Red Wine Polyphenols Inhibit Proliferation of Vascular Smooth Muscle Cells and Downregulate Expression of Cyclin A Gene

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Background—Red wine polyphenols have been shown to contribute to the “French paradox” phenomenon, which consists of lower morbidity and mortality from coronary heart disease in the French population. Although vascular smooth muscle cell (VSMC) proliferation plays an important role in the progression of atherosclerotic lesions, the effects of red wine polyphenols on VSMC proliferation have not been elucidated.

Methods and Results—We extracted the total polyphenolic fraction from red wine (RW-PF) by column chromatography. Treatment with RW-PF showed a potent inhibitory effect on the proliferation and DNA synthesis of cultured rat aortic smooth muscle cells (RASMCs). In contrast, the inhibitory effect of RW-PF on the proliferation of bovine carotid endothelial cells was observed only at much higher concentrations. To elucidate the molecular mechanisms of this antiproliferative effect of RW-PF on RASMCs, we investigated the effects of RW-PF on cell cycle regulation. RW-PF downregulated the expression of cyclin A mRNA and cyclin A promoter activity. In addition, RW-PF decreased the binding of nuclear proteins to the activating transcription factor (ATF) site in the cyclin A promoter and downregulated the mRNA levels of transcription factors, cAMP-responsive element–binding protein (CREB), and ATF-1.

Conclusions—These results suggest that the downregulation of cyclin A gene expression may contribute to the antiproliferative effect of red wine polyphenols on RASMCs through the inhibition of transcription factor expression. (Circulation. 2000;101:805-811.)

Key Words: red wine ■ polyphenol ■ atherosclerosis ■ smooth muscle cells

The French population has lower morbidity and mortality from coronary heart disease (CHD) than other Western populations despite their high-fat and high-cholesterol diet.1,2 Recently, it has been shown that this paradoxical finding, the “French paradox,” may be attributed to regular consumption of red wine and that the unique antiatherogenic effects of red wine reside in the action of polyphenols.2 LDL oxidation is known to be one of the initial events in atherogenesis,3,4 and the antioxidative effects of red wine polyphenols, which have been shown in vitro5 and in vivo,6 may be involved in the mechanism of the French paradox.7 Other aspects of atherogenesis are the dedifferentiation of vascular smooth muscle cells (VSMCs), their migration into the intima, and excessive proliferation of VSMCs in the neointima.8 Although VSMC proliferation plays an important role in the pathogenesis of atherosclerotic lesions, the effects of red wine polyphenols on VSMC proliferation in atherosclerotic lesions have not been elucidated.

Cyclin A is important in the G1/S transition and in the S and G2/M phases of the cell cycle and has a critical role in DNA replication.9 The cyclin A promoter contains activating transcription factor (ATF) or cAMP-responsive element (CRE), which are bound by cAMP-responsive element–binding protein (CREB) and ATF-1 by heterodimer formation.10 The heterodimeric binding to the cyclin A ATF site is strongly related to the induction of maximal cyclin A gene expression.10

In this study, polyphenolic substances from red wine were extracted and the effects on proliferation of VSMCs compared with those of the vascular endothelial cells investigated.

Methods

Preparation of Red Wine Polyphenolic Compounds

Pulverized red wine (10 bottles, 7600 mL in total), “Tomi-no-Oka” (Suntory Co), was fractionated by adsorption chromatography with a Diaion HP-20 column (Mitsubishi Chemical Industries) (Figure 1). The column was eluted with deionized water (150 L), and the eluent that did not contain polyphenolic substances was discarded. The total polyphenolic fraction of red wine (RW-PF, 24 g dry weight) was
Figure 1. Preparation of red wine polyphenols. Total polyphenolic fraction (RW-PF) from 10 bottles (7600 mL) of red wine was extracted with a Diaion HP-20 column. RW-PF was further separated into 6 fractions (fractions 1 through 6) with a Toyopearl HW-40ec column. Polyphenolic contents of each fraction are underlined.

obtained by eluting the column with 100% ethanol (150 L). The RW-PF fraction was further separated into 6 fractions, fractions 1 through 6, by successive column chromatography with a Toyopearl HW-40ec column (Tosoh). Elution of RW-PF with 50% ethanol (45 L) through this column resulted in the formation of 4 bands (fractions 1 through 4). The remaining compounds from RW-PF in the column were further eluted with 100% ethanol (45 L) (fraction 5). The last fraction, fraction 6, was obtained by eluting the column with 70% acetone (30 L). To measure the content of polyphenolic substances, all fractions were systematically analyzed by UV spectrophotometry according to the Folin-Denis method.11,12 The content of proanthocyanidins (polymerized anthocyanidins) was measured according to the butanol-HCl method.13 All fractions were systematically analyzed by UV spectrophotometry according to the Folin-Denis method.11,12 The content of proanthocyanidins (polymerized anthocyanidins) was measured according to the butanol-HCl method.13 All fractions were lyophilized and kept at 20°C. Just before the experiments, each compound was dissolved in 50% ethanol by ultrasonication.

Cell Culture
Rat aortic smooth muscle cells (RASMCs) were prepared from Sprague-Dawley rats and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS as described.14 Bovine carotid endothelial cells (BCECs) were cultured in the same medium without HEPES.14 Human aortic smooth muscle cells (HASMCs) or human umbilical vein endothelial cells (HUVECs) were cultured in Medium 199 (Earle’s salts, Nikken Bio Medical Laboratory) or endothelial cell basal medium (EBM, Bio Whittaker) supplemented with 10% FBS.15

[3H]Thymidine Incorporation
Cells were plated at a density of 10 000 cells/cm² in 24-well plates. After 48 hours, the cells were treated with RW-PF dissolved in 50% ethanol at final concentrations of 1 to 100 μg/mL for 72 hours, and then cell number was measured. For the thymidine incorporation study, cells were labeled with [methyl-3H]thymidine at 1 μCi/mL during the last 3 hours, and then thymidine incorporation was measured.15 The effects of RW-PF on cell proliferation and DNA synthesis were expressed as percentage of control containing 0.5% ethanol as vehicle. Moreover, we examined thymidine incorporation into RASMCs treated with 6 fractions (30 μg/mL) separated from RW-PF for 72 hours.

RNA Isolation and Northern Blot Analysis
Total RNA was extracted from the cells by guanidinium isothiocyanate extraction and centrifugation through cesium chloride.16 The cyclin A cDNA probes for each species were amplified by reverse transcription–polymerase chain reaction.17 RNA was fractionated on 1.3% formaldehyde-agarose gel and transferred to nylon filters. The filters were hybridized at 68°C for 2 hours with 32P-labeled cyclin A cDNA probe in QuickHyb solution (Stratagene) and autoradiographed. The hybridized filters were rewashed and rehybridized with 32P-labeled CREB and ATF-1 cDNA probes.18,19 To confirm the equal loading of RNA, the filters were rehybridized with a radiolabeled 18S rRNA oligonucleotide probe.20

Transfection and Luciferase Assay
Reporter constructs containing a fragment of the human cyclin A 5′-flanking region (bp -266 to +205) were inserted into the promoterless firefly luciferase reporter plasmid pGL2-Basic (Promega).21 The normal ATF consensus sequence (TGACGTCA) in the plasmid -266/+205 was mutated to TGCCCCCA by polymerase chain reaction to generate the plasmid mut -266/+205.21 RASMCs were transfected with 8 μg luciferase constructs by the DEAE-dextran method followed by a 1-minute DMSO shock.14 BCECs were also transfected with the same plasmids by a SuperFect transfection reagent (QIAGEN Japan). To correct for variability in transfection efficiency, we cotransfected 2 μg pRL-SV40 control plasmids. Twenty-four hours after transfection, the cells were incubated with RW-PF for 72 hours, and then 2 kinds of luciferase activity were measured. The ratio of firefly luciferase activity to Renilla luciferase activity in each sample was used as a measure of normalized luciferase activity.

Gel Mobility Shift Assay
Nuclear extracts from RASMCs treated with RW-PF were prepared.21 Protein concentrations were measured by the Bio-Rad protein assay system based on the Bradford method.22 A double-stranded oligonucleotide probe synthesized according to the sequence of the human cyclin A 5′-flanking region containing a typical ATF site (bp -84 to -63; 5′-TGAAATCGTCAAGGCGGAGG-3′) was radiolabeled.14 A binding reaction was performed at room temperature for 20 minutes and analyzed by 5% native polyacrylamide gel electrophoresis.15 To determine the specificity of the DNA-protein–binding complexes, a competition assay using a 100-fold molar excess of unlabelled probe encoding the wild-type ATF sequence or the mutated ATF sequence (5′-TGAAATCGTCAAGCGCCAGG-3′) was performed. The gel was dried and autoradiographed at -80°C.

Cell DNA Staining
After 24 hours of incubation of RASMCs on chamber slides, RASMCs were treated with RW-PF at the highest dose (100 μg/mL) for 12 hours. As a positive control, RASMCs were treated with the antioxidant agent pyrrolidine dithiocarbamate (PDTC; 100 μmol/L), which is known to induce apoptosis in VSMCs22 for 12 hours. Cells were washed and fixed with PBS containing 1% glutaraldehyde for 30 minutes and then incubated in PBS containing 10 μmol/L bisbenzimide (Hoechst 33258; Funakoshi Co) for 15 minutes and examined under fluorescence microscopy.

Statistical Analysis
When statistically significant effects (P<0.05) were found, the Newman-Keuls test (ANOVA) was used to isolate the differences between groups. All data in the text and figures are expressed as mean±SEM.

Results
Content of Polyphenolic Substances
The total polyphenolic contents of each fraction are shown in Figure 1. The recoveries of fractions 1 through 6 were 4.42, 5.29, 4.14, 2.30, 1.81, and 6.01 g dry weight, respectively.

Effects of RW-PF on Proliferation and DNA Synthesis
RW-PF significantly inhibited the growth of RASMCs and thymidine incorporation into RASMCs in a concentration-dependent manner (Figure 2A and 2B). RW-PF decreased the cell number of RASMCs to 94%, 93%, 87%, 77%, and 61%
of control and inhibited thymidine incorporation into RASMCs to 92%, 86%, 71%, 52%, and 18% of control at final concentrations of 1, 3, 10, 30, and 100 µg/mL, respectively. Differences between control medium containing 0.5% ethanol and ethanol-free medium were not statistically significant. These inhibitory effects of RW-PF on BCECs were observed at much higher concentrations, especially at 100 µg/mL (Figure 2C and 2D).

**Effects of Six Fractions on DNA Synthesis in RASMCs**

Six fractions (fractions 1 through 6) decreased thymidine incorporation into RASMCs to 41%, 37%, 30%, 26%, 30%, and 22% of control containing 0.15% ethanol as vehicle (Figure 3A). The inhibitory effect of each fraction was in proportion to its respective total polyphenolic content (Figure 3B).

![Diagrams](image-url)
Effects of RW-PF on Cyclin A Gene Expression

The expression of cyclin A mRNA in RASMCs was slightly decreased at 4 hours after the addition of RW-PF (30 μg/mL) and was completely suppressed at 48 to 72 hours (Figure 4A). Treatment with RW-PF for 72 hours significantly inhibited the cyclin A mRNA to 80%, 43%, 16%, and 4% of control at final concentrations of 3, 10, 30, and 100 μg/mL, respectively (Figure 4B). In contrast, RW-PF did not inhibit cyclin A gene expression in BCECs, except at a concentration of 100 μg/mL (Figure 4C).

RW-PF decreased the transcriptional activity of the cyclin A promoter in RASMCs to 87%, 71%, 69%, and 45% of control at final concentrations of 3, 10, 30, and 100 μg/mL, respectively (Figure 5A). The plasmid containing a mutated ATF site in the cyclin A promoter had 12-fold lower luciferase activity than that of the plasmid containing a normal ATF site. Luciferase activity was not affected by RW-PF. In contrast, RW-PF did not inhibit cyclin A gene expression in BCECs, except at a concentration of 10 μg/mL (Figure 4C).

In the gel mobility shift assay, specific binding of nuclear proteins extracted from RASMCs to the ATF site was confirmed by addition of a specific competitor (Figure 5B, lanes 3 and 4). RW-PF decreased the abundance of specific ATF-binding nuclear proteins in a concentration-dependent manner (lanes 2 and 5 through 10). A supershift assay using specific antibodies confirmed the existence of CREB and ATF-1 in RASMC nuclear extracts (data not shown). After repeated independent experiments, no difference was found between RASMCs in ethanol-free medium (lane 2) and RASMCs with 0.5% ethanol (lane 5) (data not shown).

Moreover, RW-PF downregulated the mRNA levels of the transcription factors CREB and ATF-1, which are known to bind to the ATF site in the cyclin A promoter with the same time course and concentration-dependence as that of cyclin A mRNA (Figure 5C).
Effects of RW-PF in Human Cells
RW-PF inhibited thymidine incorporation into HASMCs to 70% and 30% of control at 10 and 30 μg/mL (Figure 6A) and downregulated the expression of cyclin A mRNA in HASMCs in a concentration-dependent manner (Figure 6B). In contrast, the inhibitory effects of RW-PF on DNA synthesis and cyclin A gene expression in HUVECs were observed at much higher concentrations, especially at 100 μg/mL (Figure 6C and 6D).

Examination of Apoptotic Cell Death
In the fluorescence microscope study after nucleic acid staining, PDTC induced morphological changes indicative of apoptotic cell death, such as condensation of chromatin and shrinkage of the nucleus, in RASMCs (Figure 7C). However, RW-PF even at the highest concentration (100 μg/mL) did not cause any morphological changes in RASMCs (Figure 7B) compared with untreated RASMCs (Figure 7A).

Discussion
In the present study, our results demonstrated that RW-PF had antiproliferative effects on VSMCs, which might be associated with the downregulation of cyclin A gene expression through the decreased expression of transcription factors CREB and ATF-1. In contrast, RW-PF did not significantly inhibit the proliferation and DNA synthesis of vascular endothelial cells. The inhibitory effects of RW-PF on mRNA level and promoter activity of cyclin A were observed only in RASMCs, but not in BCECs. A limitation of our study is that the mechanism for these differences has not been clarified. One possible explanation is that a specific intracellular mechanism that can respond to an optimal concentration of RW-PF may exist only in VSMCs, but not in endothelial cells.

RW-PF, the total polyphenolic fraction extracted from red wine, contained a wide variety of polyphenols, including phenolic monomers and polymers, which have different molecular weights. RW-PF was broadly divided into 2 groups. One group, characterized by lower average molecular weight, consisted of 4 fractions, fractions 1 through 4, eluted first by 50% ethanol, and the other group, characterized by higher average molecular weight, consisted of 2 fractions, fractions 5 and 6, eluted by 100% ethanol and 70% acetone, respectively. The first 4 fractions (fractions 1 through 4) contained various polyphenolic monomer components, such as...
as anthocyanidins, catechins, and flavonoids (unpublished data, K.N.). Although the percentage of each monomer may be quite different among the 4 fractions, we have not found any specific polyphenolic substance that is dominant in a specific fraction. Fractions 5 and 6 contained a significant amount of specific polyphenolic compounds, proanthocyanidins, which are polymerized anthocyanidins (unpublished data, K.N.). In particular, fraction 6 contained almost 100% proanthocyanidins. The average molecular weights of fractions 1 through 4 and fraction 6 (mainly proanthocyanidins) are believed to be ~200 to 400 and 1600 to 2000, respectively. It is noteworthy that polyphenol fractions of different molecular weight showed similar potent antiproliferative effects on VSMCs.

At present, there is no method for measuring concentrations of red wine polyphenolic polymers in blood. However, Duthie et al. measured the concentration of polyphenolic monomers after the intake of red wine by healthy volunteers and demonstrated that the intake of 100 mL red wine resulted in an increase in plasma concentration of 2.5 μg/mL (gallic acid equivalents). This observation suggests that the absorption efficiency of red wine polyphenolic monomers is ~5%. If polyphenolic polymers were also absorbed through the intestine at the same level of absorption efficiency, the final blood concentration after an intake of 100 mL red wine would be ~3.2 μg/mL. Thus, it may be reasonable to suppose that the physiological concentration of RW-PF in blood after a normal intake of red wine may be 1 to 10 μg/mL. It can be assumed that the long-term (years) effect of even lower concentrations of RW-PF may have an inhibitory effect on VSMC growth in vivo.

Red wine polyphenols have been shown to have antioxidative effects in terms of LDL oxidation in vitro. Moreover, recent in vivo studies have shown that red wine polyphenols contribute to the inhibition of LDL oxidation in healthy volunteers and in apolipoprotein E knockout mice. Although antioxidant agents are reported to induce apoptosis in VSMCs, despite prolonged treatment of RASMCs with RW-PF, we did not observe any evidence of apoptosis. We suggest that the inhibitory effects of RW-PF on RASMCs may not be mediated by an apoptotic process.

It was recently been reported that resveratrol, a polyphenolic compound, is an agonist for the estrogen receptor. The agent is similar in structure to synthetic estrogen-like compounds. Estrogen, which is a ligand for the estrogen receptor expressed in VSMCs, has been shown to have an antiatherogenic effect by inhibiting the proliferation of VSMCs. This estrogen-like agonistic function of resveratrol is one possible explanation for the antiproliferative effects of RW-PF on VSMCs.

In regard to the downregulation of the cyclin A gene, it has been demonstrated that in mink lung epithelial cells, transforming growth factor-β1 treatment decreased cyclin A mRNA and promoter activity but not the abundance of the ATF-binding proteins CREB and ATF-1 at 24 hours. In a study using vascular endothelial cells, contact inhibition decreased the level of cyclin A mRNA almost completely; however, the level of ATF-1 mRNA was partially decreased and that of CREB mRNA was not changed. In the present study, RW-PF treatment completely decreased the levels of CREB and ATF-1 mRNA. It has been shown that CREB promoter contains the ATF/CRE site, which might be regulated by CRE-binding proteins, including CREB itself. Because transcription of the CREB gene may be controlled by positive autoregulation through the ATF/CRE site, it can be postulated that RW-PF may downregulate the transcription of CREB gene through some unknown mechanism first and may start to decrease the cyclin A promoter activity. The downregulation of CREB may also act on the ATF/CRE site of its promoter, and finally, CREB may become completely downregulated. The same might be true for the regulation of the ATF-1 gene.

Taken together, red wine polyphenols had potent antiproliferative effects on VSMCs, and these inhibitory effects may be associated with the downregulation of cyclin A gene expression through the inhibition of transcription factor expression. Our findings suggest that the antiproliferative effect of RW-PF may be one possible mechanism for the antiatherogenic effects of red wine, other than the antioxidative effects of red wine polyphenols on LDL. Furthermore, elucidation of the mechanisms of downregulation of cell cycle–related factors will provide important insights into the antiproliferative effects of red wine polyphenols on VSMCs.

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