Regulation of protease-activated receptor-1 in mononuclear cells by neutrophil proteases

N. Roche, R. G. Stirling, S. Lim, B. G. Oliver and K. F. Chung

Department of Thoracic Medicine, Imperial College School of Medicine at National Heart & Lung Institute, London, UK

Abstract  Neutrophils and mononuclear cells are implicated in the pathogenesis of several inflammatory conditions including chronic obstructive pulmonary disease (COPD). Neutrophil-derived serine proteases, such as cathepsin G (CG) and neutrophil elastase (NE), may interact with mononuclear cells via protease-activated receptors (PARs), which are seven-transmembrane G protein-coupled receptors activated by proteolytic cleavage of the extracellular N-terminus, and which, on activation, induce the release of several mediators and cytokines. We determined whether CG and NE could affect PAR-1 expression and function in mononuclear cells. Human blood mononuclear cells were isolated from 20 healthy donors. Surface and intracellular receptor expression and calcium mobilisation (using the calcium chelator, FLUO3-AM) were studied by fluorescence-assisted cell sorting (FACS analysis). Positive controls, i.e. thrombin (0.1–100 mU/ml) and the PAR-1-activating peptide SFLLRN (100 μM), induced a rapid and transient internalisation of PAR-1 in monocytes and lymphocytes. CG but not NE had a similar effect. By contrast, in monocytes intracellular calcium mobilisation was induced by thrombin and SFLLRN but not by CG and NE. Thus, CG can induce intracellular PAR-1 sequestration without activation of the receptor, and may act as an antagonist and prevent subsequent activation of PAR-1 in mononuclear cells. These findings may be of relevance to the pathogenesis of COPD.

Received 5 May 2002, accepted in revised form 8 August 2002
Correspondence should be addressed to: prof. K. F. Chung, National Heart & Lung Institute, Imperial College School of Medicine, Dovehouse Street, London SW3 6LY, UK. Fax: +44-0171 351 8126; E-mail: f.chung@ic.ac.uk

INTRODUCTION

Protease-activated receptors (PARs, PAR-1 to PAR-4) are seven-transmembrane G protein-coupled receptors that are activated through unique mechanism of site-specific cleavage by serine proteases (I). The main agonist of PAR-1, PAR-3 and PAR-4 is thrombin, while trypsin is the most potent agonist of PAR-2 (I–3). Several PAR-mediated effects may be relevant in airway diseases: PAR-1 and PAR-2 have been shown to regulate bronchoconstrictor responses of human airways and release of several cytokines and mediators by monocytes/macrophages, mast cells, endothelial and epithelial cells, fibroblasts and smooth muscle cells (4–10).

The site of cleavage of PARs by serine-proteases is located on the NH2-terminal exodomain of the receptor; cleavage yields a new NH2 terminus, which acts as a tethered ligand and binds to, and activates the receptor (I). Activation of these receptors is an irreversible phenomenon; cleaved receptors are phosphorylated, uncoupled from their G protein, internalised and degraded in lysosomes, while cell sensitivity to their agonists is restored by new receptors which arise from an intracellular pool (II). Some proteases can cleave PARs at sites located downstream of the tethered ligand, or cleave the tethered ligand itself, thereby rendering the receptor inactive and/or unresponsive to subsequent enzymatic activation (I). Neutrophil elastase (NE) is one of the enzymes that may inhibit activation of PAR-1 by this mechanism, while cathepsin G (CG), another neutrophil serine protease, can both activate PAR-1 by cleavage at the thrombin site and deactivate it by downstream cleavage (I).

PARs are widely distributed and are expressed in several cell types (I) including monocyte/macrophages and lymphocytes. Therefore, we hypothesised whether these cells could be affected by neutrophil serine proteases through PAR-1 cleavage. Such an interaction could occur in chronic inflammatory conditions such as chronic obstructive airways disease in which there is accumulation of monocytes/macrophages, T lymphocytes and neutrophils, and in which products of activated neutrophils such as NE and CG may play a major role (I2,13). We determined the effects of NE and CG on the expression of PAR-1 in mononuclear cells.
and function of PAR-1 in monocytes and lymphocytes, by using fluorescence-assisted cell sorting (FACS) to examine the internalisation and recycling of receptors and their coupling to intracellular calcium release.

**MATERIAL AND METHODS**

**Isolation of peripheral blood monocytes**

Peripheral venous blood from 20 healthy non-smoking volunteers was mixed with acid-citrate dextrose (1:6 vol/vol) and sedimented on dextran (6% in 0.9% NaCl) for 40 min. Mononuclear cells were separated by Ficoll-Hypaque density centrifugation. Cells were washed twice with Hanks’ balanced salt solution (HBSS) and plated at a concentration of 1 \times 10^6 monocytes/ml in 12-well plates. Cell viability was consistently >96% as assessed by trypan blue exclusion. At 24 h, cells were used to study the effect of agonists on calcium flux and PAR-1 surface expression.

**Regulation of PAR-1 expression**

PAR-1 expression was assessed by FACS. Two preliminary experiments were performed to determine the time-course of PAR-1 expression: firstly, we assessed the kinetics of the decrease in PAR-1 expression after incubation with SFLLRN (PAR-1 agonist, 100 \mu M) for 10, 30, 60, 120, and 240 min (n = 3 for each condition). Secondly, we studied the kinetics of PAR-1 re-appearance: cells were first incubated with SFLLRN during 1 h before being washed and incubated in SFLLRN-free medium for 0, 2, 4 or 24 h (n = 3 for each condition).

To study the effects of agonists and enzymes, cells were incubated for 1 h in the presence of medium alone or containing the tested agent. Optimal dosage of tested agents was first assessed by dose-response experiments with concentrations of 1, 10, and 100 \mu M for SFLLRN; 1, 10, and 100 \mu M for thrombin; 10^{-9}, 10^{-8} and 10^{-7} M for C5; 10^{-8}, 10^{-7}, and 10^{-6} M for NE; and 1, 10, and 100 \mu M for PAR-1 antagonist peptide (H-Met-Ser-Arg-Pro-Ala-Cys-Pro-Asn-Asp-Lys-Tyr-Glu-OH, negative control) (N = 3 per condition). The effect of the optimal dosage of each agent was then confirmed in three additional samples.

After incubation, cells were harvested by scraping, washed in 1 ml HBSS, and re-suspended in 100 \mu l Dulbecco’s PBS without calcium and magnesium. In order to assess surface expression of PAR-1, cells were incubated during 30 min with anti-PAR-1 phycoerythrin (PE)-conjugated antibody (7:100). To assess the intracellular pool of PAR-1, cells were first incubated with anti-CD14 antibody before being permeabilised using DAKO IntraStain Kit (Cambridge, U.K.), and incubated with anti-PAR-1 SPAN12 or WEDEIS antibodies. After a final wash, cells were re-suspended in flow cytometry buffer (FACSflow, Becton Dickinson, San Jose, CA, U.S.A.) containing 1% paraformaldehyde, and fluorescence was measured by a FACSScan flow cytometer (Becton Dickinson, San Francisco, CA, U.S.A.). Forward scatter (FSC) and side scatter (SSC) characteristics were recorded on a linear scale while fluorescence at 530 nm (FL1 channel) and 670 nm (FL2 channel) were recorded on a logarithmic scale. Monocytes and lymphocytes were identified using both the FSC–SSC and intensity of CD14 staining as recorded on the FL1 channel, in which emissions from FITC conjugates are recorded. To assess PAR-1 expression, we analysed the percentage of gated cells and the mean (MFI) and specific (SFI) fluorescence intensity of each cell type in the FL2 histograms, since PE-conjugates emit in the FL2 channel. A PE-conjugated mouse IgG2a with no known reactivity to human antigens was used as isotype control. Specific fluorescence intensity was calculated as follows: SFI = sample MFI – isotype control MFI.

**Measurement of intracellular calcium mobilisation**

Measurement of intracellular calcium mobilisation was performed using flow cytometry with Fluo3/AM, a fluorescent calcium chelator which enters cells where it is metabolised to membrane-impermeant Fluo3. The spontaneous fluorescence of Fluo3 increases after calcium chelation and can be read at 530 nm (FL1 channel) after excitation at 488 nm. Cells were first incubated during 15 min in RPMI medium containing 2 \mu M of Fluo3/AM, harvested by scraping and washed twice in HBSS before being resuspended in 0.5 ml of phenol red-free RPMI 1640 in 5-ml rounded-bottom polystyrene tubes (Falcon). Thirty minutes later, measurements were performed using a FACScan flow cytometer (Becton Dickinson); after two reproducible (<5% variation) baseline measurements, tested agents were added at the optimal concentration that induced a decrease in receptor expression (SFLLRN, 100 \mu M; thrombin, 100 \mu M; caltepsin G, 10^{-6} M; neutrophil elastase, 10^{-6} M; and PAR-1 antagonist peptide, 100 \mu M). Then, fluorescence in the FL1 channel was recorded repeatedly until baseline values were reached again. When no response was observed, recordings were repeated during 1 h. At least 5000 events were recorded for each measurement and four experiments were performed for each tested agent. For the analysis, a FSC–SSC dot plot was used to gate on monocytes and lymphocytes, and changes of mean FL1 fluorescence intensity (MFI) in each gate were analysed.
Reagents

Complete monocyte/macrophage culture medium was RPMI-1640 (ICN-Flow, High Wycombe, U.K.) containing 10% heat-inactivated fetal calf serum (Sera Lab, Crawley, U.K.), 2 mM L-glutamine (ICN-flow), and penicillin-streptomycin (100 U/ml – 100 ml, ICN flow). Culture plates were from Falcon (London, U.K.). PAR-1 agonist peptide SFLLRN (PAR-1) was from Bachem (Saffron Walden, U.K.). Thrombin (1000 NIH Units/ml) was from Sigma, elastase (150 U/mg of protein) was from Calbiochem (Nottingham, U.K.), and cathepsin G (2–4 U/mg) was from ICN-Flow. Anti-PAR-1 antibodies were from Santa Cruz Biotechnology (Calne, U.K.). FITC-conjugated monoclonal anti-CD14 mouse IgG2a was from Sigma. Fluo3/AM was from Alexis corporation (Nottingham, U.K.).

Data analysis

Results are expressed as mean ± SEM. Comparisons were performed using non-parametric tests. A threshold of $P > 0.05$ was considered for statistical significance. All statistics were performed using SPSS 7.5 software (SPSS Inc., Chicago, IL, U.S.A).

RESULTS

Regulation of PAR-1 expression

PAR-1 was expressed by 44% of lymphocytes and 28% of monocytes at baseline ($P > 0.01$) but SFI was similar in the two cell types. The decrease in PAR-1 expression after exposure to SFLLRN (100 µM) was maximal at 10 min in lymphocytes and at 60 min in monocytes (Fig 1). Therefore, subsequent experiments were studied after 1 h incubation. PAR-1 surface expression returned to baseline levels 2 h after cessation of exposure in both monocytes and lymphocytes (Fig 1).

The surface expression of intact and total (intact+cleaved) PAR-1 in lymphocytes and monocytes was markedly decreased after incubation in the presence of SFLLRN (concentration for maximal effect: 100 µM), thrombin (concentration for maximal effect: 100 U/ml), CG (concentration for maximal effect: 10 $^{-9}$M) while elastase (concentration up to 10 $^{-6}$M) and PAR-1 antagonist peptide (100 µM) had no significant effect (Fig 2). In parallel, the intracellular expression of intact and total PAR-1 was not affected by SFLLRN, thrombin and CG (data not shown).

Intracellular calcium mobilisation

In monocytes, SFLLRN and thrombin but not CG and NE elicited a transient intracellular calcium flux (Fig 3). We could not obtain reproducible results in lymphocytes, which is likely due to an insufficient sensitivity of the FACS method for assessing calcium mobilisation in these cells.

DISCUSSION

We have shown that both monocytes and lymphocytes express PAR-1, and that this expression is markedly reduced by exposure to known PAR-1 agonists such as thrombin and the peptide SFLLRN. Of the two neutrophil serine proteases, CG but not elastase also caused a reduction in PAR-1 receptor expression in both lymphocytes and monocytes. Reduction of PAR-1 expression after exposure to SFLLRN was maximal at 1 h; expression recovered 2 h after washing-off of the peptide but
remained low in case of prolonged exposure. The calcium release data indicate that both cathepsin and elastase do not induce intracellular calcium release, despite the ability of CG to induce internalisation of PAR-1 receptors. However, thrombin and the PAR-1 agonist, SFLLRN, both induce calcium mobilisation, thrombin being a more potent effector in this respect. Altogether, these data indicate that CG could act as an antagonist at the PAR-1 receptor.

Peripheral blood lymphocytes and monocytes have both been shown to express PAR-1 mRNA (14,15). Although the functional significance of PAR-1 activation in these cells is not known, this receptor seems to participate in inflammatory phenomena: in monocytes, the activation of PARs induces the release of several chemoattractants or pro-inflammatory cytokines including monocyte chemotactic protein-1, IL-1β, TNF-α and IL-6 [1,15–18]. Since lymphocytes, monocytes and neutrophils are the main inflammatory cells involved in the pathogenesis of airway inflammation and damage in chronic obstructive pulmonary disease (COPD) (12,13), our hypothesis...
was that neutrophil serine proteases could modulate mononuclear cells activation through effects on PAR-1. Studies with PAR-1 antagonists and cross-desensitisation experiments remain to be performed to test this possibility. Protease inhibitors would not be suitable in this respect since they do not avoid non-specific enzymatic effects mediated by pathways other than PAR-1.

NE and CG are both products of activated neutrophils, which are recruited to and activated in the airways of COPD patients (12,19), and produce increased amounts of NE; this enzyme is involved in the development of emphysema through its effect on the extracellular matrix (20,21) and induces goblet cell degranulation (22,23), plasma protein extravasation (24), epithelial cell detachment, and production of IL-8 and PGE$_2$ by epithelial cells (21). CG also causes mucus secretion and epithelial cell detachment (21,25) and induces airway hyperresponsiveness (26). Our data indicate that neutrophil serine proteases are not significant activators of intracellular calcium mobilisation in monocytes despite the clear expression of functional PAR-1 by these cells and the potential ability of CG to cleave this receptor at the thrombin activating site. Conversely, CG appears capable of causing intracellular sequestration of PAR-1 receptors in both monocytes and lymphocytes without receptor activation, which illustrates the dissociation between these two events. This may lead to cellular desensitisation, thereby preventing further PAR-1 activation by thrombin or CG itself.

One potential limitation of our study of PAR-1 internalisation is that, in preliminary studies, we assessed the kinetics of this phenomenon only with PAR-1 agonist peptide SFLLRN; but this may differ with CG, elastase or thrombin. However, these agonists act much more quickly than the agonist peptides, which are also less potent. The reason for this is that the 3D conformation of the uncleaved receptor, in which the unaltered NH$_2$ terminus partially masks the ligand binding site, makes it less accessible. In addition, in both monocytes and lymphocytes, a significant effect was observed between 10 min and 4 h after SFLLRN addition, making it unlikely to miss an effect of enzymatic agonists at 1 h. Finally, the lack of intracellular calcium mobilisation that we observed after stimulation with CG is unlikely to be related to a difference in kinetics since all samples were studied up to 1 h after addition of studied agents, the effect of SFLLRN and thrombin being observed within 5 min.

PAR-1 is present on both the plasma membrane and intracellularly. There is a substantial intracellular pool, from which new receptors are translocated to the plasma membrane to restore cell sensitivity to PAR-1 agonists (I). Activated cell surface PAR-1 is known to be rapidly internalised and then sorted out predominantly to lysosomes (27). This is in agreement with the decrease in PAR-1 surface expression, but not in global cellular expression that we found after exposure to thrombin and the peptide SFLLRN, which both induced cell activation as assessed by intracellular calcium mobilisation. Receptor internalisation, which is mediated by phosphorylation of the cytoplasmic tail of the receptor, has also been found to occur independently of activation, either spontaneously (as part of a partial trafficking between plasma membrane and intracellular pool) or after non-activating cleavage (II,27–31). Such a phenomenon is consistent with our findings with CG, which induced a decrease in surface PAR-1 expression in the absence of calcium signal. CG can cleave PAR-1 at the thrombin site Arg$_{41}$Ser$_{42}$ but also at two non-activating sites located downstream to the thrombin site, i.e., Phe$_{43}$Leu$_{44}$ and Phe$_{55}$Tryp$_{56}$ (32). This non-activating cleavage leads to removal of the tethered ligand and generates an unresponsive receptor that is internalised and degraded (27). Thus, our findings with CG may reflect a predominance of non-activating over activating cleavage of PAR-1 in human monocytes, subsequently rendering the cell unresponsive to activation by CG or thrombin. This is in agreement with the data of Molino et al. in platelets and endothelial cells, in which activating cleavage was prevented by removal of the tethered ligand through downstream cleavage by CG at the Phe$_{55}$Tryp$_{56}$ site (32).

We did not find any evidence of PAR-1 internalisation after exposure to elastase, which also did not induce any detectable calcium mobilisation. However, a non-activating PAR-1 cleavage site by elastase has been identified (33). Our findings suggest that such a cleavage either does not occur (e.g. because the cleavage site is not sufficiently accessible due to molecular conformation of surface PAR-1), or does not induce the signalling which is required to produce receptor internalisation (i.e. phosphorylation of intracellular carboxy-terminal tail).

In conclusion, our data indicate that the neutrophil enzymes, CG and NE, do not activate mononuclear inflammatory cells through PAR-1 cleavage.

Acknowledgements

N.R. is supported by grants from European Respiratory Society and the Societe de Pneumologie de Langue Francaise. S.L. is supported by Astra DRACO.

REFERENCES


