

Cockroach proteases increase IL-8 expression in human bronchial epithelial cells via activation of protease-activated receptor (PAR)-2 and extracellular-signal-regulated kinase

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Background: We have shown that serine proteases in German cockroach extract increase TNF- α -induced expression of IL-8 in human bronchial epithelial cells. The mechanism by which cockroach proteases regulate cytokine expression is unknown; however, protease-activated receptors (PARs) might play a role. **Objective:** We sought to determine the role of PARs and extracellular-signal-regulated kinase (ERK) in cockroach-induced regulation of IL-8 expression.

Methods: 16HBE14o- human bronchial epithelial cells were treated with the specific PAR-1 and PAR-2 agonists, TFRIFD and SLIGKV, respectively. IL-8 transcription was assessed by transiently transfecting cells with a luciferase-tagged IL-8 promoter construct, and in some cases, dominant-negative expression vectors. To block PAR cleavage, antibodies against the cleavage region of PAR-1 and PAR-2 were used. ERK phosphorylation was determined by Western blot.

Results: Although both PAR-1 and PAR-2 were endogenously expressed in 16HBE14o- cells, selective activation of PAR-2 but not PAR-1 mimicked the effect of cockroach extract on IL-8 expression. Using a blocking antibody against cleavage of PAR-2 but not PAR-1 attenuated cockroach-extract-induced responses, suggesting that cockroach proteases cleave PAR-2. Treatment of cells with cockroach extract and SLIGKV each increased phosphorylation of ERK. Chemical or genetic inhibition of Ras and mitogen-activated protein kinase/ERK (MEK), upstream activators of ERK, each attenuated cockroach- and PAR-2-induced IL-8 transcription.

Conclusion: Cockroach proteases and PAR-2 activation synergistically increase TNF- α -induced IL-8 transcription via activation of ERK. These data suggest an important role for PAR-2 and ERK activation in the regulation of cytokine expression in airway epithelium in response to cockroach proteases. (J Allergy Clin Immunol 2003;112:1112-8.)

Key words: *Blattella germanica*, mitogen-activated protein kinase, serine protease, tumor necrosis factor- α .

Abbreviations used:

AP: Activator protein
ERK: Extracellular-signal-regulated kinase
MAP kinase: Mitogen-activated protein kinase
MEK: MAP kinase/ERK
NF: Nuclear factor
PAR: Protease-activated receptor
SLIGKV: A PAR-2 agonist, ser-leu-iso-gly-lys-val
TFRIFD: A PAR-1 agonist, thr-phe-arg-iso-phe-aspartate

Many aeroallergens have been shown to possess enzymatic activity, including house dust mite (*Dermatophagoides pteronyssinus*) cysteine protease Der p1^{1,2} and serine proteases Der p3 and Der p9³; fungal extracts, including *Alternaria alternata* and *Cladosporium herbarum*⁴; and the cat allergen *Felis domesticus* (Fel d 1).⁵ In addition, this proteolytic activity has been implicated in the regulation of epithelial cell release of GM-CSF, IL-8, and IL-6 in vitro.^{2,3,5,6} We have recently shown that German cockroach (*Blattella germanica*) extract contains proteolytic activity that mediates an increase in TNF- α -induced IL-8 expression in human bronchial epithelial cells (16HBE14o-).⁷

A number of proteases have been shown to signal directly to cells by cleaving protease-activated receptors (PARs) on the cell surface. PARs are a family of G-protein-coupled receptors, and 4 family members have been cloned to date, including PAR-1,⁸ PAR-2,⁹ PAR-3,¹⁰ and PAR-4.¹¹ Cleavage of an extracellular amino portion of the molecule by thrombin (for PAR-1, PAR-3, and PAR-4) or by tryptase (for PAR-2)⁸⁻¹² leads to the creation of a neo-NH₂ terminus. This new amino terminus, or tethered ligand, binds to the activation site of the receptor to activate the heterotrimeric G-proteins in the cell membrane.¹³

All 4 members of the PAR family have been found in the A549 and BEAS-2B respiratory epithelial cell lines, as well as in primary human bronchial epithelial cells.¹⁴ PAR-2 is expressed in human intrapulmonary bronchioles,¹⁵ airway smooth muscle,¹⁶ fibroblasts,¹⁷ mast cells,¹⁸ and neutrophils.¹⁹ Activation of PAR-2 in eosinophils potentially induced superoxide anion production and degranulation, suggesting a role for PAR-2 in the inflammatory response.²⁰ Furthermore, stimulation of endothelial cell lines with TNF- α , IL-1 α , or LPS

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increased expression of PAR-2.²¹ A recent study showed that PAR-2 levels were significantly increased in asthmatic epithelium.²²

Specific activation of PAR-1, PAR-2, and PAR-4 has been shown to modulate IL-6, IL-8, and prostaglandin E₂ production in A549 and BEAS-2B cell lines and in primary human bronchial epithelial cells.¹⁴ PAR-2 has also been demonstrated to stimulate IL-8 production in a variety of cell types, including keratinocytes.²³ In addition, the house dust mite serine proteases Der p3 and Der p9 have recently been shown to activate PAR-2.³ In this study, we investigate whether the proteases in cockroach extract activate PAR-2 in human bronchial epithelial cells (16HBE14o-).

METHODS

Materials

β-Galactosidase and *Pertussis* toxin were purchased from Sigma Chemical Co (St Louis, Mo). Human TNF-α was purchased from R&D Systems (Minneapolis, Minn). Whole-body extract of German cockroach (*B germanica*) was purchased from Greer Laboratories (Lenoir, NC). Optimum and Lipofectamine were purchased from Gibco (Gaithersburg, Md). Luciferase assay buffer was purchased from Promega (Madison, Wis). Antibodies against extracellular-signal-regulated kinase (ERK) and phospho-ERK (Cell Signaling, Beverly, Mass) and against PAR-1 (H-111) and PAR-2 (SAM-11) (Santa Cruz Biotechnology, Santa Cruz, Calif) were purchased. Horseradish-peroxidase-conjugated rat anti-mouse-κ light-chain secondary antibody was from Zymed Laboratories (San Francisco, Calif). Superscript One-Step RT-PCR with Platinum *Taq* and the peptides corresponding to the tethered ligand of PAR-2 (SLIGKV) and PAR-1 (TFRIFD) were synthesized by Invitrogen (Carlsbad, Calif).

Cell culture

A simian-virus-40-transformed human bronchial epithelial cell line (16HBE14o-), provided by S. White (University of Chicago, Chicago, Ill) was studied as described previously.²⁴ Cells were grown on coated plates (10 μg/mL fibronectin, 30 μg/mL vitrogen, and 100 μg/mL BSA in minimum essential medium with 10% FBS, 1% penicillin-streptomycin, and 200 mmol/L L-glutamine).

RT-PCR

RNA was extracted using a standard TRIzol method of phenol extraction. In brief, cells were lysed in TRIzol, homogenates were transferred to microcentrifuge tubes, and chloroform was added (at room temperature for 5 minutes). After centrifugation (14,000 rpm for 15 minutes at 4°C), the aqueous phase was transferred to a new tube and isopropanol was added (10 minutes at room temperature). Tubes were centrifuged (14,000 rpm for 10 minutes at 4°C), supernatant was removed, and the pellets were washed in 75% ethanol. Superscript One-Step RT-PCR with Platinum *Taq* was used according to the company's specifications. In brief, 0.5 μg template RNA, 0.2 μmol/L sense primer, 0.2 μmol/L antisense primer, 2× reaction mix, and RT Platinum *Taq* mix was added to an amplification tube and gently mixed. cDNA synthesis and predenaturation were performed with 1 cycle of 50°C for 30 minutes, followed by 94°C for 2 minutes. PCR amplification for all primers was performed for 40 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 45 seconds, followed by 1 cycle of 72°C for 10 minutes. PAR-1 primers used were as follows: sense, 5'-TGTCTGTGTCAGCAGCATAAGC-3' and antisense, 5'-TGTTCAAGTCTAACTCTACCAAC-3' (PCR product of 657 bp). PAR-2 primers

used were as follows: sense, 5'-CTCACTGGAAGAACTGAC CACG-3' and antisense, 5'-CTTCACGACATACAAAGGGATG-3' (PCR product of 435 bp). β-Actin primers used were as follows: sense, 5'-TTCTCTGACCTGAGTCTCCTTTGG-3' and antisense, 5'-AGCACAGCCTGGATAGCAACG' (PCR product of 390 bp).

Plasmid vectors

The -162/+44 fragment of the full-length human IL-8 promoter subcloned into a luciferase reporter plasmid was obtained from A. Brasier (University of Texas, Galveston).²⁵ The dominant-negative form of MEK (pEXV-MEK 2A) and the dominant-negative form of Ras (pEXV-N17Ras)²⁶ were provided by Dr Marsha Rosner (University of Chicago, Chicago, Ill).

Transient transfection of human airway epithelial cells

Cells were grown to 70% confluence, rinsed in Optimum, and incubated with a solution of plasmid DNA (~0.5 μg total DNA per 35-mm dish), Lipofectamine (4-μL/dish), and Optimum. In some experiments, cells were transiently cotransfected with dominant-negative forms of either Ras or mitogen-activated kinase/ERK (MEK) or vector control, the IL-8 promoter, and β-galactosidase. After 5 hours, the liposome solution was replaced with 10% FBS/minimum essential medium. After overnight incubation, cells were serum-starved for 8 hours. In some experiments, cells were pretreated for 1 hour with either *Pertussis* toxin or *Pertussis* toxin B oligomer (both at 1 μg/mL), the PAR-2 amino-terminal antibody (SAM-11), or the PAR-1 amino-terminal antibody (H-111). Selected cells were then treated with cockroach extract (10 μg/mL), the PAR-2 activating peptide SLIGKV (50 μmol/L), the PAR-1 activating peptide TFRIFD (50 μmol/L), TNF-α (3 ng/mL), or a combination of the above. Sixteen hours after treatment, cells were harvested and analyzed for luciferase activity using the Promega Luciferase Assay system. β-Galactosidase activity was assessed by colorimetric assay using o-nitrophenyl-β-D-galactoside as a substrate.²⁷

Determination of the concentration response of cockroach extract and TNF-α

A dose-concentration curve of cockroach extract and TNF-α was previously shown.⁷ Concentrations of 0.3 μg/mL to 30 μg/mL cockroach extract, although insufficient to induce IL-8 expression alone, resulted in a 40% to 140% synergistic increase in TNF-α-induced IL-8 expression. A concentration of 3 ng/mL TNF-α was chosen because of the largest increase in synergy with cockroach extract with this dose.

Immunoprecipitation of PAR

Cells were extracted after 2 washes in PBS with lysis buffer (10 mmol/L Tris HCl, pH 7.5; 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L NaF, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mmol/L Na₃VO₄, and 0.288 U/mL aprotinin). Cell lysates were centrifuged (13,000g × 10 minutes), and supernatant was transferred to a fresh microfuge tube. Forty microliters of protein-G beads precoupled to either PAR-1 (H-111) or PAR-2 (SAM-11) antibodies was added to 400 μg cell lysate and incubated for 3 hours at 4°C. Samples were separated on a 10% SDS-PAGE gel. Western blot analysis using either the H-111 or SAM-11 antibody and goat anti-light chain as a secondary antibody was performed.

Immunoblot analysis

Cells were cultured in 6-well plates and serum-starved for 24 hours before treatment. Selected cells were treated with cockroach

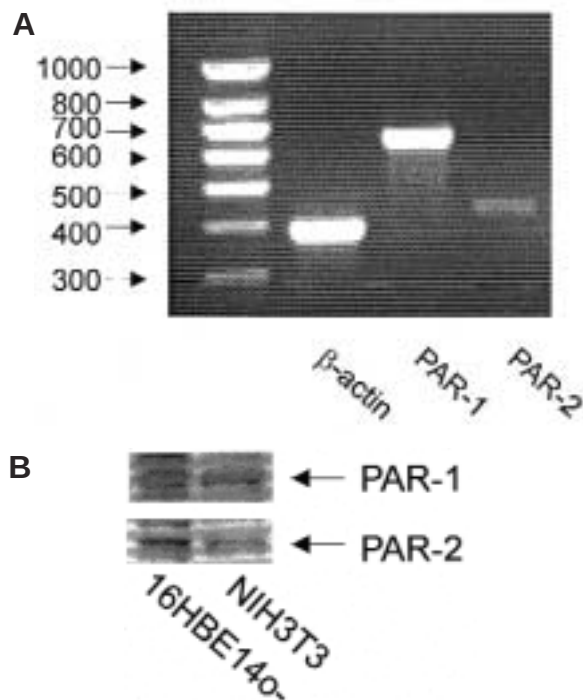


FIG 1. PAR-1 and PAR-2 are endogenously expressed in 16HBE14o- cells. **A**, RNA from growing 16HBE14o- human bronchial epithelial cells was extracted, reverse-transcribed, and amplified with specific primers for β -actin, PAR-1, and PAR-2. **B**, Growing cells were extracted and immunoprecipitated with antibodies against either PAR-1 or PAR-2. NIH3T3 cell lysate was used as a control. This experiment was performed twice.

extract (10 μ g/mL), heat-inactivated cockroach extract (10 μ g/mL; 55°C for 15 minutes), SLIGKV (50 μ mol/L), or TNF- α (3 ng/mL) for 10 minutes. Cells were washed in PBS and extracted in a lysis buffer containing 50 mmol/L Tris, pH 7.5; 40 mmol/L β -glycerophosphate, 100 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L NaF, 200 μ mol/L Na_3VO_4 , 200 μ mol/L phenylmethylsulfonyl fluoride, and 1% Triton X-100. Insoluble materials were removed by centrifugation (13,000 rpm for 10 minutes at 4°C). Extracts (15 μ g) were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. After incubation with antibody, signals were amplified and visualized by using enhanced chemiluminescence. The nitrocellulose membranes were stripped (62.5 mmol/L Tris, pH 6.8; 100 mmol/L β -mercaptoethanol, and 2% SDS at 50°C for 30 minutes) and reprobed with another antibody.

Activated Ras affinity precipitation assay

A Ras activation assay kit (Upstate Biotechnology, Lake Placid, NY) was used. In brief, 16HBE14o- cells were grown to 75% to 80% confluence, serum-deprived for 24 hours, and then treated with cockroach extract (10 μ g/mL for 15 or 30 minutes). Cells were lysed with the kit lysis buffer and affinity-precipitated with 15 μ g GST-RBD (Ras-binding domain of Raf-1) bound to glutathione-agarose (4°C for 30 minutes). The beads were washed and eluted on a 12% SDS-PAGE gel and transferred to nitrocellulose. The membrane was stained with Ponceau Red and probed with anti-Ras monoclonal antibody.

Statistical analysis

When applicable, statistical significance was assessed by 1-way ANOVA. Differences identified by ANOVA were pinpointed by the Student-Newman-Keuls multiple-range test.

RESULTS

PAR-1 and PAR-2 are endogenously expressed in human bronchial epithelium

To determine whether PAR-1 or PAR-2 was endogenously expressed in a human airway epithelial cell line (16HBE14o-), we performed RT-PCR on isolated mRNA from these cells. Both PAR-1 and PAR-2 mRNAs were detectable (Fig 1, A). Immunoprecipitation of 16HBE14o- whole-cell lysate confirmed expression of both PAR-1 and PAR-2 protein (Fig 1, B).

Inhibition of G-coupled proteins attenuates cockroach-induced synergy

Activation of PARs has been shown to regulate heterotrimeric G-proteins in the cell membrane.^{12,13} To examine the requirement of the G_i -protein subunit for cockroach-extract-mediated responses, we transiently transfected cells with the cDNA encoding the IL-8 promoter subcloned into luciferase and β -galactosidase. Selected cells were pretreated with the G_i -protein inhibitor *Pertussis* toxin or a negative-control toxin (*Pertussis* toxin B) for 1 hour before treatment with cockroach extract, TNF- α , or both together. Inhibition of G-coupled proteins before treatment abolished cockroach-induced synergy while having no effect on TNF- α -induced IL-8 transcription (Fig 2). These data are consistent with the notion that the effect of cockroach extract is mediated by PAR G_i -protein-coupled receptors.

Activation of PAR-2, but not PAR-1, is sufficient and required for cockroach-induced transcription from the IL-8 promoter

To investigate whether activation of PAR-1 or PAR-2 regulated transcription from the IL-8 promoter, cells were transiently transfected with the IL-8 promoter and β -galactosidase. Cells were then treated with the specific agonists for PAR-1 (TFRIFD), PAR-2 (SLIGKV), or cockroach extract in the presence or absence of TNF- α . The PAR agonists are peptides that correspond to the new amino terminus, which, after cleavage, activate the receptor. The value of these peptides is that they selectively activate PARs without the need for proteolytic cleavage of the receptor. Activation of PAR-2 synergistically increased IL-8 promoter activity to a level similar to that of cockroach extract, whereas activation of PAR-1 had no effect (Fig 3, A).

To block PAR-2 cleavage, we pretreated cell cultures with antibodies against the amino-terminal cleavage region of PAR-1 or PAR-2 before addition of TNF- α or cockroach extract. Addition of the antibody against the cleavage region of PAR-2, but not PAR-1, attenuated cockroach-induced increases in IL-8 promoter activation (Fig 3, B). These data suggest that PAR-2, but not PAR-1, is sufficient to induce transcription from the IL-8 promoter in the presence of TNF- α . In addition, blocking of the cleavage region of PAR-2, but not PAR-1, attenuates cockroach-induced increases in IL-8 expression.

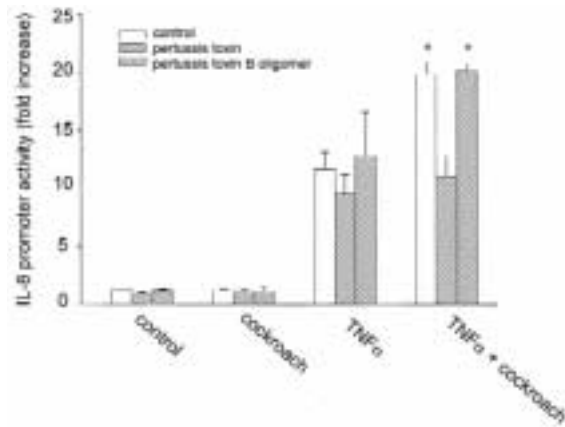


FIG 2. Inhibition of G-coupled-protein receptors attenuates cockroach-induced synergy. 16HBE14o- human bronchial epithelial cells were transfected with cDNAs encoding the IL-8 promoter and β -galactosidase. Selected cells were pretreated with *Pertussis* toxin or *Pertussis* toxin B oligomer for 1 hour before addition of TNF- α or cockroach extract. Data are expressed as luciferase/ β -galactosidase per hour (normalized to control). *Significantly greater than TNF- α alone ($n = 4$; $P < .05$, ANOVA).

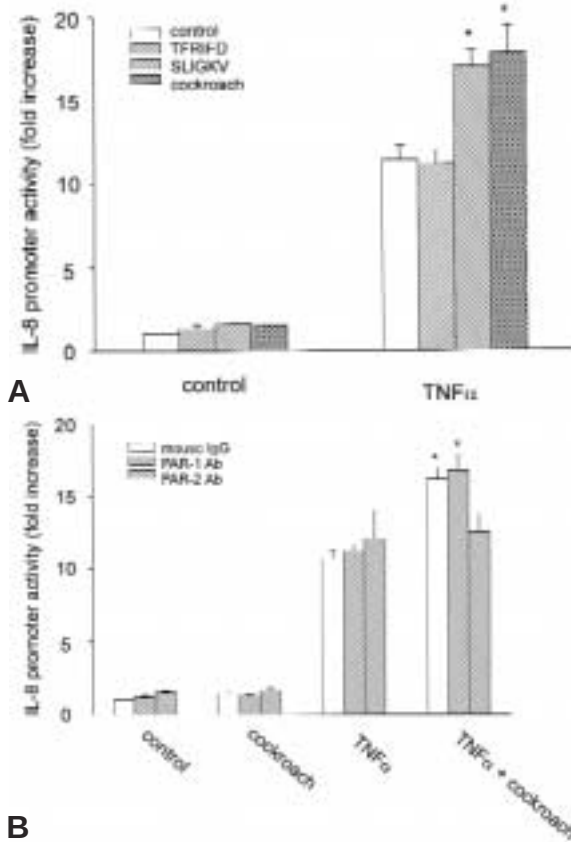
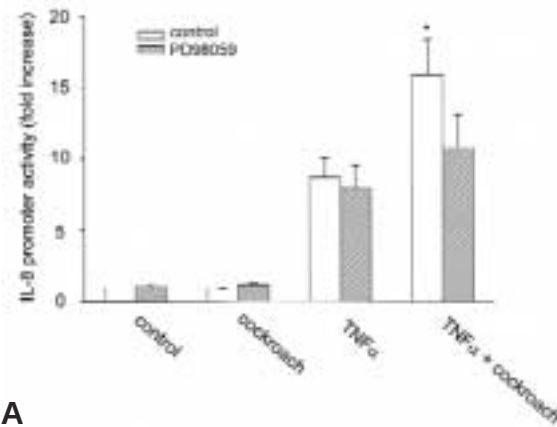
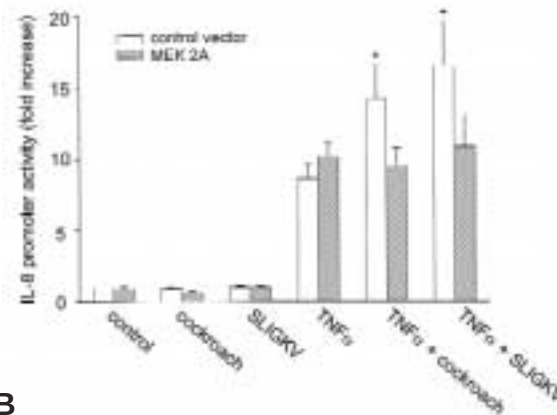


FIG 3. PAR-2 is sufficient and required for cockroach-induced transcription from the IL-8 promoter. **A**, 16HBE14o- cells were transfected with the IL-8 promoter and β -galactosidase. Selected cells were treated with the PAR agonists TFRIFD or SLIGKV, cockroach extract, or TNF- α . *Significantly greater than TNF- α alone ($n = 3-6$; $P < .05$, ANOVA). **B**, Cells were pretreated with normal mouse IgG or antibodies against the amino terminus of PAR-1 or PAR-2 for 1 hour before addition of cockroach extract and/or TNF- α . *Significantly greater than TNF- α alone ($n = 3-6$; $P < .05$, ANOVA).



A



B

FIG 4. Cockroach extract and PAR-2 activation require ERK activation to increase IL-8 promoter activity. **A**, 16HBE14o- cells were transfected with IL-8 promoter and β -galactosidase. Selected cells were pretreated with PD98059 before treatment. *Significantly higher than TNF- α alone ($n = 4$; $P < .05$, ANOVA). **B**, 16HBE14o- cells were cotransfected with vector control or dominant-negative MEK (MEK2A), the IL-8 promoter, and β -galactosidase. Selected cells were treated with cockroach extract or the PAR-2 agonist SLIGKV alone or in combination with TNF- α . *Significantly higher than TNF- α alone ($n = 4$; $P < .05$, ANOVA).

Cockroach exposure and selective activation of PAR-2 increases TNF- α -induced IL-8 promoter activity in an ERK-dependent manner

We used a battery of chemical inhibitors to determine whether specific kinases were required for the cockroach-induced synergistic responses. We found that inhibition of ERK signaling with PD98059, a chemical inhibitor of the ERK upstream activator MEK, abolished cockroach-induced IL-8 promoter responses (Fig 4, A). However, inhibition of p38 MAP kinase (using SB202190), phosphatidylinositol 3-kinase (wortmannin), and Janus kinase (AG490) had no effect (not shown). Furthermore, transient overexpression of a dominant-negative form of MEK (MEK2A) abolished the cockroach- and SLIGKV-induced synergistic effect but had no effect on TNF- α -induced activation of the IL-8 promoter (Fig 4, B).

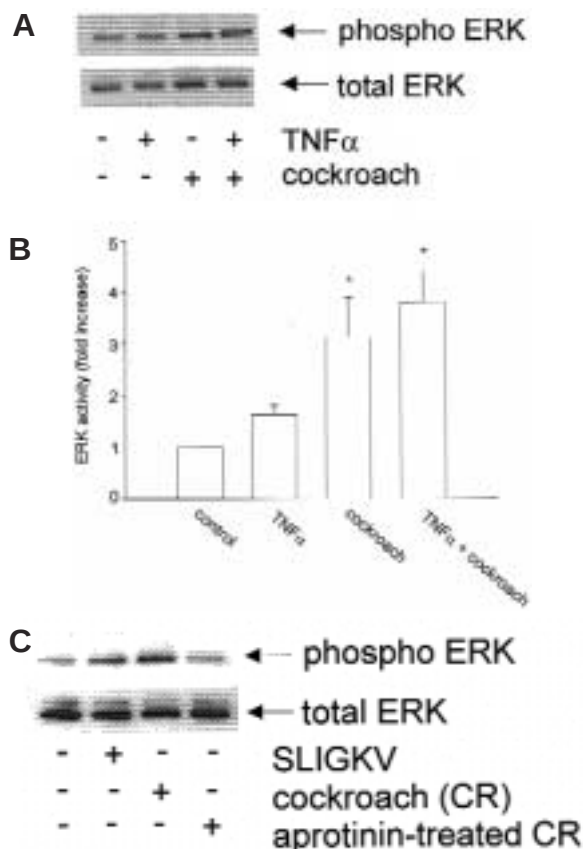


FIG 5. Cockroach extract and PAR-2 activation increase ERK phosphorylation. **A**, Cells were serum-deprived for 24 hours before treatment with TNF- α , cockroach extract, or both for 10 minutes. Immunoblot analysis using an antibody against the dually phosphorylated form of ERK is shown in the top panel. The gel was stripped and reprobed for total ERK. **B**, Analysis of phospho-ERK/total ERK. Data are mean $\pm$ SE, $n = 8$. *Significantly higher than control ($P < .05$, ANOVA). **C**, Cells were treated for 10 minutes with the PAR-2-activating peptide SLIGKV (50 μ M/L), cockroach extract, or cockroach extract that was pretreated with aprotinin. This experiment was performed twice.

We then examined the effects of cockroach extract and PAR-2 activation on ERK phosphorylation. 16HBE14o-cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with an antibody against phospho-ERK. Cockroach extract significantly increased airway epithelial cell ERK phosphorylation, whereas TNF- α alone had only modest effects (Fig 5, A and B). Cockroach proteolytic activity mediated the activation of ERK, because treatment of the cockroach extract with the serine protease inhibitor aprotinin (15 minutes at 37°C) before its addition to cells resulted in the loss of ERK activation. Furthermore, selective activation of PAR-2 resulted in phosphorylation of ERK (Fig 5, C).

Cockroach and PAR-2 signaling is mediated through Ras

To test the involvement of Ras, an upstream activator of MEK and ERK in the synergistic effect, we confirmed that cockroach extract activates Ras by using a commercially available affinity precipitation assay. Ras activa-

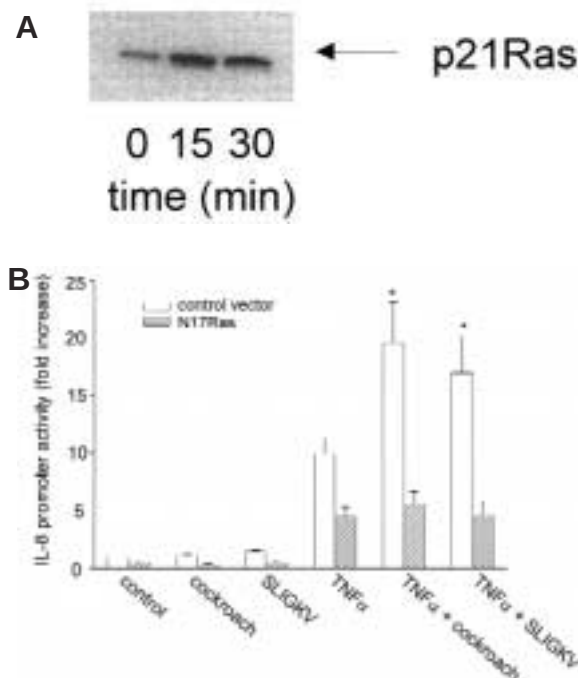


FIG 6. Cockroach extract and activation of PAR-2 require Ras to increase transcription from the IL-8 promoter. **A**, Ras activation was assessed using a commercially available affinity precipitation kit. Samples were treated with cockroach extract for the times listed. This experiment was repeated twice with similar results. **B**, 16HBE14o- cells were transiently cotransfected with vector control or dominant-negative Ras (N17Ras), the IL-8 promoter, and β -galactosidase. Selected cells were treated with cockroach extract or SLIGKV in the absence or presence of TNF- α . *Significantly higher than TNF- α alone ($n = 3-4$, $P < .05$, ANOVA).

tion is determined by binding of beads conjugated to the Ras-binding domain of Raf. Cockroach extract increased Ras activation (Fig 6, A). Next, we transiently cotransfected cells with a dominant-negative form of Ras (N17Ras) and IL-8. Overexpression of dominant-negative Ras inhibited basal and TNF- α -induced IL-8 promoter activity by ~50%. The inhibition of basal IL-8 promoter activity was most likely caused by the remaining basal level of activated Ras after 24-hour serum depletion in these cells. Despite the basal level decrease, in the presence of cockroach extract or the PAR-2 agonist SLIGKV, the synergistic increase in transcription from the IL-8 promoter was abolished (Fig 6, B).

DISCUSSION

We have recently shown that incubation of human bronchial epithelial cells with cockroach extract increases TNF- α -induced IL-8 expression in a protease-dependent manner.⁷ We examined the role of PARs in this process. We chose to study this effect in the simian virus 40-transformed human bronchial epithelial cell line 16HBE14o- because of their availability and ease of transfection. We confirmed that both PAR-1 and PAR-2 are expressed in these cells. Regulation of transcription from the IL-8 promoter in the presence of TNF- α is caused by selective

activation of PAR-2, not PAR-1. Inhibition of PAR cleavage by using blocking antibodies revealed that PAR-2, but not PAR-1, is required for cockroach-induced responses. Pertussis toxin, an inhibitor of G_i-coupled receptors, also abolished cockroach-induced regulation of IL-8. In addition, both cockroach extract and selective activation of PAR-2 induced ERK activation, and inhibition of Ras or MEK attenuated cockroach and PAR-2 responses. Together, these data suggest that cockroach-mediated regulation of IL-8 is caused by activation of PAR-2, followed by a signaling cascade involving Ras, MEK, and ERK.

PAR-2 has recently been shown to play a role in airway function. PAR-2 is expressed in airway epithelial and airway smooth muscle cells^{16,28} as well as on neutrophils.²⁹ Potential activators of PAR-2 in the airways include mast cell tryptase, airway trypsin and other trypsin-like proteases, respiratory allergens, and other exogenous proteases.³⁰ Activation of PAR-2 resulted in bronchoconstriction in the airway smooth muscle cells of anesthetized guinea pigs and isolated human bronchi.^{28,31} Selective activation of PAR-2 with a synthetic peptide increased DNA synthesis in human airway smooth muscle cells in vitro, suggesting a role in smooth muscle cell proliferation.^{32,33} In addition, activation of PAR-2 has been shown to regulate the expression of cytokines and chemokines in human airway epithelium, such as GM-CSF, IL-6, and IL-8.^{14,34}

The transcriptional regulation of IL-8 expression in lung epithelial cells is complex and involves nuclear factor NF- κ B, activator protein (AP)-1, and NF for IL-6 expression (NF-IL6; also known as c/EBP β) promoter sequences.^{25,35,36} TNF- α -induced transcription from the IL-8 promoter activates the classic NF- κ B pathway in A549³⁷ and 16HBE14o- cells.²⁴ More recently, the expression of many inflammatory cytokines and chemokines, including IL-8, has been shown to be regulated by MAP kinases. ERK has been shown to be required for IL-8 expression in multiple cell types, including A549 lung epithelial cells³⁸ and human bronchial epithelial cell lines.^{24,39} MAP kinase activation, in turn, increases the expression or *trans*-activating activity of a number of transcription factors, including the AP-1 family members c-Fos and c-Jun⁴⁰⁻⁴² and C/EBP β .⁴³ We have previously shown in 16HBE14o- cells that ERK activation is required for AP-1 *trans*-activation, and that the IL-8 promoter AP-1 site functions as a basal level enhancer.²⁴ On the other hand, unlike the IL-8 promoter NF- κ B site, the AP-1 site was not required for TNF- α responsiveness.

In this report, we found that both cockroach extract and the PAR-2 agonist significantly increase ERK activation compared with TNF- α alone. The synergistic increase of TNF- α -induced transcription from the IL-8 promoter is caused by the increase of the basal level enhancer ERK by cockroach or the PAR-2 agonist. Inhibition of ERK with chemical or genetic inhibitors had no effect on TNF- α -induced transcription from the IL-8 promoter; however, each abolished cockroach- and PAR-2-mediated synergy with TNF- α , again suggesting the importance of ERK activation in the regulation of IL-8 expression. Activation of

ERK was found to be dependent on the proteolytic activity in the cockroach extract. In a previous report, we have shown that cockroach extract-induced synergy with TNF- α was due to serine protease activity within the extract.⁷ We have confirmed that cockroach extract mediated its effects via activation of PAR-2 using a PAR-2 blocking antibody. There is at least 1 other report in rat vascular smooth muscle cells in which activation of PAR-2 resulted in phosphorylation of ERK.⁴⁴ However, in the same study, activation of ERK in vascular fibroblasts was independent of PAR-2, suggesting that PAR-2-mediated signaling is cell-type dependent. In addition, we have shown that high-level genetic overexpression of a constitutively active MEK resulted in increased transcription from the IL-8 promoter.²⁴ However, the ability of endogenous ERK to induce transcription from the IL-8 promoter is unclear. In this report, we found that cockroach extract and the PAR-2 agonist, both of which increase ERK, do not by themselves induce transcription from the IL-8 promoter. Therefore, our data suggest that activation of endogenous ERK is required but not sufficient to regulate transcription from the IL-8 promoter. Although we have previously shown that overexpression of a constitutively active MEK resulted in increased transcription from the IL-8 promoter, this does not rule out the possibility that physiologic increases in ERK are alone not sufficient to induce IL-8 transcription.

It has been previously shown that extracts of dust mite and fungus directly induce airway inflammation characterized by IL-8 and IL-6 cytokine expression in airway epithelium.^{1,2,4,6} Our data suggest that cockroach proteases might also have a direct effect on the airway epithelium, eliciting the production of a neutrophil chemoattractant, IL-8. However, the effect of aeroallergen proteases on modulating the inflammatory effect in vivo remains unknown. One recent study investigated the role of proteases in the aeroallergen *Aspergillus oryzae* in modulating the asthma phenotype. Sensitization and challenge of C57BL/6 mice with *A oryzae* resulted in allergic lung disease, as determined by airway hyperresponsiveness, airway eosinophilia, and secreted airway mucin. However, if >95% of the protease activity in *A oryzae* was inhibited, a substantial reduction in allergic lung disease phenotype was detected.⁴⁶ These data suggest that protease activity is a critical component of *Aspergillus* exposure. Aside from directly stimulating the airway epithelium, proteases could increase the penetration of allergen into the airway wall and/or play a role as an allergic adjuvant.

Overall, our data suggest that proteolytic activity in cockroach extract modulates TNF- α -induced expression of IL-8 by airway epithelium via activation of PAR-2. The synergistic increase in IL-8 is mediated by activation of ERK, a basal level enhancer of IL-8 transcription. The role of PAR-2 in modulating cytokine expression might play a very important role in the development of airway diseases such as asthma.

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