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Polyphenols and Red Wine as Antioxidants against Peroxynitrite and other Oxidants

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ABSTRACT

The antioxidant capacity of polyphenols (+)-catechin, (-)-epicatechin and myricetin, and of different types of red wines (Cabernet Sauvignon, Malbec and blended wine) was evaluated by three assays. (a) *NADH oxidation by peroxynitrite (ONOO$^-$):* the ONOO$^-$ scavenging activity was higher for myricetin (IC$_{50}$=35 µM) than for (+)-catechin (IC$_{50}$=275 µM) and (-)-epicatechin (IC$_{50}$=313 µM). (b) *Peroxynitrite initiated chemiluminescence in rat liver homogenate:* (-)-epicatechin (IC$_{50}$=7.0 µM) and (+)-catechin (IC$_{50}$=13 µM) were more potent than myricetin (IC$_{50}$=20 µM) in inhibiting the chemiluminescence signal. (c) *Lucigenin chemiluminescence in aortic rings:* (-)-epicatechin (IC$_{50}$=15 µM) and (+)-catechin (IC$_{50}$=18 µM) showed higher antioxidant capacity than myricetin (IC$_{50}$=32 µM). All the assayed *red wines* were able to scavenge the oxidants and free radical species that generate the signal in each assay. Cabernet Sauvignon was the red wine with the highest antioxidant capacity in comparison with Malbec and blended wine. It is concluded that the use of sensitive biological systems (as the aortic ring chemiluminescence) provides important information in addition to the results from chemical (NADH oxidation by peroxynitrite) and biochemical (homogenate chemiluminescence) assays and offers advances in the physiological role of polyphenols.

Key words: Antioxidant capacity, peroxynitrite, polyphenols, red wines, superoxide anion.

Abbreviations: GAE: gallic acid equivalent; GSH: glutathione; HO$^\bullet$: hydroxyl radical; NO: nitric oxide; NOS: nitric oxide synthase; ONOO$^-$: peroxynitrite; ONOOH: peroxynitrous acid; O$_2^-$: superoxide anion.

INTRODUCTION

Red wine is a rich source of phenolic compounds (flavonoids and non-flavonoids) and its antioxidant capacity has been shown in different in vitro and in vivo systems. Plant polyphenols act as reducing agents and antioxidants by the hydrogen-donating property of their hydroxyl groups (Bors et al., 1990) as well as by their metal-chelating abilities (Brown et al., 1998). A relatively high reactivity of flavonoids with radicals such as superoxide anion (O$_2^-$) (Sichel et al., 1991), hydroxyl radical (HO$^\bullet$) (Bors et al., 1990) and nitric oxide (NO) (Haenen and Bast, 1999) as well as with

oxidants such as peroxynitrite (ONOO$^-$) (Haenen et al., 1997) has been reported.

Peroxynitrite is a strong oxidizing and nitrating species that reacts with different biomolecules and is produced in vivo through the reaction of NO and O$_2^-$, both as an intracellular and as an extracellular metabolite. The physiological and pathological importance of the triad NO, O$_2^-$ and ONOO$^-$ in the vascular space is being recognized.

The aim of this work was to assess the antioxidant capacity of different wines and a variety of polyphenols ((+)-catechin, (-)-epicatechin and myricetin) and hydroxycinnamates (caffeic acid, ferulic

acid and chlorogenic acid) present in wines. Three experimental models were developed: (a) *chemical*: NADH oxidation by peroxynitrite; (b) *biochemical*: peroxynitrite initiated chemiluminescence in rat liver homogenate; and (c) *biological*: lucigenin chemiluminescence in aortic rings. The chemical model is based on an indirect fluorometric technique that involves the participation of ONOO⁻ as oxidant and NADH as target molecule. Phenolic compounds and red wines were used in competition with NADH. The biochemical model consists in the detection of the light emission triggered by the addition of ONOO⁻ in rat liver homogenate, in the absence or in the presence of phenolic compounds. The biological model determines the luminescence produced during the reaction of lucigenin with active oxygen species (mainly O_2^-) generated by aortic rings, in the absence or in the presence of polyphenols and red wines.

METHODS

Peroxynitrite synthesis

Peroxynitrite (ONOO⁻) was prepared by reacting 2 M H_2O_2 in 2 M HNO_3 with 2 M $NaNO_2$, followed by stabilization of the product with 4 M NaOH. Hydrogen peroxide was removed by the addition of granular MnO_2. The solution was frozen at -70ºC. Peroxynitrite concentration was determined spectrophotometrically at 302 nm (ε = 1670 M^{-1} cm^{-1}) and dilutions in 1 M NaOH were made in order to achieve solutions of the desired concentrations (Uppu et al., 1996).

Chemical model: NADH oxidation by peroxynitrite

The oxidation of NADH by ONOO⁻ was followed fluorometrically at 37ºC in a reaction medium consisting of 100 µM phosphate buffer, pH 7.0, 0.1 mM DTPA, 100 µM NADH and 200 µM ONOO⁻ at 340-463 nm. In competition with NADH, glutathione (GSH; 0-150 µM), plant polyphenols (0-200 µM), hydroxycinnamates (0-200 µM), and

red wines (5-50 µl of a 1/5 dilution) were used. This technique was used to estimate the rate constants of the reactions of ONOO⁻ with phenolic compounds, and the concentrations inhibiting 50 % NAD formation (IC_{50}) of the different red wines, according to Valdez et al. (2000).

Biochemical model: Peroxynitrite initiated chemiluminescence in rat liver homogenate

– *Rat liver homogenates*: Female Sprague-Dawley rats of 150 g, fed with a conventional laboratory diet and water *ad libitum* were used. The animals were sacrificed and the liver was immediately excised. The organ was homogenized in 120 mM KCl, 30 mM phosphate buffer, pH 7.4 (1:9 dilution) and centrifuged at 700*g* for 10 min at 0-4ºC. The pellet was discarded and the supernatant was used as homogenate (Boveris et al., 1972). Protein content was measured by the Folin-Ciocalteu reagent using bovine serum albumin as standard (Lowry et al., 1951).

– *Peroxynitrite initiated chemiluminescence*: It was measured in a LKB Wallac, 1209 Rackbeta liquid scintillation counter (Turku, Finland) in the out of coincidence mode. This counter has photomultipliers responsive in the range of 380-620 nm. Samples were placed in 10 mm-diameter and 35 mm-height flasks, which were placed inside 25 mm-diameter and 50 mm-height low potassium glass vials. The vials were kept in the dark up to the moment of assay and determinations were carried out in a dark room in order to avoid vial phosphorescence activated by fluorescent light. The emission from the empty flasks and vials was negligible. The experimental conditions were: 1 mg/ml of homogenate protein in a reaction medium containing 120 mM KCl, 30 mM phosphate buffer, pH 7.4, 0.1 mM DTPA, at 30ºC, 200 mM ONOO⁻ and different concentrations (5-50 µM) of myricetin, (+)-catechin and (-)-epicatechin. The wines were used in a 10-fold dilution in distilled water and were added in different volumes (5-50 µl). The emission in the absence of ONOO⁻ was 7000 ± 350

total counts. The results were expressed as total counts/mg protein (Alvarez et al., 2002).

Biological model: Lucigenin chemiluminescence in aortic rings

– *Aortic rings*: Female Sprague-Dawley rats of 200 g were injected with pentobarbital sodium (50 mg/kg) and 1000 U of heparin. Thoracic aorta was removed and placed in prewarmed Hepes-buffered salt solution (145 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.0 mM KH_2PO_4, 1.75 mM $CaCl_2$, 0.03 Na_2EDTA, 5.5 mM glucose, and 10 mM HEPES, pH 7.4) bubbled with 95 % O_2-5 % CO_2 (v/v) and added with 10 mM diethyl-dithio-carbamate (Pagano et al., 1993; Wang et al., 1997). The vessels were cleaned, cut into rings (5 mm in length) and incubated in fresh buffer at 37ºC for a 30-min equilibration period.
– *Lucigenin chemiluminescence in aortic rings:* It was measured in a LKB Wallac, 1209 Rackbeta liquid scintillation counter (Turku, Finland) in the out of coincidence mode, as described above. The reaction medium consisted in Hepes-buffered salt solution (145 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.0 mM KH_2PO_4, 1.75 µM $CaCl_2$, 0.03 Na_2EDTA, 5.5 mM glucose, and 10 mM HEPES, pH 7.4) bubbled with 95 % O_2-5 % CO_2 (v/v), 250 µM lucigenin (10,10-dimethyl-bis-9,9-bisacridinium nitrate) and 100 µM NADH (Pagano et al., 1993). The polyphenols were added at concentrations ranging from 5 to 50 µM. After the addition of polyphenols or wine, an aortic ring was placed in the reaction medium, and the luminescence recorded. The wines were used in a 10-fold dilution in distilled water and were added in different volumes (5-50 µl). The control emission (without lucigenin) was 3000 ± 180 counts. The counts were integrated over 10-s intervals. The results were expressed as total counts at 240 s/g of tissue.

Statistics

The values in the text and tables indicate mean values ± SEM. The significance of

differences between means was analyzed by the ANOVA-Dunnett´s test of variance analysis.

RESULTS

Chemical model: NADH oxidation by peroxynitrite

The addition of up to 400 µM $ONOO^-$ produced an almost stoichiometric NADH oxidation at a ratio of about 0.25 NAD formed/$ONOO^-$ added. The supplementation of the reaction medium with polyphenols and hydroxycinnamates decreased the extent of NADH oxidation by $ONOO^-$ (Figure 1A and Table I). The highest value of the rate constants was the one corresponding to the reaction of $ONOO^-$ with myricetin; the lowest values were for catechins and ferulic acid (Table I). The IC_{50} were inversely related, and in agreement with the given second order reaction constants (Fig 1A).

NADH oxidation by $ONOO^-$ was markedly influenced by pH. When pH raised from 5.5 to 7.0 an increase of 40 % in NADH oxidation was observed (66 ± 0.5 µM NAD in comparison with 90 ± 0.6 µM NAD). In addition, the protective activity of (+)-catechin and caffeic acid was also pH dependent, likely due to the ionization of phenol and carboxyl groups in flavonoids and hydroxycinnamates (Fig 2).

Biochemical model: Peroxynitrite initiated chemiluminescence

Peroxynitrite induced the light emission of rat liver homogenate. Light emission showed a maximal value at initial time, suffering then an exponential decay (Fig 3A). The supplementation of the reaction medium with 5-30 µM (+)-catechin resulted in a decrease in maximal emission (Fig 3B). The calculated IC_{50} showed that (+)-catechin and (-)-epicatechin were more potent inhibitors of peroxynitrite initiated chemiluminescence than myricetin (Fig 1B).

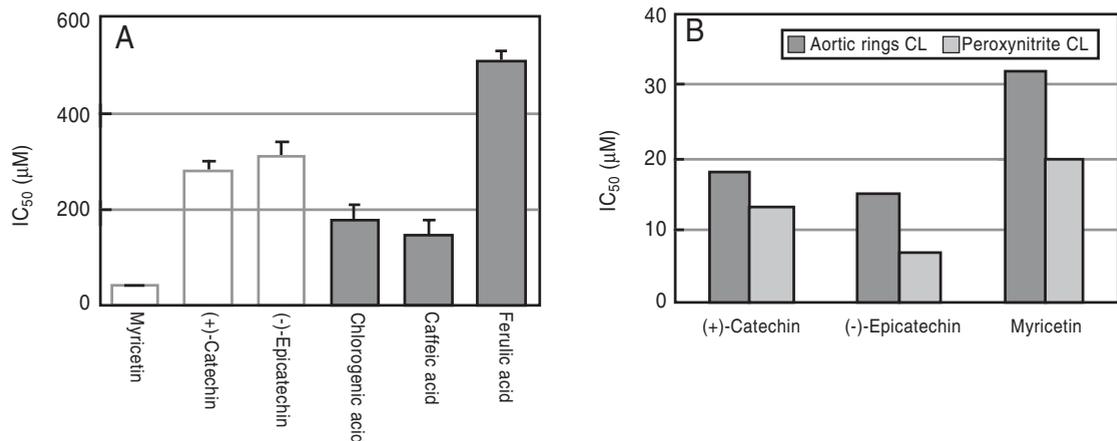Chemiluminescence initiated by $ONOO^-$ was modified by medium pH. When pH

Figure 1. Concentrations of polyphenols and hydroxycinnamates producing 50 % inhibition (IC$_{50}$) of NADH oxidation induced by peroxynitrite (A) and of light emission (B).

TABLE I

Apparent second-order rate constants of peroxynitrite reactions with plant polyphenols and hydroxycinnamates

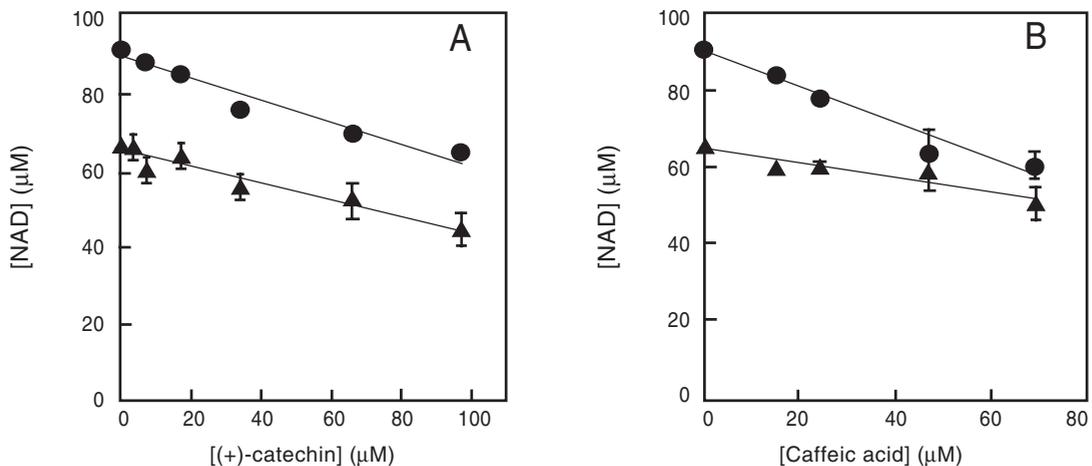|	Reductant	k $(10^3$ M^{-1} s^{-1})
Control	NADH	1.8 ± 0.1
	Glutathione	1.4*
Flavonoids	Myricetin	4.9 ± 0.3
	(+)-Catechin	0.65 ± 0.05
	(-)-Epicatechin	0.59 ± 0.05
Hydroxycinnamates	Chlorogenic acid	1.1 ± 0.2
	Caffeic acid	1.3 ± 0.3
	Ferulic acid	0.63 ± 0.05

*Quijano et al., 1997

Figure 2. Chemical model: NAD formation at pH 7.0 (●) and pH 5.5 (▲) for different concentrations of (+)-catechin (A) and caffeic acid (B).

raised from 6.0 to 7.0 an increase of 150 % in chemiluminescence signal was observed: (0.7 ± 0.1) 10^5 total counts/mg protein, in comparison to (1.7 ± 0.2) 10^5 total counts/mg protein.

Biological model: Lucigenin chemiluminescence in aortic rings

Lucigenin chemiluminescence in aortic rings showed a time dependent increase of light emission that reached a maximal response at 240 s (Fig 4A). The addition of 5-50 µM (+)-catechin (Fig 1B - Fig 4B) to the reaction medium produced a decrease in the chemiluminescence signal. In the same way, the addition of (-)-epicatechin and myricetin to the reaction medium exerted a protective effect evidenced by the inhibition of lucigenin chemiluminescence. The antioxidant capacity of catechins was approximately two-fold the myricetin one, as shown in Figure 1B.
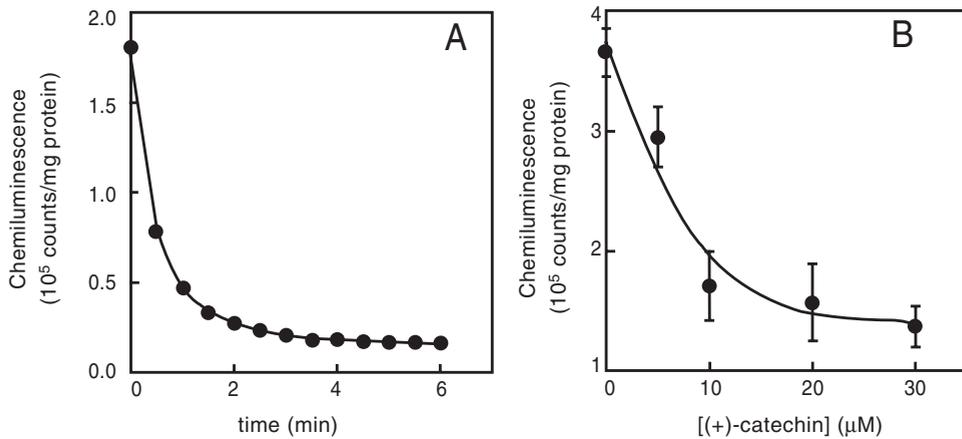

Figure 3. Biochemical model: A. Time course of peroxynitrite initiated chemiluminescence in rat liver homogenate. B. Effect of 0-30 µM (+)-catechin on maximal chemiluminescence signal.
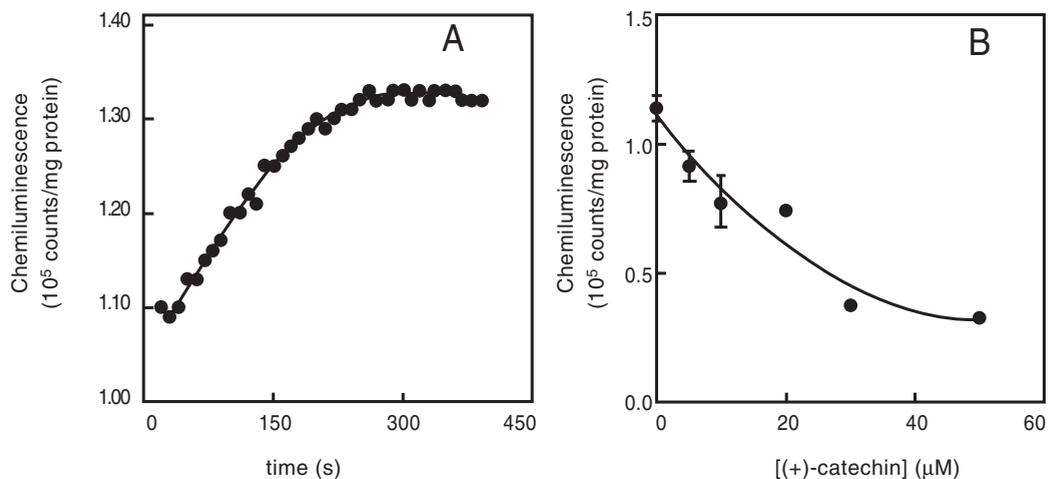

Figure 4. Biological model: A. Time course of lucigenin chemiluminescence in aortic rings. B. Effect of 0-50 µM (+)-catechin on lucigenin light emission.

Antioxidant capacity of red wines

All red wines assayed showed $ONOO^-$ and O_2^- antioxidant activities that were related to the phenolic content of the samples (Table II). This correlation was excellent ($r^2 = 0.99$) in the case of the chemical model (NADH oxidation technique). As shown in Table II, the grape strain Cabernet Sauvignon gave the more potent antioxidant effect with all three methods assayed, the Malbec strain being slightly less effective. The blended red wine was the sample with less antioxidant capacity.

TABLE II

Relationship between phenolic content and IC_{50} of wine samples

	Phenolic content	IC_{50} (µl/ml)		
	(GAE, mg/ml)	Chemical	Biochemical	Biological
Cabernet Sauvignon	2011 ± 41	22	36	21
Malbec	1441 ± 49	28	104	27
Blended	814 ± 50	44	424	19

DISCUSSION

Peroxynitrite ($ONOO^-$) is formed and mediates reactions in both the intracellular and the extracellular spaces. In the vascular space, $ONOO^-$ is produced by polymorphonuclear leukocytes (Carreras et al., 1994) and monocytes (Valdez and Boveris, 2001). In these cells, superoxide anion (O_2^-) is generated by plasma membrane-bound NADPH oxidase (Babior, 1995) and nitric oxide (NO) is produced by cytosolic nitric oxide synthase (NOS) and diffuses to the extracellular space. Phenolic compounds are bioavailable and reach plasma as glycosides and conjugates (Paganga and Rice Evans, 1997; Aziz et al., 1998) to behave as plasmatic $ONOO^-$ scavengers.

In this study we determined the scavenger properties of plant polyphenols and red wines using three experimental models. The chemical model determines the chemical trapping and the antioxidant capacity of polyphenols and red wines against $ONOO^-$. It is a sensitive method and allows the use of low concentrations of $ONOO^-$ scavengers and the estimation of reaction rate constants. The results shown in Table II indicate that the antioxidant capacity of red wines against $ONOO^-$ is strictly related to the amount of phenolic compounds. On the other hand, $ONOO^-$ is able to initiate lipid peroxidation (Radi et al., 2000). It has been shown that the products of the reaction between $ONOO^-$ and fatty acids (O´Donnell et al., 1999) appear to be highly unstable and may decompose to reinitiate radical processes. In our studies, $ONOO^-$ addition initiated rat liver homogenate light emission. Flavonoids and red wines produced a reduction of chemiluminescence, attributable not only to the direct reaction of phenolic compounds with $ONOO^-$ but also to the interference with free radical chain reactions.

Both NADH oxidation and rat liver homogenate chemiluminescence were influenced by the pH of the reaction medium. This is likely due to the ionic nature of $ONOO^-$. At lower pH, the proton catalyzed formation of peroxynitrous acid (ONOOH) is favored, leaving less peroxynitrite concentration ($ONOO^-$) able to react with reduced compounds (i.e. NADH) by one-electron oxidation reactions, or to initiate peroxidation reactions. Concerning the pH dependence of antioxidant properties showed by caffeic

acid in the NADH oxidation produced by ONOO$^-$ (Fig 2), the higher efficiency was observed at pH 7.0 at which there is a higher proportion of anionic species of the phenolic compound. The phenolate species is more readily oxidized to semiquinone than the protonated phenol.

It has been reported that the most important source of O$_2^-$ in rabbit (Pagano et al., 1993) and rat (Wang et al., 1998) thoracic aorta are the endothelial cells facing the vascular space. The O$_2^-$ generated by rat aorta, mainly derived from the adventitia, is due to the activity of NADPH oxidase. Lucigenin chemiluminescence triggered by the O$_2^-$ generated in the aorta provides another sensitive approach for the study of the antioxidant capacity of polyphenols and red wines against O$_2^-$. Fitzpatric et al. (1993) suggested that the special cardioprotective effect of wine might partly be due to the vasodilation effects of its phenolic compounds and of tannic acid. It has been reported that the exposition of isolated vascular rings to red wine leads to an increase in the vascular cGMP content (Flesch et al., 1998). In addition, it is likely that polyphenols and red wines react with O$_2^-$ (Bors et al., 1990), which would increase NO biological half-life, producing a vasodilation effect (Cuevas, 2004).

In summary, ONOO$^-$ dependent reactions (NADH oxidation and chemiluminescence) seem to be effective methods to estimate the capacity of different compounds to prevent ONOO$^-$ damage in biological systems. In addition, lucigenin chemiluminescence in aortic rings provides another sensitive approach for the study of the antioxidant capacity of polyphenols and red wines against O$_2^-$ and related free radical reactions.

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