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Non-Proteolytic House Dust Mite Allergen, Der p 2, Upregulated Expression of Tight Junction Molecule Claudin-2 Associated With Akt/GSK-3β/β-Catenin Signaling Pathway

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ABSTRACT

Non-proteolytic group 2 allergen, Der p 2 (DP2) is known as a major allergen derived from house dust mite *Dermatophagoides pteronyssinus*. Paracellular epithelial barrier, being composed of a number of tight junction (TJ) molecules, plays pivotal roles in resistance of pathogen invading. However, whether DP2 affects epithelial TJ molecules is unclear. Therefore, we aimed to investigate the effects of DP2 on epithelial TJ molecules, and the mechanism by which expression of junction molecules is regulated by DP2. Cell cycle and mRNA expression of TJ proteins of lung alveolar cell A549 were analyzed by RT-PCR and flow cytometry. Level of claudin-2, subcellular distribution of β-catenin and kinase activation was determined using immunoblot. Our findings revealed that DP2 had no significant influence on cell cycle distribution but affected mRNA expression of TJ molecules including claudin-2, occludin, and ZO-1 in A549 cells. Our results showed that DP2 significantly elevated level of claudin-2 and increased expression and nuclear translocation of β-catenin. Moreover, DP2 enhanced the phosphorylation of glycogen synthase kinase-3β (GSK-3β) and its potential upstream regulator Akt. The DP2-induced claudin-2 expression was also suppressed by GSK-3β inhibitor (lithium chloride) and phosphatidyl inositol 3-phosphate kinase (PI3K) inhibitor (wortamannin). Taken together, these findings showed that DP2 increased claudin-2 expression and its cell surface distribution in A549 cells, which may attribute to phosphorylation of GSK-3β and Akt and the consequent increase and nuclear translocation of β-catenin. It is suggested that presence of DP2 may alter epithelial junction by regulating expression of TJ molecules. J. Cell. Biochem. 112: 1544–1551, 2011.

KEY WORDS: DER P 2; CLAUDIN-2; AKT; GSK-3β; β-CATENIN

House dust mite (HDM) is considered a contributing factor to asthma. The mites commonly found in house dust belong to the genus *Dermatophagoides* of which there are two species, *D. pteronyssinus* and *D. farinae* [Bousquet et al., 2001]. Mite-derived allergens eliciting IgE immune response share structural similarities resulting in a cross-reactivity between *Dermatophagoides* spp. [Horn and Lind, 1987; Lind et al., 1988; Thomas et al., 2002]. Of mite-sensitive individuals, approximately 90% generates IgE antibody responses to well-identified HDM allergens that are categorized into 24-kDa group 1 such as Der p 1 and Der f 1 and the 14-kDa group 2 allergens like Der p 2 (DP2) and Der f 2 on basis of IgE affinity [Platts-Mills et al., 1997; Thomas and Smith, 1998]. Der p 1 exerts proteolytic activity that is proposed to be associated with allergenicity [Robinson et al., 1997]. In contrast, DP2 lacks proteolytic activity and its biological functions remains to be elucidated.

Although lacking the enzymatic activity, the immunogenic properties of HDM group 2 allergens have been widely investigated

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for the past few years. Recent studies have demonstrated that HDM group 2 allergens share not only structural homology but also functional similarity in aspect of innate immunity with MD-2, the lipopolysaccharide (LPS)-binding component of the Toll-like receptor (TLR) 4 signaling complex [Hammad et al., 2009; Ichikawa et al., 2009; Trompette et al., 2009]. DP2 recently has been considered a factor to aggravating respiratory airway disorder. It is found that interaction of DP2 with respiratory cells resulted in upregulated secretion of inflammatory cytokines and expression of intercellular adhesion molecule-1 [Osterlund et al., 2009].

Tight junction (TJ) between epithelial cells serves as a paracellular permeability barrier regulating passage of ions and small molecules [Aijaz et al., 2006]. Of respiratory epithelia, it is in a position to resist allergen penetration, whereas it is vulnerable to molecules with proteolytic activity. A TJ is composed of a number of transmembrane proteins including occludin [Furuse et al., 1993], junctional adhesion molecule [Martin-Padura et al., 1998], tricellulin [Ikenouchi et al., 2005], and the claudin family [Furuse et al., 1998]. Claudins locate on the both apicolateral and basolateral portion of epithelial cells, and their differential expressions play important roles in regulation of paracellular permeability. On basis of biological function, claudins have been divided into barrier- and channel-forming claudins [Van Itallie and Anderson, 2006]. Claudin-1 is crucial for the tightening of the epithelial barrier in native skin epithelium [Furuse et al., 2002]. In contrast, upregulation of claudin-2 markedly attenuates the tightness of the epithelial barrier [Furuse et al., 2001] and induces the paracellular cation channels [Amasheh et al., 2002]. These observations imply that difference in permeability of epithelia at various regions is attributed to the protein composition of TJ.

Appreciating the etiology of respiratory allergy such as asthma, interaction of allergen with frontline of physical barrier such as respiratory epithelia remains to be elucidated. The present study was aimed to investigate airway epithelial cells responding to DP2 with emphases on regulation of tight-junction molecules. Appreciating the role of claudin-2 in epithelia formation, mechanisms underlying regulation of claudin-2 expression through β-catenin and phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathways is of interest. The alveolar epithelial cell line A549 was used to investigate the effects of DP2 on cell cycle distribution, mRNA expression of junction and adaptor molecules, levels of claudin-2, translocation of β-catenin, phosphorylation of glycogen synthase kinase-3β (GSK-3β), and activation of Akt.

MATERIALS AND METHODS

EXPRESSION AND PURIFICATION OF RECOMBINANT DP2

Recombinant DP2 was produced as a recombinant polypeptide with a *N*-terminal glutathione *S*-transferase (GST) tag. The *Escherichia coli* BL-21 (Novagen, Madison, WI) strain containing pGES-GST-DP2 plasmid and pGES-GST (a gift from Dr. Jiunn-Liang Ko) was used for expression and purification of DP2 protein and GST control protein, respectively. For checking the expression of GST-DP2 protein and GST protein, *E. coli* was grown at 37°C and protein expression was induced with 0.1 mM isopropyl β-d-thiogalactoside.

The GST-DP2 fusion protein and the GST control protein were purified by affinity chromatography using glutathione Sepharose 4B column and by gel filtration using Superdex 75 column (Amersham-Pharmacia Biotech AB, Upsala, Sweden). The purified protein concentration was determined by BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

CELL CULTURE AND TREATMENTS

A549 cells, the human type II alveolar epithelial cell line, purchased from the American Type Culture Collection, was cultured in 10-cm sterile Petri dish in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA) and 100 U/ml penicillin–streptomycin at 37°C in a humidified atmosphere with 5% CO_2. The passage and the harvest of A549 cells were performed by using non-proteolytic CDS reagent (Sigma–Aldrich, St. Louis, MO) to detach the cells from the culture plates according to the manufacturer's instructions. Prior to allergen treatments, A549 cells were incubated with DMEM containing 1% serum for 16 h to allow cell attachment.

For DP2 treatment, the attached cells were incubated with purified GST-DP2 fusion protein at a serial concentrations for indicated times. Cells treated with purified GST protein were used as control. Inhibition of GSK-3β was performed by treating A549 cells with lithium chloride (LiCl; Sigma–Aldrich) at 5, 10, or 20 mM in DMEM for 24 h. Inhibition of ERK1/2 or Akt was performed by pretreating A549 cells with PD98059 at 20 μM or wortmannin at 100 or 200 nM for 1 h, and then treating with purified GST-DP2 fusion protein or purified GST protein as control for 24 h.

FLOW CYTOMETRY ANALYSIS

Cells synchronized at G0 phase by serum starvation for 24 h were incubated in fresh serum-containing medium to allow cell cycle progression. At various time points after release from G0 arrest, cells were analyzed by flow cytometry to determine cell cycle distribution. At the end of treatment, cells were collected, fixed with 1 ml of ice-cold 70% ethanol, incubated at −20°C for at least 24 h, and centrifuged at 380*g* for 5 min at room temperature. Cell pellets were treated with 1 ml of cold staining solution containing 20 μg/ml propidium iodide, 20 μg/ml RNase A, and 1% Triton X-100 and incubated for 15 min in dark at room temperature. Subsequently, the samples were analyzed in a FACS Calibur system (version 2.0; BD Biosciences, Franklin Lakes, NJ) using CellQuest software. Results were representative of at least three independent experiments.

RNA EXTRACTION AND RT-PCR

Total RNA was isolated from A549 cells using RNeasy min kit and treated with DNase I according to the manufacturer's instructions. First strand cDNA was synthesized from 1 μg total RNA by reverse transcription in a 20 μl reaction using SuperScript III RTS first-strand cDNA synthesis kit following the manufacturer's directions. All PCR assays were performed in a 50 μl reaction mixture containing 2.5 μl cDNA and 200 nM of each primer using PCR master mix. The temperature cycle profile for the PCR reactions was 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for

30 s followed by 72°C for 7 min. Primers used for RT-PCR were designed by using Primer Premier version 5.0 (Premier Biosoft, Palo Alto, CA) and listed in Table I. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as internal control. Agarose gel electrophoresis (2%) and direct DNA sequencing methods confirmed specificity of the PCR products.

SDS–PAGE AND IMMUNOBLOT

Cells were collected and lysed by Triton lysis buffer [10 mM Tris–HCl, pH 7.5; containing 1% v/v Triton X-100, 150 mM NaCl, 0.5 mM EDTA, 1 mM phenylmethanesulfonylfluoride (PMSF), 1 mM NaF, 1 mM $Na_4P_2O_7$, 10 μg/ml aprotinin and leupeptin] (Sigma–Aldrich). After centrifuging to remove insoluble pellet, the supernatants were collected for SDS–PAGE analysis. Protein concentration of supernatant was quantitated by BCA protein assay kit (Pierce Biotechnology). The crude proteins (30 μg/lane) were separated in 12.5% SDS–PAGE, and then transferred onto nitrocellulose membrane (Millipore, Bedford, MA). After blocking with 3% w/v skimmed milk, the membranes were incubated with antibodies against human claudin 2 (Abcam, Cambridge, UK), phosphorylated Akt (pAkt; Cell signaling, Beverly, MA), phosphorylated GSK-3β(pGSK-3β; Millipore), histone H1 (Millipore), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam), respectively. After washing with PBS containing 0.1% v/v Tween-20, the reacted membranes were incubated with anti-IgG antibodies conjugated with peroxidase (Abcam). The detection of antigen–antibody complex was performed by using ECL reagent (Millipore) and luminescence image system (LAS-4000; Fujifilm, Tokyo, Japan).

FLUORESCENCE-ACTIVATED CELL SORTER ANALYSIS

Cells were dispensed at 1×10^6 cells/ml in conical bottom 96-well plastic plates (Costar, Cambridge, MA) and were further centrifuged at $100 \times g$ at 4°C for 10 min. Cells were washed with Hanks' balanced salt solution, 0.5% bovine serum albumin supplemented with 1 mM Ca^{2+} and Mg^{2+} before adding a saturating concentration of anti-cluadin-2 antibody (5 μg/ml) or with non-immune IgG as control isotypes for 30 min at 4°C. Cells were further washed and incubated for 30 min at 4°C with the corresponding secondary FITC-conjugated antibody (10 μg/ml). Finally plates were centrifuged as above, cells were resuspended in the same buffer, and fluorescence analysis was performed using a FACScan flow cytometer (BD Biosciences).

TABLE I. Primer Sequences Used in RT-PCR.

Gene name (acc. no.)	Primer sequence
Claudin-1 (NM_021101)	(F) CAG GCC ATG TAC GAG GGG (R) GGA GGA TGC CAA CCA CCA
Claudin-2 (NM_020384)	(F) CCC CTT GTA CTT CGC TCC C (R) CTC TGC CAG GCT GAC TTC TC
Claudin-3 (NM_001306)	(F) GCT GCT CTG CTG CTC GTG (R) TCC CTG CGT CTG TCC CTT A
Claudin-4 (NM_001305)	(F) TGT GCC TTG CTC ACC GAA (R) CCA CCA CTG CCC AAA CCT
Occludin (NM_002538)	(F) AGC TAA AGG GCA TTG CTC ATC (R) ATG TCA TTG CTT GGT GCA TAA T
ZO1 (NM_003257)	(F) TGC TGA GTC CTT TGG TGA TGT (R) CAC AGT TTG CTC CAA CGA GAT
GAPDH (NM_002046)	(F) ATG CCT CCT GCA CCA CCA (R) CCA TCA CGC CAC AGT TTC C

SUBCELLULAR FRACTIONATION

Adherent A549 cells were washed with PBS and incubated with lysis buffer (10 mM HEPES, pH 7.6; containing 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.05% v/v Igepal CA-630 and 1 mM PMSF, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) for 10 min. Cell lysates were collected by a centrifugation at 2,500 g for 10 min at 4°C. The supernatant containing the cytosol was further centrifuged at 20,000*g* for 15 min at 4°C, namely cytosolic fraction. The pellets containing nuclei were washed with PBS, resuspended in nuclear buffer (25 mM HEPES, pH 7.6, 0.1% v/v Igepal CA-630, 1 M KCl, 0.1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 2 mM sodium fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), and centrifuged at 10,000*g* for 15 min at 4°C. The resulting supernatants were collected, namely nuclear fraction.

STATISTICAL ANALYSIS

Data were expressed as mean ± SEM of the three independent experiments. Statistical significance analysis was determined by using one-way ANOVA followed by Dunnett for multiple comparisons with the control or the impaired two-tailed Student's *t*-test. The differences were considered significant for $P < 0.05$.

RESULTS

DP2 SLIGHTLY AFFECTED CELL CYCLE DISTRIBUTION OF A549

To investigate whether DP2 treatments affect cell cycle distribution of epithelial cells, lung alveolar cell A549 was treated with control protein or DP2 for 24 h, and the percentage of sub-G1, G1, S, and G2/M phase was determined by flow cytometry. As shown in Figure 1, DP2 treatment up to at 30 μg/ml slightly affected the four cell cycle phases as compared to control protein treatment (Control) and medium only. Of important, sub-G1 phase, an index for cell apoptosis, was not significantly altered by DP2 treatments as compared to the control.

DP2 ALTERED mRNA EXPRESSION OF CLAUDIN-2 THROUGH TRANSCRIPTION REGULATION

As cell cycle distribution in A549 in response to DP2 was not significantly affected, it was hence postulated that expression of TJ molecules might be altered by DP2. mRNA expression of six TJ molecules, including claudin-1, 2, -3, and -4 and occludin and ZO-1 was analyzed by RT-PCR. As shown in Figure 2A, among the mRNA of six tested TJ molecules, mRNA level of claudin-2 was increased to 223% of control and ZO-1 was decreased to 72% of control by DP2 treatment. The other TJ mRNA levels were altered within 5% of control.

To further investigate whether DP2 elevated protein level of claudin-2 via upregulation of mRNA, claudin-2 level in A549 cells treated with DP2 (20 μg/ml) or pretreated with actinomycin D (Act D) and then with DP2 (20 μg/ml) was demonstrated by immunoblot. As shown in Figure 2B, DP2 alone significantly elevated level of

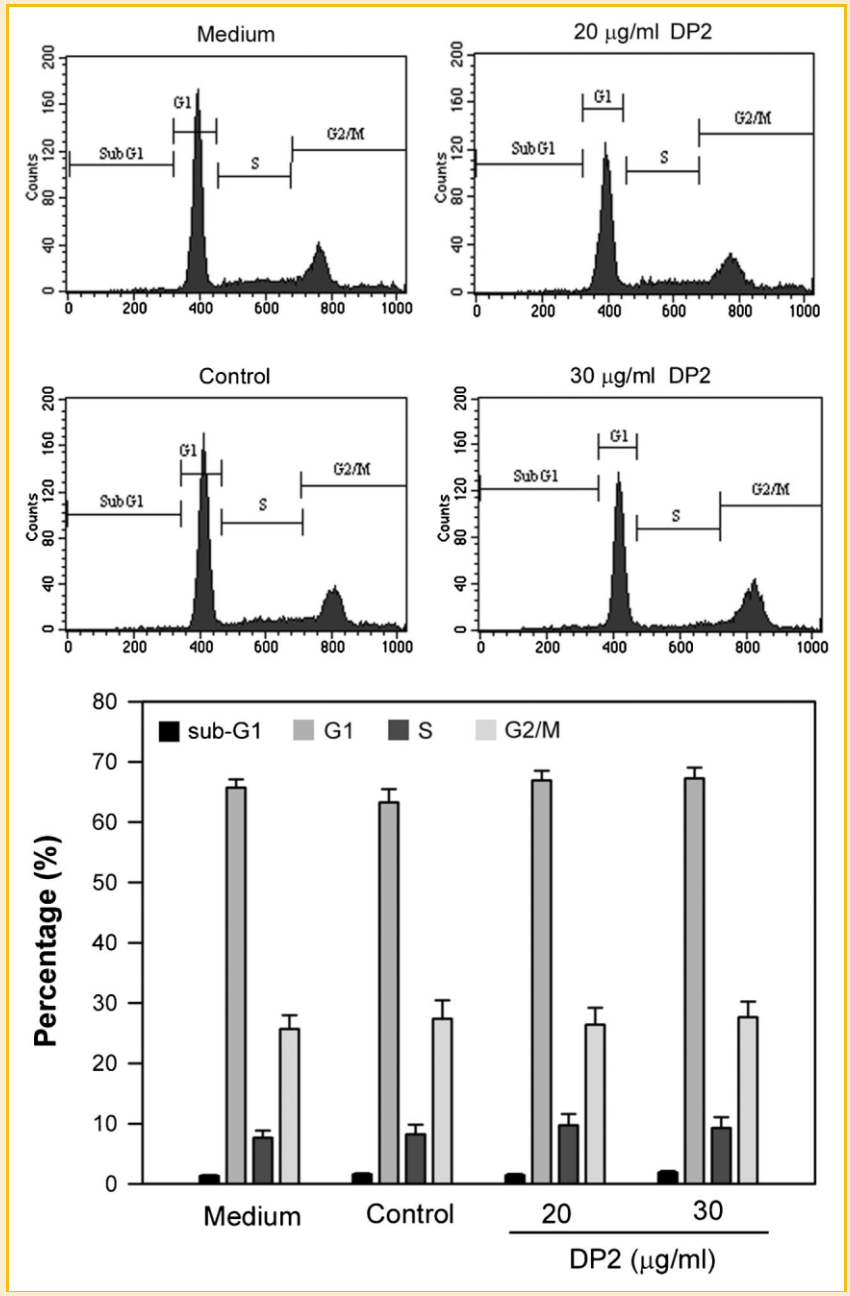

Fig. 1. Effects of DP2 treatment on cell cycle distribution of lung alveolar A549 cells. Cells were treated with DP2 at indicated concentrations or treated with control protein at 30 μg/ml for 24 h, and then the cells were analyzed by flow cytometry. Percentage of subG1, G1, S, and G2/M phase was indicated, and the ratio of each phase showed no statistic significance as compared to which of control.

claudin-2 to 262% of control in A549 cells. In addition, the increase of claudin-2 by DP2 was diminished by pretreatment of Act D, a transcription inhibitor, to 112% of control.

To investigate whether LPS affects expression level of claudin-2, the protein level of cluadin-2 in A547 cells treated with LPS alone was determined by immunoblot. As shown in Figure 2C, LPS treatment neither at 1 μg/ml nor at 10 μg/ml significantly affected the protein level of cluadin-2 and the changes of claudin-2 were <5% of control.

DP2 INCREASED PROTEIN LEVEL OF CLAUDIN-2 ON CELL SURFACE

TJ molecules play important roles in cell-to-cell adhesion; therefore, effects of DP2 on cell surface distribution of claudin-2 were investigated. Our results revealed that protein level of claudin-2 in A549 cells was increased in response to DP2 treatments in a dose-dependent manner as compared to control treatment (Fig. 3A). DP2 at 30 μg/ml significantly increased level of claudin-2 to 236% of control. Further analysis by flow cytometry showed that cell surface distribution of claudin-2 was

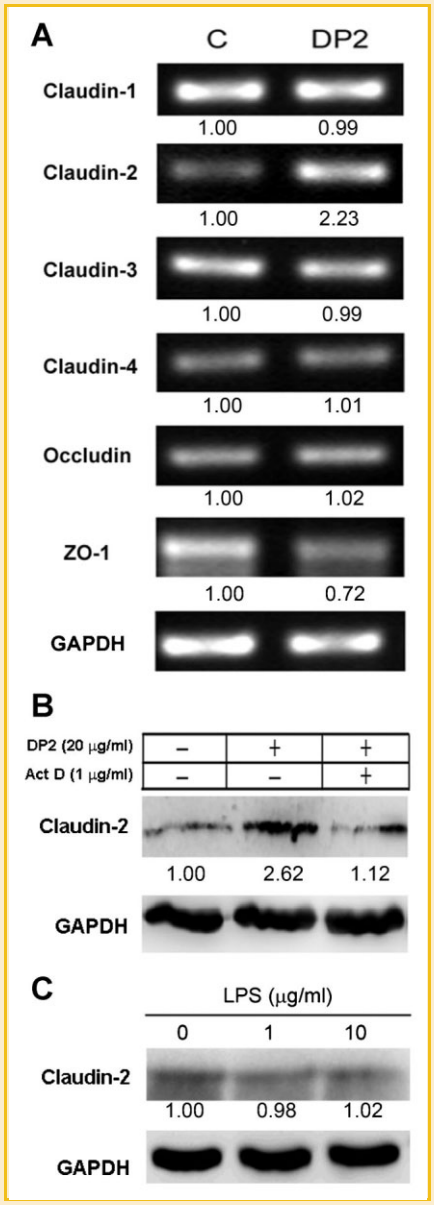

Fig. 2. Effects of DP2 on mRNA expression of tight junction molecules in A549 cells. A: Cells were treated with DP2 or GST as control protein (C) at 20 μg/ml for 24 h, and then were harvested for RT-PCR analysis. B: Cells were pretreated with actinomycin D (Act D) at 1 μg/ml for 3 h, treated with DP2 at 20 μg/ml for 24 h, and then were lysed for immunodetection of claudin-2. C: Cells were treated with LPS at 1 or 10 μg/ml for 24 h, and then were lysed for immunodetection of claudin-2. Quantitative data were acquired by densitometric analysis and level of GAPDH was used as control.

increased by DP2 treatment as compared to control treatment (Fig. 3B).

DP2 ELEVATED PROTIEN LEVEL OF β-CATENIN AND ENHANCED THE NUCLEAR TRANSLOCATION OF β-CATENIN

β-catenin has been reported for its regulatory role in mRNA expression of claudin-2 via the lymphoid enhancer factor-1/β-catenin complex [Mankertz et al., 2004]. Therefore, the level and the

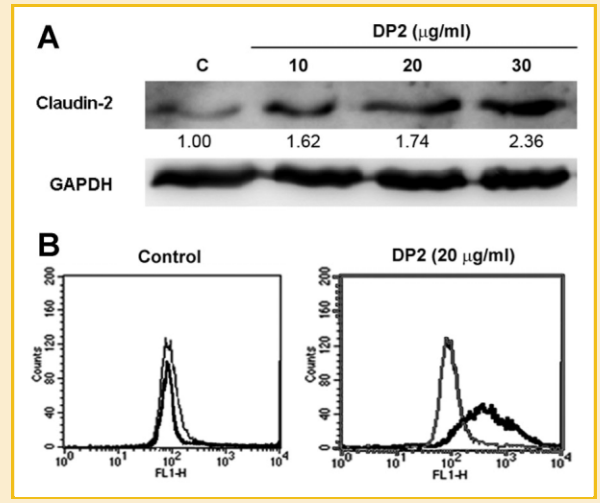

Fig. 3. Effects of DP2 on protein level of claudin-2 in A549 cells. A: Cells were treated with DP2 at indicated concentration for 24 h, and then were lysed for immunodetection of claudin-2. Quantitative data were acquired by densitometric analysis and level of GAPDH was used as control. B: Cells were treated with DP2 at 20 μg/ml for 24h, incubated with anti-claudin-2 antibodies and secondary antibodies conjugated with FITC, and then were analyzed by flow cytometry for immunodetection of claudin-2 on cell surface.

localization of β-catenin in response to DP2 exposure were investigated for its involvement in regulating claudin-2. As shown in Figure 4A, cellular level of β-catenin was elevated in response to DP2 treatments in a dose-dependent manner as compared to control treatment. DP2 at 30 μg/ml significantly increased level of

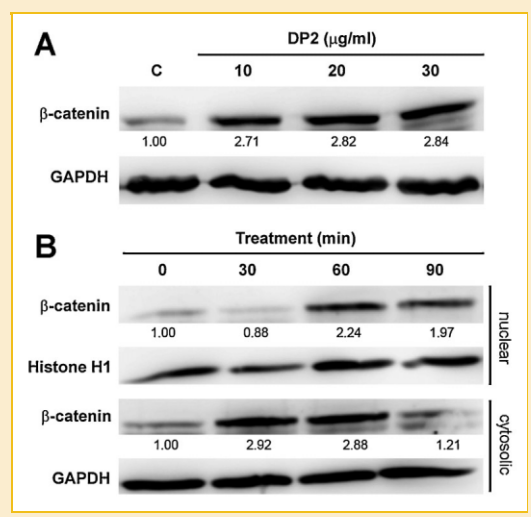

Fig. 4. Effects of DP2 on expression of β-catenin and nuclear translocation of β-catenin in A549 cells. A: Cells were treated with DP2 at indicated concentration for 16 h, and the levels of total β-catenin were determined by immunoblot. B: Cells were treated with 20 μg/ml DP2 for indicated time (min), and the levels of nuclear and cytosolic β-catenin were determined by immunoblot. Histone H1 and GAPDH was used as nuclear and cytosolic marker. Quantitative data were acquired by densitometric analysis and level of histone H1 or GAPDH was used as control.

β-catenin to 284% of control. Cytoplasmic accumulation and consequent nuclear localization of β-catenin have been reported to be correlated to the transcription activation of target genes of Wnt/β-catenin pathway [Willert and Nusse, 1998]. Therefore, nuclear-cytoplasmic distribution of β-catenin in response to DP2 treatment in A549 cells was further determined. Subcellular analysis revealed that the levels of nuclear and cytosolic β-catenin were both elevated in A549 cells exposed to DP2 at 20 μg/ml and reached a peak in 60 min (224% of control) and 30 min (292% of control) (Fig. 4B).

DP2 INCREASES SERINE-9 PHOSPHORYLATION OF GSK-3β AND PHOSPHORYLATION OF AKT INVOLVING IN UPREGULATION OF CLAUDIN-2 EXPRESSION

GSK-3β is a multifunctional protein kinase that acts as a key and negative regulator of the classical Wnt/β-catenin signaling pathway, and a primary kinase responsible for phosphorylation and down-regulation of β-catenin levels [Doble and Woodgett, 2003]. In addition, the activity of GSK-3β is differentially regulated by phosphorylation in a site-specific manner. Phosphorylation of tyrosine-216 increases the activity of GSK-3β, whereas the activity is inhibited by phosphorylation of serine-9 [Harwood, 2001]. Since accumulation and elevated nuclear localization of β-catenin were induced by DP2, effects of DP2 on inhibitory phosphorylation of GSK-3β were subsequently of interest to investigate. As shown in Figure 5A, serine-9 phosphorylation of GSK-3β in A549 cells was increased upon DP2 treatment (20 μg/ml) in 10 min and reached a peak (233% of control) in 20-min post-exposure to DP2. Moreover, our results also revealed that protein level of β-catenin was elevated by LiCl, an inhibitor of GSK-3β, in a dose-dependent manner. LiCl at 30 mM increased level of phosphorylated Ser-9 GSK-3β and β-catenin to 282% and 262% of control, respectively. Taken together, these findings showed that inhibition of GSK-3β by DP2 was associated with increase of β-catenin.

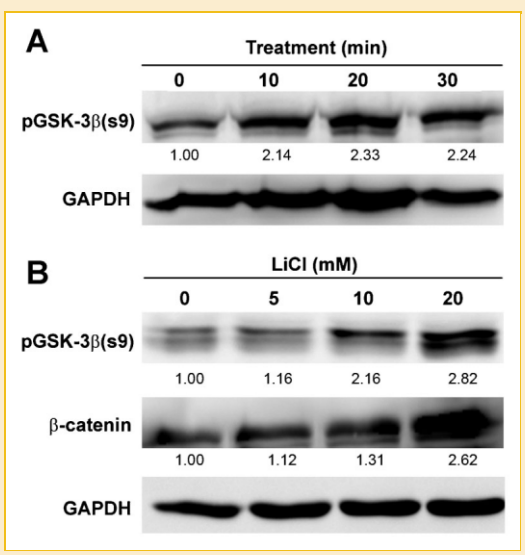

Fig. 5. Association of DP2-induced serine-9 phosphorylation of GSK-3β with elevated β-catenin in A549 cells. A: Cells were treated with DP2 at 20 μg/ml for 16 h, and then were lysed for immunodetection of phosphorylated GSK-3β at serine-9. B: Cells were treated with LiCl at indicated concentration (mM) for 16 h, and then were lysed for immunodetection of phosphorylated GSK-3β at serine-9 and β-catenin. Quantitative data were acquired by densitometric analysis and level of GAPDH was used as control.

DP2 INCREASES CLAUDIN-2 LEVEL THROUGH AKT PHOSPHORYLATION BUT NOT ERK1/2

Serine-9 of GSK-3β has been shown to be phosphorylated by extracellular signal-regulated kinase 1/2 (Erk1/2) and Akt through PI3K/Akt signal cascades [Cross et al., 1995]; therefore, activation of Erk1/2 and Akt in A549 cells upon DP2 treatment was investigated. Our result showed that DP2 treatment (20 μg/ml) increased both phosphorylation level of Erk1/2 (pErk1/2) and Akt (pAkt) in 10 min and reached a peak (234% and 242% of control) at 30 min (Fig. 6B). Further investigation revealed that inhibition of PI3K by wortamannin but not inhibition of Erk1/2 by PD98059 significantly

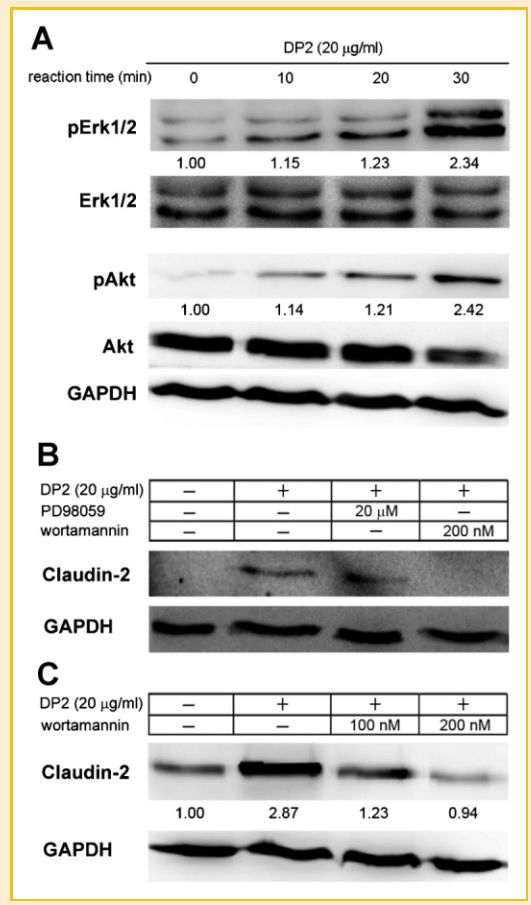

Fig. 6. Involvement of PI3K/Akt signaling in expression of claudin-2 in DP2-stimulated A549 cells. A: Cells were treated with DP2 at 20 μg/ml for 6 h, and then lysed for immunodetection of phosphorylated Erk1/2 (pErk1/2), total Erk1/2, phosphorylated Akt (pAkt) and total Akt. B: Cells were pretreated with MEK1 inhibitor PD98059 (20 μg/ml) or PI3K inhibitor wortmannin (200 nM) for 1 h, and then treated with DP2 at 20 μg/ml for 6 h. Treated cells were lysed for immunodetection of cluadin-2. C: Cells were pretreated with PI3K inhibitor wortmannin at 100 and 200 nM for 1 h, and then treated with DP2 at 20 μg/ml for 6 h. Treated cells were lysed for immunodetection of cluadin-2. Quantitative data were acquired by densitometric analysis and level of GAPDH was used as control.

diminished the elevated claudin-2 expression by DP2 treatment (Fig. 6B). Moreover, level of DP2-induced claudin-2 was decreased by inhibition of wortmannin in a dose-dependent manner (Fig. 6C).

DISCUSSION

The epithelial lining of the airways is a developmentally specialized barrier that exerts as the frontline encountering inhaled allergens. As TJs normally restrict the access of particles to paracellular channels, degree of disassociation of TJs has been found to be associated with the level of permeability of epithelial barrier, resulting in increased delivery of allergens to antigen presenting cells such as dendritic cells underneath [Wan et al., 1999]. In addition, a recent study reported that non-proteolytic aeroallergens such as DP2 may exert adjuvant-like activation of bronchial epithelial cells [Osterlund et al., 2010]. Our results indicate that DP2 alter expression of claudin-2 ad ZO-1, suggesting that regulation of TJ molecules by DP2 may at least partially contribute to the adjuvant-like function.

DP2 is known to activate respiratory epithelial cells with results of upregulated secretions of granulocyte-macrophage colony-stimulating factor, IL-6, IL-8, monocytechemotactic protein-1, and macrophage inflammatory protein-3α [Osterlund et al., 2009], which may potentiate inflammatory reactions [Conti and DiGioacchino, 2001], prolong survival of eosinophils [Lampinen et al., 2004] stimulate collagen synthesis [Gharaee-Kermani et al., 1996] and recruit dendritic cells to mucosal and epithelial surfaces [Dieu et al., 1998].

Recently, DP2 has been demonstrated to associate with innate immune responses via interaction of TLRs in the absence of LPS. Trompette et al. [2009] indicate that DP2 shows functional homology with MD-2, facilitating signaling through direct interactions with the TLR4 complex in human embryonic kidney cells. Chiou and Lin [2009] also reported that DP2 alone was able to induce production of proinflammatory cytokines in airway smooth muscle cells, which attributed to activation of TLR2 signaling, c-Jun *N*-terminal kinase (JNK), and Erk1/2. These findings provide evidences that DP2 directly binds to TLR2 or TLR4 leading to activation of TLR signaling, JNK, and Erk1/2 and the consequent induction of inflammatory responses. Our results showed that activation of PI3K/Akt but not Erk1/2 was significantly involved in claudin-2 expression in DP2-induced alveolar A549 cells. Interestingly, we also found that protein level of claudin-2 was not affected by LPS treatment (data not shown). Taken together, it is suggested that DP2 may activate an alternative PI3K/Akt signaling from LPS.

Claudin-1 and claudin-2 play important but biologically opposite roles in epithelial permeability. High expression of cluadin-1 strengthens epithelial barrier and cleavage of claudin-1 leads to a non-specific increase in epithelial permeability [Wan et al., 1999], whereas elevated expression of claudin-2 loosens epithelial integrity and causes leakage [Amasheh et al., 2002]. Recently, Peter et al. [2009] reported that claudin-2 overexpression was involved in permeability and remodeling in A549 cell model, suggesting that claudin-2 could contribute to degree of metastasis of lung cancer. In addition, our findings revealed that DP2 regulated mRNA expression of claudin-2 and ZO-1 but slightly affected that of claudin-1, -3, and -4 as well as occludin. These findings implicate that DP2 might alter permeability of lung alveolar epithelium through increase of claudin-2 and decrease of ZO-1.

β-catenin is a multifunctional protein that plays an important role in a variety of cell biological activities including cell development, cell adhesion, repair of injury, cell cycle regulation, and tumor formation [Willert and Nusse, 1998; Huang and He, 2008]. It acts as a key mediator in Wnt signaling pathway by which a variety of genes are regulated in association with its nuclear localization and interaction with transcription factor T-cell factor/lymphoid enhancer factor [Willert and Nusse, 1998; Huang and He, 2008]. We demonstrated that treatment of A549 cells with DP2 triggered cytosolic accumulation of β-catenin and promoted nuclear translocation of β-catenin. GSK-3β, a protein kinase, is known for its multifunctional activities of which the classic Wnt/β-catenin signaling pathway is negatively regulated and down-regulation of β-catenin levels through phosphorylating β-catenin and leading to its degradation [Doble and Woodgett, 2003]. We report that treatment of DP2 resulted in significantly increased serine-9 phosphorylation of GSK-3β by which kinase activity of GSK-3β is attenuated and level and phosphorylation of β-cateninare altered and affected as consequences. These findings implicate that DP2-induced signaling may be involved in or crosstalk with parts of classical Wnt/β-catenin signaling pathway.

PI3K/Akt signaling cascade is one of the signaling pathways that leads to inhibition of GSK-3β by increasing serine-9 phosphorylation [Cross et al., 1995]. Additionally, serine-9 of GSK-3β also can be phosphorylated by protein kinase C [Goode et al., 1992], p90Rsk, p70 ribosomal S6 kinase [Sutherland et al., 1993], and protein kinase A [Fang et al., 2000]. Thus, multiple mechanisms are evolved and employed to surveillance and control the activity of GSK-3β by phosphorylation. Our results showed that treatment of DP2 increased phosphorylation of Akt, and inhibition of PI3K by wortamannin significantly diminished DP2-induced claudin-2 expression, suggesting that PI3K/Akt activation is crucial for DP2-induced claudin-2 expression. However, further investigations are required to elucidate the involvement of protein kinase A, protein kinase c, and p70 ribosomal S6 kinase in DP2-induced claudin-2 expression.

The present study shows that treatment of DP2 lead to increased protein level of claudin-2 in lung alveolar cell A549, putatively resulting from, in a synergistic fashion, activation of PI3K/Akt activation, inhibition of GSK-3β, and accumulation and enhanced nuclear translocation of β-catenin to increase mRNA transcription of claudin-2. These findings provide evidences that non-proteolytic DP2 allergen can alter the TJ molecule expression through PI3K/Akt/GSK-3β/β-catenin signaling cascades.

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