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Evaluation of the cytotoxic effect of 7keto-stigmasterol and 7keto-cholesterol in human intestinal (Caco-2) cells

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ABSTRACT

The biological implications of cholesterol oxidation products have been investigated, though research on plant sterol oxidation products is scarce and in some cases contradictory.

The cytotoxicity of 7keto(k)-stigmasterol versus 7keto(k)-cholesterol at different concentrations (0–120 μM) and incubation times (4–24 h), in intestinal epithelial cells (Caco-2 cells) was evaluated. The 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide and neutral red uptake tests, mitochondrial membrane potential ($\Delta\Psi m$), and relative DNA and RNA contents in the cell cycle phases were determined. Possible interaction effects between 7k-derivatives or non-oxidized stigmasterol were monitored.

Endo/lysosomal activity was not impaired by either oxide. 7k-cholesterol showed a deleterious effect upon the mitochondrial compartment after 24 h of exposure (120 μM), as well as upon $\Delta\Psi m$ when incubated at all concentrations (12/24 h). Only cells incubated with 7k-cholesterol (120 μM) exhibited a decrease in RNA proportion in the G1 population. The presence of 7k-stigmasterol or stigmasterol with 7k-cholesterol reduced the deleterious metabolic effects upon mitochondrial functionality and integrity and the distribution of RNA contents in G1 and G2 phases. A decrease in the G1 phase proportion was detected in cells exposed to mixtures, without alterations in RNA content. The results obtained indicate the absence of 7k-stigmasterol cytotoxicity in Caco-2 cells and its capacity to reduce 7k-cholesterol toxicity.

1. Introduction

Due to their known cholesterol-lowering effect, plant sterols are used in nutritional strategies to prevent coronary and cardiovascular risks (Garcia-Llatas and Rodriguez-Estrada, 2011).

The structure of plant sterols is similar to that of cholesterol and, consequently, they are prone to oxidation during technological processes in food production and/or storage, leading to the formation of plant sterols oxidation products (POPs) with potential cytotoxic effects. There is evidence that cholesterol oxidation products (COPs) may be linked to the initiation and progression of several chronic diseases including atherosclerosis, neurodegenerative processes, diabetes, kidney failure and ethanol intoxication (Otaegui-Arrazola et al., 2010). However, research on biological activities of POPs has been more limited, and the results in some cases have been contradictory (reviewed by Hovenkamp et al., 2008; Ryan et al., 2009; Garcia-Llatas and Rodriguez-Estrada, 2011).

Previous studies have demonstrated qualitatively similar cytotoxic effects of POPs in different mammalian cell types, with differences in their susceptibility. Cholesterol oxidation products exert severe cytotoxic effects in the liver and favor the development of cardiovascular diseases - the intestinal epithelium being the first physiological barrier after the oral intake of these compounds. Acdox et al. (2001), in a cultured mouse macrophage cell line (C57BL/6) exposed to cholesterol oxides and oxides of β-sitosterol/campesterol mixture (200 μg/mL), reported similar patterns of toxicity in the form of reduced cell viability and mitochondrial dehydrogenase activity. However, the effects were less severe with the plant sterol oxides. The same conclusion was drawn by Maguire et al. (2003) in monocytic-U937 cells, after evaluating the cytotoxic effects of α-epoxysitosterol and a β-sitosterol thermooxidized mixture versus 7β-OH-cholesterol. Koschutnig et al. (2009), in hepatic-HepG2 cells, reported a decrease in cell viability when exposed to β-sitosterol oxides, but only 7k-sitosterol

Abbreviations: DHR, 1,2,3-dirhodamine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide; COPs, cholesterol oxidation products; DMEM, Dulbecco's Modified Eagle Medium; $\Delta\Psi m$, mitochondrial membrane potential; POPs, plant sterol oxidation products; PBS, phosphate buffered solution; PI, propidium iodide.

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(30 μM) induced apoptosis. Other studies have evaluated the cytotoxicity and apoptotic potential of individual β-sitosterol oxides applied to three cell lines: human monocytic line (U937), hepatoma (HepG2) and colonic adenocarcinoma (Caco-2), establishing comparisons with their corresponding COPs. 7β-OH, 7keto and triol derivatives of β-sitosterol were found to be cytotoxic (at 60 and 120 μM) for all the cell lines (in contrast to COPs, which were toxic even at 30 μM), but no evidence of apoptosis was found in either HepG2 or Caco-2 cells (Ryan et al., 2005). The mechanism by which 7β-OH derivatives of cholesterol and β-sitosterol may cause cell death in Caco-2 cells has been analyzed in more detail by Roussi et al. (2005), revealing more sensitive responses to 7β-OH cholesterol than to 7β-OH sitosterol. Both hydroxysterols (30 μM and 60 μM, respectively) reduced cell proliferation and induced apoptosis by intracellular caspase-dependent process for 7β-OH-sitosterol, and various caspase-independent factors for 7β-OH-cholesterol. Roussi et al. (2007) evaluated and compared the 7β-OH-sitosterol and 7β-OH-cholesterol effects upon mitochondrial and lysosomal integrity and several apoptosis modulators. Both compounds caused mitochondrial membrane perturbations in an early stage of the apoptotic process (12 h) after treatment. In addition, lysosomal membrane integrity was altered by both hydroxysterols, whereas 7β-OH-sitosterol was found to be more potent than its corresponding oxycholesterol.

Most of these studies reported negative effects of these compounds upon the energetic or lysosomal metabolism of the different cell cultures, sometimes associated to the appearance of apoptosis processes. However, because of their greater prevalence and also due to the lack of commercially available pure plant sterol oxides, most cytotoxicity studies have focused on β-sitosterol oxides.

β-Sitosterol, campesterol and stigmasterol are the most prevalent plant sterol in terms of dietetic intake. The average intake of plant sterols, and in particular of stigmasterol, has been estimated by several authors in different population groups (Morton et al., 1995; Vries et al., 1997; Jekel et al., 1998; Normén et al., 2001; Valsta et al., 2004). Moreover, the greater uptake efficiency of stigmasterol versus β-sitosterol has been demonstrated in Caco-2 cells (10–40 times greater, depending on the concentration involved) (1.6–12.5 μM) (Fahy et al., 2004).

Only one study has been found on the inhibitory effect of synthesized stigmasterol oxides (α-epoxide, β-epoxide, 7keto, 7β-OH, epoxydiol, diepoxide, 22*R*,23*R*-triol and 3,5,6-triol) related to the cytotoxicity and apoptotic effects upon the U937 monocytic cell line (O'Callaghan et al., 2010). These authors evaluated the viability and the mitochondrial functionality of these cells (30–120 μM) as well as the percentage of apoptotic nuclei (30–120 μM) and other indices of cell death (60 μM) - including glutathione depletion, caspase-3 activity and Bcl-2 expression levels - showing these compounds to cause different toxicities depending on their structure. In this study, they observed no decrease in cellular activity, though a decrease in mitochondrial functionality was recorded with 7k-stigmasterol (30–120 μM), without inducing apoptosis. In contrast, 7k-sitosterol was significantly ($p < 0.05$) cytotoxic and apoptotic in this cell line (Ryan et al., 2005) and in HepG2 cells (Koschutnig et al., 2009), as previously reported.

In view of the above observations, the present study was designed to evaluate the potential cytotoxicity of 7k-stigmasterol versus 7k-cholesterol in the endo/lysosomal and mitochondrial compartments, as well as the proportion of acid nuclei in intestinal epithelial cells (Caco-2 cells). Since both compounds can coexist in food, an evaluation was made of the possible effect of 7k-cholesterol in the presence of 7k-stigmasterol or stigmasterol, with evaluation of the aforementioned parameters.

2. Material and methods

2.1. Chemicals

Dulbecco's Modified Eagle Medium (DMEM + GlutaMAX™), glucose, fetal bovine serum, non-essential amino acids, Hepes, antibiotic solution (penicillin–streptomycin), fungizone, phosphate buffered solution (PBS) and trypsin–EDTA solution (2.5 g/L trypsin and 0.2 g/L EDTA) were purchased from Gibco (Scotland, UK). Ethanol 99.9%, HCl 37% were from Merck (Darmstadt, Germany). 7k-stigmasterol was from Steraloids (Newport, RI, USA). Stigmasterol, 7k-cholesterol, Neutral Red dye, 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide (MTT), triton, 1,2,3-dirhodamine (DHR), propidium iodide (PI), RNase A, trisodium citrate, Hoechst 33342 and pyronin Y were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and 2-propanol 99.9% was from Prolabo (Fontenay–Sous–Bois, France).

2.2. Caco-2 cell culture

After oral intake, the intestinal epithelium constitutes the first physiological barrier for the absorption of POPs. It is therefore interesting to study the effects of these compounds using a validated intestinal epithelial cell model (Caco-2 cells), since data in this field remain scarce. Cell cultures were used 6 days post-seeding. Cell differentiation in this cell line was assessed and confirmed by measuring brush border enzyme activities (alkaline phosphatase and sucrase–isomaltase), according to the procedure described by Jovaní et al. (2001).

Human colon adenocarcinoma (Caco-2) cells were purchased from the European Collection of Cell Cultures (ECACC 86010202, Salisbury, UK) and were used between passages 42 and 60. They were grown in 75 cm^2 Falcon flasks (IWAKI brand) in DMEM + GlutaMAX™ containing 1 g/L glucose and supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, 1% (v/v) Hepes, 1% (v/v) antibiotic solution (penicillin–streptomycin) and 0.2% (v/v) fungizone. Cells were incubated at 37 °C in a humidified atmosphere with 5% (v/v) CO_2.

For all experiments, cells were sub-cultured after trypsin treatment (2.5 g/L trypsin and 0.2 g/L EDTA) and seeded onto 24-well plates at a density of 5×10^4 cells/cm^2 with 1 mL of DMEM. Culture media were changed every two days.

2.3. Caco-2 treatment with 7keto sterols

In order to compare the cytotoxicity of 7k-stigmasterol and 7k-cholesterol, both oxides were dissolved in ethanol and used at a final concentration of 30, 60 or 120 μM in DMEM (containing 0.66% (v/v) of ethanol). One milliliter of these solutions was added to cell cultures and incubated for different times (4, 12 and 24 h).

In order to evaluate the possible effect of 7k-cholesterol in the presence of 7k-stigmasterol or stigmasterol, cell cultures were challenged to 1 mL of different solutions of stigmasterol, 7k-stigmasterol or 7k-cholesterol alone (120 μM), or to mixtures of 7k-cholesterol with stigmasterol or 7k-stigmasterol (120 μM each) in DMEM (containing 0.66% (v/v) of ethanol) for 24 h.

In both assays, control cultures were exposed to DMEM containing an equivalent volume of ethanol (0.66% (v/v).

2.4. Evaluation of endo/lysosomal activities

Following the aforementioned treatments, the supernatants were removed from the cell cultures and aliquots (1 mL) of Neutral Red dye (1/10 (v/v) in PBS) were added to each well. The plate was returned to the incubator and kept for 1 h at 37 °C and 5% CO_2 to allow for uptake of the red dye into the endo/lysosomal compartment (Borenfreud and Puerner, 1985) of viable cells. Then, the medium was removed and the red dye was redissolved with 1.5 mL of 1% glacial acetic acid/50% ethanol. After homogenization on a plate shaker, the samples were transferred to microcuvettes and absorbance was determined spectrophotometrically (540 nm with background subtraction at 570 nm).

2.5. Evaluation of mitochondrial functionality

The mitochondrial functionality of the Caco-2 cells was evaluated by using the MTT assay (Laparra et al., 2008). This colorimetric method is based on reduction of the tetrazolium ring of MTT by mitochondrial dehydrogenases (Ekmekcioglu et al., 1999), yielding a blue formazan product that is proportional to the number of viable cells and can be measured spectrophotometrically. Briefly, after incubation with the sterol oxides the culture medium was removed, MTT (0.5 mg/mL in PBS) was added to the cells, followed by incubation for 1 h at 37 °C/5% CO_2/95% relative humidity atmosphere. Then, the medium was removed and cells were harvested with 1.5 mL of a solution of acidic 2-propanol (0.1 N HCl, 0.1% w/v triton). The conversion to formazan was measured using a spectrophotometer and absorbance was measured at 570 nm with background subtraction at 690 nm.