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Quercetin Oxidation Paradoxically Enhances its Antioxidant and Cytoprotective Properties

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ABSTRACT: Quercetin oxidation is generally believed to ultimately result in the loss of its antioxidant properties. To test this assertion, quercetin oxidation was induced, and after each of its major metabolites was identified and isolated by HPLC-DAD-ESI-MS/MS, the antioxidant (dichlorodihydrofluorescein oxidation-inhibiting) and cytoprotective (LDH leakage-preventing) properties were evaluated in Hs68 and Caco2 cells exposed to indomethacin. Compared to quercetin, the whole mixture of metabolites (Q_{OX}) displayed a 20-fold greater potency. After resolution of Q_{OX} into 12 major peaks, only one (peak 8), identified as 2,5,7,3′,4′-pentahydroxy-3,4-flavandione or its 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone tautomer, could account for the antioxidant and cytoprotective effects afforded Q_{OX}. Peak 8 exerted such effects at a 50 nM concentration, revealing a potency 200-fold higher than that of quercetin. The effects of peak 8 were seen regardless of whether it was added to the cells 40 min before or simultaneously with the oxygen-reactive species-generating agent, suggesting an intracellular ability to trigger early antioxidant responses. Thus, the present study is the first to reveal that in regard to the intracellular actions of quercetin, attention should be extended toward some of its oxidation products.

KEYWORDS: *quercetin, quercetin oxidation metabolites, antioxidant, cytoprotection*

INTRODUCTION

Because of its ubiquitous distribution and abundant presence in edible plants, quercetin (3,5,7,3′,4′-pentahydroxyflavone) remains one of the most studied flavonoids. Because of its capacity to act directly as scavenger of oxygen-reactive species (ROS)[1,2] and/or indirectly, up-regulating the expression of genes that code for various ROS-removing and/or antioxidant-synthesizing enzymes,[3−5] most studies on quercetin have focused on its antioxidant properties. A commonly reported mediator of such up-regulation is the redox transcription sensor, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which binds to antioxidant response elements and initiates the corresponding gene transcription.[6,7] In addition to its antioxidant properties, several other bioactivities have been described (i.e., anti-inflammatory, antiplatelet aggregation, antiallergic, antiatherogenic, and/or antimutagenic/anticarcinogenic).[8−12] Although the existence of such activities suggests a pleiotropic potential for quercetin, broadening its spectrum for clinical applications, most of the currently available evidence still supports the contention that the health benefits associated with its dietary consumption and/or administration overall are derived from its oxidative stress-controlling capacity.[13,14] Presumably, in doing so, some of the indirect antioxidant effects of quercetin are likely to concur with its direct ROS-scavenging actions.[6,15,16] In the latter case, the quercetin molecule will inevitably undergo redox changes (i.e., electron-transfer or hydrogen atom-donation reactions) that translate into its stoichiometric oxidative consumption. During the oxidation of quercetin, some electrophilic intermediates (i.e., *o*-quinone/quinone methide) that could act as prooxidants are formed.[17,18] Although the latter species could be implied in the mutagenicity and cytotoxicity of quercetin reported by some in vitro studies,[19] the actual biological significance of such actions is still debatable and largely unsupported by most in vivo evidence.[20]

Although the oxidative changes that affect the quercetin molecule, whether chemically, electrochemically, or enzymatically induced, have been broadly studied from a chemical point of view,[21−26] only a limited number of studies have addressed the implications that such changes could have on the original antioxidant properties of the flavonoid. According to Ramos et al.[27] and Gulsen et al.,[28] certain oxidation products of quercetin could be even more active than the parent molecule when evaluated as antioxidants in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. In line with such studies, we recently reported[29] that in a cell-free system, the exposure of pure quercetin to oxidative conditions, whether chemically or enzymatically induced, leads to the formation of a quercetin-free mixture of metabolites that largely conserves the ROS-scavenging (ORAC assay) and ROS-reducing (FRAP assay) properties of the unexposed flavonoid. Based on the latter evidence, the objective of the present work was to investigate the potential that the quercetin oxidation mixture and each of its major metabolites,

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herein identified and subsequently isolated, may have as antioxidants when added to Hs68 and Caco2 cells undergoing oxidative stress, induced by indomethacin or hydrogen peroxide. The present study shows for the first time that when tested in a cellular system, the mixture of quercetin oxidation products and, in particular, one of its metabolites displays an antioxidant potency that largely surpasses that of quercetin. We believe that the findings reported here should expand our current view on the potential antioxidant implications associated with the oxidative metabolism of quercetin.

MATERIALS AND METHODS

Chemicals. Quercetin was purchased from Sigma−Aldrich (St. Louis, MO), with a purity ≥95%. Acetonitrile and ethanol were HPLC grade. Formic acid, sodium hydroxide, indomethacin, hydrogen peroxide, sodium dihydrogen phosphate, disodium hydrogen phosphate, and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich. CytoTox-One was from Promega (Madison, WI). All other reagents were of analytical grade.

Cell Culture Conditions. The human skin fibroblast cells Hs68 (ATCC CRL-1635) and the human colonic adenocarcinoma line Caco2 (ATCC HTB-37) were cultured at 37 °C (5% CO_2/95% air) in DMEM (Dulbecco's Modified Eagle's medium) supplemented with 10% fetal bovine serum. Cells were trypsinized when they reached near 90% confluence and were used for experiments.

Preparation of Nonoxidized and Oxidized Quercetin. Quercetin was initially dissolved in 500 μL of ethanol and, upon total dissolution, brought to 1 mL with the addition of sodium phosphate buffer (25 mM, pH 7.4). Prior to its addition to the cells, the former quercetin solution (10 mM) was diluted further with the same buffer at least 1000-fold, such that the cells were never exposed to a concentration of ethanol greater than 0.05% v/v. The latter solution is referred to hereafter as nonoxidized quercetin or simply as quercetin (Q). To obtain oxidized quercetin, the flavonoid was initially dissolved in NaOH (pH 12) and kept in such medium at 22 °C for 13 min. Immediately after, the solution was neutralized by the addition of a near 20 000-fold sodium phosphate buffer (75 mM, pH 7.4). Under such conditions, the resulting neutralized mixture was quercetin-free.[29] This solution is hereafter referred to as Q_{OX}.

Oxidative Status and Cell Viability Experiments. Cells were seeded (100 000 cell/well) in a 96-well plate, and after the samples were cultured for 12 h, the intracellular oxidative status and cell viability were assessed. To assess the oxidative status, DCFH-DA was used as a ROS-reactive probe.[30] Cells were loaded with DCFH-DA (50 μM) for 30 min, and after being washed with PBS, the cells were exposed to PBS, indomethacin (Indo; 275 μM), or hydrogen peroxide (1.75 mM) in the presence or absence of Q or Q_{OX} for 40 min. Cells were subsequently washed with PBS and lysed by the addition of Triton X-100 (0.03%); after 10 min, their fluorescence was measured (excitation 495 nm/emission 529 nm) using a Synergy 2 multimode reader (Biotek, Winooski, VT). A 100% oxidative damage was ascribed to the indomethacin-added cells and corresponds to the difference that results from subtracting the fluorescence emitted by PBS-exposed cells (FL_{PBS}) to that emitted by indomethacin-treated cells (FL_{Indo}). For all other cell conditions, the percentage of oxidative damage was estimated by multiplying the following equation by 100: $FL_{EXP} - FL_{PBS}/FL_{Indo} - FL_{PBS}$. FL_{EXP} corresponds to the fluorescence emitted by cells with added Q or Q_{OX}, either in the absence or presence of indomethacin. To assess cell viability, LDH leakage was evaluated in the supernatants after 40 min of exposure of the cells to either PBS or indomethacin, in the absence or presence of Q or Q_{OX}, using the CytoTox-One homogeneous membrane integrity assay (excitation 560 nm/emission 590 nm). The results are expressed as % LDH leaked into the extracellular media. A basal value of 5% was estimated in the cells with added PBS. Although most studies were carried out as coincubation experiments, namely, simultaneous exposure of the cells for 40 min to indomethacin or hydrogen peroxide and Q or Q_{OX}, some were carried out as preincubation experiments.[31] In the latter experiments, cells were preincubated with Q or Q_{OX} for 40 min, and after the cells were washed with PBS, indomethacin was added, and the cells were incubated for an additional 40 min.

HPLC-DAD Analysis of Quercetin and Its Oxidation Products. A sample of Q_{OX} was analyzed by means of a 1200 series high-performance liquid chromatography (HPLC) instrument equipped with an autosampler and a photodiode array detector (Agilent Technologies, Santa Clara, CA). The HPLC system was controlled by ChemStation software (Agilent). Quercetin and its metabolites were separated using a mobile phase mixture of (A) acetonitrile and (B) 0.1% aqueous formic acid, the composition of which was varied employing the following HPLC gradient program: 0−15.0 min, 10% A; 15.0−50.0 min, 10−60% A; and returned to starting conditions in the following 10 min. The column used was a 250 × 4.6 mm i.d., 5 μm, Kromasil 100-5-C18 (AkzoNobel, Bohus, Sweden). Other chromatographic conditions were as follows: flow rate of 0.8 mL/min and oven column set at 25 °C. The absorbance of the eluate was monitored with a diode array detector (DAD) set at 294 and 370 nm.

HPLC-ESI-MS/MS Analysis of Quercetin and Its Oxidation Products. MS/MS spectra were acquired using a triple quadrupole spectrometer employing electrospray ionization (ESI-MS/MS). HPLC-ESI-MS/MS analysis of Q_{OX} was conducted under the same chromatographic conditions as in the HPLC-DAD analysis and was performed on an Ekspert UltraLC 100 coupled to a Triple Quad 4500 tandem mass spectrometer equipped with turbo spray ionization source (AB Sciex, Ontario, Canada). The mass spectrometer was operated in negative ionization mode, and data were acquired in multiple reaction monitoring mode. The source temperature was 650 °C, and the ESI voltage and entrance potential were set at −4.5 kV and −10 V, respectively. The gas flows were as follows: ion spray gas, 50 psi; heater gas, 40 psi; curtain gas, 20 psi; and collision gas, 7 psi. The declustering potential, collision energy, and collision exit potential for each compound were adjusted at each tandem MS stage to determine the optimum parameters at which a maximum amount of signal information could be obtained, and a scanning rate of 150 ms was used. The ESI-MS/MS system was controlled by Analyst 1.6.2 software (AB Sciex, Darmstadt, Germany).

Collection of the Chromatographic Peaks That Arise from the Analysis of Oxidized Quercetin. The major peaks detected in the HPLC-DAD chromatographic analysis of Q_{OX} were collected using an automated fraction collector, coupled to the HPLC system, brought to dryness under a stream of nitrogen at 40 °C, and reconstituted in a small volume of a mixture of mobile phase whose composition was A/B (1:4, v/v). The chemical identity of the molecules present in the reconstituted peaks was confirmed by a new ESI-MS/MS analysis. To evaluate the antioxidant and cytoprotective properties, each peak was reconstituted in a small volume of PBS and subjected to HPLC-DAD analysis, employing experimental conditions identical to those described above. To define the final volume of PBS in which each peak was reconstituted, a calibration curve in which the area under the curve of each peak versus a range of concentrations of alkali-dissolved quercetin was employed.

Statistics. Data points plotted in Figures 1A,B, 4A,B, and 6 represent the means of at least three independent experiments, each conducted in sextuplicate. For the sake of simplicity, because the standard deviations represented less than 10% of the means, these were omitted from the former figures. Statistical significance of the difference between the experimental conditions was assessed with unpaired *t* tests or analysis of variance (ANOVA), as appropriate. Data were analyzed using the GraphPad Prism 5 statistical software (La Jolla, CA).

RESULTS AND DISCUSSION

Evaluation of the Antioxidant and Cytoprotective Properties of the Alkali-Oxidized Quercetin Solution in Cultured Cells. Our laboratory recently observed that after inducing the total oxidation of quercetin through its exposure to alkali, the resulting mixture of metabolites largely conserves

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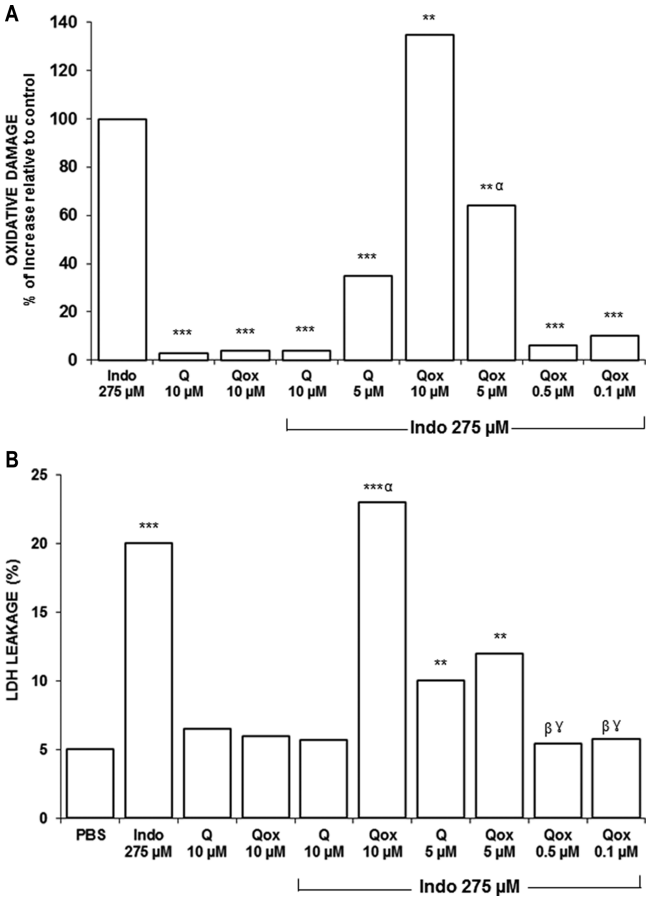

Figure 1. (A) Comparison of the antioxidant effects of quercetin and oxidized quercetin against the increase in intracellular oxidative tone induced by indomethacin. Significant differences: ** with a $p < 0.01$ or *** with a $p < 0.001$ relative to indomethacin-treated cells; α with a $p < 0.01$ relative to cells treated with indomethacin and Q 5 μM. (B) Comparison of the protective effects of quercetin and the oxidized quercetin against the cytolytic damage induced by indomethacin. Significant differences: ** with a $p < 0.01$ or *** with a $p < 0.001$ relative to PBS-treated cells; α with a $p < 0.05$ relative to indomethacin-treated cells; β with a $p < 0.01$ relative to cells treated with indomethacin and Q 5 μM; γ with a $p < 0.001$ relative to cells treated with indomethacin and Q_{OX} 5 μM.

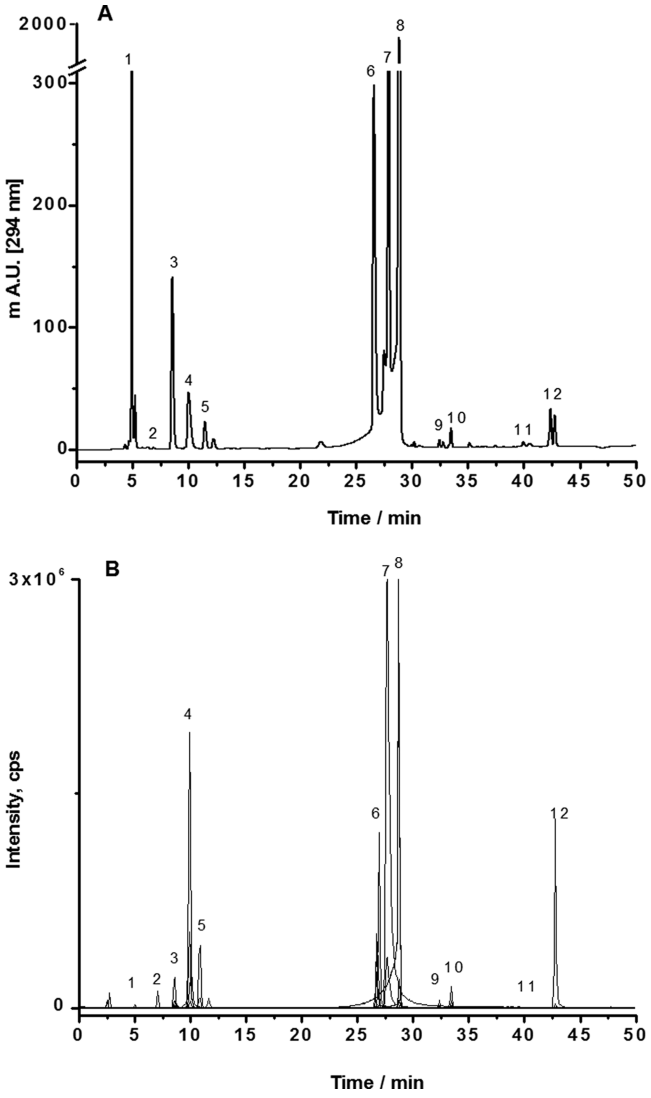

Figure 2. Chromatographic analysis of the major quercetin oxidation metabolites. (A) HPLC-DAD chromatogram of Q_{OX} (100 μM equivalent concentration). (B) HPLC-ESI-MS/MS chromatogram of Q_{OX} (100 μM equivalent concentration).

the ROS-scavenging (95%) and Folin−Ciocalteau reducing (77%) properties of the nonoxidized flavonoid.[29] Prompted by the latter findings, we evaluated the capacity of such a mixture to protect Hs68 and Caco2 cells against the increase in oxidative tone induced by either indomethacin or hydrogen peroxide. Figure 1A depicts the results from exposing Hs68 cells to indomethacin (275 μM). The 100% value corresponds to the increase in oxidative tone induced by the latter agent. In the absence of indomethacin, the sole exposure of cells to 10 μM Q or its equivalent concentration of Q_{OX}, defined based on the concentration of Q initially subjected to oxidation, resulted in no significant increase of the oxidative tone. When Q was coincubated with indomethacin-added cells, an almost full protection (over 96%) was observed at 10 μM, and a near 65% protection at 5 μM. In turn, the addition of 10 μM Q_{OX}, rather than promoting protection, led to a near 35% increase in the level of oxidative damage induced by indomethacin. However, when Q_{OX} was added at 5, 0.5, and 0.1 μM concentrations, the protection was 36%, 94% and 90%, respectively. Figure 1B depicts the results from evaluating the potential of Q and Q_{OX} to protect Hs68 cells against the lytic effects of indomethacin. Relative to the control cells (PBS added), indomethacin enhanced the basal LDH leakage by nearly 3-fold. This effect was almost totally prevented when Q or Q_{OX} were added to the cells at 10 and 0.5 μM, respectively. Compared to the degree of protection seen in Hs68 cells, the protections afforded by 10 μM Q or by 0.5 μM Q_{OX} against the oxidative and the cytolytic effects of indomethacin were almost identical when tested in Caco2 cells (data not shown). When, instead of indomethacin, hydrogen peroxide (1.75 mM) was used, the antioxidant and cytoprotective protection exerted by Q_{OX} (0.5 μM) was, in Hs68 cells, 94% and 96%, respectively, and in Caco2 cells, 92% and 95%, respectively (data not shown).

Chromatographic Separation and Identification of the Major Metabolites of the Alkali-Oxidized Quercetin Mixture. On the basis of the above results which reveal that the Q_{OX} solution possesses antioxidant and cytoprotective properties that are more potent than those exerted by Q, we addressed the chemical composition of the former. To such an end, immediately after ascertaining the total oxidative

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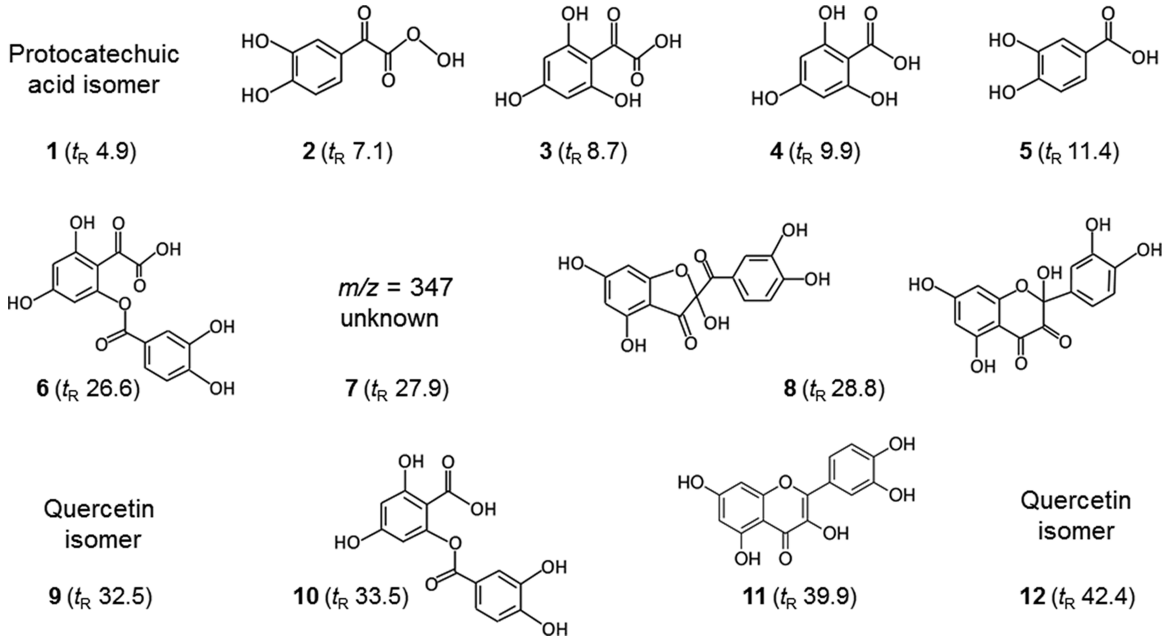

Figure 3. Chemical structures of the major quercetin oxidation products. Numbers correspond to peaks shown in Figure 2B and are accompanied by their corresponding retention times, expressed in parentheses in minutes.

disappearance of quercetin from a freshly prepared Q_{OX} solution, a sample of the latter was analyzed by HPLC-DAD. As shown in Figure 2A, under our chromatographic and detection conditions (294 nm), Q_{OX} consisted of a mixture of at least 12 major peaks. Peak 11, identified as quercetin based on its standard (t_R of 39.9 min), was virtually nonexistent in the Q_{OX} solution. Comparatively, peak 8, followed by peaks 7, 6, 1, and 3, showed the highest absorbance intensity signals. To establish the possible chemical identity of the 12 peaks obtained, HPLC-DAD fractions of each of such peaks were collected and independently subjected to ESI-MS/MS analysis. As known, the latter methodology provides m/z values which, based on the molecular ion $[M-H]^-$ and characteristic fragments, allow a tentative identification of the chemical structure of the compound present in each of such peaks. Based on the latter information, the HPLC-ESI-MS/MS analysis of Q_{OX}, conducted under the same chromatographic conditions as those employed in the HPLC-DAD analysis, generated a chromatogram (Figure 2B) that was almost identical to that obtained by HPLC-DAD in terms of both peak numbers and t_R. On the basis of the similarity between the m/z data obtained by us and those reported earlier by Zhou and Sadik[25] in their quercetin oxidation studies, we have assigned to each peak the chemical structure shown in Figure 3. The molecular ion and qualification transitions of peaks 1−12 were as follows: m/z at 153 and 109/108 for peak 1 (an isomer of protocatechuic acid, **1**); m/z at 195 and 151/127 for peak 2 (methyl 2-(3,4-dihydroxyphenyl)-2-oxoacetate, **2**); m/z at 197 and 153/151 for peak 3 (2-oxo-2-(2,4,6-trihydroxyphenyl)acetic acid, **3**); m/z at 169 and 151/83 for peak 4 (2,4,6-trihydroxy-benzoic acid, **4**); m/z at 153 and 109/79 for peak 5 (protocatechuic acid, **5**); m/z at 333 and 179/197 for peak 6 (2-(2-((3,4-dihydroxybenzoyl)oxy)-4,6-dihydroxyphenyl)-2-oxoacetic acid, **6**); m/z at 347 and 275/203 for peak 7 (unknown, **7**); m/z at 317 and 163/191 for peak 8 (2,5,7,3′,4′-pentahydroxy-3,4-flavandione or 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone, **8**); m/z at 301 and 151/273 for peak 9 (an isomer of quercetin, **9**, found also as trace in Q); m/z at 305 and 169/151 for peak 10 (2-((3,4-dihydroxybenzoyl)oxy)-4,6-dihydroxybenzoic acid, **10**); m/z at 301 and 151/273 for peak 11 (quercetin, **11**); and m/z at 301 and 273/151 for peak 12 (an isomer of quercetin, **12**, also detected in Q).

Evaluation of the Antioxidant and Cytoprotective Properties of the Major Quercetin Oxidation Metabolites in Cultured Cells. Upon addressing the identity of the metabolites associated with peaks 1−12 of the Q_{OX} solution (Figure 3), the antioxidant and cytoprotective potential of each such peak was evaluated in Hs68 cells. To this end, after a sample of Q_{OX} was subjected to HPLC-DAD analysis, the fractions corresponding to peaks 1−12 were collected, brought to dryness, and reconstituted in PBS. Because the Q_{OX} mixture had already proven (Figure 1A) to afford in cells a near total antioxidant protection at a 0.5 μM equivalent concentration of quercetin, each of the 12 peaks was independently tested at that concentration. Calibration curves were built plotting the areas of each peak against different concentrations of alkali-dissolved quercetin (not shown). As shown in Figure 4A, when added at a 0.5 μM concentration, peak 8 protected the cells against the increase in DCHF oxidation induced by indomethacin by 96%. Comparatively, peaks 1−7 and 9−12 afforded no significant protection. Thus, the results of the latter assessment are depicted in Figure 4A under the form of two columns that represent the mean of the obtained values: peaks 1−7 (P1−7) and 9−12 (P9−12). The protection afforded by P8 was not significantly diminished when its concentration was lowered to 0.05 μM, but it was slightly and significantly reduced (to near 86%) when lowered to 0.01 μM. When tested at a 0.5 μM concentration, P8 was found to fully protect against the leakage of LDH induced by indomethacin (not shown). Based on the results from applying HPLC-ESI-MS/MS analysis to P8 (Figure 2B), the chemical identity of the compound represented by the latter peak corresponded to **8**, the 2,5,7,3′,4′-pentahydroxy-3,4-flavandione or its related 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone (Figure 3). Because these compounds share the presence of at least one carbonyl moiety in their heterocyclic ring and other

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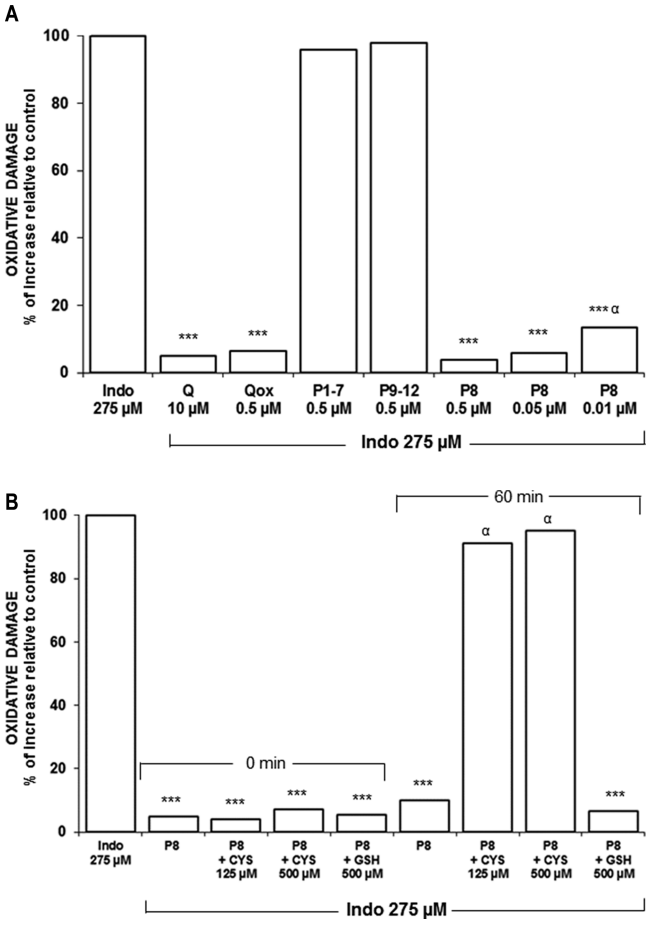

Figure 4. (A) Comparison of the antioxidant effects of quercetin and the major chromatographic peaks against the increase in intracellular oxidative damage induced by indomethacin. Significant differences: *** with a $p < 0.001$ relative to indomethacin-treated cells; α with a $p < 0.01$ relative to cells treated with indomethacin and P8 0.05 μM or 0.5 μM. (B) Effects of cysteine and glutathione on the protective action of peak 8 against the increase in intracellular oxidative damage induced by indomethacin. CYS and GSH stand for cysteine and reduced glutathione, respectively. Significant differences: *** with a $p < 0.001$ relative to indomethacin-treated cells; α with a $p < 0.001$ relative to cells treated with indomethacin and P8 0.05 μM.

quercetin oxidation products that also carry carbonyls in that ring (i.e., *o*-quinone and *p*-quinone methide) have been shown to swiftly react with cysteine and glutathione to form adducts,[32] we investigated whether these thiols could affect the antioxidant activity of P8. The increase in DCFH oxidation induced by indomethacin (275 μM) was examined after the simultaneous addition of P8 (0.05 μM quercetin equivalent concentration) plus cysteine (125−500 μM) or plus glutathione (500 μM) to Hs68 cells. In addition, similar experiments were run using a mixture of P8 plus each of such thiols incubated for 60 min at 22 °C. As shown in Figure 4B, when added simultaneously, neither cysteine nor glutathione could affect the protection afforded by P8. However, when added under the form of the mixture preformed with cysteine, P8 failed to promote that effect. In turn, glutathione exerted no effect on P8 action. Prompted by the ability of cysteine to block the effect of P8 observed with the preformed mixture, the effect of that thiol on the stability of P8 was investigated. Figure 5 depicts the HPLC-DAD chromatograms that result from analyzing samples of P8 (10 μM quercetin oxidation equivalent concentration) incubated during 0−60 min either alone (Figure 5A) or in the presence of 10 mM cysteine (Figure 5B). The chromatograms shown in panels A and B of Figure 5 reveal that independently of whether cysteine was present, the area under the curve of P8 diminished along the incubation in both cases to 8.8%, 23%, and 40% of the initial value after 20, 40, and 60 min, respectively. Concomitant to the disappearance of P8, three other minor peaks, of which the second one was only marginally detected, that were practically nonexistent at time zero emerged, and their areas under the curve started to progressively increase as the incubation progressed. The retention times of these peaks (8.7, 9.9, and 11.4 min) were similar to those exhibited by peaks 3, 4, and 5 (Figure 3) generated during the HPLC-DAD analysis of the Q_{OX} solution. As expected, after subsequent ESI-MS/MS analysis of the three former peaks, their chemical identity was determined to be 2-oxo-2-(2,4,6-trihydroxyphenyl)acetic acid, 2,4,6-trihydroxy-benzoic acid, and protocatechuic acid, respectively, identical to that of **3**, **4** and **5**. Figure 5C depicts the HPLC-DAD chromatogram that results from incubating cysteine alone for 60 min. The chromatogram reveals the presence of two peaks which, as corroborated by the use of standards, correspond to cystine and cysteine, with retention times of 3.4 and 3.7 min, respectively. Although the two latter peaks were already present at time zero in Figure 5B, in the presence of P8, the area under the curve of the peak corresponding to cystine increased with incubation at a rate substantially greater than that seen in Figure 5C. Under the chromatographic conditions employed by us (i.e., monitoring within the 200−700 nm range and for 60 min) the increase in cystine formation was not associated with the appearance of any peak other than those already seen in Figure 5A.

Comparative Antioxidant Effects of the Preincubation versus Coincubation of Hs68 Cells with Peak 8. In each of the above-described cell experiments, the antioxidant properties of Q, Q_{OX}, and P8 were tested following their addition concomitant to that of indomethacin. To gain some possible insights into the importance of the time of addition of Q, Q_{OX}, or P8 relative to indomethacin, preincubation and coincubation experiments were conducted. In the former experiments, Hs68 cells were preincubated with Q, Q_{OX}, or P8 for 40 min at the concentrations indicated in Figure 6. At the end of that incubation, the extracellular medium was removed; the cells were swiftly washed with PBS, and indomethacin was added immediately after. The antioxidant effects were assessed 40 min after indomethacin addition. In the case of the coincubation experiments, the cells were incubated with Q, Q_{OX}, or P8 together with indomethacin for 40 min. As seen in Figure 6, the antioxidant protection afforded by 10 μM Q in the preincubation experiments was only 38% of that exerted by the same in the coincubation experiments. In the case of Q_{OX} or P8, added at 0.5 μM and 0.05 μM, respectively, an almost full protection was seen independently of whether preincubation or coincubation conditions were used.

In addressing the question of whether a polyphenol necessarily loses its antioxidant properties when it gets oxidized, we recently reported that in a cell-free system, the oxidation of quercetin, whether chemically or enzymatically induced, leads to the formation of a mixture of metabolites with ROS-scavenging properties that are similar to those displayed by the nonoxidized flavonoid.[29] The present study demonstrates that the antioxidant properties of such a mixture are not only retained but markedly enhanced when Q_{OX} is tested in cells

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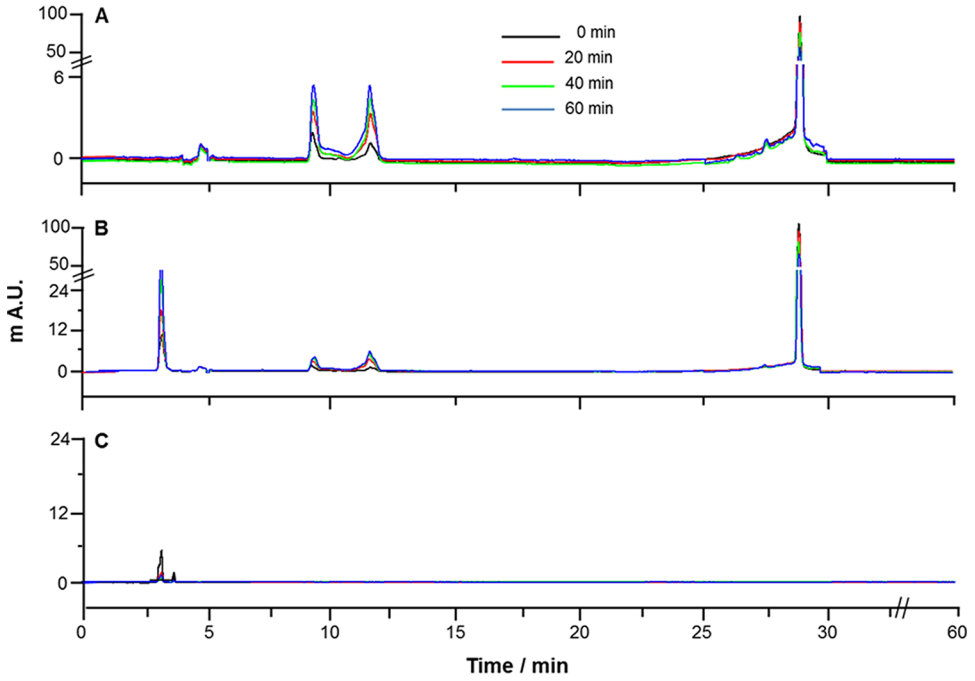

Figure 5. Chromatographic analysis of the time-dependent changes of peak 8 in the absence and presence of cysteine. (A) Incubation of peak 8 alone for 60 min (10 μM quercetin oxidation equivalent concentration); (B) incubation of peak 8 plus cysteine (10 mM); and (C) incubation of cysteine alone (10 mM) for 60 min.

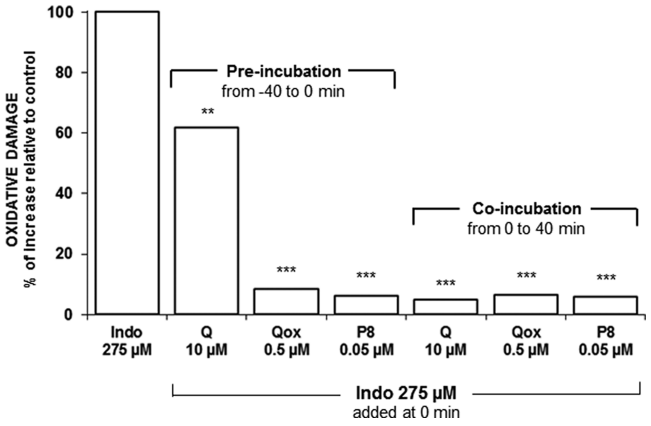

Figure 6. Comparative antioxidant effects of the preincubation versus coincubation of Hs68 cells with peak 8. Significant differences: ** with a $p < 0.01$ or *** with a $p < 0.001$ relative to indomethacin-treated cells.

exposed to an ROS-generating agent. The concentration of oxidized quercetin needed to afford full protection against the increase in oxidative tone induced by indomethacin was 0.5 μM; therefore, it was 20-fold lower than that required by the nonoxidized quercetin molecule (10 μM). However, at higher concentrations of Q_{OX} (10 μM), the mixture was found to intensify the oxidative damage induced by indomethacin. Because the latter effect was seen only in the presence of indomethacin, or hydrogen peroxide when used instead, but not in its absence, we speculate that Q_{OX} would contain certain metabolites that are not pro-oxidants as such but that, when added in high concentrations to cells facing a stringent oxidative environment, are capable of undergoing a chemical change that converts them into pro-oxidant species. The latter could also be expected to be formed when Q_{OX} is added to the cells in the 0.5−1.0 μM concentration range. We suggest, however, that when the putative pro-oxidant species are formed at lower concentrations, these would not significantly elevate the oxidative tone induced by indomethacin. In contrast, such species would act primarily as molecules capable of triggering, via a signaling pathway-mediated action, an early, effective, and fully compensatory antioxidant response which, as recently suggested by Forman et al.,[13] would render the cells with an increasing nucleophilic tone. In addition to exerting antioxidant effects, Q_{OX} was also able to effectively protect cells against the ROS-dependent cell lysis induced by indomethacin. Of note, the antioxidant and cytoprotective effects of Q_{OX} reported here occurred independent of cell type because these were seen in Hs68 and Caco2 cells and were not limited to the use of a single ROS-generating agent, as both effects were equally observed in cells treated with indomethacin or with hydrogen peroxide.

Regarding the identity of the metabolites of Q_{OX}, our HPLC-DAD and -ESI-MS/MS analyses reveal that the mixture contains at least 12 major metabolites, of which eight, corresponding to **1**−**6**, **8**, and **10**, were coincident with those reported by Zhou and Sadik[25] in their quercetin auto-oxidation study. Such similarity of composition is not surprising because previous studies aimed at establishing the identity of the metabolites generated during the oxidation of quercetin, whether electrochemical or free-radical-mediated,[21,25] chemically or enzymatically induced,[22,23] have concluded that each of such modes of oxidation yields, more or less, the same set of oxidized products. In assessing the antioxidant potential of each of the 12 quercetin oxidation products, we found that only peak 8 can protect cells against oxidation. ESI-MS/MS data revealed that such a peak corresponds to either the 2,5,7,3′,4′-pentahydroxy-3,4-flavandione or its related 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone derivative, **8**. As earlier described by Jørgensen et al.[21] and Zhou et al.,[33] and shown here in Figure 7, quercetin oxidation leads to the

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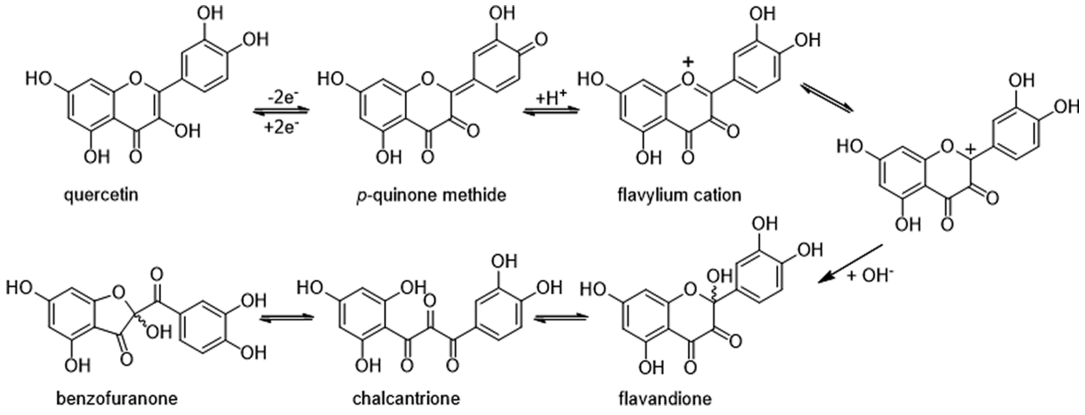

Figure 7. Sequence of chemical structures and reactions proposed to be involved in the oxidative conversion of quercetin into its flavandione and benzofuranone derivatives (based on Jørgensen et al.,[21] Zhou and Sadik,[25] and Zhou et al.[33]).

formation of a *p*-quinone-methide intermediate that, upon protonation, is converted into a flavylium cation; subsequently, the latter compound swiftly undergoes complete hydration to generate the 2,5,7,3′,4′-pentahydroxy-3,4-flavandione. Through a ring−chain tautomeric equilibrium, which leads to the formation of a 2,3,4-chalcan-trione intermediate, the flavandione coexists with 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone. Because the two latter compounds share the same *m*/*z* values,[25] it is not possible to distinguish based on the ESI/MS/MS data which compound actually accounts for the potent antioxidant activity displayed by P8. Nevertheless, clearly **8** is at least 200-fold more potent than quercetin (10 μM). The possibility that such a difference in intracellular antioxidant potency can be explained in terms of (and attributed to) a 200-fold difference in ROS-scavenging capacity is low because the flavandione and benzofuranone do not differ from quercetin in the number and position of their phenolic moieties (Figure 7), and the double bond present in ring C of quercetin, needed to delocalize the unpaired electron of its semiquinones,[34] is absent in the two former molecules. Thus, not surprisingly, Gulsen et al.[28] reported that relative to quercetin, benzofuranone has only a comparable hydrogen peroxide-scavenging capacity and a much lower hydroxyl radical-scavenging capacity.

The substantially greater antioxidant potency of **8** could alternatively involve an ROS-scavenging-independent mechanism. One such mechanism could relate to the ability of quercetin to up-regulate the antioxidant capacity of the cells via Nrf2 activation.[6,7] Although the potential of the flavandione or benzofuranone to directly or indirectly activate Nrf2 remains to be evaluated, several chalcones have already been shown to act as potent Nrf2 activators.[35,36] The electrophilic carbonyl groups of chalcones are believed to interact oxidatively with cysteinyl residues present in the regulatory sensor of Nrf2, the Kelch-like ECH-associated protein 1 (Keap 1). Addressing a possible Nrf2 activating effect of the flavandione or the benzofuranone is beyond the scope of the present study; however, we did note that when P8 was premixed with cysteine prior to its addition to the cells, its antioxidant capacity totally disappeared. Considering that the effect of cysteine was observed only 1 h after its premixing with P8, and not before, and that, under these experimental conditions, P8 markedly enhanced the conversion of cysteine into cystine (Figure 5B), it is tempting to hypothesize that, within the cells, a time-dependent redox reaction between the active component(s) of P8 and certain highly nucleophilic cysteine residues, such as those present in Keap 1, also occurs.

Recently, Carrasco-Pozo et al.[37] reported that when indomethacin-exposed cells are simultaneously incubated with quercetin, the protection against the increase in oxidative tone afforded by the flavonoid requires and relies on a prior increase in Nrf2 translocation. Because the flavandione and/or the benzofuranone are known to be generated during the interaction of quercetin with ROS,[22,25] such oxidation products could also be presumed to be generated during the oxidative stress induced by indomethacin. On the basis of our finding that **8** affords full protection against indomethacin at a concentration 200-fold lower than that of quercetin, one might speculate that to exert such protection, only one out of 200 molecules of quercetin would need to be oxidatively converted into the flavandione or the benzofuranone. Although the quinone/quinone methide precursors of such compounds are known to be generated intracellularly during the oxidative metabolism of quercetin,[38−40] the possibility also exists that the flavandione or the benzofuranone is incorporated into the cells following the intake of certain edible plants that are naturally rich in quercetin oxidation products. An example of the latter are onion peels, in which the presence of the flavandione[27] and benzofuranone[41,42] has been documented. In conclusion, given the substantially higher antioxidant efficacy of these compounds within cells, we suggest that in regard to studying the antioxidant actions of quercetin, attention should be necessarily extended toward some of its major oxidation products.

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ABBREVIATIONS USED

DCFH, 2′,7′-dichlorodihydrofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; Indo, indomethacin; Q, quercetin; Q_{OX}, oxidized quercetin

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