ROS levels with either H₂O₂ or rmEpo treatment (Fig. 2d).

Superoxide

In order to understand if Epo would mediate antioxidative mechanisms via superoxide production, we examined MitoSOX fluorescence in glial cells after rmEpo treatment following H₂O₂ injury.

While no significant changes were seen with H₂O₂ treatment in Mg, rmEpo led to significant suppression of indicator levels in high continuous H₂O₂ cultures (Fig. 2e; $p < 0.05$). In Ast cultures, no significant changes in MitoSOX indicator levels were seen with either H₂O₂ or rmEpo treatment (Fig. 2f).

Inflammatory Responses

Both Mg and Ast mediate neuroinflammation via cytokine and chemokine release. We analyzed the cytokine and chemokine secretion profile for both Mg and Ast following rmEpo treatment of H₂O₂-induced injury.

Overall, significant changes were evident in chemokine levels in Mg. Low H₂O₂ caused an increase in the levels of MIP-2 and RANTES ($p < 0.05$; Fig. 3g, h), with a similar trend but failing to reach significance in TNF α and MIP-1 α ($p = 0.06$; Fig. 3a, c). High continuous H₂O₂ suppressed MIP-1 α , MIP-1 β , and RANTES ($p < 0.05$; Fig. 3c, e, h). rmEpo treatment did not have any significant effects. There were no significant changes in any inflammatory mediator levels in Ast (Fig. 4).

Neuron-Conditioned Medium

Since cytokine/chemokine responses to H₂O₂ did not seem to be significantly altered by rmEpo in most of the paradigms tested, we speculated that the presence of at least signaling molecules from injured neurons might be necessary for glial cells to elicit significant secretory responses. To this end, we exposed cortical neurons to 30 min of 50 μ M H₂O₂, and transferred this NCM to Mg and Ast at the same time as H₂O₂ was added. Results are shown only for cytokines/chemokines exhibiting considerable changes.

Overall, there were no significant changes with the addition of NCM along with H₂O₂, nor were there any significant effects of rmEpo on chemokine/cytokine levels in either cell type (Fig. 5, Mg; Fig. 6, Ast). There was, however, a notable nonsignificant increase in secretion in the NCM wells that was suppressed in the presence of 50 μ M H₂O₂.

Fig. 2. rmEpo effects on NO, ROS, and superoxide levels following H₂O₂ injury. Cultured primary murine astrocytes (Ast) and microglia (Mg) were exposed to continuous or bolus H₂O₂ for 4 h before treatment with 10 ng/mL rmEpo. Secreted NO levels (**a, b**) in the medium were measured using the Griess assay, ROS levels (**c, d**) were measured using the H₂DCFDA indicator, and superoxide levels (**e, f**) were measured using the MitoSOX indicator at the end of the study. H₂O₂ did not have any significant effects on secreted nitrite levels in either Mg (**a**) or Ast (**b**), but Epo significantly increased the NO levels in the Ast wells. There was significant suppression of microglial ROS and superoxide with Epo treatment at the high continuous dose in Mg (**c, e**) but not in Ast (**d, f**). The results presented here are compiled from at least 5 independent assays run in duplicate. * $p < 0.05$, ** $p < 0.01$ versus control.

Discussion

Although there has been much interest in using Epo as a protective and restorative agent after injury to the central nervous system, the response of Ast and Mg to Epo after oxidative stress is largely unknown. Here, we found differential effects in these cell types in terms of viability, oxidative responses, and inflammatory mediator secretion, both without and with the presence of NCM.

Viability

In the conditions tested in this study, rmEpo did not significantly reduce H₂O₂-induced cell death in either cell

type when given 4 h after H₂O₂ initiation, even at high H₂O₂ levels. There are studies that have shown protection of Mg with Epo posttreatment up to 6 h after the insult, but they differ in terms of the type of injury as well as species [21, 23], which suggests that Epo-mediated protection in Mg depends on such factors [20, 21, 23]. In Ast, on the other hand, while there was a trend towards a decrease in cell death after high H₂O₂, this failed to reach significance.

In support of previous reports [24], pretreatment reduced LDH release in Ast, but failed to show any effect on Mg LDH release, which contrasts with previous reports [21, 25]. While it is possible that the cells were injured

Fig. 3. a–h Microglial inflammatory mediator responses to H_2O_2 stimulation and rmEpo exposure. Cultured primary murine microglia were exposed to continuous H_2O_2 for 4 h before treatment with 10 ng/mL rmEpo for 20 h. Medium was collected at the end of 20 h and inflammatory mediator levels were assessed by a 15-plex cytokine/chemokine assay. For clarity, only data with considerable changes are shown here. Final $n = 6$ (4 for some), data shown are normalized to protein. * $p < 0.05$ versus control.

beyond the possibility of repair at the higher concentrations, it is more likely that rmEpo mediates protection primarily in Ast rather than in Mg after H_2O_2 injury. Support for this hypothesis comes from several findings that suggest Epo has pleiotropic effects, depending on cell and

injury type [16]. For example, Epo activates the p38-MAPK pathway in Ast to preserve their integrity [26, 27], but blocking this pathway in vivo does not affect neuronal survival [28]. Moreover, while it has been identified that Akt1-dependent Epo signaling maintains endothelial cell

Fig. 4. a–i Astrocytic inflammatory mediator responses in response to H_2O_2 stimulation and rmEpo exposure. Cultured primary murine astrocytes were exposed to continuous H_2O_2 for 4 h before treatment with 10 ng/mL rmEpo for 20 h. Medium was col-

lected at the end of 20 h and inflammatory mediator levels were assessed by a 15-plex cytokine/chemokine assay. For clarity, only data with considerable changes are shown here. Final $n = 6$ (4 for some), data shown are normalized to protein.

[29] and Mg [21, 25] integrity, it does not appear to play a role in Ast neuroprotection [30].

Oxidative Stress

While H_2O_2 can induce cell death in the neonatal brain via the apoptotic death cascade [31], it is known especially for eliciting damaging effects via its reaction with excess free iron (Fenton reaction), leading to the generation of free radicals such as the hydroxyl radical, superoxide, and NO, all of which lead to subsequent damage [17, 32]. Al-

though there is some evidence that Epo leads to a decrease in NO levels in Mg [23, 33], most studies have shown that it has no significant effect on NO production in activated Mg cultures [23, 31–34]. Here, we actually saw an increase in nitrite levels with Epo treatment in both Mg and Ast (only significant in Ast). Wenker et al. [35], reported increased nitrite production in Mg after Epo exposure of a microglial cell line, an effect that was lost when an inhibitor of the PI3K pathway was used; a similar mode of action might have been relevant in our study.

Fig. 5. a–f Microglial inflammatory mediator responses in response to H_2O_2 stimulation and rmEpo exposure in the presence of neuron-conditioned medium (NCM). Neurons were exposed to $50 \mu M H_2O_2$ for 30 min, and the conditioned medium from these cultures was added to cultures of primary murine microglia, which were then exposed to H_2O_2 for 4 h before treatment with 10 ng/mL

rmEpo for 20 h. Medium was collected at the end of 20 h and inflammatory mediator levels were assessed by a 15-plex cytokine/chemokine assay. For clarity, only data with considerable changes are shown here. Final $n = 3$, data shown are normalized to protein. NCM0, NCM from uninjured neurons; NCM50, NCM from neurons exposed to $50 \mu M$ of H_2O_2 .

Other studies have reported a similar Epo-mediated increase in NO and NO synthase levels in various tissues [36, 37]. An increase in NO in endothelial cells is thought to maintain blood pressure homeostasis and blood flow [38], and NO induced in the central nervous system via HIF $_{1\alpha}$ -mediated Epo production has been shown to prevent axonal degeneration [39]. It is possible that the increase in NO is actually a beneficial manifestation of Epo

signaling following oxidative stress. The increase in NO levels with rmEpo treatment was observed only in the cultures exposed to continuous H_2O_2 in Ast. Glial cells have been shown to have a high antioxidative capacity [40, 41] that renders them highly tolerant to oxidative stress and capable of effectively disposing of oxidants such as H_2O_2 within a short time [42, 43], which makes sustained H_2O_2 production application more relevant

Fig. 6. a–h Astrocytic inflammatory mediator responses in response to H_2O_2 stimulation and rmEpo exposure in the presence of neuron-conditioned medium (NCM). Neurons were exposed to $50 \mu M H_2O_2$ for 30 min, and the conditioned medium from these cultures was added to the cultures of primary murine microglia, which were then exposed to H_2O_2 for 4 h before treatment with 10

ng/mL rmEpo for 20 h. Medium was collected at the end of 20 h and inflammatory mediator levels were assessed by a 15-plex cytokine/chemokine assay. For clarity, only data with considerable changes are shown here. Final $n = 3$, data shown are normalized to protein. NCM0, NCM from uninjured neurons; NCM50, NCM from neurons exposed to $50 \mu M$ of H_2O_2 .

for studies such as ours. The sustained H_2O_2 better mimics the in vivo situation following neonatal HI insult [17]. Studying the effects of Epo treatment during such an insult yields a much better understanding of the mechanisms of Epo activity.

Both ROS and MitoSOX levels were suppressed with rmEpo treatment in continuous H_2O_2 -exposed Mg, highlighting an antioxidative role for rmEpo that other studies reported previously [44]. There was, however, no significant change in the ROS levels in Ast, supporting sugges-

tions that Epo appears to act via various mechanisms depending on the individual cell and injury types [16]. This finding is in contradiction to that reported by Liu et al. [45], who showed an amelioration of MitoSOX indicator levels with Epo treatment following paraquat-induced oxidative stress in Ast. It is likely that the difference between the 2 studies regarding the type of injury accounts for these disparate findings [46].

Inflammation

rmEpo treatment failed to elicit any significant effects on the levels of secreted cytokines and chemokines in both cell types. Low continuous H_2O_2 elicited the maximal statistically significant responses in chemokine levels (MIP-2 and RANTES) in Mg, as we reported previously with similar nonsignificant effects in Ast for the same chemokines (MIP-1 α , MIP-1 β , MIP-2, and RANTES) as well as for KC and G-CSF [18]. This reflects previous findings that H_2O_2 , acting as a second messenger, induces chemokine responses [47, 48]. Chemokine expression is an important mediator of the proliferation, recruitment, and migration of Mg, as well as of other immune and stem cells which provide for the containment and resolution of injury [49–51]. It appears that low continuous H_2O_2 , which we presume is functioning as a signaling molecule, acts to provide preconditioning protection [52]. In addition to the above chemokine responses, Ast also had disparate responses to certain cytokines such as G-CSF, IL-6, and IL-4, which were unresponsive in Mg. This discrepancy in inflammatory responses is an indication of the variation in responses of both cell types to injury. For example, a previous study showed that endotoxin stimulated Mg to produce 95 times more TNF α than did Ast [53]. Ultimately, there was no apparent anti- or proinflammatory effect for either cell type in response to H_2O_2 , or, more importantly, to Epo. While the lack of an anti-inflammatory effect for Epo in Mg has already been widely established [23, 33, 34], to our knowledge, this is the first study to report cytokine/chemokine secretion by Ast in response to Epo treatment following H_2O_2 injury. This suggests that Epo-mediated neuroprotection is a result of mechanisms other than those directly mediating inflammation, at least in the paradigms tested in this study.

While the presence of NCM augmented the levels of chemokines (MIP-1 α , MIP-1 β , and RANTES) secreted in the microglial cells, the presence of H_2O_2 appeared to dampen this augmentation. This is somewhat surprising, considering that H_2O_2 has been established as a chemokine-inducing factor [47]. The fact that the presence of

NCM itself was sufficient to elicit this increase, regardless of whether it was from uninjured or injured neurons, points to the importance of cell-to-cell signaling in the brain, i.e., that Mg secretory responses are enhanced in the presence of neuronal communication (not significant with the Wilcoxon test). Chong et al. [25] showed that there was an increase in Mg proliferation and phosphatidyl serine receptor expression in response to NCM from injured neurons, a phenomenon that could be blocked by Epo pretreatment. In our study, rmEpo treatment did not appear to have any significant effect on Mg exposed to NCM, suggesting that (1) pretreatment with rmEpo would be more effective or (2) rmEpo does not modulate cytokine/chemokine responses in Mg.

A similar dampening of NCM-augmented secretory responses was evident in Ast treated with H_2O_2 , although on a much lower scale, suggesting that the mechanisms involved were similar to those in Mg, despite Mg being much more responsive to NCM than Ast. The addition of NCM suppressed MIP-2 and TNF α levels considerably, suggesting that the presence of neurons dampens H_2O_2 -induced MIP-2 and TNF α levels. Both MIP-2 and TNF α have been shown to be produced in response to injury [54], with deleterious effects. Modulation of their secreted levels in the presence of NCM suggests that neuronal communication is an important factor controlling their secretion, potentially ameliorating a harmful response.

In conclusion, it appears that rmEpo-mediated cellular effects are pleiotropic and multifaceted, depending not only on the cell type but also on the mode and level of injury and treatment. In terms of the oxidative and secretory responses of Mg and Ast, rmEpo appears to have largely similar effects on both cell types, some of which are significantly different to those reported in the neurons. Ultimately, this study highlights the importance of taking into careful consideration multiple necessary factors, which, cohesively, could predict the optimal use of Epo following injury.

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