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FLAVONOIDS AS ANTIOXIDANTS EVALUATED BY *IN VITRO* AND *IN SITU* LIVER CHEMILUMINESCENCE

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Abstract—Administration of eriodyctiol and (+)-catechin (10 mg/100 g of body weight) to mice inhibited the enhancement of *in situ* liver chemiluminescence produced by CCl_4 (0.5 ml/100 g) by 32 and 38% respectively. 3,4-Dicaffeoylquinic acid was less effective (13%), and cynarin had no effect. Previously, these compounds and other polyphenols were assayed as *in vitro* antioxidants by their abilities to inhibit the *tert*-butyl hydroperoxide (*t*-BOOH)-initiated chemiluminescence of mouse liver homogenates, and the IC_{50} (μM) values were as follows: (+)-catechin, 3; eriodyctiol, 9; myricetin and 4,2′,4′-trihydroxy-6′-metoxychalcone, 15; 3,4-dicaffeoylquinic acid, 20; isochlorogenic acid, 30; caffeic acid, 5,6,3′-trihydroxy-7,4′-dimethoxyflavone and cynarin, 50; chlorogenic acid and apigenin, 150; quercetin, pedalitin, sylimarin and quercetin-3-methyl ester, 200; 7,4′-dihydroxy-5-methoxyflavonone and kaempferol-3,7-dirhamnoside, 500; quercitrin, 900; and galangin-3-methyl ether, genkwanin, hesperidin, ombuoside, phloridzin, quinic acid, rhoifolin, rutin and sophoricoside, greater than 1 mM. The *in vitro* and *in vivo* effects of these flavonoids and polyphenols may be related to their antioxidant abilities, making them promising substances to be investigated as water-soluble protectors against lipid peroxidation and other free radical-mediated cell injury.

(+)-Catechin [(+)-cyanidanol-3] is a plant flavonoid whose protective effects against a number of hepatotoxic agents in experimental animals have been studied extensively during the last decade [1]. Flavonoids such as sylimarin [2–4] and quercetin [5], and polyphenols such as cynarin [2], caffeic acid, and chlorogenic acid [6] have also been proposed as hepatoprotective drugs. Recently, a number of flavonoids were assayed as antioxidants by their abilities to react with chemically generated peroxyl radicals [7]. Heterogenous effects of these compounds were reported when they were assayed as inhibitors of lipid peroxidation in human red cells [8]. The protective action of (+)-catechin was established by *in vivo* studies in rats in which this compound prevented increased liver chemiluminescence after acute ethanol intoxication [9], and increased ethane exhalation produced by carbon tetrachloride administration [10]. Other *in vitro* studies using (+)-catechin and other flavonoids as lipid peroxidation inhibitors have been reported [11–15].

Free radicals can be generated in biological systems either as by-products of partial oxygen reduction or by xenobiotic catabolism [16–18]. These free radicals exert various deleterious effects that are dependent upon the cellular zone where they are generated [19]. When free radical species are generated within hydrophobic domains of the cell membranes, lipid peroxidation predominates; when they are generated in a hydrophilic milieu, effects such as nucleic acid breakdown and cross-linking, protein oxidation and cross-linking, and modification of the $NADP^+$/NADPH redox state may also occur [16–19]. Damage imposed by free radicals is related not only to their reaction with biological materials, but also to delayed effects mediated by lipid peroxidation products, e.g. phospholipid hydroperoxides, aldehydes, and excited species [16, 19–21].

The proposed free-radical scavenger activity of flavonoids [20, 22] and their water solubility led to investigations of the *in vivo* antioxidant effects of these substances using chemiluminescence assays. Biological chemiluminescence can be regarded as a measure of the level of oxy-radicals generated during oxidative processes [23–26], even though the origin of the emission remains to be determined accurately. Measurements of liver surface chemiluminescence correlate with glutathione released in perfused liver [23], and *in vivo* measurements of liver chemiluminescence correspond with *in vitro* [9] methods to evaluate lipid peroxidation. Also, it was found that the activity of antioxidant enzymes diminishes and that superoxide production increases *in situ* liver emission [27–29]. Consequently, organ chemiluminescence affords a useful organ-specific and organ-noninvasive assay for physiological and non-physiological production of lipid peroxidation and other oxidative stresses by measuring the excited species generated.

In this study, flavonoids were assayed *in vivo* as liver chemiluminescence inhibitors to determine their protection against oxidative stress produced by CCl_4, a potent stimulator of lipid peroxidation [18, 29, 30]. Previously, a large number of polyphenols was screened *in vitro* for their abilities to inhibit hydroperoxide-initiated chemiluminescence of liver

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homogenates; this determination provides an integral evaluation of the level of antioxidants and prooxidants contained in or added to an assay system [27–29].

MATERIALS AND METHODS

Chemicals. The polyphenols tested were isolated through a systematic investigation carried out on Argentine medicinal plants. Isolation and identification were done using spectroscopic and chromatographic methods [31]. All of the compounds had a purity greater than 95% as determined by comparison of their physical parameters (melting point, absorbance, mass, NMR spectra, etc.) with theoretical values. Partition coefficients were determined for (+)-catechin (water/decane, 9/1) and eriodictyol (water/decane, 9.5/0.5).

CCl_4 was purchased from Mallinckrodt (Buenos Aires, Argentina). *tert*-Butyl hydroperoxide (*t*-BOOH) was from the Aldrich Chemical Co. (Phillisburg, NJ), and dimethyl sulfoxide and urethane were from BDH (Poole, England). Other reagents were of analytical grade.

Animals. Female Swiss mice (25–30 g) were fed a standard laboratory diet.

Hydroperoxide-initiated chemiluminescence of liver homogenates. The hydroperoxide-initiated chemiluminescence of liver homogenates was measured in a Packard Tri-Carb model 3320 liquid scintillation counter in the out-of-coincidence mode, at room temperature. Samples in a 4-ml final volume were placed in 25 × 58 mm glass vials. Livers were homogenized in 10 vol. of ice-cold medium consisting of 140 mM KCl, 5 mM Tris–HCl, and 1 mM EDTA, pH 7.3. After centrifugation at 600 *g* for 10 min, the supernatant fraction was used as the "homogenate" These operations were carried out at 0–2°. Protein was evaluated by the method of Lowry *et al.* [32], using bovine serum albumin as standard. The homogenates were diluted in the isolation medium to adjust the protein concentration to about 1 mg/ml. The polyphenols, previously solubilized in dimethyl sulfoxide, were added to the homogenates, and chemiluminescence was initiated immediately by addition of 3 mM *t*-BOOH. Chemiluminescence values are expressed as cps/mg protein.

Spontaneous liver chemiluminescence. In situ liver chemiluminescence was measured with a Johnson Foundation photon counter (Johnson Research Foundation, Philadelphia, PA) [23, 27]. Mice were anesthetized with 12% urethane (w/v) administered intraperitoneally at a dose of 1 ml/100 g of body weight, and the liver surface was exposed by laparotomy. After determination of the physiological emission by the liver, CCl_4 was injected intraperitoneally at a dose of 0.5 ml/100 g of body weight. The photon-counter allowed continuous monitoring of liver emission that was not interrupted by CCl_4 administration. One hour prior to addition of CCl_4, flavonoids were administered by intraperitoneal injection at a dose of 10 mg/100 g of body weight.

RESULTS AND DISCUSSION

Addition of *t*-BOOH to tissue homogenates or subcellular fractions results in chemiluminescence that is associated with lipid peroxidation resulting from formation of hemoprotein-catalyzed radicals following rupture of *t*-BOOH [27, 29, 33]. The yield of emission depends on the level of prooxidants and antioxidants in the system [27]. Vitamin E, the main *in vivo* antioxidant, inhibits *t*-BOOH-initiated chemiluminescence when added to rat liver homogenates [27] or microsomes [33]. Diethyldithiocarbamate also inhibits the emission by rat liver microsomes [34].

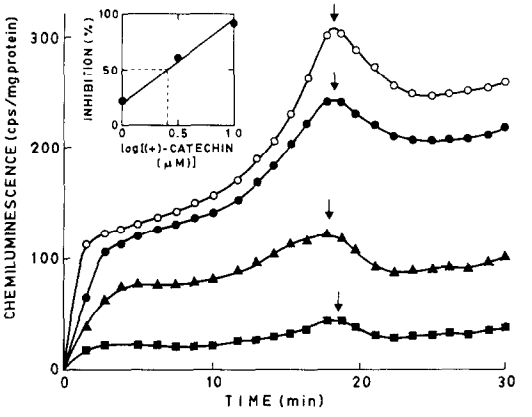

Fig. 1. Effect of (+)-catechin on *t*-BOOH-initiated chemiluminescence of mouse liver homogenates. Key: (○) no addition; (●) 1 μM, (▲) 3 μM, and (■) 10 μM (+)-catechin. Zero time was the moment of *t*-BOOH addition. Arrows indicate the times at which the values of chemiluminescence were obtained to determine IC_{50}. Inset: Determination of IC_{50}. Percent of inhibition is in reference to the value obtained for the homogenate without flavonoid addition.

In these experiments, the antioxidant activities of flavonoids and related polyphenols were evaluated *in vitro* by their abilities to inhibit *t*-BOOH-initiated chemiluminescence of mouse liver homogenates. When *t*-BOOH was added to the homogenates, the emission was enhanced (Fig. 1). Modification of emission by added polyphenols was dependent upon their antioxidant activity and their concentration. The effect of (+)-catechin concentration on *t*-BOOH-initiated emission is shown in Fig. 1. The concentration of (+)-catechin required for 50% inhibition of emission (IC_{50}) was calculated as shown in the inset of Fig. 1. Although the chemiluminescence kinetic was complex, the effects of different polyphenol concentrations were compared using the value of the first maximum of emission at 20 min. The relationship obtained at this time was not significantly different from the values calculated at other times (Fig. 1). Using the appropriate concentration, the same procedure was followed for each polyphenol tested. The calculated IC_{50} values are shown in Table 1. (+)-Catechin was the most active flavonoid. Hydroxy groups in the 3′ and 4′ positions were associated with the high antioxidant activities of (+)-catechin, eriodyctiol, myricetin, and 4,2′,4′-trihydroxy-6′-methoxychalcone. The antioxidant activity was decreased by conjugation with a sugar moiety, as occurs in rutin, rhoifolin, hesperi-

Table 1. Effects of polyphenols on *tert*-butyl hydroperoxide-initiated chemiluminescence of mouse liver homogenates

Polyphenol	IC_{50} (μM)
(+)-Catechin	3
Eriodictyol	9
Myricetin	15
4,2',4'-Trihydroxy-6'-methoxychalcone	15
3,4-Dicaffeoylquinic acid	20
Isochlorogenic acid	30
Caffeic acid	50
5,6,3'-Trihydroxy-7,4'-dimethoxyflavone	50
Cynarin	50
Chlorogenic acid	150
Apigenin	150
Quercetin	200
Pedalitin	200
Sylimarin	200
Quercetin-3-methyl ether	200
7.4'-dihydroxy-5-methoxyflavonone	500
Kaempferol-3,7-dirhamnoside	500
Quercitrin	900

Also were assayed: galangin-3-methyl ether, genkwanin, hesperidin, ombuoside, phloridzin, quinic acid, rhoifolin, rutin, and sophoricoside. All these compounds had IC_{50} values >1 mM.

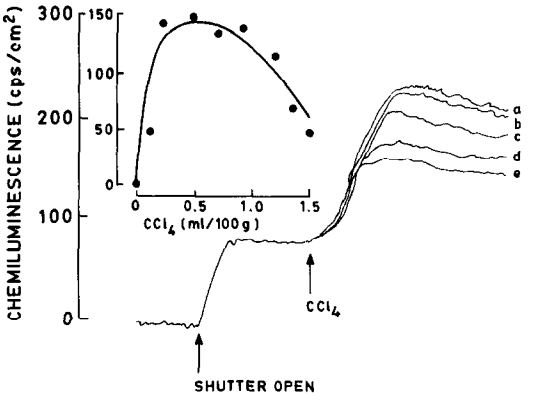

Fig. 2. Effects of flavonoids on CCl_4-stimulated liver chemiluminescence. Key: (a) none, (b) cynarin, (c) 3,4-dicaffeoylquinic acid, (d) (+)-catechin, and (e) eriodyctiol. The trace represents chemiluminescence recorded during the measurement time. Polyphenols were administered at a dose of 10 mg/100 g of body weight 1 hr before CCl_4 injection. Administration of CCl_4 increased emission almost immediately (30–80 sec); the maximum value (used to compare the effect of polyphenols) was reached at about 2–4 min after CCl_4 injection. The chemiluminescence value shown for each polyphenol is the mean of six experiments. The P values (Student's *t*-test) obtained comparing groups (d) and (e) with control (a) were: a,d, $P < 0.05$ (DF = 16); a,e, $P < 0.005$ (DF = 16); differences obtained for groups (b) and (c) were not significant. Inset: CCl_4-stimulated liver chemiluminescence as a function of CCl_4 dose.

din, kaempferol dirhamnoside, phloridzin, and sophoricoside.

The antioxidant activities of some of the compounds tested were evaluated previously by their capacity to inhibit production of thiobarbituric acid-reactive substances in rat liver homogenates [11, 15], superoxide anion production in red blood cells [8], autoxidation of linoleic acid and methyl linolenate [7], and iron-ADP or *t*-BOOH-induced microsomal lipid peroxidation [15]. The heterogeneity of the antioxidant activities reported for the flavonoids in the different studies may be related to the different types of oxidative species that are generated in each system.

Based upon *in vitro* antioxidant effectiveness of the flavonoids, the two most active flavonoids, (+)-catechin and eriodictyol, were studied *in vivo*. Also used for *in vivo* studies were 3,4-dicaffeoylquinic acid and cynarin, which are caffeoyl acid derivatives of flavonoid. These compounds were evaluated for their capacity to inhibit CCl_4-stimulated liver chemiluminescence. Injection of CCl_4 immediately increased emission (Fig. 2). Depending upon the amount of CCl_4 that was injected, the emission was increased over that of physiological emission (109 ± 6 cps/cm^2) (see inset on Fig. 2). A dose of 0.5 ml CCl_4/100 g of body weight increased emission by 150 cps/cm^2 over the control values. This increase of emission was modified by addition of the polyphenols, which had no effect on nonstimulated chemiluminescence. Eriodictyol was the most powerful antioxidant, and it inhibited the enhancement of emission by 38% (Fig. 2). (+)-Catechin was slightly less effective, and it inhibited the enhancement of emission by 32%. 3,4-Dicaffeoylquinic acid was a less effective antioxidant than the flavonoids, and it inhibited the CCl_4-enhanced emission by only 13%. Cynarin showed no inhibitory effect (Fig. 2).

In biological systems, the source of chemiluminescence seems not to be limited to reactions involving free radicals, but using CCl_4 as a stimulator, it is possible to produce free radical-related liver emission. CCl_4 reacts with cytochrome P-450 to initiate *in vivo* lipid peroxidation [18]. During this process, carbon- and oxygen-centered radicals [35] and excited species [16, 28] are formed. The observed inhibition of chemiluminescence may be explained by the chain-breaking antioxidant activity of the polyphenols through the formation of a lightly stabilized polyphenol radical that terminates the reaction chain [36]. However, a quenching of excited species cannot be disregarded, and since singlet oxygen may initiate peroxidation [24], the possibility that flavonoids can serve a double antioxidant activity, as free radical-scavenger and as excited species-quencher, remains to be corroborated.

Although lipid peroxidation is initiated in hydrophobic cell domains, if the cytochrome P-450 environment is considered to be hydrophobic, propagation reactions may reach hydrophilic domains. This would make soluble antioxidants necessary to prevent oxidative damage to soluble species, such as nucleic acids, proteins, amino acids, and nucleotides. Under physiological conditions, the concentration of reduced glutathione is high enough to cope with oxidant species, but when oxidative stress occurs, a higher level of water-soluble antioxidants may be necessary for adequate protection.

The increasing number of pathologies that have been associated with enhanced free radical production makes flavonoids and related caffeoyl acids interesting substances to test for biological antioxidant activity. These compounds have the advantage of being nontoxic and highly water soluble. Although a number of these flavonoids are ingested in a normal diet, in situations of oxidative stress, administration of both flavonoids and lipid-soluble antioxidants could be more protective than administration of a single antioxidant.

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