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Original articles

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ENDOTHELIAL NO RELEASE CAUSED BY RED WINE POLYPHENOLS

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Epidemiological studies have suggested that moderate consumption of red wine might reduce the risk of cardiovascular disease. Red Wine Polyphenolic Compounds (RWPC), a complex extract obtained from red wine, causes endothelium-dependent vasorelaxation in rat aortic rings pre-contracted with noradrenaline. This effect is associated with marked formation of NO in the vessel (directly shown by electron paramagnetic resonance spectroscopy) and it is abolished by the NO synthase inhibitor N^G-nitro-L-arginine methylester (300 μM). It is mimicked by some defined polyphenols (like the anthocyanin delphinidin) but not by others (malvidin, cyanidin, quercetin, catechin, epicatechin), despite close structures. In addition, RWPC causes an extracellular Ca^{2+}-dependent increase in $[Ca^{2+}]_i$ in endothelial but not in smooth muscle cells. The efficiency of RWPC in inducing NO production in the aorta and increase in $[Ca^{2+}]_i$, in endothelial cells is comparable to those of carbachol and bradykinine, respectively. These findings provide evidence that RWPC and polyphenols with selective structures can activate an undefined target in endothelial cells. The resulting increase in $[Ca^{2+}]_i$ activation of NO-synthase and enhanced formation of NO may be involved in cardiovascular protection.

Key words: *red wine, polyphenols, cardiovascular protection, nitric oxide, endothelium, intracellular Ca^{2+}.*

INTRODUCTION

Wine and grape extracts cause endothelium-dependent vasorelaxation (1—4). This effect may be involved in the protective action of moderate consumption of alcoholic beverage, mostly wine, on the risk of cardiovascular disease described in a number of epidemiological studies (5—8).

We previously reported that polyphenolic compounds from red wine (RWPC) are able to induce an increase in nitric oxide (NO) production in the rat aorta, accounting for endothelium-dependant relaxation in this tissue (3).

This effect seems mostly due to oligomeric tannins and anthocyanins contained in RWPC (9) and it is not due to direct oxygen radical scavenging property of polyphenols (3). It has been reported recently that alcohol free red wine extract prevents experimental thrombosis by a NO mediated mechanism in rats *in vivo* (10), supporting the view that NO is a possible mediator of the protective action of moderate consumption of red wine in cardiovascular disease.

This prompted us to further investigate the effect of RWPC on endothelial NO formation.

METHODS

All the methods have been described in a recent article (11). Briefly, vasorelaxation was studied using aortic rings with or without functional endothelium from male Wistar rats bred in our Institute. The rings were pre-contracted with noradrenaline (1 μM) in a standard organ bath 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, glucose 11) maintained at 37°C and continuously bubbled with a 95% O_2 and 5% CO_2 mixture (pH 7.4). NO formation was measured in 3 cm long aorta segments, using Fe^{2+}-diethyldithiocarbamate and electron paramagnetic resonance (EPR). EPR spectra were recorded at 77 K, 10 mW microwawe power, 0.61 mT amplitude modulation, 9.47 GHz microwave frequency.

The effect of RWPC on $[Ca^{2+}]_i$ was assessed in bovine aortic endothelial cells and in rat aorta smooth muscle cells, as described previously (11). The cells were grown in the following culture medium: 50 % Dulleco's modified Eagle's medium and 50 %Ham's F12 medium, supplemented (to final concentration) with 10 % heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 mg/ml heparin, 10,000 U/ml penicillin, 10,000 U/ml streptomycin, 10 μM vitamine C and 0,8 % amino acids (Eurobio). After the first (smooth muscle cells) or the second passage (endothelial cells), $[Ca^{2+}]_i$ was assayed using Fura-2 fluorescence at room temperature, in cells suspended in modified Krebs-Ringer bicarbonate buffer (in mM: NaCl 119, KCl 4,75, $CaCl_2$ 1,25, $MgSO_4$ 1,2, KH_2PO_4 1,2, $NaHCO_3$ 25, glucose 5, HEPES 20, pH 7,4).

RESULTS

Vasorelaxation produced by RWPC

The data illustrated in *Fig. 1* show that the efficiency of RWPC in producing maximal relaxation of rat aortic rings with functional endothelium was comparable to the one of acetylcholine. Delphinidin, which is an anthocyanin compound and is also the most active defined polyphenol causing endothelium-dependent vasorelaxation (9), was also able to cause 80 % maximal relaxation in the same conditions (9) comparable to the maximal effects of RWPC and acetylcholine.

The potency of RWPC was about 1,000 fold higher in rings with than without endothelium. The mean concentrations producing 50% relaxation,

EC_{50}, were about 0.5 and 500 mg/l (3), respectively. By comparison, the EC_{50} of delphinidin was previously found to be 10 mg/l (30 μM) in rat aorta rings with endothelium (9). Thus, RWPC apparently contain unidentified compound(s) more potent than delphinidin.

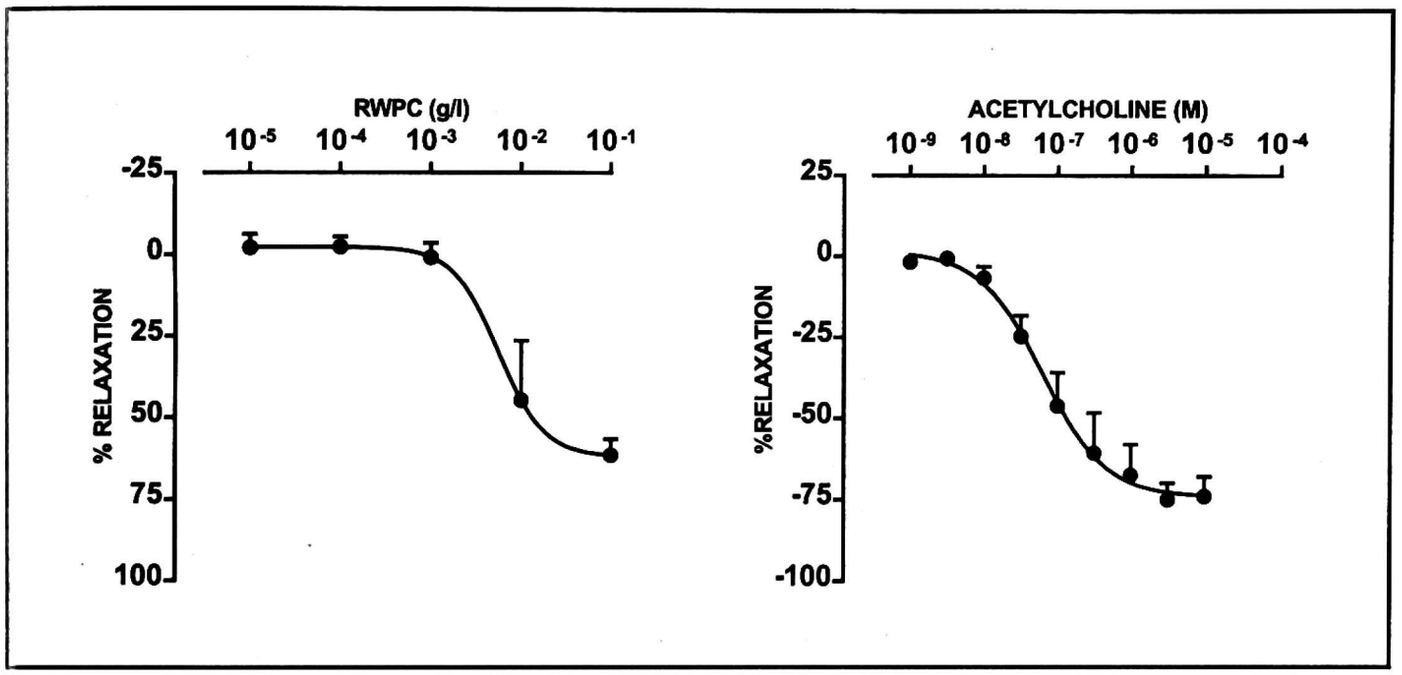

Fig. 1. Concentration-effect curves for red wine polyphenolic compounds (RWPC)- and acetylcholine-induced relaxation in rat aorta rings with functional endothelium pre-contracted with noradrenaline. Values are mean ± s.e.m. of experiments

NO formation

Direct evidence that exposure of the rat aorta with functional endothelium to RWPC results in enhanced NO formation is provided by EPR spectroscopy. Representative recordings illustrated in *Fig 2* show that maximally active concentratios of RWPC and the acetylcholine stable analog carbachol produced comparable signals, indicating that their efficiency in inducing NO formation was similar. In both cases, the signal could not be seen in arteries without endothelium. Quantification of EPR signals allowed to estimate NO formation to 1.17 ± 0.08 nmol/g wet tissue ($n = 5$) in the presence of RWPC, whereas the basal NO production was 0.55 ± 0.04 nmol/g wet tissue ($n = 6$).

Effect of RWPC on $[Ca^{2+}]_i$ in endothelial cells

As shown in *Fig 3*, RWPC elicited an increase in $[Ca^{2+}]_i$ in bovine aortic endothelial cells. This increase was comparable to the one produced by a maximally active concentration of bradykinin. It did not take place in Ca^{2+}-free medium, indicating that it relied on Ca^{2+} entry into the cells (11). By contrast, RWPC did not cause any increase in $[Ca^{2+}]_i$ in rat aorta smooth muscle cells (not shown).

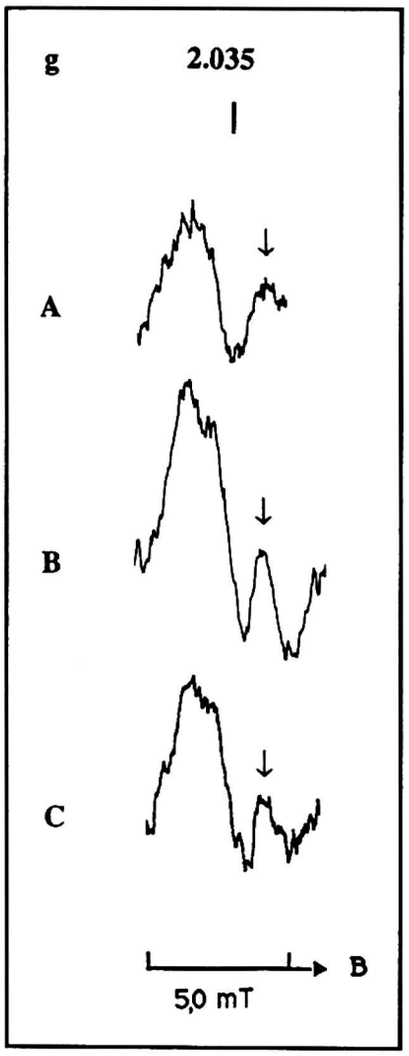

Fig. 2. Representative EPR traces showing the effects of red wine plyphenolic compounds (RWPC) and carbachol on NO production in rat aorta with functional endothelium. Aortae were incubated with the NO spin trap for 30 min under control conditions (A), in the presence of RWPC (10^{-2} g/l) (B) or carbachol (1 μM) (C). EPR features which reflect the level of NO production are indicated by an arrow.

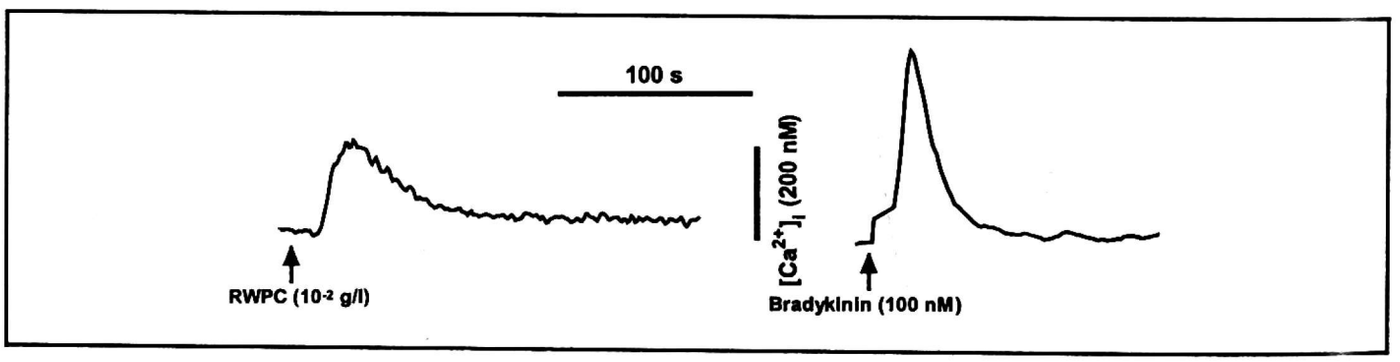

Fig. 3. Representative traces showing the increases in $[Ca^{2+}]_i$ caused by red wine polyphenolic compounds (RWPC, 10 mg/l) and bradykinin (0.1 μM) in bovine aorta endothelial cells.

DISCUSSION

The data reported above show that endothelium-dependent relaxation caused by RWPC is associated with increased NO production in the rat aorta, consistent with previous reports from our group (3, 9). In addition, they show that, at concentrations causing endothelium-dependent but not endothelium-independent vasorelaxation (10^{-2} g/L) RWPC induced an extracellular Ca^{2+}-dependent increase in $[Ca^{2+}]_i$. This is a well established

mechanism of activation of endothelial NOS and NO production in endothelial cells.

Importantly enough, RWPC was found as efficient as bradykinin or carbachol, the most efficient endothelium-dependent vasodilators, in enhancing $[Ca^{2+}]_i$ and NO production, respectively. The data are consistent with previously reported data on cyclic GMP level (3). Theoretically RWPC-induced enhanced NO formation may not only trigger vasorelaxation, but also result in longer term effects of NO due to induction of the expression of cardiovascular protective genes (for reviews see references 13 and 14). Long term prevention of thrombosis by red wine (10) or NO-triggered cardiac protection against ischaemia-reperfusion induced damages might involve such mechanisms (15).

There is evidence suggesting that only some polyphenols with specific structures are able to cause endothelium-dependent vasorelaxation (9). For example delphinidin, but not other anthocyanins with closely related structures like malvidin and cyanidin, has such effect. Flavonols like quercetin and flavanols like catechin and epicatechin do not produce endothelium-dependent vasorelaxation in the used rat aorta rings (9), despite their antioxidant properties. Thus, it seems that endothelial cells contain target(s) for polyphenols with specific structures triggering Ca^{2+} entry and increase in $[Ca^{2+}]_i$.

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