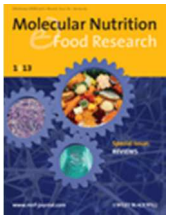

Endothelial cells ability to deconjugate resveratrol metabolites to free resveratrol: possible role in tissue factor modulation

Journal:	*Molecular Nutrition and Food Research*
Manuscript ID	Draft
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
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Keywords:	Resveratrol, Resveratrol metabolism, Piceid, Tissue Factor, Conjugation, Deconjugation

Endothelial cells ability to deconjugate resveratrol metabolites to free resveratrol: possible role in tissue factor modulation

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Keywords: Resveratrol, resveratrol metabolism, piceid, tissue factor, conjugation, and deconjugation.

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Abbreviations:

DMSO	dimethylsulfoxide
HAEC	human aortic endothelial cells
RV	Resveratrol
RV3G	RV-3-*O*-β-D-Glucuronide
RV4G	RV-4'-*O*-D-Glucuronide
RV3S	RV-3-*O*-Sulfate
RVmet	Resveratrol metabolites
TF	tissue factor
TNF-α	tumour necrosis factor-alpha

ABSTRACT

Scope. The antithrombotic effect of resveratrol (RV) and its forms remains unknown. The objective is to evaluate the effect of RV, its glucoside form piceid, and its biological metabolites (RV-3-*O*-β-D-Glucuronide, RV-4'-*O*-D-Glucuronide, and RV-3-*O*-Sulfate) on Tissue Factor (TF) modulation. Moreover, the endothelial metabolism of RV is assessed.

Methods and results. Human aortic endothelial cells were incubated with *trans*-piceid, *trans*-RV, or its biological metabolites and stimulated with TNF-α. TF activity, protein, and mRNA expression were determined in cell lysates. Moreover, RV conjugation (phase II metabolism) to its sulphated or glucuronidated metabolites and their deconjugation to their parent compound (free RV) were also assessed in cell lysates and culture media. RV decreased TF activity, protein, and mRNA expression whereas piceid and RV metabolites showed no effects. RV-3-*O*-Sulfate was the main metabolite generated in the endothelium, while RV metabolites were deconjugated to free RV. *Trans*-RV and its *trans*-metabolites isomerization was observed generating their *cis*-forms.

Conclusions. RV boasts antithrombotic effects modulating TF. RV metabolites and piceid did not show this effect. However, endothelial cells capacity to deconjugate RV metabolites to free RV indicates that RV metabolites can act as an endothelial reservoir for RV regeneration contributing to the antithrombotic effects of RV.

1. INTRODUCTION

Over the recent years, phytochemicals such as resveratrol (RV), have received increasing attention because of their potential benefits preventing and combating a wide amount of medical conditions[1–3]. RV has been traditionally under the spotlight due to its protective role in cardiovascular disease and its involvement in the "French Paradox" as phenol characteristic of red wine. This phenomenon, described by for the first time in 1992 by Renaud and Lorgeril, refers to the epidemiological observation that despite the high consumption of saturated fats, a relatively low incidence of coronary artery disease (40%) was observed in France. This effect was first attributed to the high intake of red wine, rich in RV, by the French population [4].

The polyphenol stilbene, RV (3,5,4'-trihydroxy-stilbene; **Supporting Figure S1A**) is a naturally occurring phenolic compound found in a wide range of edible fruits and vegetables (such as berries, peanuts, and grapes). Red wine is the main source of RV in the human diet, not least within the context of the Mediterranean diet [5–7], but it is also frequently consumed as a food supplement. RV and its glucoside form, called piceid (3,5,4'-trihydroxystilbene-3-β-D-glucoside; **Supporting Figure S1B**) are the main stilbenes present in red wine [6,7]. Both RV and piceid, exist in two isoforms with the *trans*-isomers being more thermodynamically stable and quantitatively more abundant in nature than the *cis*-isomers [5]. For this reason, many studies are focused on the beneficial effects of *trans*-isomers.

Clinical and preclinical studies have demonstrated that RV boasts healthy effects such as anticancer and anti-aging properties, delays age-related changes associated to obesity, and has a beneficial impact on neurodegenerative diseases mainly due to its antioxidant, anti-inflammatory, and antitumorigenic properties [3,8,9]. It is important to highlight that RV also provides a wide variety of cardioprotective effects modulating lipid profile, reducing blood pressure and atrial fibrillation, and boasting anti-inflammatory, antithrombotic, and antiplatelet aggregation effects [10–12]. The antithrombotic effect of RV is attributed to its capacity for modulating the tissue factor (TF) activity and gene expression in *in vitro* experiments [13–15]. TF, also known as thromboplastin, is the primary initiator of the extrinsic coagulation system triggering thrombosis via binding and activation of the coagulation factor VII[16]. TF is mainly expressed in the membranes of perivascular cells, platelets, neutrophils, and monocytes. Furthermore, TF is also expressed in endothelial cells in pathophysiological conditions in response to several stimuli such as tumor necrosis factor-alpha (TNF-α) [13,16,17]. However, no studies have been conducted to integrate the actions of RV on TF activity, its protein

concentration, and its mRNA expression. Moreover, piceid antithrombotic function via TF inhibition has not been reported so far.

Although oral absorption of RV in humans is about 75% [8], the presence of free RV in plasma as circulating metabolite is low as a consequence of its rapid and extensive phase II metabolism by the liver, intestinal tract, and gut microbiota [18]. Consequently, nearly twenty RV metabolites (RVmet) have been identified in plasma, urine, and in some tissues in humans and animals [3], in particular the sulfated and the glucuronidated forms RV-3-*O*-β-D-Glucuronide (RV3G), RV-4'-*O*-D-Glucuronide (RV4G), and RV-3-*O*-Sulfate (RV3S) [2,6,19] (**Supporting Figures S1C-E**). On the other hand, piceid is deglycosylated to RV by means of a glycosidase enzyme before being absorbed in the intestine and undergoing phase-II metabolism generating thus RVmet [6]. The concentration of these RVmet exceeds by far the concentration of their parent compound (free RV) in serum [20]. This evidence has aroused doubts about the clinical impact of the benefits of the native structure of RV observed in preclinical studies.

RVmet have shown to exert equal, comparable, or some degree of beneficial bioactivity to their parent compound, *i.e.* free RV [21]. In addition to RVmet bioactivity, these metabolites can be further deconjugated in the small intestine and in target tissues increasing free RV content in these tissues and exerting its reported benefits *in situ* [2,7,9,22,23]. However, to date little is known about the antithrombotic effects of RVmet and about the deconjugation process of RVmet to RV in the endothelium, as a crucial tissue involved in the onset and development of cardiovascular disease.

In the present study, we aim at discerning the antithrombotic activity of RV, piceid, and RVmet by integrating their effects on TF activity, TF protein concentration, and TF mRNA expression in human aortic endothelial cells (HAEC). Moreover, the metabolic capacity of endothelial cells on RV and RVmet has been explored in this *in vitro* model.

2. MATERIALS AND METHODS

2.1 Solvents and reagents.

Trans-RV and *trans*-piceid were provided by Sigma-Aldrich (Madrid, Spain). The *trans*-RVmet RV3G, RV4G, and RV3S were purchased from Bertin Bioreagent (Montigny le Bretonneux, France). These phenol standards were dissolved in dimethylsulfoxide (DMSO). TNF-α was purchased from Calbiochem® (Darmstadt, Germany) and dissolved in sterile water. All these reagents were stored at -70ºC protected from light. Bovine serum albumin and H_2SO_4 were also obtained from Sigma-Aldrich (Madrid, Spain). Methanol (HPLC grade),

acetonitrile (HPLC grade), and acetic acid were purchased from Scharlau Chemie (Barcelona, Spain). Ortho-phosphoric acid (85%) was acquired from Panreac (Barcelona, Spain). Ultra-pure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, USA).

2.2 Cell culture

HAEC (Cascade Biologics™, Portland, USA) at the 5th passage were seeded on NunclonTM delta-surface 12-well plates at a density of 1.1×10^4 of viable cells/cm^2 and maintained at 37ºC under 5% CO_2 atmosphere in M-200 culture medium supplemented with 2% (v/v) of low-serum growth supplement, 10 mg/mL gentamicin, 0.25 mg/mL amphotericin B (all from Life Technologies; Paisley, UK), 100 U/mL penicillin and 100 mg/mL streptomycin (both from Biowest, Barcelona, Spain). After 4 days of culture, confluent HAEC displayed a typical monolayer phenotype of quiescent endothelial cells under phase-contrast microscopy (IMT2 microscope by Olympus; Barcelona, Spain). Experiments were performed with confluent HAEC monolayers after 5 days in culture.

2.3 TF activity and protein assessment

Cells were incubated with the vehicle (DMSO), *trans*-piceid (5-50 μM), *trans*-RV (0.5-50 μM), or with *trans*-RVmet (RV3G, RV4G, or RV3S; 0.5-10 μM; 16h) and stimulated with TNF-α (10 ng/mL; 6h). Cells were then washed twice with warm phosphate-buffered saline and lysed by three repeated freeze-thaw cycles (-196ºC and 37ºC) in 110 μL/well of in-house tris-buffered saline containing 50 mM Tris (Sigma-Aldrich, Madrid, Spain), 100 mM NaCl (Panreac, Barcelona, Spain), and 0.1% Triton X-100 (Bio-Rad, Barcelona, Spain) at pH 7.4. Cells were allowed to completely lyse overnight at 4ºC. The following day cell lysates were centrifuged (10 min, 2000 *g*) and supernatants were stored at -80ºC until their use. TF activity and protein were determined in cell lysates by Actichrome ® chromogenic assay and Imubind® TF ELISA (Sekisui Diagnostics; Stamford, USA), respectively. Absorbance intensity was monitored in the multi-detection Microplate Reader Synergy HT (BioTek Instruments; Winooski, USA) at λ=405/490 nm for TF activity and at λ=450 nm for TF protein. TNF-α condition values were set at 100% and the other conditions were calculated in relation to this reference value.

2.4 TF gene expression assessment

Cells were incubated with the vehicle (DMSO), *trans*-piceid (5-50 μM), *trans*-RV (0.5-50 μM), or with *trans*-RVmet (RV3G, RV4G, or RV3S; 0.5-10 μM; 16h). Afterward, cells were stimulated with TNF-α (10 ng/mL; 6h). Total RNA was purified with PureLink® RNA Mini Kit

(Life Technologies; Paisley, UK). In short, cells were first lysed and homogenized with 600 µL of lysis buffer containing 1% 2–mercaptoethanol and guanidinium isothiocyanate. Cells were allowed to completely lyse for 1 h at 4ºC, total RNA was extracted with ethanol (70%) in spin cartridges containing a clear silica-based membrane that binds to RNA, and the purified total RNA was eluted in RNase-free water. Afterward, total RNA was quantified with NanoDrop™ spectrophotometer (Thermo Fisher Scientific; Barcelona, Spain). 0.5 µg of total RNA was reverse transcribed to cDNA (42 ºC; 50 min) using the 2720 Thermal Cycler (Applied Biosystems; Foster City, USA) with dithiothreitol random hexamers, deoxynucleotide triphosphates, SuperScript II reverse transcriptase, RNAse Out (all from Invitrogen; Paisley, UK), and $MgCl_2$ (Applied Biosystems; Foster City, USA). Quantitative real-time RT-PCR was performed with Taq polymerase and pre-designed primers for glyceraldehyde-3-phosphate dehydrogenase and TF (Applied Biosystems; Foster City, USA) using the ABI PRISM 7900 Detection System (Applied Biosystems; Foster City, USA) with the following profile: denaturing (95 ºC; 20 s) and 40 cycles of extension (95 ºC for 1 s and 60 ºC for 20 s). TF gene expression results were reported as fold-changes using the $2^{-\Delta\Delta Ct}$ mathematical method, with glyceraldehyde-3-phosphate dehydrogenase used as housekeeping gene. TNF-α condition values were set at 1 and the other conditions were calculated in relation to this value.

2.5 *Trans*-RV and *trans*-RVmet endothelial metabolism

To test the endothelial metabolic capacity to conjugate RV and deconjugate RVmet, cells were incubated with the vehicle (DMSO), *trans*-RV (0.5-10 µM), or *trans*-RVmet (0.5-10 µM; 16h). Culture media was centrifuged (500 *g*, 10 min, 4ºC) and cells were lysed with 0.5 mL of 0.5M NaOH. Culture media and cells were analysed by microelution solid-phase extraction (µSPE) in order to extract RV and RVmet, and OASIS HLB 2 mg micro-cartridges were used (Waters Corp., Milford, USA). Briefly, the micro-cartridges were sequently conditioned with 250 µL of methanol and 250 µL of 0.2% acetic acid. 350 µL of phosphoric acid 4% were added to 350 µL of culture media or cells. The solution was centrifuged (8784 *g*; 10 min, room temperature). The supernatant was loaded into the micro-cartridge. The loaded micro-cartridges were washed with 200 µL of Milli-Q water and 200 µL of 0.2% acetic acid. The retained RV and RVmet were then eluted with 2x50 µL of methanol and 2.5 µL of this solution was directly injected into the chromatographic system. Chromatographic analysis of RV and RVmet were performed with an Acquity Ultra-Performance liquid chromatography (UPLC) system equipped with a binary pump system (Waters). The analytical column was an Acquity UPLC™ Ethylene Bridged Hybrid C_{18} (100x2.1 mm, 1.7 µm) from Waters. The mobile phase was 0.2% acetic acid (eluent A) and acetonitrile (eluent B). The flow-rate and the

gradient elution was the reported in our previous study [6]. The tandem mass spectrometry analyses were carried out on a triple quadrupole mass spectrometer equipped with a Z-spray detector electrospray interface. RV and RV met were analysed in negative ion mode and the data were acquired through selected ion monitoring. The ionization source parameters were the reported in our previous study[6]. **Supporting Table S1** shows the retention time and the tandem mass spectrometry transitions for quantification and confirmation, as well as the cone voltage and collision energy values for RV and RVmet. The retention time of the *cis*-isomers were evaluated by exposing the vials of the *trans*-isomer standards compounds at UV light for 1 h. *Trans*-RV, *trans*-RV3G, *trans*-RV4G, and *trans*-RV3S were quantified by using own calibration curves. *Cis*-RV, *cis*-RV3G, *cis*-RV4G, and *cis*-RV3S were tentatively quantified by using the calibration curve of its corresponding *trans*-isomer.

2.6 Cytotoxicity

Cells were incubated with the vehicle (DMSO), *trans*-piceid (5-50 μM), *trans*-RV (0.5-50 μM), or *trans*-RVmet (RV3G, RV4G, and RV3S; 0.5-10 μM; 16h). DMSO concentrations never exceeded 0.1% (v/v) in culture medium to avoid cytotoxicity. Cells were centrifuged (500 g, 10 min, 4ºC) and supernatants were used to assess cytotoxicity. The extent of cytotoxicity in each experimental condition was determined by the colorimetric assay lactate dehydrogenase (LDH) Cytotoxicity Detection Kit (Roche Applied Science; Mannheim, Germany) and optical density absorbance was monitored at λ=492 nm. Vehicle control values were set at 100% and the other conditions were calculated in relation to this reference value.

2.7 Statistical analyses

Experiments were conducted twice on different days and conditions were run in duplicate or triplicate. Analysis of variance (ANOVA) with Bonferroni correction was used for multiple comparisons. Statistical significance was set at $P < 0.05$. Results are expressed as the mean ± standard deviation. Analyses were performed using statistical package for social sciences (SPSS) for Windows, version 25 (IBM corp., USA).

3. RESULTS

3.1 Effects of *trans*-piceid, *trans*-RV, and *trans*-RVmet on TF activity

HAEC stimulation with TNF-α (10 ng/mL; 6h) resulted in an increase in TF activity indicating HAEC activation (**Figures 1A-1C**). HAEC pre-incubation with *trans*-RV (0.5-50 μM; 16h) suppressed TF activity between 9.15% and 75.50% in a dose-dependent manner ($P<0.05$;

Figure 1B) compared to TNF-α condition. Interestingly, *trans*-RV at 50 μM completely abolished TNF-α-induced TF activity ($P<0.05$). By contrast, neither *trans*-piceid (5-50 μM) nor any *trans*-RVmet (0.5-10 μM) reduced TF activity at any of the concentrations tested (**Figures 1A** and **1C)**.

3.2 Effects of *trans*-piceid, *trans*-RV, and *trans*-RVmet on TF protein concentration

TF protein concentration in HAEC increased after TNF-α stimulation (10 ng/mL; 6h) indicating HAEC activation (**Figures 1D-1F**). HAEC pre-incubated with *trans*-RV (0.5-50 μM; 16h) resulted in a significant decrease in TF protein concentration between 13.54% and 73.19% ($P<0.05$) compared to TNF-α condition. On the contrary, neither *trans*-piceid (5-50μM) nor any *trans*-RVmet (0.5-10 μM; 16h) reduced TF protein concentration at any concentration tested, compared with the maximal stimulation triggered by TNF-α alone (**Figures 1D** and **1F**, respectively).

3.3 Effects of *trans*-piceid, *trans*-RV, and *trans*-RVmet on TF gene expression

HAEC stimulation with TNF-α (10 ng/mL; 6h) resulted in an increase in TF gene expression relative to vehicle control (**Figure 1G-1I**) indicating HAEC activation. As **Figure 1H** shows, HAEC pre-incubation with *trans*-RV at the highest doses tested (25-50 μM; 16h) resulted in a significant decrease in TF gene expression by 22.25% and 37.30%, respectively ($P<0.05$) compared to TNF-α condition. When it comes to *trans*-RVmet, out of all tested *trans*-RVmet and doses, merely RV4G at the highest dose tested (10 μM) increased TF gene expression by 47.44% (**Figure 1I**; $P<0.05$) compared to TNF-α. *Trans*-piceid (5-50 μM) did not reduce TF gene expression at any of the concentrations tested compared to TNF-α alone (**Figure 1G).**

3.4 *Trans*-RV and *trans*-RVmet endothelial metabolism

The metabolite profile obtained after cells exposure to free *trans*-RV (0.5-10 μM; 16h) was assessed in culture media and in cell lysates. RV underwent isomerization since not only *trans*- but also *cis*-RV were detected in both the media and the cells, the *cis*-isomer being predominant in both cases and at all doses tested (**Figure 2** and **Table 1**). The percentage of isomerization turned out to be constant regardless of the dose tested, ranging between 69.73 and 80.63% in media, and 40.27 and 63.49% in cells (**Table 1**). RV3G, RV4G, and RV3S were also detected in media and in cells, indicating that RV also underwent phase II

metabolism mainly to its sulfated form through a deconjugation process (**Figure 2** and **Table 1**).

The metabolite profiles obtained after cells exposure to every *trans*-RVmet (0.5-10 μM; 16h) were assessed in culture media and in cell lysates. Isomerization was observed when cells were incubated with *trans*-RVmet, the *cis*-isomers concentrations being higher than those of the *trans*-isomers (**Figure 3** and **Table 1**). Similarly to free RV, in culture media the concentrations of *cis*-isomers of RVmet were found to be dose-dependent ($P<0.05$) while the percentage of such isomerization was similar regardless the metabolite and the dose tested (71.13 to 89.90%). Conversely, in cells not only the concentration of the *cis*-RVmet but also the percentage of isomerization were found to be dissimilar according to the dose and the metabolite tested (**Table 1**). *Trans*-RV4G was the metabolite with less percentage of isomerization (36.72 to 67.90%) while *trans*-RV3G underwent total isomerization to *cis*-RV3G at the lower doses tested (0.5-1 μM) to such extent that the *trans*-isomer was not detected in cells. Interestingly, free RV was detected in media and cells exposed to every *trans*-RVmet, fact that indicates that RVmet deconjugation to RV occurred. Interestingly, RV3G, RV4G, and RV3S underwent deconjugation to their parent compound, mainly to *cis*-RV (**Figure 3** and **Table 1**).

3.5 Cytotoxicity

Neither piceid, RV, nor any RVmet were cytotoxic at any of the concentrations tested compared to vehicle control (**Supporting Figure S2**).

4. DISCUSSION

The present study confirms the hypothesis that RV modulates TF by decreasing its activity, its protein concentration, and its mRNA expression in TNF-α-activated endothelial cells, suggesting the capacity of RV to prevent the antithrombotic response in the course of atherosclerosis progression. Moreover, it is reported that neither piceid nor RVmet had such effect with regards to TF modulation at the concentrations tested. Moreover, our results indicate for the first time the HAEC capacity to conjugate RV to its main metabolites, and to deconjugate RVmet to free RV. In addition, *trans*-RV and *trans*-RVmet isomerization to their corresponding *cis*-isomers in endothelial cells is also reported.

RV effects on TF protein concentration observed in our study were comparable to those on TF activity, but not to those on TF gene expression. Since TF needs to be lipid-bounded and localized in the membrane to be active [24], the measured TF protein corresponds to active TF located at the cell surface, and not to inactive types of TF such as membrane-encrypted TF

or intracellular TF. Both TF intracellular pool and TF encrypted protein are activated and released from cells in response to cell damage by TNF-α and other stimuli [25]. In this sense, our results suggest that RV has no direct effect on activating TF protein but, rather, could be a consequence of its effect on TF protein synthesis. On the other hand, RV also modulated TF gene expression but to a lesser extent than TF protein concentration and activity. These results suggest that TF modulation by RV is accomplished at various cellular levels, *i.e.* not only inhibiting gene expression but also exerting post-transcriptional modifications. Concordantly, other authors have reported TF modulation at both transcriptional and post-transcriptional levels in response to lipopolysaccharide and cytokines in both monocytes and endothelial cells [26,27]. Pendurthi *et al.* found that RV regulates TF by inhibiting nuclear factor (NF)-kβ/Rel-dependent transcription, and impairs the transactivation potential of p65 [28]. Moreover, Houston *et al*. suggested the involvement of the transcription factor Egr-1 in mediating the response of the TF promoter to shear stress[29]. Finally, Witkowski *et al.* found that TF gene expression was also inhibited by microRNA-126 via interaction with the F3-3'-untranslated region of the TF mRNA [30].

The free RV has poor bioavailability resulting from its rapid and extensive phase II metabolism [18]. This evidence has questioned whether the benefits of RV obtained in *in vitro* experiments reflect the physiological effect of RV-containing edible items. To address this issue, in the present study the properties of the rapidly-metabolized forms of RV such as RV glucuronide and sulfate metabolites were studied. Our results indicate that any of the RVmet tested (RV3G, RV4G, and RV3S) modulated TF activity, protein, or gene expression at any of the doses tested. However, the present study reports for the first time that these metabolites underwent deconjugation to their parent form, *i.e.* free RV in endothelial cells, mainly to *cis*-RV. This finding has a significant impact because RVmet could provide a pool in endothelial cells from which the active free RV can be generated *in situ* by tissue glucuronidase and sulfatase hydrolytic enzymes, increasing the intracellular concentration of free RV in the endothelium, and therefore enhancing its beneficial effects. Thus, RV generation from its phase-II metabolites may result in the sustained exposure of tissues to the parent compound as also described in human colorectal cells [9], increasing RV benefits in the endothelium. To notice, most studies have focused on plasmatic levels of RV and its metabolites despite their beneficial effects are exerted at the tissue level. Some authors have reported that RVmet accumulate in the brain, heart, liver, colon, and kidneys in animal *in vivo* models [19] to such extent that RVmet concentrations in tissues are higher than those of free RV [22,23]. Moreover, evidence exists regarding RVmet deconjugation to parent RV in the intestine [2,7,23]. In this sense, Patel *et al.* reported that a sustained intake of 1 g of RV catered as a food supplement resulted in a concentration of 50-640 μM of RV in human colonic

tissue. Moreover, sulfate metabolites of RV contributed to the *in vivo* activity of such metabolites through regeneration of free RV in colorectal cell lines [9], supporting the idea that RVmet could serve as a reservoir for the parent compound. To the best of our knowledge, we are the first to report RVmet deconjugation to free RV, coupled to RV and RVmet accumulation in endothelial cells.

An additional novel finding of the present study is that RV conjugation to RVmet is also observed in endothelial cells, mainly to the sulfated metabolite RV3S. Concordantly, it has been previously reported that the sulfated forms of RV predominate over the glucuronidated conjugates in human plasma and urine [6,8] specifically RV3S [3,19], while mouse metabolism favors the glucuronidated forms [19]. However, little is known about the predominant metabolites accumulated in target human tissues, especially in endothelial cells.

Interestingly, our results suggest for the first time that endothelial cells conjugation and deconjugation capacity is limited, since the presence of RVmet in endothelial cells incubated with free RV, and the presence of free RV in cells incubated with RVmet were not dose-dependent. In agreement with this finding, several authors have reported that the sustained RV intake at high doses ends up in saturation of RV metabolism leading to higher circulating levels and tissue accumulation of free RV [2,23]. Moreover, Menet *et al* reported differences in RV and RVmet tissue accumulation after the acute and sustained intake of RV in mouse[18]. In this sense, a single dose of RV resulted in RVmet accumulation in heart and brain of mice, whereas the sustained intake of RV favoured the accumulation of free RV in these tissues [19], endorsing the evidence of the saturation of the RV metabolic enzymes in tissues.

Since free RV exerts TF modulation in endothelial cells, it would be expected TF modulation also in cells incubated with RVmet due to the generation of free RV resulting from RVmet deconjugation. However, in our study free RV doses with beneficial properties (0.5-50 μM) are by far higher than free RV concentrations obtained after RVmet *in situ* deconjugation, which did not exceed 120 nM in cells. Moreover, different cellular localization of RV and its metabolites may also be involved in the dissimilar effects observed in our study, as previously reported [22].

RV concentrations tested in the present study (0.5-50 μM) are higher than circulating RV levels obtained after dietary intake, which are in the nanomolar range. Within the context of the Mediterranean area, Zamora-Ros *et al*. reported that mean RV intake is 1629 μg/day in men and 235 μg/day in women in a Spanish cohort, being the red wine the most important source of RV. This RV intake is achieved by the consumption of two glasses (375 mL) or red wine with a concentration of 5 mg/L of RV, which corresponds to a dose of 27 μg/kg of RV

intake [7]. Such RV intake resulted in a peak concentration of 2.4 nM of free RV and 180 nM of RVmet in circulation [31]. Motilva *et al* has recently reported that an acute intake of 100 mL of dealcoholized red wine (containing 9.6 mg/L of RV) corresponds to 14 µg/kg of RV intake and results in a maximum concentration of 410 nM and 3.09 nM of RV sulfate and glucuronide conjugates, respectively, in human plasma, while no free RV was detected [6].

RV is not only consumed within the context of the Mediterranean diet but also is a dietary supplement widely consumed by virtue of its antioxidant and anti-cancer properties [22]. A relevant pharmacological acute dose of 100 mg/Kg of body weight was reported to result in 9 µM of free RV and 680 µM of total RVmet in circulation in humans [31], which is higher than circulating concentrations achievable with moderate red wine consumption. Moreover, repeated intake of 1 g/day of RV affords plasma concentrations of 0.6 µM of free RV, 22 µM of sulfate RVmet, and 8 µM of glucuronide RVmet [9]. Therefore, the RV and RVmet doses tested in our *in vitro* experiments are higher than the plasmatic doses achieved after moderate red wine consumption but similar to those attained after pharmacological intake of RV.

In the present study isomerization of RV and RVmet from the *trans*- to *cis*- forms is observed, being the *cis* isomers predominant. The *trans*-isomers are more common in nature and more stable [5,8], and stereoisomeric differences in their metabolism and biological activity have been described. Aumont *et al.* reported that RV metabolism is regio- and stereoselective, since glucuronidation of the *cis*-isomer is 5-10 times faster than that of the *trans*-form, and preferred the 3-position on both isomers leading to a lower bioavailability of the *cis*-isomer [32]. In spite of this, *cis*-isomers have been detected in human urine samples, specifically *cis*-RV-4-sulfate, *cis*-RV3G, and *cis*-RV4G [2]. When it comes to the biological activities of the *cis*- and *trans*-isomers of RV, some authors have reported similar anti-oxidant capacity [33], while others have reported differences in the estrogenic properties of such isomers [2].

The biological functions of piceid in the cardiovascular system have been widely explored [34–37]. To our knowledge, this is the first time that the antithrombotic activity of piceid is evaluated. In the present study, piceid exerted no modulation in TF mRNA expression, protein concentration, or activity conversely to the effects observed with RV. However, piceid is deglycosylated to RV before being absorbed in the intestine by means of a glycosidase enzyme [6,7,38], undergoing phase-II metabolism and generating thus RVmet. Therefore, the development of galenic formulations of piceid could be a good strategy to yield its absorption and therefore to promote its cardioprotective functions.

In summary, our results indicate that RV boasts antithrombotic effects by decreasing TF activity, protein concentration, and mRNA expression. Despite such benefits have not been attributed to RVmet, HAEC capacity to deconjugate RVmet to free RV indicates that RVmet can contribute to the antithrombotic effects of RV by serving as an endothelial pool for the parent compound, *i.e.* free RV. These data provide further evidence to recommend RV and RV-rich food items consumption as a possibly useful and complementary tool for the management of cardiovascular disease.

Acknowledgments and funding: This work was supported by the CENIT program from the Spanish Ministry of Science and Innovation (*Proyecto CENIT*: MET-DEV-FUN; 2006-2009). ÚC has a *Pla Estratègic de Recerca i Innovació en Salut* (PERIS) post-doctoral grant (SLT002/16/00239; Catalonia, Spain) from *Generalitat de Catalunya*.

SF-C, UC, and RS conceived and designed the experiment. SF-C and UC performed the experiment. SF-C, UC, and RS analysed the data. SF-C drafted the paper. All the authors critically interpreted the data and revised the manuscript.

Conflict of interest: None.

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FIGURE CAPTIONS

Figure 1. Effects of Piceid, RV, and RVmet on TF activity, protein concentration, and gene expression. Cells were incubated with the vehicle, trans-RV (0.5-50 μM), trans-piceid (5-50 μM), and trans-RVmet (RV3G, RV4G, and RV3S; 0.5-10 μM) for 16h and afterward, cells were stimulated with TNF-α (10 ng/mL) for 6h and lysed. In TF activity and protein concentration results, TNF-α condition values were set at 100% and the other conditions were calculated in relation to this reference value. TF gene expression results are reported as fold-changes using glyceraldehyde-3-phosphate dehydrogenase as housekeeping gene, in which TNF-α-activated cells value was set at 1 and the other conditions were calculated in relation to this reference value. Upper panel: effects of Piceid (A), RV (B), and RVmet (C) on TF activity; medium panel: effects of Piceid (D), RV (E), and RVmet (F) on TF protein concentration; lower panel: effects of Piceid (G), RV (H), and RVmet (I) on TF gene expression.* P< 0.05 versus vehicle control; † P< 0.05 versus TNF-α stimulation.

Figure 2. Presence of RV and total RVmet in endothelial cells incubated with trans-RV. Cells were incubated with the vehicle (DMSO) or trans-RV (0.5-10 μM) for 16h. Afterward, culture media were centrifuged and the presence of RV and RVmet were identified and quantified with UPLC/MS-MS in cell lysates (A) and in culture media (B). “Total RVmet” refers to the sum of the cis and trans-isomers of all the RVmet analysed (RV3G, RV3S, and RV4G). P-value specified by the legend corresponds to the P-trend between the different doses tested. * P< 0.05 versus vehicle control.

Figure 3. Presence of RVmet and total RV in endothelial cells incubated with trans-RVmet. Cells were incubated with the vehicle (DMSO) or trans-RVmet (0.5-10 μM) for 16h. Afterward, culture media were centrifuged and the presence of RV and RVmet were identified and quantified with UPLC/MS-MS in cell lysates and in cell-free supernatants. Upper panel: trans-RV3G, cis¬-RV3G, and total RV (cis- + trans- RV) identified in culture media (A) and cell lysates (B); medium panel: trans-RV4G, cis¬-RV4G, and total RV (cis- + trans- RV) identified in culture media (C) and cell lysates (D); lower panel: trans-RV3S, cis¬-RV3S, and total RV (cis- + trans- RV) identified in culture media (E) and cell lysates (F). P-value specified by the legend corresponds to the P-trend between the different doses tested. * P< 0.05 versus vehicle control.

TABLES

Table 1. Resveratrol and its metabolites and isomers concentrations (nM) detected in culture media and endothelial cells after exposure to RV, RV3G, RV4G, and RV3S.

	Exposure to RV				
	0,5 µM	**1 µM**	**5 µM**	**10 µM**	**P (trend)**
Culture Media					
trans-RV	20.36 ± 1.30	51.99 ± 11.74	822.73 ± 130.19	1804.62 ± 147.65	< 0,001
cis-RV	46.88 ± 15.75	193.27 ± 46.24	3424.05 ± 283.96	6381.48 ± 267.71	< 0,001
Total RV (cis+trans)	**67.24 ± 17.05**	**245.26 ± 57.98**	**4246.78 ± 414.15**	**8186.10 ± 415.36**	**-**
trans-RV3G	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
cis-RV3G	0.66 ± 0.93	0.00 ± 0.00	0.72 ± 0.17	0.42 ± 0.59	-
trans-RV4G	6.93 ± 1.32	6.00 ± 0.00	6.41 ± 2.05	10.03 ± 3.07	-
cis-RV4G	1.44 ± 0.00	0.00 ± 0.00	0.48 ± 0.68	1.32 ± 1.86	-
trans-RV3S	2.24 ± 2.16	3.62 ± 0.97	15.42 ± 8.60	4.05 ± 1.31	-
cis-RV3S	34.22 ± 36.75	67.00 ± 15.26	219.70 ± 64.41	58.34 ± 9.59	-
Total RVmet	**45.49 ± 41.16**	**76.62 ± 16.23**	**242.72 ± 75.91**	**74.16 ± 16.43**	**0,011**
Total RV+RVmet	**112.72 ± 58.21**	**321.88 ± 74.21**	**4489.50 ± 490.06**	**8260.26 ± 431.79**	-
Cells					
trans-RV	9.36 ± 1.00	19.86 ± 5.42	106.81 ± 6.62	230.93 ± 33.90	< 0,001
cis-RV	6.35 ± 8.98	23.33 ± 1.20	185.75 ± 46.74	377.61 ± 0.30	< 0,001
Total RV (cis+trans)	**15.71 ± 9.98**	**43.19 ± 6.62**	**292.57 ± 53.36**	**608.54 ± 34.20**	**-**
trans-RV3G	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
cis-RV3G	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
trans-RV4G	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
cis-RV4G	0.00 ± 0.00	0.67 ± 0.95	0.05 ± 0.07	0.00 ± 0.00	-
trans-RV3S	1.32 ± 0.58	1.41 ± 0.26	1.48 ± 0.43	1.23 ± 0.23	-
cis-RV3S	4.78 ± 1.70	8.28 ± 1.85	16.45 ± 3.24	10.99 ± 0.10	-
Total RVmet	**6.09 ± 2.28**	**10.36 ± 3.07**	**17.98 ± 3.74**	**12.22 ± 0.33**	**0,030**
Total RV+RVmet	**21.80 ± 12.26**	**53.56 ± 9.69**	**310.55 ± 57.10**	**620.76 ± 34.53**	**-**

(continued)

	Exposure to RV3G				
	0,5 µM	**1 µM**	**5 µM**	**10 µM**	**P (trend)**
Culture Media					
trans-RV	18.37 ± 6.72	12.70 ± 0.50	20.64 ± 2.91	29.15 ± 1.71	-
cis-RV	11.28 ± 8.73	1.84 ± 2.61	2.55 ± 3.61	3.97 ± 5.62	-
Total RV (cis+trans)	**29.65 ± 15.45**	**14.54 ± 3.11**	**23.19 ± 6.52**	**33.12 ± 7.32**	**0,054**
trans-RV3G	155.36 ± 30.17	316.66 ± 5.42	1516.00 ± 153.21	2732.12 ± 46.78	< 0,001
cis-RV3G	382.76 ± 43.56	909.21 ± 70.16	4013.62 ± 244.22	6798.55 ± 261.33	< 0,001
trans-RV4G	0.00 ± 0.00	8.17 ± 4.53	0.00 ± 0.00	9.82 ± 0.73	-
cis-RV4G	4.79 ± 2.71	2.28 ± 0.17	3.83 ± 1.69	4.07 ± 3.39	-
trans-RV3S	0.84 ± 0.05	1.42 ± 0.28	1.22 ± 1.36	0.40 ± 0.02	-
cis-RV3S	2.84 ± 0.57	4.80 ± 1.04	4.52 ± 4.59	1.24 ± 0.30	-
Total RVmet	**546.60 ± 77.06**	**1242.54 ± 81.61**	**5539.20 ± 405.06**	**9546.21 ± 312.55**	**-**
Total RV+RVmet	**576.24 ± 92.51**	**1257.08 ± 84.72**	**5562.39 ± 411.58**	**9579.33 ± 319.87**	**-**
Cells					
trans-RV	7.80 ± 1.81	12.34 ± 8.83	9.43 ± 0.50	9.22 ± 4.21	-
cis-RV	12.55 ± 5.82	10.28 ± 1.81	6.67 ± 2.91	12.06 ± 1.91	-
Total RV (cis+trans)	**20.36 ± 7.62**	**22.63 ± 10.63**	**16.10 ± 3.41**	**21.28 ± 6.12**	**0,151**
trans-RV3G	0.00 ± 0.00	0.00 ± 0.00	36.72 ± 2.37	96.64 ± 48.13	0,023
cis-RV3G	20.90 ± 4.07	30.61 ± 1.19	139.78 ± 8.81	403.42 ± 173.21	0,015
trans-RV4G	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
cis-RV4G	1.09 ± 1.54	1.40 ± 1.97	4.86 ± 3.80	6.82 ± 3.36	-
trans-RV3S	0.64 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
cis-RV3S	2.35 ± 1.39	3.13 ± 1.50	1.67 ± 0.26	2.31 ± 0.42	-
Total RVmet	**24.98 ± 7.03**	**35.14 ± 4.67**	**183.03 ± 15.24**	**509.20 ± 225.12**	**-**
Total RV+RVmet	**45.33 ± 14.66**	**57.76 ± 15.30**	**199.13 ± 18.65**	**530.48 ± 231.24**	**-**

(continued)

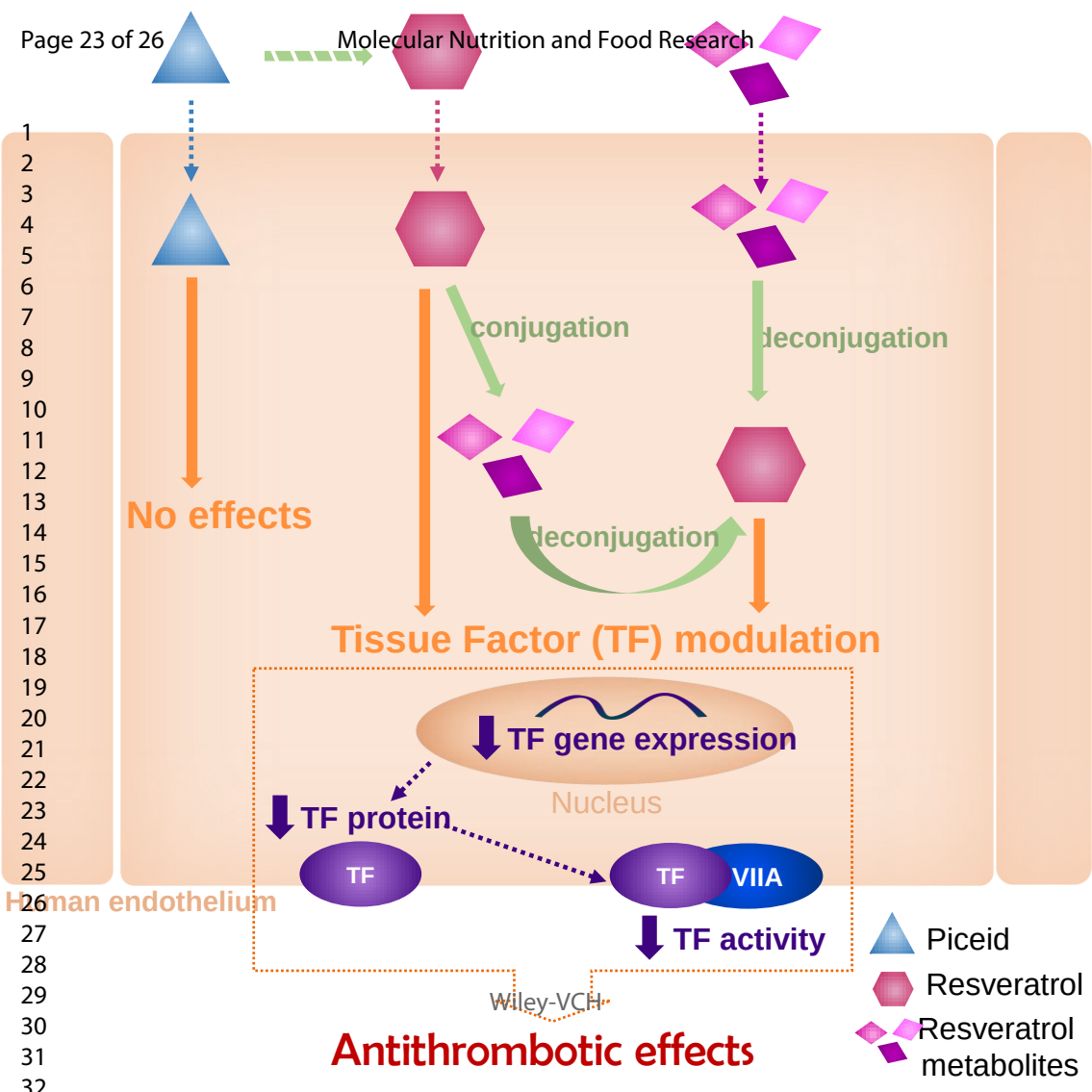
	Exposure to RV4G				
	0,5 µM	**1 µM**	**5 µM**	**10 µM**	**P (trend)**
Culture Media					
trans-RV	8.94 ± 0.20	9.43 ± 0.30	9.72 ± 1.50	16.24 ± 3.11	-
cis-RV	5.11 ± 6.42	2.70 ± 1.60	11.92 ± 2.81	9.43 ± 10.53	-
Total RV (cis+trans)	**14.04 ± 6.62**	**12.13 ± 1.91**	**21.63 ± 4.31**	**25.67 ± 13.64**	**0,029**
trans-RV3G	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
cis-RV3G	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
trans-RV4G	46.74 ± 32.47	95.96 ± 8.77	599.88 ± 56.30	2106.65 ± 13.16	< 0,001
cis-RV4G	368.74 ± 33.05	588.65 ± 31.86	3546.01 ± 44.74	8923.88 ± 142.02	< 0,001
trans-RV3S	9.14 ± 0.88	1.03 ± 0.18	0.71 ± 0.18	1.15 ± 0.26	-
cis-RV3S	6.83 ± 2.94	3.39 ± 2.06	2.24 ± 0.39	4.06 ± 0.37	-
Total RVmet	**431.45 ± 69.33**	**689.03 ± 42.88**	**4148.84 ± 101.62**	**11035.73 ± 155.82**	**-**
Total RV+RVmet	**445.50 ± 75.95**	**701.16 ± 44.79**	**4170.47 ± 105.93**	**11061.41 ± 169.46**	**-**
Cells					
trans-RV	9.36 ± 1.20	8.09 ± 1.60	11.49 ± 2.61	7.94 ± 0.80	-
cis-RV	37.02 ± 3.51	28.23 ± 11.13	69.72 ± 23.67	65.82 ± 6.32	-
Total RV (cis+trans)	**46.39 ± 4.71**	**36.31 ± 12.74**	**81.21 ± 26.28**	**73.76 ± 7.12**	**0,130**
trans-RV3G	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
cis-RV3G	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
trans-RV4G	17.89 ± 1.90	34.12 ± 1.75	261.00 ± 61.71	575.68 ± 38.46	< 0,001
cis-RV4G	37.85 ± 5.12	46.74 ± 3.07	253.97 ± 79.70	334.11 ± 165.25	0,031
trans-RV3S	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
cis-RV3S	2.57 ± 2.24	4.43 ± 1.33	5.63 ± 0.13	4.35 ± 0.09	-
Total RVmet	**58.31 ± 9.26**	**85.29 ± 6.16**	**520.60 ± 141.54**	**914.14 ± 203.81**	**-**
Total RV+RVmet	**104.69 ± 13.98**	**121.61 ± 18.90**	**601.81 ± 167.82**	**987.90 ± 210.93**	**-**

(continued)

	Exposure to RV3S				
	0,5 µM	**1 µM**	**5 µM**	**10 µM**	**P (trend)**
Culture Media					
trans-RV	12.91 ± 1.81	17.80 ± 6.52	37.66 ± 20.36	70.07 ± 34.10	-
cis-RV	17.38 ± 2.51	34.97 ± 8.53	108.30 ± 46.64	279.30 ± 60.38	-
Total RV (cis+trans)	**30.29 ± 4.31**	**52.77 ± 15.05**	**145.96 ± 67.00**	**349.38 ± 94.49**	**0,006**
trans-RV3G	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
cis-RV3G	0.18 ± 0.25	1.08 ± 0.34	0.66 ± 0.93	1.20 ± 0.51	-
trans-RV4G	10.44 ± 1.02	12.20 ± 6.43	7.55 ± 1.61	7.34 ± 1.02	-
cis-RV4G	0.00 ± 0.00	3.83 ± 0.00	2.28 ± 0.51	9.35 ± 3.05	-
trans-RV3S	56.50 ± 0.74	139.10 ± 51.39	1012.17 ± 101.09	1768.35 ± 583.12	0,004
cis-RV3S	502.79 ± 31.39	1063.36 ± 66.10	3935.82 ± 71.80	8955.03 ± 926.74	< 0,001
Total RVmet	**569.92 ± 33.41**	**1219.58 ± 124.26**	**4958.47 ± 175.94**	**10741.27 ± 1514.44**	**-**
Total RV+RVmet	**600.20 ± 37.72**	**1272.34 ± 139.31**	**5104.43 ± 242.94**	**11090.64 ± 1608.92**	**-**
Cells					
trans-RV	12.84 ± 2.71	12.48 ± 3.81	12.41 ± 0.10	30.71 ± 2.51	-
cis-RV	27.66 ± 6.52	4.89 ± 7.22	20.50 ± 12.04	83.98 ± 4.91	-
Total RV (cis+trans)	**40.50 ± 9.23**	**17.38 ± 11.03**	**32.91 ± 12.14**	**114.69 ± 7.42**	**0,001**
trans-RV3G	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
cis-RV3G	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
trans-RV4G	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
cis-RV4G	22.85 ± 2.34	12.30 ± 9.94	15.72 ± 4.83	29.37 ± 8.04	-
trans-RV3S	4.96 ± 1.54	7.17 ± 1.66	30.57 ± 2.02	102.90 ± 28.54	0,002
cis-RV3S	37.94 ± 12.09	52.81 ± 10.61	338.44 ± 42.95	1116.90 ± 171.28	< 0,001
Total RVmet	**65.75 ± 15.97**	**72.29 ± 22.22**	**384.73 ± 49.79**	**1249.17 ± 207.86**	**-**
Total RV+RVmet	**106.25 ± 25.20**	**89.66 ± 33.25**	**417.64 ± 61.93**	**1363.86 ± 215.28**	**-**

Data are expressed as mean ± standard deviation. RV, Resveratrol; RV3G, RV-3-*O*-β-D-Glucuronide; RV4G, RV-4'-*O*-D-Glucuronide; RV3S, RV-3-*O*-Sulfate. P-value corresponds to P trend.

The phenolic stilbene resveratrol (RV), its glucoside form piceid, and RV main biological metabolites (RVmet) were incubated with endothelial cells to test their antithrombotic activity and endothelial metabolism. RV was found to modulate Tissue Factor and to be conjugated to RVmet in the endothelium. Despite RVmet and piceid did not modulate Tissue Factor, endothelium capacity to deconjugate RVmet to free RV indicates that RVmet can act as an endothelial reservoir for RV regeneration contributing to the antithrombotic effects of RV.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TF gene expression
Nucleus
TF protein
TF
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Wiley-VCH
Antithrombotic effects
Piceid
Resveratrol
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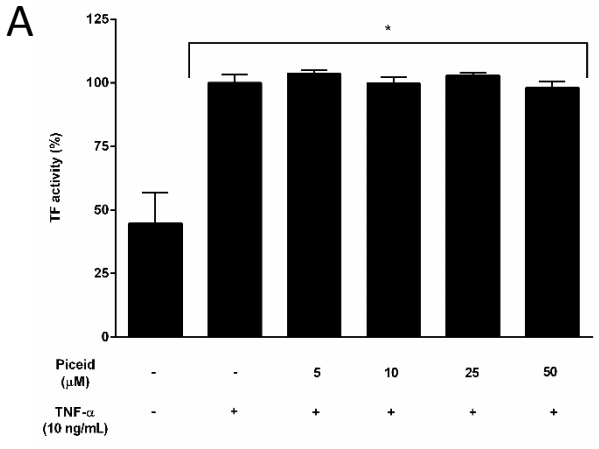
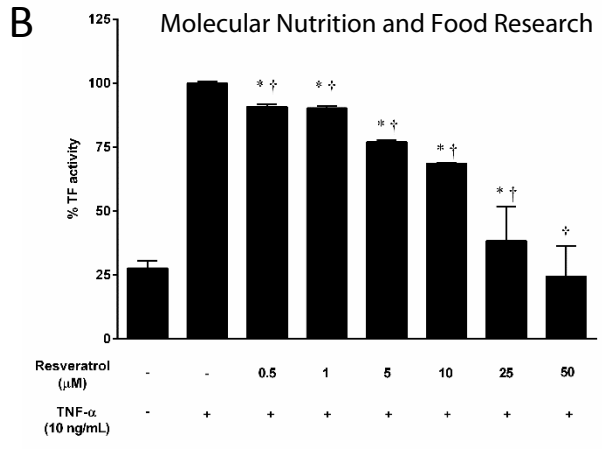
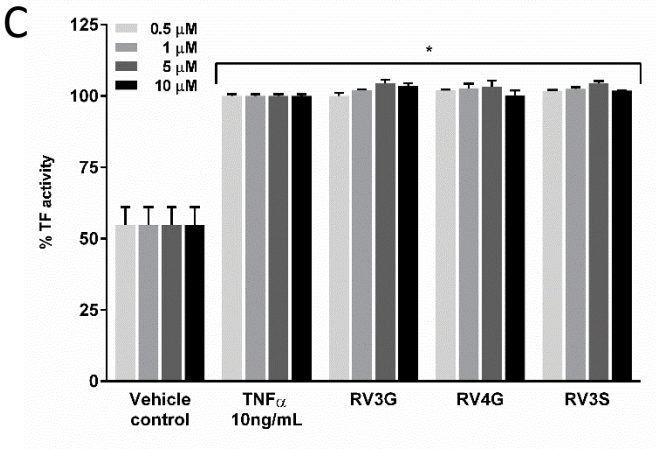
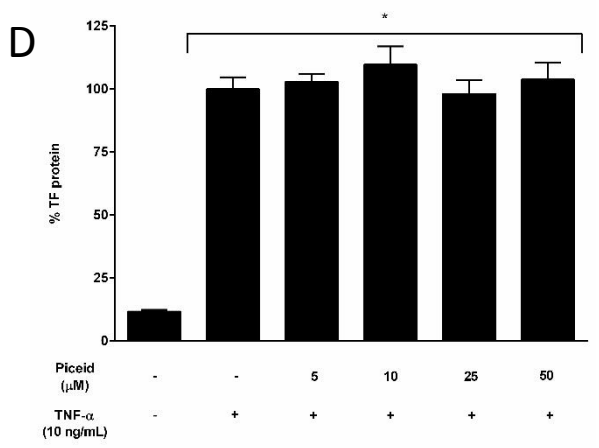
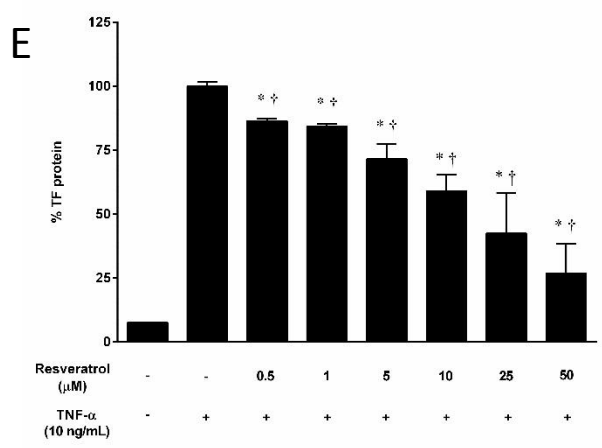
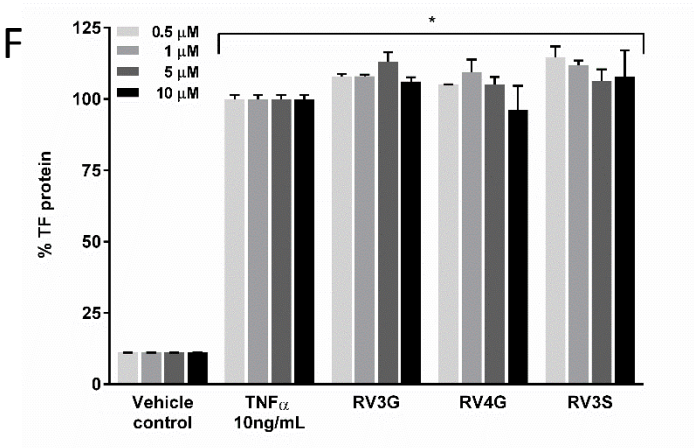

A
TF activity (%)
Piceid (μM)
TNF-α (10 ng/mL)

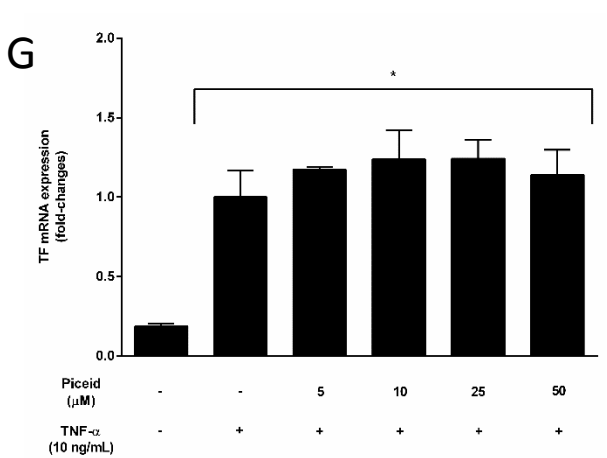

B
% TF activity
Resveratrol (μM)
TNF-α (10 ng/mL)

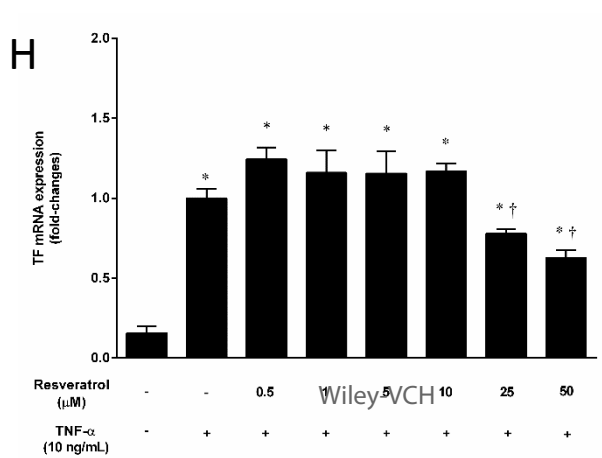

C
0.5 μM
1 μM
5 μM
10 μM
% TF activity
Vehicle control
TNFα 10ng/mL
RV3G
RV4G
RV3S

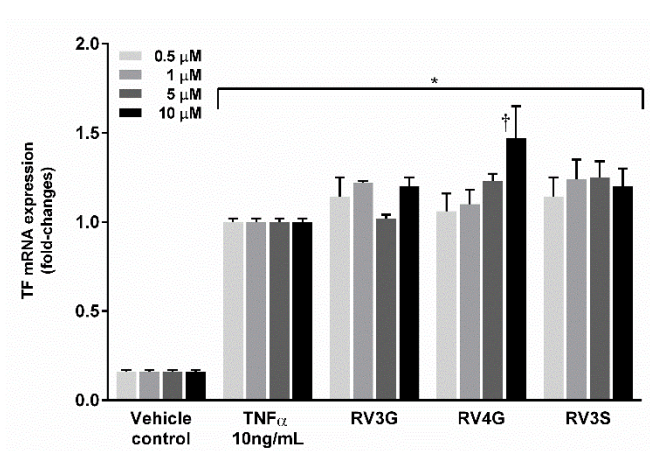

D
% TF protein
Piceid (μM)
TNF-α (10 ng/mL)

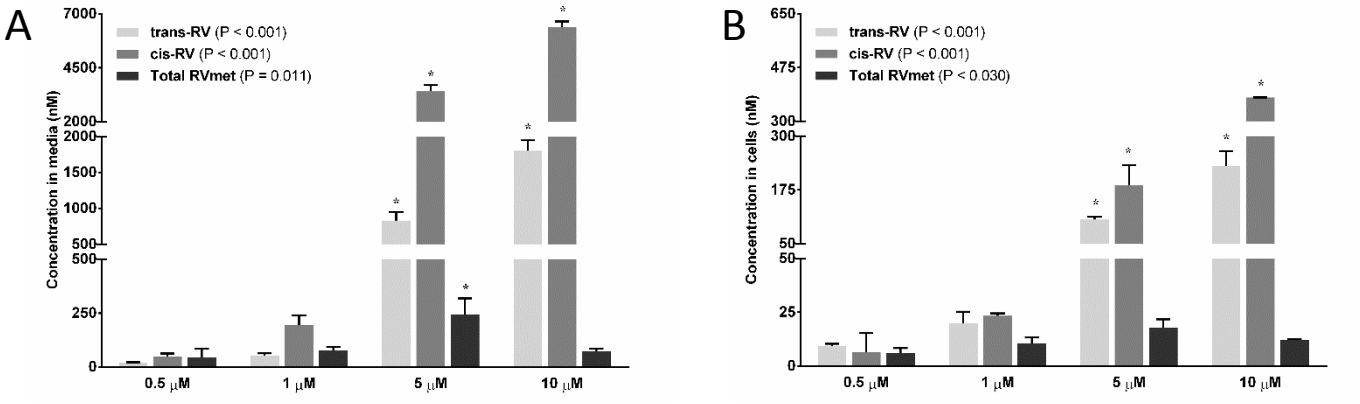

E
% TF protein
Resveratrol (μM)
TNF-α (10 ng/mL)

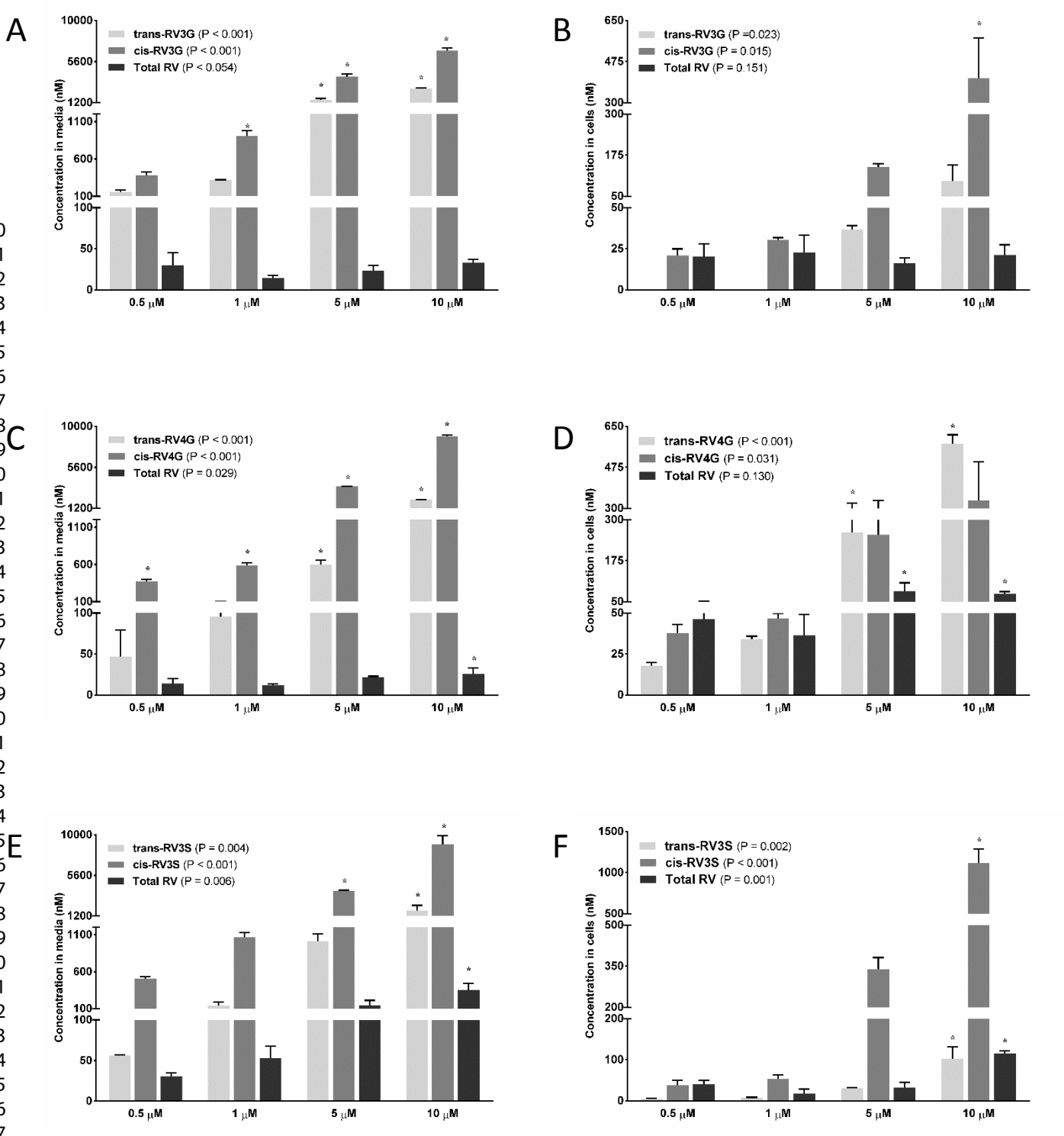

F
0.5 μM
1 μM
5 μM
10 μM
% TF protein
Vehicle control
TNFα 10ng/mL
RV3G
RV4G
RV3S


G
TF mRNA expression (fold-changes)
Piceid (μM)
TNF-α (10 ng/mL)


H
TF mRNA expression (fold-changes)
Resveratrol (μM)
TNF-α (10 ng/mL)


I
0.5 μM
1 μM
5 μM
10 μM
TF mRNA expression (fold-changes)
Vehicle control
TNFα 10ng/mL
RV3G
RV4G
RV3S


A
trans-RV (P < 0.001)
cis-RV (P < 0.001)
Total RVmet (P = 0.011)
Concentration in media (nM)
7000
4500
2000
2000
1500
1000
500
500
250
0
0.5 μM
1 μM
5 μM
10 μM
B
trans-RV (P < 0.001)
cis-RV (P < 0.001)
Total RVmet (P < 0.030)
Concentration in cells (nM)
650
475
300
300
175
50
50
25
0
0.5 μM
1 μM
5 μM
10 μM


A
trans-RV3G (P < 0.001)
cis-RV3G (P < 0.001)
Total RV (P < 0.054)
Concentration in media (nM)
0.5 μM
1 μM
5 μM
10 μM
B
trans-RV3G (P =0.023)
cis-RV3G (P = 0.015)
Total RV (P = 0.151)
Concentration in cells (nM)
C
trans-RV4G (P < 0.001)
cis-RV4G (P < 0.001)
Total RV (P = 0.029)
D
trans-RV4G (P < 0.001)
cis-RV4G (P = 0.031)
Total RV (P = 0.130)
E
trans-RV3S (P = 0.004)
cis-RV3S (P < 0.001)
Total RV (P = 0.006)
F
trans-RV3S (P = 0.002)
cis-RV3S (P < 0.001)
Total RV (P = 0.001)