Inflammatory mediators modulate thrombin and cathepsin-G signaling in human bronchial fibroblasts by inducing expression of proteinase-activated receptor-4

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Ramachandran R, Sadofsky LR, Xiao Y, Botham A, Cowen M, Morice AH, Compton SJ. Inflammatory mediators modulate thrombin and cathepsin-G signaling in human bronchial fibroblasts by inducing expression of proteinase-activated receptor-4. Am J Physiol Lung Cell Mol Physiol 292: L788–L798, 2007. First published December 1, 2006; doi:10.1152/ajplung.00226.2006.—Human lung fibroblasts express proteinase-activated receptor-1 (PAR1), PAR2 and PAR4, but not PAR3. Because PAR2 has inflammatory effects on human primary bronchial fibroblasts (HPBF), we asked (1) whether the inflammatory mediators TNF-α and LPS could modify HPBF PAR expression and (2) whether modified PAR expression altered HPBF responsiveness to PAR agonists in terms of calcium signaling and cell growth. TNF-α and LPS induced PAR2 mRNA expression (RT-PCR) at 6 h and 24 h, respectively. TNF-α and LPS also upregulated PAR2 mRNA expression with similar kinetics but had negligible effect on PAR1 and PAR3. Flow cytometry for PAR1, PAR2, and PAR3 also demonstrated selective PAR2 upregulation in response to TNF-α and LPS. Intracellular calcium signaling to SLIGKV-NH2 (a selective PAR2-activating peptide; PAR2-AP) and AYPGQV-NH2 (PAR4-AP) revealed that TNF-α and LPS induced maximal responses to these PAR agonists at 24 h and 48 h, respectively. Uprogulation of PAR2 by TNF-α heightened HPBF responses to trypsin, while PAR4 induction enabled cathepsin-G-mediated calcium signaling. Cathepsin-G also disabled PAR1 and PAR2 in HPBF, while trypsin disarmed PAR2. Induction of PAR4 also enabled thrombin to elicit a calcium signal through both PAR1 and PAR2, as determined by a desensitization assay. In cell growth assays the PAR4 agonists cathepsin-G and AYPGQV-NH2 reduced HPBF cell number only in TNF-α-treated HPBF. Moreover, the mitogenic effect of thrombin (a PAR1/PAR4 agonist) but not the PAR2-AP TFLLR-NH2 was ablated in TNF-α-treated HPBF. These findings point to an important mechanism, whereby cellular responses to thrombin and cathepsin-G can be modified during an inflammatory response.

inflammation; fibrosis; lung; trypsin

THE SERINE PROTEINASES THROMBIN, trypsin, and cathepsin-G can trigger changes in cellular behavior through the activation of one or more of a unique family of G protein-coupled receptors termed proteinase-activated receptors (PARs) (17, 34). Activation of PARs typically occurs through the proteolytic unmasking of a “tethered ligand” domain at the NH2 terminus of the receptor that initiates receptor activation. The PAR “tethered ligand” sequence can be used as a template to design small PAR-selective activating peptides (PAR-AP) that trigger PAR1, PAR2, or PAR4 activation (6, 19, 37, 38). These PAR-APs are especially useful for dissecting out a PAR response in which proteinases may activate multiple receptors within the same cell type (18, 20). The combined use of PAR-APs and PAR agonist proteinases has provided evidence suggesting an involvement of PARs in inflammatory lung diseases (24).

In addition to their role in initiating an inflammatory response, altered PAR expression has been reported in various inflammatory conditions (8, 13, 21), including asthma (21). In vitro studies have demonstrated that PAR expression can be modulated by inflammatory stimuli and growth factors. For example, constitutive PAR2 expression has been reported to be upregulated by LPS, IL-1β, and TNF-α in endothelial cells and by TNF-α in neuroblastoma cells (16, 27, 28). Growth factor- and extracellular matrix-dependent regulation of PAR2 has also been reported in skin fibroblast cell lines by platelet-derived growth factor-BB or transforming growth factor-β (TGF-β), but not by IL-1α or TNF-α (15). In addition a recent study in human synovial fibroblasts has shown that βFGF, but not TNF-α or IL-1β, upregulate PAR2 expression (1). Constitutive PAR2 and PAR4 mRNA expression has been reported to be upregulated in human coronary arteries in response to inflammatory stimuli (16). However, it is unclear whether a cell can be stimulated to express a PAR that is not constitutively present under resting conditions. An ability such as this would enable a cell to govern which proteinases can signal and thereby determine the patho/physiological outcome.

Human lung fibroblasts have been reported to express PAR1, PAR2, and PAR3, but not PAR4 (3, 30, 33). Through activation of PAR1, thrombin has been implicated in driving lung fibroblast proliferation, differentiation to myofibroblasts, PGE2 release, and procollagen release (4, 5, 9, 10, 33). The role of PAR2 in lung fibroblasts is less well known, but some studies have reported proliferation (2, 23), while others have not (30). More recently, we have reported that PAR2 agonists stimulate granulocyte colony-stimulating factor and IL-8 release and upregulation of VCAM-1 on human primary bronchial fibroblasts (HPBF), suggesting a potential proinflammatory role for PAR2 in these cells (30). Since PAR4 is not thought to be expressed by lung fibroblasts, a potential role for this receptor has understandably not been proposed. Thus, we hypothesized

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that because PAR$_2$ triggers inflammatory events in HPBF (30), inflammatory mediators may modify PAR expression in these cells. Therefore, we sought to investigate whether inflammatory mediators can 1) regulate HPBF PAR expression and 2) whether such changes in PAR expression influence calcium signaling and cell growth of HPBF in response to PAR-APs and PAR-regulating proteinases.

**MATERIALS AND METHODS**

Primers were synthesized by MWG-Biotech (Ebersberg, Germany). dNTPs and random primers, trypsin EDTA, FBS, DMEM, antibiotic-antimycotic (penicillin G sodium, streptomycin sulfate, and amphotericin B), sodium pyruvate, and enzyme-free cell dissociation fluid were supplied by Invitrogen (Paisley, UK). The human fibroblast specific marker monoclonal antibody (Clone 5B5) was purchased from DAKO (High Wycombe, UK). Fibroblast growth medium kits were obtained from TCS Cell Works (Buckinghamshire, UK), and each kit contained fibroblast basal medium, fibroblast growth supplements, 25 µg/ml gentamycin, and 50 µg/ml amphotericin B. RNeasy RNA isolation kits, gel extraction kits, and Omniscript RT-PCR kits were purchased from Qiagen (Crawley, West Sussex, UK). Taq DNA polymerase was obtained from New England Biolabs (Hitchin, Hertfordshire, UK). Anti-PAR$_2$ (SAM-11) antibody and the anti-PAR$_3$ (ATAP-2) antibody were purchased from Zymed Laboratories (San Francisco, CA), while anti-PAR$_1$, and anti-PAR$_4$ polyclonal antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). The anti-vimentin antibody and all other chemicals and reagents were from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated.

**PAR proteinases and PAR-APs.** PAR-APs were synthesized by the Peptide Synthesis Facility, University of Calgary. Alberta, Canada (peplab@ucalgary.ca) or purchased from Peptides International (Louisville, KY). Peptides were reconstituted in sterile 25 mM HEPES, pH 7.4. Human lung trypsin was purified essentially as described previously (11). Human plasma thrombin was purchased from Calbiochem (Beeston, Nottinghamshire, UK), and trypsin was purchased from Sigma. Cathepsin-G was obtained from Sigma-Aldrich and elastin products (Owensville, MO).

**Cell culture.** HPBF cultures were established as previously reported (2, 30) from conducting airway bronchial tissue explants obtained under informed consent from patients undergoing thoracotomy. Explants were dissected into small pieces of less than 1 mm$^3$ and were placed into separate wells of a 24-well culture plate with DMEM containing 20% FBS, sodium pyruvate, and antibiotic/antimycotic. The medium was then changed at least twice a week. After 4 wk, confluent fibroblast cultures were observed in the wells. The fibroblast cultures were lifted by trypsinization and transferred to 75 cm$^2$ flasks containing 10 ml of fresh HPBF medium. Confluent cultures were subsequently passaged with a split ratio of 1:3, and cells were only used between passages 1 and 10. The purity of fibroblast cultures was assessed immunocytochemically by staining for the fibroblast specific marker vimentin and prolyl-4-hydroxylase and morphologically by observing the classical spindle shape and characteristic swirls formed by fibroblasts in culture.

**PCR detection of PARs.** HPBF were placed into six-well plates and allowed to grow to confluence before quiescing in serum replacement media [DMEM supplemented with 1× serum replacement (Sigma), antibiotic/antimycotic and sodium pyruvate] for 48 h. Quiescent HPBF were subsequently treated with either TNF-α (50 ng/ml), LPS (100 ng/ml), or IL-1α (10 ng/ml) for 0, 3, 6, 24, and 48 h. At each time point, mRNA was extracted using an RNeasy minikit (Qiagen, Hilden, Germany) according to manufacturer’s protocol before being quantified using a GeneQuant spectrophotometer (Amersham Biosciences, Buckinghamshire, UK). cDNA was synthesized by reverse transcription of 1–2 µg of mRNA with an omniscript RT kit (Qiagen) and random primers (Invitrogen). The reverse transcription reaction was cycled at 24°C for 10 min, 37°C for 50 min, and 95°C for 10 min. PCR amplification of PARs was performed on 1 µl of cDNA in the presence of a master mix containing PCR buffer, 0.2 mM dNTPs and 1 U Taq DNA polymerase (New England Bioscience) with PAR and β-actin specific oligonucleotide primers as detailed elsewhere (30).

For PAR$_1$, PAR$_2$, and PAR$_3$, the PCR reactions were conducted for 35 cycles cycling at 94°C for 3 min, 55°C for 1 min, and 72°C for 1 min with a final extension for 10 min. For PAR$_4$, the PCR reaction involved 35 cycles at 94°C for 3 min, 59°C for 1 min, and 72°C for 1 min followed by a final extension of 10 min. The PCR products were resolved by running the samples on a 1.3% agarose gel and visualized by ethidium bromide under ultraviolet light. The PCR products were then purified using a gel purification kit (Qiagen) and subsequently sequenced to confirm their identities using fluorescence-based automated cycling sequencing (Qiagen sequencing service, Germany).

**Flow cytometry.** HPBF were grown to confluence in six-well culture plates before being placed in serum-free medium for 48 h. HPBF were subsequently treated with either TNF-α (50 ng/ml), LPS (100 ng/ml), or IL-1α (10 ng/ml) for 0, 6, 12, 24, 48, and 72 h. Treated cells were harvested with enzyme-free cell dissociation buffer, centrifuged and suspended in 200 µl of ice cold culture medium in the presence of PAR-specific primary antibodies and incubated on ice for 90 min. Cells were pelleted by centrifugation, resuspended in ice-cold culture medium, and incubated on ice for a further 30 min with the appropriate secondary FITC-conjugated antibody. Following a final centrifugation step, cells were resuspended in 300 µl of PBS and analyzed on a Becton Dickinson (Franklin Lakes, NJ) flow cytometer.

**Intracellular calcium mobilization.** HPBF were seeded on 12 mm glass coverslips and grown to confluence in 2 ml of HPBF medium. For time course experiments, HPBF were treated with either TNF-α (50 ng/ml) or IL-1α (10 ng/ml) for 0, 6, 12, 24, and 48 h or LPS (100 ng/ml) for 0, 12, 24, 48, and 72 h. For experiments assessing proteinase activation of PARs, HPBF were incubated with TNF-α (50 ng/ml) for 24 h before conducting experiments. After treatment of HPBF, the medium was replaced with HPBF media containing 0.25 mM sulfinpyrazone and 7 µM Flu-3-acetoxyxymethyl ester (Molecular Probes, Eugene, OR) before incubation for 30 min at 37°C. The coverslips were briefly washed in calcium assay buffer (150 mM NaCl, 3 mM KCl, 1.5 mM CaCl$_2$, 10 mM glucose, 20 mM HEPES, 0.25 mM sulfinpyrazone, pH 7.4) before mounting in a perfusion chamber (Warner Instruments, Hamden, CT) with 500 µl of calcium assay buffer. Cells were stimulated with test agonists by replacing the buffer in the perfusion chamber with agonists prediluted in calcium assay buffer. Responses were measured as a percentage of the signal obtained by the addition of 2 µM ionophore (% A23187). Flu-3 fluorescence was measured through a ×20 objective using Image Master system software and DeltaRAM rapid wavelength-switching illuminator (Photon Technology International, London, ON, Canada) with excitation at 480 nm and emission at 530 nm.

To assess whether a test proteinase was amputating (disarming) the tethered ligand from a PAR, we used an indirect approach routinely employed in the field (20, 29). Briefly, cells are incubated with the disarming proteinase for ~2 min. Successful amputation of the tethered ligand is then monitored by a subsequent application of the appropriate PAR agonist proteinase. The calcium signal in response to the PAR agonist proteinase will be ablated if the tethered ligand has been removed from the PAR by the test (disarming) proteinase. To demonstrate that the disarmed PAR is still functional and present at the cell surface, the appropriate PAR-AP is applied. A calcium signal triggered by the PAR-AP is indicative of a functional receptor at the cell surface, which is unresponsive to the PAR agonist proteinase.

**Proliferation assay.** HPBF cell growth was monitored as described previously (30). In brief, cells were plated into a 96-well culture plate in HPBF medium at a density of 8,000 cells/well and allowed to adhere for 48 h before being placed in serum replacement medium for a further 48 h. In separate experiments designed to determine PAR$_4$ effects on cell growth, serum-starved cells were treated with TNF-α (50 ng/ml) for 24 h following 24 h in serum replacement medium.
Cells were then incubated with either thrombin (0, 0.5, 1.5, 5, and 15 nM), cathepsin-G (0, 0.06, 0.18, 0.6, and 1.8 nM), TFLLR-NH$_2$ (0, 3, 10, 30, and 100 μM), AYPGQV-NH$_2$ (0, 10, 30, and 100 μM), SLIGKV-NH$_2$ (0, 10, 20, 100, and 200 μM) or trypsin (0, 1, 3, 10, and 30 nM) over 96 h before assessment of cell number using the Promega CellTiter 96 AQueous One solution proliferation assay kit. In separate experiments to assess the degree of cell growth in untreated and TNF-α-treated controls, cells were plated in six-well plates and counted using a hemocytometer following the 48 h in serum replacement medium (t = 0) and at the end of the assay (96 h). The effect of PAR agonists on the proliferation of LPS-treated HPBF was not tested.

**RESULTS**

**TNF-α and LPS, selectively upregulate PAR$_2$ and PAR$_4$ mRNA in HPBF.** We have recently reported that HPBF express mRNA and protein for PAR$_1$, PAR$_2$, and PAR$_3$, but not PAR$_4$ (30). Here, our RT-PCR data confirmed that untreated HPBF showed the same PAR profile (see NT in Fig. 1A–H). RT-PCR images are representative images, and densitometry analysis values are means ± SE of three separate experiments performed from HPBF cultures derived from three different subjects. *P < 0.05 between treated HPBF and untreated HPBF (NT).
time course experiments demonstrated that HPBF PAR2 mRNA levels were time dependently upregulated in response to challenge with TNF-α or LPS (Fig. 1, A and B, respectively), but not IL-1α (data not shown). β-actin mRNA levels remained constant across the treatments performed (Fig. 1, I and J). PAR2 mRNA upregulation with TNF-α was rapid and transient, with mRNA levels increasing at 3 h posttreatment and declining to baseline levels thereafter until the final time point tested, 48 h (Fig. 1A). LPS stimulated increases in PAR2 mRNA as early as 3 h (Fig. 1B). However, maximal PAR2 mRNA expression was not observed until 24 h post-LPS challenge. PAR2 mRNA expression declined thereafter, but remained elevated above baseline (Fig. 1B). While PAR4 mRNA was not detected in untreated HPBF (see NT in Fig. 1, C and D), both TNF-α and LPS induced mRNA expression for this receptor but with different kinetics (Fig. 1, C and D). Similar to PAR2, PAR4 mRNA induction with TNF-α was also rapid, with mRNA levels detectable as early as 3 h and reaching a maximum at 6 h posttreatment (Fig. 1C). PAR4 mRNA was still observed 24 h post-TNF-α treatment but was not detectable by 48 h (Fig. 1C). LPS was also found to stimulate PAR4 mRNA levels in HPBF (Fig. 1D), with marked induction of PAR4 mRNA expression observed at 24 and 48 h posttreatment (Fig. 1D). IL-1α (10 ng/ml) had no observable effect on PAR4 mRNA expression over the 48-h time period tested (data not shown). When HPBF were treated with either IL-1α, TNF-α, or LPS, no increases in PAR1 or PAR3 mRNA levels were observed (Fig. 1E–H, IL-1α data not shown).

**TNF-α and LPS upregulate PAR2 cell surface expression in HPBF.** FACS analysis of cell surface PAR expression was carried out to confirm results obtained from RT-PCR analysis (Fig. 2). In TNF-α-treated HPBF (Fig. 2A, i, solid bars), PAR2 cell surface expression was marginally higher than untreated HPBF at 6 h, with a marked increase detected at the 12-h time point. Levels of cell surface PAR2 peaked at 24 h (Fig. 2A, i and ii), then declined but remained higher than baseline levels at 48 h and had returned to baseline by 72 h posttreatment. LPS treatment also upregulated PAR2 cell surface expression (Fig. 2A, i, open bars), with a marginal increase observed at 12 h, peaking at 48 h (Fig. 2A, i, open bars and 2A, iii) and declining but remaining above basal expression at 72 h posttreatment. For PAR1, neither TNF-α nor LPS had a significant effect on cell surface expression at the time points tested (Fig. 2B, i–iii). No shift in either PAR2 or PAR1 cell surface expression was observed in response to IL-1α treatment for 0, 6, 12, 24, 48, or 72 h (data not shown). In keeping with the RT-PCR results, no observable increase in PAR3 cell surface expression was detectable in HPBF treated for 0, 6, 12, 24, 48, or 72 h with either TNF-α, LPS, or IL-1α (data not shown).

We next sought to visualize PAR4 cell surface expression. To determine the efficacy of the PAR4 polyclonal antibody (Santa Cruz), we tested the ability of this antibody to detect PAR4 in our permanently expressing human PAR4 cell line. The PAR4 polyclonal antibody consistently failed to detect PAR4 expression in our cell line, even though cell surface receptor could be demonstrated using an antibody directed

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**Fig. 2.** Effect of TNF-α and LPS on HPBF PAR2 (A) and PAR1 (B) cell surface expression. HPBF were treated with either TNF-α (50 ng/ml) or LPS (100 ng/ml) for up to 72 h before PAR2 and PAR1 cell surface expression was assessed by flow cytometry. A, i: time course of TNF-α (solid bars) and LPS (open bars) stimulated increases in HPBF PAR2 cell surface expression. A, ii and iii: representative histograms showing relative shifts in fluorescence after stimulation with TNF-α and LPS, respectively. B, i: time course showing the effect of TNF-α (solid bars) and LPS (open bars) on HPBF PAR1 cell surface expression. B, ii and iii: representative histograms showing relative shifts in fluorescence following stimulation with TNF-α and LPS, respectively. A, i and B, i: results are expressed as a percentage ± SE of the untreated control (NT) from three separate experiments performed with cells cultured using tissue obtained from different individuals. Stars denote significance (*P* < 0.05) between treated HPBF and untreated HPBF (NT).
aggressively against an epitope (hemagglutinin; HA) fused to the receptor (data not shown). Consequently, we were unable to detect PAR2 expression on the cell surface of either TNF-α, LPS, or IL-1α-treated HPBF (data not shown).

**TNF-α and LPS induce upregulation of functional PAR2 and PAR4 in HPBF.** Having observed the selective upregulation of PAR2 by RT-PCR and flow cytometry and PAR4 upregulation by RT-PCR, we explored whether TNF-α and LPS-treated HPBF displayed an increase in sensitivity toward PAR agonist-triggered increases in intracellular calcium (Figs. 3 and 4). Because HPBF endogenously express PAR2 and to observe clear increases in the upregulation of functional PAR2, cells were challenged with PAR2-AP SLIGKV-NH2 at 50 μM, because this concentration was below the threshold for eliciting a significant calcium response in untreated cells (see NT in Fig. 3, A and C). In contrast, SLIGKV-NH2 (50 μM) clearly triggered a calcium signal in HPBF treated for 6, 12, 24, and 48 h with TNF-α (Fig. 3, A and C). The magnitude of the calcium signal triggered by SLIGKV-NH2 relative to calcium ionophore increased time dependently from 6 h post-TNF-α treatment (Fig. 3C) and reached maximal at 24 h (see Fig. 3, A and C), before declining marginally by 48 h (Fig. 3C). For LPS-treated HPBF, a clear increase in PAR2 signaling to SLIGKV-NH2 was observed (Fig. 3, B and D). In HPBF stimulated with LPS for 12 h, the PAR2-AP SLIGKV-NH2 triggered a small calcium signal (Fig. 3D). However, a robust calcium signal in response to SLIGKV-NH2 was observed by 24 h (Fig. 3, B and D) and 48 h (Fig. 3D), which subsequently declined by 72 h but remained elevated above the untreated control (Fig. 3D).

We next investigated whether PAR4 was functionally present on HPBF following either TNF-α, LPS, or IL-1α treatment (Fig. 4) (data not shown for IL-1α). In untreated HPBF, no calcium signal was observed in response to the PAR4-AP AYPGQV-NH2 (400 μM) (Fig. 4A). TNF-α-stimulated HPBF were, however, responsive to the PAR4-AP AYPGQV-NH2 (400 μM), with a calcium signal observable at 6 h (Fig. 4C), reaching maximal at 24 h (Fig. 4, A and C) and declining, but remaining detectable at 48 h post-treatment (Fig. 4C). For LPS-treated HPBF (Fig. 4D), calcium signaling in response to AYPGQV-NH2 (400 μM) was observed to occur with slower kinetics but remaining elevated for a longer time period than that for TNF-α. AYPGQV-NH2 stimulated a minor calcium signal from as early as 12 h post-LPS treatment (Fig. 4D) with maximal signal observed at 24 h (Fig. 4, B and D), which subsequently remained maximal at the 72-h time point tested (Fig. 4D). For IL-1α-treated HPBF, no increases in intracellular calcium were observed in response to AYPGQV-NH2 (n = 3, data not shown).

**Upregulation of PAR2 sensitizes HPBF to trypsin while tryptase disarms PAR2.** Having demonstrated functionally upregulated PAR2 and PAR4 on TNF-α and LPS-treated HPBF with specific PAR2 and PAR4-APs, we assessed the ability of the PAR-activating proteinases, trypsin, and tryptase to regulate calcium signaling in TNF-α-treated HPBF (Fig. 5). In untreated HPBF, trypsin at 10 nM and 20 nM was not able to trigger a calcium signal (Fig. 5A, ND). However, trypsin stimulated clear calcium responses at 50 nM and 100 nM (Fig. 5A, open bars). In TNF-α-treated HPBF, trypsin generated a robust calcium signal at 20, 50, and 100 nM (Fig. 5A, solid bars). The positive control, thrombin at 5 nM (THR), stimulated a marked calcium response in untreated and TNF-α-treated HPBF (Fig. 5A, open and solid bars). Interestingly, the addition of tryptase 30 nM (TPZ) to TNF-α-treated HPBF triggered no observable calcium signal (Fig. 5B, ND), and the addition of trypsin 20 nM (TP20) following tryptase also failed to trigger a significant calcium signal (Fig. 5B, diagonal hatched bar), compared with the TP20 control (Fig. 5B, open bar). However, the addition of the PAR2-AP SLIGKV-NH2 200 μM (KV) following sequential application of TPZ and TP20 resulted in a calcium signal (Fig. 5B, vertical lined bar) comparable to TK alone (Fig. 5B, cross-hatched bar). Addition of TPZ before the addition of THR had no observable effect on the ability of thrombin to elicit a calcium signal (Fig. 5B, dotted bar), compared with the THR control (Fig. 5B, horizon-
Cathepsin-G disarms HPBF PAR1 and PAR2, while induction of PAR2 expression enables cathepsin-G and thrombin to trigger PAR4 dependent calcium signaling. We next assessed the ability of cathepsin-G to 1) modulate PAR2 and PAR1 signaling in untreated HPBF, and 2) trigger a PAR4-dependent calcium signal in TNF-α-treated HPBF (Fig. 6). Although 3 nM cathepsin-G (CG3) had no observable effect on calcium signaling in unstimulated HPBF (Fig. 6A, ND), addition of 50 nM trypsin (TP50) following CG3 addition resulted in a marked reduction in the response to TP50 (Fig. 6A, diagonal hatched bar) compared with its respective control (Fig. 6A, open bar). Addition of TP50 before the addition of 200 μM SLIGKV-NH2 (KV) markedly reduced the response to KV (Fig. 6A, solid bar) compared with the response obtained with KV alone (Fig. 6A, cross-hatched). In contrast, addition of KV following sequential addition of CG3 and TP50 challenge (Fig. 6A, vertical hatched bar) resulted in a response that was of similar magnitude to its respective control (Fig. 6A, cross-hatched bar).

Unlike our observations with PAR2, prior application of CG3 had little effect on the ability of 5 nM thrombin (THR) to trigger a calcium response (Fig. 6B, black dotted bar), compared with THR added alone (Fig. 6B, horizontal dashed bar). Similarly, the response to 100 μM TFLLLR-NH2 (TF) following CG3 and THR (Fig. 6B, checkered bar) was similar to that obtained following just THR addition (Fig. 6B, open bar) but significantly reduced compared with the TF control response (Fig. 6B, vertical dashed bar). Addition of 30 nM cathepsin-G (CG30) also had no observable effect on calcium signaling (Fig. 6B, ND); however, the THR response was markedly reduced following prior application of CG30 (Fig. 6B, solid bar). Although the THR response was reduced following CG30 addition, subsequent addition of TF resulted in a response (Fig. 6B, dotted bar) that was similar to TF alone (Fig. 6B, vertical dashed bar).

In TNF-α-treated HPBF CG3 stimulated a clear calcium response that was of similar magnitude to that obtained by 400 μM AYPGQV-NH2 (QV) (Fig. 6C, open bar and diagonal hatched bar, respectively). Prior addition of QV abolished the ability of CG3 to trigger a calcium response (Fig. 6C, 1st ND bar), while prior application of CG3 markedly reduced the ability of QV to stimulate a calcium response (Fig. 6C, cross-hatched bar). The THR response following addition of QV (Fig. 6C, checkered bar) was similar in magnitude to THR added alone (Fig. 6C, horizontal dashed bar). A THR response following TF application was observed in TNF-α-treated (Fig. 6C, dotted bar) but not in untreated HPBF (data not shown). Finally, no observable THR response was observed following the sequential addition of TF and QV (Fig. 6C, 2nd ND bar).

PAR4 agonists reduce cell number of TNF-α-treated, but not untreated HPBF. We next assessed whether upregulation of PAR2 and PAR4 on HPBF resulted in an altered proliferative response to a number of PAR agonists compared with untreated cells. Marked differences in cell growth were observed between TNF-α treated HPBF and untreated HPBF for the PAR4 agonists cathepsin-G and AYPGQV-NH2, the PAR1/PAR4 agonist thrombin, but not for the PAR1 agonist TFLLLR-NH2 (Fig. 7). The selective PAR1-AP TFLLLR-NH2 stimulated significant HPBF growth with similar magnitude in both TNF-α-treated HPBF and untreated HPBF (7A, ■ and ▲, respectively). For the selective PAR4-AP AYPGQV-NH2, there was a marginal reduction in cell number in untreated HPBF (7B, ▲). In TNF-α-treated HPBF, AYPGQV-NH2 reduced cell number in a concentration-dependent fashion that was maximal at 30 and 100 μM (Fig. 7B, ■). The PAR4 activating proteinase cathepsin-G stimulated marginal growth in untreated HPBF at 0.06 and 0.18 nM but had no observable effect at 0.6 and 1.8 nM (Fig. 7C, ▲). In TNF-α-treated HPBF, cathepsin-G stimulated a concentration-dependent reduction in cell number, reaching maximal reduction at 0.6 and 1.8 nM (Fig. 7C, ■). In the cathepsin-G and AYPGQV-NH2 experiments, cells were inspected morphologically at the 96-h time point and appeared healthy, with no obvious lifting and rounding of cells or significant evidence of cell debris (data not shown). We also observed no significant change in the cell number of either untreated or TNF-α-treated HPBF at the 96-h
incubation time point of the assay (untreated = 103 ± 6% and TNF-α treated = 94 ± 6% at t = 96 h, n = 3. Results expressed as a percentage of the cell number at t = 0 h). In contrast with the other PAR agonists tested, the PAR2/PAR4 agonist thrombin stimulated significant growth at all concentrations tested in untreated HPBF (Fig. 7D, △). However, in TNF-α-treated HPBF, where PAR4 expression was induced, thrombin at the same concentrations was unable to stimulate HPBF growth (Fig. 7D, ▽), although a marginal (but not significant) proliferative effect was still observed at the lowest thrombin concentration of 0.5 nM. We have previously reported that the PAR2 agonists trypsin and SLIGKV-NH2 were unable to stimulate proliferation of untreated HPBF (30). Here, we show that upregulation of PAR2 and PAR4 expression by TNF-α had minimal effect on the ability of SLIGKV-NH2 (Fig. 7E) or trypsin (0, 1, 3, 10, and 30 nM, data not shown) to stimulate HPBF proliferation.

DISCUSSION

We report for the first time that 1) LPS and TNF-α induce expression of functional PAR1 and upregulate constitutive PAR2 expression in HPBF, 2) induction of PAR4 enables cathepsin-G-mediated calcium signaling in HPBF, and 3) PAR4 counters PAR1-stimulated HPBF proliferation. Upregulation of PAR2 expression enhanced calcium responses to the PAR2 agonists trypsin and SLIGKV-NH2, while induction of PAR4 expression enabled the PAR4 agonists AYPGQV-NH2 and cathepsin-G to trigger a calcium signal. Cathepsin-G was also found to disarm both PAR1 and PAR2, while trypsin was surprisingly found to disarm PAR2. PAR4 induction also enabled thrombin to signal through both PAR1 and PAR4. In HPBF growth assays, cathepsin-G and AYPGQV-NH2 reduced HPBF cell number only when PAR4 was induced. Moreover, the mitogenic effect of thrombin but not TFLLR-NH2 was ablated when PAR4 was expressed. Thus, the upregulation of HPBF PAR2 may heighten the sensitivity of these cells to PAR2 proteinases, while the induction of PAR4 may modify the responsiveness of HPBF to thrombin, cathepsin-G, and other proteinases during an inflammatory response.

We have previously demonstrated that HPBF express PAR1, PAR2, and PAR3, but not PAR4, with only PAR1 and PAR2 able to signal through calcium (30). Others have also reported the same PAR profile on lung and synovial fibroblasts (1, 33). In this study, RT-PCR consistently showed that TNF-α and LPS selectively upregulated PAR2 and PAR4 mRNA. TNF-α induced a rapid and transient upregulation of PAR2 and PAR4 mRNA, while LPS induced a later but sustained expression, especially for PAR4, where maximal expression was observed for up to 48 h. In contrast, others have reported that TNF-α was unable to upregulate PAR2 or induce PAR4 mRNA expression in skin and synovial fibroblasts (1, 15). These differences in results may be due to the differing origins of the fibroblasts used in these studies, or because the concentrations of TNF-α utilized were too low to upregulate PAR expression. We note that similar concentrations of TNF-α (50 ng/ml) used in our study have been employed previously to upregulate PAR2 (28) and PAR4 (16). Nevertheless, flow cytometry confirmed our RT-PCR observation that PAR1 and PAR3 levels remained unchanged in response to the inflammatory mediators tested, while PAR2 cell surface expression was clearly increased in response to both TNF-α and LPS. The kinetics of PAR2 upregulation in response to TNF-α and LPS were in keeping with those of the RT-PCR, suggesting an earlier (12 h) marked upregulation of PAR2 in response to TNF-α compared with LPS (48 h), which remained elevated for up to 72 h. Unfortunately, we were unable to obtain reliable data for PAR3 by flow cytometry. The PAR3 polyclonal antibody consistently failed to detect any changes in PAR3 expression on HPBF in response to either TNF-α or LPS treatment or in our permanently expressing human PAR4 cell line. We note that others (7) have also reported inconsistencies with the specificity of the PAR3 antibody used in this study. Nevertheless, our data collectively demonstrated that TNF-α and LPS, but not IL-1α selectively upregulate PAR2 and induce PAR4 expression on HPBF.

Through measuring agonist-triggered increases in intracellular calcium as an index of receptor function, we observed a time-dependent increase in sensitivity to the PAR2-AP SLIGKV-NH2 in TNF-α and LPS-treated HPBF. These results
confirmed our RT-PCR and flow cytometry data, thus providing clear evidence for the upregulation of PAR2. Interestingly, the PAR2 activating proteinase trypsin, appeared to disarm PAR2 in both TNF-α-treated and untreated HPBF, since trypsin failed to trigger a calcium response on application, where SLIGKV-NH2 did. Tryptase’s disarming effect was shown to fail to trigger a calcium response on application, while untreated or treated for 24 h with TNF-α (50 ng/ml). Calcium signaling experiments were performed as outlined in MATERIALS AND METHODS. Disarming of PAR2 by thrombin’s ability to still elicit a calcium signal was unable to stimulate any observable calcium signal in untreated HPBF. Where two agents were added before the addition of the final agonist, arrows indicate the order of addition. Results are expressed as the means ± SE from two to six separate experiments. *P < 0.05 between indicated responses.

Because cathepsin-G has been reported to have both activating and disarming actions on PARs (12, 14, 26, 31, 32, 36), we tested the role of this enzyme in regulating PAR activity in HPBF. We found that cathepsin-G at 3 nM was unable to generate a calcium signal but could clearly disarm HPBF PAR2, preventing activation by trypsin, but not by the PAR2-AP. Uehara and coworkers (36) reported that cathepsin-G (10 μM but not 1 μM) activates PAR2 in gingival fibroblasts, while Dulon and colleagues (14) found that cathepsin-G (0.5 μM) disarmed PAR2 on airway epithelial cells. Although not tested directly, the ability of cathepsin-G to activate/disarm PAR2 would appear to be concentration dependent, with low concentrations (3–500 nM) disarming PAR2, while higher concentrations (10–100 μM) activate the receptor. In the same setting, we found that cathepsin-G (3 nM) failed to disarm PAR1, but 30 nM cathepsin-G (which was also unable to generate a calcium signal) clearly ablated thrombin responses but not that of the PAR1-AP. Our finding is in accord with previous reports that have demonstrated disarming of PAR1 by cathepsin-G (31), in addition to that of Molino and co-workers (26) who identified Phe55-Trp56 as the region cleaved within PAR1, while induction of PAR4 expression by LPS and TNF-α regulate PAR expression in these cells.

For PAR4, we also observed a clear induction of functional receptor in response to TNF-α and LPS, since the PAR4-AP was unable to stimulate any observable calcium signal in untreated cells. In accord with the RT-PCR data, calcium signaling experiments with the PAR4-AP confirmed that TNF-α stimulated the rapid induction of functional PAR4 compared with that observed for LPS, which was slow in onset and remained elevated for up to 72 h. The difference in kinetics between the ability of these mediators to induce PAR4 expression may be a result of LPS inducing the synthesis of an inflammatory mediator from HPBF, which subsequently stimulates PAR4 expression. However, further work is required to elucidate the mechanisms by which LPS and TNF-α regulate PAR expression in these cells.
specific proteinase. Similarly, using a desensitization approach we demonstrated that when PAR4 was induced, thrombin could signal through both PAR1 and PAR4. However, the PAR4 calcium signal triggered by thrombin was more modest compared with the robust PAR1 calcium signal. Regardless, the upregulation of PAR4 would not only allow HPBF to respond to additional serine proteinases during an inflammatory reaction, such as cathepsin-G and the kallikreins (29) but also enable thrombin to activate signaling pathways downstream of both PAR1 and PAR4. Furthermore, in the presence of proteinases that disarm PAR1, thrombin signaling would switch to a PAR4 signaling pathway, which could have a significant impact on modifying cellular responses. These findings may have particular relevance for the profibrotic actions of thrombin on lung fibroblasts, which have been demonstrated to be primarily mediated via PAR1 (5, 9, 10).

Finally, we assessed whether upregulation of PAR2 and PAR4 on HPBF would result in differential growth responses to a panel of PAR agonists, compared with untreated HPBF. Although PAR2 agonists had no effect on either TNF-α treated or untreated HPBF, interestingly the PAR4 agonists cathepsin-G and AYPGQV-NH₂ selectively reduced cell number in the TNF-α-treated HPBF where PAR4 was induced. The slight inhibitory effect of low concentrations of AYPGQV-NH₂ and the marginal proliferative effect of cathepsin-G observed in the untreated HPBF is suggestive that these agonists may stimulate other non-PAR4 signaling pathways. However, the striking AYPGQV-NH₂ and cathepsin-G-triggered reduction in cell number in the TNF-α-treated HPBF imply that the PAR4 signaling pathway prevails once this receptor is available at the cell surface. In the TNF-α-treated HPBF incubated with cathepsin-G or AYPGQV-NH₂, no observable lifting or rounding of cells was detected by morphological analysis at the end of the assay, even though cell number was lower than that in control samples. Further studies in our laboratory are currently assessing the mechanism of reduced cell number, including consideration of possibilities such as apoptosis. Nevertheless, in contrast to PAR4, the PAR1-AP TFLLR-NH₂ stimulated growth of both TNF-α-treated and untreated HPBF, indicating that thrombin’s proliferative effect on lung fibroblasts is PAR₁ mediated, which is in agreement with previous reports (4, 10, 35). More interesting was our observation that thrombin was unable to stimulate growth of TNF-α-treated HPBF, where PAR4 was induced. However, we note that the lowest concentration of thrombin tested (0.5 nM) triggered a minor (but not significant) proliferative response in the TNF-α-treated cells.

Fig. 7. Effect of PAR agonists on TNF-α-treated and untreated HPBF. A–E: HPBF were initially placed in serum replacement media for either 24 h (NT), or were treated with TNF-α (50 ng/ml) for 48 h in serum replacement media following 24 h in serum replacement media. HPBF were then stimulated with agonists for 96 h before assessment of cell number using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Growth responses of TNF-α-treated (●) and untreated HPBF (▲) to TFLLR-NH₂ (A), AYPGQV-NH₂ (B), cathepsin-G (C), thrombin (D), and SLIGKV-NH₂ (E). Results are expressed as the means ± SE from at least three different experiments performed in triplicate using cells derived from different subjects. *P < 0.05 between effect of test agent in untreated HPBF compared with effect in TNF-α treated HPBF.
presumably via exclusive PAR1 activation, since thrombin activates PAR1 and not PAR4 at low concentrations (19). The complete loss of thrombin’s mitogenic activity at the other concentrations tested may be the net response of this enzyme activating both PAR1 and PAR4 in these cells, implying that PAR4 counterbalances PAR1-dependent lung fibroblast proliferation. Indeed, PAR4 and PAR1 have been recently reported to counterbalance the release of proangiogenic (VEGF) and antiangiogenic (endostatin) mediators from human platelets (22), with thrombin (PAR1/PAR4 agonist) having no net effect on either endostatin or VEGF release. Thus, the ability to upregulate PAR4 in HPBF may provide insights into a novel mechanism, whereby PAR1-mediated effects by thrombin could be modified. However, further work is required to elucidate the role of PAR4 in HPBF.

We conclude that the selective upregulation of PAR2 and PAR4 on HPBF in response to inflammatory stimuli may form part of an elegant system for regulating airway fibroblast responses to specific activating and inactivating proteinases. Thus, the finding that PAR4 may play a role in regulating lung fibroblast function represents a very interesting topic with relevance to fibrotic lung disease and merits further study.

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