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Quercetin modulates OTA-induced oxidative stress and redox signalling in HepG2 cells — up regulation of Nrf2 expression and down regulation of NF-κB and COX-2

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ABSTRACT

Background: Ochratoxin A (OTA), a mycotoxin, causes extensive cell damage, affecting liver and kidney cells. OTA toxicity is fairly well characterized where oxidative stress is believed to play a role, however, the sequence of molecular events after OTA-exposure, have not been characterized in literature. Further, antidotes for alleviating the toxicity are sparsely reported. The aim of this study was to understand the sequence of some molecular mechanisms for OTA-induced toxicity and the cytoprotective effect of quercetin on OTA-induced toxicity.
Methods: Time course studies to evaluate the time of intracellular calcium release and ROS induction were carried out. The time of activation and induction of two key redox- sensitive transcription factors, NF-κB and Nrf-2 were determined by nuclear localization and expression respectively. The time of expression of inflammatory marker COX-2 was determined. Oxidative DNA damage by comet assay and micronucleus formation was studied. The ameliorative effect of quercetin on OTA-induced toxicity was also determined on all the above-mentioned parameters.
Results: OTA-induced calcium release, ROS generation and activated NF-κB nuclear translocation and expression. Pre-treatment with quercetin ameliorated ROS and calcium release as well as NF-κB induction and expression. Quercetin induced Nrf-2 nuclear translocation and expression. Quercetin's anti-inflammatory property was exhibited as it down regulated COX-2. Anti-genotoxic effect of quercetin was evident in prevention of DNA damage and micronucleus formation.
Conclusion: Quercetin modulated OTA-induced oxidative stress and redox-signaling in HepG2 cells.
General significance: The results of the study demonstrate for the first time that quercetin prevents OTA-induced toxicity in HepG2 cells.

1. Introduction

Ochratoxin A (OTA) is a ubiquitous mycotoxin produced by several food-borne fungi such as *Aspergillus* sp. and *Penicillium* sp. OTA's wide spread occurrence and persistence in the food chain may lead to a significant exposure to humans [1]. OTA has grabbed global attention over the last few years as it causes genotoxicity, carcinogenicity (group 2B human carcinogen), teratogenicity, hepatotoxicity and nephrotoxicity in both human and farm animals [2]. Toxicity studies have revealed that OTA mainly affects the kidney and liver, after absorption from the gut and circulation via the portal vein, inducing hepatotoxicity in rats and hepatocellular carcinomas in mice [3]. The mechanism of OTA toxicity includes reactive oxygen species (ROS) formation and lipid peroxidation (LPO) [4], inhibition of protein synthesis [5], disturbance of calcium homeostasis and impairment of mitochondrial oxidation reactions [6,7].

At the molecular level, OTA-induced oxidative stress results in cellular counter mechanisms involving enzymatic and non-enzymatic defense systems [8], modulation of transcription factors such as nuclear factor E2 p45-related factor 2 (Nrf-2) and nuclear factor-kappa B (NF-κB) [9,10] as well as activation of cyclooxygenase-2 (COX-2), a common inflammatory marker [11]. Although these molecular redox mechanisms are reported in literature, time course studies to evaluate time and sequence of induction have not been carried out.

Abbreviations: ANOVA, Analysis of variance; AO/EB, Acridine orange/ethidium bromide; ARE, Antioxidant responsive element; $[Ca^{2+}]_i$, Intracellular calcium level; CAT, Catalase; CCCP, Carbonyl cyanide m-chlorophenylhydrazone; COX-2, Cyclooxygenase-2; CBMN, Cytokinesis block micronucleus assay; DAB, Diaminobenzidine; DAPI, 4,4-diamidino-2-phenylindole; DCF, 2,7-dichlorofluorescein; DCF-DA, 2,7-dichlorodihydrofluorescein diacetate; DiOC6, 3,30-di-hexyloxacarbocyanine iodide; DMEM, Dulbecco's modified Eagle's medium; DTT, Dithiothreitol; EDTA, Ethylenediamine tetraacetic acid; FBS, Fetal bovine serum; GPx, Glutathione peroxidase; GSH, Glutathione; GST, Glutathione S transferase; IC20, Inhibitory concentration 20; iNOS, Inducible nitric oxide synthase; LDH, Lactate dehydrogenase; LPS, Lipopolysaccharide; LPO, Lipid peroxidation; MN, Micronucleus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, Nitric oxide; Nrf-2, Nuclear factor E2 p45-related factor 2; NF-κB, Nuclear factor-kappa B; OTA, Ochratoxin; OPT, Ortho-phthalaldehyde; PCC, Protein carbonyl content; PMSF, Phenyl methyl sulfonyl fluoride; ROS, Reactive oxygen species; SOD, Superoxide dismutase.

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In the present study, we have attempted to understand the sequence of some underlying molecular redox mechanisms of OTA toxicity. Parameters such as calcium induction, ROS generation, modulation of redox transcription factors such as NF-κB and Nrf-2 and induction of COX-2 were evaluated.

A growing body of evidence indicates that oxidative stress plays an important role in the pathogenesis of many clinical conditions involving cardiovascular diseases, liver diseases, lung diseases, gastrointestinal disorders and aging. Several environmental and food toxins also are believed to exert their detrimental effect in cells through oxidative stress. In recent years, the naturally occurring substances have been receiving increased attention by researchers and have been subject to many rigorous scientific and clinical studies. Antioxidants could be a new therapeutic tool to improve the clinical manifestation of these illnesses [12]. Quercetin, a ubiquitous flavanoid (3,3′,4′,5,7-pentahydroxyflavone) in particular has shown great promise in a number of areas relevant to human health [13]. It has been shown to be an excellent *in vitro* antioxidant through its potent scavenger of ROS, including $O2^{\cdot -}$ [14], RNS like NO [15] and ONOO— [16]. It also exerts various bioactive effects which includes, anti-inflammatory [17,18] and antimutageneic [19].

Several studies in literature have proved the efficacy of quercetin as an antioxidant and potent free radical scavenger; however, this is the first study where the cytoprotective effect of quercetin on OTA-induced toxicity has been studied in liver cells. Although the ameliorative actions of retinol, ascorbic acid, and alpha tocopherol have been studied against OTA-induced toxicity, Quercetin was chosen for this study as it is ubiquitous in nature, can be consumed a part of food and devoid of toxicity compared to fat soluble vitamins. It is suggested to substantially empower the endogenous antioxidant shield due to its contribution to the total plasma antioxidant capacity which is 6.24 times higher than the reference antioxidant trolox [20].

We present here for the first time, the sequence of some underlying molecular mechanisms for OTA-induced toxicity as well as the cytoprotective effect of quercetin on OTA-induced toxicity in HepG2 cells, with specific reference to oxidative stress, intracellular calcium flux, levels of protective antioxidant enzymes as well as nuclear localization and expression of two key transcription factors Nrf-2 and NF-κB p65. In addition, expression of inflammatory marker COX-2 as well as DNA damage and micronucleus (MN) formation was studied.

2. Materials and methods

2.1. Materials

OTA was purchased from Sigma Aldrich, Bangalore. Quercetin, Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), 3-4,5-dimethylthiazol-2-yl, 2,5-diphenyltetrazoliumbromide (MTT) and all other analytical grade chemicals were obtained from Hi-media Laboratories, Mumbai, India. Primary monoclonal antibodies for NF-κB p65 (sc-1008), COX-2 (sc-19999), Nrf2 (sc-365949), β-actin (sc-10731) and Lamin B2 (sc-58667) were obtained from Santa Cruz Biotech, CA. Western blot membranes were obtained from Whatman, USA. Dichlorofluorescein diacetate (DCF-DA), Diehxyloxacarbocyanine iodide (DiOC6), and Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (CCCP) were obtained from Calbiochem, USA. DAB/H_2O_2 color development kit for western blots was obtained from Bangalore Genei, India.

2.2. Cell culture

HepG2 (human hepatoma) cell line obtained from National centre for cell science (NCCS), Pune, was grown to confluence in 25 cm^2 flasks supplemented with DMEM and 10% FBS (v/v), containing, 100 units/ml penicillin and 30 μg/ml streptomycin in a CO_2 incubator with 5% CO_2.

2.3. Cytotoxicity assay by MTT test

Since OTA affects cells of liver, HepG2 cells were chosen for this study. Cytotoxicity was determined colorimetrically by MTT method. Cells were seeded in 96 well plate at a density of 1×10^4 cells per well, in 0.2 ml DMEM and exposed to increasing concentration of OTA (10–50 μM/ml) dissolved in 50% (*v*/*v*) ethanol (solvent concentration not exceeding 0.1%) for different time intervals (24, 48 and 72 h). The concentrations of OTA were chosen (10–50 μM) based on previous reports which reflect concentrations found in plasma and other tissues in rats after OTA was fed orally at 0.5 mg/kg [21]. Quercetin dissolved in dimethyl sulfoxide (DMSO) was added to the cells to reach a final concentration of 5, 10 and 15 μM (final solvent concentration did not exceed 0.1% v/v) to determine pre, co and post treatment efficacy. The concentrations of quercetin were based on sera concentrations of patients given an oral dose of the flavonoid [22]. Based on the dose response studies, effective OTA (10 μM for 48 h) and quercetin (10 μM pre-treatment for 24 h) concentrations were chosen as optimum which was followed for further studies. Control cells received appropriate carriers, final concentration not exceeding 0.1%. After the exposure times, MTT was added to a final concentration of 0.5 mg/ml medium and the plates were incubated for 4 h at 37 °C. The purple formazan crystals formed were dissolved in DMSO and read at 570 nm in a microquant plate reader (Bio-Tek Instruments). The results were expressed as % viability.

2.4. Measurement of intracellular calcium level ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured using fluorescent dye Fura-2 AM [23]. Cells were treated with OTA for different time intervals (10, 15, 30, 45 and 60 min). In the quercetin pre-treatment groups, cells were pre-treated with quercetin for 24 h, followed by OTA exposure. As a positive control, cells were treated with 50 μM H_2O_2 for 30 min. Cells from the treatment groups were washed in calcium buffer (HEPES 10 mM, NaCl 132 mM, KCl 3 mM, glucose 10 mM, $CaCl_2$ 1 mM and K_2HPO_4 at pH 7.4), loaded with Fura-2 AM in calcium buffer (5 μM) and incubated for 1 h at 37 °C in a CO_2 incubator. Cells were then washed with PBS, trypsinized and suspended in 1.0 ml PBS. Changes in $[Ca^{2+}]_i$ were measured in a spectrofluorometer, excitation wavelength 340 nm and 380 nm and emission wavelength 500 nm. The ratio of intensities at 340 and 380 nm is proportional to $[Ca^{2+}]_i$. The values were expressed as % relative fluorescence compared to control.

2.5. Measurement of ROS in cells with fluorescent dye DCFH-DA

OTA-induced cellular oxidative stress was evaluated by seeding cells in a 24 well plate. After overnight attachment, the cells were incubated for 30 min at 37 °C in PBS containing 25 μl of 50 μM H_2DCF-DA, [24] followed by OTA exposure (10 μM) for different time intervals (15, 30, 60, 180 and 300 min). In the quercetin pre-treatment groups, cells were pre-treated with quercetin for 24 h and then exposed to H_2DCF-DA as described above and then exposed to OTA. For positive control, 50 μM H_2O_2 for 60 min was included. The cells were centrifuged, washed and resuspended in PBS, and 2,7-dichlorofluorescein (DCF) formed was measured in a Hitachi spectrofluorometer, (excitation 480 nm, emission 520 nm). The estimations were carried out thrice in triplicate, ensuring each time that the number of cells per treatment group were the same to ensure reproducibility. The values were expressed as % relative fluorescence compared to the control.