The Enteric Nerve System as Target of Regulated and Emerging Food-Associated Mycotoxins

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Abstract: Food and feed are frequently contaminated by numerous regulated and emerging mycotoxins. Humans and animals are thus exposed daily to mycotoxins through the oral route making of the gut the first and the more exposed tissue. Although many studies have evaluated and demonstrated the impact of mycotoxins on the intestinal epithelial cells (IECs) and on the brain cells, surprisingly only few studies have investigated their impact on cells of the enteric nerve system (ENS). In the present work, we measured the impact of major regulated and emerging mycotoxins (18 mycotoxins in total) on the proliferation and viability of normal rat enteric glial cells (EGCs) in vitro. On the 18 mycotoxins tested, 12 were found toxic with anti-proliferative effects observed at doses ranging from 0.19 to 118 µM and 0.4 to 59.59 µM, respectively. It can be concluded that alterations of the EGCs caused by at least some mycotoxins may participate in their global impact on the gut and the full organism.

Keywords: food safety; food contaminants; mycotoxins; emerging mycotoxins; enteric nerve system; enteric glial cells; cyclohexadepsipeptide

Key Contribution: Mycotoxins are able to affect proliferation and viability of enteric glial cells suggesting the implication of alteration of ENS in mycotoxicosis.

1. Introduction

Mycotoxins are deleterious secondary metabolites produced by various molds belonging mainly to Aspergillus, Penicillium or Fusarium species and able to colonize plants and crops [1–4]. Food and feed are thus frequently contaminated by numerous regulated and emerging mycotoxins, humans and animals being exposed daily to those toxins through the oral route [5,6]. As a consequence, the intestine is the first and the more exposed tissue to food-associated mycotoxins. The impact of mycotoxins (regulated and emerging ones) on the viability and functions of intestinal epithelial cells (IECs) has been extensively investigated (for review [7–14]). Similarly, although less data are available compared to IECs, the impact of mycotoxins on viability and functions of brain cells has been evaluated and described (for review [12,13–21]). Although the enteric nerve system (ENS) similarly to IECs may be exposed to high doses of mycotoxins, surprisingly only few studies have investigated the impact of those toxins on cells of this system ([22–24] and for review [25]). Even less is known about the impact of mycotoxins on enteric glial cells (EGCs). EGCs are key players in the ENS formation, homeostasis and functions as...
well as more generally in gut physiology. For example, the crosstalk between EGCs and IECs plays a role in the establishment and maintain of the intestinal barrier and immune functions (for review [26–31]). In their pioneer study, Rissato et al. using rats as in vivo model were the first and only one to evaluate and demonstrate the impact of a mycotoxin (i.e., deoxynivalenol) on EGCs [32]. Based on that ascertainment, in the present work, we evaluated in vitro the impact on normal rat EGCs of 18 mycotoxins (regulated or emerging ones) known or suspected to be toxic for humans or animals and frequently found in food and feed [5]. The 6 regulated toxins tested were aflatoxin B1 (AFB1), deoxynivalenol (DON), fumonisin B1 (FB1), ochratoxin A (OTA), patulin (PAT), and zearalenone (ZEA). The 12 emerging mycotoxins tested were apicidin (API), aurofusarin (AFN), beauvericin (BEA), brevianamide-F (BRV-F), cyclo-(L-Pro-L-Tyr) (CYCLO), emodin (EMO), enniatins (ENN) (A, A1, B, B1), moniliformin (MON), and tryptophol (TRPT). Data shown that 12 out of the 18 mycotoxins tested are toxic to normal rat EGCs affecting their division and/or viability at low to high doses, suggesting a potential role for mycotoxin-induced alterations of EGCs in mycotoxicosis, API, DON, and cyclohexadepsipeptides mycotoxins (i.e., ENNs and BEA) being the more toxic.

2. Results

2.1. Impact of Regulated and Emerging Mycotoxins on EGC Proliferation

In a first series of experiments, anti-proliferative effect of mycotoxins was evaluated. Dividing normal rat EGCs were exposed to increasing concentrations of mycotoxins for 48 h before estimation of cell density using resazurin assay and determination of the IC_{50} (Figure 1 and Table 1). Among the 6 regulated mycotoxins tested, only 4, i.e., DON, OTA, PAT, and ZEA were able to inhibit the division of normal rat EGCs with IC_{50} (Figure 1A). The cyclohexadepsipeptides ENNs and BEA were all found very active with IC_{50} values ranging from 0.19 ± 0.07; 17.53 ± 7.78; 10.78 ± 1.38 and 118 ± 5.75 µM, respectively. The two other regulated mycotoxins tested (i.e., AFB1 and FB1) were found inactive, even at 100 µM (Figure 1A). The cyclohexadepsipeptides ENNs and BEA were all found very active with IC_{50} values ranging from 0.92 ± 1.07 to 1.41 ± 0.20 µM (Figure 1B). Finally, regarding the other emerging mycotoxins, AFN, API and EMO were found anti-proliferative with IC_{50} values of 79.51 ± 3.68; 1.25 ± 0.17 and 40.01 ± 2.70 µM, respectively, whereas CYCLO, BRV-F, MON and TRPT were inactive up to 100 µM (Figure 1C).

Table 1. Inhibitory effect of mycotoxins on proliferation and viability of normal rat EGCs. Normal rat EGCs were exposed to increasing concentrations of mycotoxins for 48 h before determination of their inhibitory effect on proliferation or cell viability. IC_{50} values were determined from Figures 1 and 2 using GraphPad® Prism 7 and are expressed as means ± S.D. (in µM).

|       | AFB1 | DON | FB1 | OTA | PAT | ZEA |
|-------|------|-----|-----|-----|-----|-----|
| Proliferation | >100 | 0.19 ± 0.07 | >100 | 17.53 ± 7.78 | 10.78 ± 1.38 | 118 ± 5.75 |
| Viability | >100 | 5.06 ± 0.48 | >100 | 23.88 ± 1.36 | 38.02 ± 11.37 | 31.75 ± 4.94 |

|       | BEA | ENN A | ENN A1 | ENN B | ENN B1 | CYCLO |
|-------|-----|-------|--------|-------|--------|-------|
| Proliferation | 1.41 ± 0.20 | 0.93 ± 0.13 | 1.08 ± 0.34 | 1.40 ± 0.18 | 0.92 ± 1.07 | >100 |
| Viability | 1.91 ± 0.45 | 1.05 ± 0.11 | 0.86 ± 0.09 | 0.72 ± 0.16 | 2.14 ± 0.17 | >100 |

|       | AFN | API | BRV-F | EMO | MON | TRPT |
|-------|-----|-----|-------|-----|-----|------|
| Proliferation | 79.51 ± 3.68 | 1.25 ± 0.17 | >100 | 40.01 ± 2.70 | >100 | >100 |
| Viability | >100 | 59.59 ± 10.27 | >100 | >100 | >100 | >100 |
2.2. Impact of major regulated and emerging mycotoxins on EGC viability

The cytotoxic effect of mycotoxins was then measured using non-dividing cells. Confluent normal rat EGCs were exposed for 48 h to increasing concentrations of mycotoxins before estimation of the number of living cells using resazurin assay and determination of the IC50 values (Figure 2 and Table 1). Regarding regulated mycotoxins (Figure 2A), only DON, OTA, PAT, and ZEA were found cytotoxic to EGCs with IC50 of 5.06 ± 0.48; 23.88 ± 1.36; 38.02 ± 11.37 and 31.75 ± 4.94 µM, respectively. Among the 12 emerging mycotoxins tested (Figure 2B, 2C), only API (IC50 value of 59.59 ± 10.27 µM) and the cyclohexadepsipeptides (ENNs and BEA) (with IC50 values ranging from 0.72 ± 0.16 to 2.14 ± 0.17 µM) were found cytotoxic. The other regulated or emerging mycotoxins tested (i.e., AFB1, AFN, BRV-F, CYCLO, EMO, FB1, MON, and TRPT) were all found not toxic up to 100 µM.

3. Discussion

In the present study, we evaluated the toxicity of 18 regulated or emerging mycotoxins on enteric glial cells. The eighteen toxins used were selected on the basis of their occurrence in food and feed and of their known or suspected toxicity for humans and/or animals [5]. The regulated mycotoxins tested were AFB1, DON, FB1, OTA, PAT and ZEA whereas emerging mycotoxins tested were API, AFN, BEA, BRV-F, CYCLO, EMO, ENNs (ENN A, A1, B, B1), MON, and TRPT. Among the 18 mycotoxins tested, 12 toxins were
able to inhibit or suppress the proliferation of normal rat EGCs, i.e., DON, OTA, PAT, and ZEA for the regulated ones and API, AFN, BEA, EMO, ENNs (ENN A, A1, B and B1) for the emerging ones. Anti-proliferative effect was observed at doses ranging from 0.19 to 118 µM with the following order of IC₅₀: DON < ENN A, A1, B1 < API < ENN B = BEA < OTA < EMO < AFN < ZEA (Table 1).

Regarding cytotoxicity, 10 mycotoxins were able to decrease the viability of normal rat EGCs, i.e., DON, OTA, PAT, and ZEA for the regulated ones and API, BEA, ENNs (ENN A, A1, B and B1) for the emerging ones. Cytotoxic effect was observed at doses ranging from 0.4 to 59.59 µM with the following order of IC₅₀: ENN B < ENN A1 < ENN A < BEA < ENN B1 < DON < OTA < PAT < API (Table 1). AFB1, BRV-F, CYCLO, FB1, TRPT, and MON were all found not toxic to normal rat EGCs at least for doses up to 100 µM and after 48 h treatment. Sensitivity of normal rat EGCs to mycotoxins depends on their status, i.e., proliferating / dividing or confluent / non-dividing cells. OTA, BEA and ENNs (ENN A, A1, B1) gave very similar IC₅₀ values on dividing and non-dividing cells with 0.77 to 1.36-fold difference in IC₅₀ between dividing and non-dividing EGCs. Oppositely, API was more active on dividing than non-dividing cells with IC₅₀ of 1.25 and 59.59 µM, respectively (47.6-fold difference). AFN, EMO, DON, and PAT gave the same tendency, those toxins being more active on dividing cells than on non-dividing ones (with a 3.5 and 26.6-fold difference for PAT and DON, respectively). Surprisingly, ENN B and ZEA were found more toxic for non-dividing cells than for dividing ones (1.94 and 3.71-fold difference, respectively). The reason of such differences could be related to difference in mycotoxin entry and/or detoxification or modulation of the expression of their molecular target(s) in dividing versus non-dividing EGCs. Future works will be necessary to investigate this point and as well as to identify by which mechanism(s) mycotoxins cause toxic effect on EGCs. Mycotoxins such as DON and OTA are known to alter the functions of brain glial cells (i.e., astrocytes) at sub-toxic doses [18,19]. Based on the fact that astrocytes and EGCs share many common features in term of sensitivity to drugs and physiological functions [30], it will be worthwhile to evaluate if sublethal doses of mycotoxins affect EGCs functions as observed with phycotoxins [33].

4. Conclusions

In conclusion, our data confirmed the hypothesis of Gonkowski et al. [25] about a potential alteration of ENS by at least some food-associated mycotoxins. Our data generated using rat EGCs in vitro confirmed the pioneer work of Rissato et al. [32] that showed that deoxynivalenol is able to affect proliferation and viability of EGCs in vivo in rats. In addition, our in vitro data demonstrated that 11 other regulated or emerging mycotoxins (i.e., API, AFN, EMO, ENNs (ENN A, A1, B, B1), BEA, OTA, PAT, and ZEA), are toxic for normal rat EGCs. API, DON, and cyclohexadepsipetides mycotoxins (i.e., ENNs and BEA) are the more worrying toxins with IC₅₀ as low as 0.4 to 5 µM, similar tendency being observed with IECs [5]. This study demonstrated that EGCs are the target of at least some food-associated mycotoxins. Due to their pivotal role in the gut physiology [26–31], the toxic effect of mycotoxins on EGCs could lead to alterations of both ENS and IECs homeostasis and functions, further reinforcing the hypothesis, first enounced in 2010, of a link between mycotoxins and inflammatory bowel diseases (IBD) [35]. Future works, including the evaluation of the effects of sub-toxic doses of mycotoxins on key functions of EGCs, will help confirming this hypothesis.

5. Materials and Methods

5.1. Mycotoxins

Mycotoxins were used in this study were selected based on their occurrence in food and of their known or suspected toxicity for humans and/or animals [5]. Aflatoxin B1 (AFB1) (purity > 98%), apicidin (API) (purity > 98%), beauvericin (BEA) (purity > 97%), deoxynivalenol (DON) (purity > 98%), emodin (EMO) (purity > 90%), enniatins (ENNs)
(A, A1, B, B1) (purity > 99%), fumonisin B1 (FB1) (purity > 98%), moniliformin (MON) (purity > 95%), ochratoxin A (OTA) (purity > 95%), patulin (PAT) (purity > 98%), tryptophol (TRPT) (purity > 97%) and zearalenone (ZEA) (purity > 98%) were obtained from Sigma Aldrich (Lyon, France). Cyclo(L-Pro-L-Tyr) (CYCLO) (purity > 98%), and brevianamide-F (BRV-F) (purity > 95%) were purchased from BioAustralis (Smithfield, Australia). Aurofusarin (AFN), (purity > 97%) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All mycotoxins were dissolved in ethanol or DMSO following manufacturer’s instructions to prepare stock solutions stored at −20 °C. Working dilutions were freshly prepared in cell culture medium for each experiment.

5.2. Cell Culture

Normal rat EGCs (ATCC® CRL2690™) were obtained from ATCC (LGC Standards Molsheim, France). Cells were cultured in Dulbecco’s modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotics (from Thermofisher, Illkirch-Graffenstaden, France). Cells were routinely grown on 75 cm² flasks, maintained in a 5% CO₂ incubator at 37 °C and passaged when reaching 90–95% confluence.

5.3. Anti-Proliferative and Cytotoxicity Assays

Anti-proliferative and cytotoxic effects were measured as previously described [35–40]. Briefly, rat EGCs cells grown on 75 cm² flasks were detached from flasks using a trypsin-EDTA solution (from Thermofisher, Illkirch-Graffenstaden, France). After counting using a Malassez chamber, cells were diluted in culture media and seeded into 96-well cell culture plates (Greiner bio-one, Paris, France) at the appropriate cell density depending of the assay. For the anti-proliferative assay, rat EGCs were seeded at an initial cell density of approximatively 5000 cells/well in order to let them divide. For the cytotoxicity assay, rat EGCs were seeded at an initial cell density of approximately 50,000 cells/well in order to rapidly reach confluence. In both cases, after 12 h at 37 °C in a 5% CO₂ incubator, wells were empty and the cells were exposed to increasing concentrations of mycotoxins (0 to 100 µM, ½ dilution) or the corresponding vehicle (i.e., ethanol (maximal volume of 10 %) or DMSO (maximal volume 1%), used as negative control and found not toxic) diluted in complete culture media. After 48 h incubation at 37 °C in a 5% CO₂ incubator, cell viability was evaluated using resazurin based in vitro toxicity assay kit (Sigma-Aldrich, Lyon, France) following manufacturer’s instructions. Briefly, cell wells were empty and cells were treated with 100 µL of resazurin diluted 1:10 in sterile phosphate buffer saline (PBS) containing calcium and magnesium (PBS++, pH 7.4). After 4 h incubation at 37 °C, fluorescence intensity (excitation wavelength of 530 nm / emission wavelength of 590 nm) was measured using microplate reader (Biotek, Synergy Mx, Colmar, France). The fluorescence values were normalized by the controls (vehicle treated cells) and expressed as percent of proliferation or viability. The IC₅₀ values of mycotoxins on cell proliferation or viability (i.e., the concentration of derivative causing a reduction of 50% of the cell division or survival) were calculated using GraphPad® Prism 7 software (San Diego, CA, USA). t-Test and two ways ANOVA analyses were used to address the significant differences between mean values with significance set at p < 0.05.

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