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Fumonisin B_1 induces oxidative stress in oesophageal (SNO) cancer cells

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ABSTRACT

Fumonisin B_1 (FB_1) is a ubiquitous contaminant of maize that is epidemiologically linked to oesophageal cancer (OC) in South Africa. FB_1-induced oxidative stress mediates toxicity in animals and human cell lines, but the effects relating to OC are limited. Given the species-specific effects of FB_1, this study investigated FB_1-mediated toxicity and oxidative stress in spindle-shaped N-cadherin (+) CD45 (−) osteoblastic (SNO) cells. Following exposure to FB_1 (0–20 μM) for 48 h, mitochondrial membrane potential and intracellular reactive oxygen species (iROS) were measured (flow cytometry). Malondialdehyde concentration (lipid peroxidation) was determined spectrophotometrically. ATP and reduced glutathione (GSH) concentrations were quantified using luminometry, gene expression of SOD2 by qPCR and protein expression of SOD2, GPx1, Nrf2 and HSP70 by western blotting. Mitochondrial depolarization increased at 10 μM and 20 μM FB_1, with a concomitant reduction in ATP, iROS and GSH at both concentrations. Lipid peroxidation increased at 10 μM FB_1 exposure. While transcript levels of SOD2 were significantly increased, protein levels decreased. Protein expression of GPx1, Nrf2 and HSP70 increased. In contrast to the 10 μM and 20 μM FB_1 treatment, mitochondrial depolarization decreased at 1.25 μM FB_1. Intracellular ROS and ATP were decreased and lipid peroxidation increased. Decreased GSH was accompanied by a decrease in GPx1 protein levels, and increased HSP70 and Nrf2. SOD2 expression and protein levels were significantly increased. Overall these results indicate that FB_1 caused increased ROS that were counteracted by engaging the antioxidant defense. Furthermore, the peculiar response at 1.25 μM FB_1 is noteworthy, as compared to the other two concentrations tested.

1. Introduction

Fumonisins are carcinogenic mycotoxins produced by the pathogenic fungi *Fusarium verticillioides* and *F. proliferatum* that frequently infect maize-based foods worldwide (Domijan et al., 2007; Cano-Sancho et al., 2012; Alizadeh et al., 2012; Van Der Westhuizen et al., 2010; Bittencourt et al., 2005). The high incidence of oesophageal cancer (OC) in the former Transkei region (Eastern Cape, South Africa) is associated with consumption of fumonisin B_1 (FB_1) in both home-grown maize and fermented brews (Van Der Westhuizen et al., 2010). The average daily intake of contaminating mycotoxins is higher for men (9-fold; regularly consume home brewed beer) than women (6-fold) consuming home-grown maize, 12.1 μg/kg vs 1.3 μg/kg for men and 6.7 μg/kg vs 1.1 μg/kg for women, respectively (Burger et al., 2010). FB_1 is a highly polar molecule that is poorly absorbed and rapidly eliminated in many animal species including humans (Chelule et al., 2001). However, small amounts of this toxin are retained in the liver and kidney of rats (Riley et al., 1994) and in OC tissue (Myburg et al., 2009). Thus, chronic exposure may well contribute to adverse health effects in exposed individuals. Indeed, exposure to FB_1, the most toxic and abundant of the fumonisin family, has been shown to be neurotoxic, hepatotoxic, pulmonotoxic and nephrotoxic in animals (Voss et al., 2001; Harrison et al., 1990; Ledoux et al., 2003; Gelderblom et al., 1988, 1991; Jaskiewicz et al., 1987; Marasas et al., 1988; Voss et al., 1993; Howard et al., 2001). The responses induced by FB_1 are species-, strain- and gender-specific; FB_1 induced liver and kidney cancer in rats, but failed to induce OC in various animal species (Jaskiewicz et al., 1987; Gelderblom et al., 1991; Voss et al., 1993; Howard et al., 2001; Ledoux et al., 2003). The International

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Agency for Research on Cancer has classified FB_1 as a group 2B carcinogen (IARC, 2002).

Because FB_1 is a structural analogue of sphinganine and sphingosine, the hypothesised mechanism of action involves perturbations in sphingolipid metabolism. This structural homology allows FB_1 to interfere with sphingolipid biosynthesis at the level of N-acyltransferase, resulting in increased free sphingoid bases, decreased ceramide and increased sphingosine-1-phosphate (Zitomer et al., 2009; Riley et al., 2001; Soriano et al., 2005). Ceramide and sphingosine-1-phosphate play opposing roles in mammalian cells and their relative levels may influence the ultimate fate of the cell. FB_1 toxicity manifests as increased apoptosis, inhibition of protein synthesis, cell cycle arrest and increased lipid peroxidation (Wang et al., 2014; Domijan, 2012, Stockmann-Juvala and Savolainen, 2008). Sphingolipids mediate cell-stress responses and these lipid signaling molecules may also influence mitochondria, because their presence is intrinsic to the structure and function of these organelles (Nema and Kumar, 2015, Gudz et al., 1997).

A study by Riley et al. (1994) reported that FB_1 disrupted sphingolipid metabolism and induced ultrastructural lesions in both the kidney and liver in rats (Riley et al., 1994). The major mitochondrial lesions noted in the kidney of affected animals was swelling and dilatation of the cristae while the mitochondria of affected liver often appeared swollen, the cristae were occasionally disintegrated and the mitochondrial matrix was less electron dense (Riley et al., 1994). These observations were in keeping with previous data from our laboratory that showed FB_1 induced gross changes in mitochondrial morphology that included swelling, disruption of the membrane, clearing of the matrix and re-orientation of the cristae in human oesophageal tissue and SNO OC cells (Myburg et al., 2009). Domijan and Abramov (2011) showed that FB_1 selectively targets mitochondria in primary rat astrocytes and human neuroblastoma (SH-SY5Y) cells; it decreased mitochondrial and cellular respiration by inhibiting complex I of the respiratory chain and depolarized the membrane in a dose-dependent manner (Domijan and Abramov, 2011; Domijan, 2012).

FB_1 was shown to generate reactive oxygen species (ROS) in neural, intestinal and mononuclear cells (Domijan et al., 2007; Domijan and Abramov, 2011; Stockmann-Juvala et al., 2004; Klaric et al., 2008; Mary et al., 2012). The principal generators of ROS in the cell are mitochondria, producing both superoxide anions ($O_2^{\bullet}$) and other ROS as a normal by-product of electron transfer in cells (Stockmann-Juvala and Savolainen, 2008; Rumora et al., 2007; Schieber and Chandel, 2014; Chen et al., 2016). The low levels of ROS normally produced are important second messengers in signal transduction pathways involved in cell growth, transformation and apoptosis (Chen et al., 2016). Under normal circumstances $O_2^{\bullet}$ is enzymatically reduced by superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx1) thus preventing oxidative damage. Glutathione peroxidase reduces lipid hydroperoxides to alcohol and free hydrogen peroxide (H_2O_2) to water (H_2O) using reduced glutathione (GSH) as a substrate. The pentose phosphate pathway, a generator of NADPH, plays a key role in maintaining reduced levels of GSH (Schieber and Chandel, 2014). Nuclear factor (erythroid 2) factor 2 (Nrf2) is induced by oxidative stress and activation of its signaling pathway results in the activation of antioxidant and detoxifying enzymes that confer protection from oxidative stress (Schieber and Chandel, 2014). Nrf2 controls several different antioxidant pathways including GSH production and regeneration, GSH utilization, thioredoxin production, regeneration and utilization, and NADPH production (Gorrini et al., 2013; Schieber and Chandel, 2014). Other activators of GSH synthesis include activator protein-I (AP-1) and nuclear-factor kappa B (NFκB). Heat shock protein 70 (HSP70), a stress response protein that is associated with cytoprotection (Guo et al., 2007), has been shown to upregulate antioxidant enzymes GPx1 and SOD2 during cell stress. It helps maintain a redox (reducing) environment during cell stress and facilitates the removal of misfolded redox-sensitive molecules. When the generation of ROS overrides the cell's detoxification ability, ROS concentrations increase resulting in oxidative stress. The excess ROS then oxidises lipids, DNA and proteins and results in altered cell function and genetic instability that can contribute to the initiation, development and progression of cancer.

Although oxidative stress is an established mechanism of neurotoxicity and has been demonstrated in human intestinal cells, the underlying mechanisms of toxicity in oesophageal cells have not been determined. This study aimed to determine FB_1 mediated oxidative stress and cytotoxicity in human oesophageal spindle-shaped N-cadherin (+) CD45 (−) osteoblastic (SNO) cells.

2. Methods

2.1. Preparation of cells

SNO cells were grown to 80% confluency in 25 cm^3 flasks using 10% complete culture medium (CCM; Eagles Minimum Essential Medium containing 10% foetal calf serum, 1% penstrep-fungizone and 1% L-glutamine). The cells were treated with varying concentrations (0, 1.25, 10 and 20 μM) of FB_1 (diluted in 10% CCM) for 48 h. The cells were harvested by trypsinisation and the trypan-blue assay was employed to determine cell viability and cell number.

2.2. JC-1 assay

The JC-1 mitoscreen assay was used to assess changes in mitochondrial membrane polarity ($\Delta\psi_m$) and prepared as per the manufacturer's instructions. The SNO cell suspension (1×10^6 cells) was washed and stained (300 μl JC-1 solution, 37 °C, 15 min). Cells were washed twice in 1.5 ml JC-1 wash buffer, then resuspended in 1 ml sheath fluid and analyzed using a FACS Calibur (BD Biosciences) cytometer for data acquisition and Cell Quest (BD Biosciences) for data analysis. Red JC-1 aggregates in healthy cells were detected in the FL-2 channel and green JC-1 monomers in apoptotic cells in the FL-1 channel. 15,000 events were acquired.

2.3. ATP assay

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was used to determine the viability of SNO cells based on the amount of ATP generated by metabolically active cells. Treated and control cells (1×10^6) were aliquoted into individual wells of a 96-well luminometer plate. Method control wells containing culture medium without cells were prepared to determine background luminescence. The CellTiter-Glo® Reagent (100 μl) was then added into each sample well. The plate was agitated for 2 min, incubated for 10 min at room temperature (RT) and read on a Modulus™ microplate reader (Turner Biosystems).

2.4. Intracellular ROS

Intracellular ROS (iROS) was measured using the dichlorodihydrofluorescein diacetate (DCFDA) assay. Treated cells (5×10^6) were suspended in phenol free media containing 10% FCS and 10 mM DCFDA (37 °C, 45 min). The cells were rinsed 3x with 0.1 M PBS, resuspended in 150 μl PBS and analyzed using the BD Accuri™ flow cytometer.