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The small tellurium-based compound SAS suppresses inflammation in human retinal pigment epithelium

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Purpose: Pathological angiogenesis and chronic inflammation greatly contribute to the development of choroidal neovascularization (CNV) in chorioretinal diseases involving abnormal contact between retinal pigment epithelial (RPE) and endothelial cells (ECs), associated with Bruch's membrane rupture. We explored the ability of the small organotellurium compound octa-O-bis-(R,R)-tartarate ditellurane (SAS) to mitigate inflammatory processes in human RPE cells.
Methods: Cell adhesion assays and analyses of gene and protein expression were used to examine the effect of SAS on ARPE-19 cells or primary human RPE cells that were grown alone or in an RPE-EC co-culture.
Results: Adhesion assays showed that SAS inhibited αv integrins expressed on RPE cells. Co-cultures of RPE cells with ECs significantly reduced the gene expression of PEDF, as compared to RPE cells cultured alone. Both SAS and the anti-αvβ3 antibody LM609 significantly enhanced the production of PEDF at both mRNA and protein levels in RPE cells. RPE cells co-cultured with EC exhibited increased gene expression of CXCL5, COX1, MMP2, IGF1, and IL8, all of which are involved in both angiogenesis and inflammation. The enhanced expression of these genes was greatly suppressed by SAS, but interestingly, remained unaffected by LM609. Zymography assay showed that SAS reduced the level of MMP-2 activity in RPE cells. We also found that SAS significantly suppressed IL-1β-induced IL-6 expression and secretion from RPE cells by reducing the protein levels of phospho-IkappaBalpha (pIκBα).
Conclusions: Our results suggest that SAS is a promising anti-inflammatory agent in RPE cells, and may be an effective therapeutic approach for controlling chorioretinal diseases.

Choroidal neovascularization (CNV) is the leading cause of vision loss in various pathological conditions in which the Bruch's membrane is ruptured or damaged [1-3]. CNV tends to develop under conditions in which the retinal pigment epithelium (RPE) and endothelial cells (ECs) are no longer separated by the Bruch's membrane, resulting in contact of the two cell types [4]. Indeed, ocular diseases involving a newly formed contact between RPE cells and the choriocapillaries, such as angioid streaks, irregular crack-like dehiscences in the Bruch's membrane, high myopia, inappropriate laser burn, and traumatic choroidal rupture, are all associated with CNV formation [5].

Angiogenesis is a complex process regulated by the balance between pro-angiogenic and anti-angiogenic factors. Vascular endothelial growth factor (VEGF) is the major pro-angiogenic growth factor produced and secreted by RPE cells in response to hypoxia, playing a key role in pathological angiogenesis leading to CNV [6-8]. In contrast, pigment epithelium-derived factor (PEDF), which is also produced by RPE cells, acts as an anti-angiogenic and anti-inflammatory factor [9,10]. Angiogenesis is further regulated by alpha v integrins (αvβ3 and αvβ5), which are cell adhesion molecules extensively involved in both normal and pathological angiogenesis, including tumor blood vessel growth and retinal and choroidal neovascularization [11].

Several in vitro studies have addressed the RPE-EC interaction and its potential role in the development of CNV. Both the proliferation and migration of choroidal EC are significantly increased in ECs grown in either contact or non-contact co-cultures with RPE [12,13]. RPE cells modulate tube formation by ECs embedded in type I collagen gel [14]. We previously showed that ECs grown in direct contact with RPE, a model mimicking the pathological conditions associated with Bruch's membrane rupture, exhibit enhanced angiogenic potential [15].

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Inflammatory responses contribute to the pathophysiology of numerous ocular diseases [16,17]. RPE cells play a critical role in mediating immune responses to stressors, such as bacterial endotoxins or pro-inflammatory cytokines [18,19]. Under normal conditions, the RPE is an important component in the downregulatory environment of the eye [20]. However, under inflammatory conditions, RPE cells can become activated and propagate ocular inflammation [21]. IL-1β is a major pro-inflammatory cytokine secreted by lymphocytes and macrophages during ocular inflammation [22]. IL-1β can activate RPE cells, inducing the production of pro-inflammatory and pro-angiogenic mediators, such as IL-6, IL-8, and VEGF [22-27]. Recent reports indicate that inflammatory processes contribute to the development of retinal and choroidal neovascularization [28-31]. It has been shown that IL-6 and IL-8, through the activation of nuclear factor kappa B (NFκB), participate in the pathogenesis of retinal neovascularization [32,33]. Importantly, chronic inflammation can ultimately damage the RPE and contribute to the activation of CNV, which is observed in more advanced forms of age-related macular degeneration (AMD) [34], the most common cause of severe visual loss in patients over the age of 60 in developed countries.

Our laboratory and collaborators have synthesized a group of Tellurium compounds with varied Tellurium (Te) valences. The compounds exerting the most pronounced biologic activities are AS101 [ammonium trichloro (dioxoethylene-O,O')tellurate] [35] and SAS [octa-O-bis-(R,R)-tartarate ditellurane] [36]. SAS is a new, small molecule, a Te^{IV} compound comprising two tellurium atoms, each liganded by four oxygen atoms from two carboxylates, and two alkoxides of two tartaric acids. Unlike many other Te^{IV} compounds, SAS is highly stable in aqueous solutions. SAS was shown to be non-toxic to mice, even after continuous treatment for several months (unpublished data). Accumulated evidence suggests that much of the biologic activity of SAS and AS101 is directly related to its specific chemical interactions with cysteine thiol residues. The Te^{IV}–thiol chemical bond may lead to conformational change or disulfide bond formation in a specific protein, possibly resulting in the loss of its biologic activity, if the thiol residue is essential for that function [36,37]. We have shown recently that AS101 inhibits IL-1β-induced IL-6 and IL-8 production in the human retinal pigment epithelium [38]. Moreover, we recently found that AS101 and SAS mediate the functional inhibition of specific integrins [39-41], among them the pro-angiogenic αvβ3 integrin (unpublished data). In the present study, we demonstrate that the new bioactive Te^{IV} compound SAS could mitigate inflammatory responses induced in RPE by co-culture with EC or by IL-β activity.

METHODS

Cell cultures and reagents: Human retinal pigmented epithelial (HRPE) cells obtained from an adult donor [42] and transformed retinal pigmented epithelial (ARPE 19) cells (ATCC, Manassas, VA; Appendix 1) were cultured at 37 °C in 5% CO_2 in a medium consisting of Minimum Essential Medium with Earle's salts and L-glutamine (MEM), 10% fetal bovine serum, 1x non-essential amino acids, and 1x penicillin-streptomycin (Invitrogen, Carlsbad, CA). For all experiments, the cells used were passaged fewer than 25 times. For experiments involving IL-1β-induced inflammation in RPE cells, HRPE and ARPE19 cells were seeded separately in 10% FBS MEM to form monolayers at ~50% confluency on six-well plates. Once the cells had adhered, the supernatant was replaced with serum-free MEM. The next day, the cells were treated with 0.5, 1, 2.5, or 5 µg/ml SAS (BioMAS, Jerusalem, Israel) in fresh serum-free media. After 1 h incubation, 10 or 20 ng/ml of IL-1β (Peprotech, Rock Hill, NJ) was added for the relevant incubation time. For each set of experimental cultures, control samples were cultured without either AS101 or cytokines.

Human dermal microvascular ECs, kindly donated by Dr. R. Shao [43] (Biomedical Research Institute, Baystate Medical Center/University of Massachusetts at Amherst, Springfield), were grown in EBM medium supplemented with growth factor cocktail (PromoCell, Heidelberg, Germany) and 10% FCS.

EC-RPE contact co-culture and separation of RPE cells: RPE cells and ECs were plated at 7×10^3 cells/cm^2 each, and cultured together for seven days in a mixture of RPE/EC (1:1) medium (co-culture medium), followed by the addition of either 20 µg/ml anti-integrin αVβ3 antibody (LM609; Chemicon, Temecula, CA) or SAS (BioMAS, Jerusalem, Israel) at a concentration of 1 µg/ml for 24 h. Pure monocultures of RPE cells (control samples) were established in parallel to EC-RPE co-cultures.

Following co-culture, the cells were detached by incubation with 5 mM EDTA in PBS for 30 min. ECs were separated from the cell mixture using magnetic beads coated with anti-CD-31 antibody (PlusCellect™ human CD-31 kit, R&D Systems, Minneapolis, MN). RPE cells, which do not express CD-31, remained in suspension. Separated RPE cells were further used for RNA extraction and the measurement of VEGF and PEDF levels.

Adhesion assay: A 96-well plate was coated with 80 µl/well of 10 µg/ml recombinant human vitronectin (PeproTech). Control wells were coated with 2.5% BSA (Sigma). The plate was incubated overnight at 4 °C. Then, the wells were washed

two times with 150–200 µl PBS and blocked with 80 µl of 2.5% BSA for 1 h in the incubator at 37 °C. The wells were washed again two times with 150–200 µl PBS. Next, 1×10^4 ARPE-19 cells/100 µl were seeded onto each well, and 1, 2.5, or 5 µg/ml SAS was added into the vitronectin-coated wells. After 3 h, the wells were washed twice with 150–200 µl PBS to remove the unbound cells. Then, each well was loaded with 100 µl RPMI + 50 µl XTT solution (Biologic Industries), and the plate incubated overnight in the incubator at 37 °C. The plate was read in a microplate reader (Bio-Rad) at a wavelength of 450 nm.

Semiquantitative PCR: Total RNA was extracted from ARPE-19 using Tri Reagent (Sigma, Israel) according to the manufacturer's instructions. The RNA concentrations were determined using a Nano-Drop (ND-1000) spectrophotometer (Thermo Scientific), and equal quantities of total RNA from different samples were used. Next, 2 µg of RNA were reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and then amplified with an MJ Mini Thermal Cycler PCR machine (Bio Rad). The primers for human IL-6 and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed according to their mRNA sequences. GAPDH was used as the internal control. The oligonucleotide primers used for the amplification of human IL-6 cDNA were 5′- CCT TAA AGC TGC GCA GAA TG −3′ (sense) and 5′- ATT CAA TGA GGA GAC TTG CC −3′ (antisense). The resultant PCR product was 284 bp. The oligonucleotide primers used for amplifying human GAPDH cDNA were 5′- CGA CCA CTT TGT CAA GCT CA −3′ (sense) and 5′- AGG GGT CTA CAT GGC AAC TG −3′ (antisense). The resultant PCR product was 228 bp. Each cycle consisted of 1 min at 94 °C, 2 min at 55 °C for amplifying IL-6 and 57 °C for amplifying GAPDH cDNA, and 3 min at 72 °C. All reactions were completed with an extension step of 5 min at 72 °C. All samples were amplified in the linear amplification range established using serial cDNA dilutions and varying the number of cycles (25 cycles for GAPDH and 30 cycles for IL-6). PCR products were electrophoresed onto a 2% agarose gel containing ethidium bromide (Sigma) and visualized under ultraviolet (UV) light.

Real-time PCR (RT–PCR): For the RPE-EC co-culture system, total RNA from control solo RPE cells or RPE cells separated from an EC-RPE co-culture was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were reverse-transcribed into cDNA using Moloney Mouse Leukemia Virus (MMLV) reverse transcriptase with random oligonucleotides (Invitrogen). Expression levels of genes involved in angiogenesis and inflammation were analyzed in cDNA samples by RT–PCR analysis using Sybr Green qPCR Supermix (Invitrogen Corporation) and Human Angiogenesis Primer Library (RealTimePrimers.com, Elkins Park, PA). Amplification was monitored by 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The expression levels of VEGF and PEDF genes were analyzed by RT–PCR as described above, using GAPDH and HPRT as reference genes. Primer sequences used for PCR analysis were selected using Primer3 software.

For the IL-1β-induced inflammation experiments using RPE cells, after 24 h of incubation with SAS (with or without the addition of IL1β), total RNA was purified from confluent cell cultures using an RNeasy Kit (Qiagen, Valencia, CA). Subsequently, 150 ng of total RNA was reverse-transcribed into cDNA (Reaction Ready First Strand cDNA Synthesis Kit, SABiosciences, Frederick, MD). For each RT–PCR, samples were tested in duplicate with the 96-well format PCR array and an ABI 7500 Real-Time PCR unit (Applied Biosystems). The samples were analyzed for IL-6 and IL-8 levels, normalized to GAPDH, which was used as an endogenous control (all primers were purchased from Applied Biosystems). The final results are presented as the fold expression of IL-6 or IL-8 relative to the untreated control group.

ELISA measurement of protein levels: For the IL-1β-induced inflammation experiments using RPE cells, supernatants of appropriate cultures were collected after 48 h of incubation. Subsequent analyses of samples were performed in duplicate and repeated three times. Protein levels of IL-6 were measured using ELISA kits (R&D Systems, Inc., Minneapolis, MN) following the manufacturer's instructions.

For the RPE-EC co-culture system, cellular proteins were extracted in ice-cold lysis buffer (1%-Triton X-100 in TBS (0.15 M NaCl, 20 mM TrisHCl, pH 7.5)) containing protease inhibitors (Boeringer-Manheim, Germany). Protein concentrations were measured using the Bradford assay (Bio-Rad, Germany). VEGF levels in cellular extracts were measured by commercial ELISA assay (PeproTech, London, UK) according to the manufacturer's instructions. PEDF levels in cellular extracts were measured by commercial ELISA assay (Chemicon) according to the manufacturer's instructions.

Sodium dodecyl sulfate PAGE (SDS–PAGE) and western blotting: Confluent monolayers of ARPE19 were incubated with 0.5, 1, 2.5, or 5 µg/ml of SAS in serum-free medium for 1 h, and then treated with 10 ng/ml of IL-1β. Cultures were incubated for 5 min after cytokine treatment before the monolayers of ARPE19 were lysed in ice-cold lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 1% TritonX, 1 mM EDTA, 1 mM PMSF, 1 mM sodium

vanadate, and 0.1% protease inhibitor cocktail (Calbiochem) for 30 min on ice, and then centrifuged at 15,000 × *g* for 20 min. Cell lysates were boiled for 5 min, electrophoresed on SDS–PAGE, and their membranes were incubated with anti-pIκBα (Cell Signaling) and anti-tubulin (Sigma). Blots were developed using horseradish peroxidase-conjugated secondary antibodies and the ECL detection system (Pierce).

Examination of MMP-2 activity by zymography: Media samples were centrifuged for 10 min at 310 × *g* to remove floating cells. Supernatants were resolved on 8% SDS–PAGE containing 1% gelatin. The gel was incubated in renaturing buffer (2.5% Triton X 100) for 30 min at room temperature, followed by 30 min incubation in developing buffer (Tris base 50 mM, Tris HCl 0.2M, NaCl 0.2M, $CaCl_2$ 5 mM, and Brij 35 0.02%) with gentle agitation. The gel was then incubated in a developing buffer at 37 °C overnight, and stained with Coomassie R-250 (Sigma, Rehovot, Israel) for at least 2.5 h with gentle agitation. The samples were viewed and analyzed by the LI-COR Odyssey imaging system (LI-COR Biosciences, Lincoln, NE).

Statistical analysis: Differences between groups in the adhesion assay, RT–PCR, and ELISA, as well as densitometry data for zymography, were analyzed using the Student *t* test. $p<0.05$ was considered statistically significant.

RESULTS

SAS decreases the adhesion of ARPE-19 cells to vitronectin: Cell adhesion molecules were extensively involved in both normal and pathological angiogenesis, including tumor blood vessel growth, retinal and choroidal neovascularization [11]. During experimental CNV, the binding of inhibitors to αv integrins resulted in an anti-angiogenic effect. Thus, a non-peptide antagonist specific for integrin αvβ3 attenuated experimental CNV [44,45]. We recently found that AS101 and SAS mediated the functional inhibition of specific integrins [39-41], among them the αvβ3 integrin (unpublished data). Therefore, we examined the potential inhibitory effect of SAS on the adhesion of ARPE-19 cells to vitronectin (VN), which is the ligand for αv integrins such as αvβ3 and αvβ5. ARPE-19 cells significantly attached to VN compared to plates coated with the BSA control ($p<0.001$, Figure 1). In comparison, 2.5 and 5 µg/ml of SAS significantly reduced the attachment of the cells to VN ($p<0.05$, Figure 1). Of note, SAS did not significantly affect the viability of ARPE-19 in the XTT assay (data not shown). This result suggests that SAS interrupts the interaction between αv integrins and vitronectin, and therefore may act as an inhibitory agent for αv integrins on RPE cells.

Effects of SAS and anti-αvβ3 antibody on VEGF and PEDF expression in RPE cells co-cultured with ECs: We previously demonstrated that contact co-cultures of EC with RPE cells enhances the pro-angiogenic potential of the EC [15]. In this study, we examined the effect of contact co-cultures

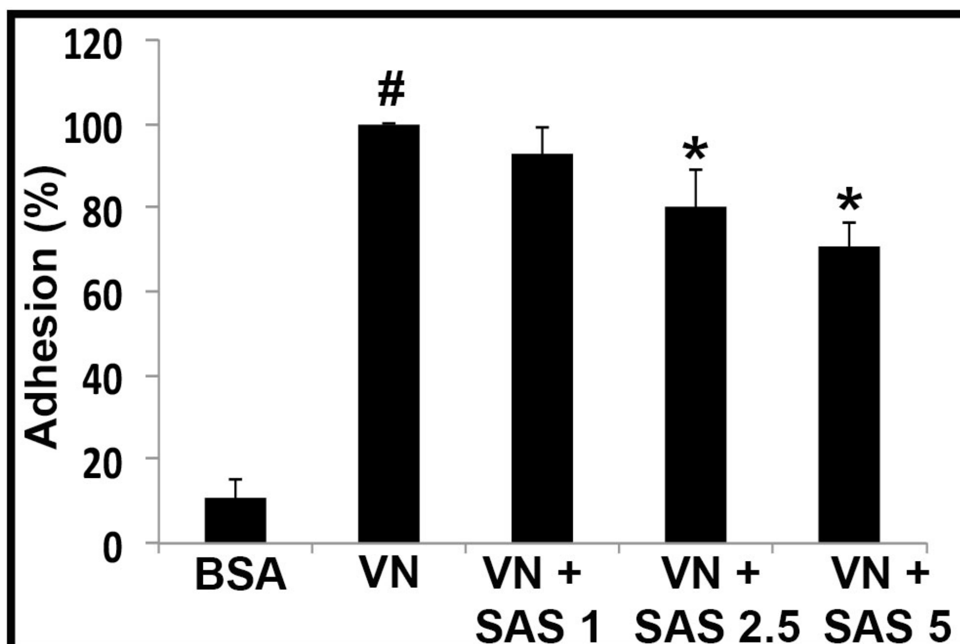

Figure 1. AS101 reduces the adhesion of ARPE-19 cells to Vitronectin. ARPE-19 cells were seeded for 3 h on vitronectin (VN) or 2.5% BSA-coated wells. Next, 1–5 µg/ml of SAS were added into VN-coated wells. After 3 h, the wells were washed, and XTT assays were performed to evaluate the number of bound cells. # $p<0.001$ increase versus BSA group, * $p<0.05$ decrease versus VN group. The O.D. value of the VN group was defined as 100%. Results shown are mean ± SEM of 3 independent experiments.

on the angiogenic and inflammatory characteristics of RPE cells. RPE and endothelial cells grown in co-cultures were separated by the removal of ECs using magnetic beads coated with the anti-CD31 antibody. Following separation, quantitative RT–PCR was used to examine the effect of the co-cultures on VEGF and PEDF mRNA levels in RPE cells. RNA extracted from RPE cell mono-cultures was used as a control (Solo RPE). The mRNA level of VEGF in RPE cells was not affected by the co-culture with EC. Furthermore, VEGF remained unaffected by the presence of either SAS or the anti-αvβ3 antibody LM609 during co-culture with EC (Figure 2). However, the mRNA level of PEDF was significantly reduced in RPE cells co-cultured with EC in comparison to RPE cells cultured alone ($p<0.05$). In addition, the mRNA level of PEDF was significantly increased by the addition of either SAS or LM609 to the co-culture medium ($p<0.05$, Figure 2). The effects of SAS and LM609 on VEGF and PEDF protein levels in RPE cells co-cultured with EC correlated with their effects on the expression of their corresponding genes ($p<0.05$, Figure 3). These results clearly suggest that αvβ3 integrin regulates the expression and secretion of PEDF from RPE cells and that SAS significantly upregulates PEDF levels in RPE cells, at least in part by the inhibition of αvβ3 integrins.

Effects of SAS and LM609 on the expression of other genes involved in angiogenesis and inflammation in RPE cells co-cultured with ECs: We further examined the effect of SAS on RPE cells co-cultured with EC by studying the expression of other genes involved in angiogenesis and inflammation. RPE and endothelial cells were grown in a co-culture and then separated using CD-31-coated magnetic beads. Following separation, RPE cells were examined for gene expression using a PCR array including 88 genes encoding proteins involved in angiogenesis and inflammation. RT–PCR analysis demonstrated that RPE cells co-cultured with EC exhibited enhanced mRNA levels of five genes: chemokine (C-X-C motif) ligand 5 [CXCL5], cyclooxygenase-1 [COX1], matrix metallopeptidase 2 [MMP-2], insulin-like growth factor 1 [IGF1], and interleukin 8 [IL-8]. All these genes are involved in the upregulation of both pro-angiogenic and pro-inflammatory processes. The enhanced expression of each of these genes was significantly reduced by SAS, but interestingly, remained unaffected by the LM609 antibody (Figure 4). These results suggest that SAS may have other anti-angiogenic and anti-inflammatory mechanisms of activity in RPE in addition to αvβ3 inhibition.

SAS reduces MMP-2 activity: MMP-2 largely contributes to CNV, as indicated by the observation of reduced CNV in MMP-2-deficient mice [46]. In view of the ability of SAS to suppress the expression of MMP-2 at the mRNA level (Figure 4), we examined the effect of SAS on MMP-2 activity observed in an RPE-EC co-culture. Conditioned media were collected from RPE-EC co-cultures grown in a control medium, or in the presence of either SAS or LM609. SAS (at 1μg/ml concentration) significantly reduced the level of MMP-2 activity by 51±13%, based on analysis by densitometry (Figure 5). MMP-2 activity in co-cultures was not affected by LM609 (data not shown). This observation is in agreement with the differential effects of SAS and LM609 on MMP-2 mRNA expression, shown in Figure 4.

SAS decreases IL-1β-induced expression of IL-6 in ARPE-19 and HRPE cells: IL-1β is known to stimulate the production of inflammatory mediators, such as IL-6 and IL-8, in RPE cells [47]. It was recently shown that the Te^{IV} compound AS101 inhibits IL-1β-activated inflammation in the human retinal pigment epithelium. To determine the potential anti-inflammatory activity of SAS on RPE cells, we used a model of IL-1β-induced inflammation in ARPE-19 and primary RPE cells (HRPE). Semiquantitative RT–PCR showed that 24 h incubation of ARPE-19 cells with IL-1β induced upregulated mRNA expression of IL-6. However, 2.5 and 5 μg/ml concentrations of SAS reduced mRNA levels of IL-6 as compared to cells treated with IL-1β alone (Figure 6A). To verify these results, we further examined the potential anti-inflammatory activity of SAS on primary RPE cells (HRPE). RT–PCR showed that 24 h incubation of HRPE cells with IL-1β induced significantly upregulated mRNA expression of IL-6 ($p<0.05$). In comparison, SAS significantly reduced mRNA levels of IL-6 compared to cells treated with IL-1β alone ($p<0.05$, Figure 6B). Of note, SAS did not significantly affect the viability of HRPE cells in the MTT assay (data not shown).

As shown in Figure 7, 48 h incubation of IL-1β alone significantly increased the secretion of IL-6 proteins in ARPE-19 cells as measured by ELISA ($p<0.01$). In comparison, high concentrations of SAS (2.5 and 5 μg/ml) significantly suppressed IL-1β-induced secretion of IL-6 from the cells ($p<0.05$, Figure 7).

SAS inhibits the NFκB pathway in ARPE-19 cells: To determine the intracellular mechanism of the anti-inflammatory activity mediated by SAS, we examined the effect of SAS on the IL-1β-induced NFκB pathway that is known to play an important role in inflammatory processes in general and that of the RPE in particular [32,33]. In the cytoplasm, NFκB is inhibited by IκB. Upstream activating signals cause phosphorylation of IκB by IKK (IκB kinase). This triggers the degradation of IκB through the ubiquitin system. The free NFκB can then translocate to the nucleus and activate

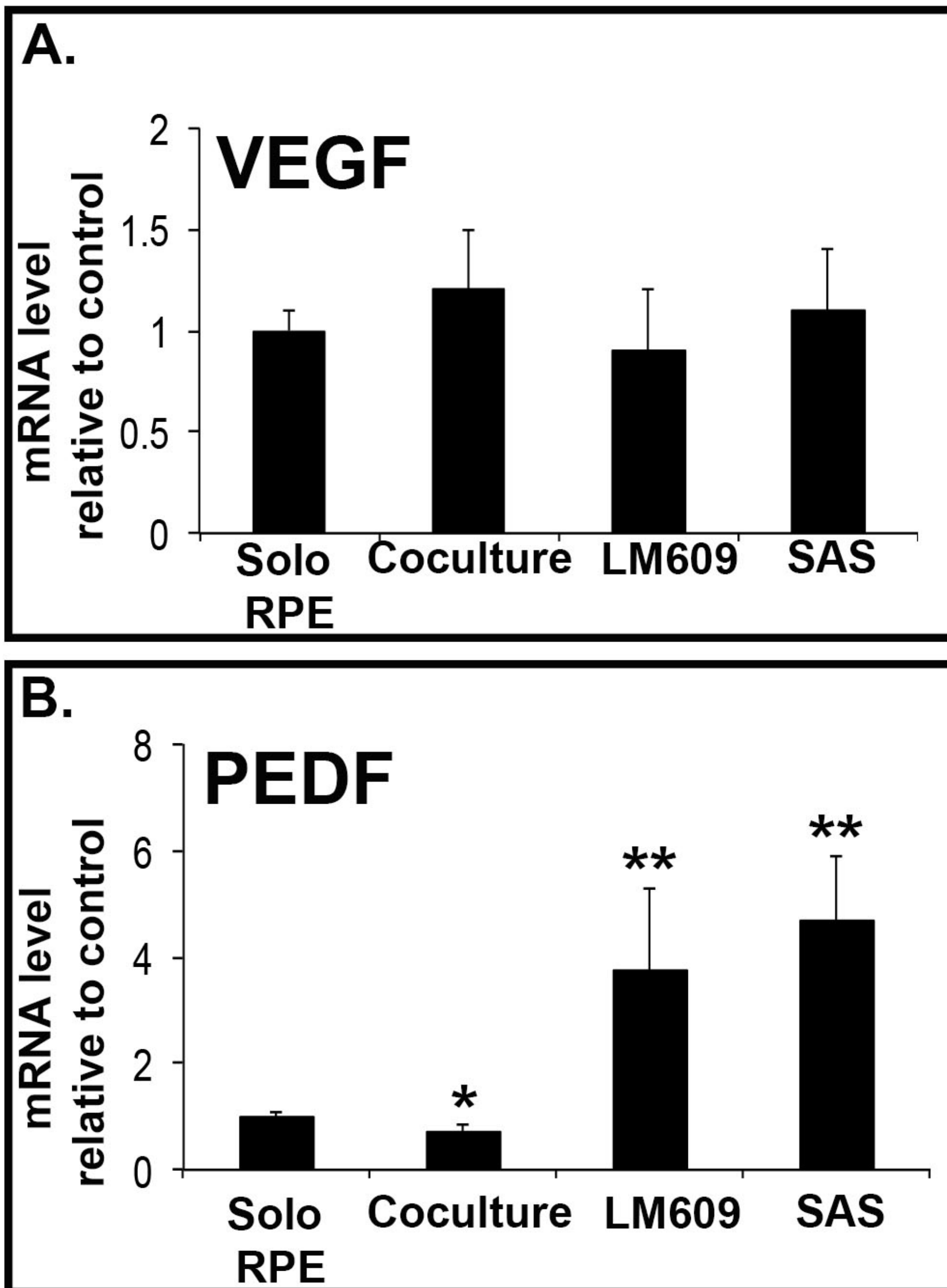

Figure 2. SAS and LM609 significantly increase PEDF mRNA levels in the RPE-EC co-culture system. RT–PCR analysis of VEGF (**A**) and PEDF (**B**) mRNA levels in pure mono-cultures of RPE alone (solo RPE) and co-cultures of RPE-EC cells (co-culture). *$p<0.05$ decreased relative to solo RPE; ** $p<0.05$ increased relative to RPE co-cultured with ECs. Results shown are mean ± SD.

transcription. Incubation of ARPE-19 with IL-1β revealed increased levels of pIκBα as compared to the control. However, 1 h pre-incubation of the cells with 2.5 or 5 µg/ml of SAS decreased the levels of pIκBα (Figure 8). This result suggests that SAS act as an anti-inflammatory agent in RPE cells at least in part through the downregulation of NFκB activity.

Collectively, our results indicate that SAS can mitigate angiogenesis and inflammatory responses induced in RPE via upregulation of PEDF levels and the suppression of pro-angiogenic and inflammatory factors. The mechanisms of these activities may be mediated by the inhibition of αv integrins and NFκB activity in RPE cells.

DISCUSSION

CNV is the most common cause of vision loss under various pathological conditions, such as AMD, in which the Bruch's membrane is ruptured or damaged. In spite of the variety of

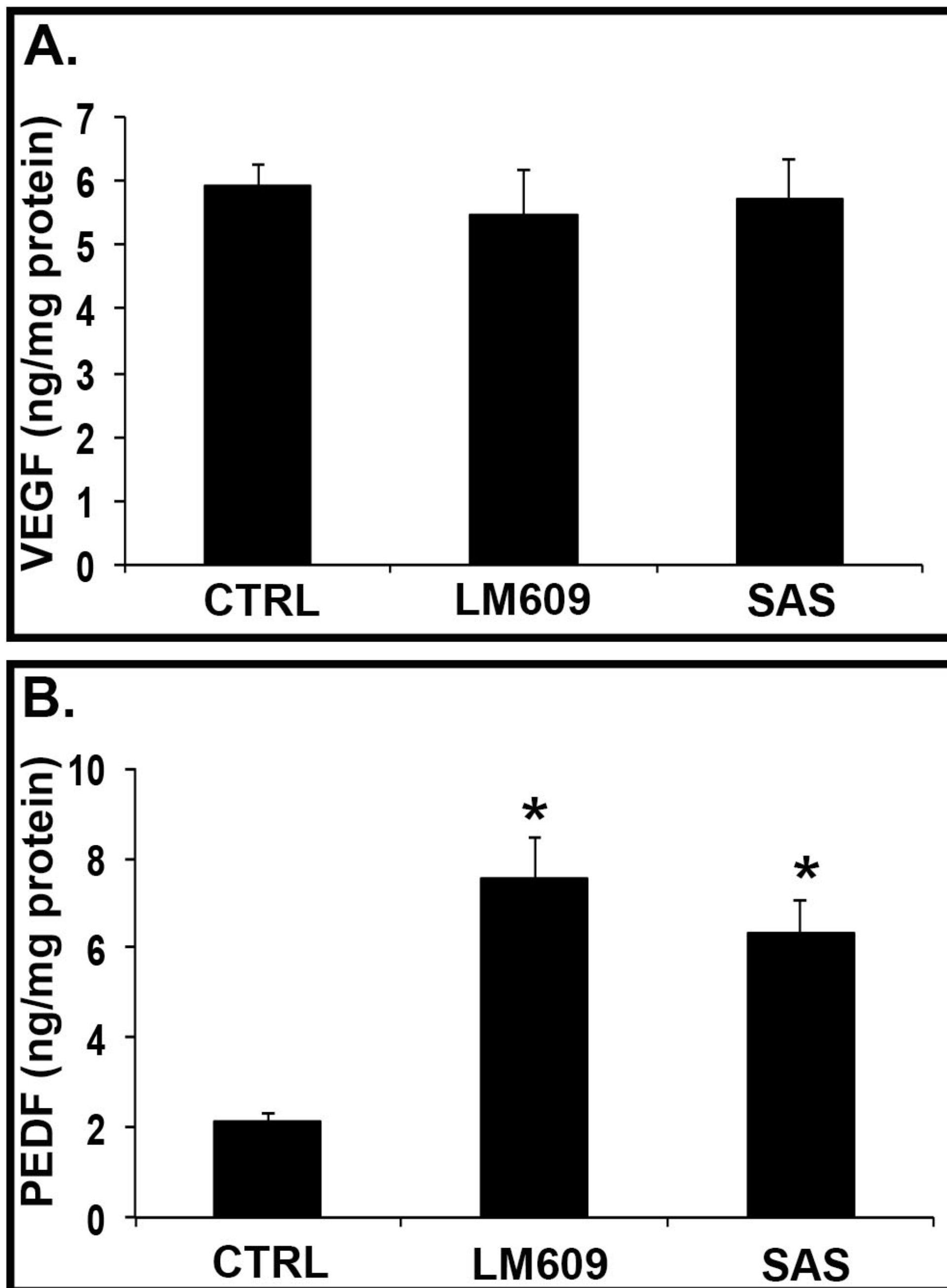

Figure 3. SAS and LM609 significantly increase PEDF protein levels in the RPE-EC co-culture system. Protein levels of VEGF (**A**) and PEDF (**B**) in RPE (lysed cells) co-cultured with ECs were measured by ELISA. *$p<0.05$ increased relative to RPE co-cultures with ECs in control medium. Results shown are mean ± SD.

diseases associated with CNV, there is significant similarity between the pathological blood vessels developing in these diseases in terms of cellular composition and morphology [2]. This observation has led to the suggestion that CNV tends to develop under conditions characterized by pathological interaction between the EC and RPE layers [3]. In an attempt to mimic such pathological interaction in vitro and to understand its molecular aspects, we designed an artificial co-culture model of ECs and RPE cells. Using this model, we have previously demonstrated that ECs grown in contact co-cultures with RPE cells exhibit enhanced pro-angiogenic potential, in contrast to ECs grown with RPE cells in non-contact co-cultures [15]. Importantly, chronic inflammation can ultimately damage the RPE and contribute to the activation of CNV [34].

AS101 and SAS are small, nontoxic tellurium^{IV}-based compounds currently being evaluated in pre-clinical and clinical trials [35,36,41,48,49]. AS101 is a potent immunomodulator (both in vitro and in vivo) with a variety of potential

therapeutic applications [35,50,51]. We previously showed that AS101 inhibits IL1-β-induced IL-6 and IL-8 expression and secretion from RPE cells [52]. Similar to AS101, SAS has been shown to selectively inhibit cysteine proteases [36] and to function as an anti-bacterial agent [53]. Based on their Te-thiol interaction, we have recently found that AS101 and SAS mediate functional inhibition of specific integrins by redox modulation, among them the pro-angiogenic αvβ3 integrin (unpublished data). Therefore, we wished to evaluate the potential anti-angiogenic and anti-inflammatory activity of SAS in two in vitro models that induce angiogenesis and inflammation in RPE cells. We show that RPE cells grown in contact with ECs demonstrate significantly reduced mRNA levels of PEDF, as compared to RPE cells grown alone. PEDF is known to act as an endogenous antagonist of VEGF [9]. In the eye, the balance between VEGF and PEDF levels plays an important role in endothelial quiescence and barrier function [54-57]. Thus, downregulation of PEDF expression in RPE by contact co-culture with EC indicates a pro-angiogenic effect, which is significantly reversed by either SAS or LM609, indicating that SAS may act by blocking αvβ3 integrin activity. In a recent study, it was found that vitronectin (the receptor for αv integrins) was important for the onset of neovascularization [58]. Moreover, retinal vasculogenesis is also sensitive to an antagonist of αvβ3 and αvβ5 [45]. This same peptide antagonist has been shown to inhibit hypoxia-induced retinal neovascularization [59], indicating the importance of αv integrins in the formation of CNV. Thus, the ability of SAS to block αv integrins on RPE cells (Figure 1) and to increase PEDF expression and secretion in the RPE-EC co-culture system (Figure 2- Figure 3) indicate its potential anti-angiogenic activity, at least in part by the blocking of the αvβ3 integrin.

Both physiologic and pathologic angiogenesis are associated with various degrees of inflammation. The coordination between angiogenesis and inflammation is mediated by the interplay of CXC chemokines with cytokines and growth factors [60,61]. We observed that RPE cells cultured in contact with ECs express elevated levels of CXCL5, COX-1, and IL-8. CXCL5 is a pro-angiogenic chemokine exerting its activity via binding to the EC receptor CXCR2 [62]. COX-1 is known to be involved in both pro-angiogenic [63] and pro-inflammatory [64] processes. Treatment with topical nepafenac, a potent COX-1 inhibitor, was shown to inhibit the development of ischemia-induced retinal and choroidal neovascularization caused by rupture of the Bruch's membrane [65]. Elevated

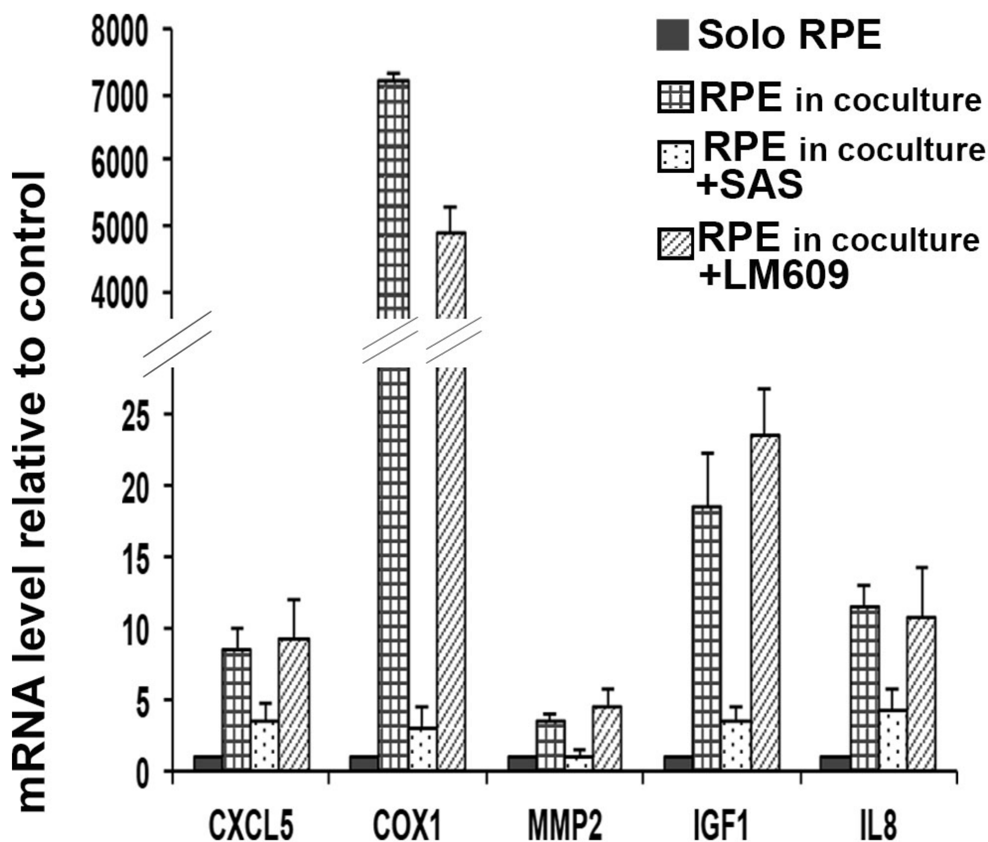

Figure 4. SAS, but not LM609, suppresses the expression of pro-angiogenic and pro-inflammatory genes in the RPE-EC co-culture system. RPE and endothelial cells were grown in co-cultures and separated using CD-31-coated magnetic beads as described in the *Materials and methods* section. Following separation, RPE cells were examined for gene expression using a PCR array including 88 genes encoding proteins involved in angiogenesis and inflammation. The mRNA levels are expressed relative to the mRNA levels observed in solo RPE cells (control; defined as 1). Results shown are mean ± SD.

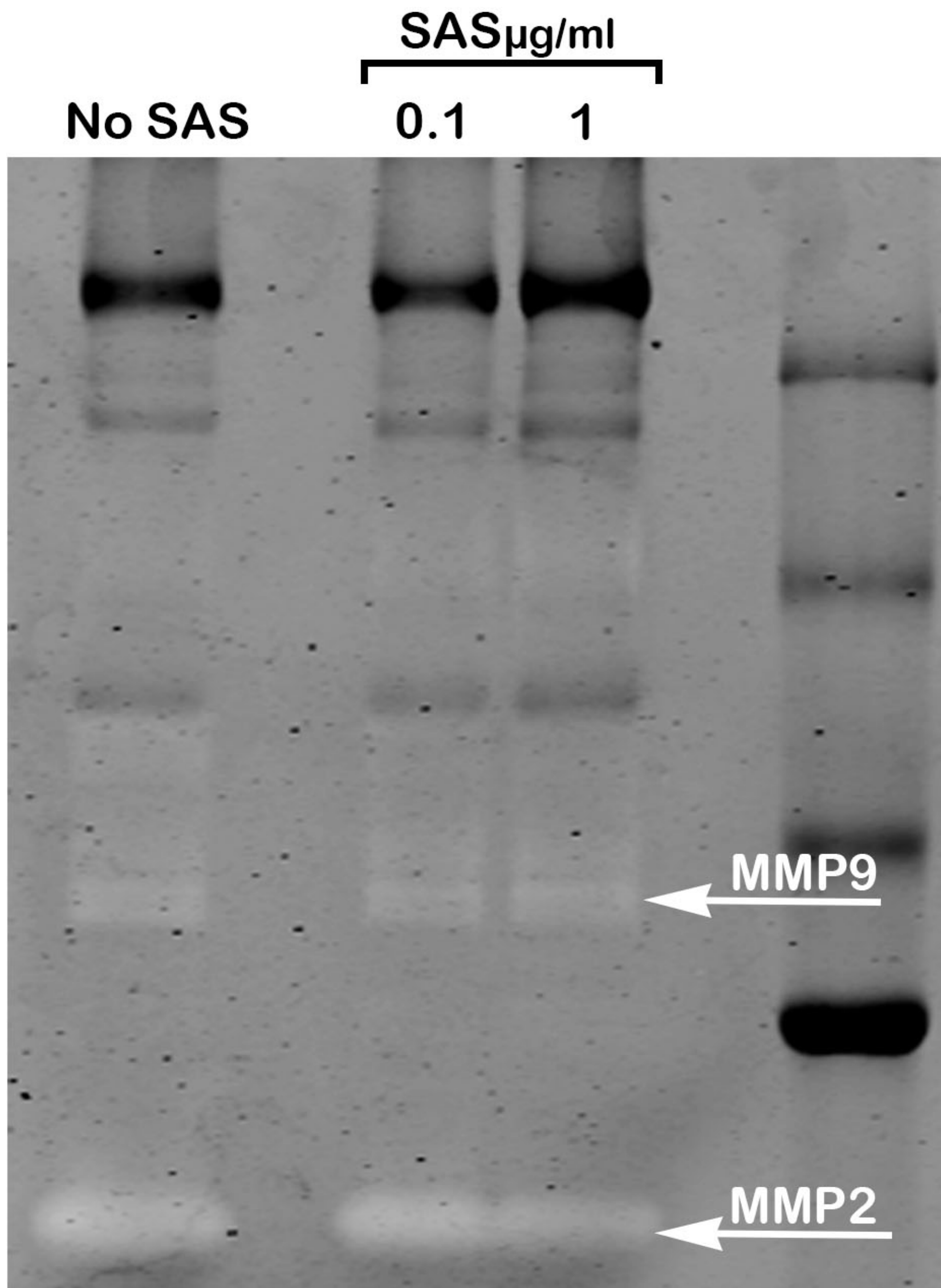

Figure 5. SAS reduces MMP-2 activity in RPE-EC co-cultures. A zymography assay was performed as described in the *Materials and methods* section to evaluate MMP-2 activity. Conditioned media were collected from RPE-EC co-cultures grown in control medium or in the presence of 0.1 and 1 µg/ml SAS.

IL-8 levels are associated with inflammation and vascular dysfunction in diabetic retinopathy [66]. In addition, the levels of IL-8 were reported to be significantly elevated in the eyes of patients with myopic CNV, as compared to high-myopia eyes without CNV [67]. IL-8 stimulates the expression of VEGF and the activation of VEGFR2 in vascular endothelial cells [68]. Our results demonstrate that the increase in the expression of CXCL5, COX1, and IL-8 is almost eliminated by SAS, but not by LM-609.

IGF-1 is a potent pro-angiogenic factor, the presence of which has been observed in neovascular membranes obtained from AMD patients [69]. Furthermore, the inhibition of the IGF-1 receptor has been shown to reduce CNV formation in vivo [70]. The ability of SAS to eliminate the increase in IGF-1 expression induced by contact co-cultures suggests a potential anti-angiogenic effect of SAS.

Proteins of the MMP family play an important role in angiogenesis in view of their ability to degrade basal

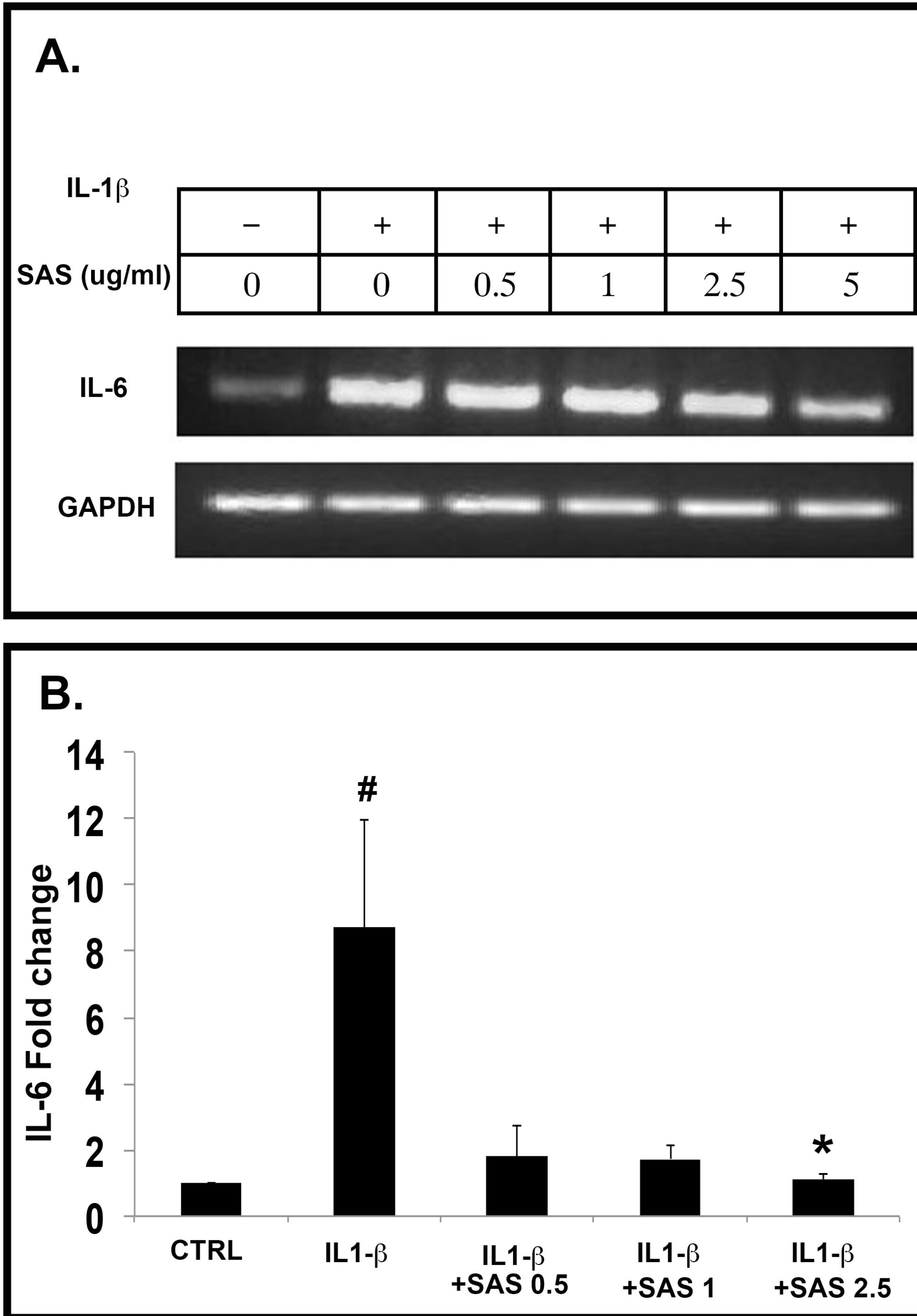

Figure 6. SAS decreases IL-1β-induced mRNA expression of IL-6 in ARPE-19 and HRPE cells. **A:** ARPE-19 cells were starved in serum-free medium overnight and then incubated for 1 h with 0.5, 1, 2.5, or 5 µg/ml of SAS before the addition of 10 ng/ml of IL-1β. After 12 h of culture, total RNA was extracted from the ARPE-19 cells, and semiquantitative RT–PCR was performed to evaluate IL-6 and GAPDH mRNA levels. PCR products on agarose gel are shown. The data represent one of three experiment performed. **B:** HRPE cells were starved in serum-free medium overnight and then incubated for 1 h with 0.5, 1, or 2.5 µg/ml of SAS before the addition of 10 ng/ml of IL-1β. After 24 h of culture, cells were processed for RNA purification and analyzed using RT–PCR. IL-6 mRNA expression levels were measured using GAPDH as an internal control. The results show the average fold change differences compared to untreated control groups. # $p<0.05$ increase versus control group, * $p<0.01$ decrease versus IL-1β group. Results shown are mean ± SEM of 3 independent experiments.

membrane and extracellular matrix proteins, thus promoting EC migration essential for blood vessel formation [71]. MMP-2 largely contributes to CNV, as indicated by the observation of reduced CNV in MMP-2-deficient mice [46]. Our findings indicate increased MMP-2 expression and activity in RPE cells grown in contact co-culture with ECs, both of which are reduced by SAS, but not by LM-609.

As PEDF was shown to act as an anti-angiogenic and anti-inflammatory agent in the eye both in vitro and in vivo [54-57,72], it is surprising that the increase in PEDF by LM-609 treatment was not able to suppress the expression of pro-angiogenic and pro-inflammatory genes in the RPE-EC co-culture system, while, in contrast, SAS treatment significantly suppressed the expression of these genes (Figure 4).

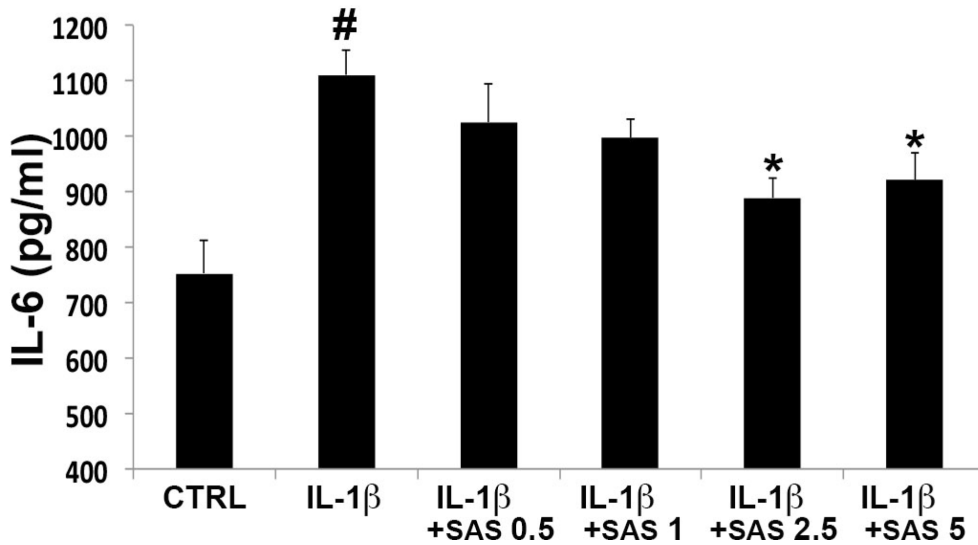

Figure 7. SAS decreases IL-1β-induced protein secretion of IL-6 from ARPE-19 cells. ARPE-19 cells were starved in a serum-free medium overnight and then incubated for 1 h with 0.5, 1, 2.5, or 5 μg/ml of SAS before the addition of 10 ng/ml of IL-1β. After 48 h, the cell supernatants were collected and analyzed for protein secretion of IL-6 using ELISA. # $p<0.01$ increase versus control group, * $p<0.05$ decrease versus IL-1β group. Results shown are mean ± SEM of 3 independent experiments.

These results may indicate other anti-angiogenic and anti-inflammatory mechanisms of activity of SAS in RPE cells beyond αvβ3 inhibition.

We previously showed that the tellurium compound AS101 inhibits IL-6 secretion from LPS-induced macrophages by regulating the NFκB pathway [47]. Importantly, we showed that AS101 inhibits IL-1β-activated inflammation in the human retinal pigment epithelium through the prevention of NFκB activation [38]. Here, we found that SAS suppresses IL-1β-induced IL-6mRNA and protein levels in RPE cells (Figure 6-Figure 7) through the inhibition of the NFκB pathway by reducing phosphorylation of IκB (Figure 8). This result suggests that the NFκB pathway is an additional target through which SAS modulates angiogenic/inflammatory factors. Thiol modulation inhibits the IL-1-mediated activation of an IL-1 receptor-associated protein kinase and NFκB [73,74]. It was further shown that free thiols in the IL-1RI complex are essential for the activation of the IL-1RI-associated protein kinase, and that this process is required for IL-1 signaling leading to NFκB activation. Moreover, based on the evidence that NFκB has a well conserved cysteine

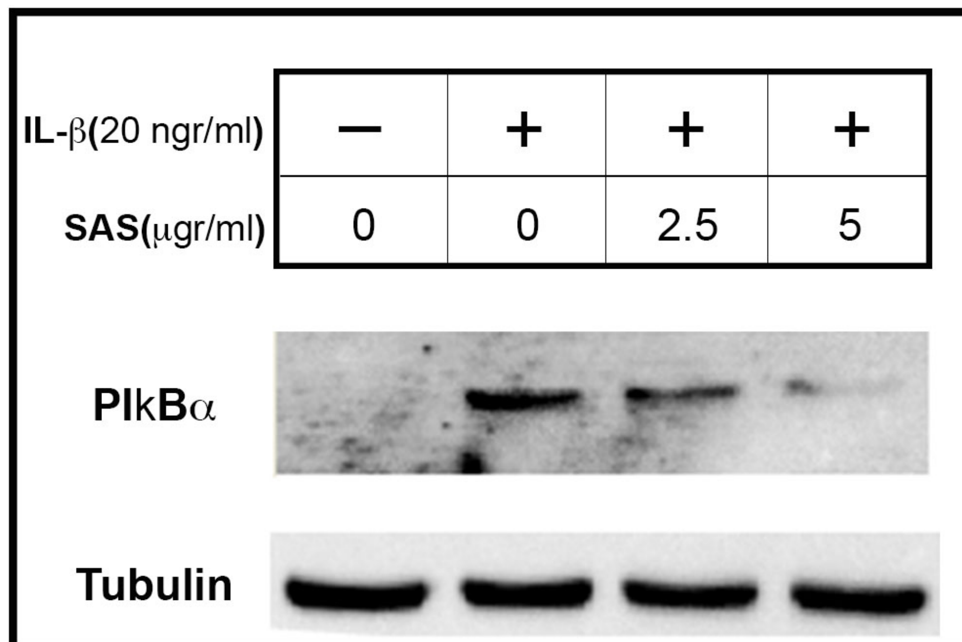

Figure 8. SAS downregulates IL-1β-induced IκBα phosphorylation in ARPE-19 cells. ARPE-19 cells were starved in serum-free medium overnight and then incubated for 1 h with 2.5 or 5 μg/ml of SAS before the addition of 20 ng/ml of IL-1β for 5 min. After 5 min, the cells were lysed as described in the *Materials and methods* section. Proteins were separated by SDS–PAGE and subjected to immunoblotting with antibodies to phospho-IκBα, with tubulin as a control. The data represent one of two independent experiments.

residue in its p50-subunit, modulation of NFκB activity may be performed by redox regulation, in great part through a decrease in DNA-binding activity due to redox-sensitive cysteine residues [75-77]. The Te^{IV} chemistry of SAS and AS101, allowing their interaction with thiols, enables them to inhibit the activity of specific proteins, in which a particular redox status of cysteine is essential for their biologic activity. Therefore, this marked redox potential of SAS may account for the beneficial anti-inflammatory effects of the compound in RPE cells via inhibition of the NFκB pathway.

PEDF is a pleiotropic glycoprotein known to exert a diversity of biologic activities in a context- and cell type-specific manner, each of which is mediated by the specific PEDF receptor. The binding of PEDF to PNPLA2 mediates neuronal survival and differentiation [78]; binding to laminin receptor is involved in the anti-angiogenic function of PEDF [79]. PEDF gene mutations resulting in complete PEDF deficiency lead to a very rare bone disease, osteogenesis imperfecta type VI, indicating a crucial developmental role for this protein [80]. PEDF was found to function as an endogenous anti-angiogenic and anti-inflammatory factor in the eye. Retinas of PEDF-deficient mice demonstrate increased microvessel density [81], whereas genetic overexpression of PEDF in mice significantly inhibits retinal neovascularization in oxygen-induced retinopathy [82]. Intravitreal injection of PEDF significantly reduces vascular hyperpermeability in rat models of diabetes- and oxygen-induced retinopathy, correlating with the decreased levels of retinal inflammatory factors, including VEGF, VEGF receptor-2, MCP-1, TNF-α, and ICAM-1. In cultured retinal capillary endothelial cells, PEDF significantly decreases TNF-α and ICAM-1 expression under hypoxia [72]. Importantly, it was recently shown that PEDF may suppress the signaling mediated by IL-1β [83], and that IL-1β dramatically downregulated mRNA and protein levels of PEDF in the HRPE cells [84]. Thus, SAS may act as an anti-inflammatory agent in experiments involving IL-1β-induced inflammation in RPE cells, at least in part by the upregulation of PEDF.

Tremendous efforts are being invested in the search for novel drugs and mechanisms to inhibit the development of pathological retinal angiogenesis. Most of these efforts are currently focused on novel VEGF pathway inhibitors, which, despite being highly effective, do not provide complete solutions, thus indicating involvement of additional pathways in retinal angiogenesis, which must be addressed by additional therapeutic agents. The results of our previous and current studies indicate that pathological interaction between the EC and RPE layers triggers both pro-angiogenic and pro-inflammatory stimuli, suggesting that a combined therapeutic approach aimed at suppressing both angiogenesis and inflammation, such as a VEGF pathway inhibitor combined with an anti-inflammatory organotellurium compound, may offer improved efficacy as compared to therapies targeting each process separately. Collectively, our results suggest the potential use of the tellurium redox-modulating small molecule compound SAS as a novel anti-inflammatory compound for the treatment of chorioretinal diseases.

APPENDIX 1. STR ANALYSIS.

To access these data, click or select the words "Appendix 1."

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