Glutathione-Dependent Generation of Reactive Oxygen Species by the Peroxidase-Catalyzed Redox Cycling of Flavonoids

Giuseppe Galati, Tom Chan, Bin Wu, and Peter J. O'Brien*

Department of Pharmacology and Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada M5S 2S2

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Catalytic concentrations of apigenin (a flavone containing a phenol B ring) and naringin or naringenin (flavanones containing a phenol B ring) caused extensive GSH oxidation at a physiological pH in the presence of peroxidase. Only catalytic H_2O_2 concentrations were required, indicating a redox cycling mechanism that generated H_2O_2 was involved. Extensive oxygen uptake ensued, the extent of which was proportional to the extent of GSH oxidation to GSSG and was markedly increased by superoxide dismutase. These results suggest that prooxidant phenoxyl radicals formed by these flavonoids co-oxidized GSH to form thiyl radicals which activated oxygen. GSH also prevented the peroxidase-catalyzed oxidative destruction of these flavonoids which suggests that phenoxyl radicals initiated the oxidative destruction. This is the first time that a group of flavonoids have been identified as prooxidants independent of autoxidation reactions catalyzed by the transition metal ions Fe^{3+}, Fe^{2+}, Mn^{2+}, and Cu^{2+}.

Introduction

Flavonoids, which are widely distributed in green vegetables and fresh fruits, have recently been identified as a major cancer-preventive component of our diet because of their antioxidative, oxygen radical scavenging, and anti-inflammatory activities (*1*). They may also be anti-atherosclerotic as they prevent Cu^{2+}-catalyzed low-density lipoprotein (LDL)[1] oxidative modification by scavenging reactive oxygen species (ROS) and eliminating LDL peroxyl and alkoxyl radicals (*2*, *3*). Many flavonoid preparations are also marketed as herbal medicines or dietary supplements for a variety of alleged nontoxic therapeutic effects which have yet to pass controlled clinical trials. However, despite all these apparently useful therapeutic properties, flavonols that contain catechol or pyrogallol B rings can autoxidize in the presence of transition metals to produce ROS which accelerate LDL oxidation during the propagation phase (*4*). The DNA strand scission (*5*) and the mutagenicity induced by flavonols such as quercetin also likely result from autoxidation that occurs during a lengthy in vitro aerobic incubation (*6*). The carcinogenicity data are conflicting (*7*), and it is possible that this prooxidant activity as a result of autoxidation may not be important in vivo, where transition metals are largely sequestered.

It is well-established that some phenolic compounds are oxidized by peroxidases to phenoxyl radicals which co-oxidize NAD(H) or GSH and cause oxygen activation (*8*). In the following, extensive oxygen activation was found when some flavones and flavanones containing phenol B rings were metabolized by peroxidases in the presence of GSH. This is the first time that a group of flavonoids have been identified as prooxidants without involving a transition metal-catalyzed autoxidation.

Materials and Methods

Flavonoids, glutathione (GSH), horseradish peroxidase (HRP) type VI, superoxide dismutase (SOD) type 1, cytochrome *c*, and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium 5-carboxanilide (XTT) were obtained from Sigma Chemical Co. Phenol was obtained from Baker Chemical Co.

Measurement of the Extent of GSH Oxygen Consumption. The reaction mixtures contained 2 mL of 0.1 M Tris-HCl/1.0 mM EDTA buffer (pH 7.4), flavonoid or phenol (25 μM), H_2O_2 (25 μM), and GSH (400 μM). Reactions were started by the addition of HRP (0.1 μM), and the extent of oxygen consumption in this reaction mixture was measured with a Clarke type electrode at 20 °C.

Measurement of the Extent of GSH Oxidation. The reaction mixtures contained 2 mL of 0.1 M Tris-HCl/1.0 mM EDTA buffer (pH 7.4), flavonoid or phenol (25 μM), H_2O_2 (1 μM), and GSH (400 μM). Reactions were started by the addition of HRP (0.1 mM). Aliquots were removed at different times for the HPLC analysis of GSH and GSSG levels after the GSH oxidation reaction was stopped with iodoacetic acid (15 mM) (*9*).

Measurement of the Kinetics of GSH Depletion. The reaction mixtures contained 2.5 mL of 0.1 M Tris-HCl/1.0 mM EDTA buffer (pH 7.4), flavonoid or phenol (25 μM), GSH (400 μM), HRP (0.1 μM), and H_2O_2 (25 μM). Aliquots of the reaction mixture were removed every 30 s for a colorimetric analysis of GSH with Ellman's reagent (2 mM dithionitrobenzoic acid in 0.5% $NaHCO_3$) after the reaction was stopped with catalase (400 units). The absorbance was followed at 412 nm using a Shimadzu UV-240 spectrophotometer (*10*).

Measurement of the Extent of Superoxide Radical Formation. The extent of superoxide radical formation was measured by determining the rate of SOD sensitive reduction of the tetrazolium salt XTT. The assay mixture contained 2.5 mL of 0.1 M Tris-HCl/1.0 mM EDTA buffer (pH 7.4), flavonoid or phenol (25 μM), H_2O_2 (25 μM), GSH (400 μM), HRP (0.1 μM), and XTT (200 μM). The absorbance of the reduced form of XTT

* Corresponding author. Phone: (416) 978-2716. Fax: (416) 978-8511. E-mail: peter.obrien@utoronto.ca.

[1] Abbreviations: LDL, low-density lipoprotein; ROS, reactive oxygen species.

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that was produced (a water-soluble formazan) was monitored at 470 nm using a Shimadzu UV-240 spectrophotometer as described previously (*11*).

Assessment of GSH Conjugates. The reaction mixtures contained 5 mL of 0.1 M Tris-HCl/1.0 mM EDTA buffer (pH 7.4), GSH (400 μM), quercetin or luteolin (100 μM), HRP (0.1 μM), and H_2O_2 (100 μM). The extent of GSH conjugate formation was determined by monitoring significant differences in the UV absorbance of the reaction mixture versus the control (absence of HRP/H_2O_2) using a Shimadzu UV-240 spectrophotometer following the extraction of flavonoid or its oxidation products from the reaction mixture with ethyl acetate.

Results

The data in Figure 1A show that catalytic amounts of apigenin, a flavone with a phenolic B ring, or naringenin, a flavanone with a phenolic B ring (Table 1), promoted GSH oxidation by peroxidase and H_2O_2 with a very high efficiency. The rate of GSH oxidation was dependent on apigenin or naringenin concentrations, indicating that the rate of peroxidase/H_2O_2-catalyzed oxidation of apigenin or naringenin was rate-limiting. GSH was also rapidly oxidized by peroxidase and H_2O_2 in the presence of catalytic amounts of naringin, a flavanone glycoside of naringenin with a phenolic B ring (Table 1). Little GSH oxidation by peroxidase and H_2O_2 occurred in the absence of flavonoid which indicates that GSH at 0.4 mM is a poor peroxidase substrate at pH 7.4, although others have reported that GSH oxidation to thiyl radicals occurred at pH 8.0 with 10 mM GSH (*12*). GSH oxidation occurred at a pseudo-first-order rate with respect to peroxidase when fixed concentrations of the other reactants were used (Figure 1B) and pseudo-first-order with respect to GSH concentration (results not shown). As shown in Figure 1C, apigenin in the absence of GSH was rapidly degraded by peroxidase and H_2O_2 but little degradation occurred in the presence of GSH. GSH also prevented naringenin oxidation by peroxidase and H_2O_2 (results not shown).

As shown in Table 2, 25 μM apigenin, naringenin, or naringin readily catalyzed the oxidation of 400 μM GSH by 1 μM H_2O_2 and 0.1 μM HRP. With apigenin or naringenin, a total of 143 or 155 mol of GSH, respectively, was oxidized per mole of added H_2O_2. The order of effectiveness of these flavonoids with respect to phenol was as follows: naringin, naringenin, and apigenin > phenol. The GSH oxidation catalyzed by naringenin was inhibited by cytochrome *c*, a superoxide radical oxidant, but was markedly stimulated by superoxide dismutase (Table 2). This suggests that superoxide dismutase generates H_2O_2 by dismutation of the superoxide anion radicals generated by the GSH oxidation. Catechol or flavanones or flavonols containing catechol B rings, i.e., luteolin, catechin, rutin, quercetin, and fisetin, did not catalyze GSH oxidation, although some GSH depletion did occur. This is likely due to GSH conjugate formation as quercetin or luteolin products formed in the presence of GSH could not be extracted by ethyl acetate (results not shown).

Extensive oxygen uptake accompanied the stoichiometric oxidation of GSH to GSSG in the peroxidase/H_2O_2 reaction system with flavones or flavanones containing phenol B rings (Table 2). No oxygen uptake occurred in the absence of flavonoids, indicating that GSH is a poor peroxidase substrate. The order of effectiveness of these flavonoids was as follows: apigenin, naringenin, and

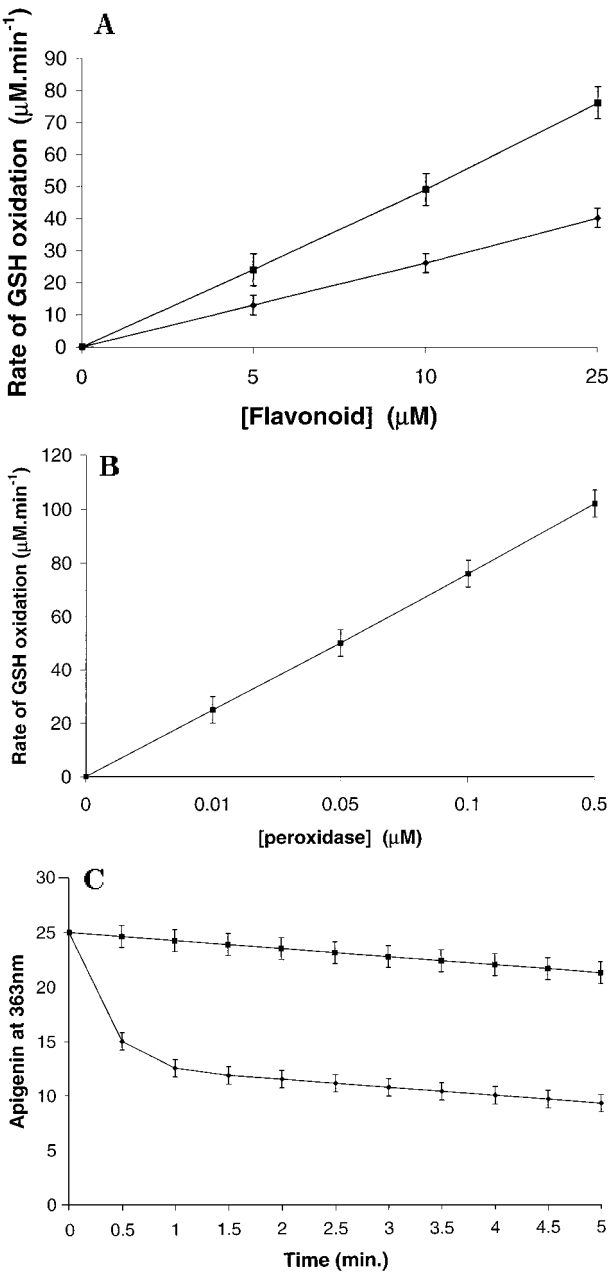
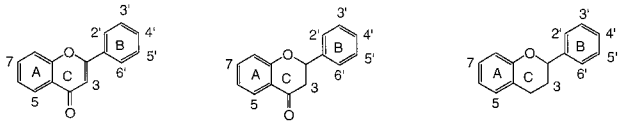


Figure 1. (A) Dependence of the rate of GSH oxidation on flavonoid concentration. The reaction vessel contained 2.5 mL of 0.1 M Tris-HCl/1.0 mM EDTA buffer (pH 7.4), GSH (400 μM), HRP (0.1 μM), H_2O_2 (25 μM), and varying concentrations of apigenin (■) and naringenin (◆) (5, 10, and 25 μM). A 25 μL aliquot was removed at various times, and the absorbance was followed at 412 nm using a Shimadzu UV-240 spectrophotometer after the addition of catalase (400 units) and dithionitrobenzoic acid (2 mM). Values represent the mean ± SE of three separate experiments. (B) Dependence of the rate of GSH oxidation on peroxidase concentration. The reaction vessel contained 2.5 mL of 0.1 M Tris-HCl/0.1 mM EDTA buffer (pH 7.4), GSH (400 μM), apigenin (25 μM), H_2O_2 (25 μM), and varying concentrations of HRP (0.01, 0.05, 0.1, and 0.5 μM). A 25 μL aliquot was removed at various times, and the absorbance was followed at 412 nm using a Shimadzu UV-240 spectrophotometer after the addition of catalase (400 units) and dithionitrobenzoic acid (2 mM). Values represent the mean ± SE of three separate experiments. (C) Protective effect of GSH on apigenin oxidative degradation. The reaction mixture contained 2.5 mL of 0.1 M Tris-HCl/1.0 mM EDTA buffer (pH 7.4), apigenin (25 μM), and HRP (0.1 μM), and the reaction was started with H_2O_2 (25 μM). The effect of 1 mM GSH (■) at preventing apigenin oxidation vs the absence of GSH (◆) was followed at 363 nm for 5 min using a Shimadzu UV-240 spectrophotometer. Values represent the mean ± SE of three separate experiments.

Table 1. Structural Formulas of the Flavonoids Used in This Study

	substituent				
	3	**5**	**7**	**3′**	**4′**
flavones					
apigenin	H	OH	OH	H	OH
luteolin	H	OH	OH	OH	OH
diosmin	H	OH	ORu[a]	OH	OMe
flavanones					
naringenin	H	OH	OH	H	OH
naringin	H	OH	ORh[b]	H	OH
hesperetin	H	OH	OH	OH	OMe
hesperidin	H	OH	ORu[a]	OH	OMe
flavanonol					
taxifolin	OH	OH	OH	OH	OH
flavan-3-ol					
catechin	OH	OH	OH	OH	OH
flavonols					
rutin	ORu[a]	OH	OH	OH	OH
quercetin	OH	OH	OH	OH	OH
galangin	OH	OH	OH	H	H
kaempferol	OH	OH	OH	H	OH
fisetin	OH	H	OH	OH	OH

[a] Ru is rutinoside [6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]. [b] Rh is rhamnoglucoside [2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl].



Schematic drawings of the different flavonoid molecules showing the A, B, C rings

naringin > phenol. No oxygen uptake occurred with catechol or flavanones or flavonols containing catechol B rings, i.e., luteolin, catechin, rutin, quercetin, and fisetin. Oxygen uptake was also stimulated by superoxide dismutase and inhibited by cytochrome *c*. A stoichiometry of approximately 0.4 mol of O_2 consumed per mole of oxidized GSH was realized. No oxygen uptake occurred in the absence of GSH under these conditions, although some oxygen uptake occurred with naringenin or apigenin at a much higher concentration (100 μM) in the absence of GSH (results not shown), indicating that some of the naringenin or apigenin breakdown products autoxidize.

The effectiveness of flavonoids at catalyzing superoxide radical formation during GSH oxidation was next investigated. As shown in Table 3, apigenin, naringenin, and naringin on metabolic oxidation by peroxidase/H_2O_2 in the presence of GSH caused XTT reduction which was inhibited by superoxide dismutase. No XTT reduction occurred in the presence of GSH alone or in the absence of HRP or H_2O_2 or GSH. Flavonoids containing catechol B rings were ineffective. Kaempferol, a flavonol, and hesperetin, a flavanone, were also ineffective, even though these flavonoids contain phenol B rings.

Discussion

These results demonstrate that some flavones or flavanones containing phenol B rings catalyze GSH oxidation in the peroxidase/H_2O_2 system and that extensive oxygen uptake accompanied the GSH oxidation. As only catalytic amounts of these flavonoids and H_2O_2 were required to catalyze the GSH oxidation, the flavonoid was oxidized by peroxidase/H_2O_2 to a product which oxidized GSH and underwent redox cycling.

Table 2. Peroxidase-Catalyzed Oxygen Activation by Flavonoids and Glutathione[a]

flavonoid	one-electron redox potential $E°$ (mV) at pH 7 ($PhO^{\bullet}/PhO^{-}$)	GSSG formed (μM GSH equiv)	total oxygen consumed (μM)[b]
none		4 ± 2	2 ± 1
phenol	860[d]	96 ± 21	20 ± 3
catechol		4 ± 1	2 ± 1
flavones			
apigenin	>1000[e]	143 ± 15	52 ± 3
luteolin	180,[e] 180,[f] 299[h]	4 ± 2	2 ± 1
diosmin		6 ± 3	3 ± 1
flavanones			
naringenin	600[e]	155 ± 16	51 ± 3
naringenin/SOD[c]		245 ± 22	98 ± 4
naringenin/cyt *c*[c]		89 ± 9	23 ± 2
naringin		173 ± 15	31 ± 3
hesperetin	440[e]	5 ± 2	3 ± 2
hesperidin	440,[e] 720[g]	6 ± 3	3 ± 1
flavanonol			
taxifolin	83[h]	4 ± 2	3 ± 2
flavan-3-ol			
catechin	160,[e] 130,[f] 570[g]	3 ± 1	2 ± 1
flavonols			
rutin	180,[e] 600,[g] 275[h]	3 ± 2	3 ± 1
quercetin	30,[e] 0.06,[f] 398[h]	5 ± 2	3 ± 2
galangin	320,[e] 340[f]	4 ± 2	2 ± 1
kaempferol	120,[e] 170,[f] 209[h]	4 ± 1	2 ± 1
fisetin	120,[e] 140,[f] 214[h]	5 ± 1	3 ± 1

[a] The reaction mixture contained in 2 mL of 0.1 M Tris-HCl/1.0 mM EDTA buffer (pH 7.4), substrate (25 μM), HRP (0.1 μM), H_2O_2 (1 μM), and GSH (400 μM) for 30 min. Reactions were started by the addition of HRP. Means ± SEM for three separate experiments are given. [b] The extent of oxygen consumption was determined as indicated in Materials and Methods. [c] SOD (1 μM) and cyt *c* (20 μM) were added where indicated. [d] E_7 (mV) (*32*). [e] $E_{p/2}$ (mV) (*33*). [f] $E_{1/2}$ (V vs SCE) (*34*). [g] E_7 (V vs NHE) (*35*). [h] $E_1°$ (mV) (*36*).

Table 3. Flavonoid-Catalyzed Superoxide Radical Formation by the H_2O_2/HRP/GSH System[a]

	rate of XTT reduction (nmol min^{-1} mL^{-1})		total XTT reduction (nmol/mL)	
	−SOD	+SOD	−SOD	+SOD
none	<1	<1	<1	<1
phenol	16 ± 2	<1	55 ± 4	<1
flavone				
apigenin	45 ± 4	4 ± 1	143 ± 11	31 ± 3
flavanones				
naringenin	27 ± 2	<1	74 ± 6	<1
naringin	3 ± 1	<1	21 ± 2	<1
hesperetin	<1	<1	<1	<1
hesperidin	<1	<1	<1	<1
flavanonol				
taxifolin	<1	<1	<1	<1
flavan-3-ol				
catechin	<1	<1	<1	<1
flavonol				
rutin	<1	<1	<1	<1
kaempferol	<1	<1	<1	<1

[a] The complete system (2.5 mL) contained 400 μM GSH, 0.1 μM HRP, 25 μM H_2O_2, 200 μM XTT, and 25 μM flavonoid or phenol in 0.1 M Tris-HCl/1.0 mM EDTA buffer (pH 7.4). SOD (1 μM) was added where indicated. The extent of XTT reduction was determined by following the increase in absorbance at 470 nm. Means ± SEM for three separate experiments are given.

Phenol also catalyzed GSH oxidation in the peroxidase/H_2O_2 system, and previously, this was attributed to redox cycling by phenoxyl radicals (*8*) in which GSH was oxidized by phenoxyl radicals to form thiyl radicals which generated reactive oxygen species (*13*). Phenol is a major benzene metabolite, and a major mechanism proposed

for benzene-induced myelotoxicity involves the oxidation of phenol by bone marrow myeloperoxidase in forming phenoxyl radicals (*14*). Incubation of bone marrow cells or HL-60 human promyelocytic leukemic cells with phenolic metabolites of benzene resulted in GSH oxidation, oxygen activation, H_2O_2 formation, oxidative DNA damage, and cytotoxicity (*15*−*17*). Phenoxyl radicals have also been shown to cause oxidative DNA damage directly or via GSH-mediated oxygen activation (*9*).

The mechanism proposed for the apigenin- or naringenin-mediated GSH oxidation is as follows. The phenoxyl radical formed by the peroxidase-catalyzed oxidation of the phenol B ring of these flavonoids oxidized GSH to a thiyl radical (reaction 1) which reacted with GSH to form a disulfide radical anion (reaction 2). The latter radical anion rapidly reduced O_2 to form GSSG and the superoxide radical anion (reaction 3). In confirmation of this mechanism, the addition of superoxide dismutase markedly increased the amount of GSH that was oxidized by apigenin presumably as a result of the formation of H_2O_2 from superoxide generated by GSH oxidation.

$$PhO^{\bullet} + GSH \rightarrow GS^{\bullet} + PhOH \quad (1)$$

$$GS^{\bullet} + GS^{-} \rightleftharpoons GSSG^{\bullet -} \quad (2)$$

$$GSSG^{\bullet -} + O_2 \rightleftharpoons GSSG + O_2^{\bullet -} \quad (3)$$

The disulfide radical, acetaminophen phenoxyl radicals, and thiyl radicals have been detected and characterized by fast flow ESR spectroscopy in a similar peroxidase system using acetaminophen as the phenol (*18*). Protein thiol oxidation by acetaminophen phenoxyl radicals has been implicated as a cause of liver necrosis induced by high doses of acetaminophen (*19*).

Only flavones and flavanones containing phenol B rings co-oxidize GSH, with resulting oxygen activation when oxidized by peroxidase. Flavonoids containing a catechol or benzene B ring did not cooxidize GSH when oxidized by peroxidase. This is surprising as most of the flavonoids in Table 1 contain a resorcinol A ring and resorcinol when oxidized by peroxidase co-oxidizes GSH with resulting oxygen activation (*8*). Furthermore, the inactivation of thyroid peroxidase and lactoperoxidase by flavonoids has been attributed to covalent binding of A ring resorcinol radicals to the amino acid radical(s) of peroxidase compound II (*20*).

This GSH prooxidant activity of flavones and flavanones seemed to partly correlate with the one-electron redox potential of their phenoxyl radicals (Table 1) as only apigenin and naringenin had redox potentials that were higher than the redox potential ($E° = 850$ mV) for the couple $GS^-/GS^{\bullet}$ (*21*). Hesperetin and kaempferol were not active presumably because the one-electron redox potential of their phenoxyl radicals was not high enough. Flavonoids containing catechol B rings also have lower one-electron redox potentials and depleted some GSH without oxygen uptake or GSSG formation, suggesting that GSH formed GSH conjugates with a B ring *o*-quinone product.

Although the flavonoid substrate specificity of HRP toward flavonoids cannot be directly transferred to myeloperoxidase as the entrance and the cleft leading to the active site is tighter and more highly constrained than HRP (*22*), we have previously demonstrated a similar GSH-dependent oxygen activation (and glutathione radical formation) with myeloperoxidase and phenolic compounds or methimazole (*23*). Others have also demonstrated a GSH-dependent oxygen activation (and glutathione radical formation) with phenolic compounds and lactoperoxidase or thyroid peroxidase, other mammalian peroxidases (*24*).

Phenoxyl radicals have also been implicated in the initiation stage of atherosclerosis as myeloperoxidase readily catalyzes the oxidation of tyrosine, a plasma phenol (*25*), to a tyrosyl radical which can co-oxidize the lipids and proteins of low-density lipoproteins (*26*, *27*). Myeloperoxidase was also found in human atherosclerotic tissue (*26*), and the level of protein-bound *o,ó*-dityrosine (a major tyrosyl radical product) was also markedly increased in LDL isolated from human atherosclerotic lesions (*28*). Interestingly, this peroxidase-catalyzed LDL oxidation mechanism, unlike previously reported LDL oxidation systems, does not require free transition metal ions (copper or iron). In the plasma, these transition metal ions are present almost exclusively in tightly bound proteins (e.g., ceruloplasmin and transferrin) which do not catalyze the oxidative modification of LDL.

The three most prooxidant flavonoids identified were naringenin, naringin, and apigenin. These particular flavonoids actually induce lipid peroxidation under the same conditions under which other flavonoids prevented lipid peroxidation (*29*). Presumably, the prooxidant phenoxyl radicals of naringenin, naringin, and apigenin catalyze lipid peroxidation as do other prooxidant phenoxyl radicals (*8*). Naringin constitutes up to 10% of the dry weight of grapefruit concentrations. Naringin concentrations in grapefruit juice are reported to range from 100 to 800 mg/L, and recently, there are concerns that naringenin, the major naringin metabolite, may contribute to intestinal cytochrome P450 3A4 inhibition that results in the reported clinical increase in the bioavailability of dihydropyridine calcium channel blockers when taken with grapefruit juice (*30*). Apigenin concentrations in celery are reported to be 108 mg/kg of fresh weight (*1*). Apigenin is also being considered as a skin cancer preventive agent in sunscreens (*31*). Further research is required to determine the consequences, if any, for activated oxygen species formation by naringenin, naringin, and apigenin in peroxidase-containing tissues such as bone marrow or thyroid or for plasma LDL oxidation.

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