6

The quercetin paradox

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Submitted

Abstract

Background

Free radical scavenging antioxidants, such as quercetin, are chemically converted into oxidation products when they protect against free radicals. The oxidation product of quercetin, however, displays a high reactivity towards thiols, which can lead to the loss of protein function. The quercetin paradox is that in the process of offering protection, quercetin is converted into a potential toxic product. In the present study this paradox is evaluated using rat lung epithelial (RLE) cells.

Methods

Markers of cellular toxicity (i.e. DNA damage, glutathione (GSH) consumption and LDH leakage) and cellular function loss (i.e. cytosolic free calcium concentration) have been measured as parameters of both the protective and the possible toxic effects of quercetin. All experiments have been performed in cultured lung cells using hydrogen peroxide (H_2O_2) as stressor.

Results

It was found that quercetin efficiently protected against H_2O_2-induced DNA damage in RLE cells, but this damage was swapped for a reduction in GSH level, an increase in LDH leakage as well as an increase of the cytosolic free calcium concentration. This so-called quercetin paradox, i.e. the exchange of damage caused by quercetin and its metabolites, became more pronounced following depletion of GSH in the cells.

Conclusions

To our knowledge, this is the first study that indicates that the quercetin paradox also occurs in intact lung cells. This paradox implies that the antioxidant directs oxidative damage selectively to thiol arylation. The more pronounced paradox after GSH depletion confirms that the formation of thiol reactive quercetin metabolites is involved. Apparently, the potential toxicity of metabolites formed during the actual antioxidant activity of free radical scavengers should be considered in antioxidant supplementation.

Introduction

Quercetin is one of the most frequently studied dietary flavonoids and is ubiquitously present in various vegetables, fruits, seeds, nuts, tea and red wine (1-3). Quercetin is an excellent free radical scavenging antioxidant (4). A diet rich in flavonoids reduces the risk for oxidative-stress related chronic diseases such as diabetes, coronary heart disease and stroke (5,6). This has been associated with the antioxidant activity of flavonoids such as quercetin.

Free radical scavenging antioxidants form products that usually have taken over some of the reactivity of the radical that has been scavenged (7). In isolated membranes and blood plasma, these products of catechol-containing antioxidants such as quercetin react with thiols and impair several enzymes (8,9). The potential toxicity of quercetin metabolites, formed during the protection offered by quercetin, in relation to the protective effect of the flavonoid itself has not been evaluated in intact cells before.

In the present study the overall effect of quercetin on oxidative stress-induced damage is determined for the first time in an integrated system, i.e. cultured lung cells. The role of glutathione (GSH) in this interaction is also considered.

Lung cells have been chosen since the oxygen tension is relatively high in the lung and because after supplementation in rats and pigs the highest quercetin concentration is found in the lung (10). Furthermore, oxidative stress plays a prominent role in the etiology of lung diseases such as emphysema (11), sarcoidosis (12) and chronic obstructive pulmonary disease (13). H_2O_2 is used as stressor because H_2O_2 levels are known to be elevated during lung pathology (14,15). Furthermore, H_2O_2 released from inflammatory cells during chronic pulmonary inflammation following inhalation of toxic air pollution particles has been implicated in lung epithelial DNA damage (16). As parameters of both the protective as well as the possible toxic effects of quercetin, markers of cellular toxicity (DNA damage, GSH consumption and LDH leakage) and cellular function (cytosolic free calcium concentration) are measured.

Materials and methods

Materials

Quercetin, hydrogen peroxide (H_2O_2), trypan blue, buthionine sulfoximine (BSO), bovine serum albumin (BSA), potassium dihydrogen orthophosphate (KH_2PO_4), di-potassium hydrogen orthophosphate (K_2HPO_4), ethylenediamine-tetra acetic acid (EDTA), sulfosalicylic acid (SSA), triton

X-100, 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB), reduced β-nicotinamide adenine dinucleotide phosphate (β-NADPH), Dulbecco's phosphate buffered saline (PBS), trypsin, agarose, low melting point (LMP) agarose, sodiumpyruvate, sodiumchlorid, sodiumhydroxid solution, dimethylsulfoxide (DMSO), sodium lauroyl sarcosinate, ethidium bromide, glutathione reductase and reduced glutathione (GSH) were purchased from Sigma (St. Louis, MO, USA). Reduced β-Nicotinamide adenine dinucleotide disodium salt (NADH) was obtained from ICN Biomedicals (Ohio, USA). Ethanol was acquired from Biosolve (Valkenswaard, the Netherlands). Hanks' Balanced Salt Solution (HBSS, cat. no. 21-022) and cell culture medium were purchased from Life Technologies (Breda, the Netherlands). 1 AM Fura-2/AM was obtained from Molecular Probes (Leiden, the Netherlands). 4-Methylcatechol quinone was synthesized according to Willstätter and Pfannenstiel by the Ag_2O-mediated oxidation of 4-methylcatechol (17).

Methods

Incubation of Rat Lung Epithelial (RLE) cells

The rat lung epithelial cell line (RLE cells) was kindly provided by K. Driscoll (Proctor and Gamble, Cincinati, USA). The RLE cells were seeded in 24-well culture dishes and cultivated in HAM's F12 medium (5% FCS, 1% penicillin, 1% streptomycin, 1% glutamine) under a 95% oxygen and 5% CO_2 atmosphere until 90% confluency was reached. In designated wells, RLE cells were treated for 24 hours with 0.1% buthionine sulfoximine (BSO) to deplete cells of GSH. The monolayers of RLE cells were treated for 30 minutes with 100 μM quercetin or HBSS as control. Subsequently 20 μM H_2O_2, or HBSS as control, was added for a further 30 minutes. Quercetin was relatively stable in HBSS over the 1 hour incubation period (data not shown). The presence of H_2O_2 did not influence the stability of quercetin and quercetin did not directly react with H_2O_2 within the 1 hour incubation period (data not shown).

Addition of quercetin, that was dissolved in ethanol, resulted in an ethanol concentration in the incubation mixture of 0.5%. Control incubations with only 0.5% ethanol were performed and showed that ethanol had no effect.

Cell viability during all treatments was measured using the trypan blue dye exclusion.

Single Cell Gel Electrophoresis

DNA strand break formation in the RLE cells was determined using the comet assay (18) as described by Schins *et al.* (19) with minor modifications. RLE cells were seeded in designated wells of 24 well tissue culture dishes and

grown further for 48 hours. After incubation, the cells were rinsed twice with PBS, treated with 500 μl trypsin-EDTA for 2 minutes and immediate after detachment of the cells 2 ml complete culture medium was added. Cells were centrifuged for 5 min at 800*g* and resuspended in 70 μl PBS. Slides previously covered with a layer of 0.65% agarose were covered with a second layer consisting of 80 μl low melting point (LMP) agarose and 30 μl cell suspension. After storage for 30 minutes at 4ºC to allow solidification of the second layer, the slides were covered with another layer of LMP agarose and stored again for at least 30 minutes at 4ºC to allow solidification. Subsequently, slides were immersed in lysis buffer (2.5 mM NaCl, 100 mM EDTA, 10 mM Tris-base, 1% sodium lauryl sarcosinate, 10% DMSO and 1% Triton X-100) and stored overnight at 4ºC.

The following day, slides were rinsed with distilled water and for 20 minutes placed in an electrophoresis tank filled with ice-cold electrophoresis solution containing 300 mM NaOH and 1 mM EDTA. Electrophoresis was conducted at 300 mA and 25 V for 15 minutes. Following three times neutralisation of the slides for 10 minutes using neutralization buffer (0.4 mM Tris, pH 7.5), slides were stained with 20 μg/ml ethidium bromide in H_2O.

All steps described above were performed in the dark or under dimmed red light to prevent additional DNA damage. Analysis of comet appearance was performed on an Olympus BX60 fluorescence microscope at 200x magnification. For each individual experiment, at least 2 times 50 cells were analysed randomly per treatment, using a comet image analysis software program (Comet Assay II, Perceptive Instruments, Haverhill, UK). DNA damage was scored by determination of Olive tail moments, defined as the product of tail length and the fraction of total DNA in the comet tail.

Measurement of total cellular glutathione (GSH + GSSG)

After incubation, the cells were washed with PBS, harvested by gentle scraping and washed again in PBS. Total intracellular glutathione was determined according to the method of Tietze (20) with DTNB and glutathione reductase in a 96-well plate (21). The total cellular concentration of glutathione in the samples was determined using linear regression to calculate the values obtained from a standard curve and expressed as nmol per mg of protein.

Measurement of LDH leakage

After incubation of the RLE cells, the incubation media were collected and stored at –80ºC. The cells were washed with PBS, harvested by gentle scraping and washed again in PBS. To lyse the cells 50 μl 1% Triton X-100 in 143 mM potassium phosphate buffer pH 7.4 was added. Samples were then vortexed and stored at –80ºC.

Before LDH analysis, all samples, i.e. media as well as lysed cells, were thawed and thoroughly vortexed. After diluting the lysed cells with 600 μl 143 mM potassium phosphate buffer pH 7.4, 50 μl of 1% Triton X-100 was added to 700 μl of each sample, followed by vortexing and centrifuging (3' at 14.000 g). Subsequently 700 μl of the obtained solutions was added to 200 μl 143 mM potassium phosphate buffer pH 7.4 containing 1.125 mg/ml natriumpyruvate and 0.9 mg/ml NADH. After mixing the samples, the decrease in NADH was measured spectrophotometrically at 340 nm for 3 minutes. Total LDH activity was calculated as the sum of LDH activity in the medium and in the cell lysate. The percentage of LDH leakage was calculated by relating the LDH activity present in the medium expressed to the total LDH activity, i.e. percentage LDH leakage=(LDH in medium/total LDH)*100%.

Measurement of cytosolic free calcium concentration ($[Ca^{2+}]_c$)

Cytosolic free concentrations of calcium were measured as described previously with minor modifications (22). Briefly, confluent monolayers of RLEs were cultured on gelatine-coated cover slips and mounted in a thermostatic (37ºC) open chamber placed on a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan). Subsequently, the cells on the cover slips were loaded with 1 AM Fura-2/AM in DMSO for 30 minutes. Quercetin or 4-methyl-ortho-benzoquinone (positive control) were added as indicated. After washing the cover slips, hydrogen peroxide or fresh medium (positive control) was added and the measurement started (t=0 min). Changes in Fura-2 fluorescence were monitored in individual cells for 30 minutes by ratio fluorometry, using a high-sensitive, camera-based Quanticell video imaging system (Visitech, Sunderland, UK). Calibration of single cell measurements to values of $[Ca^{2+}]_c$ was performed by following earlier described procedures (23).

Statistics

All experiments were performed at least in triplicate and data are presented as mean ± SEM. The SEM values corresponding to Figure 6.1 and 6.4 were low and omitted from these figures for sake of clarity, but given in the results section. Statistical analysis was carried out using the non-parametric Wilcoxon's signed rank test; the Mann Whitney U test was applied to evaluate the BSO effect. Differences were considered to be significant if $P \leq 0.05$.

Results

The effect of quercetin on control rat lung epithelial cells (RLE cells) and BSO pretreated RLE cells that are exposed to hydrogen peroxide (H_2O_2) was investigated. DNA damage, GSH consumption, LDH leakage were determined

as markers of cell toxicity, whereas cytosolic free calcium concentration was measured as marker of function loss.

DNA damage in RLE cells treated with 20 μM H_2O_2 was measured using the Comet assay. In this assay DNA damage becomes visible as a "tail" of DNA fragments behind the cell and was scored by determination of these tail moments, defined as the product of tail length and the fraction of total DNA in the comet tail. The higher the tail moment, the more DNA damage has occurred in the cell. DNA damage induced by H_2O_2 (13.0±2.0) was far greater than that occurring in control cells (0.8±0.4) as depicted in Figure 6.1. Quercetin (100 μM) did not induce DNA damage in control cells (0.8±0.2). Pre-treatment with quercetin offered full protection against H_2O_2-induced DNA damage (2.1±1.5) (P<0.01).

H_2O_2 or quercetin induced a decrease in the GSH content of the cells of 18% ± 7 or 19% ± 5, respectively. This decrease was more substantial, i.e. 50% ± 5, when RLE cells were treated with the combination of quercetin and H_2O_2 (Figure 6.1).

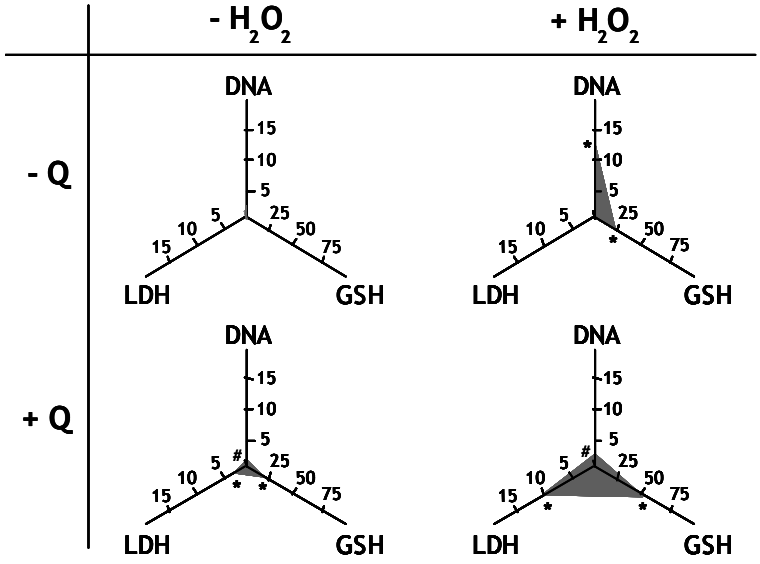

Figure 6.1 Effect of quercetin on H_2O_2-induced oxidative stress in RLE cells. The cells were pre-treated for 30 minutes with or without 100 μM quercetin (Q). Subsequently, 20 μM H_2O_2 was added for another 30 minutes. In Q control incubations no H_2O_2 was added. DNA damage, LDH leakage and GSH consumption were measured as markers of genotoxicity and/or cell toxicity. Values are expressed as mean. DNA damage is presented as Olive tail moment and both GSH consumption and LDH leakage as percentage of respectively the whole GSH or LDH content of the cells in the control incubation. * : P<0.05 compared to the incubation without quercetin and without H_2O_2; # : P<0.01 compared to the incubation without quercetin but with H_2O_2.

H_2O_2 did not induce detectable LDH leakage in control cells, as shown in Figure 6.1. Treatment with quercetin alone resulted in a cell viability of 97±1% and a small LDH leakage of 3% ± 2. The combination of H_2O_2 and quercetin lead to a cell viability of 89 ± 1% and drastically increased LDH leakage to 10% ± 2 (Figure 6.1).

Treatment of the RLE cells with H_2O_2 resulted in the occurrence of both stress fibres and membrane bleb formation (Figure 2). These morphologic changes were slightly more pronounced after treatment with both H_2O_2 and quercetin (Figure 6.2).

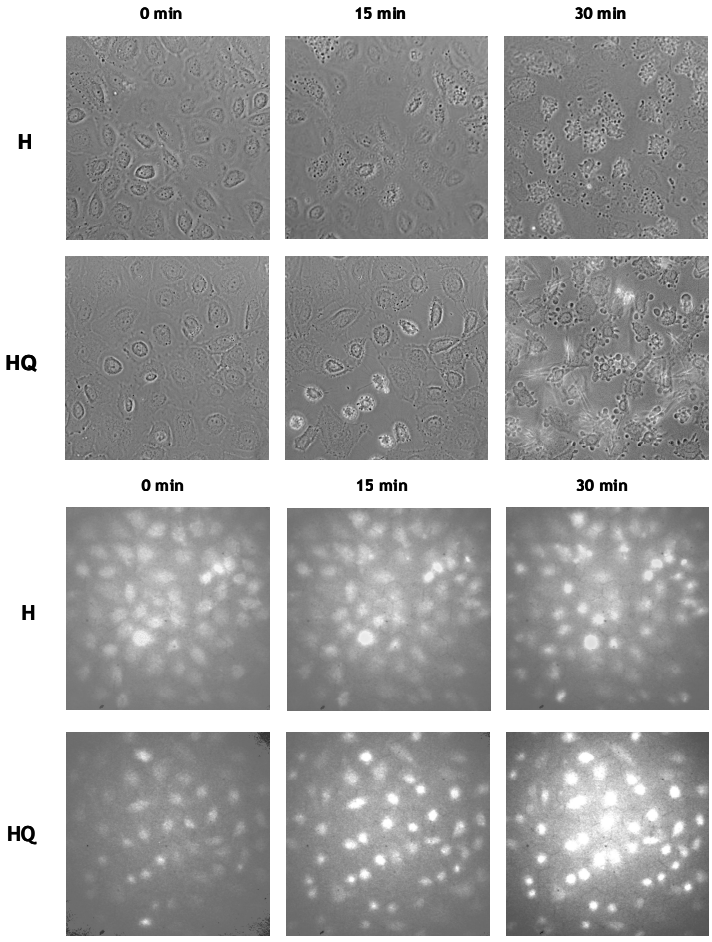

Figure 6.2 The effect of H_2O_2 and of H_2O_2 plus quercetin on RLE cells in time. (A) the morphologic changes in the cells treated with H_2O_2 (H, upper panels) or with H_2O_2 and quercetin (HQ, lower panels). (B) the fluorescent images of cells treated with H_2O_2 (H, upper panels) or with H_2O_2 and quercetin (HQ, lower panels). Images were taken before (t=0 min), half-way (t=15 min) and after H_2O_2 treatment (t=30 min).

No changes in free cytosolic calcium concentration, a marker of cell function loss, after 30 min were found following treatment with quercetin (Figure 6.3).

Hydrogen peroxide treatment of the RLE cells induced repetitive spiking, i.e. oscillation, of the cytosolic free calcium-concentration of individual cells (Figure 6.2). Combining the reactions of all individual cells, these spikes result in a significant net increase of $[Ca^{2+}]_i$ in all cells after 30 minutes (36 ± 0.1 nM vs 43 ± 0.9 nM).

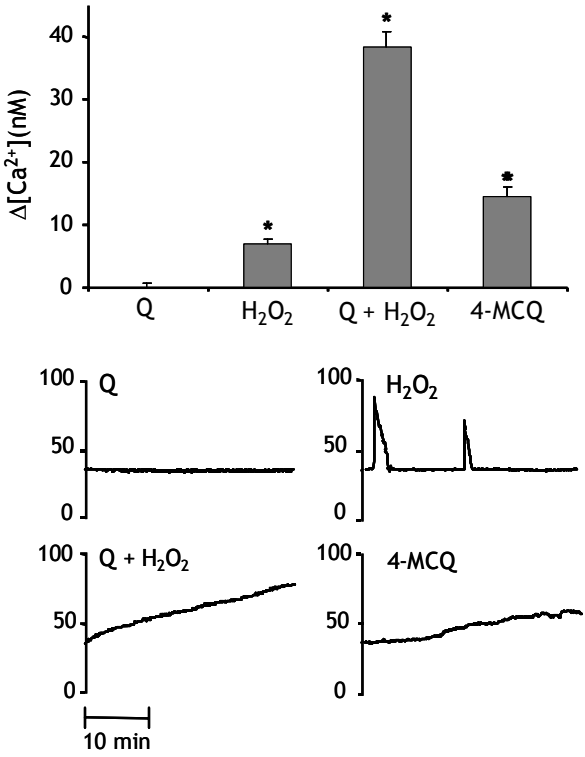

Figure 6.3 The increase in cytosolic free calcium concentration ($\Delta[Ca^{2+}]_c$) of either all RLE cells treated (upper figure) or representative single cells (lower figures) after 30 min treatment with 4-methylquinone (4-MCQ, 20 μM), H_2O_2 (20 μM), quercetin (Q, 100 μM) or both H_2O_2 (20 μM) and quercetin (100 μM).
H_2O_2 treatment induced repetitive spiking of the cytosolic free calcium concentration of individual cells rather than an increase in the total net cytosolic free calcium concentration. Data are expressed as mean ± SEM. * : $P<0.05$ compared to the incubation at t=0 min.

Treatment of the cells with both quercetin and H_2O_2 did not cause any calcium-spikes, but did significantly increase the cytosolic free calcium concentration of the cells, i.e. from 36±0.1 nM to 74±2.4 nM after 30 minutes

(Figure 6.2). This increase was more than twice that caused by 20 μM of the positive control, i.e. the thiol-reactive 4-methylortho-benzoquinone (from 35±0.1 nM to 50±1.7 nM in 30 min) (Figure 6.3).

Pre-treatment of RLE cells with the GSH synthase inhibitor BSO (0.1 mM for 24 hours) caused a 80±5% reduction in the GSH content of the cells (Figure 6.4). Treatment of the cells with H_2O_2, quercetin or both compounds together did not further reduce the GSH content of BSO-pretreated cells.

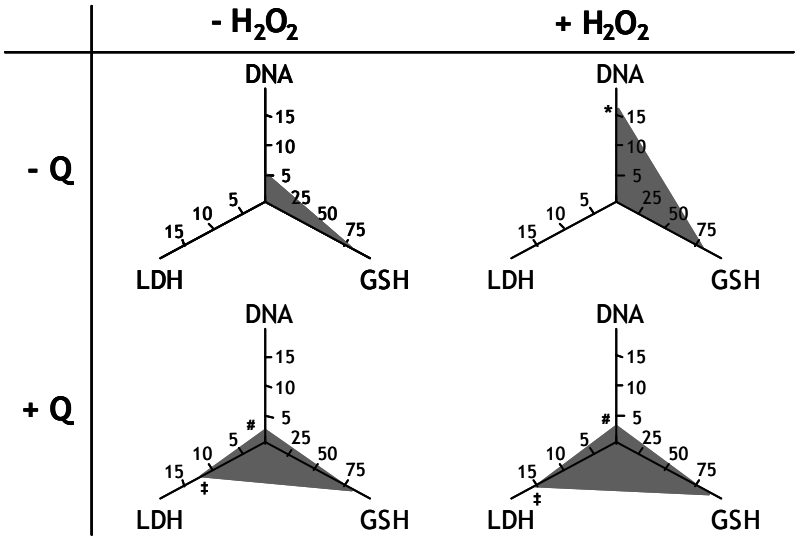

Figure 6.4 Effect of quercetin on H_2O_2-induced oxidative stress in BSO-pretreated RLE cells. The cells were pre-treated for 30 minutes with or without 100 μM quercetin (Q). Subsequently, 20 μM H_2O_2 was added for another 30 minutes. In Q control incubations no H_2O_2 was added. DNA damage, LDH leakage and GSH consumption were measured as markers of genotoxicity and cell toxicity. Values are expressed as mean. DNA damage is presented as tailmoment and both GSH consumption and LDH leakage as percentage of respectively the whole GSH or LDH content of the cells in the control incubation. * : $P<0.05$ compared to the incubation without quercetin and without H_2O_2; ‡ : $P<0.05$ compared to the corresponding incubations without BSO treatment depicted in Figure 6.1; # : $P<0.01$ compared to the incubation without quercetin but with H_2O_2.

BSO pre-treatment resulted, for all conditions tested, in a significantly increased DNA damage ($P<0.01$) compared to that in control cells (Figure 6.4). H_2O_2-induced DNA damage was slightly more pronounced after pre-treatment with BSO (13.0±2.0 for control cells vs 15.5±1.5 for cells pre-treated with BSO). Quercetin was still able to fully protect against this toxicity (3.2±2.5) (Figure 6.4).

H_2O_2 did not induce LDH leakage in BSO pre-treated cells, as shown in Figure 6.5. LDH leakage induced by quercetin in BSO pre-treated cells was 8%±1, whereas cell viability during this treatment was 92±1%. The combination of H_2O_2 and quercetin resulted in a cell viability of 83±2% and further enhanced LDH leakage to 15±2%.

Discussion

As demonstrated in this study, H_2O_2 induces DNA damage in RLE cells and quercetin completely reverses this toxicity. Similar protective effects of quercetin against H_2O_2-induced DNA damage have been obtained previously in numerous other studies (24-26). In general, the protection observed is attributed to the antioxidant activity of quercetin. H_2O_2 has been implicated in the induction of DNA damage by acting as a precursor of free radicals, including the highly reactive hydroxyl radical ($OH^{\bullet}$), that can non-selectively cause oxidative damage to virtually any molecule including DNA bases (27). Quercetin is an efficient scavenger of these radicals (4).

The paradox of quercetin is that, although a highly reactive species is being neutralized, during the same process a thiol-reactive quercetin metabolite is being formed. In this way, the aselective toxicity of the highly reactive radical is swapped for a more selective toxicity of the quercetin metabolite. This thiol reactivity manifests itself by the preferential reaction with GSH (9). The binding of oxidized quercetin with GSH is reversible (28). At low GSH concentrations, oxidized quercetin will react with protein thiols, leading to the formation of a relatively stable protein-oxidized quercetin adduct (28). Binding of oxidized quercetin to protein thiols has already been shown in isolated membranes and lymphocytes as well as in blood plasma (8, 9,29) and can result in function loss like the impairment of enzymes such as the sarco- and endoplasmatic reticulum calcium ATPase (SERCA) that is responsible for calcium sequestration (8).

The present study demonstrates for the first time that the quercetin paradox also occurs in cultured cells; through quercetin the H_2O_2-induced DNA damage in RLE cells is swapped for a reduction in GSH level and an increase in both cytosolic free calcium concentration and LDH leakage. Again, the protection offered and the toxicity caused by quercetin can be attributed to the scavenging of radicals by quercetin and the concurrent formation of a thiol-reactive oxidation product of quercetin (8,9,29). The oxidation product of quercetin is not stable and therefore the relatively stable 4-methyl-ortho-benzoquinone has been used as a reference compound that displays a similar thiol-reactivity as oxidized quercetin (28). 4-Methyl-ortho-benzoquinone induces a comparable increase in the intracellular calcium concentration as

the combined treatment with quercetin and H_2O_2. This effect of 4-methyl-ortho-benzoquinone indicates that quercetin-induced toxicity is indeed caused by the formation of thiol-reactive oxidation products of quercetin.

Since it is known that GSH efficiently protects against arylation and subsequent function loss of proteins (8), it is expected that reduction of the GSH levels will increase quercetin-induced toxicity. BSO pre-treatment of the RLE cells reduced GSH levels by 80% and indeed drastically enhanced the LDH leakage induced by the combined treatment with both quercetin and H_2O_2. This is in line with the previous finding that a loss of GSH, caused by oxidation of this thiol, is involved in peroxide-induced cytoskeletal reorganisation and bleb formation in endothelial cells (22,30).

Under normal conditions, the cytosolic free calcium concentration is kept very low by a delicate control mechanism consisting of cellular channels, transporters and large calcium stores in e.g. the endoplasmatic reticulum (31). Reduction of the calcium sequestration in these stores, for example by impairing SERCA, results in an increase in the cytosolic free calcium concentration. One of the most important targets of thiol reactive compounds is the critical sulfhydryl group of this calcium-ATPase (32). In isolated membranes, it has already been shown that catechol-containing antioxidants such as quercetin impair this enzyme (8). In the present study, H_2O_2 alone evokes sudden increases in cytosolic free calcium concentration that are followed by sharp decreases of this concentration, thus reflecting high calcium-ATPase activity. The combined treatment with quercetin and H_2O_2, however, induces a low but consistent increase in cytosolic free calcium concentration without a subsequently lowering of the calcium concentration, indicating that oxidized quercetin can also impair calcium-ATPase.

Elevated cytosolic free calcium can cause cytotoxicity or trigger apoptosis (33). Calcium can induce cell death by for example (i) mediating cytychrome c release from mitochondria via increasing the mitochondrial permeability (33), (ii) activating caspases (31,33), (iii) causing the expression of ligands that bind to and activate death receptors (31), (iv) activating hydrolytic enzymes (34) or by (v) initiating cytoskeletal degradation (34). First signs of apoptosis are the appearance of so-called stress fibres and membrane blebs at the plasma membrane, caused by a reassembly of the actin cytoskeleton (22).

In our study, the H_2O_2-induced net increase in the cytosolic free calcium concentration was indeed accompanied by concurrent signs of the formation of stress fibres and blebbing. The increase in cytosolic free calcium, and thus the resulting stress fibres and blebbing, are slightly more pronounced after combined treatment with both quercetin and H_2O_2 than with the latter alone.

Moreover, after BSO-pretreatment, the combined treatment caused a reduced cell viability. These findings suggest that, despite the direct protection against H_2O_2-induced DNA damage offered by quercetin, toxic changes that may result in LDH leakage, blebbing, decreased cell viability and eventually even apoptosis, are indirectly induced by the oxidation product of quercetin formed during this protection. In that way, the apparent protection offered by quercetin is actually a swap of one form of toxicity, i.e. DNA-damage, for another, i.e. impaired calcium sequestration.

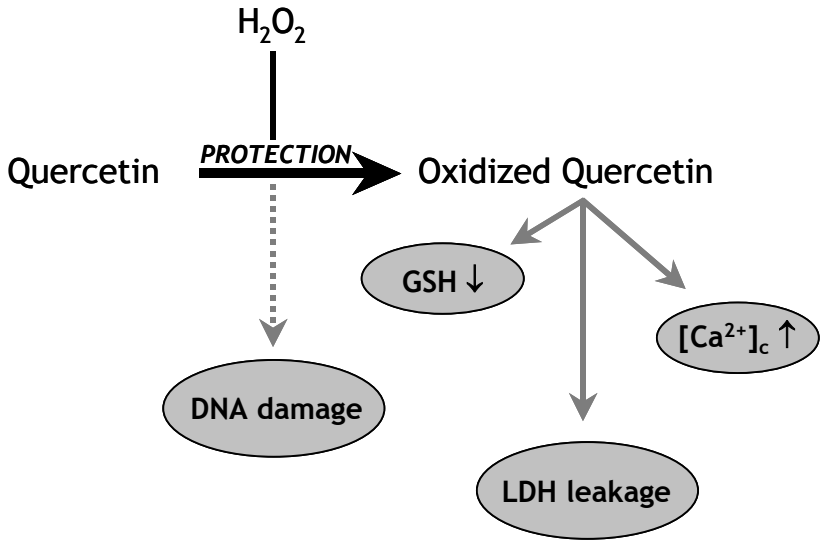

Figure 6.5 Schematic overview of the quercetin paradox. When no quercetin is present, H_2O_2 damages DNA by the formation of free radicals. When quercetin is present, it efficiently scavenges radicals and protects the cells against H_2O_2-induced DNA damage. During this protection, quercetin is converted into oxidized quercetin. Oxidized quercetin is thiol reactive and will arylate GSH as well as protein thiol groups, leading to GSH consumption, an increase in cytosolic free calcium concentration ($[Ca^{2+}]_c$) and LDH leakage.

Although the low oral bioavailability and the relatively high concentration of quercetin used in the present study hamper a straightforward extrapolation to *in vivo* conditions, our results point at some practical implications of the supplementation with quercetin. In subjects with elevated oxidative stress, i.e. subjects expected to benefit from antioxidant therapy, the formation of oxidized quercetin will be relatively high. Additionally, in these subjects GSH levels might be low, making them even more susceptible to damage by quercetin oxidation metabolites. Studies conducted with healthy volunteers on the efficacy or safety are, therefore, not representative for the actual application of quercetin. When clinical

studies with quercetin are executed, toxicity might be monitored by determining the effect of quercetin on the endogenous GSH-level.

The quercetin paradox is that, as a result of its protection, quercetin becomes toxic (depicted in Figure 6.5). Such a paradox might also apply for other free radical scavenging antioxidants. The toxicity of the metabolites that are formed during the actual antioxidant activity of free radical scavengers, might shade or even eclipse the direct positive effects of antioxidant supplementation.

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