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Involvement of Phenoxyl Radical Intermediates in Lipid Antioxidant Action of Myricetin in Iron-Treated Rat Hepatocyte Culture

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ABSTRACT. Supplementation of rat hepatocyte cultures with the flavonoid myricetin (300 μM) led to the formation of phenoxyl radical intermediates, as detected in intact cells by electron paramagnetic resonance (EPR) spectroscopy. These radicals corresponded to one-electron oxidation products of myricetin. The level of phenoxyl radicals was significantly reduced when myricetin-treated hepatocyte cultures were also supplemented with iron (Fe-NTA 100 μM). This suggested that iron could accelerate the oxidation flux of myricetin. Moreover, myricetin was found to be able to inhibit lipid peroxidation induced by iron in hepatocyte culture. Free malondialdehyde (MDA) levels and the amount of radicals derived from oxidized lipids were greatly reduced when myricetin was added to iron-treated cultures. This showed that myricetin was a good inhibitor of lipid peroxidation in this model and that the intermediate generation of phenoxyl radicals might contribute to the antioxidant mechanism of myricetin. BIOCHEM PHARMACOL **55**;9:1399–1404, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. flavonoid; myricetin; phenoxyl radical; hepatocyte; iron

Flavonoids are natural phenolic compounds recognized as potent protectors against oxidative damage. Their effects mostly result from their free radical scavenging activity towards ROS† such as hydroxyl radical [1, 2] and superoxide anion [3, 4]. It has also recently been shown that they were able to eliminate lipid peroxyl and alkoxyl radicals, demonstrating that they can act as chain-breaking antioxidants [5]. Another mechanism of their antioxidant and cytoprotective activity is their ability to chelate iron ions to form inactive complexes, which are unable to initiate lipid peroxidation [6–9]. Despite all these useful properties for combatting "free radical pathologies," flavonoids may occasionally manifest prooxidant effects. This toxicity is thought to be involved in mutagenicity [10, 11], inhibition of mitochondrial respiration [12, 13], and cytotoxicity [10]. The mechanism of this prooxidant activity may be related to the ability of flavonoids to oxidize in the presence of dissolved oxygen to produce superoxide anion, which dismutates to give hydrogen peroxide and then hydroxyl radical through Fenton chemistry [2, 14]. Simultaneously, semiquinone radicals, also called phenoxyl radicals, are formed as one-electron oxidation products of the flavonoid [14–16]. These phenoxyl radicals can be detected by EPR spectroscopy [17, 18] using, if necessary, a spin stabilization technique with Mg^{2+} as the stabilizing metal ion [19, 20]. The direct toxic effect of phenoxyl radicals on microsomes [21], mitochondria [12, 13] and sarcoplasmic reticulum has been reported [18]. However, phenoxyl radicals are also thought to participate in the antioxidant activity of flavonoids through their ability to react, like tocopherol, with reactive oxygen species [16]. Therefore, the role of phenoxyl radicals as cytotoxic or cytoprotective intermediates of flavonoids is still controversial. To elucidate these possibilities, we decided to test the flavonoid myricetin because of its structural characteristics which favour the formation of phenoxyl radicals. The effect of myricetin was studied on primary rat hepatocyte cultures that were supplemented with Fe-NTA, a potent initiator of lipid peroxidation [22, 23]. We investigated the following two points: 1) the evidence, using EPR, pointing to the formation of phenoxyl radical intermediates of myricetin in intact cells, in the presence or absence of iron; and 2) the resulting effect of myricetin towards iron-induced lipid peroxidation in hepatocyte culture. The intensity of lipid peroxidation was estimated using two markers: free MDA production and formation of carbon-centred radicals derived from oxidized cellular lipids [24]. Concomitantly, cell viability was estimated using LDH leakage.

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† *Abbreviations:* EPR, electron paramagnetic resonance; Fe-NTA, ferric iron nitrilotriacetate; LDH, lactate dehydrogenase; MDA, malondialdehyde; POBN, α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone; ROS, reactive oxygen species.

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MATERIALS AND METHODS

Materials

Myricetin, NTA, and POBN were purchased from Sigma Chemical Co. (Saint Quentin Fallavier), and ferric ammonium citrate from Merck. The iron chelator desferrioxamine B (Desferal®) was purchased from Ciba-Geigy. All chemicals and solvents used were of analytical grade.

Cell Isolation and Culture

Adult rat hepatocytes were isolated and cultured according to a method previously described [25]. Usually, cells were tested by trypan blue dye exclusion for viability that was always in the range of 85–95%, and 20×10^6 hepatocytes were plated in 175 cm^2 Nunclon® flasks. For experimental purposes, some cultures were maintained in the presence of either NTA alone (200 μM), in the control cultures, or Fe-NTA [22, 26], to give a final iron concentration of 100 μM per flask. Myricetin was dissolved in DMSO, which was maintained at a constant concentration (2%) in all samples, even in the absence of myricetin. This concentration of DMSO did not significantly affect lipid peroxidation when compared with control cultures without DMSO.

Experimental Conditions

Myricetin was added to hepatocyte cultures at a final concentration of 300 μM. Oxidative stress was induced by supplementation of the cultures with iron (100 μM) in its complexed form with NTA. In some experiments, Desferal® (100 μM) was also added to the cultures. The cultures were usually incubated for 4 hr in the presence of these various compounds, except in the case of time-course studies (2, 4 or 6 hr). Longer incubation times were not used so as to maintain cell viability, as verified by LDH leakage. The preparation of the samples for EPR analysis was carried out as follows: culture media were removed and put aside for extracellular free MDA measurement, and hepatocytes were washed three times with 0.01 M of phosphate buffer, pH 7.45. The cells were resuspended in 3 mL of the same buffer and centrifuged at 50 *g* for 2 min at 5°. The buffer was discarded and the hepatocytes kept on ice until EPR analysis. For lipid-derived radical analysis, the cells were lysed by an ultrasonic homogenizer and 160 mM (final concentration) of the spin-trap POBN was added to this cell homogenate. The mixture was then analyzed by EPR spectroscopy.

EPR Spectroscopy Measurements

EPR spectra were recorded on a Bruker ESP 106 spectrometer operating at *ca.* 9.74 GHz (X band) frequency at ambient temperature. The remaining cell suspension or homogenate was kept, at the end of analysis, for protein content estimation according to Bradford [27].

EPR Conditions for Phenoxyl Radical Analysis

The instrument conditions used were: centre field 3495 G, sweep width 50 G, microwave power 50 mW, modulation amplitude 2.5 G, modulation frequency 100 KHz, receiver gain 1×10^4, time constant 1.28 msec, sweep time 84 sec, 4 scans accumulated. The results were obtained as arbitrary units given by computer double integration of the single line of the spectrum and were related to per mg protein content in each sample.

EPR Conditions for Lipid-Derived Radical Analysis as an Index of Lipid Peroxidation

The instrument conditions used were: centre field 3495 G, sweep width 55 G, microwave power 20 mW, modulation amplitude 1.8 G, modulation frequency 100 KHz, receiver gain 1×10^6, time constant 163 msec, sweep time 335 sec, 3 scans accumulated [24]. The results were obtained as arbitrary units given by computer double integration of the low field doublet of the spectrum and were related to per mg protein content in each sample.

Free MDA and Enzyme Leakage as Indexes of Lipid Peroxidation and Cell Toxicity

Free MDA released in cell culture media was quantified according to an HPLC method previously described [22, 28]. The amount of free MDA estimated in culture media has already been shown to be representative of global lipid peroxidation in this model of iron-supplemented hepatocyte culture [22, 23]. LDH leakage into culture media was automatically measured using LDH kits (Bayer Diagnostic) adapted to an Acyon analyser.

Statistical Analysis

Experimental values are means ± SEM of the number of separate experiments indicated in the legends. Significance was assessed using the Student's *t*-test ($P < 0.01$ as significant).

RESULTS

Generation of Phenoxyl Radicals as Intermediates of Myricetin Oxidation in Hepatocyte Culture

Addition of myricetin to hepatocyte cultures led to a single broad line EPR spectrum (Fig. 1). The EPR signal (*g* value = 1.983) detected in cultures supplemented with myricetin corresponded to phenoxyl radical intermediates as previously reported for other compounds [17, 29]. No EPR signal was evident in cultures without myricetin. Because of the low sensitivity of the EPR method, a high concentration of 300 μM of myricetin was necessary to detect phenoxyl radical intermediates. At this high concentration, the incubation times with myricetin were restricted to a few hours in order to maintain a good cell

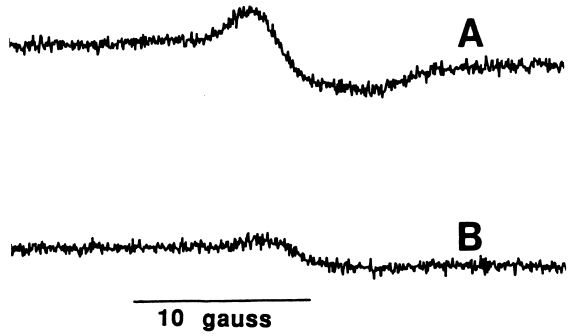

FIG. 1. EPR spectra of phenoxyl radical intermediates of myricetin oxidation produced in intact hepatocytes simultaneously treated or not with iron. Cell cultures (20 × 10^6 cells) were incubated for 4 hr in the presence of myricetin (300 μM) (A) and in the presence of both myricetin (300 μM) and Fe-NTA (100 μM), (B) (*g* value = 1.983).

viability in our experiments. Time-course studies performed from 0 to 6 hr showed that the amplitude of EPR signals corresponding to phenoxyl radicals increased with incubation times in the presence of myricetin (Fig. 2). However, when iron was also added to the cultures, amplitude of EPR signals was greatly reduced (Fig. 1). Regardless of the incubation time with both myricetin and iron, EPR signals always remained smaller than those obtained with myricetin alone (Fig. 2). When the Desferal® (100 μM) was added simultaneously to myricetin and iron in the cultures, the level of phenoxyl radicals was the same as that found in cultures treated with myricetin only (data not shown). This confirmed the role of iron in decreasing phenoxyl radical levels.

Antioxidant and Cytoprotective Effects of Myricetin towards Iron-Induced Lipid Peroxidation and Enzyme Leakage in Hepatocyte Culture

Induction of lipid peroxidation by iron was confirmed by a strong EPR signal detected in hepatocyte cultures treated with Fe-NTA (100 μM) for 4 hr (Figs. 3 and 4). This signal corresponded to POBN spin-adducts with radicals derived from oxidized lipids [24]. Extracellular free MDA levels and LDH leakage were also increased in iron-supplemented cultures (Fig. 4). However, when both myricetin (300 μM) and iron were added to the cultures, the levels of MDA and LDH leakage as well as of lipid-derived radicals remained low (Figs. 3 and 4). This demonstrated an antioxidant and cytoprotective capacity of myricetin. In cultures treated with myricetin alone, the amounts of MDA and LDH leakage and of lipid-derived radicals were not significantly higher than the basal levels found in control cultures (Figs. 3 and 4). This showed that a rather high concentration of myricetin alone was not cytotoxic and did not present any prooxidant effect towards lipids in our culture model.

DISCUSSION

This study presents data demonstrating that myricetin oxidation produced phenoxyl radical intermediates. This is the first report directly showing the formation of this type of myricetin-derived radical in an intact hepatocyte culture model. Some authors reported that phenoxyl radicals were transient, except at high pH [16], and could be stabilized by chelation with metal ions, such as Mg^{2+} or Zn^{2+}, in order to be detected by EPR [19, 20]. Chelation was not necessary in our culture system, perhaps due to the presence of small quantities of these metal ions in this model or to the structural conformation of myricetin-phenoxyl radicals. Hence, the formation of phenoxyl radicals greatly depended on the structure of the flavonoid tested. A high number of hydroxyl substituents, as for myricetin, facilitated unpaired-electron delocalization [12, 13], leading to a dipolar configuration where multiple mesomeric structures could exist [10, 16, 29]. This was probably the reason why myricetin gave a strong unresolved EPR spectrum corresponding to the envelope of several different spectra [17, 29]. Other flavonoids were tested to give phenoxyl radicals in our model, but none gave such an intense EPR signal. Phenoxyl radicals resulted from a one-electron oxidation pathway of myricetin [14, 30]. In the presence of iron, the amount of phenoxyl radical was greatly reduced, suggesting the intervention of iron redox-cycling in the intracellular oxidation of myricetin. This was confirmed by addition of

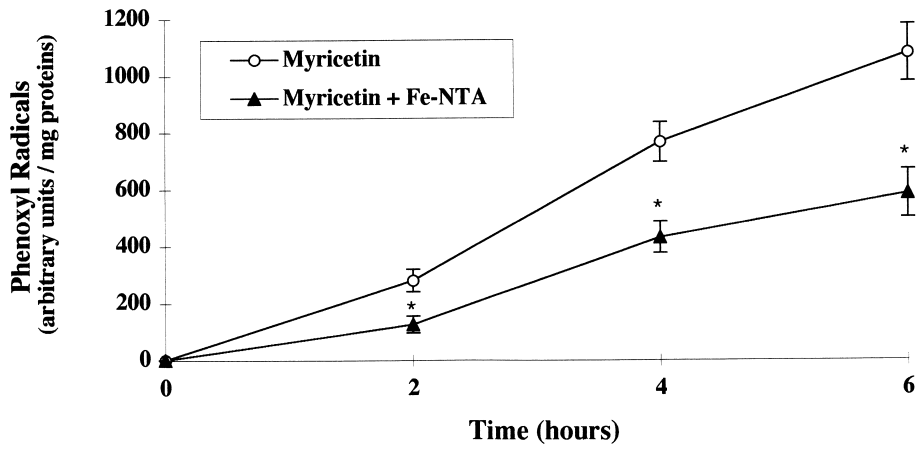

FIG. 2. Time course of phenoxyl radical generation in myricetin-treated hepatocyte cultures, in the presence or absence of iron. Cultures (20 × 10^6 cells) were supplemented from 0–6 hr with myricetin (300 μM) in the absence or presence of Fe-NTA (100 μM) (N = 3 separate experiments). Values significantly lower than those obtained in the absence of iron (*$P < 0.01$).

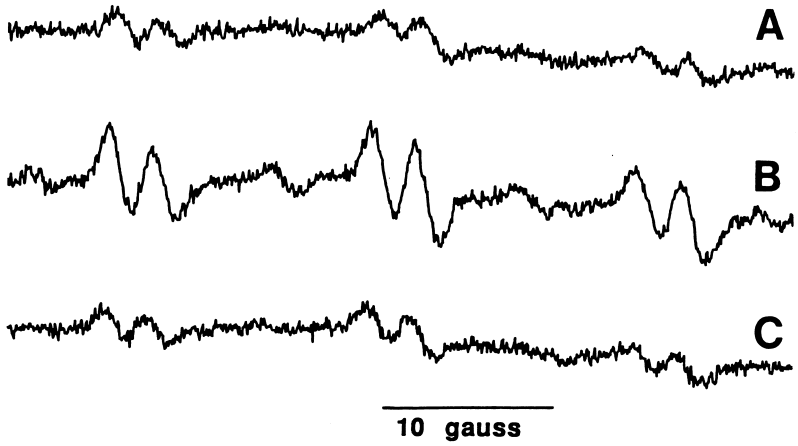

FIG. 3. Effect of myricetin on EPR signal intensities of lipid-derived radicals produced in hepatocytes. These radicals corresponded to POBN spin-adducts with oxidized cellular lipids produced during oxidative stress. They were obtained from hepatocyte control cultures showing a basal level (A), from hepatocyte cultures incubated for 4 hr either in the presence of Fe-NTA (100 μM), (B) or in the presence of both myricetin (300 μM) and Fe-NTA (100 μM) (C).

desferrioxamine, a strong iron chelator, which prevented the decrease in phenoxyl radicals observed in the presence of iron.

Two mechanisms of iron intervention in decreasing phenoxyl radical levels could be formulated: 1) Iron could act as a catalyst of electron transfer, increasing the rate of oxidation. This could lead to a more rapid transformation of phenoxyl radicals into quinonoid compounds, corresponding to the final two-electron oxidation products of myricetin [16]; 2) The decrease in phenoxyl radical levels in the presence of iron could also be ascribed to the formation of macromolecular associations between myricetin and iron. This possibility is supported by the fact that flavonoids present iron-chelating activities [7, 31]. However, in this case, iron should stabilize the radical, as previously described for metal ions [19, 20]. This implies that, instead of decreasing the EPR signal, an eventual complex between myricetin and iron might enhance it. Subsequently, the first possibility of acceleration of myricetin oxidation by iron seems most likely.

According to its polyphenolic structure, myricetin is known to produce phenoxyl radicals and ROS during an oxidation process [12, 14, 32]. These radicals might potentially be toxic for the cells. In order to elucidate this possibility, we investigated the effect of myricetin in hepatocyte cultures where oxidative stress was induced by iron supplementation. With regard to two markers of lipid peroxidation, we found an antioxidant action of myricetin in this model, despite a possible production of ROS. Therefore, the generation of phenoxyl radical intermediates and, consecutively, of ROS did not disturb the antioxidant action of myricetin. Moreover, since we found that the level of phenoxyl radical intermediates was reduced by the presence of iron in our culture model, it may be suggested that these intermediates could participate in the antioxidant action of myricetin and in its well-known free

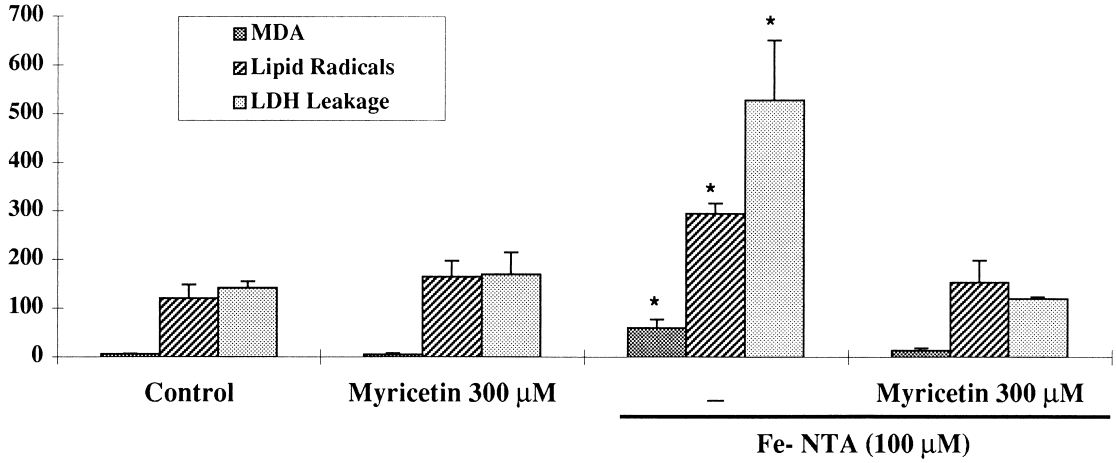

FIG. 4. Effect of myricetin on iron-induced lipid peroxidation and enzyme leakage of hepatocyte cultures. Lipid peroxidation was estimated by extracellular free MDA level (hatched bars) expressed in μM/mg of proteins and by the amplitude of the EPR signal corresponding to lipid-derived radicals (plain bars) expressed in arbitrary units/mg of proteins. These radicals were detected in cell homogenates of cultures supplemented for 4 hr with or without myricetin (300 μM) and/or Fe-NTA (100 μM). The medium of these cultures was used for extracellular free MDA and LDH leakage (mIU/mL) evaluation. (N = 3 different experiments). Significantly greater than controls (*$P < 0.01$).

radical scavenging activity, thereby eliminating toxic radical species [16]. In agreement with this, it has been reported that electrochemical properties of flavonoids could contribute to their biological activity [10]. A negative correlation between oxidation potentials and antioxidant activities has been demonstrated [8, 13, 15]. Since the oxidation potential of phenolic compounds is lowered by extended conjugation and an increased number of hydroxyl groups, myricetin could be considered as a good antioxidant. In support of this, the involvement of phenoxyl radical intermediates in the antioxidant mechanism of various compounds has already been described. This has recently been shown for melanine [33] and the antitumor drug etoposide (VP-16) [18]. In contrast, other reports suggest that drug toxicity could be mediated by the intermediate formation of phenoxyl radicals, as reported for benzene metabolites [34, 35], doxorubicine [21], and the photosensitizer phleichrome [36]. Regarding the discrepancy in beneficial or toxic effects of phenoxyl radicals, our results provide evidence that, despite the production of phenoxyl radical intermediates and probably of reactive oxygen species, myricetin possessed an antioxidant activity in hepatocyte culture. This study clearly demonstrates that myricetin protected cellular lipids against iron-induced peroxidation. Moreover, our results rule out a possible lipid prooxidant effect of these radical intermediates which might have represented damaging species in hepatocyte culture.

The involvement of the intermediate formation of phenoxyl radicals during myricetin oxidation adds to the knowledge concerning the mechanism of action of most phenolic compounds in intact cells. We found that myricetin presented a lipid antioxidant activity in hepatocyte cultures, probably by using its phenoxyl radical intermediates as electron donors. These findings could be relevant in predicting the biological effects of drugs and xenobiotics producing phenoxyl radicals during their hepatic metabolism.

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