Sigma Receptor Ligand, (+)-Pentazocine, Suppresses Inflammatory Responses of Retinal Microglia

Jing Zhao,[1–3] Yonju Ha,[*,1,3] Gregory I. Liou,[1,2] Graydon B. Gonsalvez,[3] Sylvia B. Smith,[1–3] and Kathryn E. Bollinger[1–3]

[1]James and Jean Culver Vision Discovery Institute, Georgia Regents University, Augusta, Georgia, United States
[2]Department of Ophthalmology, Medical College of Georgia, Georgia Regents University, Augusta, Georgia, United States
[3]Department of Cellular Biology and Anatomy, Medical College of Georgia, Georgia Regents University, Augusta, Georgia, United States

Correspondence: Kathryn E. Bollinger, Department of Ophthalmology, Medical College of Georgia, Georgia Regents University, 1120 15th Street, BA 2320, Augusta, GA 30912, USA; kbollinger@gru.edu.

Current affiliation: *Department of Ophthalmology and Visual Sciences, University of Texas Medical Branch, Galveston, Texas, United States.

Submitted: July 14, 2013
Accepted: April 29, 2014

Citation: Zhao J, Ha Y, Liou GI, Gonsalvez GB, Smith SB, Bollinger KE. Sigma receptor ligand, (+)-pentazocine, suppresses inflammatory responses of retinal microglia. *Invest Ophthalmol Vis Sci*. 2014;55:3375–3384. DOI:10.1167/iovs.13-12823

Purpose. To evaluate the effects of the σ 1 receptor (σR1) agonist, (+)-pentazocine, on lipopolysaccharide (LPS)–induced inflammatory changes in retinal microglia cells.

Methods. Retinal microglia cells were isolated from Sprague-Dawley rat pups. Cells were treated with LPS with or without (+)-pentazocine and with or without the σR1 antagonist BD1063. Morphologic changes were assayed. Cell viability was assessed by using MTT assay. Supernatant levels of tumor necrosis factor α (TNF-α), interleukin 10, (IL-10), monocyte chemoattractant protein-1 (MCP-1), and nitric oxide (NO) were determined. Reactive oxygen species (ROS) formation was assayed, and levels of mitogen-activated protein kinases (MAPKs) were analyzed by using Western blot.

Results. The σR1 protein was expressed in retinal microglia. Incubation with LPS and/or (+)-pentazocine did not alter cell viability or σR1 protein levels. Incubation with LPS for 24 hours induced a marked change in microglial morphology and a significant increase in secreted levels of TNF-α, IL-10, MCP-1, and NO. Pretreatment with (+)-pentazocine inhibited the LPS-induced morphologic changes. Release of TNF-α, IL-10, MCP-1, and NO was reduced with (+)-pentazocine. Intracellular ROS formation was suppressed with (+)-pentazocine. Phosphorylation of extracellular signal–regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) was reduced in the presence of (+)-pentazocine. The σR1 antagonist BD1063 blocked the (+)-pentazocine–mediated inhibition of LPS-induced morphologic changes. In addition, BD1063 treatment blocked (+)-pentazocine–mediated suppression of LPS-induced TNF-α, IL-10, MCP-1, NO, and intracellular ROS release.

Conclusions. Treatment with (+)-pentazocine suppressed inflammatory responses of retinal microglia and inhibited LPS-induced activation of ERK/JNK MAPK. In neurodegenerative disease, (+)-pentazocine may exert neuroprotective effects through manipulation of microglia.

Keywords: microglia, neuron–glia interactions, optic nerve, sigma receptor

Microglia are the resident macrophages of the retina and brain.[1] These cells are activated in response to inflammatory stimuli such as the bacterial endotoxin lipopolysaccharide (LPS). Microglia activation causes intracellular production of reactive oxygen species (ROS) and release of bioactive molecules including tumor necrosis factor α (TNF-α), nitric oxide (NO), interleukin 10 (IL-10), and monocyte chemoattractant protein-1 (MCP-1).[2] The mitogen-activated protein kinase (MAPK) subgroup members including extracellular signal–regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK have been implicated in control of expression of these immune-related factors within microglia.[3–5]

In brain and retina, microglial secreted factors cause pleiotropic effects on immunoregulation and inflammation. However, microglia activation via inflammatory stimuli can lead to chronic neuroinflammation and enhanced neuronal injury.[6,7] Several pathologic conditions are exacerbated by neuroinflammation, including stroke, Alzheimer's disease, Parkinson's disease, and traumatic brain injury.[8–10] Within the eye, disease states including glaucoma, diabetic retinopathy, and age-related macular degeneration share neuroinflammation as a common pathologic component.[11–13] In addition, many studies indicate that strategies for decreasing chronic inflammation prove therapeutic.[14,15] Therefore, microglia-mediated responses to inflammatory stimuli are treatment targets for vision-threatening diseases.

The σ receptors are classified into two subtypes (σR1 and σR2), and ligands for these receptors show variable specificity.[16] σR1 has been cloned and its sequence does not share homology with other known mammalian proteins.[16] σR1 is a transmembrane protein found in the endoplasmic reticulum that is ubiquitously expressed throughout the nervous system and visceral organs. Within the eye, σR1 is expressed in cornea, iris–ciliary body, lens, retina, and optic nerve. Cell types throughout the retina that contain σR1 include ganglion cells, inner nuclear layer cells, photoreceptors, and retinal pigment epithelium.[17,18] Within the nervous system, σR1 has been shown to regulate several processes including N-methyl-D-aspartate receptor and K^+ channel activity,[19] neuritogenesis,[20,21] L-type voltage-gated calcium channel activity,[22,23] and microglial activity.[24] However, the complete endogenous function of this protein is not known.

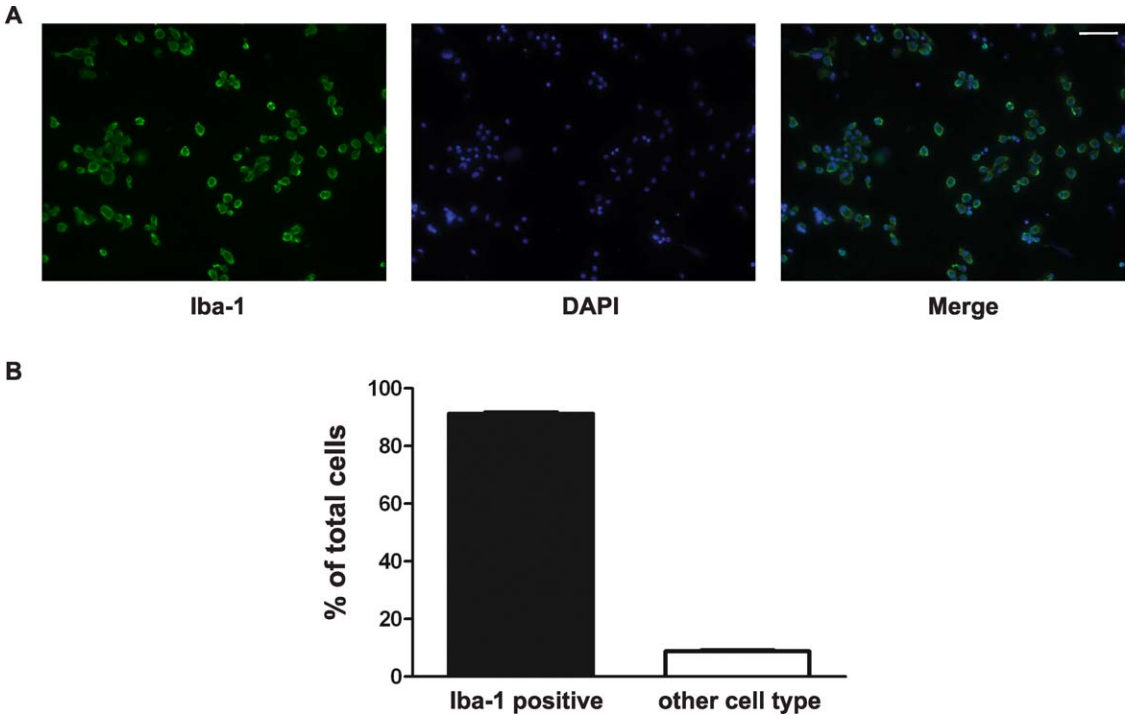

Figure 1. Characterization of cultured retinal microglia cells. (**A**) Retinas were dissected from rat pups and retinal microglia cells were purified and cultured. The cells were fixed and probed with an antibody against Iba1, a microglia-specific marker (*green*). The cells were counterstained with DAPI to label DNA (*blue*) as a marker for nuclei. A merged image is also shown. *Scale bar*: 50 μm. (**B**) Cells that stained positively for Iba1 were counted and represented as a fraction of the total cell population. More than 90% of the cells expressed Iba1.

Ligands for σR1 have shown neuroprotective properties in several animal models including amyotrophic lateral sclerosis (ALS),[25] stroke,[26] and diabetic retinopathy.[27] In addition to neuroprotection, σR1 activation has been associated with decreased reactive gliosis in both the ALS mouse model and the rat stroke injury model.[26,28] Evidence also suggests that some σR1 ligands can repress activation of the MAPK/ERK pathway within cortical neurons.[29]

Recent work has shown that σ receptors modulate the activation of CNS-derived primary microglia. Treatment with the nonspecific σR1/R2 ligand 1,3-di-o-tolylguanidine (DTG) decreased the LPS-stimulated microglial release of inflammatory mediators including NO, TNF-α, and IL-10.[24] It is not known whether this effect results from σR1 or σR2 activation. In addition, the effect of σR1 activation on neuroinflammation in the retina is not known. Further, the existence of σR1 within retinal microglia has never been reported.

The present study was undertaken to investigate the activity of the σR1-specific agonist (+)-pentazocine on retina-derived, LPS-stimulated, primary microglia. Our results showed that exposure of retinal microglia to LPS led to release of TNF-α, NO, IL-10, and ROS as well as morphologic changes in the cells, and activation of ERK/JNK MAPK pathways. These effects were inhibited by (+)-pentazocine. From these results, we suggest that the σR1 agonist pentazocine has the potential to serve as a therapeutic modulator for neuroinflammatory responses within the retina.

Methods

Materials

Sprague-Dawley (SD) rats were purchased from Harlan Laboratories (Indianapolis, IN, USA). (+)-Pentazocine and LPS (LPS from *Escherichia coli*, serotype 0111:B4) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The σR1 antagonist BD1063 was purchased from Tocris Bioscience (Bristol, UK). Dulbecco's modified Eagle's medium (DMEM)/F12 culture medium, Hanks' balanced salt solution (HBSS), 0.125% trypsin, penicillin/streptomycin, MTT Cell Proliferation Assay Kit, Griess Reagent Kit, CellROX Green Reagent, MCP-1 ELISA Kit, and Alexa Fluor 488–labeled goat anti-mouse IgG antibody were purchased from Invitrogen (Grand Island, NY, USA). Cellgro complete medium was purchased from Cellgro (Manassas, VA, USA). Tumor necrosis factor α ELISA kits and IL-10 ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). p-ERK, ERK, p-p38, p38, p-JNK, JNK polyclonal antibodies, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were purchased from Cell Signaling Technology (Danvers, MA, USA). glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody was purchased from Millipore (Billerica, MA, USA). Iba1 monoclonal antibody was purchased from Abcam (Cambridge, MA, USA). SuperSignal West Pico chemiluminescent substrate was purchased from Thermo Scientific (Waltham, MA, USA). σR1 rabbit polyclonal antibody was raised from a peptide sequence and generated within the laboratory of Sylvia Smith, PhD.[30]

Primary Rat Retinal Microglia Culture

Experiments requiring animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Microglia cells were isolated from retinas of SD rats, according to previously published protocols[31] with minor modifications. Briefly, retinas were dissected from newborn SD rat pups. Tissue was placed into ice-cold HBSS and washed, then digested with 0.125% trypsin for 5 minutes. Tissue was then triturated by passing through a disposable pipette several times and cells were dispersed in DMEM/F12 medium containing 10% FBS and 1% penicillin/streptomycin. Cells were then filtered through a 40-μm-cell strainer, collected by

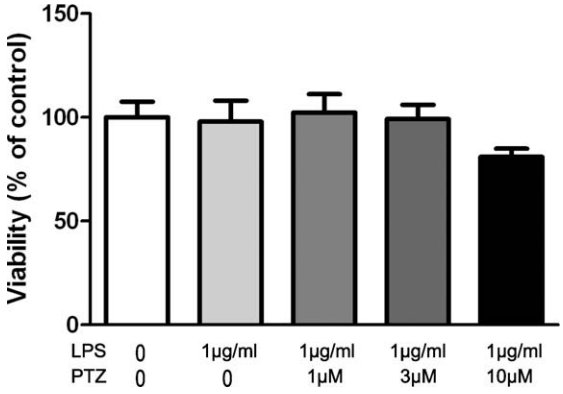

FIGURE 2. Effect of LPS and/or (+)-pentazocine [(+)-PTZ] on cell viability. Microglia cells were treated with LPS (1 μg/mL) alone or in combination with (+)-PTZ (1, 3, or 10 μM) for 24 hours. The MTT assay was used to assess viability. Treatment with LPS with or without (+)-PTZ (1 or 3 μM) did not cause a significant change in percentage viability compared to the untreated control. At high concentration of (+)-PTZ (10 μM), there was a trend toward decrease in viability that was not significant.

centrifugation, resuspended in culture medium, and plated as 1×10^7 cells per T75 cell culture flask. All cultures were maintained in a humidified CO_2 incubator at 37°C and 5% CO_2. Half of the medium was changed every 4 days. After 2 weeks, microglia cells were isolated from detached cells in the supernatant medium by shaking the T75 cell culture flasks at 100 rpm for 2 hours at 37°C. The cell suspension was centrifuged and the detached cells were seeded at a density of 1×10^5/well on coverslips in 24-well tissue culture plates, or 1×10^4 cells/well in 96-well plates with Cellgro complete medium. One day after seeding, the cells were treated with LPS (*E. coli* 0111:B4) at a final concentration of 1 μg/mL with or without (+)-pentazocine (30-minute pretreatment followed by cotreatment) at a final concentration of 3 μM. For experiments with BD1063, a 10-μM concentration of this reagent was used. When BD1063 (10 μM) was used in addition to (+)-pentazocine (3 μM), the BD1063 was given 30 or 60 minutes (depending on the experiment) before the (+)-pentazocine pretreatment.

Immunocytochemistry

Immunocytochemistry was performed to identify the purity and morphology of cultured primary rat retinal microglia. Briefly, cells were fixed with 4% paraformaldehyde at room temperature for 15 minutes, followed by washing with PBS three times. Cells were then membrane permeabilized with 0.3% Triton X-100 in PBS at room temperature for 10 minutes. Following this, the cells were blocked with Powerblock at room temperature for 1 hour, then incubated in primary antibody (Iba1 1:100), at 4°C overnight. The second day the cells were incubated in secondary antibody (Alexa Fluor 488–labeled goat anti-mouse 1:1000) at room temperature for 1 hour. After the cells were washed with PBS three times, the coverslips were mounted with Fluoroshield (Sigma-Aldrich, St. Louis, MO) with 4',6-diamidino-2-phenylindole (DAPI). Cells were observed by immunofluorescence using a Zeiss Axioplan-2 microscope (Carl Zeiss, Oberkochen, Germany) equipped with AxioVision program (version 4.6.3) and a high-resolution microscopy camera.

MTT Assay

Microglia were seeded onto 96-well plates at a density of 2×10^4 cells/well in medium (free of phenol red). After incubation

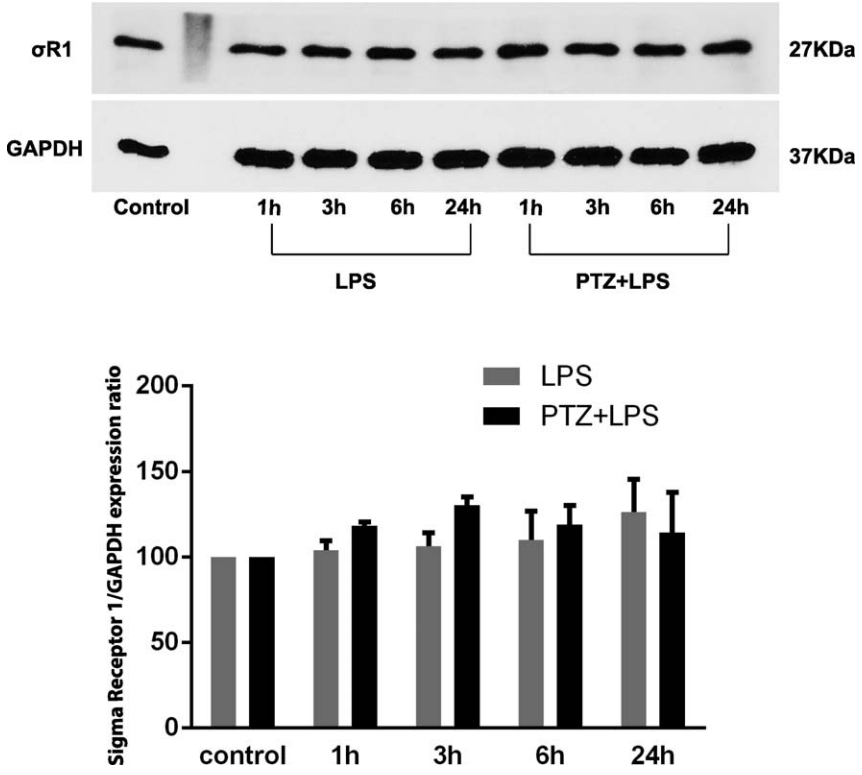

FIGURE 3. σR1 expression in retinal microglia cells. Retinal microglia cells were exposed to LPS (1 μg/mL) or a combination of (+)-PTZ (3 μM) and LPS for 1, 3, 6, and 24 hours. Lysates were prepared from the cells and analyzed by Western blot using antibodies against σR1 and GAPDH (*top panel*). There was no appreciable change in σR1 band intensity level under conditions of LPS or (+)-PTZ exposure at the time points assessed. Results from two experiments were quantified by densitometry (*bottom panel*, ImageJ).

Investigative Ophthalmology & Visual Science

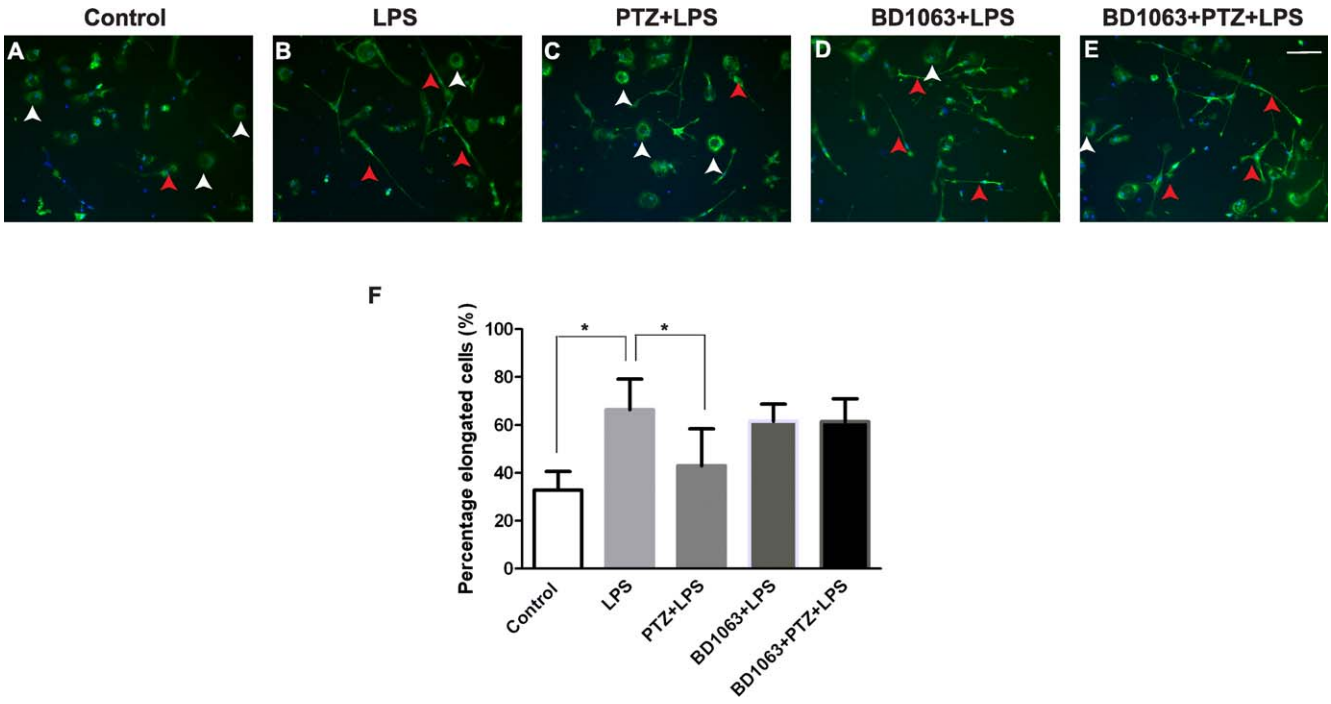

Figure 4. (+)-PTZ suppresses LPS-induced morphology change in retinal microglia cells; suppression is blocked by σR1 antagonist BD1063. Primary microglia cultures were incubated with (**A**) vehicle control, (**B**) LPS (1 μg/mL), (**C**) a combination of (+)-PTZ (3 μM, 30-minute pretreatment followed by cotreatment) and LPS, (**D**) a combination of BD1063 (10 μM, 30-minute pretreatment followed by cotreatment) and LPS, or (**E**) a combination of BD1063, (+)-PTZ, and LPS (30-minute pretreatment of 10 μM BD1063 followed by 30-minute pretreatment with 3 μM (+)-PTZ, then followed by cotreatment with LPS). After 6 hours' incubation, the cells were fixed, probed with an antibody against Iba1, and were counterstained with DAPI. Whereas the untreated cells were 60% to 70% round (*white arrowhead*) (**A**), LPS treatment induced a bipolar or multipolar morphology (*red arrowhead*), and the percentage of bipolar cells was increased to from 70% to 80% (**B**). Pretreatment with (+)-PTZ before LPS treatment suppressed the morphologic change, and the percentage of bipolar cells was decreased to 40% (**C**). While the σR1 antagonist BD1063 had no effect alone on suppression of LPS-induced morphology change (**D**), BD1063 blocked the PTZ-induced suppression of the LPS-mediated morphology change (**E**). *Scale bar*: 50 μm. (**F**) For each group, nine images were taken from each coverslip, and three coverslips were quantified for each group. Twenty to 30 cells were counted for each field. Percentage of bipolar cells was quantified by ImageJ. *Significantly different from control ($P < 0.05$).

with LPS (1 μg/mL) with or without (+)-pentazocine (3 μM, 30-minute pretreatment followed by cotreatment) for 24 hours, medium was removed and MTT (1.2 mM in culture medium) was added and incubated for 4 hours at 37°C. Solubilization with dimethyl sulfoxide was performed according to protocol directions. Absorbance was measured at 540 nm with a microplate reader (VERSA max, Molecular Devices, Sunnyvale, CA, USA).

Enzyme-Linked Immunosorbent Assay

Tumor necrosis factor α, IL-10, and MCP-1 concentrations from rat retinal microglia culture supernatants were quantified by using enzyme-linked immunosorbent assay (ELISA). The 96-well plates were provided already coated with rat monoclonal antibody. After assay diluent was pipetted into each well, standard or sample was incubated for 2 hours at room temperature. After washing, enzyme-linked polyclonal anti–TNF-α, anti–IL-10, or anti–MCP-1 was added for detection and incubated for 2 hours at room temperature. After further washing, the substrate solution was added, and the reaction was stopped. The optical density was read at 450 nm wavelength within 30 minutes.

Griess Assay

Supernatants from primary rat microglia cultures, treated in the same manner as for ELISA, were collected. Nitrite levels in the supernatants were determined by using the Griess Reagent Kit according to the manufacturer's protocol.

ROS Detection

For intracellular ROS detection, cells were washed with PBS and incubated with 5 μM CellROX Green Reagent in the dark for 30 minutes at 37°C. After sufficient washing, intracellular levels of emitted fluorescence were observed under a fluorescence microscope.

Western Blot Analysis

Rat retinal microglia were lysed in RIPA buffer (ready-to-use solution containing 150 mM NaCl, 1.0% IGEPALCA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0; Sigma-Aldrich). The cell lysates were centrifuged at 14,000*g* for 30 minutes. The protein concentration was measured by using Bradford assay (Bio-rad, Hercules, CA, USA). The proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in Tris-buffered saline—Tween 20 (TBST) for 1 hour at room temperature, then incubated overnight at 4°C with primary antibody. After three washes in TBST, the membrane was incubated for 1 hour with an appropriate HRP-conjugated secondary antibody at room temperature. The proteins were visualized by incubating with a chemiluminescent substrate (West Pico; Thermo Scientific) and quantified by densitometry (ImageJ software, http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The blots were stripped and reprobed for loading controls. p-ERK, ERK, p-p38, p38, p-JNK, JNK polyclonal antibodies and HRP-

Investigative Ophthalmology & Visual Science

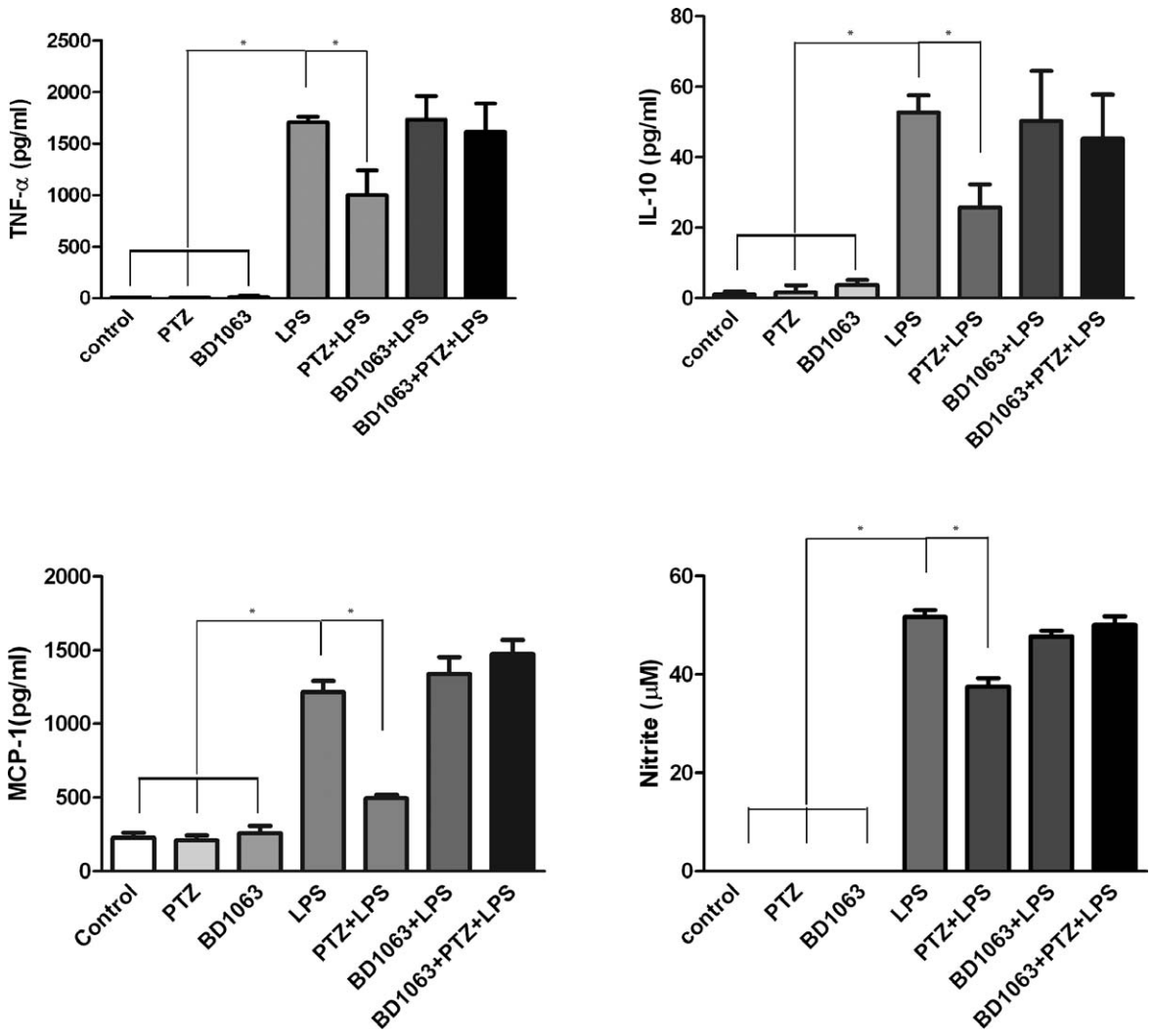

FIGURE 5. (+)-PTZ suppresses microglia release of bioactive molecules, and suppression is blocked by σR1 antagonist BD1063. Primary microglial cultures were incubated with LPS (1 μg/mL) for 24 hours in the presence/absence of BD1063 and (+)-PTZ (30-minute pretreatment of 10 μM BD1063 followed by 30-minute pretreatment with 3 μM (+)-PTZ, then followed by cotreatment with LPS). Supernatant-derived TNF-α, IL-10, and MCP-1 were measured by ELISA. NO levels were measured by using the Griess reaction. As compared to control, TNF-α, IL-10, MCP-1, and NO levels were significantly increased by LPS treatment. LPS-evoked levels of each of these four molecules were significantly decreased by (+)-PTZ pretreatment. The (+)-PTZ–mediated decreases in cytokine release were blocked by 30-minute pretreatment with BD1063. *Significantly different from control ($P < 0.05$).

conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were purchased from Cell Signaling Technology. Glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody was purchased from Millipore. σR1 rabbit polyclonal antibody was raised from a peptide sequence and generated within the laboratory of Sylvia Smith, PhD.[30]

Statistical Analysis

Data for ELISA, MTT assay, and intracellular ROS detection were analyzed by using Student's *t*-test. For Western blot analysis, two-way ANOVA was used to determine significant differences. Significance was set at $P < 0.05$ (Prism; GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

We obtained rat microglia cells from the retinas of animals between postnatal day 1 and 5. More than 90% of cells were found to express the microglia-specific markers Iba1 (Fig. 1) and CD11b (data not shown), suggesting the purity of the preparations. To determine the effect of LPS or (+)-pentazocine on microglia cell viability, we performed the MTT assay. Treatment with LPS (1 μg/mL) alone or with low concentration (+)-pentazocine (1 or 3 μM) for 24 hours did not alter viability (Fig. 2). However, high concentrations of (+)-pentazocine (10 μM) showed decreased viability. This was not statistically significant, but all further assays of microglia activity were conducted with a 3-μM concentration of (+)-pentazocine (Fig. 2). This concentration of (+)-pentazocine has been shown to be neuroprotective in primary retinal ganglion cell (RGC) cultures and in other neurologic systems.[32–35]

To determine whether σR1 was expressed within microglia cells, and whether LPS or (+)-pentazocine exposure changed σR1 levels, microglia were incubated with LPS (1 μg/mL) or (+)-pentazocine (3 μM) for 1, 3, 6, or 24 hours (Fig. 3). Western blot was performed to assay for σR1. Using our antibody against σR1, a band with the predicted molecular weight of 25 to 27 kDa was detected. Thus, σR1 protein is present in retinal

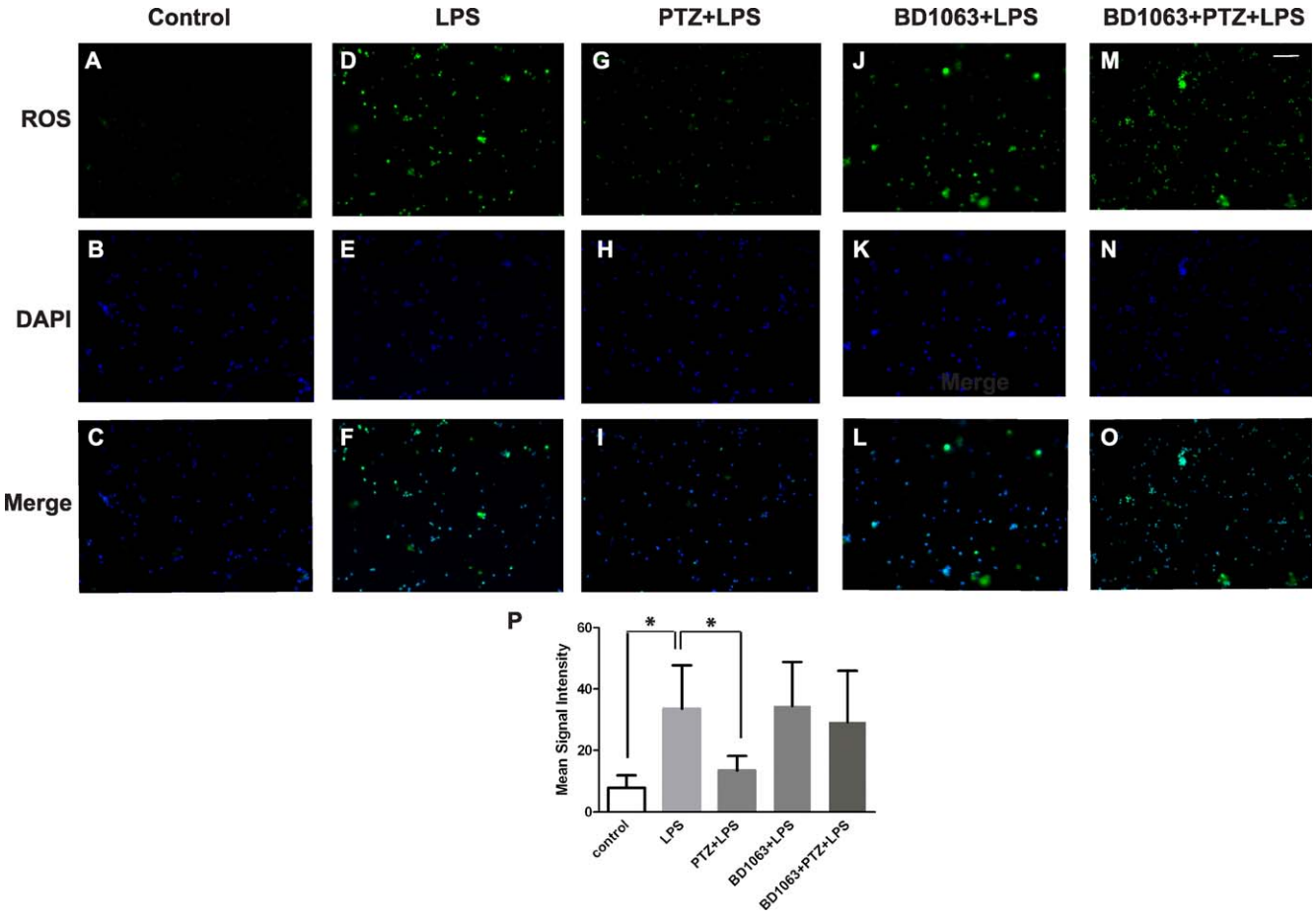

Figure 6. (+)-PTZ suppresses LPS-induced ROS generation, and suppression is blocked by σR1 antagonist BD1063. Primary microglia cultures were incubated with (**A–C**) vehicle control; (**D–F**) LPS (1 μg/mL); (**G–I**) 3 μM (+)-PTZ, 30-minute pretreatment followed by cotreatment with 1 μg/mL LPS; (**J–L**) 10 μM BD1063, 30-minute pretreatment followed by cotreatment with 1 μg/mL LPS; (**M–O**) 30-minute pretreatment of 10 μM BD1063 followed by 30-minute pretreatment with 3 μM (+)-PTZ, then followed by cotreatment with LPS. Intracellular ROS was detected by using CellROX Green Reagent (**A**, **D**, **G**, **J**, **M**). The cells were counterstained with DAPI to label DNA (*blue*) (**B**, **E**, **H**, **K**, **N**). Merged images are also shown (**C**, **F**, **I**, **L**, **O**). *Scale bar*: 50 μm. (**P**) For each group, three coverslips were quantified, and nine images were taken from each coverslip. Mean signal intensity was quantified by ImageJ. A total of 100 to 150 cells were counted in each field. ROS generation increased when cells were incubated with LPS. The LPS-induced ROS generation was inhibited by (+)-PTZ pretreatment. The inhibition was blocked by pretreatment of σR1 antagonist BD1063. *Significantly different from control ($P < 0.05$).

microglia. In addition, there was no appreciable change in σR1 band intensity under conditions of LPS or (+)-pentazocine exposure (Fig. 3).

Incubation with LPS for 6 hours (Fig. 4) and 24 hours (data not shown) induced a marked change in microglial morphology. The most dramatic effects were observed at the 6-hour time point, and LPS-exposed cells changed from a spherical shape (Fig. 4A) to a bipolar, tripolar, or rod-like shape (Fig. 4B). These changes are similar to those described by Moriyama et al.,[36] Nakamura et al.,[37] and El-Remessy et al.,[31] at 6-, 6-, and 12-hour time points, respectively. Pretreatment with (+)-pentazocine (3 μM for 30 minutes) dramatically inhibited the LPS-induced morphologic changes (Fig. 4C).

To address whether (+)-pentazocine–mediated inhibition of LPS-evoked morphologic changes occurred through σR1, we treated cells with an antagonist of this receptor, BD1063.[38] Microglia cultures were first treated with BD1063 (10 μM) for 30 minutes, followed by (+)-pentazocine (3 μM) for 30 minutes, then incubated with LPS for 6 hours (Fig. 4E). Consistent with the involvement of σR1, the (+)-pentazocine–mediated inhibition of LPS-evoked morphologic changes was blocked when microglia were treated with BD1063 (Fig. 4E).

Lipopolysaccharide is well-known to activate microglia and to cause robust cytokine and NO release. To determine whether σR1 activation could modulate these responses, we examined LPS-evoked TNF-α, IL-10, MCP-1, and NO production after pretreatment with (+)-pentazocine (3 μM for 30 minutes). Levels of MCP-1, TNF-α, and IL-10 were measured from cell culture supernatants by using ELISA, and NO levels were quantified by using the Griess reaction. Our analyses showed a statistically significant increase in cell culture supernatant levels of all four molecules when cultures were treated with LPS compared to control ($P < 0.05$). We also observed a (+)-pentazocine–mediated suppression of LPS-evoked microglial release of all three cytokines and of NO (Fig. 5). This suppression was found to be statistically significant ($P < 0.05$) (Fig. 5). In addition, consistent with involvement of σR1, the (+)-pentazocine–mediated suppression of LPS-evoked TNF-α, MCP-1, IL-10, and NO release was blocked when microglia were treated with BD1063 (10 μM) for 30 minutes, followed by (+)-pentazocine (3 μM) for 30 minutes, then incubated with LPS (Fig. 5).

Generation of ROS can lead to enhanced cellular inflammation and to cell death. Therefore, we determined intracelluar ROS formation after LPS treatment (1 μg/mL for 24 hours) in

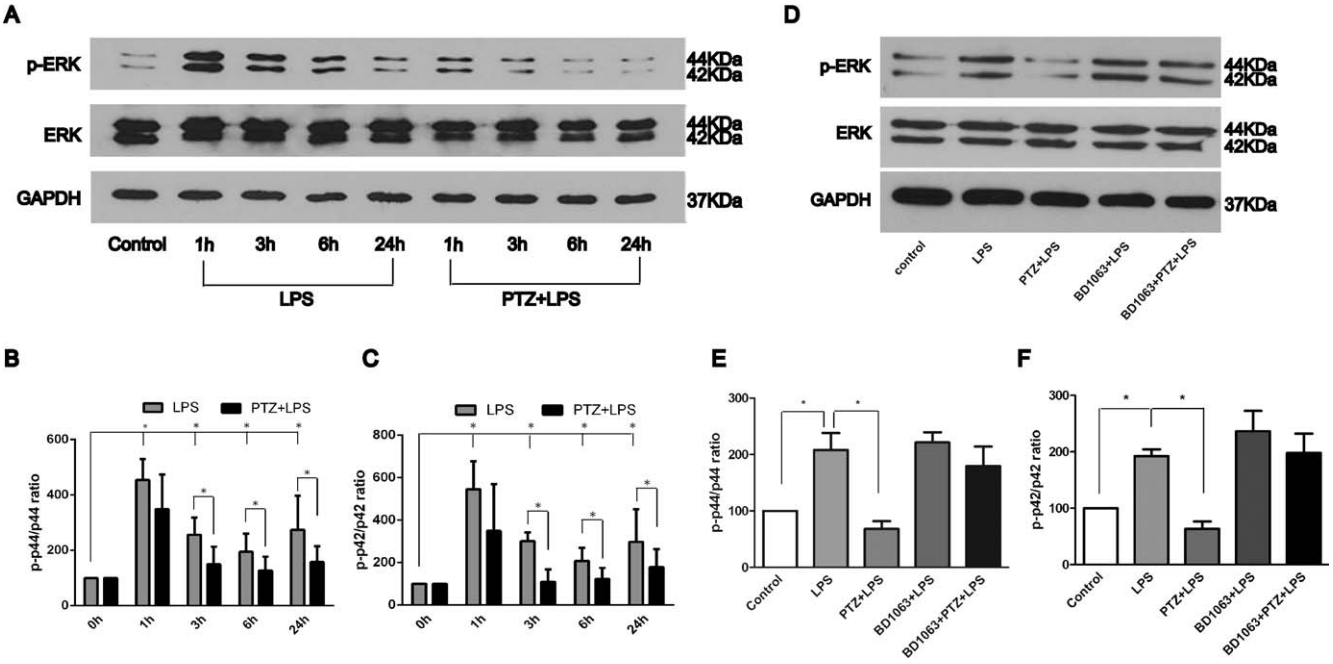

Figure 7. (+)-PTZ suppresses ERK phosphorylation in LPS-activated microglia, and the suppression is blocked by σR1 antagonist BD1063. (**A**) Retinal microglia cells were incubated with 1 μg/mL LPS at 37°C for 1, 3, 6, and 24 hours in the presence or absence of (+)-PTZ (3 μM, 30-minute pretreatment followed by cotreatment). Lysates were prepared from the cells and analyzed by Western blot using the indicated antibodies. Phosphorylation of both ERK isoforms (p44 and p42) was increased at all time points by treatment with LPS. The LPS-induced increase in phosphorylation of ERK was significantly decreased by (+)-PTZ at 3-, 6-, and 24-hour time point of LPS exposure but not in control lysates. (**D**) Retinal microglia cells were incubated with LPS (1 μg/mL) for 24 hours in the presence/absence of BD1063 and (+)-PTZ (60-minute pretreatment of 10 μM BD1063 followed by 30-minute pretreatment with 3 μM (+)-PTZ, then followed by cotreatment with LPS). Lysates were prepared from the cells and analyzed by Western blot using the indicated antibodies. Phosphorylation of both ERK isoforms (p44 and p42) was increased by treatment with LPS. The LPS-induced increase in phosphorylation of ERK was decreased by (+)-PTZ but not BD1063. BD1063 blocked (+)-PTZ–mediated suppression of ERK activation. (**B**, **C**, **E**, **F**) Results from three experiments were quantified by densitometry (ImageJ). *Significantly different from control ($P < 0.05$).

the presence and absence of a 30-minute (+)-pentazocine pretreatment. Intracellular ROS generation was measured by using CellROX Green Reagent. This is a fluorogenic, DNA-binding probe that exhibits bright green photostable fluorescence upon oxidation by ROS. Intracellular levels of ROS were examined by using fluorescence microscopy. Compared with control cells, we observed a statistically significant increase in mean intracellular fluorescence when microglia were treated with LPS. Reactive oxygen species generation was significantly inhibited when LPS-induced microglia were pretreated with (+)-pentazocine (Fig. 6). Furthermore, the effect of (+)-pentazocine on ROS generation was blocked upon pretreating cells with the σR1 antagonist BD1063 (10 μM) (Fig. 6).

Because MAPK signaling pathways play important roles in inflammatory response, we explored the effects of (+)-pentazocine treatment on activation of ERK1/2, JNK, and p38 MAPKs. In the presence of LPS alone, we observed a significant increase in ERK phosphorylation, compared with control, at all time points analyzed (Fig. 7). Pretreatment with (+)-pentazocine significantly suppressed LPS-induced phosphorylation of ERK1/2 at the 3-, 6-, and 24-hour time points (Fig. 7). Treatment with BD1063 before addition of (+)-pentazocine blocked the (+)-pentazocine–mediated suppression of LPS-induced ERK activation at the time point analyzed (24 hour) (Fig. 7). In addition, we observed a significant increase in JNK phosphorylation at 1, 3, and 6 hours after treatment with LPS alone (Fig. 8). Pretreatment with (+)-pentazocine significantly suppressed LPS-induced activation of JNK MAPK, at the 1-, 3-, and 6-hour time points. With respect to JNK, we did not appreciate consistent suppression of LPS-induced phosphorylation at the 24-hour time point (Fig. 8). Presumably, this is because JNK phosphorylation at this time point is already significantly decreased. Finally, consistent with ERK and JNK results, we observed an increase in p38 MAPK activation after 1 hour of LPS incubation (Fig. 9). This activation level decreased incrementally at the 3-, 6-, and 24-hour time points. However, in this case, pretreatment with (+)-pentazocine did not decrease (or increase) the phosphorylation status of p38 MAPK (Fig. 9).

Discussion

This study showed that σR1 is present within retinal microglia and that the σR1-specific agonist (+)-pentazocine can suppress multiple aspects of inflammatory microglia activation. These results offer the first report, to our knowledge, of the effects of σR1 activation on retinal microglia cells.

Previous reports have shown conflicting results with respect to the involvement of σ receptors in inflammatory responses. Hall et al.[24] have reported that application of the nonspecific σR1/2 ligand DTG suppressed the release of TNF-α, IL-10, and NO in LPS-activated, brain-derived microglia cells. In addition, in models of rheumatoid arthritis and sepsis, a decrease in cytokine release was observed after treatment with the σ ligands SR31747A and SSR 125329A, respectively.[39,40] However, Ruscher et al.[28] have found that exposure of rat brain–derived microglia to the σR1 ligand SA4503 did not affect the release of proinflammatory mediators after combined hypoxia/aglycemia stimulation. To our knowledge, no previous reports address the effects of (+)-pentazocine, specifically, on microglia.

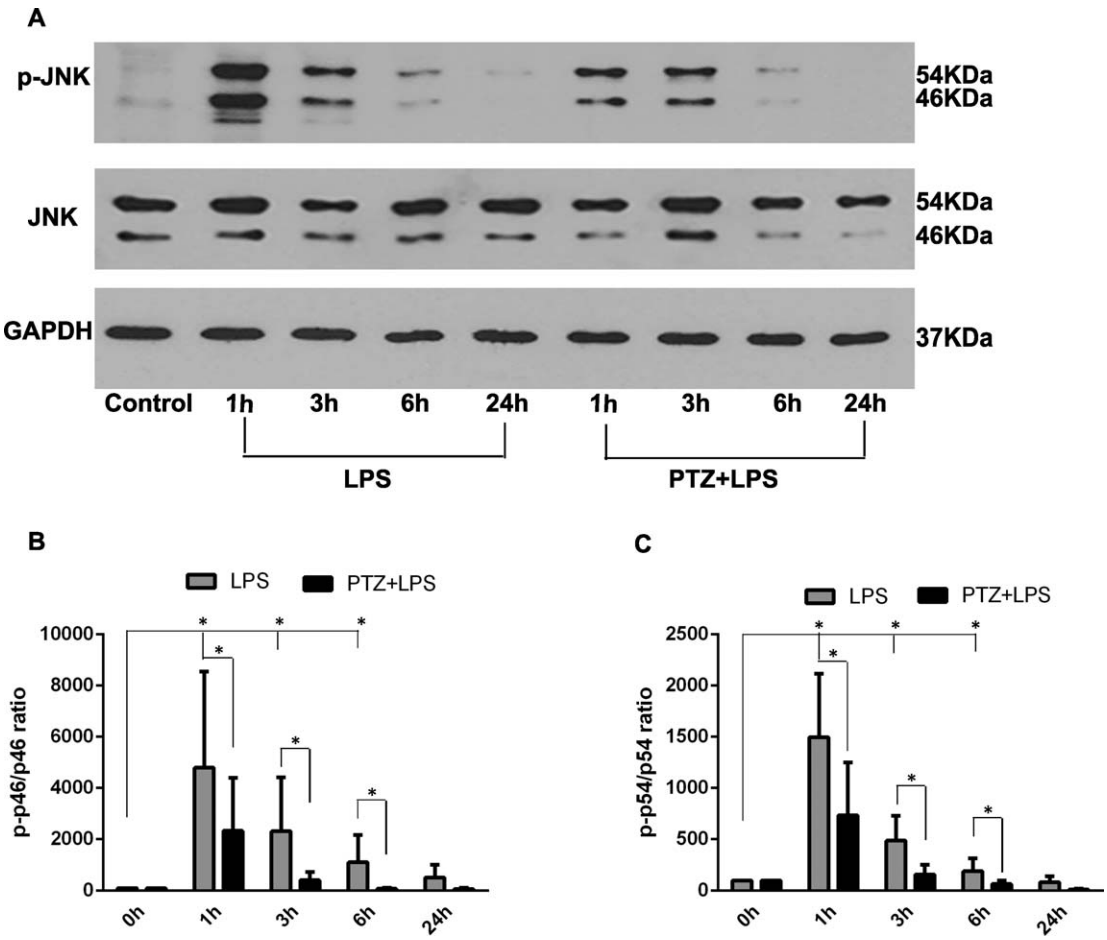

Figure 8. Effect of (+)-PTZ on phosphorylation of JNK in LPS-activated microglia. (**A**) Retinal microglia cells were incubated with 1 μg/mL LPS at 37°C for 1, 3, 6, and 24 hours in the presence or absence of (+)-PTZ (3 μM, 30-minute pretreatment followed by cotreatment). Lysates were prepared from the cells and analyzed by Western blot using the indicated antibodies. Phosphorylation of both JNK isoforms (p54 and p46) was increased by treatment with LPS at the 1-, 3-, and 6-hour time points. JNK phosphorylation returned to basal levels by 24 hours. The LPS-induced increase in phosphorylation of JNK was decreased by (+)-PTZ at the 1-, 3-, and 6-hour time points of LPS exposure but not in control lysates. (**B**, **C**) Results from three experiments were quantified by densitometry (ImageJ). *Significantly different from control ($P < 0.05$).

Pharmacologic analyses have demonstrated (+)-pentazocine to be a highly specific and potent ligand for σR1. In addition, when brain membranes derived from the σR1 knockout mouse were analyzed in binding assays using [3H] (+)-pentazocine as the radioligand, no binding activity was observed. Binding activity was reduced by half in heterozygous compared with wild-type animals.[41] We feel this is significant evidence of the specificity of (+)-pentazocine for σR1. In addition, our studies indicate that (+)-pentazocine–mediated suppression of microglia inflammatory response occurs through σR1; using the σR1 antagonist BD1063, we observed blockage of morphologic changes, attenuated bioactive molecule release, and decreased ROS production.

Although (+)-pentazocine is considered a σR1 agonist, and BD1063 is considered a σR1 antagonist, the signaling mechanisms and specific functional effects of agonists versus antagonists have not been fully characterized. For this reason, we cannot predict the effects of other compounds (presumed agonists or antagonists of σR1) on retinal microglia cells.

Ligands for σR1 have shown neuroprotective properties in several animal models of neurodegenerative disease and in a mouse model of diabetic retinopathy (DR).[22,23,25,26,42] Within the DR model, RGCs, in particular, were protected.[27] In addition, Ha et al.[17] have observed that σR1 knockout mice display a late-onset inner retinal dysfunction with some similarities to glaucoma. Further, Mavlyutov et al.[42] have found that σR1 knockout mice show accelerated RGC death under conditions of optic nerve crush injury. Whether (+)-pentazocine can provide neuroprotection in the setting of glaucomatous stress is not yet known. Interestingly, recent investigations[14] have shown that infiltration of microglia into the optic nerve and secretion, by these cells, of neurotoxic molecules such as TNF-α, is likely a very early component of glaucoma pathogenesis. So, it is possible that treatment with (+)-pentazocine could provide neuroprotection through suppression of inflammatory microglia activation.

The ERK, JNK, and p38 MAPKs have been shown to mediate microglial acquisition of inflammatory features including expression of bioactive molecules such as NO and TNF-α.[3,43,44] Consistent with this, we observed activation of each of these MAPKs upon incubation of retinal microglia with LPS. Pretreatment with (+)-pentazocine inhibited activation of ERK and JNK, while phosphorylation of p38 was not affected. With respect to ERK activation, results are consistent with those reported by Tuerxun et al.[29] showing that the σR1 ligand SA4503 prevents cultured cortical neurons from oxidative stress–induced cell death via suppression of MAPK/ERK pathway activation. However, Tuerxun and colleagues[29] have not found suppression of JNK activation. In addition, other investigations have revealed increased ERK activation (as

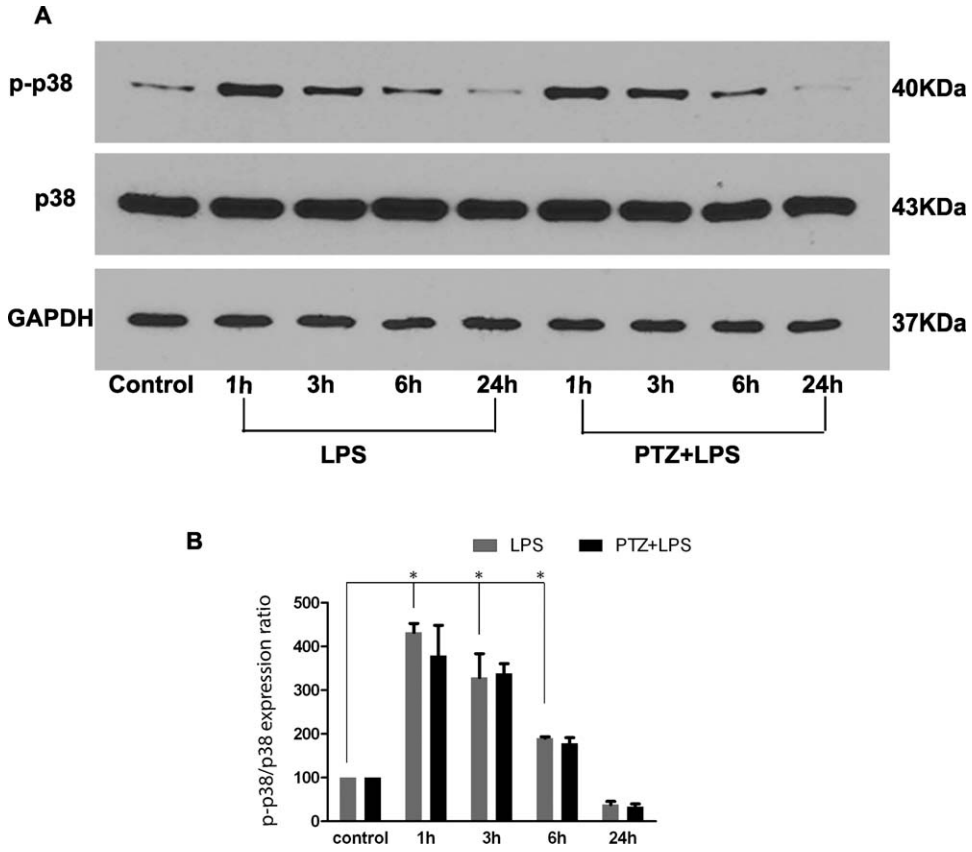

Figure 9. Effect of (+)-PTZ on phosphorylation of p38 in LPS-activated microglia. (**A**) Retinal microglia cells were incubated with 1 μg/mL LPS at 37°C for 1, 3, 6, and 24 hours in the presence or absence of (+)-PTZ (3 μM, 30-minute pretreatment followed by cotreatment). Lysates were prepared from the cells and analyzed by Western blot using the indicated antibodies. Phosphorylation of p38 was increased by treatment with LPS at the 1-, 3-, and 6-hour time points. The LPS-induced increase in phosphorylation of p38 was not reliably changed by (+)-PTZ pretreatment. (**B**) Results from three experiments were quantified by densitometry (ImageJ).

opposed to inhibition of activation as reported in this study) within hippocampal tissue[45] and within mixed cortical and hippocampal neuronal cultures[46] in the presence of σR1 ligands. The difference in effect on MAPK subgroups might be explained because of multiple cell types. For example, the effects of σR1 ligands might vary if the responsive cell is of a particular neuronal type or of glial origin. In addition, others[46] have postulated that σR1 receptors may have multiple discrete binding sites that cause different responses. We suspect that differences in effect on MAPK subgroups arise from the individual properties, including cell type–specific effects, of σR1 ligands.

(+)-Pentazocine is a small molecule that can pass through the blood–retinal barrier and shows neuroprotective effects in brain and retina. Since pentazocine has the ability to suppress microglial inflammatory responses, it could hold therapeutic potential for ophthalmic diseases such as diabetic retinopathy and glaucoma. Further work is needed to determine whether (+)-pentazocine or other σR1 ligands show disease-relevant neuroprotection. Additional studies using inducible animal models would assist with addressing this question.

Acknowledgments

Supported in part by National Institutes of Health Grants 5K08EY021758-03 and R01 EY014560, an American Glaucoma Society Young Clinician Scientist Award (KEB), and Georgia Regents University.

Disclosure: **J. Zhao**, None; **Y. Ha**, None; **G.I. Liou**, None; **G.B. Gonsalvez**, None; **S.B. Smith**, None; **K.E. Bollinger**, None

References

1. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci*. 1996;19:312–318.
2. Streit WJ, Mrak RE, Griffin WS. Microglia and neuroinflammation: a pathological perspective. *J Neuroinflammation*. 2004;1:14.
3. Bhat NR, Zhang P, Lee JC, Hogan EL. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. *J Neurosci*. 1998;18:1633–1641.
4. Waetzig V, Czeloth K, Hidding U, et al. c-Jun N-terminal kinases (JNKs) mediate pro-inflammatory actions of microglia. *Glia*. 2005;50:235–246.
5. Zhang P, Hogan EL, Bhat NR. Activation of JNK/SAPK in primary glial cultures: II, differential activation of kinase isoforms corresponds to their differential expression. *Neurochem Res*. 1998;23:219–225.
6. Langmann T. Microglia activation in retinal degeneration. *J Leukoc Biol*. 2007;81:1345–1351.
7. Zeng HY, Zhu XA, Zhang C, et al. Identification of sequential events and factors associated with microglial activation, migration, and cytotoxicity in retinal degeneration in rd mice. *Invest Ophthalmol Vis Sci*. 2005;46:2992–2999.
8. Gebicke-Haerter PJ. Microglia in neurodegeneration: molecular aspects. *Microsc Res Tech*. 2001;54:47–58.

9. Hellewell SC, Morganti-Kossmann MC. Guilty molecules, guilty minds: the conflicting roles of the innate immune response to traumatic brain injury. *Mediators Inflamm*. 2012;2012: 356494.
10. Perry VH, Nicoll JA, Holmes C. Microglia in neurodegenerative disease. *Nat Rev Neurol*. 2010;6:193–201.
11. Liou GI. Diabetic retinopathy: role of inflammation and potential therapies for anti-inflammation. *World J Diabetes*. 2010;1:12–18.
12. Tezel G. Immune regulation toward immunomodulation for neuroprotection in glaucoma. *Curr Opin Pharmacol*. 2013; 13:23–31.
13. Buschini E, Piras A, Nuzzi R, Vercelli A. Age related macular degeneration and drusen: neuroinflammation in the retina. *Prog Neurobiol*. 2011;95:14–25.
14. Roh MZY, Murakami Y, Thanos A, et al. Etanercept, a widely used inhibitor of tumor necrosis factor-α (TNF-α), prevents retinal ganglion cell loss in a rat model of glaucoma. *PLoS One*. 2012;7:e40065.
15. Cukras CA, Petrou P, Chew EY, Meyerle CB, Wong WT. Oral minocycline for the treatment of diabetic macular edema (DME): results of a phase I/II clinical study. *Invest Ophthalmol Vis Sci*. 2012;53:3865–3874.
16. Hayashi TST. Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival. *Cell*. 2007;131:596–610.
17. Ha YSA, Tawfik A, Williams C, et al. Late-onset inner retinal dysfunction in mice lacking sigma receptor 1 (σR1). *Invest Ophthalmol Vis Sci*. 2011;52:7749–7760.
18. Ola MSMP, El-Sherbeny A, Roon P, et al. Expression pattern of sigma receptor 1 mRNA and protein in mammalian retina. *Brain Res Mol Brain Res*. 2001;95:86–95.
19. Hayashi TST. The sigma receptor: evolution of the concept in neuropsychopharmacology. *Curr Neuropharmacol*. 2005;3: 267–280.
20. Nishimura TIT, Iyo M, Hashimoto K. Potentiation of nerve growth factor-induced neurite outgrowth by fluvoxamine: role of sigma-1 receptors, IP3 receptors and cellular signaling pathways. *PLoS One*. 2008;3:e2558.
21. Takebayashi MHT, Su TP. A perspective on the new mechanism of antidepressants: neuritogenesis through sigma-1 receptors. *Pharmacopsychiatry*. 2004;37(suppl 3):S208–S213.
22. Mueller BH II, Park Y, Daudt DR III, et al. Sigma-1 receptor stimulation attenuates calcium influx through activated L-type voltage gated calcium channels in purified retinal ganglion cells. *Exp Eye Res*. 2013;107:21–31.
23. Tchedre KT, Huang RQ, Dibas A, et al. Sigma-1 receptor regulation of voltage-gated calcium channels involves a direct interaction. *Invest Ophthalmol Vis Sci*. 2008;49:4993–5002.
24. Hall AAHY, Ajmo CT Jr, Cuevas J, Pennypacker KR. Sigma receptors suppress multiple aspects of microglial activation. *Glia*. 2009;57:744–754.
25. Mancuso ROS, Rando A, Casas C, Osta R, Navarro X. Sigma-1R agonist improves motor function and motoneuron survival in ALS mice. *Neurotherapeutics*. 2012;9:814–826.
26. Allahtavakoli MJB. Sigma-1 receptor ligand PRE-084 reduced infarct volume, neurological deficits, pro-inflammatory cytokines and enhanced anti-inflammatory cytokines after embolic stroke in rats. *Brain Res Bull*. 2011;85:219–224.
27. Smith SBDJ, Dun Y, Mysona B, Roon P, Martin PM, Ganapathy V. In vivo protection against retinal neurodegeneration by sigma receptor 1 ligand (+)-pentazocine. *Invest Ophthalmol Vis Sci*. 2008;49:4154–4161.
28. Ruscher KIA, Valind K, Rowshan Ravan A, Kuric E, Wieloch T. Effects of the sigma-1 receptor agonist 1-(3,4-dimethoxyphenethyl)-4-(3-phenylpropyl)-piperazine dihydro-chloride on inflammation after stroke. *PLoS One*. 2012;7:e45118.
29. Tuerxun TNT, Adachi N, Kumamaru E, Kitazawa H, Kudo M, Kunugi H. SA4503, a sigma-1 receptor agonist, prevents cultured cortical neurons from oxidative stress-induced cell death via suppression of MAPK pathway activation and glutamate receptor expression. *Neurosci Lett*. 2010;469:303–308.
30. Ola MS, Moore P, Maddox D, et al. Analysis of sigma receptor (sigmaR1) expression in retinal ganglion cells cultured under hyperglycemic conditions and in diabetic mice. *Brain Res Mol Brain Res*. 2002;107:97–107.
31. El-Remessy ABTY, Zhu G, Matragoon S, et al. Neuroprotective effects of cannabidiol in endotoxin-induced uveitis: critical role of p38 MAPK activation. *Mol Vis*. 2008;14:2190–2203.
32. Dun YTM, Prasad P, Ganapathy V, Smith SB. Prevention of excitotoxicity in primary retinal ganglion cells by (+)-pentazocine, a sigma receptor-1 specific ligand. *Invest Ophthalmol Vis Sci*. 2007;48:4785–4794.
33. Martin PMOM, Agarwal N, Ganapathy V, Smith SB. The sigma receptor ligand (+)-pentazocine prevents apoptotic retinal ganglion cell death induced in vitro by homocysteine and glutamate. *Brain Res Mol Brain Res*. 2004;123:66–75.
34. DeCoster MA, Klette KL, Knight ES, Tortella FC. Sigma receptor-mediated neuroprotection against glutamate toxicity in primary rat neuronal cultures. *Brain Res*. 1995;671:45–53.
35. Lysko PG, Gagnon RC, Yue TL, Gu JL, Feuerstein G. Neuroprotective effects of SKF 10,047 in cultured rat cerebellar neurons and in gerbil global brain ischemia. *Stroke*. 1992;23:414–419.
36. Moriyama NTM, Miyano K, Miyoshi M, Watanabe T. ANP inhibits LPS-induced stimulation of rat microglial cells by suppressing NF-kappaB and AP-1 activations. *Biochem Biophys Res Commun*. 2006;350:322–328.
37. Nakamura YSQ, Kataoka K. Lipopolysaccharide-induced microglial activation in culture: temporal profiles of morphological change and release of cytokines and nitric oxide. *Neurosci Res*. 1999;35:95–100.
38. Matsumoto RR, Bowen WD, Tom MA, et al. Characterization of two novel sigma receptor ligands: antidystonic effects in rats suggest sigma receptor antagonism. *Eur J Pharmacol*. 1995; 280:301–310.
39. Bourrié BBE, De Nys N, Esclangon M, et al. SSR125329A, a high affinity sigma receptor ligand with potent anti-inflammatory properties. *Eur J Pharmacol*. 2002;456:123–131.
40. Casellas PGS, Bourrie B, Ferrini JB, Jbilo O, Vidal H. SR31747A: a peripheral sigma ligand with potent antitumor activities. *Anticancer Drugs*. 2004;15:113–118.
41. Langa FCX, Tovar V, Lavado A, et al. Generation and phenotypic analysis of sigma receptor type I (sigma 1) knockout mice. *Eur J Neurosci*. 2003;18:2188–2196.
42. Mavlyutov TANR, Guo LW. Accelerated retinal ganglion cell death in mice deficient in the Sigma-1 receptor. *Mol Vis*. 2011; 17:1034–1043.
43. Ibrahim ASE-SM, Peña A Jr, Liou GI. Genistein attenuates retinal inflammation associated with diabetes by targeting of microglial activation. *Mol Vis*. 2010;16:2033–2042.
44. Nakajima K, Tohyama Y, Kohsaka S, Kurihara T. Protein kinase C alpha requirement in the activation of p38 mitogen-activated protein kinase, which is linked to the induction of tumor necrosis factor alpha in lipopolysaccharide-stimulated microglia. *Neurochem Int*. 2004;44:205–214.
45. Moriguchi S, Yamamoto Y, Ikuno T, Fukunaga K. Sigma-1 receptor stimulation by dehydroepiandrosterone ameliorates cognitive impairment through activation of CaM kinase II, protein kinase C and extracellular signal-regulated kinase in olfactory bulbectomized mice. *J Neurochem*. 2011;117:879–891.
46. Tan FG-AP, Downes C, Zhang M, O'Donovan L, Callaway JK, Crack PJ. The σ 1 receptor agonist 4-PPBP elicits ERK1/2 phosphorylation in primary neurons: a possible mechanism of neuroprotective action. *Neuropharmacology*. 2010;59:416–424.