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Inhibition of 15-lipoxygenases by flavonoids: structure–activity relations and mode of action

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Abstract

We have recently reported that flavonoids of cocoa inhibit the mammalian 15-lipoxygenase-1—a catalyst of enzymatic lipid peroxidation. To elucidate the structure–activity relationship of the inhibitory effect, we investigated the effects of 18 selected flavonoids of variable structure on pure rabbit reticulocyte and soybean 15-lipoxygenases using linoleic acid as substrate. Moreover, the inhibition by quercetin was studied in detail to gain insight into the mode of action. Quercetin was found to modulate the time-course of the reaction of both lipoxygenases by three distinct effects: (i) prolongation of the lag period, (ii) rapid decrease in the initial rate after the lag phase was overcome, (iii) time-dependent inactivation of the enzyme during reaction but not in the absence of substrate. A comparison of the IC_{50} for the rapid inhibition of rabbit reticulocyte 15-lipoxygenase-1 revealed that (i) the presence of a hydroxyl group in the flavonoid molecule is not essential, (ii) a catechol arrangement reinforces the inhibitory effect, (iii) in the presence of a catechol arrangement the inhibitory potency inversely correlates with the number of hydroxyl groups, (iv) a 2,3-double bond in the C ring strengthens the inhibitory effect. The flavone luteolin turned out to be the most potent inhibitor of the mammalian enzyme with an IC_{50} of 0.6 μM followed by baicalein (1 μM) and fisetin (1.5 μM).

Keywords: Antioxidants; Flavones; Flavonols; Quercetin; Luteolin; 15-Lipoxygenase

1. Introduction

Flavonoids of different chemical structure are widely distributed in plant foods and beverages and, therefore, are ingested regularly with the human diet. A large number of biological actions of flavonoids have been described so far which overall are believed to be beneficial for health [1–3]. A prominent property of flavonoids is their antioxidant capacity that may afford some protection against oxidative stress. The antioxidant capacity of flavonoids and other polyphenols appears to be to a large part due to their capability of scavenging reactive oxygen and nitrogen species [1,4]. However, inhibitory effects of these compounds on prooxidant enzymes such as xanthine oxidase, myeloperoxidase and lipoxygenases have also been reported [2], and may additionally contribute to the potential beneficial effects of dietary polyphenols.

In recent studies, we reported that flavan-3-ols occurring in cocoa products inhibited isolated mammalian lipoxygenases, in particular rabbit reticulocyte 15-lipoxygenase-1 (EC 1.13.11.33) [5] and recombinant human 5-lipoxygenase (EC 1.13.11.34) [6]. The interaction of flavonoids with mammalian 15-lipoxygenase-1 merits particular attention, since this enzyme is a potential target for the health-preserving effect of flavonoids. Thus, it has been proposed to be one of the endogenous prooxidant factors involved in oxidative modification of low-density lipoprotein (LDL) leading to formation and progression of atherosclerotic lesions [7–9]. Investigations using 12/15-lipoxygenase knock-out mice appear to support this assumption [10,11]. Despite this putative pathogenic role, its interaction with flavonoids has scarcely been investigated. da Silva *et al*. described inhibitory effects of quercetin and epicatechin on 15-lipoxygenase-1-induced oxidation of LDL [12,13]. However, this assay system is complex, because there occurs an overlap of enzymatic and nonenzymatic lipid peroxidation [14–16], so that the

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Abbreviations: GPx-1, cytosolic glutathione peroxidase; GSH, glutathione; IC_{50}, concentration to achieve half-inhibition; KP_i, potassium phosphate; LDL, low-density lipoprotein; NDGA, nordihydroguaiaretic acid.

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effects of the flavonoids observed by these authors might be accounted for by actions other than interaction with enzyme protein, such as scavenging of lipid-derived free radicals. Earlier work on flavonoid research was conducted with soybean lipoxygenase L-1 (EC 1.13.11.12) [17]. Although this plant enzyme also exhibits 15-lipoxygenase activity with arachidonic acid as substrate, its usability as model for mammalian 15-lipoxygenase-1 is limited because of large differences in both primary structure and other mechanistic features [18]. Consequently, in previous work we observed that under comparable conditions (−)-epicatechin and the fraction of procyanidin dimers from the seeds of *Theobroma cacao* inhibited rabbit 15-lipoxygenase-1 but not soybean lipoxygenase L-1 [5]. These considerations prompted us to study the inhibition of rabbit 15-lipoxygenase-1 by flavonoids in more detail. Here, we present information on structure–activity relations as well as some insight into the mode of action of the flavonol quercetin.

2. Materials and methods

2.1. Enzymes and flavonoids

15-Lipoxygenase-1 was purified from rabbit reticulocytes as described [19] and had a turnover number of 38 s^{-1}. Soybean lipoxygenase L-1, prepared by affinity chromatography, was obtained from Sigma-Aldrich, and bovine erythrocyte glutathione peroxidase (EC 1.11.1.9) from Boehringer Mannheim.

The flavonoids were purchased from Sigma-Aldrich and used as 0.1 M stock solutions in 2-methoxyethanol. All other chemicals were of analytical grade.

2.2. Lipoxygenase assays

Lipoxygenase activities were generally measured at 20° in 0.1 M air-saturated potassium phosphate (KP_i), pH 7.4, containing 0.1 mM diethylenetriamine pentaacetic acid. To assess the effect of flavonoids on rabbit 15-lipoxygenase-1, the enzyme (20 μL, diluted 1:100 in 0.01 M KP_i, pH 6.0) and 4.0 μL test compound diluted in 2-methoxyethanol were added to the assay medium (380 μL) in the oxygraphic measuring chamber (Oxygenmeter 781; Strathkelvin Instruments). After 2 min pre-incubation, the reaction was started by addition of 20 μL 5.3 mM potassium linoleate in 0.1 M KP_i, pH 7.4, containing 4% (w/v) sodium cholate unless stated otherwise. Linoleate was chosen as the simplest biologic substrate for 15-lipoxygenases since it is superior to arachidonate because of the lack of multiple reaction possibilities. The linear part of the oxygen uptake trace was evaluated and compared with the corresponding solvent control. The activity of soybean lipoxygenase L-1 (diluted 1:200) was measured in a similar way, except that sodium cholate was omitted (cholate is required to achieve optimal activity of mammalian 15-lipoxygenase-1, but it inhibits the soybean enzyme). In some experiments, lipoxygenase activities were measured by recording the formation of conjugated dienes at 234 nm (UV-Vis spectrometer Lambda 2, Perkin-Elmer).

Glutathione peroxidase activity was measured spectrophotometrically [20].

3. Results

3.1. Actions of quercetin on lipoxygenase kinetics

In Fig. 1 are shown the progress curves of the reaction of rabbit 15-lipoxygenase-1 with potassium linoleate in the absence and presence of quercetin. In this experiment the formation of conjugated dienes was recorded spectrophotometrically at 234 nm, and oxygraphic measurement of oxygen uptake yielded similar curves (not shown). As can be seen, the control curve (trace A) exhibited a typical sigmoidal shape known for this enzyme [21]: a short kinetic lag phase was followed by a linear phase of maximal activity which declined again at the chosen temperature (20°) after about 2 min. This lag phase is due to the necessary oxidation of resting ferrous enzyme to active ferric enzyme by hydroperoxy fatty acid and is overcome when the lipoxygenase has formed a sufficient amount of oxidizing reaction product. The subsequent decline of the reaction rate is due to suicide inactivation of the enzyme by its reaction product [22]. Quercetin (trace B) affected the lipoxygenase reaction in multiple way: (i) prolongation of the lag phase, (ii) reduction of the maximal rate, and (iii) stronger time-dependent inactivation of the enzyme. Parallel measurements using soybean lipoxygenase L-1 yielded quite similar curves (not shown). Rapid inhibition followed by time-dependent inactivation of soybean lipoxygenase L-1 was also observed when quercetin was added to the reaction set-up after the substrate (Fig. 2). Therefore, a stimulation of the suicide inactivation as the reason for the time-dependent inactivation of lipoxygenases by quercetin is not very likely, in as much as the suicide inactivation of soybean lipoxygenase L-1 was negligible under the conditions employed. The time-dependent inactivation of rabbit 15-lipoxygenase-1 did not occur, however, when enzyme was incubated with quercetin for the same time in the absence of substrate (Fig. 3). Consequently, it may be supposed that the inactivation is due to combined action of quercetin and intermediates of the catalytic cycle on the active site of the enzyme.

To elucidate the mode of the rapid inhibition of rabbit 15-lipoxygenase-1 by quercetin, the concentration of linoleate was varied. From Lineweaver–Burk plots it was evident that neither K_m nor V_{max} were significantly affected (data not shown). Instead, the degree of inhibition was paradoxically diminished with decreasing substrate

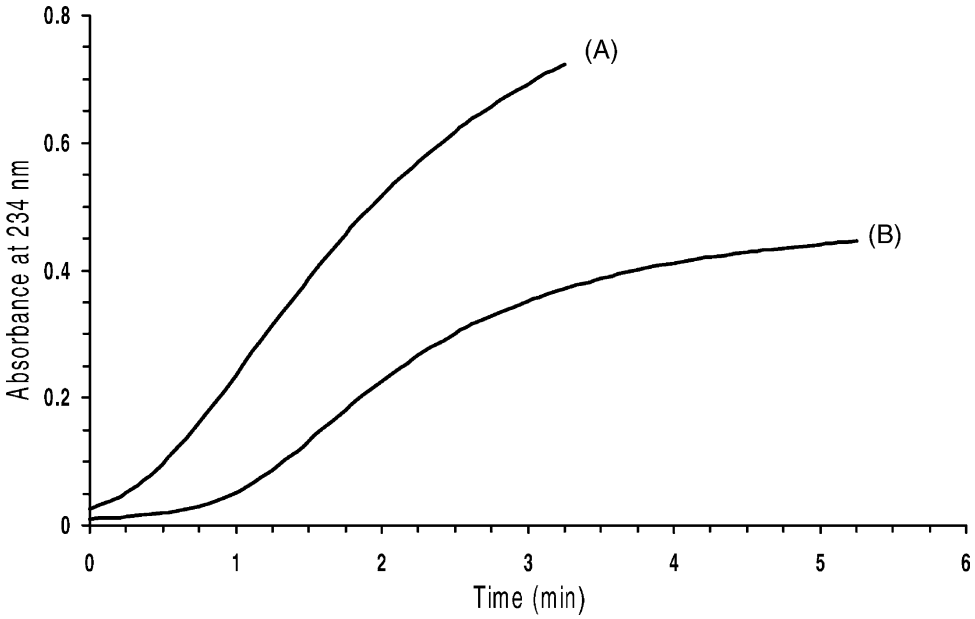


Fig. 1. Time-course of the reaction of rabbit reticulocyte 15-lipoxygenase-1 in the absence (A) or presence of quercetin (B). The samples were pre-incubated for 2 min at 20° with 10 μM quercetin or solvent and the reactions were started by addition of 0.265 mM potassium linoleate. Formation of conjugated dienoic fatty acid was recorded spectrophotometrically.

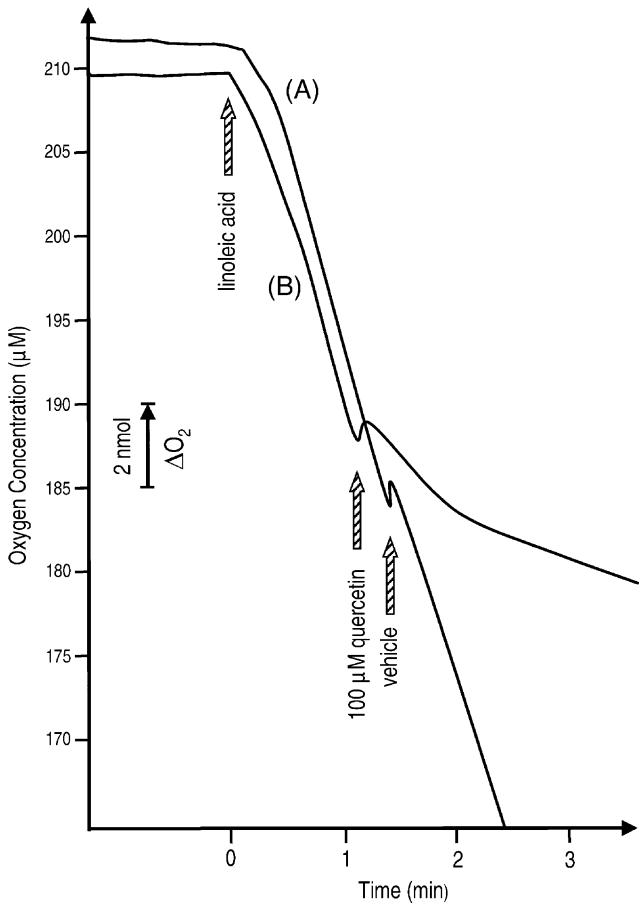


Fig. 2. Action of quercetin on the time-course of soybean lipoxygenase L-1 when added after start of the reaction. Lipoxygenase activity was measured by recording oxygen uptake. The hatched arrows indicate the times of additions (injection by Teflon tubing into the reaction vessel); (A) control; (B) with addition of quercetin.

concentration (Table 1) which reveals an unusual mode of the inhibitory effect. In any case, a competitive type of inhibition appears to be excluded.

3.2. Structure–activity relations regarding rapid inhibition

Variation of the concentrations of quercetin or other flavonoids revealed a reproducible dose-dependence of the rapid inhibition of both rabbit 15-lipoxygenase-1 and soybean lipoxygenase L-1 (Fig. 4A). In contrast, the prolongation of lag phase and the time-dependent inactivation were difficult to quantify. Therefore, we studied the structure–activity relations for the IC_{50} values regarding rapid inhibition of the two lipoxygenases by a selection of

Table 1
Unusual dependence of the inhibition by quercetin of rabbit 15-lipoxygenase-1 on the concentration of substrate

Quercetin (μM)	Linoleic acid (μM)	Activity (μkat)[a]	Percent of control
0	13.4 ± 0.4[b]	0.127 ± 0.010	100
10	11.7 ± 0.4[b]	0.119 ± 0.014	93.7 ± 11.0
100	14.4 ± 0.8[b]	0.021 ± 0.002	16.4 ± 1.4
0	139	0.229 ± 0.033	100
10	139	0.092 ± 0.007	40.2 ± 2.9
100	139	0.027 ± 0.001	11.6 ± 0.5

[a] Estimated from the slopes of the progress curves after the lag phase was overcome (cf. Fig. 1). Means ± SD of triplicate spectrophotometric measurements are given.

[b] Available concentration as estimated enzymatically from the extent of conjugated diene formation at the end of the reaction using an $\varepsilon_{234\,nm} = 25,000\ M^{-1}\ cm^{-1}$.

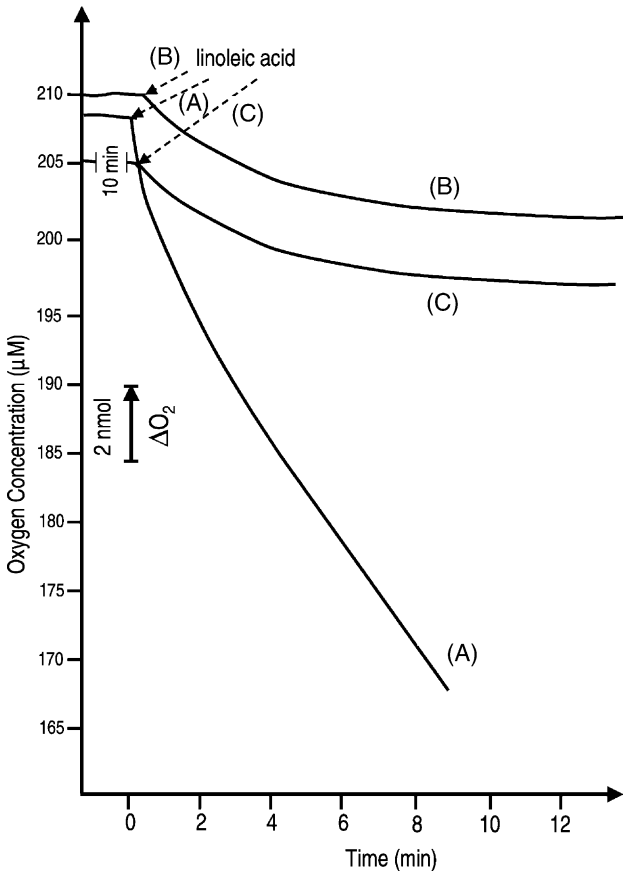


Fig. 3. Duration of the pretreatment of rabbit reticulocyte 15-lipoxygenase-1 with quercetin did not influence the time-course of the reaction with linoleic acid. Lipoxygenase activity was measured by recording of oxygen uptake. Enzyme was pre-incubated for 1.5 min without (A) or with 10 µM quercetin (B), respectively and the reaction was started by addition of potassium linnoleate as indicated by the arrows. In trace C the pre-incubation was prolonged for 10 min (recorder intermittently turned off), i.e. for the time period required to have achieved complete inhibition in the presence of substrate in trace B. The lag phase of the reactions after the start by linoleate was not so pronounced here as in the experiments in Fig. 1, since the substrate solution contained a certain amount of hydroperoxy fatty acid.

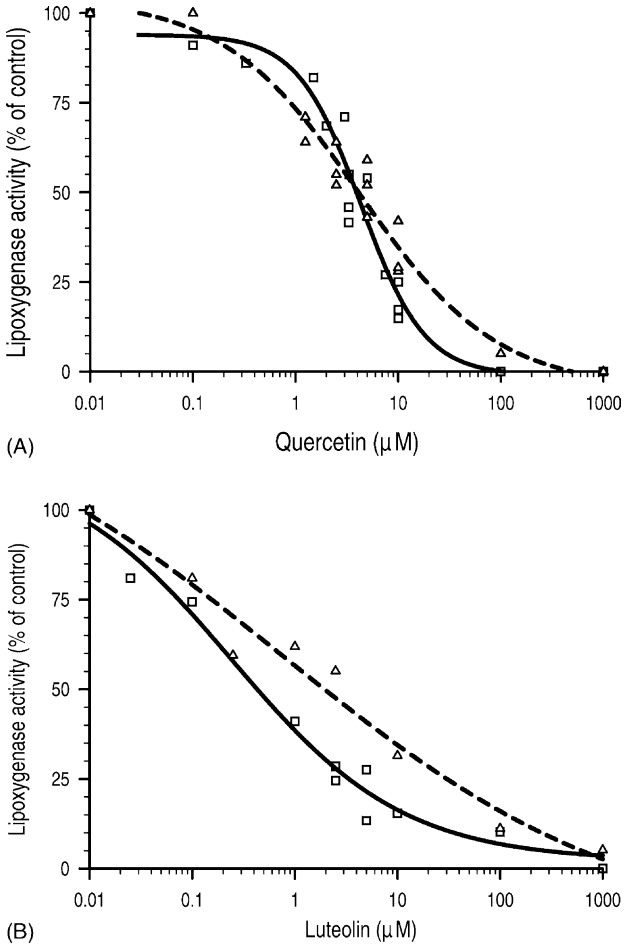


Fig. 4. Dose–response curves for the inhibition of rabbit reticulocyte 15-lipoxygenase-1 (squares, full lines) and soybean lipoxygenase L-1 (triangles, dashed lines) by quercetin (A) and luteolin (B). Lipoxygenase activities were measured oxygraphically in the standard assay (see Section 2). The data were compiled from single measurements from three independent experimental series and fitted by using the software package SigmaPlot 2001 for Windows, version 7.0 (SPSS Science Marketing).

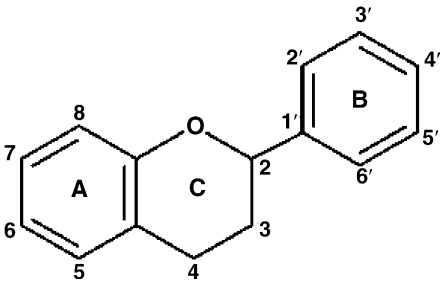
flavones, flavonols and flavanols (Table 2A). In Table 2B the data are compiled together with the structural features of the compounds.

All compounds tested inhibited the rabbit 15-lipoxygenase-1 to a variable extent. The majority of flavonoids also inhibited the soybean enzyme, albeit in most cases with lesser potency. Remarkably, even flavone was found to be inhibitory showing that the general presence of phenolic hydroxyl groups is not a prerequisite for the inhibition. In particular, the 3-OH proved to be not essential but rather interfering, as indicated by the higher potency of the flavone luteolin than that of the flavonol quercetin. Presence of a catechol arrangement in either ring A (baicalein) or ring B (cf. quercetin vs. morin) enhanced the inhibitory potency but was not essential for it. For catechols, the concentrations required to achieve 50% inhibition (IC_{50}) inversely correlated with the total number of hydroxyl groups as judged from the increase in the inhibitory potency within the series myricetin–quercetin–luteolin/fisetin. This observation contrasts the behavior of both the free radical-scavenging activity of flavonoids as expressed in the trolox equivalent antioxidant capacity (TEAC) assay, in which a pronounced maximum at quercetin has been reported [23], and the iron plus ascorbate-induced lipid peroxidation in rat liver microsomes, where the highest inhibitory potency was found for galangin [24]. The presence of a sugar moiety in the flavonoid appears to dramatically depress the inhibitory potency as evidenced by the high IC_{50} of rutin, in which the 3-OH group of quercetin is linked to rutinose. The latter effect is plausible from the higher hydrophilicity of the glycoside which is expected to give rise to a lower affinity towards the lipophilic active site of lipoxygenases.

Flavanones (naringenin, hesperetin), flavanols (epicatechin) and 2,3-dihydroflavonols = flavanolones (taxifolin) proved to be markedly less potent than quercetin. The

Table 2A
Structures of the flavonoids investigated

Numbering system of flavonoids (C=O at C-4 unless stated otherwise):



Compound	Substituents							
	3	5	6	7	2′	3′	4′	5′
Flavone	H	H	H	H	H	H	H	H
Chrysin	H	OH	H	OH	H	H	H	H
Apigenin	H	OH	H	OH	H	H	OH	H
Daidzein	–[a]	H	H	OH	H	H	OH	H
Genistein	–[a]	OH	H	OH	H	H	OH	H
Baicalein	H	OH	OH	OH	H	H	H	H
Luteolin	H	OH	H	OH	H	OH	OH	H
Fisetin	OH	H	H	OH	H	OH	OH	H
Quercetin	OH	OH	H	OH	H	OH	OH	H
Myricetin	OH	OH	H	OH	H	OH	OH	OH
Morin	OH	OH	H	OH	OH	H	OH	H
Kaempherol	OH	OH	H	OH	H	H	OH	H
Galangin	OH	OH	H	OH	H	H	H	H
Rutin	ORu[b]	OH	H	OH	H	OH	OH	H
Taxifolin	OH	OH	H	OH	H	OH	OH	H
Naringenin	H	OH	H	OH	H	H	OH	H
Hesperetin	H	OH	H	OH	H	OH	OCH[c]	H
Epicatechin[b]	OH	OH	H	OH	H	OH	OH	H

[a] Phenyl substitution (B ring) in 3-position of the C ring (isoflavone).
[b] Ru: rutinoside.
[c] CH_2 in 4-position of the C ring (flavanol).

Table 2B
Concentrations of half-inhibition (IC_{50}) for the rapid inhibition of the 15-lipoxygenases of rabbit reticulocytes and soybeans

Compound	Structural features			IC_{50} (μM)[a]	
	Number of OH	Catechol	2,3-C=C	Reticulocyte 15-lipoxygenase-1	Soybean lipoxygenase L-1
Flavone	0	−	+	320	700
Chrysin	2	−	+	1000	>1000
Apigenin	3	−	+	180	500
Daidzein	2	−	+	430	250
Genistein	3	−	+	18	1000
Baicalein	3	+	+	1	35
Luteolin	4	+	+	0.6	3
Fisetin	4	+	+	1.8	3.5
Quercetin	5	+	+	4	4.5
Myricetin	6	+	+	18	60
Morin	5	−	+	6	18
Kaempherol	4	−	+	15	50
Galangin	3	−	+	45	200
Rutin	5	+	+	1000	Inactive
Taxifolin	5	+	−	25	1000
Naringenin	3	−	−	250	Inactive
Hesperetin	3	−	−	90	Inactive
Epicatechin	5	+	−	60	Inactive

[a] Estimated from dose–response curves (see e.g. Fig. 4A and B) with 8–15 single measurements from at least two independent experimental series.

common peculiarity of these compounds is the lack of the 2,3-double bond in the C ring of the flavonoid molecule. This double bond may be important for the structure in dual way. First, it completes a conjugated binding system through all three rings as well as the carbonyl group of the C ring thus stabilizing complexes or radical intermediates formed by the flavonoid molecule as it has also been proposed earlier to explain the structure–activity relationship for the free radical-scavenging properties of flavonoids [25]. Second, it confers a spatial structure of the molecule that is largely planar so that it may more readily intercalate between aromatic or heteroaromatic amino acid residues at the active site of lipoxygenases. Interestingly, the fully planar structure is best realized with quercetin or other flavonols, whereas in luteolin there is a torsion angle of about 16° of the B ring with the rest of the molecule [26]. This slightly twisted spatial structure of luteolin together with the presence of the catechol arrangement in the B ring may contribute to its high lipoxygenase-inhibitory potency. The difference in the spatial structures of quercetin and luteolin may be also reason for the different slopes of the dose–response curves (Fig. 4) which may suggest some difference as to the mode of action.

3.3. On the mechanism of the inhibitory effects of flavonoids

Addition of catalase (8.3 μkat/mL) plus superoxide dismutase (100 units/mL) did not affect the inhibitory effects of 10 μM quercetin on rabbit 15-lipoxygenase-1 activity (data not shown) ruling out that the inhibition might be mediated by O_2^- or H_2O_2 as conceivable intermediates or side products of the reaction of dioxygen with lipoxygenase in the presence of fatty acid substrate and flavonoid.

We investigated the effects of glutathione (GSH) as well as GSH plus glutathione peroxidase (GPx-1) on the activities of the two 15-lipoxygenases and their inhibition by quercetin. As expected, the control activities of both enzymes were inhibited by the GPx-1 system in a dose-dependent manner up to completeness, the rabbit reticulocyte enzyme being markedly more sensitive (Table 3). Glutathione peroxidases are known to control lipoxygenase activities by lowering of the hydroperoxide tone [27] which is required to sustain the active ferric state of the lipoxygenases [28]. The inhibition of soybean lipoxygenase L-1 by quercetin was not affected by either GSH alone or the GSH–GPx-1 system (Table 3). This observation contrasts the results of analogous experiments with nordihydroguaiaretic acid (NDGA); here the IC_{50} was strongly lowered from 3 μM without GSH–GPx-1 to 0.3 μM in the presence of this system (data not shown). These data suggest different modes of the inhibitory actions of quercetin and NDGA despite the common presence of a catechol arrangement in both compounds.

With rabbit reticulocyte 15-lipoxygenase-1, the inhibition was prevented in the presence of 1 mM GSH (Table 3) which suggests the involvement of oxidative processes or intermediate oxidants in the inhibitory action, because GSH should not be able to directly react with quercetin. The inhibition of lipoxygenase control activity by the GSH–GPx-1 system was abolished by 10 μM quercetin (Table 3). The latter effect was, however, not due to direct inhibition of GPx-1 activity as judged by respective controls. Rather GPx-1 activity may be impaired by a species formed *via* reaction of intermediates of the catalytic cycle of 15-lipoxygenase-1 with quercetin. Taken together, the experiments with the GPx-1 system reveal different characteristics of mammalian and plant 15-lipoxygenases with respect to the inhibition by flavonoids.

Since flavonoids, in particular those containing a catechol group in the molecule, are known to chelate iron and other transition metal ions [29], and lipoxygenases contain an iron moiety at the active site [18], we addressed the issue whether the inhibitory effect of quercetin might be due to chelating of enzyme iron. Such an action has been made probable for the interaction of 4-nitrocatechol with soybean lipoxygenase L-1 [30]. If so, equimolar amounts of Fe^{3+} should abolish the inhibitory effect of the polyphenol. This was actually found with 4-nitrocatechol but not with quercetin (Table 4). With the latter compound the inhibition was even more pronounced, although the formation of the polyphenol–iron complex and pre-incubation with the enzyme were carried out under strictly anaerobic conditions in order to minimize the risk of iron-catalyzed

Table 3
Effects of glutathione (GSH) and glutathione peroxidase (GPx-1) on the activity of rabbit reticulocyte 15-lipoxygenase-1 and its inhibition by quercetin

Addition			Reticulocyte 15-lipoxygenase-1		Soybean lipoxygenase L-1	
GSH (1 mM)	GPx-1[a]	Quercetin (10 μM)	Activity (μkat)	Percent of control	Activity (μkat)	Percent of control
−	−	−	0.213 ± 0.008		0.321 ± 0.006	
+	−	−	0.155 ± 0.005		0.306 ± 0.006	
+	+	−	0.094 ± 0.006		0.168 ± 0.006	
−	−	+	0.119 ± 0.010	56	0.144 ± 0.010	45
+	−	+	0.144 ± 0.006	93	0.125 ± 0.007	41
+	+	+	0.140 ± 0.007	149	0.075 ± 0.008	45

Means ± SD of at least triplicate oxygraphic measurements (see Section 2) are given.

[a] 0.425 μkat/L in the case of reticulocyte enzyme, 5.67 μkat/L in the case of soybean enzyme; these concentration were chosen to achieve approximately half-inhibition of the controls without GPx-1 and GSH.

Table 4
Effect of Fe^{3+} on the inhibition by 4-nitrocatechol and quercetin of soybean lipoxygenase L-1

Experiment no.	Addition	Activity	
		μkat	Percent of control
I	None	0.232 ± 0.013	
	Fe^{3+} (100 μM)	0.178 ± 0.014	
	4-Nitrocatechol (100 μM)	0.035 ± 0.004	15.1
	4-Nitrocatechol (100 μM) + Fe^{3+} (100 μM)	0.125 ± 0.003	70.3
II	None	0.108 ± 0.008	
	Fe^{3+} (30 μM)	0.144 ± 0.011	
	Quercetin (10 μM)	0.046 ± 0.001	42.4
	Quercetin (10 μM) + Fe^{3+} (30 μM)	0.039 ± 0.010	27.3

Equal volumes of polyphenol dissolved in 2-methoxyethanol and an oxygen-free solution of ferric ammonium sulfate were mixed under nitrogen atmosphere and pre-incubated with 380 μL oxygen-free 0.1 M KP_i pH 7.4 and 20 μL soybean lipoxygenase (diluted 1:200) for 2 min. Then the sample was transferred to the oxygraphic vessel and stirred for a short period to equilibrate the solution with air. After reaching a constant baseline of the oxygen electrode the reaction was started by potassium linoleate as described in Section 2. Means ± SD of at least triplicate oxygraphic measurements are given.

auto-oxidation of the flavonoid, in as much as polymeric oxidation products of certain polyphenols have been reported to exhibit higher inhibitory potencies towards reticulocyte 15-lipoxygenase-1 than the parent compounds [31]. It was not feasible, however, to obviate the presence of oxygen during the lipoxygenase assay. Our observations suggest different mechanisms of the inhibitory actions of quercetin and 4-nitrocatechol despite the common presence of a catechol arrangement.

4. Discussion

This paper demonstrates that a mammalian 15-lipoxygenase-1 is inhibited by flavonoids of variable structure. Because of its unique capability of directly catalyzing enzymatic lipid peroxidation, 15-lipoxygenase-1 belongs to the endogenous prooxidants the action of which may be favored under conditions of oxidative stress [28]. Consequently, the inhibition of 15-lipoxygenase-1 may contribute to the universal antioxidant activities of dietary flavonoids.

The mode of the lipoxygenase-inhibitory action of flavonoids turned out to be more complex than expected. Three distinct actions were observed: a prolongation of the kinetic lag phase, a rapid inhibition of the reaction after the lag phase is overcome, and a time-dependent inactivation during the reaction. Generally, lipoxygenase inhibitors can act either by complexing ferric iron at the active site or by reducing the ferric enzyme species as demonstrated before for the interaction of soybean lipoxygenase L-1 with 4-nitrocatechol [30] and NDGA [32], respectively. Our data do not lend support to the assumption that flavonoids act *via* one of these mechanisms exclusively. On the other hand, these experiments were not designed to rule out the involvement of such mechanisms to produce the inhibitory effects observed. Thus, from the chemistry of flavonoids both iron-chelating and reducing properties may be expected to be involved in the interaction with lipoxygenases. Both properties may be augmented by the presence of a catechol arrangement which was shown here to enhance also the 15-lipoxygenase-inhibitory activities. Although the inhibition did not obey the kinetics for a competitive type of inhibition, the flavonoids may intercalate in the hydrophobic cavity at the active site located in the interior of the large domain of the enzyme [18] which is binding site for both hydrophobic substrate molecules and inhibitors. While the presence of the catechol arrangement is favorable, an excessive number of hydroxyl groups lowers the hydrophobicity, which may be the reason for the higher inhibitory efficacy of luteolin than quercetin.

The time-course of the lipoxygenase reactions in the presence of quercetin reveals a time-dependent inactivation of the enzymes (Figs. 1–3). A similar phenomenon has recently been described for the inhibition of DOPA decarboxylase [33] and fatty acid synthase [34] by epigallocatechin gallate suggesting covalent modification of those enzymes. During lipoxygenase catalysis enzyme-bound prooxidant intermediates such as fatty acid peroxyl radical ($ROO^{\bullet}$) are formed [28]. It is tempting to speculate, therefore, that the flavonoids are co-oxidized in this system to a semiquinone or quinone (with flavonoids containing a catechol B ring) or a phenoxy radical (with noncatechol flavonoids) which in turn may covalently bind to sulfhydryl or amino groups of the lipoxygenase, thus rendering its inhibition irreversible. In the case of quercetin and other flavonols the intermediate formation of corresponding quinone methides [35] may be involved. Such oxidation products have been reported to be formed from flavonoids in a reaction system containing horseradish peroxidase plus H_2O_2, and to react with GSH or other thiols forming thioether conjugates [35–37]. Our observation that GSH abolishes the inhibition by quercetin of 15-lipoxygenase-1 (Table 3) may be in line with the formation of such conjugates under conditions of lipoxygenase catalysis. In the absence of GSH, however, the oxidized products from catechol-containing flavonoids may react with sulfhydryl groups in the vicinity of the active site of the enzyme forming covalent adducts as the putative reason for the irreversible inactivation observed. A quite similar phenomenon has been demonstrated for the interaction of rabbit 15-lipoxygenase-1 with ebselen, one of the most potent inhibitors of this enzyme [38]. In the latter case, covalent binding of ebselen has been demonstrated [38]. In a future study, experiments with radiolabeled quercetin are planned to examine whether the time-dependent inhibition of lipoxygenases is paralleled by covalent bond formation as well.

The 15-lipoxygenase-inhibitory potency of flavonoids cannot be ascribed to their capability of scavenging lipid-derived free radicals. Although intermediate free radicals are formed during the catalytic cycle of lipoxygenases [28], they remain tightly bound at the active site, thus not being accessible for free radical scavengers such as 2,6-di-*tert*-butyl-4-methylphenol (BHT) and probucol [39] or α-tocopherol, trolox and ascorbate [40] which do not inhibit the primary lipoxygenase reaction. In certain systems, however, intermediate free radicals may escape the active site of the lipoxygenase and induce secondary nonenzymatic lipid peroxidation which is then selectively suppressed by these radical scavengers [39,40]. In conclusion, flavonoids appear to combine both lipoxygenase-inhibitory activities and free radical-scavenging properties in one agent and thus constitute a family of very effective natural antioxidants.

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