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Thrombin Stimulates RPE Cell Motility by PKC-ζ- and NF-κB-Dependent Gene Expression of MCP-1 and CINC-1/GRO Chemokines

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ABSTRACT

Retinal pigment epithelial cells (RPE) are the major cell type involved in the pathogenesis of proliferative vitreoretinopathy (PVR), which involves the epithelial-mesenchymal transition, proliferation, and directional migration of transformed RPE cells to the vitreous upon RPE exposure to serum components, thrombin among them. Although the aqueous humor and vitreous of PVR patients contain high levels of chemokines, their possible involvement in PVR development has not been explored. We here analyzed the effect of thrombin on chemokine gene expression and its correlation with RPE cell migration using rat RPE cells in culture as a model system. We demonstrated that thrombin induces RPE cell migration through the dose-dependent stimulation of MCP1 and GRO expression/release, and the autocrine activation of CXCR-2 and CCR-2 chemokine receptors. Whereas inhibition of CXCR2 by Sb-225002 and of CCR2 by Rs-504393 partially prevented hirudin-sensitive cell migration, the joint inhibition of these receptors abolished thrombin effect, suggesting the contribution of distinct but coincident mechanisms. Thrombin effects were not modified by Ro-32-0432 inhibition of conventional/novel PKC isoenzymes or by the MAPkinase pathway inhibitor U0126. MCP1 and GRO expression/secretion, and cell migration were completely prevented by the inhibitory PKC-ζ pseudosubstrate and by the nuclear factor-kappa B (NF-κB) inhibitor BAY11-7082, but not by wortmannin inhibition of PI3K. Results show that signaling pathways leading to RPE cell migration differ from the MEK–ERK–PI3K-mediated promotion RPE of cell proliferation, both of which concur at the activation of PKC-ζ. J. Cell. Biochem. 110: 948–959, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: RPE; THROMBIN; MCP-1; GRO; PAR-1

Proliferative vitreoretinopathy (PVR) is the most common cause of failed surgical repair of rhegmatogenous retinal detachment [Conrath, 2007]. Occurrence of a retinal break induces an initial inflammatory phase accompanied by the breakdown of the blood–ocular barrier. As a consequence of retinal detachment, PVR derives from the trans-differentiation, proliferation, and migration of transformed retinal pigment epithelial (RPE) cells, which form traction-generating cellular membranes in the vitreous and inner and/or outer surfaces of the retina, where contraction of RPE cells causes retinal re-detachment [Campochiaro, 1997].

The serine-protease thrombin has been shown to affect a wide span of processes such as platelet aggregation, endothelial cell activation, proliferation, and cytokine release within diverse cell types [Crook et al., 1992; Yang et al., 1995; Yoshida et al., 2001; Wang et al., 2007]. The generation of thrombin from prothrombin following vascular insult is known to promote blood–retinal barrier breakdown and to be involved in angiogenesis, wound healing, and the development of tumor stroma, among other physiologic and pathological processes. In RPE cells, the expression of prothrombin and its enhancement in inflammatory processes has been recently reported [Rehak et al., 2009]. Moreover, work from Hollborn et al. [2009] demonstrating the expression of different coagulation factors in RPE cells, indicates that these cells may form thrombin from prothrombin via the extrinsic coagulation pathway.

Intracellular thrombin signaling is triggered by the activation of the protease-activated receptors (PARs), a family of G-protein-coupled receptors (GPCRs) activated by proteolytic cleavage of the extracellular NH_2-domain, which unmasks a new sequence that

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functions as an intramolecular ligand. Synthetic ligands corresponding to the cleaved N-terminus can displace the tethered ligand from the binding site, and fully activate PAR-1 in an intermolecular mode [Macfarlane et al., 2001]. To date, four members of the PAR family have been identified, all expressed in a cell-type-specific manner: PAR-1, -3, and -4, activated by thrombin, and the close-related PAR-2, sensitive to trypsin [Coughlin, 2000]. PAR-1 is the prototype of this receptor family, and its cleavage at the Arg41–Ser42 bond by thrombin exposes a fresh 42SFLLRN47 N-terminus that acts as a tethered ligand [Coughlin, 2000]. Recent studies have demonstrated that thrombin involvement in a variety of biological processes such as coagulation, inflammation, chemotaxis, mitogenesis, apoptosis, and angiogenesis, are mediated by PAR-1 [Ramachandran and Hollenberg, 2008]. Among this wide range of effects, thrombin has been shown to stimulate chemo-attractant chemokine expression in distinct cells capable of directional migration, including RPE cells [Allen et al., 2007].

Chemokines are included in the cytokine superfamily, mainly involved in leukocyte locomotion. Human chemokines have been subdivided in four subfamilies based on the position of either one or two cysteine residues neighboring the N-terminus of the protein: C (lymphotactin), CC (MIP-1α, MCP-1, -2, -3, RANTES, and eotaxin), CXC (IL-8, MIP-2, NAP-2, GRO-α, and IP-10), and CX3C (fraktalkine). The CXC chemokines are classified according to the presence of an ELR motif (Glutamic acid–Leucine–Arginine) preceding CXC at the N-terminus, shown to relate to target specificity [Allen et al., 2007].

Chemokines act through GPCRs in the target cells, which trigger cell migration by dynamical assembly/disassembly of integrin adhesions to the extracellular matrix (ECM) [Li et al., 2005]. The human CC-class chemokine MCP-1 signals through hCCR-2 receptor, whereas the human CXC-class chemokine GRO-α activates hCXCR-1 and hCXCR-2 receptors [Allen et al., 2007]. As for its human counterpart, rat MCP-1 binds to rat CCR-2 receptor, however, as a difference from human, the rat equivalent of GRO-α, CINC-1, binds exclusively to CXCR-2 [Viola and Luster, 2008].

In contrast with the classical two-cell system described within the immune system, an additional autocrine mechanism for chemokine action has been suggested by findings showing that microglia, astrocytes, oligodendrocytes, and neurons both, release and express the cognate receptors for specific chemokines [Bajetto et al., 2001; Banisadr et al., 2003; Miller et al., 2008]. Increased cytokine levels in the aqueous ocular fluid have been observed in retinal vascular diseases and diabetic retinopathy, shown to reflect the level in the vitreous [Noma et al., 2008]. IL-8, MIP-1α, and IP-10 elevation in the aqueous humor from patients suffering from intraocular inflammation has been observed [Abu El-Asrar et al., 2004] and particularly, higher expression of MCP-1 and IP-10 has been detected in the vitreous humor from patients suffering from proliferative diabetic retinopathy and PVR, associated with clinical grades of the disease, suggesting the participation of chemokines in proliferative eye diseases [Capeans et al., 1998; Abu El-Asrar et al., 2006].

RPE cells have been shown to produce the chemokines MCP-1 [Elner et al., 1991], IL-8 [Elner et al., 1990], RANTES [Crane et al., 1998], and SDF-1 [Crane et al., 2000a] upon in vitro stimulation by pro-inflammatory cytokines including interleukin-1 (IL-1), gamma interferon (IFN-γ), and tumor necrosis factor-α (TNF-α) [Viola and Luster, 2008]. However, the involvement of cytokines in the development of PVR under pro-inflammatory conditions which allow the access of thrombin to immune privileged eye tissue due to traumatic injury or surgical procedures which compromise the integrity of the blood–retina barrier (BRB), has not been explored.

Our previous work demonstrated that thrombin activation of PAR-1 induces RPE cell proliferation mediated by PKCζ-dependent ERK1/2 MAPK activation [Palma-Nicolas et al., 2008], suggesting thrombin involvement in the onset of proliferative eye diseases. In order to further support a role for thrombin in PVR pathogenesis, in the present work we investigated the effect of thrombin on the expression of chemokines and the induction of RPE cell migration. Our results show for the first time that thrombin activation of PAR-1 induces RPE cell migration by an autocrine action of CINC-1/GRO and MCP-1 chemokines, under the control of atypical PKC-ζ and nuclear factor-kappa B (NF-κB).

MATERIALS AND METHODS

REAGENTS

All reagents used were cell culture grade. The PAR-1 peptide (Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe), thrombin, the conventional/novel PKC inhibitor Ro32-0432, the NF-κB inhibitor BAY11-7082, the PI3K inhibitor Wortmannin, and the inhibitory PKC-ζ pseudosubstrate (PS) (Myr-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu) were obtained from Calbiochem (San Diego, CA). The CXCR2 inhibitor Sb-225002, the CCR2 inhibitor Rs-504393, and the MEK inhibitor U0126 were purchased from Tocris (Ellisville, MO). PAR-3 (Ser-Phe-Asn-Gly-Gly-Pro) and PAR-4 (Gly-Tyr-Pro-Gly-Lys-Phe) peptide agonists were obtained from Bachem (Torrance, CA). The PLA-2 inhibitor Quinacrine was obtained from Sigma (St. Louis, MO). Serum-free Opti-MEM (Invitrogen, Carlsbad, CA) was used as the standard medium for all assays.

LONG-EVANS RAT RPE CELL CULTURE

Retinal pigment epithelium cells were isolated as previously described [Palma-Nicolas et al., 2008]. Briefly, 8- to 10-day-old Long-Evans rats were anesthetized by inhaled chloroform and sacrificed following the animal care and use guidelines established by our institution. The eyes were enucleated, rinsed in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) containing penicillin (100 U/ml) and streptomycin (100 μg/ml), and incubated for 30 min at 37°C in the presence of dispase (2%, v/v). After removal of the sclera and the choroid, the RPE was detached from the neural retina in calcium- and magnesium-free Hanks'-balanced salt solution (HBSS), and incubated in the presence of trypsin (0.1%) for 5 min at 37°C. Trypsin digestion was stopped by 1:1 dilution with Opti-MEM containing 4% fetal bovine serum (FBS). The dissociated cells were suspended in Opti-MEM containing 4% FBS, and seeded at a density of 50,000 cells/cm^2 in six-well format culture plates. Cell viability (>90%) was assessed by Trypan-blue exclusion.

WOUND HEALING ASSAY

RPE cells were grown at confluence in six-well plates (Costar, Corning, Inc., Washington, DC), in Opti-MEM containing 4% FBS. After serum-deprivation for 24 h, RPE cell monolayers were scratched mechanically with a plastic cell scraper drawing a 1.2 mm line. Scratched monolayers were washed twice with Krebs Ringer Bicarbonate buffer (KRB: 118 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1 mM $NaHPO_4$, 5.6 mM glucose, 25 mM $NaHCO_3$) and incubated with thrombin (2 U/ml) in serum-free Opti-MEM. Four percent FBS in Opti-MEM and serum-free Opti-MEM were added to positive or negative control wells, respectively. After incubation at 37°C (4–24 h), cells were visually examined by phase-contrast microscopy (100×). When indicated, RPE cells were pre-incubated with Sb-225002 (10 μM), Rs-504393 (10 μM), PKC-ζ inhibitor (10 μM), and BAY11-7082 (5 μM). After 1 h of incubation at 37°C, the inhibitors were washed-out, and the cells were stimulated with thrombin (2 U/ml in serum-free Opti-MEM) for 24 h.

TRANS-WELL MIGRATION ASSAYS

Confluent RPE cells grown in Opti-MEM supplemented with 4% FBS were trypsin-detached and re-plated on the upper chamber of a Boyden Transwell migration plate (Falcon™ Multiwell Cell Culture Plates, BD Biosciences, Franklin Lakes, NJ) at 50,000 cell/cm^2. Cells were allowed to attach for 4 h in Opti-MEM (4% FBS), and to migrate through a polycarbonate mesh (8 μm pore size). Cell chemotaxis was induced by the inclusion of Thrombin (2 U/ml), 4% FBS in Opti-MEM or serum-free Opti-MEM in the inferior chamber of the migration plate. When indicated, the lower chamber was filled with filtered (0.22 μm pore) conditioned medium from RPE cultures. Conditioned medium was obtained by incubating 24 h serum-starved RPE cultures with 2 U/ml thrombin (CM-T2) or in the absence of thrombin (CM) for 48 h in serum-free Opti-MEM. After incubation for 12 h at 37°C, non-migrating cells were removed from the upper side of the membrane insert with a sterile cotton swab, and the cells, which migrated to the inferior face of the membrane, were trypsin-detached. Detached cells were incubated at 37°C with the MTS reagent (3-(4,5-dimethyl thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Cell Titer 96, (Promega, Madison, WI), and cell reactivity was read at 490/630 nm in a Microtiter Plate Reader (Opsys MR, Dinex. Technologies, Inc. Chantilly, VA). Cell migration index was defined as the ratio of absorbance for cells migrating with the stimulus/cells migrating in serum-free Opti-MEM. When indicated, the CXCR2 inhibitor Sb-225002, and the CCR2 inhibitor Rs-504393 inhibitors (10 μM) were added to the RPE cell monolayer located in the upper chamber 1 h prior to CM-T2 addition, after allowing cell attachment to the membrane inserts.

RT-PCR ANALYSIS OF CHEMOKINE GENE EXPRESSION

RPE cells were grown at confluence in six-well plates (Costar, Corning, Inc.) in Opti-MEM containing 4% FBS. After serum deprivation for 24 h, cells were stimulated with 2 U/ml thrombin in serum-free Opti-MEM. Negative control wells were kept in serum-free Opti-MEM. After thrombin stimulation for 12–72 h, or PAR agonist (25 μM) for 24 h, cells were harvested and total RNA extraction was carried out by using the TRIzol reagent (Sigma).

cDNA was synthesized from 3 μg of total RNA using Molony Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) and oligo dT_{12-18} primer, following the recommendations of the supplier (Invitrogen). Three microliters of the reverse transcriptase reaction were amplified in a final volume of 20 μl including 1.5 mM $MgCl_2$, 300 μM dNTPs, 0.5 μM of each primer, 1.25 U of Taq DNA polymerase, and the corresponding buffer. The sequence for the primers used are listed in Table I. The thermocycler (Master Cycler Personal, Eppendorf, Hamburg, Germany) program consisted of an initial step at 94°C for 5 min, 28 cycles (each consisting of a denaturation step at 94°C for 30 s, an annealing step at 55°C for 30 s, an extension step at 72°C for 1 min), and a final extension step at 72°C for 5 min. PCR products were resolved by agarose gel electrophoresis (0.8%), and chemokine gene expression relative to β-actin was quantified by optical densitometry (Fluor-S, Multi-Imager System, and Quantity One Software, BioRad, Hercules, CA). When indicated, RPE cells were pre-incubated with PKC-ζ inhibitor (10 μM), U0126 (20 μM), Ro32-0432 (20 μM), BAY11-7082 (5 μM), or wortmannin (1 μM). After 1 h of incubation at 37°C, the inhibitors were washed-out, and the cells were stimulated with thrombin (2 U/ml in serum-free Opti-MEM) for 24 h.

CHEMOKINE QUANTIFICATION

RPE cells were grown at confluence in Opti-MEM containing 4% FBS, in six-well plates (Costar, Corning, Inc.). Following serum deprivation for 24 h, cells were stimulated for 24–72 h with 2 U/ml thrombin, or PAR agonist (25 μM) for 48 h in serum-free Opti-MEM. Negative control wells were kept in serum-free Opti-MEM. After thrombin or PAR stimulation, supernatants were collected and stored at −20°C until chemokine quantification by ELISA.

CINC-1/GRO and MCP-1 chemokine release, was quantified using the Quantikine™ ELISA assay and the Rat MCP-1 ELISA kit, following the manufacturer's instructions (R&D Systems, Minneapolis, MN, and Thermo Scientific, Rockford, IL, respectively). Briefly, 50 μl/well of 1:2 serial dilutions of standards or 1:4 dilutions of the culture supernatants were added, and the pre-coated 96-well ELISA plates were incubated for 2 h at 25°C. Plates were rinsed with 0.05% PBS–Tween-20, and incubated for 2 h at 25°C with a polyclonal

TABLE I. Primers Used in This Study

Gene	Primer sequence	Product (bp)
MCP-1	5′-TGTTGTTCACAGTTGCTGCCTG-3′ 5′-GTGCTGAAGTCCTTAGGGTTGATG-3′	312
IP-10	5′-AGTGCTGCTGTCGTTCTCTGCCTCGTGCTG-3′ 5′-GGGCATGGCACATGCTGAAGAGATTAGTAC-3′	600
CINC-1	5′-GGTGTCCCCAAGTAATGGAGAAAG-3′ 5′-CATAGCCTCTCACACATTTCCTCAC-3′	390
RANTES	5′-CATCCCTCACCGTCATC-3′ 5′-CTTCTCTGGGTTGGCAC-3′	218
SDF-1	5′-GTCAGCCTGAGCTACAGATGC-3′ 5′-CACTTTAATTTCGGGTCAATG-3′	164
CCR2	5′-GTT TGCCTCTCTACCAGGAATC-3′ 5′-CTCCCCAGTAGAAGGGGTAAAT-3′	533
CXCR2	5′-CCGGTGCTTCTGCCCCATGT-3′ 5′-CACCGATGTCTACCTGCTGAACCT-3′	499
CXCR4	5′-ATGTAAGGCTGTGCATATCAT-3′ 5′-GCGCTTCTG GTGGCCCTTGGAGTGTG-3′	382
β-ACTIN	5′-GCTCGTC GTCGACAACGGCT-3′ 5′-CAAACATGATCTGGGTCATCTTCTC-3′	353

antibody against rat CINC-1 or rat MCP-1 conjugated to horseradish peroxidase (HRP-conjugate). Plates were rinsed and incubated with supplied substrate solution for HRP (H_2O_2, and tetramethylbenzidine (TMB) as color reagent). The reaction was stopped with 1.0 M hydrochloric acid, and absorbance was measured at 450 nm in a Microtiter Plate Reader (Opsys MR, Dinex. Technologies, Inc.). Detection limit for CINC-1/GRO assay was 4.0 pg/ml, and 8.0 pg/ml for MCP-1.

WESTERN BLOT ANALYSIS FOR IκB-α

RPE cells from confluent six-well plates were serum-deprived for 24 h and washed three times with RPE-Krebs Ringer Bicarbonate buffer. Cultures were then incubated in thrombin-supplemented serum-free Opti-MEM (2 U/ml) or PAR agonist-1, -3, and -4 (25 μM). At 15 min after stimulation, cells were washed twice with Krebs Ringer bicarbonate Buffer, and disrupted in lysis buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.1% SDS, 1%, Triton X-100, 1% CHAPS, 0.5% NP40, 0.1% BSA, 40 mM β-glycerophosphate, 10 mM sodium pyrophosphate, and a protease inhibitor cocktail (10%; Sigma). Proteins in total cell lysates (30 μg) were resolved by 6–12% SDS–PAGE and electro-transferred to PVDF membranes.

After blocking for 60 min at room temperature (25°C) with 4% non-fat milk, the PVDF membranes were probed overnight at 4°C with the following primary antibodies: 1:1,000 rabbit anti-IκB-α (90 mg/ml), Calbiochem; and 1:5,000 mouse anti-actin (clone C4), Chemicon (Temecula, CA). Secondary HRP-conjugated antibodies, raised in the corresponding species, were used at the same dilution as primaries and developed using the Immobilon Western AP Chemiluminescent Substrate (Millipore, Billerica, MA). Kodak® film images were digitized using an Alpha Digi-Doc system (Alpha-Innotech, San Leandro, CA), and densitometric analysis was performed using the Quantity One Software v4.6 from BioRad.

STATISTICAL ANALYSIS

Raw data for analysis were obtained from pooled RPE cells of 10–15 Long Evans Rats in three independent experiments. One-way ANOVA and Bonferroni post hoc test was applied for statistical analysis, using the Prism v4.0 program from GraphPad (La Jolla, CA). Statistical significance was depicted in graphs as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

RESULTS

THROMBIN INDUCES RPE CELL MIGRATION

We analyzed the effect of thrombin on RPE cell migration in monolayers of 24 h serum-deprived RPE cultures, using an in vitro scratch assay. Following the addition of 2 U/ml thrombin or 4% FBS (positive control) for 24 h at 37°C, visual inspection under light phase-contrast microscopy (100×) revealed that thrombin-treated cells undergo cell proliferation/migration to fill the gap in scratched monolayers (Fig. 1b), to an extent comparable to that induced by 4% FBS (positive control, Fig. 1c), whereas in negative control wells incubated in serum-free Opti-MEM, the scratched area remained cell-free up to 24 h incubation (Fig. 1a).

THROMBIN INDUCES CELL MIGRATION BY RELEASING A CHEMOTACTIC COMPOUND

In order to exclude cell proliferation contribution to the effect observed in the wound-healing assay, cell migration was determined in a Boyden chamber transwell system. RPE cells in the upper chamber were maintained in serum-free Opti-MEM but stimulated to migrate into the lower chamber by inclusion of medium containing 4% FBS or 2 U/ml thrombin. Cells were allowed to migrate into the lower chamber for 12 h at 37°C. Migration index was calculated by quantifying the reactivity of migrated cells to MTS reagent, as described in the Materials and Methods Section. Results showed that thrombin itself was unable to induce cell migration; however, when conditioned media from cultures pre-treated with 2 U/ml thrombin for 48 h (CM-T2) was included in the lower chamber, cell migration was in the same range as that induced by 4% FBS (Fig. 1d). Although conditioned medium from cultures incubated for 48 h in the absence of thrombin (CM) promoted RPE cell migration, this effect was significantly lower than for conditioned medium from thrombin-stimulated cultures. These results indicate that thrombin induces RPE cell migration indirectly, through the release of chemotactic factors into the culture media.

THROMBIN UP-REGULATES THE EXPRESSION OF MCP-1 AND CINC-1/GRO IN RPE CELLS

To determine if thrombin stimulates chemokine gene transcription in RPE cells, the time course for MCP-1, IP-10, CINC-1/GRO, RANTES, and SDF-1 mRNA expression was determined using the primers listed in Table I. mRNA levels were semi-quantified following 12–72 h stimulation with 2 U/ml thrombin. Figure 2a shows that thrombin up-regulates MCP-1 mRNA expression. Consistent with this result chemokine quantification by ELISA from culture supernatants at same time points showed that chemokine secretion was up-regulated by fivefold (19,500 ± 500 pg/ml). These effects were prevented by the thrombin specific blocker hirudin (Fig. 4). By contrast, the expression of mRNA for IP-10 and RANTES was negligible or absent, respectively, whereas SDF-1 mRNA was constitutively expressed throughout the 72h time-course assay (not shown). In order to identify the receptor subtype responsible for thrombin effect, cells were stimulated for 24 h with PAR-specific agonists. Figure 2c,d shows that treatment with PAR-1 agonist peptide up-regulates MCP-1 gene expression and secretion, whereas peptides mimicking the putative tethered ligand sequence of PAR-3, or PAR-4 had no effect.

In addition to MCP-1 chemokine up-regulation by PAR-1 cleavage, thrombin stimulated the mRNA expression and secretion of CINC-1/GRO chemokine (Fig. 3) by twofold (to 680 ± 20 pg/ml) following 72 h treatment. An unexpected result from this analysis was the finding that, in contrast with MCP1, thrombin up-regulation of gene expression and release of CINC-1/GRO was not mimicked by any of the two available PAR-1 agonists (SFFLRN, or SFLLRNPNDKYEPF) alone or together with the PAR-3 agonist peptide; the same lack of effect was obtained for the PAR-2 agonist (not shown).

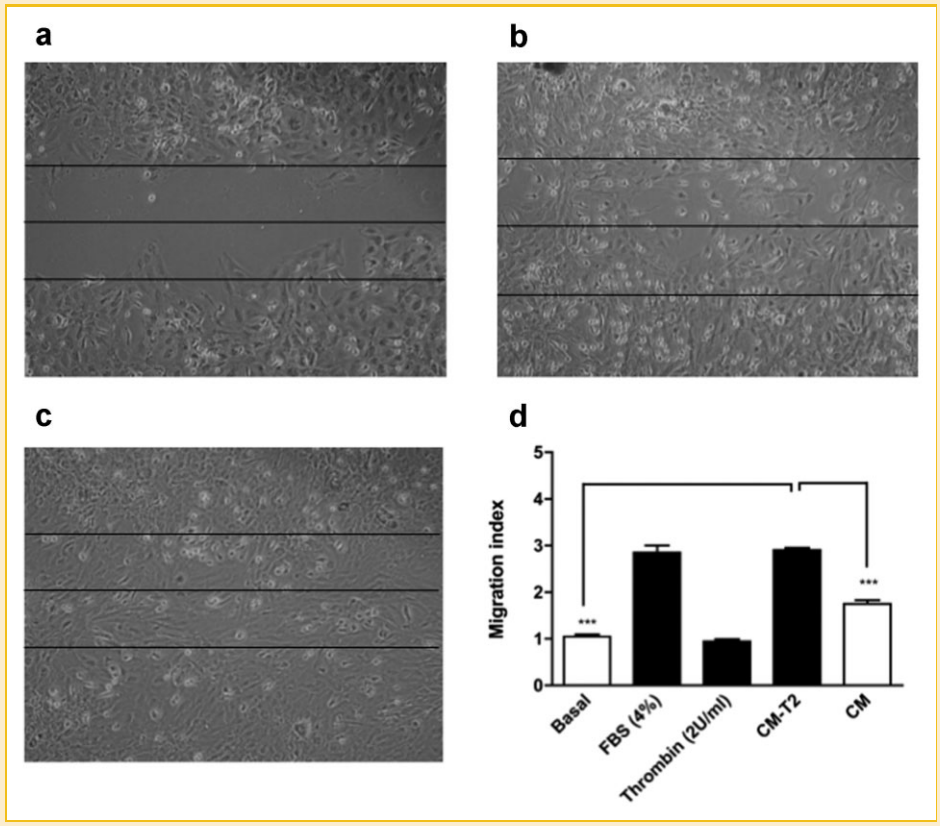

Fig. 1. Thrombin induces RPE cell migration by releasing a chemotactic compound. Confluent rat RPE cell cultures were serum-starved for 24 h, and in vitro "scratch" wounds were created by scraping a 1.2 mm line with a sterile plastic policeman. Cells at the same spot were photographed by phase-contrast microscopy at 100× magnification following 24 h in the presence of thrombin. a: Cultures in serum-free Opti-MEM were used as negative control. b: The addition of thrombin (2 U/ml) promoted cell migration into the wounded area. c: As a positive control, 4% fetal bovine serum was added, and cell migration following 24 h is depicted. External solid lines indicate the original margin of the scratched area. d: For Boyden chamber assays, RPE cells were seeded into the upper chamber of a transwell plate, as described in the Materials and Methods Section. Migration was induced by inclusion in the lower chamber of either 2 U/ml thrombin in serum-free Opti-MEM, conditioned medium from cultures maintained in the absence of thrombin (CM), or conditioned medium from cultures stimulated with 2 U/ml thrombin for 24 h (CM-T2). For positive and negative controls, medium containing 4% FBS or serum-free Opti-MEM, respectively, were included in the inferior chamber of the plate. After 12 h, cells that migrated through the filter were trypsin-detached from the inferior face of the membrane insert (pore size 8 μm) and the migration index relative to basal migration was calculated using the MTS reagent, as described in the Materials and Methods Section. Results are the mean ± SEM of three independent experiments performed in triplicate; one-way ANOVA plus Bonferroni post hoc test was depicted as: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

INHIBITION OF CCR AND CXCR2 CHEMOKINE RECEPTORS PREVENTS THROMBIN-INDUCED RPE CELL MIGRATION

In order to determine if RPE cell migration is a consequence of MCP-1/CCR-2 and CINC-1/CXCR-2 signaling, we blocked the activation of CCR-2 using Rs-504393 and the activation of CXCR-2 receptor using Sb-225002 both, in the in vitro wound healing and the transwell migration assays.

As depicted in Figure 5a–d, although each of these inhibitors significantly prevented thrombin-induced migration, only the joint addition of Rs-50439 and Sb-225002 completely inhibited migration (Fig. 5e). These results demonstrate that thrombin induces RPE cell migration by an autocrine loop involving MCP-1/CCR-2 and CINC-1/CXCR-2 signaling.

THROMBIN-INDUCED MCP-1 AND CINC-1/GRO EXPRESSION DEPENDS ON PKC-ζ ACTIVITY

We next inhibited key isoenzymes known to be coupled to PAR-1 activation in order to identify the downstream signaling pathway leading to the up-regulation of MCP-1 and CINC-1/GRO in response to thrombin. Results showed that neither the inhibition of MEK by U0126 (20 μM) nor that of conventional/novel (c/n) PKC isoforms by Ro32-0432 (20 μM) impaired thrombin-induced up-regulation of chemokines, but the specific PKC-ζ peptide inhibitor (PS; 25 μM) fully prevented thrombin-induced mRNA expression of MCP-1 (Fig. 6a) and CINC-1/GRO (Fig. 6b), indicating that a pathway different from ERK1/2 and c/n PKC, but dependent on PKC-ζ activity is involved in the migratory response of RPE cells to thrombin.

THROMBIN-INDUCED CHEMOKINE EXPRESSION DEPENDS ON IκB-α/NF-κB ACTIVATION

The interaction of the transcription factor NF-κB with cytokine gene promoters has been shown to play an essential role in the control of inflammatory cytokine synthesis [Karin and Lin, 2002; Hayden et al., 2006]. We investigated if thrombin-induced promotion of PKCζ-dependent MCP-1 and CINC-1/GRO transcription is related to

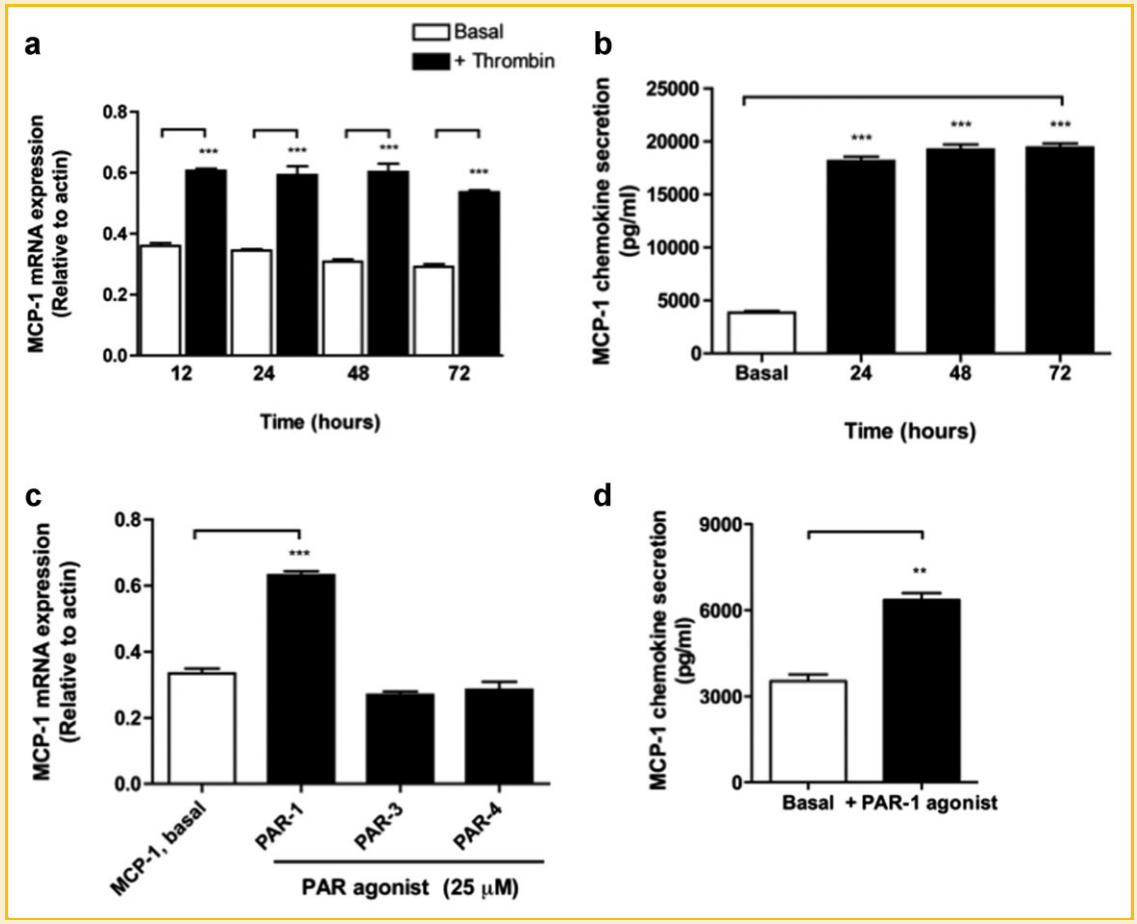

Fig. 2. Thrombin up-regulates the expression/secretion of MCP-1 chemokine in RPE cells. Confluent monolayers of RPE cells were serum-starved for 24 h and then stimulated with 2 U/ml thrombin (a,b) or 25 μM PAR agonists (c,d). Cells were harvested in Trizol for RNA isolation at the indicated time points (a) or at 24 h post-stimulation with PAR agonists (c). The expression of mRNA for MCP-1 was quantified by RT-PCR, as described in the Materials and Methods Section. MCP-1 chemokine secretion was determined by ELISA in culture supernatants collected at equivalent time points after stimulation with thrombin (b) or PAR-1 agonist (d). Results are expressed as the mean ± SEM of three independent experiments; one-way ANOVA plus Bonferroni post hoc test was depicted as: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Basal (white bars) indicates the mRNA expression or chemokine secretion in the absence of thrombin.

NF-κB activation, by measuring IκB-α degradation. Our results showed that thrombin acting through PAR-1 (but not PAR-3 or -4) promotes IκB-α phosphorylation/degradation, the first step in the activation of the canonical NF-κB signaling pathway (Fig. 7a). Also we showed that the inhibition of IκB-α phosphorylation/degradation by BAY11-7082 completely prevented thrombin-induced MCP-1 (Fig. 7b) and CINC-1/GRO (Fig. 7c) mRNA expression. Moreover, treatment of RPE cells with either BAY11-7082 or the PKC-ζ inhibitor-PS prior to stimulation inhibited thrombin-induced RPE cell migration in a wound-healing assay (Fig. 8).

DISCUSSION

The directional migration of RPE cells into the vitreous is a major feature in the pathogenesis of PVR which in ~90% of cases develops as a consequence of ocular trauma involving recurrent rhegmatogenous damage and the exposure of eye immunologically privileged environment to serum pro-inflammatory components, thrombin among them [Campochiaro, 1997]. In previous work, we showed that thrombin induces RPE cell proliferation through ERK1/2 signaling triggered by the joint activation of PLC-β/cPKC and PKC-ζ [Palma-Nicolas et al., 2008]. We here analyzed the effect of thrombin on RPE cell migration, in order to further support its possible involvement in PVR pathogenesis.

Results from the present study demonstrate that thrombin promotes the migration of RPE cells by an indirect mechanism mediated through an increase in MCP-1 and CINC-1/GRO gene expression and chemokine release, followed by the interaction of these chemokines with their specific receptors CCR2 and CXCR2 in an autocrine fashion, since conditioned media from thrombin-treated cultures induced cell migration whereas thrombin itself had no effect. On this matter, whereas a direct chemotactic effect of thrombin on cell migration has been suggested [Karp et al., 2005; Kaufmann et al., 2007], thrombin has also been shown to induce the migration of different cell types indirectly, by promoting the release

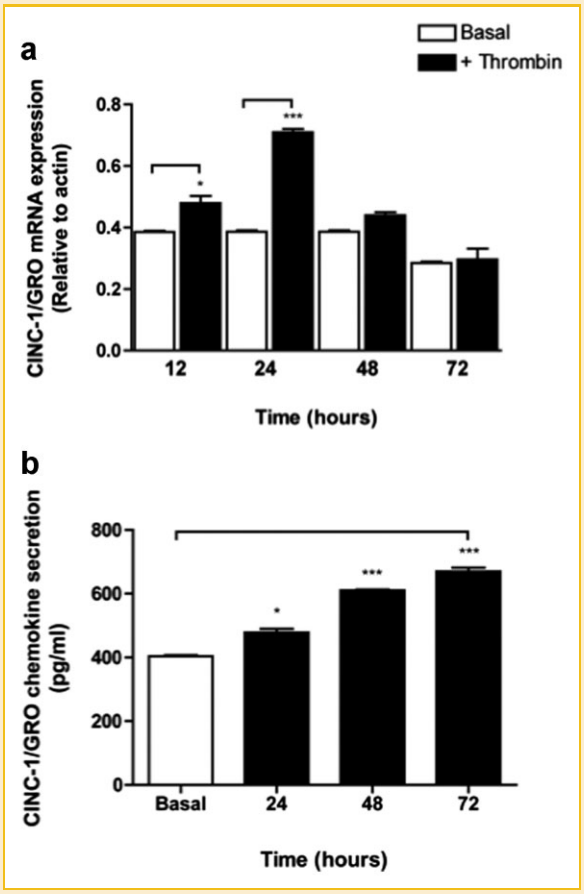

Fig. 3. Thrombin up-regulates the expression/secretion of CINC-1/GRO chemokine in RPE cells. Confluent monolayers of RPE cells were serum-starved for 24 h and then stimulated with 2 U/ml thrombin. a: Cells were harvested in Trizol for RNA isolation at the indicated time points and the expression of mRNA for CINC-1/GRO was quantified by RT-PCR, as described in the Materials and Methods Section. b: CINC-1/GRO secretion was determined by ELISA in culture supernatants collected at equivalent time points after stimulation. Results are expressed as the mean ± SEM of three independent experiments; one-way ANOVA plus Bonferroni post hoc test was depicted as: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Basal (white bars) indicates the mRNA expression or chemokine secretion in the absence of thrombin.

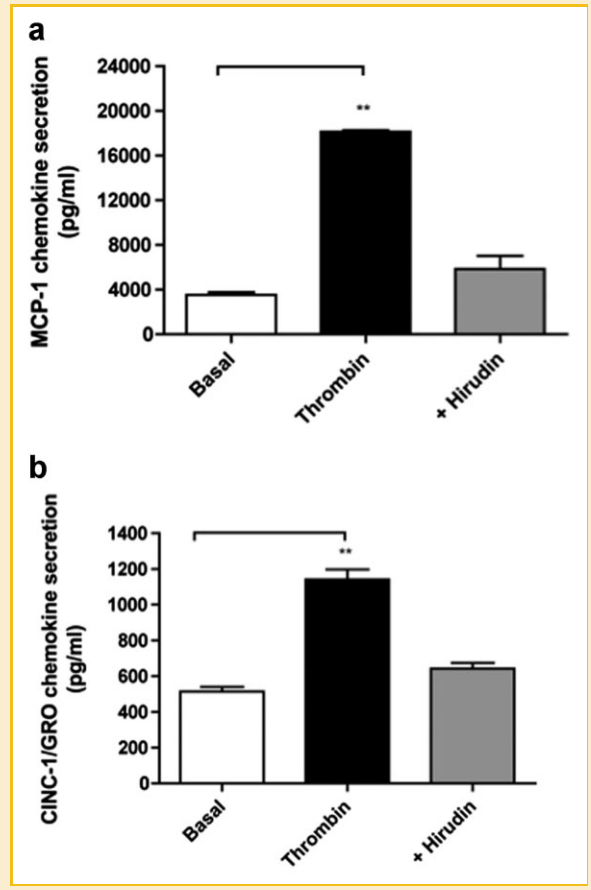

Fig. 4. Thrombin-induced chemokine gene expression is prevented by hirudin. Confluent monolayers of RPE cells were serum-starved for 24 h and then stimulated with 2 U/ml thrombin or equivalent 1:1 thrombin/hirudin neutralized complex. MCP-1 and CINC-1/GRO chemokine secretion was determined by ELISA in culture supernatants after 48 h of stimulation. Results are expressed as the mean ± SEM of three independent experiments; one-way ANOVA plus Bonferroni post hoc test was depicted as: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Basal (white bars) indicates the chemokine secretion in the absence of thrombin.

of growth factors such as FGF-1 and FGF-2, (basic fibroblast growth factors 1 and 2) [Cao et al., 2006; Duarte et al., 2006], in agreement with our present findings. This controversy may be explained by recent evidence from studies in the CNS showing that chemokines are selectively expressed according to specific developmental or repair stages, indicating that the induction of migration by chemokines may be involved in physiological conditions requiring tissue remodeling, in addition to their role in pathological proliferative conditions [Miller et al., 2008].

We showed that, among the chemokines known to be expressed by RPE [Crane et al., 1998, 2000b; Elner et al., 2006], thrombin stimulation specifically up-regulates MCP-1 and CINC-1/GRO mRNA expression and subsequent release (Figs. 2 and 3). RPE cells have been shown to express the thrombin-activated receptors PAR-1 and -3, but not PAR-4 [Hollborn et al., 2009; Parrales et al., 2010]. By stimulation of the cells with specific PAR agonists, we demonstrated that thrombin effect on MCP1 expression/secretion is mediated by PAR-1 receptor. In contrast, PAR-1 agonist failed to increase GRO expression/secretion, indicating a different regulatory mechanism for both chemokines. Among alternative mechanisms, PAR-1 signaling in endothelial cells has been shown to require PAR-1/PAR-3 dimerization [McLaughlin et al., 2007]. Also, the interaction of the tethered ligand domain of PAR-1 with uncleaved PAR-2 to transduce the signal has been proposed [O'Brien et al., 2000]. Since PAR-2 has been shown to induce GRO/CINC-1 release from rat astrocytes [Wang et al., 2007], we tested the effect of PAR-2 agonist on RPE cells, and found that neither PAR-2 agonist nor the joint addition of PAR-1 and -3 agonists increased GRO gene transcription and secretion (not shown). Although the mechanism involved in thrombin effect on CINC-1/GRO remains to be established, the inhibition of thrombin actions by hirudin (Fig. 4) rules-out a non-specific effect being responsible for this outcome.

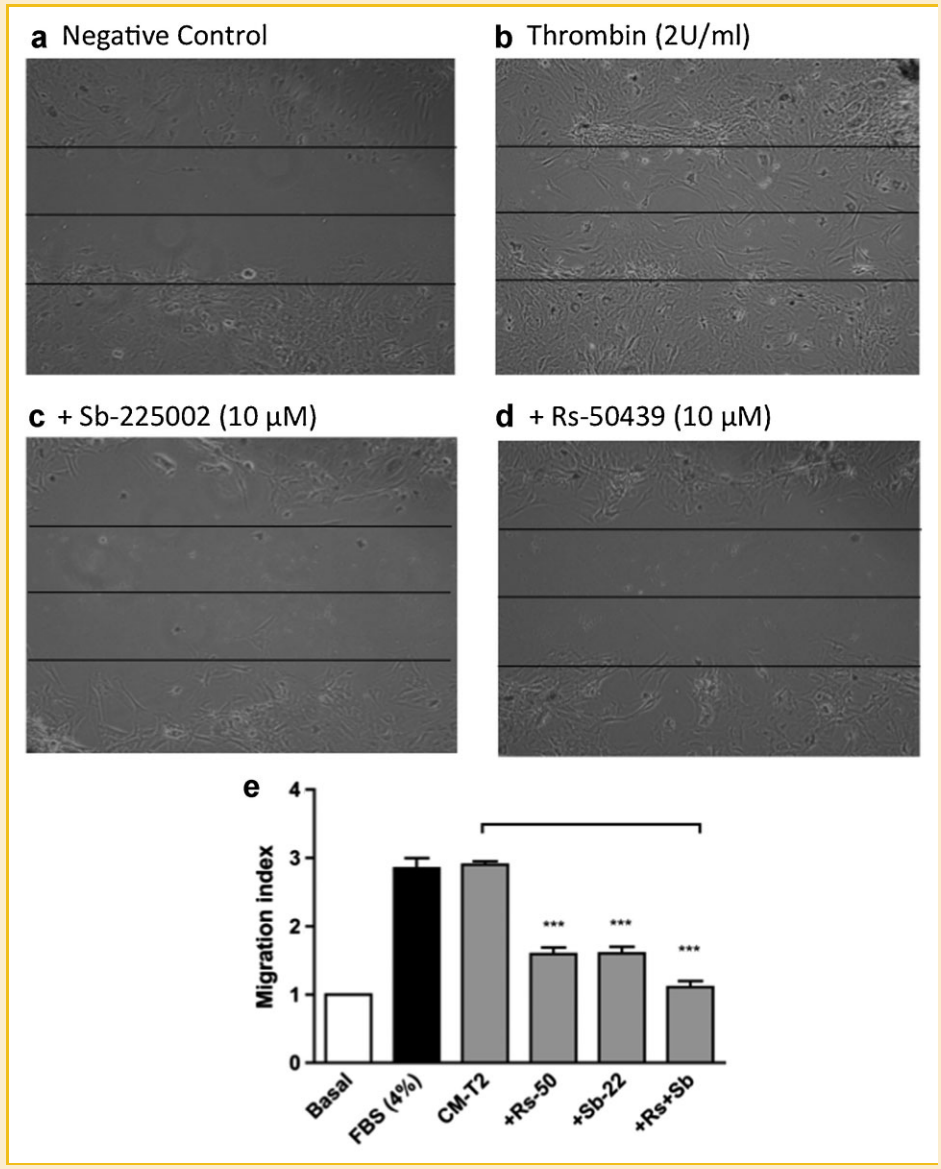

Fig. 5. Inhibition of CCR2 and CXCR2 chemokine receptors prevents thrombin-induced RPE cell migration. Confluent RPE cell cultures were serum-starved for 24 h, and mechanically scratched, as described for Figure 1. a: Cells were kept in serum-free Opti-MEM as negative control; (b–d) cells were incubated in the presence of 5 μM CXCR2 inhibitor Sb-225002 or 5 μM CCR2 inhibitor Rs-504393 for 1 h prior to the addition of 2 U/ml thrombin for 24 h. Photographs from the cultures at 100× magnification were obtained 24 h after wound infliction. External solid lines indicate the original margin of the scratch. e: Cell migration was quantified in transwell assays, as described for Figure 1d and the Materials and Methods Section. The lower well contained: 4% FBS in Opti-MEM as positive control (black bar), serum-free Opti-MEM as negative control (white bar), or conditioned media (CMT2, gray bars). Five millimolars of Rs-504393 (Rs-50) and Sb-225002 (Sb-22) were added 1 h prior to stimulation with conditioned media (CM-T2). Cell migration was quantified as described for Figure 1d. Results are the mean ± SEM of three independent experiments; one-way ANOVA plus Bonferroni post hoc test was depicted as: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

An autocrine loop involving chemokine/chemokine-receptor expression has been recently reported for GRO-α/CXCR-2 [Wang et al., 2006], and SDF-1/CXCR-4 signaling in cancer cells [Uchida et al., 2007; Barbieri et al., 2008]. We demonstrated that CCR-2 (the MCP-1 receptor) and CXCR-2 (the CINC-1/GRO receptor) are expressed by RPE cells and furthermore, that their corresponding specific inhibitors Rs-504393 and Sb-225002 partially inhibited thrombin-induced RPE cell migration. Since the simultaneous inhibition of both receptors completely prevented migration, our results suggest that these two chemokines activate distinct intracellular pathways which converge in cell migration through a functional MCP-1/CCR-2 and CINC-1/CXCR-2 signaling axis.

The activation of PARs by thrombin has been linked to diverse physiologic responses involving distinct signaling pathways, among them PKC and MAPK [Ramachandran and Hollenberg, 2008]. In contrast with IL-8/CXCL8 release from fibroblasts, mediated by PAR-induced activation of conventional PKC isoforms [Jordan et al., 1996], our present results show that thrombin stimulation of

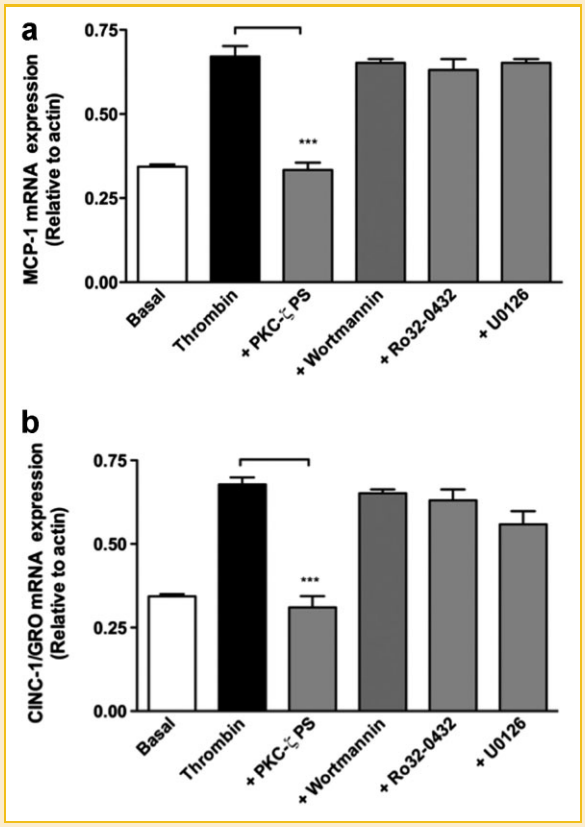

Fig. 6. PI3K-independent PKC-ζ activation is required for thrombin-induced chemokine gene expression. Confluent monolayers of RPE cells were serum-starved for 24 h and then stimulated with thrombin (2 U/ml). At 24 h post-stimulation cells were harvested in Trizol for RNA isolation and quantification of mRNA for MCP-1 (a) and CINC-1/GRO (b) by RT-PCR. When indicated, PKC-ζ pseudosubstrate (25 μM), wortmannin (1 μM), the classic/novel PKC inhibitor Ro32-0432 (20 μM), or MEK/ERK inhibitor U0126 (20 μM) were added to the culture 1 h prior to thrombin stimulation. Results are the mean ± SEM of three independent experiments; one-way ANOVA plus Bonferroni post hoc test was depicted as: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Basal (white bar) indicates the mRNA expression in the absence of thrombin.

chemokine gene expression/secretion and cell migration is independent from classic/novel PKC and MAPK ERK1/2 activity, since the pharmacological inhibition of these pathways by Ro32-0432 and U0126, respectively, did not modify thrombin effect. In contrast, thrombin-induced MCP-1 and CINC-1/GRO expression and cell migration were prevented by the inhibitory peptide PS of atypical PKC-ζ. Surprisingly, the inhibition of the classical activator of PKC-ζ PI3K by wortmannin did not inhibit thrombin effect (Fig. 6), indicating a distinct signaling mechanism as responsible for the activation of PKC-ζ. In this regard, the identification of a protein–protein interaction module named PB1 in atypical PKCs, suggests that the formation of PKC-ζ complexes with different adapters and scaffold proteins could confer distinct specificity to the actions of PKC-ζ [Moscat et al., 2009], in agreement with PI3K/PKC-ζ control of RPE cell proliferation through the MEK-ERK1/2 pathway [Palma-Nicolas et al., 2008] and the regulation of chemokine gene expression and cell migration through PI3K-independent signaling, as shown here.

Clinical trials have shown that NF-κB mRNA expression and protein levels are significantly increased in patients suffering from PVR compared to controls [Harada et al., 2004], suggesting that this transcription factor could be involved in the development of the pathology. Additionally, thrombin has been shown to activate distinct signaling pathways leading to NF-κB activation [Barnes and Karin, 1997]. In resting cells, NF-κB is maintained in the cytoplasm through its interaction with inhibitory IκB-α, which masks NF-κB nuclear localization signals and DNA-binding domains. Upon stimulation, IκB protein subunits are phosphorylated by IκB kinases (IKKs) and rapidly degraded through the proteosome. This process releases NF-κB, allowing its translocation to the nucleus, where it binds to DNA promoters and activates transcription [Karin and Ben-Neriah, 2000].

Molecular studies of MCP-1 and GRO-α gene promoters have revealed the existence of a single NF-κB binding site with high affinity for p65 in the human Gro-α promoter [Anisowicz et al., 1991] whereas two NF-κB binding sites are present in the human MCP-1 promoter [Bian et al., 2004]: the A2 site which has a high affinity for c-Rel/p65, and the A1 site, shown to bind p50/p65 NF-κB dimers [Ueda et al., 1997]. Evidence has been provided indicating the involvement of PKC-ζ in the control of NF-κB pathway [Leitges et al., 2001]. Importantly, recent findings have shown that PKC-ζ may regulate NF-κB transcriptional activity by the direct phosphorylation of the NF-κB Rel A (p65) subunit at Ser311 once released from IκB-α, or by the activation of IKKβ [Duran et al., 2003; Moscat et al., 2009]. Consistent with these findings, we showed that thrombin induces the degradation of IkB-α, hence activating the canonical NF-κB signaling pathway (Fig. 7a). Moreover, the specific inhibition of NF-κB signaling by BAY11-7082 prevented the PKCζ-dependent transcription of MCP-1 and CINC-1/GRO and the consequent promotion of RPE cell migration (Fig. 8), indicating that NF-κB activation is required for thrombin-induced RPE cell motility. Although the activation of NF-κB by TNF-α has been implicated in IL-8 (the human counterpart of GRO) and MCP-1 gene expression in human RPE cells [Bian et al., 2004], to our knowledge, the activation of NF-κB by thrombin stimulation has not been reported.

Inflammation appears to be important mechanistically in the pathogenesis of PVR, in which RPE, glia, and fibroblast proliferation leading to the formation of epi-retinal membranes follows an initial inflammatory stage [Campochiaro, 1997]. We demonstrated that the exposure of RPE cells to thrombin in vitro induces cell migration through a chemokine-mediated autocrine process. Although the involvement of this mechanism in the migration of RPE cells into the vitreous after traumatic injury in vivo remains to be established, recurrent findings regarding the high levels of chemokines in the vitreous from PVR patients suggest that this could be the case [Capeans et al., 1998; Abu El-Asrar et al., 2004, 2006].

The present findings, together with our previous study [Palma-Nicolas et al., 2008], demonstrate that thrombin induces two well-defined aspects of PVR pathogenesis: the proliferation and migration of RPE cells, and because both of them are under the control of PKC-ζ, this study strongly suggests a central role for thrombin in the pathophysiology of proliferative vitreoretinopathy,

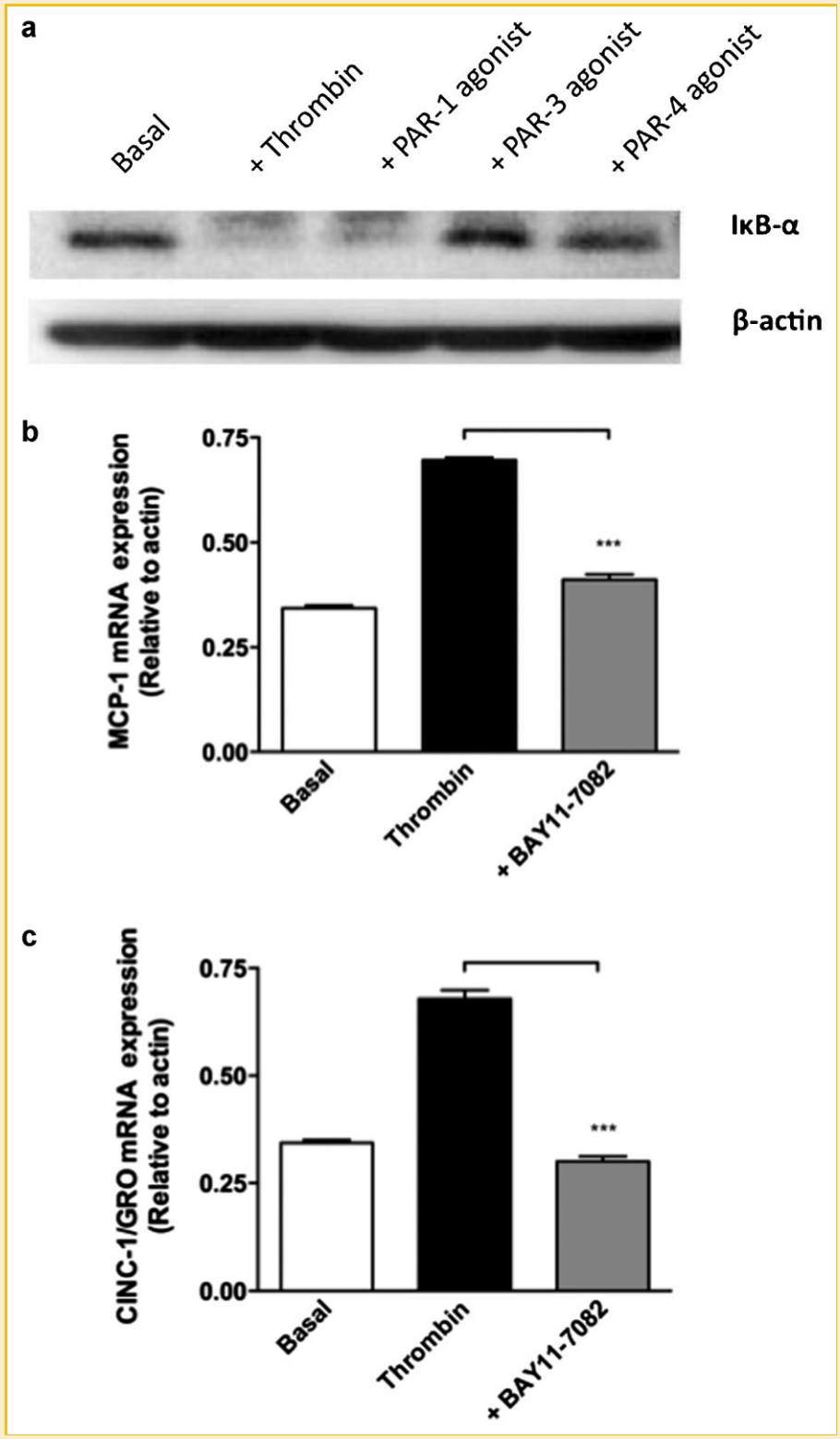

Fig. 7. Thrombin interaction with PAR-1 induces chemokine expression by activating NF-κB. Confluent RPE cultures were serum-deprived for 24 h prior to thrombin (2 U/ml) or PAR-agonist inclusion (25 μM). a: The promotion of IκB-α degradation by thrombin stimulation was measured, as an indication of NF-κB activation. Cell lysates were obtained at 15 min post-stimulation, and a 25 μg sample of total protein was resolved by SDS–PAGE. PVDF membranes were probed with: 1:1,000 rabbit anti-IκB-α (C21) and 1:5,000 mouse anti-β actin (clone C4). The gel shows a representative experiment from three independent determinations. b,c: Cells were harvested in Trizol for mRNA extraction and chemokine gene expression quantification by RT-PCR after 24 h of thrombin stimulation. When indicated, the NF-κB inhibitor BAY11-7082 (5 μM) was added to cultures 1 h prior to thrombin stimulation. Results are the mean ± SEM of three independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Basal (white bars) indicates the mRNA expression in the absence of thrombin.

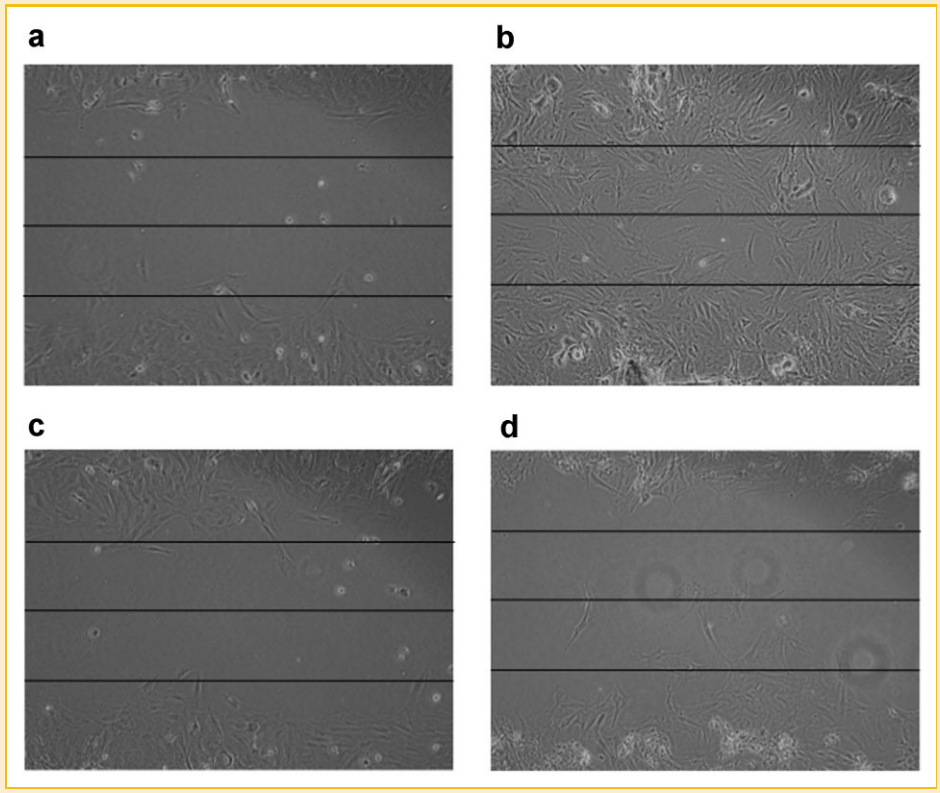

Fig. 8. Inhibition of PKC-ζ or NF-κB prevents thrombin-induced RPE cell migration. Confluent rat RPE cell cultures were serum-starved for 24 h, and in vitro "scratch" wounds were created as described for Figure 1. Cells at the same spot were photographed by phase-contrast microscopy at 100× magnification following 24 h in the presence of thrombin. a: Cultures in serum-free Opti-MEM were used as negative control. b: The addition of thrombin (2 U/ml) promoted cell migration into the wounded area. c: Cells were incubated in the presence of the NF-κB inhibitor BAY11-7082 (5 μM) or (d) the PKC-ζ PS inhibitor peptide (25 μM) for 1 h, and then stimulated with 2 U/ml thrombin for 24 h and photographed at 100× magnification. External solid lines indicate the original margin of the scratch.

and support this enzyme as a pharmacological target for the prevention of PVR.

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