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Effect of nitric oxide and plant antioxidants on microsomal content of lipid radicals

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ABSTRACT

The antioxidant ability of nitric oxide (NO) generated by a chemical donor and of commercially available antioxidant preparations was assayed. SNAP (S-Nitroso-N-acetylpenicilamine) was used as the NO donor, and Ginkgo biloba, wheat and alfalfa preparations were tested. Lipid peroxidation was assayed by EPR employing a reaction system consisting of rat liver microsomes, ADP, $FeCl_3$, NADPH and POBN in phosphate buffer, pH=7.4. *In vitro* NO exposure decreased microsomal lipid peroxidation in a dose-dependent manner. The dose responsible for inhibiting the microsomal content of lipid radical adducts by 50% (LD_{50}) for SNAP was 550 μM (NO generation rate 0.1 μM/min). The addition of 50 μM hemoglobin to the incubation media prevented NO effect on lipid peroxidation. The addition of an amount of the antioxidant preparations equivalent to the LD_{50} doses inhibited lipid peroxidation by 21, 15, and 33% for wheat, alfalfa, ginkgo biloba preparations respectively in the presence of 550 μM SNAP. We detected a decrease in the content of lipid radical adducts after simultaneous supplementation, although it was less than 50%, even when LD50 doses of the products were added. This suggests that NO and the natural antioxidants inhibit lipid peroxidation by a mechanism that has both common and non-shared features.

KEY TERMS: antioxidants, nitric oxide, plants extracts, lipid radicals.-

INTRODUCTION

The discovery that the molecular free radical nitric oxide (NO) is endogenously formed in biological systems regulating a wide variety of physiological functions has led to the investigation of the role that this molecule plays in various toxicological mechanisms (Moncada et al., 1991; Ignarro et al., 1990; Feldman et al., 1992). NO produced by a variety of mammalian cells, including endothelium, neuronal cells, smooth muscle cells, macrophages, neutrophils, platelets, fibroblasts, and type II pneumocytes, mediates a variety of biological actions ranging from vasodilatation, neurotrasmission, inhibition of platelet adherence and aggregation, and macrophage- and neutrophil-mediated killing of pathogens (Moncada et al., 1991). As has been extensively described (Wink et al., 1995), this molecule forms various reactive nitrogen oxide species in the presence of oxygen, which can be a damaging factor to biological systems. Contrary to the deleterious effects of the reactive nitrogen oxide species formed from either NO/O_2 and NO/O_2^-, NO showed antioxidant properties and played a beneficial role in diseases such as atherosclerosis (Hogg et al., 1993; Rubbo et al., 1994). The biological chemistry of this molecule is dominated by free-radical reactions, and interaction of NO with other free-radical species leads to either inhibition or potentiation of oxidative damage (Beckman et al., 1990). One mechanism by which NO can inhibit lipid peroxidation has been postulated to involve reaction between NO and lipid-derived radicals (e.g. peroxyl, alkoxyl radicals) (Hogg et al., 1993; Rubbo et al., 1994).

In addition to NO, numerous free radical scavengers and antioxidants can reduce lipid peroxidation and the generation of reactive oxygen species. The protection that

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fruits and vegetables provide against diseases, including cancer, cardio and cerebrovascular diseases, has been attributed to the various antioxidants they contain (Ames, 1983; Gey, 1990; Steinberg et al., 1989; Wang et al., 1996). Thus, the supplementation of natural antioxidants in addition to a balanced diet has been suggested to protect the body against oxidative damage under different conditions. The ability of wheat bran, alfalfa and ginkgo biloba extracts to inhibit rat liver microsomal NADPH-dependent lipid peroxidation was previously reported (Boveris and Puntarulo, 1998).

The goal of this study employing EPR detection techniques was to evaluate NO ability to decrease the lipid radical content in biological membranes after *in vitro* exposure. Effectiveness of commercially available plant antioxidants to protect against lipid peroxidation was studied in the presence of a nitric acid source.

MATERIALS

Wheat bran and alfalfa tablets by Natura-Vigor (Arnet Pharmaceutical Corp., Hialeah, Florida, USA) and Ginkgo biloba by Japan Greenwave (Jarrow Formulas) were employed. Extracts were prepared by dissolving the commercially available capsules in destilled water. Livers from male Wistar rats (200 g) were excised and microsomes were prepared isolated by differential centrifugation as previously described (Klein et al., 1983).

METHODS

EPR-spin trapping of lipid radicals

Rat liver microsomes prepared by differential centrifugation were incubated for 20 min at 37°C in the presence of α-(4-pyridyl 1-oxide)-N-t-butyl nitrone (POBN), 100 mM; ADP, 5.5 mM; $FeCl_3$, 0.1 mM; NADPH, 1 mM; buffer phosphate 100 mM, pH=7.4. EPR instrument settings were microwave power, 20 mW; modulation amplitude, 1.232 G; time constant, 81.92 ms; receiver gain, 2 x 10^4 (Jurkiewicz and Buettner, 1994). All EPR spectra were obtained at room temperature using a Bruker ECS 106 ESR with a ER4102ST cavity.

NO detection by EPR

SNAP (S-Nitroso-N-acetylpenicilamine) was used as the NO donor. Different amounts were prepared in MGD-Fe^{2+}(10:1), pH=7.4. The EPR settings were microwave power, 20 mW; modulation amplitude, 6.175 G; time constant, 164 ms; modulation frequency, 50 kHz; receiver gain, 2.10^4; sweep with 150 G; conversion time 163 ms and microwave frequency, 9.81 Ghz (Komarov and Lai, 1995). Quantitation of the spin adduct was performed using an aqueous solution of 2,2,5,5-tetramethyl piperidine 1-oxyl (TEMPO) (Kotake et al., 1996).

Statistical Analysis

Data in the text and figures are expressed as means ± SEM of 4 to 6 independent experiments. Statistical tests were carried out using Statview SE+, version 1.03 (Abacus Concepts Inc., Berkeley, CA, USA).

RESULTS

EPR spin trapping measurements using the NO trap MGD-Fe^{2+} were recorded at room temperature in the presence of SNAP as the NO source. The EPR signal is characterized by an isotropic triplet signal at g = 2.03 and a^N= 12.5 G; its features are unique and enable a fingerprint-like identification of NO (Fig. 1A). The amount of spin adduct is calibrated using an aqueous solution of TEMPO, introduced into the same cell used for spin trapping. EPR spectra of spin adduct solution and TEMPO solution were recorded at exactly the same spectrometer settings. The first-derivative EPR spectra were double integrated by a computer attached to the EPR spectrometer to obtain the area intensity, and the concentration of

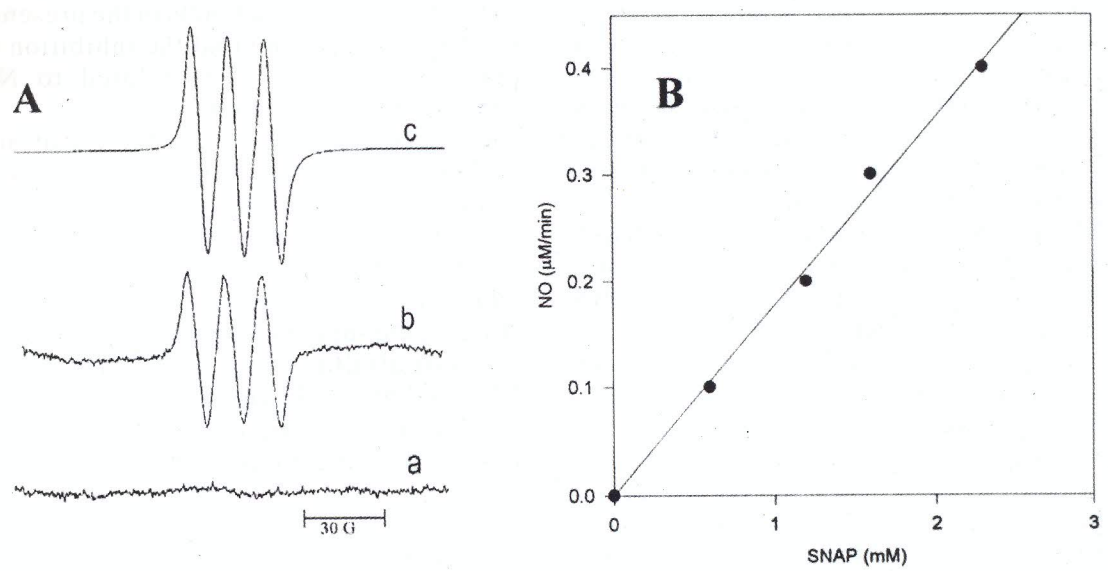

Figure 1: EPR detection of NO. **A.** (a) control spectrum in the presence of 10 mM MGD, and 1 mM $FeSO_4$; (b) typical EPR spectra of the MGD-Fe-NO adduct in a reaction medium consisting of 10 mM MGD, and 1 mM $FeSO_4$ and 1 mM SNAP; (c) computer-simulated spectrum of MGD-Fe-NO adduct. **B.** NO generation rate as a function of the concentration of the NO-donor SNAP. No signal was detected in the absence of SNAP and the presence of NAP, the product of SNAP reaction to generate NO.

spin adduct was then calculated using the ratio of these areas. It has been described previously (Caro and Puntarulo, 1998) that the area intensity of the EPR signal was proportional to NO concentration, and the efficiency of NO trapping (concentration ratio NO adduct vs. added NO) was 90% within the NO concentration range from 0.5 to 10 µM (Caro and Puntarulo, 1998). Figure 1B shows lineal dependence (up to 2.5 mM SNAP) of NO generation rate with SNAP concentration added to the incubation medium.

The decomposition of hydroperoxides formed during NADPH-dependent peroxidation in rat liver microsomes supplemented with iron led to the generation of lipid radicals such as peroxyl (ROO•), alkoxyl (RO•) and alkyl (R•) radicals. To characterize the antioxidant ability of NO, the membrane content of these radicals was measured by EPR in the presence of ferric chloride as the iron catalyst and NADPH as the reductant for the microsomal electron transfer system. Lipid radicals combined with the spin trap POBN resulted in adducts that gave a characteristic EPR spectrum with hyperfine coupling constants of a^N= 15.8 G and a^H= 2.6 G (Fig. 2), in agreement with computer spectral simulated signals obtained using those parameters. Even

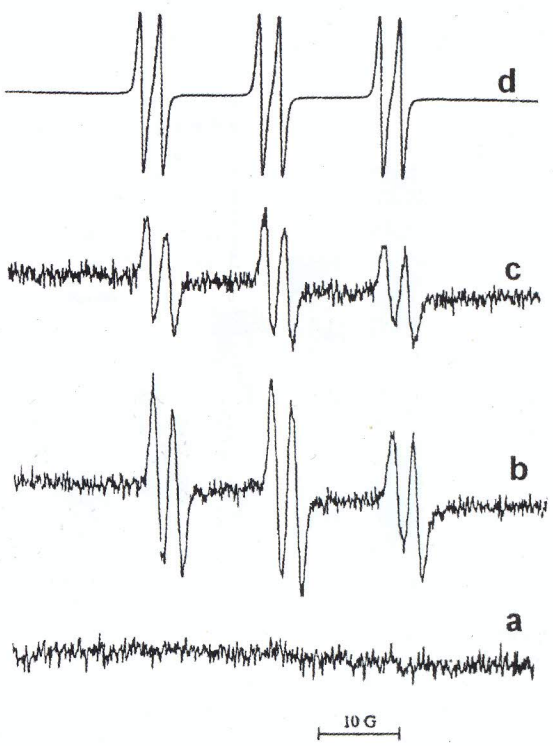

Figure 2: EPR spectra of POBN-lipid radical adducts generated by rat liver microsomes. (a) spectrum in the presence of 100 mM POBN, phosphate buffer 100 mM (pH=7.4), 1 mM NADPH, 100 mM $FeCl_3$ and 5.5 mM ADP; (b) basal system consisting of reaction medium a and rat liver microsomes (2 mg protein/ml); (c) idem b, but in the presence of 550 mM SNAP; and (d) computer-simulated spectrum of POBN-lipid radical adducts.

though these constants could be assigned to lipid radicals (Buettner, 1987), spin trapping studies cannot readily distinguish between ROO•, RO• and R• adducts, owing to the similarity of the corresponding coupling constants. The supplementation to the incubation medium with 550 μM SNAP significantly decreased the POBN-lipid radical EPR signal in comparison with the control, which represents 100% POBN-lipid radical adduct (Fig. 2).

A significant decrease in POBN-lipid radical adducts was detected in microsomal membranes exposed to 0.14, 0.28, 0.55 and 1.1 mM SNAP during 20 min (Fig. 3). NO generation rates, assessed as indicate in Figure 1, were 23, 47, 92 and 183 nM/min. Under these conditions, the LD50 for SNAP calculated from the activity-concentration curve was 550 μM, as it was SNAP concentration required for inhibiting lipid radical content by 50% (Fig. 3, inset). The supplementation of 25 μM hemoglobin to the incubation medium completely prevented the decrease in the content of POBN-lipid radical adducts in the presence of SNAP, suggesting that the inhibition of peroxidation showed is related to NO generation (Fig. 3, inset).

The LD50 doses for alfalfa, wheat and ginkgo biloba preparations as inhibitors of lipid peroxidation were previously reported as 12.4 ± 0.2, 7.7 ± 0.3, and 1.20 ± 0.06 mg/ml, respectively (Boveris and Puntarulo, 1998). In the presence of 550 μM SNAP (LD50), the supplementation with the LD50 doses of alfalfa, wheat and ginkgo biloba inhibited by 30, 42 and 65% the content of POBN-lipid radical adducts in the microsomal membranes (Fig. 4).

DISCUSSION

The EPR data presented here indicate that NO can abate lipid peroxidation, perhaps by intercepting various lipid radicals forming ROONO adducts, which results in the termination of the chain propagation reactions, as was previously suggested by

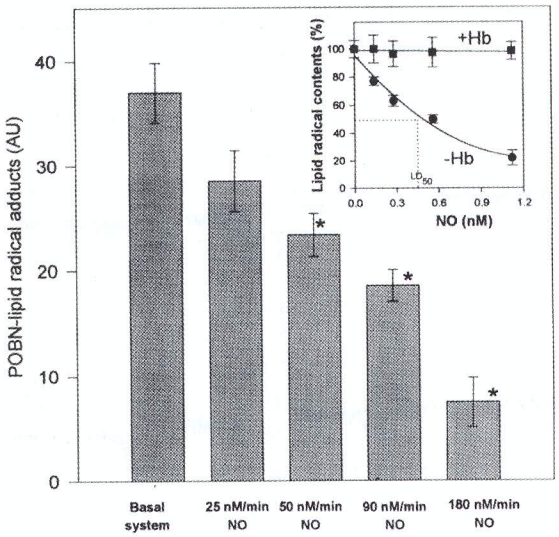

Figure 3: Effect of SNAP supplementation on microsomal content of lipid radicals.
Basal system consisted of 100 mM POBN, phosphate buffer 100 mM (pH=7.4), 1 mM NADPH, 100 mM $FeCl_3$, 5.5 mM ADP, and rat liver microsomes (2 mg protein/ml). The required SNAP concentration was supplemented during 20 min.
Inset: Hemoglobin effect on NO dependent inhibition of lipid peroxidation. Hemoglobin (25 mM) was added to the incubation medium during the 20 min reaction. LD_{50} stands for the low doses that decreased the content of lipid radical adducts by 50%.
*Significantly different respect to basal system. ANOVA ($p<0.05$).

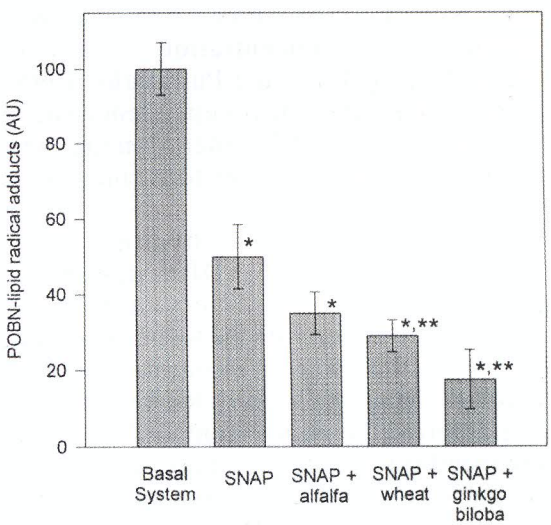

Figure 4: Effect on the content of lipid radical adducts in microsomal membranes of the supplementation of commercially available antioxidants in the presence of SNAP. SNAP was supplemented to a final concentration of 550 mM (LD_{50}). LD_{50} doses of SNAP were supplemented with either 12.4 mg/ml alfalfa, 7.7 mg/ml wheat, or 1.2 mg/ml ginkgo biloba (LD_{50} of each preparation, respectively).
* significantly different with respect to basal system. ANOVA ($p<0.05$).
**significantly different with respect to SNAP supplemented system. ANOVA ($p<0.05$)

indirect measurements (Wink et al., 1995). NO is an iron ligand that contains an unpaired electron and has been used as a spin labeled ligand for probing the electronic structure of the heme group and heme environments (Kanner et al., 1991). Thus, NO could modulate oxidative reactions, particularly the generation of cytotoxic oxygen species or tetravalent iron. Kanner et al. (1991) also proposed that these reactions included (i) modulation of the reactivity of iron-heme and non-heme compounds; (ii) chelation of *free* iron in a form that prevents or decreases its potential to generate cytotoxic oxygen species; and (iii) scavenging of free radicals. Moreover, it was suggested that NO can protect cells from the deleterious effects of the reactive oxygen species generated through the reaction of ferryl intermediates with NO to avoid the formation of ferric species (Ames et al., 1993). An alternative to this explanation is that lipid peroxidation in biological membranes is decreased after NO exposure as a result of a different mechanism that involves cytochrome P_{450} inactivation. Puntarulo and Cederbaum (1997) showed that NO-related decrease in P_{450} content in microsomal membranes affected O_2^- production and iron release from ferritin. Moreover, the interaction between an iron compound and NO has complementary effects that change the reactivity of both the iron ion and NO toward other molecules. Since the rate constant of NO with iron-heme and non-heme compounds seems to reach a diffusion controlled limit (Kanner et al., 1991), once the NO-iron adducts are generated they are very stable (Moore and Gibson, 1976). Thus, it might be possible that the interaction NO-iron are the main reactions responsible for NO inhibition of peroxidation in microsomal membranes after exposure to SNAP.

The role of iron and superoxide anion in the initiation step of lipid peroxidation have been discussed extensively (Aust et al., 1985; Puntarulo and Cederbaum, 1988; Ursini et al., 1989). Regarding the commercially-available antioxidants, it was suggested that ginkgo biloba is a scavenger of peroxyl radicals generated in both lipid and aqueous environments (Maitra et al., 1995; Boveris and Puntarulo, 1998), but iron reduction did not seem to be a critical factor in the scavenging mechanism of ginkgo biloba. It has been reported that the iron reduction rate by rat liver microsomes was not affected by antioxidant supplementation (Boveris and Puntarulo, 1998). However, the ability of ginkgo biloba preparations to limit microsomal O_2^- radical generation could be of interest in terms of its scavenging activity. Even though the O_2^- radical is not particularly reactive, by removing it, ginkgo biloba would reduce or eliminate the formation of H_2O_2 and the reactive and toxic hydroperoxyl radicals derived from O_2^-. Reducing the formation of H_2O_2 would also reduce the formation of $^{\bullet}OH$ generated by Fenton (1894) or Haber and Weiss (1934) reactions that could participate in chain reactions. In this regard, it was shown that ginkgo biloba was active as an inhibitor of $^{\bullet}OH$ production by rat liver microsomes (Boveris and Puntarulo, 1998). On the other hand, the ability of wheat and alfalfa preparations to inhibit lipid peroxidation seems to be related to their ability of scavenging lipid radicals, since O_2^- and $^{\bullet}OH$ radical generation, as well as iron reduction by rat liver microsomes were not affected after *in vitro* supplementation with these antioxidants (Boveris and Puntarulo, 1998).

The results presented here show that the content of POBN-lipid radical adducts in the membranes significantly decreased after exposure to either SNAP or plant preparations. However, in the presence of SNAP, the supplementation with the plant antioxidants was not able to give the same degree of protection that was shown in its absence. Thus, even though NO and the commercially available products are efficient inhibitors of lipid peroxidation *in vitro*, a different reactivity to iron could be the distinctive characteristic between the plant products and NO. Regarding mechanisms of action, all of the tested substances seemed to share, at least partially, common features such as scavenger properties towards lipid radicals. Ginkgo biloba showed a more significant effect than the other tested products as an

inhibitor of lipid peroxidation, probably due to the broader spectrum of reactions in which it is involved.

This information must be taken into consideration for further biotechnological developments of protective antioxidants, which could have important applications in human diseases accompanied by free radical injury. Since any biologically active compound supplemented in the diet must appear in the target tissues in significant amounts to elicit bioprotective effects, future studies should consider interactions of the dietary supplement with endogenous antioxidants (such as NO), as well as tissue specificity, compartmentalization, and concentration levels of the active compound(s) in target organs after supplementation in order to appropriately assess effectiveness *in vivo* (which could be even higher than expected) and to define the appropriate pathological or physiological situations to be used.

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