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INFLAMMATORY RESPONSES OF HUMAN EOSINOPHILS TO COCKROACH ARE MEDIATED THROUGH PROTEASE- DEPENDENT PATHWAYS

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To the Editor:

Exposure to cockroach is one of the major risk factors for developing asthma (1). Among inner-city children, cockroach allergen has an important role in increasing asthma morbidity. The molecular mechanism for this association between cockroach exposure and asthma is unclear. Cockroaches are complex organisms, and the presence and activities of proteases in cockroach extracts have been controversial. In general, the individual allergen proteins, whether purified or recombinant, do not show proteolytic activity (2). However, protease activities were detected in German cockroach frass and whole body extract (3). Eosinophils are likely involved in the pathophysiology of asthma, atopic dermatitis, and certain gastrointestinal diseases (4). Herein, we examined the effects of cockroach products on the activation and effector functions of human eosinophils, and we investigated the biologically active molecules in cockroach extract.

As previously described (5), 12.5 µg/ml German or 6.2 µg/ml Oriental cockroach extracts incubated for 3 h with isolated eosinophils released the eosinophil granule protein, eosinophil-derived neurotoxin (EDN), (see Figure E1 in this article's Online Repository at www.jacionline.org). German cockroach extract induced superoxide anion production after 15 minutes of exposure, and Oriental cockroach extract induced superoxide anion production after 30 minutes (Figure E1). Both German and Oriental cockroach extracts treated at 4 °C or 37 °C potentially induced EDN release (Figure 1A). In contrast, inactivating the extracts at 56 °C decreased their ability to stimulate eosinophils (p<0.05, n=4), and treatment at 100 °C almost abolished the activity. To verify the importance of heat-labile molecule(s) in eosinophil activation induced by cockroach extracts and to demonstrate their lack of direct toxicity to eosinophils, we examined IL-8 production. After 24 hr, both German and Oriental cockroach

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extracts induced IL-8 production by eosinophils ($p < 0.05$, $n = 4$) (Figure 1B); their stimulatory effects were partially heat-inactivated at 56 °C and abolished at 100 °C. Thus, heat-labile molecules, likely proteases, in cockroach extracts are involved in eosinophil activation.

PARs, in particular PAR-2, likely play a major role in eosinophil activation in response to proteases (6). Therefore, we investigated whether cockroach extracts contain proteolytic activities that activate PAR-2. An authentic ligand for PAR-2, namely trypsin, cleaves the extracellular N-terminus of PAR-2 between R³⁶ and S³⁷ and exposes a tethered “neo-ligand” (i.e. S³⁷LIGKV-) that binds intramolecularly to PAR-2 and triggers cellular activation (7). To mimic the proteolytic activation of PAR-2, we used a fluorogenic peptide substrate, Abz-SKGRSLIGKdD, which encompasses the trypsin cleavage site of human PAR-2 (from Ser³³ to Lys⁴¹) (8); the Abz group fluoresces only after release of the KdD group, following cleavage of internal peptides. As expected, trypsin cleaved the PAR-2 peptide, increasing the fluorescence intensity over time (Figure 2A). German and Oriental cockroach extracts also cleaved the PAR-2 peptide in a concentration-dependent manner. To characterize the cockroach proteases cleaving this PAR-2, we used protease inhibitors. Pepstatin A specifically inhibits acid proteases, in particular aspartate proteases. Absorption of a complex protease mixture with pepstatin A agarose removes aspartate-like proteases. Treatment of German and Oriental cockroach extracts with pepstatin A agarose decreased PAR-2 cleavage by 70% and 55 %, respectively ($p < 0.05$, $n = 5$); treatment with control agarose showed no effects (Figure 2B). Pepstatin A treatment showed no effects on trypsin-mediated PAR-2 cleavage, demonstrating the inhibitor’s specificity. A serine protease inhibitor, APMSF, and a cysteine protease inhibitor, E64, did not inhibit PAR-2 cleavage by cockroach extracts (Figure 2C); APMSF significantly inhibited trypsin-induced PAR-2 cleavage. Furthermore, after pretreatment with pepstatin A, both German and Oriental cockroach extracts induced less EDN release compared to the untreated extracts (Figure 2D, $p < 0.05$, $n = 5$). Pretreatment of PMA with pepstatin A did not affect eosinophil degranulation. In addition, preincubating eosinophils with the PAR-2 antagonistic peptide, L³⁶SIGKV, before exposure to German cockroach extract inhibited degranulation by an average of 37% ($p < 0.01$, $n = 9$). Overall, the cockroach extracts likely contain pepstatin A-sensitive protease(s) that activate PAR-2 and induce eosinophil degranulation.

In general, trypsin and trypsin-like proteases cleave PAR-2 specifically between Arg³⁶ and Ser³⁷ (7). To investigate the PAR-2 site cleaved by cockroach extracts, we analyzed the peptide fragments by reverse-phase-HPLC-ESI-MS. The same peptide substrate, as used in the PAR-2 cleavage enzymatic assay (Abz-SKGR³⁶S³⁷LIGKdD), was incubated with cockroach extracts, which had been pretreated with medium, pepstatin A agarose or control agarose. As expected, trypsin cleaved the peptide substrate between Arg³⁶ and Ser³⁷ and produced the S³⁷LIGKdD fragment (Figure 3); pretreatment of trypsin with pepstatin A agarose showed no effect on the cleavage (see Table E1 in this article’s Online Repository at www.jacionline.org). Both German and Oriental cockroach extracts cleaved the peptide substrate between Arg³⁶ and Ser³⁷, producing an S³⁷LIGKdD fragment (Figure 3 and Table E1). Pretreatment of German and Oriental cockroach extracts with pepstatin A inhibited production of the S³⁷LIGKdD peptide fragment by about 50~70%. Thus, these cockroach extracts likely contain pepstatin A-sensitive protease(s), which cleaves PAR-2 peptide similarly to the authentic PAR-2 ligand, trypsin.

While none of the well-characterized cockroach allergens showed proteolytic activity, the midgut of cockroaches contains trypsin, chymotrypsin, subtilisin, and cysteine protease-like proteases (9). Typically, PAR-2 is cleaved and activated by serine proteases, such as trypsin (7). However, in a human epithelial cell line stimulated with German cockroach extract, the aspartate protease inhibitor, pepstatin A, and the cysteine protease inhibitor, E64, inhibited phospho-p44 MAP kinase levels, suggesting a role for cysteine proteases or aspartate proteases.

Furthermore, exogenous chitinase from a bacterium, *Streptomyces griseus*, cleaved human PAR-2 peptide and induced a PAR-2-dependent $[Ca^{2+}]_i$ response. These previous observations and our current findings suggest that PAR-2 recognizes both conventional trypsin-like proteases and perhaps other proteases and glycosidases derived from microbes, fungi and insects. Understanding these cockroach protease molecules and their receptors on immune cells may create novel strategies to prevent and to treat bronchial asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

APMSF	4-Amidinophenylmethanesulfonyl fluoride hydrochloride
EDN	eosinophil-derived neurotoxin
HPLC-ESI-MS	electrospray ionization mass spectrometry
PAR	protease-activated receptor
PMA	Phorbol-12-Myristate-13-acetate

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Figure 1. The eosinophil-stimulating activities of cockroach extracts are heat-sensitive
German and Oriental cockroach extracts (50 μ g/ml) were heat-treated at 37, 56 and 100 °C or kept at 4 °C for 30 min. (A) Human eosinophils were incubated with these stimuli for 3 hr at 37 °C; EDN concentrations in the cell-free supernatants were measured by ELISA. (B) Eosinophils were incubated with stimuli for 24 hr at 37 °C; IL-8 concentrations were measured by ELISA. Results show the means $\pm$ SEM from 5 (A) or 4 (B) different donors. *, ** significant differences compared with 4 °C-treated extract ($P < 0.05$, < 0.01 , respectively). # significant difference compared to IL-8 production with medium alone.

Figure 2. Cockroach extracts have pepstatin-sensitive aspartate protease activity that is involved in PAR-2 cleavage and eosinophil degranulation

(A) German and Oriental cockroach extracts (10 or 50 μ g/ml) and trypsin (1 nM) were incubated with a PAR-2 fluorogenic peptide substrate, Abz-SKGRSLIGKdD (50 μ M), for 60 min at 37 °C. The figure is representative of three experiments having similar results. (B) German (5 μ g/ml) and Oriental (50 μ g/ml) cockroach extracts or trypsin (1 nM) were incubated with pepstatin A agarose, control agarose or medium only for 15 min at 4 °C. After centrifugation, supernatants were incubated with Abz-SKGRSLIGKdD (50 μ M) as above. Results show the means $\pm$ SEM from 3 experiments. *; significant differences compared with non-treated extracts ($P < 0.05$). (C) German (5 μ g/ml) and Oriental (50 μ g/ml) cockroach

extracts or trypsin (1 nM) were incubated with buffer alone (control), 200 μ M AMPSF or 10 μ M E64 for 30 min at room temperature and were incubated with Abz-SKGRSLIGKdD (50 μ M) as above. Results show the means $\pm$ SEM from 3 experiments. *, significant differences compared with control-treated extracts ($P<0.05$). (D) Cockroach extracts (50 μ g/ml) and PMA (1 ng/ml) were preincubated with pepstatin A agarose, control agarose or medium for 15 min at 4 °C. Eosinophils were incubated with these treated extracts for 3 hr at 37 °C. EDN concentrations were measured by ELISA. Results show the means $\pm$ SEM from 6 donors. *, significant differences compared with non-treated extracts ($P<0.05$).

Figure 3. German cockroach extract cleaves PAR-2 peptide substrate between arginine and serine
 German cockroach extract samples were pretreated with pepstatin A agarose, control agarose or untreated for 15 min at 4 °C. Next, the fluorogenic peptide substrate, Abz-SKGRSLIGKdD, was incubated for 30 min at 37 °C with these cockroach samples (10 µg/ml) or trypsin (1 nM). Peptide products were analyzed by reverse-phase-HPLC-ESI-MS. The results are representative of four different experiments.