



Mycotoxin-induced depletion of intracellular glutathione and altered cytokine production in the human alveolar epithelial cell line A549[☆]

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Abstract

Mould exposure has been associated with asthma and other inflammatory airway conditions. However, cellular effects of inhaled mould components are not well understood. We hypothesised that host defence mechanisms, such as production of cytokines (TGFβ1, IL-6 and IL-8) and the intracellular antioxidant glutathione (GSH), could be adversely affected by different concentrations of mycotoxins. We studied the effects of citrinin and gliotoxin on lipopolysaccharide (LPS)-stimulated alveolar epithelial cells (A549). Cytokines in cell culture supernatants were analysed by ELISA and levels of GSH were measured by colorimetric (absorbance) determination. We found that GSH decreased in a dose- and time-dependent manner when cells were exposed to citrinin in particular. TGFβ1 was moderately reduced at low mycotoxin concentrations but elevated at higher sub-toxic concentrations. A tendency for an inverse relationship between TGFβ1 and GSH levels was observed. IL-6 and IL-8 were not significantly reduced at non-toxic mycotoxin concentrations. Thus, reduced epithelial GSH and TGFβ1 levels combined with elevated IL-6 and IL-8 levels may result in increased pro-inflammatory activity during mycotoxin exposure. We suggest that this mechanism can contribute to inflammation in mould exposure.

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1. Introduction

An association between indoor mould exposure and inflammatory airway responses such as asthma has frequently been reported (Douwes and Pearce, 2003; Norbäck et al., 1999; Peat et al., 1998; Zock et al., 2002).

The exposure situation is complicated by the presence of several different compounds produced by moulds, along with the presence of bacterial endotoxins (Thorn, 2001). Indoor moulds may be a source of potentially inflammatory, allergenic and toxic compounds, e.g. cell wall polysaccharides such as β-glucans, enzymes such as glucoamylases, allergenic proteins, and also several volatile organic compounds (MVOC's) that could act additively or synergistically to induce and increase inflammation and immune responses (Beijer et al., 2003; Kreja and Seidel, 2002; Mari et al., 2003; Rylander and Rong-Hwa, 2000). Some studies indicate that adverse health

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effects in mouldy buildings could in part be related to toxic metabolites (mycotoxins) produced by moulds (Bunger et al., 2005; Fischer and Dott, 2003; Jarvis and Miller, 2005). Several mycotoxins have been associated with health effects seen in humans due to mould exposure (Bennett and Klich, 2003). Common mould genera such as *Aspergillus* and *Penicillium* are frequently isolated from water-damaged buildings and, depending on growth conditions, they may produce mycotoxins. One mycotoxin that is often produced by *A. fumigatus* on water damaged building materials is the immunosuppressive compound gliotoxin (Nieminen et al., 2002; Schulz et al., 2004; Watanabe et al., 2004). Citrinin, which is known to be nephrotoxic, has been isolated from mouldy building materials with growth of *Penicillium* sp. and *Aspergillus* sp. among other mould species (Tuomi et al., 2000).

Host defence mechanisms against inhaled microbes include production of cytokines and chemokines by several different cell types including epithelial cells in the airway mucosa. The normal regulation of an immune reaction is dependent on the balance between pro-inflammatory mediators, such as IL-1, TNF- α , IL-6, IL-8 and IL-12, and regulatory cytokines such as IL-10 and TGF β 1. In this study, we explored the effects of mycotoxins on production of IL-6, IL-8 and TGF β 1. IL-6 is a pleiotropic pro- and anti-inflammatory cytokine involved in cell proliferation, antibody production and acute-phase protein formation. Depletion of IL-6 may also be important during pro-apoptotic processes (Hodge et al., 2002). IL-8 is a chemokine generally important in chemotaxis during inflammation. Production of transforming growth factor beta 1 (TGF β 1) is important in the development of several chronic lung diseases, including asthma (Howell and McAnulty, 2006; Redington et al., 1997; Tillie-Leblond et al., 1999). Altered TGF β 1 production has also been associated with chronic inflammatory pulmonary diseases with the induction of oxidative stress in alveolar epithelial cells (Mastruzzo et al., 2002). Normal host defence mechanisms include the constant production of relatively high amounts of the antioxidant glutathione (GSH). GSH is present in all mammalian cells and provides reducing equivalents for the redox-reaction catalysed by the reducing enzyme glutathione peroxidase. During this process GSH becomes oxidised glutathione (GSSG). The GSSG is then recycled into GSH by glutathione reductase and 2-nicotinamide adenine dinucleotide phosphate (NADPH). It has been shown that adequate production of GSH controls and regulates inflammatory processes in the lung (Rahman and MacNee, 2000). Hence, alteration in alveolar GSH pro-

duction is recognised as a central feature of progressive inflammatory lung diseases, such as asthma.

We hypothesised that mycotoxins may interfere with the normal levels of GSH and the balance of cytokines produced during inflammatory responses triggered by LPS. The aim of this study was to explore whether mycotoxins affect the production of some of the cytokines involved in lung inflammation (IL-6, IL-8 and TGF β 1) and also whether oxidative stress occurred in alveolar epithelial cells when they were exposed to mycotoxins.

2. Materials and methods

2.1. Experimental design

The human alveolar epithelial carcinoma cell line A549 (Lieber et al., 1976) was cultured with or without lipopolysaccharide (LPS) to induce production of IL-6, IL-8 and TGF β 1. After exposure to citrinin or gliotoxin, cytokine levels were analysed in cell culture supernatants. Further, cell viability was measured to provide information on whether or not LPS and mycotoxins had cytotoxic effects in the concentrations used. To investigate oxidative stress in cells when exposed to mycotoxins, intracellular reduced glutathione levels (GSH) and oxidised glutathione levels (GSSG) were determined.

2.2. Culture of alveolar epithelial cells

The cell line A549 (ATCC CCL-185) was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured at 37 °C and 5% CO₂ in Ham's F12K growth medium with L-glutamine (Gibco Life Tech., Eggenstein, Germany) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (EuroClone, Milano, Italy) and 0.5% gentamicin (Gibco).

2.3. Mycotoxins

Citrinin from *Penicillium citrinum* and gliotoxin from *Gliocladium virens* (Sigma–Aldrich, Steinheim, Germany) were dissolved in 96% ethanol and serially diluted in cell culture medium as described below. The final concentration of ethanol in the cell culture experiments was maximum 1% (v/v) for the highest mycotoxin concentrations.

2.4. Cell culture assay

A549 cells were adjusted to 10⁵ cells/mL and 400 μ L was added into three separate 24-well culture plates (Costar, Corning, NY, USA) for each of the mycotoxins. Cells were allowed to rest overnight at 37 °C and 5% CO₂. Then, 50 μ L lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* (Sigma) was added to a final concentration of 10 μ g/mL. The mycotoxins were added in concentrations ranging from 0 to 50 μ g/mL citrinin and 0 to 100 ng/mL gliotoxin in dilution

steps of 1:2. The exposed and non-exposed cells were incubated for 0 h, 1 h, 6 h, 24 h, 48 h and 72 h (for GSH and viability analysis) or 48 h and 72 h (for cytokine and viability analyses). All experiments were repeated on two (GSH) or three (cytokine) different occasions.

2.5. Analysis of cytokines

A quantitative sandwich ELISA cytoset (BioSource Europe, Nivelles, Belgium) was used for measurement of the cytokines IL-6, IL-8 and TGF β 1 in cell culture supernatants and the analysis was performed according to the manufacturer's recommendations. Briefly, 96-well ELISA plates (NUNC, Roskilde, Denmark) were coated with murine monoclonal anti-human IL-6 antibody, anti-human IL-8 antibody or anti-multispecies TGF β 1 antibody. After addition of the cell culture supernatants, incubation and washing, biotinylated murine anti-IL-6 antibody, anti-IL-8 antibody or anti-TGF β 1 antibody was added. Recombinant human IL-6, IL-8 and multispecies TGF β 1 proteins were used as standards. After incubation, HRP-streptavidine enzyme was added, followed by substrate solution containing tetramethylbenzidine (Sigma). The optical density was read in a microplate reader (BioRad, Hercules, CA, USA) at 450 nm. Concentrations of cytokines were calculated from standards ranging from 1000 to 7.8 pg/mL (IL-6), 800 to 7.8 pg/mL (IL-8) or 4000 to 7.8 pg/mL (TGF β 1) in dilution steps of 1:2. The results are presented as mean and standard deviation (S.D.) of duplicate ELISA samples (two samples from each of three separate culture plates in each of three independent experiments).

2.6. Cell viability testing

To investigate the time- and dose-dependent effects of the mycotoxins on A549 cells, Alamar Blue dye (BioSource) was used as recommended by the manufacturer. Cells were incubated with increasing concentrations of mycotoxins (with or without LPS) for 0 h, 1 h, 6 h, 24 h, 48 h and 72 h (GSH analysis) or 48 h and 72 h (cytokine analysis). Cells in the culture plates were spun down, and the cells were provided with fresh culture media before 10% Alamar Blue dye was added and the cultures were incubated further for 3 h at 37 °C and 5% CO $_2$. Change in cell metabolic activity was registered as a colour change in the wells. Aliquots of 100 μ L were taken from cell culture supernatants and added into 96-well plates (Costar), and fluorometric determinations were made in a Fluoroscanner II plate reader (Lab-systems, Stockholm, Sweden) at emission wavelength 590 nm and excitation wavelength 544 nm. Cell viability after mycotoxin/LPS exposure was calculated as percent of controls (cells with no mycotoxin added).

2.7. GSH/GSSG assay

Analysis of GSH and GSSG levels was performed according to a modified form of the Tietze method (Tietze, 1969) by using the Bioxytech GSH/GSSG-412 kit (Oxis Research,

Portland, OR, USA) which utilises the change in colour development during the reaction. The reaction rate is proportional to the GSH or GSSG concentrations in each sample. Experiments were performed according to the manufacturer's recommendations, with slight modifications. Briefly, cells were exposed to mycotoxins (with or without LPS) as described above. After 0 h, 1 h, 6 h, 24 h, 48 h or 72 h incubation, cell culture plates were centrifuged at 500 $\times$ g for 5 min and supernatants were gently removed without removing unattached cells in the cell culture. Then, 100 μ L ice cold PBS was added to the wells, and additionally 10 μ L of scavenger reagent (M2VP) was added to the GSSG samples to scavenge GSH and prevent oxidation of GSH and hence an overestimation of GSSG in the samples. Cell culture plates were then immediately frozen at –80 °C until GSH/GSSG analysis. Before analysis, samples were thawed for 2 min, and 300 μ L ice cold 5% metaphosphoric acid (MPA) (Sigma) was added. Then, the samples were sonicated in the cell culture plates for at least 15 min keeping the water in the sonicator cold by adding ice. The lysed cells were mixed gently and the culture plates were centrifuged for 10 min at 1000 $\times$ g and 4 °C before adding 50 μ L of MPA extract (cell culture supernatant) to 700 μ L of ice-cold GSSG or GSH buffer provided with the kit. Final dilution of samples was 1:60. The samples were kept on ice until final preparation and analysis of absorbance at 412 nm in a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan).

2.8. Data presentation and statistical analysis

Analysis of cytokines, GSH/GSSG and viability are presented as means and standard deviation (S.D.) from two (GSH/GSSG with viability testing) or three (cytokine with viability testing) separate experiments. Statistical analysis was performed using unpaired two-tailed Student's *t*-test to compare the exposed cultures (LPS/mycotoxin) with the non-exposed cultures (no mycotoxin). For comparison of relative TGF β 1 and GSH curves, a two-way analysis of variance of means (ANOVA) was used. A probability value of 0.05 or less was considered statistically significant in all experiments.

3. Results

3.1. Cell viability

Cell viability after mycotoxin exposure was determined by the Alamar Blue dye method. The mycotoxins generally reduced cell viability in a dose- and time-dependent manner both for citrinin and gliotoxin, as seen in Figs. 1 and 2. Citrinin at 50 μ g/mL reduced cell viability to 80% of control values after 6 h incubation (Fig. 1C) and after 24 h incubation, viability was reduced to approximately 40% of control values (Fig. 1D). The majority of cells did not survive 50 μ g/mL citrinin after 72 h incubation as cell viability dropped to approximately 20% of control values (Fig. 1F). Cell viability

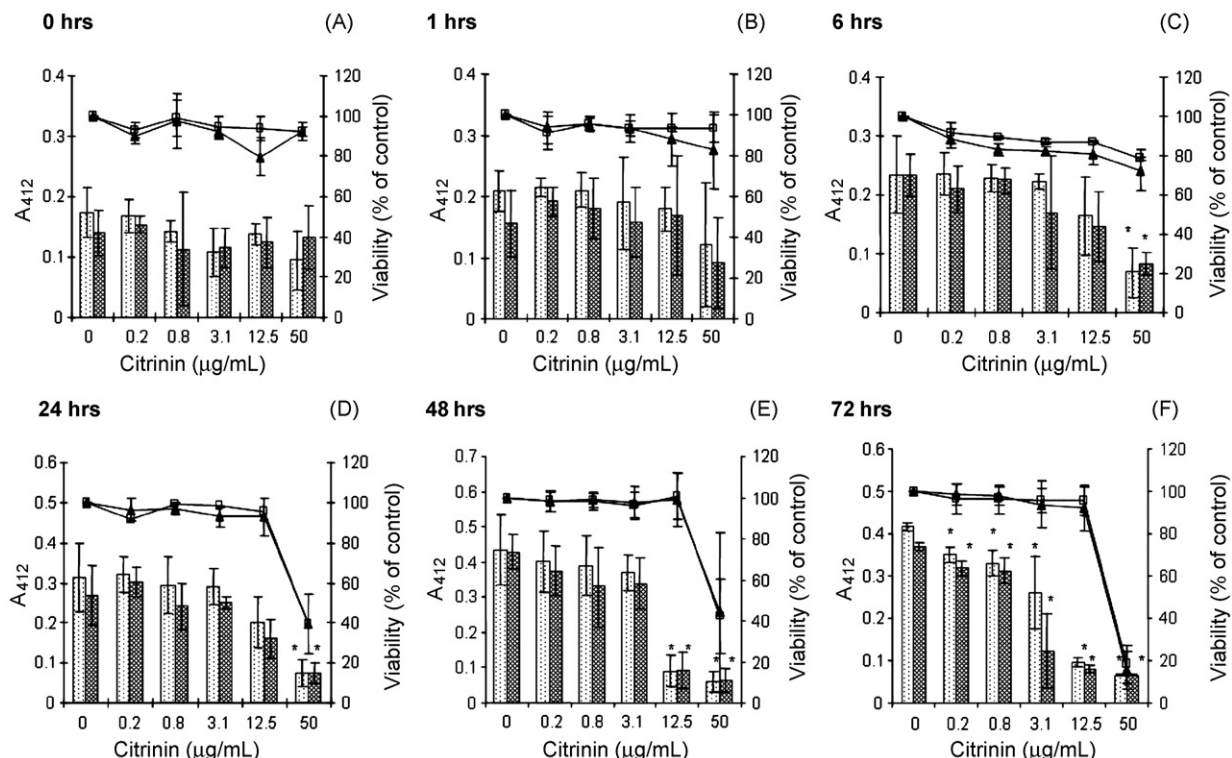


Fig. 1. GSH levels (A_{412}) and cell viability of A549 cells after incubation for 0 h (A), 1 h (B), 6 h (C), 24 h (D), 48 h (E) and 72 h (F) with or without *Pseudomonas aeruginosa* LPS (10 µg/mL) and different concentrations of citrinin. GSH values are given as net absorbance (subtraction of blanks) at 412 nm and are the means $\pm$ S.D. of two separate experiments. (*) Significant decreased GSH levels compared to control ($P < 0.05$). (□) GSH (without LPS); (▨) GSH (with LPS); (▲) viability (without LPS); (□) viability (with LPS).

was reduced to approximately 40% after 6 h (Fig. 2C) and to approximately 20% after 24 h and 48 h when cells were incubated with 100 ng/mL gliotoxin (Fig. 2D and E). In contrast, cell viability was not significantly reduced after incubation with 100 ng/mL gliotoxin for 72 h (Fig. 2F). Exposure to mycotoxin with LPS in the cell culture medium did not reduce cell viability to a greater extent than mycotoxin without LPS. Ethanol (as solvent for the mycotoxins) in the concentrations used (maximum 1% (v/v)) did not exert any significant effect on cell viability (results not shown).

3.2. GSH production and cell viability

A time-dependent increase in relative GSH levels in non-exposed control cultures was observed (Figs. 1 and 2). With increasing doses of mycotoxins, GSH levels were reduced. Notably, for citrinin, a significant reduction of GSH occurred at concentrations below toxic doses at incubation times 48 h and 72 h (Fig. 1E and F), while this was not seen for gliotoxin (Fig. 2E and F). GSH levels were not significantly affected by LPS

alone. The oxidised form of glutathione (GSSG) was not increased with decreasing GSH levels, and GSSG levels were not elevated above baseline levels at any mycotoxin concentrations (results not shown). Ethanol (as solvent for the mycotoxins) in the concentrations used (maximum 1% (v/v) and maximum 72 h incubation) did not exert any significant effect on GSH levels (results not shown).

3.3. Cytokine production and cell viability

Production of IL-6, IL-8 and TGF β 1 from A549 cells was induced by the presence of *P. aeruginosa* LPS (Figs. 3 and 4). In contrast, LPS from *Escherichia coli* (serotype 026:B6) did not induce detectable amounts of IL-6, IL-8 or TGF β 1 (results not shown). None of the LPS preparations induced detectable production of TNF- α , IL-12, IL-10 or IL-4 from A549 cells (results not shown). Mycotoxin exposure without LPS did not induce cytokine production above baseline levels at the concentrations used (Fig. 3; results not shown for IL-6/IL-8 analysis in Fig. 4).

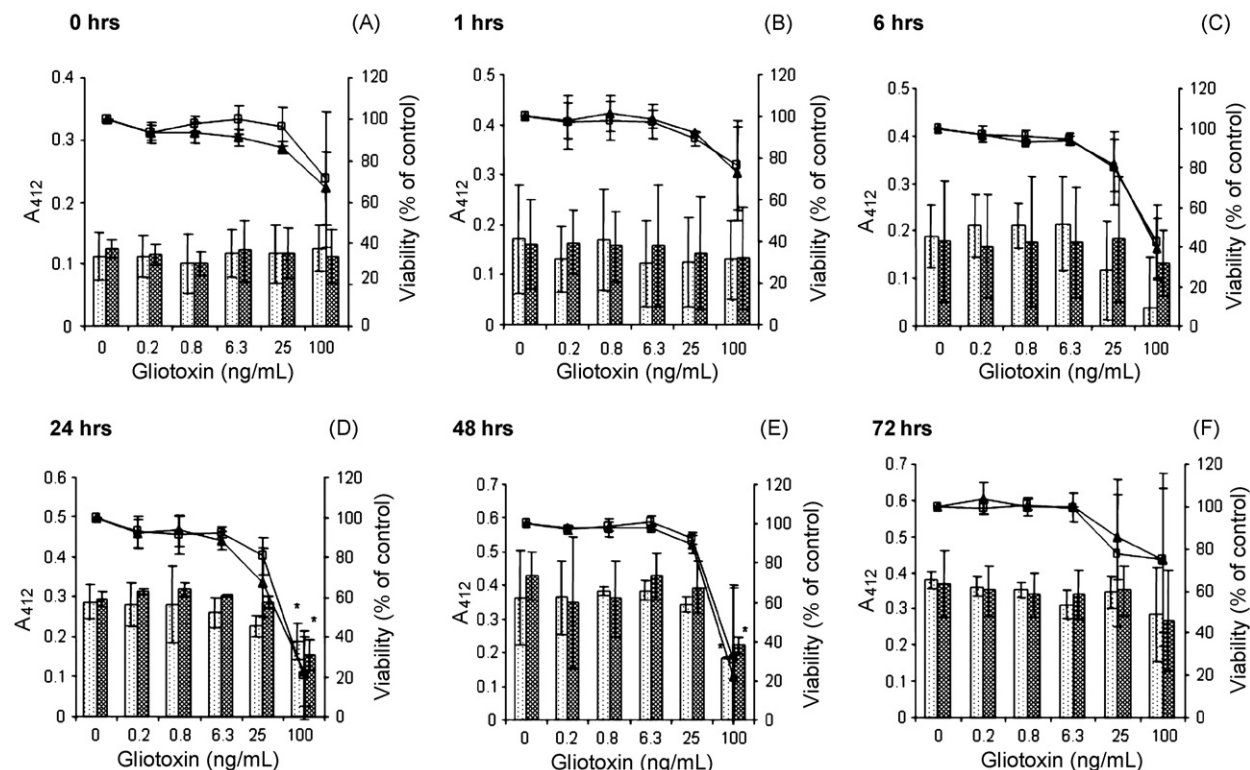


Fig. 2. GSH levels (A_{412}) and cell viability of A549 cells after incubation for 0 h (A), 1 h (B), 6 h (C), 24 h (D), 48 h (E) and 72 h (F) with or without *P. aeruginosa* LPS (10 μ g/mL) and different concentrations of gliotoxin. GSH values are given as net absorbance (subtraction of blanks) at 412 nm and are the means $\pm$ S.D. of two separate experiments. (*) Significant decreased GSH levels compared to control ($P < 0.05$). (▨) GSH (without LPS); (▤) GSH (with LPS); (▲) viability (without LPS); (□) viability (with LPS).

3.3.1. TGF β 1

Although not consistently statistically significant, TGF β 1 levels seemed to be overall reduced at the lower mycotoxin concentration range (below 0.8 μ g/mL citrinin and below 1.6 ng/mL gliotoxin) as shown in Fig. 3. A tendency to increased TGF β 1 levels was consistently observed at higher mycotoxin concentrations (1.6–12.5 μ g/mL citrinin and 3.1–50 ng/mL gliotoxin) in the presence of LPS. Citrinin at 25 μ g/mL (with and without LPS) and 48 h incubation reduced TGF β 1 abruptly along with a small reduction of cell viability to approximately 90% of control values. Citrinin at 50 μ g/mL significantly reduced TGF β 1 levels both after 48 h and 72 h along with reduction of cell viability to about 40% and 20% of control values. The highest concentration of gliotoxin (100 ng/mL) did not reduce TGF β 1, in contrast to the findings with citrinin.

3.3.2. IL-6 and IL-8

The concentration of IL-6 was reduced after 48 h incubation when cells were exposed to 25 μ g/mL cit-

rinin in the presence of LPS while cell viability was reduced to about 90% (Fig. 4A). The highest concentration of citrinin (50 μ g/mL) reduced IL-6 levels with a simultaneous reduction of cell viability to approximately 40%. A similar tendency was observed at 72 h incubation, although not statistically significant. Gliotoxin at 50 ng/mL and 48 h incubation reduced IL-6 levels significantly (Fig. 4B), while gliotoxin at the highest concentration after 72 h incubation did not reduce IL-6 levels significantly. IL-8 was significantly reduced after 72 h incubation with 25 μ g/mL citrinin, with a simultaneous reduction of cell viability to approximately 80% (Fig. 4A), while 50 μ g/mL citrinin reduced IL-8 along with loss of cell viability to about 40% of control values after 48 h and to about 20% after 72 h incubation. Gliotoxin reduced IL-8 production significantly at 100 ng/mL after 48 h and caused reduction in cell viability to approximately 40% of control values (Fig. 4B). IL-8 was not reduced after 72 h incubation with any of the gliotoxin concentrations, and also cell culture viability was maintained after 72 h.

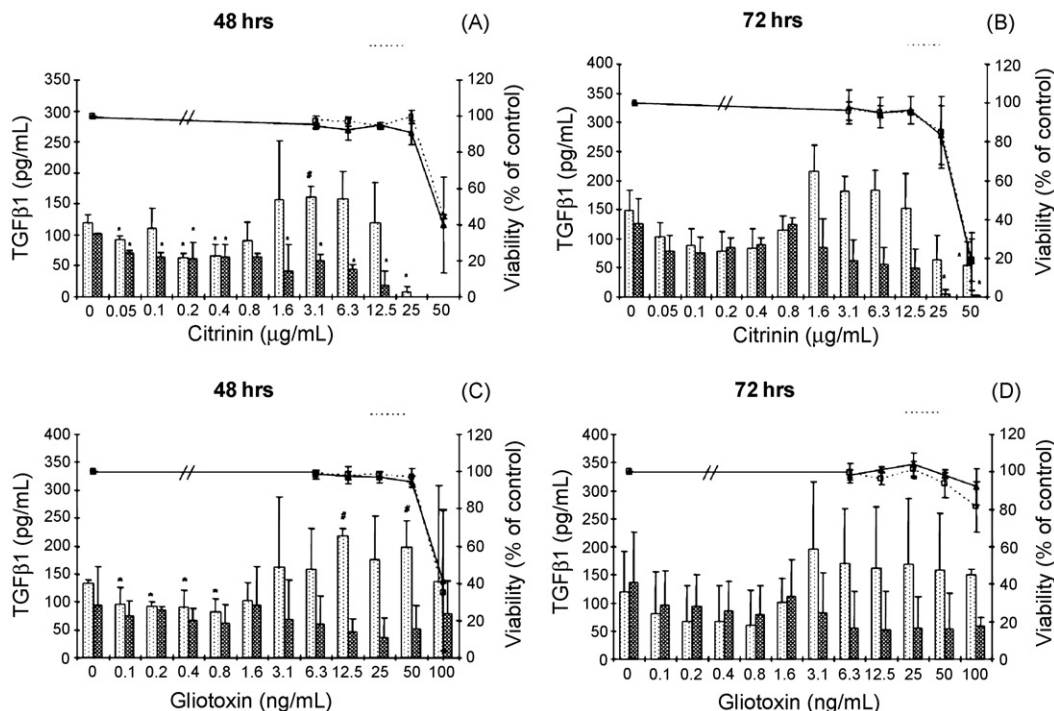


Fig. 3. TGFβ1 production and cell viability of A549 cells after incubation for 48 h or 72 h with or without *P. aeruginosa* LPS (10 μg/mL) and different concentrations of citrinin (A and B) or gliotoxin (C and D). Values are the means ± S.D. of three separate experiments. (*) Significantly decreased TGFβ1 levels compared to control ($P < 0.05$). (#) Significantly increased TGFβ1 levels compared to control ($P < 0.05$). (.....) TGFβ1 (with LPS); (.....) TGFβ1 (without LPS); (▲) viability (with LPS); (□) viability (without LPS).

3.4. Inverse co-variation between TGFβ1 and GSH

Fig. 5 shows the relative (compared to control) levels of GSH and TGFβ1 after 48 h or 72 h incubation of A549 cells with mycotoxin and LPS. A tendency to an inverse co-variation between GSH and TGFβ1 can be observed. Comparison of GSH curves with TGFβ1 curves at citrinin concentration steps identical to those used in the GSH assay, shows a significant difference between the curves at concentrations from 0.8 to 12.5 μg/mL (Fig. 5A and B). In the lower concentration range (below 0.8 μg/mL citrinin), relative GSH levels were higher than relative amounts of TGFβ1. The highest dose of citrinin (50 μg/mL) reduced both GSH and TGFβ1 corresponding to decreased cell viability. At 0.8–3.1 μg/mL citrinin, TGFβ1 levels increased while GSH levels were decreased. Gliotoxin did not affect GSH and TGFβ1 levels in the same manner as citrinin. However, concentrations from 0.8 to 100 ng/mL gliotoxin seemed to increase TGFβ1 slightly (Fig. 5C and D), while GSH was not significantly affected by gliotoxin in this concentration range.

4. Discussion

The mycotoxins gliotoxin and citrinin are produced by common indoor moulds such as *Aspergillus* and *Penicillium*. These toxins have been shown to exhibit diverse cellular effects, including cytotoxicity. The aim of this study was to investigate the dose- and time-dependent effects of citrinin and gliotoxin on the alveolar epithelial cell line A549. Both citrinin and gliotoxin are known to be toxic to various types of cells, and the cytotoxicity is known to be due to apoptosis (Johannessen et al., 2005; Waring et al., 1988; Yu et al., 2006; Zhou et al., 2000). Apoptosis is an efficient cellular mechanism to remove damaged cells without causing sustained organ inflammation. However, very high doses of these mycotoxins will kill A549 cells directly by necrosis (unpublished observations). Our hypothesis was that non-cytotoxic concentrations of mycotoxins would affect intracellular oxidant/antioxidant balance mechanisms and/or interfere with inflammatory cytokine responses triggered by LPS.

We found that the mycotoxins, in a dose- and time-dependent manner, decreased cell viability of A549 cells.

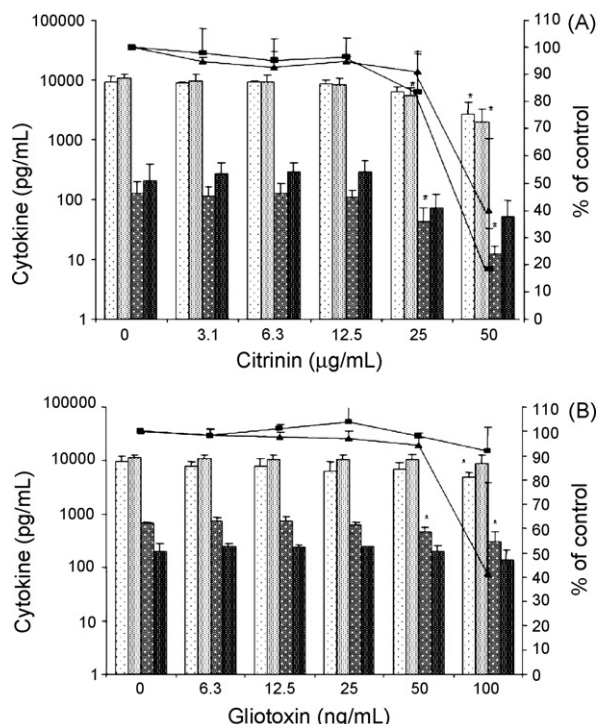


Fig. 4. IL-6 and IL-8 production and cell viability of A549 cells after incubation for 48 h or 72 h with *P. aeruginosa* LPS (10 µg/mL) and different concentrations of citrinin (A) or gliotoxin (B). Values are the means $\pm$ S.D. of three separate experiments. (*) Significant reduction compared to control ($P < 0.05$). (□) IL-8 (48 h); (▨) IL-8 (72 h); (▤) IL-6 (48 h); (■) IL-6 (72 h); (▲) viability (48 h); (■) viability (72 h).

After 6 h incubation, viability was reduced to 80% (citrinin) and to approximately 40% (gliotoxin) when the highest mycotoxin concentrations were used (50 µg/mL citrinin or 100 ng/mL gliotoxin). After 72 h incubation, 50 µg/mL citrinin reduced cell viability to about 20% of control values. Gliotoxin at 100 ng/mL seemed not to be completely cytotoxic to A549 cells as we observed a population of surviving cells after 72 h incubation. Doubling time of A549 cells is about 22 h (Lieber et al., 1976) and we suggest that some cells after 72 h may have proliferated, hence the maintenance of cell culture viability. Possibly, this indicates some heterogeneity of the cell line, with selection of gliotoxin-resistant variants.

We now tested if oxidative stress could be induced by sub-toxic doses of the mycotoxins. The levels of reduced glutathione (GSH) and oxidised glutathione (GSSG) were measured in relation to dose and time of exposure for both mycotoxins. Under normal conditions, cellular redox- and defence-mechanisms include action of regulatory enzymes keeping the oxidised form of glutathione (GSSG) at low intracellular levels, while the reduced form of glutathione (GSH) is produced in high

amounts (Rahman and MacNee, 2000). GSH depletion is in some cases associated with GSSG elevation and this is known to occur in response to some oxidative stimuli, such as H_2O_2 , while GSH depletion in parallel with unchanged and low GSSG levels have been described as a response to other oxidative stimuli, such as cigarette smoke condensate (CSC) (Rahman et al., 1995). First, we observed a time-dependent increase of GSH levels in the non-exposed control cultures, suggesting a time-dependent increase of cell density. Then, we found that citrinin, in a dose- and time-dependent manner, reduced GSH levels before any extensive loss of cell viability, while GSH reduction by gliotoxin was minimal before loss of cell viability. Neither of the mycotoxins caused elevated GSSG levels above baseline. Reactive oxygen species (ROS) may cause direct oxidising of GSH and form GSSG, while other types of oxidative agents may form conjugates with GSH, and temporarily prevent re-synthesis of GSH rather than elevating the intracellular GSSG levels (Rahman et al., 1995). We speculate that GSH may counteract the damaging effects of citrinin by forming GSH-citrinin conjugates, although this has to be confirmed by further studies. Such formation of GSH-citrinin conjugates has to our knowledge not been described in the literature. Recently, it has been suggested that apoptosis in human promyelocytic leukemia cells after citrinin exposure is not associated with formation of cellular oxidative stress (Yu et al., 2006). Here, we report that citrinin significantly reduce the antioxidant glutathione at sub-toxic doses, therefore we suggest that induction of oxidative stress may be part of citrinin cytotoxicity in alveolar epithelial cells.

Gliotoxin exists naturally in an oxidised form and cellular uptake is a glutathione-dependent mechanism, as shown in a murine tumour cell line (Bernardo et al., 2003). The toxin is rapidly reduced by GSH after cellular internalisation. However, induction of reactive oxygen species (ROS) followed by apoptosis have been observed in many cell types, including renal cells at a relatively high dose of gliotoxin (1200 ng/mL) (Zhou et al., 2000). We observed no decrease in GSH levels at concentrations below 100 ng/mL gliotoxin, indicating that no extensive oxidative stress occurs in gliotoxin-exposed alveolar epithelial cells below toxic concentrations.

We wanted to test if the mycotoxins affected the release of cytokines from epithelial cells triggered by LPS. First, we tested the ability of LPS from *E. coli* (serotype 026:B6) to trigger cytokine release, and then LPS from *P. aeruginosa*. No cytokine production was detected with LPS from *E. coli*. We found that LPS from *P. aeruginosa* at a relatively high dose (10 µg/mL) stimulated A549 cells to release IL-6, IL-8 and TGFβ1, but not

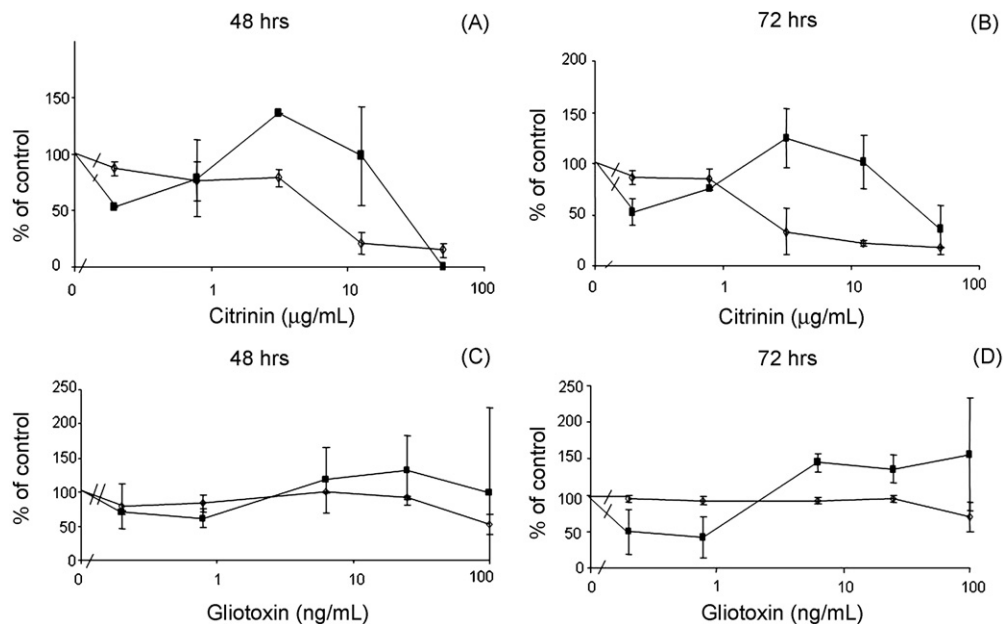


Fig. 5. Relative GSH (◇) and TGFβ1 (■) production from A549 cells after incubation for 48 h or 72 h with *P. aeruginosa* LPS (10 μg/mL) and different concentrations of citrinin (A and B) or gliotoxin (C and D). Statistical calculations were performed by two-way ANOVA.

TNF-α, IL-12, IL-10 or IL-4. Previously, similar results on the ability of different LPS preparations to stimulate A549 cells have been reported (Koyama et al., 2000). It is likely that different bacterial LPS have somewhat different potentials to cause inflammatory reactions in various cells and organs. Thus, *P. aeruginosa*, being a pathogen of the lung, may be more likely than *E. coli* to induce LPS-induced inflammatory reactions in alveolar epithelial cells.

We found that the pleiotropic cytokine TGFβ1 and the pro-inflammatory cytokines IL-6 and IL-8 were affected by increasing mycotoxin doses, although IL-6 and IL-8 largely remained elevated at non-toxic concentrations. IL-6 was slightly reduced at sub-toxic doses of 25 μg/mL citrinin and at 50 ng/mL gliotoxin after 48 h incubation, while IL-8 remained elevated until the highest mycotoxin concentrations. Although not consistently significant, we found that TGFβ1 was slightly reduced at the lower mycotoxin concentration range, while a tendency to increased levels was observed at higher mycotoxin doses. Asthma, and other inflammatory diseases, is characterised by high levels of bio-active TGFβ1 *in vivo* (Redington et al., 1997), but also by even higher levels of pro-inflammatory cytokines (Tillie-Leblond et al., 1999).

Regulation of TGFβ1 expression in the cell still remains largely unclear. Epithelial TGFβ1 production may not depend on the induction of pro-inflammatory mediators such as TNF-α, IL-1β and IL-8, but rather

TGFβ1 production may involve pro-inflammation independent pathways (Kwong et al., 2004). Interestingly, we found that there seems to be an inverse co-variation between levels of GSH and production of TGFβ1 at sub-toxic mycotoxin concentrations. This effect was most clearly seen with citrinin. At low citrinin concentrations (<0.8 μg/mL) GSH levels were relatively higher than TGFβ1. At higher citrinin concentrations (from 3.1 to 12.5 μg/mL citrinin) TGFβ1 was elevated along with a corresponding decrease in GSH.

Previously, it has been reported that TGFβ1 is a potent inhibitor of GSH, and that GSH depletion in A549 is executed by TGFβ1 by transcriptional down-regulation of the enzyme responsible for GSH formation, γ-glutamylcysteine synthetase (Arsalene et al., 1997; Jardine et al., 2002). Although it seems that citrinin reduces GSH in parallel to elevated TGFβ1 production in our experiments, it cannot be ruled out that TGFβ1 may be the true inhibitor of GSH. In this context, elevated TGFβ1 may be the response to sub-toxic doses of citrinin, while depletion of GSH will be the indirect consequence. However, this cannot explain the situation after gliotoxin exposure as non-toxic doses did not deplete GSH levels significantly, in spite of elevated TGFβ1 production.

Our experiments showed that increased TGFβ1 production occurred at relatively high mycotoxin concentrations, although well below concentrations causing extensive cell death. Previously, it has been shown that

human bronchial epithelial cells that underwent apoptosis up-regulated TGF β 1, while IL-6 was down-regulated (Hodge et al., 2002). Cytotoxicity in A549 cells is likely to proceed by apoptosis and the elevated TGF β 1 levels and the decreased IL-6 observed may have been pro-apoptotic effects of the mycotoxins. However, at prolonged incubation times, the tested sub-toxic exposure doses did not cause further reduction of cell viability, as would have been expected with an induction of apoptosis, but rather cell viability remained constant after 48 h and 72 h incubation.

The mechanisms behind health effects caused by inhalation of toxigenic mould spores are yet to be further investigated. In this study we demonstrated that citrinin, but not gliotoxin, reduced glutathione levels in A549 cells at non-toxic concentrations. An inverse co-variation between TGF β 1 and GSH levels was also demonstrated. Further, the levels of IL-6 and IL-8 were largely maintained in the non-toxic concentration range.

The levels of airborne mycotoxins that readily may be inhaled in mouldy environments must be assumed to vary depending on the amount of toxigenic moulds present. However, in a case of favourable growth of toxigenic moulds, we speculate that the observations in this study may reflect an ability of gliotoxin and citrinin in particular, to render human alveolar epithelial cells susceptible to inflammatory trigger agents in the environment, such as other mould components and bacterial LPS. Also, a susceptibility to environmental oxidative agents may develop. We suggest that this may facilitate asthma and other inflammatory lung diseases experienced by individuals in mouldy environments.

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