Crocin and Quercetin protect HCT116 and HEK293 cells from Zearalenone-induced apoptosis by reducing endoplasmic reticulum stress

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Abstract Mycotoxins are considered to be significant contaminants of food and animal feed. Zearalenone (ZEN) is a non-steroidal estrogenic mycotoxin produced by several species of *Fusarium* in cereals and agricultural products. ZEN has been shown to be cytotoxic, genotoxic, and mutagenic in different cell types. In the present study, we investigated the involvement of endoplasmic reticulum (ER) stress in ZEN-mediated toxicity in human intestine (HCT116) and kidney (HEK293) cells and evaluated the effects of the two common dietary compounds Quercetin (QUER) and Crocin (CRO). We show that ZEN treatment induces ER stress and activates the unfolded protein response (UPR) as evidenced by XBP1 mRNA splicing and upregulation of GRP78, ATF4, GADD34, PDIA6, and CHOP. Activation of the ER stress response is associated with activation of the mitochondrial pathway of apoptosis. This apoptotic process is characterized

by an increase in ROS generation and lipid peroxidation, a loss of mitochondrial transmembrane potential ($\Delta\Psi_m$), and an activation of caspases and DNA damages. We also demonstrate that the antioxidant properties of QUER and CRO help to prevent ER stress and reduce ZEN-induced apoptosis in HCT116 and HEK293 cells. Our results suggest that antioxidant molecule might be helpful to prevent ZEN-induced ER stress and toxicity.

Keywords Zearalenone · Crocin · Quercetin · Oxidative stress · Endoplasmic reticulum stress · Apoptosis

Abbreviations

ZEN	Zearalenone
CRO	Crocin
QUER	Quercetin
ER	Endoplasmic reticulum
$\Delta\Psi_m$	Mitochondrial transmembrane potential
mtO ₂ ^{•−}	Mitochondrial superoxide anion

Highlights

Zearalenone triggers ER stress in human intestinal and kidney cells
Zearalenone induces apoptosis through ER stress and activation of the mitochondrial pathway
Crocin and Quercetin protect cells against ZEN-induced ER stress and apoptosis

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Introduction

Molds synthesize secondary metabolites, which are toxic compounds known as mycotoxins. The global contamination by these natural products in food, feed, and environment, including indoor surfaces and particles, form a health risk to both animals and humans (Zain 2011). Zearalenone (ZEN) [6-(10-hydroxy-6-oxo-trans-1-undecenyl)-bresorcylic acid lactone] is a mycotoxin produced by various *Fusarium* fungi, including *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium equiseti*, and *Fusarium cerealis* (Habschied et al. 2011; Rodrigues and Naehrer 2012) and found in grains and animal feeds (Kuiper-Goodman et al. 1987; Bryden 2012).

ZEN occurs naturally all over the world in a variety of food products designed for human and animal consumption, and potentially high concentrations are encountered as contamination in many important crops (Bennett and Klich 2003). The main route of human exposure to ZEN is through ingestion of contaminated food products, such as maize, wheat, rye, and other cereals (Zinedine et al. 2007). Spontaneous outbreaks of *Fusarium* mycotoxicosis in humans and animals have been reported worldwide (Fung and Clark 2004).

ZEN and its metabolites exhibit potent estrogenic activity and are thus often referred to as mycoestrogens. ZEN is implicated in reproductive disorders of farm animals and occasionally in hypoestrogenic syndromes in human (Zinedine et al. 2007). In addition, cytotoxic and genotoxic activities of ZEN, which are independent of its binding affinity to estrogen receptor, have also been reported. For instance, ZEN has been demonstrated to be hepatotoxic (Maaroufi et al. 1996; Obremski et al. 1999; Conkova et al. 2001; Bouaziz et al. 2008), hematotoxic (Maaroufi et al. 1996; Murata et al. 2003; Abbes et al. 2006a, b), nephrotoxic (Ouanes et al. 2003; Abbes et al. 2006a; Liang et al. 2010), and toxic toward the intestinal tract (Abid-Essefi et al. 2003). Different reports have shown in vitro and in vivo that ZEN induces cytotoxicity through reactive oxygen species (ROS) production leading to lipid peroxidation, DNA damages, and apoptosis by the mitochondrial pathway (Abid-Essefi et al. 2004; Hassen et al. 2007; Abbes et al. 2007; Bouaziz et al. 2008). ZEN was also shown to induce endoplasmic reticulum (ER) stress-mediated apoptosis in leukemic cells (Banjerdpongchai et al. 2010).

The ER plays crucial roles in various cellular processes including protein folding, protein trafficking, and intracellular Ca^{2+} regulation. Impairment of the physiological function of the ER, such as accumulation of unfolded proteins, disturbance of luminal calcium homeostasis, and disruption of redox status, initiates ER stress which in turn triggers the unfolded protein response (UPR) (Szegezdi et al. 2006). The UPR is an adaptive response that acts to restore ER homeostasis by activating the three proximal sensors IRE1 α (inositol-requiring enzyme 1 α), PERK (PKR-like endoplasmic reticulum kinase), and ATF6 (activating transcription factor 6). Nevertheless, if ER stress is too severe or prolonged, the UPR leads to apoptosis by activating downstream effectors including CHOP (C/EBP homologous protein), JNK, caspases, and members of Bcl2 family (Ron and Walter 2007; Akazawa et al. 2004).

The prevention of ZEN toxicity involves reduction of mycotoxin levels in foodstuffs and increasing the intake of diet components such as vitamins and antioxidants. We have demonstrated that almost all ZEN toxic effects are prevented in vitro and in vivo using vitamin E (Abid-Essefi et al. 2003; Ouanes et al. 2003; 2005; El Golli et al. 2006; Hassen et al. 2007). Furthermore, a strong protection against ZEN-induced toxicity has been demonstrated to be conferred by extracts

from cactus cladodes (Zourgui et al. 2008; 2009), radish (*Raphanus sativus*) (Ben Salah-Abbes et al. 2009), or garlic (*Allium sativum*) (Abid-Essefi et al. 2012). Studies on the effect of antioxidants, especially those consumed in food, thus appear of great interest to prevent ZEN-induced toxicity.

Crocin is a pharmacologically active compound of *Crocus sativus* L. (saffron) (Rios et al. 1996). The high antioxidant capacity of Crocin has been reported in vitro and in vivo (Ochiai et al. 2004a, b, 2007; Hosseinzadeh et al. 2009, 2010; Mousavi et al. 2010). For example, Crocin can decrease lipid peroxidation in kidney (Hosseinzadeh et al. 2005) and skeletal muscle (Hosseinzadeh et al. 2009) during ischemia-reperfusion-induced oxidative damage in rats. In addition, this carotenoid increases cell viability in PC12 cells upon serum deprivation by inducing glutathione (GSH) synthesis, increasing glutathione reductase (GR), and c-glutamylcysteinyl synthase (c-GCS) activities, and decreasing ceramide formation (Ochiai et al. 2004a, b).

Quercetin (3,5,7,3',4'-Pentahydroxy flavon), a typical member of the flavonoid family, is one of the most widely recognized dietary polyphenolic compounds. It is ubiquitously present in foods and exhibits a broad spectrum of properties, i.e., antioxidant, anti-inflammatory, and immunomodulatory (Kobylińska and Janas 2015). The protective activity of Quercetin has been largely associated with its antioxidant and anti-inflammatory properties. Indeed, within the flavonoid family, Quercetin is proven to be the most potent scavenger of free radicals (Hanasaki et al. 1994). There is evidence that Quercetin reduces low-density lipoprotein oxidation (Loke et al. 2008) and prevents the development of atherosclerotic lesions (Loke et al. 2010). It has also been reported that Quercetin inhibits the production of superoxide anion ($\text{O}_2^{\cdot-}$) in rat aorta and decreases protein expression of the NADPH oxidase subunit, p47phox (Sanchez et al. 2006; Romero et al. 2009).

The present study was designed to investigate whether ZEN toxicity involves ER stress induction in human large intestine (HCT116) and kidney (HEK293) cells and to determine the effect of the antioxidant molecules Crocin and Quercetin.

Materials and methods

Chemicals

Zearalenone, Crocin, and Quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle medium-F12 (DMEM-F12), fetal bovine serum (FBS), phosphate buffer saline (PBS), trypsin-EDTA, penicillin and streptomycin mixture, dichlorodihydrofluorescein diacetate (DCFH-DA), propidium iodide (PI), fluorescein diacetate (FDA), 30-dihexyloxycarbocyanin iodide (DiOC6(3)),

MitoSOX™ Red and Hanks' balanced salt solution (HBSS) were supplied by Invitrogen (Saint Aubin, France). All other compounds were purchased from Sigma–Aldrich, and all the used chemicals were of analytical grade.

Cell culture and treatment

Human colon carcinoma cells HCT116 and embryonic kidney cells HEK293 were cultured in DMEM-F12 and supplemented with 10 % FBS, 1 % L-glutamine (200 mM), 1 % of mixture penicillin (100 IU/ml), and streptomycin (100 lg/ml) at 37 °C with 5 % CO₂. ZEN was dissolved in pure DMSO. To obtain the studied concentrations in the cell culture media, the mycotoxin treatment volume was negligible and represented about 0.025 % of the total medium volume. In these conditions, untreated cells and cells receiving this low vehicle volume responded in the same manner. For this reason, we have chosen untreated cells as control.

Flow cytometry analysis

For flow cytometry analysis of mitochondrial transmembrane potential ($\Delta\Psi_m$), cells were stained with 10-nM DiOC6(3) for 20 min at 37 °C. Necrosis was estimated by adding 10 µg/ml of propidium iodide (PI) just before analysis. The fluorescent probe fluorescein diacetate (FDA) was used to assess cell viability. After treatment, cells were incubated for 5 min at 37 °C with FDA at 0.2 µg/ml. To measure the relative levels of mitochondrial superoxide anion, O₂^{•−}, the probe MitoSOX™ Red was used. Once in the mitochondria, MitoSOX™ Red reagent is oxidized by superoxide anion and exhibits red fluorescence detected by flow cytometry. Cells were centrifuged, washed with PBS, and incubated with 2 µM of MitoSOX™ Red for 10 min at 37 °C. MitoSOX™ Red fluorescence was immediately analyzed by flow cytometry (10,000 cells). Caspase-3 activation was assessed using DEVD-NucView 488 caspase-3 substrate. After treatment, cells were incubated with 5 µM DEVD-NucView 488 for 30 min at RT before cytometric analysis. Fluorescence of cells was analyzed on a Cell Lab Quanta MPL cytometer (Beckman Coulter, Villepinte, France).

Western blot analysis

Cells were lysed in RIPA lysis buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 1 % Triton, 1 mM EDTA, 0.1 % SDS, 0.5 % deoxycholic acid) plus a cocktail of protease inhibitors (Roche) and PMSF for 30 min at 4 °C. Proteins (30 µg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Merck Millipore). Membranes were incubated overnight at 4 °C with the following antibodies: anti-GRP78 (sc-1050), anti-GADD34 (sc-8327), anti-PDIA6 (sc-107533), and anti-β-actin (sc-47778)

from Santa Cruz. Proteins were detected on a ChemiDoc XRS (BioRad) by using the ECL method according to the manufacturer's instructions (Merck Millipore). To calculate the relative density (RD), ImageJ software was used and the intensity of each protein was normalized to β-actin. The data obtained were then expressed as the ratio of the intensity of the protein in treated cells to that of the corresponding protein in untreated cells.

RNA isolation and real-time reverse transcription polymerase chain reaction

RNA was isolated from cultured cells using Zymo Research QuickRNA MiniPrep according to the manufacturer's instructions. Total RNA (1 µg) was reverse transcribed using BioRad iScript reverse transcription kit. For real-time PCR, cDNA was amplified by the “two-step” SsoFast EvaGreen supermix (BioRad), heated at 95 °C for 30 s, then 50 cycles of denaturation at 95 °C during 2 s and hybridization/elongation at 60 °C during 5 s. The PCR primers were obtained from Eurofins: 5' GTCCCTCCAACAACAGCAAG3'(F) and 5' AGGTCATC TGGCATGGTTTC3'(R) for ATF4; 5'TGCTGAGTCCGC AGCAGGTG3'(F) and 5'GCTGGCAGGCTCTGGGGAAG 3'(R) for spliced XBP1 as described by van Schadewijk et al. (2012); 5'AGCGACAGAGCCAAAATCAG3'(F) and 5'ACAAGTTGGCAAGCTGGTCT 3'(R) for CHOP. The results were quantified according to the Cq value method, where Cq is defined as the quantification cycle of PCR at which the amplified product is detected. The ratio $(1+E_{\text{target gene}})^{-(Cq_{\text{sample}}-Cq_{\text{control}})}$ target gene/ $(1+E_{\text{reference gene}})^{-(Cq_{\text{sample}}-Cq_{\text{control}})}$ reference gene was calculated, where E represents the efficiency of the quantitative PCR reaction. With this calculation, expression of control was equivalent to one.

Measurement of reactive oxygen species production

The intracellular amounts of ROS were measured by a fluorometric assay with 2,7-dichlorofluorescein diacetate (DCFH-DA) to detect intracellular ROS. The probe, after diffusing in the cell membrane, is hydrolyzed by intracellular esterases to non-fluorescent dichlorofluorescein (DCFH), which is trapped inside the cells then oxidized to fluorescent DCF through the action of peroxides in the presence of ROS (Le Bel et al. 1992). HCT116 and HEK293 cells were seeded on 24-well culture plates (Polylabo, France) at 10⁵ cells/well for 24 h. After incubation, cells were treated with 20 µM DCFH-DA. Intracellular production of ROS was measured after 30 min incubation at 37 °C by fluorometric detection of DCF oxidation on a fluorimeter (Biotek FL 800×) with an excitation wavelength of 485 nm and emission wavelength of 522 nm. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly.

Lipid peroxidation

Lipid peroxidation was assayed by the measurement of malondialdehyde (MDA) according to the method of Ohkawa et al. (1979). Cells were seeded on 6-well plates at 7.5×10^5 cells/well. After 24 h of incubation, they were exposed to ZEN in the absence or the presence of CRO and QUER for 24 h at 37 °C. The cells were then washed with cold PBS, scraped, and lysed by homogenization in ice-cold 1.15 % KCl. Samples containing 0.1 ml of cell lysates were combined with 0.2 ml of 8.1 % SDS, 1.5 ml of 20 % acetic acid adjusted to pH 3.5, and 1.5 ml of 0.8 % thiobarbituric acid. The mixture was brought to a final volume of 4 ml with distilled water and heated to 95 °C for 120 min. After cooling to room temperature, 5 ml of mixture of n-butanol and pyridine (15:1, v/v) was added to each sample and the mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the supernatant fraction was isolated and the absorbance measured at 546 nm. The concentration of MDA was determined according to a standard curve.

DNA damage assessed by the comet assay

Single cell gel electrophoresis (SCGE) is a visual and sensitive technique for measuring DNA breakage in individual mammalian cells. HCT116 and HEK293 cells were seeded on 6-well culture plates (Polylabo, France) at 7.5×10^5 cells/well for 24 h of incubation and were re-incubated as described above in the presence of ZEN in the absence or the presence of QUER/CRO for 24 h at 37 °C. Approximately 2×10^4 cells were mixed with 1 % low melting point (LMP) agarose in PBS and spread on a microscope slide previously covered with a 1 % normal melting agarose (NMP) in PBS layer. After agarose solidification, cells were treated with an alkaline lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10, 1 % (v/v) Triton X-100, and 10 % (v/v) DMSO) for 1 h at 4 °C, then the DNA was allowed to unwind for 40 min in the electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH>13). The slides were then subjected to electrophoresis in the same buffer for 30 min at 25 V and 300 mA. Slides were then neutralized using a Tris buffer solution (0.4 M Tris, pH 7.5) for 15 min. After staining the slides with ethidium bromide (20 µg/ml), the comets were detected and scored using a fluorescence microscope. The experiment was done in triplicate. The damage is represented by an increase of DNA fragments that have migrated out of the cell nucleus during electrophoresis and formed an image of a “comet” tail. A total of 100 comets on each slide were visually scored according to the intensity of fluorescence in the tail and classified by one of five classes as described by Collins et al. (1996). The total score was evaluated according to the following equation: (% of cells in class 0×0)+(% of cells in class 1×1)+(% of cells in class 2×2)+(% of cells in class 3×3)+(% of cells in class 4×4).

Statistical analysis

Each experiment was done three times separately. Values were presented as means±SD. One-way ANOVA was used to assess differences among groups followed by Dunnett's post hoc test. When two groups were compared, differences were assessed by Student's *t* test. Differences were considered significant at $P<0.05$.

Results

ZEN induces cell death and ER stress in HCT116 and HEK293 cells

To determine the cytotoxicity of Zearalenone, HCT116 and HEK293 cell lines were exposed to increasing concentrations of ZEN ranging from 25 to 200 µM for 24 h and cell viability was determined by FDA assay. Our results indicate that ZEN induced a marked decrease in cell viability in a dose-dependent manner in the two cell lines (Fig. 1a). The IC₅₀ values, determined after 24 h of cell treatment, were about 100 and 75 µM, respectively, in HCT116 and HEK293 cells. We next investigated whether ER stress and UPR are activated in response to ZEN. HCT116 cells were incubated with ZEN, and the expression of ER stress markers and UPR target genes was measured by western blot and quantitative RT-PCR (Fig. 1b). After treatment with ZEN, an increase in the level of GRP78 and GADD34, two well-known ER stress markers was observed (Fig. 1b, left panel). ZEN also induced the up-regulation of ATF4, involved in the PERK branch of the UPR, increased the expression of PDIA6, suggesting the induction of ATF6 branch and induced the splicing of XBP1 (XBP1-s), indicative of the activation of IRE1 branch (Fig. 1b, right panel). In addition, ZEN increased by about 8.45-fold the expression of CHOP, a major component of the ER stress-mediated apoptosis pathway. Taken together, these results indicate that ZEN induces ER stress and activates different branches of the UPR in HCT116 cells. Similarly, ZEN-induced ER stress in HEK293 cells as shown by the increase in the level of GRP78 and GADD34 (Fig. 1c). To confirm the relationship between ZEN-induced ER stress and cell death, cells were pre-incubated for 2 h with the chemical chaperone 4-phenylbutyric acid (PBA) before ZEN treatment and cell mortality (FDA negative cells) and expression of GRP78 were analyzed after 24 h. By stabilizing protein conformation, PBA is known to alleviate ER stress (Morris et al. 1997; Ozcan et al. 2006). As expected, we observed that PBA greatly decreased protein level of the ER stress marker GRP78 (Fig. 2a) in HCT116 and HEK293 cells. In addition, PBA significantly decreased cell mortality induced in response to ZEN (Fig. 2b).

Fig. 1 ZEN induces ER stress and cell death in HCT116 and HEK293 cells. **(a)** Cells were treated with ZEN at the indicated concentrations for 24 h. Cell viability was determined using the FDA assay and expressed as percentages of FDA positive cells (viable cells). Data are expressed as the mean±SD of three independent experiments. Values are significantly different ($P<0.05$) from control. **(b)** HCT116 cells were incubated for 24 h with ZEN (100 μM), and protein levels of the ER stress markers GRP78, GADD34, and PDIA6 were analyzed by western blot. The

relative mRNA levels of the UPR target genes ATF4, spliced XBP1 (XBP1-s), and CHOP were quantified by qRT-PCR and expressed as fold change over untreated controls. Data are expressed as the mean±SD of three separate experiments. $^{####}P<0.001$ vs. control **(c)** HEK293 cells were incubated for 24 h with ZEN (75 μM), and protein levels of the ER stress markers GRP78 and GADD34 were analyzed by western blot. β-actin was used as a loading control. RD relative density

Effect of Quercetin and Crocin on ZEN-induced ROS generation and lipid peroxidation

The toxicity of ZEN has been linked to the generation of oxidative stress in different cells (Hassen et al. 2007). Therefore, we evaluate whether Quercetin (QUER) and Crocin (CRO) exert their proposed antioxidant properties and modulate the level of ROS induced by ZEN in HCT116

and HEK293 cells. The level of intracellular ROS was measured after ZEN treatment in the absence or presence of QUER (5 μM) or CRO (250 μM) by recording the fluorescence of DCF, which is the result of DCFH oxidation mainly by H_2O_2 . As shown in Fig. 3a, ZEN treatment induced an important increase in ROS production to about 2.57- and 1.74-fold to control, respectively, in HCT116 and HEK293 cells. Pretreatment with QUER or CRO totally abolished the

Fig. 2 PBA inhibits ZEN-induced ER stress and cell death. Cells were pretreated for 2 h with 1 mM PBA before treatment with ZEN at the indicated concentrations for 24 h. **(a)** The protein level of the ER stress marker GRP78 was analyzed by western blot. **(b)** Cell death was measured using FDA assay. Data are expressed as the mean±SD of three separate experiments. $^{####}P<0.001$ vs. control, $^{**}P<0.01$ vs. ZEN alone

Fig. 3 Effects of Quercetin and Crocin on ZEN-induced ROS generation. HCT116 and HEK293 cells were pretreated with QUER (5 μ M) or CRO (250 μ M) for 2 h before ZEN treatment for 24 h (100 μ M for HCT116, 75 μ M for HEK293) **(a)** The relative intracellular ROS production was evaluated by recording the fluorescence of DCF, the product of DCFH oxidation mainly by H_2O_2 . **(b)** The production of

mitochondrial anion superoxide was analyzed by flow cytometry using MitoSOXTM Red probe. MitoSOX high cells represent cells with a high level of mitochondrial anion superoxide. **(c)** The peroxidation of lipids was recorded by measuring the accumulation of MDA. Data are expressed as the mean $\pm$ SD of three separate experiments. ### P <0.001 and ## P <0.01 vs. control, *** P <0.001 and ** P <0.01 vs. ZEN alone

intracellular ROS generated by ZEN in the two cell lines. Mitochondrial superoxide anion ($O_2^{\cdot -}$) levels were examined by staining cells with the MitoSOXTM Red probe. Mitochondrial $O_2^{\cdot -}$ level increased in HCT116 cells from 5.64 ± 1.12 % in control to 40.85 ± 3.5 % in the presence of ZEN and from 4.65 ± 1.32 % in control to 42.32 ± 3.37 % in HEK293-treated cells. The presence of QUER or CRO significantly reduced the percentage of MitoSOX high cells induced by ZEN (Fig. 3b), reflecting a marked decrease in the level of $O_2^{\cdot -}$. Lipid peroxidation was also measured by MDA assay in response to ZEN. After 24 h incubation with ZEN, the MDA level was 1.20 ± 0.11 μ mol MDA/mg of protein and 0.88 ± 0.081 μ mol MDA/mg of protein, respectively in HCT116 and HEK293 cells as compared to the control (Fig. 3c), indicating that ZEN treatment significantly increased the

peroxidation of lipids. The addition of QUER or CRO decreased the level of MDA induced by ZEN both in HCT116 and HEK293 cell lines. Altogether, these data indicate that QUER and CRO exert antioxidant activity and limit ZEN-induced oxidative stress in intestinal and renal cells.

Quercetin and Crocin inhibit ER stress and cell death induced by ZEN

ER stress response has been suggested to be a consequence of ROS generation (Santos et al. 2009). We thus investigated whether the antioxidant activity of QUER and CRO modulates ER stress and cell death induced by ZEN. As shown in Fig. 4, pretreatment of cells with QUER or CRO prevented the increase in the level of the ER stress markers GRP78 and

Fig. 4 Quercetin and Crocin inhibits ZEN-induced ER stress. Cells were pretreated with QUER (5 μ M) or CRO (250 μ M) for 2 h before ZEN treatment for 24 h (100 μ M for HCT116, 75 μ M for HEK293). Protein levels of the ER stress markers GRP78 and GADD34 were detected by

western blot in HCT116 (a) and HEK293 cells (b). β -actin was used as a loading control. Values represent mean $\pm$ SD of three independent experiments. ### P <0.001 and ## P <0.01 vs. control, *** P <0.001 and ** P <0.01 vs. ZEN alone

GADD34 induced by ZEN in the two tested cell lines. In addition, while QUER and CRO alone exhibited no toxicity towards HCT116 or HEK293 cells, pretreatment with these antioxidant molecules significantly decreased ZEN-mediated cytotoxicity. Indeed, in HCT116, cell mortality was decreased from 50.71 ± 2.19 % with ZEN alone to 26.97 ± 2.3 % and 25.83 ± 2.84 %, in the presence of QUER or CRO, respectively (Fig. 5). Similarly, in HEK293, cell viability was increased from 51.14 ± 3.12 % after treatment by ZEN to 30.86 ± 2.58 % in the presence of QUER and to 29.61 ± 3.23 % in the presence of CRO (Fig. 5). Collectively, our results indicate that QUER and CRO inhibit ER stress and protect HCT116 and HEK293 cells from cell death.

Quercetin and Crocin decrease mitochondrial alterations induced by ZEN

Severe ER stress is known to induce apoptosis through the mitochondrial pathway to eliminate damaged cells (Sharaf el dein et al., 2009). In addition, we have previously reported that ZEN induces cell death by activating the mitochondrial pathway of apoptosis (Bouaziz et al., 2008). We thus assessed the effect of QUER and CRO on the mitochondrial alterations induced by ZEN. To this end, cells were analyzed by flow cytometry after staining with propidium iodide (PI) to evaluate necrosis and DiOC6(3) to measure mitochondrial transmembrane potential ($\Delta\Psi$ m). As shown in Fig. 6, the

Fig. 5 Quercetin and Crocin reduce ZEN-induced cytotoxicity in HCT116 and HEK293 cells. Cells were pretreated for 2 h with QUER (5 μ M) or CRO (250 μ M) before ZEN treatment for 24 h (100 μ M for HCT116, 75 μ M for HEK293). Cell viability was determined using the FDA assay and expressed as percentages of FDA positive cells (viable cells). Data are expressed as the mean $\pm$ SD of three separate experiments. ### P <0.001 vs. control, ** P <0.01 vs. ZEN alone

Fig. 6 Quercetin and Crocin protect cells from ZEN-induced loss of mitochondrial membrane potential. HCT116 (a) and HEK293 (b) cells were pretreated for 2 h with QUER (5 μ M) or CRO (250 μ M) before ZEN treatment for 24 h (100 μ M for HCT116, 75 μ M for HEK293). After treatment, cells were stained with the mitochondrial

transmembrane potential ($\Delta\psi_m$)-sensitive dye DiOC6(3) and the necrosis/late apoptosis probe propidium iodide (PI) and analyzed by flow cytometry. The data are expressed as the mean $\pm$ SD of three separate experiments. ## P < 0.01 and # P < 0.05 vs. control, * P < 0.05 vs. ZEN alone

percentage of PI high cells remained very low (less than 20 %), excluding the involvement of necrosis in ZEN-induced cell death. The percentage of DiOC6(3) low cells, after 24 h of mycotoxin exposure, reached about 57.74 ± 3.12 % and 55.33 ± 3.05 % in HCT116 (Fig. 6a) and HEK293 (Fig. 6b) cells, respectively. Pretreatment of cells by QUER or CRO significantly decreases the percentage of DiOC6(3) low cells as compared to cells treated by ZEN alone. These results indicate that QUER and CRO reduce the mitochondrial alterations induced by ZEN.

Quercetin and Crocin reduce ZEN-induced caspase activation and DNA fragmentation

We next examined the ability of Quercetin and Crocin to modulate ZEN-induced caspase activation and DNA fragmentation, two apoptotic hallmarks. Cells were incubated with ZEN for 24 h, labeled with the DEVD-NucView 488 caspase-3 substrate, which becomes fluorescent when cleaved by active caspase-3, and analyzed by flow cytometry. We observed that ZEN increases the percentage of cells with activated caspase-3, suggesting that caspases are involved in the apoptotic process triggered by ZEN. Pretreatment of cells with QUER or CRO reduced caspase-3-activation induced by ZEN both in HCT116 and HEK293 cells (Fig. 7a). DNA damages were analyzed using the alkaline Comet assay. The high sensitivity of the Comet assay allows measurement of DNA fragmentation in individual cells. As shown in Fig. 7b, ZEN induced 235.8 ± 12.2 and 198 ± 11.57 DNA fragmentations, as compared to 29 ± 6 and 19 ± 6.3 DNA fragmentations in controls, in HCT116 and HEK293 cells, respectively. The addition of QUER or CRO significantly decreased the DNA

fragmentation induced by ZEN in the two tested cell lines. Together, these data demonstrate that the antioxidant molecules Quercetin and Crocin protect HCT116 and HEK293 cells from ZEN-triggered caspase activation and DNA fragmentation.

Discussion

The main focus of this study was to explore the involvement of ER stress in ZEN-induced apoptosis and to evaluate the protective effects of the two common food components Quercetin and Crocin in HCT116 and HEK293 cells. Our results show that ZEN induces cell death in the two human cell lines tested, at least in part, by inducing ER stress-associated activation of the mitochondrial pathway of apoptosis. In addition, we demonstrate that Quercetin and Crocin reduce the level of ROS produced by ZEN, inhibit ER stress, and protect cells from apoptosis.

Our data show that cell treatment with ZEN results in induction of ER stress as demonstrated by robust increase in the expression of the ER resident chaperone GRP78. ER stress is sensed by three upstream proteins IRE1, ATF6, and PERK, which activate different signaling pathways collectively termed UPR (Tabas and Ron 2011). In HCT116 cells, we showed that ZEN induces an increase in the expression of ATF4 and GADD34, two markers of the PERK pathway, and the splicing of XBP1 (XBP1-s). XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress (Yoshida et al. 2001), and ATF6 is required for optimal expression of GRP78 (Baumeister et al. 2005). Our results thus indicate that PERK/ATF4, IRE1, and ATF6 arms of the UPR

Fig. 7 (a) Quercetin and Crocin reduce caspase 3 activation induced by ZEN. HCT116 and HEK293 cells were pretreated for 2 h with QUER (5 μ M) or CRO (250 μ M) before ZEN treatment for 24 h (100 μ M for HCT116, 75 μ M for HEK293). The percentage of cells with activated caspase-3 was analyzed by flow cytometry after staining with the NucView 488 caspase-3 substrate. Data are expressed as the mean $\pm$ SD of three separate experiments. ### P <0.001 and ## P <0.01 vs. control, ** P <0.01 and * P <0.05 vs. ZEN alone. **(b)** Quercetin and Crocin reduce ZEN-induced DNA fragmentation in HCT116 and HEK293. Cells were pretreated for 2 h with QUER (5 μ M) or CRO (250 μ M) before ZEN treatment for 24 h (100 μ M for HCT116, 75 μ M for HEK293). DNA damages were analyzed by the comet assay as described in the “Materials and methods”. Data are expressed as the mean $\pm$ SD of three separate experiments. ## P <0.01 vs. control, ** P <0.01, and * P <0.05 vs. ZEN alone

are activated upon ZEN treatment. Different reports have shown that treatment with other mycotoxins such as deoxynivalenol and trichodermin also induce ER stress in different cell systems (Yang et al. 2010; Su et al. 2013). This suggests that induction of ER stress may be a common feature of mycotoxin toxicity. In response to ER stress, the UPR is initially induced to restore ER homeostasis by inhibiting general protein translation, increasing chaperones and folding enzymes in the ER, and eliminating unfolded proteins. In the case of severe or sustained ER stress, the pro-survival function of the UPR turns into an apoptotic signal to eliminate stressed cells (Tabas and Ron 2011). Our results show that the expression of the pro-apoptotic factor CHOP is increased after ZEN treatment and that reduction of ER stress by PBA reduces

GRP78 expression and ZEN-induced cell mortality indicating that the ER stress induced by this mycotoxin is sufficient to activate the pro-apoptotic pathway of the UPR. The transcription factor CHOP was the first protein identified as a key mediator of ER stress-induced apoptosis and is upregulated by the three branches of the UPR (Zinszner et al. 1998). CHOP acts through downregulation of the anti-apoptotic protein Bcl-2 and upregulation of the BH3-only protein Bim, which is necessary for Bax-mediated mitochondrial permeabilization and apoptosis (Tabas and Ron 2011). In agreement, we observed in HCT116 and HEK293 cells that ZEN induces the dissipation of $\Delta\Psi_m$, indicative of mitochondrial membrane permeabilization, the activation of caspases, and the fragmentation of DNA with a relative persistence of plasma

membrane integrity. These data indicate that ER stress elicited by ZEN is associated with an induction of apoptosis through a mitochondria-mediated caspase-dependent pathway.

ZEN is known as a potent inducer of ROS in mammalian system. Indeed, different reports have demonstrated that ZEN induces ROS production (Bouaziz et al. 2009; Jia et al. 2014; Zhou et al. 2015) and lipid peroxidation in vitro and in vivo and can engender oxidized DNA bases (Abid-Essefi et al. 2004; Hassen et al. 2007; Abbes et al. 2007). Results of our study provide evidence that ZEN triggers ROS generation in HCT116 and HEK293 cells. ZEN-induced oxidative stress was evidenced by production of intracellular and mitochondrial ROS and accumulation of MDA, an end product of lipid peroxidation, considered as a late biomarker of oxidative stress and cellular damages (Vaca et al. 1988). The toxicity of ZEN thus appears to be related to the substantial degree of intracellular oxidative stress generated. Therefore, preventing ROS production or scavenging of free radicals may be an effective strategy for limiting the toxicity of ZEN.

We demonstrate here that Quercetin and Crocin decrease the level of ROS and the peroxidation of lipids induced by ZEN and reduce the toxicity of this mycotoxin in HCT116 and HEK293 cells. Quercetin is one of the most frequently studied dietary flavonoids, distributed in vegetables, fruits, and many other dietary sources (Bhatt and Flora 2009). This flavonoid has been reported to scavenge free radicals directly to inhibit lipid peroxidation and to strengthen antioxidant defense pathway in vivo and in vitro (Anjaneyulu and Chopra 2004). Crocin, a major carotenoid of saffron, exhibits high antioxidant and scavenging properties in vitro and in vivo (Ochiai et al. 2004a, b, 2007; Hosseinzadeh et al. 2009, 2010; Mousavi et al. 2010). For instance, Crocin has been shown to attenuate lipid peroxidation in kidney (Hosseinzadeh et al. 2005) and skeletal muscle (Hosseinzadeh et al. 2009) during ischemia-reperfusion-induced oxidative damage in rats and to promote cell viability in PC12 cells (Mousavi et al. 2010). Our results indicate that, as effective free-radical scavengers and antioxidants, Quercetin and Crocin reduce ZEN toxicity in HCT116 and HEK293 cells.

An excess ROS production can induce ER stress (Santos et al. 2009) and initiate the mitochondrial pathway of apoptosis (Le Bras et al. 2005). Indeed, even if the ER is one of the most oxidizing intracellular compartments to allow disulfide formation and proper protein folding, an oxidative stress will lead to excessive oxidative modifications to proteins and hence to ER stress and UPR activation. Here, we report for the first time that scavenging of ROS by the antioxidants Quercetin and Crocin inhibits the ER stress response and protects cells from ZEN-induced mitochondrial apoptosis. These results suggest that ROS are initiators of ER stress and mitochondrial damages in the signalling cascade induced by ZEN.

In conclusion, this study provides insight into ways to reduce the toxicity of the commonly encountered mycotoxin

Zearalenone. We show that ZEN-induced mitochondrial apoptotic cell death is associated with ER stress in HCT116 and HEK293 cells. We also demonstrated that the two common antioxidant food components Quercetin and Crocin protect cells from ZEN by inhibiting ER stress, suggesting that antioxidant treatment might be helpful to prevent ZEN-related toxicity.

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Conflict of interest The authors declare that they have no competing interests.

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