Short Communication

A novel model to study the biological effects of red wine at the molecular level

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Several food items of plant origin, and in particular red wine, have been reported to protect from cardiovascular disease (CVD) development, thanks to their polyphenol components. Polyphenols undergo complex metabolic transformation during digestion and intestinal absorption. Here we report a novel model to study the effects of complex food matrices, applied to red wine, on gene expression in cultured primary human endothelial cells that takes into account the polyphenol metabolic transformation. Red wine was administered to human volunteers acting as 'bio-reactors'. Serum (RWS) obtained after 40 min was utilized to enrich endothelial cell culture media. The expression of specific genes involved in cell adhesion (vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM) and monocytes chemoattractant protein (MCP-1)) and fibrinolysis (tissue-plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2)) was considered as a molecular marker of cell function and related to the effects of RWS. The gene expression profile determined by RWS incubation was significantly different from that observed after the addition of red wine. Data obtained by this approach indicate the importance of taking into account the complex metabolic transformation of polyphenols that occurs during absorption when studying their effect on human health.

Red wine: Human serum: Endothelial cells: Gene expression

Dietary factors play an important role in the risk of degenerative diseases. Several reports indicate that light-to-moderate wine consumption reduces the risk of mortality by CVD (Gronbaek *et al.* 2000). This activity has been attributed to its main component, the polyphenol fraction (Sato *et al.* 2002). Polyphenols have been shown to possess multiple biological activities including antioxidant capacity, protection of LDL from oxidation, inhibition of platelet aggregation and vasorelaxation (da Luz & Coimbra, 2004). Moreover, polyphenols have been proposed to regulate the adhesion process (Ludwig *et al.* 2004) and promote fibrinolysis (Abou-Agag *et al.* 2001). The comprehensive understanding of the protective mechanisms exerted by polyphenols is hindered by the lack of complete knowledge on their bioavailability. Information about absorption, distribution, metabolism and excretion of individual flavonoids are scarce. Moreover, most *in vitro* studies addressing the molecular basis of polyphenol activity on the endothelium have been designed and performed on the basis of the addition to experimental cultured cell models 'as they are in the food', either as glycone or in their aglycone form. This approach is obviously unable to take into account the extensive metabolism of polyphenols during gastrointestinal absorption and often excludes any possibility to assess a possible synergic/co-operative activity between different molecules. In addition, the majority of the studies (Ludwig *et al.* 2004) considered concentrations in the 10–100 μM range, which is largely too high to be achieved in circulation in physiological conditions. Indeed, at least in theory, these concentrations could be obtained in specific tissues such as skin, where high amounts of polyphenols can be topically applied, or in the gastrointestinal tract after a meal. The obvious consequence of their complex metabolism and poor bioavailability is that the direct transfer of *in vitro* observations to *in vivo* conclusions should be made with caution. In fact, effects of polyphenols in the form found in food, detected *in vitro*, could be not necessarily relevant *in vivo* as previously suggested by our group (Totta *et al.* 2005) and by others (Rimbach *et al.* 2004).

Overall, these considerations strongly suggest the need for a model able to mimic the complex metabolism as it occurs in man, to be applied to cellular models to study the molecular mechanisms of complex food matrices undergoing significant

modification during metabolism. Here we propose a novel model to study the effect of red wine (possibly also other complex food matrices) on molecular aspects of cell function with a more physiological approach. In this model we utilized healthy subjects as 'bioreactors': after red wine consumption, blood was withdrawn and serum utilized to enrich the medium of human primary endothelial cells, *in vitro*. The serum isolated immediately before red wine supplementation was used as negative control. The effect of serum containing red wine metabolites on endothelial cells was evaluated by assessing the expression of selected genes involved in CVD, especially in the atherosclerotic process.

Materials and methods

Experimental model

A limited number of healthy men aged 35–40 years (*n* 3) were recruited and asked to consume an acute dose of red wine (5 ml red wine/kg body weight) in fasting conditions. Teroldego Rotaliano wine was selected among others due to its high content of polyphenols (Mattivi *et al.* 2002). The phenolic compound content of the wine is shown in Table 1. Blood was withdrawn before and 40 min after red wine supplementation and serum was isolated by centrifugation at 1800 *g* for 10 min. This time-point was chosen on the basis of previous studies demonstrating that this is the average peak time for polyphenols and alcohol absorption (Abu-Amsha Caccetta *et al.* 2000; Goldberg *et al.* 2003). Ethanol level was measured after serum isolation, using alcohol dehydrogenase enzymatic assay (Sigma Chemical Co., St Louis, MO, USA). Fasting serum (CS) and serum enriched of red wine metabolites (RWS) were used to supplement the culture medium (20 %) of primary human vascular endothelial cells (human umbilical vein endothelial cells (HUVEC)), in absence of bovine serum. HUVEC were incubated with CS and RWS for 16 h.

Phenolic compounds analysis

Analytical characterization of phenolic compounds in Teroldego Rotaliano wine was analysed as follows: total polyphenols, vanillin index, proanthocyanidins and total anthocyanins, using spectrophotometric assays (Rigo *et al.* 2000); resveratrol, hydroxycinnamic acids, flavonol aglycons and anthocyanins by HPLC (Mattivi, 1993; Spagna *et al.* 1996; Franco *et al.* 2002; Rossetto *et al.* 2004). Finally, catechin and epicatechin by LC–MS according to Mattivi *et al.* (2005). Moreover, total phenolic compounds in human serum were estimated by using the Folin-Ciocalteu method according to Serafini *et al.* (1998). Total phenols were expressed as (+)-catechin equivalent.

Endothelial cell culture

HUVEC were obtained from the umbilical cord as described previously (Jaffe *et al.* 1973). The nursery of 'Annunziatella' hospital of Rome kindly provided umbilical cords. HUVEC were grown on gelatin-coated tissue culture plates in 199 medium (Sigma) containing 20 % bovine serum (Sigma), HEPES (20 mM), heparin (50 U/ml; Sigma), L-glutamine

Table 1. Phenolic compound content of the red wine Teroldego Rotaliano

Phenolic compound	Content (mg/l)
Total polyphenols*	1748
Index vanillin*	762
Proanthocyanidins†	2301
Total anthocyanins‡	386
Malvidin 3-monoglucoside	123·0
Delphinidin 3-monoglucoside	12·7
Cyanidin 3-monoglucoside	3·4
Petunidin 3-monoglucoside	19·6
Peonidin 3-monoglucoside	7·2
Malvidin 3-monoglucoside acylated	51·6
Delphinidin 3-monoglucoside acylated	6·4
Cyanidin 3-monoglucoside acylated	0·8
Petunidin 3-monoglucoside acylated	6·5
Peonidin 3-monoglucoside acylated	5·1
Malvidin 3-monoglucoside *p*-coumarate	13·1
Delphinidin 3-monoglucoside *p*-coumarate	3·4
Cyanidin 3-monoglucoside *p*-coumarate	1·5
Petunidin 3-monoglucoside *p*-coumarate	0·5
Peonidin 3-monoglucoside *p*-coumarate	2·0
Quercetin	1·9
Miricetin	11·8
Kaempferol	0·15
trans-Resveratrol glucoside	0·6
cis-Resveratrol glucoside	ND
trans-Resveratrol	0·6
cis-Resveratrol	0·2
Catechin	37·0
Epicatechin	79·0
Mean degree of polymerization of proanthocyanidins	2·3
trans-Caftaric acid	23·4
cis-Coutaric acid	2·5
trans-Coutaric acid	10·7
Fertaric acid	1·6
trans-Caffeic acid	ND
trans-*p*-Coumaric	4·1
trans-Ferulic acid	0
2-S-glutathionylcaftaric acid	4·9
Tirosol	25·8

ND, not detectable.
Data as *(+)-catechin; †cyanidin; ‡malvidin 3-glucoside chloride.

(1 %; Sigma), penicilline/streptomicine (1 %; Sigma) under 5 % CO_2 at 37°C. Cells were utilized for experiments at 90–100 % apparent confluence within the third to fourth passages. Passages were performed according to standardized protocols and by diluting the cell population 1:3. Cultures were made from at least three different preparations from different umbilical vein cords pooled together.

Cell viability

Cytotoxicity of RWS on endothelial cells was assessed by the Trypan blue dye exclusion method. The percentage of cells excluding Trypan blue was taken as a measure of cell viability (Carluccio *et al.* 2003).

Real-time PCR

At the end of the incubation, RNA was extracted using TRI® reagent (Sigma) and quantified by spectrophotometry. Gene expression at the level of mRNA was assessed by real-time quantitative PCR utilizing an ABI PRISM® 7900 HT

Instrument (Applied Biosystem, Foster City, CA, USA) coupled with the Sybr green JumpStart™ Taq Ready Mix kit (Sigma). The specific primers set for the target genes are as shown in Table 2.

Data were collected and processed with SDS2.2 software (Applied Biosystems, Foster City, CA, USA) and given as threshold cycle (C_t). The C_t values for each target and reference gene were obtained and their difference was calculated (ΔC_t). Primer efficiencies for the test genes were comparable to those for G3PDH (reference gene). The last step in quantification was the conversion of C_t to absolute values. Results are expressed as fold of increase or decrease compared to control.

Statistical analysis

RWS and their respective CS isolated from three subjects were independently used for at least three separate experiments. Each experiment was repeated three times. Data presented on the effect of native wine are based on at least three different experiments. Values are presented as means and standard deviations of the fold of changes of the gene expression compared to control. Multifactorial ANOVA was used to test the significance between differences taking into account the variability within the experiments and among the treatments. $P < 0.05$ was considered the threshold level for significance.

Results

Cell viability

RWS cytotoxicity was assessed by means of Trypan blue permeability assay. RWS contained 0·077 (SD 0·01) % (w/v) of alcohol and 5·6 (SD 2·07) µg/ml of total phenolic compounds. Therefore, after appropriate dilution (20 %), endothelial cells were exposed to about 0·015 % (w/v) and 1·09 µg/ml of alcohol and phenolic compounds, respectively. No difference in living cell number was observed in any treatments, compared with bovine serum incubation (98·7 (SD 1·2) % of viable cells).

Gene expression

Real-time PCR was used to address whether RWS was able to modulate vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), and monocytes chemoattractant protein (MCP-1) or tissue-plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2) gene expression in endothelial cells as representative genes involved in cell adhesion and fibrinolysis, respectively. Gene expression was assessed in HUVEC after 16 h of incubation with RWS. The effect of each RWS treatment was compared with its corresponding negative control (CS).

RWS enrichment was associated with a down-regulation of the expression of the genes considered, with the exception of MCP-1. VCAM and ICAM mRNA levels decreased by 1·78-fold ($P < 0.05$) and 1·15-fold ($P < 0.01$), respectively. t-PA and PAI-2 decreased by 1·39-fold and 1·82-fold ($P < 0.01$), respectively. On the other hand, MCP-1 increased by 1·48-fold ($P < 0.01$). Data are expressed as fold of changes in comparison with CS (Fig. 1).

In order to provide a comparison of RWS effect on HUVEC with a 'classical' experimental approach, the effect of a direct addition of wine to the culture medium of endothelial cells for 16 h was also considered. The volume of wine to be provided to cultured cells was calculated in order to reach an amount of alcohol (0·015 %, w/v) in the same range of that utilized in experiments conducted with RWS. On this basis, the final concentration of alcohol provided to the cells with red wine corresponds to wine intake of about one standard glass (100–120 ml) in an adult individual. The resulting volume of wine also provided 2·5 µg/ml total polyphenols, 3·7 ng/ml quercetin, 0·48 ng/ml resveratrol, 72 ng/ml catechin and 153 ng/ml epicatechin. According to the studies available in the literature, these amounts appear from 'low' to 'very low' for *in vitro* experiments (Kondo *et al.* 2002; Leikert *et al.* 2002; Kuhlmann *et al.* 2005). Table 3 summarizes the total polyphenol and alcohol concentration provided in *in vivo* and *in vitro* experiments.

Incubation with native wine was associated with a very different gene expression profile compared to RWS: the levels of mRNA encoding for VCAM, ICAM and MCP-1 were all significantly up-regulated (770-, 120- and 650-fold increase compare to control, respectively; $P < 0.01$). On the other hand, t-PA and PAI-1 mRNA levels showed a 7·7-fold decrease ($P < 0.01$) and 1·8-fold increase ($P < 0.01$), respectively, relative to the control (Fig. 2).

Discussion

Here we present an original model to study the biological effects of food matrices at the molecular level. The model was utilized to study the effect of red wine on the expression of genes involved in atherogenesis in primary human endothelial cells. This approach allows the food matrix to be

Table 2. Specific primers set for the target genes

Gene	Forward	Reverse
VCAM	GAATGGGAGCTCTGTCACTGTAAGC	GACCAAGACGGTTGTATCTCTGGG
ICAM	GGGAGCTTCGTGTCCTGTATGGCC	AGTCTGTATTTCTTGATCTTCCGCTGGC
MCP-1	TTCTCAAACTGAAGCTCGCACTCTCGCC	TGTGGAGTGAGTGTTCAAGTCTTCGGAGTT
t-PA	TGTGGAGTGAGTGTTCAAGTCTTCGGAGTT	GTCGGGTGTTCCTGGTCACGGTCG
PAI-1	AGATCGAGGTGAACGAGAGTGGCACG	TTTGTCCCAGATGAAGGCGTCTTTCC
PAI-2	CTTTCCGTGTAAACTCGGCTCAGCGC	GAAATTGGCCCGTCCCTTGTTGAAGG
G3PDH	GCTCTCCAGAACATCATCCCTGC	GGGTGTCGCTGTTGAAGTCAGAGG

G3PDH, glyceraldehyde 3-phosphate; ICAM, intercellular adhesion molecule; MCP-1, monocytes chemoattractant protein; PAI-1, plasminogen activator inhibitor-1; PAI-2, tissue-plasminogen activator inhibitor-2; t-PA, tissue-plasminogen activator; VCAM, vascular cell adhesion molecule.
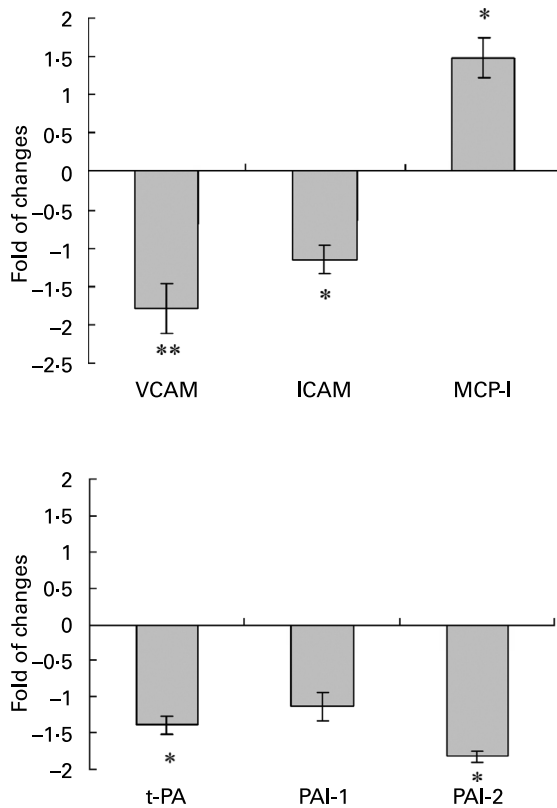
Fig. 1. Effect of human serum obtained after red wine consumption (RWS) on mRNA expression in human umbilical vein endothelial cells. The culture medium was enriched with RWS at a concentration of 20 %. Cells were incubated for 16 h. At the end of the incubation time RNA was isolated and gene expression was assessed by real-time PCR. For details of procedures, see p. 1054. Values are means of the fold of changes of gene expression compared to control, with their standard deviations depicted by vertical bars. Mean values were significantly different from those of the control group: *$P < 0.01$; **$P < 0.05$. ICAM, intercellular adhesion molecule; MCP-1, monocytes chemoattractant protein; PAI-1, plasminogen activator inhibitor-1; PAI-2, plasminogen activator inhibitor-2; t-PA, tissue-plasminogen activator; VCAM, vascular cell adhesion molecule.

metabolized and administered to cells in a fashion much closer to a 'real physiological' event. The observed effects of red wine on the gene expression profile should be, therefore, more genuine and reliable. We arbitrarily selected two

Table 3. Total polyphenols and alcohol concentration provided in *in vivo* and *in vitro* experiments

HUVEC treatment	*In vivo**	*In vitro*	
		RWS†	Red wine‡
Total polyphenols (mg/ml)	1·748	0·001	0·0025
Alcohol (% w/v)	10·03	0·015	0·015

HUVEC, human umbilical vein endothelial cells; RWS, human serum obtained after red wine consumption.

* Total polyphenol and alcohol concentrations in red wine. Wine load to human subjects corresponded to about three standard glasses, depending on the weight of the subjects.

† Total polyphenol and alcohol concentrations in the medium of cultured cells treated with RWS.

‡ Total polyphenol and alcohol concentrations in the medium of cultured cells treated with red wine. The volume of wine was calculated in order to reach the same range of alcohol measured in human serum after wine consumption and corresponds to about one standard glass.
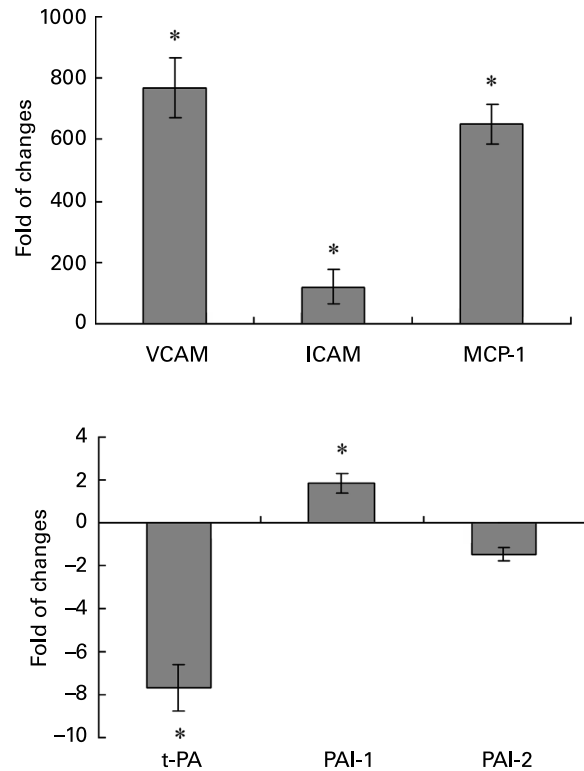
Fig. 2. Effect of direct wine addition on mRNA expression in human umbilical vein endothelial cells. Wine was added to a standard culture medium to reach a final concentration of 0·015 % ethanol. Cells were incubated for 16 h. At the end of the incubation time RNA was isolated and gene expression was assessed by real-time PCR. For details of procedures, see p. 1054. Values are means of the fold of changes of gene expression compared to control, with their standard deviations depicted by vertical bars. Mean values were significantly different from those of the control group: *$P < 0.01$. ICAM, intercellular adhesion molecule; MCP-1, monocytes chemoattractant protein; PAI-1, plasminogen activator inhibitor-1; PAI-2, plasminogen activator inhibitor-2; t-PA, tissue-plasminogen activator; VCAM, vascular cell adhesion molecule.

groups of genes known to be involved in the early stages and progression of atherosclerosis. Endothelial cell dysfunction plays a key role in atherosclerotic lesion formation. It is characterized by a proinflammatory, proliferative and procoagulatory status that favours all stages of atherogenesis (Bonetti *et al.* 2003). Early stages of atherosclerosis show the interaction of blood leucocytes to endothelial cells mediated by an increase in adhesion molecule levels. Furthermore, endothelial dysfunction is characterized by a reduction in the anticoagulatory potential of the endothelium and an increase in endothelial production of pro-coagulatory mediators (plasminogen activator inhibitors) resulting in a thrombogenic vascular environment. This relationship between endothelial dysfunction and atherosclerosis reflects the propensity of an individual to develop atherosclerosis (Dessein *et al.* 2005). To assess the effect of RWS on endothelial function we measured the expression of genes involved in endothelial activation and dysfunction (VCAM, ICAM and MCP-1 or t-PA, and PAI). The cardioprotective effect of red wine has been proposed to be mediated by an increase in fibrinolytic activity. Purified polyphenols and alcohol were shown to increase t-PA in HUVEC (Abou-Agag *et al.* 2001), but they did not determine any effect on the expression of adhesion molecules, in

the absence of pro-inflammatory stimuli (Ludwig *et al.* 2004; Saeed *et al.* 2004). The present data obtained by supplementing cultured HUVEC with red wine components contained in human serum, after red wine consumption, have been compared to those obtained by directly adding red wine to the culture medium. The volume of wine administered to cells was calculated in order to equalize alcohol concentration measured in RWS after wine drinking. We have chosen this approach considering that wine polyphenols are a heterogeneous group of compounds undergoing differential metabolic processing during absorption and transport, and therefore are not appropriate to be utilized to standardize the volume of wine used in the tissue culture model. Incubation of endothelial cells with RWS leads to changes in the expression of specific genes that cannot be simply attributed to alcohol. In fact, endothelial cells incubated with a volume of wine containing a comparable amount of alcohol induced a totally different profile in gene expression than that induced by RWS. This difference suggests that the gene expression profile induced by RWS results from a combined effect of wine components (mainly modified polyphenols) and alcohol. Incubation of endothelial cells with RWS is associated with a down-regulation of all genes with the exception of MCP-1. The addition of red wine 'as it is' induced a very strong and evidently not physiological, inflammatory or pro-coagulant pattern of expression in endothelial cells. The evidence points at the important difference in biological activity of red wine components 'as they are in food' compared to red wine metabolized during gastrointestinal absorption.

The metabolism of wine components different from alcohol, and in particular polyphenols, is still poorly understood. With very few exceptions, the overall result of the extensive metabolism of polyphenols is that the predominant forms in plasma are sulphates and glucuronide or methyl conjugates (Manach *et al.* 2004; Nardini *et al.* 2006). Conjugates differ in size, polarity and ionic form from their parent molecule. Consequently, their physiological effect is likely to be different from that of native compounds. In addition, there are different sites of possible conjugation, and not all the possible existing metabolites have been identified, so far. For example, plasma samples from volunteers receiving quercetin orally contained twelve distinct conjugated forms of quercetin not present in the original food (Day & Williamson, 2001). According to these considerations, the major issue still open for the understanding of the molecular mechanism underlying the effect of red wine on human health is in the effects of metabolism on the biological activities of polyphenols. Different studies have addressed the effect of bio-transformed polyphenols on endothelial response to pro-atherogenic stimuli (Rimbach *et al.* 2004). However, it is important to remark that no studies addressing the cellular effects of wine metabolites in the form circulating in the body, once ingested, absorbed, modified and distributed to target tissues and organs, are available at present. All the evidence suggests a very difficult picture. The aim of the present report is not to provide a complete description of the mechanism underlying the effect of red wine on endothelial cell function. However, it underscores the importance of utilizing an appropriate model to study the biological effects of complex food matrices at the molecular level. The model described herein can contribute to the understanding of the biological properties of the complex food matrix in *in vitro* experiments, taking into account bio-transformation and possible synergism between different components.

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