

# Pressure-Induced Regulation of IL-6 in Retinal Glial Cells: Involvement of the Ubiquitin/Proteasome Pathway and NF $\kappa$ B

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**PURPOSE.** To investigate how hydrostatic pressure influences regulation of interleukin (IL)-6 by retinal glia and whether this regulation is associated with the ubiquitin/proteasome pathway (UPP) and activation of the transcription factor nuclear factor (NF) $\kappa$ B.

**METHODS.** Astrocytes and microglia isolated from rat retina were maintained in vitro, and the IL-6 concentration in the media at ambient and elevated pressure were compared, with and without the proteasome inhibitor MG132 (10  $\mu$ M). Immunocytochemistry was used to correlate translocation of NF $\kappa$ B with pressure.

**RESULTS.** Exposure to elevated pressure for 24 hours maximally altered the concentration of media IL-6 of glia cultures, where IL-6 concentrations decreased in astrocyte cultures and increased in microglia cultures. These pressure-induced changes in IL-6 were largely insensitive to MG132 in astrocytes, but were largely MG132-sensitive in microglia. Like IL-6 regulation, pressure-induced activation of NF $\kappa$ B also differed between the two glial cell types, where nuclear localization of NF $\kappa$ B was transient in astrocytes, but sustained in microglia. Elevated pressure also increased MG132-sensitive expression of IL-6 mRNA by microglia.

**CONCLUSIONS.** Though pressure-induced regulation of IL-6 by astrocytes is preceded by NF $\kappa$ B translocation, it is not altered by MG132 and therefore is not likely to be regulated by NF $\kappa$ B or the UPP. In contrast, pressure-induced regulation of IL-6 protein and mRNA by microglia is preceded by NF $\kappa$ B translocation and is sensitive to MG132. Together with precedence in the literature, these data suggest that pressure-induced activation of the UPP leads to transcription of IL-6 driven by NF $\kappa$ B. (*Invest Ophthalmol Vis Sci.* 2006;47:3860-3869) DOI:10.1167/iovs.05-1408

**G**laucoma is characterized by progressive loss of retinal ganglion cells (RGCs) and damage to the optic nerve that is often associated with increases in intraocular pressure.<sup>1-3</sup> Although the symptoms of glaucoma are attributable to degeneration of RGCs, the processes underlying this degeneration

are largely unknown. In recent years, astrocyte glia and microglia have emerged as significant players in RGC survival.<sup>4-8</sup> We recently demonstrated that retinal astrocytes and microglia differentially affect pressure-induced death of RGCs, where astrocytes decrease survival and microglia increase survival.<sup>9</sup> The inflammatory cytokine interleukin (IL)-6 is a key component of microglia-RGC signaling, reducing pressure-induced death.<sup>9</sup> While exposure to 24 hours of elevated pressure alters IL-6 release by both astrocytes and microglia, there is a dramatic 12-fold difference between the concentration of IL-6 released by astrocytes and that released by microglia.<sup>9</sup> These data suggest that the cellular processes regulating IL-6 during exposure to elevated pressure also differ between these two glial cell types.

In other systems, regulation of IL-6 in astrocytes, microglia, and macrophages is linked to the transcription factor nuclear factor (NF) $\kappa$ B, whose translocation from the cytosol to the nucleus depends on the sequestration of its inhibitor (I) $\kappa$ B in the ubiquitin/proteasome pathway (UPP).<sup>10-14</sup> Stimuli that modulate NF $\kappa$ B-dependent production of IL-6 include exposure to viral and bacterial infection, toxins, and other cytokines.<sup>15-20</sup>

To determine whether the UPP and NF $\kappa$ B also contributes to pressure-modulated regulation of IL-6, we examined the temporal pattern of IL-6 released by purified retinal glia in vitro under conditions of controlled hydrostatic pressure and evaluated whether this release is associated with the UPP and activation of NF $\kappa$ B. Our results indicate that pressure-induced regulation of IL-6 by astrocytes and microglia differed dramatically in the direction of regulation (decreased concentration versus increased) and in sensitivity to the proteasome inhibitor MG132, with astrocyte regulation of IL-6 largely insensitive to MG132 and microglia regulation largely sensitive. These pressure-induced changes in IL-6 were also accompanied by nuclear translocation of NF $\kappa$ B in both astrocytes and microglia. However, nuclear localization of NF $\kappa$ B was transient in astrocytes and sustained in microglia. These data suggest that astrocytes and microglia differ not only in their responses to elevated pressure, but also in the mechanisms that lead to the response. Despite evidence for pressure-induced activation of NF $\kappa$ B in both astrocytes and microglia, the inability of MG132 to alter astrocyte-derived IL-6 suggests that the UPP and NF $\kappa$ B are not likely to contribute to the IL-6 response. In contrast, NF $\kappa$ B activation that precedes changes in IL-6 and sensitivity to MG132 suggest that the UPP is likely to regulate the IL-6 response in microglia and that NF $\kappa$ B-induced gene transcription may also contribute to the response.

## MATERIALS AND METHODS

### Animals

This study was conducted in accordance with regulations set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animal protocols were approved by the Institutional Animal Care and Use Committees of the University of Rochester Medical Center and the Vanderbilt University Medical Center.

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## Cell Separation and Culture

Primary cultures of retinal astrocytes and retinal microglia were generated as previously described.<sup>9</sup> Briefly, retina from postnatal day-2 to -4 Sprague-Dawley rats were dissociated by trituration and incubation at 37°C in 1 mg/mL papain (Worthington, Lakewood, NJ) and 0.005% DNase I in Earle's balanced salt solution. Viability was assessed by trypan blue exclusion and found to be greater than 98%. The astrocytes and microglia were purified by incubation with a monoclonal anti-human astrocyte antibody (4 µg/mL; cat. no. MAB5314; Chemicon, Temecula, CA) and a monoclonal anti-rat RT 1a/OX18 antibody (5 µg/mL; cat. no. CBL1519; Chemicon), respectively. The suspension was incubated in the magnetic bead-conjugated secondary antibody and loaded into a pre-equilibrated column in the presence of a magnetic field (Miltenyi Biotec, Auburn, CA). Cells positively selected by either anti-OX18 or anti-astrocyte antibodies were eluted, plated at a density of  $5 \times 10^4$  (1 mL/well) on two-chamber glass slides (Nalge-Nunc, Rochester, NY) and maintained in a 50:50 mixture of Dulbecco's modified Eagles' medium and F12 medium (DMEM/F12; Invitrogen, Grand Island, NY) plus 2 mM L-glutamine, 15 mM HEPES buffer, 0.1% gentamycin, and 1% G<sub>5</sub> supplement (insulin 500 µg/mL, transferrin 5 mg/mL, selenite 520 ng/mL, biotin 100 µg/mL, hydrocortisone 360 ng/mL, FGF 500 ng/mL, and EGF 100 ng/mL; Invitrogen). Cultures were grown to approximately 80% confluence (10–14 days) in a standard incubator with 5% CO<sub>2</sub> before our timed experiments. During this time, 50% of the culture medium was replaced every 48 hours. As previously reported, the purity of our glial cultures was determined by immunocytochemistry and PCR against cell-type-specific markers.<sup>9</sup>

## Hydrostatic Pressure Experiments

For the pressure experiments, we exposed astrocyte and microglia cultures to either ambient or +70-mm Hg pressure for 0 to 72 hours. This magnitude of pressure was chosen to compliment our previous work, where the rationale for using 70 mm Hg is described in detail.<sup>9</sup> We used a humidified pressure chamber equipped with a regulator and gauge, whose design and utility for measuring cellular responses to hydrostatic pressure has been well-established, not only for retina and optic nerve studies,<sup>6,21–32</sup> but also for many other systems.<sup>33–35</sup> For elevated pressure, the entire chamber was placed in a 37°C oven, and an air mixture of 95% air and 5% CO<sub>2</sub> was pumped into the chamber to obtain +70-mm Hg pressure, which was maintained by the regulator. For ambient pressure experiments, cells were kept in a standard incubator.

We took many precautions and designed the experiments to limit artifact from the experimental method. We addressed the questions of pH, dissolved oxygen, and carbon dioxide content, and culture nutrition. Throughout the pressure experiments, we monitored pH with phenol red in the media. At no time during the incubations did the medium change in color. We confirmed this with a series of preliminary experiments using a pH meter and also measured the dissolved oxygen content in the medium to rule out a state of hyperoxia. After the cultures were exposed to 24 hours of either ambient or 70-mm Hg pressure, we used an oxygen electrode to measure the dissolved oxygen content in the medium. We found that the oxygen content in neither the ambient medium (21.88%) nor the 70-mm Hg medium (21.28%) differed from the expected value of 21% ( $\chi^2 = 0.04$ ). These data suggest that the change in the dissolved oxygen content of the media predicted by Boyle's law is small enough to be below the sensitivity threshold of the probe. Similarly, the change in CO<sub>2</sub> is fractional and undetectable, consistent with other published control studies.<sup>21,25,34,35</sup> To avoid artifacts due to on-off changes in pressure, the culture media were not changed during the experiments. For all cultures, including those maintained at ambient pressure, 100% of the media were replaced immediately before experimentation. As stated in the section on cultures, 50% of the media were replaced every 48 hours during the growth and equilibration stages. As such, 72 hours without feeding should not be a confounding factor.

## Phorbol Ester Experiments

To verify our ability to detect nuclear translocation of NFκB and secretion of IL-6 by our astrocyte and microglia cultures, we exposed cells to the phorbol ester, phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO). PMA is known to induce cytokine production in a proteasome-dependent manner in glial cells as well as macrophages.<sup>13,15–19</sup> In addition, PMA induces nuclear translocation of NFκB that precedes the production of cytokines in U-937, HeLa, and Jurkat cells.<sup>14,36</sup> After 10 to 14 days in culture, cells were exposed to 1 µM PMA in a standard incubator for 7 hours in the presence or absence of the proteasome-inhibitor carbobenzyoxy-L-leucyl-L-leucyl-L-leucyl (MG132; Sigma-Aldrich; see the following section).<sup>37–39</sup> To achieve a final concentration of 1 µM, we added 1 µL PMA in dimethyl sulfoxide (DMSO; Sigma-Aldrich) solvent to 1 mL of culture medium. To control for solvent effects, all cultures not exposed to either PMA or MG132 were treated with an equal volume of DMSO (described in the next section).

## Proteasome Inhibition

To assess the role of NFκB in pressure- and PMA-induced release of IL-6 by astrocytes and microglia, we exposed cells to elevated pressure or PMA in the presence of the proteasome-inhibitor MG132.<sup>37–39</sup> For MG132 experiments, cells were exposed to 70 mm Hg hydrostatic pressure or 1 µM PMA in the presence or absence of MG132 for 8 to 72 hours. In all experiments, MG132 or DMSO was added to the cultures 1 hour before experiments.

To achieve a final concentration of 10 µM, 1 µL MG132 in DMSO solvent was added to 1 mL of culture medium. To control for solvent effects, all cultures incubated in the absence of MG132 were treated with an equal volume of DMSO. As such, most of the cultures received 1 µL of DMSO in a volume of 1 mL medium; however, cultures receiving both PMA and MG132 received 2 µL of DMSO in 1 mL of medium. The vehicle-only control cultures in these experiments were treated with 2 µL of DMSO.

## Enzyme-Linked Immunosorbance Assay

We used ELISA assays to measure the concentration of IL-6 secreted by astrocytes and microglia in response to elevated pressure and PMA. The culture medium was prepared for analysis, as previously described.<sup>9</sup> Briefly, it was first centrifuged at low speed to remove intact cells and then centrifuged again at a high speed, to remove cellular debris. ELISAs were conducted with anti-rat IL-6 ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's recommendations and as previously described.<sup>9</sup> We used a microplate reader to determine the optical density of each well (Molecular Devices, Sunnyvale, CA). The concentration of IL-6 in each sample was determined by comparison of the optical densities in the sample wells with that of the standard curve. To determine the sensitivity of the IL-6 ELISA kit, we calculated the minimum concentration of cytokine necessary for detection. Based on the experimental ODs, we performed a back calculation of cytokine concentration for the zero standards in 21 separate assays. These concentrations were averaged, and the standard deviation was multiplied by 2 to obtain the minimum reliable detection of 3.2430 pg/mL. All results from the ELISA assays are the average of at least three individual experiments.

## Immunocytochemistry

Immunolabeling of NFκB in glia cultures was performed with slight modification, as previously described.<sup>9</sup> Briefly, cells were fixed in 100% acetone for 10 minutes and air dried. To quench autofluorescence, samples were treated with 0.1% sodium borohydride for 30 minutes at room temperature, washed, and incubated in a blocking solution containing 5% normal goat serum (NGS) and 0.1% Triton X-100 in PBS for 2 hours at room temperature. Incubation in mouse anti-NFκB p65 (cat. no. 610868; BD-PharMingen, San Diego, CA) was performed overnight at 4°C in a solution containing 3% NGS and 0.1% Triton

X-100 in PBS. Samples were then incubated for 2 hours at room temperature in secondary antibody solution containing goat anti-mouse secondary antibody conjugated with Alexa 488 dye (10  $\mu\text{g}/\text{mL}$ ; cat. no. A11017; Invitrogen, Eugene, OR), 1% NGS, and 0.1% Triton X-100 in PBS. Cultures were then counterstained with DAPI (4',6'-diamino-2-phenylindole; 50  $\mu\text{g}/\text{mL}$ ; cat. no. D29410; Invitrogen) in ddH<sub>2</sub>O for 5 minutes at room temperature and coverslipped with an antifade mounting medium (EM Sciences, Fort Washington, PA). Controls for ICC experiments were conducted with no primary antibody and the appropriate IgG isotypes.

### Light Microscopy

For assessment of morphologic properties and NF $\kappa$ B immunofluorescence, cultures were examined with an inverted microscope equipped with phase-contrast optics and an upright microscope equipped with differential interference contrast (DIC), a fluorescent lamp, and four wavelength cubes (Olympus, Melville, NY). Both microscopes were digitally interfaced with semicooled charge-coupled device (CCD) cameras and image-capture software (Diagnostic Instruments, Sterling Heights, MI).

### Reverse Transcription–Polymerase Chain Reaction

RT-PCR was performed as previously described with slight modification.<sup>9</sup> Briefly, total RNA was isolated, according to the manufacturer's instructions, from cultured cells with a commercially available RNA isolation kit (RNeasy Micro Kit; Qiagen, Valencia, CA). Isolated RNA was incubated with oligo dT primer (1  $\mu\text{M}$ ) in a solution containing 100 mM dNTPs and HB<sub>20</sub>O at 65°C for 5 minutes before the first-strand reaction, which was performed with reverse transcriptase for 1 hour at 50°C and 1 hour at 55°C (Invitrogen). The second-strand synthesis was performed with DNA polymerase (Invitrogen) using a primer that contained (5'-3'): a 20-mer heel sequence absent from the mammalian database, a stretch of five random nucleotides, and a defined pentameric sequence at the 3' end (Invitrogen). Gene-specific PCR for IL-6 was conducted in a 25- $\mu\text{L}$  reaction (5  $\mu\text{L}$  RT product, 10 $\times$  polymerase buffer, polymerase (Invitrogen), and 1  $\mu\text{M}$  forward and reverse primers) for 30 cycles with the following primers: 5'-GGA CAT TCC TCA CTG TGG TCA GAA-3' and 5'-TGC AAG AAA CCA TCT GGC TAG G-3'. These primers were designed to span an intron of gene sequence for rat IL-6 (accession no. M26745) to produce a PCR product of 276-bp (Integrated DNA Technologies, Coralville, IA). These PCR products were separated on an agarose gel stained with ethidium bromide and digitally imaged on a gel reader (Alpha Innotech, San Leandro, CA). To evaluate contamination of RNA samples by genomic DNA, PCR was conducted on RNA samples from each culture.

### Statistical Analysis

For comparison of ELISA data between treatment groups, we compared the mean concentration of IL-6 from a minimum of three separate experiments by using Student's *t*-test, with  $P < 0.05$  considered statistically significant. All data are reported as the mean  $\pm$  SD.

## RESULTS

### Modulation of IL-6 Release in Both Astrocytes and Microglia by Elevated Pressure

We have demonstrated that exposure to +70 mm Hg of hydrostatic pressure for 24 hours dramatically increases IL-6 release by microglia, but decreases IL-6 release by astrocytes.<sup>9</sup> For a more thorough examination of the effect of elevated pressure on IL-6 release by these two cell types, we exposed astrocyte and microglia cultures to ambient or elevated pressure at intervals up to 72 hours and measured the concentration of IL-6 in the culture media. Elevated pressure only altered IL-6 in culture medium from astrocytes after 24 hours of expo-

sure, when the concentration of IL-6 in the medium was four times less than that of the ambient cultures (Fig. 1A). Between 24 and 72 hours of pressure, the concentration of IL-6 was equivalent to that in the ambient cultures (Fig. 1A). In microglia, elevated pressure induced an early 32% decrease in the concentration of IL-6 in the medium, compared with ambient levels (Fig. 1A). This was reversed to levels two times above ambient levels after 24 hours (Fig. 1A). Between 48 and 72 hours of elevated pressure, the concentration of IL-6 decreased with respect to the concentration measured after 24 hours of exposure. However, these levels remained above the IL-6 concentrations measured at ambient pressure (Fig. 1A, insert). We also noted a significant increase in the concentration of IL-6 in the astrocytes and microglia cultures maintained at ambient pressure for 24 hours and slight fluctuations across all other time points. Changes in IL-6 concentration at ambient pressure could reflect either constitutive regulation of IL-6 or an artifact of culture. Regardless, ambient and elevated cultures at each time point were conducted with cells from the same isolation and plating. As such, comparison within each time point is an accurate reflection of pressure-induced effects. However, these questions are addressed further in the Discussion section.

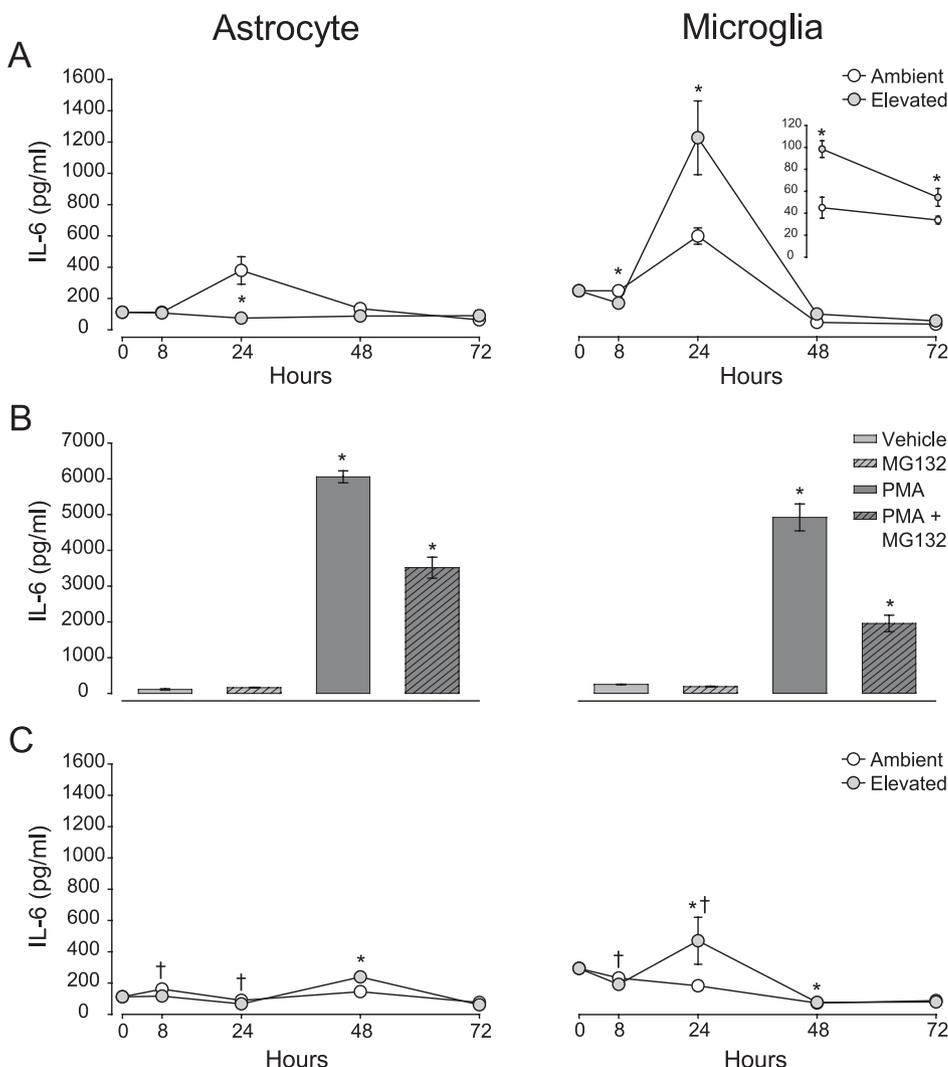
### Potential Contribution of the UPP to Pressure-Induced IL-6 Release

The UPP is associated with the production of many cytokines and diffusible factors, including IL-6.<sup>10–14</sup> To determine whether the UPP also contributes to pressure-induced release of IL-6 in retinal glia, we examined changes in the concentration of IL-6 in the culture medium of astrocytes and microglia exposed to ambient or elevated pressure in the presence of the proteasome inhibitor MG132.

Although MG132 is generally considered a strong proteasome inhibitor that has a high affinity for all subunits of the proteasome, it can also, although less efficiently, inhibit calpains and some lysosomal proteases.<sup>37–42</sup> In an attempt to reduce the probability of nonspecific effects of MG132, we treated our cultures with 10  $\mu\text{M}$  MG132, which does not inhibit lysosomal proteases<sup>40</sup> and is considered a low dose when compared with literature precedence.<sup>43–45</sup> To evaluate further the ability of 10  $\mu\text{M}$  MG132 to inhibit UPP processes in our studies, we tested the ability of this dose to inhibit phorbol ester-induced increases in IL-6 release by astrocytes and microglia (Fig. 1B). The production of cytokines induced by exposure to the phorbol ester PMA is a classic example of UPP activation in many cell types, including astrocytes and microglia.<sup>10–13,46</sup> We exposed astrocytes and microglia to 1  $\mu\text{M}$  PMA for 7 hours in the presence or absence of MG132 and measured the concentration of IL-6 in the medium. For both astrocytes and microglia, a short exposure to PMA induced an order of magnitude increase in the concentration of medium IL-6 (Fig. 1B). Treatment with MG132 alone did not alter secretion of IL-6 by either astrocytes or microglia. However, treatment with both PMA and MG132 reduced the concentration of IL-6 in the medium by 42% in astrocytes and 60% in microglia, compared with PMA alone (Fig. 1B). The inability of MG132 treatment to inhibit the PMA response completely is most likely due to our dose of 10  $\mu\text{M}$ , which was intentionally low, to reduce nonspecific inhibition of calpains and lysosomal proteases. These data strongly suggest that the 10  $\mu\text{M}$  dose of MG132 significantly inhibits the UPP, but is not high enough to completely abolish the pathway and potentially interact with other proteases.

To examine the potential contribution of UPP on pressure-induced increases in media IL-6, we exposed astrocytes and microglia to ambient or elevated pressure up to 72 hours in the presence or absence of 10  $\mu\text{M}$  MG132. At ambient pressure,

**FIGURE 1.** Elevated pressure altered IL-6 regulation in retinal glia through mechanisms that were MG132 sensitive. ELISA measurements of IL-6 concentrations in the media of retinal astrocytes and microglia exposed to various conditions. \*Significant change from ambient pressure ( $P \leq 0.05$ ); error bars, SD. (A) IL-6 concentrations in astrocyte and microglia cultures maintained at ambient or +70 mm Hg pressure for 0 to 72 hours. Elevated pressure reduced IL-6 concentrations in astrocyte cultures after 24 hours, followed by a rebound to ambient levels with longer exposures. In microglia cultures, elevated pressure dramatically increased IL-6 after 24 hours and decreased by 48 hours, but remained elevated through 72 hours (*inset*), compared with ambient pressure. (B) Treatment with the PMA (1  $\mu\text{M}$ ) for 7 hours significantly increased the concentration of IL-6 in the media of both astrocytes and microglia. Treatment with the proteasome inhibitor MG132 (10  $\mu\text{M}$ ) 1 hour before and during the 7-hour PMA exposure (1  $\mu\text{M}$ ), reduced PMA-induced secretion of IL-6 significantly for both astrocytes and microglia. (C) IL-6 concentrations in astrocyte and microglia cultures maintained at ambient or +70 mm Hg pressure in the presence or absence of 10  $\mu\text{M}$  MG132. At ambient pressure, treatment with MG132 for shorter exposures, but not longer exposures, altered the IL-6 concentration in astrocyte medium. In contrast, IL-6 concentrations in microglia medium decreased after both 8- and 24-hour exposures to MG132. Longer exposures did not alter microglia-derived IL-6 from baseline levels. At elevated pressure, treatment with MG132 had no effect on astrocyte release during shorter pressure exposures, but increased secretion at 48 hours. Similarly for microglia, MG132 had little effect on IL-6 secretion during shorter exposures, but produced a steady reversal of the pressure-induced increase during longer exposures. In (C) only, \* $P \leq 0.05$  for elevated versus elevated+MG132; † $P \leq 0.05$  for ambient versus ambient+MG132.



treatment with MG132 for 8 hours increased the concentration of IL-6 by 50% in astrocyte medium, while treatment for 24 hours decreased the IL-6 concentration by 50%. However, treatment with MG132 for 48 to 72 hours did not alter the concentration of IL-6 from baseline levels. In contrast, treatment with MG132 for 8 and 24 hours decreased the IL-6 concentration in microglia medium by 25% and 75%, respectively (Fig. 1C compared with Fig. 1A). These levels returned to baseline after 48 and 72 hours of treatment with MG132.

At elevated pressure, treatment with MG132 altered the concentration of IL-6 in astrocyte medium IL-6 only after 48 hours of elevated pressure, when the concentration of IL-6 increased by fourfold (Fig. 1C compared with Fig. 1A). All other exposure times with MG132 were identical with pressure alone. For microglia, MG132 induced a slow decrease in the concentration of IL-6, dropping by approximately 75% after both 24 and 48 hours of elevated pressure (Fig. 1C compared with Fig. 1A). Treatment for 72 hours also reduced IL-6, but was not as significant ( $P = 0.07$ ). To ensure that the effects of MG132 were not an artifact of toxicity, we also evaluated astrocyte and microglia cultures for cell death, by using cell counts and TUNEL labeling. We did not observe a significant decrease in cell density or an increase in TUNEL labeling at any of the time points (data not shown). These data suggest that

the UPP is likely to contribute partially to pressure-induced changes in IL-6 regulation by astrocytes and microglia. However, the ability of MG132 to both increase and decrease the concentration of medium IL-6 for each cell type makes it unclear whether the UPP contributes to the production, uptake, degradation or release of IL-6.

### Effect of Elevated Pressure on Differential Activation of NF $\kappa$ B in Retinal Glia

Modulation of IL-6 in other systems is linked to the transcription factor NF $\kappa$ B, whose activation requires translocation to the cell nucleus.<sup>15-20</sup> The activation and translocation of NF $\kappa$ B requires phosphorylation of I $\kappa$ B proteins by I $\kappa$ B kinase, which tags I $\kappa$ B for degradation in the proteasome and releases NF $\kappa$ B.<sup>47,48</sup> Therefore, nuclear translocation of NF $\kappa$ B is dependent on the UPP and can be diminished by MG132.<sup>36-37</sup> To determine whether contribution of the UPP to pressure-induced regulation of IL-6 by retinal glia also involves activation of NF $\kappa$ B, we compared localization of the p65 subunit of NF $\kappa$ B at ambient and elevated pressure for both astrocytes and microglia.

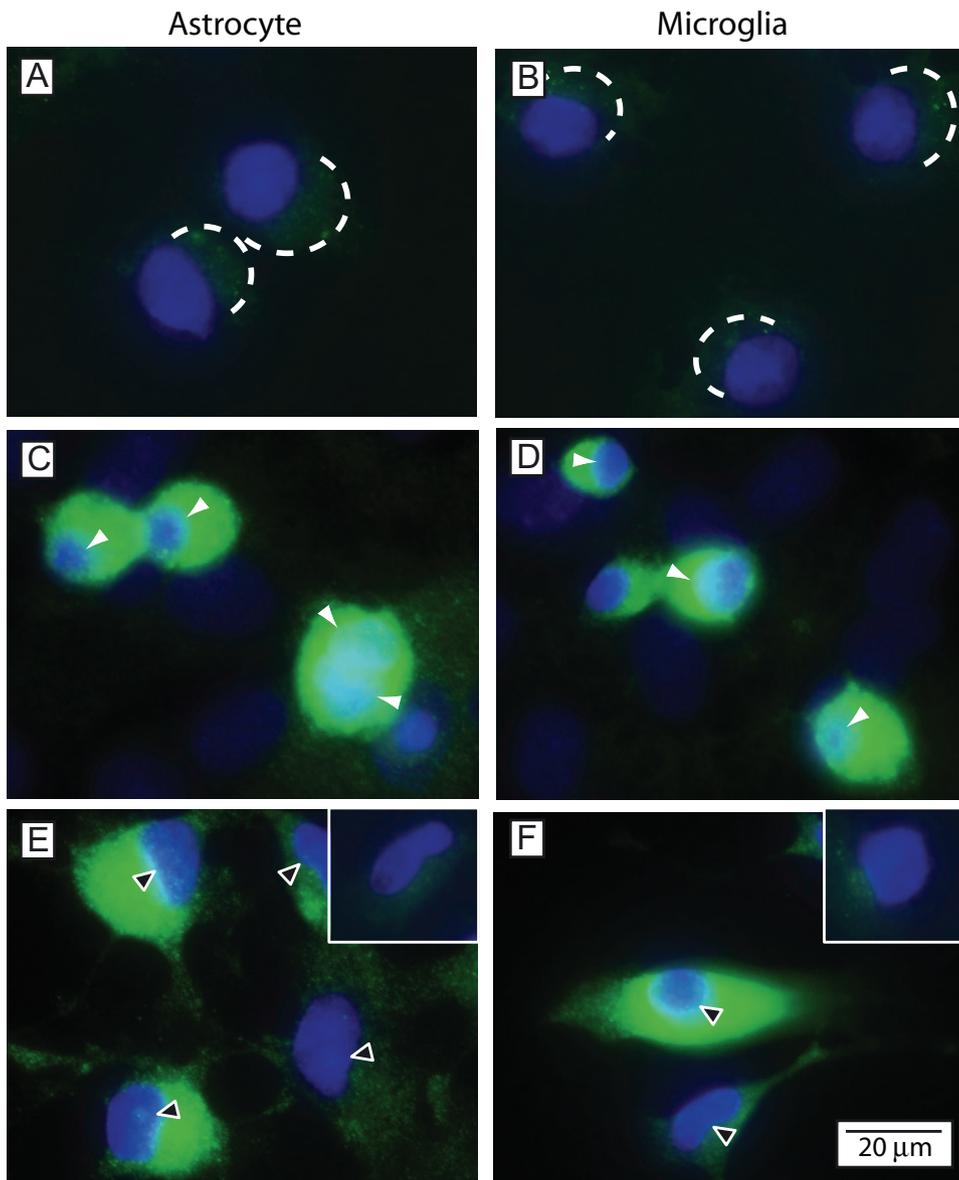
To verify our ability to detect nuclear translocation of NF $\kappa$ B in our cultures, we again used PMA as a positive control.

Activation and nuclear translocation of NF $\kappa$ B precedes PMA-induced production of cytokines, is linked to transcription of these cytokines, and is the source of proteasome dependence in the PMA response.<sup>14,36</sup> We again exposed astrocyte and microglia to PMA for 7 hours in the presence or absence of MG132 and examined colocalization of NF $\kappa$ B with the nuclear stain DAPI. In both astrocytes and microglia without PMA treatment, NF $\kappa$ B was expressed only slightly, was diffusely localized to the cytosol, and was absent from the nucleus (Figs. 2A, 2B). Exposure to PMA induced an increase not only in cytosolic expression of NF $\kappa$ B, but also a dramatic increase in localization of NF $\kappa$ B to the nucleus (Figs. 2C, 2D). Treatment with MG132 prevented PMA-induced localization of NF $\kappa$ B to the nucleus (Figs. 2E, 2F). Treatment with MG132 alone induced no change in NF $\kappa$ B localization (Figs. 2E, 2F insets). Thus, the localization of NF $\kappa$ B to the nucleus with PMA treatment is sensitive to treatment with MG132, and we interpret the absence of nuclear NF $\kappa$ B with MG132 as indicative of effective prevention of translocation and inhibition of the UPP.

To determine whether elevated pressure also induces activation of NF $\kappa$ B in retinal glia, we exposed astrocytes and microglia to 8 and 24 hours of elevated pressure in the pres-

ence or absence of MG132. At 8 hours of ambient pressure, NF $\kappa$ B was only weakly expressed, diffusely localized to the cytosol, and absent from the nucleus in both astrocytes and microglia (Figs. 3A, 3B). As with PMA treatment, exposure to elevated pressure for 8 hours induced nuclear expression of NF $\kappa$ B, as well as a robust increase in cytosolic expression (Figs. 3C, 3D). Treatment with MG132 inhibited this nuclear localization in both astrocytes and microglia, but did not reduce cytosolic labeling (Figs. 3E, 3F). Again, treatment with MG132 alone did not alter labeling or localization of NF $\kappa$ B (Figs. 3E, 3F insets). These data suggest that exposure to 8 hours of elevated pressure induces activation of NF $\kappa$ B in both astrocytes and microglia, as determined by the qualitative increase in cytosolic labeling and apparent nuclear localization of p65.

For both astrocytes and microglia, NF $\kappa$ B staining in cultures maintained at ambient pressure for 24 hours was similar to that observed at 8 hours (Figs. 4A, 4B). Exposure to elevated pressure for 24 hours effectively eliminated NF $\kappa$ B from the cytosol of astrocytes with no evidence of nuclear translocation (Fig. 4C). In the microglia, exposure to 24 hours of pressure induced not only a clear increase in cytosolic NF $\kappa$ B, but also robust labeling in the nucleus that was comparable to that at 8



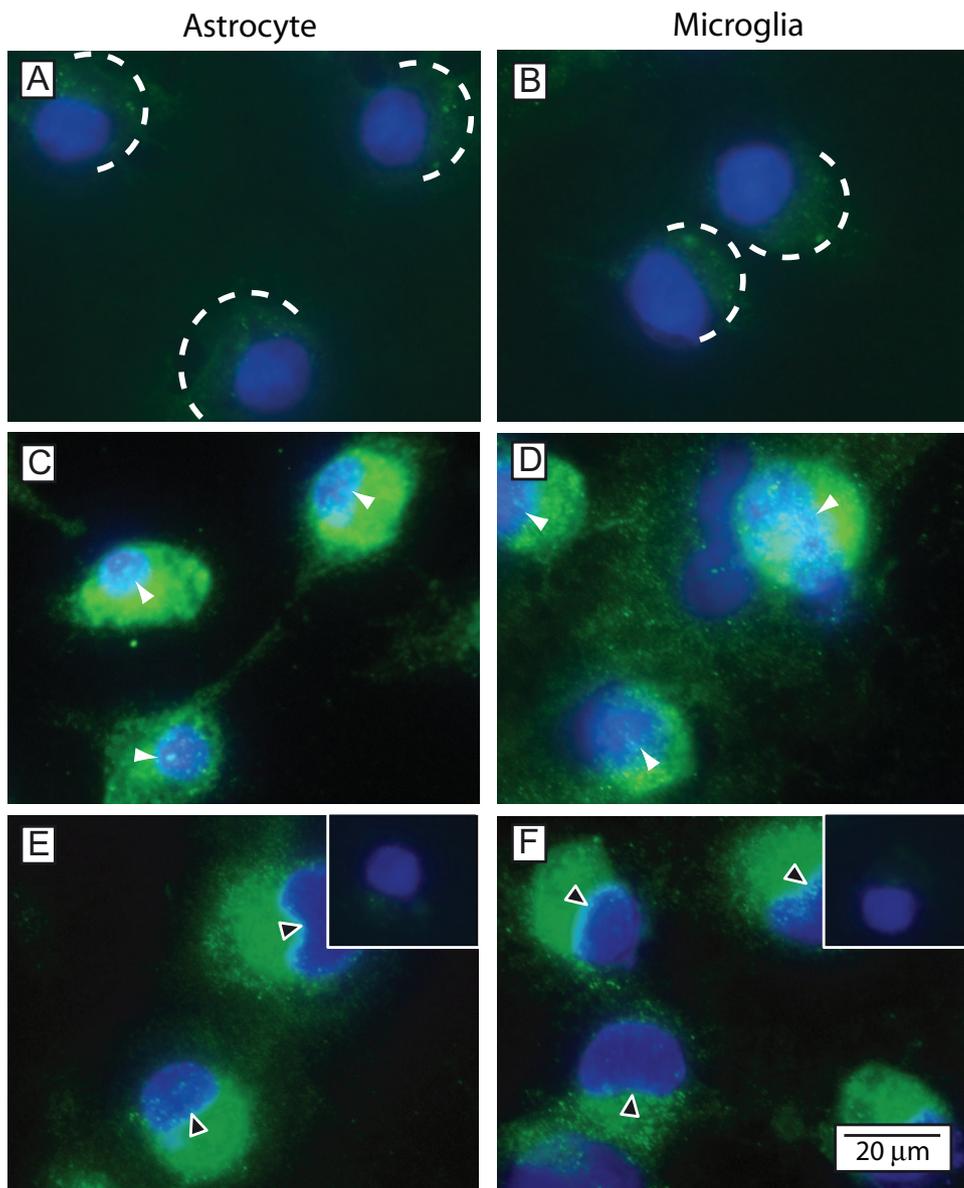
**FIGURE 2.** PMA induced MG132-sensitive nuclear translocation of NF $\kappa$ B. Fluorescence micrographs of astrocytes (A) and microglia (B) at ambient pressure for 7 hours immunolabeled against the p65 subunit of NF $\kappa$ B (green) and counterstained with the nuclear label DAPI (blue). NF $\kappa$ B was weakly expressed, diffusely localized to the cytosol, and absent from the nucleus (dashed outline). (C, D) Exposure to 1  $\mu$ M PMA for 7 hours increased cytosolic expression of NF $\kappa$ B and induced its nuclear localization, as indicated by the white appearance of the nucleus (white arrowheads). (E, F) Treatment with 10  $\mu$ M MG132 1 hour before and during the 7-hour PMA exposure, did not significantly alter the PMA-induced increase in cytosolic NF $\kappa$ B, but does diminish its nuclear localization (black arrowheads). MG132 alone did not alter NF $\kappa$ B localization, compared with ambient (insets).

hours of pressure (compare Figs. 4D and 3C). Treatment with MG132 at elevated pressure did not alter NF $\kappa$ B expression in astrocytes (Fig. 4E), but inhibited nuclear NF $\kappa$ B in microglia and tended to reduce cytosolic expression (Fig. 4F). As previously noted, treatment with MG132 alone at ambient pressure did not alter labeling or localization of NF $\kappa$ B (Figs. 4E, 4F, insets). These data suggest that NF $\kappa$ B activation is a component of early responses by retinal glia to elevated pressure. In addition, the continued activation of NF $\kappa$ B in microglia after 24 hours of exposure suggests that the response of microglia to elevated pressure requires NF $\kappa$ B activation beyond that of astrocytes.

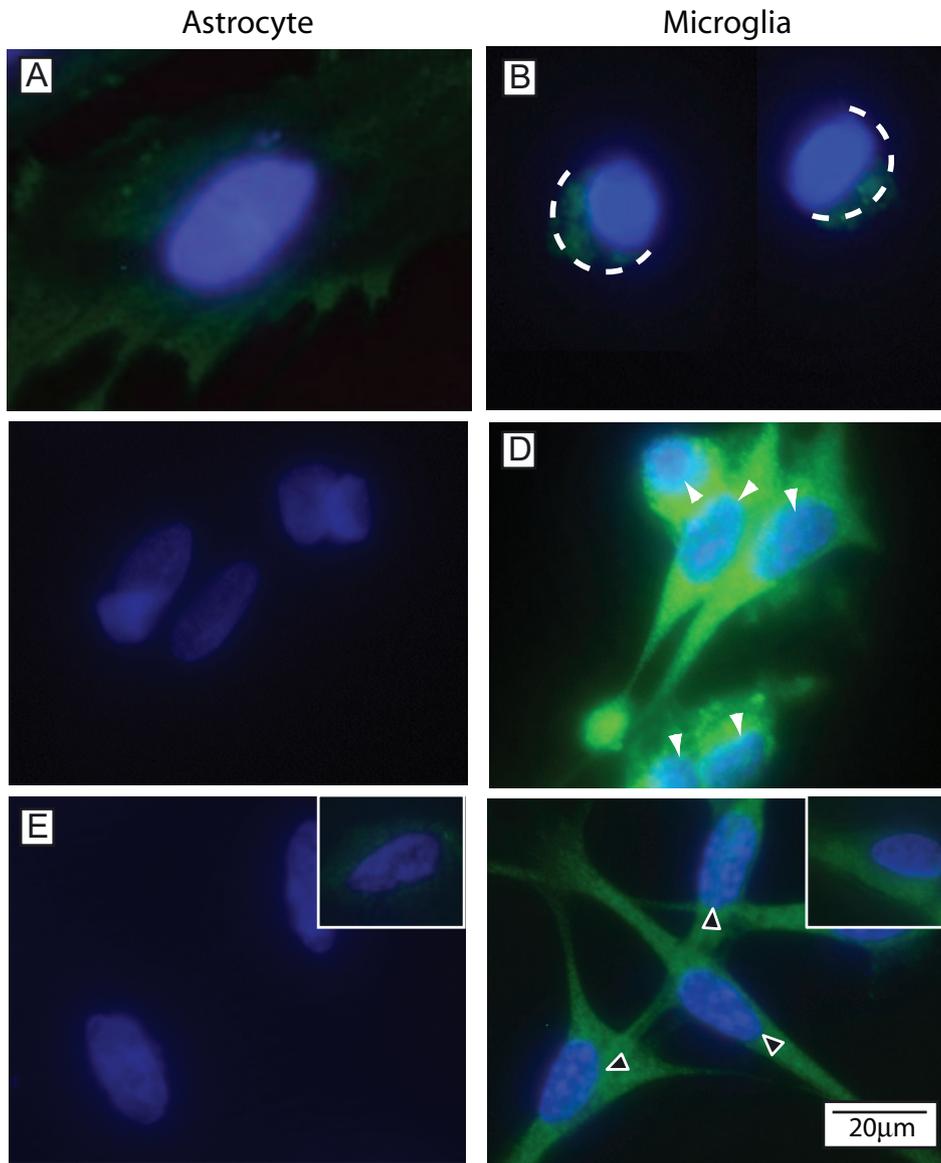
### Effect of Elevated Pressure and UPP Inhibition on Expression of IL-6 mRNA by Microglia

The timeline of events leading to pressure-induced changes in IL-6 regulation by microglia suggests a link between NF $\kappa$ B and the UPP and increased IL-6, where MG132-sensitive increases in IL-6 concentration coincide with NF $\kappa$ B translocation. To determine whether NF $\kappa$ B could in fact be driving transcription

of IL-6, we exposed microglia cultures to 12 hours of ambient or elevated pressure in the presence or absence of MG132 and examined expression of IL-6 mRNA by RT-PCR. We evaluated expression after 12 hours of elevated pressure based on our observations of NF $\kappa$ B translocation and pressure-induced changes in IL-6, where NF $\kappa$ B translocation occurred at both 8 and 24 hours and the most dramatic increase in IL-6 concentrations also occurred between 8 and 24 hours. To allow for qualitative analysis of mRNA expression, we processed the same quantity of cDNA for each sample. As can be expected with induced expression of mRNA, there was some variability between the three preparations we examined. However, exposure to elevated pressure appeared to increase expression of IL-6 mRNA in two of three cultures (Fig. 5A). In each culture where IL-6 mRNA was expressed, treatment with 10  $\mu$ M MG132 greatly diminished its expression (Fig. 5B). This is true of cultures maintained at ambient pressure as well as those exposed to elevated pressure. In combination with ELISA measurements taken after 24 hours at both ambient and elevated pressures, these data suggest that the MG132-sensitive increases in IL-6 concentration not only involve the UPP, but that



**FIGURE 3.** Elevated pressure induced early, MG132-sensitive translocation of NF $\kappa$ B. As in Figure 2, for both astrocytes (A) and microglia (B) maintained at ambient pressure for 8 hours, NF $\kappa$ B (green) is diffusely localized to the cytosol (dashed outlines) and absent from the DAPI-stained nucleus (blue). (C, D) Exposure to elevated pressure for 8 hours induced both an increase in cytosolic NF $\kappa$ B and localization to the nucleus, as indicated by its whiter appearance (arrows). (E, F) Treatment with 10  $\mu$ M MG132 1 hour before and during pressure exposure, did not alter the pressure-induced increase in cytosolic NF $\kappa$ B, but diminished nuclear localization (black arrowheads). MG132 alone did not alter NF $\kappa$ B localization, compared with ambient pressure (insets).



**FIGURE 4.** Elevated pressure induced MG132-sensitive nuclear translocation of NF $\kappa$ B that was sustained in microglia, but not in astrocytes. After 24 hours at ambient pressure, NF $\kappa$ B (green) was weakly expressed in astrocytes (A), but more intensely expressed in microglia (B). For both cells, NF $\kappa$ B was restricted to the cytosol and absent from the nucleus (blue). (C, D) Exposure to elevated pressure for 24 hours substantially reduced cytosolic NF $\kappa$ B and did not induce nuclear localization in astrocytes, but increased cytosolic NF $\kappa$ B and nuclear localization in microglia (white arrowheads). (E, F) Treatment with 10  $\mu$ M MG132 before and during exposure to elevated pressure for 24 hours does not alter NF $\kappa$ B in astrocytes, compared with pressure alone (C), but may decrease cytosolic NF $\kappa$ B in microglia, as well as preventing nuclear localization (black arrowheads). MG132 did not alter NF $\kappa$ B localization, compared with ambient pressure (insets).

the UPP modulates IL-6 regulation upstream of gene transcription rather than posttranslationally.

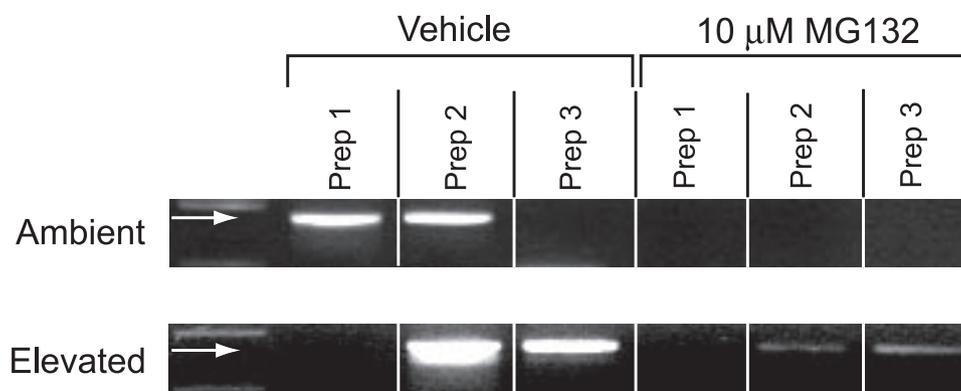
## DISCUSSION

We examined the regulation of IL-6 by retinal astrocytes and microglia exposed to either ambient or elevated hydrostatic pressure in vitro for 0 to 72 hours and determined the potential contribution of the UPP and NF $\kappa$ B to this pressure-induced regulation. We found that (1) elevated pressure altered the regulation of IL-6 in a manner that transiently decreases IL-6 in astrocyte medium, but persistently increased IL-6 in microglia medium, (2) the UPP is likely to contribute directly to pressure-induced regulation of IL-6 in microglia, and (3) elevated pressure induced nuclear translocation of NF $\kappa$ B in astrocytes and microglia that is transient and sustained, respectively.

In concurrence with our previous study, exposure to 24 hours of elevated pressure dramatically decreases (400–1000 pg/mL) IL-6 in astrocyte medium, but dramatically increases (600–1200 pg/mL) IL-6 in medium from microglia. Of note, other exposures did not alter the concentration of IL-6 in astrocyte medium, suggesting that 24 hours of elevated pres-

sure is necessary to achieve a relevant decrease in IL-6 and that the processes that underlie this regulation are transiently active (Fig. 1A). In contrast, elevated pressure transiently decreases IL-6 concentrations in microglia medium before the dramatic increase in IL-6 after 24 hours of elevated pressure. This elevated level of IL-6 is sustained through 72 hours of exposure (Fig. 1A). These data suggest that early regulation of IL-6 by elevated pressure acts to inhibit IL-6 signaling from microglia, whereas regulation during longer exposures acts to increase signaling. Together, these data indicate that elevated pressure activates regulatory processes that act to reduce transiently the amount of IL-6 available for astrocyte signaling, but generally increases the amount of IL-6 available for microglial signaling. Therefore, elevated pressure induces changes in IL-6 regulation by retinal astrocytes and microglia that not only differ in the amount of IL-6 available for signaling, but are also likely to differ in the processes that underlie the regulation.

As suggested by our ELISA data, the contribution of the UPP to pressure-induced regulation of IL-6 in retinal glia, as determined by the proteasome inhibitor MG132, is significantly different in astrocytes and microglia. Although MG132 can also inhibit calpains and lysosomal proteases, inhibition of



**FIGURE 5.** Elevated pressure qualitatively increased mRNA expression of IL-6 through a mechanism that was sensitive to MG132. Expression of mRNA encoding IL-6 in microglia from three individual cultures exposed to ambient (*top*) or elevated (*bottom*) pressure for 12 hours in the presence or absence of 10  $\mu$ M MG132. All lanes were loaded with equivalent concentrations of cDNA, making qualitative comparison possible. Although expression of mRNA by microglial cultures varied between preparations (Prep), there was a general trend toward increased expression of IL-6 mRNA after exposure to elevated pressure. At both ambient and elevated pressures, IL-6 mRNA clearly decreased with MG132 treatment. Arrows: expected product size of 276 bp.

these additional proteases would most likely be in addition to proteasome subunits, as suggested by the high affinity of MG132 for the proteasome.<sup>37-42</sup> Thus, an inability of MG132 to alter pressure-induced changes in IL-6 concentration can be interpreted as a definitive lack of modulation by the UPP. For astrocytes, the pressure-induced decrease in medium IL-6 between 0 and 24 hours is insensitive to MG132, whereas the return to baseline levels from 24 to 72 hours is largely MG132-sensitive (Fig. 1C). These data suggest that the UPP is unlikely to contribute to pressure-induced regulation of IL-6 in astrocytes, but is likely to contribute to the recovery from this regulation. In microglia, the initial decrease in IL-6 concentration after 8 hours of pressure was MG132 insensitive, whereas the sustained twofold increase in IL-6 at longer exposures was largely sensitive to MG132 (Fig. 1C). This suggests that in microglia, pressure-induced regulation of IL-6 occurs through different mechanisms that depend on the length of exposure, where short exposures do not regulate IL-6 through the UPP, and longer exposures are very likely to involve the UPP. Of note, pressure-induced changes in IL-6 concentrations for astrocytes and microglia are both increased and decreased by treatment with MG132, suggesting both negative and positive contributions to IL-6 regulation (Fig. 1C). Together, these data indicate a role for the UPP in pressure-induced regulation of IL-6 by retinal glia that differs not only in its contribution to regulation in each cell type, but also in the purpose of the regulation.

Although these data clearly indicate that elevated pressure alters the regulation of IL-6 by retinal glia and suggest that the UPP plays a role in this regulation, they also raise questions as to the nature of IL-6 regulation at both ambient and elevated pressure. Pressure-induced changes in the concentration of IL-6 and in the contribution of the UPP to these changes could be attributed to multiple processes, including changes in production and release, reuptake, and degradation. Although we cannot definitively ascribe a mechanism to all alterations in IL-6 concentration, we can determine that increases in IL-6 concentration are at least in part attributable to an increase in release. Before each pressure exposure, the medium for cultures maintained at ambient pressure and those exposed to elevated pressure was entirely replaced. Therefore IL-6 present in the medium can only result from processes occurring during the time of exposure. Because we were able to measure IL-6 above background levels at all time points in cultures at both ambient

and elevated pressures, we can deduce that IL-6 was actively being released at ambient pressure and that any increase in IL-6 noted with elevated pressure is a direct result of an increase in secretion. However, we cannot rule out the possibility that changes in reuptake and/or degradation of IL-6 are occurring simultaneously with increases in the release. In contrast, decreases in IL-6 concentration are far more difficult to interpret, as they could be the result of changes in production and release, reuptake, or degradation in any combination of these.

In addition to pressure-induced changes in IL-6 concentration, we also noted significant fluctuations in IL-6 in cultures maintained at ambient pressure. For both astrocytes and microglia, the concentration of IL-6 in the culture medium increased significantly at 24 hours and decreased significantly at 48 hours. These changes could represent either culture artifact or a true phenomenon of constitutive regulation of IL-6 in retinal glia. Although we cannot state for certain whether these changes represent a constitutive, time-dependent regulation of IL-6, we can determine that the processes that underlie the changes is at least partially dependent on the UPP in both astrocytes and microglia, as determined by sensitivity to MG132. If these time-dependent changes are in fact representative of a true phenomenon, they could, like pressure-induced changes, be attributed to changes in production and release, reuptake, or degradation. Although all these permutations of mechanism are possible for changes in IL-6 regulation at both ambient and elevated pressure, we should note that there is no precedence in the literature for a specific mechanism of IL-6 degradation or reuptake by the producing cell type.

Given that NF $\kappa$ B is both dependent on the UPP pathway and associated with IL-6 production in glia and related cell types,<sup>14-20</sup> we identified the transcription factor NF $\kappa$ B as a potential component of the processes that underlie pressure-induced regulation of IL-6 in retinal glia. Using immunocytochemistry, we demonstrated that elevated pressure induces nuclear translocation of NF $\kappa$ B in both retinal astrocytes and microglia. Although pressure-induced translocation of NF $\kappa$ B occurs during short exposures in both cell types, it is transient in astrocytes and sustained through longer exposures in microglia (Figs. 3, 4). In both astrocytes and microglia, localization of NF $\kappa$ B to the nucleus is inhibited by MG132, confirming not only adequate assessment of nuclear translocation by immunocytochemistry, but also inhibition of the proteasome by MG132. These data suggest that, although astrocytes and mi-

croglia differ on many levels in their response to elevated pressure, NF $\kappa$ B translocation and the presumed induction of gene transcription are common components of the response pathway for both cell types. Furthermore, the reduction in pressure-induced transcription of IL-6 by MG132 together with precedence in the literature strongly suggests that activation of NF $\kappa$ B in microglia drives transcription of IL-6 specifically (Fig. 5).

Although the present study is the first to describe pressure-induced activation of NF $\kappa$ B and UPP-related regulation of IL-6 in retinal glia maintained in vitro, previous studies describe activation of NF $\kappa$ B in both the human disease and in glaucoma models.<sup>49,50</sup> There is no doubt that the biomechanics of exposure to regulated hydrostatic pressure in vitro differs greatly from those involved in chronic, progressive exposure to variations in elevated ocular pressure in vivo. We based the design of our chamber on those commonly used to study how hydrostatic pressure influences cellular proliferation and membrane elasticity.<sup>33</sup> When changes in pH, temperature, and the partial pressures of O<sub>2</sub> and CO<sub>2</sub> are carefully monitored, controlled for, or made negligible, as we and others have done (see the Methods section),<sup>21,25,34,35</sup> the most likely sources of cellular perturbation are distortional strain, compression, and the gradient of liquid potential across the cell membrane.<sup>34</sup> A common misconception is that, in a liquid environment, cells are incompressible and therefore do not experience a pressure gradient. However, for cells plated on an inelastic surface exposed to a uniform load (i.e., air pressure distributed equally above a small-volume liquid column), a careful mathematical analysis indicates that the gradient between the plates and the liquid column is substantial, and the most likely source of the cellular response, even for less elastic tissue such as cartilage.<sup>33,34</sup> So although the in vivo case is bound to differ, the in vitro pressure chamber is certainly a pertinent and highly useful model for isolating the influence of pressure-induced stresses.

Our data not only identify components of the mechanisms responsible for regulation of IL-6 in retinal glia, they also suggest that regulation of gene expression by NF $\kappa$ B may be a common pathway of response for multiple cell types in the glaucomatous retina. Although activation of NF $\kappa$ B previously described in RGCs most likely regulates transcription of anti-apoptotic factors,<sup>49-51</sup> the NF $\kappa$ B activation in microglia noted herein most likely regulates cytokine production. As previously demonstrated, the difference in IL-6 concentration for astrocytes and microglia under pressure underlies their different capacities to protect RGCs from pressure-induced death. However, the differences in the temporal pattern and mechanism of IL-6 regulation between astrocytes and microglia may also be significant in determining an additional role for glia-derived IL-6 in vivo.<sup>4,5</sup> Furthermore, these data indicate that retinal glia can produce neuroprotective cytokines in response to elevated pressure and that this production is likely to occur through mechanisms that mirror those described elsewhere in the nervous system.<sup>16,18,20,46,52,53</sup>

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