Red Wine Polyphenolic Compounds Inhibit Vascular Endothelial Growth Factor Expression in Vascular Smooth Muscle Cells by Preventing the Activation of the p38 Mitogen-Activated Protein Kinase Pathway

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Objective—Moderate consumption of red wine has a beneficial effect on the cardiovascular system. This study examines whether red wine polyphenolic compounds (RWPCs) affect vascular endothelial growth factor (VEGF) expression, a major angiogenic and proatherosclerotic factor in vascular smooth muscle cells (VSMCs).

Methods and Results—VEGF mRNA expression was assessed by Northern blot analysis and the release of VEGF by immunoassay in cultured VSMCs. Short-term and long-term exposure of VSMCs to RWPCs inhibited VEGF mRNA expression and release of VEGF in response to platelet-derived growth factor AB (PDGF_AB), transforming growth factor-β1, or thrombin. The PDGF_AB-induced expression of VEGF was markedly reduced by SB203580 (inhibitor of p38 mitogen-activated protein kinase [MAPK]), antioxidants, and diphenylene iodonium (inhibitor of flavin-dependent enzymes), slightly reduced by PD98059 (inhibitor of MEK), and not significantly affected by wortmannin (inhibitor of PI-3-kinase) and L-JNKI (inhibitor of JNK). Short-term and long-term treatment of VSMCs with RWPCs markedly reduced PDGF_AB-induced production of reactive oxygen species and phosphorylation of p38 MAPK.

Conclusions—These data indicate that RWPCs strongly inhibit growth factor–induced VEGF expression in VSMCs by preventing the redox-sensitive activation of the p38 MAPK pathway. The potential antiangiogenic and antiatherosclerotic properties of RWPCs are likely to contribute to cardiovascular protection by preventing the development of atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2003;23:1001-1007.)

Key Words: red wine polyphenolic compounds ■ vascular endothelial growth factor ■ vascular smooth muscle cells ■ atherosclerosis ■ angiogenesis

Epidemiological studies have suggested that light to moderate consumption of alcoholic beverages, particularly red wine, is associated with a reduction in overall mortality, and this effect is attributable primarily to a reduced risk of coronary heart disease.1,2 Although the exact nature of the protective effect of red wine on coronary diseases is unclear, it might be attributable, in part, to its ability to reduce the progression of early atherosclerotic lesions, as observed in human coronary arteries at childhood, to advanced plaques, which are prone to rupture with superimposed thrombosis. This is consistent with the findings that the consumption of red wine reduced the progression of lesions in experimental models of atherosclerosis.3–5 The protective effect of red wine seems to be attributable, at least in part, to polyphenols, because nonalcoholic wine products and the red wine polyphenolic compounds (RWPCs) quercetin and catechin also prevented the progression of atherosclerotic lesions.3–5 The beneficial effect of RWPCs might be related to their ability to prevent oxidation of LDL,6 activation of platelets,7 and expression of tissue factor and monocyte chemoattractant protein-1.8,9

Recent findings have indicated that vascular endothelial growth factor (VEGF) is strongly expressed in human atherosclerotic plaques.10,11 The cellular sources of VEGF in plaques are predominantly vascular smooth muscle cells (VSMCs) and to some extent foamy macrophages.10,11 Besides being the major inducer of angiogenesis and vasculogenesis, VEGF is also known to induce vascular permeability and to function as a powerful endothelial mitogen and chemoattractant.12 In addition, VEGF stimulates gene expression of several endothelial proteins involved in prothrombotic and proatherosclerotic responses, including tissue factor,13 adhesion molecules,14 and monocyte chemoattractant protein-1.15 Moreover, VEGF induces monocyte procoagulant activ-
phenolic compounds were described previously. In brief, phenolic compounds were prepared and analyzed RWPCs from red French wine (Corbières A.O.C.) provided by Dr M. Moutounet (Institut National de la Recherche Agronomique, Montpellier, France) and analyzed by Dr P.-L. Teissedre (Département D’Oenologie, Université de Montpellier, France). One liter of red wine produced 2.7 g of RWPCs, which contained 471 mg/g of total phenolic compounds expressed as gallic acid. The extract contained 8.6 mg/g catechin, 8.7 mg/g epicatechin, anthocyanins (malvidin-3-glucoside, 11.7 mg/g; peonidin-3-glucoside, 0.66 mg/g; and cyanidin-3-glucoside, 0.06 mg/g) and phenolic acids (gallic acid, 5.0 mg/g; caffeic acid, 2.5 mg/g; and caftaric acid, 12.5 mg/g).

Cell Culture
Rat aortic VSMCs were cultured in MEM containing 10% FCS and antibiotics. Human aortic VSMCs were obtained from Clonetics and cultured as recommended. All experiments were performed with VSMCs from passages 5 to 15, which were serum-deprived for 24 hours.

Northern Blot Analysis
The cellular RNA from VSMCs was prepared by isothiocyanate and phenol extraction. VEGF mRNA level was assessed by Northern blot analysis as described. A 350-bp-long restriction fragment obtained from the cloned rat VEGF cDNA (provided by Dr C. Frelin, Université de Nice, France) was labeled with ³²P-α-dCTP using the Random Primer labeling kit from Stratagene. Autoradiographs were analyzed by scanning densitometry. VEGF mRNA levels were normalized to their respective 18S ribosomal RNA levels and expressed in arbitrary units as a fold increase of the signal obtained with untreated cells.

Western Blot Analysis
A commercially available human VEGF immunoassay (R&D system) was used for the determination of VEGF content in human VSMC-conditioned medium (24 hours).

Determination of VEGF
The medium of treated and untreated VSMCs grown in 96-well plates was replaced by Hanks’ balanced salt solution (HBSS), and the cells were loaded with dichlorofluorescein (DCF) diacetate (10 μmol/L; Molecular Probes) dissolved in HBSS for 30 minutes at 37°C. The extracellular dye was removed, and HBSS containing PDGFAB (30 ng/mL) or solvent was added. DCF fluorescence was measured in a Wallac Victor 1420 fluorescence plate reader (EG&G Wallac) at 37°C at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

Statistical Analysis
Results are shown as mean±SEM. Statistical analyses were performed using ANOVA followed by Fisher’s protected least-significant difference test to compare 2 treatments. P<0.05 was considered statistically significant.

Methods
Preparation of RWPC
RWPCs dry powder from red French wine (Corbières A.O.C.) was provided by Dr M. Moutounet (Institut National de la Recherche Agronomique, Montpellier, France) and analyzed by Dr P.-L. Teissedre (Département D’Oenologie, Université de Montpellier, France). The procedures used to prepare and analyze RWPCs have been described previously. In brief, phenolic compounds were adsorbed on a preparative column, and then alcohol was desorbed; the alcoholic-eluent was gently evaporated; the concentrated residue was lyophilized and finely sprayed to obtain RWPCs dry powder.

Figure 1. RWPCs inhibit growth factor-induced VEGF mRNA expression in VSMCs. A, Rat VSMCs were exposed to either solvent (0.1% ethanol) or RWPC for 15 minutes before the addition of a growth factor for 1 hour. Thereafter, VEGF mRNA levels were assessed by Northern blot analysis. B, Concentration-dependent inhibitory effect of RWPC on PDGFAB (1 hour)-induced VEGF mRNA expression in rat VSMCs. Depicted are representative Northern blots showing VEGF mRNA (top), 18S ribosomal RNA (center), and cumulative data (bottom). Results represent a fold increase of the signal obtained with untreated cells.

Results
Exposure of rat VSMCs either to PDGFAB, transforming growth factor (TGF)-β, or α-thrombin for 1 hour markedly increased the low basal steady state-level of VEGF mRNA (Figure 1A). The stimulatory effect of the 3 growth factors was markedly reduced by exposure of VSMCs to RWPCs 15 minutes before addition of a growth factor (Figure 1A). RWPCs alone also slightly but significantly reduced the basal expression of VEGF mRNA (Figure 1A). Similar findings...
were obtained with human VSMCs (data not shown). To characterize the inhibitory effect of RWPCs on VEGF expression, all subsequent experiments were performed with PDGF_AB, because this growth factor induced the strongest expression of VEGF mRNA (Figure 1A). RWPCs inhibited PDGF_AB-induced VEGF mRNA expression in a concentration-dependent manner with a statistically significant inhibition at concentrations of ≥10 μg/mL (Figure 1B). In addition to the 15-minute pretreatment period, exposure of VSMCs to RWPCs for either 24 or 18 hours followed by washout and an additional incubation period with serum-free medium for 6 hours also significantly reduced both the basal and PDGF_AB-induced VEGF mRNA expressions (Figures 2A and 2B).

Figure 2. Long-term inhibitory effect of RWPCs on PDGF_AB-induced VEGF mRNA expression in VSMCs. Rat VSMCs were exposed to either solvent (0.1% ethanol) or RWPCs for 24 hours (A) or 18 hours followed by washout and an additional 6-hour incubation period with serum-free culture (B) before the addition of PDGF_AB (1 hour). Thereafter, VEGF mRNA levels were assayed by Northern blot analysis. Depicted are representative Northern blots showing VEGF mRNA (top), 18S ribosomal RNA (center), and cumulative data (bottom). Results are shown as mean±SEM of 4 different experiments. #Treatment vs control; *Treatment vs PDGF_AB treatment alone.

Figure 3. RWPCs inhibit PDGF_AB-induced release of VEGF protein into conditioned medium of VSMCs. Human VSMCs were exposed to either solvent (0.1% ethanol) or RWPCs for 15 minutes (A), 18 hours followed by washout and an additional 6-hour incubation period in serum-free culture medium (B), or 24 hours before the addition of PDGF_AB for 24 hours. Thereafter, the amount of VEGF protein into conditioned medium was determined by immunoassay. A, Results are shown as mean±SEM of 3 different experiments performed in triplicate. #Treatment vs control; *Treatment vs PDGF_AB treatment alone.

RWPCs treatment did not affect cell viability as assessed by CellTiter 96 MDSU aqueous one solution cell proliferation assay (Promega; the values were 93.5±0.8% and 95.5±1.9% in control [0.1% ethanol] and RWPCs [30 μg/mL]-treated cells after a 24-hour incubation period, respectively, n = 6).

To demonstrate that the inhibitory effect of RWPCs on VEGF mRNA expression was followed by a reduction of the secretion of VEGF protein, human VSMCs were exposed to either vehicle or PDGF_AB for 24 hours, and thereafter the amount of VEGF in conditioned medium was determined by immunoassay. PDGF_AB markedly increased the modest basal release of VEGF, and this response was significantly inhibited by pretreatment of VSMCs with RWPCs for 15 minutes before the addition of PDGF_AB (Figure 3A). The inhibitory effect of RWPCs was concentration-dependent and was statistically significant at concentrations of ≥3 μg/mL (Figure 3A). The PDGF_AB-stimulated release of VEGF was also reduced by pretreatment of VSMCs with RWPCs for either 24 or 18 hours followed by washout and a subsequent 6-hour incubation period with serum-free culture medium (Figure 3B). In addition, exposure of VSMCs to RWPCs alone either for 24 or 18 hours followed by washout slightly but significantly reduced the basal release of VEGF, whereas the
PDGFAB-induced VEGF expression was examined. The results showed in Figure 4A indicate that PDGFAB caused the formation of ROS as assessed by DCF fluorescence; this response was abolished by N-acetylcysteine. In addition, N-acetylcysteine and vitamin C (two antioxidants) and diphenylene iodonium (an inhibitor of flavin-dependent enzymes such as the NAD(P)H oxidase) significantly reduced the PDGFAB-induced expression of VEGF mRNA, indicating the involvement of a redox-sensitive event (Figure 4B). In the absence of PDGFAB, N-acetylcysteine, vitamin C, and diphenylene iodonium alone affected the basal expression of VEGF mRNA only minimally (Figure 4B). Immunoblot analysis indicated that PDGFAB caused a transient phosphorylation of p38 MAPK, ERK1/2, JNK, and Akt with a maximal response occurring within 10 minutes (Figures 5A and 5B). Next, the involvement of these protein kinase–dependent pathways in the PDGFAB-induced expression of VEGF was assessed using SB203580, an inhibitor of p38 MAPK; PD98059, an inhibitor of MEK; L-JNKI, an inhibitor of JNK; and wortmannin, an inhibitor of phosphoinositide 3-kinase. The stimulatory effect of PDGFAB was abolished by inhibition of the p38 MAPK pathway, significantly reduced by inhibition of the ERK1/2 kinase pathway, and minimally affected by inhibition of the JNK and phosphoinositide 3-kinase pathways (Figure 6). Neither SB203580 nor PD98059 and L-JNKI alone affected significantly the basal release of VEGF, whereas a slight but significant increase was obtained in response to wortmannin (Figure 6). Because phosphorylation of p38 MAPK and ERK1/2 in VSMCs is markedly induced by H2O2 (Figure 5C), the possibility that ROS mediate the PDGFAB-induced phosphorylation of p38 MAPK and ERK1/2 was evaluated. N-acetylcysteine and diphenylene iodonium significantly attenuated the PDGFAB-induced phosphorylation of p38 MAPK and had only minor effects on that of ERK1/2 (Figure 5C). In addition, the stimulatory effect of PDGFAB on p38 MAPK was also reduced by the combination PEG-superoxide dismutase (500 U/mL) and PEG-catalase (500 U/mL, data not shown).

Next, the possibility that RWPCs inhibit the PDGFAB-induced expression of VEGF by preventing the generation of ROS or subsequent activation of the p38 MAPK pathway was determined. Exposure of VSMCs to RWPCs for either 15 minutes or 24 hours significantly blunted the PDGFAB-induced generation of ROS (Figure 4A) and phosphorylation of p38 MAPK (Figures 5A and 5B). In contrast to p38 MAPK, RWPCs did not significantly affect the phosphorylation of ERK1/2, JNK, and Akt (Figure 5A). In the absence of PDGFAB, RWPCs alone affected the basal generation of ROS and phosphorylation of p38 MAPK and ERK1/2 only minimally (Figures 4A, 5A, and 5B). RWPCs (30 µg/mL) also reduced the H2O2 (100 µM)-stimulated release of VEGF from 304±18% to 239±8% (n=3) after a 24-hour incubation period.

Discussion

The present findings indicate that RWPCs strongly inhibit VEGF expression in VSMCs in response to PDGFAB, TGF-β1, and α-thrombin. These 3 growth factors are all potential endogenous stimuli of VEGF expression in atherosclerotic lesions, because an increased expression of PDGF-B and TGF-β has been found in human atherosclerotic lesions, and...
procoagulant and prothrombotic responses have been observed at the surface of plaques.\textsuperscript{24,25} The inhibitory effect of RWPCs is detected at concentrations of \( \leq 3 \mu g/mL \). Although the concentration of RWPCs in blood after intake of red wine remains unknown, a previous study has indicated that intake of 100 mL of red wine by healthy volunteers caused an increase in plasma concentration of polyphenolic monomers of 2.5 \( \mu g/mL \) (gallic acid equivalents).\textsuperscript{26} Thus, the inhibitory effect of RWPCs on VEGF expression observed in the present study occurs at concentrations that are likely to be achieved in blood after moderate consumption of red wine. The present findings also indicate that RWPCs caused a sustained inhibition of VEGF expression in VSMCs that lasts several hours after their removal from the incubation medium. Such a long-lasting effect of RWPCs might reflect their association with VSMCs or the possibility that RWPCs induce the production of one or several peptides/proteins, which, in turn, contribute to prevent the expression of VEGF.

In addition to growth factors, VEGF expression can also be strongly stimulated by ROS such as \( H_2O_2 \) in most types of cells, including VSMCs.\textsuperscript{27} More recent findings have suggested that ROS can also act as key signaling molecules controlling VEGF expression in response to growth factors. This is supported by the fact that exposure of VSMCs to thrombin or PDGF\textsubscript{AB} caused the generation of substantial amounts of ROS via activation of a \( p22^{phox} \)-containing NAD(P)H oxidase.\textsuperscript{19,28} Moreover, prevention of the generation of ROS by either antioxidant treatment or diphenylene iodonium, a nonselective inhibitor of NAD(P)H oxidase,\textsuperscript{19,28,29} ROS seem to control the expression of VEGF by activating the heterodimeric transcription factor hypoxia-inducible factor-1.\textsuperscript{28} RWPCs are known to have antioxidant properties most likely attributable to their ability to scavenge ROS, such as hydroxyl radical and superoxide anion.\textsuperscript{30–33} Altogether, these previous findings suggest that the inhibitory effect of RWPCs on the expression of VEGF involves their antioxidant properties. Consistent with such an idea, both short- and long-term exposure of VSMCs to RWPCs prevented the PDGF\textsubscript{AB}-induced generation of ROS.

To further characterize the signaling pathways involved in the PDGF\textsubscript{AB}-induced expression of VEGF, the activation of several protein kinases, including members of the mitogen-activated protein kinases and Akt, has been assessed by immunoblot analysis. Consistent with previous findings,\textsuperscript{22,34,35} PDGF\textsubscript{AB} caused a transient phosphorylation of ERK1/2, p38 MAPK, and Akt. Because activation of these protein kinase pathways has been involved in the upregulation of VEGF expression in several cell types,\textsuperscript{36–38} their role in the PDGF\textsubscript{AB}-induced expression of VEGF was determined using specific pharmacological inhibitors.
findings indicate that the stimulatory effect of PDGFAB is critically dependent on the activation of the p38 MAPK pathway and also, to some extent, ERK1/2 but not JNK and phosphoinositide-3-kinase/Akt. ROS seem to mediate the phosphorylation of p38 MAPK in response to PDGFAB, because this effect is attenuated by antioxidant treatments and by diphenylene iodonium. Moreover, H2O2 markedly increased the phosphorylation level of p38 MAPK. The present findings also indicate that RWPCs selectively prevent the PDGFAB-induced phosphorylation of the p38 MAPK without affecting those of ERK1/2, JNK, and Akt. These findings are in good agreement with previous ones showing that red wine polyphenols inhibited PDGFAB-induced activation of p38 MAPK whereas higher concentrations were also able to inhibit the activity of phosphoinositide-3-kinase in VSMCs.35 Although red wine has been shown to inhibit PDGFAB binding to PDGF β receptor and PDGF β receptor phosphorylation,39 such explanations are unlikely to account for the present findings, because PDGFAB-induced phosphorylation of ERK1/2, JNK, and Akt was unaffected by RWPCs. Altogether, these findings suggest that RWPCs inhibit growth factor–induced VEGF expression by preventing the redox-sensitive activation of the p38 MAPK, which in turn upregulates VEGF gene expression.

Previous studies have indicated that resveratrol, a polyphenolic compound found in wine, suppresses the growth of new blood vessels in several in vivo models of angiogenesis36 and inhibits tumor growth and tumor-induced neovascularization in vivo.41,42 Although the molecular mechanism of the in vivo antiangiogenic activity of wine-derived polyphenolic compounds remains unknown, it could be attributable to their direct inhibitory effect on endothelial cell growth43,44 and migration45 and, as suggested by the present findings, inhibition of the endogenous production of VEGF.

In conclusion, RWPCs strongly inhibit growth factor–induced VEGF expression by preventing the redox-sensitive activation of the p38 MAPK pathway in VSMCs. These effects are observed at concentrations that are likely to be achieved in blood after moderate wine consumption. Therefore, the antiangiogenic and antiatherosclerotic properties of RWPCs could contribute to explain the reduced risk of coronary heart disease and cancer mortality after moderate consumption of red wine for long-term periods.

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References

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