Internalization of Aspergillus fumigatus into pulmonary epithelial cells: joint action of host and pathogen
Han, Xuelin

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 05-10-2019
Chapter 4

Gliotoxin promotes *Aspergillus fumigatus* internalization into type II human pneumocyte A549 cells by inducing host phospholipase D activation

Xiaodong Jia¹, Fangyan Chen¹, Weihua Pan¹, Rentao Yu¹,³, Shuguang Tian¹,
Gaige Han¹, Haiqin Fang¹, Shuo Wang¹, Jingya Zhao¹, Xianping Li¹,
Dongyu Zheng¹, Sha Tao¹, Wanqing Liao², Xuelin Han*¹, Li Han*¹

¹ Department for Hospital Infection Control & Research, Institute of Disease Control & Prevention of PLA, Academy of Military Medical Sciences, Beijing, China.
² Shanghai Key Laboratory of Molecular Mycology, Department of Dermatology, Shanghai Changzheng Hospital, Second Military Medical University, Shanghai, China.
³ Patent Examination Cooperation Center of the Patent Office, Beijing, China.

*Corresponding author:
hanli@bmi.ac.cn or hanlicdc@163.com (Li Han);
yilin791228@126.com (Xuelin Han)

Abstract

The internalization of *Aspergillus fumigatus* into lung epithelial cells is critical for the infection process in the host. Gliotoxin is the most potent toxin produced by *A. fumigatus*. However, its role in *A. fumigatus* internalization into the lung epithelial cells is still largely unknown. In the present study, the deletion of the *gliP* gene regulating the production of gliotoxin in *A. fumigatus* suppressed the internalization of conidia into the A549 lung epithelial cells, and this suppression could be rescued by the exogenous addition of gliotoxin. At lower concentrations, gliotoxin enhanced the internalization of the conidia of *A. fumigatus* into A549 cells; in contrast, it inhibited the phagocytosis of J774 macrophages in a dose-dependent manner. Under a concentration of 100 ng/ml, gliotoxin had no effect on A549 cell viability but attenuated ROS production in a dose-dependent manner. Gliotoxin significantly stimulated the phospholipase D activity in the A549 cells at a concentration of 50 ng/ml. This stimulation was blocked by the pretreatment of host cells with PLD1- but not PLD2-specific inhibitor. Morphological cell changes induced by gliotoxin were observed in the A549 cells accompanying with obvious actin cytoskeleton rearrangement and a moderate alteration of phospholipase D distribution. Our data indicated that gliotoxin might be responsible for modulating the *A. fumigatus* internalization into epithelial cells through phospholipase D1 activation and actin cytoskeleton rearrangement.
Gliotoxin promotes Aspergillus fumigatus internalization.

Introduction

Aspergillus fumigatus is one of the major fungal pathogens which cause allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis (1). Its airborne conidia are inhaled from the environment and then colonize within the lung alveoli. Like many intracellular bacterial pathogens (2), A. fumigatus conidia are able to bind to and internalize into lung type II alveolar epithelium cells. They can then survive within these normally non-phagocytic host cells. This brings the possibility of immune evasion as well as the possibility of dissemination (3-5). To date, the mechanism associated with A. fumigatus internalization into type II lung epithelial cells remains unclear.

Gliotoxin is one of the well-known members of the epipolythiodioxopiperazine class of metabolites produced by A. fumigatus. It is characterized by a disulfide bridge across a piperazine ring which is essential for its toxicity (6). It should be noted that gliotoxin possesses multiple immuno-suppressive activities (7). In murine models of invasive aspergillosis (IA), gliotoxin has been shown to inhibit specifically the nuclear transcription factor NF-κB (8,9), which consequently induces host cell apoptosis (10,11) and suppresses the cytotoxic T-cell response (12,13). Moreover, gliotoxin is also able to inhibit macrophage and polymorphonuclear cell function, including phagocytosis (14) and respiratory bursts (15,16). It has been demonstrated that gliotoxin from A. fumigatus affects the process of phagocytosis and actin cytoskeleton rearrangement of human neutrophils through distinct signaling pathways, which involve cAMP and arachidonic acid signals, respectively (17). Recently, an immune-active role of gliotoxin was reported that gliotoxin can activate the platelets and its antifungal function (18). For epithelial cells, it has been shown that gliotoxin can reduce the ciliary movement of epithelial cells and alter membrane permeability leading to damage of the epithelial cells (14,19,20). Gliotoxin may also reduce TGFβ1, IL-6 and IL-8 levels in A549 lung epithelial cells at lower concentrations (below 50 ng/ml) (20). However, much less is known about the effect of gliotoxin on A. fumigatus internalization into lung epithelial cells.

It has been generally accepted that the internalization of A. fumigatus conidia into type II A549 lung epithelial cells is closely related to host actin dynamics including invagination of the membrane and formation of the pseudopods that engulf the conidia (3,5,21). Phospholipase D (PLD) is an enzyme that catalyzes the hydrolysis of the most abundant membrane phospholipids, phosphatidylcholine (PC) to produce phosphatidic acid (PA) and choline (22). In mammalian cells PLD activity has been found to be closely related to actin dynamics (23-25). In our previous study, it was demonstrated that the internalization of A. fumigatus conidia into epithelial cells was regulated by β-1,3-glucan-induced PLD activation in epithelial cells (26). However, block of dectin-1, the receptor on lung epithelial cells for β-1,3-glucan, could not fully inhibit the internalization of conidia or conidia-induced PLD activation. This implied that some other factors may also be involved in the regulation of A. fumigatus internalization into lung epithelial cells.
Therefore, we hypothesized that gliotoxin from *A. fumigatus* may be involved in regulating the conidial internalization into lung epithelial cells. In present study, we discovered that gliotoxin facilitates the internalization of *A. fumigatus* conidia by stimulating PLD activity in A549 cells.

**Materials and Methods**

*A. fumigatus* strains, Cell Lines

*A. fumigatus* wild type strain B5233, its mutant strain *gliPΔ* (deletion of the peptide synthetase *gliP*), and the *gliP* reconstituted strain *gliPR* were generously provided by Dr. K.J. Kwon-Chung (National Institute of Health, Bethesda, Maryland) (27). Except where indicated, all of the *A. fumigatus* strains were propagated on Sabouraud dextrose agar slants (10 g/l peptone, 10 g/l glucose, 15 g/l agar, pH6.0) for 5–8 days at 37°C. The type II human pneumocyte cell line A549 and murine macrophage cell line J774 were obtained from the ATCC and the 16HBE14o human bronchial epithelial cell line was purchased from shanghai Beinuo life science company. These three kinds of cells were cultured in DMEM supplemented with a 10% fetal calf serum (GIBCO, Germany), 100 mg/l streptomycin, 16 mg/l penicillin at 37°C in 5% CO₂. The human monocytic leukemia cell line THP-1 was gift from Beijing Proteome Research Center and cultured in suspension cultures in RPMI-1640 (GIBCO, Germany) supplemented with 10% fetal calf serum (GIBCO, Germany), 100 mg/l streptomycin, 16 mg/l penicillin at 37°C in 5% CO₂. THP-1 cells were differentiated into macrophages by 100 ng/ml phorbol myristate acetate (PMA) for 60 h. Afterwards, the macrophages were cultured in DMEM/F12 1:1 medium (GIBCO, Germany) supplemented with 10% fetal calf serum (GIBCO, Germany), 100 mg/l streptomycin, 16 mg/l penicillin at 37°C in 5% CO₂.

Preparation of the Conidia

*A. fumigatus* conidia were harvested and prepared in the same way as described in a previous study (28). After 5–8 days of cultivation, *A. fumigatus* conidia were dislodged from agar plates with gentle washing. They were then re-suspended in sterile phosphate-buffered saline supplemented with 0.1% Tween 20 (PBST). The conidia were passed through 8 layers of sterile gauze in order to remove hyphal fragments. At this point, they were enumerated on a hemacytometer and the resting conidia were washed twice and stored at 4°C to be used within 48 h. For preparation of swollen conidia, the resting conidia were incubated at 37°C in DMEM for the indicated periods. The preparations which were incubated for 5 h contained swollen conidia and early germlings with < 5 μm hyphal extensions.

Chemical Reagents and Antibodies

The gliotoxin was purchased from Sigma-Aldrich and the [³H] oleic acid (33.4 Ci/mmol) was obtained from PerkinElmer, Inc. The PLD1-specific inhibitor VU0359595 and the
Gliotoxin promotes Aspergillus fumigatus internalization.

PLD2-specific inhibitor VU0285655-1 were purchased from Avanti Polar Lipids. Both the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and the ROS assay kit were purchased from Applygen Co. Ltd. (C1300, China).

For the immunofluorescence analysis, both the rabbit polyclonal anti-PLD1 antibody (sc-25512), and the secondary antibody tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG were purchased from Southern Biotech. For the western blot analysis, the rabbit anti-PLD1-41 and rabbit anti-PLD2-41 antibodies were gifts presented by Dr. Sylvain Bourgoin, Canada. The mouse monoclonal anti-β-actin antibody (sc-47778) was obtained from Santa Cruz Biotechnology Inc. Both the HRP-conjugated goat anti-rabbit IgG, and the HRP-conjugated goat anti-mouse IgG antibodies were obtained from ZSGB-BIO, China.

Analysis of A. fumigatus Internalization
The nystatin protection assay to determine the A. fumigatus internalization into host cells were performed as previously described and this method were well correlated with immunofluorescence assay in determining the internalization of A. fumigatus (5). Both methods were used to analyze the effect of extracellular gliotoxin on A. fumigatus internalization in A549 cells and nice correlation was verified in present study (Figures S2 and S3). For the nystatin protection assay, the cells (alone or pretreated with specific PLD inhibitors) were seeded at 2 × 10^4 cells/well in 96-well plates (Corning). After 16 h growth, the host cells were incubated with A. fumigatus swollen conidia at the indicated MOI under centrifugation at 200 g for 5 min at 4°C. Internalization was performed by incubation at 37°C for the indicated times (1 h for epithelial cells or 0.5 h for macrophages) in 5% CO_2. The wells were subsequently washed 3 times with PBST and then incubated with nystatin (20 μg/ml) in DMEM for 3 h at 37°C. Afterwards, the monolayers were washed twice with PBST and lysed by incubating in 0.25% Triton X-100 for 15 min. The released conidia were then diluted and plated onto SDA agar (3 replicate plates/well) and incubated at 37°C for 24 h. The colonies were counted to determine the total amount of intracellular conidia. The internalization capacity has been expressed as a percentage of the initial inoculum.

For the immunofluorescence assay, cells were seeded on 12-mm-diameter coverslips in 24-well plates at 1 × 10^5 cells/well (Corning) and grown for 16 h. The cells were infected with 1 ml of 1 × 10^6 conidia/ml in DMEM for the indicated times at 37°C. After infection, unbound conidia were removed by washing 3 times with PBST, and the cells were incubated for 1 h in PBS containing 0.1% (w/v) BSA (Roche 738328) at 37°C. Afterwards, extracellular conidia were labeled with the anti-A. fumigatus rabbit antibody (1:20) and goat anti-rabbit TRITC-conjugated secondary antibody (1:100). Then, the wells were washed and fixed for 15 min with 4% (w/v) PFA (pH 7.4)/PBS. Finally, the coverslips were mounted on a slide and observed under a BX51 fluorescent microscope (Olympus). To analyze the uptake of conidia, 10 fields per coverslip were captured with an Olympus DP71 camera using Image Pro Express (IPE) for image capturing (Media Cybernetics Inc., MA, USA). The extracellular and total conidia (extracellular
Chapter 4

+ intracellular) were enumerated under the red and green channels in IPE, respectively. The number of internalized conidia was calculated by the subtraction of the extracellular conidia from the total conidia. The internalization index determined by the immunofluorescence assay is the number of internalized conidia divided by the number of total conidia per field ×100.

**Assay of PLD Activity in Intact Cells**

For the measurement of cellular PLD activity, the cellular phospholipids were labeled by incubating the cell monolayer for 20–24 h with [3H] oleic acid (2 μCi/ml) in the growth medium. Afterwards, the cells were washed twice in Hank’s balanced salt solution (HBSS) containing 118 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM d-glucose and 15 mM HEPES at pH 7.4. Then, the cells were stimulated with gliotoxin at the indicated concentration in the presence of 2% ethanol at 37°C for 30 min. The reaction was stopped by adding ice-cold methanol/chloroform (1:1, v/v). The labeled phospholipids (including the specific PLD product [3H] phosphatidylethanol ([3H] PtdEtOH)) were isolated as previously described (26). The formation of [3H] PtdEtOH has been expressed as a percentage of the total amount of labeled phospholipids.

**Immunofluorescence Analysis and Immunoblotting**

The A549 cells (5 × 10⁴ cells/ml) were seeded onto coverslips in 24-well plates. They were then grown for 24 h. The gliotoxin was added to the wells at the indicated concentrations. In order to stop the treatment, the cells were washed twice with ice-cold PBS and then fixed in ice-cold 4% PFA in a PBS buffer for 20 min at room temperature. The cells were then permeabilized for 4 min with 0.05% Triton X-100 in a PBS buffer. Finally, they were stained with the indicated antibodies. The primary antibodies used for PLD1 staining were anti-PLD1 (sc-25512, 1:100) and the secondary antibodies used for PLD1 were TRITC-conjugated goat anti-rabbit IgGs (1:50). The actin-filaments (F-actin) were stained with the fluorescein isothiocyanate (FITC)-conjugated molecular probe (code number F432, 1:100 dilution, Invitrogen) for 30 min. Horchest was then added in order to stain the nuclei. The preparations were observed with a laser scanning confocal microscope (Olympus FluoView FV1000). Green fluorescence was captured with a 515- to 540-nm band pass filter. Red fluorescence was captured with a 590- to 610-nm band pass filter. These images were later processed using Olympus FluoView ver. 1.6.

For immunoblotting, equal amounts of protein from the cell lysates were separated by SDS-PAGE and then transferred to a PVDF membrane. Next, the membrane was incubated for 3 h with the appropriate primary antibodies. It was then subsequently incubated for 3 h with the HRP-conjugated secondary antibody. The proteins were visualized with enhanced chemiluminescence (Santa Cruz Biotechnology Inc.). A densitometric analysis of the immunoblots was performed with Image J2x (Wayne Rasband, National Institutes of Health, USA).
Intracellular ROS Measurement
Cells were seeded at 2 × 10⁴ cells/well in 96-well plates (Corning). They were then grown for 16 h. The cultures were stimulated with gliotoxin (at the indicated concentration) at 37°C for 30 min. Then, the cells were incubated with a medium containing DCFH-DA (1:1250 dilution) for 30 min and washed twice with PBS. The presence of ROS was detected under a Leica DMI 3000B fluorescence microscope as a green signal (excitation wavelength 490 nm, emission wavelength 520 nm). The corresponding fluorescence intensity was analyzed using an Image-Pro Plus 6.0.

Cell Viability Determination
The cells were seeded inside multi-well dishes and covered with 200 μl of the medium. They were treated with gliotoxin dissolved in 100 μl DMEM for increasing time periods. Then MTT (microculture tetrazolium) was added with a final concentration of 0.25 mg/ml, and incubated at 37°C for 4 h. The medium was then removed and replaced with 150 μl of dimethylsulfoxide (DMSO). This was done in order to dissolve the blue formazan crystals. The absorbance at 490 nm was measured with a microplate reader. The data was then normalized to measurements from the control cultures which were considered to have a 100% cell survival Figure.

Statistical Analysis
The data shown in the Figures is from a representative experiment or from a mean ± SE of 3–4 independent experiments which were performed in triplicate. Except where otherwise indicated, a student’s paired t-test was used to compare the differences between the groups. Values of P < 0.05 were considered to be statistically significant.

Results

1. Deficiency in Gliotoxin Production of A. fumigatus was Associated with a Decrease in the Internalization of Conidia into A549 Epithelial Cells
We initially compared the internalization capacity into A549 epithelial cells among wild type A. fumigatus B5233, its mutant (gliPΔ), and the complemented strain gliPR. The gliP gene is the largest gene in the gene cluster for gliotoxin production in A. fumigatus. It catalyzes the first step in gliotoxin biosynthesis. A deletion of the gliP abolishes gliotoxin production in A. fumigatus (27). We incubated the three different conidia of A. fumigatus with the A549 cells at a multiplicity of infection (MOI) of 10 (conidia : cells) to detect the internalization efficiency. As illustrated in Figure 1A, the gliPΔ conidia internalization decreased about by 40% compared to the wild type conidia, whereas the conidia of gliPR internalized the A549 cells as effectively as the wild type strain. The similar result was also demonstrated in another airway epithelial cell, 16HBE14o human bronchial epithelial cells (Figure S1A). These results were a
bit surprising since it is known that gliotoxin is immuno-suppressive for several physiological processes. Therefore, as a control, we also checked the phagocytosis of these three different conidia by J774 macrophages. Compared to the conidia of wild type B5233, the conidia of the \(\text{gliP}\Delta\) mutant were engulfed by J774 macrophages significantly easier, about 60% increase. No significant difference in phagocytosis was found between the wild type and the \(\text{gliP}\text{R}\) \(\text{A. fumigatus}\). Similarly, it was found that the phagocytosis of \(\text{gliP}\Delta\) mutant was significantly higher than wild type B5233 by human THP-1 derived macrophage (Figure S1B). These data indicated that the gliotoxin produced by \(\text{A. fumigatus}\) was definitely an inhibitor on the phagocytosis of macrophages, which was in line with the previous reports; however, gliotoxin might be a positive factor for the internalization of \(\text{A. fumigatus}\) into the A549 epithelial cells.

![Figure 1. The deficiency of gliotoxin production of \(\text{A. fumigatus}\) reduces the internalization of conidia into epithelial cells.](image)

A549 cells (A) and J774 macrophages (B) were infected with the swollen conidia from the wild type, gliotoxin-production deficient mutant (\(\text{gliP}\Delta\)), and gliP-complemented mutant (\(\text{gliP}\text{R}\)) of \(\text{A. fumigatus}\) B5233, at an MOI of 10, respectively. The internalization of \(\text{A. fumigatus}\) into A549 cells and phagocytosis by J774 macrophages were analyzed with the nystatin protection assay. Differences in \(\text{A. fumigatus}\) internalization into A549 cells or phagocytosis by J774 macrophages between the wild type strain and the mutants were compared. Data were shown as the mean ± SE (n = 3–4). *\(P < 0.05\).
2. Gliotoxin Promoted the Internalization of *A. fumigatus* into A549 Epithelial Cells
Consequently, we investigated the effect of exogenous gliotoxin on the internalization of *A. fumigatus* into A549 epithelial cells, as well as its toxicity to the host cells. As shown in Figure 2A, gliotoxin promoted the conidial internalization of wild type B5233 *A. fumigatus* in a concentration-dependent manner, peaking at the concentration of 50 ng/ml and then declined. In 16HBE14o human bronchial epithelial cells, 10 ng/ml gliotoxin could obviously improve the internalization of conidial internalization of wild type B5233 *A. fumigatus* and this increase peaked at the concentration of 20 ng/ml (Figure S2A). The cell viability of A549 cells and 16HBE14o human bronchial epithelial cells was not affected by gliotoxin, even at a concentration of 100 ng/ml (Figure 2C and Figure S2C). In contrast, 20 ng/ml gliotoxin obviously suppressed the phagocytosis of the J774 macrophages to the conidia. This suppression was not altered by 50 ng/ml gliotoxin. In the presence of 100 ng/ml gliotoxin, the phagocytosis was inhibited even more significantly (Figure 2B); however, a cytotoxic effect on the *J774* macrophages was also observed under this concentration (Figure 2D). The similar inhibition on phagocytosis and unaffected cell viability were also found in human THP-1 derived macrophage (Figure S2B and S2D). Moreover, at the presence of 50 ng/ml gliotoxin the internalization capacity of the *gliPΔ* conidia into A549 epithelial cells was fully restored to the level of wild type B5233 conidia (Figure 2E). On the contrary, the increase of phagocytosis by J774 macrophages to *gliPΔ* conidia (compared to wild type conidia) was not fully blocked by the presence of 50 ng/ml gliotoxin (Figure 2F). Taken together, it can be concluded that gliotoxin might promote the internalization of *A. fumigatus* conidia into epithelial cells and suppress the phagocytosis by macrophages.
Figure 2. Gliotoxin enhances the internalization of the conidia of *A. fumigatus* into A549 epithelial cells.

A549 cells (A, C, E) and the J774 macrophages (B, D, F) were infected with the swollen conidia of wild type *A. fumigatus* B5233 or the gliotoxin-production deficient mutant (gliPΔ) at an MOI of 10 in the absence or presence of gliotoxin at the indicated concentrations. The internalization of *A. fumigatus* into A549 cells and phagocytosis by J774 macrophages was analyzed with the nystatin protection assay. Cell viability for both cells was measured with the MTT assay in parallel. Differences in *A. fumigatus* internalization, phagocytosis and host cell viability between the group without gliotoxin ("concentration"=0) and the other groups, and between the wild type strain and mutant, were then compared. Data were shown as the mean ± SE (n = 3–4). *P < 0.05.
3. Gliotoxin Suppressed ROS Production in A549 Epithelial Cells and J774 Macrophages

It has been reported that gliotoxin suppresses the ROS production of neutrophils. The inhibitory effect of gliotoxin on NADPH-oxidase is one of the anti-microbial activities that can be observed at micromolar concentrations (16). Here, we found that gliotoxin could significantly reduce (by about 4-fold) the release of superoxide in the epithelial cells at a rather low concentration of 50 ng/ml (Figure 3A). This inhibition became stronger at a concentration of 100 ng/ml. However, no further inhibition was found at 200 ng/ml. In contrast, 50 ng/ml gliotoxin was not enough to suppress the ROS release in J774 macrophages, and the significant suppression of ROS release by gliotoxin was found at a concentration of 100 ng/ml. Despite this, no further suppression was shown in J774 macrophage at a concentration of 200 ng/ml. These data implied that the lung epithelial cells might respond in a more sensitive way to gliotoxin inhibition on ROS production compared to macrophages and neutrophils.

Figure 3. The effect of gliotoxin on ROS production in A549 epithelial cells and J774 macrophages.

A549 cells (A) and the J774 macrophages (B) were incubated with gliotoxin at the indicated concentration for 30 min. The generation of intracellular ROS was then measured with the fluorescence assay. Differences in ROS generation between the groups without gliotoxin ("concentration"= 0) and the other groups were compared. Data were shown as the mean ± SE (n = 3-4). *P < 0.05.

4. Gliotoxin-induced A. fumigatus Internalization into A549 Epithelial Cells was Dependent on Host PLD Activity

The stimulation of PLD has been described in many cellular systems in response to a large variety of agonists (29). Host PLD activity is involved in the process of A. fumigatus internalization in lung epithelial cells (26). For these reasons, it was interesting to investigate whether gliotoxin-regulated A. fumigatus internalization is related to host PLD activity. First, we checked whether gliotoxin stimulates PLD activity in A549 lung epithelial cells. As shown in Figure 4A, gliotoxin did significantly induce PLD activation at a concentration of 10 ng/ml, about two times greater
than the untreated cells. This PLD activation became stronger as the gliotoxin concentration increased, and it reached a peak at a concentration of 50 ng/ml. In contrast, no obvious PLD activation was detected in J774 macrophages with gliotoxin stimulation (Figure 4B). All the treatments with gliotoxin did not affect the endogenous expression of the two PLD isoenzymes, PLD1 and PLD2 in A549 cells (Figure 4C).

**Figure 4. Gliotoxin-induced internalization is dependent on PLD activity in A549 cells.**

A549 cells (A) and J774 macrophages (B) were pre-labeled with [3H] oleic acid and then stimulated with gliotoxin at the indicated concentration for 30 min. Then, ethanol was added to determine the PLD activity (A, B). In parallel, the cells were lysed for immunoblotting with the indicated antibody. The densitometric analysis of the immunoblots for the three independent experiments was shown (C). A549 cells were incubated for 30 min with 2 nM VU0359595, a PLD1-specific inhibitor (D) or 100 nM VU0285655-1, a PLD2-specific inhibitor (E). Subsequently, the untreated or treated A549 cells were infected with swollen conidia of *A. fumigatus* B5233 at an MOI of 10 for 1 h in the absence or presence of 50 nM gliotoxin. The internalization of *A. fumigatus* conidia was analyzed with the nystatin protection assay. Differences in [3H] PtdEtOH formation or *A. fumigatus* internalization between the untreated (control) cells and the other groups of cells were compared. Data were shown as the mean ±SE (n = 3–4). *P < 0.05.
We further investigated the role of host PLD activity on gliotoxin-induced *A. fumigatus* internalization into A549 cells. A pretreatment of A549 cells with the PLD1-specific inhibitor VU0359995 was shown to completely block the increase of *A. fumigatus* internalization induced by 50 ng/ml gliotoxin (Figure 4D). In contrast, the pretreatment with the PLD2-specific inhibitor VU0285655-1 only partially inhibited the gliotoxin-induced increase of *A. fumigatus* internalization (Figure 4E). These data suggested that the gliotoxin-induced *A. fumigatus* internalization into A549 cells might be dependent on host PLD activation and PLD1 might play more critical role than PLD2 during this process.

5. Gliotoxin Induced Obvious Cell Morphology Alteration in the A549 Epithelial Cells

In terms of neutrophils, gliotoxin induced a significant re-organization of the actin cytoskeleton which collapsed around the nucleus leading to cell shrinkage and the disappearance of filopodia (17). In order to investigate the effect of gliotoxin on the cellular morphology of A549 cells, we stained the actin cytoskeleton with TRITC-phalloidin. The gliotoxin-untreated A549 cells showed a characteristic spread shape with lamellipodia at the cell periphery along with stress fiber (Figure 5A, A549 cells panel). In the presence of 10 ng/ml gliotoxin, a pronounced change in morphology was observed in more than 15% of the A549 cells (Figure 5A). The cells became rounded, and typical cell shrinkage was observed. This morphological change in the A549 cells became stronger as the gliotoxin concentration increased. However, there was no significant difference between 50 ng/ml and 100 ng/ml (Figure 5B). In contrast, the gliotoxin did not induce a significant morphological change in J774 macrophages, even at a concentration of 100 ng/ml (Figure 5A and 5C).

In order to clarify the effect of gliotoxin on host actin cytoskeleton rearrangement and distribution of the PLD1 enzyme in A549 cells, we labeled the F-actin with FITC-conjugated molecular probe and PLD1 with fluorescent antibody. The cell morphological alteration and the subcellular distribution of PLD1 were observed under confocal microscopy. As illustrated in Figure 5D, the actin cytoskeleton spreaded across the whole cell and formed lamellipodia and fibers (green) in the resting A549 cells. Meanwhile, PLD1 localized generally to the intracellular compartments and the plasma membrane (red). In the presence of 50 ng/ml gliotoxin, the A549 cells were compacted and shrunken. This was in sharp contrast to the resting cells. The F-actin fibers largely disappeared in cytosol and localized more to the plasma membrane (green), which was distinct from the untreated cells. The subcellular distribution of PLD1 was not significantly altered by gliotoxin treatment; however, it appeared that PLD1 became more distributed around the nucleus shown as large purple patches (merged by red and blue) which was different from the untreated cells. These images indicated that gliotoxin was able to induce obvious cell morphological change in the A549 epithelial cells, featuring strong actin cytoskeleton rearrangement, and a minor alteration of PLD1 subcellular distribution.
Figure 5. The effect of gliotoxin on the cell morphology is different between epithelial cells and macrophages.

A549 cells and J774 macrophages were treated with gliotoxin at the indicated concentrations for 30 min. The control samples were not treated. The cells were then fixed, permeabilized, and incubated with TRITC-coupled phalloidin to stain F-actin. They were then examined by fluorescence microscopy. A representative (×100-magnified) image for the three was shown (A). White arrows indicated some A549 cells with the typical morphological changes that occurred after the gliotoxin treatment. The A549 cells and J774 macrophages with clear morphological changes (shrinkage and compaction) were counted and quantified (B, C). Differences in cell morphological changes between the group without gliotoxin (“concentration” = 0) and the other groups were then compared. Data were shown as the mean ± SE (n = 3–4). *P < 0.05. D, A549 cells were treated with 50 ng/ml gliotoxin for 30 min. The control samples were not treated. Thereafter, the distribution of the actin filament and the PLD1 enzyme was monitored by immunofluorescence using the laser confocal microscopy Olympus FluoView FV1000 (green, actin filament; red, PLD1). The images were processed with Olympus Fluoview ver. 1.6. The merged fluorescence images were shown. Data were representative of 3 similar experiments. Scale bar, 10 μm.
Discussion

It had been thought that gliotoxin contributes to virulence by primarily targeting the activity of immune cells present in non-neutropenic mice, namely neutrophils or other phagocytes (9,27). However, less was known about the role of lung epithelial cells in the pathogenicity of gliotoxin. Our findings showed that the deficiency in gliotoxin-production of the \textit{A. fumigatus} mutant (\textit{gliPΔ}) resulted in less internalization of \textit{A. fumigatus} conidia into the A549 cells than the wild type strain (Figure 1) and by contrast, an increase of phagocytosis by J774 macrophages. At rather low concentrations ranging from 10–50 ng/ml which were not cytotoxic to the A549 cells, gliotoxin was shown to promote \textit{A. fumigatus} internalization into A549 cells in a dose-dependent manner but inhibit the macrophage phagocytosis (17,20,30,31). Interestingly, gliotoxin has been addressed as an important component associated with the hazards of indoor air pollution and exposure to inhaled moulds (14,20,32). The vast majority (>96%) of both environmental and clinical \textit{A. fumigatus} has the ability to produce gliotoxin (31). In the case of cystic fibrosis (CF), \textit{A. fumigatus} colonization is associated with increased gliotoxin in the bronchoalveolar lavage fluid (BALF) (33). On the other hand, it has been proven that gliotoxin is produced in experimental animal aspergillosis as well as in human IA, with serum concentrations of 166–785 ng/ml in 80% of patients with IA (30,34). Thus, concerning that the internalization of \textit{A. fumigatus} into the lung epithelium allows them to protect themselves from the phagocytosis by macrophages or neutrophils, it seemed physiologically feasible that a low amount of gliotoxin produced by \textit{A. fumigatus} from mouldy environments or lung secretions may help \textit{A. fumigatus} internalization and evade further immune attacks at the initial step of infection.

Another critical finding in the present study was that gliotoxin is able to stimulate the PLD activity of A549 lung epithelial cells. In this way, it can promote the efficient internalization of \textit{A. fumigatus} into the cells. This seemed to be complementary to another PLD signal pathway, β-1,3-glucan-dectin-1 cascade which was demonstrated in our previous report (26). To some extent, this finding was surprising because gliotoxin is a well-known immunosuppressive agent in many immune cells including neutrophils, macrophages, and thymocytes (14,35). It inhibits several important enzymes, such as NADPH oxidase of neutrophils (15,16), and the nuclear transcription factor NF-κB and 20S proteasome activity (35). However, gliotoxin was recently shown to activate the platelets (18). It was also reported that gliotoxin can increase the intracellular cAMP concentration (11), and induce Ca$^{2+}$ release from the mitochondria of intact rat livers (36,37). Both cAMP and Ca$^{2+}$ are the putative agonists for PLD stimulation in several signaling events (38,39), which may also be involved in a gliotoxin-induced PLD activation pathway. Therefore, PLD may be a novel intracellular target of gliotoxin. For this reason, the detailed mechanism for PLD activation by gliotoxin in epithelial cells should be investigated further.
Furthermore, the control of PLD activity on the gliotoxin-induced *A. fumigatus* internalization was in line with the previous findings that PLD is a critical regulator in many phagocytosis or internalization processes in response to a large variety of agonists. The regulation of PLD on pathogen internalization mostly resulted from its close interaction with host actin dynamic induced by the pathogens. In mammalian cells, elements of the actin dynamic system interact directly or indirectly with PLD enzymes and its related phospholipid metabolism (23,29,40). Inversely, the phosphatidic acid produced by PLD catalysis could induce stress fiber formation and deliberately regulate the formation of phagocytic cups (41). In the present study, gliotoxin induced significant actin cytoskeleton rearrangement (Figure 5) along with the promotion of *A. fumigatus* internalization. Therefore, the stimulated PLD activity by gliotoxin may interact with actin cytoskeleton rearrangement in order to control *A. fumigatus* internalization.

Compared to PLD2, PLD1 played a more important role in gliotoxin-induced *A. fumigatus* internalization into epithelial cells. This discrepancy might be gliotoxin-specific because it has been previously reported that both PLD1 and PLD2 contribute coordinately to phagosome formation and development during the internalization of *A. fumigatus* into A549 lung epithelial cells (24,26). Moreover, since the subcellular distribution of PLD1 was not significantly altered by gliotoxin in A549 cells (Figure 5), the gliotoxin-induced PLD activation may not be associated with the subcellular translocation of PLD1. Therefore, it is interesting to analyze the precise function and distribution of PLD isotypes during gliotoxin-induced *A. fumigatus* internalization.

Finally, gliotoxin significantly blocked the ROS production in A549 epithelial cells at low concentrations (from 10 to 100 ng/ml). In contrast, in human neutrophils, the inhibition of ROS production by gliotoxin has been reported only at higher concentrations (above 1 μg/ml (15, 16)) and no inhibition of NADPH-oxidase activity was observed at the low concentrations of gliotoxin (from 10 to 100 ng/ml) (17). As NADPH-oxidase is one of major modulators responding to oxidative stress in the cell, it can be deduced that NADPH oxidase complex of epithelial cells might be more sensitive to gliotoxin than that of J774 macrophages and neutrophils. In addition, as the epithelial cells constitute the first barrier against *A. fumigatus*, the low threshold value of gliotoxin concentration required for enough inhibition on ROS response in lung epithelial cells might facilitate the internalization and intracellular living of *A. fumigatus*.

In addition, concerning that the nystatin protection assay is an indirect and error-prone approach, we carefully repeated the internalization experiments, even for several times in order to get the reliable and stable results. We had also certified the correlation of internalization results between nystatin protection assay and immunofluorescence assay (Figure S3). Certainly, more accurate assay would be expected for further interpretation on the exact role of gliotoxin on *A. fumigatus* internalization into lung epithelium.

In conclusion, we reported an important finding that gliotoxin is able to regulate *A. fumigatus* internalization into epithelial cells by inducing host PLD activation. However, the
Gliotoxin promotes *Aspergillus fumigatus* internalization.

detailed function and regulatory mechanism of gliotoxin on *A. fumigatus* entry into epithelial cells need to be further clarified.

**Acknowledgements**

We would like to thank Dr. KJ. Kwon-Chung for kindly providing the various *A. fumigatus* strains. This project was supported by grants from 973 Program (No.2013CB531606) and from the Chinese National Scientific Foundation Committee (No.30772029 and No.81172801).
Supplementary Materials

Figure S1. The deficiency of gliotoxin production of A. fumigatus reduces the internalization of conidia into 16HBE14o human bronchial epithelial cells.

The 16HBE14o human bronchial epithelial cells (A) and human THP-1 derived macrophages (B) were infected with the swollen conidia from the wild type, gliotoxin-production deficient mutant (gliPΔ), and gliP-complemented mutant (gliPR) of A. fumigatus B5233, at an MOI of 10, respectively. The internalization of A. fumigatus into 16HBE14o human bronchial epithelial cells and phagocytosis by THP-1 derived macrophages were analyzed with the nystatin protection assay. Differences in A. fumigatus internalization into 16HBE14o human bronchial epithelial cells or phagocytosis by THP-1 derived macrophages between the wild type strain and the mutants were compared. Data were shown as the mean ± SE (n = 3–4). *P < 0.05.
Gliotoxin promotes Aspergillus fumigatus internalization.

Figure S2. Gliotoxin enhances the internalization of the conidia of A. fumigatus into 16HBE14o human bronchial epithelial cells.

The 16HBE14o human bronchial epithelial cells (A, C) and the THP-1 derived macrophages (B, D) were infected with the swollen conidia of wild type A. fumigatus BS233 at an MOI of 10 in the absence or presence of gliotoxin at the indicated concentrations. The internalization of A. fumigatus into 16HBE14o human bronchial epithelial cells and phagocytosis by THP-1 derived macrophages was analyzed with the nystatin protection assay. Cell viability for both cells was measured with the MTT assay in parallel. Differences in A. fumigatus internalization, phagocytosis and host cell viability between the group without gliotoxin (“concentration” = 0) and the other groups were then compared. Data were shown as the mean ± SE (n = 3–4). *P < 0.05.
Figure S3. Determination on the effect of gliotoxin on internalization of the conidia of *A. fumigatus* into epithelial cells with immunofluorescence assay.

The 16HBE14o human bronchial epithelial cells were infected with the swollen conidia of wild type *A. fumigatus* 13073 at an MOI of 10 in the absence or presence of gliotoxin at the indicated concentrations. The internalization of *A. fumigatus* into cells was analyzed with immunofluorescence assay. Differences in *A. fumigatus* internalization between the group without gliotoxin ("concentration" = 0) and the other groups were then compared. Data were shown as the mean ± SE (n = 3-4). *P < 0.05.*
Gliotoxin promotes *Aspergillus fumigatus* internalization.

**References**


