Toxicology 288 (2011) 43–48

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

# Olive oil hydroxytyrosol reduces toxicity evoked by acrylamide in human Caco-2 cells by preventing oxidative stress

Ildefonso Rodríguez-Ramiro, María Ángeles Martín, Sonia Ramos, Laura Bravo, Luis Goya*

*Department of Metabolism and Nutrition, ICTAN, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain*

ARTICLE INFO

*Article history:*
Received 13 June 2011
Accepted 6 July 2011
Available online 12 July 2011

*Keywords:*
Olive oil antioxidants
Antioxidant defences
Oxidative stress
Reactive oxygen species
Jun kinases
Colon toxicity

ABSTRACT

Humans are exposed to dietary acrylamide (AA) during their lifetime, it is therefore necessary to investigate the mechanisms associated with AA-induced toxic effects. Accumulating evidence indicates that oxidative stress contributes to AA cytotoxicity, thus, dietary antioxidants might have a protective role in colonic cells against AA toxicity. We have recently reported that hydroxytyrosol (HTy), a natural antioxidant abundant in olive oil, is able to enhance the cellular antioxidant defence capacity, thereby protecting cells from oxidative stress.

In this study, we evaluate the protective role of HTy on alterations of the redox balance induced by AA in Caco-2 intestinal cells. AA cytotoxicity was counteracted by HTy by powerfully reducing ROS generation, recovering the excited enzyme antioxidant defences and decreasing phospho-Jun kinase concentration and caspase-3 activity induced by AA. Therefore, AA-induced cytotoxicity and apoptosis are closely related to oxidative stress in Caco-2 cells and the olive oil natural dietary antioxidant HTy was able to contain AA toxicity by improving the redox status of Caco-2 cells and by partly restraining the apoptotic pathway activated by AA.



## 1. Introduction

The presence of acrylamide (AA) in commonly ingested foods has originated a great concern because humans could be exposed to significant quantities of this toxin during their life-span. Relevant amounts of AA have been identified in heat-treated carbohydrate-rich foods such as fried potatoes, cookies, bread and breakfast cereals (Tareke et al., 2002). Most investigations of AA toxicity have mainly focused on their genotoxic and carcinogenic properties; however, accumulating evidences seem to indicate that AA also possesses cytotoxic properties by affecting the redox status of the cells (Naruszewicz et al., 2009; Park et al., 2010; Parzefal, 2008; Yousef and El-Demerdash, 2006; Zhang et al., 2009).

AA is readily absorbed into the intestinal cells and it is usually non-enzymatically and enzymatically conjugated with reduced glutathione (GSH) resulting in a depletion of cellular GSH stores (Lash, 2006; Pernice et al., 2009; Zödl et al., 2007). As occurs under various pathological states (Lash, 2006), the decreased GSH levels evoke the overproduction of reactive oxygen species (ROS) which activates signalling cascades involving members of the mitogen-activated protein kinase (MAPK) family, such as Jun kinases (JNKs), that play a key role in the regulation of many cellular processes including apoptosis (Li et al., 2006; Valko et al., 2007). Studies in intestinal cells have demonstrated that loss of cellular glutathione redox balance is an important player in apoptotic signalling and cell death (Circu et al., 2008; Wang et al., 2000). In line with this, we have recently shown that AA-induced cytotoxicity and apoptosis are closely related to oxidative stress in Caco-2 cells (Rodríguez-Ramiro et al., 2011), a human cell line originating from the gastrointestinal tract that retains many of the morphological and enzymatic features typical of normal human colonocytes. Therefore, depletion of GSH levels favouring cellular oxidative stress and apoptosis may be suggested as a potential mechanism for AA toxicity in gastrointestinal tract.

The use of natural compounds derived from the diet with antioxidant effects might provide a strategy to reduce AA toxicity. Indeed, we have recently shown the protective effect of cocoa flavanols against an induced AA toxicity in colonic cells Caco-2 (Rodríguez-Ramiro et al., 2011). In line with this, olive oil, the major fat source of Mediterranean diet, is recognized for its antioxidant properties and its positive effects on oxidative stress associated processes (Fitó et al., 2007). The phenolic fraction of virgin olive oil has proved to have antioxidant activity in vitro, scavenging peroxyl radicals (Saija et al., 1998), other free radicals (Gordon et al., 2001) and reactive nitrogen species (De la Puerta et al., 2001), or breaking peroxidative

* Corresponding author at: Department of Metabolism and Nutrition, ICTAN (former Instituto del Frío), Consejo Superior de Investigaciones Científicas, José Antonio Novais 10, Ciudad Universitaria, 28040 Madrid, Spain. Tel.: +34 91 544 56 07; fax: +34 91 549 36 27.
*E-mail address:* luisgoya@ictan.csic.es (L. Goya).

0300-483X/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.tox.2011.07.002

chain reactions and preventing metal ion catalyzed production of reactive oxygen species (Manna et al., 1997; Mateos et al., 2003). The main phenolic compounds in virgin olive oil are secoiridoid derivatives of 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol) (HTy) and of 2-(4-hydroxyphenyl)ethanol or tyrosol, and 2-(3,4-dihydroxyphenyl)ethyl acetate (Mateos et al., 2001). In particular, HTy is considered one of the most abundant and representative olive oil phenols and its protective effect against an oxidative stress-induced cytotoxicity has been systematically demonstrated (Goya et al., 2007; Martín et al., 2010).

Biological actions of phenolic compounds have been commonly related to their free radical scavenging activities, but current evidence strongly support that natural biophenols may also offer an indirect protection by increasing the endogenous antioxidant defence system (Masella et al., 2004, 2005). In fact, we have recently shown an additional mechanism of action of HTy to prevent oxidative stress damage in hepatic cells through the modulation of signalling pathways involved in antioxidant/detoxifying enzymes regulation (Martín et al., 2010). This double mechanism of action confers HTy a great chemo-protective potential to prevent oxidative stress-associated cell damage. Therefore, the aim of the present study was to investigate whether HTy is able to protect Caco-2 cells against oxidative stress and apoptosis induced by AA.

## 2. Materials and methods

### 2.1. Materials and chemicals

O-phthalaldehyde (OPT), gentamicin, penicillin G, streptomycin, acrylamide and the rest of chemicals for all analysis were purchased from Sigma Chemicals (Madrid, Spain). The fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR). Anti-JNKs, antiphospho-JNK (p-JNKs) and anti-β-actin were obtained from Cell Signaling Technology (Izasa, Madrid, Spain). Caspase-3 substrate (Ac-DEVD-AMC) was purchased from Pharmingen (San Diego, CA). Materials and chemicals for electrophoresis and the Bradford reagent were from BioRad (Madrid, Spain). Cell culture dishes were from Falcon (Cajal, Madrid, Spain) and cell culture medium and foetal bovine serum (FBS) from Biowhittaker Europe (Lonza, Madrid, Spain).

### 2.2. Cell culture and AA and hydroxytyrosol treatment

Human Caco-2 cells were grown in a humidified incubator containing 5% $CO_2$ and 95% air at 37 °C. They were grown in DMEM F-12 medium from Biowhitaker (Lonza, Madrid, Spain), supplemented with 10% Biowhitaker foetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin. Plates were changed to FBS-free medium the day before the assay. HTy was obtained by chemical synthesis from 3,4-dihydroxyphenylacetic acid by reduction with $LiAlH_4$ and was a kind gift from Dr. José Luis Espartero (Facultad de Farmacia, Universidad de Sevilla, Spain). The different concentrations of HTy, 5, 10, 20, 40 (and 100 for the viability assay) μM were dissolved in serum-free culture medium and added to the cell plates for 20 h. To evaluate the protective effect of HTy against AA toxicity, concentrations of HTy were diluted in serum-free culture medium and added to the cell plates for 20 h. After that, the medium was discarded and fresh medium containing 5 mM of AA was added for different incubation times; this AA concentration was selected from our previous study (Rodríguez-Ramiro et al., 2011).

### 2.3. Cytotoxicity assays

Cellular damage induced by AA was evaluated by crystal violet assay (Granado-Serrano et al., 2007) and by lactate dehydrogenase (LDH) leakage (Goya et al., 2007). For the crystal violet assay, Caco-2 cells were seeded at low density ($10^4$ cells per well) in 96-well plates, grown for 20 h with the different treatments and incubated with crystal violet (0.2% in ethanol) for 20 min. Plates were rinsed with distilled water, allowed to dry, and 1% sodium dodecyl sulfate (SDS) added. The absorbance of each well was measured using a microplate reader at 570 nm (Bio-Tek, Winooski, VT, USA). For LDH assay, the culture medium and the cells scraped in PBS were collected after the different treatments. Cells were first sonicated to ensure breaking down the cell membrane to release the total amount of LDH; then, after centrifugation ($1000 \times g$, 15 min) to clear up the cell sample, 10 μL were placed into a well of a 96 multiwell for the assay. In the same manner, 10 μL of each culture medium were also deposited into a well of a 96-well multiwell. The LDH leakage percentage was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content.

### 2.4. Evaluation of ROS generation

Cellular ROS were quantified by the DCFH assay using a microplate reader as previously described (Goya et al., 2007). For the assay, cells were plated in 24-well multiwells at a rate of $2 \times 10^5$ cells per well and changed to FBS-free medium and the different treatments the day before the assay. After that, 5 μM DCFH was added to the wells for 30 min at 37 °C. Then, cells were washed twice with PBS and 0.5 mL of serum-free medium or serum-free medium with 5 mM AA were added per well. After being oxidized by intracellular oxidants, DCFH will become dichorofluorescein (DCF) and emit fluorescence. ROS generation was evaluated at different times in a fluorescent microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Bio-Tek, Winooski, VT, USA).

### 2.5. Determination of caspase-3 activity

Apoptosis was evaluated as activation of caspase-3 (Granado-Serrano et al., 2006). Cells were lysed in a buffer containing 5 mM Tris (pH 8), 20 mM EDTA, and 0.5% Triton X-100. The reaction mixture contained 20 mM HEPES (pH 7), 10% glycerol, 2 mM dithiothreitol (DTT), and 30 μg of protein per condition, and 20 μM Ac-DEVDAMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) as substrate. Enzymatic activity was determined by measuring fluorescence at an excitation wavelength of 380 nm and an emission wavelength of 440 nm (Bio-Tek, Winooski, VT, USA).

### 2.6. Determination of GSH concentration

The content of GSH was quantified by the fluorometric assay of Hissin and Hilf (1976). The method takes advantage of the reaction of GSH with OPT at pH 8.0. After the different treatments, the culture medium was removed and cells were detached and homogenized by ultrasound with 5% trichloroacetic acid containing 2 mM EDTA. Following centrifugation of cells for 30 min at 3000 rpm, 50 μL of the clear supernatant were transferred to a 96 multiwell plate for the assay. Fluorescence was measured at an excitation wavelength of 340 nm and an emission wavelength of 460 nm. The results of the samples were referred to those of a standard curve of GSH. The precise protocol has been described elsewhere (Goya et al., 2007).

### 2.7. Determination of glutathione peroxidase (GPx) and glutathione reductase (GR) activities

Cells were collected in PBS and centrifuged at low speed ($300 \times g$) for 5 min to pellet cells. Cell pellets were resuspended in 20 mM Tris containing 5 mM EDTA and 0.5 mM mercaptoethanol, sonicated and centrifuged at $3000 \times g$ for 15 min. Enzyme activities were measured in the supernatants. Determination of GPx activity is based on the oxidation of GSH by GPx, using *tert*butyl hydroperoxide as a substrate, coupled to the disappearance of NADPH by GR (Gunzler et al., 1974). GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione (Goldberg and Spooner, 1987). Protein was measured by the Bradford reagent.

### 2.8. Preparation of cell lysates for Western blotting

To detect the levels of JNKs and p-JNKs, cells were lysed at 4 °C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.1% Triton X-100, 200 mM β-glycerolphosphate, 0.1 mM $Na_3VO_4$, 2.5 μg/mL leupeptin, 2.5 μg/mL aprotinin and 1 mM phenylmethylsulfonyl fluoride. The supernatants were collected, assayed for protein concentration by using the Bradford reagents, aliquoted and stored at −80 °C until used for Western blot analyses.

### 2.9. Protein determination by Western blotting

Equal amounts of protein (100 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) filters (Protein Sequencing Membrane, Millipore). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated antirabbit Ig (GE Healthcare, Madrid, Spain). Blots were developed with the ECL system (GE Healthcare). Normalization of Western blot was ensured by β-actin and bands were quantified by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA).

### 2.10. Statistics

Statistical analysis of data was as follows: prior to analysis the data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. The level of significance was $P < 0.05$. A SPSS version 19.0 program has been used.