JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of the Citrus Flavanone Naringenin on Oxidative Stress in Rats

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Flavonoids are non-nutrient plant phenolic compounds proposed to provide health benefits in humans. The antioxidant and prooxidant effects of the citrus flavanone naringenin have been tested only in vitro. The dose−response effect of naringenin consumption was tested in weanling rats ($n = 6-8$/group) with a 2×4 factorial design using high or low oxidative stress (Hox or Lox, respectively) diets, created by adequate or deficient amounts of vitamin E and selenium, with three increasing naringenin concentrations (30, 60, or 120 mg/kg of diet). Hox compared to Lox rats exhibited reduced growth and liver hypertrophy, which was not prevented by naringenin consumption. Also, Hox rats exhibited severalfold higher liver NAD(P)H:quinone oxidoreductase-1 activity, which was further elevated in proportion to naringenin intake, but this was not sufficient to protect against oxidative stress indicated by higher liver total aldehydes. In addition, dietary naringenin did not affect antioxidant nutrient status or physiological markers of growth under Lox conditions. Thus, dietary naringenin did not exhibit antioxidant or prooxidant effects in vivo in this rat model.

KEYWORDS: Naringenin; flavonoid; vitamin E deficiency; oxidative stress; antioxidant; in vivo; rat; liver

INTRODUCTION

Reactive oxygen species (ROS) are generated by normal cellular metabolism and by exogenous agents. Even though ROS may serve as cell signaling or bactericidal agents, excess ROS can cause damage to cellular macromolecules such as polyunsaturated lipid, protein, and DNA, leading to a process called oxidative stress (*1*). Oxidative stress is implicated in the development of many diseases such as macular degeneration, cancer, cardiac disease, and premature aging; however, its exact role in a given disease is often unclear (*2*).

Diets rich in fruits, vegetables, and herbs have been negatively correlated with the risk for a variety of diseases, particularly cardiovascular disease and some types of cancer (*3*). These positive health effects are largely attributed to their high dietary fiber, vitamins, and non-nutrient flavonoid content. Flavonoids, ubiquitously found in the plant kingdom, are proposed to elicit their beneficial effects in vivo in part through their ability to scavenge oxygen free radicals, quench transition metals, and/or boost the endogenous antioxidant defense system (*4*). Although the antioxidant properties of flavonoids support a positive role in human nutrition and disease prevention, some reports have described their prooxidant activity in vitro. Because extracts of flavonoid-rich plants are sold as supplements to different population groups with various disease conditions, any relevant information about the toxic effects of flavonoids through the enhancment of oxidative damage is of great concern (*5*). Most of the studies have identified flavonoids with multiple hydroxylations in the B-ring to have stronger prooxidant activities. However, these activities are mostly present in conditions of high concentration of transition metals such as iron and copper (*6*). Higher iron and copper concentrations under physiological conditions are not likely due to their selective chelation by specialized storage proteins (*2*). Therefore, the role of flavonoids as prooxidant molecules in vivo is uncertain.

Consumption of citrus plants has been proposed to provide multiple beneficial properties such as anticarcinogenic, antiviral, and antiinflammatory, among others, which are elicited, in part, through their high content of flavonoid antioxidants (*7*). The flavanone naringenin and its glucosides are widespread in nature and can reach significant concentrations in commonly consumed citrus juices (e.g., in grapefruit from 100 to 500 mg/L) (*8*). Multiple in vitro studies using different cell models have identified naringenin (**Figure 1**), the most predominant flavanone in grapefruit (*Citrus paradisi*), as an effective antioxidant (*9*). In vivo, narigenin, at 50 mg/kg of body weight, was found to partially protect against oxytetracycline-induced oxidative stress in rat liver (*10*). Both partial protection against increased lipid peroxidation and the decline of antioxidant enzymes were observed, suggesting multiple antioxidant mechanisms. However, prooxidant effects of narigenin have been reported as well (*11*). This discrepancy could arise from the limited information on the role of naringenin in vivo, where both its antioxidant

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10.1021/jf061714h CCC: $37.00
Published on Web 02/22/2007

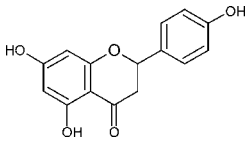

Figure 1. Naringenin structure.

and prooxidant activities may be modified due to extensive metabolism from conjugative enzymes (*12*, *13*).

In the present study, we have examined the prooxidant and antioxidant activities of the grapefruit flavanone, naringenin, in vivo. We tested the hypothesis that increased concentrations of naringenin will not affect antioxidant nutrient status under normal conditions and, instead, that such increased doses can protect rats under oxidative stress by enhancing the endogenous antioxidant defense system.

MATERIALS AND METHODS

All of the chemicals used in the experiments, unless otherwise specified, were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions were made within the week of the experiments and stored in the dark at either 4 or −20 °C.

Animals and Experimental Design. Fifty (40−50 g) male Long−Evans hooded rats (Harlan, Indianapolis, IN) were received after weaning and placed on one of eight experimental diets for 6 weeks. The basal diet was made with torula-yeast to control Se, and the oils were tocopherol-stripped to control vitamin E. A low oxidative stress diet (Lox) was supplemented with α-tocopheryl acetate (30 mg/kg; ICN Biomedicals, Cleveland, OH) and sodium selenite (0.5 mg/kg), and the high oxidative stress diet (Hox) was deficient in both antioxidant nutrients. The animals and dietary composition were as previously described (*14*, *15*). To ensure proper mixture, pure naringenin was added to 500 g of the sugar and used at increasing concentrations: 0, 30, 60, and 120 mg/kg of diet. Then, individual mixtures were added to the remaining dry ingredients according to the diet composition. The present concentrations were selected because they correspond to amounts that an individual would consume from drinking grapefruit juice daily (1−2 cups) (*16*). The animals were housed individually in stainless steel cages and maintained at 23 ± 1 °C with 12 h light−dark cycles. Rats had free access to food and deionized water at all times and were fed the diets for 6 weeks. For food intake compliance, food consumption was measured biweekly using a small electronic scale. Body weight was measured weekly throughout the study. At the end of the experimentation, the animals were fasted for 12 h before euthanasia (via exsanguination after anesthesia). Livers and other tissues were perfused with physiological saline, removed, weighed, frozen in liquid nitrogen, and stored at −80 °C until further analysis. The Purdue University Animal Care and Use Committee approved all of the experimental procedures.

Tissue Preparation. Representative liver samples were thawed on ice, weighed, and homogenized (20%, w/v) in ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM potassium chloride and 1 mM sodium EDTA for the analysis of fat-soluble antioxidants, quinone reductase (NQO1) activity, and total aldehydes (type A homogenates) or in ice-cold 15% perchloric acid containing 1 mM EDTA for water-soluble antioxidant determination (type B homogenates). The two types of liver homogenates were made on ice using a Potter-Elvehjem apparatus attached to a rotatory device. After six strokes at medium-high speed, type A liver homogenates were centrifuged at 1000*g* for 15 min at 4 °C. Upon protein precipitation type B homogenates were centrifuged at 1000*g* for 15 min at 4 °C. Supernatants were immediately transferred, vortexed, and aliquoted into amber vials, snap-frozen in liquid nitrogen, and kept frozen at −80 °C until further analysis. Clear supernatants were immediately transferred, vortexed, and aliquoted into small amber vials, snap-frozen in liquid nitrogen, and stored at −80 °C until further analysis.

Determination of Fat-Soluble Antioxidants. Extraction of tocopherols and ubiquinones from type A homogenates was done as described by McCrehan (*17*) with minor changes. Briefly, a ratio of 2:1 ethanol to tissue homogenates was used and spiked with 10 μL of 100 μM δ-tocopherol as internal standard. Fat-soluble antioxidants were extracted using hexane. The upper hexane layer was evaporated under nitrogen. After resuspension in ethanol, samples were filtered using 0.22 μm nylon syringe filters (Osmonics Inc., Minnetonka, MN) directly into HPLC vials.

Simultaneous separation and quantification of α-tocopherol, γ-tocopherol, δ-tocopherol, CoQ_9 reduced and oxidized, and CoQ_{10} reduced and oxidized were carried out by reverse phase HPLC with electrochemical detection using the CoulArray (model 5600A) with a binary pump system (ESA, Inc., Chelmsford, MA). The MD-150 column (C18, 150 × 3.2 mm, 3 μm particle size; ESA, Inc.) was equilibrated in mobile phase A, methanol/0.2 mM acetate buffer, pH 4.0 (90:10 v/v). Fat-soluble antioxidants were gradient-eluted with mobile phase B, methanol/*n*-propanol/1.0 mM acetate buffer pH 4.0 (78:20:2 v/v). Monitoring of antioxidants was carried out with an electrochemical detector as described in CoulArray Applications (*18*). Briefly, five different in-line potentials were used to selectively oxidize α-, γ-, and δ-tocopherol and reduced CoQ_9 and CoQ_{10}. Oxidized CoQ_9 and CoQ_{10} were detected by applying a negative in-line potential of −1000 mV at channel 6 and reoxidized at channels 7 and 8, 300 and 700 mV, respectively. Eluted compounds were quantified using HPLC grade external standards. Results were expressed per milligram of protein.

Determination of Water-Soluble Antioxidants. Simultaneous determination of ascorbate, glutathione (GSH), and uric acid from type B homogenates was adapted from the methods of Stempal et al. (*19*) and Kristal et al. (*20*). Briefly, type B homogenates were diluted with double-deionized water and filtered directly into HPLC vials with 0.45 μm RC-membrane filters (Ministart RC 4; Sartorius AG, Göttingen, Germany).

Separation and quantification of liver water-soluble antioxidants were carried out by reverse phase HPLC with electrochemical detection using the CoulArray (model 5600A) with a binary pump system (ESA, Inc.). The SupelcosilTM LC-18-T-150 column (150 × 4.6 mm, 3 μm particle size; Supelco-Sigma, St. Louis, MO) was equilibrated in mobile phase A, 50 mM sodium phosphate buffer, pH 3.0. Water-soluble antioxidants were gradient-eluted with mobile phase B, acetonitrile/50 mM sodium phosphate buffer, pH 3.0/methanol (50:30:20 v/v). Detection was carried out using the eight-channel CoulArray electrochemical system with electrode potentials set at 100, 200, 300, 400, 500, 600, 700, and 800 mV. Eluted compounds were quantified using HPLC grade external standards. Results were expressed in grams of fresh liver.

Total Aldehyde Analysis. Analyses of liver total aldehydes, malondialdehyde (MDA), and 4-hydroxynonenal (4-HAE), as markers of oxidative stress, were carried out using type A liver homogenates and a kit from OxisResearch (*21*), following the manufacturer's instructions, and the results were expressed per milligram of protein.

Quinone Reductase Activity. Determination of NAD(P)H:quinone oxido-reductase (NQO1, DT-diaphorase, EC 1.6.99.2) activity as a marker of oxidative stress and adaptation was measured using type A liver homogenates and a plate reader (PowerWaveX, BIO-TEK Instruments, Inc., Winooski, VT) as previously described by Prochaska and Santamaria (*22*).

Protein Concentration. Protein was determined by the bicinchoninic acid method of Smith et al. (*23*) using type A liver homogenates and reagents supplied by Sigma (Sigma Chemical Co., St. Louis, MO).

Statistical Analysis. Data are shown as the means ± standard errors (SEM) of six to eight measurements/group. Either two-way ANOVA or one-way ANOVA was used to analyze two- or one-factor effects at $P < 0.05$ using the SAS software (SAS Institute, Cary, NC). Variances were isolated and analyzed using the F test ($P < 0.05$) for the main effects of oxidative stress, Lox versus Hox, dose effects of selected naringenin concentrations, and main effects interactions. Data were subjected to logarithmic transformation when necessary to achieve homogeneity of variances. Proper analysis of statistical assumptions such as homogeneity of variances and uniformity of residuals, for one-way ANOVA and two-way ANOVA, were also performed. Post-hoc analysis of each mean treatment was compared to the controls using Tukey or LSD, and differences were considered to be significant when $P < 0.05$. The repeated-measures ANOVA approach was used to determine any difference in the food consumption among groups.

Table 1. Effects of Naringenin Supplementation and Oxidative Conditions on Rat Weight, Living Performance, and Liver Antioxidants at the End of the Study

		naringenin[a] (mg/kg of diet)			
	diet	0	30	60	120
		Changes in Body and Tissue Weights, Growth, and Food Intake[b]			
total weight (g)	Lox	246 ± 10	227 ± 6	244 ± 12	235 ± 9
	Hox[c]	236 ± 5	235 ± 7	227 ± 9	211 ± 6
weight gain (g)	Lox	27.6 ± 1.3	26.3 ± 0.8	29.8 ± 2.1	29.7 ± 1.2
	Hox[c]	23.5 ± 2.1	24.6 ± 0.8	24.1± 2.5	23.7 ± 1.0
food intake (g/day/rat)	Lox	13.9 ± 0.8	12.9 ± 0.7	14.5 ± 0.8	13.6 ± 0.7
	Hox	13.8 ± 0.8	14.0 ± 0.7	13.3 ± 0.5	13.2 ± 0.6
liver index (g/100 g of BW[d])	Lox	3.00 ± 0.10	2.78 ± 0.12	2.88 ± 0.05	2.91 ± 0.09
	Hox[c]	3.33 ± 0.06	3.47 ± 0.19	3.38 ± 0.09	3.42 ± 0.12
parametrial fat (g/100 g of BW)	Lox	1.14 ± 0.10a	0.93 ± 0.03 ab	0.93 ± 0.07 ab	0.70 ± 0.04 b
	Hox[c]	0.77 ± 0.08	0.71 ± 0.07	0.76 ± 0.07	0.79 ± 0.09
		Fat-Soluble Antioxidants			
α-tocopherol (pmol/mg)	Lox	307 ± 13	289 ± 12	277 ± 16	310 ± 30
	Hox[c]	19.9 ± 1.0	25.3 ± 2.8	20.2 ± 1.4	18.2 ± 1.2
γ-tocopherol (pmol/mg)	Lox	1.05 ± 0.09	1.09 ± 0.07	1.18 ± 0.11	1.03 ± 0.11
	Hox[c]	1.68 ± 0.08	1.93 ± 0.16	2.08 ± 0.27	1.85 ± 0.21
total CoQ_9 (nmol/mg)	Lox	2.57 ± 0.09	2.69 ± 0.16	2.39 ± 0.19	2.65 ± 0.26
	Hox[c]	1.36 ± 0.07	1.66 ± 0.11	1.33 ± 0.07	1.42 ± 0.04
total CoQ_{10} (pmol/mg)	Lox	86.7 ± 3.8	102 ± 9.4	109 ± 13.4	121 ± 18
	Hox[c]	76.1 ± 7.4	91.4 ± 8.3	76.9 ± 8.9	87.0 ± 9.0
		Water-Soluble Antioxidants			
ascorbate (μmol/g)	Lox	2.33 ± 0.11	2.37 ± 0.09	2.70 ± 0.14	2.59 ± 0.08
	Hox[c]	2.40 ± 0.10	2.19 ± 0.04	2.22 ± 0.06	2.07 ± 0.10
GSH (μmol/g)	Lox	4.10 ± 0.14	3.74 ± 0.18	3.94 ± 0.43	3.55 ± 0.18
	Hox[c]	3.55 ± 0.14	3.44 ± 0.20	3.35 ± 0.24	3.50 ± 0.30
uric acid (μmol/g)	Lox	0.28 ± 0.02	0.30 ± 0.02	0.28 ± 0.02	0.25 ± 0.02
	Hox	0.31 ± 0.03	0.21 ± 0.02	0.22 ± 0.02	0.23 ± 0.01

[a] Values are presented as means ± SEM, $n = 6–8$ for each treatment group. [b] Data reprinted with permission from ref *50*. Copyright 2006 American Chemical Society. [c] Represents statistical differences between oxidative conditions after two-way ANOVA, $P < 0.05$. Letters represent statistical differences among the Lox treatment groups after one-way ANOVA and post-hoc analysis (Tukey; $P < 0.05$). [d] BW, body weight.

RESULTS

Effect of Naringenin Supplementation on Growth, Tissue Weight, and Food Intake. At the end of the 6 weeks of dietary treatment, consumption of the Hox diet led to significantly reduced growth, enlarged liver to body weight ratio, and a marked increase in physical symptoms of oxidative stress compared to consumption of the Lox diet that was sufficient in vitamin E and selenium. Rats consuming the deficient diet presented significantly lower total body weights and reduced weight gains compared to rats fed the Lox diet ($P < 0.05$; **Table 1**). Moreover, Hox animals showed 10% higher liver weights expressed as a percentage of body weight compared to Lox animals, 3.33 versus 3.0%, respectively ($P < 0.05$; **Table 1**). Naringenin supplementation did not protect rats from anthropometric changes resulting from nutrient antioxidant deficiency ($P > 0.05$). Lox animals supplemented with increasing flavanone amounts did not show differences in weight gain or the liver to body weight ratio, but did have less parametrial fat ($P < 0.05$; **Table 1**). Although naringenin is a bitter component that provides the characteristic flavor to grapefruit juice, the concentrations used in this study did not influence food consumption, which during the 6 weeks of the study and for all of the groups averaged 13.6 g/day/rat (**Table 1**).

Effect of Naringenin Supplementation on Fat-Soluble Antioxidants. The concentrations of liver fat-soluble antioxidants are presented in **Table 1**. As expected, liver α-tocopherol concentrations in Hox animals were <10% of the concentration measured in Lox animals ($P < 0.01$), and naringenin supplementation had no effect. In contrast, liver γ-tocopherol concentrations were about 50% higher in Hox rats compared to Lox rats, with no naringenin effect observed. Total coenzyme Q concentrations for CoQ_9 and CoQ_{10} were calculated by adding reduced and oxidized concentrations of liver homogenates. The liver total CoQ_9, but not CoQ_{10}, concentrations were reduced in Hox compared to Lox animals, and naringenin supplementation did not prevent the CoQ_9 reduction. The molar ratio of CoQ_{10} to CoQ_9 was determined on the basis of the total liver homogenate concentrations, and this result is presented in **Figure 2**. Hox rats had higher total CoQ_{10}/CoQ_9 ratios compared to Lox rats, which was not influenced by naringenin intake. However, in Lox rats naringenin intake at 60 and 120 mg/kg showed significantly higher CoQ_{10}/CoQ_9 ratios compared to control ($P < 0.05$).

Effect of Naringenin Supplementation on Water-Soluble Antioxidants. We have previously shown that rats maintained on these torula-yeast-based diets without added selenium become deficient in this nutrient after 3 weeks of basal diet consumption as assessed by measuring selenium-dependent GSH peroxidase (GPx) activity of whole blood and tissue cytosol (*14*). On the basis of GPx activity, selenium status was previously shown to be unaffected by vitamin E status and maintained at 5% of selenium-sufficient levels through 6 weeks of basal diet consumption (*14*, *15*). Naringenin consumption was not expected to affect selenium status, but water-soluble antioxidants may have been affected, so ascorbic acid, GSH, and uric acid concentrations were simultaneously determined using HPLC-EC in type B liver homogenates. The results are presented in **Table 1**. Liver concentrations of ascorbate and GSH were at least 10% lower in Hox rats compared to Lox rats ($P < 0.05$), and supplemental naringenin showed no effect under either condition.

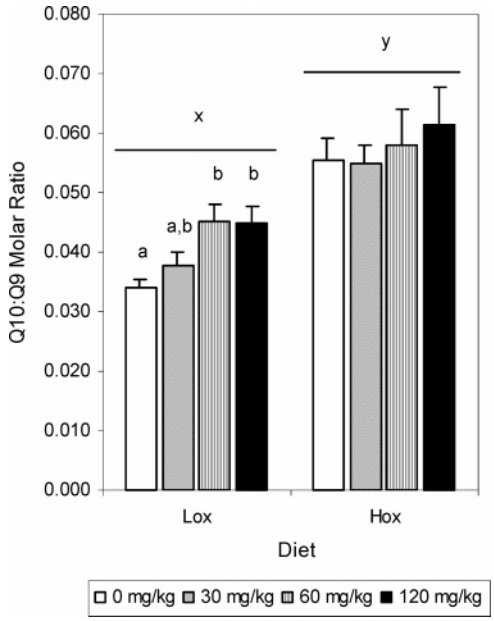

Figure 2. Effect of oxidative stress and naringenin supplementation on CoQ_{10}/CoQ_9 molar ratio in rat liver homogenates. Data are means ± SEM (error bars), $n = 6–8$ for each treatment group. x and y represent statistical differences between oxidative conditions after two-way ANOVA, $P < 0.05$. a and b represent statistical differences among Lox experimental groups after one-way ANOVA and post-hoc analysis (Tukey; $P < 0.05$).

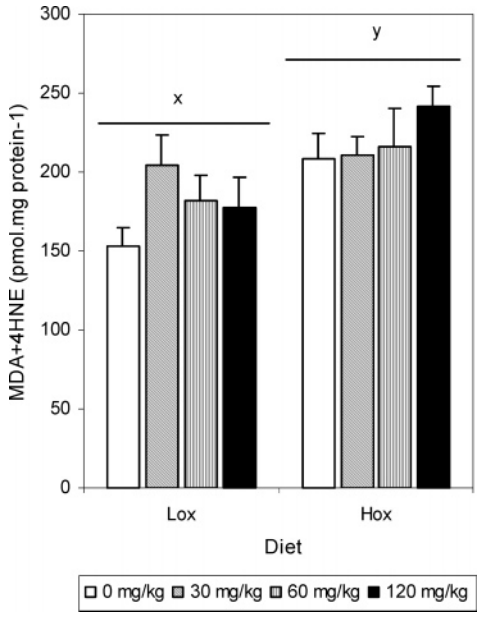

Figure 3. Effect of oxidative stress and naringenin supplementation on total aldehydes (MDA + 4-HAE) in rat liver homogenates. Data are means ± SEM (error bars), $n = 6–8$ for each treatment group. x and y represent statistical differences between oxidative conditions after two-way ANOVA, $P < 0.05$. Reprinted with permission from ref *50*. Copyright 2006 American Chemical Society.

Effect of Naringenin Supplementation on Lipid Oxidation Markers. To characterize the degree of oxidative stress present in animals under Lox and Hox conditions, we determined total aldehyde concentrations in liver homogenates. Aldehydes, such as MDA and 4-HAE, are byproducts of lipid peroxidation. The results showed that consumption of the Hox diet led to significantly greater concentrations of total aldehydes (4-HAE and MDA) in liver homogenates than consumption of the Lox diet (**Figure 3**). Moreover, naringenin intake did not affect the accumulation of lipid peroxidation byproducts in rat livers in either treatment group.

Effect of Naringenin Supplementation on Quinone Reductase Activity. NQO1 activity was used as a marker of adaptation to oxidative stress and measured in liver homogenates. Consumption of Hox diets led to higher liver NQO1 activities ($P < 0.01$; **Figure 4**), and naringenin-fed rats did not show differences in liver NQO1 activity under Lox conditions. In contrast, Hox rats fed increasing levels of naringenin showed significantly higher hepatic NQO1 activities (**Figure 4**).

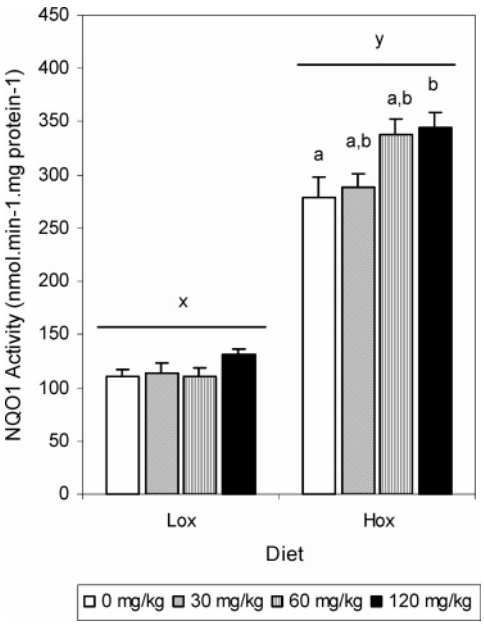

Figure 4. Effect of oxidative stress and naringenin supplementation on NQO1 activity in rat liver homogenates. Data are means ± SEM (error bars), $n = 6–8$ for each treatment group. x and y represent statistical differences between oxidative conditions after two-way ANOVA, $P < 0.05$. a and b represent statistical differences among Hox experimental groups after one-way ANOVA and post-hoc analysis (Tukey; $P < 0.05$). Reprinted with permission from ref *50*. Copyright 2006 American Chemical Society.

DISCUSSION

Consumption of fruits and vegetables, rich in phytochemicals, is inversely associated with the risk for a variety of chronic diseases in which oxidative stress is strongly implicated (*3*). Studies to understand how mammals can use phytochemicals to prevent, delay, manage, adapt to, or even promote ROS formation are, therefore, of interest. A 2 × 4 factorial design was used to determine the effect of consuming increasing amounts of naringenin on growing rats under low oxidative stress (Lox) and high oxidative stress (Hox) conditions created by modifying the amounts of vitamin E and Se in their diets. Liver vitamin E and Se status are significantly reduced as early as 3 weeks into diet intervention, and symptoms of deficiency can be reversed by adding both nutrients back to the diet (*15*, *24*). Further development of the deficiency drastically attenuates normal growth and finally leads to death. This double micronutrient deficiency model has been proven effective to provoke accelerated oxidative stress in growing rats, whereas single antioxidant nutrient deficiencies result in less-pronounced and often insignificant effects (*14*, *15*, *25*, *26*). Oxidative stress symptoms are manifested by changes in body weight and growth, biochemical markers of tissue damage, and biochemical markers of adaptation of the endogenous antioxidant defense system.

Rat growth deceleration and liver to body weight ratio gain are assessments of the physiological effect of oxidative stress caused by vitamin E and Se deficiency (*24*). Consumption of the Hox, but not the Lox, diet led to reduced growth rate and liver hypertrophy, a visible maker of liver damage. Although enzyme markers of liver damage such as alanine and aspartate transaminases were not measured, they are known to be increased under oxidative stress created from Se deficiency (*27*). Rat growth deceleration and liver to body weight ratio gain were not prevented by naringenin consumption at any dose, whereas we had previously shown that consumption of *tert*-butyl hydroquinone at 200 mg/kg of diet did prevent these deficiency signs (*28*). Additionally, food consumption was not associated with the changes in body weight and growth. Although naringenin is known to provide the bitter taste to grapefruit juice, this organoleptic property did not affect overall food consumption in any of the treatments.

The extent of oxidative stress can be assessed in vivo directly, by measuring biochemical markers of free radical damage to macromolecules such as lipids, proteins, and carbohydrates, and indirectly, by evaluating the tissue concentrations of endogenous antioxidants such as vitamin E, ascorbic acid, GSH, and coenzyme Q (*2*). Accumulations of the aldehydes malondialdehyde and 4-hydroxynonenal and of F_2-isoprostanes are qualified markers of oxidative damage to lipid molecules (*26*, *29*). The administration of the Hox diet led to increased levels of oxidative stress reflected by higher amounts of total aldehydes in the liver, which was not prevented by naringenin consumption. Our results agree with those from studies performed by Willcox and colleagues (*30*) using a similar vitamin E deficiency model to test the antioxidant effects of polyphenols in vivo. The authors did not observe any protective effect of different flavonoids against F_2-isoprostane accumulation, a sensitive marker of lipid peroxidation in rat hearts and plasma, even at the high doses used. It is possible that we failed to observe antioxidant protection, in part, due to low bioavailability of the citrus flavanone. It is known that chronic naringenin intake leads to steady flavanone metabolite concentrations in the blood and to accelerated degradation by the adapted gut microflora (*13*). Moreover, flavonoid metabolism and ultimate effect will rely on the organism's overall condition. The absorption and disposition of flavonoids are expected to be altered during pathological conditions, when oxidative stress could be an underlying cause (*31*); nonetheless, whether the absorption and metabolism of flavonoids are enhanced or reduced during different conditions of oxidative stress is still largely unknown.

Animals consuming the Hox diet had lower concentrations of hepatic GSH and ascorbate, and naringenin supplementation did not affect these important antioxidants. The concentrations of cytosolic antioxidants such as GSH and ascorbate could be compromised under oxidative stress conditions not only due to their function scavenging ROS but also due to their role in recycling plasma membrane antioxidants such as vitamin E and CoQ (*32*). Furthermore, naringenin intake neither affected the amounts of liver tocopherols nor prevented the reduction of liver total CoQ_9 in Hox animals. The higher concentrations of γ-tocopherol observed in Hox compared to Lox livers were most likely due to the absence of α-tocopherol, which has previously been shown to affect liver γ-tocopherol concentrations (*33*). In mammals, dietary vitamin E and endogenous CoQ play an important role in protecting biological membranes from ROS attack (*34*, *35*). CoQ in its reduced state has been associated with antioxidant protection (*36*), working synergistically with vitamin E at the plasma membrane by regenerating the active form from the tocopheroxyl radical (*37*). As reported by van Acker and colleagues (*38*), it is possible that flavonoids can be incorporated into lipid membranes, where they can interact with endogenous antioxidants such as α-tocopherol. Contrary to these in vitro findings, our results demonstrated that even at the highest dose, naringenin did not elicit antioxidant activities in vivo that could have resulted in a positive or negative interaction with water- and fat-soluble antioxidants under Hox conditions.

The potential prooxidant impact of flavonoids is considered to be directly proportional to the degree of hydroxylation in their respective A- and B-rings and to occur in the presence of high concentrations of transition metals such as Fe^{2+} and Cu^{+} (*6*). Some authors have suggested that higher concentrations of transition metals are unlikely to occur under normal conditions in vivo. However, Galati and colleagues (*11*) have reported the potential prooxidant effect of naringenin in vitro following the oxidation of water-soluble antioxidants independent of the presence of transition metals. To date, however, this reaction has not been shown to occur in vivo. A potential prooxidant effect of naringenin in vivo might be suggested by the observed higher molar ratios of CoQ_{10}/CoQ_9 in Lox animals consuming 60 and 120 mg/kg of this flavanone. As proposed by De Cabo and colleagues (*39*), a higher CoQ_{10}/CoQ_9 ratio suggests an adaptive response to oxidative stress as demonstrated in their studies with caloric-restricted animals. They demonstrated that a higher ratio of CoQ_{10}/CoQ_9 in the plasma membrane is related to a beneficial adaptation process that aged animals develop against oxidative stress under caloric restriction conditions. Indeed, in our study Hox rats showed higher liver CoQ_{10}/CoQ_9 ratios compared to Lox control rats; however, it was observed that this higher ratio was due to a disproportionate reduction in CoQ_9 under oxidative stress, not an enhanced production of CoQ_{10}. In contrast, the greater molar ratio of CoQ_{10}/CoQ_9 in Lox rats consuming 60 and 120 mg/kg naringenin was not accounted for by a decrease in CoQ_9 concentrations. All naringenin-consuming Lox groups had mean CoQ_{10} values that were higher than the control, but these were not significantly different. Thus, the higher calculated molar ratios of CoQ_{10}/CoQ_9 in Lox rats were probably an artifact and not an indication of adaptation to oxidative stress. This conclusion, which is negative for a prooxidant effect of naringenin in vivo, is supported by several additional observations. First, naringenin consumption did not affect the weight gain of rats after 6 weeks of diet consumption. Rat growth is negatively influence by oxidative stress, and this was not observed with naringenin consumption. Second, naringenin intake did not affect the concentrations of liver fat- and water-soluble antioxidant nutrients. The status of endogenous antioxidants is an indirect marker of oxidative stress. It has been postulated that the redox environment of a cell is directly affected by the redox status of cytosolic compounds such as GSH (*40*). Finally, and more definitively, naringenin did not increase liver total aldehydes or NQO1 activities in Lox animals, which are among the more sensitive markers reflecting ROS damage to lipids and adaptation to excess ROS formation. Taken together, the results did not indicate that naringenin exhibited prooxidant effects in growing rats under the Lox experimental conditions employed.

Animals are capable of up-regulating enzymatic systems that allow for their survival during exposure to high oxidative stress conditions. Some of these adaptive enzymes are involved in the synthesis of endogenous antioxidants (*41*), in the detoxification of free radicals or oxidation products (*42*), or as support systems for the recycling of membrane antioxidants (*43*). The up-regulation of supportive enzymatic systems has been associ-

ated, in part, with changes in gene expression, protein synthesis, and function. Deficiency of selenium alone leads to increased transcription of the *nqo1* gene product (*44*). Nevertheless, deficiency of vitamin E alone indirectly leads to similar effects, but it takes longer times to observe significant changes in NQO1 activity (*24*). Navarro and colleagues (*45*) demonstrated that vitamin E and Se deficiency increased the activities and expression of cytochrome *b*5 reductase and NQO1 at the plasma membrane. Our results agreed with previous papers with further demonstration that liver cytosolic NQO1 activity was significantly increased in proportion to the amount of naringenin, ~23% at the highest dose, consumed by rats under the Hox condition only.

Induction of NQO1 activity is widely used as a test to screen potential anticarcinogenic molecules in vitro (for a review see ref *46*). Although it has been reported that a variety of flavonoids can induce NQO1 in cells in culture, multiple studies have predicted and demonstrated that due to its structural characteristics (*47*) naringenin and its chalcone are poor inducers (*48*). More conclusively, Breinholt and colleagues (*49*) demonstrated that gavage-fed naringenin, among other flavonoids, did not induce the activity of phase I and II enzymes, including NQO1, in the liver, heart, and colon of weaned female rats treated with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, a carcinogen that induces ROS formation and oxidative DNA damage. In contrast with all of this evidence, here we show that naringenin promoted the activity of liver NQO1 only under Hox conditions. This implies that, at least in rats, chemically induced oxidative stress provokes metabolic changes different from those derived from antioxidant nutrient deficiency. The latter could affect the absorption and metabolism of xenobiotics in unknown ways that could possibly modify the effect of flavonoids on enzymatic adaptation systems.

In summary, the present study directly addressed the following questions: does naringenin act as an antioxidant or prooxidant in vivo, and does its dietary consumption affect antioxidant defense systems in rats under Lox or Hox conditions? Our results provided evidence that this highly consumed citrus flavanone does not exhibit antioxidant or prooxidant effects when fed to rats at physiological concentrations. Although naringenin intake led to higher liver NQO1 activities, this effect on the endogenous antioxidant defense system was not strong enough to prevent the symptoms of oxidative stress in Hox animals. It is suggested that the naringenin effect on NQO1 is due to alterations in the metabolism of flavonoids under Hox conditions. In conclusion, dietary naringenin neither protects rats against oxidative stress induced by nutrient antioxidant deficiency nor interferes with their normal nutrient antioxidant status.

ABBREVIATIONS USED

ANOVA, analysis of variance; CoQ, coenzyme Q; Hox, high oxidative stress; Lox, low oxidative stress; 4-HNE, 4-hydroxynonenal; MDA, malondialdehyde; NQO1, NAD(P)H:quinone oxidoreductase-1; ROS, reactive oxygen species.

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Received for review June 19, 2006. Revised manuscript received January 4, 2007. Accepted January 18, 2007. This work was supported by The Botanicals Center for Age Related Diseases at Purdue University, West Lafayette, IN.

JF061714H