

Coagulation Factor Xa Modulates Airway Remodeling in a Murine Model of Asthma

Kazuhiko Shinagawa, J. Andrew Martin, Victoria A. Ploplis, and Francis J. Castellino

W.M. Keck Center for Transgene Research and Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana

Rationale: Previous studies have demonstrated that dysregulated coagulation and fibrinolysis contribute to the pathogenesis of asthma.

Objective: The role of procoagulant factor X in a murine model of ovalbumin (OVA)-induced asthma was investigated.

Methods: Biochemical, cellular, and physiologic *in vivo* and *in vitro* approaches were used to determine effects of factor X on the asthmatic response in mice.

Measurements and Main Results: Factor X transcript levels and factor Xa activity were increased in lungs of asthmatic mice challenged with OVA, compared with controls treated with phosphate-buffered saline. Factor X was highly expressed in bronchoalveolar lavage fluid macrophages from asthmatic mice. Treatment of mice with the factor Xa inhibitor fondaparinux during the last 4 wk of OVA challenge resulted in the attenuation of airway hyperresponsiveness but did not alter infiltration of inflammatory cells into the lung. There was a significant decrease in the thickness of the mucosal layer and in lung collagen deposition in fondaparinux-treated mice. *In vitro* investigations using human mucus-producing NCI-H292 cells indicated that exogenous factor Xa enhanced mucin production in a dose-dependent manner. Levels of amphiregulin, a protein that induces mucin production, were also increased in cells stimulated by factor Xa.

Conclusions: The results of this study introduce a novel participant in the asthmatic response and indicate that factor Xa functions in airway remodeling in asthma by stimulating mucin production, through regulation of amphiregulin expression and collagen deposition.

Keywords: coagulation protein; airway hyperresponsiveness; mucus production; amphiregulin

Previous investigations have indicated that coagulation- and fibrinolysis-related proteins contribute to the pathogenesis of asthma. Plasminogen activator inhibitor-1 levels are increased during asthma and contribute to fibrosis (1–3). Furthermore, activated protein C is associated with the chronic asthmatic response (4, 5). Recently, it was reported that fibrin deposition and abnormalities in the coagulation and fibrinolytic pathways in the distal airways of the lung significantly contribute to airway hyperresponsiveness and airway closure in asthma (6). In that study, fibrin deposits were found on the luminal surface of the airway epithelium in a patient who died in status asthmaticus. In related work, extravascular fibrinogen and thrombin have

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Previous results implied that an imbalance in coagulation and fibrinolysis contributes to the pathologic events associated with asthma, but their mechanistic roles in these processes have not been elucidated.

What This Study Adds to the Field

This study suggests that coagulation protein factor Xa may be involved in the regulation of asthma.

been identified in the sputum of patients with asthma (7, 8). Thrombin activity in bronchoalveolar lavage fluid (BALF) was increased by segmental antigen provocation in patients with asthma, and BALF from these patients was able to induce proliferation of fibroblasts (9). These results suggest an association between coagulation events and asthmatic responses. It is not known whether coagulation factors are directly associated with the asthmatic response. Fibrin formation is the result of thrombin generated by factor Xa (FXa), a key component of the intrinsic and extrinsic coagulation pathways. As a result, we hypothesize that FXa plays a role in asthmatic responses and tested this hypothesis using an ovalbumin (OVA) allergen mouse model (10, 11). Some of the results of these studies have been previously reported in the form of an abstract (12).

METHODS

Animals

All experimental procedures were approved by the Laboratory Animal Care and Use Committee of the University of Notre Dame. Male A/J mice (7–10 wk old) were used in this study because of the sensitive airway responses in this mouse strain.

Induction of Eosinophilic Inflammation

Mice were anesthetized by isoflurane inhalation, after which OVA (1 mg/ml) was administered intranasally (50 μ l) 3 d/wk for 16 wk. Phosphate-buffered saline (PBS) was administered to mice as a vehicle control. At 24 h after the last OVA dose, the mice were anesthetized, and the lungs were lavaged with sterile PBS. Cell counts were performed on bronchoalveolar lavage cells. BALF was collected for analyses of FXa activity.

Isolation of Macrophages, Lymphocytes, and Eosinophils

Macrophages were purified from BALF by adhesion to a plastic surface, and T and B lymphocytes were purified from the peribronchial lymph nodes using Dynabeads specific for T and B cells (Dynal Biotech, Oslo, Norway). Eosinophils were purified from BALF by lectin affinity negative selection as previously reported (13). Additional details on these methods are provided in the online supplement.

(Received in original form August 5, 2006; accepted in final form October 27, 2006)

Supported by grant HL73750 from the National Institutes of Health and the Kleiderer-Pezold endowed professorship (F.J.C.).

Correspondence and requests for reprints should be addressed to Francis J. Castellino, Ph.D., 230 Raclin-Carmichael Hall, W.M. Keck Center for Transgene Research, University of Notre Dame, Notre Dame, IN 46556. E-mail: fcastell@nd.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 175, pp 136–143, 2007

Originally Published in Press as DOI: 10.1164/rccm.200608-1097OC on November 2, 2006
Internet address: www.atsjournals.org

Quantitative Real-time Reverse Transcriptase–Polymerase Chain Reaction

Quantitative reverse transcriptional–polymerase chain reaction (RT-PCR) was performed as previously described (14). Total RNA was isolated from lungs, BALF, and NCI-H292 cells by the RNeasy total RNA purification kit (Qiagen, Valencia, CA). For determining if FXa-mediated induction of mucin production is regulated through amphiregulin, a mouse monoclonal antibody to human amphiregulin (10 µg/ml) (R&D Systems, Minneapolis, MN) was added to NCI-H292 cells before the addition of FXa. Quantitative RT-PCR was performed with the primers and probes listed in Table 1 for FX, protein-activated receptor (PAR)1, PAR2, amphiregulin, MUC5AC, and the housekeeping gene RPL19.

Assay of FXa Activity

FXa activity was determined using a chromogenic assay with S-2222 (Chromogenix, Lexington, MA) as the substrate. Human FXa (Enzyme Research Laboratories, South Bend, IN) was used as the standard over the concentration range of 0.001–1 U/ml.

Fondaparinux Levels in Blood as a Function of Time

Fondaparinux (FPX) (GlaxoSmithKline, Triangle Park, NC) was administered subcutaneously to mice at doses of 10 or 30 µg. A standard curve of FXa inhibition was generated using FPX at 0.01–1 µg/ml and FXa at 0.2 U/ml in an FXa chromogenic assay to determine FPX levels in test serum samples at various times after its administration.

Assessment of Airway Responsiveness

Airway hyperresponsiveness was measured by lung resistance in anesthetized mice at 24 h after the last intranasal antigen challenge as previously reported (10). Increasing doses of methacholine (1–16 mg/ml) (Sigma-Aldrich, St. Louis, MO) were administered by inhalation. Additional details on this method are provided in the online supplement.

FPX Treatment

FPX was administered subcutaneously to a population of OVA-treated mice, from Week 13 to 16 during challenge, at a dose of 10 or 30 µg for 6 d/wk. Fluticasone (TOCRIS Bioscience, Ellisville, MO) was used as a positive control and administered intranasally at 10 µg/mouse for 6 d/wk.

Collagen Deposition, Mucus Production, and Smooth Muscle Cell Hyperplasia in Lung Tissue

Lungs were obtained at 24 h after the final OVA administration, fixed with periodate-lysine-paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 4 µm. Slides were stained with periodic acid–Schiff or Masson's trichrome. Smooth muscle α-actin immunostains were performed as previously described (15).

Measurement of Airway Wall Thickness

Lung tissue was prepared as described previously, and slides were stained with Elastica van Gieson. Cross-sections of airways were used for measuring the airway wall thickness. Additional detail on the methods for making these measurements is provided in the online supplement.

Hydroxyproline Assay

Total lung collagen was determined by analysis of hydroxyproline (10).

Mucin Production from NCI-H292 Cells

NCI-H292 cells were seeded in 96-well plates and cultured for 2 d in RPMI 1640 media containing 10% fetal bovine serum. After reaching confluence, the cells were washed twice with PBS and incubated with fetal bovine serum-free RPMI 1640 for 2 h. NCI-H292 cells were then stimulated for 48 h with various concentrations of FXa, thrombin (ERL), epidermal growth factor (EGF) (PeproTech, Rocky Hill, NJ), or activation peptides for PAR1 and PAR2 (TOCRIS). After fixation,

TABLE 1. SEQUENCES OF PRIMERS AND PROBES USED FOR QUALITATIVE REVERSE TRANSCRIPTIONAL–POLYMERASE CHAIN REACTION

Gene and Type	Sequence	Position	Size (bp)
RPL19 (NM_009078)			
Forward	ATGTATCACAGCCTGTACCTG	355–375	
Probe*	TTTCGTGCTTCCTTGGTCTTAGACCT (AS)	487–512	233
Reverse	TTCTTGGTCTCCTCCTCCTG	567–587	
FX (NM_007972)			
Forward	CTGCTGCCCACTGTCTCCAT	809–828	
Probe†	TCAAGGTGAGGGTAGGTGATCGGAACA	842–868	101
Reverse	GTCACCTCGTGCACCATCT	890–909	
PAR-1 (NM_010169)			
Forward	CAGCCAGAATCAGAGAGGACAGA	141–163	
Probe*	CGGTGAACCCCGCTCATCTTTCTAA (AS)	169–195	136
Reverse	CCAGCAGGACGCTTTCATTT	257–276	
PAR-2 (NM_007974)			
Forward	AGCCGGACCGAGAACCTT	165–182	
Probe*	CTTCCTTACTGTTGTTGCGTCCCGGT (AS)	185–211	101
Reverse	GGAACCCCTTCCCACTGATT	245–265	
MUC5AC (XM_001130382)			
Forward	TACTCCACAGACTGCACCAACTG	1272–1294	
Probe*	TGTGCTTGAGGTGCCCACTTCTCAA	1352–1377	129
Reverse	CGTGTATTGCTTCCCGTCAA	1381–1400	
Amphiregulin (NM_001657)			
Forward	AGGCCATTATGCTGCTGGAT	270–289	
Probe*	ACCTCAATGACACCTACTCTGGGAAGCGT	293–321	74
Reverse	TGTGGTCCCCAGAAAATGGT	324–343	

Definition of abbreviation: AS = antisense.

* Probes were labeled at the 5' end with 6-FAM and at the 3' end with BHQ1 or TAMRA.

assays for MUC5AC production were performed. For FPX studies in FXa-stimulated cells, 12.8 $\mu\text{g/ml}$ of antithrombin-III was added. Mouse-anti-human MUC5AC (45M1; Lab Vision, Fremont, CA) was used as the primary antibody. Maximal production of MUC5AC (100%) was identified as the absorbance obtained from cells stimulated with 1 $\mu\text{g/ml}$ EGF.

Amphiregulin Protein Levels in the Supernatant of NCI-H292 Cells

Human amphiregulin levels in supernatants were obtained from the culture medium of NCI-H292 cells that were incubated with 0–30 U/ml of FXa and were measured using the DuoSet ELISA Development System kit (R&D Systems, Minneapolis, MN).

Data Analysis

Data were analyzed using the Student's *t* test and are represented as the mean \pm SEM. Analysis of variance, with Dunnett's test for multiple comparisons, was used for determining significance (Excel Statistics, version 5.0; Esumi Co. Ltd., Tokyo, Japan). A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Increase of Coagulation FX mRNA Level in the Lungs of Asthmatic Mice

After mice were exposed to OVA for 16 wk, their lungs were removed, and total RNA was isolated. The mRNA levels of FX were compared with PBS-exposed mice (Figure 1A). FX

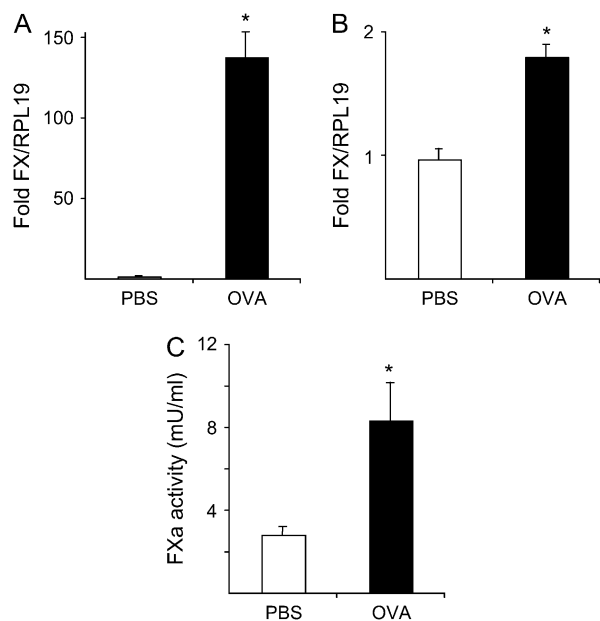


Figure 1. (A) Quantitative reverse transcriptional–polymerase chain reaction (RT-PCR) analysis of factor X (FX) mRNA in lungs obtained from ovalbumin (OVA)-exposed mice (solid bar, *n* = 6) at 16 wk of treatment versus phosphate-buffered saline (PBS)-treated mice (open bar, *n* = 6). The values indicate the fold difference relative to RPL19 mRNA. Data represent the mean \pm SEM. **p* = 0.004 versus PBS-treated mice. (B) Quantitative RT-PCR analysis of FX mRNA in macrophages obtained from bronchoalveolar lavage fluid (BALF) of OVA-exposed mice (solid bar, *n* = 4) versus PBS-treated mice (open bar, *n* = 4). Values indicate the fold difference relative to the RPL19 housekeeping gene. Data represent the mean \pm SEM. **p* < 0.001 versus PBS-treated mice. (C) FXa activity in BALF from OVA-exposed mice (solid bar, *n* = 5) versus PBS-treated mice (open bar, *n* = 5) determined by a S-2222–based chromogenic assay. **p* = 0.04 versus PBS-treated mice.

transcripts were significantly elevated in OVA-exposed mice relative to PBS controls (137.26 ± 16.29 [*n* = 6] vs. 1.03 ± 0.09 [*n* = 6], fold change relative to RPL19, respectively; *p* = 0.0019). In BALF, FX message was only detected in macrophages, and its level was increased in OVA-challenged mice relative to PBS-challenged mice (1.79 ± 0.10 [*n* = 4] vs. 0.96 ± 0.09 [*n* = 4], fold change relative to RPL19, respectively; *p* = 0.0009) (Figure 1B). FX mRNA levels were undetectable in the human epithelial cell line NCI-H292, eosinophils from BALF, and T and B cells purified from OVA-challenged mouse lung lymph nodes (data not shown).

Increase of Coagulation FXa Activity in the BALF of Asthmatic Mice

FXa activity was measured in BALF from mice exposed to OVA for 12 wk, along with similarly treated PBS controls. The FXa activity was increased in OVA-challenged mice in comparison to PBS-challenged mice (8.30 ± 1.86 mU/ml [*n* = 5] vs. 2.80 ± 0.42 mU/ml [*n* = 5], respectively; *p* = 0.04) (Figure 1C). To confirm that FXa is the enzyme responsible for hydrolysis of S-2222 in BALF, tick anticoagulant peptide (TAP) (a generous gift from Dr. S. Krishnaswamy, Children's Hospital of the University of Pennsylvania), specific for inhibition of FXa, was added to the reaction mixture. Studies using purified human FXa and murine FXa (Hematologic Technologies, Burlington, VT) demonstrated an approximately 10-fold weaker inhibition of murine FXa relative to human FXa. In the interest of BALF sample stability, it was required that a concentration of TAP be used at which the inhibition of mouse FXa is immediate. Under these experimental requirements, at 10^{-4} M TAP, more than 80% of the S-2222 hydrolysis due to murine FXa was inhibited when mouse BALF, TAP, and S-2222 were added together (data not shown). Thus, at least 80% of the increased S-2222 activity in BALF was attributed to FXa.

Effect of FPX on Respiratory Function

To investigate the effects of FXa inhibition on asthmatic responses, FPX, a selective FXa inhibitor, was administered subcutaneously to mice 6 d/wk for 4 wk during 12–16 wk of OVA challenge (Figure 2A). This time frame was chosen because eosinophilic inflammation, airway wall thickness, and airway hyperresponsiveness plateaued at these time points (10). Serum concentrations of FPX were monitored by the ability of test sera to inhibit FXa activity after a single injection of FPX (Figure 2B). The serum concentration of FPX was 0.913 and 1.229 $\mu\text{g/ml}$, at a dose of 10 μg or 30 μg , respectively, at 1 h after injection. FPX levels were detectable after 8 h (0.194 and 0.312 $\mu\text{g/ml}$, at a dose of 10 or 30 μg , respectively), and these values were higher than the IC_{50} value (0.20 $\mu\text{g/ml}$) for inhibiting FXa activity (Figure 2C). The FPX concentrations in the BALF obtained 1 h after its injection were 0 and 0.358 ± 0.002 ng/ml in vehicle (PBS)- and FPX-injected mice, respectively.

Respiratory function was determined 24 h after the final antigen provocation, and the lung resistance (R_L) was found to be significantly increased in OVA-challenged mice after 16 wk of challenge. R_L was determined by exposing the mice to increasing concentrations of aerosolized methacholine. FPX (30 μg) attenuated the increase of R_L on OVA-challenged mice, compared with OVA-challenged mice not receiving FPX, although this agent was not effective at lower doses of 10 μg (Figure 3). The effect of FPX at 30 μg was slightly less than fluticasone (10 μg), a glucocorticoid frequently used to treat asthma, but it still significantly attenuated airway hyperresponsiveness.

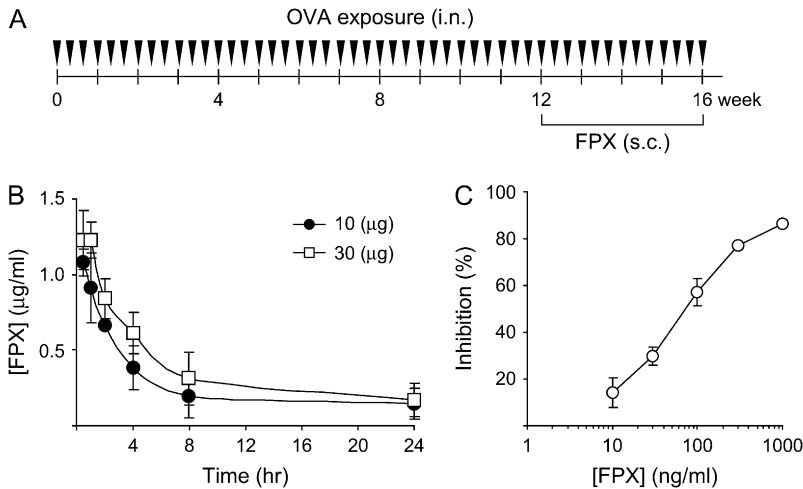


Figure 2. (A) Timeline showing the protocols used for OVA exposure (instillation of OVA solution intranasally 3 d/wk for 16 wk). Fondaparinux (FPX) was administered at 10 and 30 μg by subcutaneous injection 6 d/wk from Week 13 to Week 16 after initiation of OVA challenge. Fluticasone was given at a dose of 10 μg , intranasally, 6 d/wk, from Week 13 to Week 16. Mice were killed 24 h after the final OVA challenge. (B) Serum FPX concentration in mice as a function of time after a single dose of 10 μg or 30 μg of FPX. (C) Factor Xa inhibitory activity of FPX as a function of FPX concentration. Data represent the mean \pm SEM.

Effect of FPX on Cell Numbers in BALF

Inflammatory cells in BALF were increased after OVA exposure for 16 wk and were compared with PBS-treated mice. Among the inflammatory cells, macrophages, eosinophils, and lymphocytes were increased in OVA-challenged mice. FPX treatment for 4 wk, from Week 13 to Week 16 after initiation of the OVA challenge, did not affect the total cell numbers in the BALF (Figure 4). Specifically, eosinophils, macrophages, and lymphocytes were not affected by treatment with this drug. In contrast, fluticasone decreased the numbers of eosinophils and lymphocytes and the total number of cells in BALF.

Effect of FPX on Goblet Cell Hyperplasia, Airway Wall Thickness, Collagen Deposition, and Mucus Production

Periodic acid–Schiff staining (goblet cells) was increased in lung tissue from OVA-challenged mice compared with FPX-treated,

OVA-challenged mice (Figures 5A and 5B). Mucus secretion was also observed in OVA-challenged mice, which was not present in FPX-treated, OVA-challenged mice (Figures 5A and 5B). In addition, collagen deposition (Figure 5C) and the presence of smooth muscle cells (Figure 5E) were increased in OVA-challenged mice relative to FPX-treated, OVA-challenged mice (Figures 5D and 5F, respectively). To quantify the thickness of the mucosal layer, cross-sectional areas were obtained using computer graphic analysis of Elastica van Gieson–stained sections as previously reported (10). The thickness of the mucosal layer was increased approximately 2.5 \times in 16-wk OVA-challenged mice in comparison to PBS-challenged mice (Figure 6A and Table E1 in the online supplement). The thickness of the smooth muscle layer was also increased by approximately 2.2 \times (Figure 6B and Table E1). FPX treatment of OVA-challenged mice, at a dose of 30 μg , significantly reduced the thickness of the mucosal layer compared with nontreated, OVA-challenged mice (Figures 6A and 6B and Table E1).

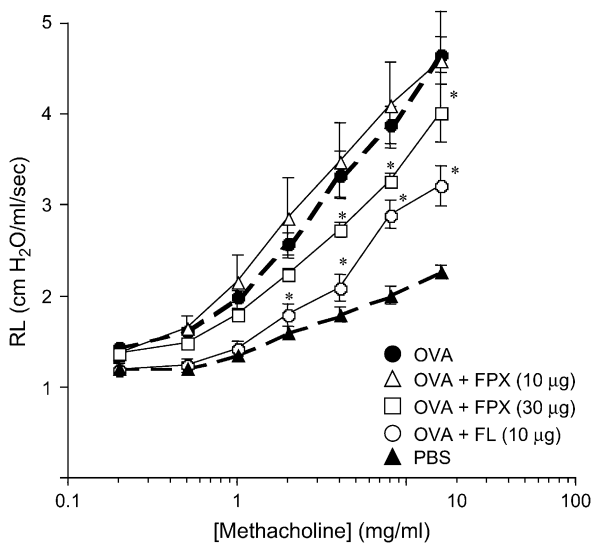


Figure 3. Effects of FPX and the positive control, fluticasone (FL), on respiratory function in mice exposed to OVA for 16 wk. Airway hyperresponsiveness (AHR) was induced by methacholine inhalation (1–16 mg/ml). AHR was monitored 24 h after the final OVA challenge. Data represent the mean \pm SEM ($n = 6$ –9). * $p < 0.05$ versus OVA-challenged mice not treated with FPX.

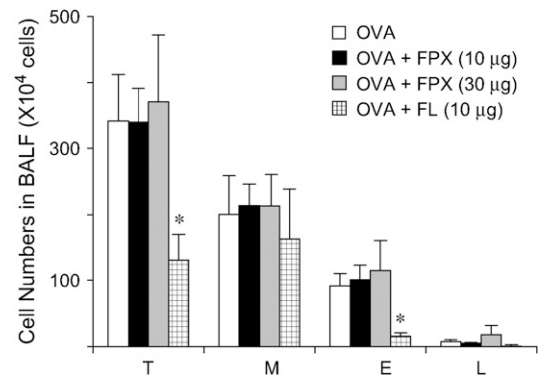


Figure 4. Effects of FPX on cell numbers in the BALF of OVA-exposed mice after 16 wk of OVA challenge. BAL was performed 24 h after the final challenge of OVA. BALF cells were counted and dispersed onto microscope slides using a cytopsin. Cytopsin preparations were stained with DiffQuick to obtain differential leukocyte counts. Total cell (T), macrophage (M), eosinophil (E), and lymphocyte (L) counts in BALF samples obtained from mice exposed to OVA for 16 wk. Data represent the mean \pm SEM ($n = 5$ –6). * $p < 0.05$ versus OVA-challenged mice not treated with FPX.

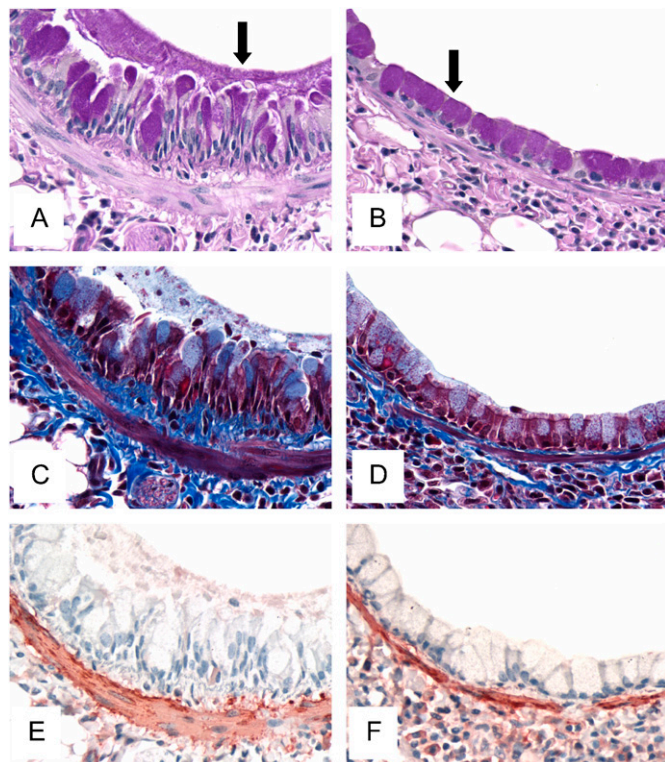


Figure 5. Periodic acid-Schiff (A and B), Masson trichrome (C and D), and α -smooth muscle actin (E and F) staining of whole lung sections from mice challenged with OVA for 16 wk. The photomicrographs are representative of lungs removed from OVA-challenged controls (A, C, E) and FPX-treated, OVA-challenged mice (B, D, F). Goblet cell hyperplasia and mucus plugs in control mice were observed around the airways (arrow in A), but goblet cell hyperplasia was decreased and mucus plugs not evident in mice treated with 30 μ g FPX (arrow in B). Enhanced collagen deposition (C, dark-blue areas) and smooth muscle cell hyperplasia (E, brown areas) were observed in control mice relative to FPX-treated, OVA-challenged mice (D and F, respectively). Original magnification: $\times 400$.

Effect of FPX on Hydroxyproline Contents in the Lung

The hydroxyproline content in the lungs was determined as a marker of fibrosis. This value was significantly increased in OVA-challenged mice compared with PBS-challenged mice (421.8 ± 26.6 [n = 10] vs. 122.6 ± 12.9 [n = 6] μ g/lung, respectively; $p < 0.000001$) (Figure 6C). FPX treatment of OVA-challenged mice significantly decreased the hydroxyproline content in the lungs in comparison to OVA-challenged mice (316.5 ± 39.9 [n = 10] vs. 421.8 ± 26.6 [n = 10] μ g/lung, respectively; $p = 0.04$) (Figure 6C).

Effect of FPX on mRNA Levels of FX, PAR-1, and PAR-2 in the Lung

The mRNA levels of FX, PAR1, and PAR2 were analyzed in lung tissue by quantitative RT-PCR (Figure 7). The levels of FX mRNA were increased in 16-wk OVA-challenged mice in comparison to PBS-challenged mice (Figure 7A and Table E2). FPX treatment of OVA-challenged mice, at a dose of 30 μ g for 4 wk, significantly decreased the FX mRNA levels relative to nontreated OVA-challenged mice (Figure 7A and Table E2). Furthermore, mRNA levels of PAR-1 and PAR-2 were increased in OVA-challenged mice in comparison to PBS-challenged mice (Figures 7B and 7C and Table E2). These levels were decreased after FPX treatment (Figures 7B and 7C and Table E2).

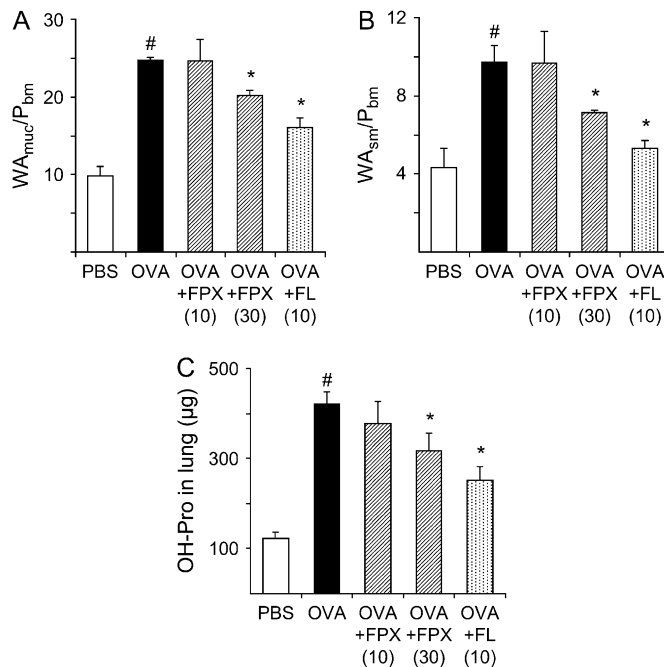


Figure 6. Effects of FPX and the positive control FL on airway wall thickness in bronchi in mice challenged with OVA for 16 wk. Values for cross-sectional areas of (A) mucous layer and (B) smooth muscle cell layer. Data represent the mean \pm SEM (n = 3–4) for each group. [#] $p < 0.05$ versus PBS-treated mice; ^{*} $p < 0.05$ versus OVA-challenged mice not treated with FPX. (C) Hydroxyproline (OH-Pro) levels in lung tissue were measured and expressed as micrograms per lung. Data represent the mean \pm SEM (n = 5–10). ^{*} $p < 0.05$ versus OVA-challenged mice not treated with FPX. [#] $p < 0.05$ versus PBS-treated mice.

Effect of FXa, Thrombin, and PAR1-AP and PAR2-AP on Mucin Production in NCI-H292 Cells

NCI-H292 cells, a human mucin-producing cell line, were seeded in 96-well plates, and mucin production was investigated after stimulation with EGF or FXa. MUC5AC production was increased in a dose-dependent manner by the addition of EGF (Figure 8A). MUC5AC production was also significantly increased by stimulation with FXa in a dose-dependent manner (Figure 8B). In an additional set of experiments in which 30 μ g of FPX and 12.8 μ g/ml of antithrombin-III were added to the cells stimulated with 30 U/ml thrombin, mucin levels diminished from $51.35 \pm 2.93\%$ of maximum (n = 4) to $4.74 \pm 1.70\%$ of maximum (n = 4) ($p < 0.00001$). Thrombin also increased mucin production in a dose-dependent manner (Figure 8B), although activation of PAR1 or PAR2 did not seem to have a significant affect on mucin production (Figure 8C) as previously observed (16).

Effect of FXa on Amphiregulin mRNA and Protein Levels in NCI-H292 Cells

The mRNA levels of amphiregulin were significantly elevated in NCI-H292 cells after incubation with 30 U/ml of FXa relative to nonstimulated cells (80.78 ± 4.34 -fold [n = 4] vs. 1.08 ± 0.24 -fold [n = 4], relative to RPL19; $p = 0.0003$, respectively) (Figure 9A). In addition, protein levels in the supernatant increased in an FXa dose-dependent manner and were significantly higher than nonstimulated cells at 10 U/ml and 30 U/ml of FXa ($p < 0.01$) (Figure 9B). The increase in FXa-induced MUC5AC mRNA levels in NCI-H292 cells was decreased by incubating with an anti-amphiregulin antibody (Figure 9C).

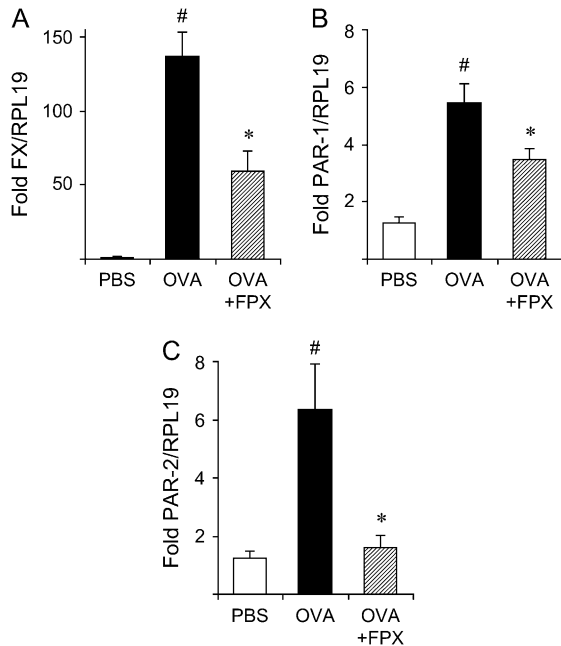


Figure 7. Effects of FPX on mRNA levels of (A) FX, (B) PAR1, and (C) PAR2 in the lungs of mice challenged with OVA for 16 wk. The values indicate the fold difference relative to RPL19. Data represent the mean \pm SEM (n = 5–6). [#]p < 0.05 versus PBS-treated mice; ^{*}p < 0.05 versus OVA-challenged mice not treated with FPX.

DISCUSSION

The results from this study identify FXa as a novel participant in airway remodeling in asthma and link FXa with amphiregulin production for the first time. The observation that FX mRNA expression was present in the lung tissue and FXa activity in BALF in OVA-challenged mice supports a role for FXa in the

asthmatic response. Further studies indicated that FX was highly expressed in purified alveolar macrophages from BALF. This is consistent with other studies in which FX was shown to be expressed in murine peritoneal macrophages (17). mRNA levels of FX were also elevated in lung tissue, most probably from type II alveolar epithelial cells and/or bronchial epithelial cells (18). FPX attenuated the airway hyperresponsiveness induced by OVA challenge, although it did not affect the recruitment of inflammatory cells in BALF. FPX treatment decreased the thickness of the airway wall and the hydroxyproline content, a measure of fibrosis, in the lungs. In experiments using NCI-H292, a human-derived mucus-producing cell line, FXa induced mucin production. These results indicate that FXa is associated with asthma by increasing airway wall thickness and exacerbating asthma.

In the present study, FPX attenuated the airway remodeling, thereby ameliorating the airway hyperresponsiveness in the chronic asthma model under conditions in which airway changes have already been established. Although FPX is less effective than fluticasone, which is one of the most efficient drugs for treating asthma, FPX ameliorated the airway remodeling process. FPX is a synthetic antithrombin-III (AT-III) binding pentasaccharide and shows a therapeutic advantage in preventing thromboembolism (19). The mechanism by which FPX inhibits FXa is by enhancing the reactivity of AT-III to FXa. FPX potentiates the neutralization of FXa by AT-III approximately 300 \times greater than heparin, but several reports indicate that it also inactivates other enzymes (20). However, the concentration of FPX for effective inhibition of FXa is approximately 10-fold less compared with that for other enzymes. Therefore, we propose that the effects we observed in this study are due primarily to inhibition of FXa, and this was confirmed by a similar use of another specific FXa inhibitor, TAP.

Several studies have demonstrated that FXa stimulates fibroblast proliferation and induces collagen production through the PAR1 pathway (21, 22). However, up to now, it has not been reported that FXa is associated with mucin production. In this study, mucin production (MUC5AC) was analyzed by using

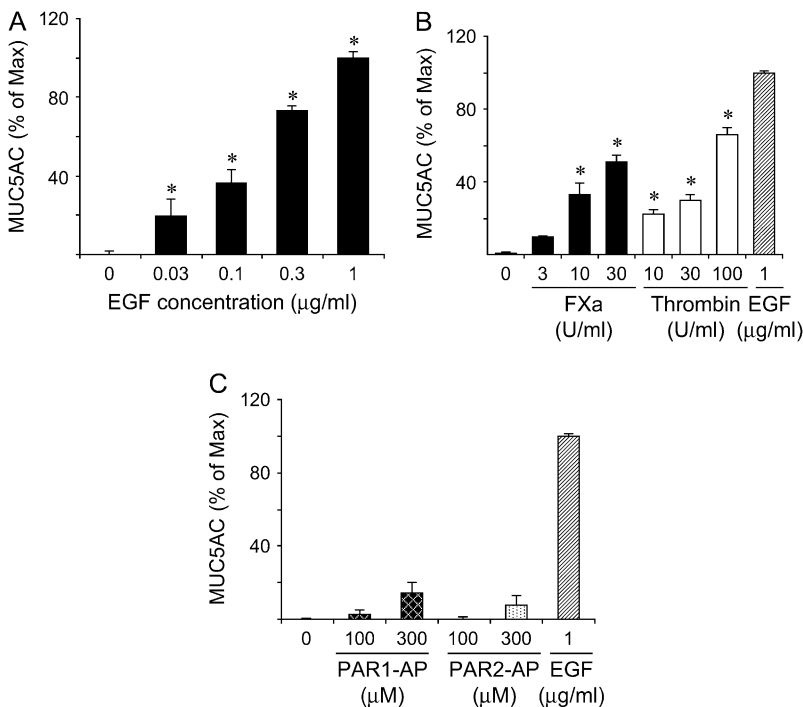


Figure 8. MUC5AC production in NCI-H292 cells. NCI-H292 cells were cultured in 96-well plates and stimulated with (A) epidermal growth factor (EGF); (B) FXa, thrombin, or EGF; or (C) PAR1-activating peptide (PAR1-AP) or PAR2-AP at the indicated concentrations. MUC5AC production was determined as percentage relative to maximum expression (stimulation with EGF at 1 μ g/ml).

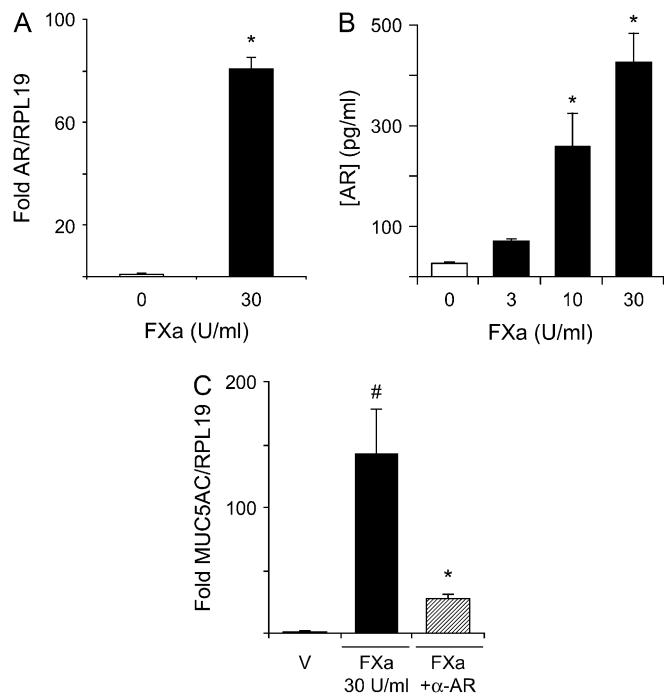


Figure 9. (A) Amphiregulin (AR) mRNA and (B) protein levels in NCI-H292 cells stimulated with FXa. Amphiregulin mRNA levels were significantly increased after stimulation with 30 U/ml FXa ($n = 4$); $*p = 0.0003$ (A). This correlated with effects on protein levels, which increased in an FXa dose-dependent manner ($n = 4$); $*p < 0.01$ (B). Anti-amphiregulin antibody (α -AR) reduced the mRNA levels of FXa-induced MUC5AC in NCI-H292 cells (C).

NCI-H292 cells that were derived from human mucoepidermoid carcinoma cells. Although there are several differences between this cell line and primary lung epithelial cells, this cell line has been used by other investigators to study mucin production, and EGF is a strong inducer of mucin in this cell line. MUC5AC production in NCI-H292 cells was up-regulated by FXa in a dose-dependent manner. Additional studies demonstrated that mucin production by NCI-H292 cells was induced by thrombin and FXa. It was therefore speculated that mucin production was mediated by PAR1 or PAR2 because both are expressed in the epithelial and smooth muscle cells of the respiratory system (23, 24). Indeed, studies have implicated coagulation FVIIa and FXa as possible activators of PAR2 in the airway (24). However, neither PAR1 nor PAR2 activating peptides stimulated mucin production. Recent studies have indicated that a trypsin-like protease (human airway trypsin-like protease [HAT]) can enhance mucin production through an amphiregulin/EGFR pathway, and activation of PAR2 is not sufficient for HAT-induced release of amphiregulin or induction of mucin production (16). In addition, it has been shown that FVIIa/tissue factor (TF) complex up-regulates mRNA levels of amphiregulin in a human keratinocyte cell line (25). Amphiregulin is a member of the EGF family and, like other members of this family, has been reported to play an important role in cell proliferation (26, 27), cell survival (28), and cell differentiation (26). Additional studies have demonstrated that amphiregulin increases mucin production in airway epithelial cells and increases expression in mast cells from patients with asthma, events that were correlated with goblet cell hyperplasia in the mucosa (29). In the current study, FXa significantly increased amphiregulin mRNA and protein levels in NCI-H292 cells, and the addition of a specific antibody

to amphiregulin significantly attenuated FXa-mediated mucin production. Therefore, FXa may induce mucin production by regulating amphiregulin production directly or indirectly through activation/membrane release of HAT from NCI-H292 cells and potentially *in vivo* through a murine homolog of HAT (30).

Although this investigation demonstrated that activation of PAR1 and PAR2 did not seem to play a significant role in mucin production, these receptors may contribute to other events associated with airway remodeling. PAR comprises a unique family of G-protein-coupled receptors, which are cleaved at an activation site within the N-terminal exodomain by serine proteinases. After cleavage, the new N-terminal sequence functions as a tethered ligand that binds intramolecularly to activate the receptor (24, 31). Four PARs have been identified and several studies have shown that PAR1 and PAR2 are expressed in the airway epithelial cells (32–38). Activation of PAR2 *in vivo* leads to a proinflammatory responses, such as neutrophil and eosinophil infiltrations (36, 39), and bronchoconstriction (35). Serine proteases (e.g., trypsin, mast cell tryptase, and FVIIa and FXa) have been shown to activate PAR2 (40). In addition to PAR2, FXa has also been shown to activate PAR1 (41). In the present study, mRNA levels of PAR1 and PAR2 were increased in 16-wk-challenged mice, and these levels decreased after FPX treatment. Previous studies have identified a role for PAR1 in tissue fibrosis in lung (21, 22) and activation of these receptors, potentially by FXa, may contribute to airway remodeling by regulating fibroblast proliferation and collagen deposition. Therefore, FXa could regulate asthmatic events through its participation in a number of different pathways.

In conclusion, it has been demonstrated that FXa is involved in airway remodeling. The presence of FXa led to increased mucin production through increased amphiregulin, although FXa did not affect inflammatory cell infiltration into the lung in OVA-challenged mice. Other contributions by FXa during the remodeling phase of this disease may involve activation of PARs and resultant increased fibroblast proliferation and collagen production.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Ms. Deborah Donahue for the animal work, Ms. Melanie DeFord for assistance with cell culture, and Ms. Mayra J. Sandoval-Cooper for assistance with histology.

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