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Objective—Red wine (RW) consumption has been associated with a reduction of cardiovascular events, but limited data are available on potential mediating mechanisms. This study tested the hypothesis that intake of RW may promote the circulating endothelial progenitor cell (EPC) level and function through enhancement of nitric oxide bioavailability.

Methods and Results—Eighty healthy, young subjects were randomized and assigned to consume water (100 mL), RW (100 mL), beer (250 mL), or vodka (30 mL) daily for 3 weeks. Flow cytometry was used to quantify circulating EPC numbers, and in vitro assays were used to evaluate EPC functions. After RW ingestion, endothelial function determined by flow-mediated vasodilation was significantly enhanced; however, it remained unchanged after water, beer, or vodka intake. There were significantly increased numbers of circulating EPC (defined as KDR^+CD133^+, $CD34^+CD133^+$, $CD34^+KDR^+$) and EPC colony-forming units only in the RW group (all $P<0.05$). Only RW ingestion significantly enhanced plasma levels of nitric oxide and decreased asymmetrical dimethylarginine (both $P<0.01$). Incubation of EPC with RW (but not beer or ethanol) and resveratrol in vitro attenuated tumor necrosis factor-α–induced EPC senescence and improved tumor necrosis factor-α–suppressed EPC functions and tube formation. Incubation with nitric oxide donor sodium nitroprusside significantly ameliorated the inhibition of tumor necrosis factor-α on EPC proliferation, but incubation with endothelial nitric oxide synthase inhibitor L-NAME and PI3K inhibitor markedly attenuated the effect of RW on EPC proliferation.

Conclusion—The intake of RW significantly enhanced circulating EPC levels and improved EPC functions by modifying nitric oxide bioavailability. These findings may help explain the beneficial effects of RW on the cardiovascular system. This study demonstrated that a moderate intake of RW can enhance circulating levels of EPC in healthy subjects by increasing nitric oxide availability. Direct incubation of EPC with RW and resveratrol can modify the functions of EPC, including attenuation of senescence and promotion of EPC adhesion, migration, and tube formation. These data suggest that RW ingestion may alter the biology of EPC, and these alterations may contribute to its unique cardiovascular-protective effect. (*Arterioscler Thromb Vasc Biol*. 2010;30:869-877.)

Key Words: endothelial function endothelial progenitor cell ■ nitric oxide ■ red wine

Epidemiological evidence confirms that light to moderate alcohol consumption reduces the risk of morbidity and mortality from cardiovascular disease.[1,2] Regular consumption of any type of alcoholic beverage appears to confer healthy benefits, but red wine (RW), with its abundant antioxidant contents, seems to offer additional healthy benefits.[3,4] Recent studies indicated that the beneficial effects of RW are derived from increased endothelium-derived nitric oxide (NO),[5] implying that enhanced NO bioavailability may mediate the cardiovascular protection provided by RW.

During the past decades, endothelial function has been shown to play an important role in the clinical manifestations of established atherosclerotic lesions.[6] Endothelial dysfunction is characterized by a reduction of the bioavailability of vasodilators, particularly NO, and can predict future cardiovascular events.[7] Increasing evidence suggests that the in-

jured endothelial monolayer is regenerated by circulating bone marrow-derived endothelial progenitor cells (EPC), which accelerate reendothelialization and protect against the initiation and progression of atherosclerosis.[8,9] Levels of circulating EPC reflect vascular repair capacity and have been shown to be associated with endothelial function.[10] Moreover, EPC are significantly downregulated in patients with coronary artery disease or risk factors for coronary artery disease.[11] Clinical evidence showed that the number of circulating EPC predicts the occurrence of cardiovascular events and death from cardiovascular causes and may help to identify patients at increased cardiovascular risk.[12] A progressive reduction in circulating EPC when risk factors are elevated may contribute to a vicious cycle, resulting in endothelial dysfunction and the rapid progression of atherosclerosis.[13] Although many epidemiological studies have indicated that light to moderate consumption of RW can reduce the incidence of coronary artery disease, the direct effect of RW consumption on circulating EPC and their functions remains unclear. Therefore, we designed this study to investigate whether intake of RW could enhance the number and function of EPC through increasing NO bioavailability.

Subjects and Methods

Human Study Protocol

The human study was a prospective, randomized, diet-controlled, open-label trial in which 80 young, healthy subjects were enrolled (please see Supplemental material available online at http://atvb.ahajournals.org).

Endothelium-Dependent Flow-Mediated Vasodilation

Endothelium-dependent flow-mediated vasodilation was assessed using a 7.5-MHz linear array transducer (Hewlett-Packard Sonos 5500) as previously described (available online at http://atvb. ahajournals.org).[14]

Laboratory Measurements

After 8 hours of overnight fasting, all subjects had a venous blood sample taken for measurement of high-sensitivity C-reactive protein, tumor necrosis factor-α (TNF-α), NO, asymmetrical dimethylarginine, adiponectin, and oxidized low-density lipoproteins (please see http://atvb.ahajournal.org).

Flow Cytometry

A volume of 100 μL peripheral blood was incubated for 15 minutes in the dark with monoclonal antibodies against human *KDR* (R&D) followed by PE-conjugated secondary antibody, with the FITC-labeled monoclonal antibodies against human CD45 (Becton Dickinson), with the PE-conjugated monoclonal antibody against human CD133 (Miltenyi Biotec, Germany), and with FITC-conjugated or PE-conjugated monoclonal antibodies against human CD34 (Serotec; Supplementary Figure I).[11]

Human Early and Late EPC Cultivation

Peripheral blood samples (20 mL) were obtained from healthy, young volunteers, and total mononuclear cells were isolated by density gradient centrifugation with Histopaque-1077 (Sigma).[15]

Human EPC Colony-Forming Assay

Isolated mononuclear cells were resuspended in growth medium (EndoCult liquid medium; StemCell Technologies), and 5×10^6 mononuclear cells were preplated in a fibronectin-coated 6-well plate in duplicate (please see http://atvb.ahajournals.org).[16]

EPC Number and Proliferation Assay

The number of early EPC and the proliferation of late EPC was determined by direct staining of 6 random high-power microscopic fields ($\times 100$) and by 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyltetrazolium bromide assay, respectively (supplemental material).

EPC Senescence Assay

Cellular aging was determined as previously described (supplemental material).[15]

EPC Fibronectin Adhesion Assay

EPC fibronectin adhesion test was assessed as previously described (supplemental material).[16]

EPC Migration Assay

The migratory function of EPC was evaluated by a modified Boyden chamber assay (Transwell; Coster; please see supplemental material).[17]

EPC Tube Formation Assay

EPC tube formation assay was performed with an in vitro angiogenesis assay kit (Chemicon; supplemental material).[17]

Western Blotting Analysis and Measurement of Nitrite Levels

Protein extracts were prepared as previously described (supplemental material).[18]

Statistical Analysis

All data were expressed as the mean$\pm$SEM for continuous variables and as the number (percent) for categorical variables. Intergroup comparisons were performed by 1-way ANOVA and a Bonferroni test. $P<0.05$ was considered statistically significant. The SPSS 9.0 (version 12; SPSS) software package was used for all statistical analyses.

Results

Baseline Characteristics of Study Subjects and Effects of RW on Endothelial Function in Human Study

Eighty healthy subjects participated in the study (mean age, 34 ± 1 years) and were randomized into 4 groups that consumed pure water, RW, beer, or vodka daily for 3 weeks. The baseline characteristics of 80 participants are presented in Supplemental Table I. No significant differences of baseline characteristics were noted among the 4 groups. After pure water, RW, beer, or vodka consumption for 3 weeks, there were no significant changes in body weight, blood pressure, and serum levels of total cholesterol, high-density lipoproteins, triglycerides, and fasting glucose levels. Endothelial function evaluated by endothelium-dependent flow-mediated vasodilation was assessed before and after the study. The baseline diameters of the brachial artery did not differ from each other. However, 3 weeks of RW consumption was associated with a significant enhancement of flow-mediated vasodilation (from 7.4%$\pm$0.6% to
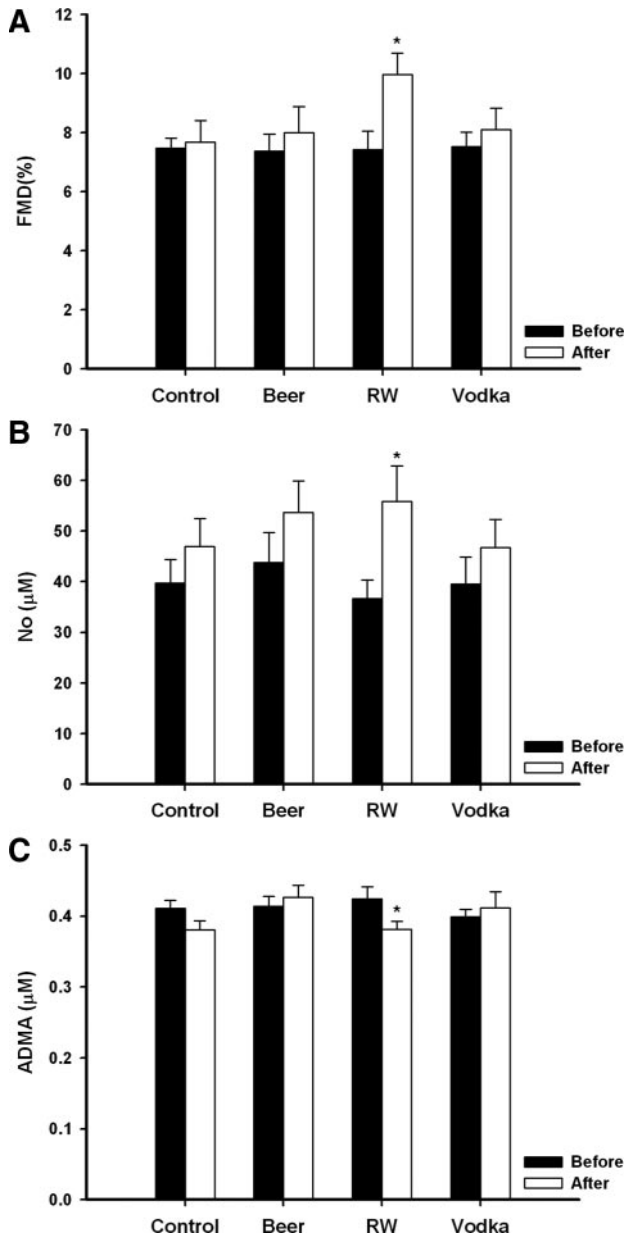
Figure 1. Effects of RW on endothelial function, NO, and asymmetrical dimethylarginine (ADMA). Comparison of percent changes of endothelium-dependent flow-mediated vasodilation (FMD) (A) and plasma concentrations of NO (B) and ADMA (C) before and after 3 weeks of pure water, RW, beer, or vodka consumption. Data are means±SEM.

$10.0\% \pm 0.7\%$; $P=0.008$); however, it remained unchanged in the pure water, beer, and vodka groups (Figure 1A).

We further determined the plasma levels of NO and asymmetrical dimethylarginine at baseline and after water, RW, beer, or vodka ingestion. As shown in Figure 1B and 1C, RW intake significantly augmented NO concentration (37 ± 4 vs 56 ± 7 mmol/L; $P=0.008$) and downregulated plasma asymmetrical dimethylarginine levels (0.42 ± 0.02 vs 0.38 ± 0.01 μmol/L; $P=0.009$). In contrast, subjects in the pure water, beer, or vodka groups did not show significant changes in plasma NO or asymmetrical dimethylarginine concentrations.

The effects of RW or beer consumption on systemic inflammatory markers were determined. No significant differences were observed in plasma levels of high-sensitivity C-reactive protein or TNF-α after 3 weeks of pure water, RW, beer, or vodka intake (Figure 2A, B). We also assessed the effects of RW, beer, or vodka consumption on plasma adiponectin levels and oxidized low-density lipoproteins status, which are biomarkers of lipolysis and oxidative stress. However, there were no significant changes in adiponectin and oxidized low-density lipoproteins responses after RW, beer, or vodka consumption for 3 weeks (Figure 2C, D).

RW Ingestion Increases EPC Colony-Forming Units and Circulating EPC Levels In Vivo

As shown in Figure 3A, there was no significant change in the water, beer, or vodka groups; however, there was a significant increase in the number of EPC colony-forming units noted in the RW group (from 41 ± 9 to 48 ± 8 colony-forming units/well; $P=0.001$). Flow cytometry was used to determine the numbers of circulating EPC, defined as KDR$^+$CD133$^+$, CD34$^+$CD133$^+$, and CD34$^+$KDR$^+$ double-positive cells. There were no significant differences at baseline in the circulating EPC numbers among the 4 groups. After intake of RW for 3 weeks, the numbers of circulating EPC significantly increased (all $P<0.05$), but the numbers of circulating EPC remained unchanged in the pure water, beer, and vodka groups (Figure 3B, D).

RW Enhances EPC Number and Reverses TNF-α–Suppressed EPC Proliferation and Colony-Forming Capacity In Vitro

In an in vitro study, we assessed the direct effects of RW on EPC number, proliferation, and colony-forming capacity. After seeding mononuclear cells on 6-well plates, early EPC were incubated with RW (1%), beer (2%), ethanol (0.15%), or resveratrol (50 μmol/L) for 4 days. Incubation of mononuclear cells with RW significantly increased the number of differentiated early EPC, characterized by DiI-acLDL and fluorescein isothiocyanate lectin staining compared with that in the control group (control vs RW, 51 ± 3 vs 71 ± 3/high-power field; $P=0.008$; Figure 4A), but no significant difference was found between the control and beer or vodka groups. Administration of resveratrol was noted to increase early EPC numbers (control vs resveratrol, 51 ± 3 vs 67 ± 5/high-power field; $P=0.003$).

The effect of RW on late EPC proliferation was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyltetrazolium bromide assay. As shown in Figure 4B, incubation with TNF-α (T20; 20 ng/mL) in culture medium markedly reduced late EPC proliferation (control vs T20, 1.00 ± 0.03 vs 0.85 ± 0.04; $P=0.038$). However, administration of RW and resveratrol significantly reversed the reduction in EPC proliferation in the presence of TNF-α (T20 vs T20+RW and T20+resveratrol, 0.85 ± 0.04 vs 1.08 ± 0.04 and 1.04 ± 0.05; both $P<0.05$), but this effect was not observed with beer or ethanol. Additionally, the number of EPC colony-forming unit was markedly suppressed by TNF-α, and this suppression was reversed by treatment with RW (T20 vs T20+RW,
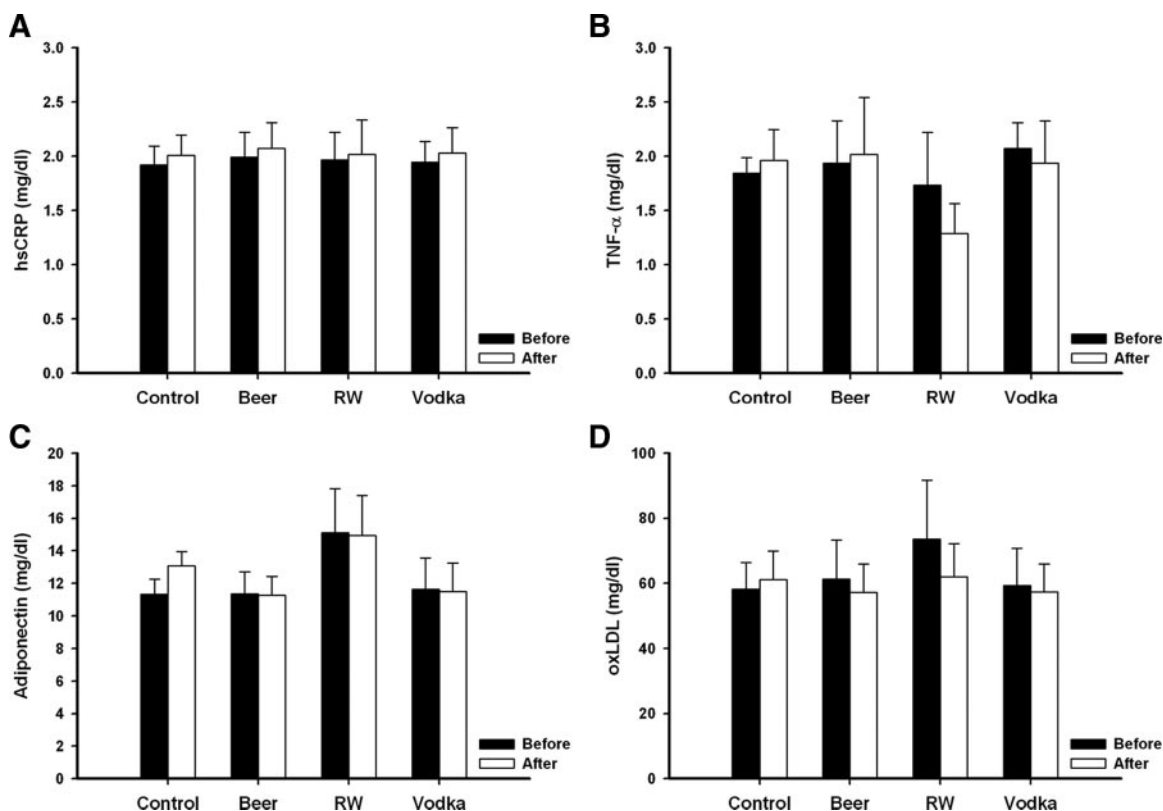
Figure 2. Effects of RW on inflammatory biomarkers, adiponectin and oxidized low-density lipoproteins (ox-LDL). Comparison of plasma levels of high-sensitivity C-reactive protein (hsCRP) (A), tumor necrosis factor-α (TNF-α) (B), adiponectin (C), and ox-LDL (D) before and after pure water, RW, beer, or vodka ingestion. Data are means±SEM.

26 ± 2 vs 39 ± 2 colony-forming units/well; $P=0.002$) but not with beer or ethanol (Figure 4C).

RW Upregulates Phosphorylation of Endothelial NO Synthase and Akt and Promotes Bioavailable NO in EPC

We evaluated the effects of RW on endothelial NO synthase (eNOS) and Akt activities by Western blotting in cultured late EPC. After 4 days of incubation, the eNOS phosphorylation at Ser[1177] and Akt phosphorylation at Ser[473] shown by immunoblotting were significantly upregulated by treatment with RW in late EPC (Figure 5A). This augmentation of eNOS and Akt phosphorylation was associated with an increase in EPC-derived NO production (nitrite levels; T20+RW, 1% vs T20; increased 89.6%; Figure 5B). Coincubation with NO donor sodium nitroprusside significantly ameliorated the inhibition of TNF-α on EPC proliferation (T20+sodium nitroprusside vs T20, increased 57.6%; $P=0.001$). In contrast, coincubation with eNOS inhibitor L-NAME (100 μmol/L) and PI3K inhibitor (LY294002; 5 μmol/L) markedly attenuated the effect of RW on EPC proliferation (all $P<0.001$; Figure 5C). These data indicated that RW may augment EPC function by modulating PI3K/Akt-mediated, and NO-mediated mechanisms.

Effects of RW on EPC Senescence, Adhesion, Migration, and Tube Formation In Vitro

Compared with the control group, incubation of late EPC with TNF-α significantly increased the percentage of senescence-associated β-galactosidase–positive EPC (control vs T20; $P=0.001$; Figure 6A). Administration of RW and resveratrol for 4 days significantly attenuated the percentage of senescence-associated β-galactosidase–positive EPC in the presence of TNF-α (T20 vs T20+RW and T20+resveratrol, both $P<0.001$); however, beer or ethanol treatment had no significant effect. Administration of L-NAME abolished the antisenescence effect of RW.

As shown in Figure 6B, TNF-α markedly suppressed late EPC adhesion, and administration of RW and resveratrol significantly augmented TNF-α–suppressed EPC adhesion (T20 vs T20+RW and T20+resveratrol, 13 ± 2 vs 39 ± 2 and 35 ± 3 cells/high-power field; both $P<0.001$), and this effect was inhibited by treatment with L-NAME. However, there was no significant effect of beer or ethanol on recovery of EPC adhesion in the presence of TNF-α ($P=0.526$ and 0.197, respectively).

A modified Boyden chamber assay with vascular endothelial growth factor as a chemoattractic factor was used to evaluate EPC migration. As shown in Figure 3C, incubation with RW and resveratrol for 4 days significantly enhanced TNF-α–suppressed late EPC migration (T20 vs T20+RW and T20+resveratrol, 37 ± 2 vs 67 ± 3 and 62 ± 2 cells/high-power filed; both $P<0.001$), but this benefit was not observed after incubation with beer or ethanol. In addition, administration of eNOS inhibitor significantly diminished the effect of RW on EPC migration.

An in vitro angiogenesis assay was performed with late EPC to investigate the effect of RW on EPC neovascular-
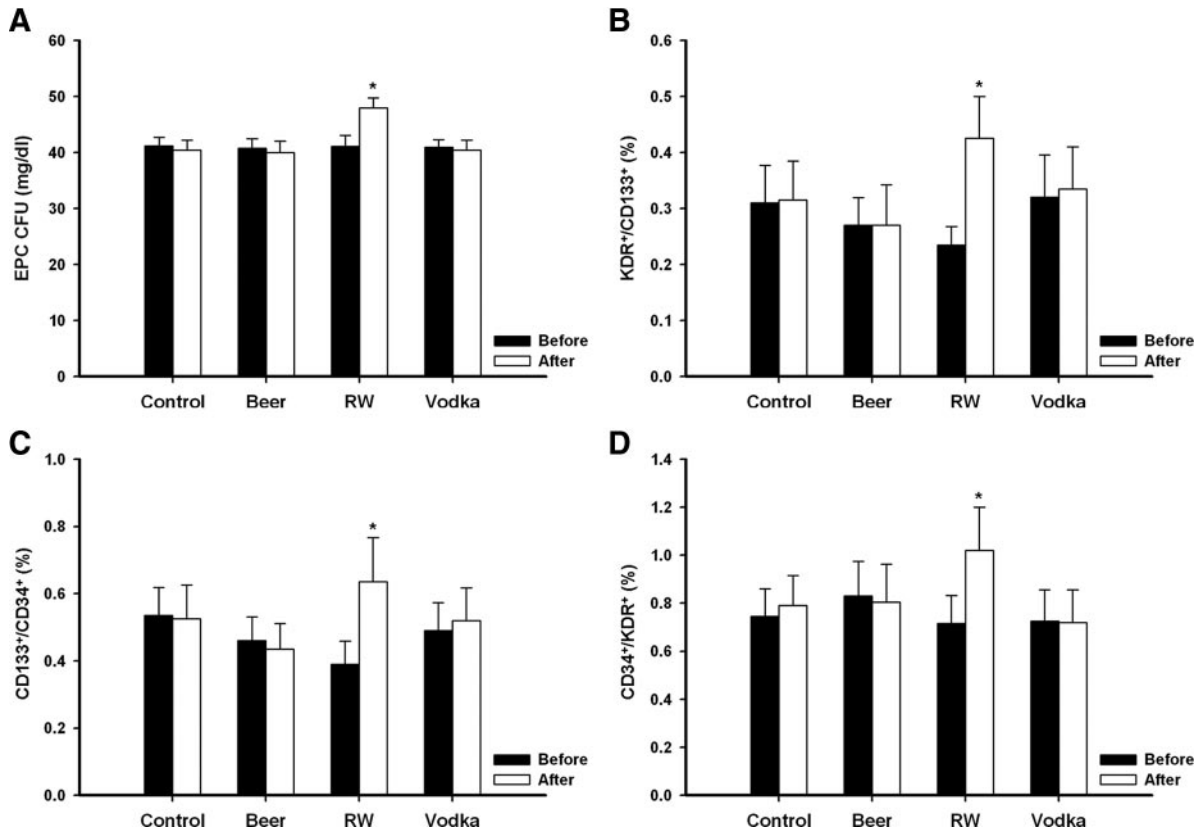
Figure 3. Effects of RW on EPC colony-forming units (CFU) and circulating EPC in vivo. Circulating EPC CFU (A) and EPC numbers (B–D) were determined before and after RW or beer consumption. Flow cytometry was used to determine the numbers of circulating EPC defined as KDR$^+$CD133$^+$, CD34$^+$CD133$^+$, and CD34$^+$KDR$^+$ double-positive EPC. Data are means±SEM.

ization. After 4 days of culturing, the functional capacity for tube formation of late EPC on ECMatrix gel was significantly reduced in the presence of TNF-α compared with the control group, whereas incubation with RW and resveratrol promoted tube formation in EPC (both $P<0.001$; Figure 3D) but incubation with beer or ethanol did not. Administration of L-NAME attenuated the effect of RW on EPC tube formation.

Discussion

The major new findings of this study are that RW consumption increases circulating EPC levels, and this effect seems to be independent of improvement in lipid profiles, inflammation, adiponectin, and oxidized low-density lipoproteins levels. The beneficial effects exerted by RW appear to be related to upregulation of NO bioavailability. In vitro studies support these clinical observations. Incubation of EPC with RW and RW content, resveratrol, can modify the function of EPC, including attenuation of senescence and promotion of EPC adhesion, migration, and tube formation. These data suggest that intake of RW may alter the biology of EPC, and these alterations may contribute to its unique cardiovascular protective effect.

Light to moderate ethanol intake from any type of beverage has been shown to improve lipoprotein metabolism and lower cardiovascular mortality.[19,20] However, RW, with its abundant content of phenolic acids, polyphenols, and flavonoids, appears to confer additional healthy

benefits.[3,4] The protective effect of RW is supported by epidemiological data that show a significantly reduced incidence of coronary artery disease in certain areas of France, despite a high-fat diet, little exercise, and widespread cigarette smoking. This phenomenon has been termed the "French paradox."[21] Evidence for a more pronounced cardioprotective effect of RW as compared with other alcoholic beverages first emerged from the Copenhagen City Heart Study, in which 13 285 men and women were observed for 12 years.[22] The results from this study suggested that patients who drank wine had half the risk of death from coronary heart disease or stroke compared with those who never drank wine. Those who drank beer and spirits did not experience this advantage. The additional benefit of RW is supported further by an analysis of 13 studies involving 209 418 participants. This analysis showed a 32% risk reduction of atherosclerotic disease with RW intake, which was >22% risk reduction for beer consumption.[23] However, other studies and reviews have not observed the dominant effect of wine. Explanations for these contradictory results may include differences in the risk factors patterns among beer, spirit, and wine drinkers, the pattern of alcohol consumption, the presence of other confounding lifestyle factors, or, alternatively, differences in the type of wine consumed.

A number of mechanisms have been proposed for the beneficial effect of RW, including favorable changes in lipid metabolism, antioxidant effects, changes in hemostasis and
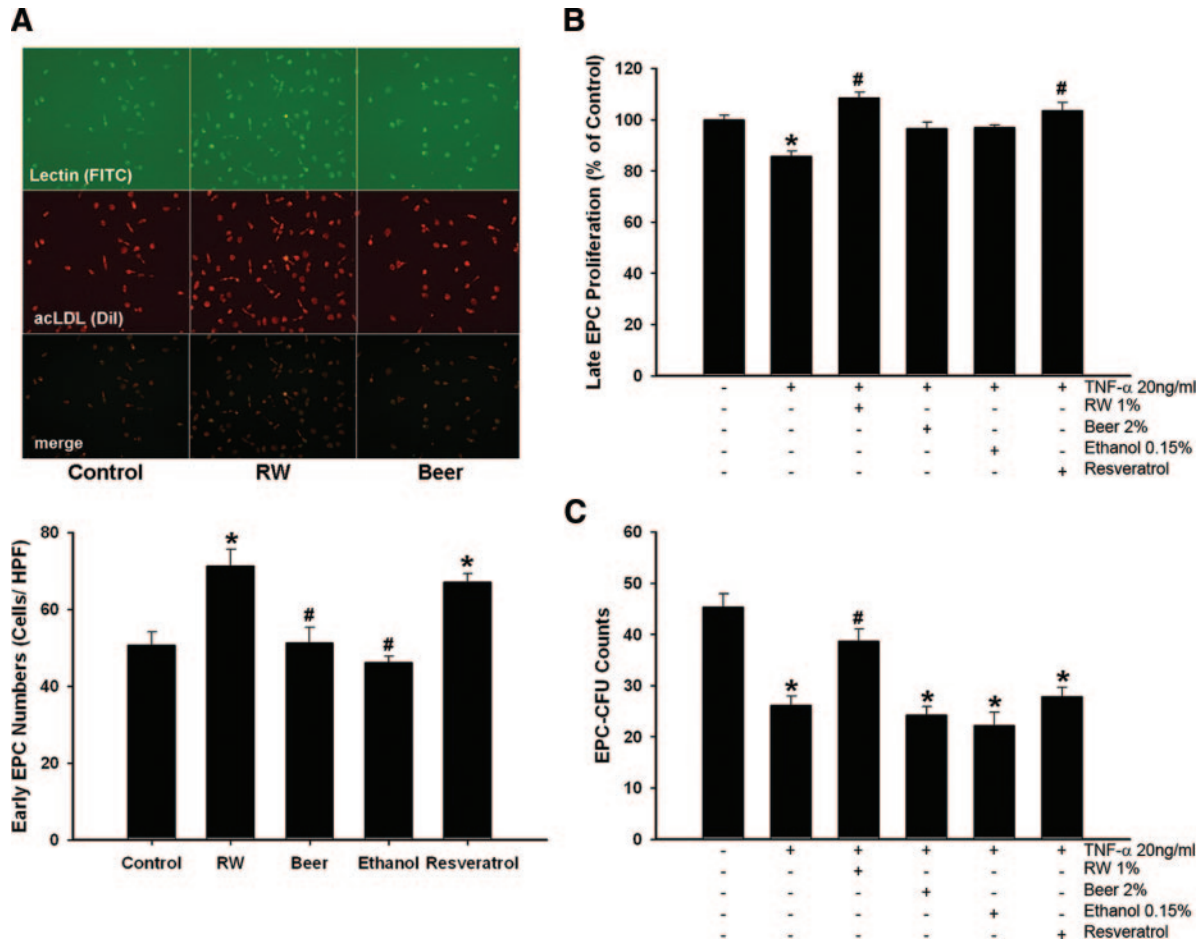
Figure 4. Effects of RW on EPC number, proliferation, and colony-forming capacity in vitro. The number of early EPC was determined by direct staining of 6 random high-power microscopic fields ($\times$100). After culturing for 4 days, the number of early EPC was counted and phenotyping of endothelial characteristics of EPC by indirect immunostaining was performed with 1,1′-dioctadecyl-3,3,3′,3′-tetra-methylindocarbocyanine perchlorate-acetylated low-density lipoprotein (Dil-acLDL) and FITC-labeled lectin from Ulex europaeus (UEA-1) (A). *$P<0.05$ compared with control. #$P<0.05$ compared with RW. The effect of RW on late EPC proliferation was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyltetrazolium bromide (MTT) assay (B). A colony of EPC was defined as a central core of round cells with elongated sprouting cells at the periphery. After culturing for 4 days, the number of CFU per well was counted (C). Data are means$\pm$SEM; n=5 in each experiment. *$P<0.05$ compared with control. #$P<0.05$ compared with TNF-α 20 ng/mL.

platelet aggregation, arterial vasodilation mediated by NO release, insulin sensitization, and lower levels of inflammatory markers.[24] However, mechanistic studies suggested that many of these beneficial effects of RW are compatible with the action of endothelium-derived NO,[5] implying that NO may be a critical mediator of the cardiovascular protection provided by RW.

A large body of evidence indicates that the integrity and functional activity of the endothelial monolayer play an important role in atherogenesis.[25] In humans, extensive endothelial cell damage by cardiovascular risk factors can result in endothelial cell apoptosis with subsequent loss of integrity of the endothelium. The extent of endothelial injury may represent a balance between the magnitude of injury and the capacity for repair and predicts cardiovascular event rates.[26] The traditional view suggests that endothelial cell repair is exclusively mediated by the adjacent endothelial cells. However, a series of basic and clinical studies prompted by the discovery of bone marrow-derived EPC have provided new insights into

these processes and indicate that circulating EPC play a pivotal role in endothelial cell regeneration.[27] Reduced levels of circulating EPC independently predict atherosclerotic disease progression and future cardiovascular events,[12] thus supporting an important role for endogenous vascular repair by EPC to modulate the clinical course of coronary artery disease. Therefore, enhancement of circulating EPC level and its functional capacity by lifestyle modification or pharmacological strategies may be of clinical importance and therapeutic potential.

In animal studies, moderate intake of RW promoted EPC numbers in wild-type mice subjected to physical exercise.[28] Furthermore, RW improved ischemia-induced neovascularization in hypercholesterolemic apolipoprotein E-deficient mice after hindlimb ischemia by increasing the number and functional activity of EPC.[29] Balestrieri et al[30] reported that low doses of RW in vitro upregulated the number of circulating EPC during treatment with TNF-α. However, the relationship between RW consumption and circulating EPC remains unclear in humans. In our pro-
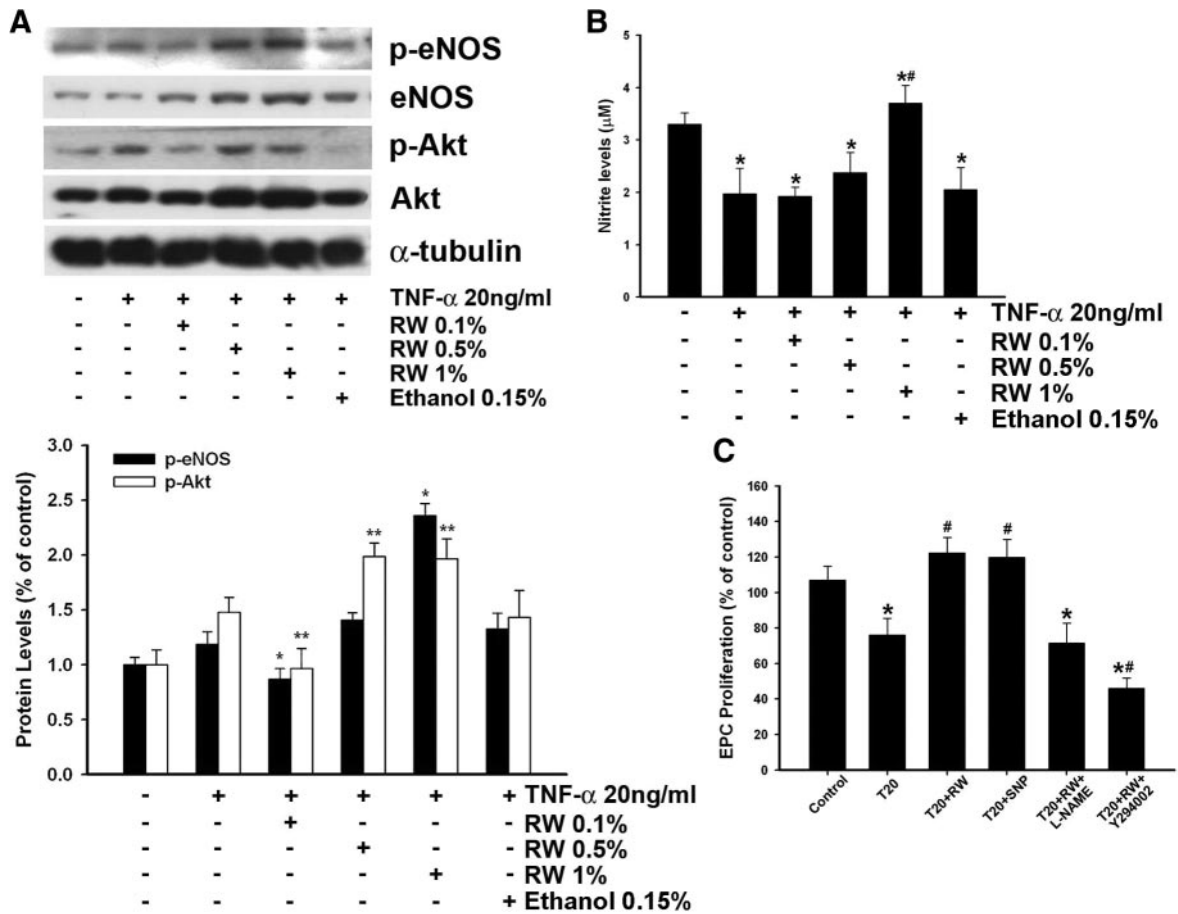
Figure 5. RW upregulated eNOS, Akt phosphorylation, and increased bioavailability of NO in EPC. Effects of RW on eNOS, Akt activities, and nitrite levels were determined in cultured late EPC. Administration of RW in the presence of TNF-α dose-dependently upregulated eNOS phosphorylation (p-eNOS) and p-Akt (A). Each bar graph shows the summarized data from 4 separate experiments by densitometry after normalization (p-eNOS, *$P<0.05$ compared with TNF-α 20 ng/mL; p-Akt, **$P<0.05$ compared with TNF-α 20 ng/mL). Nitrite production (as NO content) in culture medium was measured by the Griess reagent (B). Late EPC proliferation was analyzed after 4 days of culture in RW, sodium nitroprusside (SNP) (NO donor; 25 μmol/L), L-NAME (NOS inhibitor; 100 μmol/L), and LY294002 (PI3K/Akt inhibitor; 5 μmol/L) in the presence of TNF-α (C). Data are means±SEM; n=5 in each experiment. *$P<0.05$ compared with control. #$P<0.05$ compared with TNF-α 20 ng/mL.

spective, randomized study, it was interesting to find that even light consumption of RW for 3 weeks significantly increased circulating EPC levels, implying an additional beneficial effect of RW on EPC and endothelial repair capability. We further confirmed these findings in in vitro study and demonstrated that RW increased EPC numbers and improved TNF-α–suppressed EPC proliferation and colony-formation capacity. RW and resveratrol were also shown to attenuate EPC senescence and restored EPC adhesion, migration, and tube formation, which were suppressed by TNF-α, suggesting resveratrol might be at least partly responsible for the beneficial cardiovascular effects of RW. Besides, the suppressive effect of TNF-α could be ameliorated by coincubation with the NO donor, sodium nitroprusside. In addition, administration of an NOS inhibitor or PI3K inhibitor abolished the beneficial effect of RW on EPC proliferation. These data indicate that RW may promote EPC function by modulating PI3K/Akt-related and NO-related mechanisms.

There is increasing evidence for an interaction of RW and RW polyphenolic compounds with the endothelial NO system. Wallerath et al[5] demonstrated that RW upregulated eNOS expression and activity in human endothelial cells by both transcriptional and posttranscriptional (mRNA-stabilizing) mechanisms. Ndiaye et al[31] reported that RW polyphenolic compounds induced the redox-sensitive activation of the PI3-kinase/Akt pathway in endothelial cells, which, in turn, caused phosphorylation of eNOS and resulted in an increased formation of NO. Recent evidence suggested that mobilization and differentiation of EPC are modified by NO, and eNOS expressed by bone marrow is essential for the mobilization of stem and progenitor cells.[32] In addition, endogenous NOS inhibitors, such as asymmetrical dimethylarginine, were shown to suppress EPC differentiation and function and contributed to impaired endothelial function.[33] Additionally, low-density lipoprotein oxidation is a key event in atherogenesis, and previous studies indicated that consumption of RW or its polyphenols was associated with reduced low-density lipoprotein oxidation, low-density lipoprotein aggregation, and decreased foam cell formation.[34] However, in this study, there was no significant change in oxidized low-
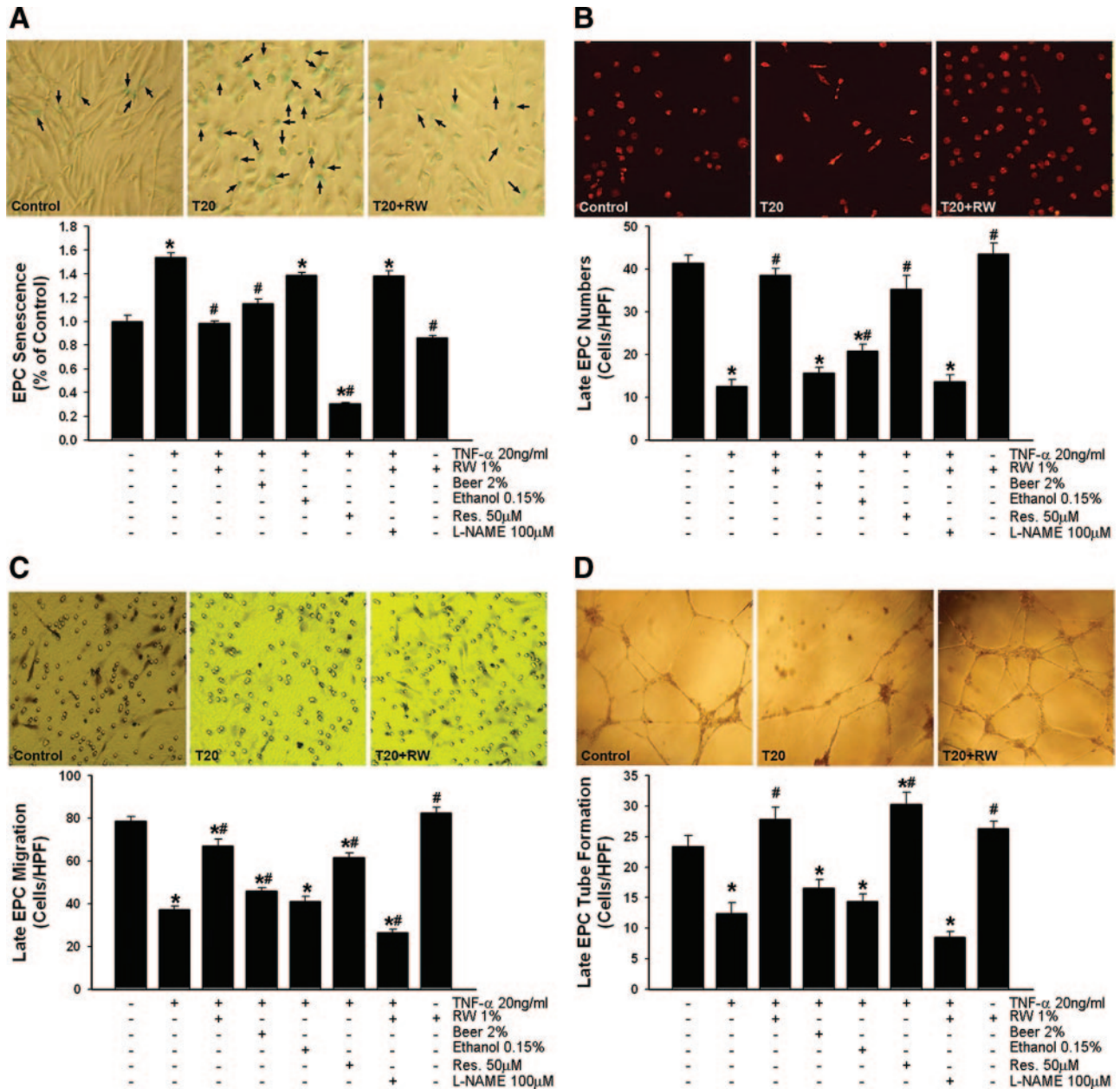
Figure 6. RW attenuated senescence and improved adhesion, migration, and tube formation of EPC in vitro. Acidic β-galactosidase was detected as a biochemical marker for acidification typical of EPC senescence (A). Fibronectin adhesion assay was used to evaluate the effect of RW on EPC adhesive function. Phenotyping of endothelial characteristics of adherent cells by indirect immunostaining was performed with Dil-acLDL and BS-1 lectin (B). A modified Boyden chamber assay using vascular endothelial growth factor as a chemoattractic factor was performed to assess the effect of RW on EPC migration. The migrated cells were stained with hematoxylin and counted under a microscope (C). An in vitro angiogenesis assay for late EPC was used with ECMatrix gel. Representative photos for in vitro angiogenesis are shown. The cells were stained with crystal violet, and the averages of the total area of complete tubes formed by cells were compared by computer software (D). Data are means±SEM; n=4 in each experiment. *$P<0.05$ compared with control. #$P<0.05$ compared with TNF-α 20 ng/mL.

density lipoproteins levels in response to the 3-week intake of RW. The current results may be explained by the short intervention period and the small amount of RW consumed.

Conclusions

This study clearly demonstrated that a low-dose intake of RW can significantly enhance circulating levels of EPC in healthy young subjects by increasing NO bioavailability. These

findings may explain the underlying mechanism responsible for the beneficial effects of RW ingestion on the cardiovascular system.

Disclosures

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Supplement material

Intake of red wine increases the number and functional capacity of circulating endothelial progenitor cells by enhancing nitric oxide bioavailability

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METHODS

Human study protocol

The human study was a prospective, randomized, diet-controlled, open-label trial, in which eighty young healthy subjects were enrolled. Exclusion criteria included a history of smoking, hypertension, diabetes mellitus, and regular alcohol consumption (drinking more than 20 g of ethanol per week). In addition, subjects receiving any regular medications or antioxidant supplements were also excluded. All subjects were asked not to change their lifestyle and were prohibited from consuming grape juice, tea, or other alcoholic beverages during the study period. All subjects were randomized into a group that consumed 100 ml/day of pure water (n=20), 100 ml/day of RW (n=20), 250 ml/day of beer (n=20), or a group that consumed 30 ml/day of vodka (n=20). The daily dose of alcohol was equivalent in the RW, beer and vodka groups.

The RW used in this study was "Réserve Maison Nicolas" (12.5% ethanol), a cabernet sauvignon from Languedoc-Roussillon, France, that contains a relatively high concentration of resveratrol.[1] The beer used was Taiwan Beer (5% alcohol) from Taiwan, and the vodka used was Smirnoff vodka from Russia (37.5% ethanol). The study design included a 2-week run-in period, during which all subjects abstained from alcohol and all medications. To ensure subject's compliance with the protocol, the dietary instructions were reinforced during weekly contact (by telephone) with the subjects throughout the study period. All patients gave informed consent, and the study was approved by the local research ethics committee (VGHIRB: 96-01-11A). The study protocol was consistent with the ethical guidelines provided in the 1975 Helsinki Declaration.

Endothelium-dependent flow-mediated vasodilation (FMD)

Endothelium-dependent FMD was assessed using a 7.5-MHz linear array transducer (Hewlett-Packard Sonos 5500, Andover, MA, USA) to scan the brachial artery in longitudinal section.[2] Briefly, a baseline image was acquired, and blood flow was estimated by time-averaging the pulsed Doppler velocity signals obtained from a midartery sample volume. The cuff was inflated to at least 50 mmHg above systolic pressure to occlude the brachial artery for 5

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minutes and released abruptly. A mid-artery pulsed Doppler signal was obtained immediately upon cuff release and no later than 15 seconds after cuff deflation to assess hyperemic velocity. Post-occlusion diameters were obtained at 60, 100 and 120 seconds after deflation. Endothelium-dependent FMD was calculated as the maximal post-occlusion diameter relative to the averaged pre-occlusion diameter.

Laboratory measurements

After 8 hours of overnight fasting, all subjects had a venous blood sample taken for measurement of high-sensitive C-reactive protein (hsCRP), tumor necrosis factor-α (TNF-α), NO, asymmetric dimethylarginine (ADMA), adiponectin, and oxidized low-density lipoproteins (ox-LDL). The blood samples were centrifuged at 3000 rpm for 10 minutes immediately after collection, and the plasma samples were kept frozen at -70°C until analysis. Determination of hsCRP level was performed with use of a latex-enhanced immunophelometric assay (Dade Behring, Marburg, Germany). Plasma TNF-α level was determined by a quantitative sandwich enzyme immunoassay technique with a Quantikine TNF-α HS immunoassay kit (R&D Systems, Inc., Minneapolis, USA). NO was measured by chemiluminescence with a Sievers

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Nitric Oxide Analyzer, model 280 (Sievers, Boulder, CO, USA). Plasma ADMA level was determined by a quantitative sandwich enzyme immunoassay technique with an ADMA enzyme-linked immunosorbent assay kit (DLD DIAGNOSTIKA GMBH, Hamburg, Germany). The plasma concentration of adiponectin was measured using an enzyme-linked immunosorbent assay kit (Linco Research, Inc., St. Charles, MO, USA). Ox-LDL levels were assessed with the ox-LDL ELISA kit (Biomedica, San Diego, CA, USA). Each standard and plasma samples were analyzed twice, and the mean value was used in all subsequent analyses. The intra-assay and inter-assay variation coefficients of the tests were less than 10%.

Flow cytometry

A volume of 100 µL peripheral blood was incubated for 15 minutes in the dark with monoclonal antibodies against human KDR (R&D, Minneapolis, MN, USA) followed by PE-conjugated secondary antibody, with the FITC-labeled monoclonal antibodies against human CD45 (Becton Dickinson), with the PE-conjugated monoclonal antibody against human CD133 (Miltenyi Biotec, Germany), and with FITC- or PE-conjugated monoclonal antibodies against human CD34 (Serotec, Raleigh, NC, USA).[3] Isotype-identical antibodies

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served as controls (Becton Dickinson, Franklin Lakes, NJ, USA). After incubation, cells were lysed, washed with phosphate-buffered saline (PBS), and fixed in 2% paraformaldehyde before analysis. Each analysis included 100,000 events. To assess the reproducibility of EPC measurements, circulating EPCs were measured from 2 separate blood samples in 10 subjects, and there was a strong correlation between the two measurements ($r = 0.86$, $P < 0.001$).

Human early and late EPC cultivation

Peripheral blood samples (20 ml) were obtained from healthy young volunteers, and total mononuclear cells (MNCs) were isolated by density gradient centrifugation with Histopaque-1077 (Sigma, St. Louis, MO, USA).[4] Briefly, MNCs (5×10^6) were plated in 2 ml endothelial growth medium (EGM-2 MV Cambrex, East Rutherford, NJ, USA) on fibronectin-coated 6-well plates. After 4 days of culturing, the medium was changed and nonadherent cells were removed; attached early EPCs appeared elongated with a spindle shape. A certain number of MNCs were allowed to grow into colonies of late EPCs, which emerged 2-4 weeks after the start of the MNC culture. The late EPCs

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exhibited a "cobblestone" morphology and monolayer growth pattern typical of mature endothelial cells at confluence. Both early and late EPCs were collected and used for the functional assays in this study.

Human EPC colony-forming assay

Isolated MNCs were resuspended in growth medium (EndoCultTM liquid medium, StemCell Technologies, Vancouver, Canada), and 5×10^6 MNCs were preplated in a fibronectin-coated 6-well plate in duplicate.[5] After 48 hours, the nonadherent cells were collected by pipetting the medium in each well up and down 3 times, and 1×10^6 cells were replated onto a fibronectin-coated 24-well plate. Cultured cells were then treated with RW, beer or medications for 72 hours. On day 5 of the assay, the number of colony-forming units (CFUs) per well for each sample was counted. All colonies were counted manually in a minimum of 3 wells by two independent observers.

EPC number and proliferation assay

The number of early EPCs and the proliferation of late EPCs were determined by direct staining of 6 random high-power microscopic fields ($\times 100$) and by the

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3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyltetrazolium bromide (MTT) assay, respectively.[4] After culturing for 4 days with RW, beer or medications, the number of early EPCs was counted and phenotyping of endothelial characteristics of EPCs by indirect immunostaining was performed with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-acetylated low-density lipoprotein (Dil-acLDL, Molecular Probe) and FITC-labeled lectin from Ulex europaeus (UEA-1) (Sigma). After incubation with RW, beer or medications for 4 days, late EPCs were supplemented with MTT (0.5 mg/ml, Sigma) and incubated for 4 hours for the proliferation assay. The blue formazen was dissolved with dimethyl sulfoxide and measured at 550/650 nm.

EPC senescence assay

Cellular aging was determined with a Senescence Cell Staining kit (Sigma).[4] Cultured late EPCs were treated with RW, beer or medications for 4 days. After washing with PBS, late EPCs were fixed for 6 minutes in 2% formaldehyde and 0.2% glutaraldehyde in PBS, and then incubated for 12 hours at 37°C without CO_2 with fresh X-gal staining solution. After staining, green-stained cells and total cells were counted and the percentage of β-galactosidase-positive cells was calculated.

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EPC fibronectin adhesion assay

EPC fibronectin adhesion test was assessed as previously described.[5] Briefly, after centrifugation and resuspension in basal medium with 5% fetal bovine serum, late EPCs (1×10^4 cell per well) were placed on fibronectin-coated 6-well plates and incubated with RW, beer or medications for 30 minutes at 37 ºC. Gentle washing with PBS 3 times was performed after 30 minutes' adhesion, and adherent cells were counted by independent blinded investigators. Phenotyping of endothelial characteristics of adherent cells by indirect immunostaining was performed with Dil-acLDL and BS-1 lectin.

EPC migration assay

Cultured late EPCs were treated with RW, beer or medications for 4 days, and a modified Boyden chamber assay (Transwell, Coster) was used to evaluate the migratory function of EPCs.[6] Briefly, isolated late EPCs were detached as described above with trypsin/EDTA, and then 4×10^4 late EPCs were placed in the upper chambers of 24-well transwell plates with polycarbonate membrane (8-µm pores) that contained serum-free endothelial growth medium; VEGF (50 ng/ml) was added to medium placed in the lower chambers. After incubation

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for 24 hours, the membrane was washed briefly with PBS and fixed with 4% paraformaldeyde. The upper side of membrane was wiped gently with a cotton ball. The membrane was stained using hematoxylin solution and carefully removed. The magnitude of migration of late EPCs was evaluated by counting the migrated cells in six random high-power ($\times$100) microscopic fields.

EPC tube formation assay

Cultured late EPCs were treated with RW, beer or medications for 4 days, and an EPC tube formation assay was performed with an In Vitro Angiogenesis Assay Kit (Chemicon).[6] ECMatrix gel solution was thawed at 4°C overnight, mixed with ECMatrix diluent buffer, and placed in a 96-well plate at 37°C for 1 hour to allow the matrix solution to solidify. Late EPCs were harvested as described above with trypsin/EDTA, and then 1×10^4 EPCs were placed on a matrix solution with EGM-2 MV medium and incubated at 37°C for 16 hours. Tubule formation was inspected under an inverted light microscope ($\times$100). Six representative fields were taken, and the average of the total area of complete tubes formed by cells was compared by using computer software, Image-Pro Plus.

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Western blotting analysis and measurement of nitrite levels

Cultured late EPCs were treated with RW or ethanol for 4 days, and protein extracts were prepared as previously described.[7] Briefly, EPCs were lysed in a buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.5 mM PMSF, 2 µg/ml aprotinin, pepstatin, and leupeptin), and the protein lysates were subjected to SDS-PAGE, followed by electroblotting onto a PVDF membrane. Membranes were probed with monoclonal antibodies that directed to phosphorylated endothelial NO synthase (p-eNOS), eNOS, p-Akt, Akt (Cell Signaling), and α-tubulin (Chemicon). Bands were visualized by chemiluminescence detection reagents. Densitometric analysis was conducted with ImageQuant (Promega) software. After incubation of EPCs with RW or ethanol for 4 hours, the conditioned medium was measured for nitrite level by the Griess reagent [1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine in 2% phosphoric acid].

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Table 1. Baseline characteristics, hs-CRP and EPC levels of all subjects in the four groups

	Control (n=20)		RW (n=20)		Beer (n=20)		Vodka (n=20)	
	Before	After	After	Before	Before	After	Before	After
Age (years)	34±1		33±1		34±1		33±1	
Female	3 (15%)		6 (30%)		5 (25%)		3 (15%)	
Body mass index (kg/m^2)	24±1	24±1	25±1	24±1	25±1	25±1	24±1	24±1
Body weight (kg)	69±3	70±3	71±4	70±4	71±4	71±4	69±3	69±3
Waist circumference (cm)	34±3	35±3	33±1	32±1	33±1	33±1	35±3	35±3
Systolic blood pressure (mm Hg)	120±2	120±2	121±2	120±2	121±2	121±2	125±2	122±2
Diastolic blood pressure (mm Hg)	75±1	75±1	76±1	75±1	76±1	76±1	73±1	73±1
Heart rate (/min)	73±1	73±1	75±1	73±1	75±1	75±1	72±1	71±1
Lipid profile (mg/dl)								
Total cholesterol	185±6	185±6	183±7	185±6	183±7	183±7	179±8	181±7
Triglycerides	113±16	113±16	115±16	113±16	115±16	115±16	106±13	101±12
High-density lipoproteins	43±2	43±2	45±2	43±2	45±2	45±2	43±2	43±2
Low-density lipoproteins	117±5	117±5	114±6	117±5	114±6	114±6	115±6	118±6

Fasting serum glucose (mg/dl)	91±2	91±2	90±2	91±2	90±2	90±2	91±2	95±2
EPCs (%)								
KDR$^+$/CD133$^+$	0.3 ± 0.1	0.8 ± 0.1	0.2 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
CD133$^+$/CD34$^+$	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
CD34$^+$/KDR$^+$	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	0.8 ± 0.1	0.8 ± 0.2	0.7 ± 0.1	0.7 ± 0.1
hs-CRP (mg/l)	1.9 ± 0.2	2.0 ± 0.2	2.0 ± 0.3	2.0 ± 0.3	2.0 ± 0.2	2.1 ± 0.2	1.9 ± 0.2	2.0 ± 0.2

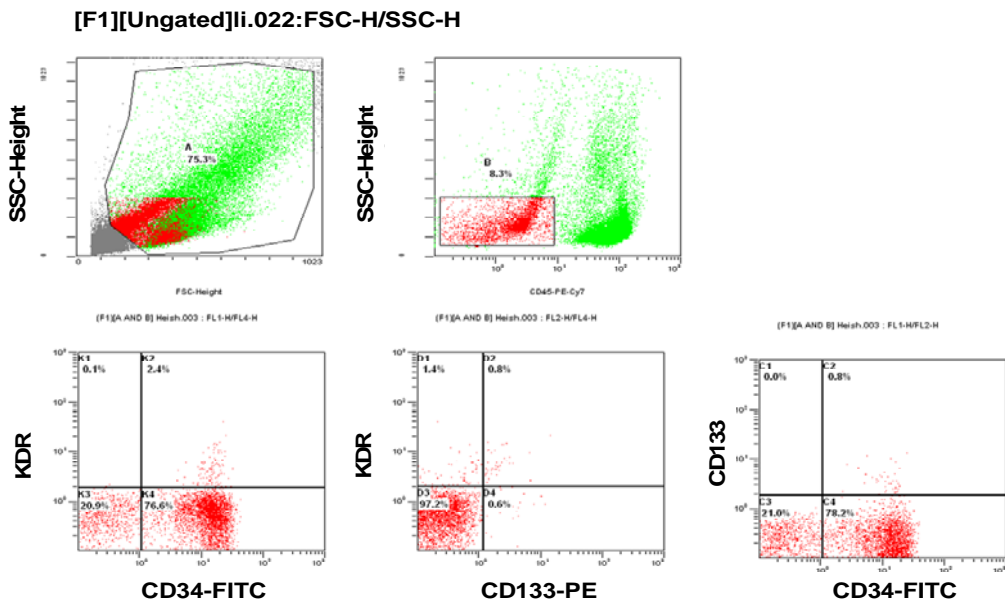RW: red wine; values are mean±SEM or number (%).

A
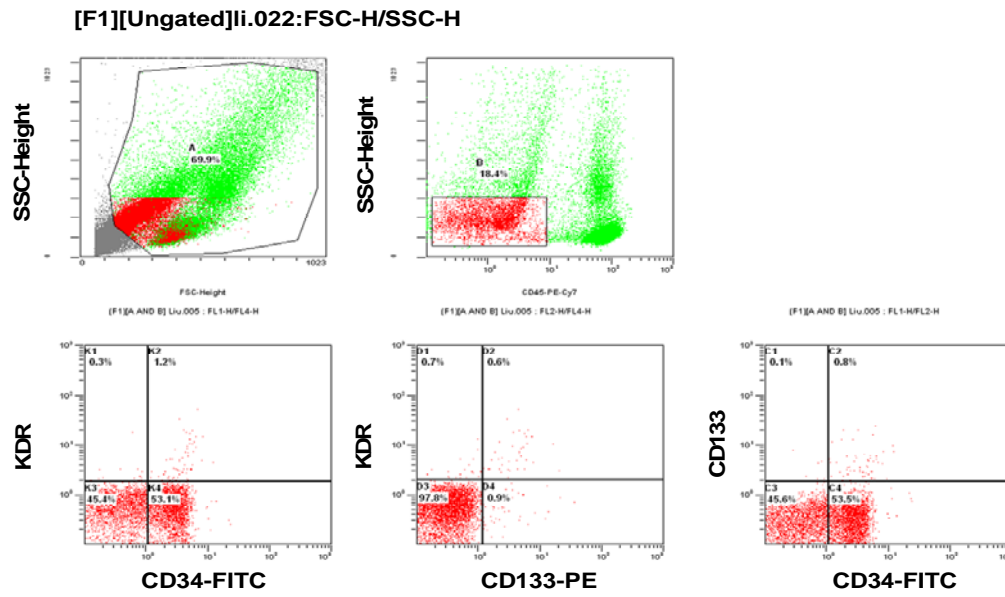
B

Figure I. Representative flow cytometry analysis for quantifying the number of circulating endothelial progenitor cells (EPCs). Mononuclear cells (MNCs) were gated by forward/sideward scatter (FSC/SSC), and the

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numbers of circulating EPCs were defined as CD34$^+$KDR$^+$CD45^{low}, CD34$^+$CD133$^+$CD45^{low}, and KDR$^+$CD133$^+$CD45^{low}, respectively. Circulating EPCs detected by FACS were presented before (A) and after (B) red wine intake.

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