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A cell culture model for the assessment of the chemopreventive potential of dietary compounds

Luis Goya, M. Angeles Martín, Sonia Ramos, Raquel Mateos and Laura Bravo

Departamento de Metabolismo y Nutrición; Instituto del Frío (CSIC); José Antonio Novais, 10; 28040 Madrid, Spain

*Corresponding author: Luis Goya, Departamento de Metabolismo y Nutrición, Instituto del Frío (CSIC); José Antonio Novais, 10; 28040 Madrid, Spain

Phone: +34.91.544.56.07
Fax: +34.91.549.36.27

e-mail: luisgoya@if.csic.es

Abstract

The study of the mechanisms involved in cell damage mediated by oxidative compounds as well as the evaluation of biomarkers of the cellular antioxidant defence system in such conditions could greatly help to prevent appearance and development of oxidative stress related diseases. The present overview describes a model of oxidative stress in cultured cells based on the evaluation of cellular antioxidant defences and suitable to assay the possible chemopreventive effect of dietary compounds.

Since the liver is the major place for xenobiotic metabolism, research on chemopreventive compounds should focus on the response of liver cells. Human HepG2, a differentiated cell line from hepatic origin, is a reliable model widely used for biochemical and nutritional studies where many compounds and conditions can be assayed with minor interassay variations

The products selected for this overview are representative of different constituents included in the human diet and possess diverse structures and mechanisms of antioxidant activity: phenolic compounds such as quercetin and hydroxytyrosol, a Maillard reaction product such as coffee melanoidin and an oligoelement such as a selenium derivative. The results confirm the reliability of the model and give more insight into the specific mechanisms involved in the biological activity of the tested compounds.

Keywords: antioxidant defences, biomarkers of oxidative stress, dietary antioxidants, polyphenols, melanoidins, selenium

Introduction

Oxidative stress caused by oxygen radicals damages cellular DNA, proteins and lipids and is widely recognized as one of the causes of the development of chronic disease [1,2]. Under normal circumstances the levels of reactive oxygen species (ROS) are low enough to be effectively removed by the natural defence mechanisms of the cell. There are, however, many conditions that enhance the production of ROS to such an extent that cellular defences are overwhelmed and the cell is injured [3]. The clinical implications of these alterations can be severe; in fact, the accumulation of ROS in several cellular components is thought to be a major cause of molecular injury leading to cell aging and to age-related degenerative diseases such as cancer, brain dysfunction and coronary heart disease [1,2,4].

There is substantial evidence that antioxidative food components have a protective role against oxidative stress-induced atherosclerosis, degenerative and age-related diseases, cancer and aging [5]. Among dietary components considered for chemopreventive activity, plant-derived compounds have shown early and continued promise [4,5]. The present overview describes a model of oxidative stress in cultured cells based on evaluation of cellular antioxidant defences and suitable to assay the possible chemopreventive effect of natural dietary compounds.

In order to establish a model of oxidative stress in cultured cells it is necessary to assess: a) cell viability, b) an index of cellular oxidative stress, c) the response of the non-enzymatic and enzymatic antioxidant defences, and d) biomarkers of oxidative damage to molecules [3-6]. The potential protective effect of different chemopreventive compounds has been tested in a cell culture model of human HepG2. The products selected for this overview are representative of different antioxidant groups included in human diet: phenolic compounds such as quercetin (Q, plant flavonoid) and

hydroxytyrosol (HTy, olive oil phenolic), a digested coffee melanoidin (DCM, Maillard reaction product) and selenomethyl selenocystein (Se-MeSeCys, selenium derivative). All four products present rather different structures and mechanisms of chemopreventive activity, but treatment of cultured HepG2 cells with each one evoked a significant protective effect against a chemically-induced condition of oxidative stress. The present overview summarizes the most remarkable results obtained with this cell culture model and discusses the different antioxidant mechanisms involved in the chemoprotective process.

Interest and setting of the model

In order to unravel the intimate mechanisms that regulate the antioxidant defences, it is necessary to study the response of such defence system to external signals that modify the intracellular prooxidant-antioxidant balance. The liver is not only the main target for antioxidant compounds once absorbed from the gastrointestinal tract but is also the major place for xenobiotic metabolism; therefore, studies dealing with the effect of dietary compounds at a physiological level in the liver of live animals and at a cellular level in cultured cells from hepatic origin should be encouraged.

Both experimental animal [7,8] and cell culture [9-13] approaches have been used by researchers to carry out such studies in vivo. On the one hand, the simultaneous fluctuations of fuels and hormones and the complexity of tissue and organ organization in experiments in live animal models make it very difficult to demonstrate molecular mechanisms of specific regulation [7]. On the other hand, the cell model should be strong enough to go through a process of response to oxidative stress by activating defence mechanisms to face and endure it, and only if the insult is too high or too long the cell will die and detach from the cell culture plate. Moreover, the cell model should

have strong antioxidant defences so that measurable changes in the antioxidant responses are observed. Therefore, the cells should be able to stay in culture for a long period both to allow long treatments with high stability and to ensure a constant and reliable response to a repeated condition. All these handicaps frequently preclude the use of cultured hepatocytes as a model for oxidative stress in cell culture.

The study of the regulation of antioxidant defence mechanisms at the cellular level may benefit from the use of an established cell culture line. Human HepG2, a well differentiated transformed cell line, is a reliable model, easy to culture, well characterized and widely used for biochemical and nutritional studies where many antioxidants and conditions can be assayed with minor interassay variations [14-20].

Among the array of products that induce pharmacological oxidative stress, hydrogen peroxide (H_2O_2) [21-27] and *tert*-butyl hydroperoxide (t-BOOH) [28-31] are the most frequently used compounds for this type of study in cell culture experiments. Hydrogen peroxide is a non-radical ROS produced in living cells as a result of cell metabolism. As such, it can directly damage DNA, lipids and other macromolecules causing oxidative injury to the cell [32,33]. Similarly, *tert*-butyl hydroperoxide, as other organic peroxides, can decompose to other alkoxyl and peroxyl radicals in a reaction aided by metal ions that can generate ROS, including H_2O_2. Decomposition of t-BOOH will accelerate lipid peroxidation chain reactions, induce cell toxicity by damage to DNA, and deplete cell GSH and protein thiols resulting in the alteration of intracellular calcium homeostasis and in a general cell damage and apoptosis [32,34,35].

The response of the antioxidant defence enzymes to a range of doses of t-BOOH or H_2O_2 has been tested in cultured HepG2 cells [36], and the results demonstrate that a condition of cellular stress is evoked when HepG2 cells in culture are treated with t-

BOOH but not with H_2O_2. Therefore, treatment of human HepG2 with t-BOOH yields an excellent model of oxidative stress in cell culture.

Adequacy of the model

1) Generalities of the cell culture

HepG2 cells initially isolated from a liver biopsy in a 15-year-old Caucasian male [37], were grown in DMEM F-12 medium from Biowhitaker (Lonza, Madrid, Spain), supplemented with 2.5% foetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin (all from Sigma, Madrid, Spain). The same medium deprived of serum plus the antibiotic mixture was used in all experiments. The serum added to the medium favors growth of most cell lines but might interfere in the running of the assays and affect the results. Moreover, it has been observed a fairly good growth of HepG2 cells in FBS-free DMEM-F12 [38].

2) Uptake and metabolism of dietary compounds

The HepG2 cell line is widely used to study the biotransformation of different drugs and chemicals as a model system of the human liver [14,39,40]. Genotyping of phase I and phase II enzymes, and drug transporter polymorfisms in these cells confirmed HepG2 as a suitable model for metabolic studies [14]. Only low levels of sulfotransferase and N-acetyltransferase were reported in this cell line, yet they were still high enough to allow metabolic assays by these enzymes [14].

In this cell culture model we have shown active uptake and metabolism of HTy and other olive oil phenolics [39] and hydroxycinnamic acids [40], as well as a remarkable UDP-glucuronil transferase activity for Q in a comparative study between rat microsomes and HepG2 in culture [41]. Glucuronidated and methylated conjugates

were the main derivatives formed, resembling the metabolic profile of polyphenols observed in vivo [42-49].

Assessment of Biomarkers in the cell culture model

1) Indicator of cell integrity

1.1) Cell viability

LDH leakage from the cell cytosol to the culture medium has been generally used as an indicator of cell integrity. LDH leakage can be estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content [50,51].

Although flavonols such as Q may have potent antioxidant and anticarcinogenic effects in cell culture, elevated doses of these dietary compounds can also be toxic and mutagenic in cell culture systems and excess consumption by mammals could cause adverse metabolic reactions [52,53]. In this context, Q has been shown to protect against both chemically induced and spontaneous formation of tumors in animals [2,54] and arrest cell proliferation in a variety of transformed cell lines in culture [55-58]. Indeed, we have recently reported that Q induces apoptosis via caspase activation, regulation of Bcl-2 and inhibition of PI-3-kinase/AKT and ERK pathways in HepG2 cells [59,60]. Generally, elevated doses of dietary polyphenolic compounds [52,53,61] or Maillard reaction products [62] can behave as pro-oxidants and induce mutagenesis in cell culture systems. In a similar context, deficiency of selenium induces pathological conditions which have been related to oxidative processes, such as cancer, coronary heart disease, and liver necrosis [63], but dietary levels above 1 mg kg^{-1} will lead to toxic manifestations [64].

A wide concentration range of the four compounds was tested and the final concentrations were selected based on the lack of cytotoxic effect and a realistic concentration range in human diet. Pharmacological doses of Q that evoke biological effects (10-50 μM) could be achieved with dietary supplements and their continuous daily intake result in a steady-state concentration [65]. The range of concentrations of HTy was finally selected after an extensive search in the literature showing that only concentrations of and above 10 μM HTy were effective in most conditions [66]. The concentration range of DCM used for the experiments is realistic at the physiological level since a cup of espresso coffee provides between 6.25 up to 18.75 mg/mL of melanoidin [67]. Finally, steady-state concentrations around 0.7 μM of all selenium species have been reported in human serum [68]. Although a fair range of concentrations were used for the performed experiments, only a representative dose of each compound was selected for this overview, 5 μM Q, 10 μM HTy, 0.5 μg/mL DCM and 1 μM Se-MeSeCys.

In our experimental conditions a 3 h treatment with 200 μM t-BOOH evoked a great increase in LDH activity in the cell culture medium indicating cell death in HepG2 (Table 1). Pre-treatment for 2 or 20 h of HepG2 cultures with 5 μM Q greatly reduced cell damage induced by t-BOOH to values around and below 10 % of LDH activity in the culture medium. Pre-treatment for 2 or 20 h of HepG2 cultures with 10 μM HTy, 0.5 μg/mL DCM or 1 μM Se-MeSeCys completely prevented cell damage induced by t-BOOH (Table 1), indicating that integrity of the cells was fully protected against the oxidative insult.

Table 1. ***Protective effect against oxidative stress on cell viability***. HepG2 were treated with the noted concentrations of the compounds for 2 or 20 h or left untreated (controls and t-BOOH), then the cultures were washed and 200 µM t-BOOH was added to all the cultures except controls for 3 h. Results of lactate dehydrogenase (LDH) leakage are expressed as percent of LDH activity in the culture medium of the total activity, culture medium plus intracellular. Results of control and t-BOOH were always significantly different. Letters upon symbols indicate statistically significant differences (P < 0.05) when those data are compared to control (letter a) or to t-BOOH (letter b). Values are means ± SD of 6-8 data

	LDH Leakage (% LDH)	
	2 hours	**20 hours**
Control	4,5 ± 1,7	4,0 ± 0,3
t-BOOH	35,3 ± 5,4	36,0 ± 10,0
Q (5µM)	8,0 ± 2,1[ab]	10,1 ± 3,4[ab]
HTy (10µM)	7,4 ± 1,1[ab]	2,4 ± 0,5[b]
DCM (0,5µg/ml)	7,3 ± 2,1[ab]	9,0 ± 0,5[ab]
Se-MeSeCys (1µM)	2,7 ± 0,9[b]	3,0 ± 0,4[b]

2) Biomarker of oxidative status

2.1) Reactive Oxygen Species (ROS)

Evaluation of the intracellular reactive oxygen species (ROS) yields an index of the overall oxidative stress in living cells [69,70]. By reporting a direct measurement of the intracellular concentration of ROS (hydrogen peroxide, superoxide anion, single oxygen and hydroxyl radical) not neutralized by the cell antioxidant defences, this method [70] is based upon the fact that non-fluorescent 2',7'-dichlorofluorescin diacetate (DCFH-DA) crosses cell membranes, is deacetylated by intracellular esterases to non-fluorescent DCFH and then is oxidized by intracellular ROS to highly fluorescent dichlorofluorescein (DCF).

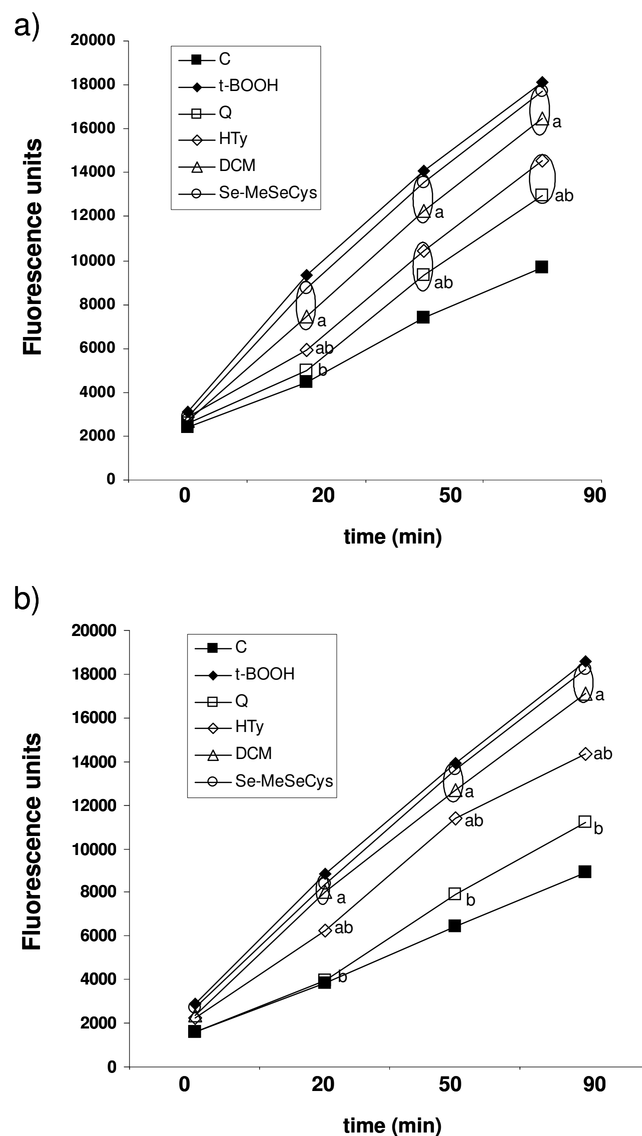

Figure 1.- Protective effect against oxidative stress on intracellular reactive oxygen species (ROS) generation. HepG2 cultures were treated with the concentrations of the four compounds noted in the text for 2 (a) or 20 (b) h, or left untreated (controls and t-BOOH), then the cultures were washed and 200 μM t-BOOH was added to all cells except controls and intracellular ROS production was expressed as fluorescence units. Letters upon symbols indicate statistically significant differences ($P < 0.05$) when those data or groups of data are compared to control (letter a) or to t-BOOH (letter b). Values are means ± SD of 7-8 different samples per condition. SD values were not included due to intense bar overlapping.

A significant increase in ROS production was observed over time in the presence of 200 μM t-BOOH as compared to unstressed controls (Figure 1). On the one hand, pre-treatment of HepG2 cultures with Q or HTy evoked a partial reduction of the t-BOOH-induced ROS production. Besides, the inhibitory effect of the antioxidant

phenolic HTy was not time-dependent since the ROS quenching effect was similar both in the short (2 h) and long (20 h) treatment. However, the longest treatment with Q resulted more efficient to reduce ROS generation evoked by t-BOOH. On the other hand, pre-treatment of HepG2 cultures with DCM or Se-MeSeCys for either 2 or 20 h did not prevent ROS production induced by t-BOOH (Figure 1).

The results indicate that the high levels of ROS generated during the stress period are being more efficiently quenched in cells pretreated with the two polyphenols Q [71] and HTy [66], an effect that by itself could explain the reduced cell damage. However, other mechanisms must be involved in the cell protective effect depicted by DCM and Se-MeSeCys.

3) Assessment of the antioxidant defence system

3.1) Non-enzymatic defence: concentration of reduced glutathione

Reduced glutathione is the main non-enzymatic antioxidant within the cell and plays an important role in the defence against oxidative stress, as a substrate in glutathione peroxidase-catalysed detoxification of organic peroxides, by reacting with free radicals and by repairing free-radical-induced damage through electron-transfer reactions [3,5,6,36,38]. It is usually assumed that GSH depletion reflects intracellular oxidation. On the contrary, an increase in GSH concentration could be expected to prepare the cell against a potential oxidative insult [3,36,38,72,73].

The content of reduced glutathione was quantified by the fluorometric assay of Hissin and Hilf [74]. The method takes advantage of the reaction of reduced glutathione with o-phthalaldehyde (OPT) at pH 8.0. Addition to the cell culture of 200 μM t-BOOH evoked a dramatic decrease of cytosolic GSH which was overcome by a pretreatment for 2 or 20h with 5 μM Q (Figure 2). An increase in intracellular GSH concentration

induced by Q has been previously found in MCF7 human breast cancer cells [61], monkey kidney derived COS1 cells [72] and HepG2 [38,73]. Those studies report that the increase in GSH is preceded by stimulation by Q of the g-glutamylcysteine synthetase, the enzyme involved in glutathione synthesis. Perhaps the concentration of GSH increases during the pretreatment of cells with Q preventing its decrease below steady-state levels in the presence of t-BOOH [38].

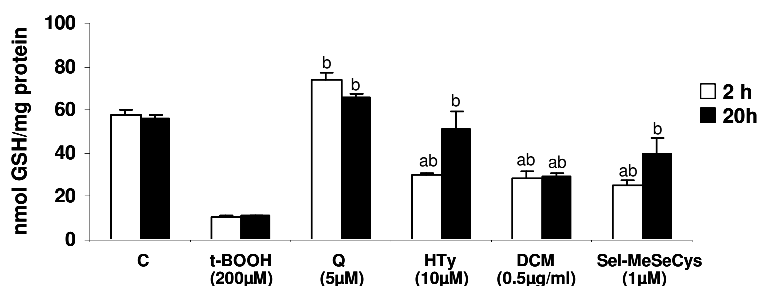

Figure 2.- Protective effect against oxidative stress on intracellular concentration of reduced glutathione. HepG2 were treated with the noted concentrations of the compounds for 2 or 20 h or left untreated (controls and t-BOOH), then the cultures were washed and 200 µM t-BOOH was added to all the cultures except controls for 3 h. The results of the fluorescent analysis for reduced glutathione are means of 4-5 different samples per condition. Letters upon symbols indicate statistically significant differences ($P < 0.05$) when those data are compared to control (letter a) or to t-BOOH (letter b). Values are means ± SD of 6-8 data.

The response of the GSH content to the oxidative insult was time-dependent when cells were pretreated with HTy or Se-MeSeCys, showing a recovery of 50 % with the short treatment and almost 100 % with 20 h. A steady recovery to about 50 % of the control values was observed when cells were pretreated with 0.5 µg/mL DCM for 2 or 20 h. In the case of HTy, the partial recovery of GSH is consistent with the partial reduction of ROS generation which may be explained by an increased consumption of GSH in the enzymatic and non-enzymatic quenching of reactive oxygen species generated by t-BOOH. Although the enhanced consumption of GSH in cells pretreated with DCM and Se-MeSeCys was not enough to reduce ROS levels when submitted to t-

BOOH, at least the storage of reducing power was not overly wasted. Maintaining GSH concentration above a critical threshold while facing a stressful situation represents an advantage for cell survival [3,5,6,36,38,72,73].

3.2) Enzymatic defence: activity of antioxidant enzymes

In the defence against oxidative stress, the cellular antioxidant enzyme system plays a crucial role. The system includes SOD, CAT, GPx and GR. There are three forms of SOD in mammals that catalyze the dismutation of the superoxide radical anion, i.e., MnSOD located in mitochondria, Cu/Zn SOD found mainly in cytosol and an extracellular SOD [3,6,12,75]. Catalase converts H_2O_2 to H_2O [3,5,6,76]; GPx catalyzes GSH oxidation to oxidized glutathione (GSSG) at the expense of H_2O_2 or other organic peroxides [3,5,6,77] and GR recycles GSSG back to reduced glutathione using NADPH [2,3,5,6]. The consequences of oxidative stress are serious and, in many cases, are manifested by increased activity of enzymes involved in oxygen detoxification [1-6,78]. Oxidative challenges lead to an increase in activity of antioxidant enzymes, particularly GPx and GR, in cultured cells such as mouse skeletal muscle cells [79], primary rat hepatocytes [9], and human hepatoma HepG2 [80]. For the purpose of this overview, attention on the antioxidant enzyme system has only been focussed on the response of GPx and GR.

The determination of GPx activity is based on the oxidation of reduced glutathione by GPx, using t-BOOH as a substrate, coupled to the disappearance of NADPH by GR, which was monitored by following absorbance decrease at 340 nm [36,38,66,77]. GR activity was determined by following the decrease in absorbance at 340 nm due to the oxidation of NADPH utilized in the reduction of oxidized glutathione [36,38,66,71].

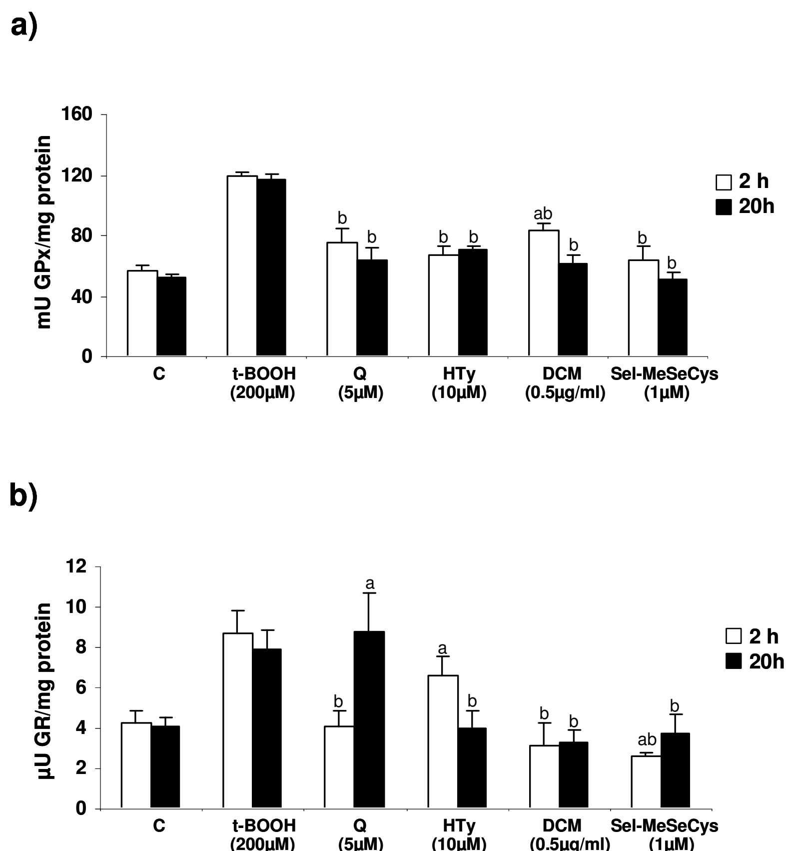

Figure 3.- Protective effect against oxidative stress on the activity of (a) GPx and (b) GR evaluated in cultures of HepG2. HepG2 cells were treated with the noted concentrations of the compounds for 2 or 20 h or left untreated (controls and t-BOOH), then the cultures were washed and 200 µM t-BOOH was added for 3 h to all cultures except controls. Letters upon symbols indicate statistically significant differences ($P < 0.05$) when those data are compared to control (letter a) or to t-BOOH (letter b). Values are means ± SD of 4-5 different samples per condition.

The presence of 200 µM t-BOOH in the culture medium for 3 h induced significant increases in the enzyme activities of GPx, GR (Figure 3), which clearly indicates a positive response of the cell defense system to face an oxidative insult [36,38,66,71,81]. The response of GPx and GR to the oxidative insult was dependent on the concentration and time of exposure to the chemopreventive compounds; i.e. pretreatment for 2 or 20 h with 5 µM Q completely prevented the t-BOOH induced increase in GPx activity of HepG2 cells, whereas only the shortest treatment with Q prevented the t-BOOH induced rise in GR activity (Figure 3). When cells were pretreated for 2 or 20 h with 10 µM HTy, the t-BOOH-induced increase in enzyme

activity of GPx was thoroughly suppressed whereas only the longest treatment with HTy reduced GR activity to that of control untreated cells (Figure 3). The activity of GPx in cells pretreated 2 h with 0.5 μg/mL DCM and submitted to t-BOOH was between that of control cells and those only submitted to the t-BOOH, whereas the t-BOOH-induced increase in GPx activity was totally suppressed when cells were pretreated for 20 h with the same dose of DCM. A pretreatment of cells with DCM for either 2 or 20 h completely prevented the increase in GR induced by t-BOOH (Figure 3). Finally, t-BOOH-induced GPx and GR activities were completely overcome by a pre-treatment for 2 or 20 h with 1 μM Se-MeSeCys. Only a previous cell culture study has shown a specific effect of a selenium compound on the GPx response to an oxidative insult in experimental conditions similar to those of the present study [82]. In summary, activity of GPx showed the highest sensitivity to all four antioxidant compounds, whereas GR activity depicted higher sensitivity to DCM and Se-MeSeCys and was time-dependent with Q and HTy.

Other dietary compounds have been tested by other authors and significant changes in the enzyme activity of the antioxidant enzymes have been observed only at very high doses [2,17,61,75]. In the present cell model we have shown that physiologically realistic concentrations of the flavonoid quercetin [71], olive oil phenol hydroxytyrosol [66], a coffee melanoidin [81] and the selenium derivative Se-MeSeCys [83] protect cell damage by preventing the severely increased activity of antioxidant enzymes induced by t-BOOH. The results indicate that at the end of a chemically-induced stress period the antioxidant defence system of cells that had been pretreated with the chemopreventive compounds has more efficiently returned to a steady-state activity diminishing, therefore, cell damage and enabling the cell to cope in better conditions with further oxidative insults.

4) Biomarkers of oxidative damage

4.1) Damage to lipids: malondialdehyde

Identification of proper biomarkers of oxidative damage to macromolecules would afford information on the predisposition and prognosis of certain pathologies and it is pivotal to evaluate the effect of interventions with antioxidants on the incidence of diseases associated with oxidative stress [84,85].

An important step in the degradation of cell membranes is the reaction of ROS with the double bonds of polyunsaturated fatty acids (PUFAs) to yield lipid hydroperoxides. On breakdown of such hydroperoxides a great variety of aldehydes can be formed [85-87]. MDA, a three-carbon compound formed by scission of peroxidized PUFAs, mainly arachidonic acid, is one of the main products of lipid peroxidation [85-87]. Since MDA has been found elevated in various diseases thought to be related to free radical damage, it has been widely used as an index of lipoperoxidation in biological and medical sciences [88,89]. We have established a new method of evaluation of MDA that is sensitive enough to detect a significant increase in MDA concentration in response to either an oxidative stress induced by t-BOOH in cell cultures [90] or to a hypercholesterolemic condition in rat serum and liver [91]. By using this method we have found that the t-BOOH-induced increase of MDA was completely avoided when cells were pretreated for 2 or 20 h with Q and Se-MeSeCys [71,83] as well as with HTy for 20 h and DCM for 2 h [66,81] (Figure 4). Other than our results, determination of MDA levels in cell culture conditions is extremely scant in the literature [92]. This protection by dietary bioactive compounds against an induced lipid peroxidation in a cell culture is in line with previous studies by other researchers that showed a similar effect by other melanoidins in primary cultures of hepatocytes

[13] as well as by tea catechins [15,17], beta carotene and lutein [15] in the same cell line, human HepG2.

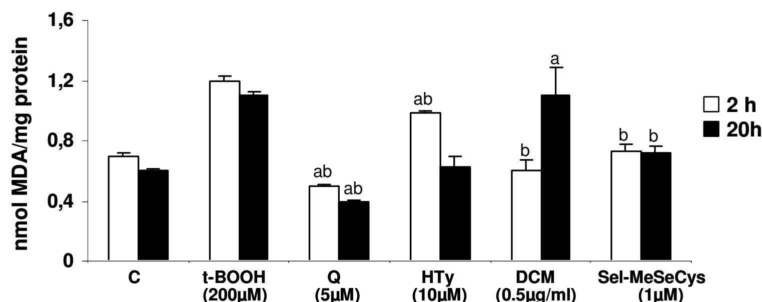

Figure 4.- Protective effect against oxidative stress on cellular malondialdehyde. HepG2 were treated with the noted concentrations of the compounds for 2 or 20 h or left untreated (controls and t-BOOH), then the cultures were washed and 200 µM t-BOOH was added to all the cultures except controls for 3 h. Malondialdehyde in cytoplasmic contents of HepG2 are means ± SD, n= 4. Letters upon symbols indicate statistically significant differences ($P < 0.05$) when those data are compared to control (letter a) or to t-BOOH (letter b).

Concluding remarks

The study of the mechanisms involved in cell damage mediated by oxidative compounds as well as the evaluation of biomarkers of the cellular antioxidant defence system in such conditions could greatly help to identify potential strategies for the prevention or disappearance of oxidative stress related diseases. In this line, nutritional sciences would greatly benefit from the setting of an in vivo cellular model where the biological effect of potential chemopreventive compounds can be rapidly assayed. Human HepG2, is an easy to culture, well characterized and reliable model used for biochemical and nutritional studies where conditions can be tested with minor interassay variations.

The results obtained in the cell culture model presented in this overview support previous data on the chemoprotective effect of some dietary phenolic compounds and

extend the preventive effect against oxidative stress to other bioactive molecules included in the human diet. Besides, the results confirm the reliability of the model and give more insight on the specific mechanisms involved in the biological activity of the tested compounds, showing that concentrations within a realistic range evoke a favourable response. Therefore, all tested bioactive compounds may contribute to the protection afforded by fruits, vegetables and plant-derived beverages against diseases for which excess production of ROS has been implicated as a causal or contributory factor.

Abbreviations

CAT, catalase; DCF, dichlorofluorescein; DCFH-DA, 2',7'-dichlorofluorescin diacetate; DCM, digested coffee melanoidin; GPx, glutathione peroxidasa; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; HTy, hydroxytyrosol; LDH, lactate dehydrogenase; MDA, malondialdehyde; OPT, o-phthalaldehyde; Q, quercetin; ROS, reactive oxygen species; Se-MeSeCys, selenomethyl selenocystein; SOD, superoxide dismutase; t-BOOH, *tert*-butyl hydroperoxide.

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