Insulin-like Growth Factor-binding Protein-3 (IGFBP-3) Blocks the Effects of Asthma by Negatively Regulating NF-κB Signaling through IGFBP-3R-mediated Activation of Caspases

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Insulin-like growth factor-binding protein-3 (IGFBP-3) is a multifunctional protein known for modulating mitogenic and metabolic actions of IGFs as well as exerting a variety of biological actions not involving IGFs. Here, we show that IGFBP-3 blocks specific physiological consequences of asthma in an IGF-independent manner in vitro and in vivo. IGFBP-3 treatment effectively reduced all physiological manifestations of asthma examined in vivo (airway hyper-responsiveness, cellular and pathological changes in bronchoalveolar lavage fluid and lung tissue, and expression of numerous proinflammatory molecules). These unique IGFBP-3 effects were further confirmed in IGFBP-3-transgenic mice, thus strengthening the notion of IGFBP-3 actions within the respiratory system. Using human epithelial cells, we demonstrated the following: 1) IGFBP-3 blocks TNF-α-induced expression of proinflammatory molecules; 2) IGFBP-3 attenuates the TNF-α-induced migratory response of eosinophils; and 3) IGFBP-3 negatively regulates TNF-α-induced expression of the key NF-κB regulatory molecules IkBα and p65-NF-κB at the post-translational level. We identified that IGFBP-3 degrades IkBα and p65-NF-κB proteins through IGFBP-3 receptor (IGFBP-3R)-mediated activation of caspases thereby inhibiting TNF-α-induced activation of NF-κB signaling cascades. This unique IGFBP-3/IGFBP-3R action was further confirmed by demonstrating complete inhibition of IGFBP-3 action in the presence of caspase inhibitors as well as IGFBP-3R siRNAs. Non-IGF-binding IGFBP-3 mutants further proved the IGF-independent action of IGFBP-3. Our findings indicate that IGFBP-3 inhibits airway inflammation and hyper-responsiveness via an IGF-independent mechanism that involves activation of IGFBP-3R signaling and cross-talk with NF-κB signaling. The IGFBP-3/IGFBP-3R system therefore plays a pivotal role in the pathogenesis of asthma and can serve as a newly identified potential therapeutic target for this debilitating disease.

The IGFBP-3 system, which includes ligands (IGF-I and IGF-II), receptors (IGF-IR and IGF-IIR), and a family of binding proteins (IGFBPs1–6), plays a significant role not only in somatic growth but also in diseases such as cancer, diabetes, and malnutrition (1–4). IGFBPs are essential to transport IGFs, prolong their half-lives, and regulate the availability of free IGFs for interaction with IGF receptors, thereby modulating the effects of IGFs (5, 6). Recent studies provide ample evidence that IGFBPs have unique activities besides interaction with the IGF/IGF-IR axis (7, 8). In particular, IGF/IGF-IR-independent actions of IGFBP-3 have been shown to contribute to the pathophysiology of a variety of human diseases such as cancer, diabetes, ischemia, and Alzheimer disease (9–14). Furthermore, a novel IGFBP-3 receptor (IGFBP-3R) has been recently identified and demonstrated to mediate IGF-independent anti-tumor effects of IGFBP-3 in human cancer (15). However, the functional significance of IGFBP-3R for the biological actions of IGFBP-3 in other diseases has yet to be unraveled.

Bronchial asthma is one of the most common chronic inflammatory diseases in modern society, and yet, despite the availability of current drugs, its incidence is increasing (16). It is characterized by airway eosinophilia, goblet cell hyperplasia with mucus hypersecretion, and hyper-responsiveness to inhaled allergens (17). Eosinophil accumulation and activation in bronchial tissues play critical roles in the pathophysiology of bronchial asthma (18–20). Bronchial epithelial cells also perpetuate airway inflammation by producing pro-inflammatory cytokines, such as interleukin (IL)-1, -5, -6, and -8, granulocyte macrophage-colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), macrophage chemotactic protein-1 (MCP-1), and regulated on activation, normal T-cell expressed and secreted (RANTES), thereby contributing to the local accumulation of inflammatory cells in bronchial asthma (21).

Despite the critical role of the IGF system in the pathophysiology of many diseases, limited information is available for its role in bronchial asthma. A few studies demonstrated that...
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IGF-I may be involved in the inflammatory process associated with bronchial asthma (22, 23). However, the underlying mechanism of the biological actions in the IGF system is largely unknown. In this study, we utilized both in vitro (human lung epithelial cell line) and in vivo (a mouse model of asthma) experimental systems to examine the involvement of IGFBP-3 in the pathogenesis of bronchial asthma.

Our findings indicate that IGFBP-3 inhibits airway inflammation and hyper-responsiveness in an IGF-independent manner. Furthermore, this action involves cross-talk with the NF-κB signaling pathway whereby IGFBP-3 not only inhibits NF-κB signaling but is also responsible for the degradation of key NF-κB signaling proteins through activation of caspases. We further describe that the newly identified IGFBP-3R mediates inhibitory effects of IGFBP-3 on NF-κB activity.

EXPERIMENTAL PROCEDURES

Mice and Experimental Protocol—Female C57BL/6 mice, 8–10 weeks of age and free of murine-specific pathogens, were obtained from the Orientbio Inc. (Seoungnam, Korea). IGFBP-3 transgenic mice were generated and maintained as described previously (24). All experimental animals used in this study were treated according to guidelines approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. Mice were sensitized on days 1 and 14 by intraperitoneal injection of 20 μg of OVA (Sigma) emulsified in 1 mg of aluminum hydroxide (Pierce) in a total volume of 200 μl, as described previously (supplemental Fig. S1) (25). On days 21–23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (w/v) OVA in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12; Omron Corp., Japan). Bronchoalveolar lavage (BAL) was performed 72 h after the last challenge. At the time of lavage, the mice (n = 6) were sacrificed with an overdose of sodium pentobarbitone (pentobarbital sodium, 100 mg/kg body weight, administered intraperitoneally). For IGFBP-3 treatments, either recombinant human IGFBP-3-1 and 10 μg/kg body weight/day; Upstate) or adenoviral plasmids (10^9 plaque-forming units) were administered intratracheally two times to each treated animal, once on day 21 (1 h before the first airway challenge with OVA) and the second time on day 23 (3 h after the last airway challenge with OVA) (supplemental Fig. S1).

Collection of BAL Fluids and Inflammatory Cells—The chest cavity was exposed to allow for expansion, after which the trachea was carefully incubated and the catheter secured with ligatures. Prewarmed 0.9% NaCl solution was slowly infused into the lungs and withdrawn. The aliquots were pooled and centrifuged. The supernatants were kept at −70 °C until use. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared by cytoSpin (Shandon Scientific, United Kingdom). The smears were stained with Diff-Quik solution (Dade Diagnostics, Puerto Rico) to examine the cell differentials. Two investigators blinded to treatments and genotypes counted the cells independently using a microscope. Approximately 400 cells were counted in each of four different random locations. Variation of results between investigators was less than 5%. The mean of the values from the two investigators was used for each cell count.

Preparation and Administration of Adenoviral Plasmids—We used the AdEasy system (Quantum Biotechnologies) to generate AdIGFBP-3 and AdIGFBP-3GGG as described previously (26). Ligation and recombination of plasmids and purification of adenovirus are described in the supplemental “Experimental Procedures.”

Measurement of IGFBP-3 Protein in BAL Fluids—Western blot analysis was performed for the quantification of IGFBP-3 in human BAL fluids. Each supernatant of BAL fluids was recovered, and BAL protein concentration was quantified using the Bradford reagent (Bio-Rad). Fifteen micrograms of samples were resolved on 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with the specific antibodies against IGFBP-3 antibody (Santa Cruz Biotechnology) and actin antibody (Sigma) separately. The blots were washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally detected by ECL reagent (Amersham Biosciences) according to the manufacturer’s instructions.

Determination of Airway Responsiveness—Airway responsiveness was also assessed as a change in airway function after challenge with aerosolized methacholine via airways, as described previously (27). In brief, mice were anesthetized by intraperitoneal injection with 80 mg/kg pentobarbital sodium. The trachea was then exposed through midcervical incision and tracheostomized, and an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small animal ventilator (FlexiVent, SCIREQ, Canada). The mouse was quasi-sinusoidally ventilated with nominal tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm of H_2O to achieve a mean lung volume close to that during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to the water column. Methacholine aerosol was generated with an in-line nebulizer and administered directly through the ventilator. To determine the differences in airway response to methacholine, each mouse was challenged with methacholine aerosol in increasing concentrations (2.5–50 mg/ml in saline). After each methacholine challenge, the data of calculate R_l was continuously collected. Maximum values of R_l were selected to express changes in airway function, which was represented as a percentage change from base line after saline aerosol.

Measurement of Cytokines and Chemokines from Mice—Levels of IL-1β, TNF-α, IL-4, IL-5, IL-13, and RANTES were quantified in the supernatants of BAL fluids by enzyme immunoasays according to the manufacturer’s protocol (IL-1β, TNF-α, IL-4, and IL-5 from Endogen and IL-13 and RANTES from R&D Systems). Levels of IL-6, IL-8, RANTES, and MCP-1 were quantified in the supernatants of BEAS-2B-treated and -untreated cells using a Bio-Plex multiplex assay (Bio-Rad) according to the manufacturer’s instructions.

Eosinophil Isolation—Freshly prepared buffy coats were obtained from the Virginia Blood Services (Richmond, VA). The contents of the buffy coats (34–40 ml) were further anti-coagulated with 0.01 mol/liter EDTA, pH 7.6, and diluted 1:1 with 1× Hanks’ buffered saline solution, pH 7.4. Cells (~20 ml)
were layered over 20-ml cushions of 1.10 g/ml density Percoll in 50-ml tubes. After centrifugation at 700 × g for 15 min at room temperature, leukocytes were collected from the Percoll/cell medium interface, and erythrocytes were found at the bottom of the tube. Leukocytes were retrieved, diluted 1:1 with 1× Hanks’ buffered saline solution, and layered (10–12 ml) over 12 ml of 1.077 g/ml density Percoll. Densities were confirmed with a Leica refractometer (Buffalo, NY). After centrifugation at 700 × g for 15 min at room temperature, the eosinophils, neutrophils, and remaining red blood cells were collected as a pellet at the bottom of the tube. Percoll-enriched cells were washed once (10 min at 500 × g at 4 °C) with cold PBS containing 1% BSA and 0.01 mol/liter EDTA. The cell pellet was next resuspended in ammonium chloride erythrocyte lysis solution (0.8% NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) for 10 min on ice. Cells were centrifuged at 500 × g at 4 °C for 10 min. The supernatant was removed, and cells were washed three times (10 min at 500 × g at 4 °C). Total cell numbers (hemocytometry) and viabilities (trypsin blue exclusion test) were determined. Eosinophil purity was calculated after assessing at least 300 cells on cytospin preparations labeled with Giemsa stain. Further purification was performed with the magnetic cell separation eosinophil isolation kit according to the manufacturer’s protocol by using negative selection with antibodies against CD2, CD14, CD16, CD19, CD56, CD123, and CD235a (glycophorin A) using an LS column. Unbound cells, mostly eosinophils, were washed four times with 3 ml of ice-cold PBS/BSA/EDTA. Eosinophil numbers, purities, and viabilities were evaluated as described above.

Eosinophil Chemotaxis Assay—The migration of eosinophils was measured using the ChemoTx system (Neuro Probe) as described previously (29). Samples to be tested (the conditioned media of cells treated with TNF-α, TNF-α + AdIGFBP-3 m.o.i. of 100; TNF-α + AdIGFBP-3 m.o.i. of 250; TNF-α + mutant-AdIGFBP-3GGG m.o.i. of 100; TNF-α + AdIGFBP-3 m.o.i. of 250; TNF-α + AdEV m.o.i. of 100; TNF-α + AdEV m.o.i. of 250; TNF-α + IKK inhibitor 2.5 μM; TNF-α + IKK inhibitor 5.0 μM; and appropriate no treatment control) as well as the eosinophil preparations were prewarmed at 37 °C before assay. 30–μl aliquots of each sample (three times) were loaded per well. Aliquots (25 μl) of purified eosinophils (>95%, 1 × 10⁵) in DMEM containing 1% serum were placed into the inserts on top of the wells. The plates were incubated for 90 min. Eosinophils that migrated through the filter into the lower chamber were collected, resuspended, with Giemsa, and counted. To determine eosinophil number attached to the membrane, the membrane was also dried and stained, and cell number was determined. Chemotaxis was expressed as a migration index (the ratio of migrated cell numbers from experimental groups divided by those from control groups).

BEAS-2B Cell Culture, Treatment and Harvesting—BEAS-2B is an adenovirus 12-SV40 virus hybrid (Ad12SV40)-transformed human epithelial cell line, which was isolated from normal human bronchial epithelium obtained from autopsy of noncancerous individuals. It was purchased from the American Type Culture Collection (ATCC) (Manassas, VA). The cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) in 5% CO₂, 95% humidified air at 37 °C. Before each experiment, cells were plated into 6-well plates (12-well plates were also used in some studies) overnight into an almost confluent monolayer. The next day, the cells were washed with PBS, and the media were changed to serum-free media (800 μl). Infection was then conducted in appropriate wells with the adenovirus (AdIGFBP-3, mutant-AdIGFBP-3GGG, or AdEV) at m.o.i. of 100 (m.o.i. of 250 also used in some studies). After 24 h, TNF-α (100 ng/ml) (Sigma) was added in appropriate wells for 24 h (sustained response). In the case where the acute response to TNF-α (5 min) was studied, infection with adenoviruses was conducted for 48 h prior to TNF-α treatment. For studies conducted with recombinant IGFBP-3, IGFBP-5, pyrrolidine dithiocarbamate, and the IKK-2 inhibitor IV (Calbiochem), treatments were conducted twice (1 h prior to TNF-α as well as 6 h following TNF-α treatment).

Harvesting Conditioned Media and Analysis of IL-6, IL-8, RANTES, and MCP-1 Used in Eosinophil Chemotaxis Assay Experiments—Conditioned media were collected, spun down to remove debris, aliquoted, and frozen at −70 °C until ready for use.

Harvesting Cell Lysates and Analysis of ICAM-1, p65-NF-κB, IkBa, and α-Tubulin—Cell lysates were harvested using 200 μl of HBSS/T lysis buffer (Hanks’ buffered saline solution containing 1 mM MgSO₄, 1 mM CaCl₂, 4 mM NaHCO₃, 0.5% Triton X-100, protease inhibitor mixture). Cell debris was removed by centrifugation, and protein concentration was determined by BCA protein assay (Pierce) according to the manufacturer’s instructions.

NF-κB Reporter Gene Expression—NF-κB reporter gene expression was determined as outlined previously (28). Briefly, BEAS-2B cells (2 × 10⁵) were co-transfected with 0.5 μg of firefly pNF-κB-luciferase vector as well as 0.1 μg of Renilla luciferase gene pRL-TK. Transfection with the reporter plasmid was conducted alone (control) or together with TNF-α. Adenoviruses were added at the same time of transfection. TNF-α treatment was done following 24 h of transfection/infection. After a further 24 h, cells were lysed and spun down, and activity was measured as recommended in the manufacturer’s protocol.

RT-PCR Analysis of ICAM-1, IL-6, IL-8, RANTES, and MCP-1—Total RNA was extracted from cells treated with the following: TNF-α, TNF-α + AdIGFBP-3 m.o.i. of 100; TNF-α + AdIGFBP-3 m.o.i. of 250; TNF-α + mutant-AdIGFBP-3GGG m.o.i. of 100; TNF-α + AdIGFBP-3 m.o.i. of 250; TNF-α + AdEV m.o.i. of 100; TNF-α + AdEV m.o.i. of 250; TNF-α + IKK inhibitor 2.5 μM; TNF-α + IKK inhibitor 5.0 μM; and appropriate no treatment control as well as the eosinophil preparations were prewarmed at 37 °C before assay. 30–μl aliquots of each sample (three times) were loaded per well. Aliquots (25 μl) of purified eosinophils (>95%, 1 × 10⁵) in DMEM containing 1% serum were placed into the inserts on top of the wells. The plates were incubated for 90 min. Eosinophils that migrated through the filter into the lower chamber were collected, resuspended, stained with Giemsa, and counted. To determine eosinophil number attached to the membrane, the membrane was also dried and stained, and cell number was determined. Chemotaxis was expressed as a migration index (the ratio of migrated cell numbers from experimental groups divided by those from control groups).

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IGFBP-3 levels were measured in the lung tissue of C57BL/6 mice after OVA challenge. IGFBP-3 expression in the lung tissue of OVA-induced asthmatic mice show decreased IGFBP-3 expression (Fig. 1A). In contrast, no significant changes in IGFBP-3 protein level were observed after saline inhalation. IGFBP-3 was significantly decreased in normal mice but not in transgenic mice after OVA sensitization and challenge (Fig. 1B). Immunoreactive IGFBP-3, normally localized in the epithelial layers around the bronchioles of control mice, was not detectable in allergen-induced asthmatic lungs and was further restored by intratracheal administration of wild type-IGFBP-3 (WT-AdIGFBP-3) but not AdLacZ (control adenovirus) (Fig. 1C).

Effect of IGFBP-3 and IGFBP-3<sup>GFG</sup> Mutant on Pathological Changes of OVA-induced Asthma—Because inflammatory cell recruitment is a hallmark phenomenon of all inflammatory diseases, including asthma (30), we next assessed the population of total cells, lymphocytes, neutrophils, and eosinophils in BAL fluids obtained from normal (Fig. 2A) and IGFBP-3 transgenic mice (Fig. 2B). All cell populations increased significantly at the lung tissues of normal mice significantly decreased following 1, 6, 24, 48, and 72 h of the last challenge with OVA, compared with levels before OVA inhalation or in the control group (Fig. 1A). In contrast, no significant changes in IGFBP-3 protein level were observed after saline inhalation. IGFBP-3 was significantly decreased in normal mice but not in transgenic mice after OVA sensitization and challenge (Fig. 1B). Immunoreactive IGFBP-3, normally localized in the epithelial layers around the bronchioles of control mice, was not detectable in allergen-induced asthmatic lungs and was further restored by intratracheal administration of wild type-IGFBP-3 (WT-AdIGFBP-3) but not AdLacZ (control adenovirus) (Fig. 1C).

**RESULTS**

**Decreased Expression of IGFBP-3 in OVA-induced Asthma**—To investigate the role of IGFBP-3 in the pathogenesis of bronchial asthma, a standard mouse model of asthma was used, and normal as well as IGFBP-3 transgenic mice were sensitized and challenged to OVA (supplemental Fig. S1). IGFBP-3 levels in the lung tissues of normal mice significantly decreased following 1, 6, 24, 48, and 72 h of the last challenge with OVA, compared with levels before OVA inhalation or in the control group (Fig. 1A). In contrast, no significant changes in IGFBP-3 protein level were observed after saline inhalation. IGFBP-3 was significantly decreased in normal mice but not in transgenic mice after OVA sensitization and challenge (Fig. 1B). Immunoreactive IGFBP-3, normally localized in the epithelial layers around the bronchioles of control mice, was not detectable in allergen-induced asthmatic lungs and was further restored by intratracheal administration of wild type-IGFBP-3 (WT-AdIGFBP-3) but not AdLacZ (control adenovirus) (Fig. 1C).

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72 h after OVA inhalation. The increased numbers of these cells were significantly reduced by the administration of WT-AdIGFBP-3. In IGFBP-3 transgenic mice, infiltration of inflammatory cells was significantly decreased compared with that of the WT control mice after OVA inhalation, although the basal level of the total number of cells was similar. Histological anal-
yses in normal mice revealed typical pathological features of asthma in the OVA-exposed mice, i.e. numerous inflammatory cells, including eosinophils, infiltrated around the bronchioles (Fig. 2C). Mice treated with WT-AdIGFBP-3 showed marked reductions in the infiltration of inflammatory cells in the peribronchial and perivascular regions. The scores of peribronchial, perivascular, and total lung inflammation were increased significantly at 72 h after OVA inhalation compared with the scores after saline inhalation (Fig. 2D). To elucidate whether the mechanism involves IGF-independent actions of IGFBP-3, studies were simultaneously conducted using a mutant-IGFBP-3GGG, which has no binding capacity for IGFs (31). Increased lung inflammation was significantly reduced by the administration of mutant-AdIGFBP-3GGG, suggesting that inhibitory effect of IGFBP-3 on antigen-induced inflammation in the lungs is independent of IGFs (Fig. 2, A and C).

Effect of IGFBP-3 and IGFBP-3GGG Mutant on Airway Hyper-responsiveness and Expression of Pro-inflammatory Proteins Induced by OVA in Mice—To investigate the impact of IGFBP-3 on airway function, airway responsiveness was assessed after challenge with aerosolized methacholine. Methacholine administration caused an increase of the percent airway resistance ($R_g$) in a dose-dependent manner with a 200% increase at the concentration of 50 mg/ml in control mice (Fig. 3A). OVA treatment resulted in a further substantial increase of the percent $R_g$ with a 2-fold increase at a dose of 50 mg/ml compared with the controls administered the same amounts of methacholine. However, OVA-induced mice treated with WT-AdIGFBP-3 and mutant-AdIGFBP-3GGG significantly inhibited the OVA-induced increase of percent $R_g$, indicating that IGFBP-3 reduces OVA-induced AHR. This effect was further confirmed in the IGFBP-3 transgenic mice (Fig. 3B). Furthermore, intratracheal administration of recombinant human IGFBP-3 (1 and 10 µg/kg body weight/day) results in identical inhibitory effects on the infiltration of inflammatory cells as well as AHR (supplemental Fig. S2, A and B).

We next investigated the effect of IGFBP-3 on an array of pro-inflammatory molecules known to be crucial in the process of asthma. OVA-induced mice resulted in a significant increase of IL-4, IL-5, IL-13, TNF-α, IL-1β, RANTES, eotaxin, VCAM-1, and ICAM-1 protein levels in lung tissues at 72 h after OVA inhalation, compared with control mice (Fig. 3C). Furthermore, enzyme immunoassay analysis of these pro-inflammatory molecules in BAL fluids yielded similar results (Fig. 3D). The increased cytokine levels at 72 h after OVA inhalation in lung tissue and BAL fluid were reduced in a similar fashion by the administration of WT-AdIGFBP-3 and mutant-AdIGFBP-3GGG. IGFBP-3 transgenic mice showed no significant change in IL-4, IL-5, and IL-13 after OVA inhalation (Fig. 3E).

Cross-talk between IGFBP-3- and TNF-α-activated NF-κB Signaling Cascades in Human Bronchial Epithelial Cells—Because TNF-α contributes to the dysregulated inflammatory response in the asthmatic airway, in part by induction of epithelial expression of pro-inflammatory cytokines and adhesion molecules (32, 33), potential cross-talk between IGFBP-3 and the TNF-α-NF-κB signaling cascade was examined using a bronchial inflammation mimic cell system (34). Pretreatment with recombinant IGFBP-3 resulted in a dose-dependent decrease in TNF-α-induced ICAM-1 expression, whereas IGFBP-5 pretreatment resulted in no change in BEAS-2B human bronchial epithelial cells (Fig. 4A). IGFBP-3 inhibited TNF-α-induced NF-κB-dependent reporter gene expression; TNF-α stimulated a 17-fold increase of luciferase activity, whereas IGFBP-3 and mutant-IGFBP-3GGG (but not AdEV) inhibited TNF-α-induced luciferase activity in an m.o.i.-dependent manner (Fig. 4B). IGFBP-3 and mutant-IGFBP-3GGG also inhibited TNF-α-induced IL-6, IL-8, MCP-1, and RANTES in a dose-dependent manner at both the protein and mRNA levels (supplemental Fig. S3, A and B). Because the eosinophil is regarded as the central effector cell that is responsible for ongoing airway inflammation (18, 19), we proceeded to evaluate the effect of IGFBP-3 on TNF-α-induced eosinophil chemotaxis to bronchial epithelial cells. TNF-α increased eosinophil chemotaxis up to 700%, whereas IGFBP-3 and mutant-IGFBP-3GGG significantly attenuated TNF-α-induced eosinophil chemotaxis similar to the effect observed with an IKK inhibitor (Fig. 4C).

Potential Mechanism for the Inhibitory Effect of IGFBP-3 on TNF-α-activated NF-κB Activity—We next tested whether IGFBP-3 had any effect on TNF-α-activated NF-κB activity by monitoring both the phosphorylated and total protein levels of two key NF-κB regulatory molecules, IkBα and p65-NF-κB. We investigated the effect of IGFBP-3 on both acute (5 min) and sustained (24 h) responses to TNF-α. Our results show that IGFBP-3 inhibited TNF-α-activated phosphorylation of IkBα (Fig. 5A) and p65-NF-κB (Fig. 5B). In particular, analysis of phosphorylated p65-NF-κB by confocal microscopy upon short term treatment with TNF-α (5 min) shows rapid phosphorylation and translocation to the nucleus. Prior treatment with AdIGFBP-3 or IKK inhibitor before TNF-α treatment resulted in inhibition of NF-κB phosphorylation (Fig. 5B). Unlike the IKK inhibitor, not only was phosphorylation inhibited but IGFBP-3 also caused a decrease in the total levels of IkBα and p65-NF-κB regardless of whether TNF-α treatment was acute (5 min) or sustained (24 h) (Fig. 5, A, C, and D). In contrast, IGFBP-3 did not induce any change in mRNA levels of IkBα and p65-NF-κB in bronchial epithelial cells, suggesting post-transcriptional effects (supplemental Fig. S4). Mutant-IGFBP-3GGG yielded similar results, supporting mechanisms of actions independent of IGFs (Fig. 5, B, C, and D). These results suggest that IGFBP-3 plays a functional role in degradation of proteins, rather than inhibiting their phosphorylation.

Further results demonstrated that IGFBP-3 degraded IkBα and p65-NF-κB proteins through activation of caspases, thereby inhibiting TNF-α-activated activation of NF-κB signaling cascades. Overexpression of IGFBP-3 resulted in a significant decrease of total IkBα and p65-NF-κB thereby inhibiting TNF-α-activated ICAM-1 expression (Fig. 5E). Concomitantly, IGFBP-3 induced significant cleavage of poly(ADP-ribose) polymerase (PARP) protein, which is indicative for caspase activation. This unique caspase-dependent action of IGFBP-3 was further confirmed by treatment of caspase inhibitors. In the presence of Z-VAD-fmk, a pan-caspase inhibitor (50 µM), IGFBP-3 resulted in no cleavage of the PARP protein, suggesting complete inhibition of IGFBP-3-induced caspase activities. The IGFBP-3-induced decrease of IkBα, p65-NF-κB total pro-
teins, and TNF-α-induced ICAM-1 expression was blocked by
Z-VAD-fmk treatment, whereas AdEV-infected cells showed
no effects on those proteins in the presence of Z-VAD-fmk (Fig.
5E). We further investigated which caspases were involved in
the IGFBP-3 action using specific caspase inhibitors (Fig. 5F).
Because our previous studies demonstrated that IGFBP-3 can
activate the initiator caspase, caspase-8, and its downstream
executioner caspase, caspase-3 (10), we treated cells with the
caspase-8 inhibitor Z-IETD-fmk or the caspase-3/7 inhibitor
Z-DEVD-fmk. As seen with Z-VAD-fmk treatment, we
observed similar effects with individual inhibitor treatment.
Those specific caspase inhibitors completely blocked IGFBP-3-
induced PARP degradation as well as the decrease of IκBα, p65-
NF-κB total proteins, and TNF-α-induced ICAM-1 expression.
Furthermore, IGFBP-3 treatment results in a 5-fold increase
of caspase-3/-7 activity, and co-treatment of caspase inhibitors
completely suppresses IGFBP-3-induced caspase-3/-7 activity
(Fig. 5G). Intriguingly, IGFBP-3-induced activation of caspases
results in no induction of apoptosis in human bronchial epithe-

dial cells (Fig. 5G).

Furthermore, the caspase-dependent effect of IGFBP-3 on
IκBα and p65-NF-κB appears to be specific because other crit-
ical signaling proteins known to be substrates of caspases in
a variety of cell systems such as ERK1/2, p38 MAPK, c-Fos, cyclin

FIGURE 3. Effect of IGFBP-3 on airway responsiveness and expression of pro-inflammatory proteins in OVA-sensitized and -challenged mice. A, airway
responsiveness was measured at 72 h after the last challenge in saline-inhaling C57BL/6 mice administered saline (SAL + SAL) as well as OVA-inhaling mice
administered saline (OVA + SAL), WT-AdIGFBP-3 (OVA + WT-AdIGFBP-3), mutant-AdIGFBP-3GGG (OVA + AdIGFBP-3GGG), or AdLacZ (OVA + AdLacZ). Rl,
values were obtained in response to increasing doses (2.5–50 mg/ml) of methacholine. Data represent mean ± S.E. from six mice per group. #, p < 0.05 versus
SAL + SAL; *, p < 0.05 versus OVA + SAL. B, assessment of airway responsiveness in transgenic mice sensitized and challenged with OVA (TG-OVA) versus saline
control (TG-Saline). Comparative studies were conducted with OVA-induced wild type mice (WT-OVA) and respective WT-saline control. #, p < 0.05 versus
WT-Saline. *, p < 0.05 versus CMVBP-3-Saline. §, p < 0.05 versus WT-OVA. C, densitometric analyses are presented as the relative ratio of cytokines to actin. The relative
ratio of cytokines in the lung tissues of mice inhaling saline is arbitrarily presented as 1. Data represent mean ± S.E. from six mice per group. #, p < 0.05 versus
SAL + SAL; *, p < 0.05 versus OVA + SAL. D, enzyme immunoassay analysis of BAL from C57BL/6 mice. The mean of the values from the two investigators was
used for each cell count. Bars represent mean ± S.E. from six mice per group. #, p < 0.05 versus SAL + SAL; *, p < 0.05 versus OVA + SAL. E, immunoblot analysis
of cytokines from the lung tissue of transgenic mice. Results were similar in six different experiments.
D1, and NFATc4 showed no significant changes after IGFBP-3 treatment in human bronchial epithelial cells (supplemental Fig. S5). In addition, our results demonstrate that IGFBP-3 activates initiator caspase-8 and executioner caspase-3/-7 but not initiator caspase-9 in BEAS-2B cells. Infection of AdIGFBP-3 at m.o.i. of 100 resulted in a 2.8-fold increase of caspase-3/-7 activities in the presence of TNF-α/H9251, whereas treatment of TNF-α/H9251 alone showed no changes in caspase-3/-7 activities. However, IGFBP-3 had no significant impact on activation of caspase-9 activity in BEAS-2B cells (supplemental Fig. S6A).

Furthermore, the inhibitory effect of IGFBP-3 on TNF-α-induced ICAM-1 expression was blocked by treatment with Z-VAD-fmk pan-caspase inhibitor as well as Z-IETD-fmk caspase-9 inhibitor (supplemental Fig. S6B).

Recently, we have identified a novel IGFBP-3 receptor (IGFBP-3R) that mediates IGFBP-3-induced growth inhibition and apoptosis via activation of caspases in human cancer cells (15). To determine whether this newly identified IGFBP-3R is required for the observed IGFBP-3 functions, we used IGFBP-3R siRNAs to knock down endogenous IGFBP-3R and tested the inhibitory effects of IGFBP-3 on TNF-α-induced activation of NF-κB signaling cascades in BEAS-2B cells. Transfection of IGFBP-3R siRNAs but not control siRNAs resulted in significant knockdown of endogenous IGFBP-3R by IGFBP-3R siRNA at the mRNA and protein levels (Fig. 5H), following significant inhibition of IGFBP-3-induced caspase activities (measured by cleaved PARP), p65 NF-κB degradation, and suppression of ICAM-1 expression in the presence of TNF-α. IGFBP-3-induced degradation of p65-NF-κB and subsequent suppression of ICAM-1 expression were determined (Fig. 5I). These data collectively suggest that IGFBP-3 specifically regulates NF-κB signaling cascades through IGFBP-3R-mediated activation of caspases thereby inhibiting TNF-α-induced activation of NF-κB signaling cascades.

**DISCUSSION**

Recent clinical studies suggested a role for IGFBP-3 on the pathogenesis of inflammatory diseases by demonstrating that serum IGFBP-3 was significantly decreased in patients with a variety of inflammatory diseases, including juvenile idiopathic arthritis, rheumatoid arthritis, pulmonary sarcoidosis, cystic fibrosis, Crohn disease, and inflammatory bowel disease (35–40), and when disease was in remission, the serum IGFBP-3 reached normal levels (39, 40). Furthermore, it has been reported that IGF-I plays an important role in the late stage of inflammation and remodeling of the asthmatic airway (41), and IGFBP-3 may modulate the biological effects of IGF-I in allergic airway remodeling in humans (42). Thus, these studies support our findings and provide evidence for relevance to asthma in humans.
IGFBP-3 Blocks the Effects of Asthma

A + TNF-α

Control  + TNF-α  + AdiIGFBP-3  + AdiIGFBP-3-MOI 100  + AdiEV  + AdiEV-MOI 100

IκBα-phos  IκBα-total  p65-NF-κB (total)  α-tubulin

B + TNF-α (100 ng/ml)

Control  + TNF-α  + AdiIGFBP-3  + AdiIGFBP-3 ε  + AdiEV  + AdiEV-MOI 100  + IKK inhibitor  + Blocking peptide

Hoechst’s  p65-NF-κB (phos)  Overlay

C + TNF-α (100 ng/ml)

Control  + TNF-α  + AdiIGFBP-3 (100)  + AdiIGFBP-3 ε (100)  + AdiEV (100)  + AdiEV-MOI 250  + IKK IV inhibitor (2.5)  + Blocking peptide

IκBα-phos  IκBα-total  p65-NF-κB (total)  α-tubulin

D + TNF-α (100 ng/ml)

Control  + TNF-α  + Ad:IGFBP-3  + AdiIGFBP-3-35G  + Ad:EV  + IKK inhibitor  + Blocking peptide

Hoechst’s  p65-NF-κB (total)  Overlay

E + TNF-α (50 ng/ml)

Control  + TNF-α  + Ad:EV  + AdiIGFBP-3  + zVAD

PARP  IκBα  p65-NF-κB  ICAM-1  α-tubulin

F + TNF-α  + zVAD  + NETD  + DEVD

AdiIGFBP-3  TNF-α  PARP  Cleaved PARP  IκBα  p65-NF-κB  ICAM-1  α-tubulin

G

Caspase-3/7 activity (pmol/mg/min)

Con  +  +  +  +  +  +  +
+z-VAD  +ETD  +DEVD  AdiIGFBP-3  TNF-α

H

IGFBP-3R mRNA (fold change)

Control  +  +  +  +  +  +
24 hr  48 hr  4 day  6 day  IGFBP-3R siRNA

I + TNF-α

Ad:LacZ  AdiIGFBP-3  IGFBP-3R siRNA

PARP  Cleaved PARP  p65-NF-κB  ICAM-1  IGFBP-3  IGFBP-3R  α-tubulin

Cell death (fold of control)

control  AdiIGFBP-3
This study demonstrates the physiological significance of IGFBP-3 in bronchial epithelial cells for airway inflammation and airway hyper-responsiveness. First, IGFBP-3 was suppressed in bronchial epithelial cells from OVA-exposed mice. Second, restoration of IGFBP-3 either by recombinant IGFBP-3 treatment or adenoviral IGFBP-3 gene transfer resulted in a marked inhibition of antigen-induced inflammation in the lungs, including the influx of eosinophils. Third, IGFBP-3 inhibits OVA-induced pro-inflammatory cytokines in lung tissue and BAL fluid. Finally, IGFBP-3 treatment restores airway functions as demonstrated by the reduction of OVA-induced AHR. These unique IGFBP-3 effects were further confirmed in IGFBP-3 transgenic mice by demonstrating significant reduction of infiltration of inflammatory cells, cytokine production, and OVA-induced AHR compared with that of the WT-control mice after OVA inhalation.

In vitro studies further provide convincing evidence for the underlying mechanisms of action of IGFBP-3 in a human cell system, in particular cross-talk with the pro-inflammatory NF-κB signaling pathway. Three lines of evidence suggest a central role of NF-κB in the pathogenesis of asthma as follows: 1) activated NF-κB has been identified in key locations such as lung epithelial cells and infiltrated immune cells in the airways of asthmatic patients (43); 2) agents such as allergens and viral infections, which are associated with exacerbation of asthma, stimulate activation of NF-κB (44); 3) the major effective treatment for asthma, corticosteroids, is a potent blocker of NF-κB activation (45); and 4) TNF-α-blocking monoclonal antibodies (etanercept and infliximab) have considerable anti-inflammatory effects on allergen-induced lung inflammation in an animal model of acute asthma (33, 46). NF-κB is normally found in its inactive form in the cytosol as the heterodimer p50/p65 bound to its inhibitory unit IκBα. Activation of NF-κB requires the phosphorylation and ubiquitin-mediated degradation of IκBα by the IKK complex. This is followed by the phosphorylation of the p65 unit of the heterodimer and the subsequent translocation of unmasked NF-κB to the nucleus where it binds to the enhancer or promoter regions of target genes and regulates their transcription (47). Our in vivo and in vitro data clearly demonstrate that IGFBP-3 is potentially functioning as an NF-κB inhibitor as follows: 1) IGFBP-3 inhibits OVA-induced increase of p65-NF-κB in lung tissue; 2) IGFBP-3 inhibits TNF-α-induced NF-κB-dependent reporter gene expression; 3) IGFBP-3 suppresses NF-κB-regulated inflammatory molecules induced by TNF-α; and 4) IGFBP-3 significantly blocks TNF-α-stimulated eosinophil migratory response. These data clearly indicate that IGFBP-3 inhibits NF-κB activity thereby inhibiting inflammatory effects of TNF-α.

Regarding the underlying mechanisms for IGFBP-3 action, we have identified that IGFBP-3 degrades IκBα and p65-NF-κB but not IKK proteins through activation of caspases, in particular caspase-8 and caspase-3/-7, thereby inhibiting TNF-α-induced activation of NF-κB signaling cascades. This unique caspase-dependent action of IGFBP-3 is clearly different from the actions of IKK inhibitors that inhibit only IKK-mediated phosphorylation and ubiquitin-mediated degradation of IκBα, resulting in no changes on total IκBα and p65-NF-κB proteins. Caspases, the cysteinyI aspartate-specific proteases, are key players during apoptotic cell death and are divided into initiator caspases (caspase-2 and -8–10) and executioner caspases (caspase-3, -6, and -7) (48). During apoptosis, activated apoptotic caspases cleave selected target proteins, thereby resulting in the degradation of chromosomal DNA and death of the cell. These caspase-dependent apoptotic signals can be triggered by internal signals (the intrinsic or mitochondrial pathway) or external signals (the extrinsic or death receptor pathway) (49). The initial caspase in the mitochondrial pathway is caspase-9, which subsequently activates the executioner caspases -3, -6, and -7. In contrast, the initial caspase of the death receptor pathway is caspase-8. Caspase-8 (like caspase-9) further activates the cascade of executioner caspases. Interestingly, recent studies have provided increasing evidence that apoptotic caspases also participate in several nonapoptotic cellular processes such as cell proliferation, cell cycle regulation, and differentiation (50–53).

In this respect, several studies have reported the interaction between caspases and the NF-κB signaling pathway. Caspase-3 cleaves IκBα regardless of phosphorylation status and results in inhibition of NF-κB activity, which is different from the typical proteasome-mediated degradation of IκBα (54, 55). Activated caspase-8 also cleaves specific kinases involved in NF-κB activation such as receptor-interacting protein and NF-κB-inducing kinase but not IKK, thereby inhibiting NF-κB activity (56, 57). These studies strongly support our findings that IGFBP-3 blocks the effects of asthma.

FIGURE 5. Post-translational and caspase-dependent suppression of IκBα and p65-NF-κB expression by IGFBP-3/IGFBP-3R. The effect of IGFBP-3 on IκBα and p65-NF-κB levels was induced on both acute (A and B) and sustained (C and D) response treatments with TNF-α. Immunoblot analysis of the effect of IGFBP-3 on IκBα and p65-NF-κB levels after 5 min (A) or 24 h (C) of treatment with TNF-α is shown. Infection was conducted with AdIGFBP-3 m.o.i. of 100, AdIGFBP-3-m.o.i. of 250, mutant-AdIGFBP-3 m.o.i. of 100, mutant-AdIGFBP-3-m.o.i. of 250, AdEV m.o.i. of 100, and AdEv m.o.i. of 250 and Ikk-2 inhibitor IV at concentrations of 2.5 and 5.0 μM. Confocal microscopy analysis of the effect of IGFBP-3 on p65-NF-κB levels after 5 min (B) or 24 h (D) of treatment with TNF-α is shown. Cells were immunostained for either phosphorylated (B) or total (D) p65-NF-κB (red) and Hoechst (blue) and analyzed by confocal microscopy. E, effect of Z-VAD-fmk, a pan-caspase inhibitor on the IGFBP-3-induced suppression of the NF-κB signaling cascade. Total IκBα and p65-NF-κB, ICAM-1, and PARP levels were determined by immunoblotting. F, effect of Z-VAD-fmk (pan-caspase inhibitor), Z-IE TD-fmk (caspase-8 inhibitor), and Z-DEVD-fmk (caspase-3/7 inhibitor) on the IGFBP-3-induced suppression of the NF-κB signaling pathway. Total IκBα and p65-NF-κB, ICAM-1, PARP, and cleaved PARP levels were determined by immunoblotting. G, caspase-3/-7 activity and cell death ELISAs. Left panel, effect of caspase inhibitors on the IGFBP-3-induced caspase-3/-7 activity was measured with caspase fluorescence kits. Right panel, IGFBP-3-induced apoptosis in BEAS-2B cells. Cells were infected with either AdEv m.o.i. of 100 or AdIGFBP-3 m.o.i. of 100 for 2 days. Apoptotic cell death was measured by the cell death detection ELISA kits. H, knockdown of endogenous IGFBP-3R by treatment with IGFBP-3R siRNAs. Twenty nM IGFBP-3R siRNAs or nontransfected siRNAs were transfected for the indicated time in BEAS-2B cells. Total RNA and cell lysates were collected and subjected to RT-PCR and Western immunobots, respectively (inset). IGFBP-3R siRNAs but not control siRNAs inhibited endogenous IGFBP-3R up to 90% at the mRNA and protein levels. I, specific knockdown of IGFBP-3R inhibited IGFBP-3-induced suppression of the NF-κB signaling cascade. Treatment with IGFBP-3R siRNAs resulted in complete suppression of IGFBP-3-induced caspase activities (measured by cleaved PARP), p65-NF-κB degradation, and suppression of ICAM-1 expression in the presence of TNF-α. Cells were transfected with 20 nM siRNAs or control siRNAs on days 1 and 2.5. On day 3, infection conducted with AdIGFBP-3 m.o.i. of 100. On day 4, cells were treated with 100 ng/ml TNF-α for 4 h and harvested.
IGFBP-3 activates caspase-8 and caspase-3/-7 and subsequently degrades IkBα and p65-NF-κB, thereby inhibiting TNF-α induced activation of NF-κB signaling cascades.

Ample evidence indicates that IGFBP-3 has unique intrinsic biological activities beyond its ability to interact with the IGF/IGF-IR axis, termed the “IGF-independent” actions (7–14). IGFBP-3 also shows unique biological functions in a variety of noncancerous cells in addition to its growth inhibitory effects and induction of apoptosis observed in cancer cells. These include the following: 1) IGFBP-3 has proangiogenic effects on endothelial precursor cells inducing migration, tube formation, and differentiation of these cells into endothelial cells, thereby promoting proper revascularization and repair after ischemic injury (12, 14, 58); 2) extracellular matrix-bound IGFBP-3 stimulates adhesion of breast epithelial cells and activation of p44/42 MAPK (59); 3) IGFBP-3 induces replicative senescence in human umbilical vein endothelial cells (60); and 4) IGFBP-3 inhibits human preadipocyte differentiation and differentiated adipocyte function (61). These studies indicate that IGFBP-3 enhances cell growth or other cell functions depending upon cell types and specific conditions. However, the underlying molecular mechanisms involved in these biological actions of IGFBP-3 are largely unknown.

We have recently identified a novel IGFBP-3R, a single span membrane receptor that mediates IGFBP-3-induced growth inhibition and apoptosis via activation of caspases in human prostate and breast cancer cells (15). Because IGFBP-3-activates caspase-8 that is usually induced by a variety of activated receptors on the cell membrane and because exogenously added IGFBP-3 (either recombinant IGFBP-3 or secreted IGFBP-3 from the AdIGFBP-3-infected cells) shows the same biological effect, it strongly suggests that the observed IGFBP-3 action in asthma may be mediated through the newly identified IGFBP-3R. Indeed, our data further demonstrated that IGFBP-3 degrades IkBα and p65-NF-κB proteins through IGFBP-3R-mediated activation of caspases thereby inhibiting TNF-α-induced activation of NF-κB signaling cascades, whereas the knockdown of endogenous IGFBP-3R completely negates the biological effect of IGFBP-3. Interestingly, IGFBP-3 induces activation of caspases and inhibits NF-κB activity similar to the findings in cancer cells, but no induction of apoptosis was observed in human bronchial epithelial cells. These data strongly suggest that the IGFBP-3/IGFBP-3R axis exerts different biological functions depending upon cell types, and IGFBP-3R may mediate the observed IGF-independent effects of IGFBP-3 on AHR and bronchial inflammation. This is further supported by our findings that a mutant-IGFBP-3<sup>3GAGG</sup>, which has no binding capacity for IGFs but retains IGFBP-3R binding, exhibits a similar effect as wild type IGFBP-3. The mutant-AdIGFBP-3<sup>3GAGG</sup> treatment reduces eosinophilic inflammation, OVA-induced pro-inflammatory cytokines in lung tissue, and BAL fluid and airway hyper-responsiveness in vivo as well as TNF-α-induced NF-κB activation, eosinophil migratory response, and degradation of IkBα and p65-NF-κB in vitro. Taken together, these data strongly suggest that IGFBP-3 inhibits many aspects of the inflammatory processes in asthma via activation of IGFBP-3R and cross-talk with NF-κB signaling. It is of note that we have also observed that IGFBP-3/IGFBP-3R system appears to negate elevated NF-κB activity in human adipocytes without induction of apoptosis.<sup>3</sup>

In conclusion, our findings clearly indicate that IGFBP-3 is capable of blocking crucial physiological manifestations of asthma, such as AHR and bronchial inflammation, via activation of IGFBP-3R leading to inhibition of the NF-κB signaling pathway. These newly discovered insights into the actions of IGFBP-3 have now opened an avenue for exploration of the IGFBP-3/IGFBP-3R system and may lead to new approaches to treating asthma and possibly function in situations where steroids and other current treatments are less effective.

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