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Research Article

The protease-activated receptor-3 (PAR-3) can signal autonomously to induce interleukin-8 release

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Abstract. Protease-activated receptors (PARs) play a clear role in the burst of inflammatory reactions and immune responses. However, for PAR-3, the most elusive member of the PAR family, the functional role is still largely unclear. It has been claimed that PAR-3 does not signal autonomously, although the wide expression of human PAR-3 indicates its important physiological roles. We demonstrate that in HEK-293 cells, stably transfected with human PAR-3, thrombin induced calcium signaling, IL-8 gene expression and IL-8 release. We confirmed this finding using human lung epithelial and human astrocytoma cells that express endogenous PAR-3. Moreover, thrombin exposure of HEK-293 cells resulted in ERK1/2 activation coinciding with IL-8 release. The effects of thrombin were not dependent on PAR-1 activation, as confirmed by PAR-1 gene silencing. Thus, we propose that PAR-3 is able to signal autonomously to induce IL-8 release mediated by ERK1/2 phosphorylation, which contributes actively to inflammatory responses.

Keywords. Protease-activated receptors, epithelial cells, interleukin-8, inflammatory mediators.

Introduction

Protease-activated receptors (PARs) belong to a subfamily of proteins with seven transmembrane domains, the G protein-coupled receptors, which are activated *via* proteolytic cleavage by serine proteases. There are four types of PARs, named in the chronological order of their discovery as PAR-1, -2, -3, and -4. Three of them (PAR-1, -3 and -4) are thrombin receptors, whereas the fourth one, PAR-2, is not activated by thrombin but by other proteases such as trypsin, tryptase and factor Xa. The role of PARs has been extensively investigated, and it has become clear that PARs, besides other functions, constitute part of the body's defense system, and that they actively participate in the development of inflammation through inducing the release of pro- and anti-inflammatory mediators [1–3].

However, the knowledge about PAR-3 signaling and its functional role, in contrast to the other members of PAR family, is still unclear and confined to relatively few findings that have been published until now. A specific feature of this receptor is its different species-specific susceptibility and consequently physiological function in mouse and human. The responsiveness of human PAR-3 to thrombin was confirmed in heterologous expression systems, in COS7 cells and *Xenopus* oocytes [4]. However, the experiments on murine PAR-3, overexpressed in COS7 cells as well as platelets from PAR-4-deficient mouse, demonstrated that murine PAR-3 itself does not mediate intra-

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cellular signaling but serves as a cofactor for PAR-4 cleavage [5, 6]. Additionally, the PAR-3 expression displays different patterns in these two species, human and mouse, further indicating differences in the functional role of PAR-3. Particularly platelets, where thrombin is a potent activator, represent a good model of PAR-3 signaling. Consistently, human and mouse platelets display distinct PAR-3 expression as well as signaling. Murine PAR-3 is necessary for complete thrombin-mediated platelet activation; however, its role seems to be restricted to act as cofactor for PAR-4 [7, 8], whereas, in human platelets, thrombin triggers signaling through the activation of PAR-1 and PAR-4, but not PAR-3 [9, 10].

The presence of a considerably shorter C-terminal tail in PAR-3 (15 amino acids for human PAR-3) than in other PARs (about 40 amino acids) suggests that PAR-3 signaling and desensitization differs from that of other PARs, although there have been no investigations of this aspect available until now. Furthermore, the great obstacle in the extension of studies on PAR-3 signaling is the lack of PAR-3-selective agonists or antagonists. There are studies reporting that synthetic peptides that mimic the putative tethered ligand of human and murine PAR-3 are inactive on PAR-3, but may activate PAR-1 and PAR-2 [11, 12]. Nevertheless, our and other groups previously showed that the PAR-3-activating peptide is able to induce cellular responses in rat astrocytes [13], human smooth muscle cells [14] and Jurkat T cells [15]. Therefore, the issue of selective agonists for PAR-3 remains controversial.

A most recent report discloses interesting findings about the function of endothelial PAR-3 [16]. The authors show that PAR-3 can form heterodimers with PAR-1. These PAR-1/3 dimers are formed constitutively and, upon activation, induce signaling distinct from PAR-1/1 homodimers, involving coupling to $G\alpha_{13}$. PAR-3 functions as an allosteric regulator of PAR-1 signaling, leading to increased endothelial permeability.

However, PAR-3, particularly from human origin, has remained largely unexplored and leaves plenty of questions open. The abundant distribution of this receptor in human tissues indicates the high biological relevance of PAR-3 activation. Therefore, unequivocal establishment of a signaling cascade connected to PAR-3 without cross-talk to other PARs would underline the eminent importance of PAR-3.

In our present study we aimed to answer the question of whether PAR-3, similarly to the other members of the PAR family, is able to participate in inflammatory reactions by mediating secretion of the cytokine IL-8. Many reports showed that activation of PAR-1, PAR-2 and PAR-4 induces the release of IL-8, a potent chemoattractant for neutrophils, that comprises part of the innate immune response [17–24]. However, the putative signaling of PAR-3 and its role in inflammatory reactions is still elusive. Here, we demonstrate that human PAR-3 is able to trigger signals independent from other thrombin receptors. We show that thrombin-mediated PAR-3 activation results in extracellular signal-regulated kinase (ERK)1/2 phosphorylation and increased production of IL-8.

Materials and methods

Materials. Thrombin was purchased from Sigma (Taufkirchen, Germany), Fura-2/AM was from Molecular Probes (MoBiTec, Göttingen, Germany), and the synthetic thrombin receptor agonist peptide (TRag; Ala-pFluoro-Phe-Arg-Cha-homoArg-Tyr-NH_2) was from Neosystems Laboratories (Strasbourg, France). PAR-4-activating peptide (GYPGQV) was from Bachem (Heidelberg, Germany). U0126, SP600125, SB203580 and G418 were from Calbiochem (La Jolla, CA, USA). Human IL-8 enzyme-linked immunosorbent assay (ELISA) kit was from Amersham Biosciences (Freiburg, Germany). Antibodies against PAR-3 (H-103) were from Santa Cruz (Heidelberg, Germany) and anti-myc were from Invitrogen. Anti-phospho-JNK (Thr183/Tyr185), anti-JNK, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-p38 MAPK, anti-phospho-p44/42 MAPK (Thr202/Tyr204) and anti-p44/42 MAPK antibodies were from New England Biolab (Beverly, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were from Dianova (Hamburg, Germany). pcDNA3.1Myc-His was purchased from Invitrogen (Karlsruhe, Germany) and pEGFP-N1 from Clontech. The cell culture medium, Dulbecco's modified Eagle's F-12 1:1 medium, fetal calf serum (FCS), and antibiotics (penicillin and streptomycin) were from Biochrom KG (Berlin, Germany).

Cell culture and transfection. Human embryonic kidney (HEK)-293 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 1:1 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Cells were passaged using Accutase (PAA, Pasching, Austria), to minimize the proteolytic activation of PARs.

HEK-293 cells were transfected with PAR3-green fluorescent protein (GFP), GFP or PAR3-Myc-His (MH) using DOTAP (liposome formulation for transfection of DNA into eukaryotic cells) according to the

manufacturer's protocol (Roche Diagnostics). To generate stable clones, the cells were selected with 500 μg/ml G418 sulfate.

The human bronchial epithelial (HBE) cells, were kindly provided by Prof. Dr. L. Pott (Institut für Physiologie, Ruhr-Universität Bochum, Germany). HBE cells were cultured in DMEM-Hams's F-12 (1:1) supplemented with 50 μg/ml gentamicin, 50 μg/ml kanamycin, 10 μg/ml ITS (insulin, transferrin, selenite), 1 μM hydrocortisone, 3.75 μg/ml pituitary extract, 25 ng/ml EGF, 30 nM T_3 and 10 ng/ml cholera toxin. Human astrocytoma cells, 1321N1, and human airway epithelial cells, A549 cells, were cultured in DMEM supplemented with 10 % FCS and 100 U/ml penicillin and 100 μg/ml streptomycin and kept at 37°C in a humidified atmosphere of 5 % (A549 cells) and 10 % (1321N1 cells) CO_2. HEK-293 cells, A549 cells, and 1321N1 cells were all from ATCC (Wesel, Germany).

The HEK-PAR3-GFP and 1321N1 cells were transfected with small interfering RNA (siRNA) using Magnet Assisted Transfection (MATra-A) reagent for adherent cells according to the manufacturer's protocol (IBA GmbH, Göttingen, Germany). After 48 h of incubation cells were used for experiments.

Cytosolic Ca^{2+} measurement. The free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using the Ca^{2+}-sensitive fluorescent dye fura-2/AM. For dye loading, the cells grown on a coverslip were placed in 1 ml HEPES-buffered saline (NaHBS, containing 20 mM HEPES, pH 7.4, 145 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 25 mM glucose) for 30 min at 37°C, supplemented with 2 μM fura-2/AM. Loaded cells were transferred into a perfusion chamber with a bath volume of about 0.2 ml and mounted on an inverted microscope (Axiovert 135; Zeiss, Jena, Germany). During the experiments the cells were continuously superfused with NaHBS heated to 37°C. Single-cell fluorescence measurements of $[Ca^{2+}]_i$ were performed using an imaging system from T.I.L.L. Photonics GmbH (Munich, Germany). Cells were excited alternately at 340 and 380 nm for 25–75 ms at each wavelength with a rate of 0.33 Hz and the resultant emission was collected above 510 nm. Images were stored on a personal computer, and subsequently the changes in fluorescence ratio (F340 nm/F380 nm) were determined from selected regions of interest covering a single cell.

Real-time RT-PCR analysis. Total RNA was isolated from the cells with the RNeasy Kit (Qiagen, Hilden, Germany). The isolation included DNase treatment. Reverse transcription was carried out with 1 μg of each RNA with iScript™ cDNA synthesis kit (Bio-Rad) in a final volume of 20 μl according to the manufacturer's protocol. Real-time PCR was performed on the iCycler (Bio-Rad) in 25-μl reaction volume using SYBR green PCR Master Mix (Bio-Rad) and the following primers pairs: PAR-1 sense 5′-CGCCTGCTTCAGTCTGTGCGGC-3′ antisense 5′-GGCCAGGTGCAGCATGTACACC-3′; PAR-3 sense 5′-TTGTCAGAGTGGCATGGAA-3′, antisense 5′-TGGCCCGGCACAGGACCTCTC-3′; IL-8 sense 5′-CTAGGACAAGAGCCAGGAAG-3′, antisense 5′-GTGTGGTCCACTCTCAATC-3′; GAPDH sense 5′-TCCAAAATCAAGTGGGGC-GATGCT-3′, antisense 5′-ACCACCTGGTGCT-CAGTGTAGCCC-3′. The usage of intron-flanking primers, additionally to DNase treatment during RNA isolation, excludes the possibility of genomic DNA amplification. The thermal cycling conditions included a denaturation step at 95°C for 3 min, followed by 40 cycles at 94°C for 30 s, 60°C for 90 s, 72°C for 1 min and the final melting curve program with a ramp rate of 0.5°C/s from 60°C to 95°C. Amplification specificity of PCR products was confirmed by melting curve analysis and agarose gel electrophoresis. All mRNA measurements were normalized to the GAPDH mRNA level, which was unchanged in control and treated cells.

IL-8 release. According to the manufacturers' protocol, IL-8 protein levels were determined using human IL-8 ELISA kits. Briefly, serum-starved cells were stimulated with TRag or thrombin for 6 h and then the supernatant was collected for ELISA analysis. Absorbance for IL-8 was measured at 450 nm. For inhibitor studies, cells were pre-treated with the inhibitors for 30 min prior to stimulation with thrombin. Cells that were treated only with thrombin, without pre-treatment with inhibitors, served as baseline (100 %).

Western blot. Serum-starved cells were treated with thrombin for the times indicated. Next, cells were washed with ice-cold phosphate-buffered saline and lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % Igepal, 0.25 % sodium deoxycholate, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitor cocktail from Roche Molecular Biochemicals, Mannheim, Germany). Cell suspensions were rotated for 30 min at 4°C and centrifuged at 14 000 *g* for 15 min at 4°C. The protein concentration was determined by the Bradford method (Bio-Rad protein Assay, Bio-Rad, Munich, Germany) using bovine serum albumin as standard.

Samples containing equal amounts of protein were separated on 10 % SDS-PAGE, transferred to nitrocellulose membranes (Hybond C; Amersham Bio-

sciences) and blocked with 3 % bovine serum albumin. The blots were developed by incubation with antibodies against phospho-p44/42 MAPK (1:2000), phospho-JNK (1:2000), and phospho-p38 MAPK (1:1000), overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:10 000) for 60 min at 25°C. Bands were visualized by enhanced chemiluminescence (Super-Signal West Pico; Pierce, Rockford, IL) and Hyperfilm™ ECL (Amersham Biosciences). After stripping, the membranes were re-probed with anti-p44/42 MAPK (1:1000), JNK (1:1000), and p38 MAPK (1:1000) antibody. Quantification of the band densities was carried out using a GS-800 calibrated densitometer and Quantity One software (Bio-Rad).

Generation of plasmid constructs. The full-length human PAR-3 was cloned as described previously [25]. Briefly, PAR cDNA was amplified from human fibroblasts by RT-PCR. The primer sequences were as follows: sense 5′-GTCATCCTCGAGAAAATGAAAGCCCTC-3′, antisense 5′-ATTTCACTAAAGTCTTTTTTGTAAGGTAAGC-3′. The product was digested with *Xho*I and *Hin*dIII and cloned into pEGFP-N1 vector generating PAR3-GFP. Additionally PAR-3 was subcloned into pcDNA3.1/Myc-His (MH) using *Eco*RI and *Xho*I generating PAR3-MH.

Small interfering RNA. siRNA was synthesized by Qiagen. The sequences of human PAR-1 siRNA were: sense 5′-GGGACUGCUGGGAGGUUAA-3′; antisense 5′-UUAACCUCCCAGCAGUCCC-3′ and the DNA target sequence: AAGGGACTGCTGGGAGGTTAA. The sequences of human PAR-3 siRNA were: sense 5′-AGCCAACCUUACCCAUUAA-3′; antisense 5′-UUAAUGGGUAAGGUUGGCU-3′ and the DNA target sequence: AAAGCCAACCTTACCCATTAA. AllStars negative control siRNA served as control siRNA.

Statistical analysis. Statistical evaluation was carried out by *t*-test and multiple comparisons by one-way ANOVA with Dunnett's correction with $p<0.05$ considered as significant.

Results

Functional expression of PAR-3 in HEK-PAR3-GFP cells. To assess the ability of thrombin to activate PAR-3-mediated intracellular signaling, we used cell lines that overexpressed human PAR-3. For this we engineered exogenous PAR-3 expression. The full-length human PAR-3 was cloned by PCR amplification into the pEGFP-N1 vector and also subcloned into pcDNA3.1/Myc-His (+) vector (see Materials and methods). This GFP- and MH-tagged PAR-3 was used for stable transfection of HEK-293 cells. HEK-293 cells transfected with the pEGFP-N1 plasmid DNA (HEK-GFP) and HEK-293 wild-type cells (HEK-WT) served as a reference.

Quantification of relative expression levels of PAR-3 in HEK-PAR3-GFP showed significant expression of PAR-3 in both HEK-PAR3-GFP and HEK-PAR3-MH, whereas in HEK-WT and HEK-GFP cells PAR-3 expression was below the threshold level of detection. It is important to mention that in HEK-PAR3-GFP cells mRNA expression of PAR-3 was 3 times higher than in HEK-PAR3-MH. When compared with other cells that express endogenous PAR-3, the PAR-3 mRNA level in HEK cells overexpressing PAR-3 was 5–15 times higher. Furthermore, transfection of the cells had no influence on the PAR-1 mRNA level (data not shown). Additionally, localization of the receptor was determined using confocal microscopy. PAR3-GFP fusion protein (Fig. 1A) and PAR3-MH (data not shown) were localized on the plasma membrane with little cytoplasmic distribution, whereas GFP in HEK-GFP cells was visible in the whole cell without staining of particular organelles. The localization of PAR3-GFP was detected on the basis of green fluorescence of the GFP vector and of PAR3-MH after immunocytochemistry with antibodies against PAR-3 or Myc.

To confirm the functional expression of PAR-3, we examined the effect of thrombin on $[Ca^{2+}]_i$. Since HEK-293 cells endogenously express another thrombin receptor, PAR-1, HEK-GFP cells were used in parallel for evaluation of the results.

The concentration-response curves were obtained by analyzing thrombin-induced Ca^{2+} mobilization for both cell lines. The results are represented in Figure 1B. In the receptor-transfected cells, the stimulation with thrombin resulted in significantly higher amplitude of Ca^{2+} responses (~3-fold higher) than in HEK-GFP cells. This indicates that PAR-3 used in our studies is functionally expressed and mediates cellular Ca^{2+} signaling, upon stimulation with thrombin.

HEK-293 cells expressing PAR-3 secrete IL-8 upon stimulation with thrombin. To address the question of whether activation of PAR-3 can lead to cellular inflammatory responses, we studied the effect of thrombin on IL-8 production in HEK-293 cells stably expressing PAR-3. As shown in Figure 2, IL-8 synthesis was greatly enhanced in HEK-PAR3-GFP as well as in HEK-PAR3-MH cells upon stimulation with 1 and 10 U/ml thrombin. The increase was seen already after 3 h of stimulation (data not shown).

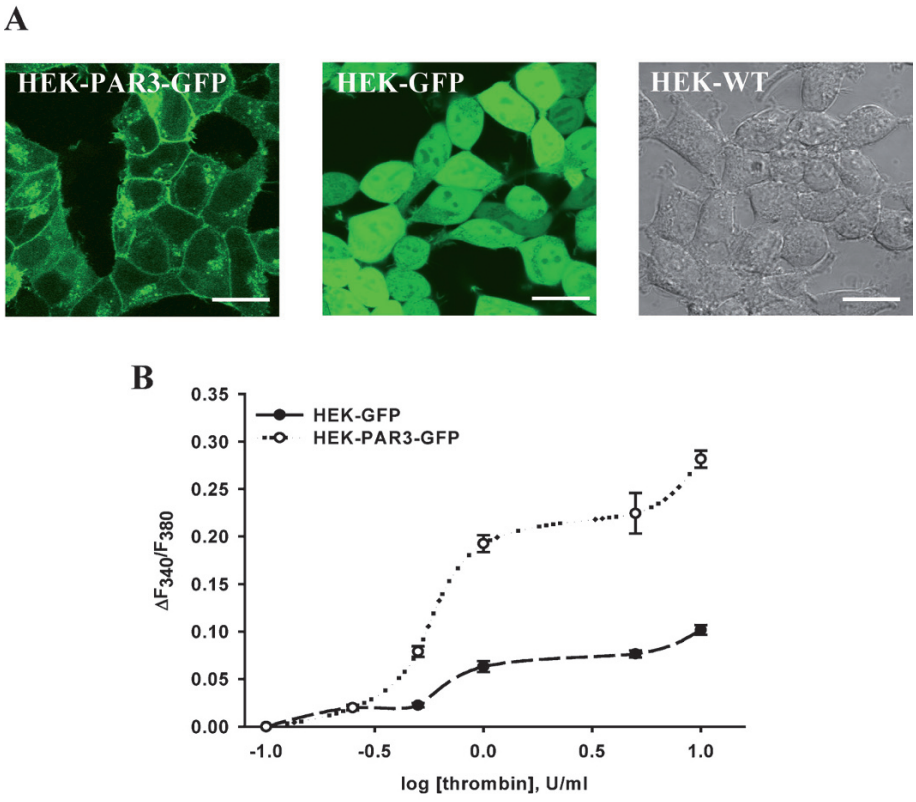

Figure 1. Localization and functional expression of protease-activated receptor (PAR)-3 in PAR-3-transfected HEK-293 cells. (*A*) PAR-3-green fluorescent protein (GFP) and GFP were detected by GFP fluorescence with LSM510 confocal laser scanning microscope (Carl Zeiss, Germany). Images are representative for three different experiments. Scale bar is 20 μm. (*B*) Concentration-effect curves for $[Ca^{2+}]_i$ rise induced by thrombin in HEK-PAR3-GFP and HEK-GFP cells. The fura-2-AM loaded cells were stimulated with varying concentrations of thrombin and the change in ratio of fluorescence at 340 and 380 nm was measured. Data represent the mean ± SE from 70 to 150 single cells investigated in at least three separate experiments (when the error bars are not shown, they are smaller in size than the symbols used).

This effect was seen for both thrombin concentrations that were used, indicating that PAR-3 overexpression does not alter the avidity with which thrombin evokes IL-8 release. IL-8 mRNA level was up-regulated up to 30-fold in HEK-PAR3-GFP and 10-fold in HEK-PAR3-MH after stimulation with thrombin. In HEK-GFP and HEK-WT cells that served as control, the IL-8 mRNA level did not change significantly (Fig. 2A). A similar effect was seen on protein level. The IL-8 release was enhanced only in HEK cells transfected with PAR-3, about 23-fold in HEK-PAR3-GFP and 4-fold in HEK-PAR3-MH (Fig. 2B). The IL-8 production by HEK-WT and HEK-GFP was not influenced by exposure to thrombin demonstrating that PAR-3-deficient cells do not respond to thrombin to induce IL-8 synthesis. Moreover, upon stimulation of the cells with 50 μM PAR-1-activating peptide (TRag), the IL-8 expression did not differ from that of non-treated cells, on either the mRNA level (Fig. 2A) or the protein level (data not shown). TRag used at a concentration of 10 μM showed the same inability to induce production of IL-8 (data not shown).

PAR-1 gene silencing by RNA interference. Gene silencing of PAR-1 was performed using target sequence specific siRNA. HEK-PAR3-GFP cells were co-transfected with 100 nM PAR-1 siRNA or with AllStars negative control siRNA. As shown in Figure 3A, transfection of HEK-PAR3-GFP cells with PAR-1 siRNA for 48 h resulted in highly suppressed (by about 80%) PAR-1 transcription level. The control siRNA did not interfere with PAR-1 mRNA expression, confirming the specificity of PAR-1 siRNA. Additionally, the rise in $[Ca^{2+}]_i$ after PAR-1 activation was measured to confirm the attenuation of PAR-1 expression in terms of functionality. Specific activation of PAR-1 with TRag induced significantly lower $[Ca^{2+}]_i$ responses in cells transfected with PAR-1 siRNA than in cells transfected with control siRNA or in cells that were not co-transfected (Fig. 3B). Therefore, we can conclude that transfection of HEK-PAR3-GFP cells with PAR-1 siRNA significantly decreased PAR-1 expression and function in terms of Ca^{2+} signaling.

Suppression of PAR-1 expression does not interfere with PAR-3-mediated IL-8 production. Our next aim was to exclude the possibility that thrombin-mediated IL-8 release is due to activation of PAR-1 or that PAR-3 participates in a dual receptor system involving PAR-1. For this purpose the cells were incubated for 6 h with 10 U/ml thrombin or 10 μM TRag as a control. Figure 4A demonstrates that stimulation with thrombin, but not with TRag, significantly elevated the synthesis of IL-8 mRNA by 20- and 22-fold ($p<0.05$) in HEK-PAR3-GFP cells transfected

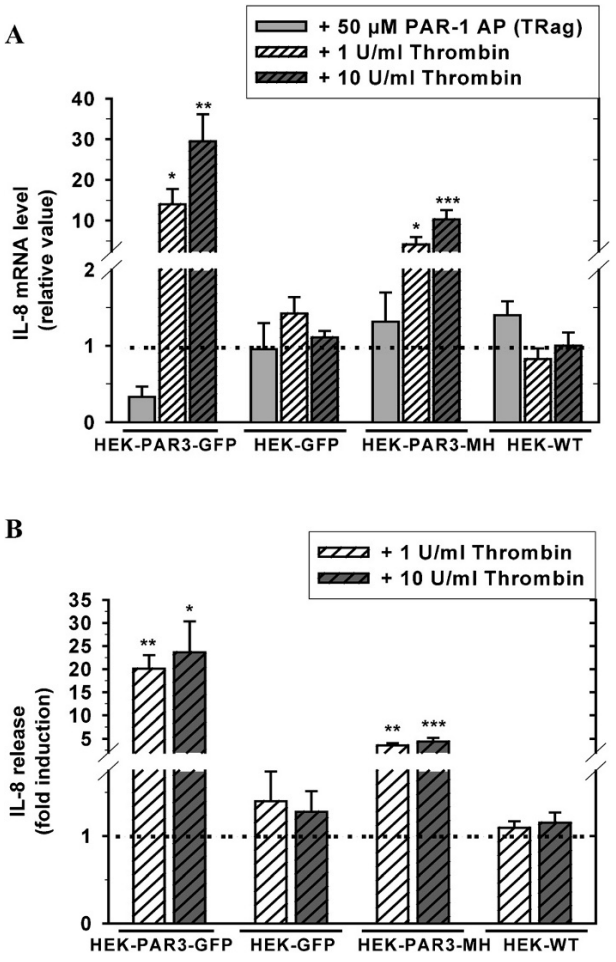

Figure 2. Effect of thrombin receptor agonist peptide (TRag) and thrombin on the IL-8 synthesis in HEK-293 cells expressing PAR-3. The HEK-PAR3-GFP and HEK-PAR3-MH as well as HEK-GFP and HEK wild-type (WT) cells were incubated with agonists as indicated. (*A*) Changes in IL-8 mRNA level after 6 h of stimulation with 50 µM TRag, 1 or 10 U/ml thrombin were estimated by real-time PCR. Modulation of mRNA expression was calculated using GAPDH as a reference gene. (*B*) IL-8 secreted into the cell culture medium after 6 h of incubation with 1 or 10 U/ml thrombin was quantified by ELISA. Data are means ± SE of three independent experiments. Dotted line at "1" indicates control level. ***$p<0.001$, **$p<0.01$, *$p<0.05$ as compared to the respective untreated cells.

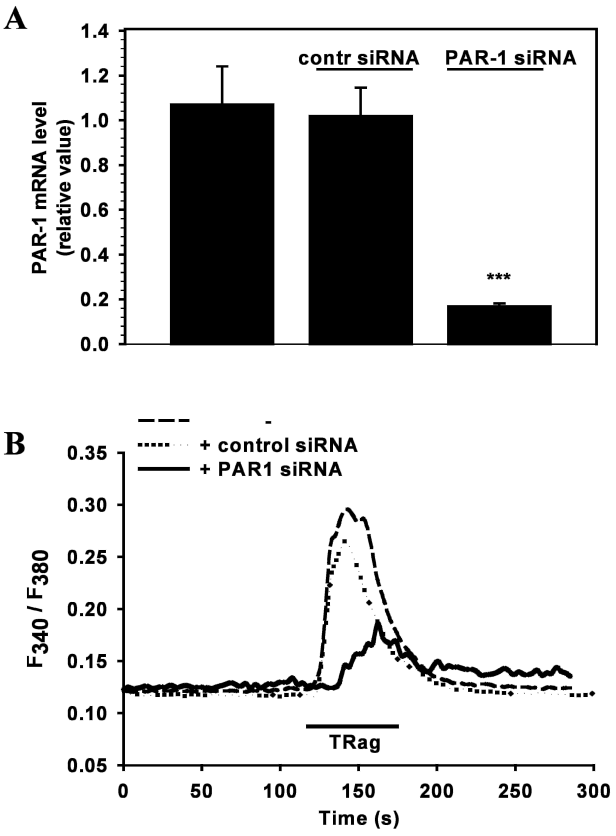

Figure 3. Suppression of PAR-1 expression by small interfering RNA (siRNA). HEK-PAR3-GFP cells were transiently transfected with either PAR-1 siRNA or control siRNA as mentioned in Materials and methods. (*A*) Quantification of PAR-1 expression was assessed by real-time PCR. Measurements were normalized to the GAPDH mRNA level. The values given are means ± SE of three independent determinations. The PAR-1 expression level is expressed relative to the PAR-1 mRNA expression in cells, which were transfected with control siRNA. This was chosen as a reference value of 1. ***$p<0.001$ as compared to the non-transfected cells. (*B*) $[Ca^{2+}]_i$ rise in HEK-PAR3-GFP induced by PAR-1-activating peptide (TRag). The fura-2-AM-loaded HEK-PAR3-GFP cells, HEK-PAR3-GFP additionally transfected with PAR1 siRNA or control siRNA were exposed to TRag (10 µM) and the changes in fluorescence ($\Delta F_{340\,nm}/F_{380\,nm}$) were detected. The traces are the mean value of at least 25 single cells measured in one experiment and are representative for at least three different experiments.

with PAR-1 siRNA and in cells transfected with control siRNA, respectively. Consequently, thrombin induced a significant increase of IL-8 in the cell culture supernatant of these cells (13- and 17-fold, respectively) (Fig. 4B). This highly enhanced the amount of IL-8, which was released from the HEK cells expressing PAR-3 upon exposure to thrombin, did not differ significantly between the cells co-transfected with PAR-1 siRNA and control siRNA. Therefore, it can be concluded that knock-down of endogenous PAR-1 in HEK-PAR3-GFP cells had no significant influence on the mRNA synthesis and protein secretion of IL-8 induced by thrombin.

Lung epithelial cells, A549 cells, endogenously expressing PAR-3, release IL-8 after stimulation with thrombin. To extend our investigation on the role of PAR-3 to determine whether it can induce production of IL-8, we used other human cells in our experiments. We compared two different lung epithelial cell lines, A549 and HBE, that both express PAR-1 mRNA at a similar level. A549 cells, but not HBE cells, express PAR-3 mRNA [25]. As shown in Figure 5, stimulation with 5 µM TRag for 4 h had no influence on IL-8 mRNA synthesis in either type of epithelial cells. However, the exposure of the cells to 5 U/ml thrombin resulted in enhanced IL-8 synthesis in A549 cells, but

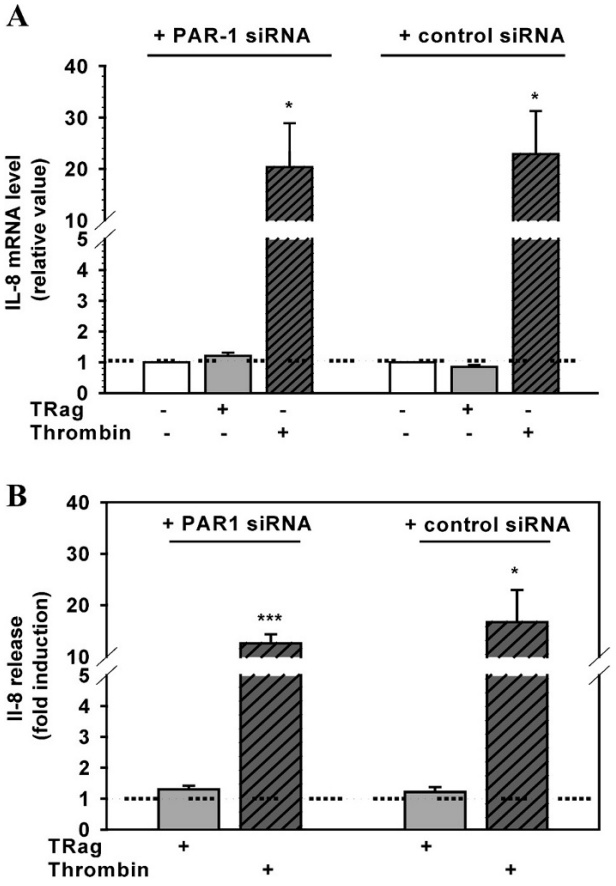

Figure 4. Effect of TRag and thrombin on the IL-8 mRNA expression level and IL-8 protein secretion in HEK-PAR3-GFP cells transfected with PAR1 siRNA. The HEK-PAR3-GFP cells transfected either with PAR-1 siRNA or control siRNA were incubated for 6 h with 10 μM TRag or 10 U/ml thrombin. (*A*) Changes in IL-8 mRNA level were determined by real-time PCR. Modulation of mRNA expression was calculated using GAPDH as a reference gene. (*B*) IL-8 secreted into the cell culture medium was quantified by ELISA. Data are means ± SE of three independent experiments. Dotted line at "1" indicates control level.***$p<0.001$, *$p<0.05$ as compared with the respective untreated cells.

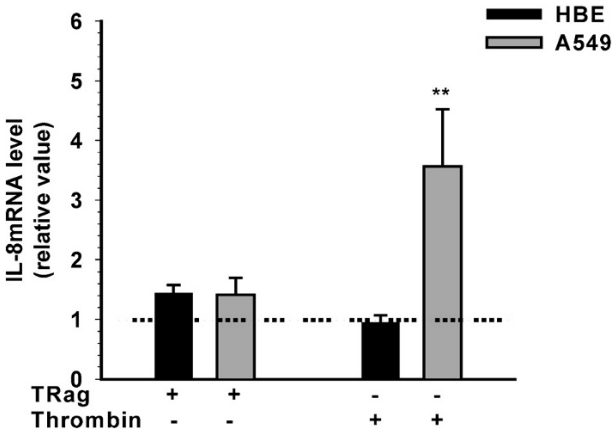

Figure 5. Effect of TRag and thrombin on the IL-8 mRNA expression level in A549 and HBE cells, human lung epithelial cells. Changes in IL-8 mRNA level were determined by real-time PCR after incubation of both cell lines with 5 μM TRag or 5 U/ml thrombin for 4 h. Modulation of mRNA expression was calculated using GAPDH as a reference gene. Data are means ± SE of three independent experiments. Dotted line at "1" indicates control level.**$p<0.01$ as compared with the respective untreated cells.

not in HBE cells. The protein amount of IL-8 released into culture medium corresponded to the mRNA level (data shown).

PAR-1 silencing does not affect IL-8 production in human astrocytoma cells 1321N1. The next step in investigating the intriguing role of PAR-3 in production of IL-8 was to knock-down endogenous PAR-1 and PAR-3 in 1321N1 cells. These cells express similar levels of both PARs [25]. As shown in Figure 6A, after transfection of 1321N1 cells with PAR-1 siRNA, the effect of exposure of the cells to PAR-1-activating peptide, TRag, on IL-8 mRNA level was significantly attenuated when compared to the same stimulation of the cells transfected with control siRNA. The thrombin-mediated IL-8 mRNA level in cells transfected with PAR-1 siRNA was not significantly reduced compared to cells treated with control siRNA and reached a level of about 20- to 10-fold. However, PAR-3 knock-down, in addition to PAR-1 silencing, significantly diminished the IL-8 mRNA level after stimulation with thrombin by about 80 % compared to the IL-8 mRNA level in cells treated with control siRNA. A similar effect of PAR-1 and PAR-3 silencing was observed on protein level (Fig. 6B). Here, the silencing of PAR1 attenuated only the TRag-mediated IL-8 synthesis *via* PAR1, but upon exposure to thrombin the IL-8 release reached almost the same level. However, the amount of IL-8 released from cells treated with PAR-1 and PAR-3 siRNA and exposed to thrombin was dramatically reduced, when compared to the amount of IL-8 released by cells transfected with control siRNA. In the cells that were transfected with control siRNA, thrombin-mediated IL-8 synthesis was almost 45-times higher than in non-stimulated cells, whereas in cells with PAR-1 and PAR-3 knock-down the IL-8 release was only 3.5-fold higher after stimulation with thrombin, compared with cells without stimulation.

Thrombin activates PAR-3-mediated ERK1/2 phosphorylation. It has been shown that PAR-1 and PAR-2 can affect cellular functions through different signal transduction pathways, including the MAPK pathway. Furthermore, MAPK cascades are involved in PAR-1- and PAR-2-induced IL-8 production [21, 22, 24, 26, 27]. However, until now, no data were available showing the participation of MAPKs in PAR-3 signaling. In this work, the involvement of ERK1/2, JNK

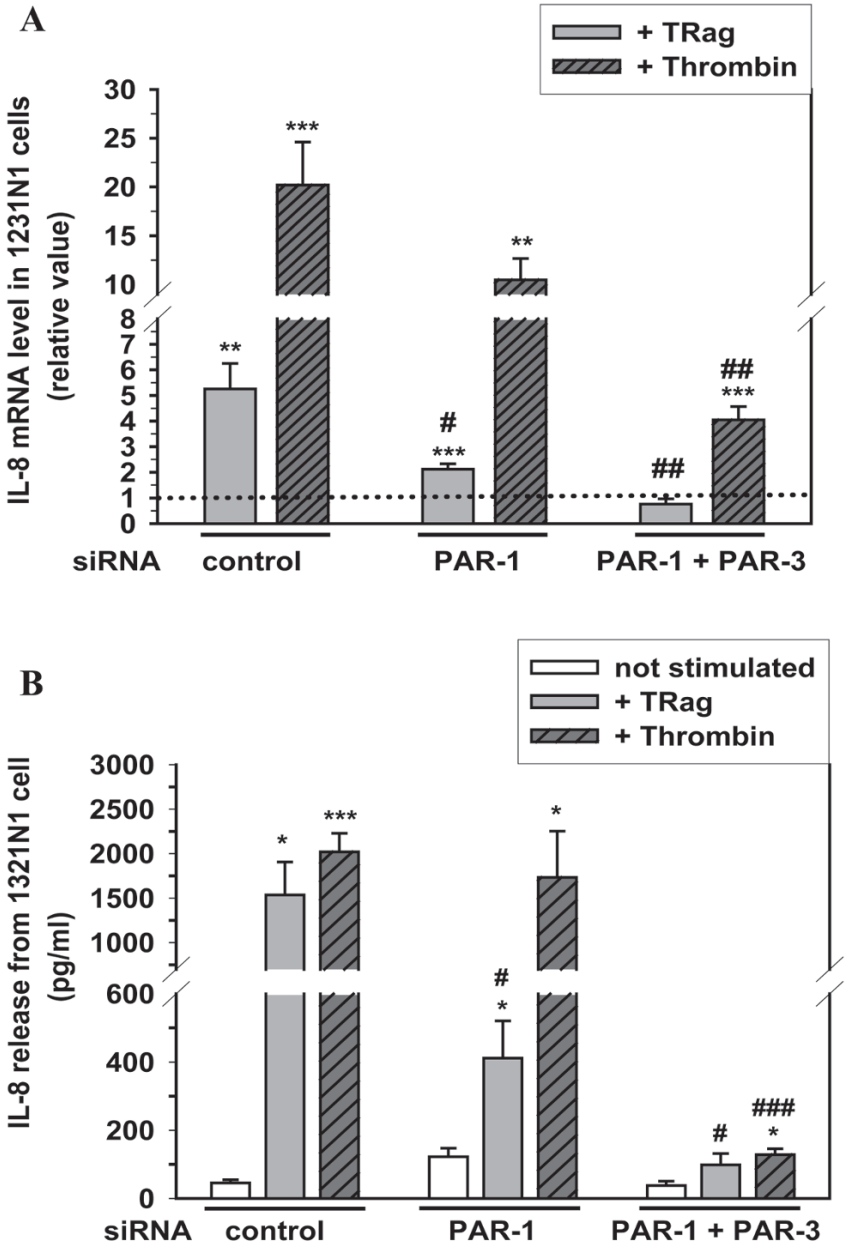

Figure 6. Effect of TRag and thrombin on the IL-8 synthesis in 1321N1 human astrocytoma cells. 1321N1 cells were transfected with siRNA as mentioned in Materials and methods. (*A*) Changes in IL-8 mRNA level after 6 h of stimulation with 10 μM TRag or 10 U/ml thrombin were estimated by real-time PCR. Modulation of mRNA expression was calculated using GAPDH as a reference gene. (*B*) IL-8 secreted into the cell culture medium after 6 h of incubation with 10 μM TRag or 10 U/ml thrombin was quantified by ELISA. Data are means ± SE of three independent experiments. Dotted line at "1" indicates control level. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ as compared to the untreated cells transfected with respective siRNA. $^{###}p<0.001$, $^{##}p<0.01$, $^{#}p<0.05$ as compared to the same corresponding stimulation of the cells transfected with control siRNA.

and p38 MAPK, in PAR-3-mediated production of IL-8 was investigated using specific inhibitors. The HEK-293 cells overexpressing PAR-3 were preincubated with 10 μM p38 MAPK inhibitor (SB203580), 10 μM JNK inhibitor (SP600125) and 20 μM ERK1/2 inhibitor (U0126) followed by the stimulation with 10 U/ml thrombin. The application of all these inhibitors has been established previously in our laboratory [28, 29]. As shown in Figure 7, a blockade of JNK and ERK1/2 reduced the IL-8 secretion induced by thrombin by 40 % and by 77 %, respectively. Pretreatment of the cells with the p38 MAPK inhibitor did not significantly affect the IL-8 production.

By Western blot analysis we confirmed that thrombin activates ERK1/2 in HEK-PAR3-GFP cells. Figure 8 demonstrates that incubation of these cells with thrombin (10 U/ml) highly induced the time-dependent phosphorylation of ERK1/2 (up to 26-times) in HEK-PAR3-GFP cells expressing endogenous PAR-1. In cells transfected with PAR-1 siRNA there was also an increase of up to 21-times above the control level. However, after 5 min of incubation with thrombin, there seemed to be a difference in phosphorylation levels between HEK-PAR3-GFP cells and the cells with silenced PAR-1. The ERK1/2 activation was 50 % higher in HEK-PAR3-GFP cells expressing

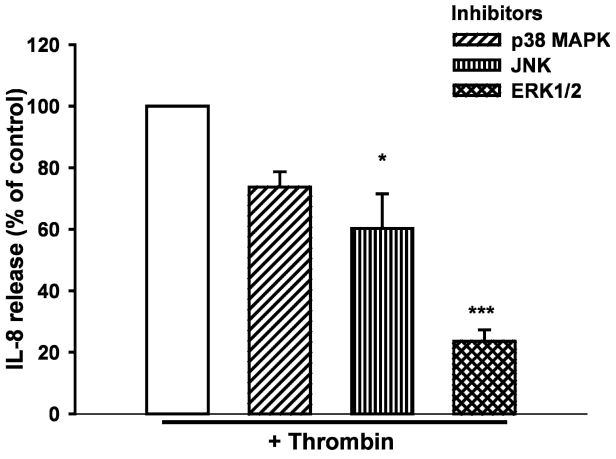

Figure 7. Effect of MAPK inhibitors on thrombin-induced IL-8 release in HEK-PAR3-GFP cells. Serum-starved cells were pre-treated with either p38 MAPK inhibitor SB203580 (10 μM), JNK inhibitor SP600125 (10 μM), or ERK1/2 inhibitor U0126 (20 μM) for 30 min, followed by incubation with 10 U/ml thrombin for 6 h. An ELISA was used to quantify the amount of released IL-8. The results are means ± SE of three independent experiments.***$p<0.001$, *$p<0.05$ as compared with the cells treated only with thrombin.

endogenous PAR-1 than in cells co-transfected with PAR-1 siRNA. After 10 min of incubation the ERK1/2 phosphorylation in these two cell lines reached a similar level. In HEK-GFP cells, ERK1/2 was activated 2-fold. A similar tendency was observed in human dermal fibroblasts expressing endogenous PAR-1 [24]. These results are presented in Figure 8A as a time course showing representative blots, which are quantified in Figure 8B.

JNK phosphorylation was considerably smaller than that of ERK1/2 and reached a maximal level of 200 % of control value in HEK-PAR3-GFP cells after thrombin treatment. In the HEK-PAR3-GFP cells transfected with PAR-1 siRNA, this effect was reduced by about 40–80 % (Fig. 8C). p38 MAPK was not activated by exposure to thrombin in HEK-293 cells expressing PAR-3 (data not shown).

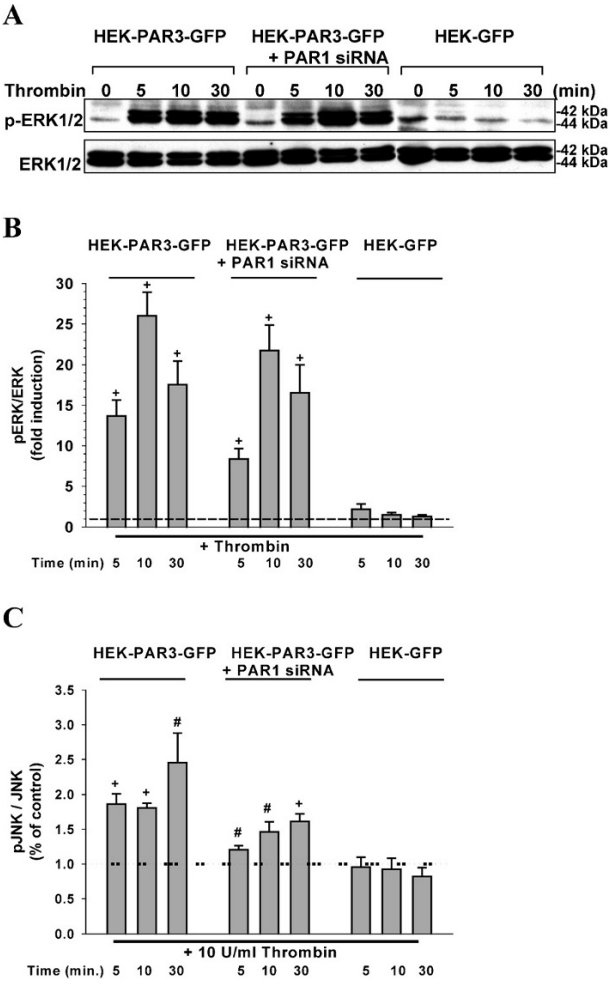

Figure 8. Activation of ERK1/2 and JNK by thrombin in HEK-PAR3-GFP cells, HEK-PAR3-GFP cells deficient of PAR-1 and HEK-GFP cells. Serum-starved cells were exposed to thrombin (10 U/ml) for 5, 10 and 30 min, as indicated. Phosphorylation of ERK1/2 and JNK was analyzed by Western blotting using the anti-phospho-ERK1/2 or anti-phospho-JNK antibodies. Equal amounts of protein loading were confirmed with the antibodies specific for total ERK1/2 or JNK. The representative blot from three independent experiments for ERK1/2 activation is shown (*A*). The quantification of ERK1/2 (*B*) and JNK (*C*) was done by densitometry and normalized by referring to the corresponding total amount. Cells without treatment with thrombin served as control (100 %), marked as dashed line at "1". The results are means ± SE of three independent experiments. $^{+}p<0.001$, $^{\#}p<0.005$ as compared to the cells without treatment with thrombin.

Discussion

The pathophysiological roles of human PAR-3 are still largely unknown. The aim of this study was to verify whether PAR-3 participates in inflammatory reactions, similarly to other PARs, by mediating IL-8 production. Furthermore, an important question to be answered was whether human PAR-3 can signal autonomously or only in cooperation with another thrombin receptor. Our investigations show that human PAR-3 might signal after activation by thrombin independently from PAR-1 and PAR-4. Thrombin-mediated PAR-3 activation enhances IL-8 production, besides the mobilization of Ca^{2+}.

In our studies, the HEK-293 cells transfected with the PAR-3-GFP construct showed clearly a significant rise in Ca^{2+} response compared to those cells transfected only with GFP. These data confirm that the PAR-3 used in our system was functional. Indeed, PAR-3 can be activated by thrombin, thereby triggering increase in $[Ca^{2+}]_i$. Our findings are in line with data obtained from PAR-3-expressing COS-7 cells and *Xenopus* oocytes, where thrombin induced phosphoinositide

hydrolysis and ^{45}Ca release, respectively [4]. However, in other systems expressing PAR-3 thrombin did not trigger PAR-3 signaling. The group of Hollenberg [12, 30] reported the inability of thrombin to induce Ca^{2+} mobilization after desensitization of PAR-1 by its specific agonist in cells expressing PAR-3, *i.e.*, Jurkat and HEK-293 cells. Similar results were obtained in human brain microvascular endothelial and in human umbilical vein endothelial cells [31, 32]. This inconsistency in the calcium signaling between endogenous und exogenous PAR-3 may be due to the respective expression level of the receptor.

Further, the results presented here demonstrate that thrombin mediates IL-8 production in HEK-293 cells stably expressing PAR-3. The cytokines are the main effector substances of the inflammatory and immune response. Many common human diseases are characterized by a dysregulation of the balance between pro- and anti-inflammatory cytokines. Therefore, this ability of PAR-3 to mediate increased IL-8 release suggests that human PAR-3 is an important player in the inflammatory state.

Concerning the issue of whether PAR-3 is able to autonomously mediate intracellular signaling, it should be noted that PAR-4 was not detected in all cells used in this study. This was confirmed by RT-PCR and measurement of Ca^{2+} mobilization after stimulation with PAR-4-activating peptide. Therefore, it can be concluded that, in contrast to the mouse system in which PAR-3 acts as a cofactor for PAR-4 activation, in humans PAR-3 can generate an intracellular signal independently from PAR-4. Additionally, our investigations on PAR-1-deficient HEK-293 cells expressing PAR-3 suggest that the presence of PAR-1 is also not necessary for PAR-3-mediated production of IL-8. The silencing of the PAR-1 expression in HEK-PAR3-GFP cells had no significant influence on IL-8 production upon thrombin stimulation. Moreover, the application of TRag, the PAR-1-activating peptide, did not trigger the IL-8 synthesis in HEK-PAR3-GFP cells co-transfected with control siRNA, which thus express functional PAR-1. This finding underlines PAR-1 not being involved in the IL-8 synthesis and that PAR-3, independently from PAR-1, autonomously mediates IL-8 release in HEK-PAR3-GFP cells upon exposure to thrombin.

A similar effect of thrombin was seen in other human cell lines. Lung epithelial A549 cells that endogenously express PAR-3 responded to this protease with enhanced IL-8 release. In contrast, HBE cells that lack PAR-3 expression failed to respond to thrombin with increasing IL-8 production. Although both cell lines express PAR-1, PAR-1 activation by its activating peptide was not able to induce IL-8 synthesis. These finding give supportive evidence that endogenous PAR-3 is susceptible to thrombin by enhancement of IL-8 production. However, it has to be mentioned that in other systems, such as human dermal fibroblasts or human luteinized granulosa cells (LGC), thrombin stimulation resulted in increased IL-8 release *via* PAR-1 activation, which was shown using the PAR-1 agonist [18, 24]. This PAR-1-mediated IL-8 synthesis might be due to the relatively high expression level of PAR-1 in these cells. Since besides PAR-1 both cells also expressed PAR-3, the second alternative might be that, when endogenously expressed, both receptors could in part form heterodimers, according to a recent report [16]. It is possible that PAR-1/PAR-3 heterodimers might also be able to induce IL-8 release.

Additional support was given by investigations on the human astrocytoma 1321N1 cells. These cells express similarly high levels of PAR-1 and PAR-3 and respond to both TRag and thrombin inducing IL-8 production. In these cells, PAR-1-knock-down reduced only PAR-1-mediated production of IL-8. Thrombin-induced synthesis of IL-8 was drastically diminished only when expression of PAR-3 was silenced. Therefore, it is clear that PAR-3, overexpressed as well as expressed endogenously, mediates IL-8 release upon activation by thrombin.

We next investigated the involvement of MAPKs in IL-8 release induced by thrombin from HEK-293 cells expressing PAR-3. The MAPKs have been shown to regulate the IL-8 expression and secretion induced by PAR activation in many different cells. Here, we demonstrated for the first time that activation of PAR-3 by thrombin mediates IL-8 synthesis *via* ERK1/2 phosphorylation, whereas the other members of the MAPK family, JNK and p38 MAPK, were not significantly involved in thrombin-mediated PAR-3 activation. We confirmed these observations by phosphorylation analysis and inhibitor studies. Silencing of PAR-1 in HEK-PAR3-GFP cells had no significant effect on the level of ERK1/2 phosphorylation. These experiments confirm that PAR-3-mediated signaling does not require the presence of PAR-1. However, the existence of some proportion of PAR-1/PAR-3 heterodimers cannot be excluded. Moreover, the relatively weak but significant JNK activation and its involvement in thrombin-mediated IL-8 secretion might be due to PAR-1/PAR-3 co-activation. Therefore, the important concept of receptor dimerization has to be followed further in exploring PAR-3 signaling. It has been shown already that PAR-1 and PAR-4 are able to form stable heterodimers on human platelets and also when expressed in COS-7 fibroblasts, where PAR-1 acts as a cofactor and promotes the cleavage and activation of PAR-4 [10].

In conclusion, our findings presented here give new insights into the signaling and function of human

PAR-3. We have demonstrated unequivocally that the functional consequence of PAR-3 activation *per se* by thrombin is elevation in ERK1/2 phosphorylation and increase in production of IL-8. Furthermore, PAR-3 responsiveness to thrombin is generally not dependent on the presence of PAR-1. Therefore, as a receptor that mediates cytokine production, PAR-3 can be considered as an important modulator in the burst of inflammatory reactions.

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