Wine Polyphenols Decrease Blood Pressure, Improve NO Vasodilatation, and Induce Gene Expression

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Abstract—The effects of short-term oral administration of red wine polyphenolic compounds on hemodynamic parameters and on vascular reactivity were investigated in rats. Endothelial function and vascular smooth muscle contractility were studied in association with the induction of gene expression in the vascular wall. Rats were treated daily for 7 days by intragastric administration of either 5% glucose or red wine polyphenolic compounds (20 mg/kg). Administration of these compounds produced a progressive decrease in systolic blood pressure, which became significantly different on day 4. Aortas from rats treated with red wine polyphenolic compounds displayed increased endothelium-dependent relaxation to acetylcholine that was related to increased endothelial NO activity and involved a mechanism sensitive to superoxide anion scavengers. However, no increase in whole-body oxidative stress has been observed in rats treated with red wine polyphenolic compounds, as shown by plasma glutathione assay. Also, in the aorta, red wine polyphenolic compounds increased the expression of cyclooxygenase-2 and increased the release of endothelial thromboxane A2, which compensated for the extraendothelial NO-induced hyporeactivity in response to norepinephrine, resulting from enhanced inducible NO synthase expression. The present study provides evidence that short-term oral administration of red wine polyphenolic compounds produces a decrease in blood pressure in normotensive rats. This hemodynamic effect was associated with an enhanced endothelium-dependent relaxation and an induction of gene expression (of inducible NO synthase and cyclooxygenase-2) within the arterial wall, which together maintain unchanged agonist-induced contractility. These effects of red wine polyphenolic compounds may be a potential mechanism for preventing cardiovascular diseases. (Hypertension. 2001;38:159-165.)

Key Words: endothelium ■ arteries ■ nitric oxide ■ cyclooxygenase

Epidemiological studies have suggested that dietary factors, including moderate red wine consumption, might reduce the risk of cardiovascular diseases. In vitro studies have shown that the beneficial effect of fruits, vegetables, or red wine may be in part explained by the presence of polyphenols. Indeed, polyphenols possess a multitude of biological activities, including antioxidant and free radical–scavenging properties, leading to a decrease in LDL oxidation and decreased platelet aggregation. Very recently, these biological properties of polyphenols have been reported after in vivo treatment with either red wine or other dietary sources, such as grape juice. In addition, red wine supplementation of drinking water has been shown to modulate homeostasis and prevent experimental thrombosis in animals, independently of its alcohol content. Another therapeutically relevant effect of polyphenols on the cardiovascular system may be their ability to interact with the pathway leading to the generation of NO from vascular endothelium. This pharmacological effect of polyphenols is of importance because NO has vasorelaxant and antiaggregatory properties, and in the longer term, it induces the expression of genes protective of the cardiovascular system. Also, NO is able to limit the flux of atherogenic plasma proteins into the artery wall. In previous studies, we have reported that red wine polyphenol compounds (RWPCs) from different sources, including dry powder from red wine (Provinol) and RWPC1, were able to produce ex vivo endothelium-dependent relaxation in rat aortic rings by enhanced NO synthesis rather than by enhancing the biological effectiveness of NO or by protecting it against breakdown by superoxide anions (O2·−). However, few studies have reported the cardiovascular effects of RWPCs, inasmuch as it is not known whether a sufficient level of polyphenols can be reached in the circulation after oral administration of RWPCs. Mizutani et al have recently reported that in vivo administration of an extract of polyphenolic compounds from wine attenuates the elevation of blood pressure in spontaneously hypertensive rats, possibly by improving the biomechanical properties of the aorta.

The aim of the present study was to determine the effects of short-term oral administration of RWPCs on hemodynamic parameters, on vascular reactivity, and on the induction of gene expression in the vascular wall in normotensive rats.
Methods

Animals
This investigation conforms to authorization No. 01918 given by the Department of Agriculture of the French government. Rats had free access to pellets with the following composition in percentages: cereals and cereal produce, 88%; vegetable proteins (meal soy bean, yeast), 7%; vitaminized mineral mixture, 3%; and animal proteins (fish), 2%. Twenty-four male Wistar rats weighing 350 to 400 g (12 weeks old) were used in the present study and were equally distributed in 3 groups of rats. The first group (control) received the vehicle containing 5% glucose, the second and third groups received 2 varieties of RWPCs, Provinol (20 mg/kg) and RWPC1 (20 mg/kg), respectively, dissolved in 5% glucose. The rats were treated daily for 7 days by intragastric gavage with 2 mL/kg of either 5% glucose, Provinol, or RWPC1. The dose of RWPC corresponded to 10 times the maximal response obtained with norepinephrine (0.3 mol/L) and the COX inhibitor indomethacin (10 mol/L). Both the reduced and oxidized forms of blood NO synthase inhibitor were present 30 minutes before vasoactive agonist except for O_2^- scavengers, which were added 15 minutes before agonist application.

TXB_2 Production
The production of thromboxane B_2 (TXB_2) was determined in 1 mL physiological salt solution collected from aortic rings with endothelium in the absence or presence of norepinephrine added for 10 minutes. TXB_2 was measured by enzyme-immunoassay system (Research & Development Systems). TXB_2 production was expressed as picograms per microgram protein.

Immunoblotting With Anti-iNOS and Anti–Inducible COX-2 Antibodies
Aortas were homogenized, and ~100 μg of total protein from the supernatant fraction was loaded on 7% and 10% SDS-PAGE to separate inducible NO synthase (iNOS) and COX-2 proteins, respectively. After electrophoresis, proteins were transferred onto the nitrocellulose membrane. Immunostaining of iNOS and COX-2 was achieved by use of specific monoclonal mouse anti-iNOS and anti–COX-2 antibodies (Transduction Laboratories) and reacted with peroxidase-conjugated antimusle antibody. The blots were detected by using an enhanced chemiluminescence assay (ECL, Amersham).

Expression of Results and Statistical Analysis
The SBP of each animal is expressed in millimeters of mercury, and the heart rate is in beats per minute. Tension is expressed as grams per milligram of dry tissue, and relaxations are expressed as a percentage of the level of precontraction. A Student's unpaired t test or ANOVA was used for statistical analysis.

Drugs
The Provinol dry powder from red wine was provided by D. Ageron (Société Française de Distillerie, Vallont Pont d’Arc, France), and the RWPC1 (Cabernet Sauvignon grape variety) was from Dr M. Moutouet (Institut National de la Recherche Agronomique, Montpellier, France). RWPC1 and Provinol polyphenol contents have been already described as RWPC1 and RWPC2, respectively. All the chemicals were purchased from Sigma Chemical Co, except for MnTMPyP (Alexis Corp).

Results
The SBP and heart rate of rats receiving the vehicle, Provinol, or RWPC1 were not significantly different before the beginning of the treatment. Treatment of the rat for 7 days with the vehicle did not significantly alter the SBP (Figure 1) and heart rate. However, intragastric administration of either Provinol (Figure 1A) or RWPC1 (Figure 1B) produced a progressive decrease of SBP during the treatment. Compared with SBP in rats receiving the vehicle, the decreases in SBP produced by Provinol or RWPC1 were significantly different on days 4, 5, and 6 of the treatment. It should be noted that in anesthetized rats on day 7, the mean arterial pressure was significantly reduced in Provinol-treated rats compared with control rats, being 129±4 mm Hg (n=8 rats) and 141±2 mm Hg (n=7 rats), respectively.

In contrast, the heart rates of the rats receiving the vehicle, Provinol, or RWPC1 were not significantly different during the treatment both within the group and between the groups (not shown). Both the reduced and oxidized forms of blood
glutathione were not significantly different between control and RWPC1-treated rats (Figure 2).

**Vasoreactivity, Western Blot Analysis, and TXB₂ Production of Thoracic Aorta**

Acetylcholine produced concentration-dependent relaxation in aortas from either control or Provinol-treated rats (Figure 3A). Acetylcholine failed to produce relaxation in aortas without functional endothelium or aortas with functional endothelium in the presence of the NO synthase inhibitor L-NA (100 μmol/L) from both control and Provinol-treated rats. Acetylcholine-induced relaxation was significantly potentiated in aortas taken from either Provinol-treated \( (P < 0.05) \) or RWPC1-treated \( (P < 0.05, \text{not shown}) \) rats compared with the response obtained in aortas from control rats. The response to acetylcholine obtained in aortic rings taken from Provinol-treated rats was significantly reduced in the presence of SOD (100 IU) plus catalase (1000 IU) \( (P < 0.05, \text{Figure 3A}) \), such that the response to acetylcholine was not significantly different from that of aortas taken from control rats. Also, the response to acetylcholine was significantly reduced in the presence of MnTMPyP (100 μmol/L, \( P < 0.05 \)) in aortic rings from RWPC1-treated rats (not shown). Neither SOD plus catalase nor MnTMPyP significantly reduced the acetylcholine-induced relaxation in aortas taken from control rats (not shown).
The inhibitor of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, thapsigargin, and the Ca\(^{2+}\) ionophore, ionomycin, produced concentration-dependent relaxations of aortas with intact endothelium that were not significantly different in control or Provinol-treated rats (Figure 3B and 3C).

Norepinephrine produced concentration-dependent contractions of aortas with or without functional endothelium from both control and Provinol-treated rats (Figure 4). In the absence of functional endothelium, the contractile response to norepinephrine was significantly reduced in vessels taken from either control rats or rats treated with Provinol. In the absence of functional endothelium, the contractile response to norepinephrine was significantly reduced in vessels taken from Provinol-treated rats compared with vessels taken from control rats ($P < 0.05$, Figure 4B). The maximal response to norepinephrine was significantly reduced (ie, from $3.67 \pm 0.12$ to $2.67 \pm 0.43$ g/mg of dry tissue; $P < 0.05$) without any alteration of the pD\(_2\) values (ie, $8.42 \pm 0.07$ and $8.32 \pm 0.36$) in aortas taken from control and Provinol-treated rats, respectively. However, in endothelium-denuded aortic rings taken from Provinol-treated rats, blockade of NO synthesis with the NO synthase inhibitor L-NAME (300 \(\mu\)mol/L) restored the contractile response to norepinephrine toward that of aortic rings taken from control rats (Figure 5A). Furthermore, Western blot analysis of inducible NO synthase showed that although there was a weak expression of the 140-kDa form of iNOS in aortas from control rats, the expression of this isoform was markedly enhanced in aortas removed from Provinol-treated rats (Figure 5B). The iNOS expression was 2- to 3-fold higher in aortas from Provinol-treated rats compared with aortas from control rats.

Finally, in aortic rings with functional endothelium, the COX inhibitor indomethacin (10 \(\mu\)mol/L) did not significantly alter the contractile response to norepinephrine in the control group (not shown), but it markedly reduced the norepinephrine-induced contraction in vessels taken from Provinol-treated rats (Figure 6A, $P < 0.05$). The maximal response to norepinephrine was significantly reduced from $3.09 \pm 0.27$ to $1.66 \pm 0.20$ g/mg of dry tissue ($P < 0.01$) without any significant change in pD\(_2\) values (ie, $7.54 \pm 0.34$ and $7.40 \pm 0.12$) in aortas taken from control and Provinol-treated rats, respectively. Furthermore, there was a marked stimulation of TXB\(_2\) production by norepinephrine in aortic rings from Provinol-treated but not from control rats (Figure 6B). In the absence of norepinephrine, TXB\(_2\) production was not significantly different in aortas taken from the 2 groups. Moreover, aortas from Provinol-treated rats but not aortas from control rats displayed a marked expression (3-fold increase compared with control) of the 70-kDa isoform of COX-2 (Figure 6C).

**Discussion**

The present study shows that oral administration of RWPC from 2 different sources (ie, Provinol and RWPC1) to the rat modifies hemodynamic parameters and vascular reactivity. In vivo treatment of normotensive rats with RWPC decreased arterial blood pressure without any change in heart rate. In addition, RWPC treatment is associated with changes in the vascular reactivity of the thoracic aorta. RWPC treatment enhanced endothelium-dependent relaxation in response to acetylcholine but also induced increased expression of COX-2 with subsequent increased release of endothelial thromboxane A\(_2\) (TXA\(_2\)), which opposes the NO-induced hyporeactivity of the aorta to norepinephrine, which is consequent on increased iNOS expression.
In previous studies, we reported that RWPCs from both Provinol and RWPC1 were able to produce ex vivo endothelium-dependent relaxation in rat aortic rings as a result of enhanced NO synthesis. The present study provides evidence that in vivo administration of these 2 sources of RWPCs induced in the rat a progressive decrease in blood pressure without any alteration in heart rate. The 2 preparations of RWPCs have similar contents of polyphenols but contain a large number of compounds, including phenolic acids, flavonoids, anthocyanins, and tannins. The molecular identity of the compounds responsible for the in vivo effect described in the present study has not been assessed. However, it is clear that daily feeding of rats for 1 week with 20 mg/kg RWPC is adequate to produce a sufficient circulating concentration of compounds to induce cardiovascular effects. In our previous study, the compounds from RWPCs that meditated the ex vivo endothelial NO–induced relaxation of rat aortic rings included oligomeric-condensed tannins and anthocyanins but not polymeric-condensed tannins. Similar polyphenols might be implicated in the ex vivo effect of RWPCs and, hence, might act on the endothelium in vivo. In the present study, RWPCs decreased blood pressure in normotensive rats. It should be noted that Provinol was also able to prevent the increase in blood pressure in NO-deficient hypertensive rats (O. Pechanova, I. Bernatova, R. Andriantsitohaina, unpublished data, 2001).

The mechanism involved in the decrease in blood pressure produced by in vivo treatment with RWPCs is unknown. Very recently, it has been reported that the antithrombotic properties of red wine in vivo involve NO, inasmuch as these effects are prevented by the NO synthase inhibitor L-NAME. However, in the latter study, the origin of the NO production has not been established. In the present study, it can be hypothesized that NO, probably from the endothelium, might account for the decrease in blood pressure because RWPCs are able to stimulate its release in both in vitro and ex vivo experiments. Very recently, it has been reported that short-term ingestion of red grape juice (whose constituents include anthocyanins and tannins) improves flow-mediated endothelium-dependent vasodilation in patients with coronary artery diseases despite the use of antioxidant vitamins. These authors advanced the hypothesis that the mechanisms involved NO production.

With regard to endothelium-dependent vasodilation, it was found that acetylcholine-induced relaxation was increased in aortas taken from either Provinol- or RWPC1-treated rats. The activity of the NO pathway is probably increased in this phenomenon, inasmuch as the acetylcholine-induced relaxation in the aorta from either group was completely prevented by the NO synthase inhibitor L-NA. This could result either from augmented NO generation or from reduced NO breakdown by O2. The latter hypothesis is unlikely because relaxation produced by 3-morpholino-sydnonimine, which spontaneously releases NO and O2−, was not significantly different in aortas taken from control and RWPC-treated rats (not shown). Thus, the increased acetylcholine-induced relaxation is probably due to enhanced NO generation. The results also show that although the relaxing effect of acetylcholine was increased, the relaxation to the sarco-plasmic reticulum Ca2+-ATPase inhibitor, thapsigargin, or to the Ca2+ ionophore, ionomycin, was not modified by RWPC treatment. It is well established that an increase in cytosolic Ca2+ ([Ca2+]i) within the endothelial cell is the principal intracellular step for the activation of NO synthase. In contrast to thapsigargin and ionomycin, which increase [Ca2+], by direct action on Ca2+ handling, acetylcholine acts via second messengers. Therefore, it can be hypothesized that RWPC treatment improves endothelium-dependent vasodilatation by amplifying the pathway leading to second-messenger production, such as the activity of phospholipase C. On the other hand, it is interesting to note that the potentiating effect of RWPCs on acetylcholine-induced relaxation is attenuated in the presence of O2 scavengers MnTMPyP or SOD plus catalase. These results suggest that O2 production is involved in the effect of RWPC treatment. Glutathione is the most abundant antioxidant in the cell, in which it is formed predominantly in 2 redox forms: reduced glutathione and oxidized glutathione. It plays a central role in cellular defense against oxidative stress. Blood glutathione levels may reflect glutathione status in other less accessible tissues. Plasma measurement of both reduced glutathione and oxidized...
glutathione has been considered essential as an index of several physiological and pathophysiological situations and provides a sensitive index of whole-body oxidative stress. In the present study, no increase in the levels of GSSG was observed in the plasma from RWPC-treated rats. Thus, the involvement of O$_2^−$ scavenger–sensitive mechanisms in the improved relaxation to acetylcholine in vessels from RWPC-treated rats was not the consequence of an enhanced production of reactive oxygen species in the circulation. This observation is surprising, because O$_2^−$ is known to be implicated in the breakdown of NO. However, these results are in accordance with the in vitro effect of RWPCs in cultured bovine endothelial cells, in which O$_2^−$ production is implicated in the RWPC-induced rise in [Ca$^{2+}$]. This observation is also consistent with data reported in the literature, showing that O$_2^−$ induces an increase of [Ca$^{2+}$], in human umbilical vein and aortic endothelial cells. Therefore, the results from the present study suggest that increase of O$_2^−$ plays a role in the enhanced acetylcholine-induced vasodilatation that is due to endothelial NO after treatment of the rat with RWPCs. These results may explain in part the cardioprotective effect of moderate red wine consumption.

Turning now to the effect of RWPC treatment on vascular reactivity in response to a vasoconstrictor agonist, we note that the contractions to norepinephrine were unchanged by RWPCs in aortas with functional endothelium. In endothelium-denuded aortic rings from RWPC-treated rats, the norepinephrine–induced contraction was reduced; this hyporeactivity was prevented by the NO synthase inhibitor L-NAME and was associated with an increased expression of iNOS in the vessel. Taken together, these data are consistent with the hypothesis that RWPC treatment elicits extraendothelial production of NO from iNOS, which leads to vascular hyporeactivity toward norepinephrine. Meanwhile, the hyporeactivity to norepinephrine did not occur in RWPC-treated aortas that contained a functional endothelium; in these vessels, the COX inhibitor indomethacin decreased the contraction to norepinephrine. The inhibitory effect of indomethacin may be explained by the observation that there was an increase in TXB$_2$ production, the stable product of TXA$_2$, in aortic rings stimulated by norepinephrine from RWPC-treated rats. Moreover, aortas from RWPC-treated rats but not those from control rats displayed a marked expression of the COX-2 isofrom. Taken together, these results suggest that RWPC treatment is associated with increased participation in vascular regulation of endothelial vasoconstrictor products from COX, probably TXA$_2$, arising from the induction of COX-2. Thus, the present study highlights the induction of gene expression (ie, of iNOS and COX-2) by RWPCs, which allows maintenance of norepinephrine–induced contraction. This implies that there is a subtle balance between extraendothelial NO production, inducing vascular hyporeactivity, and increased endothelial release of vasoconstrictor products from COX (ie, TXA$_2$) in aortas taken from RWPC-treated rats.

In conclusion, the present study provides evidence that short-term oral administration of RWPC produces a decrease in blood pressure in normotensive rats. This hemodynamic effect of RWPC was associated with an augmentation of endothelium-dependent relaxation and a modest induction of gene expression of iNOS and COX-2 within the arterial wall, which together maintain unchanged agonist-induced contractility. These cardiovascular effects of RWPC may contribute to the beneficial effects of moderate red wine consumption against coronary artery disease.

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