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Effect of *Penicillium roqueforti* mycotoxins on Caco-2 cells: Acute and chronic exposure

Nolwenn Hymery*, Jérome Mounier, Emmanuel Coton

Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France

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ABSTRACT

Penicillium roqueforti is a common food and feed contaminant. However, it is also worldwide renowned for its use as a technological culture responsible for the typicity of blue-veined cheese. Members of the *P. roqueforti* species are also known to be able to produce secondary metabolites including mycophenolic acid (MPA) and roquefortine C (ROQ C) mycotoxins. In order to more closely simulate the reality of mycotoxin exposure through contaminated food consumption, this work investigated the toxicological effects of MPA and ROQ C not only in acute but also in chronic (*i.e.* 21-days continuous exposure) conditions on Caco-2 cells. Acute exposure to high MPA or ROQ C concentrations induced an increase of IL-8 secretion. Effects of 21-days continuous exposure on barrier integrity, based on concentrations found in blue-veined cheese and mean of blue cheese intake by French consumers, were monitored. Concerning exposure to ROQ C, no alteration of the intestinal barrier was observed. In contrast, the highest tested MPA concentration (780 µM) induced a decrease in the barrier function of Caco-2 cell monolayers, but no paracellular passage of bacteria was observed. This study highlighted that exposure to MPA and ROQ C average concentrations found in blue-veined cheese does not seem to induce significant toxicological effects in the tested conditions.

1. Introduction

Over the past 20 years, exposure to mycotoxin has been recognized as a significant health risk. Mycotoxins are secondary metabolites produced by different fungal species belonging in particular to the *Aspergillus*, *Penicillium* and *Fusarium* genera that exhibit toxic effects in human or animals (Bahat et al., 2009). These compounds are excreted into the matrices (including food or feed) on which the fungi grow (Jestoi et al., 2004; Zöllner and Mayer-Helm, 2006; Meca et al., 2010). Consumption of mycotoxin-contaminated commodities has been associated with several chronic diseases in humans and animals with many different adverse health effects, such as carcinogenic, mutagenic and estrogenic effects, as well as other systemic disorders (Speijers and Speijers, 2004; Kouadio et al., 2007; Ruiz et al., 2011). Some mycotoxins are also immunosuppressive reducing resistance to infectious diseases (Zain, 2011).

Penicillium roqueforti is the main silage spoiler (Schneweis et al., 2000) and is also used as ripening cultures in blue-veined cheeses in which it contributes to their organoleptic typicity. In the silage context, it has been shown to produce roquefortine C (ROQ C), isofumigaclavines A and B, PR toxin, macrofortines, and mycophenolic acid (MPA) (Cole and Cox, 1981; Frisvad and Thrane, 1996; Auerbach et al., 1998; Nielsen et al., 2006; Hymery et al., 2014). In the blue-veined cheese context, it has been shown to produce MPA and ROQ C (Engel et al., 1982; Fontaine et al., 2015) while PR toxin is unstable (Chang et al., 2003). Among 86 analyzed blue-veined cheeses, Fontaine et al. (2015) showed that a large concentration range of mycotoxin could be observed (mean levels of 841 ± 1271 mg/kg and 848 ± 1670 mg/kg for MPA and ROQ C respectively, with 75% of cheese samples containing < 705 mg/kg MPA and 792 mg/kg ROQ C).

MPA [6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl)-4-methyl-4 hexenoic acid] is a mycotoxin with antifungal, antibacterial and antiviral properties. Toxicity studies showed a lethal dose 50 (LD50) for rats and mice of 700 and 2500 mg/kg, respectively. On ruminants, Gallo et al. (2015) suggested that MPA and ROQ C could interfere with digestive processes and might represent a potential risk for ruminants. On human, MPA is an immunosuppressant which is frequently used for prevention of acute transplant rejection. MPA blocks proliferative response of T and B lymphocytes, and inhibits production of cytotoxic T cells (Eugui et al., 1991) as it is a noncompetitive

Abbreviations: DMSO, dimethylsulfoxide; DON, deoxynivalenol; EC, European Commission; FB1, fumonisin B1; FITC, fluorescein isothiocyanate; IC, inhibitory concentration; LD, lethal dose; MPA, mycophenolic acid; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); OTA, ochratoxin A; ROQ C, roquefortine C; TEER, transepithelial electrical resistance

* Corresponding author.
E-mail address: nolwenn.hymery@univ-brest.fr (N. Hymery).

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inhibitor of eukaryotic inosine monophosphate dehydrogenase. To explain adverse effects of MPA on gastrointestinal tract, Qasim et al. (2014) indicated that Caco-2, exposed to MPA at therapeutic concentrations (10 μM), exhibited functional alterations in tight junctions. As for ROQ C, a compound belonging to the 2,5-diketopiperazine class, several studies described neurotoxic properties (Nielsen et al., 2006). Animal studies, after intraperitoneal (IP) administration of 10 mg/kg of ROQ C, reported muscle contractions and ataxia in mice. LD50 in male mice was estimated to be 15–20 mg/kg. Reversible paralytic effects were reported in cows that ingested *P. roqueforti* contaminated feed grains containing an average ROQ C concentration of 25.3 mg/kg (Haggblom, 1990). While MPA and ROQ C are not regulated in feed and food, their presence has raised toxicological question when ingested.

The intestine represents the major site of exposure to xenobiotics, like food contaminants (including mycotoxins), drugs, pollutants or other additives present in food and water. The Caco-2 cell line, first isolated in the 70's from a human colon adenocarcinoma (Fogh et al., 1977), forms a monolayer of highly polarized cells, joined by functional tight junctions, with well-developed and organized microvilli on the apical (AP) membrane. This cell line has proved to be a good choice for studies of intestinal absorption and toxicity of xenobiotics. While the main human and veterinary health burden of mycotoxin exposure is related to chronic exposure, very few study reported the *in vitro* toxicity on Caco-2 after 21-days continuous exposure. Moreover, human exposure to MPA and ROQ C has not been extensively studied.

In this context, the aim of this study was to evaluate these mycotoxin effects on intestinal epithelial cells. In a first step, evaluation of acute effect was assessed on proliferating and mature enterocytes. In a second step, effect on barrier integrity after 21-days continuous exposure to these two mycotoxins, based on concentrations found in blue-veined cheese and mean of blue cheese intake by French consumers (CREDOC/CNIEL, personal communication; Fontaine et al., 2015), were studied (Tables 1 and 2).

2. Material and methods

2.1. Chemicals

ROQ C standard (CAS#58735-64-1), 99.9% purity, produced by *P. roqueforti*, was purchased from Cfm Oskar Tropitzsch e.K. (Marktredwitz, Germany). MPA standard (CAS#24280-93-1), purity ≥98%, obtained from *P. brevicompactum*, was purchased from Sigma-Aldrich (St. Louis, USA). Standards were dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored at −20 °C, in order to obtain final concentrations for cytotoxicity assays ranging from 0.001 to 100 μM and from 0.0078 to 780 μM for ROQ C and MPA, respectively. For 21-days continuous exposure, tested concentrations were calculated from both the highest, median and lowest quantity of MPA and ROQ C encountered in blue cheese based on results reported by Fontaine et al. (2015) and the median blue-veined cheese consumption of 10 g/day by French consumers, as reported by the French "Centre de Recherche pour l'Etude et l'Observation des Conditions de vie" (CREDOC, personal communication). The selected concentrations were thus as follows: 0.47, 11.8 and 190 nM for MPA and 29, 94 and 360 nM for ROQ C (Tables 1 and 2).

Table 1
Cheese consumption in France (CREDOC/CNIEL, personal communication).

	Children (3–17 years)		Children and adults (3–75 years)	
	N	Mean ± SD (g/day)	N	Mean ± SD (g/day)
Cheese consumption	968	34.2 ± 0.6	2162	20.9 ± 0.6
Blue-veined cheese consumption (g/day)	62	13.2 ± 0.8	360	10.7 ± 1.0

N: number of respondents; SD: standard deviation.

Table 2
Calculation of tested mycotoxin concentrations based on mycotoxin concentrations encountered in blue-veined cheeses (Fontaine et al., 2015) and mean blue cheese consumption of children and adults.

	Mycophenolic acid			Roquefortine C		
	Min	Median	Max	Min	Median	Max
Mycotoxin content (μg/kg)	15	378	6190	11	366	14,125
Quantity in 10 g (μg)	0.15	3.78	61.9	0.11	3.66	141.25
Tested concentrations (nM) for chronic study (quantity in 10 g/mycotoxin MW)[a]	0.47	11.8	190	29	94	360

Min: minimal concentration; Max: maximal concentration; MW: molecular weight.
[a] We hypothesized that 1 kg of small intestine might correspond to 1 L of culture medium.

2.2. Cell culture

Caco-2 cells, derived from a human colorectal adenocarcinoma, were purchased from ECACC (number 88081201, Salisbury, UK). Cells were cultured at 37 °C and 5% (v/v) CO_2 in DMEM supplemented with penicillin (100 UI/mL), streptomycin (100 μg/mL) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 4.5 g/L glucose, 25 mM HEPES, 2% (v/v), 2 mM of L-glutamine and 1% (v/v) of non-essential amino acids (NEAA) (Invitrogen, Carlsbad, CA) with weekly passage. The cells were removed from the flasks by incubating the monolayers with 0.25% trypsin in 1 mM EDTA (Sigma-Aldrich) solution for 10 min at 37 °C. Cells between passage 30 and 50 were seeded at a density of 80×10^3 cells/cm^2 (Sigma–Aldrich). For continuous exposure, the Caco-2 cells were seeded on 3 μM pore Transwell inserts (4.71 cm^2 area) at a density of 3×10^5 cell/cm^2 and maintained for 21 days in complete medium; the medium was changed three times a week (Sambuy et al., 2005). In these conditions, cells reach confluence in 3 days and differentiate completely in 21 days. For experiment, Caco-2 cells were grown for 48 h (proliferating cells) or until 21 days post-confluency (differentiated cells) in culture medium in the presence of MPA or ROQ C.

2.3. Acute exposure on proliferating and mature enterocytes

2.3.1. Cytotoxicity: evaluation of mitochondrial activity

Cells were plated in 96-well tissue culture plates at a density of 3×10^4 cells/well to perform the experiments. The effects on the mitochondrial activity of Caco-2 cells exposed to MPA and ROQ C at concentrations ranging from 0.001 to 100 μM for ROQ C or 0.0078 to 780 μM for MPA during 48 h of exposure, were studied using the MTS assay kit on undifferentiated cells (proliferating cells) and 21 days post-confluency cell (differentiated cells). Control cultures without mycotoxin but with solvent (DMSO) were included.

The assay was composed of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) and phenazine methosulfate (PMS), an electron coupling reagent. MTS is reduced by dehydrogenase enzymes in metabolically active cells into a formazan product that is soluble in tissue culture medium. After 48 h of exposure, the cells were washed with PBS and 20 μL of a freshly prepared MTS/PMS solution was added to the wells. The cells were further incubated for 3 h. The amount of soluble formazan (MTS metabolite) was then quantified by reading the absorbance at 490 nm on a Multiskan FC plate reader (Thermo Scientific, Madison, WI, USA). Cell viability obtained for the negative control (cell

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