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INHIBITION OF GLUTATHIONE REDUCTASE BY FLAVONOIDS

A STRUCTURE–ACTIVITY STUDY

ANDREW J. ELLIOTT, SANDRA A. SCHEIBER, CARIN THOMAS and RONALD S. PARDINI*
Allie M. Lee Laboratory for Cancer Research, Department of Biochemistry, University of Nevada, Reno, NV 89557, U.S.A.

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Abstract—A structure–activity study of fourteen chemically related flavonoids was conducted to evaluate their abilities to inhibit glutathione reductase (GR). By comparing the I_{50} values of flavonoids from different classes possessing an identical hydroxyl configuration, we determined the following order of potency for inhibition of GR: anthocyanidin > dihydroflavonol = chalcone > flavonol > catechin. Enzyme inhibition by delphinidin chloride and myricetin was partially prevented in a N_2 atmosphere which implicates a role for oxygen in the mechanism of inhibition. To determine the role of oxygen species in enzyme inhibition, GR was preincubated with either mannitol, diethylenetriaminepenta-acetic acid (DETAPAC), superoxide dismutase (SOD), catalase (CAT), or SOD and CAT prior to assays for enzyme inhibition by flavonoids. Enzyme inhibition by delphinidin chloride and myricetin was suppressed by the addition of SOD, suggesting that superoxide ($O_2^{\cdot}$) is involved. However, inhibition by quercetin and morin was not sensitive to antioxidants. To further investigate the role of $O_2^{\cdot}$ in GR inhibition, a superoxide generating system was utilized in the presence and absence of flavonoid. The $O_2^{\cdot}$ generating system failed to inhibit GR in the absence of flavonoid but enhanced the inhibition by myricetin, indicating that the $O_2^{\cdot}$ did not directly inhibit GR but reacted directly with certain flavonoids to form a reactive intermediate which, in turn, inhibited GR. These findings suggest that the mechanism of inhibition of GR by flavonoids is complex and may have oxygen-dependent and oxygen-independent components.

Flavonoids are a class of naturally occurring pigments with ubiquitous distribution in the plant kingdom [1]. They have been reported to possess widespread biological activities [1] especially toward enzymes that catalyze an oxidation–reduction reaction including mitochondrial succinoxidase [2], and NADH-oxidase [3], enzymes involved in arachidonic acid metabolism [1], neutrophil NADH-oxidase [4], aldose reductase [5], as well as several other oxido-reductases [6]. In a structure–activity investigation of fourteen chemically similar flavonoids, four flavonoids, quercetagetin, quercetin, myricetin, and delphinidin chloride, were shown to generate a cyanide-insensitive respiratory burst in the presence of isolated mitochondria and to auto-oxidize in buffer alone [2]. Subsequently, these same flavonoids were shown to auto-oxidize with the concomitant production of semiquinone radicals, superoxide anion radical ($O_2^{\cdot}$),† hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) [2, 7, 8]. Furthermore, in an electrochemical investigation employing cyclic voltammetry, flavonoids that underwent auto-oxidation with concomitant production of toxic oxygen species, possessed significantly lower oxidation potentials ($E_{1/2}$: −30 to +60 mV vs saturated calomel electrode (SCE)) than the flavonoids that did not (+130 to +340 mV vs SCE) [9]. Thus, the electrochemical properties of flavonoids are important in their abilities to produce oxygen radicals and may contribute to their biological activities.

Quercetin, a flavonol, is one of the most abundant flavonoids found in plants and is therefore a common constituent in the diet of phytophagous insects [10]. Accordingly, the toxicity of quercetin toward an insect model comprised of the three species, *Trichoplusia ni*, *Spodoptera eridania*, and *Papilio polyxenes*, was compared to their endogenous antioxidant enzyme levels [11–13]. Quercetin toxicity was shown to be inversely proportional to endogenous superoxide (SOD) and glutathione reductase (GR) activities. Moreover, the GR levels of *P. polyxenes*, the black swallowtail butterfly, are depressed following quercetin feeding [11], a finding which prompted us to evaluate the *in vitro* effects of flavonoids on glutathione reductase (EC 1.6.4.2).

METHODS

Reagents. Bovine serum albumin (BSA), diethylenetriaminepenta-acetic acid (DETAPAC), mannitol, myricetin, NADPH, nitroblue tetrazolium (NBT), oxidized glutathione (GSSG), quercetin,

* Corresponding author: Dr. Ronald S. Pardini, Department of Biochemistry, Mail Stop 330, University of Nevada-Reno, Reno, NV 89557. Tel. (702) 784-4107; FAX (702) 784-1419.

† Abbreviations: BSA, bovine serum albumin; CAT, catalase; DETAPAC, diethylenetriaminepenta-acetic acid; DMSO, dimethyl sulfoxide; $E_{1/2}$, oxidation potential; GR, glutathione reductase; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; $^{\cdot}OH$, hydroxyl radical; NBT, nitroblue tetrazolium; SCE, saturated calomel electrode; SOD, superoxide dismutase; $O_2^{\cdot}$, superoxide anion radical; and XOD, xanthine oxidase.

xanthine, and the purified enzymes catalase (CAT), glutathione reductase (Type IV from Baker's yeast), superoxide dismutase, and xanthine oxidase (XOD) were obtained from the Sigma Chemical Co. (St. Louis, MO). The remaining flavonoids were purchased from Roth (Atomergic Chemetals, Plainview, NY) and Pfaltz & Bauer, Inc. (Waterbury, CT). All other chemicals were of reagent grade.

Determination of glutathione reductase activity. GR activity was assayed according to Racker [14]. The reaction mixture consisted of 0.1 M potassium phosphate buffer, pH 7.6, with 1% (w/v) BSA with a final concentration of 3.4 mM GSSG and 0.1 mM NADPH. Flavonoids were solubilized in 100% ethanol or 99.9% dimethyl sulfoxide (DMSO). GR inhibition was determined as follows: five volumes of buffer containing the enzyme were incubated with one volume of inhibitor for 90 sec and a 0.5-mL aliquot of the preincubated sample was added to 2.5 mL of the reaction mixture in a 3.0-mL quartz cuvette. Control (no inhibitor added) and test incubation mixtures contained equal volumes of solvent. Oxidation of NADPH was monitored at 25° on an Aminco DW-2 spectrophotometer at 340 nm. The I_{50} values, the flavonoid concentration required to inhibit GR activity by 50%, were interpolated from inhibition curves and are reported as micromolar concentrations.

GR inhibition under hypoxic conditions was measured using delphinidin chloride, morin, quercetin and myricetin at their estimated I_{50} concentrations by the method described above with modifications as follows. Purified GR was diluted 1:500 in 0.1 M potassium phosphate buffer, pH 7.6, with 0.1% (w/v) BSA. To achieve a hypoxic environment, enzyme and reaction mixtures were flushed with N_2 for 2 hr prior to assay. Incubation of the GR–flavonoid mixture was conducted under a flow of N_2 for 80 sec at 25° followed by injection into a hypoxic cuvette. NADPH oxidation was then monitored at 340 nm on a Beckman DU-70 spectrophotometer at 25°. The spectrophotometer was calibrated initially with the appropriate flavonoid dissolved in DMSO and diluted by buffer to its approximate I_{50} concentration in all the following experiments.

The effects of antioxidants and antioxidant enzymes on flavonoid-mediated GR inhibition were investigated. Catechin, delphinidin chloride, morin, myricetin, and quercetin were tested for GR inhibition at their I_{50} concentrations with the addition of antioxidants or antioxidant enzymes. Mannitol (50 μM), DETAPAC (1.33 mM), the antioxidant enzymes CAT (0.5 μg/mL), SOD (2.5 μg/mL), as well as CAT + SOD (0.5 and 2.5 μg/mL, respectively) were each preincubated with enzyme mixture for 60 sec folowed by incubation with each flavonoid for 80 sec. Amounts of flavonoids, antioxidants, and antioxidant enzymes represent the final concentration utilized in assay.

Effects of superoxide on GR activity. Superoxide was generated by xanthine and XOD as described by Oberley and Spitz [15] in the GR phosphate-buffered reaction mixture [14]. The XOD concentration was adjusted to give a rate of NBT reduction [15, 16] between 0.0125 and 0.0250 absorbance units/min at 560 nm on a Beckman DU-70 spectrophotometer at 25°. The ability of SOD to interrupt the rate of reduction of NBT by scavenging $O_2^{\cdot}$ produced by the final XOD concentration was verified by the addition of SOD (2.5 μg/mL).

Purified GR was diluted 1:500 as previously described. GR was incubated with the xanthine/XOD mixture for 80 sec or preincubated with SOD for 60 sec followed by incubation with xanthine/XOD mixture for 80 sec. Oxidation of NADPH was then monitored at 340 nm on a Beckman DU-70 as described previously. Test and control mixtures contained equal volumes of buffer.

Inhibition of GR activity by myricetin and superoxide. Superoxide was generated as described above by xanthine and XOD and tested for NBT reduction at 560 nm on a Beckman DU-70 spectrophotometer at 25° to determine sufficient $O_2^{\cdot}$ production.

Purified GR was diluted 1:500 as previously described. GR was incubated with 110 μM myricetin, xanthine/XOD mixture, or 110 μM myricetin and xanthine/XOD mixture for 80 sec. NADPH oxidation was then monitored as described above. Test and control mixtures contained equal volumes of solvent and buffer.

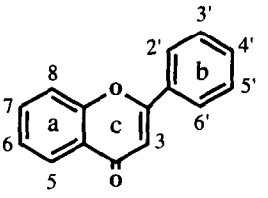
Determination of flavonoid log P values. Log P values, the water/1-octanol partition coefficients for each flavonoid representing transport of drug *in vivo*, were calculated using the fragment values published by Rekker and de Kort [17]. Values range from negative to positive, and represent a range from hydrophilic to hydrophobic properties of the test compounds. The anthocyanidins undergo several structural changes [9, 18], and these compounds presumably exist in their deprotonated forms at the assay pH, 7.5 [9]. Therefore, lipophilicity was approximated for the deprotonated forms using the formula $\log P^{+} = \log P - 3\ (\pm 1)$, which is based on the initial calculation of the fully protonated structures [17].

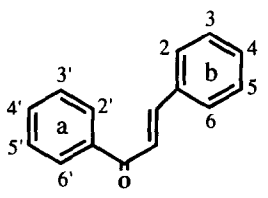
Statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA). If the analysis exhibited a significant difference, they were further analyzed by the Waller–Duncan k-ratio t test to discern significant differences among the means ($\alpha = 0.05$).

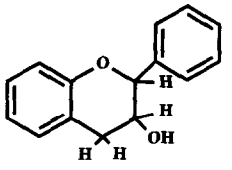
RESULTS

A structure–activity study of fourteen structurally related flavonoids was conducted to evaluate the structural requisites for GR inhibition. I_{50} values interpolated from the concentration–response curves are shown in Table 1 and indicate that flavonoid classes exhibited the following order of potency for GR inhibition: anthocyanidin > dihydroflavonol = chalcone > flavonol > catechin. This order of potency was based upon comparisons of selected flavonoids which belong to different classes but possess identical hydroxyl configurations. For example, the anthocyanidin, delphinidin chloride, was a more potent inhibitor of GR than myricetin, the structurally analogous flavonol ($I_{50} = 60$ and 110 μM, respectively). In addition, the anthocyanidin, cyanidin chloride ($I_{50} = 120$ μM) was a

Table 1. Inhibition of glutathione reductase by selected flavonoids







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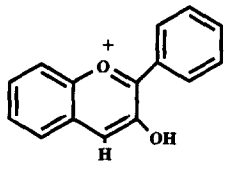
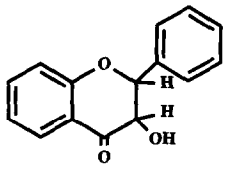


anthocyanidins



dihydroflavonols

Name	Class	Hydroxylation pattern	No. of -OH Groups	GR I_{50}*	Log P†	$E_{1/2}$‡
Delphinidin chloride	Anthocyanidin	3,5,7,3',4',5'	6	60	−2.660	NA§
Myricetin	Flavonol	3,5,7,3',4',5'	6	110	−0.779	0.06
Cyanidin chloride	Anthocyanidin	3,5,7,3',4'	5	120	−2.170	NA
Luteolin	Flavone	5,7,3',4'	4	170	+1.369	0.18
Fustin	Dihydroflavonol	3,7,3',4'	4	190	+0.577	0.15
Butein	Chalcone	3,4,2',4'	4	210	+1.305	0.13
Taxifolin	Dihydroflavonol	3,5,7,3',4'	5	220	+0.081	0.15
Quercetin	Flavonol	3,5,7,3',4'	5	280	−0.283	−0.03/0.06
Fisetin	Flavonol	3,7,3',4'	4	280	+0.213	0.14
Quercetagetin	Flavonol	3,5,6,7,3',4'	6	300	−0.597	0.06/0.25
Kaempferol	Flavonol	3,5,7,4'	4	330	+0.213	0.17
Morin	Flavonol	3,5,7,2',4'	5	370	−0.283	0.14
Galangin	Flavonol	3,5,7	3	>330	+0.709	0.34
Catechin	Catechin	3,5,7,3',4'	5	>830	+1.376	0.15

* I_{50}: Concentration (μM) required to inhibit GR activity by 50%. Values are means of 4–18 independent determinations for each concentration of flavonoid. The specific activity of GR controls ranged from 6.63 to 9.03×10^5 U/mg protein.

† Water/1-octanol partition coefficient as calculated using the fragment values published by Rekker and de Kort [17].

‡ $E_{1/2}$ values are from Ref. 9.

§ Data not available.

more potent inhibitor than taxifolin ($I_{50} = 220\ \mu M$), quercetin ($I_{50} = 280\ \mu M$), and catechin ($I_{50} > 830\ \mu M$), the structurally analogous dihydroflavonol, flavonol, and catechin derivatives, respectively. Comparisons between the dihydroflavonols, fustin and taxifolin, and the corresponding flavonols, fisetin and quercetin (I_{50} = 190, 220, 280, and 280 μM, respectively), revealed that the dihydroflavonols exhibited lower I_{50} values.

Since the flavonol myricetin was found to be a potent inhibitor of GR, a detailed structure–activity study was performed on a series of 3,5,7-trihydroxy flavonols containing various hydroxyl configurations in the B-ring. The following order of potency (from the lowest I_{50} value to the highest) was observed for the B-ring hydroxyl group configuration (Table 1): myricetin, a pyrogallol (3',4',5'-trihydroxy), > quercetin, a catechol (*ortho*-dihydroxy), > kaempferol, a 4'-hydroxy (monohydroxy), > morin, a *meta*-dihydroxy, > galangin, unhydroxylated. The midpoint potentials and log P values of these five flavonols paralleled their respective I_{50} values toward GR, indicating that the more hydrophilic flavonols with lower midpoint potentials were better inhibitors.

Flavonoid log P values were inversely related to the number of B-ring hydroxyl groups which are polar and contribute to the hydrophilicity of the molecule. Overall, hydrophilicity of the flavonoid (Table 1) did not appear to be related to its inhibitory capacity except in the series of 3,5,7-trihydroxy flavonols with modified B-rings.

The findings by Hodnick *et al.* [7] that delphinidin chloride, myricetin, quercetin, and quercetagetin undergo auto-oxidation in aqueous buffer, pH 7.4, introduced the possibility that oxygen radicals are involved in the inhibition of GR by flavonoids. To

Table 2. Flavonoid-induced inhibition of glutathione reductase under hypoxic conditions*

	% Inhibition			
	Delphinidin chloride	Myricetin	Morin	Quercetin
Aerobic	30.2 ± 4.0[a]	40.7 ± 7.2[a]	42.8 ± 5.5[a]	42.1 ± 5.1[a]
Hypoxic	15.7 ± 7.2[b]	19.6 ± 4.9[b]	45.4 ± 3.8[a]	45.0 ± 4.9[a]

* Percent inhibition values are means ± SD of 9–11 independent determinations of GR activity. Flavonoids were tested at their calculated I_{50} concentrations (Table 1). Specific activity of GR controls ranged from 2.95 to 3.62 × 10^5 U/mg protein. ANOVA of percent inhibitions: delphinidin chloride, $F_{6,61} = 8.01$, $P > F = 0.0001$; myricetin, $F_{6,72} = 23.37$, $P > F = 0.0001$; morin, $F_{5,52} = 1.55$, $P > F = 0.1775$; quercetin, $F_{5,72} = 4.04$, $P > F = 0.0028$. Values not accompanied by the same letter were significantly different ($P < 0.005$) by the Waller–Duncan k-ratio t test.

Table 3. Effects of antioxidants on flavonoid inhibition of glutathione reductase*

	% Inhibition				
	Delphinidin chloride	Myricetin	Morin	Quercetin	Catechin
Flavonoid	30.2 ± 4.3[a]	40.7 ± 7.2[a]	42.8 ± 5.5[a]	42.1 ± 5.1[ab]	−0.7 ± 0.8[a]
Mannitol	27.6 ± 3.6[ab]	40.3 ± 6.5[ab]	44.2 ± 2.3[a]	36.8 ± 4.7[c]	−11.4 ± 11.3[b]
CAT	25.6 ± 3.7[ab]	42.9 ± 5.7[a]	47.2 ± 3.1[a]	35.1 ± 2.9[c]	1.5 ± 3.2[a]
SOD	20.5 ± 3.5[c]	24.9 ± 3.3[c]	46.4 ± 4.1[a]	37.9 ± 7.5[bc]	0.1 ± 9.1[a]
CAT + SOD	26.4 ± 3.4[ab]	36.0 ± 6.2[b]	46.8 ± 3.7[a]	39.0 ± 6.5[bc]	3.0 ± 4.9[a]
DETAPAC	24.5 ± 7.3[bc]	39.6 ± 5.6[ab]	44.8 ± 5.3[a]	ND†	−1.5 ± 8.9[a]

* Percent inhibition values are means ± SD of 9–21 independent determinations of GR activity. Flavonoids were tested at their calculated I_{50} concentrations (Table 1), except for catechin which was tested at 830 μM. Specific activity of GR in controls ranged from 2.95 to 3.62 × 10^5 U/mg protein. ANOVA of percent inhibitions: delphinidin chloride, $F_{6,61} = 8.01$, $P > F = 0.0001$; myricetin, $F_{6,72} = 23.37$, $P > F = 0.0001$; morin, $F_{5,52} = 1.55$, $P > F = 0.1775$; quercetin, $F_{5,72} = 4.04$, $P > F = 0.0028$; catechin, $F_{5,61} = 4.76$, $P > F = 0.0010$. Percent inhibition values not accompanied by the same letter were significantly different ($P < 0.05$) by the Waller–Duncan k-ratio t test.

† ND: Addition of DETAPAC caused a precipitate and enzyme activity could not be determined.

assess this possibility, we monitored the inhibition of GR by flavonoids under both hypoxic and aerobic conditions. Data obtained from this study clearly indicated that for the two most potent inhibitors of GR, delphinidin chloride and myricetin, inhibition was 2-fold greater in the presence of air than under N_2 (Table 2). Thus, the inhibition by these two flavonoids was enhanced in the presence of oxygen. In contrast, the inhibition of GR by quercetin and morin was similar under aerobic and hypoxic conditions, suggesting that these two flavonoids do not require oxygen for maximum inhibition.

The data in Table 3 reveal that the addition of SOD, a $O_2^{\cdot}$ scavenger, protected GR from inhibition by delphinidin chloride and myricetin, suggesting a role for $O_2^{\cdot}$ in the oxygen-enhanced inhibition of GR by these flavonoids. Surprisingly, CAT, a scavenger of H_2O_2, did not protect against GR inhibition by delphinidin chloride or myricetin. Also unexpectedly, CAT and SOD together did not yield superior protection against delphinidin chloride and myricetin to that of SOD alone, and in fact was less. Thus, $O_2^{\cdot}$ appears to play a role in the inhibition of GR by myricetin and delphinidin chloride. GR inhibition by morin was not affected by the addition of antioxidants or antioxidant enzymes which is consistent with the previous observation that oxygen is not required for maximum GR inhibition by morin.

To test the importance of $O_2^{\cdot}$ in direct inhibition of GR, a xanthine and XOD $O_2^{\cdot}$ generating system was employed in the absence of flavonoids [15, 16, 19]. At a final concentration of 0.0075 U/mL XOD, this xanthine/XOD $O_2^{\cdot}$ generating system yielded a rate of 3.4 μM $O_2^{\cdot}$ produced/min as calculated from NBT reduction [20]. NBT reduction was inhibited 94.5% in the presence of SOD (2.5 μg/mL). These parameters indicate that sufficient $O_2^{\cdot}$ was produced by the xanthine/XOD system to test the direct effects of $O_2^{\cdot}$ on GR activity. Incubation of the $O_2^{\cdot}$ generating system with GR failed to inhibit enzyme activity and consequently the addition of SOD to the system had little effect as well. We observed 3.5 ± 8.5% inhibition in the presence of

the $O_2^{\cdot}$ generating system and 4.1 ± 5.0% inhibition in the presence of the $O_2^{\cdot}$ generating system plus SOD. These inhibition levels were not significantly different at $P < 0.05$ by the Waller-Duncan k-ratio t test. The superoxide generating system was further tested in the presence of myricetin at its I_{50} concentration. NBT reduction was checked for sufficient $O_2^{\cdot}$ production (3.4 μM min^{-1}) by the xanthine/XOD mixture. Myricetin was found to inhibit GR 52.50 ± 2.4% in the absence of $O_2^{\cdot}$ and 61.06 ± 1.2% in the presence of the $O_2^{\cdot}$ generating system. These inhibition values were found to be significantly different ($P < 0.05$) by the Waller–Duncan k-ratio t test.

DISCUSSION

Results from this structure–activity study demonstrated that flavonoids from several different classes inhibited GR at micromolar concentrations. The relative potency of the flavonoid classes towards GR was in contrast to a similar flavonoid structure–activity study previously conducted against mitochondrial succinoxidase and NADH-oxidase activity [2, 3]. The salient feature of this difference is that anthocyanidins are only marginally active in the mitochondrial enzymes, whereas against GR they are the most potent inhibitors tested. However, the order of potency toward GR for 3,5,7-trihydroxy flavonols possessing a modified B-ring, specifically myricetin, quercetin, kaempferol, morin, and galangin, was directly analogous to the order of potency observed for mitochondrial succinoxidase [2].

The B-ring hydroxyl configuration of flavonoids is important to GR inhibition. Both myricetin and quercetagetin possess a pyrogallol configuration. For myricetin, a potent GR inhibitor, the pyrogallol is in the B-ring, whereas for quercetagetin, which is less potent, it is in the A-ring. Delphinidin chloride, the most potent GR inhibitor observed, possesses the same B-ring pyrogallol structure as myricetin. Pyrogallol itself has been reported to undergo auto-oxidation similar to delphinidin chloride and myricetin [21]. This suggests that the ability to undergo a redox reaction may provide preferential inhibition toward GR. However, a trihydroxy configuration is not required for effective GR inhibition because cyanidin chloride and luteolin, which lack this structural feature, were more effective inhibitors of GR than the trihydroxylated quercetagetin.

The underlying feature in inhibition of GR by flavonoids cannot be attributed to the B-ring structure alone. Delphinidin chloride and cyanidin chloride possess oxonium ions in the C-ring (Table 1). These anthocyanidin oxonium ion species may be inhibitory towards GR which may account for the observed potency of anthocyanidins towards GR. They exist in their deprotonated forms at the experimental pH and presumably go through several structural changes similar to that of the flavonoid pelargonidin [9]. It is possible that one of these structural intermediates, common to both delphinidin chloride and cyanidin chloride, is the inhibiting species. In addition, it must be noted that myricetin, a flavonol, must have a unique mode of action in that it was as inhibitory toward GR as the anthocyanidin, cyanidin chloride. That is, inhibition of GR is not a characteristic of anthocyanidins alone but may involve more than one mechanism of inhibition. Two possible mechanisms include direct enzyme–flavonoid interaction and the auto-oxidation of certain flavonoid species resulting in production of toxic oxygen species and/or reactive intermediates.

The ability of pyrogallols to redox cycle, in addition to the observation that certain flavonoids undergo auto-oxidation with concomitant production of semiquinone intermediates [7, 8, 21], supports an oxygen-dependent mechanism in enzyme inhibition by flavonoids. Delphinidin chloride, myricetin, and quercetin have been shown to produce a substrate-independent cyanide-insensitive respiratory burst in isolated mitochondria and to auto-oxidize in aqueous buffer [2]. They also possess significantly lower oxidation potentials than the flavonoids that do not auto-oxidize [2, 9]. In addition to exhibiting less GR inhibition under hypoxic conditions, myricetin and delphinidin chloride were less effective GR inhibitors in the presence of SOD, a scavenger of $O_2^{\cdot}$. This directly implicates a role for oxygen and superoxide in the mechanism of enzyme inhibition by these flavonoids. SOD inhibits the auto-oxidation of pyrogallol [21] and myricetin, and $O_2^{\cdot}$ has been reported to increase the rate of auto-oxidation of myricetin [2]. Since the auto-oxidation of myricetin produces an *o*-semiquinone free radical [7], it is likely that superoxide reacts directly with myricetin to form a reactive intermediate. This reactive intermediate could then directly interact with the key disulfide/thiol group of GR, depending on enzyme state, resulting in disruption of the active site and inhibition of enzyme activity. This proposed mechanism is consistent with the observations in Tables 2 and 3, that oxygen enhanced the inhibition of GR by myricetin and that SOD protected GR from myricetin inhibition. This mechanism is further supported by the ability of myricetin, but not $O_2^{\cdot}$, to directly inhibit GR while the addition of the $O_2^{\cdot}$ generating system to myricetin increases myricetin inhibition of GR by approximately 10%. The fact that catalase, mannitol, and DETAPAC did not affect myricetin inhibition of GR significantly supports this conclusion, since H_2O_2, and $^{\cdot}OH$ are not apparently involved in the mechanism of inhibition whereas $O_2^{\cdot}$ is involved. Since enzyme inhibition is evident under hypoxic conditions, however, the proposed oxygen-dependent mechanisms do not work alone.

The finding that quercetin was a moderate inhibitor of GR is consistent with the observed decrease in GR activity in black swallowtail butterflies that were fed quercetin [11]. Polyphenolic compounds, especially flavonoids, are known to complex with proteins through hydrogen and covalent bonds causing precipitation and/or enzyme inhibition [22]. Morin, which possesses a *meta*-dihydroxyl configuration in the B-ring, does not auto-oxidize to produce toxic oxygen species [2, 7, 8, 23], and inhibited GR equally in hypoxic and aerobic conditions, apparently through such an oxygen-independent mechanism. Since quercetin is acting

through an oxygen-independent mechanism, binding could preclude GR from participating in the antioxidant defense of the host. Thus, the ability of quercetin to inhibit GR and its separate ability to produce $O_2^{\cdot}$, H_2O_2, and $^{\cdot}OH$ [2, 7] could have greater toxicological implications by inhibiting a key antioxidant enzyme and producing toxic oxygen species and thereby promoting extensive oxidative stress *in vivo*.

In conclusion, our studies have clearly demonstrated that flavonoids are capable of inhibiting GR by both an oxygen-dependent and -independent mechanism. The biological implications of these findings could be important not only in understanding plant–insect interactions, but also in understanding the cytotoxic and antineoplastic activities of flavonoids [5].

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