Effect of red wine on endothelium-dependent relaxation in rabbits

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(Received 28 July/11 September 1997; accepted 17 September 1997)

1. Published data on the effects of red wine, ethanol and flavonoids on endothelium-dependent relaxation are equivocal. The present study was undertaken to determine the effects of red wine, ethanol and selected flavonoids present in red wine on endothelium-dependent relaxation.

2. Aortic rings from New Zealand White rabbits were set up in organ baths (20 ml) and contracted with noradrenaline (10⁻⁶ mol/l). An attempt was made to elicit dose-dependent relaxant responses to red wine (15, 30, 40, 80 or 120 µl), ethanol (5.4, 10.8 and 16.2 µl) and the flavonoids catechin, epicatechin, quercetin and polymeric phenols (10⁻⁷ to 10⁻⁴ mol/l). In some experiments, endothelium-dependent relaxation to cumulative doses of acetylcholine (10⁻⁴ to 10⁻⁶ mol/l) was determined before and after incubating the rings for 15 min with red wine (120 µl), ethanol (16.2 µl), quercetin (10⁻⁵ mol/l), catechin (10⁻⁵ mol/l), epicatechin (10⁻⁵ mol/l) and PPs (10⁻⁵ mol/l) respectively. cGMP was also measured in some rings in the control state and after addition of 120 µl of red wine, sodium nitroprusside (10⁻⁴ mol/l) and polymeric phenols (10⁻² mol/l).

3. Red wine evoked a dose-dependent relaxation in aortic rings. The highest volumes of wine (120 µl) relaxed the vessels by 71.35 ± 7.89% of the maximal contraction (8.95 ± 0.97 g). Polymeric phenols also relaxed the precontracted rings. These responses were abolished by L-NAME and by removal of endothelium. Addition of red wine, polymeric phenols and sodium nitroprusside increased the cGMP content of the rings. In tissues previously incubated with red wine and polymeric phenols, endothelium-dependent relaxation in response to acetylcholine was attenuated. Ethanol had no such effect.

4. Acute exposure of aortic rings to red wine and polymeric phenols evokes an endothelium-dependent relaxation which is mediated by nitric oxide. However, prior exposure to both red wine and polymeric phenols has a second effect in that it attenuates the endothelium-dependent relaxation evoked by acetylcholine. Since this effect is restored by arginine, it is likely to be due to depletion of substrate for nitric oxide synthase.

INTRODUCTION

Relatively few studies have examined the effects of red wine on endothelium-dependent relaxation (EDR) [1, 2]. Studies have been concerned primarily with the effects of acute exposure of aortic rings to wine, grape skin extracts and selected flavonoids and their findings have been inconsistent. The purpose of the present study was to identify the effects of red wine, ethanol and selected flavonoids present in wine on EDR in aortic rings.

METHODS

The investigation was conducted on New Zealand White rabbits using protocols approved by the Animal Care and Use Committee of the University of California, Davis.

Demonstration of EDR in isolated aortic rings

Male New Zealand White rabbits weighing 2.5–3.5 kg were terminally anaesthetized with pentobarbital (50 mg/kg). The thoracic aorta was removed, cut into rings (4–5 mm long) and suspended from force displacement transducers (Grass

Key words: endothelium-dependent relaxation, flavonoids, red wine.

Abbreviations: EDR, endothelium-dependent relaxation; L-NAME, N⁶-L-arginine methyl ester; NO, nitric oxide; PP, polymeric phenols.

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Instrument Co.; Quincy MA Model FT03C) in 20 ml organ chambers containing Krebs buffer (37°C), bubbled with 95% O₂/5% CO₂ gas. Basal tension was set at 8 g. In some rings, the endothelium was removed by rubbing the lumen of the vessel with a wooden stick for 30 s and functional removal of the endothelium was established by the failure of the ring to relax in response to acetylcholine (10⁻⁷ mol/l). The effects of prostanoids and nitric oxide (NO) were inhibited by adding the cyclo-oxygenase inhibitor indomethacin (10⁻⁵ mol/l) and the NO synthase inhibitor N⁶-(1-arginine methyl ester (l-NAME) (10⁻⁵ mol/l), respectively, as required to the organ bath. These methods have been published in detail previously [3].

All rings were contracted with noradrenaline (10⁻⁶ mol/l) before administration of putative relaxing agents. Red wine (15, 30, 40, 80 or 120 μl) and ethanol (5.4, 10.8 and 16.2 μl) were added as single doses to individual rings. These volumes of ethanol were chosen as the equivalent volumes present in the three highest doses of red wine. Catechin, epicatechin, quercetin and polymeric phenols (PPs) (10⁻⁷ to 10⁻⁴ mol/l) were used on precontracted vessels also. Rings were washed repeatedly between additions of agonists and allowed to re-equilibrate back to their original resting tension before further manipulations. All the concentrations of alcohol used were lower than 1.6 × 10⁻⁴ mol/l (which is the legal limit for operators of motor vehicles).

In some experiments, EDR to cumulative doses of acetylcholine (10⁻⁹ to 10⁻⁶ mol/l) was determined before and after incubating the rings for 10–15 min with red wine (120 μl), ethanol (16.2 μl), quercetin (10⁻⁵ mol/l), catechin (10⁻⁵ mol/l), epicatechin (10⁻⁵ mol/l) and PPs (10⁻⁵ mol/l) derived from grape seeds. Additionally the effects of sodium nitrite (10⁻⁴ mol/l) and noradrenaline (10⁻⁹ to 10⁻³ mol/l, n = 4) were examined in tissues which were incubated with flavonoids. Flavonoids were extracted from grape seeds using HPLC [4] (Vinox; Polyphenolics LLC, Canandaigua, New York). Flavonoids extracted from grape seeds are similar to those present in red wine.

cGMP determinations [5]

Aortic rings were equilibrated for 90 min with no tension in oxygenated Krebs buffer. The phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) (10⁻³ mol/l) was added to the bath during the final 30 min of this period. After addition of red wine (120 μl), sodium nitroprusside (10⁻⁴ mol/l) and PPs (10⁻⁵ mol/l), respectively, the vessels were removed from the organ chamber and frozen in liquid nitrogen. cGMP was extracted and measured using an enzyme immunoassay kit (Amersham, U.S.A.). Each sample was measured in duplicate with a corresponding pair of controls. The assays were carried out in three batches (i.e. red wine, sodium nitroprusside and PPs). The protein content of each ring was measured [6], and the cGMP content was expressed as pmol/mg protein.

Reagents and chemicals

A single vintage of red wine (Ravenswood 1993 Vintner's Blend Merlot, 13.5% ethanol by volume) was used throughout all experiments. Reagents and chemicals for Krebs buffer were obtained from Sigma (St. Louis, MO, U.S.A.).

Statistical methods

Group data were expressed as means±SEM and paired data were compared using Student's t-test. When dose–response curves were analysed, the curves were linearized and compared by an analysis of covariance. A P value of <0.05 was taken to indicate statistical significance.

RESULTS

Effect of acute exposure of aortic rings

Red wine and EDR. In preliminary experiments, it was found that aortic rings failed to respond in a reproducible manner on repeated exposure to the same quantity of red wine. As such, dose–response curves were obtained in parallel rings with time controls. Addition of increasing volumes of red wine to the organ chamber resulted in a dose-dependent relaxation of precontracted rings (Fig. 1). The

![Fig. 1. Effect of red wine on tension in aortic rings – acute exposure.](image)

Relaxation in response to red wine administration (% maximal contraction).

<table>
<thead>
<tr>
<th>Volume Red Wine added (μl)</th>
<th>15</th>
<th>30</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relaxation</td>
<td>90</td>
<td>80</td>
<td>70</td>
<td>60</td>
<td>50</td>
</tr>
</tbody>
</table>

Data are plotted as means ± SEM.
highest volumes of wine (120 µl) relaxed the vessels by 71.35 ± 7.89% of the maximal contraction (8.95 ± 0.97 g). This response was not seen in rings denuded of endothelium. In the presence of L-NAME, the maximum relaxation observed with 120 µl of red wine was 4.3 ± 0.6%. Indomethacin had no effect on the response to red wine.

**Ethanol and EDR.** Exposure to ethanol did not produce a relaxation of the precontracted vascular rings. At the higher volumes, ethanol induced small transient contractions.

**Flavonoids and EDR.** Catechin, epicatechin and quercetin had no effect on vascular tension of precontracted tissues (n = 4 for each). However, PPs relaxed the precontracted rings (maximum relaxation: 81.25 ± 7.11%, n = 4). In the presence of L-NAME the maximum relaxation observed with 3 × 10⁻⁶ mol/l PPs was significantly reduced (maximum relaxation: 13.97 ± 7%, n = 4). Removal of the endothelium abolished the effect of PPs. These findings were similar to those obtained with red wine.

**Effects on cGMP content of aortic rings.** Exposure of aortic rings to red wine (120 µl), sodium nitroprusside and PPs resulted in significant increases in cGMP compared with untreated controls. These results are summarized as follows: red wine: 39.0 ± 10.6 versus 8.90 ± 2.66 pmol/mg protein (n = 4, P < 0.035); sodium nitroprusside: 56.3 ± 12.1 versus 9.46 ± 2.36 pmol/mg protein (n = 4, P < 0.014); PPs: 11.2 ± 2.7 versus 2.19 ± 0.4 pmol/mg protein (n = 4, P < 0.04).

**Incubation of aortic rings**

*Red wine incubation and EDR.* Red wine (120 µl) was added to the organ baths for the final 15 min of the equilibration period. The tissues were washed five to six times and precontracted as described previously. EDR evoked by acetylcholine (10⁻⁶ mol/l) was measured. The findings were compared with time controls which were not incubated in this way. There was a significant attenuation in the relaxations evoked by graded doses of acetylcholine (10⁻⁹ to 10⁻⁶ mol/l) in the tissues which had been exposed previously to red wine (Fig. 2). Responses to sodium nitrite (10⁻⁵ mol/l) were unaffected by previous incubation with PPs. In contrast, incubation with catechin, epicatechin and quercetin had no effect on the responses to acetylcholine (n = 3 for each).

**Incubation with PPs**

*Indomethacin incubation and EDR.* After incubation with PPs (3 × 10⁻⁶ mol/l), the responses to acetylcholine were markedly diminished in a manner similar to that observed with red wine (Fig. 3). It was possible to partially restore responses to PPs by subsequent incubation with 10⁻⁴ mol/l arginine for 30 min (maximum relaxation to PPs before incubation was 54 ± 5%, after incubation with PP 19 ± 3%, after re-incubation with arginine 33 ± 2%; n = 4). Responses to sodium nitrite (10⁻⁵ mol/l) and noradrenaline (10⁻⁸ to 10⁻³ mol/l, n = 4) were unaffected by previous incubation with PPs. In contrast, incubation with catechin, epicatechin and quercetin had no effect on the responses to acetylcholine (n = 3 for each).
DISCUSSION

There have been several recent reports on the effects of flavonoids on vascular smooth muscle [1, 2, 7]. Fitzpatrick et al. [1, 7] reported that aqueous extracts of grape skins, and a variety of wines, vegetables and plants, evoked an EDR in the rat aorta. The EDR evoked by these agents was abolished by L-NAME and restored in part by L-arginine. It was argued that the EDR observed with red wine was the result of an increase in the production of NO as opposed to its preservation by antioxidants in wine. However, in a comparable study in rats, Duarte et al. [2] observed that several flavonoids present in red wine evoked a relaxation which was not dependent upon the presence of endothelium. There is no explanation for this apparent discrepancy. The present study, undertaken in New Zealand White rabbits, has shown that red wine caused a dose-dependent EDR which was abolished by exposure to L-NAME and unaffected by indomethacin. Relaxation was also associated with an increase in the content of cGMP in aortic rings. These findings suggest that EDR induced by red wine is mediated by NO.

With respect to the active ingredient present in red wine we examined the effects of ethanol and several flavonoids. Flavonoids are diphenylpropanes which are ubiquitous in plants [7]. The basic C15 unit consists of two benzene rings, A and B, connected by a three-carbon chain. The latter is closed in most flavonoids, forming the heterocyclic C ring; the various subclasses of flavonoids are based upon the oxidative state of the C ring (see below).

![Structure of a diphenylpropane](image)

Quercetin, catechin and epicatechin are monomers and PPs are 4–8-linked oligomers and polymers of these basic units. In our experimental model these three monomers did not evoke an EDR whereas the polymers did. Ethanol in quantities proportional to those of the flavonoids did not evoke an EDR.

Flavonoids have several biological properties which are potentially beneficial in a variety of clinical syndromes that affect the cardiovascular system. In particular, they inhibit oxidation of low-density lipoproteins [8], and aggregation of platelets [9]. It has been argued that these properties are the mechanisms which mediate the so-called French Paradox [10]. Many of these beneficial effects have been attributed to the antioxidant, free-radical-scavenging properties of these compounds [11]. Studies reported by Saija et al. [12] and Teissedre et al. [4] have shown that catechin, epicatechin and quercetin are potent antioxidants. However, the present study has shown that these three compounds do not evoke an EDR on aortic rings. Therefore it is suggested that the effect of the PPs could be mediated via an alternate mechanism.

There are at least three mechanisms by which PPs could influence NO production and release: (i) release of NO from intracellular stores, (ii) preservation/stabilization of NO released under basal conditions and (iii) stimulation of NO synthase activity. The data presented show that the EDR evoked by PPs is abolished by inhibition of NO synthase. If physiologically significant quantities of NO are released by a direct action of PPs, precontracted vessels would show a relaxation to PPs after administration of L-NAME. A similar relaxation effect would be evident if the PPs acted by stabilizing basally released NO. An example of the latter phenomenon would be the effect of superoxide dismutase on precontracted vessels treated with L-NAME. It has been shown that under these conditions superoxide dismutase produces a dose-dependent relaxation [13]. These considerations provide strong support for the claim that acute exposure of rabbit aortic rings to PPs releases NO by stimulating NO synthase.

The other novel finding of this study is that the EDR induced by acetylcholine was abolished by prior incubation with red wine and PPs. This effect was not due to failure of the vascular smooth muscle to relax as shown by the responses to sodium nitrite. Since arginine restored the response to acetylcholine, it appears that incubation with red wine and PPs depletes the supply of substrate for NO synthase, i.e. arginine.

Preliminary data obtained in our laboratory (C. T. Kappagoda, M. Karim and J. B. German, unpublished work) have shown that atropine abolished EDR to acetylcholine while the effect of PPs was preserved. This observation suggests that PPs do not activate muscarinic receptors. It is not known at this time whether this response is mediated by another receptor or by a direct action on the NO pathway. It is also possible that the two main effects observed with the PPs are not due to the actions of a single molecule. Additional investigations are required to elucidate these issues.

In summary we have observed three new findings with respect to red wine and rabbit aortic rings: (i) separation of the effects of alcohol per se and the non-alcoholic components of wine, (ii) identification of differences between the effects of monomeric and polymeric phenols and (iii) the two effects of polyphenolics on smooth muscle. The first is that acute exposure of the blood vessels to PPs produces an EDR. The second is that when the tissues are incubated with PPs these compounds have the ability to abolish the acute response and the EDR evoked by acetylcholine. Our findings suggest a new area for
research into flavonoids which may have clinical implications.

REFERENCES


