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The Possible Implication of *trans*-Resveratrol in the Cardioprotective Effects of Long-Term Moderate Wine Consumption

FRANCISCO ORALLO, EZEQUIEL ÁLVAREZ, MERCEDES CAMIÑA, JOSÉ MANUEL LEIRO, EVA GÓMEZ, and PILAR FERNÁNDEZ

Departamentos de Farmacología (F.O., E.Á., M.C.) y Microbiología y Parasitología (J.M.L.), Facultad de Farmacia; Departamento de Farmacología, Facultad de Medicina y Odontología (E.G., P.F.), Universidad de Santiago de Compostela, Santiago de Compostela (La Coruña), España

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ABSTRACT

trans-Resveratrol (*t*-RESV; 1–10 μM), a phenolic component of wines, had no effect on phenylephrine-(PE; 1 μM) and high KCl-(60 mM) induced contractions in endothelium-denuded rat aortic rings. However, it relaxed the contractile response produced by these vasoconstrictor agents in intact rat aorta. The vasorelaxing effects of *t*-RESV were completely inhibited by N^G-nitro-L-arginine (L-NOARG; 0.1 mM) and methylene blue (10 μM), but they were unaffected by atropine (10 μM) and yohimbine (1 μM). The reversal effect produced by L-NOARG was antagonized by L-arginine but not by D-arginine (0.1 mM). *t*-RESV (1–10 μM) did not significantly modify rat aorta constitutive nitric-oxide synthase activity. However, this natural compound decreased NADH/NADPH oxidase activity in rat aortic homogenates. In addition, *t*-RESV (1–10 μM) was ineffective in scavenging superoxide anions ($O_2^{\cdot-}$) generated enzymatically by a hypoxanthine/xanthine oxidase (HX/XO) system and/or to inhibit XO. The above data demonstrate that the characteristic endothelium-dependent vasorelaxant effect of *t*-RESV in rat aorta seems to be caused by the inhibition of vascular NADH/NADPH oxidase and the subsequent decrease of basal cellular $O_2^{\cdot-}$ generation and, therefore, of NO biotransformation. Under the assumption that *t*-RESV exhibits a similar behavior in human blood vessels and bearing in mind that an overactivity of NADH/NADPH oxidase has been found in a number of cardiovascular pathologies, the results obtained in this work suggest that *t*-RESV could play an important role in the cardioprotective effects induced by the long-term moderate wine consumption.

Recently, studies on wine consumption have received considerable attention in both the scientific community and the general public. The lower incidence of coronary artery disease in the Southern French and other Mediterranean populations, despite a diet rich in saturated fat and high smoking habits (the so-called *French paradox*), has been attributed to the prolonged and moderate wine consumption by these populations, especially of red wine (St Leger et al., 1979; Renaud and de Lorgeril, 1992).

For certain wines, grape juices, and grape skin extracts, Fitzpatrick et al. (1993) described an endothelium-dependent vasorelaxant activity on rat aortic rings that seems to be independent of the alcohol content of the wine, probably because of as-yet-unidentified active constituents of grape skins and possibly mediated by NO release from endothelial cells. This endothelium-dependent vasorelaxation could explain, at least in part, the beneficial role of some of the above beverages in the so-called *French paradox* described above.

Although it has been reported that a number of flavonoids (some of the most prominent components of wines) have antioxidant activity (Hertog et al., 1993), they do not seem to be responsible for the endothelium-dependent vasorelaxant effects of wine, because they inhibit, with almost equal effectiveness, the contractions induced by several vasoconstrictor

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Some of the data obtained in this work and the effects of *t*-RESV on norepinephrine-induced contractions in rat aorta have already been published in abstract form: Orallo F and Camiña M (1998) Study of the endothelium-dependent and endothelium-independent vasodilator effects of resveratrol in rat aorta. *Br J Pharmacol* **124:**108P; Orallo F, Álvarez E, Leiro J, González S, and Fernández MP (2000) Inhibitory effects of resveratrol, a natural phenolic component of wines, on superoxide anion production in rats. *Methods Find Exp Clin Pharmacol* **22:**417.

ABBREVIATIONS: NOS, nitric-oxide synthase; *t*-RESV, *trans*-resveratrol (*trans*-3,4′,5-trihydroxystilbene); PE, phenylephrine; DMSO, dimethyl sulfoxide; L-NOARG, N^G-nitro-L-arginine; SOD, superoxide dismutase; ecNOS, endothelial constitutive nitric-oxide synthase; $O_2^{\cdot-}$, superoxide anions; HX, hypoxanthine; XO, xanthine oxidase; NBT, nitro blue tetrazolium; lucigenin, *N*′,*N*′-dimethyl-9,9′-biacridinium dinitrate; DPI, diphenyleneiodonium.

agents in intact and endothelium-denuded rat aortic rings, and their vasorelaxant effects are not reversed by NOS inhibitors (Fitzpatrick et al., 1993; Herrera et al., 1996).

trans-resveratrol (*t*-RESV; Fig. 1) is a phenolic natural component of *Vitis vinifera* L. (Vitaceae), mainly abundant in the skin of the grapes and found to be present in higher concentration in red than in white wines (Siemann and Creasy, 1992). This natural compound displays in the in vitro, ex vivo, and/or in vivo experiments a number of pharmacological effects, including modulatory lipoprotein metabolism, anti-inflammatory, platelet antiaggregatory, and antifungal properties, as reviewed previously (Soleas et al., 1997; Frémont, 2000). Recently, the therapeutic interest in *t*-RESV has increased considerably given that this drug may have a potential cancer chemopreventive activity in humans, as widely described in many articles published during the last few years (see, for example, Jang et al., 1997; Chun et al., 1999; Nakagawa et al., 2001).

Despite the above considerations, the in vitro vasodilator activity of this natural compound has not been studied extensively. In this context, Jager and Nguyen-Duong (1999) and Naderali et al. (2000) have described the vasorelaxant effects of *t*-RESV on porcine coronary arteries and on mesenteric and uterine arteries from female guinea pigs, respectively. Furthermore, Li et al. (2000) have demonstrated that this drug can enhance the activity of Ca^{2+}-activated K^+ channels (K_{Ca}) in endothelial cells derived from human umbilical veins, which may underlie the mechanism of *t*-RESV–induced vasorelaxation.

In rat aorta, on the other hand, controversial results have been reported. Thus, Fitzpatrick et al. (1993) have described that *t*-RESV (at concentrations up to 0.1 mM) has no effects on PE-induced contractions in endothelium-containing rat aorta, whereas Chen and Pace-Asciak (1996) have shown that *t*-RESV (at > 30 μM) causes relaxation of PE precontracted endothelium-intact rat aorta and at higher concentrations (>60 μM) also relaxes the endothelium-denuded rat aortic rings.

Given these apparent discrepancies and to provide new data for determining the possible implication of *t*-RESV in the protective effects of long-term moderate wine consumption against the incidence of cardiovascular diseases, we now report for the first time a detailed study of the possible endothelium-dependent vasodilator effects of this natural compound in rat aorta and the possible action of *t*-RESV on the L-arginine–NO–cGMP pathway, using different experimental protocols.

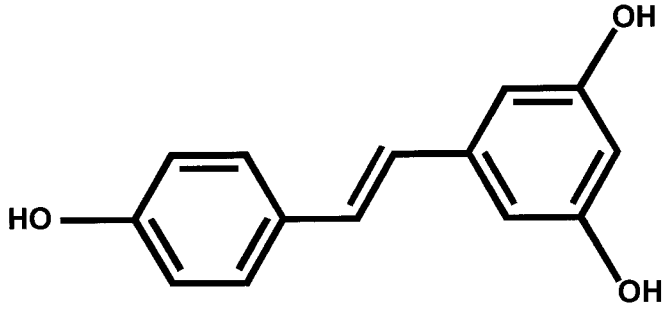

Fig. 1. Chemical structure of *t*-RESV (*trans*-3,4′,5-trihydroxystilbene).

Materials and Methods

Animals. Male Wistar rats (Iffa-Credo, L'Arbresle, Lyon, France), purchased from Criffa (Barcelona, Spain), were used throughout this study. They were housed (groups of five) in Makrolon cages (Panlab, Barcelona, Spain) on poplar shaving bedding (B&K Universal; G. Jordi, Barcelona, Spain) in a standard experimental animal bioclean room, illuminated from 8:00 AM to 8:00 PM (12-h/12-h light/dark cycle) and maintained at a temperature of 22 to 24°C. The animals had free access to food pellets (B&K Universal), drinking fluid (tap water), and were allowed to acclimatize for 1 week before the experiments.

Ethical Approval. The studies reported in this work have been carried out in accordance with the European regulations on the protection of animals (Directive 86/609), the Declaration of Helsinki and/or the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health (NIH Publication 85–23, revised 1996). In this context, all experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela, Spain.

Functional (Contraction/Relaxation) Studies in Isolated Rat Thoracic Aorta Rings. Vascular rings were prepared from the aortas of male Wistar rats weighing 250 to 300 g, essentially as described elsewhere (Orallo, 1997). In addition, contraction/relaxation studies were performed following the general method indicated in Orallo et al. (1998). Before initiating specific experimental protocols in the presence of the tested compounds, rat aortic rings were equilibrated at a resting tension of 2 g for at least 1 h. Thereafter, three consecutive isometric contractions induced by PE (1 μM) or extracellular high KCl (60 mM), instead of the equivalent amount of NaCl to maintain the osmolarity constant, were obtained in each ring at approximately 60-min intervals (time necessary to achieve a complete relaxation and to recover the basal tone), during which the physiological solution was replaced every 10 min to allow washout. Usually, the first contraction differed from the last two, which were reproducible. For this reason, when the third contraction of the vascular tissue in response to the corresponding vasoconstrictor agent stabilized (after approximately 10–15 min for PE and 15–20 min for KCl), a single concentration of acetylcholine (1 μM) was added to the bath to assess the endothelial integrity of the preparations. Endothelium was considered to be intact when this drug produced a strong vasorelaxation of precontracted vascular rings (see *Results*). On the other hand, the absence of acetylcholine relaxant action in the vessels indicated the total removal of endothelial cells.

Vasorelaxant Activity in Precontracted Rat Aortic Rings. Once the presence or absence of functional endothelium was verified and after a new washout and equilibration period of at least 60 min, the aortic rings were precontracted again with PE or high KCl. When the contractile response induced by these vasoconstrictor agents reached a stable value (plateau), increasing cumulative concentrations of *t*-RESV were added to the bath at approximately 25-to 30-min intervals (time necessary to obtain a steady-state relaxation). Control tissues were subjected to the same procedures simultaneously, but omitting the drug and adding the vehicle [appropriate DMSO dilutions].

In other experiments, 20 min before initiating the above experimental protocol, L-NOARG (0.1 mM), methylene blue (10 μM), atropine (10 μM), or yohimbine (1 μM) were included in the bath, to analyze the effects of these drugs on the vasorelaxation induced by *t*-RESV. In a different series of experiments, the ability of SOD to relax rat aortic rings was also studied and compared with that produced by *t*-RESV and acetylcholine.

In another series of aortic rings, the vasorelaxant effects caused by *t*-RESV (10 μM) were evaluated. Thereafter, a single dose of L-NOARG (0.1 mM) was included in the bath to study the reversal by this NOS inhibitor of the *t*-RESV–induced vasorelaxation. Finally, when the contraction produced by L-NOARG reached a steady state,

L-arginine (0.1 mM) or D-arginine (0.1 mM) was added to each ring to investigate the potential actions of these drugs on the L-NOARG-induced increase in vascular tone.

Determination of ecNOS Activity in Rat Aortic Homogenates. Male Wistar rats (250–300 g) were killed by cervical dislocation and immediate bleeding. Segments (2–3 cm) of rat thoracic aorta were rapidly removed and placed in a Petri dish with Krebs-HEPES solution at room temperature of the following composition: 135 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM K_2HPO_4, 10 mM HEPES, 11 mM glucose, pH 7.4, and cleaned of adherent fat and connective tissue. These vascular segments were frozen in liquid nitrogen, weighed and stored at −70°C for several weeks without loss of NOS activity.

Frozen rat aortic segments were homogenized in ice-cold buffer (pH 7.2 at 24°C) that contained 0.5 mM EDTA-Na_2, 20 mM HEPES, 300 mM sucrose, 1 mM dithiothreitol (Sigma, Alcobendas, Spain) and the protease inhibitor cocktail Complete mini (Roche Diagnostics GmbH, Mannheim, Germany), using first a glass Potter-Elvehjem tube with a manual glass pestle for mechanical disruption and, thereafter, a Polytron homogenizer (Kinematica, Littau-Luzern, Switzerland) for 15 s, at setting 7. One tablet of Complete mini was sufficient to inhibit the proteolytic activity of 10 ml of the homogenate solution.

This homogenate solution was centrifuged at 750*g* for 10 min at 4°C in a refrigerated centrifuge Hettich Universal 30F/RF (Hettich, Tuttlingen, Germany). The pellet was discarded and the supernatant was subsequently used for determination of ecNOS activity. The protein concentration in this supernatant was measured by the Bradford method (1976), using a protein assay kit from BioRad Laboratories (Alcobendas, Spain).

ecNOS activity was determined by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, as we reported previously (Fernández et al., 1998) and according to the method described by Bredt and Snyder (1990) with modifications.

Briefly, ecNOS activity under steady-state conditions was assayed at pH 7.2 in test solutions of 1 ml that contained 0.1 mM EDTA-Na_2, 0.45 mM $CaCl_2$, 1 mM tetrasodium salt of NADPH, 20 mM HEPES, 60 mM sucrose, 0.2 mM dithiothreitol, 0.25–20 μM purified L-[^{3}H]arginine, 250 to 350 μg of homogenate protein, and different concentrations of *t*-RESV (1, 3, or 10 μM). The incubations were carried out for 10 min at room temperature (22–24°C). Control experiments were performed in parallel by replacing *t*-RESV by its vehicle (corresponding dilutions of DMSO in deionized water). In some test solutions, supramaximal concentrations (2 mM) of L-NOARG, a well-known competitive inhibitor of NOS, were used (in place of *t*-RESV) to determine nonspecific binding of L-[^{3}H]arginine, which was approximately 50% of the total binding.

The reaction was stopped in each sample by the addition of 150 μl of 3.4% (w/v) of SDS in sodium citrate (0.05 M, pH 2.5). Thereafter, L-[^{14}C]citrulline (10 mM) was added to each tube and the samples were loaded on Dowex AG50 W-X4 (Bio-Rad counter-Na^+) cation-exchange resin columns (0.75 ml bed, 200–400 mesh; Bio-Rad Laboratories, Alcobendas, Spain). The effluent was collected into 20-ml scintillation vials. L-[^{3}H]Citrulline formed was eluted from the columns using 3 ml of distilled water.

Radioactivity was measured by a dual-label DPM program in an LKB Wallac 1409 Counter (PerkinElmer Wallac, Turku, Finland). The recovery of L-[^{14}C]citrulline was usually greater than 90%.

Generation of $O_2^{\cdot-}$ by Use of the HX-XO System. $O_2^{\cdot-}$ were produced enzymatically in a HX-XO system and estimated by the spectrophotometric measurement of the product of the reduction of NBT (Sigma, Alcobendas, Spain), essentially as described elsewhere (Robak and Gryglewski, 1988). Briefly, $O_2^{\cdot-}$ were generated by the reaction between HX and XO. Test solutions of 250 μl contained, in a phosphate buffer (50 mM KH_2PO_4-KOH, pH 7.4), the following: 1 mM EDTA-Na_2, 100 μM HX, 100 μM NBT, and *t*-RESV in various concentrations (1, 3, or 10 μM). Control experiments were carried out simultaneously by substituting *t*-RESV by its vehicle. In addition, the possible ability of this natural compound to directly reduce NBT was determined by adding *t*-RESV to solutions containing only NBT in a phosphate buffer.

Reaction was started with test solutions already in an AutoAnalyzer Cobas Fara 22–3123 (Roche, Barcelona, Spain), by adding XO in phosphate buffer (final concentration, 0.066 U/ml) and continued at room temperature (22–24°C) for 10 min. The rate of NBT reduction was calculated from the differential absorbance at 560 nm with a blank solution in which the XO was replaced by buffer solution. In some experiments, the sensitivity of the method was evaluated by measuring the influence of various concentrations of SOD, a known scavenger of $O_2^{\cdot-}$, on enzymatic reduction of NBT under the conditions described above.

Determination of XO Activity by Use of the Xanthine-XO System. The potential action of *t*-RESV on XO activity was tested by measuring uric acid formation (Robak and Gryglewski, 1988). Test solutions of 1 ml in a phosphate buffer (50 mM KH_2PO_4-KOH, pH 7.4) containing different concentrations of *t*-RESV (1, 3, or 10 μM), EDTA-Na_2 (1 mM), and 0.066 U of XO were incubated for 15 min at room temperature (22–24°C). Reaction was started by adding xanthine in phosphate buffer (final concentration, 100 μM), and the rate of uric acid production was calculated from the differential absorbance at 295 nm [measured at room temperature for 10 min in an UV-visible absorption spectrophotometer (Shimadzu UV-240; Shimadzu Corporation, Kyoto, Japan) against a blank solution in which XO was replaced by buffer solution. In some tests, the validity of the method was checked by measuring the influence of various concentrations of allopurinol (a well-known XO inhibitor reference product) on uric acid formation under the conditions given above. Similarly to $O_2^{\cdot-}$ scavenging studies, control experiments were performed by replacing *t*-RESV by its vehicle.

Determination of NADH/NADPH Oxidase Activity in Rat Aortic Homogenates. Rat aortic segments were prepared as described for the determination of ecNOS activity (see above), frozen in liquid nitrogen, and stored at −70°C until their use.

On the day of the experiments, these frozen rat aortic segments were homogenized in ice-cold phosphate buffer (50 mM KH_2PO_4-KOH, pH 7.4), which contained 0.01 mM EDTA-Na_2 and a protease inhibitor cocktail (Complete mini, Roche Diagnostics), as indicated in the determination of ecNOS activity.

The homogenate was centrifuged at 1,000*g* for 10 min at 4°C in a refrigerated centrifuge Hettich Universal 30F/RF. The pellet was discarded and the supernatant was subsequently used for determination of vascular NADH/NADPH oxidase activity. Protein content in this supernatant was measured by the Bradford method (1976), as indicated above. Vascular NADH/NADPH oxidase activity was evaluated by lucigenin (*N*′,*N*′-dimethyl-9,9′-biacridinium dinitrate; Sigma)-enhanced chemiluminescence according to the general procedure described by Zalba et al. (2000) with several modifications.

The reaction between $O_2^{\cdot-}$ and lucigenin was detected in a luminometer (BG-250; Optocomp II, MGM Instruments, Inc., Hamden, CT). Test solutions of 0.5 ml containing *t*-RESV in various concentrations (1, 3, or 10 μM) and 50 μg of homogenate protein (as source of the enzyme) in phosphate buffer were incubated for 15 min at room temperature (22–24°C) in appropriate vials already placed into the dark luminometer chamber. After this incubation period, lucigenin in phosphate buffer was directly injected in the vials (final concentration, 250 μM) using the system of direct injection incorporated in the luminometer, to minimize potential photochemical reactions of lucigenin mediated by natural light. Reaction was started 20 s later by adding 100 μM NADH (disodium salt) or NADPH (tetrasodium salt; Sigma) as substrates (freshly diluted in phosphate buffer). The chemiluminescence signal produced by 100 μM NADPH was very low and inappropriate to evaluate the potential inhibitory effects of *t*-RESV on vascular NADH/NADPH oxidase activity (Zalba et al., 2000).

Control experiments were performed in parallel by replacing *t*-RESV by its vehicle. In addition, the possible ability of this natural

compound to directly react with lucigenin was determined by adding *t*-RESV to solutions containing only lucigenin in a phosphate buffer.

The luminometer was set to record the development of chemiluminescence in terms of relative light units emitted over 0.5 s at 5 s intervals for 1 min (time sufficient to achieve the plateau phase). The specific light emission (used to obtain the final results) was calculated after subtraction of background activity which was determined from vials containing all components with the exception of the homogenate, which was replaced by a phosphate buffer solution.

In some experiments, the influence of different concentrations of DPI, a well known NADH/NADPH oxidase inhibitor, on the specific chemiluminescence signal was tested under the conditions given in the above paragraphs, to check the validity of the method and to compare the potency of *t*-RESV against DPI as possible inhibitor of vascular NADH/NADPH oxidase activity.

Data Presentation and Statistical Analysis. Unless otherwise specified, results shown in the text and figures are expressed as means ± S.E.M. Significant differences between two means ($p < 0.05$ or $p < 0.01$) were determined by Student's two-tailed *t* test for paired or unpaired data, where appropriate.

In the experiments carried out in precontracted rat aortic rings, contractile responses to vasoconstrictor agents are expressed as a percentage of the maximal contraction (E_{max} = 100%) produced by the corresponding vasoconstrictor agent before the addition of *t*-RESV. Concentration-response curves for the vasorelaxant effects of this natural compound were analyzed using a sigmoidal curve-fitting analysis program (Origin 5.0; Microcal Software, Inc., Northampton, MA) and the IC_{50} values of this natural compound were calculated.

Rat aorta ecNOS activity is expressed as picomoles of L-[^{3}H]citrulline formed per minute per milligram of protein (V_{max}). For the determination of this kinetic constant (V_{max}), the effect of different concentrations (0.25–20 μM) of L-[^{3}H]arginine on the ecNOS activity was evaluated in rat aortic homogenates. The V_{max} values were calculated by a computer-assisted curve fitting program (Kaleidagraph 3.08; Synergy Software, Reading, PA) using a predefined Michaelis-Menten equation.

In the experiments carried out to study the possible $O_2^{\cdot-}$ scavenging properties of *t*-RESV by use of the HX-XO system and the potential effects of this drug on XO activity, results are expressed as rate of NBT reduction in terms of variation of absorbance at 560 nm per unit of time or as rate of uric acid production in A_{295} units per minute, respectively. In these experiments, IC_{50} values for SOD and allopurinol were calculated by the least-squares linear regression, using a fitting analysis program (Origin 5.0), of log molar concentration of the tested compound on percentage of maximal pharmacological response obtained with each concentration.

NADH/NADPH oxidase activity in rat aortic homogenates is expressed as a variation of the specific chemiluminescence per unit of time (relative light units per second). In these experiments, the inhibitory effects of DPI and *t*-RESV are expressed as IC_{50} values, which were calculated as described above.

Drugs, Chemicals, and Radioisotopes. The drugs used in our experiments were: *t*-RESV, L-arginine, D-arginine, L-PE hydrochloride, HX, xanthine, methylene blue, acetylcholine hydrochloride, atropine sulfate, L-NOARG, yohimbine hydrochloride, SOD (from bovine erythrocytes), DPI chloride, allopurinol, and XO (from buttermilk), all purchased from Sigma. The radioisotopes L-[2,3-^{3}H]-arginine (40 Ci/mmol) and L-[ureido-^{14}C]citrulline (57.80 mCi/mmol) were obtained from PerkinElmer Life Sciences (Pacisa and Giralt, Madrid, Spain).

The appropriate dilutions of the above drugs were prepared every day immediately before use, in phosphate buffer (for the experiments involving the HX-XO system and the vascular NADH/NADPH oxidase) or in deionized water (for the other experiments), from the following concentrated stock solutions (0.1 M unless otherwise specified) kept at −20°C: *t*-RESV in DMSO (Sigma); PE in deionized water [sodium bisulfite (0.2% w/v) was added to the PE stock solution to prevent oxidation]; acetylcholine (to test the presence and integrity of the endothelium), methylene blue, atropine, L-NOARG, L-arginine, D-arginine, yohimbine, DPI, allopurinol, and SOD 20 kU/ml in deionized water; HX and xanthine (10 mM) in a potassium hydroxide (0.1% w/v) solution (10 mM).

XO was dissolved daily before the experiments in a phosphate buffer. In all tests carried out in this work, deionized water and the appropriate dilutions of the different vehicles used had no significant pharmacological effects. Because of the photosensitivity of *t*-RESV, all experiments were performed in the dark.

The specific chemicals and materials used in the different tests were purchased from suppliers indicated in the corresponding sections. All the other chemicals, including the reagents used in the preparation of different buffers and physiological solutions, were of the best quality available commercially.

Results

Effects on Resting Tension in Rat Aorta. In our experiments, the rat aortic rings lacked spontaneous activity, as we have reported previously (Orallo, 1997). The resting tone was unaffected by DMSO (0.14–1.40 mM), atropine (10 μM), yohimbine (1 μM), and *t*-RESV (1–10 μM) in endothelium-denuded and/or intact rat aortic rings ($n = 5$; $p > 0.05$; data not shown).

Vasorelaxant Activity of *t*-RESV in Precontracted Rat Aortic Rings. PE (1 μM) produced a sustained contraction in the rat isolated aortic rings with or without endothelium. The maximal tensions reached were 1954 ± 78 and 2821 ± 87 mg, respectively ($p < 0.01$, $n = 20$). Extracellular high KCl concentration (60 mM) caused a tonic contraction in intact and endothelium-denuded preparations. The maximum tensions generated were 2907 ± 90 and 3895 ± 104 mg, respectively ($n = 20$, $p < 0.01$). The differences between the contractions induced by both vasoconstrictors in endothelium-containing and/or endothelium-denuded rat aortic rings were significant ($n = 20$, $p < 0.01$). These contractile effects were maintained without significant tension changes in control rings for at least 90 min. DMSO (0.14–1.40 mM) had no significant effect on PE- and extracellular high KCl-induced contractions in endothelium-denuded and/or intact rat aorta ($n = 5$, $p > 0.05$).

t-RESV (1–10 μM) relaxed, in a concentration-dependent fashion, the contractions induced by PE (1 μM) or by a high KCl concentration (60 mM) in endothelium-containing rat aortic rings but had no effect in endothelium-denuded rat aorta (Fig. 2). The difference between the IC_{50} values obtained with PE and KCl were significant ($n = 5$, $p < 0.01$; Table 1). The time necessary to obtain a steady-state relaxation with each concentration of *t*-RESV (31.4 ± 2.8 min) was similar to that exhibited by SOD (28.6 ± 2.4, $n = 5$, $p > 0.05$) and clearly higher than that of acetylcholine (2.4 ± 0.2 min, $n = 5$, $p < 0.01$).

The maximum percentage of relaxation (R_{max}) of *t*-RESV against PE- and KCl-induced contractions was similar to that caused by supramaximal concentrations of SOD (300 U/ml) but less than that produced by high concentrations of acetylcholine (Table 2). Furthermore, the NOS inhibitor L-NOARG (0.1 mM) reversed the relaxation induced by *t*-RESV; i.e., it caused the vascular tissue to contract to a level of tone close to that present before *t*-RESV addition [maximal tensions reached: 2214 ± 198 (PE) and 3112 ± 273 (KCl) mg, $n = 5$, $p > 0.05$ with respect to control values, see above]. The L-NOARG-induced increase in tone was reversed,

in turn, by L-arginine (0.1 mM; Table 2) but not by D-arginine (0.1 mM), indicating the competitive and enantioselective nature of the interaction. A representative record showing these effects on PE-induced contractions is shown in the Fig. 3.

Preincubation of endothelium-containing aortic rings with atropine (10 μM) or yohimbine (1 μM) had no significant action on the magnitude of high KCl and PE-induced sustained tension (Table 3; $n = 5$, $p > 0.05$). In addition, pretreatment of rat aorta rings with these drugs did not significantly modify the corresponding IC_{50} values for *t*-RESV-induced vasorelaxation (Table 1; n = 5, $p > 0.05$).

L-NOARG (0.1 mM) and methylene blue (10 μM) caused a slow onset (of about 15–20 min) and sustained contraction of endothelium-containing rat aorta rings. Subsequent addition of PE and extracellular high KCl concentration (60 mM) produced an additional increase in tension ($n = 5$, $p < 0.01$; Table 3). Cumulative addition of *t*-RESV (1–10 μM) did not relax L-NOARG + PE- or L-NOARG + KCl-induced contractions ($n = 5$, $p > 0.05$).

Potential Effects of *t*-RESV on ecNOS Activity in Rat Aortic Homogenates. Rat aorta ecNOS activity in control group was 20.4 ± 0.89 pmol of L-[^{3}H]citrulline/min per mg of protein ($n = 20$). *t*-RESV (1–10 μM) did not significantly modify this value: 19.81 ± 1.52, 21.01 ± 1.62 and 20.10 ± 1.48 pmol of L-[^{3}H]citrulline/min per mg of protein in the presence of *t*-RESV 1, 3 and 10 μM, respectively ($n = 5$, $p > 0.05$).

Potential Effects of *t*-RESV as Scavenger of $O_2^{\cdot-}$. We investigated the ability of *t*-RESV to scavenge $O_2^{\cdot-}$ generated

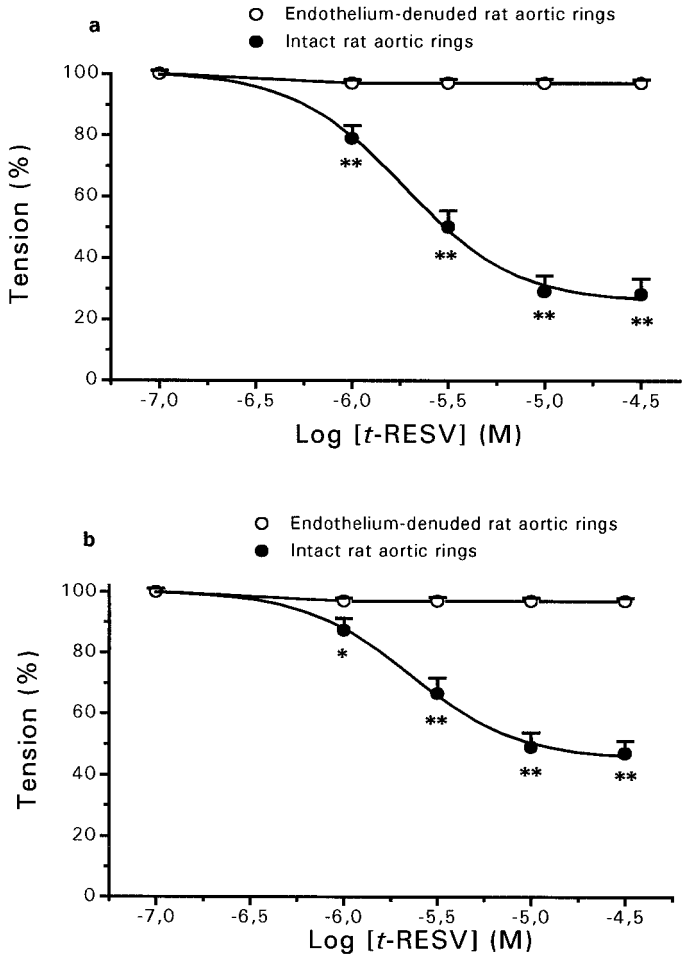

Fig. 2. Concentration-relaxation curves for *t*-RESV (1–30 μM) in endothelium-denuded and intact rat thoracic aortic rings precontracted with PE (1 μM; a) or extracellular high KCl (60 mM; b). Each point represents the mean value ± S.E.M. (indicated by vertical bars) from five experiments. Level of statistical significance: *, $p < 0.05$ or **, $p < 0.01$ with respect to the maximal tension (100%).

TABLE 1

IC_{50} values for *t*-RESV-induced vasorelaxation in endothelium-containing rat aortic rings precontracted with PE (1 μM) or extracellular high KCl concentration (60 mM) in the absence and presence of atropine (10 μM) or yohimbine (1 μM). Each value represents the mean ± S.E.M. from five experiments

Protocols	PE	KCl
	μM	
t-RESV	3.12 ± 0.26	9.60 ± 0.87
Atropine + *t*-RESV	2.94 ± 0.23	9.33 ± 0.76
Yohimbine + *t*-RESV	3.05 ± 0.21	8.97 ± 0.88

TABLE 2

Maximum percentage of relaxation (R_{max}) produced by different treatments in endothelium-containing rat aortic rings precontracted with PE (1 μM) or extracellular high KCl (60 mM). Each value represents the mean ± S.E.M. from five experiments

Protocols	PE	KCl
t-RESV (10 μM)	72.3 ± 4.8	53.2 ± 3.7
SOD (300 U/ml)	74.1 ± 5.0*	52.6 ± 3.6*
Acetylcholine (1 μM)	94.5 ± 6.9**	65.8 ± 4.4**
L-NOARG + *t*-RESV + L-arginine	75.1 ± 5.2*	54.6 ± 3.9*

* $p > 0.05$ versus the corresponding R_{max} for *t*-RESV.
** $p < 0.05$ with respect to the corresponding R_{max} values of *t*-RESV.

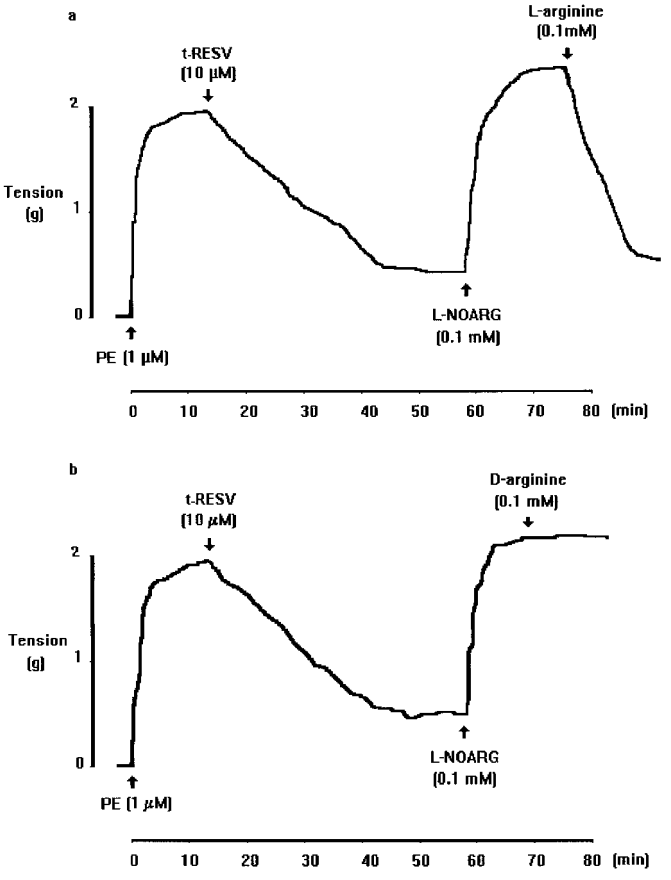

Fig. 3. Representative records showing the effects of *t*-RESV (10 μM) on endothelium-containing rat aortic rings precontracted with 1 μM PE. *t*-RESV-induced vasorelaxation was reversed by the NOS inhibitor L-NOARG (0.1 mM). This L-NOARG-induced contraction was, in turn, reversed by the normal substrate L-arginine (0.1 mM) (a) but not by D-arginine (b). Arrows indicate time of application of the corresponding drug.

by an enzymic system (HX-XO). *t*-RESV itself was unable to directly reduce NBT. On the other hand, the control activity of the generating system alone was 0.067 ± 0.003 A_{560} U/min (n = 20). This value was unaffected by *t*-RESV (1–10 μM); consequently, it had no effect as a selective scavenger of $O_2^{\cdot-}$ (Fig. 4a). SOD (0.1–10 U/ml), however, was capable of removing $O_2^{\cdot-}$ generated by the enzymatic HX-XO system (Fig. 4b). The IC_{50} value was 1.02 ± 0.06 U/ml (n = 5).

Potential Effects of *t*-RESV on XO Activity. The effects of *t*-RESV on XO activity were determined by measuring its ability to affect the production of uric acid from xanthine. The control activity of the generating system alone was 0.333 ± 0.016 A_{295} U/min (n = 20). *t*-RESV (1–10 μM) did not significantly modify this value (Fig. 5a); therefore, it did not inhibit XO activity.

Allopurinol (1–10 μM), however, decreased, in a concentration-dependent manner, the generation of uric acid and, therefore, XO enzymatic activity (Fig. 5b). The IC_{50} value was 3.63 ± 0.29 μM (n = 5).

Effects of *t*-RESV on NADH/NADPH Oxidase Activity in Rat Aortic Homogenates. *t*-RESV itself was unable to directly reduce lucigenin. On the other hand, NADH/NADPH oxidase activity in control rat aortic homogenates (using NADH 100 μM as the substrate) was 3.53 ± 0.12 relative light units/s (n = 20). *t*-RESV (1–10 μM) and DPI (0.5–3 μM) decreased, in a concentration-dependent manner, this control enzymatic activity (Fig. 6). The corresponding IC_{50} values were 4.81 ± 0.37 and 1.46 ± 0.10 μM (n = 5), respectively.

Discussion

In this work, the mechanism of the potential endothelium-dependent vasorelaxant activity of *t*-RESV, a natural product mainly derived from grapes of *Vitis vinifera* L., was studied in rat aorta.

The results presented in the above section clearly show that *t*-RESV (1–10 μM) produced a powerful concentration-dependent relaxation of endothelium-containing rings of rat aorta but had no effect on endothelium-denuded aortic rings. These results disagree with those reported by Fitzpatrick et al. (1993) for this natural compound on PE-induced contractions in rat aorta with endothelium (see the introduction), probably because of the different conditions used by Fitzpatrick et al. in their experiments, such as the presence of light (which degrades *t*-RESV), the use of anesthetics for tissue isolation, the use of a different rat strain and buffer, the low number of observations (n = 2) and possibly others.

This characteristic endothelium-dependent vasorelaxation caused by *t*-RESV seems to be mediated by an enhancement of the L-arginine-NO-cGMP pathway because: 1) It was blocked by L-NOARG (an inhibitor of NO synthesis) and methylene blue, an inhibitor of soluble guanylate cyclase (Kuriyama et al., 1995; Moncada et al., 1997). In addition, the relaxation induced by *t*-RESV was reversed by L-NOARG and this reversal was antagonized by L-arginine but not by D-arginine. 2) The vasorelaxant effects of *t*-RESV on high KCl-induced contractions were significantly smaller than those obtained on PE-induced contractions, as described previously for NO (Furchgott, 1983).

Two main mechanisms may be basically implicated in the enhancement of the L-arginine-NO-cGMP pathway by *t*-RESV: 1) the increase of the synthesis/release of NO from endothelial cells and/or 2) the decrease of NO inactivation. Thereafter, the process includes the diffusion of NO into the smooth muscle cells of the media and the stimulation of the soluble (cytosolic) guanylate cyclase, leading to a higher ac-

TABLE 3

Maximum tension values produced by PE (1 μM) or extracellular high KCl concentration (60 mM) in endothelium-containing rat aortic rings in the absence and presence of atropine (10 μM), yohimbine (1 μM), L-NOARG (0.1 mM), and methylene blue (10 μM). Each value represents the mean ± S.E.M. from the number of experiments shown in parentheses

Protocols	Maximum Tension Values
	mg
PE	1954 ± 78 (n = 20)
KCl	2907 ± 90 (n = 20)
Atropine + PE	1826 ± 114 (n = 5)
Atropine + KCl	2839 ± 132 (n = 5)
Yohimbine + PE	1863 ± 115 (n = 5)
Yohimbine + KCl	2935 ± 138 (n = 5)
L-NOARG	1295 ± 103 (n = 5)
L-NOARG + PE	2704 ± 129 (n = 5)
L-NOARG + KCl	3586 ± 181 (n = 5)
Methylene blue	1830 ± 118 (n = 5)
Methylene blue + PE	3396 ± 176 (n = 5)
Methylene blue + KCl	4478 ± 217 (n = 5)

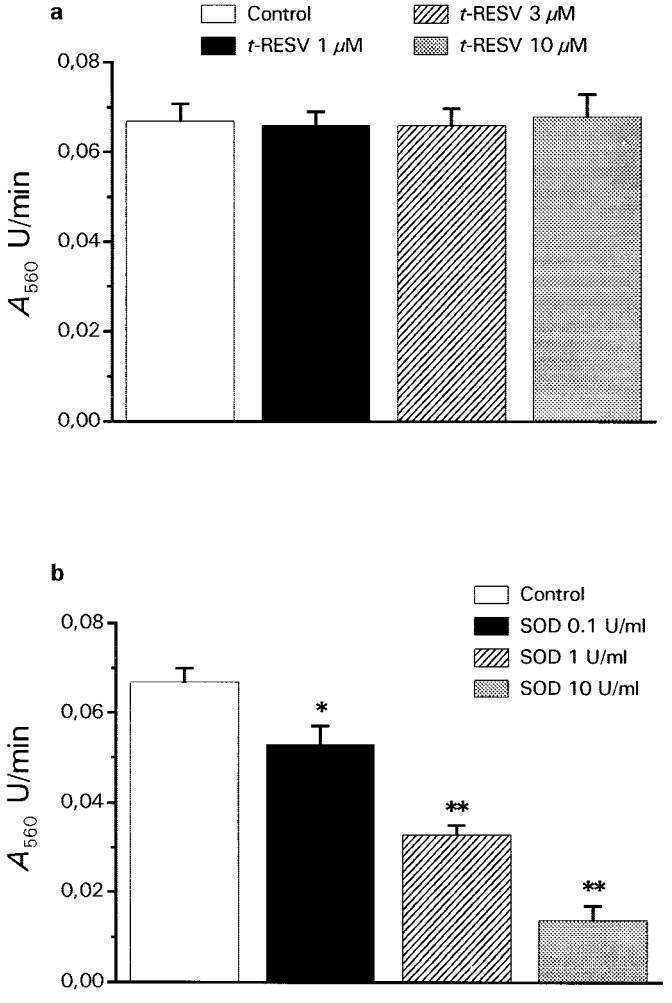

Fig. 4. The influence of *t*-RESV (1–10 μM) (a) and SOD (0.1–10 U/ml) (b) on reduction of NBT by $O_2^{\cdot-}$ generated by XO in the presence of 100 μM HX. Values are expressed as mean (n = 5) with S.E.M. shown by vertical lines. *, $p < 0.05$ and **, $p < 0.01$ with respect to control values from 20 experiments.

cumulation of cGMP, which could relax vascular smooth muscle via different mechanisms (Kuriyama et al., 1995; Orallo, 1996). Therefore, to provide new data to clarify this enhancement of the L-arginine-NO-cGMP pathway by *t*-RESV, different series of experiments were designed.

To investigate the potential effects of *t*-RESV on the synthesis/release of NO from endothelial cells, we have studied the possible direct or indirect activation by this drug of ecNOS. The potential direct effects of *t*-RESV on the activity of this enzyme were evaluated in rat aorta. Our results clearly demonstrate that this natural compound did not increase ecNOS enzymatic activity in rat aortic homogenates, which suggests that its characteristic endothelium-dependent vasodilator effects are not caused by a direct activation of this enzyme and, consequently, by an increase of NO biosynthesis. In this context, it is interesting to note that previous studies of the potential direct and acute action of *t*-RESV on ecNOS have not yet been reported. However, Hsieh et al. (1999) described that *t*-RESV, only at high concentrations (50–100 μM), may increase, after chronic treatment (3 days), the expression of this enzyme in cultured bovine pulmonary artery endothelial cells.

It has been extensively reported that, in rat aorta, the activation of endothelial muscarinic receptors and α_2-adrenoceptors by acetylcholine and α_2-adrenoceptor agonists, respectively, produces an elevation in the cytosolic free Ca^{2+} concentration, which stimulates the Ca^{2+}/calmodulin-dependent ecNOS and the subsequent and rapid production/release of NO (Eglème et al., 1984; Boulanger et al., 1994). Selective antagonists of the above receptors specifically block these effects.

The finding that the endothelium-dependent vasorelaxant action of *t*-RESV on PE or KCl-precontracted rat aortic rings is not antagonized by atropine (a known muscarinic receptor blocking agent) and yohimbine (a selective α_2-adrenoceptor antagonist) suggests that the *t*-RESV-induced NO release is not due to an indirect stimulation of the Ca^{2+}/calmodulin-

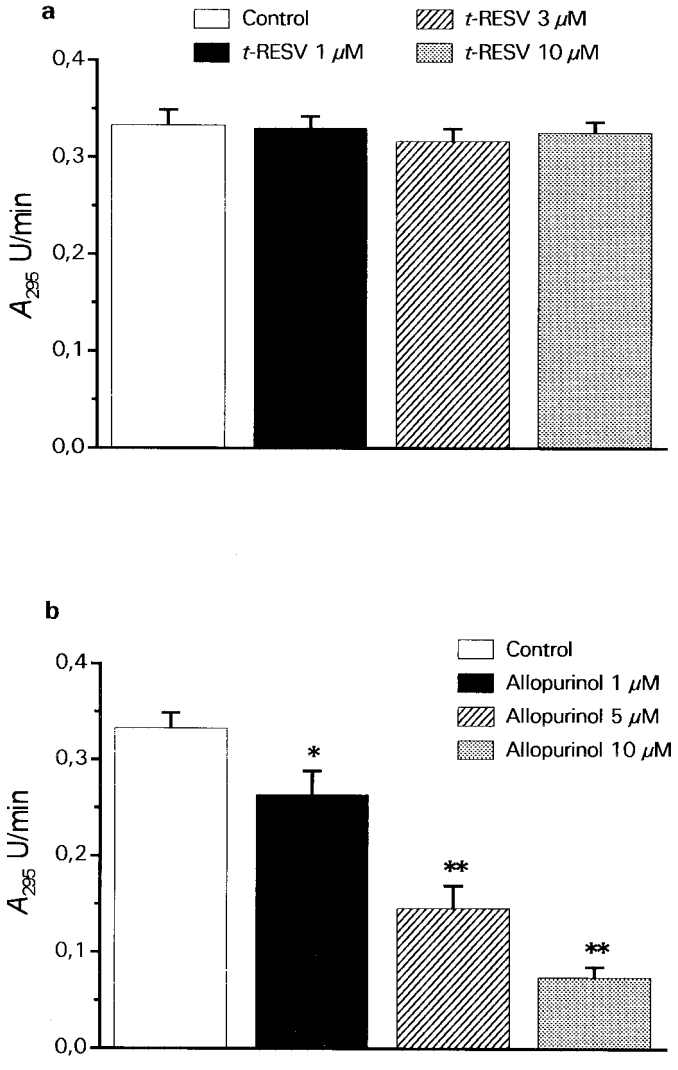

Fig. 5. The influence of *t*-RESV (1–10 μM) (a) and allopurinol (1–10 μM) (b) on production of uric acid by XO in the presence of 100 μM xanthine. Values are expressed as means (n = 5) with S.E.M. shown by vertical lines. * $p < 0.05$ and ** $p < 0.01$ with respect to control values from 20 experiments.

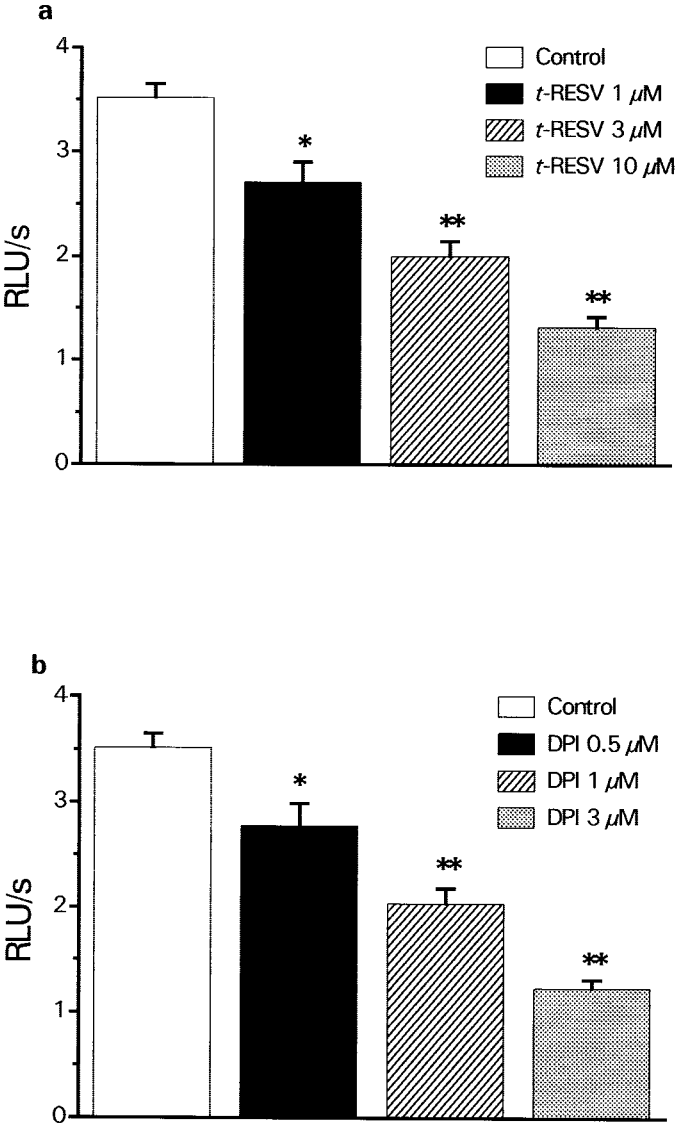

Fig. 6. Effects of *t*-RESV (1–10 μM) (a) and DPI (0.5–3 μM) (b) on NADH/NADPH oxidase enzymatic activity (using NADH 100 μM as the substrate) in rat aortic homogenates. Each column represents the mean value ± S.E.M. (indicated by vertical bars) from five experiments. *, $p < 0.05$ and **, $p < 0.01$ with respect to control values (n = 20). RLU, relative light units.

regulated rat aorta ecNOS (Moncada et al., 1997), via the activation by *t*-RESV of endothelial muscarinic receptors and α_2-adrenoceptors. This is supported by the fact that the time necessary to obtain a steady-state relaxation with each concentration of *t*-RESV was clearly longer than that exhibited by acetylcholine.

To study the possible inhibition by *t*-RESV of the biotransformation of NO, we have investigated the potential $O_2^{\cdot-}$ scavenging properties of this drug as well as its possible inhibitory effects on the cellular production of $O_2^{\cdot-}$.

The possible effects of *t*-RESV as selective scavenger of $O_2^{\cdot-}$ were evaluated by using the enzymic HX-XO system. It has been reported that XO converts HX or xanthine to uric acid, H_2O_2, and $O_2^{\cdot-}$ (see, for example, Cos et al., 1998). $O_2^{\cdot-}$ generated by this system react with NBT to produce formazan (NBT is reduced in the reaction). The formation of this colored compound (formazan) and, therefore, the amount of $O_2^{\cdot-}$ generated enzymatically, may be measured spectrophotometrically (see, for example, Robak and Gryglewski, 1988). When a drug lowers the amount of $O_2^{\cdot-}$ (i.e., the reduction of NBT) and at the same time does not affect the formation of uric acid, it is considered to be a selective scavenger of $O_2^{\cdot-}$. On the other hand, if a drug inhibits XO activity, both uric acid and $O_2^{\cdot-}$ concentrations are diminished.

In our experiments and unlike SOD, *t*-RESV did not modify formazan generation produced by the reaction of $O_2^{\cdot-}$ (formed by the HX-XO system) with NBT. In addition, unlike allopurinol, *t*-RESV had no effects on the enzymatic oxidation of xanthine to uric acid catalyzed by XO. These results clearly indicate that *t*-RESV does not display selective $O_2^{\cdot-}$ scavenging and/or direct inhibitory XO properties and, consequently, that these properties are not implicated in the endothelium-dependent vasorelaxant effects of *t*-RESV in rat aorta. In principle, the incapacity of *t*-RESV to selectively remove $O_2^{\cdot-}$ shown in this work does not seem to correlate well with the results obtained by Fauconneau et al. (1997) and Basly et al. (2000), who have previously reported that *t*-RESV has a direct scavenging effect on a stable free radical, 1,1-diphenyl-2-picryl-hydrazyl. However, it is interesting to note that the concentrations of *t*-RESV used by these authors were very high (>50 μM). In addition, the 1,1-diphenyl-2-picryl-hydrazyl tests are not specific and, therefore, not the most suitable to demonstrate the potential ability of *t*-RESV to selectively scavenge $O_2^{\cdot-}$.

The investigation of the potential inhibitory effects of a drug on cellular $O_2^{\cdot-}$ generation is basically possible by studying the possible inhibition of XO enzymic activity (see above) and/or NADH/NADPH oxidase. Therefore, we also studied the possible direct inhibitory effects of *t*-RESV on the activity of this latter enzyme (measured by lucigenin-enhanced chemiluminescence) in rat aorta.

It has been described that, in different cells of this vascular tissue, the plasma membrane-bound NADH/NADPH oxidase catalyzes the synthesis of $O_2^{\cdot-}$ from oxygen and NADH (the preferred substrate) or NADPH (Zalba et al., 2000).

The results presented in this work clearly demonstrate that *t*-RESV (like DPI) decreased the specific chemiluminescence signal emitted by the reaction between lucigenin and $O_2^{\cdot-}$ generated from NADH in rat aortic homogenates. This suggests that the enhancement of the L-arginine-NO-cGMP pathway produced by *t*-RESV and responsible for its characteristic endothelium-dependent vasodilator effects may be basically caused by the inhibitory action of this natural compound on basal cellular $O_2^{\cdot-}$ generation (via a direct inhibition of vascular NADH/NADPH oxidase enzymatic activity) and, therefore, on NO inactivation. This conclusion is supported by the fact that the percentage of relaxation produced by *t*-RESV on PE- and high KCl-induced contractions in rat aortic rings was similar to that caused by supramaximal concentrations of SOD (300 U/ml) but less than the maximum percentage of relaxation evoked by high concentrations of acetylcholine (1 μM).

t-RESV was found to be present at higher concentrations in red than in white wines, possibly because in the preparation of many white and rosé wines, even though red grapes are sometimes used, the grape skins (principal source of *t*-RESV) are removed before fermentation, allowing very little time for extraction of the vasoactive grape skin component(s) (Siemann and Creasy, 1992; Fitzpatrick et al., 1993). In addition, it is known that red wines are far superior vasorelaxants and/or protectants against cardiovascular diseases than white wines (St Leger et al., 1979; Renaud and de Lorgeril, 1992).

On the other hand, it is also interesting to note that 1) NO has been described to have important vasodilator, platelet antiaggregatory, and inhibitory effects on low-density lipoprotein oxidation (Radomski and Moncada, 1993; Kuriyama et al., 1995; Moncada et al., 1997). 2) A defective L-arginine–NO–cGMP pathway (altered ratio of NO/$O_2^{\cdot-}$ production) seems to exist in cardiovascular pathologies/conditions such as atherosclerosis, hypertension, etc. (see, for example, Marín and Rodríguez-Martínez, 1997; Hamilton et al., 2001), probably via an overactivity of NADH/NADPH oxidase (Meyer and Schmitt, 2000; Zalba et al., 2000). 3) The *t*-RESV concentrations reached in plasma and tissues after the oral administration of this natural compound to rats and humans (see, for example, Bertelli et al., 1998; Soleas et al., 2001) seem to approach the in vitro active concentrations (usually in the range 1–30 μM; see, for example, Bertelli et al., 1998; Wu et al., 2001).

Bearing in mind the above reports and under the assumption that *t*-RESV exhibits a similar behavior in human blood vessels, our results could explain, at least in part, the protection induced by the prolonged consumption of moderate amounts of wine, especially of red wine, against the incidence of cardiovascular diseases (mainly coronary heart disease).

Finally, taking into account the vascular effects of *t*-RESV described in the present work, it can also be concluded that: 1) *t*-RESV, a natural and typical phenolic component of wines, may have interesting therapeutic potential as an original chemical model for the development of new, selective, and efficient drugs, capable of inhibiting vascular NADH/NADPH oxidase activity, of protecting NO from inactivation by $O_2^{\cdot-}$ (via inhibition of $O_2^{\cdot-}$ generation catalyzed by the above enzyme) and, consequently, useful for reducing the risk of mortality from ischemic cardiopathy and other cardiovascular disorders and/or for improving their pharmacological treatment. 2) Because long-term large amounts of alcohol consumed (e.g., wine) can produce a number of adverse side effects in humans, foods and nonalcoholic beverages prepared from grape skins may be more beneficial and a better choice for *t*-RESV as alternative dietary source to be used for protecting against the incidence of cardiovascular pathologies.

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Address correspondence to: Dr. Francisco Orallo Cambeiro, Departamento de Farmacología, Facultad de Farmacia, Universidad de Santiago de Compostela, Campus Universitario Sur, E-15782 Santiago de Compostela (La Coruña), España. E-mail: fforallo@usc.es