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ORIGINAL CONTRIBUTION

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Influence of quercetin and rutin on growth and antioxidant defense system of a human hepatoma cell line (HepG2)

Summary *Background* Dietary polyphenols like quercetin and rutin are considered beneficial because of their potential protective role in the pathogenesis of multiple diseases associated to oxidative stress such as cancer, coronary heart disease and atherosclerosis. However, many of these effects may depend on the concentration of the polyphenol utilized since high doses of some phenolic compounds may be prooxidant and negatively affect cell growth and viability. *Aim of the study* To test the potential chemoprotective effects of quercetin and rutin, two flavonols with high antioxidant capacity, on cell growth, viability and the response of the antioxidant defense system of a human hepatoma cell line (HepG2). *Methods* Cell growth was measured by diaminobenzoic acid and bromodeoxyuridine assays, cell toxicity by lactate dehydrogenase leakage assay, reduced glutathione was quantified by a fluorimetric assay, cellular malondialdehyde was analyzed by high-performance liquid chromatography, reactive oxygen species were quantified by the dichlorofluorescein assay, antioxidant enzyme activities were determined by spectrophotometric analysis and their gene expression by northern blot. *Results* Short-term exposure (4 h) to these flavonols had no antiproliferative nor cytotoxic effect. High doses of quercetin (50–100 μM) increased glutathione concentration and gene expression of Cu/Zn superoxide dismutase and catalase inhibiting the activity of the latter enzyme, whereas lower doses (0.1–1 μM) decreased gene expression of Cu/Zn superoxide dismutase and increased that of glutathione peroxidase. All doses of quercetin and rutin diminished reactive oxygen species and high doses (10–100 μM) decreased malondialdehyde concentration. *Conclusion* The results indicate that both natural antioxidants induce favorable changes in the antioxidant defense system of cultured HepG2 that prevent or delay conditions which favor cellular oxidative stress.

Key words oxidative stress – polyphenols – dietary antioxidants – glutathione – malondialdehyde – antioxidant enzymes – reactive oxygen species

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Introduction

Dietary antioxidants, including polyphenolic compounds, are considered beneficial because of their potential protective role in the pathogenesis of multiple diseases associated to oxidative stress such as cancer, coronary heart disease and atherosclerosis. Flavonoids, and specifically flavonols such as quercetin, have been reported to exhibit a wide range of biological activities [1–3], including anticarcinogenic, anti-inflammatory and antiviral actions. The flavonoids exert these effects as antioxidants, chelators of divalent cations and free radical scavengers and thus may be involved in preventing free radical mediated cytotoxicity and lipid peroxidation which are associated with cell aging and chronic diseases [4, 5].

The flavonoids used in this study, quercetin and its glycoside derivative rutin, are two common dietary flavonols with a well characterized in vitro antioxidant activity. Quercetin is the predominant flavonol found in foods, and intakes of between 6 to 31 mg per day have

been reported [6, 7]. The study of the effect of dietary polyphenols on the regulation of antioxidant defense mechanisms at the molecular level may benefit from the use of an established cell culture line. Human hepatoma HepG2 is a well differentiated transformed cell line that meets all biochemical requirements for the present study. This cell line has been widely used in biochemical and nutritional studies because it is considered one of the experimental models that more closely resemble the human hepatocyte in culture [8, 9]. In addition, this continuously growing transformed cell line permits the study of antiproliferative factors for liver cancer research.

The objective of the present study was to investigate the effect of different quercetin and rutin concentrations (0.1–100 μM) on the growth, cell viability (cytotoxicity) and redox status of HepG2 cells in culture. As indexes of cellular oxidative stress, the generation of reactive oxygen species (ROS), content of malondialdehyde (MDA), and the activity and gene expression of antioxidant enzymes were determined in the presence of different doses of quercetin and rutin.

Material and methods

Chemicals and cell culture

The cell proliferation ELISA BrdU (colorimetric) assay kit was from Roche Diagnostics (Roche Molecular Biochemicals, Barcelona, Spain), and the kit for the determination of superoxide dismutase activity (Bioxytech SOD-525) from Oxis International Inc. (Portland, OR, USA). Cell culture medium DMEM F-12 and fetal bovine serum were from BioWhittaker Europe (Innogenetics, Madrid, Spain). All other reagents were from Sigma-Aldrich Química S. A. (Madrid, Spain). Human hepatoma HepG2 cells [10] were a gift from Dr. Paloma Martin-Sanz (Instituto de Bioquimica, CSIC, Madrid, Spain). Cells were defrosted and passed no more than 10 times until they were used for any experiment. They were grown in DMEM F-12 medium supplemented with fetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin. Preliminary experiments to determine the percentage of FBS required for optimal growth of HepG2 cells were carried out. Cells were incubated in DMEM F-12 culture medium devoid of FBS or supplemented with increasing percentages of serum (0.5, 1, 2.5, 5, 10 %). Considering the results (data not shown), cells were routinely grown in 2.5 % FBS and all experiments to test the effect of flavonoids were carried out in serum-free medium. The same medium deprived of serum plus the antibiotic mixture was used in experiments. Fetal serum favors growth of most cell lines but might interfere in the running of the assays and affect the results. This cell line was grown in a humidified incubator containing 5 % CO_2 and 95 % air at 37 °C. Doubling time of this cell line was of 24 h.

Cell proliferation assays and lactate dehydrogenase leakage assay

A colorimetric immunoassay (ELISA) was used for the quantification of cell proliferation. This method is based on the measurement of BrdU incorporation into genomic DNA during DNA synthesis of proliferating cells. HepG2 cells were seeded at low density (10^4 cells/well) in 96-well multiwells, grown 20 h and labeled by the addition of 5-bromo-2'-deoxyuridine (BrdU) for 4 h. Then the anti-BrdU antibody was added and the immune complexes were detected by the subsequent substrate (tetramethylbenzidine) reaction and quantified by measuring the absorbance at 620 nm using a scanning multiwell spectrophotometer (ELISA reader). DNA concentration in HepG2 cultures was measured by the fluorimetric method of Hinegardner [11].

The potential cytotoxic effect of quercetin and rutin on HepG2 cells was determined by the lactate dehydrogenase leakage (LDH) assay [12, 13]. LDH leakage was estimated from the ratio between the LDH activity in the culture medium and that of the whole LDH activity, medium plus cell content.

Determination of reduced glutathione, malondialdehyde levels and reactive oxygen species

The content of reduced glutathione was quantified by the fluorimetric assay of Hissin and Hilf [14]. The method takes advantage of the reaction of reduced glutathione with o-phthalaldehyde (OPT) at pH 8.0. Fluorescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm. The results of the samples were referred to those of a standard curve of reduced glutathione (5 ng–1μg).

Cellular MDA is analyzed by high-performance liquid chromatography (HPLC) as its 2,4-dinitrophenylhydrazone (DNPH) derivative [15]. The amount of MDA was calculated from the calibration curve $y = 47.651\ x + 0.679$; where y is the peak area corresponding to the 2,4-dinitrophenylhydrazone derivative and x the concentration of MDA. Values are expressed as nmol of MDA/mg protein.

Cellular oxidative stress was quantified by the dichlorofluorescein assay to determine the effect of quercetin and rutin on the intracellular generation of ROS [16]. After being oxidized by intracellular oxidants, DCFH-DA will become dichlorofluorescein (DCF) and fluoresce. Multiwell plates were measured in a fluorescent microplate reader at excitation wavelength of 485 nm and

emission wavelength of 530 nm. Time points were taken at 0, 20, 50 and 90min.

Determination of antioxidant enzymes activities

After incubation of cells with conditions the medium was aspirated, cells washed twice with PBS, scraped and collected in 3 mL PBS. Cells were centrifuged (1350 rpm, 4 °C), supernatants removed and the pellet suspended in PBS. Then, cells were sonicated for 7 min, centrifuged again (5000 rpm, 4 °C) and supernatants collected and stored frozen until analysis. All enzyme activities were measured in the supernatants. Catalase (CAT) activity was determined by following the decomposition of H_2O_2 measured as a decrease in absorbance at 240 nm [17]. Glutathione reductase (GR) activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione [18]. The determination of glutathione peroxidase (GPx) activity is based on the oxidation of reduced glutathione by GPx coupled to the disappearance of NADPH by GR [19]. Superoxide dismutase (SOD) activity was determined by the Oxis commercial kit Bioxytech SOD-525. Enzyme activities are expressed as units per mg of protein, determined by the Bradford method [20].

Preparation of RNA and Northern blot analysis

Cultured cells were separated from the plastic substrate by trysinization and total cell RNA was prepared with Rneasy kit (IZASA, Barcelona, Spain). A 498 bp human Cu/Zn SOD, a 424 bp human catalase insert and a 316 bp human GPx insert were used as probes. All probes were cloned into HindIII-BamHI sites of the pGEM4z (Promega Biotech, Madison, WI) and kindly provided by Dr. Jonathan L. Tilly (Vincent Center for Reproductive Biology, Massachusetts General Hospital, Boston, MS, USA) and Dr. M. Cascales (Instituto de Bioquimica, CSIC, Madrid, Spain). A β-actin probe (0.6 Kb EcoRI/HindIII fragment isolated from VC18 vector kindly provided by Dr. P. Martin-Sanz from Instituto de Bioquimica, CSIC, Madrid, Spain) was used for loading normalization. ^{32}P-labeled probes were generated using a random prime labeling kit (Amersham Pharmacia Biotech, Barcelona, Spain).

Statistics

To contrast groups, one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test were used. Homogeneity of variances was evaluated by the Cochran's test, and to discriminate among means the Fisher's least significant difference procedure was applied. The level of significance was $P < 0.05$. A Statgraphics Plus program version 2.1 (Statistical Graphics Corp., Rockville, MD) was used. At least two separate experiments were carried out for each condition and assay, with four replicates analyzed in each experiment (n = 8).

Results

Effect of polyphenols on cell proliferation

BrdU assay showed no statistically significant differences when cells were treated 24 h with rutin; however, concentrations of 50 and 100 μM quercetin caused a significant arrest of HepG2 cell growth (15 and 60 % respectively) after 24 h in the culture medium (data not shown). The study of cell proliferation measuring DABA incorporation into DNA confirmed the cell growth arrest of HepG2 in culture caused by high quercetin doses (Fig. 1a). Statistical differences were observed as soon as day 1 and a clear dose-response inhibition of cell growth by this flavonol was observed after 48 h. Quercetin concentrations over 10 μM totally inhibited replication of HepG2 cells. The effect of rutin on cell proliferation evaluated by the DABA assay confirmed the lack of effect of this flavonol glycoside after 24 h of treatment (Fig. 1b). However, at longer incubation times (48 h) a significant inhibition of cell growth was observed for the high (100 μM) and intermediate (10–50 μM) rutin concentrations, while doses of 0.1 and 1 μM showed no effect on cell growth. Nevertheless, the extent of cell growth inhibition by rutin was lower than with quercetin.

Cytotoxic effect of quercetin and rutin on HepG2 cells

Leakage of intracellular lactate dehydrogenase (LDH) into the culture medium was used as an indicator of cytotoxicity. Treatment for 4 h with different quercetin and rutin concentrations had no effect on LDH leakage (Table 1). After 24 h, cell damage was apparent when cells were treated with quercetin. A trend to increase LDH leakage was observed with low doses (0.1–5 μM) of quercetin, whereas higher quercetin doses (50–100 μM) were clearly cytotoxic, causing a 4- to 5-fold increase of LDH liberation into the culture medium (Table 1). On the contrary, rutin had no cytotoxic effect on HepG2 cells at any dose after 24 h exposure to this flavonol. Considering the results with longer exposures of high doses of the flavonols, the remaining assays were focused on the short-term effect of quercetin and rutin, that is, in conditions of high cell viability.

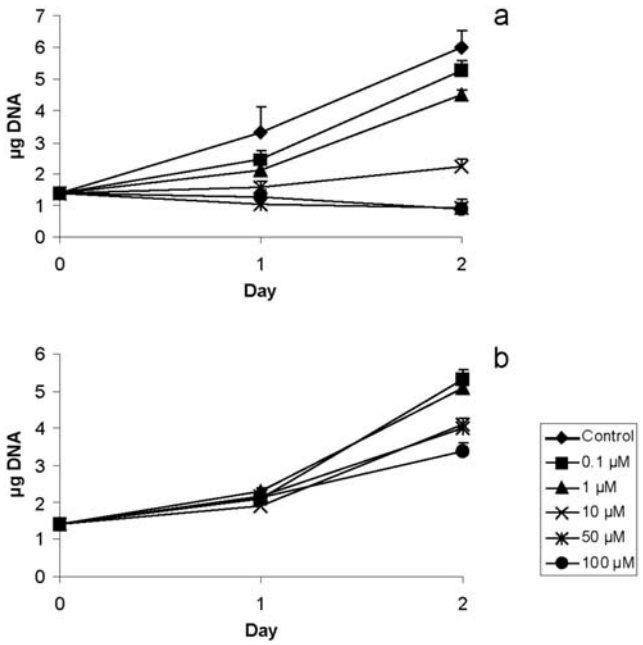

Fig. 1 Effect of quercetin and rutin on HepG2 proliferation as measured by the diaminobenzoic assay. Cells were grown with different concentrations of quercetin (**a**) and rutin (**b**). Plates were stopped at fixed times and incorporation of DABA to DNA determined by a standard DNA curve. Standard points and samples were measured at an excitation wavelength of 405 nm and emission wavelength of 500 nm in a fluorimeter. Values are presented as means, SD is not shown due to bar overlapping. Four different wells (samples) for each experiment and two different experiments (n = 8) were used for statistical analysis. A significant decrease in cell growth compared to controls was observed at days 1 and 2 for doses of 1–100 µM quercetin and only at day 2 for 10–100 µM rutin ($P < 0.05$)

Table 1 Effect of quercetin and rutin on cell viability (as % lactate dehydrogenase in culture of the total activity), and intracellular concentration of reduced glutathione (ng GSH/mg cell protein) and malondialdehyde (nmol MDA/mg cell protein). Asteriscs indicate statistically significant differences ($P < 0.05$) as compared to controls. Values are means ± SD of 4–6 data from 2–3 different assays (*C* control)

	LDH (% in medium)		GSH (ng/mg prot)	MDA (nmol/mg prot)
	4h	24 h	4h	4h
Quercetin				
C	12.6±2.1	13.8±4.0	63±3	1.09±0.09
0.1 µM	13.6±2.5	13.0±0.5	70±6	1.08±0.11
1 µM	12.6±1.5	11.0±2.5	66±2	1.06±0.12
5 µM	13.0±1.0	16.4±6.0	69±8	1.05±0.13
50 µM	16.0±3.5	42.3±3.0*	76±6*	0.87±0.07*
100 µM	16.0±4.0	38.8±6.0*	83±7*	0.72±0.27*
Rutin				
C	12.6±2.1	13.8±4.0	63±3	1.13±0.06
0.1 µM	13.4±2.8	12.3±1.5	55±7	1.00±0.08
1 µM	12.3±2.5	11.0±0.5	58±3	0.92±0.07
5 µM	12.1±1.8	11.0±1.0	58±4	0.96±0.08
50 µM	12.3±0.5	10.1±1.5	68±5	0.54±0.14*
100 µM	13.2±0.8	10.0±1.0	52±2*	0.60±0.11*

Effect of quercetin and rutin on reduced glutathione concentration, lipid peroxidation (MDA) and intracellular generation of reactive oxygen species (ROS)

As an index of the intracellular non-enzymatic antioxidant defenses, the concentration of reduced glutathione was measured in cells treated for 4 h with 0.1, 1, 5, 50 and 100 µM quercetin or rutin. After 4 h of treatment, the highest doses of quercetin, 50 and 100 µM, evoked a significant increase in the concentration of intracellular glutathione, whereas 100 µM rutin evoked a decrease (Table 1).

As a biomarker for lipid peroxidation the cytoplasmic concentration of MDA was measured in cells treated 4 h with 0.1, 1, 5, 50 and 100 µM quercetin or rutin. A significant decrease in the intracellular concentration of MDA was found with doses of 50 and 100 µM quercetin or rutin (Table 1). As a positive control for ROS generation 200 µM t-BOOH was used. A great increase in ROS production was observed over time in the presence of the stressor as compared to control. Interestingly, ROS generation was significantly decreased in the presence of 1–100 µM quercetin and all four doses of rutin (Fig. 2).

Effect of quercetin and rutin on the activity of antioxidant enzymes

GPx, GR, CAT and SOD activity was tested in HepG2 cells in culture in the presence of different doses of quercetin and rutin. As a positive control, the presence of 200 µM t-BOOH in the culture medium for 3 h induced significant increases in the enzyme activities of GPx, GR, CAT and SOD (data not shown). None of the doses of either polyphenol induced significant modifications in the activity of GPx and SOD (Figs. 3a, d). The activity of GR in cultured cells was modified by the highest concentration of rutin, 100 µM (Fig. 3b). Finally, the activity of CAT was significantly reduced by the two highest doses of quercetin (Fig. 3c).

Effect of quercetin on the gene expression of antioxidant enzymes

Gene expression of the antioxidant enzymes Cu/Zn SOD, CAT and GPx was determined in cultures of HepG2 treated for 4 h with a range of doses of quercetin. The results, referred to a constitutive control for RNA expression such as β-actin, show that 0.1 and 1 µM quercetin evoked a decrease whereas 10, 50 and 100 µM evoked a significant increase in RNA levels of Cu/Zn SOD. Only the highest dose (100 µM) of quercetin evoked a slight but significant increase in RNA for CAT in cultured cells.

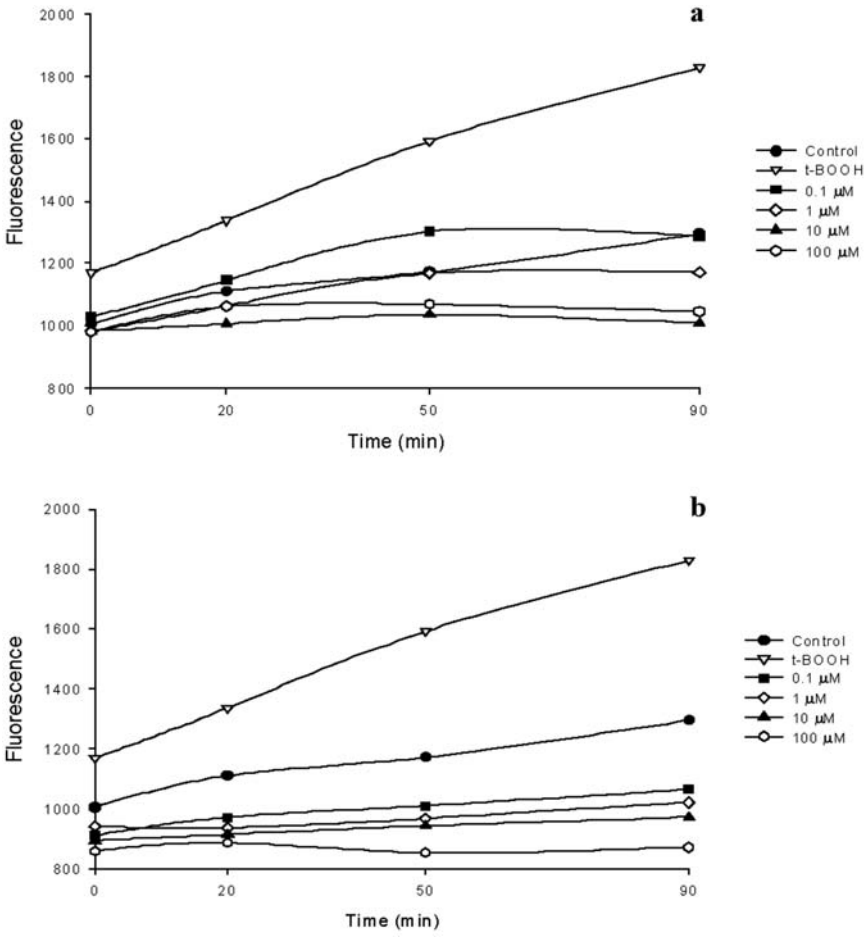

Fig. 2 Effect of quercetin and rutin on intracellular reactive oxygen species (ROS) generation. Cultures of HepG2 cells were treated with vehicle (control), 200 µM tert-butylhydroperoxide (t-BOOH) and the noted concentrations of quercetin (**a**) or rutin (**b**) and intracellular ROS production was evaluated at 0, 20, 50 and 90 min and expressed as fluorescence units. Statistical analysis showed a significant decrease in ROS concentration at 20, 50 and 90 min in cells treated with all four doses of both polyphenols as compared to cells treated with t-BOOH ($P < 0.05$). All doses of rutin reduced ROS production at all time points as compared to control cells. Values are means ± SD of 8 different samples per condition

Finally, treatment of HepG2 with 0.1 and 1 µM quercetin induced a significant increase in the RNA concentration of GPx (Fig. 4).

Discussion

Quercetin is a flavonoid present in varying amounts in most fruits and vegetables and is often present as glycoside derivatives, such as rutin (the rutinose glycoside of quercetin), or as glucosides in onions, apples, etc. While quercetin can be absorbed in the upper portion of the gastrointestinal tract, rutin is a prodrug that is metabolized to the active form, quercetin, by the intestinal microflora in the colon where it can be absorbed [21]. Concentrations of flavonols in general do not exceed 1–5 µmol/L plasma. Quercetin has been reported to be a potent inhibitor of LOX and COX, Na-K-ATPase, protein kinase C, various tyrosine kinases and other enzymes [21]. Rutin seems to present similar effects but is less active than quercetin, which may be related to its lower bioavailability.

Through one or more of the above biochemical mechanisms, quercetin has been reported to protect against both chemically induced and spontaneous formation of tumors in animals [21, 22] and arrest cell proliferation in a variety of transformed cell lines in culture [22–26]. Certain polyphenols, in particular quercetin, can inhibit oxidative damage to DNA and, therefore, prevent or reduce cellular oxidative stress and cell overgrowth [27, 28]. In this study, we show that elevated concentrations of quercetin and rutin affect in vitro growth of HepG2. Interestingly, quercetin doses that significantly decrease HepG2 cell proliferation have been previously reported to have similar effects on other cancer cells in vitro, such as human adenocarcinoma [29], human promyelocytic leukemia [30, 31], human colon cancer [23, 24, 32], and murine hepatoma [33]. Thus, our results with quercetin support a generalized growth inhibitory effect of this flavonol on multiple cancer cell lines in culture derived from human and other species. The inhibitory effect of rutin on HepG2 growth was smaller, indicating a lower activity or accessibility to the cultured cell of this compound.

Although flavonols such as quercetin may have potent antioxidant and anticarcinogenic effects in cell cul-

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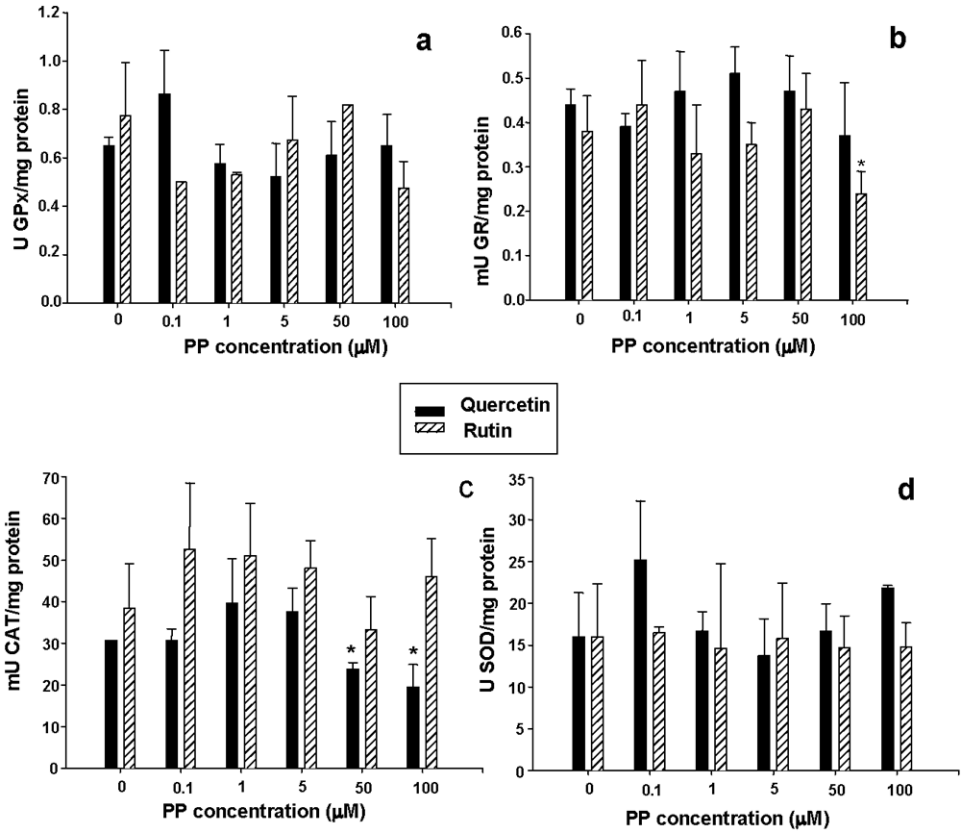

Fig. 3 Effect of quercetin and rutin on the activity of antioxidant enzymes. Activity of GPx (**a**), GR (**b**), CAT (**c**) and SOD (**d**) was evaluated in cultures of HepG2 cells treated for 4 h with noted polyphenol concentrations (PP) of quercetin, rutin or vehicle (noted as 0). Statistical differences compared to control ($P < 0.05$) are noted with asteriscs. Specific enzyme activity was expressed as indicated in each panel and represents the means ± SD of 3–4 different experiments per condition

ture, 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 [27, 34, 35]. In fact, some of the mutagenic and genotoxic responses to polyphenols by cells may reflect pro-oxidant rather than antioxidant activity of these compounds. Cell toxicity can be evaluated in cell culture by the leakage of the intracellular enzyme lactate dehydrogenase (LDH) into the culture medium. As a positive control for the assay, we have observed that treatment of HepG2 with concentrations of t-BOOH of 100 μM or above for 3 h evoked a great increase in LDH activity in the cell culture medium indicating an unequivocal cell damage (unpublished data from our lab). The high increase in the percent of LDH activity in the culture medium after treatment of HepG2 cells for 24 h with the highest doses of quercetin indicates a significant cytotoxicity in HepG2 in culture. Thus, the cell growth arrest observed in HepG2 after a 24 h exposure to quercetin could be explained by the cytotoxic effect of the same doses. The same would apply in the case of rutin, since no cytotoxic effect was observed with any dose of rutin after 4 and 24 h of treatment, in concert with the lack of effect of rutin on HepG2 cell growth. In order to test the response of the antioxidant defense system to quercetin and rutin in conditions of high cell viability, the longer exposures to both flavonols were deliberately avoided in the rest of the study.

Reduced glutathione is the main non-enzymatic antioxidant defense within the cell, reducing different peroxides, hydroperoxides and radicals (alkyl, alkoxyl, peroxyl, etc.) [36]. 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 [37–39]. In our experimental conditions, the higher doses of quercetin induced a significant rise in intracellular GSH concentration, whereas rutin had the opposite effect only at its highest concentration. An increase in GSH concentration induced by quercetin has been previously found by other authors in MCF7 human breast cancer cells [37], monkey kidney derived COS1 cells [38] and HepG2 [39].

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 [40]. MDA, a three-carbon compound formed by scission of peroxidized PUFAs, mainly arachidonic acid, is one of the main products of lipid peroxidation [41]. Since MDA has been found elevated in various diseases thought to be related

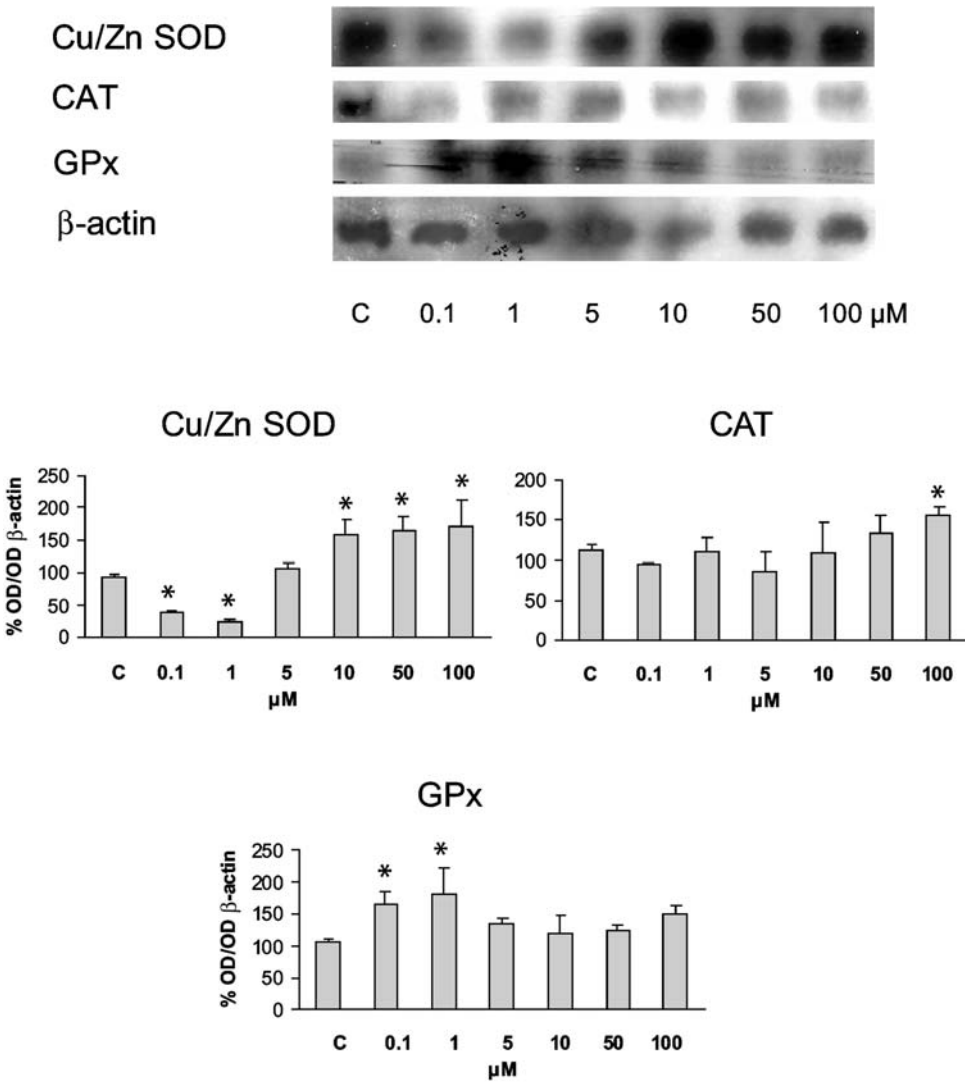

Fig. 4 Effect of quercetin on gene expression of antioxidant enzymes. RNA expression of SOD, CAT and GPx was determined in cultures of HepG2 cells treated for 4 h with the noted concentrations of quercetin or vehicle (C). Specific RNA bands from representative northern blot experiments are shown. Densitometric quantitation of the bands expressed as the means ± SD of 2–3 different experiments (4–6 samples per condition) is depicted below. A β-actin probe was used for loading normalization, and the results are expressed as percent of optical density of the specific band referred to optical density of β-actin in the same sample. This ratio is then referred to the ratio obtained for control samples which is assigned a value of 100 %. Statistical differences compared to control ($P < 0.05$) are noted with asterisc

to free radical damage, it has been widely used as an index of lipoperoxidation in biological and medical sciences [42]. By using a new method recently established in our lab [15], we have demonstrated an increase of MDA concentration in HepG2 cells treated with 200 µM t-BOOH, indicating a significant degree of lipid peroxidation [15]. In our experimental conditions, the decrease of MDA levels observed in cells treated with 10–100 µM quercetin or rutin might suggest a certain degree of protection against the steady-state lipid peroxidation.

Although there are various methods to assess oxidative damage of cells [43], a direct evaluation of the reactive oxygen species (ROS) yields a very good indication of the oxidative damage to living cells [16]. Based upon the fact that non-fluorescent 2',7'-dichlorofluorescein (DCFH) crosses cell membranes and is oxidized by intracellular ROS to highly fluorescent DCF, the intracellular DCF fluorescence can be used as an index to quantify the overall oxidative stress in cells [16, 44]. The antioxidant and free radical activity of quercetin and rutin determined by other methods has been shown in different experimental models such as cell-free in vitro assays, primary cultures, transformed cell lines and experimental animals [45]. However, direct evaluation of intracellular ROS after treatment of human cell lines with dietary polyphenols is not easily found in the literature. In this article, we show the significant increase of ROS production in cells treated with 200 µM t-BOOH as a positive control for the assay, as well as a significant inhibition of ROS generation in cultured HepG2 in the presence of quercetin or rutin. This result clearly shows that by decreasing the steady-state generation of ROS by HepG2 in culture, these flavonoids prevent or delay conditions which favor oxidative stress in the cell.

In the defense against oxidative stress, the antioxi-

dant enzyme system of cells (SOD, GR, GPx and CAT) plays an important role. There are three forms of SOD in mammals that catalyze the dismutation of the superoxide radical anion [46]. Catalase and GPx convert H_2O_2 to H_2O [17, 47] and GR recycles oxidized glutathione back to its reduced form [18]. Scientific literature dealing with the effect of quercetin or rutin on the activity or gene expression of antioxidant enzymes in cultured cells is scarce, but it has been shown that the intracellular antioxidative function of flavonoids requires the interaction with GPx, at least in the cells expressing the enzyme [48]. Interestingly, in that study with cultured rat hepatocytes, quercetin clearly enhanced activity of GPx. Other natural antioxidants have been tested and significant changes in the enzyme activity or gene expression of the antioxidant enzymes have been observed only at very high doses [22, 46, 49].

As a positive control for our experiments, cells were treated with a potent cell oxidant such as *tert*-butylhydroperoxide (t-BOOH). A significant increase in the activity of all four antioxidant enzymes was observed after a 3 h treatment with 200 μM t-BOOH (data not shown), indicating a positive response of the cell defense system to face an oxidative insult. When a wide range of doses of quercetin or rutin was tested in cultured hepatoma HepG2, only the highest doses of quercetin (50–100 μM) and rutin (100 μM) significantly reduced CAT and GR activity respectively. This negative result seems to indicate that, although the enzyme defense system of cultured human hepatoma HepG2 is active and responsive to potent oxidative insults, the activity of the antioxidant enzymes is not sensitive to short-term changes in the concentration of the two flavonols in the culture media. Perhaps the presence of any of the polyphenolic compounds does not represent a major change in the redox status in order to trigger the antioxidant enzyme response. In fact, in view of the results of ROS and MDA, the cell treated with these flavonoids might be better prepared to face an oxidative insult. To test this possibility, experiments are in progress in our laboratory.

Although enzyme activity was mostly not affected by the presence of the flavonoids, perhaps the short-term treatment with these compounds could affect the enzyme gene expression. Two different characteristic regulatory elements, the antioxidant responsive element (ARE) and xenobiotic responsive element (XRE), have been located in the promoter region of the Cu/Zn superoxide dismutase in human liver cells [50]. In addition, sequence analysis of the mouse GPx and CAT genes revealed putative binding motifs for NF kappa B and AP-1, transcriptional regulators that are activated in response to oxidative stress in various tissues [51]. These findings imply that the antioxidant enzymes can be transcriptionally induced by the above factors in combination with or independently of the two regulatory elements to effectively defend cells from oxidative stress. By mechanisms yet unknown, the doses of quercetin tested seem to be able to up- or down-regulate gene expression of the main antioxidant enzymes. However, in our experimental conditions, the significant changes in RNA levels of Cu/ZnSOD and GPx found in cells treated 4 h with quercetin might not be relevant enough to significantly affect enzyme concentration and activity. Therefore, transcriptional regulation of antioxidant enzymes by quercetin cannot be ruled out, although its biological relevance might be uncertain.

In summary, the potential antioxidant effects of quercetin and rutin, two dietary flavonols with high antioxidant capacity, have been tested in a human hepatoma cell line. Although no relevant changes in the activity of the antioxidant enzymes were observed, both natural antioxidants reduced generation of ROS and MDA concentration in cultured HepG2, thus preventing or delaying conditions which favor oxidative stress in the cell. Finally, these results make it highly interesting to investigate the potential protective effect of this range of doses of quercetin and rutin on HepG2 in conditions of oxidative insults, experiments that are currently in progress in our laboratory.

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