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Influence of Dietary Quercetin on Glutathione Redox Status in Mice

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Flavonoids such as quercetin have been shown to serve as a protective defense against oxidative damage *in vivo*. However, the bioavailability of quercetin depends on the food source and type of glycosidic moiety linked to the molecule. In this study, mice were fed 1 mg/day quercetin in the form of quercetin aglycone, rutin, apple, or onion, and reduced glutathione (GSH), oxidized glutathione (GSSG), and protein-GSH mixed disulfides were determined to investigate the influence of dietary quercetin on the GSH redox status in metabolically active tissues, mitochondria, and plasma of mice. All quercetin treatment groups produced increases in the GSH:GSSG ratio and decreases in mixed disulfide levels in hepatic tissue. Cardiac tissue did not change in response to dietary quercetin; however, cardiac mitochondria demonstrated a reduction in the GSH:GSSG ratio and an increase in protein mixed disulfide levels. No significant changes were observed in the plasma GSH:GSSG ratio, but mixed disulfide levels were decreased for all of the diets. The changes in plasma redox status did not parallel the changes in the tissues. Onion fed mice demonstrated the greatest increases in GSH:GSSG ratios and the greatest decreases in protein mixed disulfide levels of all diets compared. For all treatment groups, increases in the GSH:GSSG ratios corresponded with decreases in protein mixed disulfide levels. The results of this study indicate that quercetin influences GSH:GSSG ratios and protein thiolation in a tissue-specific manner and that these effects are dependent on food source and bioavailability.

KEYWORDS: Glutathione; GSH; quercetin; quercetin glycosides; rutin; onion; apple; S-glutathiolation; S-thiolation; mixed disulfides; oxidative stress; redox potential; diet; mouse

INTRODUCTION

Oxidative stress is generally defined as a shift in the balance between antioxidants and pro-oxidants in the cellular environment toward the pro-oxidant state. When the cellular redox homeostasis becomes pro-oxidizing, the antioxidant repair and defense mechanisms of aerobic cells are unable to cope with the reactive oxygen and nitrogen species (ROS/NOS) that accumulate during normal cellular metabolism. ROS/NOS are believed to contribute to progressive host inflammatory-immune responses as well as cellular and tissue damage as these compounds can readily oxidize critical cellular macromolecules including proteins, lipids and nucleic acids. An accumulation of oxidized cellular materials occurs with age and is associated with chronic and degenerative diseases such as cancer and cardiovascular disease (*1*). Increasing the consumption of fruits and vegetables rich in vitamins E and C and polyphenolics is proposed as a strategy to help moderate oxidative damage and prevent or attenuate diseases associated with oxidative stress. Vitamins C and E as well as numerous flavonoids demonstrate the ability to reduce the accumulation of oxidative damage (*2*) and are epidemiologically associated with a decreased risk of chronic disease associated with oxidative damage including cardiovascular disease (*3–5*).

Quercetin is a polyhydroxylated flavonol with antioxidant activity (**Figure 1a**). It is the most prevalent flavonoid in the Western diet, and its consumption is estimated at 10–20 mg/day (*6*). The antioxidant activities of quercetin are attributed to many factors including the ability to inhibit pro-oxidant enzymes, up-regulate enzymes involved in drug metabolism, form complexes with pro-oxidant redox-cycling cations such as iron or copper, and trap ROS/NOS by direct hydrogen ion donation (*7, 8*). Many flavonols have higher antioxidant activity than vitamins C and E as a result of their 4-carbonyl (or 4-oxo) and 5- or 3-hydroxyl groups. Such structural features allow quercetin to donate hydrogens to scavenge free radicals while maintaining the stability of the resultant aryloxyl radical (*8*). Typical physiological levels of flavonoids are as much as 1000-fold lower that those of either vitamins C or E, and it is now recognized that the biological activity of flavonoids such as quercetin likely arise from more specific interactions with biological targets and less from their ability to act as a general antioxidant in vivo. For example, quercetin has been shown to

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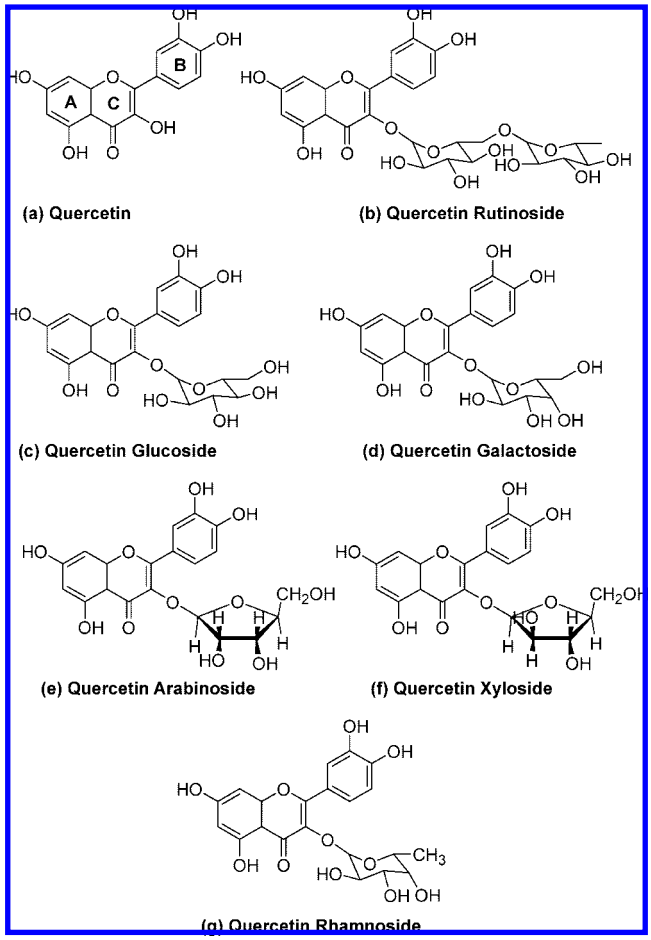

Figure 1. Structures of (**a**) quercetin aglycone, (**b**) quercetin rutinoside (rutin), (**c**) quercetin glucoside, (**d**) quercetin galactoside, (**e**) quercetin arabinoside, (**f**) quercetin xylosides, and (**g**) quercetin rhamnoside.

upregulate enzymes involved in antioxidant defense (*7*), such as γ-glutamylcysteine synthetase, the rate limiting enzyme in glutathione (GSH) synthesis (*9*), GSH peroxidase, GSH reductase (*10*), and catalase (*11*). There is also evidence that certain flavonoids, including quercetin, may have a sparing effect on tocopherols (vitamin E) and carotenoids (vitamin A) (*12*).

GSH is the primary endogenous antioxidant responsible for the maintenance of an intracellular redox balance and detoxification of electrophilic xenobiotics and reactive oxygen species (*13*). GSH is present in millimolar concentrations in most cells. GSH is sensitive to changes in redox potential and can form oxidized GSH (GSSG) in the presence of free radicals. Although there are several possible biochemical indicators of oxidative stress, the ratio of reduced to oxidized GSH (GSH:GSSG) has been widely used as a biomarker of redox status for its sensitivity, abundance, and reliability (*14, 15*). Several studies indicate that quercetin has the ability to increase the GSH:GSSG ratio and consequently lower cellular oxidative status. For example, studies by Molina et al. indicated that quercetin protects against ethanol induced oxidative stress in liver tissue by augmenting the levels of GSH (*16*). In other studies, Ishige et al. (2001) demonstrated that quercetin protects against glutamate induced oxidative stress in neurons through GSH modulation (*17*).

The GSH:GSSG redox ratio is generally viewed as a representative biomarker of oxidative stress, but a more sensitive biomarker may be protein mixed disulfides formed from the reaction of oxidized GSH and cysteinyl thiols on select proteins. This reaction, referred to as S-glutathiolation or S-thiolation, occurs during periods of oxidative stress and is proposed to be more physiologically relevant and sensitive than measuring GSH:GSSG alone (*18, 19*). The formation of protein mixed disulfides has been proposed to function in the protection of protein thiol groups from irreversible cysteine oxidation (*20, 21*), in the reversible post-translational modification of various enzymes (*18*), and as a reserve of GSH when levels are depleted (*22*). Mixed protein disulfides have been shown to form as markers of in vivo oxidative damage in rat lungs exposed to cigarette smoke and cataracts in eye lenses, and to correlate with shifts in the GSH:GSSG ratio (*23*). A recent review on GSH biochemistry indicates that the majority of publications describing the alteration of cellular GSH levels only discuss the effect on the antioxidant capacity of the cell instead of all redox equilibria between free GSH and mixed disulfides (*19*).

Since mitochondria are highly predisposed to oxidative damage and are involved in redox signaling, the formation of mixed disulfides within mitochondria is particularly important (*24*). Most ROS are generated in the mitochondria and therefore pose an oxidative challenge to this organelle. The function of mitochondria is sensitive to both redox status and the formation of protein mixed disulfides (*25, 26*). A pro-oxidizing mitochondrial environment is associated with aging (*27*) and other degenerative diseases (*24*). Shifting the redox environment toward a more reducing one, through dietary manipulation, may potentially attenuate the degenerative processes associated with oxidative stress. The effect of quercetin on mitochondrial redox status has not yet been investigated. However, as the mitochondrial GSH pool is separate from the intracellular GSH pool, it may respond differently to dietary antioxidants than the cytoplasmic pool (*24*).

The effect of quercetin on GSH redox status and protein S-glutathiolation is dependent on quercetin bioavailability. Quercetin exists in plants as glycosides, and the glycosidic form of quercetin varies widely between species. The small intestine is the primary site of absorption of quercetin glycosides (*28*), and it appears that the sugar moiety of the glycoside is an important determinant in the absorption process (*29*). The absorption of quercetin glycosides is thought to occur via interactions with epithelial brush border membrane transporters, such as the sodium-dependent glucose transporter-1 [SGLT-1], and subsequent deglycosylation by cytosolic beta-glucosidase (*30*). Additional studies indicate that flavonoids may also be absorbed after deglycosylation by lactose−phloridzin hydrolase located at the intestinal brush border membrane (*31*). Although these absorption models vary by mechanism, they both demonstrate the importance of the type of sugar moiety attached to quercetin in recognition and bioavailability.

Previous studies on the effect of quercetin supplementation on redox status have used quercetin aglycone (*16, 17*). However, as quercetin is not found in foods as the aglycone, it is unclear how this data translates to more typical dietary exposures. Data show that absorption of the quercetin aglycone is only 20%, whereas 52% of quercetin-4′-*O*-glucoside (Q4′-G) is absorbed from the gut (*30*). Interestingly, significant differences in the absorption of quercetin from two main dietary sources, onions and apples, were also demonstrated. The predominant form of quercetin in onions is Q4′-G, whereas apples contain a mixture of galactosides, arabinosides, rhamnosides, xylosides, and glucosides (**Figure 1b−g**). Hollman et al. (*28*) determined that the time to peak plasma concentration levels for quercetin from onions was 42 min as compared to 151 min for quercetin from apples, and the area under the curve indicated that the bioavail-

ability from onions was more than 2-fold higher than that for apples. This result suggests that Q4′-G is more rapidly and completely absorbed than quercetin galactosides. In addition, both food sources had higher bioavailability compared to that of purified quercetin-3′-rutinoside (rutin), the main glycoside found in tea, which reached peak plasma concentration at 558 min, indicating absorption from the colon as opposed to the stomach or small intestine and possible prerequisite hydrolysis of the rutinoside by gut microflora (*28*).

The aims of the present study were to (1) investigate the influence of dietary quercetin on GSH redox status, as measured by the GSH:GSSH ratio, and protein S-glutathiolation in metabolically active tissues (liver and heart), mitochondria, and plasma of mice and (2) to determine whether the GSH redox status is influenced by the type of quercetin glycoside present in different dietary sources. To achieve these aims, animals were fed diets standardized to give 1 mg of quercetin equivalents per day as either the quercetin aglycone, rutin, apples, or onions.

METHODS AND MATERIALS

Reagents. Glacial acetic acid, sucrose, potassium phosphate dibasic, acetonitrile, and methanol were obtained from Fisher Scientific (Houston, TX). Diethylenetriaminepentaacetic acid (DEPA), dithiothreitol, GSH, oxidized GSH, gammaglutamatyl glutamate, magnesium chloride, boric acid, potassium chloride, nicotinamide, potassium tetraborohydrate, *N*-morpholinopropane sulfonic acid (MOPS), *m*-cresol purple, cresol red, L-serine, sodium chloride, quercetin, rutin, sodium acetate trihydrate, β-glucuronidase with sulfatase activity from *Helix pomatia*, and dansyl chloride were purchased from Sigma (St. Louis, MO). Potassium hydroxide was purchased from EMD Chemicals (Gibbstown, NJ). Tris base was purchased from Boehringer Manheim (Indianopolis, IN). Reagent-grade water was generated by a Barnstead E-pure deionization system (Dubuque, IA).

Animals. Male Swiss Webster mice (25–30 g) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Animals were housed in wire bottom cages and fed Laboratory Rodent Diet 5001* (Laboratory Diet, St. Louis, MO) ad libitum for 2 days to acclimate them to their new environment, then switched to Purified Rodent Diet AIN-76A (Dyets, Inc., Bethlehem, PA) for 5 days prior to dietary manipulation. Animals (12/group) were then fed either the purified control diet or the purified diet plus quercetin, rutin, dried onion, or dried apple for 7 days. Each treatment diet was normalized to contain 200 μg of quercetin equivalents per gram. The quercetin concentration of the dried onion and apple was determined by HPLC (*32*). Animals were euthanized on the morning of the eighth day with carbon dioxide, blood was drawn from the portal vein, and livers were perfused with ice-cold phosphate buffered saline. Tissues were removed, rinsed with ice-cold 0.9% sodium chloride, immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Blood was placed in tubes containing 100 μL of 100 mM serine borate containing 0.5 mg/mL sodium heparin and 1 mg/mL DEPA at pH 8.5 as described previously (*33*). Blood was then centrifuged at 200*g*, and the plasma fraction was added to an equal volume of protein precipitating solution containing 5% perchloric acid, 0.2 M DEPA, 0.2 M boric acid, and 5 mg/mL cresol red before freezing at −80 °C as described previously (*34*).

Sample Preparation. Tissues were homogenized in 10 volumes (w/v) of the following tissue-specific isolation buffers in a Teflon glass Dounce homogenizer: liver (0.25 M sucrose, 10 mM Tris buffer, 2 mM DEPA, 3 mM potassium phosphate dibasic, 20 mM potassium chloride, and 5 mM magnesium chloride at pH 7.4) and heart (0.3 M sucrose, 0.03 M nicotinamide, and 0.02 M DEPA at pH 7.4). Tissue homogenization procedures and mitochondrial extraction by differential centrifugation procedures were published previously (*35*). Although the methods of Lash extracted mitochondria from fresh tissue, the stability of soluble mitochondrial enzymes has been shown to be unaffected by one freeze–thaw cycle, indicating the suitability of frozen mitochondria for measurement of glutathione (*35*). Sample preparation was performed according to a previous method (*34*). Briefly, 750 μL of each homogenate and 350 μL of each mitochondrial preparation was added to an equal volume of protein precipitating solution, frozen at −80 °C, thawed, and centrifuged at 10,000*g* for 5 min. The pellet was saved for mixed disulfide analysis. A 300 μL aliquot of supernatant was added to 5 μL γ-glutamyl glutamate, the internal standard, and 60 μL of 100 mM iodoacetic acid in 0.2 mM *m*-cresol purple, and adjusted to pH 9.0 ± 0.2 with 1 M potassium hydroxide in saturated potassium tetraborohydrate solution. After 2 h, 100 μL of 20 mg/mL dansyl chloride in acetone was added. After 24 h in a dark at room temperature, 500 μL of chloroform was added, and the samples were mixed vigorously and centrifuged at 500*g* for 5 min, and 25 μL of the inorganic layer was injected onto the HPLC. The pellet was prepared for mixed disulfide analysis according to a prior method (*37*). Briefly, 1 mL of ice-cold ethanol was added to the pellet, the sample was mixed and centrifuged at 10,000*g* for 5 min, and the supernatant was discarded. The pellet was washed a second time, resuspended in 500 μL MOPS buffer, pH 8, containing 25 mM dithiothreitol, incubated at 37 °C with shaking for 60 min, added to 500 μL of protein precipitating solution, frozen at −80 °C, thawed, and centrifuged at 10,000*g* for 5 min. The supernatant was then derivatized for analysis in the same manner as that used for the homogenate and mitochondria samples. The protein content of homogenates and mitochondria were determined by the Bradford assay according to the manufacturer's instructions (BioRad, Hercules, CA).

Quercetin Excretion. A separate subset of mice in each treatment group ($n = 2$) was kept in metabolic cages and fed as described above, and total urine and feces were collected for a period of 2 days. The quercetin metabolites in the urine and feces samples were hydrolyzed enzymatically with β-glucuronidase and sulfatase, prior to analysis for total quercetin aglycone. Samples were analyzed by HPLC using external standardization (*32*). Briefly, feces samples were homogenized in 4 mL of 0.2 M acetate buffer, and urine was added to 2 mL of acetate buffer. A 30 μL aliquot of β-glucuronidase with sulfatase activity (92,500 unit/mL glucuronidase and 7,500 unit/mL sulfatase) was added to both feces and urine samples, and the samples were incubated at 37 °C for 16 h. Next, 5 mL of ethyl ether was added, and the samples were centrifuged at 3000*g* for 10 min. The ethyl ether layer was removed and dried under nitrogen. A 100 μL aliquot of methanol was added to the samples, and 25 μL was injected onto the HPLC column. Quercetin concentration was quantified using a standard curve ranging from 1 to 50 μg/mL. The percent quercetin excreted was calculated by dividing the amount of quercetin in urine by the amount of quercetin consumed. The amount of quercetin consumed was determined by weighing the amount of food consumed and multiplying by 200 μg quercetin/g diet.

Analysis by HPLC. Chromatographic analyses were performed on a Waters 2690 Alliance system using reversed ion-exchange HPLC with a 250 × 3.0 mm i.d., 5 μm aminopropyl column (Phenomenex, Torrance, CA) using a modified method described previously (*38*). Dansyl derivatives were detected using a Waters 474 scanning fluorescence detector recording at excitation wavelength 328 nm and emission wavelength 541 nm. The mobile phase consisted of (A) 80% acetonitrile/water (v/v) and (B) an acetate buffered acetonitrile solution prepared by adding 1100 mL of acetonitrile, 346 mL of glacial acetic acid, and 554 mL of sodium acetate stock solution. The sodium acetate stock solution was made by adding 544 g of sodium acetate trihydrate, 224 mL of water, and 756 mL of glacial acetic acid. Separations were achieved at a flow rate of 0.6 mL/min with the following linear gradient: 20% B in A, 0–5 min and 20–100% B in A, 5–35 min. GSH, GSSG, and γ-glutamyl glutamate were quantified using standard curves from 0.1 to 3 μM. The limit of detection of this method is 1 pmol for GSH and 2 pmol for GSSG (*34*).

Statistics. All data except for the bioavailability data are presented as the mean ± SD for 3 replications of homogenates and mitochondria from the pooled organs of four animals in each treatment group. Bioavailability data are presented as the mean ± SD for 2 replications. Statistical analysis was performed with SigmaStat 3.5 software (Systat Software, San Jose, CA). Statistical significance was determined by ANOVA and Fisher LSD tests with $p < 0.05$ considered to be significant. Data represented by different letters were determined to be significantly different (i.e., data marked with ab is not significantly

Table 1. Concentration of GSH and GSSG in Tissue Homogenates from Mice Fed Quercetin, Rutin, Apple Peels, or Onions[a]

	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSH:GSSG ratio
		liver	
control	140.27 ± 32.7 a	4.18 ± 1.20 ab	29.81 ± 0.39 ab
quercetin	178.74 ± 19.3 b	4.77 ± 1.35 a	47.56 ± 17.7 a
rutin	142.57 ± 44.2 a	3.35 ± 0.86 bc	47.37 ± 29.1 a
apple	93.53 ± 14.2 c	4.13 ± 0.20 abc	22.65 ± 3.19 b
onion	139.90 ± 5.03 a	2.86 ± 0.76 c	51.68 ± 15.9 a
		heart	
control	16.00 ± 2.35	0.62 ± 0.02	25.81 ± 3.61
quercetin	15.94 ± 5.99	0.59 ± 0.23	27.39 ± 2.78
rutin	16.50 ± 7.10	0.55 ± 0.20	29.51 ± 3.44
apple	12.79 ± 4.26	0.48 ± 0.16	26.43 ± 1.29
onion	18.82 ± 2.29	0.66 ± 0.06	28.90 ± 4.55
		plasma	
control	5.38 ± 0.80 ab	1.01 ± 0.14	5.33 ± 0.24 a
quercetin	7.07 ± 1.88 b	1.96 ± 1.04	4.22 ± 1.76 ab
rutin	4.73 ± 2.40 ab	1.43 ± 1.20	4.08 ± 1.80 ab
apple	4.14 ± 3.71 a	1.15 ± 1.06	3.72 ± 0.27 b
onion	5.22 ± 0.25 ab	0.94 ± 0.01	5.57 ± 0.34 a

[a] Data are presented as the mean ± standard deviation for $n = 4$ with each sample consisting of tissues pooled from 4 animals. Data represented by different letters are significantly different by the Fisher LSD test at $p < 0.05$.

Table 2. Concentration of GSH and GSSG in Tissue Mitochondria from Mice Fed Quercetin, Rutin, Apple Peels, or Onions[a]

	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSH:GSSG ratio
		liver	
control	8.715 ± 1.443 a	0.58 ± 0.093 ab	15.28 ± 3.40 ab
quercetin	6.349 ± 1.169 ab	0.76 ± 0.485 a	10.11 ± 4.68 c
rutin	5.720 ± 2.338 bc	0.41 ± 0.125 b	13.61 ± 1.88 bc
apple	3.579 ± 0.224 c	0.34 ± 0.028 b	10.46 ± 1.55 c
onion	8.069 ± 3.172 ab	0.43 ± 0.098 b	18.36 ± 3.58 a
		heart	
control	0.354 ± 0.009 ab	0.021 ± 0.003 a	16.79 ± 1.89 a
quercetin	0.434 ± 0.100 a	0.032 ± 0.007 b	13.72 ± 0.99 bc
rutin	0.446 ± 0.038 a	0.030 ± 0.003 b	15.11 ± 2.10 ab
apple	0.327 ± 0.087 bc	0.024 ± 0.002 a	13.52 ± 3.05 bc
onion	0.248 ± 0.101 c	0.020 ± 0.004 a	12.06 ± 2.83 c

[a] Data are presented as the mean ± standard deviation for $n = 4$ with each sample consisting of tissues pooled from 4 animals. Data represented by different letters are significantly different by the Fisher LSD test at $p < 0.05$.

different from data marked with a or b, but is statistically different from data marked with c.

RESULTS AND DISCUSSION

Table 1 demonstrates the GSH, GSSG, and GSH:GSSG values in liver, heart, and plasma of animals fed the control diet and quercetin, rutin, apple, or onion fortified diets. Levels of GSH varied in tissues of the control animals and ranged from 16 nmol/mg protein in the heart to 140 nmol/mg protein in the liver. Similar findings were reported previously (*39*), and the variation is thought to be caused by differences in the rate of production of free radicals and the levels of antioxidants in these different tissues (*40*). Levels of GSH, GSSG, and GSH:GSSG in the mitochondria of the same tissues are given in **Table 2**. The results indicate that mitochondrial GSH levels are significantly lower than corresponding tissue levels and are tissue-dependent, ranging from 354 μmol/mg protein in the heart to 8.71 nmol/mg protein in the liver. These values were similar to values previously reported for healthy mice (*39*). Protein mixed disulfide levels, which reflect protein thiolation, (**Figure 2**) ranged from 503.4 pmol/mg protein for heart to 2329.4 pmol/mg protein for liver. Protein mixed disulfides were at least 5-fold

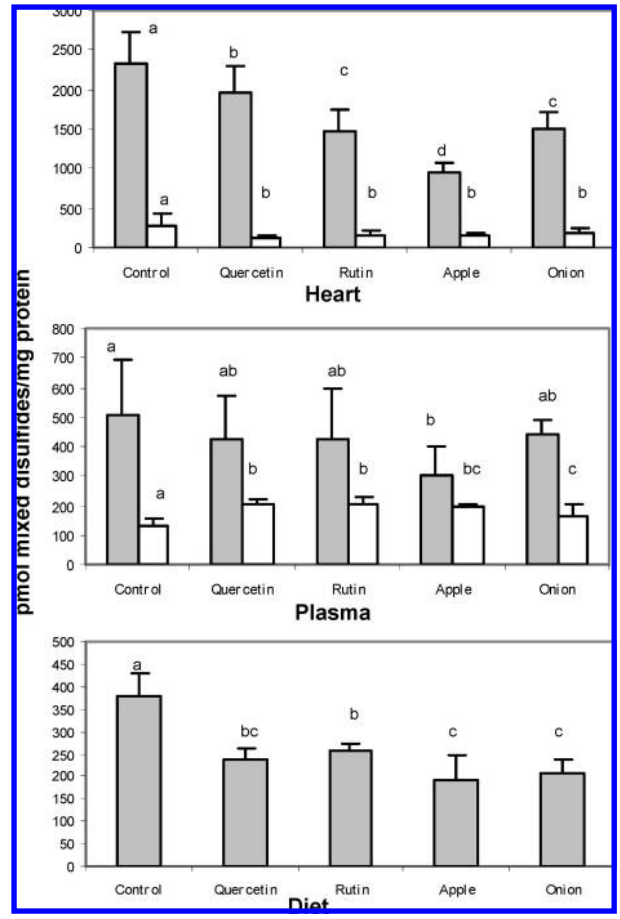

Figure 2. Protein-GSH mixed disulfide concentration in mouse tissue homogenates (gray) and mitochondria (white) in response to dietary flavonoids. Data are presented as the mean ± standard deviation for triplicate samples with each sample consisting of tissues pooled from 4 animals. Data represented by different letters are significantly different by the Fisher LSD test at p 0.05.

higher in tissues than in the corresponding mitochondria. The finding that mitochondrial levels of protein mixed disulfides were lower than the levels in corresponding tissues is in contrast to previous studies of Rebrin et al. and Park who reported that mitochondrial levels of protein mixed disulfides were 3–4-fold higher and 100-fold higher, respectively, than the levels in corresponding tissues (*39, 41*).

Comparisons of the dietary treatments indicate that effects on GSH:GSSG ratios are tissue- and source-dependent (**Table 1**). In hepatic tissues, the GSH:GSSG ratio increased in the quercetin (60%), rutin (60%), and onion (73%) fed animals as compared to the control animals, although the increases were not significant. Apple consumption reduced the GSH:GSSG ratio by 24% of the control value. This reduction was due to a reduction in GSH levels rather than an increase in GSSG. Interestingly, our data (**Table 3**) indicates that a higher quantity of quercetin was consumed and excreted in the apple fed mice as compared to the onion fed mice, likely due to the taste of the onion fortified diet. This may have affected the GSH/GSSG values since caloric restriction has been shown to improve redox status in mice (*39*). However, the quercetin glycosides found in apple are absorbed more slowly, and the plasma C_{max} is generally lower as compared with that of onion glucosides, which may help to explain the reduced GSH:GSSG ratio in this tissue. A reduction in overall food consumption may be a confounding factor affecting GSH redox status since caloric

Table 3. Consumption and Bioavailability of Quercetin in a Subset of Mice Fed Quercetin, Rutin, Apple Peels, or Onions[a]

diet	food consumed (g)	quercetin consumed (mg)	quercetin in 24 h urine (μg)	quercetin in 24 h feces (μg)	% quercetin excreted
quercetin	4.84	0.967	9.39	19.4	0.984
rutin	3.53	0.706	0.74	4.87	0.108
apple	3.72	0.743	4.40	7.06	0.593
onion	2.10	0.420	0.01	0.40	0.002

[a] Data represent the mean of $n = 2$.

restriction has been shown to reduce oxidative stress in mice (*39*). No significant differences were observed in GSH, GSSG, or GSH:GSSG values in cardiac tissue for any treatments. One could speculate that the redox status of cardiac tissue is less affected by quercetin treatment because the heart is exposed to a lower dose of quercetin metabolites due to extensive first pass metabolism, secretion of metabolites into bile, binding to serum albumin and/or the indiscriminate engulfing of quercetin metabolites by active macrophages within the cardiac vasculature.

Changes in mitochondrial GSH:GSSG ratios (**Table 2**) did not parallel the changes observed in the tissue in response to the various diets. For example, hepatic mitochondria from quercetin (10.11 ± 4.68 nmol/mg protein) and apple (10.46 ± 1.55 nmol/mg protein) fed mice had significantly lower GSH:GSSG ratios as compared to that of control animals (15.28 ± 3.40 nmol/mg protein). The reduction in the GSH:GSSG ratio in the apple group resulted from a significant reduction in GSH concentration. In the quercetin group, it was caused by a concomitant and nonsignificant reduction in GSH and an increase in GSSG. No significant difference was found for mitochondrial GSH:GSSG ratios in the onion or rutin fed groups. The cardiac mitochondrial GSH:GSSG ratio decreased in all of the treatment groups with the exception of rutin, which did not change significantly. Rutin is absorbed in the colon more slowly than the other glycosides compared in this study. Decreases in cardiac GSH:GSSG ratios were due to increased GSSG in the quercetin group and decreased GSH in the apple and onion groups. The different effects of the diets on tissue and mitochondria GSH:GSSG ratios may be due to the fact that the mitochondrial GSH pool is separate from the intracellular GSH pool (*25*). A lack of similarity between tissue and mitochondrial redox values after dietary antioxidant intervention has been reported previously (*42*).

Differences in the effect of quercetin treatments on tissues may be due to variability in the metabolic activity of these tissues. During absorption, quercetin is metabolized in intestinal enterocytes by UDP-glucuronyl transferases, sulfotransferases, methyl transferases forming numerous glucuronidated, sulfated, methylated, and under certain conditions glutathionylated conjugates prior to reaching portal or systemic circulation (*43, 44*). These metabolites along with a small fraction of absorbed glycoside can be further metabolized in hepatic tissues. The liver, therefore, is exposed to a larger complement of quercetin metabolites and in the highest concentration as compared to other tissues. Metabolites that enter the systemic circulation have lower redox potentials than the parent compound and are subject to binding to serum albumin (*45*). Distinctions in the metabolic activity of tissues and distribution of quercetin metabolites could explain why larger increases were observed with GSH:GSSG ratios in hepatic tissues and mitochondria than in the heart. Glutathione-S-transferase activity also varies by tissue, with hepatic tissue having higher activity than cardiac tissue (*46*). Variability in the tissue levels of enzymes that utilize GSH may also impact the modulation of GSH concentration.

The plasma GSH levels are shown in **Table 1**. No changes were observed for plasma GSH:GSSG ratios in the quercetin, rutin, or onion fed groups. The plasma GSH:GSSG ratio of the apple fed group decreased by 30%; however, this change was due to a nonsignificant increase in GSH and a concomitant decrease in GSSG. GSH in tissues is continually being exchanged with plasma GSH (*47*), and the release of GSH and GSSG varies as a function of tissue concentration (*48*). However, transient changes in tissue GSH levels do not necessarily correspond to plasma levels, which would be expected to be controlled very tightly (*49*). Plasma contains numerous antioxidants that tightly regulate a consistent redox level including ascorbic acid, uric acid, tocopherols, carotenoids, and glutathione (*50*). The physiologically relevant doses of quercetin given to the animals in this study would not be expected to disrupt the well-maintained redox balance of plasma.

Protein mixed disulfide levels (**Figure 2**) decreased significantly in hepatic tissues for all treatment groups as compared to those in the control ($p < 0.05$). The mixed disulfide levels were 16%, 36%, 59%, and 36% lower compared to the control value of 2329.4 ± 393.0 pmol/mg protein, for quercetin, rutin, apple, and onion fed animals, respectively. Hepatic mitochondrial protein mixed disulfides also decreased significantly ($p < 0.05$) for all treatment groups. In cardiac tissue, the only significant difference in protein mixed disulfide concentration was an observed 1.7-fold decrease in the apple fed mice. In cardiac mitochondria, all treatment groups demonstrated significantly higher levels of protein mixed disulfides than the control. Whereas in plasma, all treatment groups showed significantly lower levels of mixed protein disulfides as compared with those of the control group (control, 377.1 ± 51.7 pmol/mg protein; rutin, 258 ± 11.7 pmol/mg protein; quercetin, 239.0 ± 21.2 pmol/mg protein; onion, 192.6 ± 30.4 pmol/mg protein; and apple, 192.6 ± 54.3 pmol/mg protein).

The differences in the effects caused by each dietary treatment may be attributed to the resultant plasma concentration of quercetin from each source (**Table 3**). Hydrolyzed quercetin metabolites were quantified in urine to calculate the percent excreted, which in turn reflects the plasma area under the curve. A strong correlation between urinary quercetin metabolite concentration and plasma area under the curve has been observed in healthy human subjects (*28*). The percent quercetin excreted was the highest from quercetin aglycone (0.984%), followed by apple (0.593%), rutin (0.108%), and onion (0.002%). On the basis of the levels of excreted quercetin measured in this study, it would be expected that the effect of quercetin on GSH redox status would be greatest for the quercetin aglycone and the least for onion. However, our results show that onion had the greatest effect on GSH:GSSG ratios. The bioavailability of quercetin glycosides from different foods may not compare to that of the pure glycosides because in a complex food matrix, there may be interactions between quercetin and other food components that may enhance or limit bioavailability. Studies have shown that other phytochemicals in the food matrix of

apples and onions that have antioxidant activity may have synergistic or additive effects with quercetin (*51*). Nonquercetin compounds may also have negative effects on redox status. Our data indicate that the apple treatment had variable effects, both increasing and decreasing markers of oxidative stress in different tissues. In general, decreases in GSH:GSSG ratio in apple fed mice could be attributed to decreased GSH levels. This paradox could possibly be explained by an inhibition of the GSH synthesizing enzyme, γ-glutamylcysteine synthetase and by other phytochemicals present in the apples. Moreover, apples have the greatest variability in the range of glycosidic conjugates, and not all would be expected to have the same bioavailability. The apple treatment may also have had a negative effect on GSH redox status in certain tissues because of its higher sugar content since high sugar intakes are pro-oxidative (*52*).

The seemingly dissimilar results seen with quercetin treatment may be explained by quercetin's dual nature as both an anti and pro-oxidant. Quercetin can be oxidized to an electrophilic quinone compound that can undergo redox cycling at physiologic pH and thus act as a pro-oxidant in the semiquinone radical form (*53*). GSH conjugation to quinones through the action of glutathione-*S*-transferases effectively inhibits further reactivity of these compounds (*15*). However, flavonoid metabolism by glutathione-*S*-transferases has been shown to expend GSH reserves (*54*). During GSH depletion, an upregulation of mRNA levels and transcription rates of the catalytic and regulatory subunits of γ-glutamylcysteine synthetase are observed (*55*). γ-Glutamylcysteine synthetase is the first and rate-limiting enzyme in the pathway for GSH synthesis. Quercetin can also directly upregulate the production of GSH by activation of the γ-glutamylcysteine synthetase promoter (*9*). The changes in GSH:GSSG ratios in this study were due to changes in GSH levels rather than GSSG levels. This pattern suggests utilization of GSH and regeneration by γ-glutamylcysteine synthetase or direct upregulation of γ-glutamylcysteine synthetase activity rather than reduction of GSSG.

The results of this study indicate that quercetin influences GSH:GSSG ratios and protein mixed disulfides in a tissue-specific manner, which is dependent on food source. All quercetin treatment groups produced increases in the GSH:GSSG ratio in hepatic tissues as well as a reduction of protein mixed disulfides. Cardiac tissue displayed no change in response to dietary quercetin, yet cardiac mitochondria demonstrated a reduction in the GSH:GSSG ratio and an increase in protein mixed disulfide levels. No significant changes were observed in the plasma GSH:GSSG ratio, but mixed disulfide levels were decreased for all of the diets. Onion fed mice demonstrated the greatest increases in GSH:GSSG ratios and the greatest reduction in protein mixed disulfide levels, despite having the lowest quercetin intake of all the diets. In all cases, increases in the GSH:GSSG ratios corresponded with decreases in protein mixed disulfide levels. The data suggests that quercetin reduces oxidative stress indirectly by increasing the GSH:GSSG ratio and by reducing the levels of protein mixed disulfides.

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