

Dioscorin protects tight junction protein expression in A549 human airway epithelium cells from dust mite damage

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Background and purpose: In addition to being an allergen, the trypsin activity of dust mite extract also destroys the tight junctions of bronchial epithelium. Such damage can lead to airway leakage, which increases airway exposure to allergens, irritants, and other pathogens. Dioscorin, the storage protein of yam, demonstrates anti-trypsin activity, as well as other potential anti-inflammatory effects. This study investigated the protective role of dioscorin for tight junctions.

Methods: The immunofluorescence stains of zonula occludens (ZO-1), E-cadherin (EC) and desmoplakin (DP) proteins were compared. A cultured A549 cell line was used as a control and A549 cells were incubated with mite extract 100 mg/mL for 16 h, with or without dioscorin 100 mg/mL pretreatment for 8 h and with dioscorin 100 mg/mL alone for 16 h. Western blot was performed to detect changes in ZO-1, EC, and DP in the treated A549 cell lines.

Results: Loss of tight junction protein expression (ZO-1, EC, DP) was demonstrated after 16-h mite extract incubation. The defect could be restored if cells were pretreated with dioscorin for 8 h. In addition, dioscorin did not cause damage to the A549 cell lines in terms of cell survival or morphology. Western blot showed no change in the amount of tight junction protein under various conditions.

Conclusion: Dioscorin is a potential protector of airway damage caused by mite extract.

Key words: Cadherins; Desmoplakins; *Dermatophagoides pteronyssinus*; dioscorin protein, *Dioscorea cayenensis*; Tight junctions; zonula occludens-1 protein

Introduction

Airway epithelial cells play a key role in the pathophysiology of airway diseases. Airway epithelial cells act as a physical barrier separating the external environment and the internal milieu of the airway. In pulmonary diseases such as asthma, damage to the bronchial epithelium is often associated with disruption of the underlying basement membrane and cell-cell interactions [1]. Impairment of the protective function of the epithelium in asthma was originally

suggested by the extent of epithelial damage that was evident in asthmatic airways at autopsy [2]. That epithelial shedding is an important feature of asthma is supported by the presence of increased numbers of epithelial cell clumps (Creola bodies) in sputum, and by disruption of the bronchial epithelium. Those events are frequently observed in mucosal biopsies obtained from asthmatic airways by either rigid [3] or fiberoptic [1] bronchoscopy.

Disruption of the bronchial epithelia causes important structural and functional changes, leading to disruption of mucociliary clearance and loss of barrier function, enabling tissue-damaging molecules to pass unimpeded through the lumen into the airway wall. This disruption also triggers the bronchial epithelia

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to secrete inflammatory molecules. Several tight junction proteins have been shown to be implicated in bronchial epithelium damage. E-cadherin (EC), a cell-cell adhesion molecule attaching to the tight junction of epithelium [4], and zonula occludens (ZO-1), a component of the tight junction [5], have been noted to have decreased expression in bronchial lavage samples from asthma patients and in vitro studies. Desmosomal protein desmoplakin (DP) is a key component of cellular adhesion junctions known as desmosomes, which are button-like structures that hold cells together and provide anchoring sites for intermediate filaments [6]. Peptidase from dust mite has been shown to increase the permeability of epithelial cells in vitro due to its ability to degrade the tight junction proteins ZO-1, DP [7,8], and EC [7].

The data on tight junctions of bronchial epithelium in asthma pathogenesis are limited. A MEDLINE search for the years 1966 to 2004, using the key words 'tight junction', ZO-1, EC, DP, and 'mite' found only 13 articles. However, these limited references showed evidence of decreased expression of ZO-1, EC, and DP after different stresses were tested, such as cytokines [9,10] and allergens [4,5,11,12], including *Dermatophagoides pteronyssinus* [13]. The major allergens of mites are digestion enzymes from their feces. The proteolytic activity can break the tight junction of bronchial epithelial cells [13], which results in the changes mentioned above. In previous studies, 16HBE14o⁻ [14] and Calu-3 [7] cells were used as in vitro models. This study tested the A549 cell line, which shows fair expression of ZO-1, EC, and DP.

Dioscorin from yam, one of the 5 tuber storage proteins, is a carbonic anhydrase [15]. Dioscorin also exhibits antioxidant radical-scavenging activities and biological activities that could contribute to resistance to pests, pathogens, or abiotic stresses, indicating that it may have a dual role in the tubers. Dioscorin also demonstrates angiotensin-converting enzyme inhibitory activities [16], and carbonic anhydrase and trypsin inhibitor activities [17]. This protein was first purified in 1995 [18]. Currently, reports about its application are scarce. Mite allergens, including *Der p1*, *Der f1*, *Dm*, *Ds*, and *Em* all have the biochemical function of cysteine protease [19]. This information suggests that it is constructive to study the protective role exhibited by dioscorin through its antiprotease activities in tight junction damage triggered by mite allergens.

Methods

Cell culture

A human A549 cell line was used for this study. Culture materials and methods followed the authors' previous study [20]. The study cells were treated as follows: cells were incubated with mite extract 100 µg/mL (Allergon AB, Ängelholm, Sweden) for 16 h; with dioscorin 100 µg/mL pretreatment for 8 h and mite extract 100 µg/mL for 16 h; or with dioscorin alone for 16 h. The control cells were untreated.

The survival curves were measured as previously reported [21]. The tested dioscorin concentrations were 10, 50, 100, 200, 500, and 1000 µg/mL, which were incubated individually for 16 h. The tested mite extract concentrations included 10, 50, 100, 250, 500, and 1000 µg/mL, incubated individually for 16 h.

Morphological observation and immunofluorescence staining

A549 cells (2×10^5 cells/well) were seeded into a 6-well plate and allowed to attach, then the old medium was removed and the cells were washed with fresh medium 3 times. For the control group (A549 cells only), 1 mL serum-free medium (RPMI-1640) was added to each well, and for the dioscorin group and the mite extract plus dioscorin group, 0.9 mL serum-free medium was added. These 2 plates were incubated for 8 h. Mite extract 100 µg/mL was added to the cultures of the mite extract and mite extract plus dioscorin groups and the plates were cultured overnight. The old medium was removed and the cells were washed with phosphate-buffered saline (PBS), before adding 1 mL -20°C ethanol to each well and then refrigerating at -20°C for 1 h. The cells were then washed again with PBS and blocked by adding 1 mL fetal bovine serum per well and incubated at 4°C for 2 h. After washing with PBS, all cells were incubated with primary and secondary antibodies.

Photographs were taken with a phase contrast microscope, then 1 mL of the primary monoclonal antibody (ZO-1, EC, DP; BD Pharmingen, Franklin Lakes, NJ, USA) was added to each well. The wells were put on an orbital shaker (100 rpm) for 1 h before the secondary fluorescein-conjugated antibody (ICN/Cappel, Aurora, OH, USA) 1 mL/well was added, and the plates returned to the orbital shaker for 1 h. The staining of secondary antibodies was visualized by fluorescence microscope (Axioskop 21 plus; Zeiss, Jena, Germany) and photographs were taken immediately.

ZO-1 was used to visualize tight junctions, EC was used for adherens junctions, and DP was used for desmosomes.

Western blot

A549 cells (2×10^5 cells/well) were seeded in 6-well plates for 16 to 18 h, and treated with serum-free medium, mite extract, dioscorin, and mite extract plus dioscorin. The cells were washed with PBS and treated with sample buffer (0.5M Tris, pH 6.8). After boiling for 5 min, the cells were collected by cell scraper and cell lysates were centrifuged at $450 \times g$ for 5 min. A gel was soaked in 1X Tris/boric acid/ethylenediaminetetraacetic acid buffer. The protein lysates were loaded and analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). This was then incubated with blocking buffer solution (2.5% low fat milk blocking buffer in PBS Tween 20 [PBST; 1X PBS/500 mL Tween 20]) for 30 min on a shaker (TKS, Model-RS01; Kansin Instruments Co., Ltd., Sunnyvale, CA, USA). Then 20 mL of the primary antibody (ZO-1, EC, or DP at a concentration of 1:500) was added to each centrifuge tube with 10 mL 2.5% milk block buffer in PBST, and shaken at 35 rpm for 3 h. The primary antibodies were then removed, followed by 3 washes, and the membranes were incubated with secondary fluorescence-conjugated antibody (concentration 1:2500; 30 mL blocking buffer). Protein bands were visualized by using an infrared imaging system

(LI-COR Biosciences, Lincoln, NE, USA). Actin was used as the internal control. Bands of tight junction proteins (ZO-1, DP, EC) were visualized using an infrared imaging system.

Statistical analyses

Data are presented as mean \pm standard deviation (SD). *t* Tests were used to compare differences. A *p* value < 0.05 was considered to be statistically significant.

Results

Cytotoxicities of mite extract and dioscorin

Dioscorin was not significantly cytotoxic to A549 cells at the tested concentrations (Fig. 1A). Concentrations of mite extract higher than 250 $\mu\text{g}/\text{mL}$ had significant cytotoxic activity on A549 cells ($p < 0.01$) [Fig. 1B].

Morphological observation

Morphological changes are shown in Fig. 2. A549 cells shrank, lost some contact between the cells and became discrete after treatment with mite extract 100 $\mu\text{g}/\text{mL}$ for 16 h (Fig. 2C). A549 cells treated with dioscorin or dioscorin plus mite extract showed no morphological changes, nor did the control cells (Fig. 2A, Fig. 2B, and Fig. 2D).

Immunofluorescence staining

The control group (Fig. 3A, Fig. 3B, and Fig. 3C) showed intact integrity structure of tight junctions in A549 cells. The dioscorin group (Fig. 3D, Fig. 3E,

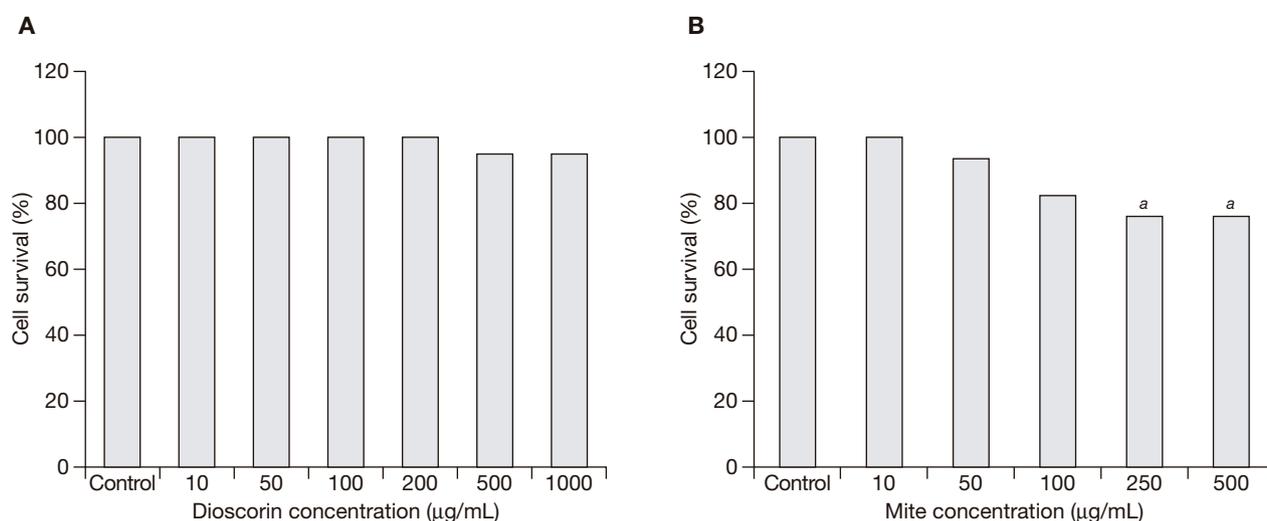


Fig. 1. Cytotoxicities of dioscorin and of mite extract. Percent cell survival under various concentrations of (A) dioscorin and (B) dust mite extract.

^a $p < 0.01$

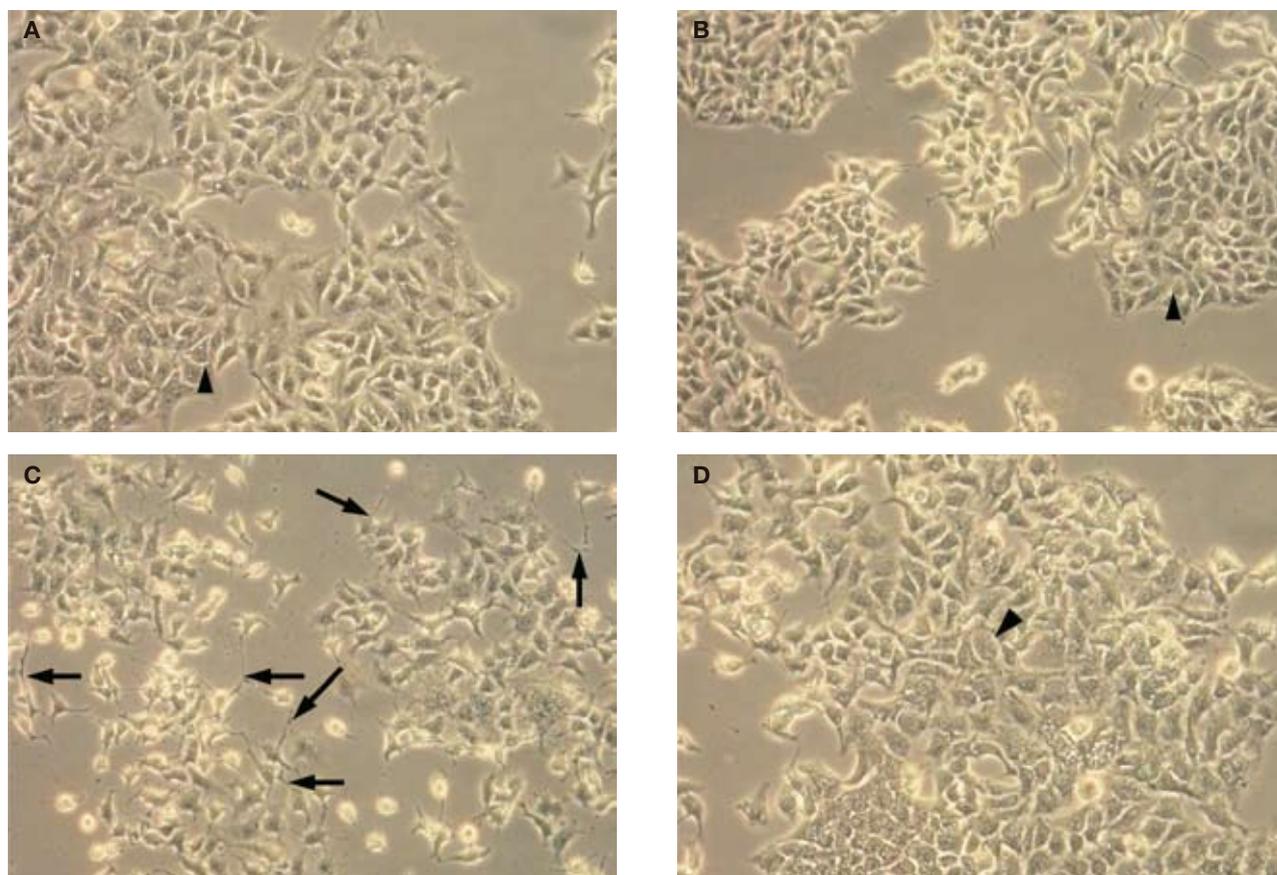


Fig. 2. Images of A549 cells under various conditions. (A) Control; (B) dioscorin-treated cells; (C) dust mite extract-treated cells; and (D) dioscorin plus dust mite extract-treated cells (phase contrast microscopy, x 100).

and Fig. 3F) showed no significant changes in ZO-1, EC, or DP expression on cells, similar to the control group. Cells treated with mite extract showed loss of ZO-1, EC, and DP immunostaining (Fig. 3G, Fig. 3H, and Fig. 3I). Cells treated with dioscorin 8 h before the introduction of mite extract showed intact integrity structure of tight junctions in the epithelium (Fig. 3J, Fig. 3K, and Fig. 3L). The dioscorin group and the mite plus dioscorin group were similar to the control group.

Western blot

Fig. 4 illustrates EC protein bands, which showed no differences between the 4 groups (control, dioscorin only, mite only, and mite plus dioscorin). Actin served as the internal control. There were no differences in the amount of either ZO-1 or DP protein bands under various conditions.

Discussion

This study suggests that dioscorin can protect tight junction protein expression in A549 human airway

epithelial cells from dust mite damage. Although good expression of ZO-1, EC, and DP on Calu-3 cells has been demonstrated [7], similar results were not found in the preliminary experiments of this study (data not shown); as cell growth and expression of these proteins was better in A549 cells than in Calu-3 cells, A549 cells were used in the study. Interestingly, there is evidence that A549 lung epithelial cells do not form functional tight junctions, failing to show significant immunostaining against the tight-junction protein ZO-1 [12]. This study suggests that ZO-1, DP, and EC tight-junction proteins were destroyed by mite extract alone, but remained intact after treatment with dioscorin and dioscorin plus mite extract (Fig. 3). House dust mite allergen Der p1 is a cysteine peptidase, which could contribute to sensitization and an allergic response by degrading the function of the airway epithelial barrier. Immunoblotting demonstrates that the disruption of tight junction morphology is associated with cleavage of ZO-1, DP, and EC proteins [5].

In Asia, Chinese yam tubers are frequently made into dried chips for desert and storage. Both dried

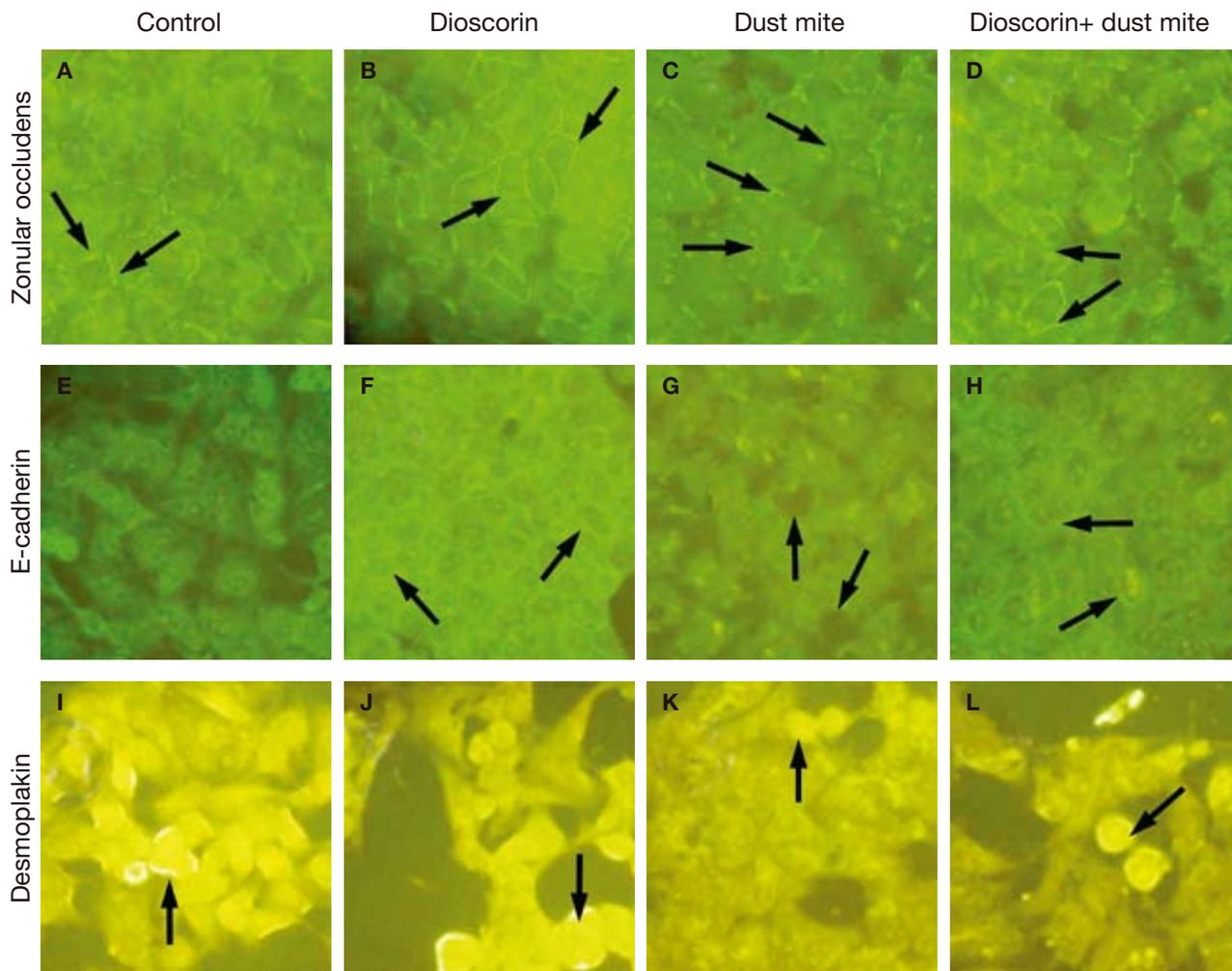


Fig. 3. Immunofluorescent labeling of the intercellular junction structure of zonula occludens, E-cadherin, and desmoplakin.

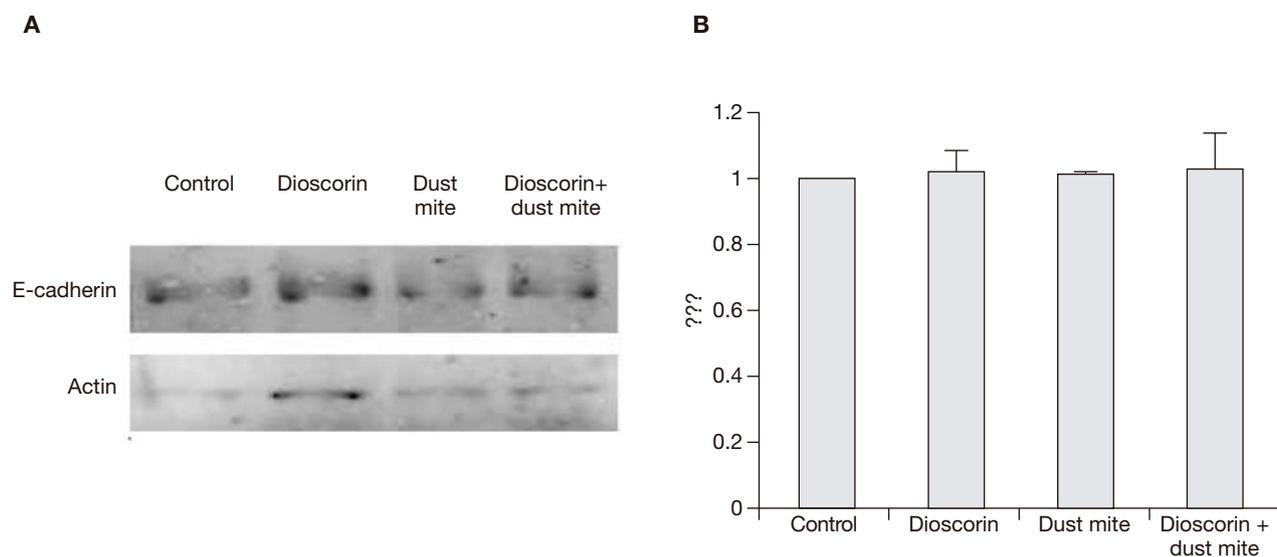


Fig. 4. Western blot analyses of E-cadherin, with actin as an internal control for dioscorin only, dust mite extract only, and dust mite extract plus dioscorin (original magnification, $\times 400$).

chips and fresh tubers are considered to be healthy foods. The immune-modulating activities of yam-derived mucopolysaccharide have been reported [22]. This molecule has previously been shown to have carbonic-anhydrase, trypsin-inhibitor, and angiotensin-converting enzyme inhibitory activities [16,23]. Dioscorin is able to activate Toll-like receptor 4-dependent signaling pathways and induce cytokine expression in macrophages [24].

Dioscorin caused no damage to A549 cells, as demonstrated by the survival curve and morphology in this study. Immunofluorescence staining showed that dioscorin protected cells from mite damage. However, Western blot showed no difference in the cells. To the authors' knowledge, there are no data on the ratio of these proteins in tight junctions to their cytoplasmic amount in either bronchial epithelial or other cell types.

The molecular weight of dioscorin is 31,000, making it a relatively large molecule. The key antitrypsin functional site is still unknown. Further studies of dioscorin, incorporating techniques from proteomics, are warranted in the future. Whether dioscorin induces cell inflammation is a relationship that needs further evaluation.

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