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L-2-oxothiazolidine-4-carboxylic acid attenuates oxidative stress and inflammation in retinal pigment epithelium

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Purpose: Oxidant- and inflammation-induced damage to retinal pigment epithelial (RPE) cells is central to the pathogenesis of age-related macular degeneration (AMD). Thus, developing novel strategies to protect these cells is important. We reported previously on the robust antioxidant and therefore cell-protective effects of the cysteine pro-drug L-2-oxothiazolidine-4-carboxylic acid (OTC) in cultured human RPE cells. New reports citing a novel anti-inflammatory role for OTC in addition to the known glutathione-stimulating and antioxidant properties emerged recently; however, this role has not been evaluated in RPE cells or in intact retina. Given the crucial causative roles of oxidative stress and inflammation in AMD pathogenesis, knowing whether OTC might exhibit a similar benefit in this cell and tissue type has high clinical relevance; thus, we evaluated OTC in the present study.

Methods: ARPE-19 and primary RPE cells isolated from wild-type, *Gpr109a*$^{-/-}$, or *Slc5a8*$^{-/-}$ mouse eyes were exposed to TNF-α in the presence or absence of OTC, followed by analysis of IL-6 and Ccl2 expression with real-time quantitative polymerase chain reaction or enzyme-linked immunosorbent assay. Cellular and molecular markers of inflammation and oxidative stress (i.e., IL-1β, TGF-β, ABCG1, ABCA1, reduced glutathione, and dihydroethidium) were evaluated in *Ccl2*$^{-/-}$*/Cx3cr1*$^{-/-}$ double knockout mice on rd8 background (DKO rd8) treated with OTC (10 mg/ml) in drinking water for a period of 5 months.

Results: OTC treatment significantly inhibited the expression and secretion of IL-6 and Ccl2 in TNF-α-stimulated ARPE-19 cells. Studies conducted using DKO rd8 animals treated with OTC in drinking water confirmed these findings. Cellular and molecular markers of inflammation were significantly suppressed in the retinas of the OTC-treated DKO rd8 animals. Subsequent in vitro and in vivo studies of the possible mechanism(s) to explain these actions revealed that although OTC is an agonist of the anti-inflammatory G-protein coupled receptor GPR109A and a transportable substrate of the sodium-coupled monocarboxylate transporter SMCT1 (SLC5A8), these properties may play a role but do not explain entirely the anti-inflammatory effects this compound elicits in cultured RPE cells and the intact mouse retina.

Conclusions: This study represents, to our knowledge, the first report of the suppressive effects of OTC on inflammation in cultured RPE cells and on inflammation and oxidative stress in the retina in vivo.

Age-related macular degeneration (AMD) is a leading cause of blindness worldwide [1-3]. The pathogenesis of the disease is multifactorial and complex; thus, the task of elucidating mechanisms and developing novel strategies for treating and preventing AMD involves significant challenges. However, several major findings related to the disease, based upon an abundance of clinical and experimental evidence, are relatively indisputable. First, as the name implies, AMD is a disease of aging; clinical symptoms begin to appear only at relatively older ages (>60 years). Second, oxidative stress and inflammation are crucial players, both in disease development and progression. Last, retinal pigment epithelial (RPE) cells, cells crucial for normal retinal health and visual function, are highly susceptible to damage or dysfunction and therefore represent a primary site of pathology in the disease. Our focus in this study centers on the latter two points, as our aim is to explore a novel means of limiting oxidative stress and inflammation not only in cultured RPE cells but also in the eyes of the living animal. Specifically, we evaluate the efficacy of L-2-oxothiazolidine-4-carboxylic acid (OTC) as a dual antioxidant and anti-inflammatory agent in cultured RPE cells (ARPE-19 and primary mouse RPE cells), and in the eyes of the *Ccl2*$^{-/-}$*/Cx3cr1*$^{-/-}$ mouse, a murine model predisposed to increased oxidative stress and inflammation in the retina [4].

OTC is a prodrug of cysteine. Upon entering cells, the compound is cleaved by the ubiquitous intracellular enzyme 5-oxoprolinase, readily generating cysteine, the limiting amino acid in glutathione (GSH) biosynthesis [5].

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The beneficial effects of OTC in terms of augmenting levels of this major cellular antioxidant have been documented in several cell and tissue types and confirmed by studies in animals and humans [6-13]. Congruent with this is our recent report demonstrating for the first time the robust antioxidant and cell-protective properties of this compound in cultured human RPE cells [14]. However, whether this benefit can be extrapolated to the intact retina in vivo is unknown.

Regarding AMD pathogenesis, oxidative stress and inflammation go hand in hand; inflammation is a common consequence of increased oxidative stress in RPE cells and the retina, and once initiated, inflammation further potentiates reactive oxygen species (ROS) production in this cell and tissue type [15-17]. This may be reflective directly of the fact that RPE cells are exposed to considerable amounts of oxidative stress continuously, even in the absence of disease [18]. Additionally, RPE cells represent a major source of cytokines in the retina and therefore are a critical regulator of inflammation in this tissue [18-20]. Thus, in aging, when the antioxidant capacity of RPE cells decreases and the balance between anti- and pro-oxidant factors tips in favor of increased oxidative stress, the production of proinflammatory cytokines also increases, and inflammation ensues [21]. Under such conditions, a compound such as OTC, capable of modulating both factors, oxidative stress and inflammation, in RPE cells would be highly desirable.

Several reports have emerged citing a novel anti-inflammatory role for OTC in addition to its known GSH-stimulating and antioxidant properties [22-24]. However, these studies stem primarily from a single research group and have for the most part involved non-retinal cell and tissue types. Therefore, confirming these findings in a different system is important. Given the crucial causative roles of oxidative stress and inflammation in AMD pathogenesis, determining whether OTC might exhibit a similar benefit in the ocular environment is clinically relevant and important. Here, we report the first evidence of the suppressive effects of OTC on inflammation and oxidative stress in cultured RPE cells and in intact mouse retinas. Our subsequent investigation of the underlying mechanisms responsible shows for the first time that OTC is an agonist of the anti-inflammatory G-protein coupled receptor GPR109A, a receptor expressed robustly in RPE cells [25,26]. Interestingly, however, we found that despite OTC's ability to activate GPR109A, the anti-inflammatory and antioxidant effects of OTC in RPE cells are not entirely GPR109A-dependent.

METHODS

Materials: OTC and nicotinic acid (NA) were purchased from Sigma-Aldrich (St. Louis, MO). Pertussis toxin (PTX) was from EMD Chemicals (Gibbstown, NJ). The antibodies used were obtained from the following sources: rabbit polyclonal anti-adenosine triphosphate-binding cassette sub-family A member 1 (ABCA1) and rabbit polyclonal anti-ABCG1 antibodies (Novas Biologic, Littleton, CO) and goat anti-rabbit IgG coupled to Alexa Fluor 568 (Invitrogen, Grand Island, NY). TRIzol, cell culture media, and all other supplements were also from Invitrogen.

Animals: Gpr109a$^{-/-}$ and *Slc5a8*$^{-/-}$ mice have been described previously [27,28]. The genotypes of the animals used in the study were confirmed with PCR using specific primer pairs. Age-matched *Gpr109a*$^{+/+}$ and *Gpr109a*$^{-/-}$ or *Slc5a8*$^{+/+}$ and *Slc5a8*$^{-/-}$ mice were used to prepare total RNA from primary mouse RPE (mRPE) cells or isolated retinal tissues. *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice on *rd8* mutation (DKO rd8) animals have also been described; age- and gender-matched C57BL/6N on *rd8* mutation mice served as controls [4]. C57BL/6J (non-rd8 wild-type) mice were purchased from Jackson Laboratories (Bar Harbor, MN) and used as controls to detect possible effects due to the rd8 mutation itself. For in vivo studies using OTC, DKO rd8 mice were paired for breeding and maintained either on regular drinking water (controls) or OTC water (10 mg/ml) from the day of pairing until litters were generated. Male breeders were removed, and dams and resultant offspring kept together and maintained on the respective drinking water regimen (regular water or OTC water) up until the time of weaning (approximately 3 weeks of age). Following weaning, pups were segregated according to gender to eliminate the possibility of additional breeding, and maintained on OTC water or regular water for 5 months. Fresh drinking water, with or without OTC, was prepared and given daily. At the end of the treatment period, the animals were euthanized by carbon dioxide asphyxiation followed immediately by cervical dislocation, and sera and eyes were collected for analyses. The care and use of the animals in these studies adhered to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85–23) and was approved by the Institutional Committee for Animal Use in Research and Education of the Georgia Regents University.

Cell culture and treatments: ARPE-19 cells, a human retinal pigment epithelial cell line, were obtained from American Type Culture Collection (Manassas, VA). The cells were maintained as described previously [25]. Primary mRPE cells were isolated from 3-week-old *Gpr109a*$^{+/+}$, *Gpr109a*$^{-/-}$, *Slc5a8*$^{+/+}$, or *Slc5a8*$^{-/-}$ mouse retinas and maintained in culture according to our published methods [14,25]. Passage 2–4

mRPE cells were used for all experiments. For treatments, cells (ARPE-19 and mRPE) were seeded at a density of 2×10^5 cells/well in six-well culture plates. Twenty-four hours after seeding, the cells were treated with varying concentrations of OTC and NA for 24 h in the presence or absence of tumor necrosis factor-α (TNF-α; 10 ng/ml) according to our previously published method [26]. When the cells were harvested, RNA or protein was prepared and the respective cell culture media collected for enzyme-linked immunosorbent assay (ELISA). Findings obtained using this cell culture model system were confirmed with additional experiments performed using fully differentiated and polarized ARPE-19 cells obtained by maintaining confluent monolayers of ARPE-19 cells, according to Bridges et al.'s method [29], in culture for a period of 4–6 weeks. Identical treatments (varying concentrations of OTC or NA for 24 h in the presence or absence of 10 ng/ml TNF-α) were then performed using these cells.

Real-time quantitative PCR: Total RNA was isolated from ARPE-19 and mRPE cells prepared from *Gpr109a*$^{+/+}$, *Gpr109a*$^{-/-}$, *Slc5a8*$^{+/+}$, or *Slc5a8*$^{-/-}$ mouse eyes, as well as from the neural retina (NR) and RPE cells and eyecup obtained from the C57BL/6N rd8 and DKO rd8 mouse eyes (treated with or without OTC) using TRIzol. RNA (1 µg) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time quantitative PCR (qPCR) was used to monitor steady-state mRNA levels of interleukin-6 (IL-6), chemokine (C-C) motif ligand 2 (Ccl2; also referred to as monocyte chemotactic protein or MCP-1), IL-1β, transforming growth factor-β (TGF-β), ABCA1, and ABCG1 using human- or mouse-specific primer sets as appropriate. Primer sequences for mouse GPR109A and human and mouse IL-6 and Ccl2 have been reported previously [25,26]. Information relevant to all other primers used in the study is provided in Table 1. Hypoxanthine phosphoribosyltransferase 1 (HPRT) or 18S was used as the internal control. Real-time qPCR amplifications, using detection chemistry (SYBR Green; Applied Biosystems, Foster City, CA), were run in triplicate on 96-well reaction plates. Gene expression was calculated by comparing Ct values (ΔΔCt).

Bioluminescence resonance energy transfer assay: Bioluminescence resonance energy transfer (BRET) was used to monitor the dissociation of G-protein heterotrimers as an indicator of GPR109A activation following our previously published method [30-32]. Briefly, HEK-293 cells were transfected overnight with human GPR109A cDNA along with constructs coding for $G\alpha_{i1}$, venus155–239-$G\beta_1$, venus1–155-$G\gamma_2$, and G-protein-coupled receptor kinase 3-C-terminus luciferase. Cells were then harvested in PBS (0.01 M phosphate buffer, 0.137 M NaCl, 0.0027 M KCl, pH 7.4) containing 5 mM EDTA, and an equal amount of cells was transferred to the wells of a 96-well plate followed by exposure to various concentrations of OTC or NA, a prototypic agonist for GPR109A, in the presence or absence of pertussis toxin (PTX; 0.5 µg/ml), a well-characterized, specific, and irreversible inhibitor of G_i-linked G-protein coupled receptors (GPCRs). The luciferase substrate benzyl coelenterazine (5 µM) was then added to the wells in the dark. Under these conditions, the activation of GPR109A by an agonist results in the dissociation of the Gβγ dimer containing the complementary fragments of the yellow fluorescent protein venus (the 1- to 155-amino acid fragment and the 155- to 239-amino acid fragment) from $G_i\alpha1$. The dimer then binds to the G-protein-coupled receptor kinase 3-C-terminus-luciferase fusion protein, enabling BRET in the presence of benzyl colenterazine. Steady-state BRET measurements were made within 15 min of exposure of the cell suspensions to ligands using a photon-counting multimode plate reader (Mithras LB940; Berthold Technologies GmbH, Bad Wildbald, Germany). The BRET signal was calculated as the ratio of the emission intensity at 520 to 545 nm to the emission intensity at 475 to 495 nm.

cAMP measurement: As an additional means of assessing GPR109A functional activity, intracellular levels of cyclic adenosine monophosphate (cAMP) were monitored in the ARPE-19 cells treated with or without OTC following our published method [25]. Briefly, ARPE-19 cells were seeded at a density of 1×10^5 cells/well in a 24-well plate. On the 3rd day after seeding, the culture medium was removed and replaced with culture medium containing 10 µM forskolin (FSK) in

Table 1. PCR Primers

Gene	Primer sequence (5′-3′)	Expected product size
Mouse IL-1β	F: CGAGGCTAATAGGCTCATCT R: GTTTGGAAGCAGCCCTTCAT	177
Mouse TGF-β	F: CTCCCGTGGCTTCTAGTGC R: GCCTTAGTTTGGACAGGATCTG	133
Mouse ABCA1	F: GCTAGAGATGACAAGGAGGATGGA R: CGTTTCCGGGAAGTGTCCTA	79
Mouse ABCG1	F: ACCTACCACAACCCAGCAGACTTT R: GGTGCCAAAGAAACGGGTTCACAT	160

the presence of increasing concentrations of OTC (1 h treatment). NA was used as a positive control. The cAMP levels were measured using the cAMP Assay kit (Assay Designs, Ann Arbor, MI), following the manufacturer's instructions. Experiments were repeated three times with independent cell cultures, and cAMP was measured in triplicate in each experiment.

Radioligand binding assay: Monolayers of GPR109A-overexpressing MB231 cells were chilled on ice, washed with ice-cold PBS (pH 7.5), and lysed with 5 mM KPO_4 buffer (pH 7.5). The suspension was centrifuged for 30 min at 56,800 g; the final membrane pellets were rinsed and suspended in 5 mM KPO_4 buffer and homogenized 20 times using a 25-gauge needle. Protein concentration in the final membrane preparation was measured with the protein assay kit (Bio-Rad Protein Assay Reagent; Bio-Rad). [^{3}H]-NA (10 nM) binding to membrane preparations was assayed in the presence of increasing concentrations of unlabeled NA (positive control) or OTC (0.1–10,000 µM) prepared in 250 µl of 5 mM KPO_4 buffer, pH 7.5. Reactions were allowed to proceed at 25 °C for 90 min. Binding was terminated by adding ice-cold binding buffer, and the mixture was filtered on a Whatman GF/F glass fiber filter, presoaked in 0.3% polyethylenimine. The filter was washed three times with ice-cold binding buffer, and radioactivity associated with the filter was determined with liquid scintillation spectrometry. The theory behind this method is that if OTC, similar to NA, is a GPR109A ligand, then OTC should compete with [^{3}H]-NA in a dose-specific manner for binding to GPR109A-expressing cell membranes. Therefore, decreased levels of [^{3}H]-NA in this assay are synonymous with increased binding of the unlabeled ligand.

Western blot analysis: Protein was extracted from retinal tissues isolated from control and OTC-treated DKO rd8 mouse eyes. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Rockford, IL). Twenty µg of protein were subjected to SDS–PAGE, transferred to nitrocellulose membranes, and then incubated with primary antibodies (rabbit polyclonal anti-ABCG1, or rabbit polyclonal anti-ABCA1) overnight at 4 °C. Secondary detection was done using horseradish peroxidase–conjugated secondary antibodies (anti-rabbit immunoglobulin antibody; Promega, Madison, WI). After washing, the proteins were visualized using the enhanced chemiluminescence (ECL) western blot detection system (Thermo Fisher Scientific). β-actin served as the loading control.

Enzyme-linked immunosorbent assay analysis: Commercially available ELISA assay kits (see Materials) were used to monitor the concentration of cytokines (IL-6, Ccl2, IL-1β, and TGF-β) in culture media obtained from ARPE-19 and mRPE cell cultures or in isolated retinal tissues (RPE cells and eyecup and neural retina) harvested from control and OTC-treated DKO rd8 mouse eyes. In all cases, assays were performed in strict accordance with the manufacturers' directions. ELISA plates were read spectrophotometrically at 450 nm using a SpectraMAX 190 plate reader (Molecular Devices, Sunnyvale, CA).

Measurement of cellular levels of glutathione: Glutathione levels in the sera and samples of the NR and RPE cells and eyecup obtained from control and OTC-treated DKO rd8 mouse eyes were measured using the GSH Glo assay kit (Promega) following the manufacturer's instructions and our previously published method [14]. Briefly, the assay involves a series of two reactions. Luciferin is generated first from a luminogenic substrate, catalyzed by glutathione-S-transferase (GST) in the presence of glutathione. This luciferin is then detected as a luminescent signal that is directly proportional to the amount of luciferin formed and therefore to the amount of GSH present in the sample. GSH levels in tissues were expressed as nmol/mg tissue.

Measurement of superoxide production: Superoxide production was evaluated in retinal cryosections obtained from control (regular drinking water) DKO rd8 and OTC-treated DKO rd8 mice using dihydroethidium (DHE) as described previously [33]. Briefly, frozen sections were incubated with DHE (2 µM) for 30 min at 37 °C. DHE is oxidized upon reaction with superoxide to ethidium bromide, which binds to DNA in the nucleus and fluoresces red. DHE images were obtained using a Leica DM5500B fluorescent microscope (Leica Microsystems, Buffalo Grove, IL) equipped with a CoolSNAP HQ^2 digital camera (Photometrics, Tucson, AZ) and Leica Application Suite imaging software version 2.5.0.6735. DHE was excited at 488 nm with an emission spectrum of 610 nm. The relative fluorescence intensity within the images obtained was determined via automated image analysis or ImageJ software.

Data analysis: All experiments were repeated three to five times with independent cell or tissue preparations and samples run in duplicate. Data are presented as mean±standard error of the mean (SEM). Statistical significance was determined with the Student *t* test, and one-way ANOVA with Tukey–Kramer's post-hoc tests for comparisons between two groups or multiple groups, respectively. Differences were considered statistically significant at $p<0.05$.

RESULTS

In vitro suppression of inflammatory molecules by treatment with OTC: In keeping with the role as a key regulator of retinal immunity and inflammation, RPE cells express and secrete several cytokines, both pro- and anti-inflammatory in nature, in response to various stimuli [18,20]. Here, we asked whether OTC treatment modulates the RPE-specific expression and secretion of IL-6 and Ccl2, two proinflammatory molecules critically implicated in the pathogenesis of AMD [34-36]. Total RNA was prepared and culture media collected from ARPE-19 cells cultured in the presence of TNF-α and in the presence or absence of 0.5 mM OTC, a concentration that we previously showed to be effective at protecting RPE cells from oxidant-induced damage [14]. Cells treated identically with NA (1 mM), a compound and concentration also shown previously to confer anti-inflammatory protection [26], instead of OTC, served as positive controls in the experiment. As expected, TNF-α significantly induced IL-6 and Ccl2 mRNA expression (approximately two-fold and approximately 50-fold, respectively), an effect that was significantly suppressed by NA or OTC (Figure 1A,B). ELISA analyses performed using the culture medium obtained from these respective cell treatments revealed a corresponding effect regarding the attenuation of TNF-α-induced IL-6 and Ccl2 protein secretion. The secretion of IL-6 and Ccl2 by ARPE-19 cells significantly increased with TNF-α. Treatment of the cells with NA significantly attenuated the TNF-α-induced increase in the IL-6 and Ccl2 proteins (17% and 25% inhibition, respectively); an even greater inhibitory effect was observed in the presence OTC (67% and 70% inhibition, respectively; Figure 1C,D). The findings observed in our cell culture model system were confirmed with identical experiments performed using confluent monolayers of fully differentiated and polarized ARPE-19 cells (Figure 2). TNF-α treatment significantly increased the expression of IL-6 and Ccl2 (Figure 2A,B, respectively). These increases were significantly attenuated and in a dose-dependent manner by OTC.

IL-1β, TGF-β, ABCG1 and ABCA1 expression in DKO rd8 retina: OTC has been used previously in humans and found to be relatively safe in that it is non-toxic and produces minimal unwanted effect; however, the use of OTC, or its efficacy, in treating indications involving the eye has not been tested. Preliminary studies in non-human models are therefore highly warranted. Along these lines, we chose the *Ccl2/Cx3cr1*-deficient mouse (DKO rd8). The DKO rd8 is a model that,

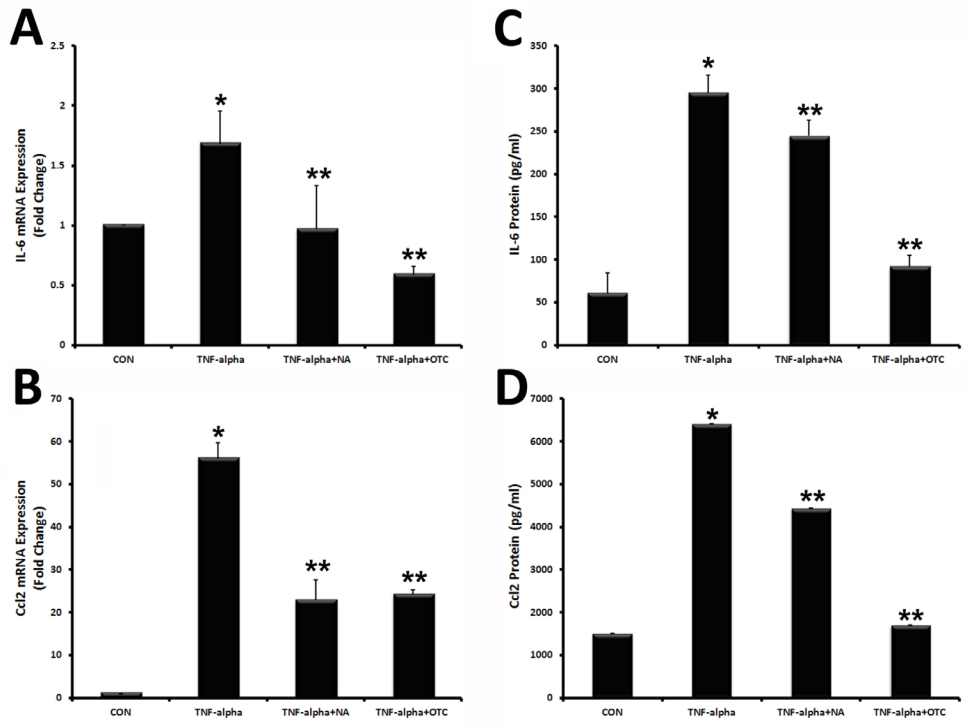

Figure 1. Suppression of TNF-α-induced IL-6 and ccl2 mRNAand protein by OTC. ARPE-19 cells were exposed to tumor necrosis factor-α (TNF-α; 10 ng/ml; 24 h incubation) in the presence or absence of L-2-oxothiazolidine-4-carboxylic acid (OTC; 0.5 mM) and nicotinic acid (NA; 1 mM; positive control). Quantitative polymerase chain reaction (qPCR) analysis of (**A**) interleukin-6 (IL-6) and (**B**) chemokine (C-C) motif ligand 2 (Ccl2) mRNA expression. The cell culture medium was then collected and used for enzyme-linked immunosorbent assay (ELISA) analysis of the (**C**) IL-6 and (**D**) Ccl2 proteins. *p<0.01 compared to control cells; ** p<0.01 compared to TNF-α-treated cells.

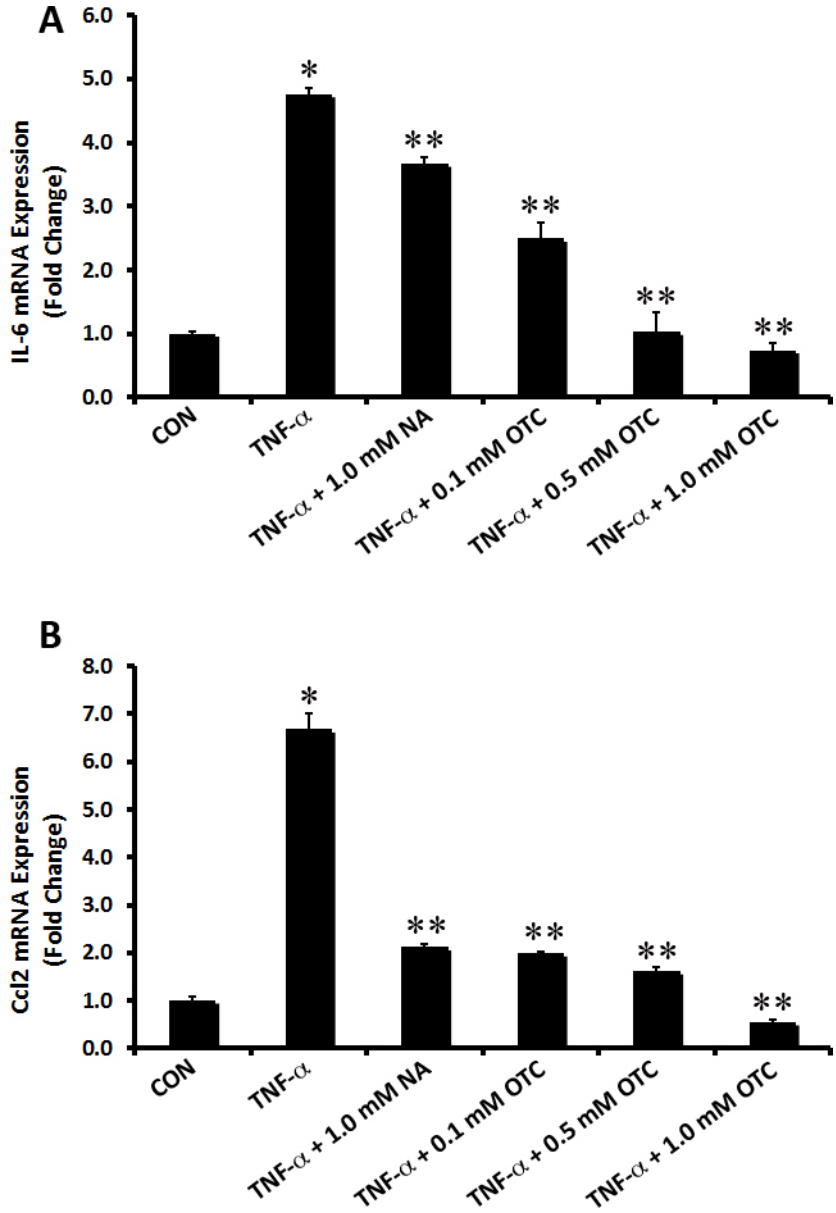

Figure 2. Suppression of TNF-α-induced IL-6 and Ccl2 mRNA by OTC in polarized, fully differentiated ARPE-19 cells. ARPE-19 cells were cultured to a state of confluency and then maintained in culture for an additional for 6 weeks to facilitate their polarization and differentiation. The cells were then exposed to tumor necrosis factor-α (TNF-α; 10 ng/ml; 24 h incubation) in the presence or absence of L-2-oxothiazolidine-4-carboxylic acid (OTC; 0.5 mM) and nicotinic acid (NA; 1 mM; positive control). Quantitative polymerase chain reaction (qPCR) analysis of (**A**) interleukin-6 (IL-6) and (**B**) chemokine (C-C) motif ligand 2 (Ccl2) mRNA expression. *p<0.01 compared to untreated, control; **p<0.01 compared to TNF-α-treated cells.

owing to the presence of the rd8 mutation within the strain, may not be ideal for use in studies of morphological features characteristic of human AMD and the ability of agents to prevent or slow down the progression thereof. However, the DKO rd8 mouse model is predisposed, as reported by others, to increased oxidative stress and inflammation in the retina and, is accordingly, a suitable model in which to evaluate these parameters at the molecular level [4,37]. Increased or upregulated expression of a high number of molecules that are proinflammatory has been implicated in the pathogenesis of AMD [38]. Here, we chose specifically to evaluate the DKO rd8 mouse retina in terms of the expression of four molecules: IL-1β, TGF-β, ABCA1, and ABCG1 (Figure 3). The rationale for choosing these four molecules in particular was the following: (a) ABCA1 and ABCG1 have been implicated strongly in regulating cholesterol efflux, with decreased expression of these transport proteins associated in turn with increased cholesterol accumulation and consequent inflammation in tissues, factors highly relevant to the metabolism and handling of lipids by RPE cells and therefore to drusen biogenesis in AMD [39,40]. (b) Increased TGF-β expression is associated strongly with increased vascular endothelial growth factor (VEGF) production, a factor that contributes significantly to the development or progression of choroidal neovascularization characteristic of neovascular "wet" AMD [41]. (c) Upregulated IL-1β expression is associated strongly

with NLRP3 inflammasome activation in RPE cells, a phenomenon highly relevant to geographic atrophy (dry) and neovascular (wet) AMD in human patients [16,42]. To determine whether the presence of the rd8 mutation itself influenced the expression of the molecular parameters evaluated in our experimental system, preliminary experiments were performed using retina samples obtained from wild-type C57BL/6N (rd8 positive) and wild-type C57BL/6J (non-rd8) mouse eyes. No significant differences were detected in the expression of any of the molecules evaluated in these animals, confirming as reported by others that the rd8 mutation, although associated with the development of a degenerative retinal phenotype, is in itself not inflammatory [37]. Thus, all remaining studies were conducted using only wild-type C57BL/6N rd8 and DKO rd8 animals. TGF-β expression was significantly increased in the DKO rd8 retina compared to the control, C57Bl/6N rd8 retina (Figure 3A). IL-1β expression was not significantly changed (Figure 3B). ABCG1 and ABCA1 expression was significantly downregulated in the DKO rd8 retina (Figure 3C,D, respectively), a phenomenon consistent with increased cholesterol accumulation and therefore inflammation in DKO rd8 eyes.

OTC suppresses pro-inflammatory molecule expression in vivo: Our in vitro studies strongly suggest a positive association between OTC treatment and decreased inflammation. Additionally, studies in DKO rd8 mice have confirmed the increased expression of proinflammatory molecules in the DKO rd8 retina and therefore the high suitability of this model for studying parameters relevant to increased inflammation or altered cytokine regulation in the retina and novel mechanisms or therapeutic strategies for countering, our goal in the present study. Using this rationale, we evaluated the efficacy of OTC as an anti-inflammatory agent in vivo. To do so, DKO rd8 animals were treated with 10 mg/ml OTC provided daily in drinking water for a period of 5 months. The dosage and regimen for administering OTC used in the present study were similar to those used by others to study the effectiveness of OTC for treating non-retinal related diseases in humans and in animal models [5,43,44]. Age- and gender-matched DKO rd8 animals maintained on regular drinking water served as controls. At the end of the 5-month treatment period, the animals were euthanized, and blood and tissue samples collected and used to assess the effect of OTC on molecular parameters associated with inflammation (IL-1β, TGF-β, ABCG1, and ABCA1 expression) and oxidative stress (levels of tissue glutathione and superoxide; Figure 4 and Figure 5, respectively).

IL-1β and TGF-β protein expression was examined in samples of the NR and RPE cells and eyecup obtained from OTC-treated DKO rd8 and control DKO rd8 mouse eyes

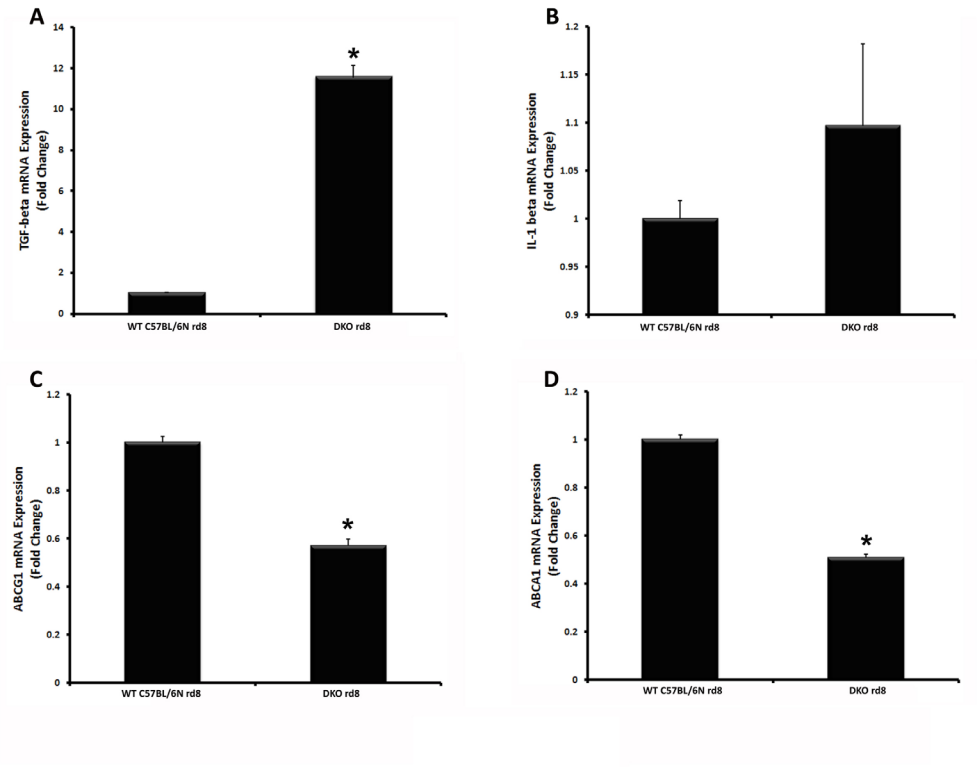

Figure 3. Expression of proinflammatory cytokines and cholesterol efflux pumps in the Ccl2$^{-/-}$/Cx3cr1$^{-/-}$ double knockout mouse retina. Quantitative polymerase chain reaction (qPCR) analysis of (**A**) transforming growth factor-β (TGF-β), (**B**) interleukin-1β (IL-1β), (**C**) adenosine triphosphate-binding cassette sub-family G member 1 (ABCG1), and (**D**) adenosine triphosphate-binding cassette sub-family A member 1 (ABCA1) expression in the wild-type (WT) C57BL/6N rd8 and double knockout (DKO rd8) mouse retina.

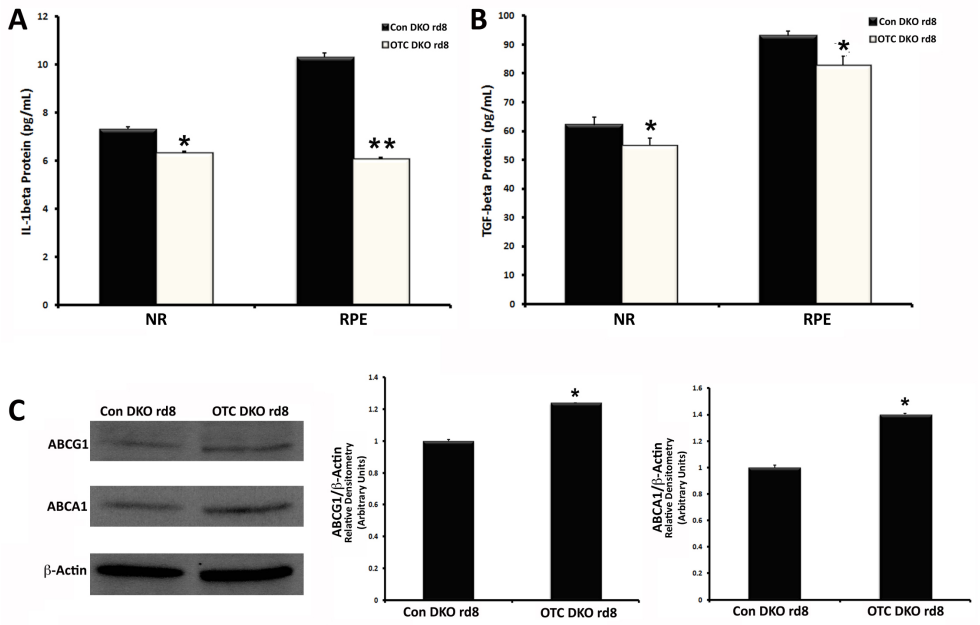

Figure 4. OTC attenuates the expression of IL-1β and TGF-β, and enhances expression of ABCA1 and ABCG1 in the Ccl2$^{-/-}$/Cx3cr1$^{-/-}$ double knockout mouse retina. Enzyme-linked immunosorbent assay (ELISA) analysis of (**A**) interleukin-1β (IL-1β) and (**B**) transforming growth factor-β (TGF-β) protein; western blot analysis of (**C**) adenosine triphosphate-binding cassette sub-family G member 1 (ABCG1) and adenosine triphosphate-binding cassette sub-family A member 1 (ABCA1) proteins in the neural retina (NR) and retinal pigment epithelial (RPE) and eyecup samples were obtained from double knockout (DKO rd8) mice treated with L-2-oxothiazolidine-4-carboxylic acid (OTC DKO rd8) or regular drinking water (Con DKO rd8) for a period of 5 months. *p<0.05 compared to corresponding control; **p<0.01 compared to corresponding control.

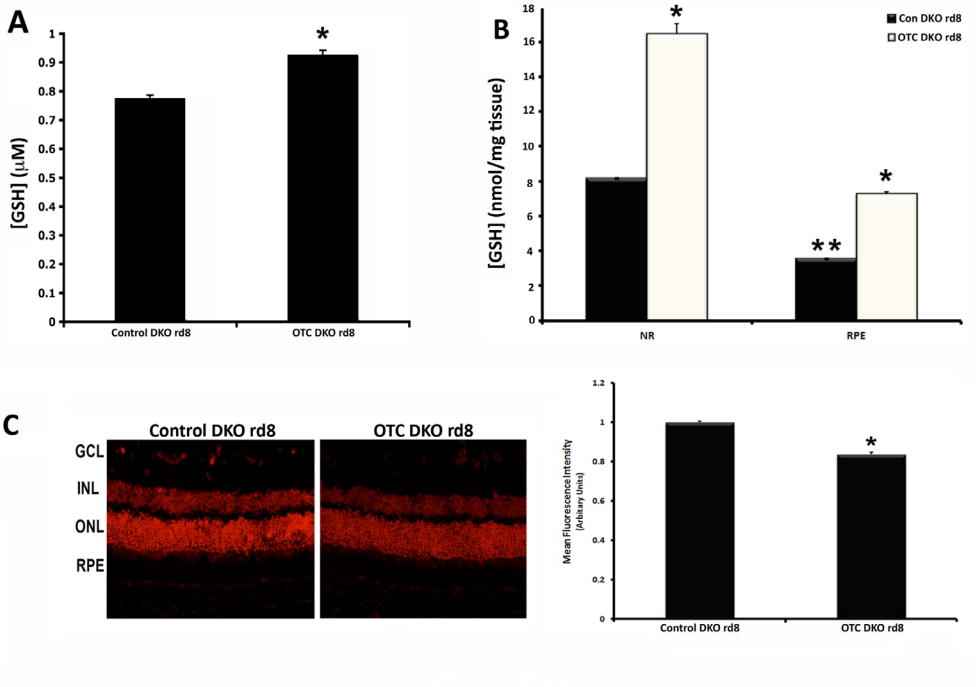

Figure 5. Antioxidant effects of OTC in the Ccl2$^{-/-}$/Cx3cr1$^{-/-}$ double knockout mouse retina. Intracellular concentrations of glutathione (GSH) were measured in (**A**) sera and (**B**) samples of the neural retina (NR) and retinal pigment epithelium (RPE) and eyecup obtained from double knockout (DKO rd8) mice treated with L-2-oxothiazolidine-4-carboxylic acid (OTC DKO rd8) or regular drinking water (Con OTC DKO rd8) for a period of 5 months. (**C**) Superoxide generation was also assessed in these animals with the fluorescent indicator dihydroethidium (DHE). *p<0.01 compared to corresponding Con DKO rd8 NR or RPE; **p<0.01 compared to Con DKO rd8 NR.

with ELISA (Figure 4A,B, respectively). Consistent with the role of RPE cells as a key regulator of retinal immunity and inflammation and therefore a major producer of cytokines, levels of IL-1β and TGF-β protein were significantly higher in the RPE cells and eyecup compared to the NR in the control, untreated DKO rd8 animals. The protein levels of these molecules were significantly decreased, however, in association with OTC treatment, in the NR and the RPE cells and eyecup. Congruent with the OTC-mediated suppression of inflammation in the DKO rd8 retina as shown by the significant decrease in retinal proinflammatory cytokine production, ABCG1 and ABCA1 expression was also increased in the OTC-treated animals (Figure 4C).

OTC is most noted for its GSH-stimulating and antioxidant properties. Here, we show the compound to be effective as an anti-inflammatory agent in cultured RPE cells and in the intact retina. We reported previously on the antioxidant and therefore cell-protective property of OTC in RPE cells in culture [14]; however, whether this can be extrapolated to the eyes of living animals requires further investigation. To address this issue, GSH concentrations were measured in the serum and retinal tissue samples obtained from OTC-treated and control DKO rd8 animals. GSH levels were significantly elevated in the sera and tissue (the NR and RPE cells and eyecup) of the OTC-treated DKO rd8 animals (Figure 5A,B, respectively). Retinal levels of superoxide, as determined with DHE staining and subsequent quantification of fluorescence intensity, were evaluated in cryosections of OTC-treated and control DKO rd8 retinas as an additional means of assessing the overall impact of OTC on the antioxidant capacity of DKO rd8 retinas. If OTC serves as expected as a potent antioxidant in this tissue, then the production of free radicals like superoxide should also be reduced in association with the administration of this compound; we found that this was indeed the case (Figure 5C).

Anti-inflammatory effect of OTC is independent of SLC5A8: Consistent with reports by others, we found that OTC elicits robust anti-inflammatory and antioxidative effects in cultured RPE cells and in the retinas of living animals. However, the underlying mechanisms that explain the dual-actions remain to be determined. Thus, we examined what we believed were various possible avenues based upon our prior work. We reported previously on the GSH-stimulating effects of OTC in RPE cells, a phenomenon that was dependent upon sodium-coupled monocarboxylate transporter 1 (SMCT1; SLC5A8)–mediated transport of the compound across the plasma membrane into the cell [14]. To determine whether SLC5A8 has a role in the anti-inflammatory effects of OTC observed in the present study, we evaluated the effects of the compound on TNF-α-induced IL-6 and Ccl2 expression in primary RPE cells isolated from *Slc5a8*$^{+/+}$ and *Slc5a8*$^{-/-}$ mouse eyes using methods identical to those used in our studies of ARPE-19. NA and OTC significantly inhibited the TNF-α-induced upregulation of IL-6 and Ccl2 expression in *Slc5a8*$^{+/+}$ RPE cells (Figure 6A,B, black bars). The same did not hold true, however, in the case of *Slc5a8*$^{-/-}$ RPE cells treated under identical conditions, particularly regarding NA. In these cells, the suppressive effects of NA on TNF-α-induced IL-6 and Ccl2 expression were lost (Figure 6A,B, gray bars). Interestingly, however, the attenuation of the TNF-α-induced upregulation of IL-6 and Ccl2 by OTC persisted even in the absence of SLC5A8 (*Slc5a8*$^{-/-}$ RPE). This finding was corroborated with ELISA analyses of IL-6 protein secretion into the media (Figure 6C). *Slc5a8*$^{+/+}$ cells responded similarly to ARPE-19; NA and OTC treatment significantly inhibited the TNF-α-induced secretion of IL-6 protein by these cells (23% and 92% inhibition, respectively). However in the *Slc5a8*$^{-/-}$ RPE cells, only OTC was effective in suppressing TNF-α-induced IL-6 secretion (approximately 75% inhibition); NA had no significant effect.

OTC is a GPR109A agonist: The studies, conducted using *Slc5a8*$^{-/-}$ RPE cells, indicate that the anti-inflammatory effects of OTC occur independent of SLC5A8 expression and therefore possibly independent also of the compound's entry into the intracellular compartment of the RPE cell. Thus, we postulated that the anti-inflammatory effects of OTC might be mediated instead via OTC's action upon or interaction with a target present on the surface of the plasma membrane; this interaction could initiate signaling events that lead ultimately to suppressing proinflammatory cytokine production and release by RPE cells. GPCRs, a large protein family of transmembrane receptors, do just that: interact with ligands extracellularly to mediate an intracellular effect [45,46]. We reported recently on the expression of a G_i-coupled receptor termed GPR109A that, when activated, promotes anti-inflammatory signaling in RPE cells [25,26]. Furthermore, studies by others indicate that at higher doses, OTC administration in humans is associated with a phenomenon known as flushing [5]. Interestingly, the activation of GPR109A by pharmacologic agents such as nicotinate induces a similar response [47]. With this in mind, we used three methods, each commonly used to evaluate GPCR activity, to evaluate the possible interaction between OTC and GPR109A, namely, BRET, cAMP, and radioligand binding assays (Figure 7). The BRET assay revealed the rapid increase in the BRET signal (indicating the dissociation of the α from the βγ subunits and therefore the activation of GPR109A) in the presence of increasing concentrations of OTC; NA served as a positive control (Figure 7A). The OTC-induced increase

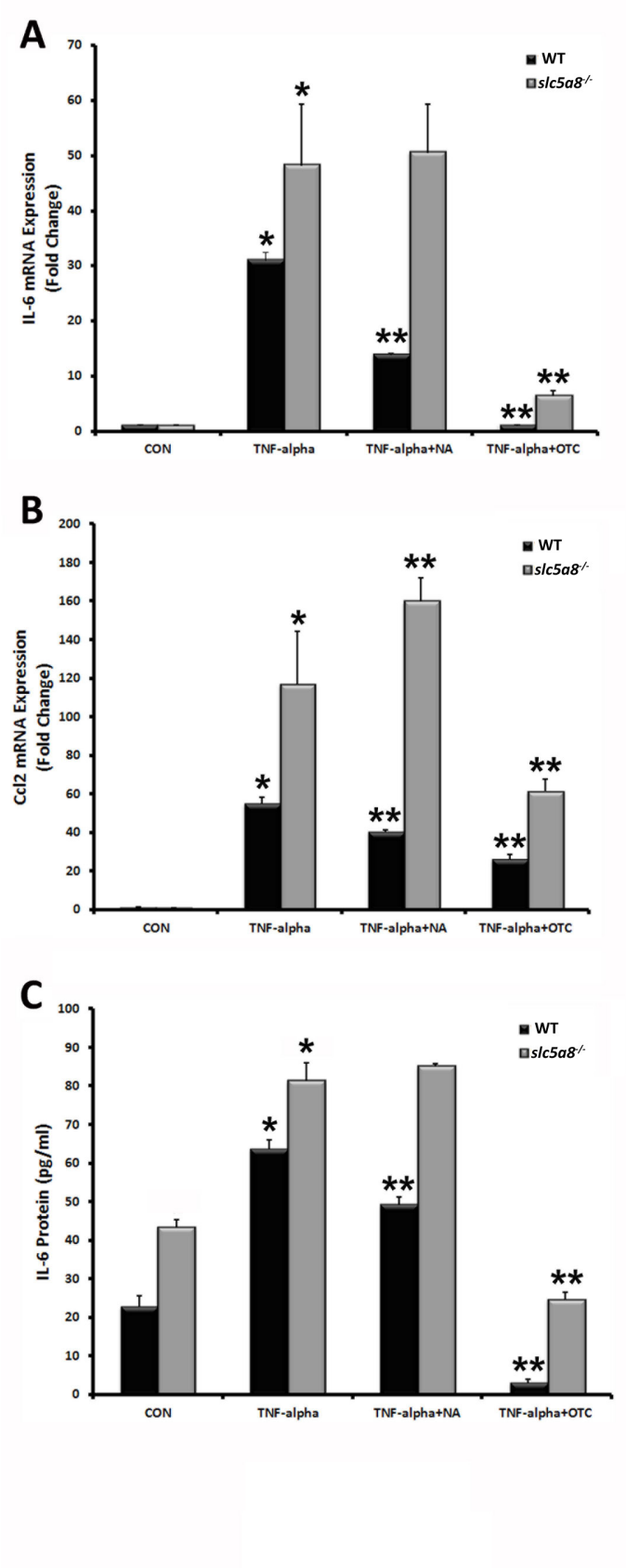

Figure 6. The anti-inflammatory effect of OTC in cultured RPE occurs independent of SLC5A8 expression. Mouse primary retinal pigment epithelial (mRPE) cells were prepared from *Slc5a8*$^{+/+}$ (wild-type, WT) and *Slc5a8*$^{-/-}$ mouse retinas and exposed to tumor necrosis factor-α (TNF-α; 10 ng/mL; 24 h incubation) in the presence or absence of L-2-oxothiazolidine-4-carboxylic acid (OTC; 0.5 mM) and nicotinic acid (NA; 1 mM; positive control). Quantitative polymerase chain reaction (qPCR) analysis of (**A**) interleukin-6 (IL-6) and (**B**) chemokine (C-C) motif ligand 2 (Ccl2) mRNA expression. Cell culture medium was then collected and used for enzyme-linked immunosorbent assay (ELISA) analysis of (**C**) IL-6 protein. *p<0.01 compared with control cells; **p<0.01 compared to TNF-α-treated cells.

in BRET activity was significantly inhibited by PTX, indicating that the BRET signal observed in the presence of OTC was due specifically to its activation of a G_i-linked receptor. Although OTC, similarly to NA, induced BRET activity and by this measure this is a GPR109A agonist, there was a large difference in the EC_{50} value (the concentration required to reach half-maximal activation of the receptor) between OTC and NA (200 µM for OTC and 0.5 µM for NA). A similar phenomenon was observed when the interaction of OTC with GPR109A was evaluated with a radioligand binding assay (Figure 7B). This competition-based assay showed that, similarly to unlabeled NA, OTC competed with [^{3}H]-NA for binding to GPR109A in a dose-dependent manner. Again, however, the affinity of the receptor for OTC was relatively lower compared to that for NA; EC_{50} values were approximately 900 µM for OTC and 3.0 µM for NA. Collectively, the BRET and radioligand binding assays show that although OTC can activate GPR109A, the affinity of the receptor for OTC is 300–400-fold lower than that for NA. To confirm this in RPE cells specifically, we compared the activation of GPR109A by OTC and NA in ARPE-19 cells. The activation of inhibitory GPCRs (G_i-linked GPCRs) similarly to GPR109A is associated with a decrease in the intracellular levels of cAMP [25,43,48]. Congruent with this was the OTC-induced reduction of FSK-induced elevations in cAMP observed in our experimental system (Figure 7C). Similarly to NA, OTC reduced cAMP levels in a dose-dependent manner; however, as was the case in our previous two assay systems, OTC was less effective than NA in this regard.

The anti-inflammatory effect of OTC is not solely dependent upon the activation of GPR109A: Thus far, we have shown OTC to be a potent and effective anti-inflammatory agent in cultured RPE cells and in live mice, in addition to its GSH-stimulating and antioxidant properties. We speculated that the anti-inflammatory effects of OTC might be mediated via GPR109A based on our discovery that OTC is an agonist for this receptor. The studies demonstrated that although OTC can activate GPR109A, OTC is not as effective in doing so as NA. This is interesting given that in terms of limiting inflammation, OTC appears to be the most effective agent. Thus, we cannot state with certainty that the anti-inflammatory effects elicited by this agent in the DKO rd8 mouse retina actually stemmed from GPR109A activation. The fact that in all of our functional assays OTC interacted with GPR109A but much less effectively than NA further fueled our desire to evaluate directly the potential involvement of GPR109A in the anti-inflammatory effects elicited by OTC. To do so, we went back to our cell culture model system, this time isolating mRPE cells from *GPR109a*$^{+/+}$ and *GPR109a*$^{-/-}$ mouse retinas. *Gpr109a*$^{+/+}$ and *Gpr109a*$^{-/-}$ cells were exposed to TNF-α in the presence or absence of OTC or NA in a fashion identical to that described previously. The exposure of *Gpr109a*$^{+/+}$ and *Gpr109a*$^{-/-}$ mRPE cells to TNF-α was associated with robust increases in IL-6 mRNA and protein expression (Figure 8A,B, respectively); a similar phenomenon was observed for Ccl2 (Figure 8C,D, respectively). OTC significantly inhibited the TNF-α-induced increase in the expression of both proinflammatory molecules in *Gpr109a*$^{+/+}$ mRPE cells, and interestingly also in *Gpr109a*$^{-/-}$ mRPE. NA, however, was effective only in *Gpr109a*$^{+/+}$ mRPE cells. These data suggest that although OTC, similar to NA, is an agonist for GPR109A, activation of the receptor by this compound is not likely to be solely responsible for the anti-inflammatory effects elicited by OTC in cultured RPE cells or the intact retina.

DISCUSSION

Oxidative stress and inflammation are crucial in degenerative diseases of the retina; this is particularly true regarding AMD, a disease in which these factors have been implicated as major players. Thus, developing and testing novel strategies for regulating oxidative stress and inflammation in the retina are important and have broad clinical relevance. In the present study, we evaluated OTC for this purpose. The robust antioxidant properties of OTC are well known; however, recent studies suggest added benefit in terms of modulating inflammation [22-24]. This, to date, has not been evaluated in the retina. Expanding upon our prior study demonstrating the antioxidant and therefore cell-protective properties of OTC in cultured RPE cells [14], we evaluated the compound for its ability to modulate inflammatory processes in these cells and in the intact retina. We found that OTC performed quite effectively in this regard, a phenomenon consistent with reports by others touting the compound as a dual antioxidant and anti-inflammatory agent. Studies conducted using cultured RPE cells revealed that the doses of OTC required for the anti-inflammatory action are quite similar to those we reported previously that induced the GSH-stimulating or anti-oxidative effect [14]. Interestingly, however, the exact mechanism(s) responsible for OTC's newly discovered anti-inflammatory properties in RPE cells and the retina remain to be ascertained. Along these lines, we evaluated whether the transport of OTC via SLC5A8 or its interaction with the anti-inflammatory receptor GPR109A might be possible underlying mechanisms. Studies involving *slc5a8*-deficient primary RPE cells showed that although OTC is indeed a transportable substrate of SLC5A8 and transport by this monocarboxylate transporter may be needed for OTC's antioxidant actions [14], it is not required for the anti-inflammatory effects observed in our present experimental

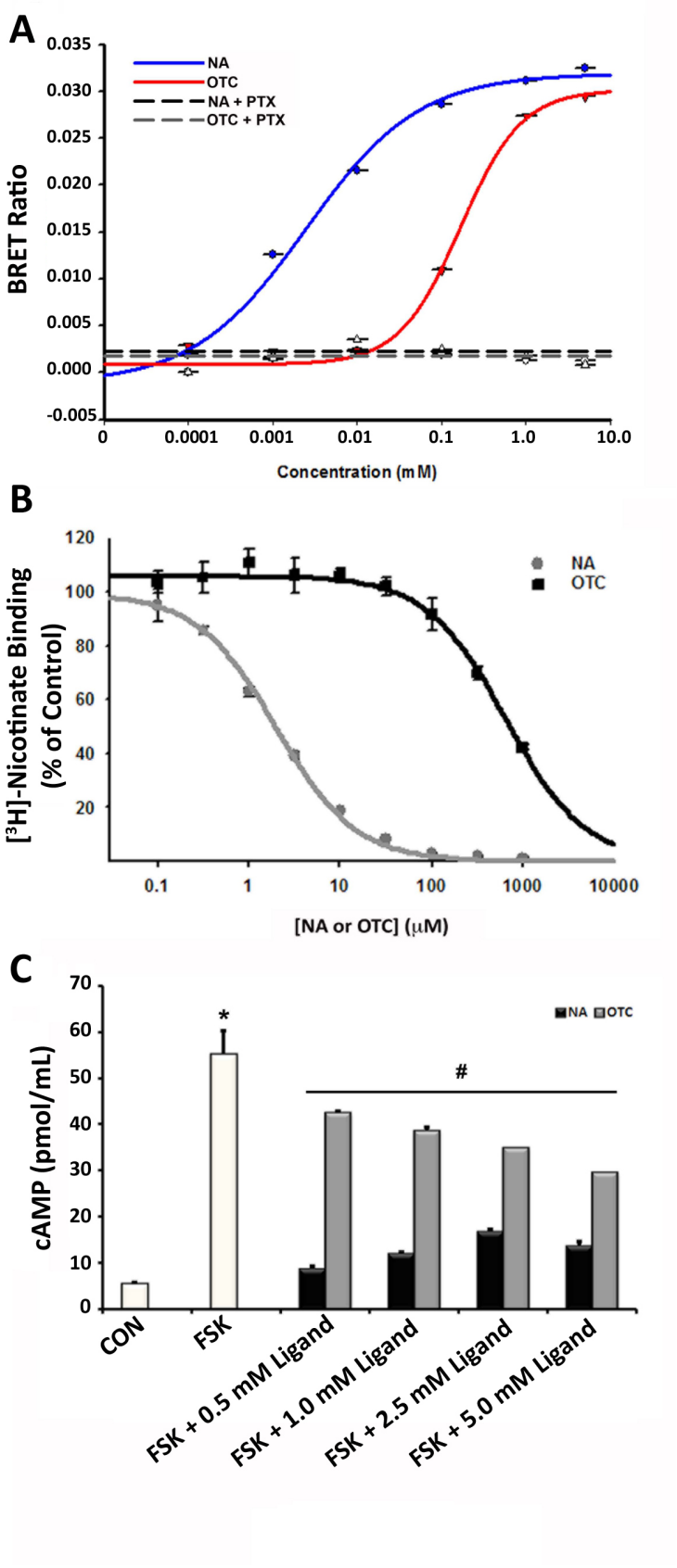

Figure 7. OTC is an agonist for GPR109A. **A**: Bioluminescence resonance energy transfer (BRET) analysis of L-2-oxothiazolidine-4-carboxylic acid (OTC)-induced GPR109A activation in the presence or absence of pertussis toxin (PTX, 0.5 μg/ml). NA served as a positive control. **B**: Radiolabeled competition binding assay was used to evaluate the binding of [^{3}H]nicotinate to MDA-MB-231 cells overexpressing human GPR109A in the presence or absence of increasing concentrations of unlabeled OTC or NA (positive control). **C**: Analysis of the effects of OTC or nicotinic acid (NA; positive control) on forskolin (FSK)-induced elevations in cAMP. *p<0.01 compared to control cells; #p<0.01 compared to FSK-treated cells.

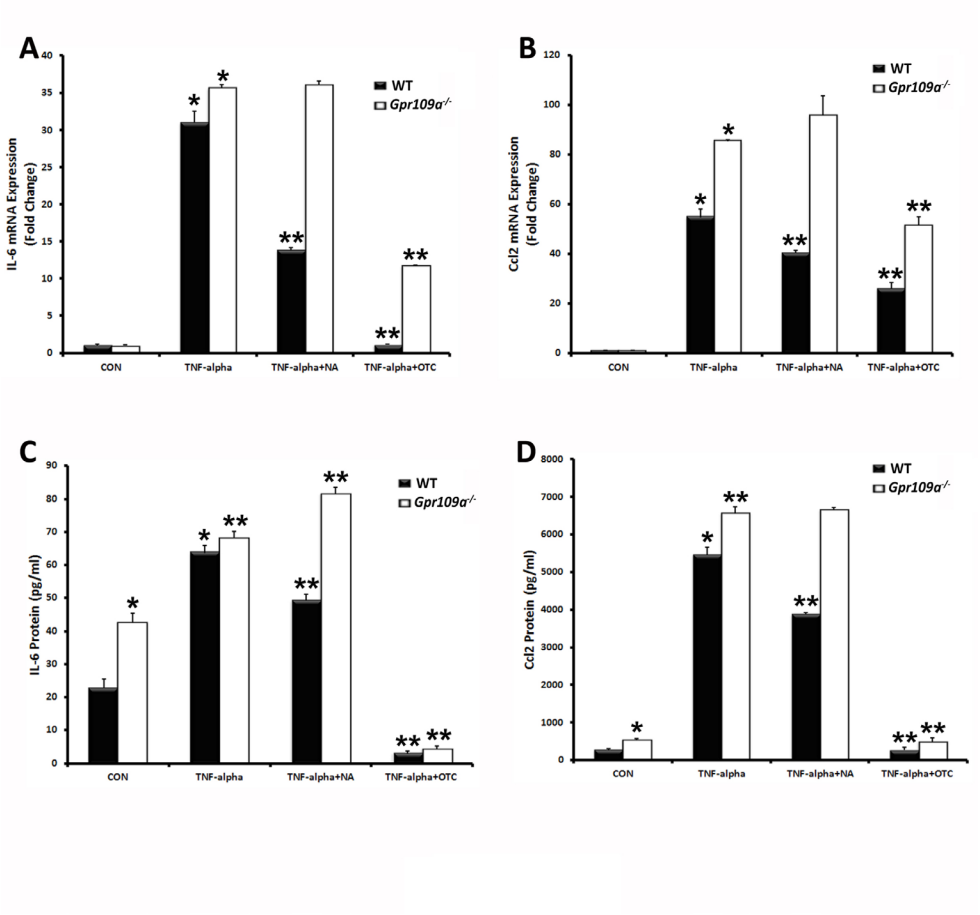

Figure 8. The anti-inflammatory effects of OTC are not solely dependent upon GPR109A. Mouse primary retinal pigment epithelial (mRPE) cells were prepared from *Gpr109a*$^{+/+}$ (wild-type, WT) and *Gpr109a*$^{-/-}$ mouse retinas and exposed to tumor necrosis factor-α (TNF-α; 10 ng/ml; 24 h incubation) in the presence or absence of L-2-oxothiazolidine-4-carboxylic acid (OTC; 0.5 mM) or nicotinic acid (NA; 1 mM; positive control). Quantitative polymerase chain reaction (qPCR) analysis of (**A**) interleukin-6 (IL-6) and (**B**) chemokine (C-C) motif ligand 2 (Ccl2) mRNA expression. Cell culture medium was then collected and used for enzyme-linked immunosorbent assay (ELISA) analysis of (**C**) interleukin-6 (IL-6) and (D) Ccl2 protein. *p<0.01 compared to respective WT or *Gpr109a*$^{-/-}$ untreated, control cells; **p<0.01 compared to respective TNF-α-treated WT or *Gpr109a*$^{-/-}$ cells.

systems. Therefore, we predicted that the anti-inflammatory effects of OTC must be mediated by its interaction with a receptor present on the membrane of the cell. Given our recent identification of GPR109A as an anti-inflammatory G-protein coupled receptor in RPE cells [26], we immediately tested the interaction of OTC with this receptor. To date, none have evaluated whether OTC can activate GPR109A. Thus, in the present study we investigated this phenomenon using multiple assays and cell culture model systems. Specifically, we used HEK-293 cells, cells that do not express GPR109A endogenously and are therefore suitable for transfection with exogenous GPR109A and the other components required to measure quantitatively the dissociation of α from βγ subunits of the G-protein coupled receptor in the presence of OTC as an indicator of receptor activation. MDA-MB-231, a mammary gland cell line that expresses GPR109A robustly, was also used as were ARPE-19 cells, cells that express GPR109A endogenously at basal levels, and *Gpr109A*$^{-/-}$ primary RPE cells, cells devoid of GPR109A expression. Collectively, studies using these cell types and assays demonstrated the following definitively: (a) OTC can activate GPR109A but not as effectively as nicotinic acid, the protypic GPR109A agonist, and (b) although NA and OTC can suppress inflammation in our experimental system, they do so by different means, NA via GPR109A and OTC via some other unknown mechanism. Despite our inability to find a clear-cut mechanism to explain the anti-inflammatory actions of OTC, the fact remains that OTC has been used successfully in patients for various clinical indications in which oxidative stress and inflammation are majorly involved; OTC appears to be superior in its ability to raise intracellular glutathione levels and protect cells from oxidative stress [7,8,13]. Additionally, OTC is well tolerated and associated with low toxicity [5,49-53]. Therefore, we investigated the in vivo efficacy of the compound in the DKO rd8 mouse, a model of chronic, low-grade retinal inflammation. The expression of molecular markers of inflammation and oxidative stress was significantly suppressed in DKO rd8 animals treated with OTC in drinking water for a period of 5 months. This result strongly supports the possible extrapolation of OTC use to therapeutically manage retinal diseases such as AMD.

This study represents, to our knowledge, the first report of the suppressive effects of OTC on inflammation in cultured RPE cells, and on inflammation and oxidative stress in the retina in vivo. Furthermore, in addition to adding a new tissue, the retina, to the existing repertoire of tissues and pathologies in which OTC use confers benefit, our present demonstration that positive outcomes (the modulation of parameters associated with increased oxidative stress and inflammation) can be achieved in the retina following the oral administration of this compound to animals has strong implications in terms of the possible circumvention of challenges associated with delivering drugs to the posterior segment [54,55]. This is particularly relevant to dry AMD, a type of AMD in which oxidant- and inflammation-induced damage to RPE is a primary pathologic feature and one for which, at present, there is a critical lack of effective strategies for treatment and prevention [56]. Gamcsik et al. [57] have shown convincingly that following the infusion of [^{13}C]-labeled OTC into the brain, incorporation of ^{13}C-label into glutathione, taurine, hypotaurine, and lactate is readily detectable. Given the known benefits of these compounds in the retina regarding energy metabolism, blood flow regulation, and antioxidant and anti-inflammatory signaling, any increase in cellular levels or the stabilization of these compounds in the retina and RPE cells normally and under pathologic conditions such as AMD, would likely yield substantial benefit [58-62]. However, whether similar phenomena occur in the retina and RPE cells following the oral administration of OTC and thus explain, at least in part, the robust antioxidant and anti-inflammatory effects elicited by this compound in this tissue is a subject worthy of further investigation. Additionally, before testing OTC clinically in patients with AMD, comparative analyses of the effects of the compound alone and in combination with other antioxidant or anti-inflammatory supplements in animal models of AMD-like retinal disease may be warranted.

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