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Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta

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1 The aim of this work was to investigate the mechanism of vasorelaxation induced by red wine polyphenolic compounds (RWPC) and two defined polyphenols contained in wine, leucocyanidol and catechin. The role of the endothelium, especially endothelium-derived nitric oxide (NO), was also investigated.

2 Relaxation produced by polyphenols was studied in rat aortic rings with and without functional endothelium, pre-contracted to the same extent with noradrenaline (0.3 and 0.1 μM, respectively). RWPC and leucocyanidol, but not catechin, produced complete relaxation of vessels with and without endothelium. However, 1000 fold higher concentrations were needed to relax endothelium-denuded rings compared to those with functional endothelium.

3 High concentrations of catechin (in the range of 10^{-1} g l^{-1}) only produced partial relaxation (maximum 30%) and had the same potency in rings with and without endothelium.

4 The NO synthase inhibitor, N^{ω}-nitro-L-arginine-methyl-ester (L-NAME, 300 μM) completely abolished the endothelium-dependent but not the endothelium-independent relaxations produced by all of the polyphenolic compounds.

5 In contrast to superoxide dismutase (SOD, 100 u ml^{-1}), neither RWPC nor leucocyanidol affected the concentration-response curve for the NO donor, SIN-1 (3-morpholino-sydnonimine) which also produces superoxide anion (O_2^-).

6 In aortic rings with endothelium, RWPC (10^{-2} g l^{-1}) produced a 7 fold increase in the basal production of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) which was prevented by L-NAME (300 μM).

7 Electron paramagnetic resonance (e.p.r.) spectroscopy studies with Fe^{2+}-diethyldithiocarbamate as an NO spin trap demonstrated that RWPC and leucocyanidol increased NO levels in rat thoracic aorta about 2 fold. This NO production was entirely dependent on the presence of the endothelium and was abolished by L-NAME (300 μM).

8 These results show that RWPC and leucocyanidol, but not the structurally closely related polyphenol catechin, induced endothelium-dependent relaxation in the rat aorta. They indicate that this effect results from enhanced synthesis of NO rather than enhanced biological activity of NO or protection against breakdown by O_2^-. It is concluded that some polyphenols, with specific structure, contained in wine possess potent endothelium-dependent vasorelaxing activity.

Keywords: Polyphenols; endothelium; nitric oxide; superoxide anion; electron paramagnetic resonance sprectroscopy; rat thoracic aorta

Introduction

Polyphenols are believed to be the active principles of a wide range of medicinal plants used to produce vascular protection, but their mechanism(s) of action is (are) not fully elucidated, although, such substances decrease serum low density lipoproteins (LDL) and platelet aggregation (Seigneur *et al*., 1990; Frankel *et al*., 1993) and have well established anti-oxidant properties (Uchida *et al*., 1987; Huguet *et al*., 1990).

Very recently it was shown that extracts from grape and wine which are known to contain polyphenols, can induce endothelium-dependent vasorelaxation probably via nitric oxide (NO) release, or enhanced biological activity of NO, leading to an elevated accumulation of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Fitzpatrick *et al*., 1993). However, the mechanisms by which polyphenolic compounds produce their vasorelaxant effects are still unknown, inasmuch as the structure of polyphenols responsible for the vasorelaxant activity of wine remains to be identified. Catechin (flavan-3-ol) is one of the polyphenolic compounds found in grapes as well as in wine and leucocyanidol (flavan-3,4-ol), which has also been shown to be present in wine (Michaud *et al*., 1971), have a closely related structure. These compounds display comparable anti-oxidant activity (Mora *et al*., 1990; Ricardo da Silva *et al*., 1991; Polette *et al*., 1996).

The aim of the present study was to characterize the vasorelaxant effects of the red wine polyphenol compounds (RWPC), catechin and leucocyanidol on rat aortic rings and to study the underlying mechanism(s) of action. The involvement of the endothelium and endothelial NO were investigated by use of the NO-synthase inhibitor, N^{ω}-nitro-L-arginine-methyl-ester (L-NAME). Direct detection of NO in vascular tissue was performed with electron paramagnetic resonance (e.p.r.) techniques with Fe^{2+}-diethyldithiocarbamate complex as specific NO trap (Vanin *et al*., 1984; Mülsch *et al*., 1992). As NO induces vascular smooth muscle relaxation through activation of guanylyl cyclase leading to accumulation of cyclic GMP, the involvement of this pathway was also studied. Finally, we investigated whether polyphenolic compounds enhanced the release or the biological activity of NO in smooth muscle cells or

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protected NO from breakdown by O_2^-. For this purpose the effects of polyphenols on the vasorelaxation induced by 3-morpholino-sydnonimine (SIN-1), which releases both NO and O_2^- (Feelisch *et al*., 1989) were investigated.

Methods

Aortic preparation and mounting

Male Wistar rats (12–14 weeks old) bred in our institute from genitors provided by Iffa Credo (Abresle, France) were killed by cervical dislocation and then exsanguinated by carotid artery transection. The thoracic aorta was removed and carefully cleaned of adhering fat and connective tissue, and cut into rings (2–3 mm length). The rings were then mounted in standard organ baths filled with a physiological salt solution (PSS) (composition in mM: NaCl 119, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.17, KH_2PO_4 1.18, $NaHCO_3$ 25 and glucose, 11), maintained at 37°C and continuously bubbled with a 95% O_2-5% CO_2 mixture. Resting tension was adjusted to 2 g. Tension was measured with an isometric force transducer.

After an equilibration period of 90 min, the vessels were maximally contracted with noradrenaline (1 μM) in order to test their contractile capacity. In some experiments, the endothelium was removed by gently rubbing the intima surface with curved forceps. The presence of functional endothelium was assessed in all preparations by determining the ability of acetylcholine (1 μM) to induce more than 50% relaxation of rings pre-contracted with noradrenaline (1 μM). Vessels were considered to be denuded of functional endothelium when there was no relaxation response to acetylcholine.

Characterization of the relaxant effect of polyphenolic compounds

Aortic rings with and without functional endothelium were pre-contracted to the same tension with noradrenaline (i.e. 80% of maximal response obtained in vessels with functional endothelium) by 0.3 or 0.1 μM noradrenaline, respectively. When the contraction reached a steady state, increasing concentrations of polyphenolic compounds were added cumulatively.

In order to characterize the involvement of NO, some arteries with functional endothelium were exposed to the NO synthase inhibitor, L-NAME (300 μM), added to the bath 15 min before noradrenaline. In this case, the concentration of noradrenaline was adjusted in order to obtain the same level of pre-contraction as in the absence of L-NAME.

Effect of polyphenolic compounds on relaxation produced by the NO donor, 3-morpholino-sydnonimine

To test the hypothesis whether or not polyphenolic compounds increase the biological activity of NO, concentration-response curves to SIN-1 were performed in vessels without endothelium, in the absence or presence of polyphenolic compounds. Concentration-response curves to SIN-1 were constructed in aortic rings without endothelium by cumulative addition of the NO donor (from 0.1 nM to 10 μM) on noradrenaline pre-contracted vessels.

In order to test the effect of extracellular O_2^- on the response to SIN-1, the same experimental protocols were carried out in the absence or in the presence of superoxide dismutase (SOD, 100 u ml^{-1}).

Assay of tissue cyclic GMP content

Rat thoracic aortic rings with endothelium were incubated for 5 min in the absence and in the presence of a single concentration of RWPC (10^{-2} g l^{-1}), either alone or with L-NAME (300 μM), in Krebs solution containing isobutylmethylxanthine (IBMX, 100 μM), SOD (100 u ml^{-1}) and catalase (100 u ml^{-1}) bubbled with 95% O_2/5% CO_2 mixture and kept at 37°C. IBMX was added in order to inhibit cyclic GMP degradation through cyclic nucleotide phosphodiesterases. SOD and catalase were added to prevent NO degradation by O_2^- and to remove H_2O_2, respectively. L-NAME was added 5 min before the exposure to RWPC. In parrallel, the same protocol was used on some rings in the presence of acetylcholine (1 μM). The reaction was stopped by addition of ice-cold HCl (0.1 N). Following homogenization, the cyclic GMP content of the tissue was determined by radioimmunoassay according to the method of Cailla *et al*. (1976), modified as described previously (Andriantsitohaina *et al*., 1995).

NO spin trapping and electron paramagnetic resonance (e.p.r.) studies

Freshly isolated thoracic aortae (3 cm length) were opened, the blood washed away and then incubated in Krebs solution at 37°C for 15 min for equilibration. The aortic preparations were then exposed to the NO spin trap components; firstly, sodium diethyldithiocarbamate (DETC; 5 mM), then $FeSO_4$ (50 μM), and incubated for 30 min in the absence (control) or in the presence of either RWPC (10^{-3} g l^{-1}), RWPC plus L-NAME (300 μM) or leucocyanidol (10^{-3} g l^{-1}). After the incubation period, the aortae were frozen in liquid N_2. In some experiments the same protocol was carried out in aortae without functional endothelium. E.p.r. investigations were performed with a Bruker 300E spectrometer at 77K (10 mW microwave power, 0.61 mT amplitude modulation, 9.47 GHz microwave frequency and 100 kHz modulation frequency).

Expression of results and statistical analysis

Since the aortae were pre-contracted to the same extent with noradrenaline whether the endothelium was present (i.e. 3.31 ± 0.25 g with 3 μM noradrenaline, $n = 28$) or not (3.41 ± 0.15 g with 1 μM noradrenaline, $n = 22$), relaxations were expressed as a percentage of the level of pre-contraction. The sensitivity of the vessels to vasodilator agents was expressed as the EC_{50} value (i.e. the concentration of vasodilator agent required to produce 50% of the maximal relaxation). Cyclic GMP content was expressed as fmol μg^{-1} DNA, DNA content being measured as described previously by Brunck *et al*. (1976). All results are expressed as mean ± s.e.mean of n experiments. Student's unpaired t test was used for statistical analysis. Analysis of variance was used to compare the concentration-curves to vasodilator agents between each groups preparations. A P level of 0.05 or less was considered significant.

Drugs

The RWPC dry powder from red wine (Cabernet-Sauvignon grape variety) was provided by Dr M. Moutounet (Institut National de la Recherche Agronomique, Montpellier, France). Briefly, the procedure involved three steps: (1) adsorption of phenolics on a preparative column, (2) alcoholic desorption and (3) gentle evaporation of the alcoholic-eluent. The concentrated residue was finally sprayed in order to obtain the RWPC dry powder. One litre of red wine produced 1.3 g of RWPC which contained 100 mg g^{-1} of total catechins plus procanthocyanidins expressed as cathechin (with only 1.0% of each monomeric form of catechin and epicatechin), 64 mg g^{-1} of total anthocyanins (including 36% of malvidin-3-glucoside), 5 mg g^{-1} of total flavonols and 8.7 mg g^{-1} of total phenolic acids (including 19.5% of caftaric acid).

Acetylcholine, catalase, (+)-catechin (wt.: 290.3), DETC, iron (II) sulphate heptahydrate, L-NAME, noradrenaline, SOD were purchased from Sigma Chemical Co. (Grenoble, France). Leucocyanidol was a generous gift from Laboratoire Pharmafarm (Courbevoie, France). SIN-1 was a generous gift from Hoechst (Paris, France). SIN-1 was diluted in deionized

water containing 5% glucose. All other drugs were diluted in deionized water (Millipore, Q10). The paramagnetic mononitrosyl iron complex with DETC ($NO\text{-}Fe(DETC)_2$) was prepared in dimethylsulphoxide as described previously (Vanin *et al*., 1984).

Results

Relaxant effect of polyphenolic compounds

All of the polyphenolic compounds and extract used relaxed aorta with or without functional endothelium (Figure 1). The mean EC_{50} and maximal relaxation values are given in Table 1. At a concentration lower than 10^{-2} g l^{-1}, relaxations induced by RWPC and leucocyanidol were exclusively dependent on the presence of a functional endothelium. In endothelium denuded vessels, relaxation occured at a concentration 1000 fold higher than that needed to obtain relaxation in vessels with functional endothelium. In vessels with an endothelium, blockade of endothelial NO synthesis by L-NAME (300 μM) completely abolished endothelium-dependent relaxation but did not affect the endothelium-independent response to these agents. In contrast to RWPC and leucocyanidol, catechin produced partial relaxation with the same potency, in rings with and without endothelium. This effect occured only at high concentrations (0.3 g l^{-1}). The solvent used for catechin (i.e. 1% ethanol of stock solution of catechin 0.1 M) did not produce a relaxant effect.

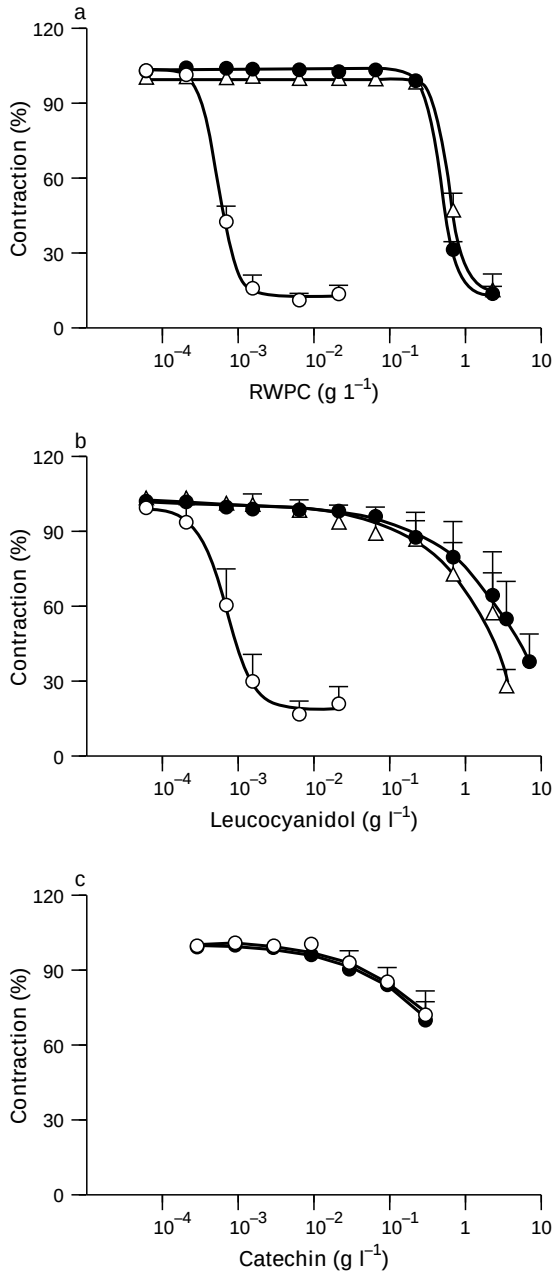

Figure 1 Concentration-response curves for red wine polyphenolic compound (RWPC, a), leucocyanidol (b) and catechin (c) in noradrenaline pre-contracted rat thoracic aortic rings with (○) or without functional endothelium (●), or with functional endothelium in the presence of L-NAME 300 μM (△). Values are mean of 6 experiments, s.e.mean shown by vertical lines.

Effect of polyphenolic compounds on the relaxation produced by the NO donor, SIN-1

As shown in Figure 2a, SIN-1 produced a concentration-dependent relaxation in endothelium-denuded aorta which was potentiated in the presence of SOD (100 u ml^{-1}). SOD produced a leftward shift in the concentration-response curve to SIN-1, the EC_{50} value being significantly decreased from 0.15 ± 0.04 μM to 0.02 ± 0.00 μM ($P < 0.05$).

Figure 2b and c illustrate the effect of polyphenolic compounds. These compounds were used at concentrations producing maximal endothelium-dependent relaxation without showing any relaxant effect in endothelium-denuded rings. In contrast to SOD, none of the polyphenolic compounds significantly affected the concentration-response curves to SIN-1 in endothelium-denuded preparations.

Tissue cyclic GMP content

In aortic rings with a functional endothelium, RWPC (10^{-2} g l^{-1}) produced a 7 fold increase in cyclic GMP content (Figure 3) which was abolished by L-NAME (300 μM). As a reference, acetylcholine (1 μM) which produced comparable endothelium-dependent relaxation (more than 80%) of the aorta also increased the cyclic GMP content of the aortic rings by 8 fold.

NO spin trapping and electron paramagnetic resonance (e.p.r.) studies

Control aortae with endothelium which had been incubated with DETC and $FeSO_4$ exhibited an e.p.r. feature (Figure 4a) that can be superimposed on the two signals derived from $NO\text{-}Fe(DETC)_2$ (Figure 4b) and Cu^{2+}-DETC (Figure 4c). Ac-

Table 1 pD_2 values and maximum relaxation (E_{max}) obtained with RWPC, leucocyanidol and catechin in rat thoracic aorta

Products	*Protocols*	n	*pD_2 value* (g l^{-1})	E_{max} *(%) relaxation*
RWPC	+E	6	3.27 ± 0.02	87.4 ± 3.14
	–E	6	0.31 ± 0.02***	86.8 ± 3.01
	+E+L-NAME	6	0.21 ± 0.01***	84.3 ± 5.91
Leucocyanidol	+E	6	2.75 ± 0.15	82.6 ± 5.68
	–E	6	0.18 ± 0.06***	64.4 ± 10.81
	+E+L-NAME	6	-0.14 ± 0.06***	94.7 ± 10.81
Catechin	+E	6	ND	27.0 ± 9.32
	–E	6	ND	29.5 ± 7.10

Values are means ± mean of 6 experiments. +E and –E indicate respectively with and without a functional endothelium. **$P < 0.01$; ***$P < 0.001$ are the statistical differences versus +E. ND indicates not determined.

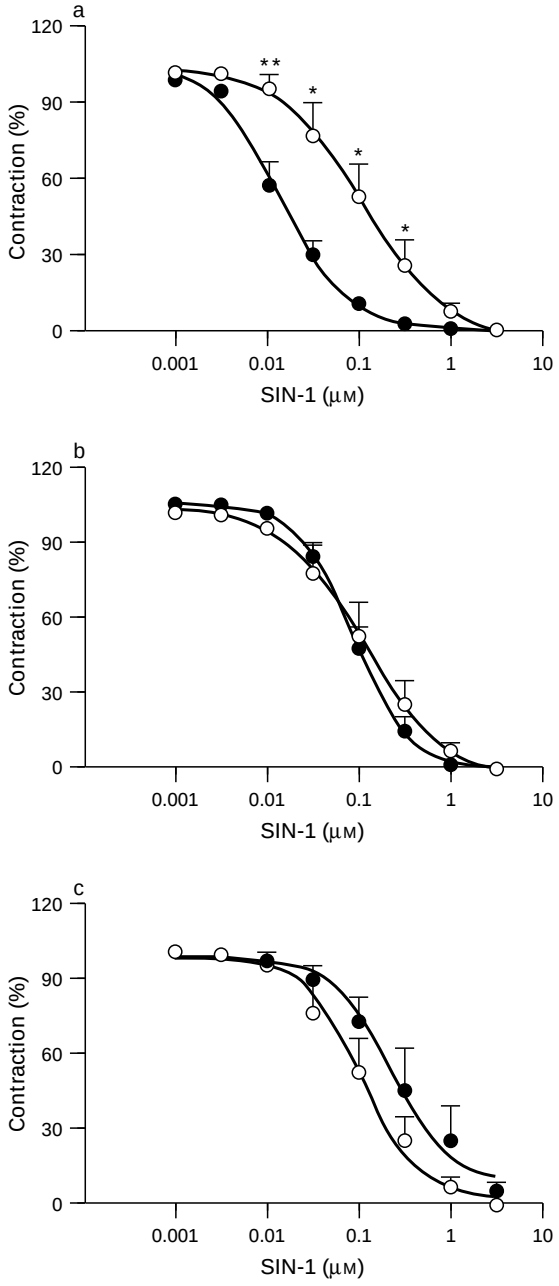

Figure 2 Concentration-response curves for SIN-1-induced relaxation in aortic rings without endothelium pre-contracted with noradrenaline in the absence (○) and in the presence (●) of SOD (100 u ml^{-1}, a), red wine polyphenolic compound (RWPC, 10^{-2} g l^{-1}, b), leucocyanidol (10^{-2} g l^{-1}, c). Values are mean of 5 experiments; s.e.mean shown by vertical lines. $^{**}P<0.01$, $^{*}P<0.05$ significantly different as compared to controls, by Student's unpaired *t* test.

cording to Mülsch *et al*. (1992; 1995), the third component of the NO-Fe(DETC)$_2$ e.p.r. signal is not overlapped by the background Cu^{2+}-DETC signal and indicates the generation of NO in the given system. Thus, in the non-stimulated aorta this component probably reflects basal NO synthesis by the endothelium.

Incubation of the aorta with endothelium with the spin trap components and RWPC (10^{-3} g l^{-1}) produced about a two fold increase in the NO-Fe(DETC)$_2$ e.p.r. signal, the shape of which became almost identical to the signal exhibited by the model NO-Fe(DETC)$_2$ (Figure 4d). The same observation was made with RWPC (10^{-2} g l^{-1}) (not shown). The appearance of the NO-Fe(DETC)$_2$ feature was completely prevented by the NO synthase inhibitor, L-NAME (300 μM) (Figure 4e).

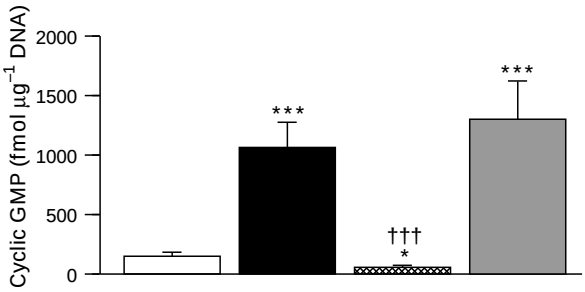

Figure 3 Histograms showing the cyclic GMP content of rat thoracic aorta with endothelium in the absence (open column) or in the presence of polyphenolic red wine extracts (RWPC, 10^{-2} g l^{-1}, solid column), RWPC plus L-NAME (cross-hatched column, 300 μM) or acetylcholine (grey column, 1 μM). Values are mean ± s.e.mean of 6 experiments. $^{***}P<0.001$, $^{*}P<0.05$ significantly different compared to controls, $^{†††}P<0.001$ significantly different vs vessels treated with RWPC, by Student's unpaired *t* test.

Also, this signal was not detected in the endothelium-denuded aorta incubated with the trap components and RWPC (Figure 4f).

Aortae, with endothelium, incubated with the trap components and leucocyanidol (10^{-3} g l^{-1}) exhibited an increase in the formation of NO-Fe(DETC)$_2$ adducts comparable with those observed after additon of RWPC (Figure 4g). As with RWPC, the appearance of the NO-Fe(DETC)$_2$ signal was completely dependent on the presence of endothelium and prevented by L-NAME (not shown).

Discussion

The above results provide direct evidence that a mixture of polyphenols contained in wine and also a defined polyphenol, leucocyanidol, can cause an endothelium-dependent increase in NO content in the rat aorta. They show the involvement of NO in endothelium-dependent vasorelaxation induced by low concentrations of leucocyanidol and red wine polyphenols (RWPC). In addition they demonstrate that 1000 fold higher concentrations of RWPC and leucocyanidol are needed to cause endothelium-independent vasorelaxation via another, undetermined, mechanism.

RWPC contains many different types of polyphenols including catechins, proanthocyanidins, anthocyanins, flavonols and phenolic acids. The finding that the defined polyphenol, leucocyanidol, but not the closely related structure catechin, mimicked the effects of RWPC suggests that a restricted polphenol structure supports the relaxant activity of RWPC. Catechin only differs from leucocyanidol by the lack of hydroxyl substituent in position 4 in the pyrane nucleus.

An increased aortic NO content, shown by e.p.r. experiments, and enhanced NO biological activity shown by the relaxation study and cyclic GMP accumulation, could result either from augmented NO generation or from diminished NO breakdown. The latter hypothesis was investigated with SIN-1, which spontaneously releases NO and O_2^- and therefore mimics the release of these agents by endothelial cells. It is well known that NO interacts with O_2^- to produce peroxynitrite (Blough & Zafiriou, 1985), and that this mechanism decreases the vasodilator effect of both SIN-1 and NO released by the endothelium, as shown in cascade bioassays (Gryglewski *et al*., 1986; Rubanyi & Vanhoutte, 1986) Indeed, it was verified here that the addition of SOD enhanced the relaxing effect of SIN-1, showing the result of scavenging O_2^- in the experimental conditions. However, at the concentrations producing maximal endothelium-dependent relaxation, neither RWPC nor leucocyanidol caused any modification of SIN-1 concentration-effect curve, showing that polyphenols did not modify the relaxant effect of NO. Furthermore, RWPC increased aortic

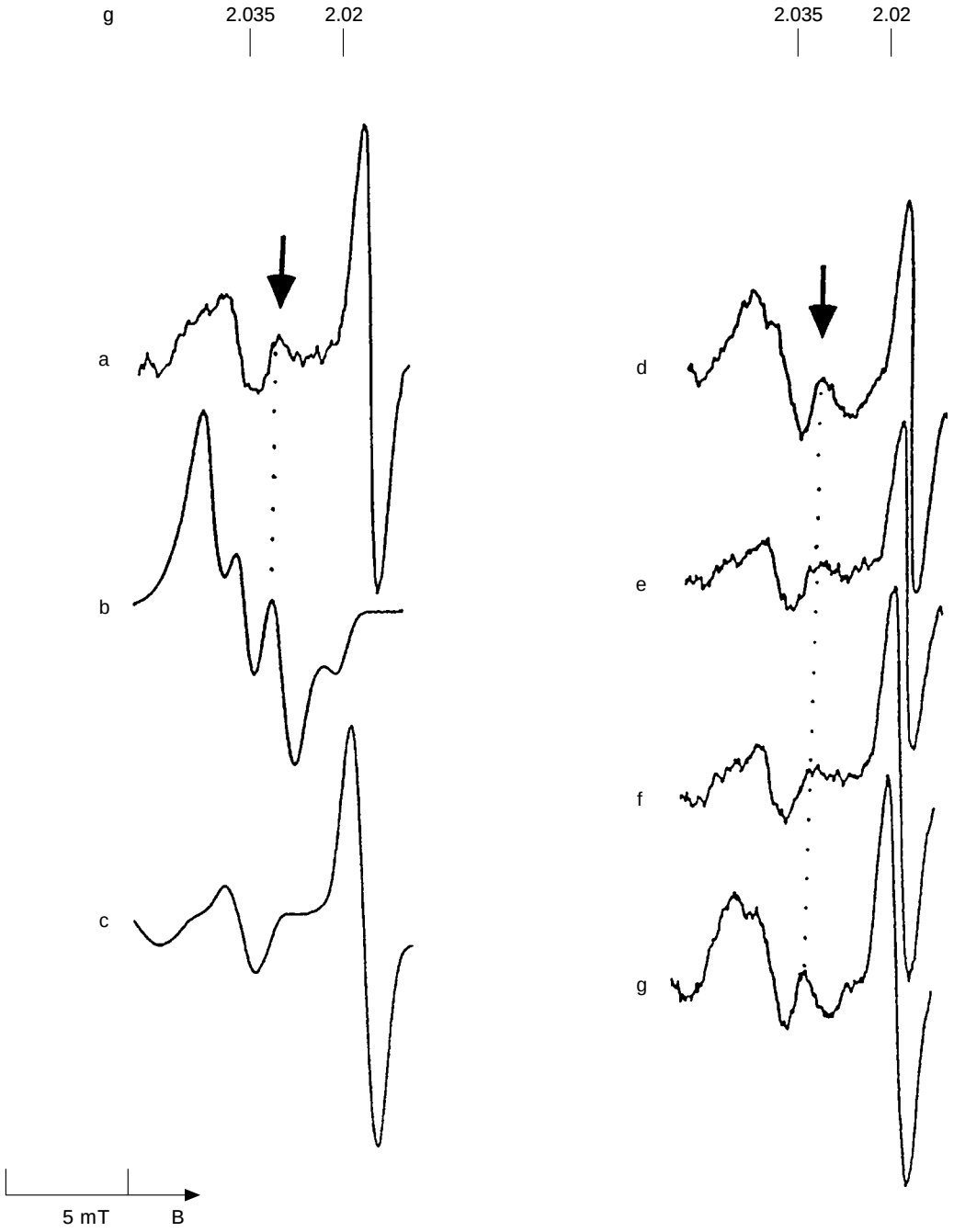

Figure 4 E.p.r. spectra of rat thoracic aortae treated with sodium diethyldithiocarbamate (DETC, 5 mM) and $FeSO_4$ (50 μM) and incubated at 37°C for 30 min under control conditions (a). E.p.r. spectra of NO-Fe(DETC)$_2$ (b) and Cu^{2+}-DETC (c). Aortae incubated with RWPC (10^{-3} g l^{-1}) (d,e,f) or leucocyanidol (10^{-3} g l^{-1}) (g). (e) Incubation was performed in the presence of L-NAME (300 μM). (f) Incubation of aorta without endothelium. E.p.r. settings were as described in the Methods section. The third component of the NO-Fe(DETC)$_2$ e.p.r. signal which is not masked by the signal from Cu^{2+}-DETC is indicated by (↓). The spectra are representative of 3 independent experiments.

cyclic GMP content in the presence of SOD and catalase (to remove O_2^- and H_2O_2, respectively). Conversely, aortic NO production (monitored by e.p.r.), cyclic GMP accumulation and relaxation elicited by RWPC were all abolished by L-NAME and by removal of the endothelium. These findings indicate that RWPC causes NO synthase activation, and that NO is involved in the subsequent cyclic GMP accumulation and endothelium-dependent relaxation. This conclusion is further supported by the finding that RWPC and acetylcholine produced comparable elevations in aortic cyclic GMP levels at concentrations at which they also elicited comparable relaxations (more than 80%, in the present experimental conditions). Catechin and leucocyanidol have been shown to have comparable anti-oxidant activity. However, in contrast to leucocyanidol, catechin was not able to produce an endothelium-dependent relaxation. A similar effect of catechin has been obtained in the same type of vessel (Duarte *et al*., 1993). Taken together, these data do not support the view that an anti-oxidant property of polyphenols is involved in the endothelium-dependent effects of this class of compound.

The present work also shows that RWPC and polyphenolic compounds produced, at high concentrations, relaxation of aortic rings in the absence of a functional endothelium. Several studies have shown that polyphenols such as flavonoids can inhibit cyclic nucleotide phosphodiesterases, a family of enzymes involved in the breakdown of the vasorelaxants ade-

nosine 3′:5′-cyclic monophosphate (cyclic AMP) and cyclic GMP (Beretz *et al*., 1983; 1986). In addition, inhibition of cyclic nucleotide phosphodiesterases and the subsequent rise in cyclic AMP and cyclic GMP contents produced relaxation of aortic rings without endothelium (Komas *et al*., 1991; Eckly & Lugnier, 1994). Such a mechanism might be implicated in the endothelium-independent relaxation produced by these polyphenols but remains to be elucidated.

In conclusion, the present work shows that some polyphenols, with specific structure requirement, induce an endothelium-dependent relaxation via an enhancement of endothelial NO synthesis. The anti-oxidant properties of polyphenols is unlikely to be involved in this effect. Furthermore, NO has been shown to have platelet antiaggregatory properties (Yao *et al*., 1992) and to limit the flux of the atherogenic plasma proteins into the artery walls (Cardona-Sanclemente & Born, 1995). Therefore some of the polyphenols present in red wine may produce therapeutically relevant effects. Anti-oxidant effects after oral administration of RWPC have been demonstrated in human volunteers (Carbonneau *et al*., 1996), indicating oral absorption of some of the anti-oxidants contained in wine. Whether polyphenols are able to reach blood vessels and therefore produce endothelium-dependent vasorelaxation after oral ingestion awaits further investigation.

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