

Endoplasmic reticulum stress influences bronchial asthma pathogenesis by modulating nuclear factor κ B activation

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Background: Despite many studies on endoplasmic reticulum (ER) stress in patients with various inflammatory diseases, there is scarce information on ER stress in patients with bronchial asthma.

Objective: In this study we aimed to elucidate the role of ER stress in the pathogenesis of bronchial asthma.

Methods: Using mice sensitized with ovalbumin (OVA) and LPS and challenged with OVA (OVA_{LPS}-OVA mice), as well as mice sensitized and challenged with OVA (OVA-OVA mice), we investigated whether ER stress is involved in the pathogenesis of bronchial asthma. Moreover, we also determined the levels of ER stress markers in blood and bronchoalveolar lavage fluid from asthmatic patients.

Results: The OVA_{LPS}-OVA mice showed that the expression of ER stress markers and the protein levels of unfolded protein response-related markers in lung tissue were significantly increased after OVA challenge. Moreover, we found that ER stress markers in PBMCs and bronchoalveolar lavage fluid from human asthmatic patients were dramatically increased compared with those from healthy control subjects. In OVA_{LPS}-OVA mice 4-phenylbutyric acid (4-PBA), a chemical chaperone, significantly reduced the increases in ER stress, nuclear translocation of nuclear factor κ B, inflammatory cytokine levels, dendritic cell infiltration, Toll-like receptor 4 expression, airway inflammation, and bronchial hyperresponsiveness, whereas it further enhanced the increase in IL-10 levels.

Additionally, the established asthmatic features of OVA-OVA mice were substantially attenuated by 4-PBA administered after completion of OVA challenge.

Conclusion: These results indicate that ER stress might be implicated in the pathogenesis of bronchial asthma at least

in part through modulation of nuclear factor κ B activation. (J Allergy Clin Immunol 2013;132:1397-408.)

Key words: Bronchial asthma, endoplasmic reticulum stress, phenylbutyric acid, Toll-like receptor 4, IL-10, nuclear factor κ B

Endoplasmic reticulum (ER) is a specialized organelle that plays a central role in the biosynthesis, correct protein folding, and posttranslational modifications of secretory and membrane proteins.¹ However, when the process of protein synthesis and protein folding is out of balance, unfolded and misfolded proteins are accumulated in the ER lumen, which is referred to as ER stress.^{2,3} Cells initiate an adaptive response called the unfolded protein response (UPR) to maintain homeostasis of ER function against an excess of ER stress. The UPR is mediated by 3 ER membrane-associated proteins: PKR-like eukaryotic initiation factor 2 α kinase, inositol-requiring enzyme 1, and activating transcription factor 6 (ATF6). When these protein sensors recognize enhanced ER stress, they activate the UPR process, such as increases in expression of glucose-regulated protein 78 (GRP78), a prominent ER-resident chaperone, and CCAAT/enhancer-binding protein-homologous protein (CHOP), an apoptotic transcriptional factor induced in response to ER stress.⁴⁻⁹ Several studies have demonstrated that excess ER stress interferes with protein synthesis and secretion, induces reactive oxygen species generation, increases inflammation through nuclear factor κ B (NF- κ B) activation, and mediates apoptotic cell death through expression of CHOP,¹⁰ thereby being involved in various disorders, including neurodegenerative disorders, metabolic disorders, inflammatory diseases, and malignancies.¹¹⁻¹³

Classically, asthma is a chronic airway disease with its origins in childhood; is related to allergies, dysregulated T_H2 inflammatory response, and eosinophils; and is best treated by targeting inflammation. However, there is mounting evidence for the complexity of some asthmatic populations (ie, steroid-resistant asthma and difficult or refractory asthma) that do not exhibit typical asthmatic features compared with classic allergic asthma.¹⁴⁻¹⁹ In fact, severe and fatal asthma might be mediated by neutrophils.²⁰⁻²² Because current treatment guidelines for asthma are based on the very typical phenotypic group of T_H2-predominant asthma,²³ the major unmet needs of current therapies include better treatment of patients with severe and refractory forms of asthma, such as neutrophilic asthma.

Considering the link between ER stress and various inflammatory responses, ER stress can be one of the most promising targets for the treatment of bronchial asthma. However, to date, there is no report regarding the role of ER stress in the pathogenesis of bronchial asthma, specifically neutrophil-dominant asthma.

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Abbreviations used

ATF6:	Activating transcription factor 6
BAL:	Bronchoalveolar lavage
CHOP:	CCAAT/enhancer-binding protein-homologous protein
DC:	Dendritic cell
DMSO:	Dimethyl sulfoxide
eIF2 α :	Eukaryotic initiation factor 2 α
ER:	Endoplasmic reticulum
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GRP78:	Glucose-regulated protein 78
KC:	Keratinocyte-derived chemokine
MPO:	Myeloperoxidase
NF- κ B:	Nuclear factor κ B
OVA:	Ovalbumin
OVA-OVA mice:	Mice sensitized and challenged with OVA
OVA _{LPS} -OVA mice:	Mice sensitized with OVA and LPS and challenged with OVA
PAA:	Phenylacetic acid
4-PBA:	4-Phenylbutyric acid
Penh:	Enhanced pause
R _{rs} :	Respiratory system resistance
SAL-SAL mice:	Saline-sensitized and saline-challenged mice
SV mice:	SAL-SAL mice administered drug vehicle
TLR:	Toll-like receptor
UPR:	Unfolded protein response
XBP-1:	X-box binding protein 1

In this study we investigated an association of ER stress with inducing/maintaining bronchial asthma, especially neutrophil-dominant asthma, using a murine model. The association was assessed by evaluating changes in ER stress markers and the effects of 4-phenylbutyric acid (4-PBA), a chemical chaperone. In addition, the therapeutic potential of 4-PBA was evaluated by using an ovalbumin (OVA)-induced ongoing and established eosinophil-dominant asthma model of mice.

METHODS

The details of the methods used in this study can be found in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS**ER stress markers are increased in our murine model of asthma and the patients with asthma**

Expression levels of GRP78 and CHOP in lung tissue were determined by using RT-PCR and real-time RT-PCR to evaluate the excess of ER stress in mice sensitized with OVA and LPS and challenged with OVA (OVA_{LPS}-OVA mice). The results revealed that mRNA levels of GRP78 and CHOP in lung tissue of OVA_{LPS}-OVA mice were gradually increased up to 24 hours after the last challenge with OVA, and the increased levels of mRNAs were then maintained up to 72 hours ([Fig 1, A-D](#)). Western blot analyses revealed that protein levels of GRP78 and CHOP in lung tissue of OVA_{LPS}-OVA mice were also increased in patterns similar to those seen with the expression of GRP78 and CHOP mRNA ([Fig 1, E-H](#)).

Interestingly, Western blot analyses showed that the levels of GRP78 and CHOP in PBMCs ([Fig 1, I-K](#)) and bronchoalveolar lavage (BAL) fluid ([Fig 1, L and M](#)) from asthmatic patients were significantly higher than the levels in healthy subjects (see [Table E1](#) in this article's Online Repository at www.jacionline.org).

4-PBA reduces ER stress and UPR in lung of OVA_{LPS}-OVA mice

To further assess the involvement of ER stress and UPR in patients with neutrophilic asthma, we used a chemical chaperone, 4-PBA. The effects of 4-PBA on the protein levels of ER stress markers and UPR-related proteins in lung tissue or BAL fluid of OVA_{LPS}-OVA mice were evaluated by means of Western blotting, immunohistochemical analysis, or immunofluorescence staining.

Confocal microscopic analyses revealed that all BAL cells from OVA_{LPS}-OVA mice showed a significant increase in immunofluorescence of GRP78 and CHOP, predominantly in cytoplasmic areas ([Fig 2, A](#)). In addition, immunofluorescence intensities of GRP78 and CHOP in lung tissue of OVA_{LPS}-OVA mice were markedly increased at 48 hours after OVA challenge, especially in bronchiolar epithelium and inflammatory cells around the bronchioles of lung tissue compared with those in saline-sensitized and saline-challenged mice (SAL-SAL mice; [Fig 2, B](#)). 4-PBA substantially reduced the increases in immunofluorescence intensities of GRP78 and CHOP in BAL cells and lung tissue of OVA_{LPS}-OVA mice. Consistent with these observations, the results from immunohistochemical ([Fig 2, C-E](#)) and Western blot analyses showed that protein levels of GRP78 or CHOP in lung tissue ([Fig 2, F-I](#)) or primary cultured tracheal epithelial cells ([Fig 2, J-M](#)) were substantially increased in OVA_{LPS}-OVA mice compared with the levels in SAL-SAL mice. The OVA-induced increases in GRP78 and CHOP protein levels in lung tissue of OVA_{LPS}-OVA mice were significantly reduced by administration of 4-PBA.

In addition, to investigate the effects of 4-PBA on UPR-related markers, we measured levels of ATF6 and X-box binding protein-1 (XBP-1) and phosphorylation of eukaryotic initiation factor 2 α (eIF2 α) in lung tissue of OVA_{LPS}-OVA mice. The increases in ATF6 and XBP-1 protein levels in nuclear protein extracts from lung tissue of OVA_{LPS}-OVA mice were substantially reduced by administration of 4-PBA ([Fig 2, N-Q](#)). In the case of phospho-eIF2 α , the increase in levels of this protein in lung tissue from OVA_{LPS}-OVA mice was markedly reduced by administration of 4-PBA ([Fig 2, R and S](#)). However, no significant changes in eIF2 α levels were observed in any of the groups tested.

Effect of 4-PBA on cellular changes in BAL fluid from OVA_{LPS}-OVA mice

The increase in the numbers of cells, especially neutrophils, in BAL fluid from OVA_{LPS}-OVA mice was significantly reduced by administration of 4-PBA ([Fig 3, A](#)).

4-PBA ameliorates pathologic features of OVA_{LPS}-OVA mice

Histologic assessment showed that numerous inflammatory cells infiltrated into the bronchioles, and the airway epithelium was thickened in the lungs of OVA_{LPS}-OVA mice ([Fig 3, C](#)) compared with SAL-SAL mice ([Fig 3, B](#)). OVA_{LPS}-OVA mice treated with 4-PBA showed marked reduction in thickening of the airway epithelium. A considerable decrease was also observed in the infiltration of inflammatory cells into the peribronchiolar region of the lung ([Fig 3, D](#)). The scores of peribronchial, perivascular, and total lung inflammation in OVA_{LPS}-OVA mice were significantly increased compared with those in SAL-SAL mice

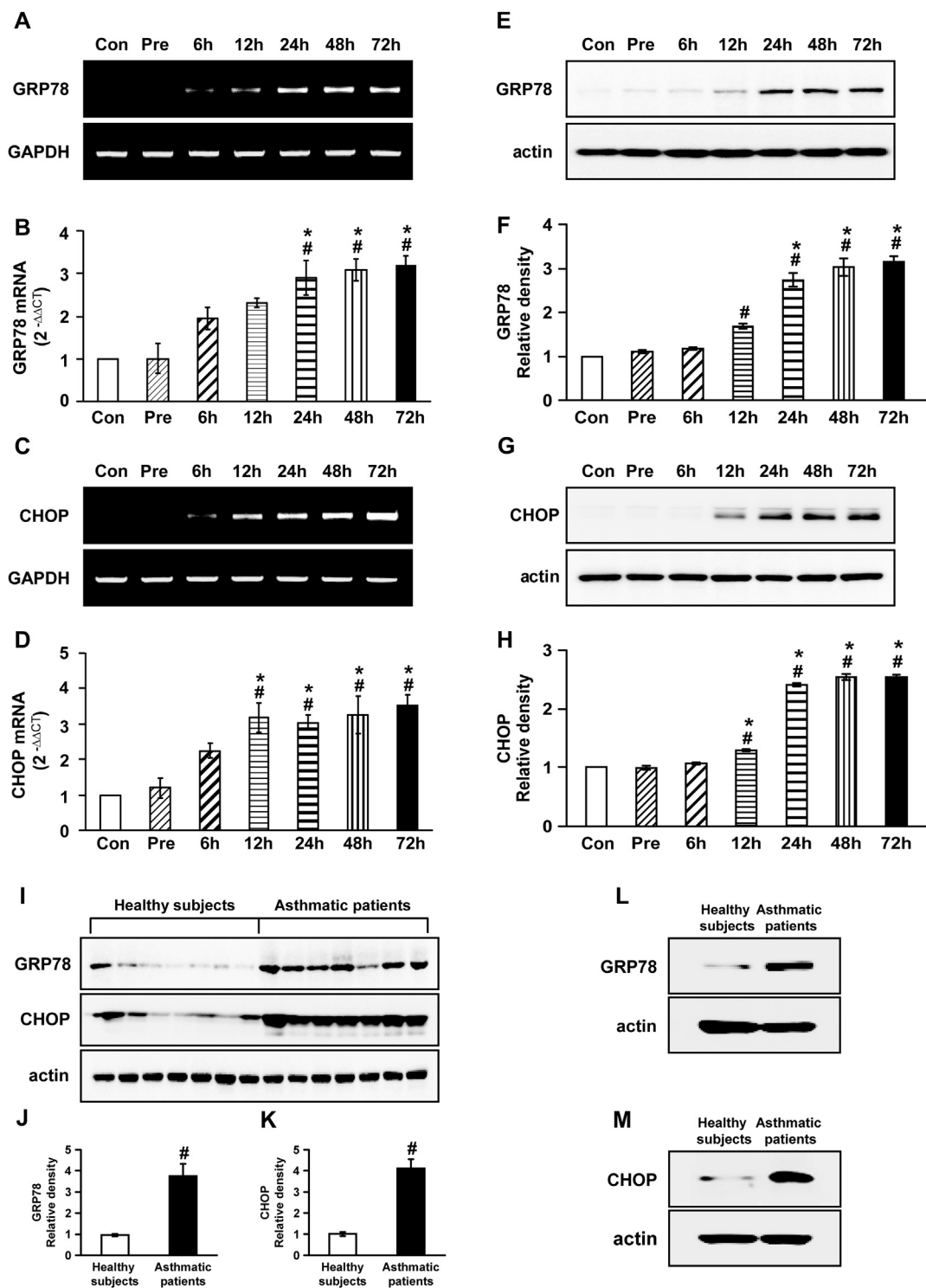


FIG 1. A-D, Representative RT-PCR of GRP78 (Fig 1, A) and CHOP (Fig 1, C) mRNA expression and real-time RT-PCR of GRP78 (Fig 1, B) and CHOP (Fig 1, D) mRNA expression. E-H, Representative Western blot of GRP78 (Fig 1, E) and CHOP (Fig 1, G) protein expression and densitometric analyses of GRP78 (Fig 1, F) and CHOP (Fig 1, H). Bars represent means $\pm$ SEMs from 7 mice per group. Con, No treatment; Pre, 1 hour before the first challenge with OVA. # P < .05 versus Con and * P < .05 versus Pre. I-K, Representative Western blot of GRP78 and CHOP in PBMCs from healthy subjects and asthmatic patients (Fig 1, I) and densitometric analyses of GRP78 (Fig 1, J) or CHOP (Fig 1, K). The relative ratio of each protein in the first healthy subject (ie, first left lane) is arbitrarily standardized as 1. # P < .05 versus healthy subjects. L and M, Representative Western blots of GRP78 (Fig 1, L) and CHOP (Fig 1, M) in human BAL fluid from asthmatic patients and healthy subjects (n = 3 per group).

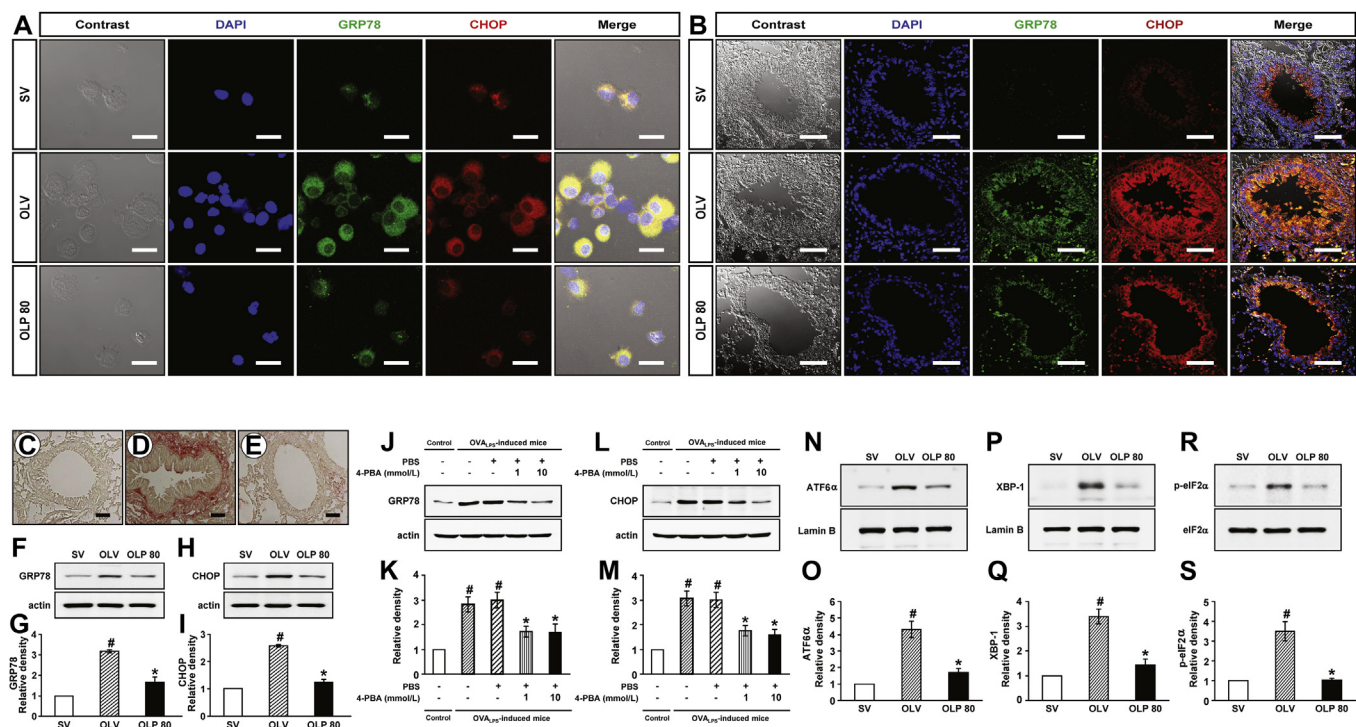


FIG 2. A and B, Representative confocal laser immunofluorescence photomicrographs of BAL cells (Fig 2, A) and lung tissue (Fig 2, B) in SAL-SAL mice administered drug vehicle (SV mice), OVA_{LPS}-OVA mice administered drug vehicle (OLV), or OVA_{LPS}-OVA mice administered 4-PBA (OLP 80). Bars indicate 20 μ m (Fig 2, A) or 50 μ m (Fig 2, B). C-E, Representative immunohistochemical staining of GRP78 in the lung tissue of SV (Fig 2, C), OLV (Fig 2, D), and OLP 80 (Fig 2, E) mice. Bars indicate 20 μ m. F-I, Representative Western blots of GRP78 (Fig 2, F) and CHOP (Fig 2, H) in lung tissue and densitometric analyses of GRP78 (Fig 2, G) or CHOP (Fig 2, I). Bars represent means $\pm$ SEMs from 7 mice per group. # P < .05 versus SV mice and * P < .05 versus OLV mice. J-M, Representative Western blot of GRP78 (Fig 2, J) and CHOP (Fig 2, L) in primary cultured tracheal epithelial cells and densitometric analyses of GRP78 (Fig 2, K) or CHOP (Fig 2, M). Bars represent means $\pm$ SEMs from 4 independent experiments. # P < .05 versus control and * P < .05 versus OVA_{LPS}-OVA mice administered PBS only. N-S, Representative Western blot of ATF6 α (Fig 2, N), XBP-1 (Fig 2, P), and phospho-eIF2 α (Fig 2, R) in lung tissue and densitometric analyses of ATF6 α (Fig 2, O), XBP-1 (Fig 2, Q), and phospho-eIF2 α (Fig 2, S). Bars represent means $\pm$ SEMs from 7 mice per group. # P < .05 versus SV mice and * P < .05 versus OLV mice.

(Fig 3, E). The increased peribronchial, perivascular, and total lung inflammation in OVA_{LPS}-OVA mice was significantly decreased by administration of 4-PBA.

Inhibition of ER stress decreases myeloperoxidase activity in lung tissue of OVA_{LPS}-OVA mice

Myeloperoxidase (MPO) is a major constituent of neutrophil cytoplasmic granules, and therefore its activity is a direct measure of the presence of neutrophils. As expected, MPO activity was significantly increased in lung tissue of OVA_{LPS}-OVA mice (Fig 3, F). The increase in MPO activity was substantially reduced by administration of 4-PBA.

4-PBA reduces airway hyperresponsiveness in OVA_{LPS}-OVA mice

Airway responsiveness was assessed through both noninvasive (enhanced pause [Penh]; Fig 3, G) and invasive (respiratory system resistance [R_{rs}]; Fig 3, H) measurements. In OVA_{LPS}-OVA mice the dose-response curve of the percentage of Penh or R_{rs} shifted to the left compared with that of SAL-SAL mice. Administration

of 4-PBA reduced substantially the Penh or R_{rs} values observed at 50 mg/mL methacholine in OVA_{LPS}-OVA mice.

4-PBA decreases production of inflammatory cytokines in OVA_{LPS}-OVA mice

To investigate whether ER stress evokes inflammatory responses in patients with bronchial asthma, we examined the effects of 4-PBA on inflammatory cytokine levels in the lungs of OVA_{LPS}-OVA mice. Levels of IL-4, IL-5, IL-13, TNF- α , IL-1 β , IFN- γ , IL-17, and keratinocyte-derived chemokine (KC) protein in lung tissue, BAL fluid, or both of OVA_{LPS}-OVA mice were significantly increased compared with those seen in SAL-SAL mice (Fig 4, A-N). Administration of 4-PBA reduced significantly the OVA-induced increases of these inflammatory mediators in the lung.

4-PBA prevents degradation of I κ B α and nuclear localization of NF- κ B p65 in OVA_{LPS}-OVA mice

Western blot data showed that I κ B α protein levels in lung tissue of OVA_{LPS}-OVA mice were significantly decreased at 48

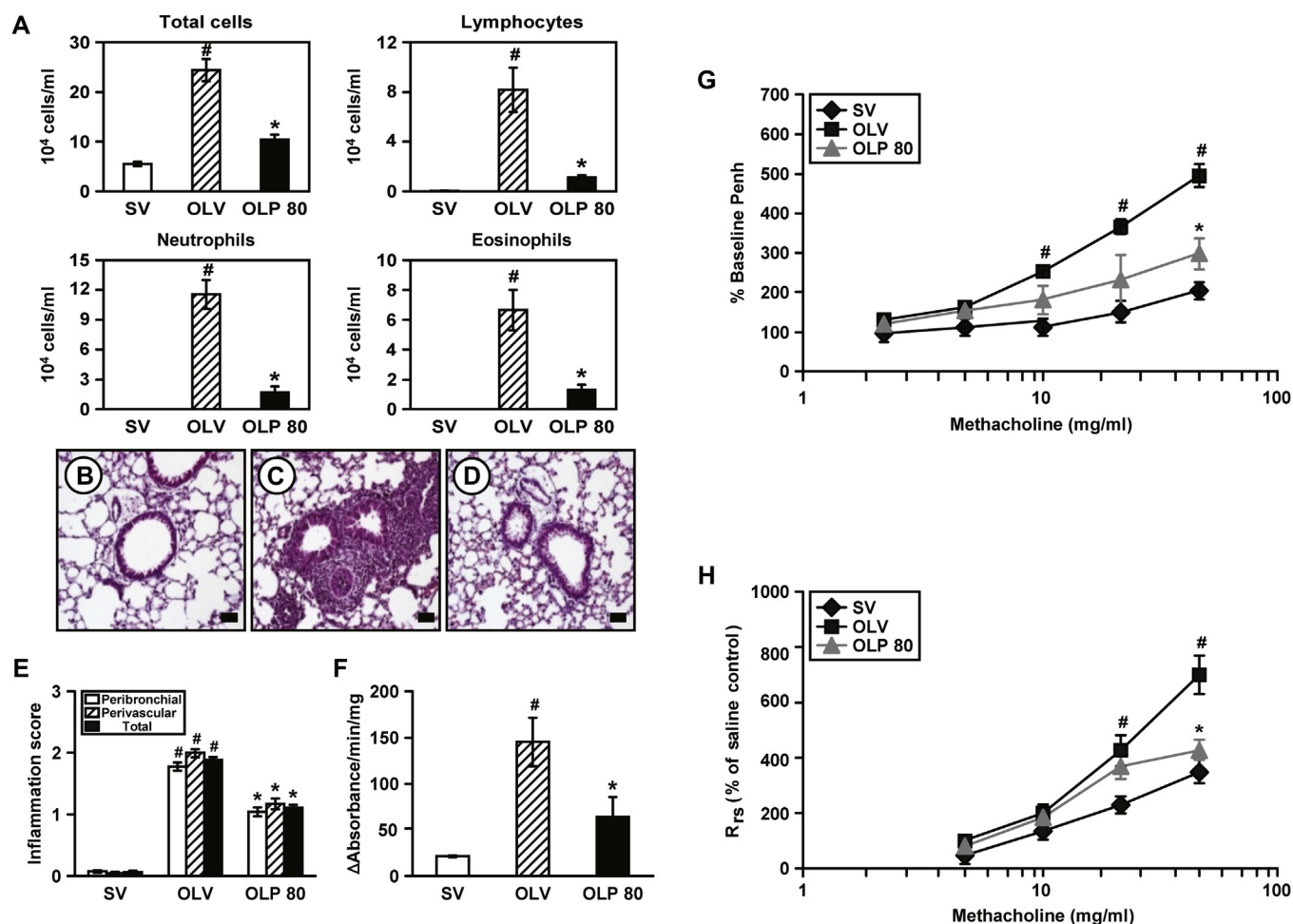


FIG 3. A, Cellular changes in BAL fluid of OVA_{LPS}-OVA mice. B-D, Representative hematoxylin and eosin-stained sections of lungs from SAL-SAL mice administered drug vehicle (SV mice; Fig 3, B), OVA_{LPS}-OVA mice administered drug vehicle (OLV; Fig 3, C), and OVA_{LPS}-OVA mice administered 4-PBA (OLP 80; Fig 3, D). Bars indicate 20 μm. E, Inflammation scores. F, MPO activity in lung tissue. Airway responsiveness assessed by noninvasive (percentage Penh in Fig 3, G) and invasive (R_{rs} in Fig 3, H) measurements. Bars represent means ± SEMs from 7 mice per group. [#]*P* < .05 versus SV mice and ^{*}*P* < .05 versus OLV mice.

hours after the last challenge compared with levels in SAL-SAL mice (Fig 4, O and P). The decrease in IκBα protein levels in OVA_{LPS}-OVA mice was markedly attenuated by administration of 4-PBA. The NF-κB p65 level in nuclear extracts of lung tissue was significantly increased in OVA_{LPS}-OVA mice compared with the level in SAL-SAL mice. The OVA-induced increase in nuclear NF-κB p65 levels was significantly reduced by administration of 4-PBA (Fig 4, Q and R).

Effects of dexamethasone on ER stress and asthmatic features of both murine model of asthma

Western blot analyses showed that protein levels of GRP78, CHOP, and ATF6α in lung tissue were substantially increased in OVA_{LPS}-OVA mice (Fig 5, A-F) and mice sensitized and challenged with OVA (OVA-OVA mice; Fig 5, K-P) compared with the levels in SAL-SAL mice. Treatment of OVA_{LPS}-OVA mice with dexamethasone did not show any effect on the OVA-induced increases in GRP78, CHOP, and ATF6α protein levels (Fig 5, A-F). On the other hand, treatment of OVA-

OVA mice with dexamethasone substantially reduced the increases in GRP78, CHOP, and ATF6α levels (Fig 5, K-P). In addition, histologic assessment showed that numerous inflammatory cells were significantly infiltrated into the bronchioles in the lungs of OVA_{LPS}-OVA (Fig 5, H) and OVA-OVA (Fig 5, R) mice compared with the lungs of SAL-SAL mice (Fig 5, G). Interestingly, OVA_{LPS}-OVA mice treated with dexamethasone showed similar pathologic features to OVA_{LPS}-OVA mice treated with drug vehicle only (Fig 5, J). However, the peribronchial and perivascular inflammation in the lungs of OVA-OVA mice was dramatically improved by treatment with dexamethasone (Fig 5, S). Consistent with these results, increases in the numbers of total cells, lymphocytes, and neutrophils in BAL fluid from OVA_{LPS}-OVA mice were not changed significantly by administration of dexamethasone (Fig 5, J), whereas OVA-OVA mice showed a significant reduction in the numbers of BAL cells, including eosinophils, by administration of dexamethasone (Fig 5, T).

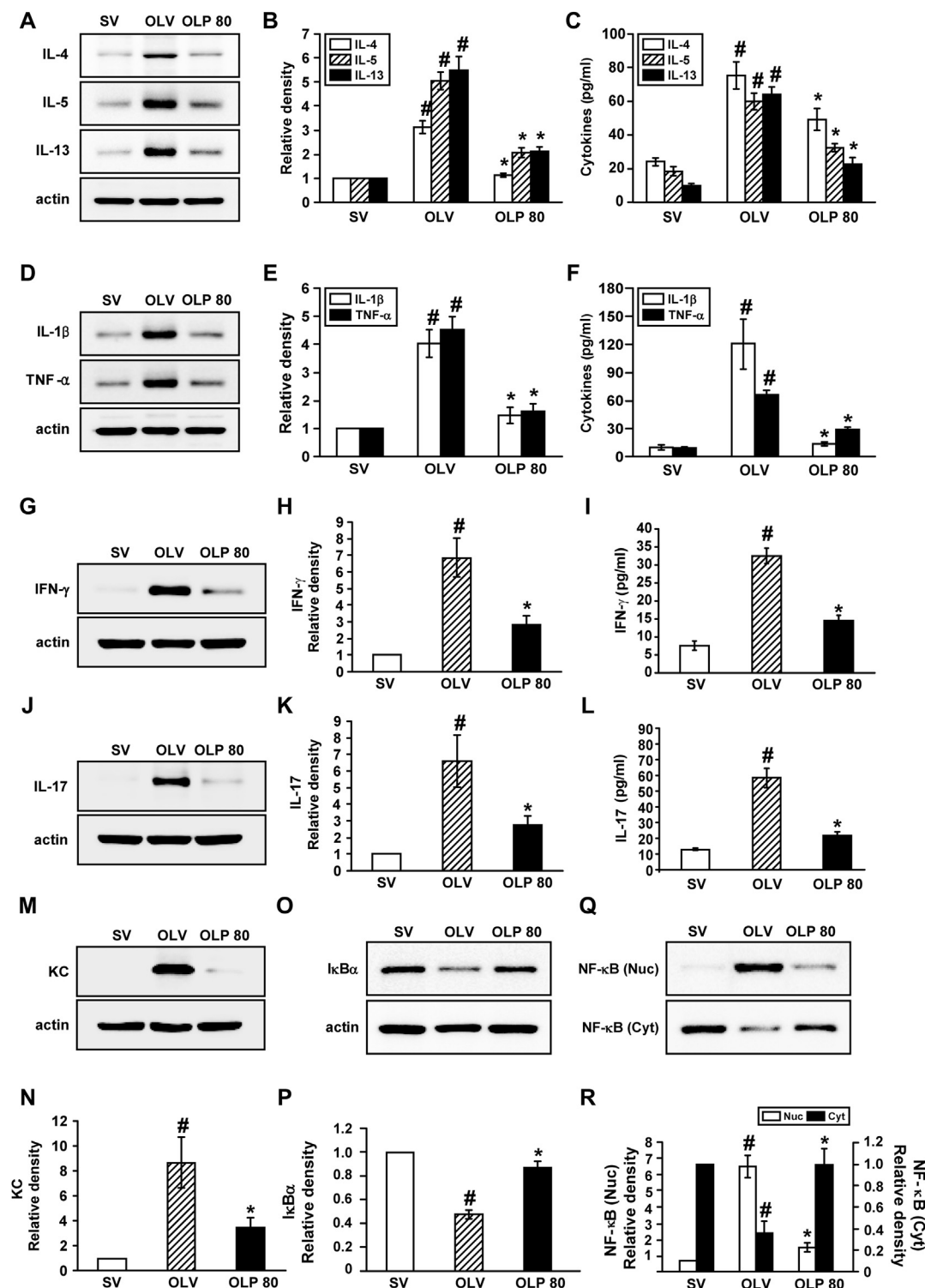


FIG 4. A and B, Representative Western blots of IL-4, IL-5, and IL-13 in lung tissue (Fig 4, A) and densitometric analysis of IL-4, IL-5, and IL-13 (Fig 4, B). C, Enzyme immunoassay of IL-4, IL-5, and IL-13 in BAL fluid. D and E, Representative Western blots of IL-1 β and TNF- α in lung tissue (Fig 4, D) and densitometric analysis of IL-1 β and TNF- α (Fig 4, E). F, Enzyme immunoassay of IL-1 β and TNF- α in BAL fluid. G, H, J, K, M, and N, Representative Western blot of IFN- γ (Fig 4, G), IL-17 (Fig 4, J), and KC (Fig 4, M) in lung tissue and densitometric analysis of IFN- γ (Fig 4, H), IL-17 (Fig 4, K), and KC (Fig 4, N). I and L, Enzyme immunoassay of IFN- γ (Fig 4, I) and IL-17 (Fig 4, L) in BAL fluid. O-R, Representative Western blot of I κ B α (Fig 4, O) and NF- κ B (Fig 4, Q) in lung tissue and densitometric analyses of I κ B α (Fig 4, P) and NF- κ B p65 (Fig 4, R) levels. Bars represent means $\pm$ SEMs from 7 mice per group. OLP 80, OVA_{LPS}-OVA mice administered 4-PBA; OLV, OVA_{LPS}-OVA mice administered drug vehicle; SV, SAL-SAL mice administered drug vehicle. # P < .05 versus SV mice and * P < .05 versus OLV mice.

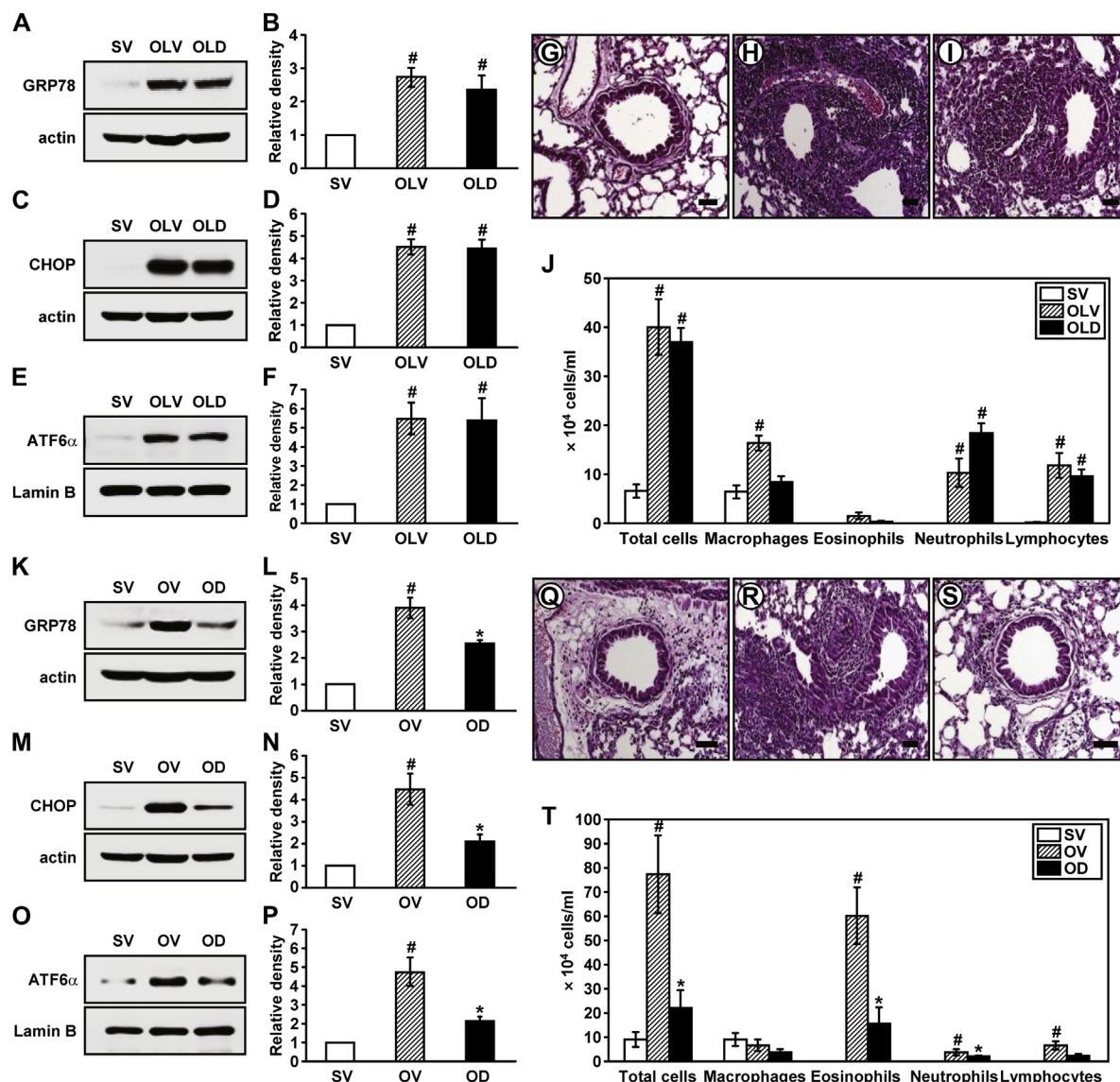


FIG 5. A-D, E, F, and K-P, Representative Western blots of GRP78 (Fig 5, A and K), CHOP (Fig 5, C and M), and ATF6 α (Fig 5, E and O) in lung tissue of SAL-SAL mice administered drug vehicle (SV), OVA_{LPS}-OVA mice administered drug vehicle (OLV), OVA_{LPS}-OVA mice administered dexamethasone (OLD), OVA-OVA mice administered drug vehicle (OV), or OVA-OVA mice administered dexamethasone (OD) and densitometric analyses of GRP78 (Fig 5, B and L), CHOP (Fig 5, D and N), and ATF6 α (Fig 5, F and P) to lamin B. G-I and Q-S, Representative hematoxylin and eosin-stained sections of lungs from SV (Fig 5, G and Q), OLV (Fig 5, H), OLD (Fig 5, I), OV (Fig 5, R), and OD (Fig 5, S) mice. Bars are 50 μ m. J and T, Cellular changes in BAL fluid of OVA_{LPS}-OVA mice (Fig 5, J) and OVA-OVA mice (Fig 5, T). Bars represent means $\pm$ SEMs from 6 mice per group. # P < .05 versus SV mice and * P < .05 versus OV mice.

4-PBA regulates infiltration of dendritic cells and expression of Toll-like receptor 4 in lung tissue of OVA_{LPS}-OVA mice

Toll-like receptors (TLRs) are critical for host defense because of their role in sensing pathogen invasion and triggering innate immune responses. Among them, TLR4-mediated recognition of bacterial LPS by various cells, including dendritic cells (DCs) and airway epithelial cells, plays a key role in immune and inflammatory responses.²⁴ To determine the role of LPS sensitization and its relationship with ER stress in the pathogenesis of neutrophil-dominant asthma, we analyzed the effects of 4-PBA on infiltration of DCs into lung tissue and TLR4 expression.

Numbers of DCs were remarkably increased in the lungs of OVA_{LPS}-OVA mice compared with numbers in SAL-SAL mice. The increase in DC numbers was significantly blocked by treatment with 4-PBA (Fig 6, A and B). Western blot analysis showed that the TLR4 protein level in lung tissue of OVA_{LPS}-OVA mice was significantly increased compared with that seen in SAL-SAL mice. The increase in TLR4 expression was substantially reduced by administration of 4-PBA (Fig 6, C and D).

In addition, the immunofluorescence intensity of TLR4 in lung tissue of OVA_{LPS}-OVA mice was markedly increased, predominantly in bronchiolar epithelium and inflammatory cells around the bronchioles of lung tissue compared with that seen in

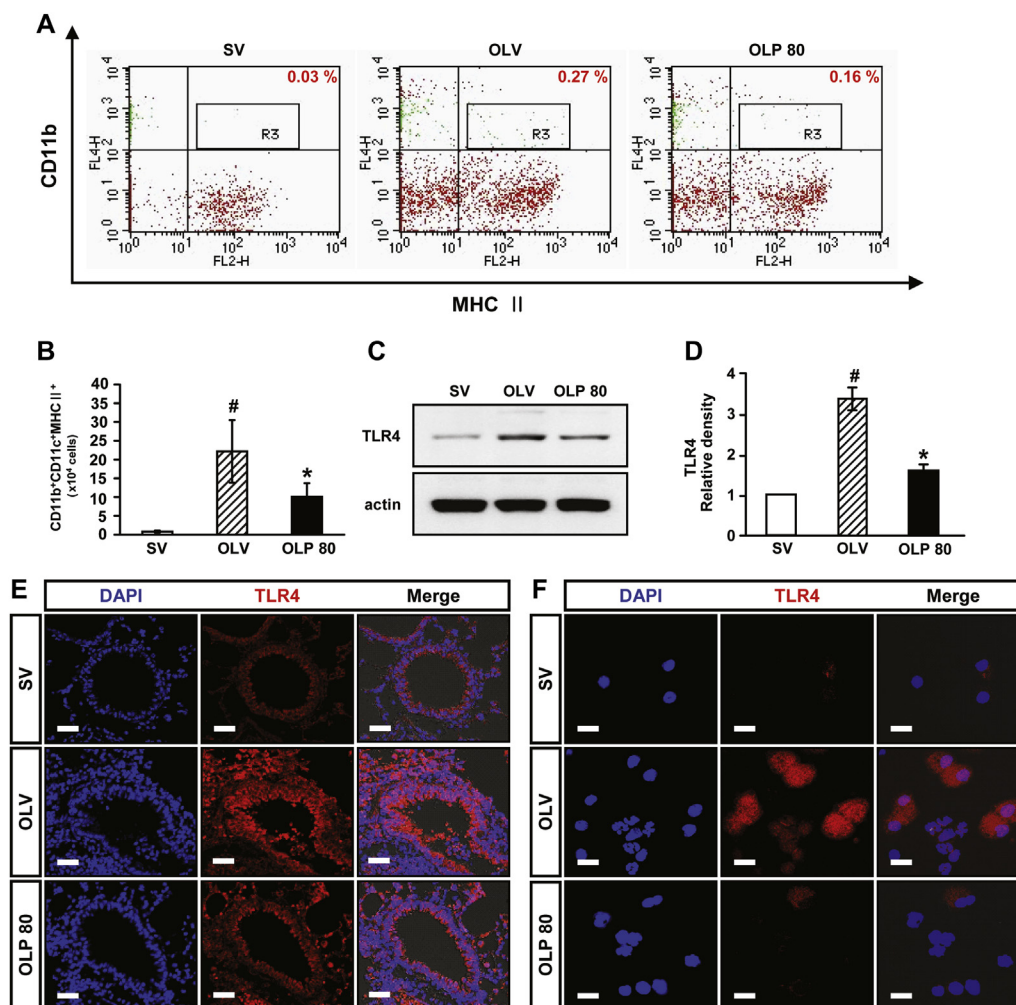


FIG 6. Effect of 4-PBA on infiltration of CD11b⁺CD11c⁺ cells and TLR4 protein levels in lung tissue or BAL cells of OVA_{LPS}-OVA mice. **A**, Dot plot analysis of CD11b⁺CD11c⁺MHC class II⁺ cells infiltrated into lung tissue in SAL-SAL mice administered drug vehicle (SV mice), OVA_{LPS}-OVA mice administered drug vehicle (OLV), and OVA_{LPS}-OVA mice administered 4-PBA (OLP 80). **B**, Number of infiltrated DCs. **C** and **D**, Representative Western blot of TLR4 in lung tissue (Fig 6, C) and densitometric analysis of TLR4 (Fig 6, D). Bars represent means $\pm$ SEMs from 7 mice per group. [#]*P* < .05 versus SV mice and ^{*}*P* < .05 versus OLV mice. **E** and **F**, Representative confocal laser immunofluorescence photomicrographs of TLR4 expression in lung tissue (Fig 6, E) and BAL cells (Fig 6, F). Bars indicate 25 μ m (Fig 6, E) and 20 μ m (Fig 6, F).

SAL-SAL mice (Fig 6, E). Confocal microscopic analyses also revealed that all BAL cells from OVA_{LPS}-OVA mice showed increased immunofluorescence intensity of TLR4 (Fig 6, F). Administration of 4-PBA substantially reduced increases in the immunofluorescence intensity of TLR4 in BAL cells and lung tissue of OVA_{LPS}-OVA mice.

4-PBA modulates production of IL-10 in lung tissue and BAL cells of OVA_{LPS}-OVA mice

IL-10 is a regulatory cytokine produced by several cell types and exhibits antiallergic inflammatory properties.^{25,26} Confocal microscopic analyses revealed that all BAL cells, including macrophages, from OVA_{LPS}-OVA mice showed increased immunofluorescence intensity of IL-10, predominantly in the cytoplasmic area (Fig 7, A and B). Administration of 4-PBA further enhanced the increases in

immunofluorescence intensity of IL-10 in BAL cells from OVA_{LPS}-OVA mice. In addition, IL-10 protein levels in lung tissue or BAL fluid of OVA_{LPS}-OVA mice was increased significantly compared with that seen in SAL-SAL mice (Fig 7, C-G). The increased IL-10 levels in lung tissue or BAL fluid of OVA_{LPS}-OVA mice was further increased by administration of 4-PBA.

Effects of 4-PBA following completion of OVA inhalation on ER stress in OVA-OVA mice

To investigate the involvement of ER stress in patients with eosinophilic asthma, we measured levels of GRP78 and CHOP in lung tissue of OVA-OVA mice after completion of the last challenge. As expected, the GRP78 and CHOP protein levels in lung tissue of OVA-OVA mice were markedly increased at 48 hours after the last challenge compared with the levels seen in

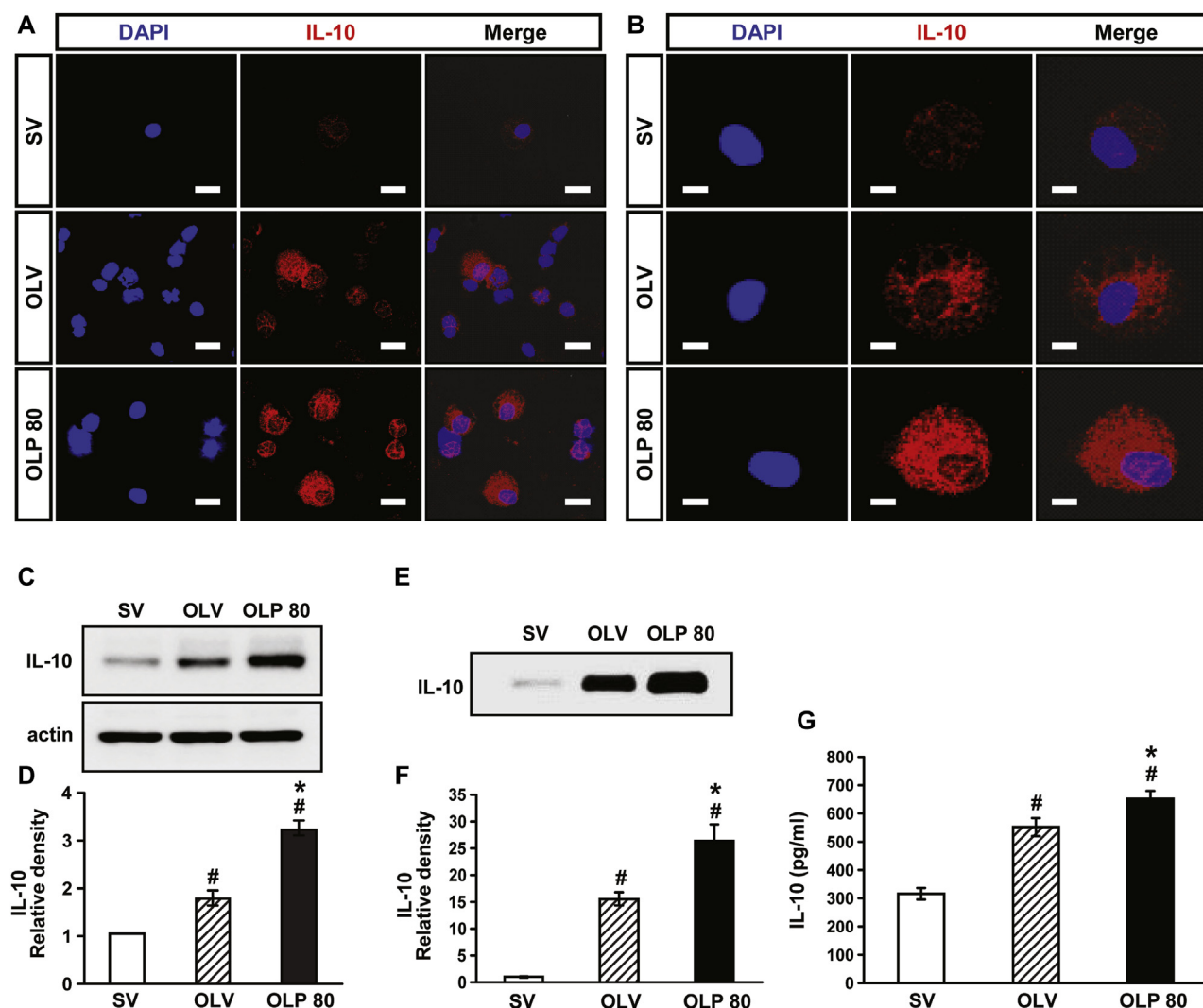


FIG 7. Expression of IL-10 protein in lung tissue and BAL fluid from OVA_{LPS}-OVA mice. **A** and **B**, Representative confocal laser immunofluorescence photomicrographs of BAL cells (Fig 7, A) and a macrophage (Fig 7, B) showed the localization of IL-10. Bars indicate 20 μ m. **C-F**, Representative Western blots of IL-10 protein in lung tissue (Fig 7, C) and BAL fluid (Fig 7, E) and densitometric analyses of IL-10 in lung tissue (Fig 7, D) and BAL fluid (Fig 7, F). **G**, Enzyme immunoassay of IL-10 in BAL fluid. Bars represent means $\pm$ SEMs from 6 or 7 mice per group. OLP 80, OVA_{LPS}-OVA mice administered 4-PBA; OLV, OVA_{LPS}-OVA mice administered drug vehicle; SV, SAL-SAL mice administered drug vehicle. # P < .05 versus SV mice and * P < .05 versus OLV mice.

SAL-SAL mice (see Fig E1 in this article's Online Repository at www.jacionline.org). The OVA-induced increase in GRP78 and CHOP protein levels was significantly reduced by administration of 4-PBA.

Effects of 4-PBA following completion of OVA inhalation on asthmatic features and nuclear localization of NF- κ B p65 in OVA-OVA mice

Effects of the drug on changes in BAL cells, airway hyperresponsiveness, and protein levels of T_H2 cytokines were evaluated by using OVA-OVA mice to verify the therapeutic potential of 4-PBA for ongoing and established asthma (see Fig E2, A-F, in this article's Online Repository at www.jacionline.org). The increased numbers of airway inflammatory cells, increased T_H2 cytokine levels, and enhanced airway

hyperresponsiveness after completion of OVA challenge were substantially reduced by administration of 4-PBA.

To investigate how 4-PBA works to attenuate established airway inflammation, we determined the levels of NF- κ B and degradation of I κ B α in lung tissue using Western blotting. The OVA-induced decreased I κ B α levels in lung tissue were restored by treatment with 4-PBA (see Fig E2, G and H), whereas the increase in nuclear NF- κ B levels after OVA challenge was significantly reduced by treatment with 4-PBA (see Fig E2, I and J).

Additionally, protein levels of IL-10, IL-17, and TLR4 in lung tissue of OVA-OVA mice were significantly increased at 48 hours after the last challenge compared with levels seen in SAL-SAL mice (see Fig E3 in this article's Online Repository at www.jacionline.org).

Expression of ER stress markers in lung tissue isolated from control groups

In Western blotting data we found that enhancement of the ER stress markers GRP78 and CHOP was observed only in the OVA_{LPS}-OVA mice, whereas other groups showed no significant increase in protein levels of the ER stress markers in lung tissue (see Fig E4 in this article's Online Repository at www.jacionline.org).

DISCUSSION

To the best of our knowledge, the current study has dealt with the implication of ER stress in the pathogenesis of bronchial asthma, specifically of neutrophilic asthma, for the first time. Our data using the pharmacologic intervention with 4-PBA, an ER stress regulator, have been concentrated to support the hypothesis that ER stress plays an important role in inducing and maintaining bronchial asthma and therefore the modulation of ER stress can be a valuable pharmacologic target for therapeutic strategies of bronchial asthma.

Asthma is a heterogeneous disease with variable clinical presentations. By understanding this heterogeneity of asthmatic inflammation, the roles of innate immunity have been highlighted in asthma pathogenesis.¹⁴⁻¹⁹ Given this recent concept, in this study we used 2 types of murine models for bronchial asthma: OVA_{LPS}-OVA mice and OVA-OVA mice, which are represented as a neutrophilic asthma animal model and a classic eosinophilic asthma model, respectively. In fact, there is increasing evidence that a significant proportion of human asthma might be driven by alternative forms of inflammation. In particular, studies on the cellular components of airway inflammation in asthmatic patients suggest distinct eosinophilic and noneosinophilic asthma phenotypes.²⁷⁻²⁹ Neutrophil-dominant asthma has been reported to be presented as severe, refractory, or fatal asthma clinically, regardless of the chronologic phase (ie, acute or chronic state), which is quite different from the classical eosinophilic form that is relatively well controlled by current pharmacologic agents. Our results revealed that both animal models of asthma showed enhanced airway hyperresponsiveness and inflammation, although the inflammatory cellular components were different in each model (ie, neutrophil vs eosinophil dominance). In addition, we have found that the representative standard therapeutic agent, the glucocorticoid, was effective for the asthmatic features of OVA-OVA mice; however, there is no significant effect of this agent on the neutrophil-dominant asthmatic features of OVA_{LPS}-OVA mice. These findings suggest that OVA_{LPS}-OVA mice display the characteristics of refractory or severe asthma.

A more interesting finding in our study is the significant increase in the ER stress markers GRP78 and CHOP in lung tissue of neutrophilic and eosinophilic asthmatic mice, as well as in PBMCs or BAL fluid from asthmatic patients. These findings imply the interaction between ER stress and asthmatic pathogenic features in mice and human subjects. In this regard one question has been raised: Why did the patients show persistently increased levels of ER stress markers despite receiving medical treatment with inhaled corticosteroids? In this study these patients could not be confirmed as having clear neutrophilic asthma, with relatively normal FEV₁ values and controlled symptom status in two thirds of patients. However, we found that there was no statistical correlation among FEV₁, the status of symptom control, and ER stress marker expression levels in human subjects. This finding suggests

that not all of the patients represent classic eosinophilic asthma. In addition, as is well known, real human allergen is not as pure as in the experimental OVA challenge; in fact, in human diseases the predominant eosinophilic manifestations are complicated with various factors, including simultaneous neutrophilic inflammation, life environment, age-related immune responses, and so on. Therefore it is likely that these reasons seem to be associated with the persistently increased levels of ER stress markers in PBMCs and BAL fluid from asthmatic patients treated with inhaled corticosteroids. In fact, ER stress has been suggested to be involved in the inflammatory component of chronic diseases.⁴ Although inflammation might contribute to the induction of ER stress markers in affected tissues, recent evidence suggests that ER stress, by triggering specific ER signaling pathways, might promote inflammation *per se*.³⁰ Supporting these concepts, our results revealed that enhanced expression of ER stress markers and UPR-related proteins, increases in airway hyperresponsiveness and inflammation, and production of inflammatory cytokines in the lung were suppressed in a dose-dependent manner by 4-PBA (see Fig E5 in this article's Online Repository at www.jacionline.org). Moreover, the increase of GRP78 and CHOP protein levels in primary tracheal epithelial cells was also blocked by pretreatment with 4-PBA. In the setting of bronchial asthma, these data indicate that the enhanced ER stress can induce asthmatic inflammation and airway hyperresponsiveness, although the roles of many other contributing factors associated with the pathogenesis of bronchial asthma cannot be excluded.

Supporting our contention, when OVA_{LPS}-OVA mice were treated with tunicamycin, which is an antibiotic that induces ER stress by blocking N-linked protein glycosylation,³¹ we found that use of tunicamycin resulted in somewhat more severe asthma phenotypes, including pathologic changes and airway hyperresponsiveness, than those seen in OVA_{LPS}-OVA mice treated with vehicle only (see Fig E6 in this article's Online Repository at www.jacionline.org). In fact, asthma is not caused by specific protein structural abnormalities, and asthmatic inflammatory conditions seem to increase the production of complex proteins, such as immunoglobulins, cytokines, and chemokines, which can lead to protein misfolding/unfolding. Therefore these findings suggest that accumulation of the various misfolded/unfolded proteins (ie, ER stress evoked by the airway inflammatory condition) might induce and aggravate asthmatic features.

Innate immunity has evolved the capacity to detect specific pathogenesis and interrogate cell and tissue integrity to mount an appropriate immune response.²⁴ Therefore recent studies of mice with airway delivery of native allergens or respiratory tract viruses have underscored the critical role of innate signaling in the generation of allergic pulmonary inflammation and complex immune responses.¹⁴⁻¹⁹ An immunologic role of ER stress was first recognized when XBP-1 was identified as an essential transcription factor involved in the differentiation of plasma B cells.³² UPR has also been shown to be essential for the survival and development of DCs.³³ Furthermore, ER stress seems to be a positive regulator for the immune response in patients with inflammatory pathologies.³⁴⁻³⁶ Actually, UPR activation has been reported to display a dramatically enhanced inflammatory response to TLR4 and TLR2 activation (ie, induction of IL-23 and type I interferon).^{37,38} Consistent with these observations, our results revealed that activation of TLR4 and increases in infiltration of DCs into the lung tissue of OVA_{LPS}-OVA mice were significantly reduced by inhibiting ER stress with 4-PBA. We

have also found that the increase in TLR4 expression occurring after OVA challenge in various inflammatory cells infiltrating into lung tissue, airway epithelial cells, and several BAL cells, including macrophages, was substantially reduced by treatment with 4-PBA. These findings suggest that ER stress-induced TLR4 expression occurs in various cells.

4-PBA treatment blocked the nuclear translocation of NF- κ B and degradation of I κ B α , which are implicated in inflammatory responses, thereby reducing production of IFN- γ , IL-4, IL-5, IL-13, TNF- α , IL-1 β , KC, or IL-17 in the lungs of OVA_{LPS}-OVA and OVA-OVA mice. As is well known, NF- κ B, a multiprotein complex, is involved in early cellular defense reactions in higher organisms and plays a pivotal role in immune and inflammatory responses.³⁹⁻⁴¹ Moreover, the expression of IL-17, which is one of the effector cytokines in neutrophilic inflammation, as well as T_H2 cytokines, is controlled by NF- κ B signaling.^{42,43} Recent studies have demonstrated that ER stress results in greater translocation of NF- κ B to the nucleus, indicating that ER stress activates NF- κ B signaling.^{44,45} Taken together, these findings suggest that ER stress boosts inflammatory responses through NF- κ B activation in both types of bronchial asthma, even in the setting of established asthmatic inflammation.

IL-10 is a representative regulatory cytokine produced by several cell types, including monocytes and, to a lesser extent, lymphocytes, mastocytes, regulatory T cells, and a certain subset of activated T and B cells.^{46,47} IL-10 can suppress airway inflammation and airway hyperresponsiveness in patients with allergic airway diseases.⁴⁷⁻⁴⁹ Although ER stress plays a role in regulatory T-cell differentiation and induces IL-10 production,⁵⁰ there is, to date, little information on the relationship between ER stress and IL-10, especially in patients with airway disorders. Interestingly, our mouse model of neutrophilic asthma showed a substantial increase in IL-10 levels in lung tissue or BAL fluid cells, and the IL-10 level was further enhanced by administration of 4-PBA, suggesting that induction of IL-10 expression is one of the molecular bases of 4-PBA in ameliorating allergic airway disease.

Meanwhile, 4-PBA can have various off-target effects. In fact, 4-PBA is an orally bioavailable terminal aromatic substituted fatty acid that is used in the treatment of urea cycle disorders and possesses chemical chaperone activity. In addition, 4-PBA works as a histone deacetylase inhibitor and is able to penetrate the blood-brain barrier, exerting significant neuroprotective effects in some animal models.⁵¹⁻⁵⁷ Specifically, given the potential therapeutic effects of histone deacetylase inhibitor, which are mostly well known in bronchial asthma, in our current study these off-target effects of 4-PBA, as well as the inhibitory effect on ER stress, are considered to contribute to improvement in the pathogenic phenotypes of bronchial asthma.

More importantly, these inhibitory effects of 4-PBA on airway inflammation and hyperresponsiveness were also observed when 4-PBA was administered to mice after completion of OVA inhalation. In addition, 4-PBA works well on the suppression of ER stress and airway inflammation in both neutrophilic and eosinophilic asthma models; however, glucocorticoid treatment showed inhibitory effects on ER stress and asthmatic features only in the eosinophilic asthma model. These findings provide a distinction between 4-PBA and the pharmacologic agents currently used for the treatment of bronchial asthma.

In summary, we have investigated the role of ER stress in experimental bronchial asthma, as well as the blood or BAL fluid

of asthmatic patients. Our results have shown that inhibition of ER stress with 4-PBA, a potent ER stress inhibitor, attenuates both neutrophilic and eosinophilic asthmatic inflammation and airway hyperresponsiveness effectively through the inhibition of nuclear translocation of NF- κ B. Moreover, 4-PBA decreases TLR4 expression in various inflammatory cells, such as DCs, macrophages, and airway epithelial cells, whereas it increases the production of IL-10 in various airway inflammatory cells. Our results also have shown that 4-PBA is superior for managing neutrophilic asthma compared with dexamethasone, a current pharmacologic agent. This study suggests not only a new concept of the pathogenesis of bronchial asthma involving ER stress but also a novel potential for 4-PBA as an effective therapeutic candidate for asthma, including the neutrophilic inflammatory type.

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Clinical implications: This study suggests not only a new concept of the pathogenesis of bronchial asthma involving ER stress but also a novel potential for 4-PBA as an effective therapeutic candidate for asthma, including the neutrophilic inflammatory type.

REFERENCES

- Gaut JR, Hendershot LM. The modification and assembly of proteins in the endoplasmic reticulum. *Curr Opin Cell Biol* 1993;5:589-95.
- Hosoi T, Ozawa K. Endoplasmic reticulum stress in disease: mechanisms and therapeutic opportunities. *Clin Sci (Lond)* 2009;118:19-29.
- Kaufman RJ. Orchestrating the unfolded protein response in health and disease. *J Clin Invest* 2002;110:1389-98.
- Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell* 2010;140:900-17.
- Hampton RY. ER stress response: getting the UPR hand on misfolded proteins. *Curr Biol* 2000;10:R518-21.
- Sidrauski C, Chapman R, Walter P. The unfolded protein response: an intracellular signaling pathway with many surprising features. *Trends Cell Biol* 1998;8:245-9.
- Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* 2007;8:519-29.
- Li J, Wang JJ, Yu Q, Wang M, Zhang SX. Endoplasmic reticulum stress is implicated in retinal inflammation and diabetic retinopathy. *FEBS Lett* 2009;583:1521-7.
- Endo M, Mori M, Akira S, Gotoh T. C/EBP homologous protein (CHOP) is crucial for the induction of caspase-11 and the pathogenesis of lipopolysaccharide-induced inflammation. *J Immunol* 2006;176:6245-53.
- Kincaid MM, Cooper AA. Eradicate ER stress or die trying. *Antioxid Redox Signal* 2007;9:2373-87.
- Toth A, Nickson P, Mandl A, Bannister ML, Toth K, Erhardt P. Endoplasmic reticulum stress as a novel therapeutic target in heart diseases. *Cardiovasc Hematol Disord Drug Targets* 2007;7:205-18.
- Kelsen SG, Duan X, Ji R, Perez O, Liu C, Merali S. Cigarette smoke induces an unfolded protein response in the human lung: a proteomic approach. *Am J Respir Cell Mol Biol* 2008;38:541-50.
- Poppek D, Grune T. Proteasomal defense of oxidative protein modifications. *Antioxid Redox Signal* 2006;8:173-84.
- Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen induced airway hyperreactivity. *Nat Med* 2003;9:582-8.
- Bartlett NW, Walton RP, Edwards MR, Anisenco J, Caramori G, Zhu J, et al. Mouse models of rhinovirus-induced disease and exacerbation of allergic airway inflammation. *Nat Med* 2008;14:199-204.
- Grayson MH, Cheung D, Rohlfing MM, Kitchens R, Spiegel DE, Tucker J, et al. Induction of high-affinity IgE receptor on lung dendritic cells during viral infection leads to mucous cell metaplasia. *J Exp Med* 2007;204:2759-69.
- Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 2009;15:410-6.

18. Kim EY, Battaile JT, Patel AC, You Y, Agapov E, Grayson MH, et al. Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. *Nat Med* 2008;14:633-40.
19. Phipps S, Lam CE, Kaiko GE, Foo SY, Collison A, Mattes J, et al. Toll-interleukin-1 signalling is critical for house dust mite-specific Th2 and Th17 responses. *Am J Respir Crit Care Med* 2009;179:883-93.
20. Maud T, Dolhnikoff M. Pathologic similarities and differences between asthma and chronic obstructive pulmonary disease. *Curr Opin Pulm Med* 2008;14:31-8.
21. Lamblin C, Gosset P, Tillie-Leblond I, Saulnier F, Marquette CH, Wallaert B, et al. Bronchial neutrophilia in patients with noninfectious status asthmaticus. *Am J Respir Crit Care Med* 1998;157:394-402.
22. Tsokos M, Paulsen F. Expression of pulmonary lactoferrin in sudden-onset and slow-onset asthma with fatal outcome. *Virchows Arch* 2002;441:494-9.
23. Shirtcliffe P, Weatherall M, Travers J, Beasley R. The multiple dimensions of airways disease: targeting treatment to clinical phenotypes. *Curr Opin Pulm Med* 2011;11:272-8.
24. Martinon F, Glimcher LH. Regulation of innate immunity by signaling pathways emerging from the endoplasmic reticulum. *Curr Opin Immunol* 2011;23:35-40.
25. Hobbs K, Negri J, Klinnert M, Rosenwasser LJ, Borish L. Interleukin-10 and transforming growth factor-beta promoter polymorphisms in allergies and asthma. *Am J Respir Crit Care Med* 1998;158:1958-62.
26. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001;19:683-765.
27. Haldar P, Pavord ID. Noneosinophilic asthma: a distinct clinical and pathologic phenotype. *J Allergy Clin Immunol* 2007;119:1043-52.
28. Douwes J, Gibson P, Pekkanen J, Pearce N. Non-eosinophilic asthma: importance and possible mechanisms. *Thorax* 2002;57:643-8.
29. Wenzel SE. Asthma: defining of the persistent adult phenotypes. *Lancet* 2006;368:804-13.
30. Zhang K, Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. *Nature* 2008;454:455-62.
31. Reimertz C, Kögel D, Rami A, Chittenden T, Prehn JH. Gene expression during ER stress-induced apoptosis in neurons: Induction of the BH3-only protein Bbc3/PUMA and activation of the mitochondrial apoptosis pathway. *J Cell Biol* 2003;162:587-97.
32. Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomlanyi-Tsuda E, Gravalles EM, et al. Plasma cell differentiation requires the transcription factor XBP-1. *Nature* 2001;412:300-7.
33. Iwakoshi NN, Pypaert M, Glimcher LH. The transcription factor XBP-1 is essential for the development and survival of dendritic cells. *J Exp Med* 2007;204:2267-75.
34. Xu Y. DNA damage: a trigger of innate immunity but a requirement for adaptive immune homeostasis. *Nat Rev Immunol* 2006;6:261-70.
35. Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxid Redox Signal* 2007;9:2277-93.
36. Todd DJ, Lee AH, Glimcher LH. The endoplasmic reticulum stress response in immunity and autoimmunity. *Nat Rev Immunol* 2008;8:663-74.
37. Martinon F, Chen X, Lee AH, Glimcher LH. TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages. *Nat Immunol* 2010;11:411-8.
38. Smith JA, Turner MJ, Delay ML, Klenk EI, Sowders DP, Colbert RA. Endoplasmic reticulum stress and the unfolded protein response are linked to synergistic IFN-beta induction via Xbp1 binding protein 1. *Eur J Immunol* 2008;38:1194-203.
39. Richardson CE, Kooistra T, Kim DH. An essential role for XBP-1 in host protection against immune activation in *C. elegans*. *Nature* 2010;463:1092-5.
40. Urade R. The endoplasmic reticulum stress signaling pathways in plants. *Biofactors* 2009;35:326-31.
41. Tateda C, Ozaki R, Onodera Y, Takahashi Y, Yamaguchi K, Berberich T, et al. NtbZIP60, an endoplasmic reticulum-localized transcription factor, plays a role in the defense response against bacterial pathogens in *Nicotiana tabacum*. *J Plant Res* 2008;121:603-11.
42. Kim SR, Lee KS, Park SJ, Min KH, Lee KY, Choe YH, et al. PTEN down-regulates IL-17 expression in a murine model of toluene diisocyanate-induced airway disease. *J Immunol* 2007;179:6820-9.
43. Park SJ, Lee KS, Kim SR, Min KH, Choe YH, Moon H, et al. Peroxisome proliferator-activated receptor gamma agonist down-regulates IL-17 expression in a murine model of allergic airway inflammation. *J Immunol* 2009;183:3259-67.
44. Jiang HY, Wek SA, McGrath BC, Scheuner D, Kaufman RJ, Cavener DR, et al. Phosphorylation of the alpha subunit of eukaryotic initiation factor 2 is required for activation of NF-kappaB in response to diverse cellular stresses. *Mol Cell Biol* 2003;23:5651-63.
45. Deng J, Lu PD, Zhang Y, Scheuner D, Kaufman RJ, Sonenberg N, et al. Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2. *Mol Cell Biol* 2004;24:10161-8.
46. Said EA, Trautmann L, Dupuy F, Zhang Y, Ancuta P, El-Fari M, et al. PD-1 induced IL10 production by monocytes impairs T-cell activation in a reversible fashion. *Nat Med* 2010;16:452-9.
47. Robinson DS. Regulatory T cells and asthma. *Clin Exp Allergy* 2009;39:1314-23.
48. Borish L. Interleukin-10: evolving concepts. *J Allergy Clin Immunol* 1998;101:293-7.
49. Kim SR, Lee KS, Park HS, Park SJ, Min KH, Jin SM, et al. Involvement of IL-10 in peroxisome proliferator-activated receptor gamma-mediated anti-inflammatory response in asthma. *Mol Pharmacol* 2005;68:1568-75.
50. Franco A, Almanza G, Burns JC, Wheeler M, Zanetti M. Endoplasmic reticulum stress drives a regulatory phenotype in human T-cell clones. *Cell Immunol* 2010;266:1-6.
51. Brusilow SW, Maestri NE. Urea cycle disorders: diagnosis, pathophysiology, and therapy. *Adv Pediatr* 1996;43:127-70.
52. Collins AF, Pearson HA, Giardina P, McDonagh KT, Brusilow SW, Dover GJ. Oral sodium phenylbutyrate therapy in homozygous beta thalassemia: a clinical trial. *Blood* 1995;85:43-9.
53. Mercuri E, Bertini E, Messina S, Pelliccioni M, D'Amico A, Colitto F, et al. Pilot trial of phenylbutyrate in spinal muscular atrophy. *Neuromuscul Disord* 2004;14:130-5.
54. Carducci MA, Gilbert J, Bowling MK, Noe D, Eisenberger MA, Sinibaldi V, et al. A phase I clinical and pharmacological evaluation of sodium phenylbutyrate on an 120-h infusion schedule. *Clin Cancer Res* 2001;7:3047-55.
55. Inden M, Kitamura Y, Takeuchi H, Yanagida T, Takata K, Kobayashi Y, et al. Neurodegeneration of mouse nigrostriatal dopaminergic system induced by repeated oral administration of rotenone is prevented by 4-phenylbutyrate, a chemical chaperone. *J Neurochem* 2007;101:1491-504.
56. Ono K, Ikemoto M, Kawarabayashi T, Ikeda M, Nishinakagawa T, Hosokawa M, et al. A chemical chaperone, sodium 4-phenylbutyric acid, attenuates the pathogenic potency in human alpha-synuclein A30P+A53T transgenic mice. *Parkinsonism Relat Disord* 2009;15:649-54.
57. Ricobaraza A, Cuadrado-Tejedor M, Pérez-Mediavilla A, Frechilla D, Del Río J, García-Osta A. Phenylbutyrate ameliorates cognitive deficit and reduces tau pathology in an Alzheimer's disease mouse model. *Neuropsychopharmacology* 2009;34:1721-32.

METHODS

Animals and experimental protocol

Female C57BL/6 mice 8 to 10 weeks of age and free of murine-specific pathogens were obtained from Orient Bio (Seoungnam, Korea), housed throughout the experiments in a laminar flow cabinet, and maintained on standard laboratory chow *ad libitum*. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University.

For the neutrophilic asthma model, mice were sensitized intranasally with OVA (Sigma-Aldrich, St Louis, Mo) and LPS (Sigma-Aldrich; OVA_{LPS}; 10 µg of OVA combined with 1 µg of LPS in saline) on days 1, 2, 3, and 14. On days 21, 22, and 23 after initial sensitization, the mice were challenged for 30 minutes with an aerosol of 3% (wt/vol) OVA in saline (or with saline as a control) by using an ultrasonic nebulizer (NE-U17; Omron, Kyoto, Japan).

For the investigation of the role of ER stress in ongoing or established asthma, we used OVA-OVA mice, which were intraperitoneally sensitized on days 1 and 14 with 10 µg of OVA (Sigma-Aldrich) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical, Rockford, Ill) in a total volume of 200 µL, as previously described but with some modifications.^{E1} On days 21, 22, and 23 after initial sensitization, the mice were challenged for 30 minutes with an aerosol of 3% (wt/vol) OVA in saline (or with saline as a control) by using an ultrasonic nebulizer (NE-U17, Omron). BAL was performed at 48 hours after the last challenge with OVA in OVA_{LPS}-OVA or OVA-OVA mice. At the time of lavage, the mice (5–7 mice in each group) were killed by means of cervical dislocation. The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter was secured with ligatures. Prewarmed 0.9% NaCl solution was slowly instilled into the lung and withdrawn. The collected solutions were pooled and kept at 4°C. A part of each pool was then centrifuged, and the supernatants were kept at –70°C until use. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared by using a cytospin (Thermo Electron, Waltham, Mass) and stained with Diff-Quik solution (Dade Diagnostics of Puerto Rico, Aguada, Puerto Rico) to examine cell differentials. Two independent blinded investigators counted the cells with a microscope. Approximately 400 cells were counted in each of 4 different random locations. Interinvestigator variation was less than 5%. The mean number from the 2 investigators was used to estimate cell differentials.

Administration of the chemical chaperone 4-PBA, dexamethasone, and tunicamycin

4-PBA (80 mg/kg body weight per day; Calbiochem, San Diego, Calif) diluted with PBS was administered by means of intraperitoneal injection 2 times to each treated animal, once at 1 hour before the first challenge with OVA and again at 6 hours after the last challenge in OVA_{LPS}-OVA mice. To examine the therapeutic effects of 4-PBA on ongoing or established asthma, the same dose of 4-PBA was administered by means of intraperitoneal injection 2 times to each treated animal, once at 2 hours after the last challenge with OVA and again at 24 hours after the last challenge in OVA-OVA mice. The administration time of 4-PBA in animal models was determined based on our data obtained from pharmacokinetic analyses of PBA and its metabolite, as reported by Marahatta et al.^{E2} In the study pharmacokinetic analyses of PBA and phenylacetic acid (PAA) in mouse plasma and tissue revealed that maximum concentrations of PBA in plasma and tissue were seen at 15 minutes after administration, whereas the highest concentration of PAA was found at 45 minutes. In addition, the half-life of PBA was less than 30 minutes in plasma and tissue. The half-life of PAA after intraperitoneal injection of PBA ranged from 0.080 to 0.455 hours. To compare the effectiveness of 4-PBA with that of the current standard treatment, dexamethasone (1 mg/kg body weight per day, Sigma-Aldrich) was administered by means of oral gavage 2 times to each treated animal, once at 24 hours before and again at 2 hours before the first OVA challenge in both animal models of bronchial asthma.

Tunicamycin (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) and diluted to 20 µg/mL in 20% DMSO diluted in PBS, and then

100 µL of solution was delivered to mice intratracheally. A similar volume of 20% DMSO diluted in PBS was used as a vehicle control.

RNA isolation and RT-PCR

Levels of mRNA expression were analyzed with the RT-PCR assay, with total RNA isolated from lung tissue by using a rapid extraction method (TRI-Reagent), as previously described.^{E3} RNA was quantified by measuring absorption at 260 nm and stored at –70°C until use. One microgram of total RNA was reverse transcribed to cDNA by using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland). The primers used were as follows: GRP78, sense 5'-GAAAGGATGGTTAATGATGCTGAG-3' and antisense 5'-GTCTTCAATGT CCGCATCCTG-3'; CHOP, sense 5'-CATACACCACCACACCTGAAAG-3' and antisense 5'-CCGTTTCCTAGTTCCTCCTTGC-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense 5'-GCCATCAACGACCCCTTCATTGAC-3' and antisense 5'-ACGGAAGGCC ATGCCAGTGAGCTT-3'. PCR reactions were performed in a thermocycler (GeneAmps PCR System 2400; Applied Biosystems, Foster City, Calif). The amplified PCR products were electrophoresed by using 1.6% agarose gels stained with ethidium bromide. DNA bands were visualized by using Fuji film LAS-3000 (Fujifilm, Tokyo, Japan).

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR analysis was performed by using the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics). The Δ cycle threshold method was used for the calculation of relative changes of mRNA with LightCycler 480 Multiple Plate Analysis Software (Roche Diagnostics).^{E4} Data were normalized to the expression of GAPDH and confirmed by using quantitative real-time RT-PCR to represent a ubiquitously and consistently expressed gene among all groups analyzed. The results of real-time RT-PCR were expressed as fold changes. The normalized value of the target mRNA of the control group is arbitrarily presented as 1. The sequences of primers used were as follows: GRP78, sense 5'-GAAAGGATGGTTAATGATGCTGAG-3' and antisense 5'-GTCTTCAATGTCCGCATCCTG-3'; CHOP, sense 5'-CATACACCACCAC ACCTGAAAG-3' and antisense 5'-CCGTTTCCTAGTTCCTCCTTGC-3'; and GAPDH, sense 5'-GCCATCAACGACCCCTTCATTGAC-3' and antisense 5'-ACGGAAGGCCATGCCAGTGAGCTT-3'.

Western blot analysis

Lung tissue or PBMCs were homogenized in the presence of protease inhibitor cocktail (Sigma-Aldrich), and protein concentrations were determined by using Bradford reagent (Bio-Rad Laboratories, Hercules, Calif). Samples were loaded onto an SDS-PAGE gel. After electrophoresis at 120 V for 90 minutes, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories) at 250 mA for 90 minutes by using a wet transfer method. Nonspecific sites were blocked with 5% nonfat dry milk in Tris-buffered saline/Tween 20 (25 mmol/L Tris [pH 7.5], 150 mmol/L NaCl, and 0.1% Tween 20) for 1 hour, and the blots were then incubated overnight at 4°C with an anti-IL-4 antibody (Serotec, Oxford, United Kingdom), anti-IL-5 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), anti-IL-10 antibody (R&D Systems, Minneapolis, Minn), anti-IL-13 antibody (R&D Systems), anti-IL-17 antibody (R&D Systems), anti-KC antibody (BioVision, Mountain View, Calif), anti-IFN- γ antibody (Santa Cruz Biotechnology), anti-IL-1 β antibody (Thermo Scientific, Waltham, Mass), anti-TNF- α antibody (Thermo Scientific), anti-I κ B α antibody (Santa Cruz Biotechnology), anti-TLR4 antibody (Santa Cruz Biotechnology), anti-GRP78 antibody (Cell Signaling Technologies, Beverly, Mass), anti-CHOP antibody (Santa Cruz Biotechnology), anti-phospho-eIF2 α (Ser51) antibody (Cell Signaling Technologies), anti-eIF2 α antibody (Cell Signaling Technologies), and anti-actin antibody (Sigma-Aldrich). Anti-rabbit, anti-mouse, or anti-rat horseradish peroxidase-conjugated IgG (Cell Signaling Technologies) was used to detect antibody binding. The binding of the specific antibody was visualized by using Fujifilm LAS-3000 (Fujifilm) after treating with ECL system reagents (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Densitometric analysis was performed on the relative intensity of each band by using the Multi Gauge

program, version 3.0 (Fujifilm). For the quantification of specific bands, a square with same size was drawn around each band to measure the density, and then the value was adjusted by the density of the background near that band. The results of densitometric analysis were expressed as a relative ratio of the target protein to reference protein. The relative ratio of the target protein of the control group is arbitrarily presented as 1.

Histology and immunohistochemistry

At 48 hours after the last OVA challenge, mice were killed for histologic assessment, and the lungs and tracheas of mice were filled with 10% (vol/vol) neutral buffered formalin intratracheally and then removed. Neutral buffered formalin was also used for fixation. Specimens were dehydrated and embedded in paraffin. For histologic examination, 4- μ m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica Microsystems Nussloch GmbH, Wetzlar, Germany), placed on glass slides, deparaffinized, and stained sequentially with hematoxylin and eosin (Richard-Allan Scientific, Kalamazoo, Mich). Stained slides were analyzed with a light microscope (Axio Imager M1, Carl Zeiss, Goettingen, Germany) under identical conditions, including magnification ($\times 20$), gain, camera position, and background illumination.^{E5} The inflammation score was graded by 3 independent blinded investigators. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3, as described elsewhere.^{E6} A value of 0 was considered when no inflammation was detectable, a value of 1 was selected for occasional cuffing with inflammatory cells, a value of 2 was selected when most bronchi or vessels were surrounded by a thin layer (1-5 cells) of inflammatory cells, and a value of 3 was selected when most bronchi or vessels were surrounded by a thick layer (>5 cells) of inflammatory cells.

For immunohistochemistry of GRP78, the deparaffinized 4- μ m sections were incubated sequentially in accordance with instructions for the R. T. U. Vectastain Universal Quick kit (Vector Laboratories, Burlingame, Calif). Briefly, the slides were incubated in Endo/Blocker (Biomed Corp, Foster City, Calif) for 15 minutes and in proteinase K (Dako, Glostrup, Denmark) for 15 minutes at 37°C. The slides were incubated in normal horse serum for 30 minutes at room temperature. The slides were probed with anti-GRP78 antibody (Santa Cruz Biotechnology) for 2 hours at room temperature and then incubated with prediluted, biotinylated panspecific IgG for 30 minutes. The slides were incubated in streptavidin/peroxidase complex reagent for 15 minutes and then in a 3-amino-9-ethylcarbazole substrate kit for 5 minutes. Controls consisted of sections of lung tissue from mice incubated without the primary antibody. After immunostaining, the slides were photomicrographed (Axio Imager M1, Carl Zeiss).

Immunofluorescence staining for GRP78, CHOP, TLR4, and IL-10

Paraffin-embedded lung tissue sections were deparaffinized and hydrated. The sections or smeared BAL cells were fixed with ice-cold methanol and permeabilized in PBS containing 0.25% Triton X-100 for 10 minutes at room temperature and washed 3 times with PBS. Subsequently, after antigen retrieval for 15 minutes at 37°C in proteinase K (Dako), nonspecific bindings were blocked with 1% BSA (Sigma-Aldrich) in PBS containing 0.05% Tween 20 (0.05% PBS-T) for 1 hour. Specimens were then incubated in a humidified chamber for 2 hours at room temperature with an anti-GRP78 antibody (Santa Cruz Biotechnology), anti-CHOP antibody (Santa Cruz Biotechnology), anti-TLR4 antibody (Santa Cruz Biotechnology), and anti-IL-10 antibody (Abcam, Cambridge, United Kingdom). For the detection of each binding antibody to GRP78, CHOP, TLR4, or IL-10, Alexa Fluor 488 (green)-labeled donkey anti-goat IgG and Alexa Fluor 546 (red)-labeled goat anti-rabbit IgG (Invitrogen, Carlsbad, Calif) in 1% BSA were loaded for 1 hour at room temperature in the dark. After the specimens were washed, nuclei were stained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen). Stained tissues were mounted on slides with fluorescent mounting medium (Golden Bridge International, Mukilteo, Wash) and then visualized with a confocal microscope (Zeiss LSM 510 Meta, Carl Zeiss) equipped with a C-Apochromat 63 $\times$ /1.20W Korr UV-VIS-IR M27 water immersion objective. Phase-contrast microscopy of each group was used for morphologic analysis of lung tissue.

PBMC isolation and BAL procedure

Heparinized venous blood was obtained from healthy volunteers ($n = 14$) and asthmatic patients ($n = 20$) in accordance with a protocol approved by the Institutional Review Board for Chonbuk National University Hospital. Fully informed written consent was obtained from each subject. PBMCs were isolated by using dextran sedimentation and Ficoll-Hypaque density-gradient separation, followed by hypotonic lysis of erythrocytes, as previously described.^{E7,E8} Generally, monocytes comprise approximately 10% of the isolated PBMCs. Purified PBMCs were suspended in PBS and kept on ice until they were used. The additional study subjects (3 asthmatic patients and 3 healthy subjects) were pretreated intramuscularly with atropine (0.5 mg), and 4% lidocaine was used as a local anesthetic immediately before the BAL procedure, which was performed in the segmental bronchus of the right middle lobe. Fifty milliliters of warm 0.9% NaCl solution was instilled into the bronchial tree 5 times, followed by gentle suction with a negative pressure of 80 to 100 mm Hg by using a flexible fiberoptic bronchoscope (Olympus BF-260; Olympus Optical, Tokyo, Japan). No complications occurred during the BAL procedures. The total volume of recovered fluids was measured. The aspirated fluid was collected into sterile siliconized containers at 4°C and then filtered through sterile gauze and separated into fluid and cellular components by means of centrifugation at 400g for 10 minutes at 4°C.

MPO assay

MPO was extracted from each homogenized lung tissue sample by suspending the sample in 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical, St Louis, Mo) in 50 mmol/L potassium phosphate buffer (pH 6.0) before sonication in an ice bath for 20 seconds. The samples were freeze-thawed 2 times, after which sonication was repeated. Suspensions were then centrifuged at 13,200 rpm for 15 minutes, and the supernatant was assayed. MPO activity was determined by mixing 1 μ L of supernatant with 299 μ L of the above potassium phosphate buffer containing 0.167 mg/mL o-dianisidine dihydrochloride (Sigma Chemical) and 0.0005% hydrogen peroxide (Sigma Chemical). The change in absorbance at 450 nm was measured. MPO activity was then derived from the observed change in absorbance per minute. The activity was expressed as the observed change in absorbance per minute per milligram of protein.

Isolation and primary culture of murine tracheal epithelial cells

Murine tracheal epithelial cells were isolated under sterile conditions, as described previously.^{E1} The epithelial cells were seeded onto 60-mm collagen-coated dishes for submerged culture. The growth medium, Dulbecco modified Eagle medium (Invitrogen) containing 10% FBS, penicillin, streptomycin, and amphotericin B, was supplemented with insulin, transferrin, hydrocortisone, phosphoethanolamine, cholera toxin, ethanolamine, bovine pituitary extract, and BSA. The cells were maintained in a humidified 5% CO₂ incubator at 37°C until they adhered.

4-PBA treatment on murine tracheal epithelial cells from OVA_{LP}-OVA mice

Cells were seeded in culture dishes and grown until 70% confluence. The medium was then replaced with a new medium containing vehicle (PBS) or 4-PBA (1 or 10 mmol/L, Calbiochem) and incubated overnight (15 hours) at 37°C.^{E9}

Cytosolic or nuclear protein extractions

Lungs were removed and homogenized in 2 volumes of buffer A (50 mmol/L Tris-HCl [pH 7.5], 1 mmol/L EDTA, 10% glycerol, 0.5 mmol/L dithiothreitol, 5 mmol/L MgCl₂, and 1 mmol/L phenylmethylsulfonyl fluoride) containing protease inhibitor cocktails. The homogenates were centrifuged at 1000g for 15 minutes at 4°C. The supernatants collected were incubated on ice for 10 minutes and centrifuged at 100,000g for 1 hour at 4°C to obtain cytosolic proteins for analysis of NF- κ B p65. The pellets

were washed twice in buffer A, resuspended in buffer B (1.3 mol/L sucrose, 1.0 mmol/L $MgCl_2$, and 10 mmol/L potassium phosphate buffer [pH 6.8]) and then pelleted at 1000g for 15 minutes. The pellets were suspended in buffer B with a final sucrose concentration of 2.2 mol/L and centrifuged at 100,000g for 1 hour. The resulting pellets were washed once with a solution containing 0.25 mol/L sucrose, 0.5 mmol/L $MgCl_2$, and 20 mmol/L Tris-HCl (pH 7.2) and centrifuged at 1000g for 10 minutes. The pellets were solubilized with a solution containing 50 mmol/L Tris-HCl (pH 7.2), 0.3 mol/L sucrose, 150 mmol/L NaCl, 2 mmol/L EDTA, 20% glycerol, 2% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktails. The mixture was kept on ice for 1 hour with gentle stirring and centrifuged at 12,000g for 30 minutes. The resulting supernatant was used as soluble nuclear proteins for analysis of NF- κ B p65, ATF6, and XBP-1. Protein levels were analyzed by means of Western blotting with anti-NF- κ B p65 antibody (Upstate Biotech, Lake Placid, NY), anti-ATF6 α antibody (Santa Cruz Biotechnology), anti-XBP-1 antibody (Santa Cruz Biotechnology), and anti-lamin B antibody (Santa Cruz Biotechnology), as described above.

Determination of airway responsiveness to methacholine

Both noninvasive and invasive measurements of airway responsiveness were used in this study. For noninvasive measurement, airway responsiveness was assessed in unrestrained and conscious mice at 48 hours after the last challenge with OVA, as described previously.^{E10,E11} Mice were placed in a barometric plethysmographic chamber (All Medicus, Seoul, Korea), and baseline readings were taken and averaged for 3 minutes. Aerosolized methacholine in increasing concentrations (2.5–50 mg/mL) was nebulized through an inlet of the main chamber for 3 minutes. Readings were taken and averaged for 3 minutes after each nebulization. Penh, which was calculated as

$$\frac{(\text{Expiratory time/Relaxation time} - 1) \times (\text{Peak expiratory flow/Peak inspiratory flow})}{\text{Peak expiratory flow/Peak inspiratory flow}}$$

according to the manufacturers' protocol, is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and a function of the timing of expiration. Penh was used as a measure of airway resistance to methacholine. Results were expressed as the percentage increase in Penh after challenge with each concentration of methacholine, where the baseline Penh value (after saline challenge) was expressed as 100%. Penh values averaged for 3 minutes after each nebulization were evaluated.

Invasive measurement of airway responsiveness was performed, as described elsewhere.^{E12} Anesthesia was achieved through intraperitoneal injection of 45 mg/kg body weight sodium pentobarbital. The trachea was then exposed through a midcervical incision and tracheostomized, and an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small-animal ventilator (flexiVent; SCIREQ, Montreal, Quebec, Canada). The mouse was quasisinusoidally ventilated with a nominal tidal volume of 10 mL/kg body weight at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H_2O to achieve a mean lung volume close to that occurring during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to water column. Methacholine aerosol was generated with an inline nebulizer and administered directly through the ventilator. Each mouse was challenged with methacholine aerosol in increasing concentrations to determine the differences in airway response to methacholine (5.0–50 mg/mL in saline). After each methacholine challenge, the data of calculated R_{rs} values were continuously collected. Maximum R_{rs} values were selected to express changes in airway function, which were represented as percentage changes from baseline after saline aerosol.

Measurements of cytokine levels in BAL fluid

IL-4, IL-5, IL-13, IL-17, IL-1 β , IL-10, TNF- α , and IFN- γ levels in BAL fluid were quantified in supernatants of BAL fluid by using an enzyme

immunoassay, according to the manufacturer's protocol (IL-4, IL-5, IL-13, IL-1 β , IFN- γ [R&D Systems], IL-10, and IL-17 [eBioscience, San Diego, Calif]). Sensitivities for the IL-4, IL-5, IL-13, IL-17, IL-1 β , IL-10, TNF- α , and IFN- γ assays were 2.0, 7.0, 1.5, 1.6, 3.0, 5, 5.1, and 2 pg/mL, respectively.

Measurement of DC numbers in lungs

Lung DCs were measured, as described previously, with some modifications.^{E13} Lung tissue was homogenized in RPMI culture medium containing 0.1% collagenase (Type IV; Sigma-Aldrich), 0.01% hyaluronidase (Sigma-Aldrich), and 0.002% DNase (Sigma-Aldrich) and passed through a 40- μ m cell strainer. Red blood cells were lysed with 0.165 mol/L ammonium chloride (NH_4Cl) in PBS for 10 minutes at room temperature. Single-cell suspensions of lung tissue were washed with PBS supplemented with 1% FBS. Lung cells were incubated for 30 minutes in PBS supplemented with 1% BSA to block nonspecific interactions. Lung cells were stained with fluorescence isothiocyanate-conjugated anti-CD11c (HL3), allophycocyanin-conjugated anti-CD11b (M1/70), and phycoerythrin-conjugated anti-MHC class II (2G9) for 30 minutes on ice. The antibodies were purchased from BD PharMingen (San Diego, Calif). Lung cells were washed 2 times with PBS supplemented with 1% FBS and analyzed with a FACSCalibur apparatus (BD Biosciences). After classic quadrant gating, we used the additional gating strategy for more specific identification of DCs to restrict high-level MHC class II expression ($>10^2$ on the log FL-2 fluorescence scale) in the CD11b⁺ and CD11c⁺ cell population. Data were analyzed with CellQuest Pro software (BD Biosciences) and presented as a representative dot plot graph, indicating the number of DCs in a total of 10,000 cells per group.

Statistics

We used SPSS statistical software (version 16.0; SPSS, Chicago, Ill). Data are expressed as means $\pm$ SEMs. Statistical comparisons were performed by using 1-way ANOVA, followed by the Scheffe test. Statistical differences between 2 groups were determined by using the unpaired Student *t* test. A *P* value of less than .05 was considered statistically significant.

RESULTS

Clinical characteristics of human subjects

For collection of PBMCs, 20 asthmatic patients and 14 healthy volunteers were recruited from the Chonbuk National University Hospital. The asthmatic patients had received diagnoses of asthma and had asthma treatment for more than 3 months with an inhaled corticosteroid or a combined inhaled corticosteroid and β_2 -agonist; asthma was diagnosed according to the criteria of the American Thoracic Society.^{E14} Mean ages of patients and healthy control subjects were 52.2 ± 4.4 and 44.6 ± 3.3 years, respectively (means $\pm$ SEMs, *P* = .254). Sexual distribution did not differ significantly between the 2 groups (male vs female: patients, 12 vs 8; control subjects, 5 vs 9; *P* = .066). Eight (40%) of 20 patients were classified as having "uncontrolled" disease based on Asthma Control Test scoring, and mean FEV₁ was $96.9\% \pm 4.6\%$ of predicted value (mean $\pm$ SEM). These characteristics are summarized in Table E1.

Additionally, BAL fluid from 3 asthmatic patients and 3 healthy subjects was analyzed for ER stress markers by using Western blotting. These subjects were all male, with the following phenotypes: mean age of 54.0 ± 2.5 years (healthy subjects) and 62.0 ± 1.5 years (asthmatic patients; mean $\pm$ SEM, *P* = .066); uncontrolled asthma in 1 of 3 patients; and mean FEV₁ in asthmatic patients of $76.0\% \pm 16.2\%$ of predicted value (mean $\pm$ SEM).

Dose-response effects of 4-PBA on protein levels of ER stress marker and various inflammatory cytokines in lung tissue and on cellular changes of BAL fluid from OVA_{LPS}-OVA mice

Effects of the drug on expression of GRP78 and CHOP protein, changes in BAL cell numbers, and protein levels of various inflammatory cytokines were evaluated by using OVA_{LPS}-OVA mice to verify the dose-response effects of 4-PBA on neutrophilic asthma. The enhanced ER stress, increased airway inflammatory cell numbers, and increased inflammatory cytokine levels after OVA challenge were reduced in a dose-dependent manner by 4-PBA (Fig E5).

Induction of ER stress through tunicamycin administration is associated with asthmatic airway inflammation in OVA_{LPS}-OVA mice

Histologic assessment showed that numerous inflammatory cells infiltrated into the bronchioles in the lungs of OVA_{LPS}-OVA mice treated with drug vehicle or tunicamycin (Fig E6, B and C) compared with the numbers seen in SAL-SAL mice treated with drug vehicle (Fig E6, A). Interestingly, OVA_{LPS}-OVA mice treated with tunicamycin appeared to show somewhat more severe pathologic features than OVA_{LPS}-OVA mice treated with drug vehicle only. Consistent with these pathologic findings, in OVA_{LPS}-OVA mice treated with tunicamycin, the percentage of Penh exhibited an inclination toward increase compared with that of OVA_{LPS}-OVA mice treated with drug vehicle only (Fig E6, H). These findings support our contention that ER stress is associated with the pathogenesis of bronchial asthma.

REFERENCES

- E1. Kwak YG, Song CH, Yi HK, Hwang PH, Kim JS, Lee KS, et al. Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma. *J Clin Invest* 2003;111:1083-92.
- E2. Marahatta A, Bhandary B, Lee MR, Kim DS, Lee YC, Kim SR, et al. Determination of phenylbutyric acid and its metabolite phenylacetic acid in different tissues of mouse by liquid chromatography with tandem mass spectrometry and its application in drug tissue distribution. *J Chromatogr B Analyt Technol Biomed Life Sci* 2012;903:118-25.
- E3. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-9.
- E4. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25:402-8.
- E5. Cho JY, Miller M, Baek KJ, Han JW, Nayar J, Lee SY, et al. Inhibition of airway remodeling in IL-5-deficient mice. *J Clin Invest* 2004;113:551-60.
- E6. Tournay KG, Kips JC, Schou C, Pauwels RA. Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness. *Clin Exp Allergy* 2000;30:79-85.
- E7. Blomkalns AL, Stoll LL, Shaheen W, Romig-Martin SA, Dickson EW, Weintraub NL, et al. Low level bacterial endotoxin activates two distinct signaling pathways in human peripheral blood mononuclear cells. *J Inflamm (Lond)* 2011;8:4.
- E8. Böyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand J Clin Lab Invest Suppl* 1968;97:77-89.
- E9. Wiley JC, Meabon JS, Frankowski H, Smith EA, Schecterson LC, Bothwell M, et al. Phenyl butyric acid rescues endoplasmic reticulum stress-induced suppression of APP proteolysis and prevents apoptosis in neuronal cells. *PLoS One* 2010;5:e9135.
- E10. Zerfaoui M, Naura AS, Errami Y, Hans CP, Rezk BM, Park J, et al. Effects of PARP-1 deficiency on airway inflammatory cell recruitment in response to LPS or TNF: differential effects on CXCR2 ligands and duffy antigen receptor for chemokines. *J Leukoc Biol* 2009;86:1385-92.
- E11. Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, et al. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997;156:766-75.
- E12. Takeda K, Hamelmann E, Joetham A, Shultz LD, Larsen GL, Irvin CG, et al. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J Exp Med* 1997;186:449-54.
- E13. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 2009;15:410-6.
- E14. American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. *Am Rev Respir Dis* 1987;136:225-44.

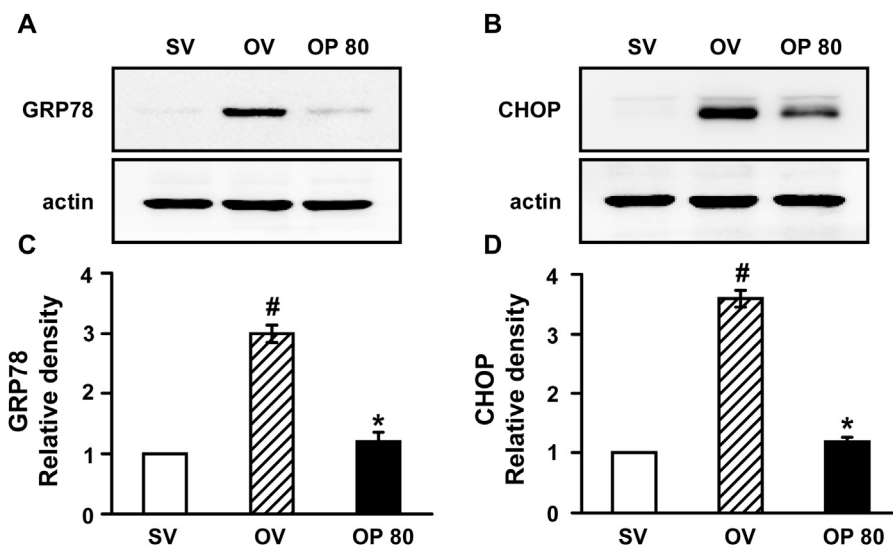


FIG E1. Effects of 4-PBA administered after completion of OVA challenge on GRP78 and CHOP protein levels in lung tissue from OVA-OVA mice. Sampling was performed at 48 hours after the last challenge in SAL-SAL mice administered drug vehicle (*SV mice*), OVA-OVA mice administered drug vehicle (*OV mice*), and OVA-OVA mice administered 80 mg/kg 4-PBA (*OP 80*). **A** and **B**, Representative Western blots of GRP78 (Fig E1, **A**) and CHOP (Fig E1, **B**) in lung tissue of OVA-OVA mice. **C** and **D**, Densitometric analysis data are presented as the relative ratio of GRP78 (Fig E1, **C**) or CHOP (Fig E1, **D**) to actin. The relative ratio of GRP78 and CHOP in the lung tissue of SV mice are arbitrarily presented as 1. Bars represent means $\pm$ SEMs from 7 mice per group. [#] $P < .05$ versus SV mice and ^{*} $P < .05$ versus OV mice.

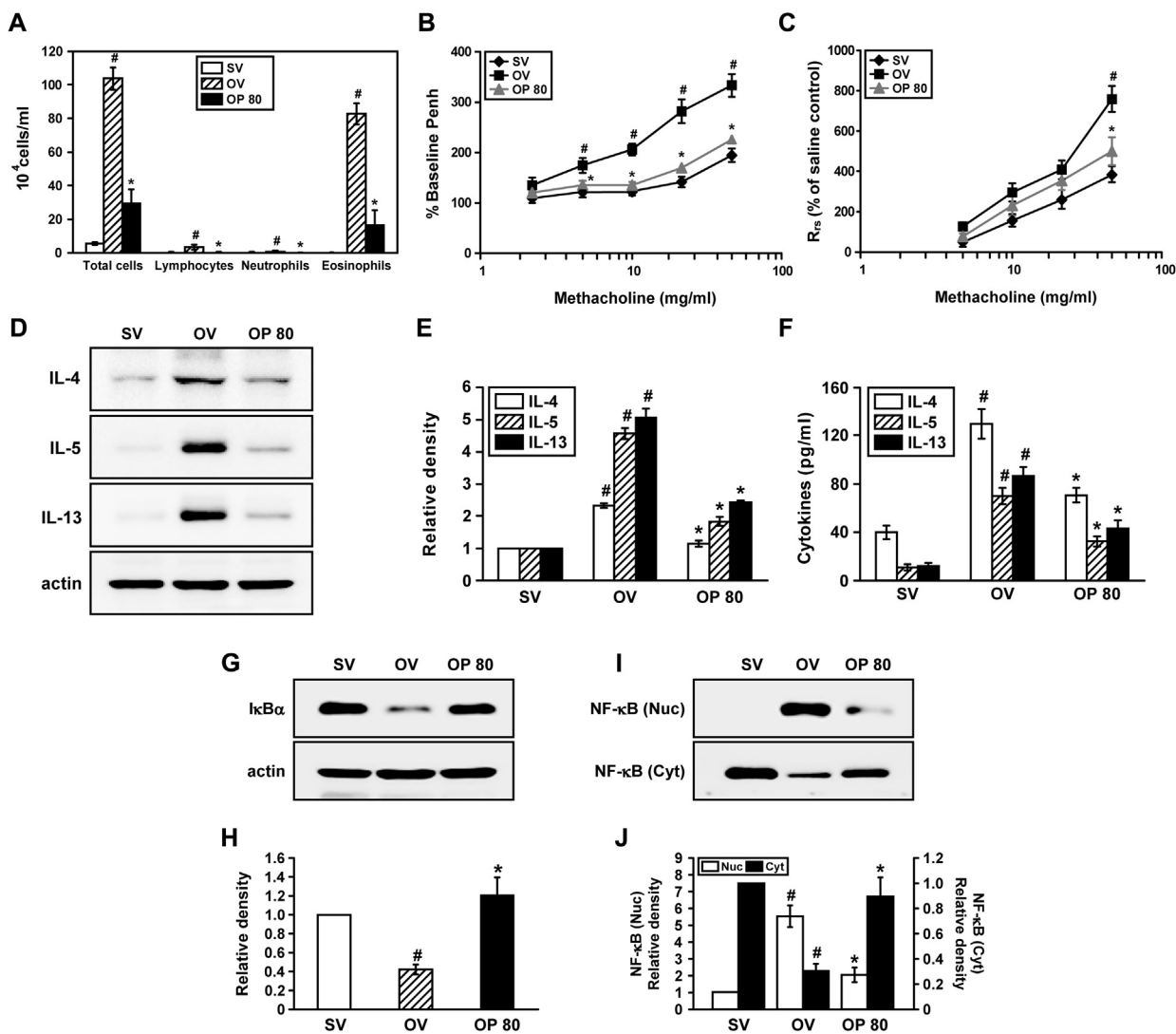


FIG E2. Therapeutic effects of 4-PBA on cellular changes in BAL fluid, airway responsiveness, T_H2 cytokine levels, and activation of NF- κ B/I κ B α in lungs of OVA-OVA mice. All parameters were measured at 48 hours after completion of OVA challenge in SAL-SAL mice administered drug vehicle (SV mice), OV, and OVA-OVA mice administered 80 mg/kg of 4-PBA (OP 80). **A**, Total and differential cellular components from BAL fluid of OVA-OVA mice. **B** and **C**, Airway responsiveness was assessed by using both noninvasive (percentage Penh; Fig E2, B) and invasive (R_{rs} ; Fig E2, C) measurements. **D**, Representative Western blots of IL-4, IL-5, and IL-13 in lung tissue. **E**, Densitometric analysis data are presented as the relative ratio of each molecule to actin. The relative ratio of each molecule in the lung tissue of SV mice are arbitrarily presented as 1. **F**, Enzyme immunoassay of IL-4, IL-5, and IL-13 in BAL fluid. Bars represent means $\pm$ SEMs from 7 mice per group. **G** and **I**, Representative Western blots of I κ B α (Fig E2, G) and NF- κ B (Fig E2, I) in lung tissue. **H**, Densitometric analysis data are presented as the relative ratio of I κ B α to actin. **J**, For NF- κ B p65, densitometric analysis data are presented as the relative ratio of NF- κ B p65 levels in OV or OP 80 to the level in SV. The relative ratio of each protein in the lung tissue of SV mice is arbitrarily presented as 1. Bars represent means $\pm$ SEMs from 6 mice per group. # P < .05 versus SV mice and * P < .05 versus OV mice.

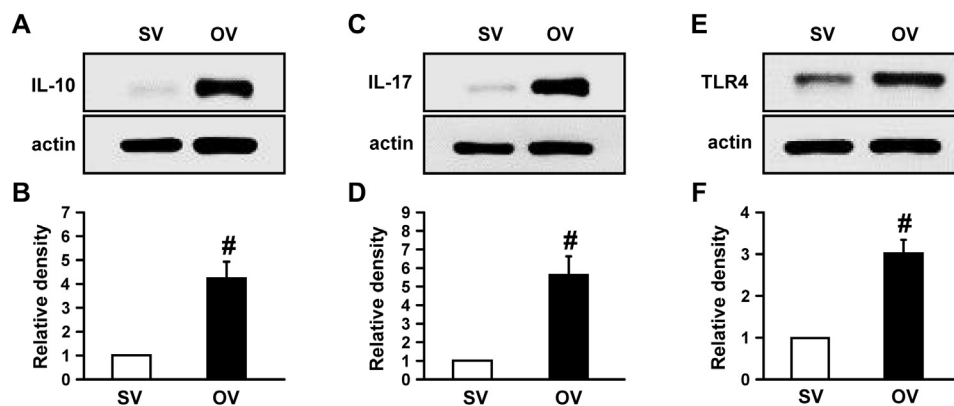


FIG E3. Protein levels of IL-10, IL-17, and TLR4 in lung tissue of OVA-OVA mice. **A**, **C**, and **E**, Representative Western blots of IL-10 (Fig E3, **A**), IL-17 (Fig E3, **C**), and TLR4 (Fig E3, **E**) in lung tissue. **B**, **D**, and **F**, Densitometric analysis data are presented as the relative ratio of IL-10 (Fig E3, **B**), IL-17 (Fig E3, **D**), and TLR4 (Fig E3, **F**) to actin. The relative ratio of each protein in the lung tissue of SV mice is arbitrarily presented as 1. Bars represent means $\pm$ SEMs from 5 mice per group. # $P < .05$ versus SV mice.

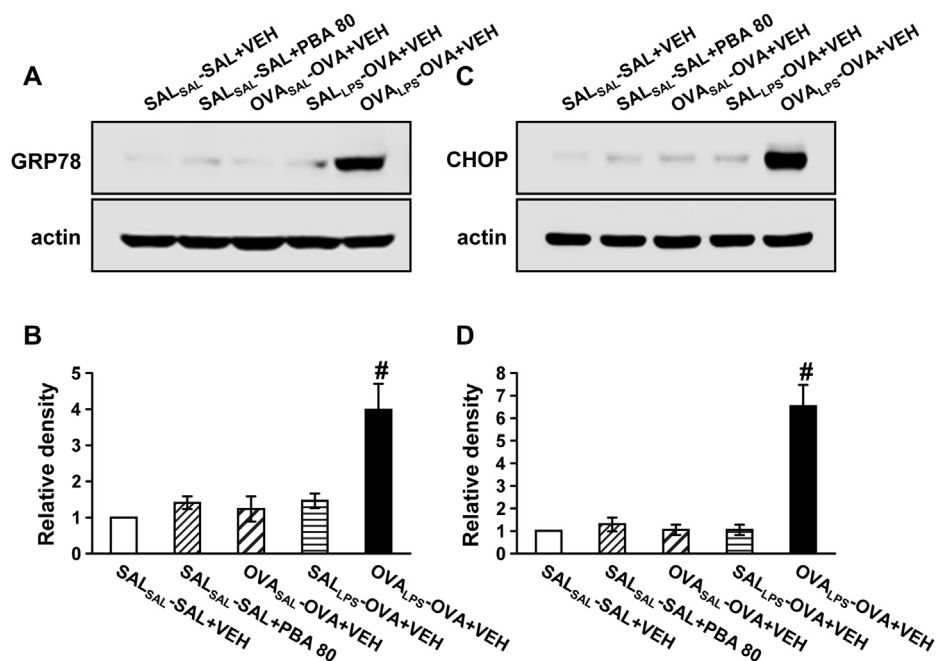


FIG E4. **A** and **C**, Representative Western blots of GRP78 (Fig E4, A) and CHOP (Fig E4, C) in lung tissue of SAL-SAL mice treated with drug vehicle, SAL-SAL mice treated with 4-PBA, intranasal OVA-sensitized and OVA-challenged mice treated with drug vehicle, intranasal LPS-sensitized and OVA-challenged mice treated with drug vehicle, and OVA_{LPS}-OVA mice treated with drug vehicle. **B** and **D**, Densitometric analysis data are presented as the relative ratio of GRP78 (Fig E4, B) or CHOP (Fig E4, D) to actin. The relative ratio of GRP78 and CHOP in the lung tissue of SAL-SAL mice administered drug vehicle (SV) are arbitrarily presented as 1. Bars represent means $\pm$ SEMs from 3 mice per group. # $P < .05$ versus SV mice.

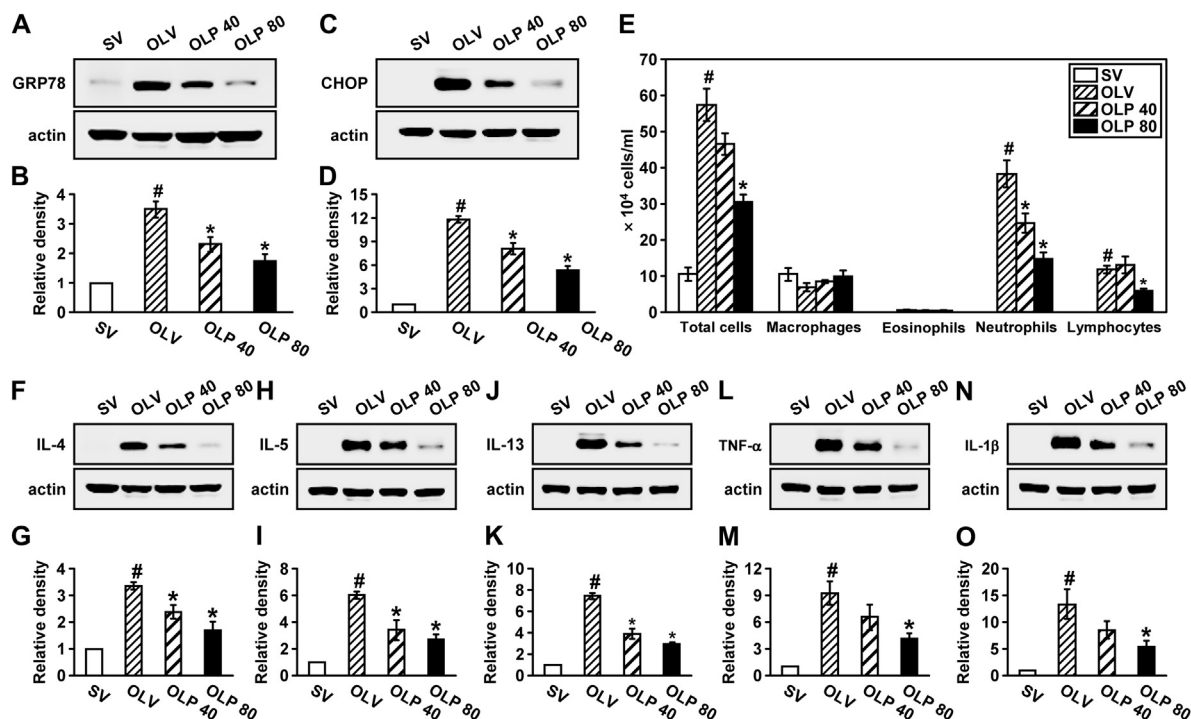


FIG E5. Dose-response effects of 4-PBA on ER stress and asthmatic features of OVA_{LPS}-OVA mice. All parameters were measured at 48 hours after OVA challenge in SAL-SAL mice administered drug vehicle (SV), OVA_{LPS}-OVA mice administered vehicle (OLV), OVA_{LPS}-OVA mice administered 40 mg/kg 4-PBA (OLP 40), and OVA_{LPS}-OVA mice administered 80 mg/kg 4-PBA (OLP 80). **A** and **C**, Representative Western blots of GRP78 (Fig E5, A) and CHOP (Fig E5, C) in lung tissue of OVA_{LPS}-OVA mice. **B** and **D**, Densitometric analysis data are presented as the relative ratio of GRP78 (Fig E5, B) or CHOP (Fig E5, D) to actin. The relative ratio of GRP78 and CHOP in the lung tissue of SV mice are arbitrarily presented as 1. **E**, Total and differential cellular components from BAL fluid of OVA_{LPS}-OVA mice. **F**, **H**, **J**, **L**, and **N**, Representative Western blots of IL-4 (Fig E5, F), IL-5 (Fig E5, H), IL-13 (Fig E5, J), TNF-α (Fig E5, L), and IL-1β (Fig E5, N) in lung tissue. **G**, **I**, **K**, **M**, and **O**, Densitometric analysis data are presented as the relative ratio of each molecule to actin. The relative ratio of each molecule in the lung tissue of SV mice are arbitrarily presented as 1. Bars represent means ± SEMs from 6 mice per group. #*P* < .05 versus SV and **P* < .05 versus OLV.

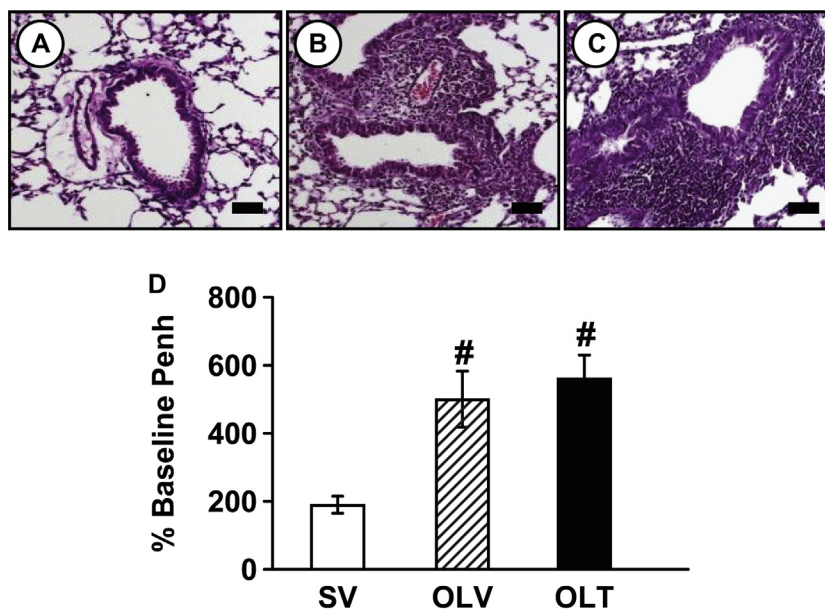


FIG E6. Effects of tunicamycin on pathologic changes and airway hyperresponsiveness in OVA_{LPS}-OVA mice. Sampling was performed at 48 hours after the last challenge in SAL-SAL mice administered drug vehicle (SV), OVA_{LPS}-OVA mice administered drug vehicle (OLV), and OVA_{LPS}-OVA mice treated with tunicamycin (OLT). **A-C**, Representative hematoxylin and eosin-stained sections of the lungs isolated from SV (Fig E6, A), OLV (Fig E6, B), and OLT (Fig E6, C) mice. Bars indicate 50 μ m. **D**, Airway responsiveness at 50 mg/mL methacholine in OVA_{LPS}-OVA mice was assessed by means of noninvasive (percentage Penh) measurement. Bars represent means $\pm$ SEMs from 6 mice per group. # $P < .05$ versus SV mice and $P < .05$ versus OLV mice.

TABLE E1. Basic characteristics of subjects

	Asthmatic patients (n = 20)	Healthy control subjects (n = 14)
Sex*		
Female (persons)	12	5
Male (persons)	8	9
Age (y [mean ± SEM])†	52.2 ± 4.4	44.6 ± 3.3
Asthma control status‡		
Controlled	12	
Uncontrolled	8	
FEV ₁ (% [mean ± SEM])	96.9 ± 4.6	
Atopy§ (persons)	9 (45%)	

**P* = .066.

†*P* = .254.

‡Asthma control status was assessed by using the Asthma Control Test scoring system. Uncontrolled asthma was defined as a score of less than 19.

§Atopic status was confirmed based on allergic skin prick test responses.