Free Radicals in Licorice-Flavored Sweets Can Be Detected Noninvasively Using Low Frequency Electron Paramagnetic Resonance after Oral Administration to Mice

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ABSTRACT The observation of the fate of free radicals coming from food after oral administration could be important in evaluating their reactivity in vivo. The aim of this study was to demonstrate that it is feasible to detect directly in vivo free radicals coming from food with the use of low frequency electron paramagnetic resonance (EPR) spectroscopy. Because polyphenols are easily oxidized into stable radicals, we assumed that these radicals could be detected in food. We chose licorice, which contains several types of polyphenols. The presence of free radicals was demonstrated in licorice-flavored sweets. Using low frequency EPR spectroscopy, we detected these free radicals directly and noninvasively after oral administration to mice. These radicals were rather stable in the guts of the mice. This study is the first report demonstrating noninvasively the presence of free radicals in vivo coming from food. J. Nutr. 130: 1831–1833, 2000.

KEY WORDS: • electron paramagnetic resonance • licorice • food • gut • mice • cachou

Recent progress in the development of electron paramagnetic resonance (EPR) spectroscopy techniques now permits sensitive measurements of viable biological systems, including intact living animals. EPR is a magnetic resonance technique based on the detection and characterization of molecules with unpaired electrons. The use of low frequency EPR spectrometers permits an increased wave penetration into tissues and has led to an increasing number of applications in the detection of free radicals directly in vivo. including measurements of partial pressure of oxygen (Gallez et al. 1999, Goda et al. 1996), monitoring of pharmacokinetics of paramagnetic spin labels (Gallez et al. 1996), detection of short-lived radicals by spin-trapping techniques (Jiang et al. 1995) and formation of free radicals from therapeutic drugs (Mäder et al. 1995). To date, most EPR studies carried out with animals have involved the use of spectrometers operating at 1 GHz, which constitutes a good compromise between the sensitivity of the detection (signal-to-noise ratio) and the depth of wave penetration into tissues (1 cm). Such studies could be applied to larger samples or even to humans, thanks to the development of EPR spectrometers operating at lower frequency, typically in the MHz range with a higher wave penetration (several centimeters) (Halpern and Bowman, 1991).

Observing the fate of free radicals arising from food after oral administration could be important in evaluating their reactivity in vivo. However, no report exists to date in the literature that relies on applications of in vivo EPR to detect the fate of free radicals coming from food. The aim of this study was to demonstrate that it is feasible to detect directly in vivo free radicals coming from food using low frequency EPR spectroscopy. Because polyphenols are easily oxidized into stable radicals, we assumed that these radicals could be detected in food. We chose licorice, which contains several types of polyphenols, compounds that are easily oxidized into radicals (Hatano et al. 1997). We first demonstrate that stable free radicals were present in the licorice-flavored sweets. Then, using low frequency EPR spectrometer, we were able to detect these free radicals directly and noninvasively after oral administration to mice.

MATERIALS AND METHODS

A total of eleven preparations containing licorice were tested, i.e., pieces and powder of licorice, and nine commercial licorice-flavored sweets. The samples analyzed in this study are shown in **Table 1**; they were analyzed using Bruker ER-200 tt and a EMX-320 X-Band (9 GHz; Bruker, Ettlingen, Germany) EPR spectrometers for the characterization of the radicals by their g-value and peak-to-peak linewidth. The g-value is related to the line position through the resonance condition $h\nu = g\beta B$, where h is Planck's constant, ν is the microwave frequency, β is the Bohr magneton and B is the applied magnetic field. The linewidth is the distance between the maximum peak and the minimum peak of the first derivative spectrum.

Sweet 11 was used for further in vivo studies of male NMRI mice (Animalerie Facultaire, Brussels) (30–40 g). The mice were deprived of food and water for at least 12 h before the experiments were conducted. The mice were anesthetized by intramuscular injection of a mixture containing xylazine (20 mg/kg) and ketamine (50 mg/kg). A 1-mL suspension (containing 1 g of finely divided cachou) was administered to four mice with a thin animal feeding needle inserted into the stomach. EPR spectra were recorded noninvasively using an EPR spectrometer (Magnettech, Berlin, Germany) with a low frequency microwave bridge operating at 1.1 GHz and equipped with an extended loop resonator (Nilges et al. 1989). Great sensitivity of detection was obtained by laying the mouse stomach down on top of the coil so that it was close to the area of reception. The mouse was placed on the same spot during the course of the study. Because the sensitivity of the measurement decreases dramatically when the distance from the resonator increases, and because the stomach region was put directly on the loop resonator, the measurements essentially reflect the fate of these radicals in the stomach region. Animal protocols were approved by the local ethics committee.

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TABLE 1

Electron paramagnetic resonance (EPR) spectroscopy parameters of the preparations containing licorice used in the study

Sample	Origin	g-Value[1]	Linewidth[2]
			mT
1	Licorice root, Laboratory of Pharmacognosy, Université Catholique de Louvain, Brussels, Belgium	2.0046	0.59
2	Licorice powder, Laboratory of Pharmacognosy, Université Catholique de Louvain, Brussels, Belgium	2.0047	0.7
3	Licorice root, Carafa, Italy	2.0039	0.6
4	Potter's, S-C sa, Herentals, Belgium	2.0032	0.5
5	Dol's, Cinfa, Huarte-Pamplona, Spain	2.0047	0.82
6	Curix, Warnimont, Brussels, Belgium	2.0038	0.65
7	JoJo Réglisse, Look-o-Look, Van Melle sa, Antwerpen, Belgium	2.0034	0.5
8	Réglisse Sirea, Montecchio, Italy	2.0037	0.82
9	Killtoïds, Société Dubois, Guerville, France	2.0038	0.73
10	Cachou Ducardon, Mons, Belgium	2.0041	0.82
11	Cachou Lajaunie, Toulouse, France	2.0029	0.05

[1] The g-value is related to the line position through the resonance condition $h\nu = g\beta B$, where h is Planck's constant, ν is the microwave frequency, β is the Bohr magneton and B is the applied magnetic field.

[2] The linewidth is the distance between the maximum peak and the minimum peak of the first derivative spectrum.

RESULTS

All samples contained a free radical with a g-value of 2.003–2.005. The peak-to-peak linewidth of the EPR signals was generally ~0.5–0.8 mT, except for sample 11 for which it was 0.05 mT (Table 1). For a given spin concentration, narrow lines imply large amplitudes and hence a higher detectability compared with broad lines. Therefore, sample 11, which presented a relatively strong EPR signal, was used further for in vivo experiments. Only for this sample was it possible to record an EPR signal with a reasonable signal-to-noise ratio using the 1.1-GHz EPR spectrometer. A typical EPR spectrum recorded in vivo and the kinetic monitoring of the EPR signal intensities are shown in **Figure 1**. The spectrum (*upper panel*, A) is compared with an EPR spectrum recorded from the stomach of a food-deprived mouse (*upper panel*, *B*). No EPR signal was observed in the latter case. After administration of sample 11, the signal intensity (height of the first derivative peak) recorded from the stomach region was rather stable because the signal decreased only ~20% over the time of observation (Fig. 1, *lower panel*).

DISCUSSION

Licorice, the underground part of the *Glycyrrhiza* species, is one of the most frequently used constituents in natural medicine, but is also a flavor used in many sweets (cachou). The phenolic constituents of licorice exhibit a variety of effects such as antioxidant activity and inhibition of oxidative enzyme activity. A recent study elucidated the phenolic constituents of licorice with radical-scavenging activities (Hatano et al. 1997). These compounds, called chalcones, were able to oxidize into stable radicals as demonstrated by EPR spectroscopy (Hatano et al. 1997). Because polyphenols are easily oxidized into radicals, we assumed that free radicals could be detected in preparations containing licorice. A first aim of this study was to demonstrate the presence of free radicals in licorice preparations, particularly in sweets.

In the study of Hatano et al. (1997), which elucidated the structure of the chalcones, the EPR spectrum (obtained after air-oxidation of licorice phenolics in alkaline solution) presented a hyperfine splitting pattern (coupling of the unpaired electron to nuclei in the molecule). Using our preparations, neither the crude preparations nor the air-oxidized alcaline solutions of sweets presented a hyperfine splitting. The EPR spectrum obtained in our case could be the sum of different oxidized phenolics leading to an unresolved EPR spectrum with a broad line. One of the samples (sample 11) presented a

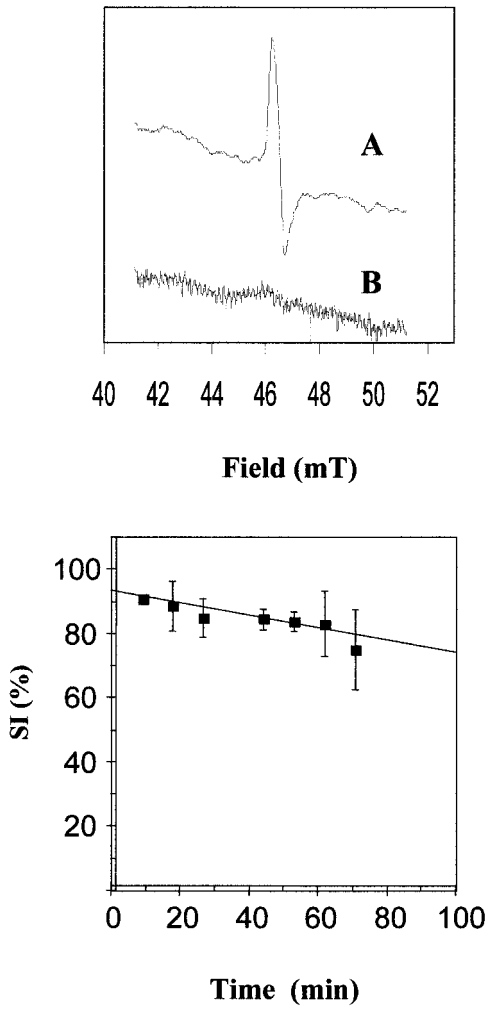

FIGURE 1 In vivo electron paramagnetic resonance (EPR) spectroscopy experiments carried out on mice administered a suspension of licorice-flavored sweet. *Upper panel*: typical EPR spectra recorded in vivo in anesthetized mice using an EPR spectrometer with a low frequency microwave bridge operating at 1.1 GHz and equipped with a surface coil resonator placed on the stomach region. (*A*) Spectrum after oral administration of a licorice-flavored sweet (sample 11) and (*B*) in food-deprived mouse (control). *Lower panel*: evolution with the time of the EPR signal intensities (SI) recorded from the stomach region. The SI corresponds to the height of the peak. For each mouse, the SI was measured immediately after the gavage. The results are expressed as the percentage of this first measurement (mean ± SD, $n = 4$).

narrow EPR line. The EPR spectrum is very intense and sharp, probably due to intermolecular electron spin exchange. This narrow EPR signal could be the result of modification of the microenvironment of the paramagnetic centers during the preparation of these sweets.

Direct evidence of the presence of radicals was obtained in vivo using low frequency EPR spectrometers. Low frequency permits measurement in live animals due to the increased microwave penetration. A great sensitivity of detection was obtained by laying the mouse stomach down on top of the coil so that the organ under study was close to the area of reception. It was possible to record an EPR signal in vivo with a reasonable signal-to-noise ratio. The signal intensity recorded from the stomach region was rather stable because the signal decreased only ~20% over the time of observation (Fig. 1). That indicates a very low reactivity of these radicals in the biological media. It is likely that the decrease in this signal was due to the gastric emptying rather than a reactivity of the radical toward biological components present in the stomach.

In conclusion, this study is the first report demonstrating noninvasively and in vivo the presence of free radicals coming from food. We are further investigating the possibility of monitoring, using low-frequency EPR, the fate of other polyphenols present in food (e.g., fruits, vegetables or tea) and of free radicals coming from irradiated food. This study emphasized that the noninvasive detection of free radicals in the gut using low frequency EPR is not limited to the use of spin labels (Gallez et al. 1996).

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