ORIGINAL RESEARCH

Dissociation of FK506-Binding Protein 12.6 kD from Ryanodine Receptor in Bronchial Smooth Muscle Cells in Airway Hyperresponsiveness in Asthma

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Abstract

Airway hyperresponsiveness (AHR) in asthma is predominantly caused by increased sensitivity of bronchial smooth muscle cells (BSMCs) to stimuli. The sarcoplasmic reticulum (SR)-Ca^{2+} release channel, known as ryanodine receptor (RyR), mediates the contractive response of BSMCs to stimuli. FK506-binding protein 12.6 kD (FKBP12.6) stabilizes the RyR2 channel in a closed state. However, the interaction of FKBP12.6 with RyR2 in AHR remains unknown. This study examined the interaction of FKBP12.6 with RyR2 in BSMCs in AHR of asthma. The interaction of FKBP12.6 with RyR2 and FKBP12.6 expression was determined in a rat asthma model and in BSMCs treated with inflammatory cytokines. The calcium responses to contractile agonists were determined in BSMCs with overexpression and knockdown of FKBP12.6. Asthmatic serum, IL-5, IL-13, and TNF-α enhance the calcium response of BSMCs to contractile agonists and cause dissociation of FKBP12.6 from RyR2 and a decrease in FKBP12.6 gene expression in BSMCs in culture and in ovalbumin (OVA)-sensitized and -challenged rats. Knockdown of FKBP12.6 in BSMCs causes a decrease in the association of RyR2 with FKBP12.6 and an increase in the calcium response of BSMCs. Overexpression of FKBP12.6 increases the association of FKBP12.6 with RyR2, decreases the calcium response of BSMCs, and normalizes airway responsiveness in OVA-sensitized and -challenged rats. Dissociation of FKBP12.6 from RyR2 in BSMCs is responsible for the increased calcium response contributing to AHR in asthma. Manipulating the interaction of FKBP12.6 with RyR2 might be a novel and useful treatment for asthma.

Keywords: asthma; airway hyperresponsiveness; calcium; RyR2; FKBP12.6

Clinical Relevance

Although enhanced calcium response of bronchial smooth muscle to stimuli has been reported to be involved in asthmatic airway hyperresponsiveness, an interaction of FKBP12.6 with RyR2 on sarcoplasmic reticulum in asthmatic airway hyperresponsiveness has not been investigated. We have shown that increased responsiveness of bronchial smooth muscle in asthma is attributed to the dissociation of FKBP12.6 from ryanodine receptor 2, which promotes calcium release from the sarcoplasmic reticulum.

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Asthma is a chronic, inflammatory disease of the lung characterized by intermittent airway obstruction, airway hyperresponsiveness (AHR), the presence of activated inflammatory cells, inflammatory mediators, and remodeling in the airway (1). AHR is a characteristic feature of asthma and is predominantly caused by increased sensitivity of the bronchial smooth muscle to nonspecific stimuli and increased excretion of airway glands, which lead to bronchial contraction and airway narrowing. The mechanisms involve increased release of inflammatory cytokines and enhanced sensitivity of airway sensory nerves and augmented output of airway vagal outflow. In response to inflammatory stimuli, large amounts of cytokines, such as IL-5, IL-13, and TNF-α, are released (2–6). These cytokines play pivotal roles in the pathogenesis of AHR. For example, IL-13 enhances calcium oscillation in airway smooth muscle, and inhibition of IL-13 completely blocks airway hyperreactivity in mouse asthma models (2, 7). IL-5 is a key factor for eosinophilia and could therefore be responsible for some of the tissue damage seen in chronic asthma (3). TNF-α is a proinflammatory cytokine that has been implicated in many aspects of airway pathology in asthma and has recently been highlighted as a potentially important contributing factor in refractory asthma (4).

Bronchial smooth muscle cells (BSMCs) are the major effector cells involved in AHR. The contractility of BSMCs is significantly increased in subjects with asthma (8–10). Calcium mediates smooth muscle contraction. The response of BSMCs to diverse stimuli is controlled by changes in the concentration of free cytosolic calcium ($[Ca^{2+}]_i$) and myosin light chain phosphatase. Alterations in agonist-induced calcium responses in BSMCs contribute to a stronger contractile response of the airways, a characteristic feature of asthma. Elevation of $[Ca^{2+}]_i$ can result from increased Ca^{2+} influx from the extracellular fluid and Ca^{2+} release from the sarcoplasmic reticulum (SR) (11). In smooth muscle cells, excitation–contraction coupling involves depolarization of the plasma membrane to open voltage-gated calcium (Ca^{2+}) channels. However, the voltage-dependent calcium channels do not appear to strongly influence BSMC contraction (12, 13). Agonist-induced calcium release is largely from the SR (12, 14). Ca^{2+} efflux from the SR is mediated by the SR-Ca^{2+} release channel, known as ryanodine receptor (RyR), and inositol 1,4,5-trisphosphate receptors (11). However, to what extent such spontaneous calcium release is involved in the physiological importance of RyRs in airway smooth muscle contraction is unclear. A recent report indicated that a 12.6-kD FK506-binding protein (FKBP12.6) is associated with type 2 RyR (RyR2) protein (15). FK506-binding proteins (FKBPs) are intracellular receptors for the immunosuppressant drug FK506 (15). FKBP12.6 stabilizes the RyR2 channel in the closed state and reduces its activity. Removal of FKBP12.6 from the RyR2 by FK506 or rapamycin increases the probability of an open channel and induces the appearance of long-lasting subconductance states (15, 16). The endogenous ligand cADPR and down-regulation of FKBP12.6 might induce the activation of RyR2 by dissociating FKBP12.6 from the RyR2 complex. FKBP12.6 and RyR2 are predominantly expressed in cardiac muscle and vascular smooth muscle. Activation of RyR2 by dissociation of FKBP12.6 from the RyR2 complex has been reported in tissues such as cardiac muscle (17), coronary arterial smooth muscle cells (18), pulmonary arterial smooth muscle cells (19), and pancreatic islets (20). In tracheal smooth muscle, FKBP12.6 associates with and regulates RyR2. FKBP12.6 binds to RyR2 but not to other RyRs or inositol 1,4,5-trisphosphate receptors. cADPR can enhance Ca^{2+} release, which is mediated through FKBP12.6 (21). Recently, it has been shown that FKBP12.6 translocates from SR to the cytosol in hypoxic pulmonary arterial smooth muscle, resulting in Ca^{2+} release (19). It is unclear how FKBP12.6 interacts with RyR2 in response to inflammatory stimuli in AHR of asthma.

In the present study, we investigated the effects of IL-5, IL-13, and TNF-α on the calcium response of BSMCs to contractile agonists and on the interaction of FKBP12.6 with RyR2 in asthmatic AHR *in vivo* and *in vitro*. We found, for the first time, that chronic airway allergic inflammation causes dissociation of FKBP12.6 from RyR2 in BSMCs and enhances Ca^{2+} release response from the SR, leading to AHR in asthma.

Materials and Methods

Further details about the methods used in this study are provided in the online supplement.

Asthma Animal Model

All experiments involving Sprague-Dawley rats and guinea pigs were approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology. Rats were sensitized with ovalbumin (OVA) plus aluminum hydroxide and challenged with OVA. Measurement of airway responsiveness was performed at Day 75. Guinea pigs were sensitized and challenged with OVA, and measurement of bronchial tension and serum collection were performed at Day 75.

FKBP12.6 Small Interfering RNA and Overexpression of Lentivirus Plasmids

Recombinant lentivirus vectors containing the overexpression plasmid of FKBP12.6 gene (lenti-FKBP12.6) or the small interfering RNA plasmid of FKBP12.6 gene (lenti-FKBP12.6 small interfering RNA [siRNA]) were prepared by Genechem (Shanghai, China). FKBP12.6 overexpression lentivirus plasmid was delivered to rats transnasally under anesthesia at Day 55. BSMCs were transfected with lenti-FKBP12.6 RNAi or lenti-FKBP12.6 for 12 hours.

Measurement of Airway Responsiveness

Airway responsiveness was measured as described previously (22). Rats were anesthetized, intubated, administered with a paralytic agent, and ventilated mechanically. Airway opening pressure was recorded under stepwise increased acetylcholine (0.3–3 mg/ml) using the PowerLab/4SP data acquisition system (AD Instrument, Bella Vista, Australia). The data were expressed as enhanced airway intrapressure.

Tension Measurement of Bronchial Ring

Bronchial ring preparations of guinea pigs and *ex vivo* tension measurements were performed using a modified protocol similar to that described previously with some modification (23).

Isolation, Primary Culture, and Treatment of Rat BSMCs

Rat BSMCs were isolated from smooth muscle bundles of the large bronchial airways of rats (24). Smooth muscle bundles were isolated, minced into 1-mm^3 pieces,

and plated onto flasks in DMEM/F12 with 10% FBS in a CO_2 incubator at 37°C. Cells grew from the border of the smooth muscle bundles at Day 3 and grew to confluence over 6 days. Passage 2, 3, and 4 BSMCs were used for experiments. BSMCs were treated with 20 ng/ml cytokines and asthmatic serum from guinea pigs or from patients with chronic asthma in acute exacerbation for 22 hours. The Institutional Review Board of Tongji Medical College, Huazhong University of Science and Technology for human studies approved the protocols for collection of serum from patients with asthma.

Immunofluorescence Staining and Confocal Microscopy

The lung sections were stained with a mouse monoclonal antibody specific to FKBP12.6 and rabbit anti-RyR2 polyclonal antibody.

$[Ca^{2+}]_i$ Measurements

BSMC $[Ca^{2+}]_i$ was measured after loading with the acetoxymethyl ester form of Fura-2. The fluorescence of Fura-2 was recorded from BSMCs on coverslips in a perfusion chamber mounted on the stage of a modified Olympus inverted fluorescence microscope (Tokyo, Japan) after excitation at 340 ± 10 and 380 ± 10 nm. The relative $[Ca^{2+}]_i$ level was presented with the F340:F380 ratio (25).

Single Cell Contraction Measurement

BSMC contraction in response to 10^{-5} mol/L bradykinin was measured by an inverted microscope. The maximum capacity of shortening was presented the length:width ratio, and the velocity of shortening was presented by the slopes of the time-length:width ratio curve (8).

Coimmunoprecipitation and Western Blots

BSMC lysates were precleared and incubated with rabbit anti-RyR2 polyclonal antibody overnight at 4°C. The antibody–protein complexes were captured by incubating with protein G-plus agarose beads for 1 hour at 4°C. The pull-down protein levels of FKBP12.6 with RyR2 were detected with Western blots.

Determination of FKBP12.6 mRNA Expression

FKBP12.6 mRNA level was measured by quantitative real-time PCR using SYBR (TAKARA Biotechnology, Dalian, China). An internal control (β-actin, 4352341E; Life

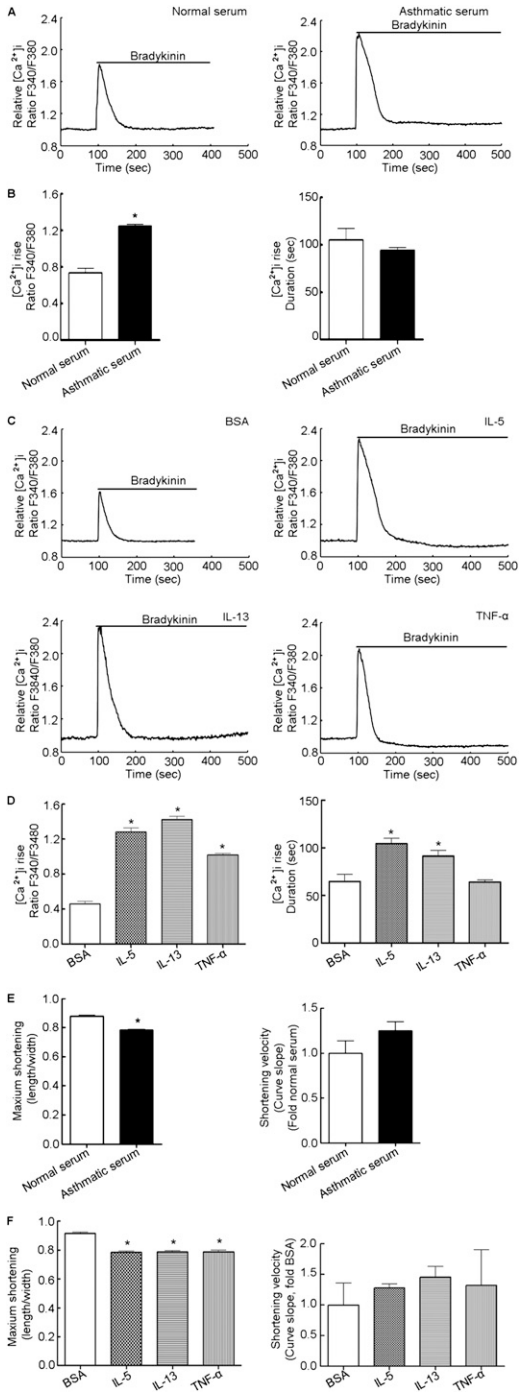

Figure 1. Asthmatic serum and cytokines IL-5, IL-13, and TNF-α enhance calcium response and contraction to bradykinin in bronchial smooth muscle cells (BSMCs). BSMCs were treated with 10% serum from ovalbumin (OVA)-sensitized and -challenged guinea pigs (asthmatic serum) or control guinea pigs (normal serum) (*A*, *B*, and *E*) and with 20 ng/ml IL-5, IL-13, TNF-α, or BSA (*C*, *D*, and *F*) for 22 hours. Intracellular Ca^{2+} levels were recorded using Fura-2/AM after stimulation with 10^{-5} mol/L bradykinin. At least 10 cells were recorded for calculating the fluorescence intensity ratio F340/F380. (*A* and *C*) Representative trace before and after stimulation with bradykinin. (*B* and *D*) Changes in ratio F340/F380 and duration before and after stimulation with bradykinin. (*E* and *F*) BSMC shortening was recorded using microscopy before and after stimulation with 10^{-5} mol/L bradykinin. The maximum shortening was presented by the length:width ratio. The velocity of shortening was presented by the slopes of time-length:width ratio curve. Results are expressed as mean ± SD ($n = 3$ experiments). *$P < 0.05$ versus normal serum or BSA.

Technologies, Grand Island, NY) was used to normalize expression.

Statistical Analysis

Results are shown as the mean ± SD for *n* experiments. Differences between groups were analyzed using unpaired *t* tests or two-way ANOVA. A *P* value less than 0.05 was considered to be statistically significant.

Results

Asthmatic Serum, IL-5, IL-13, and TNF-α Enhance the Calcium Response and Contraction of BSMCs to Contractile Agonists

Intracellular calcium response is a critical process in AHR. To investigate the effect of asthmatic inflammation on calcium signaling, we used 10% serum from OVA-sensitized and -challenged guinea pigs and 20 ng/ml asthma-related cytokines IL-5, IL-13, and TNF-α to make asthma airway inflammation cell models. We found that the increase in $[Ca^{2+}]_i$ in response to 10^{-4} mol/L acetylcholine (*see* Figures E1A and E1B in the online supplement) or 10^{-5} mol/L bradykinin (Figures 1A and 1B) were significantly higher in asthmatic serum–treated BSMCs compared with those treated with normal control serum. Asthmatic serum treatment resulted in increases in the calcium response to acetylcholine by approximately 250% in terms of magnitude and duration (Figures E1A and E1B) and to bradykinin by approximately 70% in terms of magnitude (Figures 1A and 1B). Similarly, the increases in $[Ca^{2+}]_i$ in response to acetylcholine (Figures E1C and E1D) or bradykinin (Figures 1C and 1D) in terms of magnitude and duration were significantly higher in BSMCs treated with IL-5, IL-13, and TNF-α compared with those treated with normal BSA ($P < 0.05$). Treatment with IL-5, IL-13, and TNF-α resulted in increases in the calcium response to acetylcholine by approximately 220, 200, and 70%, respectively (Figures E1C and E1D), and to bradykinin by approximately 180, 210, and 120%, respectively (Figures 1C and 1D). These results indicate that airway inflammation enhances the increase in $[Ca^{2+}]_i$ in BSMCs in response to agonists.

In bovine tracheal smooth muscle cells, Ca^{2+} released from the ryanodine-sensitive Ca^{2+} store can induce the activation of Ca^{2+}–activated K^+ channels rather than cell contraction directly (14). To confirm whether

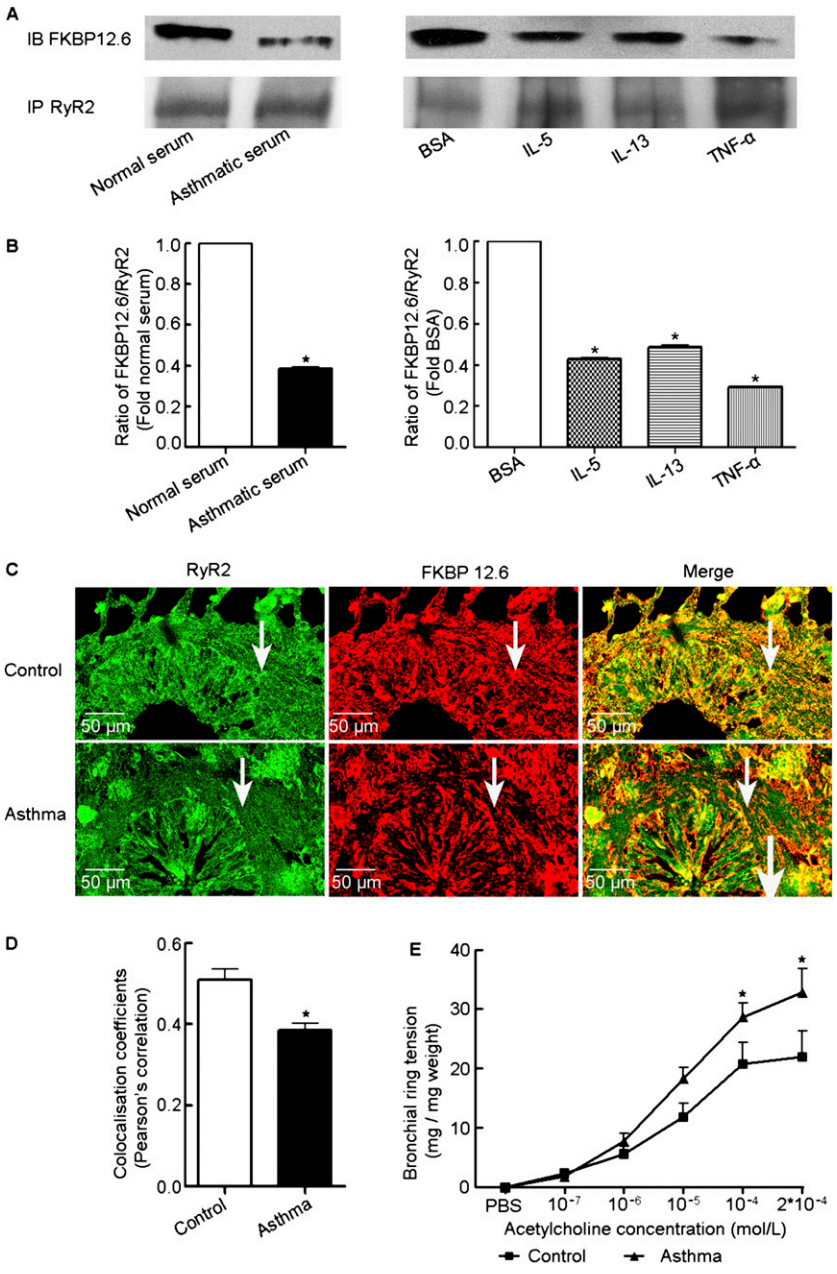

Figure 2. Asthmatic inflammation induces dissociation of FKBP12.6 from ryanodine receptor (RyR)2 in BSMCs and guinea pig asthma models. The interaction of FKBP12.6 with RyR2 in asthmatic serum and cytokine-treated BSMCs were measured using coimmunoprecipitation (*A* and *B*). (*A*) Representative images detected with anti-FKBP12.6 and anti-RyR2 antibodies after immunoprecipitation with anti-RyR2 antibodies. (*B*) The relative binding of FKBP12.6 to RyR2. Results are expressed as mean ± SD ($n = 3$ experiments). *$P < 0.05$ versus normal serum or BSA. Colocalization of FKBP12.6 with RyR2 in asthmatic guinea pig bronchial myocytes was measured using immunofluorescence (*C* and *D*). (*C*) Representative images of colocalization of FKBP12.6 with RyR2 in asthma and normal guinea pigs using FITC-conjugated RyR2 antibody (*green*) and Cy3-conjugated FKBP12.6 antibody (*red*). *White arrow* shows bronchial myocytes. *Scale bar* = 50 μm. (*D*) Changes in coefficients of colocalization of FKBP12.6 with RyR2. The increased airway responsiveness of guinea pig was measured by bronchial ring tension [(peak tension − baseline)/ring weight] of isolated bronchial rings (*E*). Results are expressed as mean ± SD ($n = 5$ experiments). *$P < 0.05$ versus normal control.

the increased calcium response leads to enhanced cell contraction, we measured BSMC contraction in response to bradykinin after treatment with asthmatic serum or cytokines. Treatment with asthmatic serum, IL-5, IL-13, and TNF-α resulted in cell shortening in response to bradykinin by approximately 89, 86, 84, and 88%, respectively, but no difference of shortening velocity was found (Figures 1E and 1F).

Taken together, these results indicate that airway inflammation enhances the calcium response and contraction of BSMCs to contractile agonists.

Asthmatic Airway Inflammation Decreases the Association of FKBP12.6 with RyR2 in BSMCs and Guinea Pigs

To determine whether asthmatic airway inflammation affects the interaction of FKBP12.6 with RyR2, the association of FKBP12.6 with RyR2 was studied in untreated BSMCs and BSMCs treated with IL-5, IL-13, and TNF-α. The amounts of FKBP12.6 pulled down by an antibody against RyR2 were less in BSMCs treated with asthmatic serum, IL-5, IL-13, and TNF-α than BSMCs treated with normal serum or BSA (Figures 2A and 2B). These results suggest that airway inflammation causes dissociation of FKBP12.6 from RyR2 in BSMCs.

To confirm the dissociation of FKBP12.6 from RyR2 in *in vivo* asthma model, we assessed the association of FKBP12.6 with RyR2 in the airway of guinea pigs with asthma by detecting the colocalization of FKBP12.6 with RyR2 using immunofluorescence confocal microscopy. The colocalization of FKBP12.6 with RyR2 (Figures 2C and 2D) was reduced in bronchial smooth muscle in guinea pigs with asthma, which have increased airway responsiveness (Figure 2E), suggesting that FKBP12.6 association with RyR2 is decreased in AHR in asthma. We also assessed the expression of FKBP12 and RyR1. The protein levels of RyR1 in bronchial smooth muscles are very low. However, in guinea pigs (Figure E2A) and rats (Figure E2B), there are significantly higher tissue concentrations of FKBP12 in bronchial smooth muscles than in other lung tissues, but the levels in bronchial smooth muscles are not significantly different between normal and asthmatic animals.

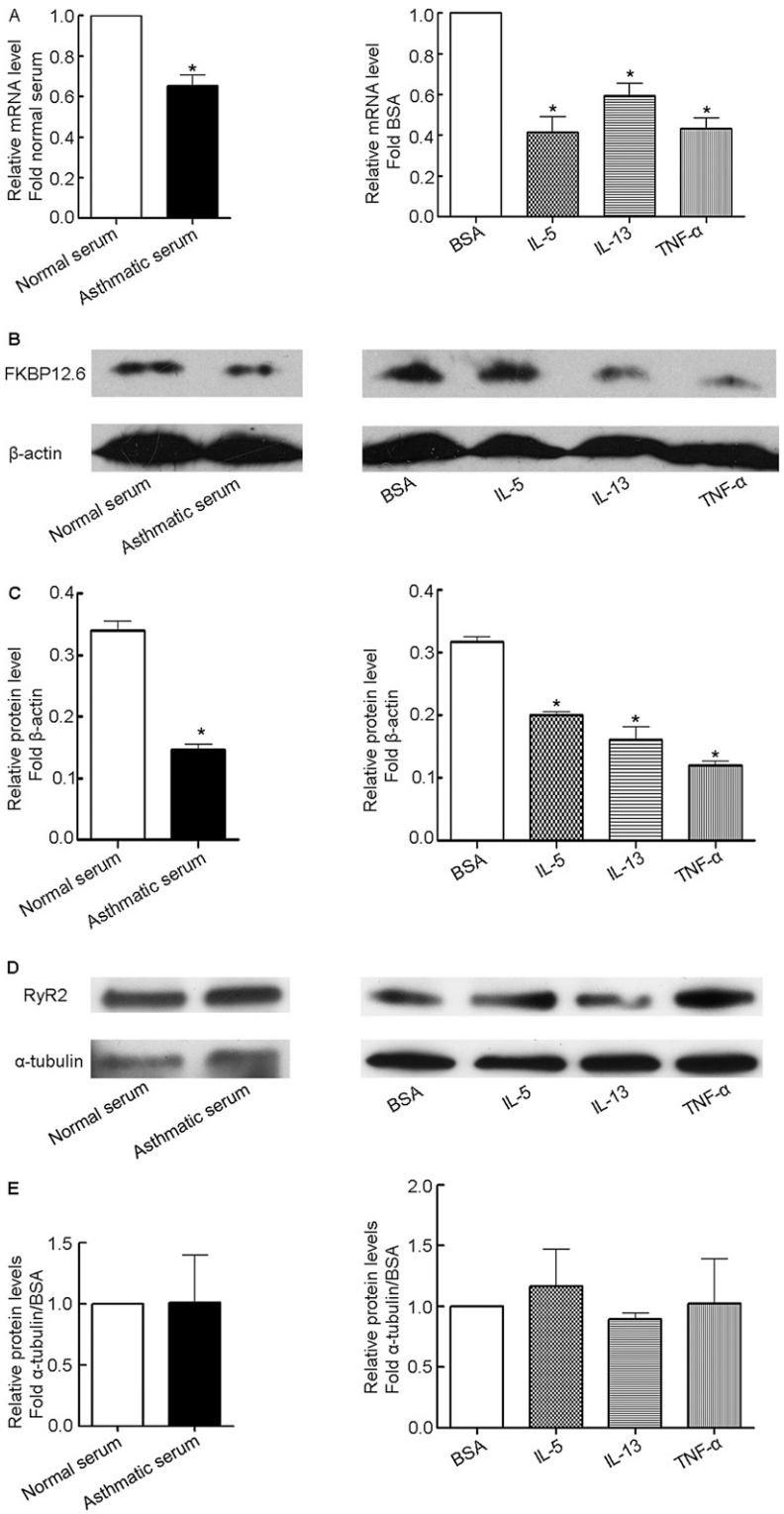

Figure 3. The effects of asthmatic serum or cytokines on FKBP12.6 and RyR2 expression in BSMCs. BSMCs were treated with 10% serum from OVA-sensitized and -challenged guinea pigs (asthmatic serum) or control guinea pigs (normal serum) for 22 hours. FKBP12.6 mRNA levels were measured using RT-PCR (*A*), and FKBP12.6 (*B* and *C*) and RyR2 protein levels (*D* and *E*) were measured by Western blots. (*B* and *D*) Representative images of a Western blot analysis of FKBP12.6 with RyR2 from three separate experiments. (*C* and *D*) *Bar graphs* depicting changes in FKBP12.6 with RyR2 protein levels. Results are expressed as mean ± SD (n = 3 experiments). *$P < 0.05$ versus normal serum or BSA.

Asthmatic Airway Inflammation Down-Regulates FKBP12.6 Expression in BSMCs

We further investigated whether the decreases in the association of FKBP12.6 with RyR2 induced by asthmatic serum, IL-5, IL-13, and TNF-α are due to a decrease in FKBP12.6 expression. The levels of mRNA (Figure 3A) and protein (Figures 3B and 3C) of FKBP12.6 in BSMCs treated with asthmatic serum, IL-5, IL-13, and TNF-α were significantly lower than those without treatment. However, RyR2 protein levels were comparable in control BSMCs and BSMCs treated with asthmatic serum, IL-5, IL-13, and TNF-α (Figures 3D and 3E).

FKBP12.6 Knockdown Enhances Calcium Response of BSMCs to Bradykinin

To investigate the role of FKBP12.6 in calcium response to contractile agonist, FKBP12.6 was knocked down by transfecting BSMCs with lentivirus plasmids containing FKBP12.6 siRNA (Figures E3A and E3B). We found that the duration of the $[Ca^{2+}]_i$ rise in response to bradykinin was significantly longer in BSMCs transfected with plasmids containing FKBP12.6 siRNA than in those with control vector, although the magnitude of $[Ca^{2+}]_i$ rise in response to bradykinin was comparable in BSMCs transfected with plasmid containing FKBP12.6 siRNA and those with control vector (Figures E3C and E3D). These data show that FKBP12.6 knockdown enhances calcium response of BSMCs to contractile agonist.

FKBP12.6 Overexpression Prevents Alterations in the Association of RyR2 with FKBP12.6 and Calcium Response to Bradykinin of BSMCs Induced by Asthmatic Serum, IL-5, IL-13, and TNF-α

To confirm whether the decrease in the association of FKBP12.6 with RyR2 induced by asthmatic serum, IL-5, IL-13, and TNF-α is caused by a decrease in FKBP12.6 availability due to a reduction of FKBP12.6 level, we measured the association of FKBP12.6 with RyR2 in BSMCs in which FKBP12.6 was overexpressed by transfecting BSMCs with lentivirus plasmids containing the FKBP12.6 gene. Transfection of BSMCs with lenti-FKBP12.6 viral vectors resulted in a dramatic increase in FKBP12.6 level (Figures 4A and 4B). KBP12.6 overexpression prevented the decrease in the association of FKBP12.6 with RyR2 induced by asthmatic serum (Figures 4C and 4D) and by IL-5, IL-13, and TNF-α (Figures 4E and 4F), indicating that the decrease in the association of FKBP12.6 with RyR2 induced by asthmatic serum, IL-5, IL-13, and TNF-α is caused by a decrease in FKBP12.6 availability due to a reduction of FKBP12.6 level. More importantly, KBP12.6 overexpression reduced the duration and magnitude of $[Ca^{2+}]_i$ rise and the quasi–steady-state intracellular $[Ca^{2+}]$ in response to bradykinin and prevented the increase in calcium response of BSMCs to bradykinin when BSMCs were pretreated with asthmatic

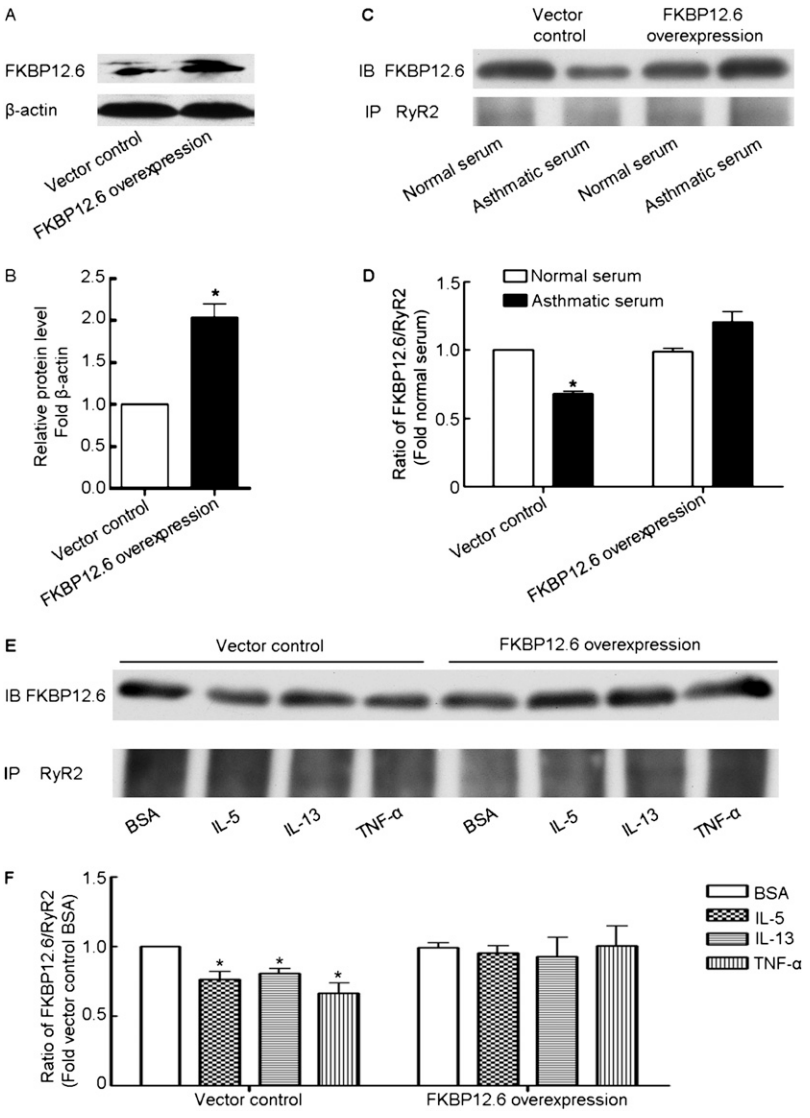

Figure 4. FKBP12.6 overexpression prevents the alterations in the association of RyR2 with FKBP12.6 induced by asthmatic serum, IL-5, IL-13, and TNF-α. BSMCs were transfected with lenti-FKBP12.6 viral vectors (FKBP12.6 overexpression) or lentivirus vehicle (vector control) and incubated with asthmatic serum, IL-5, IL-13, and TNF-α for 22 hours, after which FKBP12.6 levels (*A* and *B*) and FKBP12.6 association with RyR2 (*C–F*) were measured. (*A*) Representative images of FKBP12.6 level. (*B*) Changes in FKBP12.6 levels. Data were normalized to levels of the housekeeping gene β-actin. The interaction of FKBP12.6 with RyR2 was measured using coimmunoprecipitation (*C–F*). (*C* and *E*) Representative images detected with anti-FKBP12.6 and anti-RyR2 antibodies after immunoprecipitation with anti-RyR2 antibodies after treatment with asthmatic serum (*C*) or cytokines (*E*). (*D* and *F*) *Bar graphs* depicting the relative binding of FKBP12.6 to RyR2. Results are expressed as mean ± SD (n = 3 experiments). *$P < 0.05$ versus control.

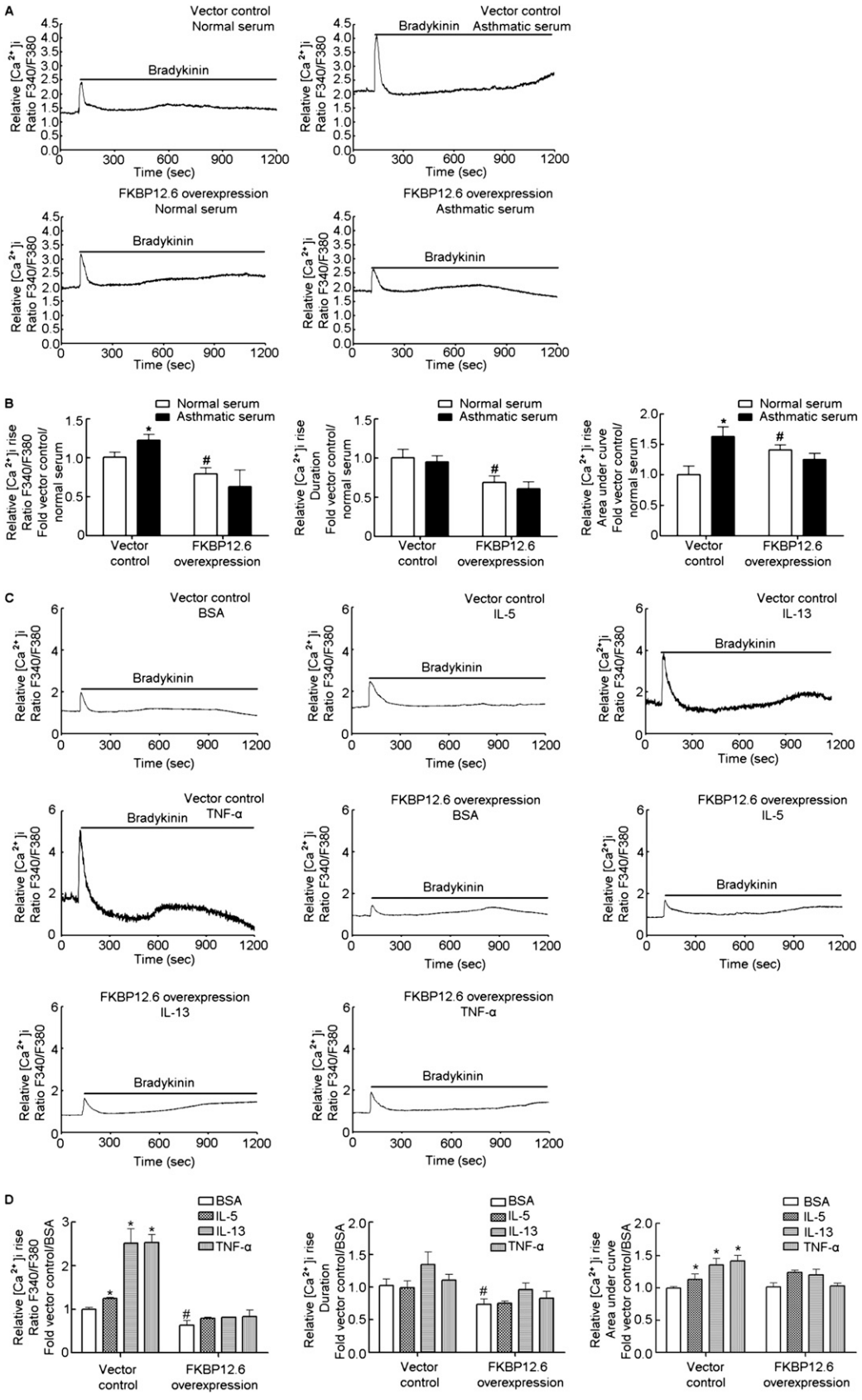

Figure 5. (See figure legend on following page)

serum (Figures 5A and 5B) and with IL-5, IL-13, and TNF-α (Figures 5C and 5D). These results indicate that asthmatic serum–, IL-5–, IL-13–, and TNF-α–induced decrease in the association of FKBP12.6 with RyR2 is responsible for the increase in calcium response of BSMCs to bradykinin.

FKBP12.6 Overexpression Attenuates the Decreased Colocalization of FKBP12.6 with RyR2 and Normalizes Airway Responsiveness in a Rat Asthma Model

To further confirm the role of FKBP12.6 in AHR, we assessed the association of FKBP12.6 with RyR2 in the airway of control and asthmatic rats with and without FKBP12.6 overexpression by detecting the colocalization of FKBP12.6 with RyR2 using immunofluorescence confocal microscopy. FKBP12.6 was successfully overexpressed in rats transfected with lenti-FKBP12.6 viral vectors, and FKBP12.6 overexpression increased the colocalization of FKBP12.6 with RyR2 (Figures E4A and E4B). We then evaluated airway responsiveness to acetylcholine in control and asthmatic rats with and without FKBP12.6 overexpression. Airway responsiveness in asthmatic rats was significantly higher than that in control rats (Figures 6A and E5). The airway responsiveness was comparable in asthmatic rats with FKBP12.6 overexpression and in control rats without FKBP12.6 overexpression (Figures 6A and E5). Taken together, these results indicate that FKBP12.6 overexpression prevents the decrease in the association of FKBP12.6 with RyR2 and restores the increased airway responsiveness in asthma to a normal level.

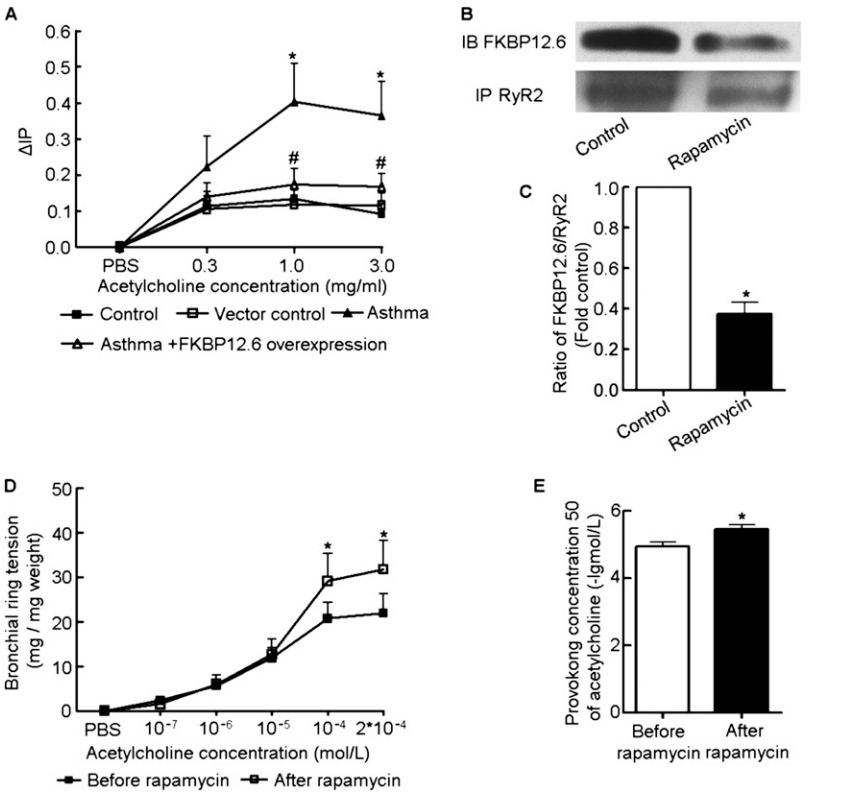

Figure 6. FKBP12.6 overexpression normalizes airway responsiveness in a rat asthma model, and dissociation of FKBP12.6 from RyR2 induced by rapamycin increases airway responsiveness in guinea pigs. Asthmatic rats were transfected with lenti-FKBP12.6 viral vectors transnasally (asthma + FKBP12.6 overexpression). Normal control rats were administered lentivirus vehicle transnasally (vector control) or PBS (control). Airway responsiveness represented by enhanced airway intrapressure [ΔIP = (peak pressure − baseline)/baseline] to acetylcholine were measured in intubated mechanically ventilated animals (*A*). The interaction of FKBP12.6 with RyR2 was measured using coimmunoprecipitation (*B* and *C*). The contraction of bronchial rings of asthmatic guinea pigs is represented by enhanced bronchial ring tension [(peak tension − baseline)/ring weight] measured in isolated bronchial rings (*D*). (*B*) Representative images detected with anti-FKBP12.6 and anti-RyR2 antibodies after coimmunoprecipitation with anti-RyR2 antibodies. (*C*) Bar graph depicting relative binding of FKBP12.6 to RyR2. (*D*) Dose-response curve of bronchial ring tension before and after rapamycin treatment. (*E*) Provoking concentration 50 of acetylcholine before and after rapamycin treatment. Results are expressed as mean ± SD (n = 5 experiments). *$P < 0.05$ versus normal control.

Dissociation of FKBP12.6 from RyR2 Induced by Rapamycin Increases Airway Responsiveness in Guinea Pigs

It has been shown that dissociation of FKBP12.6 from the RyR2 by rapamycin increases the open probability of the channel and induces the appearance of long-lasting subconductance states (26). To further confirm the role of the dissociation of FKBP12.6 from RyR2 in AHR, rapamycin was used to dissociate FKBP12.6 from RyR2 in normal rat BSMCs and bronchial rings from normal guinea pigs. Treatment of BSMCs with rapamycin induced the dissociation of FKBP12.6 from RyR2 (Figure 6B–6C). The responses to acetylcholine (10^{-4} and 2×10^{-4} mol/L) and sensitivity (provoking concentration 50) are significantly higher in the guinea pig bronchial ring treated with rapamycin than in those without rapamycin (Figures 6D and 6E). These results provide further evidence confirming that dissociation of FKBP12.6 from RyR2 induces AHR in asthma.

Figure 5. (Continued). FKBP12.6 overexpression prevents alterations in calcium response of BSMCs to bradykinin induced by asthmatic serum, IL-5, IL-13, and TNF-α. BSMCs were transfected with lenti-FKBP12.6 viral vectors (FKBP12.6 overexpression) or lentivirus vehicle (vector control) and incubated with asthmatic serum, IL-5, IL-13, and TNF-α for 22 hours, after which intracellular Ca^{2+} levels were recorded using Fura-2/AM after stimulation with bradykinin. At least 10 cells were recorded for calculating the fluorescence intensity ratio F340:F380. (*A* and *C*) Representative traces before and after stimulation with bradykinin. (*B* and *D*) *Bar graphs* depicting changes in the F340:F380 ratio and duration and area under curve of the response. Results are expressed as mean ± SD (n = 3 experiments). *$P < 0.05$ versus normal serum or BSA; #$P < 0.05$ versus normal serum or BSA of vector control.

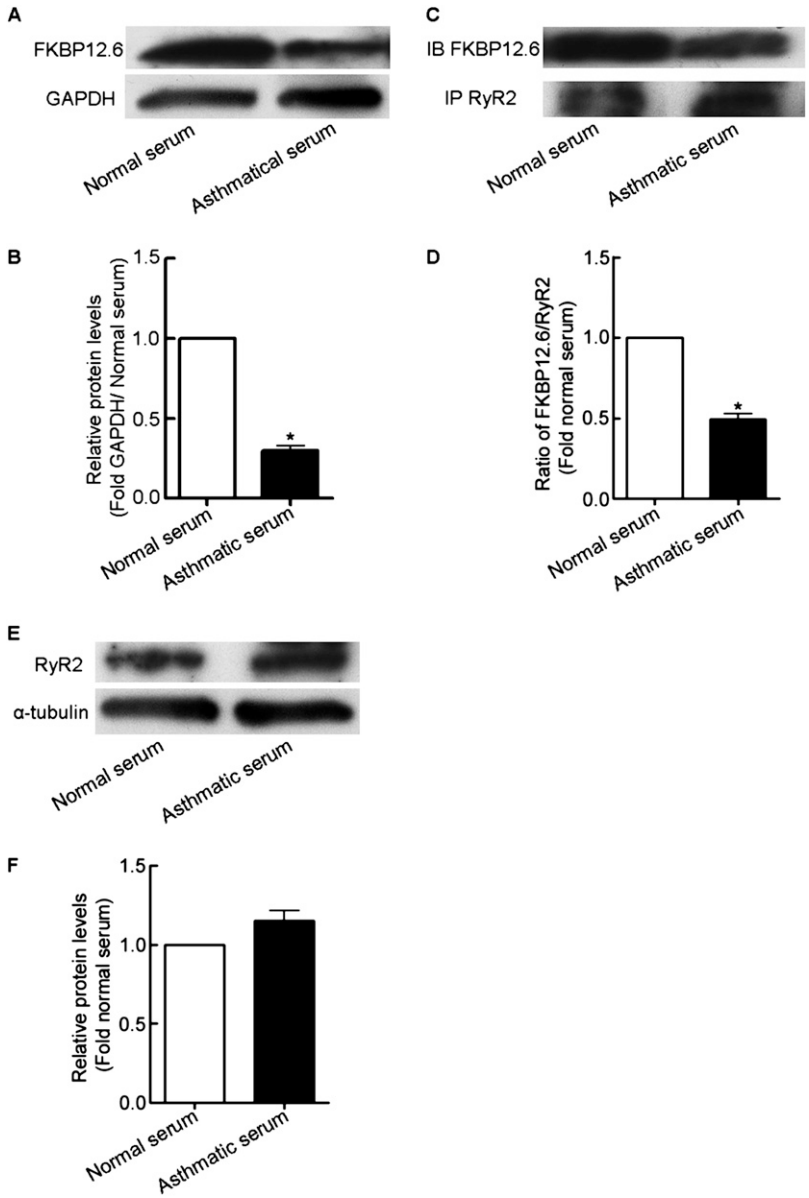

Figure 7. Serum from patients with asthma induces down-regulation of FKBP12.6 and decreased association of FKBP12.6 from RyR2 in BSMCs. BSMCs were treated with 10% serum from patients with chronic asthma in acute exacerbation (asthmatic serum) or from healthy subjects (normal serum) for 22 hours. FKBP12.6 (*A* and *B*) and RyR2 protein levels (*E* and *F*) were measured by Western blotting. (*A* and *E*) Representative images of a Western blot analysis of FKBP12.6 with RyR2 from three separate experiments. (*B* and *F*) *Bar graphs* depicting changes in FKBP12.6 with RyR2 protein levels. The interaction of FKBP12.6 with RyR2 was measured using coimmunoprecipitation (*C* and *D*). (*C*) Representative images detected with anti-FKBP12.6 and anti-RyR2 antibodies after immunoprecipitation with anti-RyR2 antibodies. (*D*) The relative binding of FKBP12.6 to RyR2. Results are expressed as mean ± SD (n = 3 experiments). *$P < 0.05$ versus normal serum.

Serum from Patients with Chronic Asthma in Acute Exacerbation Induces FKBP12.6 Down-Regulation and Decreased Association of FKBP12.6 with RyR2 in BSMCs

To address the clinical relevance of these observations in animal models, we collected serum from patients with chronic asthma in acute exacerbation and treated BSMCs with these sera. Treatment of BSMCs with serum from patients with asthma resulted in down-regulation of FKBP12.6 levels (Figures 7A and 7B). The association of FKBP12.6 with RyR2 was decreased in BSMCs with serum from patients with asthma (Figures 7C and 7D). However, RyR2 protein levels were comparable (Figures 7E and 7F). These results confirm our findings that asthmatic airway inflammation caused the dissociation of FKBP12.6 from RyR2.

Discussion

The major new finding of the present study is that asthmatic serum, IL-5, IL-13, and TNF-α enhance the calcium response and contraction of BSMCs to contractile agonists and cause dissociation of FKBP12.6 from RyR2 and a decrease in FKBP12.6 gene expression in BSMCs. Moreover, the association of FKBP12.6 with RyR2 and FKBP12.6 gene expression is decreased in BSMCs in OVA-sensitized and -challenged rats. Knockdown of FKBP12.6 in BSMCs causes a decrease in the association of FKBP12.6 with RyR2 and an increase in the calcium response of BSMCs to acetylcholine and bradykinin, mimicking the AHR in asthma. Furthermore, overexpression of FKBP12.6 increases the association of FKBP12.6 with RyR2, decreases the calcium response of BSMCs to acetylcholine and bradykinin, and normalizes airway responsiveness in OVA-sensitized and -challenged rats. These data represent the first evidence showing that dissociation of FKBP12.6 from RyR2 in BSMCs is responsible for an increased calcium response to agonists and contributes to AHR in asthma.

We found that asthmatic serum, IL-5, IL-13, and TNF-α enhance the calcium response and contraction of BSMCs to acetylcholine and bradykinin. These observations are consistent with the findings reported by Tao and colleagues (27, 28). Nevertheless, the mechanism for the modulation of calcium responses of BSMCs to inflammatory cytokines is largely unexplored. In muscle cells, the release of Ca^{2+} from the SR before muscle contraction occurs through RyR2 (8). FKBP12.6 is associated with RyR2 protein and stabilizes the RyR2 channel in the closed state and reduces its activity. We found that asthmatic serum, IL-5, IL-13, and TNF-α cause dissociation of FKBP12.6 from RyR2 in the *in vitro* cell model. Moreover, our observations show that rapamycin-induced dissociation of FKBP12.6 from RyR2 is closely correlated with increased airway responsiveness. In addition, the association of RyR2 with FKBP12.6 is also decreased in

BSMCs in OVA-sensitized and -challenged rats. Taken together, our data indicate that dissociation of FKBP12.6 from RyR2 is related to the increased calcium response in AHR in asthma.

We then studied the mechanism for the dissociation of FKBP12.6 from RyR2 in BSMCs. Our data show that the FKBP12.6 level is lower in BSMCs in OVA-sensitized and -challenged rats and in BSMCs treated with asthmatic serum, IL-5, IL-13, and TNF-α. Moreover, knockdown of FKBP12.6 in BSMCs causes a decrease in the association of RyR2 with FKBP12.6 and a concomitant increase in the calcium response of BSMCs to acetylcholine and bradykinin, mimicking the AHR in asthma. Consistent with our findings, Wehrens and colleagues (29) reported that FKBP12.6 is deficient in the RyR2 macromolecular complex and that RyR2 channels exhibit a 10-fold increase in the opening probability in the cardiac myocytes of FKBP12.6$^{-/-}$ mice compared with age- and sex-matched wild-type control mice. Therefore, reduction of FKBP12.6 availability could result in dissociation of FKBP12.6 from RyR2, leading to an increase in the opening probability and calcium response. We further overexpressed FKBP12.6 in BSMCs *in vitro* using recombinant lentivirus vectors containing the gene for FKBP12.6. Our results indicate that overexpression of FKBP12.6 prevents the decrease in the association of RyR2 with FKBP12.6 induced by asthmatic serum, IL-5, IL-13, and TNF-α. Moreover, overexpression of FKBP12.6 reduced the duration and magnitude of the $[Ca^{2+}]_i$ rise in response to bradykinin and prevented the increase in calcium response of BSMCs to bradykinin induced by asthmatic serum, IL-5, IL-13, and TNF-α. Therefore, the decrease in the association of RyR2 with FKBP12.6 due to a reduction of FKBP12.6 expression is responsible for the increase in calcium response of BSMCs to contractile agonists.

Because the endogenous ligand cADPR might induce the dissociation of FKBP12.6 from the RyR2, we studied the role of cADPR in airway inflammation–induced dissociation of FKBP12.6 from the RyR2. We found that asthmatic airway inflammation enhanced cADPR production by increasing ADP-ribosyl cyclase activity, but when we treated BSMCs with 8-Br-cADPR, a cADPR antagonist, we observed no difference in calcium responses to bradykinin (data not shown). These results indicate that cADPR does not contribute to airway inflammation–induced dissociation of FKBP12.6 from the RyR2.

Ca^{2+} mobilization from SR in BSMCs in response to contractile agonists such as bradykinin or acetylcholine is an important component of smooth muscle contraction (12, 28, 30). When dissociated from FKBP12.6, the RyR2 calcium channel on the SR membrane has more opening probability and becomes sensitized to intracellular activator signals. Thus, dissociation of FKBP12.6 from RyR2 induced by inflammatory cytokines renders BSMCs more sensitive to contractile agonists and leads to AHR in asthma. In the present study, our data show that overexpression of FKBP12.6 in the airway *in vivo* using lentivirus vector prevents the dissociation of FKBP12.6 from RyR2 in BSMCs in OVA-sensitized and -challenged rats. Furthermore, overexpression of FKBP12.6 in the airway normalizes the increased airway responsiveness in asthmatic rats. Together, these data provide solid evidence showing that dissociation of FKBP12.6 from RyR2 due to a decreased FKBP12.6 level is responsible for the increased calcium response contributing to AHR in asthma.

The regulatory mechanism for FKBP12.6 level is not clear. We found in the present study that FKBP12.6 mRNA is down-regulated in BSMCs treated with asthmatic serum, IL-5, IL-13, and TNF-α, suggesting the possibility that regulation of FKBP12.6 synthesis may occur at the transcriptional or mRNA level. FKBP12.6 mRNA levels represent the balance between gene transcription and mRNA degradation. The promoter of the human FKBP12.6 gene has been previously characterized (31). Further studies are needed to clarify the regulatory mechanism for FKBP12.6 gene transcription and the kinetics of mRNA degradation in chronic asthmatic inflammation.

In asthma models in guinea pigs and rats, profiles of inflammatory cells and cytokines are comparable to those of humans. Rat and guinea pig models of asthma have the classic Th2 asthmatic phenotype, which is characterized by high levels of antigen-specific IgE, eosinophil-dominated airway inflammation, and a pattern of Th2 cytokines including IL-4, IL-5, and IL-13 (32–34). In our studies, we found that treatment of BSMCs with serum from patients with asthma induced FKBP12.6 down-regulation and decreased association of FKBP12.6 from RyR2. These observations verify our findings in animal models and provide further evidence that FKBP12.6 association with RyR2 is decreased in AHR in asthma.

Calcium-dependent AHR induced by inflammatory cytokines is a hallmark of asthma. In the present study, we have demonstrated, for the first time, that chronic asthmatic inflammation causes dissociation of FKBP12.6 from RyR2 due to down-regulation of FKBP12.6 expression, which contributes to increased calcium response of BSMCs to stimuli in AHR in asthma. Manipulating the interaction of FKBP12.6 with RyR2 might be a novel and useful treatment for bronchial asthma. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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References

1. Postma DS, Kerstjens HA. Characteristics of airway hyperresponsiveness in asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;158:S187–S192.
2. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science* 1998;282:2258–2261.
3. Simon HU, Yousefi S, Schranz C, Schapowal A, Bachert C, Blaser K. Direct demonstration of delayed eosinophil apoptosis as a mechanism causing tissue eosinophilia. *J Immunol* 1997;158:3902–3908.
4. Adner M, Rose AC, Zhang Y, Swärd K, Benson M, Uddman R, Shankley NP, Cardell LO. An assay to evaluate the long-term effects of inflammatory mediators on murine airway smooth muscle: evidence that TNFalpha up-regulates 5-HT(2A)-mediated contraction. *Br J Pharmacol* 2002;137:971–982.
5. Moynihan BJ, Tolloczko B, El Bassam S, Ferraro P, Michoud MC, Martin JG, Laberge S. IFN-gamma, IL-4 and IL-13 modulate responsiveness of human airway smooth muscle cells to IL-13. *Respir Res* 2008;9:84.
6. Jain D, Keslacy S, Tliba O, Cao Y, Kierstein S, Amin K, Panettieri RA Jr, Haczku A, Amrani Y. Essential role of IFNbeta and CD38 in TNFalpha-induced airway smooth muscle hyper-responsiveness. *Immunobiology* 2008;213:499–509.

7. Matsumoto H, Hirata Y, Otsuka K, Iwata T, Inazumi A, Niimi A, Ito I, Ogawa E, Muro S, Sakai H, *et al*. Interleukin-13 enhanced Ca^{2+} oscillations in airway smooth muscle cells. *Cytokine* 2012;57:19–24.
8. Ma X, Cheng Z, Kong H, Wang Y, Unruh H, Stephens NL, Laviolette M. Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L1181–L1189.
9. Bullimore SR, Siddiqui S, Donovan GM, Martin JG, Sneyd J, Bates JH, Lauzon AM. Could an increase in airway smooth muscle shortening velocity cause airway hyperresponsiveness? *Am J Physiol Lung Cell Mol Physiol* 2011;300:L121–L131.
10. Mitchell RW, Rühlmann E, Magnussen H, Leff AR, Rabe KF. Passive sensitization of human bronchi augments smooth muscle shortening velocity and capacity. *Am J Physiol* 1994;267:L218–L222.
11. Mahn K, Ojo OO, Chadwick G, Aaronson PI, Ward JP, Lee TH. Ca^{2+} homeostasis and structural and functional remodelling of airway smooth muscle in asthma. *Thorax* 2010;65:547–552.
12. Sanderson MJ, Delmotte P, Bai Y, Perez-Zogbhi JF. Regulation of airway smooth muscle cell contractility by Ca^{2+} signaling and sensitivity. *Proc Am Thorac Soc* 2008;5:23–31.
13. Janssen LJ. Ionic mechanisms and Ca^{2+} regulation in airway smooth muscle contraction: do the data contradict dogma? *Am J Physiol Lung Cell Mol Physiol* 2002;282:L1161–L1178.
14. Tao L, Huang Y, Bourreau JP. Control of the mode of excitation-contraction coupling by Ca^{2+} stores in bovine trachealis muscle. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L722–L732.
15. Xiao RP, Valdivia HH, Bogdanov K, Valdivia C, Lakatta EG, Cheng H. The immunophilin FK506-binding protein modulates Ca^{2+} release channel closure in rat heart. *J Physiol* 1997;500:343–354.
16. Kaftan E, Marks AR, Ehrlich BE. Effects of rapamycin on ryanodine receptor/Ca^{2+}-release channels from cardiac muscle. *Circ Res* 1996; 78:990–997.
17. Wehrens XH, Lehnart SE, Reiken SR, Deng SX, Vest JA, Cervantes D, Coromilas J, Landry DW, Marks AR. Protection from cardiac arrhythmia through ryanodine receptor-stabilizing protein calstabin2. *Science* 2004;304:292–296.
18. Tang WX, Chen YF, Zou AP, Campbell WB, Li PL. Role of FKBP12.6 in cADPR-induced activation of reconstituted ryanodine receptors from arterial smooth muscle. *Am J Physiol Heart Circ Physiol* 2002;282: H1304–H1310.
19. Liao B, Zheng YM, Yadav VR, Korde AS, Wang YX. Hypoxia induces intracellular Ca^{2+} release by causing reactive oxygen species-mediated dissociation of FK506-binding protein 12.6 from ryanodine receptor 2 in pulmonary artery myocytes. *Antioxid Redox Signal* 2011;14:37–47.
20. Noguchi N, Yoshikawa T, Ikeda T, Takahashi I, Shervani NJ, Uruno A, Yamauchi A, Nata K, Takasawa S, Okamoto H, *et al*. FKBP12.6 disruption impairs glucose-induced insulin secretion. *Biochem Biophys Res Commun* 2008;371:735–740.
21. Wang YX, Zheng YM, Mei QB, Wang QS, Collier ML, Fleischer S, Xin HB, Kotlikoff MI. FKBP12.6 and cADPR regulation of Ca^{2+} release in smooth muscle cells. *Am J Physiol Cell Physiol* 2004;286: C538–C546.
22. Kibe A, Inoue H, Fukuyama S, Machida K, Matsumoto K, Koto H, Ikegami T, Aizawa H, Hara N. Differential regulation by glucocorticoid of interleukin-13-induced eosinophilia, hyperresponsiveness, and goblet cell hyperplasia in mouse airways. *Am J Respir Crit Care Med* 2003; 167:50–56.
23. Hua X, Chason KD, Fredholm BB, Deshpande DA, Penn RB, Tilley SL. Adenosine induces airway hyperresponsiveness through activation of A3 receptors on mast cells. *J Allergy Clin Immunol* 2008;122:107–113, 113. e1–7.
24. Hirst SJ. Airway smooth muscle cell culture: application to studies of airway wall remodelling and phenotype plasticity in asthma. *Eur Respir J* 1996;9:808–820.
25. Zhu L, Luo Y, Chen T, Chen F, Wang T, Hu Q. Ca^{2+} oscillation frequency regulates agonist-stimulated gene expression in vascular endothelial cells. *J Cell Sci* 2008;121:2511–2518.
26. Long C, Cook LG, Hamilton SL, Wu GY, Mitchell BM. FK506 binding protein 12/12.6 depletion increases endothelial nitric oxide synthase threonine 495 phosphorylation and blood pressure. *Hypertension* 2007;49:569–576.
27. Tao FC, Tolloczko B, Mitchell CA, Powell WS, Martin JG. Inositol (1,4,5)trisphosphate metabolism and enhanced calcium mobilization in airway smooth muscle of hyperresponsive rats. *Am J Respir Cell Mol Biol* 2000;23:514–520.
28. Tao FC, Shah S, Pradhan AA, Tolloczko B, Martin JG. Enhanced calcium signaling to bradykinin in airway smooth muscle from hyperresponsive inbred rats. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L90–L99.
29. Wehrens XH, Lehnart SE, Huang F, Vest JA, Reiken SR, Mohler PJ, Sun J, Guatimosim S, Song LS, Rosemblit N, *et al*. FKBP12.6 deficiency and defective calcium release channel (ryanodine receptor) function linked to exercise-induced sudden cardiac death. *Cell* 2003; 113:829–840.
30. Moynihan B, Tolloczko B, Michoud MC, Tamaoka M, Ferraro P, Martin JG. MAP kinases mediate interleukin-13 effects on calcium signaling in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L171–L177.
31. Nakazawa T, Takasawa S, Noguchi N, Nata K, Tohgo A, Mori M, Nakagawara K, Akiyama T, Ikeda T, Yamauchi A, *et al*. Genomic organization, chromosomal localization, and promoter of human gene for FK506-binding protein 12.6. *Gene* 2005;360: 55–64.
32. Kucharewicz I, Kasacka I, Pawlak D, Tankiewicz-Kwedlo A, Mroczko B, Buczko W, Bodzenta-Lukaszyk A. The concentration of kynurenine in rat model of asthma. *Folia Histochem Cytobiol* 2008; 46:199–203.
33. Takayama S, Tamaoka M, Takayama K, Okayasu K, Tsuchiya K, Miyazaki Y, Sumi Y, Martin JG, Inase N. Synthetic double-stranded RNA enhances airway inflammation and remodelling in a rat model of asthma. *Immunology* 2011;134:140–150.
34. Mahajan SG, Mehta AA. Suppression of ovalbumin-induced Th2-driven airway inflammation by β-sitosterol in a guinea pig model of asthma. *Eur J Pharmacol* 2011;650:458–464.