

Effect of acute and chronic inflammatory stimuli on expression of protease-activated receptors 1 and 2 in alveolar macrophages

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Background: Protease-activated receptors 1 and 2 (PAR-1 and PAR-2) are 7-transmembrane G protein-coupled receptors activated by serine proteases in many cell types, including monocytes-macrophages, leading to the production of pro-inflammatory mediators, cytokines, and growth factors.

Objective: We determined the influence of chronic smoking and asthma on the expression of PAR-1 and PAR-2 receptors on alveolar macrophages (AMs).

Methods: We used RT-PCR and immunocytochemistry with confocal microscopy to determine mRNA and protein expression of PAR-1 and PAR-2 in AMs obtained from healthy smokers, asthmatic patients, and healthy subjects. In addition, we examined the effect of IL-1 β and LPS.

Results: *PAR1* mRNA was decreased, whereas *PAR2* mRNA was increased in 24-hour cultured AMs from smokers when compared with values in AMs from healthy subjects. Paradoxically, there was a higher degree of PAR-1 protein staining in AMs from smokers, whereas PAR-2 staining was similar in smokers and healthy subjects. PAR-1 and PAR-2 mRNA and protein expression were similar in asthmatic patients and control subjects. IL-1 β and LPS had no effect on *PAR1* and *PAR2* gene expression by AMs.

Conclusions: There is a dissociation between gene and protein expression of PAR-1 and PAR-2. PAR-1 protein overexpression in AMs from smokers might be important in the pathophysiology of chronic airways disease. (*J Allergy Clin Immunol* 2003;111:367-73.)

Key words: Protease-activated receptors, chronic airways disease, asthma, alveolar macrophages

Abbreviations used

AM:	Alveolar macrophage
BAL:	Bronchoalveolar lavage
COPD:	Chronic obstructive pulmonary disease
GAPDH:	Reduced glyceraldehyde-phosphate dehydrogenase
PAR:	Protease-activated receptor

Protease-activated receptors (PARs) are widely distributed 7-transmembrane G protein-coupled receptors that are activated through site-specific cleavage by serine proteases and have been implicated in numerous processes, including regulation of smooth muscle contraction, inflammation, healing, and remodeling.¹ Four members of this family of receptors have been identified thus far and identified as PAR-1 to PAR-4. The main agonist of PAR-1, PAR-3, and PAR-4 is thrombin, whereas trypsin is the main agonist of PAR-2 and also activates PAR-1 and PAR-4.¹ The site of receptor cleavage 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. Activation of these receptors is an irreversible phenomenon; cleaved receptors are internalized and degraded by lysosomes, whereas cell sensitivity to their agonists is restored by new receptors, which arise from an intracellular pool.²

PARs are expressed by several cell types, including platelets, endothelial cells, epithelial cells of several organs (including the lung), smooth muscle cells, fibroblasts, and inflammatory cells (including monocytes, lymphocytes, and neutrophils).¹ In guinea pigs and human subjects, thrombin induces bronchoconstriction through PAR-1 activation, with this effect being mediated by the release of platelet mediators in guinea pigs.^{3,4} Conversely, activation of airway epithelial PAR-2 induces cyclooxygenase-dependent relaxation of airway preparations from human subjects and rodents.^{5,6} In addition, these receptors are involved in inflammatory processes because PAR-1 and PAR-2 activation increases the release of mediators and cytokines by several cell types, including mononuclear cells, and increases vascular paracellular permeability.⁷⁻¹¹ Chronic airway inflam-

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mation is a major characteristic of asthma and cigarette smoking. However, the relevance of the potential pro-inflammatory roles of PARs in chronic airways diseases is unclear, and the effect of inflammatory factors on the expression of PARs in airway cells is not known.

The pathophysiology of cigarette smoke-induced airways disease and of asthma involves several serine proteases secreted by neutrophils (ie, elastase, cathepsin G, and proteinase 3) and mast cells (ie, tryptase and chymase), respectively; in addition, some allergens (ie, *Der-matophagoides pteronyssinus*) also have protease activity.¹²⁻¹⁴ Interaction between these enzymes and PARs might lead to airway damage, inflammation, and hyper-responsiveness. Because macrophages appear to be key cells in the inflammation that characterizes asthma and chronic obstructive pulmonary disease (COPD),^{15,16} we hypothesized that PAR expression by alveolar macrophages (AMs) might be different, differentially regulated, or both in asthmatic patients and smokers. Therefore we studied the expression of *PAR1* and *PAR2* mRNA (by using RT-PCR) and protein (by using immunocytochemistry and Western blotting) in AMs from healthy smokers, asthmatic subjects, and nonsmokers. We also assessed the regulation of *PAR1* and *PAR2* gene expression by the pro-inflammatory products IL-1 β and LPS, which are activators of macrophages.

METHODS

Subject recruitment

Current smokers with a cumulative smoking history of 5 to 10 pack-years, patients with mild asthma using short-acting β_2 -agonists less than 3 times per week,¹⁷ and nonsmokers were recruited for the study. Asthma was defined by a history of reversible airflow obstruction with increased airway responsiveness to methacholine ($PC_{20} < 8$ mg/mL).¹⁷ All subjects were free of any significant comorbid condition or recent (< 3 months) acute respiratory tract infection and had normal lung function. None of the subjects were receiving any long-term treatment, especially with corticosteroids (inhaled or systemic) or other anti-inflammatory agents. Smokers had to be free of chronic bronchitis. All patients provided written informed consent before participating in the study, which was approved by the Royal Brompton Hospital Ethics committee.

Fiberoptic bronchoscopy

After an overnight fast, subjects attended our bronchoscopy suite. They were pretreated with atropine (0.6 mg administered intravenously) and midazolam (5-10 mg administered intravenously). Oxygen (3 L/min) was administered through nasal prongs throughout the procedure, and oxygen saturation was monitored with a digital oximeter. After local anesthesia with lidocaine (4%) to the upper airways and larynx, a fiberoptic bronchoscope (Olympus BF10) was passed into the trachea, and bronchoalveolar lavage (BAL) was performed from the right middle lobe with 4 successive aliquots of 60 mL of warmed 0.9% NaCl.¹⁸

Alveolar macrophages

BAL fluid was filtered through sterile gauze and centrifuged at 500g for 10 minutes. Cells were washed twice in HBSS and counted. For each sample, 4 to 10 million fresh BAL cells were kept for RT-PCR: 0.5×10^6 were used to prepare cytospin preparations, 6×10^6 were plated in 12-well plates at a concentration of 1×10^6 /mL

in complete culture medium, and the remaining cells were kept for Western blot analysis. For each cytospin slide, 50,000 cells in 50 μ L of HBSS were centrifuged at 500 rpm for 5 minutes. Then slides were dried overnight at room temperature, embedded in foil, and kept at -70°C until staining was performed. Because it was not possible to obtain a large number of cells (10×10^6) to perform all these analyses in all subjects, we did not perform RT-PCR on fresh cells from the asthmatic patients, and Western blot analysis could be performed in only 3 subjects from each group.

The medium in which BAL cells were cultured was replaced with fresh complete medium after 24 hours of culture. Then adhering cells (ie, macrophages) from each subject were cultured for a further 24 hours in the presence of medium alone or containing either IL-1 β (10 ng/mL) or LPS (10 μ g/mL). Each condition was tested in duplicate in AMs from each subject. Finally, medium was removed, and cells were disrupted by buffer (RLT, Qiagen), harvested, and kept at -70°C until RT-PCR was performed.

RT-PCR

Messenger RNA was extracted with ethanol by using the RNeasy kit (Qiagen). After DNase I treatment, reverse transcription was performed with AMV reverse transcriptase. PCR protocol was performed in duplicate for each sample and included 36 cycles with 30 seconds of denaturation (94°C), 30 seconds of annealing (at 59°C , 68°C , and 70°C for reduced glyceraldehyde-phosphate dehydrogenase [*GAPDH*], *PAR1*, and *PAR2* mRNA, respectively), and 30 seconds of extension (72°C) in the presence of magnesium, primers, deoxyribonucleoside triphosphates, and TAQ polymerase. Primers were designed from *GAPDH*, *PAR1*, and *PAR2* mRNA sequences (GenBank accession nos.: X01677, M62424, and U34038, respectively) and were as follows: *GAPDH* forward, CCACCCATGGCAAATTCATGGCA; *GAPDH* reverse, TCTA-GACGGCAGGTCAGGTCCACC; *PAR1* forward, CTCAAGGAG-CAAAACCATCC; *PAR1* reverse, TGGCACTCAGAGGAAGCGT; *PAR2* forward, TCATGACCTGCCTCAGTGT; and *PAR2* reverse, TCAGGTACATGGCCAGGAC. The size of the PCR products were 578 bp, 422 bp, and 400 bp for *GAPDH*, *PAR1*, and *PAR2*, respectively. PCR products were separated by means of electrophoresis on a 2% agarose gel and visualized by means of ethidium bromide staining. The gel was photographed by using UV luminescence, and the relative abundance of product was assessed by using laser densitometry measured from the photographic negative. Results are expressed as the ratio of *PAR* mRNA to *GAPDH* mRNA. Two sets of gels were run simultaneously to obtain optimal conditions for comparisons between patients and control subjects: one with control subjects and smokers and one with control subjects and asthmatic patients.

Immunocytochemistry

Frozen slides were left at room temperature for 20 minutes before fixation in 2% paraformaldehyde (10 minutes of incubation at 4°C). Then they were incubated with 5% blocking rabbit serum for 20 minutes and with anti-PAR-1 or anti-PAR-2 polyclonal goat IgG at a dilution of 1:50 for 1 hour. These antibodies recognize both cleaved and uncleaved receptors. Biotinylated polyclonal rabbit anti-goat IgG (1:100, 45 minutes of incubation at room temperature) was used for labeling, followed by FITC-conjugated streptavidin (1:100, 45 minutes of incubation at room temperature). Between steps, slides were repeatedly washed with PBS (pH 7.4). Control slides were stained by using the same protocol, with the primary antibody being replaced by mouse IgG1 κ isotype antibody. Finally, slides were dried overnight at room temperature and examined with a Biorad MRC1000 confocal scanning laser system. Samples were excited at 488 nm, and emission (green fluorescence) was

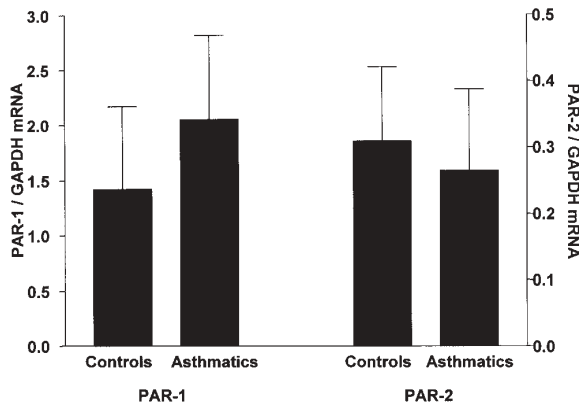


FIG 1. *PAR1* and *PAR2* mRNA levels in unstimulated, 24-hour cultured AMs from control subjects and asthmatic patients ($n = 6$ subjects per group, mean of duplicate samples for each subject). Results (mean $\pm$ SEM) are expressed as the ratio of *PAR* mRNA to *GAPDH* mRNA, as assessed by means of OD measurement after ethidium bromide staining. No significant difference was observed.

collected at 520 nmol/L. Positive and control slides were imaged with identical confocal settings. For each slide, representative images were acquired for subsequent analysis by 2 investigators (NR and RGS) who were not aware of the origin of the samples by using Scion Image Software (Scion Corp). This software allows quantitative assessment of the OD of individual cells. For each image, OD of 20 to 50 cells was measured, and the mean value was calculated. Staining was considered positive when this value was greater than the mean and 2 SDs of the negative control values (ie, 40 arbitrary OD units).

Western blot analysis

Total cell lysates were obtained by means of incubation of cell samples in ice-cold lysis buffer (1 mmol/L EDTA, 0.2 mmol/L phenylmethylsulfonyl fluoride) with protease inhibitors (1.1 μ mol/L leupeptin and 1 μ mol/L pepstatin) and centrifugation to remove debris (12,000g for 10 minutes at 4°C). An equal volume of Laemmli sample buffer (2 $\times$ concentration) was added to the final volume of the sample. Proteins were separated by 45 minutes of electrophoresis on a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane blocked by 45 minutes of incubation with 15% nonfat milk and 4% rabbit serum. Membranes were then incubated for 45 minutes with anti-PARs or anti- β -actin antibodies (1:1000) and further incubated for 45 minutes with rabbit anti-goat IgG conjugated to horseradish peroxidase (1:4000). Detection was performed by using enhanced chemiluminescence reagents (ECL-plus, Amersham).

Reagents

Complete macrophage culture medium was RPMI-1640 (ICN-Flow) containing 10% heat-inactivated FCS (Sera Labs), 2 mmol/L L-glutamine (ICN-flow), and penicillin-streptomycin (100 U/mL-100 mL, ICN flow). Culture plates were from Falcon. LPS (*Escherichia coli*) was from Sigma. Recombinant human IL-1 β was purchased from R&D Laboratories. Anti-PAR-1 and anti-PAR-2 antibodies were from Santa Cruz Biotechnology. Secondary antibodies and FITC-conjugated monoclonal anti-CD14 mouse IgG2a were from Sigma. Primers for RT-PCR were from Amersham-Pharmacia. AMV reverse transcriptase was from Promega. Other RT-PCR reagents were from Biotline.

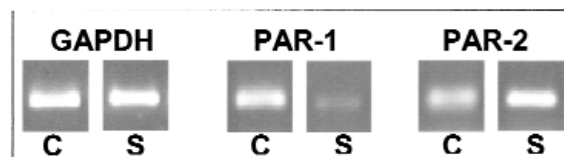
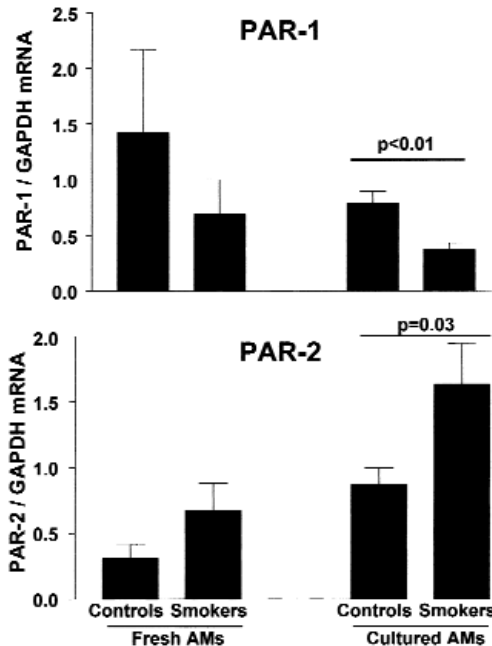


FIG 2. Levels of *PAR1* and *PAR2* mRNA in fresh and cultured AMs from control subjects and smokers ($n = 6$ subjects per group, mean of duplicate samples for each subject). Results (mean $\pm$ SEM) are expressed as the ratio of *PAR* mRNA to *GAPDH* mRNA. Gels of *GAPDH*, *PAR1*, and *PAR2* RT-PCR of pooled mRNA from control subjects (C) and smokers (S) are shown at the bottom.

Statistical analysis

Agreement between investigators for quantitative image analysis of immunostained cytospin preparations was assessed by using the κ concordance coefficient. Because other studied variables could not be considered as normally distributed according to the Shapiro-Wilks test, nonparametric tests were used: comparisons between smokers, asthmatic patients, and control subjects were performed by using the Mann-Whitney *U* test, and the effects of IL-1 β , LPS, and culture were studied by using the Wilcoxon signed-rank test. A *P* value threshold of .05 was considered for statistical significance. All statistics were performed with SASS 7.5 software (SASS Inc). Results are expressed as means $\pm$ SEM.

RESULTS

The characteristics of the 6 current smokers, 6 patients with mild asthma, and 6 healthy nonsmoking volunteers are shown in Table I.

PAR1 and *PAR2* gene expression in fresh BAL cells and cultured AMs

PAR1 and *PAR2* mRNA levels were similar in asthmatic patients and control subjects (Fig 1). By contrast, *PAR1* mRNA was lower and *PAR2* mRNA was higher in

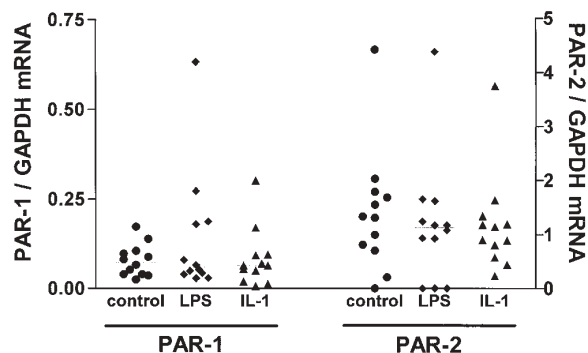


FIG 3. Effect of 24-hour culture in the presence of IL-1 β (10 ng/mL) and LPS (10 μ g/mL) on *PAR1* and *PAR2* mRNA levels in AMs from nonsmokers (n = 6) and smokers (n = 6). Both groups of subjects are combined because there was no difference in the effect of pro-inflammatory mediators between them. There were no significant differences for all comparisons.

DISCUSSION

Our data show that the expression profile of PAR-1 and PAR-2 in AMs is different between smokers and control subjects: for PAR-1, AMs of smokers express lower levels of mRNA but higher levels of protein, whereas for PAR-2, they express higher levels of mRNA but similar levels of protein. Conversely, levels of PAR-1 and PAR-2 mRNA and protein expression in AMs do not differ between asthmatic patients and control subjects. Finally, levels of *PAR1* and *PAR2* gene expression are not influenced by IL-1 β and LPS. Thus PAR-1 might play a role in the pathogenesis of cigarette smoke-induced bronchopulmonary disease, although this putative role remains to be explored in functional studies; in addition, control of PAR-1 and PAR-2 protein expression differs from that of gene expression, which is not influenced by acute exposure to some pro-inflammatory stimuli.

The increase in PAR-1 protein expression in AMs from smokers supports a possible participation of this receptor in the pathogenesis of COPD. Only a few studies assessed PAR expression in mononuclear phagocytes.¹⁹⁻²² We found PAR-1 mRNA but not protein expression in AMs from control subjects and asthmatic patients, whereas PAR-1 protein was detected in AMs from all but one smoker. This suggests that PAR-1 protein is not expressed in resting AMs but is triggered by agents contained in cigarette smoke or released in the context of cigarette smoke-induced (but not asthma-induced) airway inflammation. In parallel, PAR-2 protein and mRNA were detected in AMs, with similar levels of protein in all groups and higher levels of mRNA in smokers, indicating no abnormality in the regulation of PAR-2 protein expression by AMs in asthma- and cigarette smoke-induced airway inflammation. The potential functional significance of the increase in *PAR2* mRNA in smokers with no change in protein expression is uncertain. Differences in *PAR1* and *PAR2* mRNA levels between smokers and nonsmokers were significant only after culture, although similar trends were observed in freshly isolated macrophages. This is likely to be due to a decreased interindividual variation of PAR expression after culture, as demonstrated by the smaller SDs.

Because of the differences in *PAR1* and *PAR2* gene expression between AMs from smokers and control subjects, we determined the acute effect of IL-1 β and LPS, 2 known pro-inflammatory macrophage activators, on *PAR1* and *PAR2* gene expression in cultured AMs. Few studies have assessed the effect of pro-inflammatory stimuli on PAR expression,²²⁻²⁴ and none focused on macrophages. In our study there was no effect of 24 hours of culture on the presence of IL-1 β and LPS on mRNA expression of PARs. This and the lack of difference between asthmatic patients and control subjects suggests that the changes in PAR-1 and PAR-2 expression that we found in smokers are not observed in all the conditions in which AMs are activated.

Our results also show a dissociation between the regulation of PAR-1 and PAR-2 protein expression and that of

smokers when compared with that seen in healthy subjects (Fig 2). These differences were significant only in cultured AMs but not in fresh BAL cells. The level of *PAR1* mRNA decreased and the level of *PAR2* mRNA increased after culture of AMs for 48 hours ($P < .01$ and $P < .05$, respectively). IL-1 β (10 ng/mL) and LPS (10 μ g/mL) had no effect on the level of *PAR1* and *PAR2* gene expression in smokers, control subjects and asthmatic patients (Fig 3).

PAR-1 and PAR-2 immunocytochemistry

PAR-1 and PAR-2 staining was observed in the cytoplasm and cell membrane but not in nuclei. OD measurements obtained by the 2 observers unaware of the origin of the slides were highly concordant (κ coefficient = 0.966). The mean + 2 SDs OD of negative control slides was 50 arbitrary units. Control values from MOPC-stained slides were similar in samples from smokers and nonsmokers and did not differ between PAR-1 and PAR-2 immunocytochemistry. All slides from control subjects and asthmatic patients showed negative results, whereas 5 of 6 slides from smokers showed positive results for PAR-1 ($P < .01$; Fig 4, A). Four of 6 slides showed positive results for PAR-2 in all groups of subjects, with no difference in the intensity of staining (Fig 4, B).

Western blot analysis

Individual studies of PAR-1 and PAR-2 protein expression were performed in 3 smokers, 3 control subjects, and 3 asthmatic patients because of an insufficient number of available cells in the other subjects. Similar to the data shown in Fig 4, the level of PAR-1 protein expression was increased in 2 of 3 smokers and none of the asthmatic patients when compared with that seen in the 3 control subjects (Fig 5). No difference in the level of PAR-2 protein expression was observed among the groups.

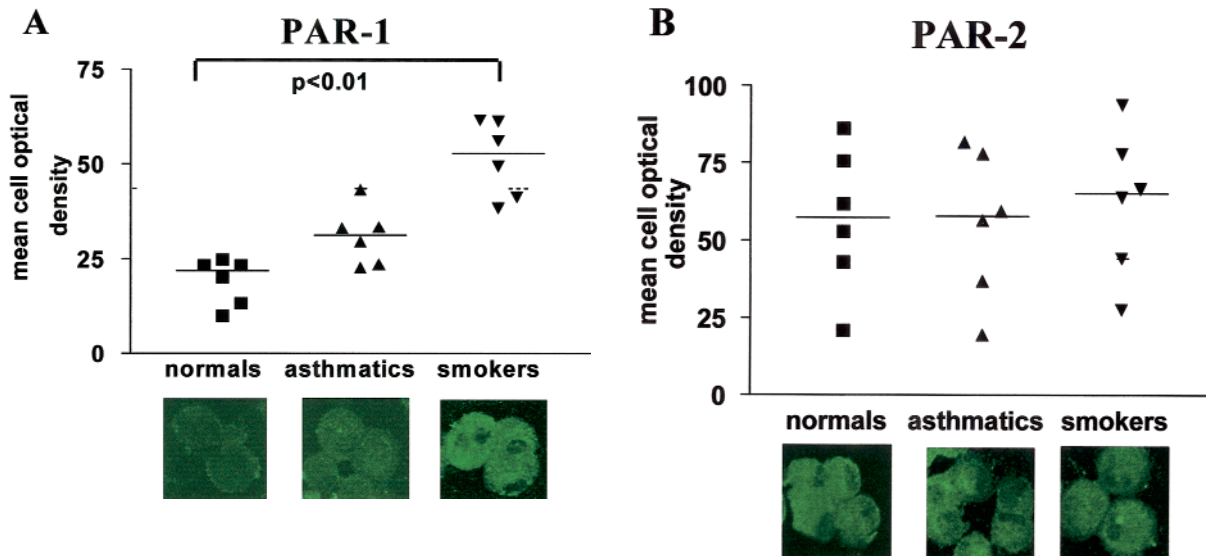


FIG 4. Intensity of PAR-1 (**A**) and PAR-2 (**B**) immunostaining in AMs from control subjects, asthmatic patients, and smokers ($n = 6$ per group). Horizontal lines represent medians. Representative examples of immunostaining are shown at the bottom of each graph for PAR-1 and PAR-2 staining and on the right of the dotted lines for the negative control.

TABLE I. Characteristics of control subjects, smokers, and asthmatic patients

	Control subjects ($n = 6$)	Smokers ($n = 6$)	Asthmatic patients ($n = 6$)
Age (y)	26.2 ± 1.2	34.1 ± 4.2	36.5 ± 1.82
Sex (M/F)	3:3	2:4	3:3
FEV ₁ (% predicted)	98.3 ± 2.9	98.9 ± 1.8	92.1 ± 8.7
Cellular content of BAL fluid			
Total cell count ($\times 10^6$)	11.2 ± 1.9	$56.1 \pm 5.6^*$	9.8 ± 1.2
Macrophages (%)	83.3 ± 0.8	$93.5 \pm 1.4^*$	82.1 ± 1.4
Lymphocytes (%)	14.3 ± 0.9	$2.5 \pm 0.8^*$	12.9 ± 2.2
Neutrophils (%)	1.6 ± 0.2	2.9 ± 1.0	2.0 ± 0.5
Eosinophils (%)	0.2 ± 0.1	0.9 ± 0.7	$3.4 \pm 0.9^*$

Data are shown as means $\pm$ SEM.

* $P < .01$, comparison with control group.

gene expression. Protein expression of PARs is influenced by binding to and cleavage of the receptor. Unlike other G protein-coupled receptors, activation of PARs by enzymatic cleavage is irreversible, so that cell resensitization requires that new receptors are translocated to the surface.¹ Indeed, both activating and nonactivating cleavage and binding to agonist peptides induce receptor internalization and degradation.¹ Cell resensitization comes from (1) trafficking between the cell surface and the intracellular pool²⁵ and (2) an increase in translation but not transcription.²⁶ Using confocal microscopy, we found an increase in PAR-1 surface expression but a decrease in its mRNA expression in AMs from smokers compared with that seen in control subjects. By means of Western blotting of a limited number of macrophage samples, we also found an increase in PAR-1 expression in smokers, although this is likely to represent PAR-1 recovered from both cytosol and cell membrane fractions. Increased cleavage by neutrophil

enzymes, such as elastase, cathepsin G, and proteinase 3, might stimulate trafficking of intact receptors from the intracellular pool, increase translation to resensitize the cell, or both but downregulate transcription, as seen with β_2 -adrenergic receptors.²⁷ However, both downregulation and increased trafficking, translation, or both of PAR-1 after enzymatic stimulation of AMs remains to be formally demonstrated. Increased trafficking, translation, or both and decreased transcription could also be due to an effect of pro-inflammatory stimuli, such as TNF- α , as found in endothelial cells.²⁴ Interestingly, increased trafficking of PAR-1 has also been suggested by immunohistochemical data obtained in epithelial cells from steroid-naïve asthmatic patients, in whom the receptor is diffusely present in the cytoplasm, whereas it is predominantly located at the apical side of the cell in healthy subjects and steroid-treated asthmatic patients.²⁸ In parallel, we found an increase in PAR-2 mRNA but not protein expression in smokers;

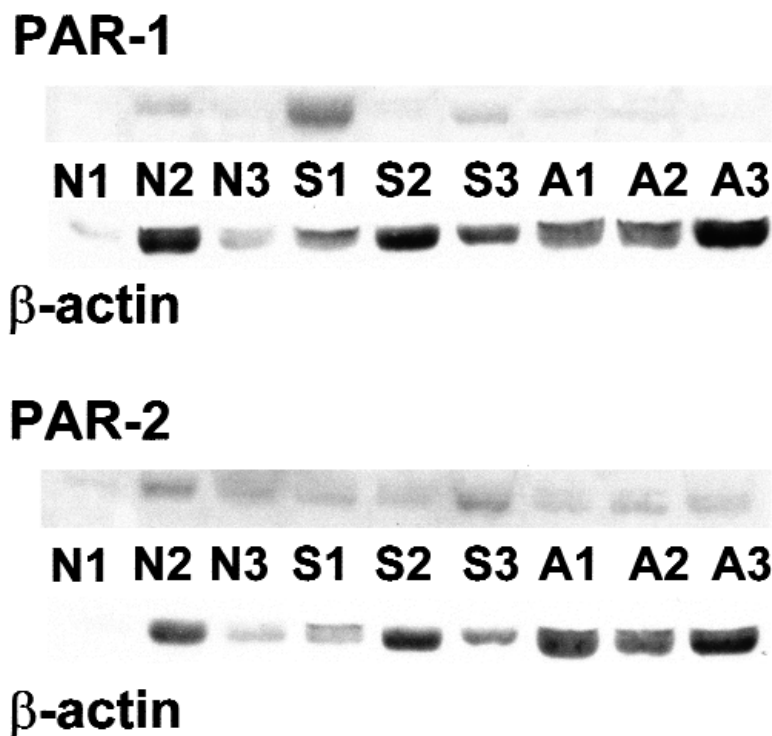


FIG 5. Western blot analysis of PAR-1 and β -actin and of PAR-2 and β -actin protein expression in AMs obtained from 3 control subjects (N1, N2, N3), 3 asthmatic patients (A1, A2, A3), and 3 smokers (S1, S2, S3).

the increase in *PAR2* mRNA might be due to an effect of pro-inflammatory mediators, as shown by Nystedt et al²³ in endothelial cells. We did not recover enough bronchoalveolar macrophages to perform permeabilization for immunocytochemical or western blot studies, and we are therefore unable to determine whether the differences in total protein expression were related to changes in the number of surface receptors and to differences in intracellular stores. We presume that because no permeabilization was performed before immunostaining, the detectable receptors should consist mostly of surface receptors.

In addition to their main agonists (ie, thrombin and trypsin, respectively), PARs can be cleaved by several enzymes that are known to play a role in inflammatory obstructive airways diseases, including the neutrophil serine proteases cathepsin G, neutrophil elastase, and proteinase 3 for PAR-1 and mast cell tryptase for PAR-2.¹ PAR-1 cleavage by neutrophil enzymes appears to be predominantly nonactivating, although cathepsin G is also capable of cleaving the receptor at the activating thrombin site.^{1,29} Neutrophil elastase and cathepsin G are known to play an important role in the pathophysiology of COPD, in which neutrophils are recruited to and activated in the airways.¹³ Increased PAR-1 activation in AMs might be involved in airways inflammation because PAR-1 stimulation in monocytes has been shown to induce release of several mediators, such as IL-6, IL-1 β , and TNF- α .^{1,22,30}

In conclusion, PAR-1 but not PAR-2 protein expression in AMs is upregulated by cigarette smoking, despite lower expression of *PAR1* mRNA. Upregulation at the protein level might be due to increased stimulation of receptor trafficking and translation by neutrophil enzymes or pro-inflammatory stimuli. Downregulation of *PAR1* mRNA could result from a persistent increase in PAR-1 cleavage by such enzymes. PAR-1 might play a role in cigarette smoke-induced lung disease. Expression of PAR-1 and PAR-2 in AMs was not different between asthmatic patients and control subjects.

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