Red wine extract protects against oxidative-stress-induced endothelial senescence


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A B S T R A C T

Red wine polyphenols may preserve endothelial function during aging. Endothelial cell senescence enhances age-related endothelial dysfunction. We investigated whether RWE (red wine extract) prevents oxidative-stress-induced senescence in HUVECs (human umbilical-vein endothelial cells). Senescence was induced by exposing HUVECs to tBHP (t-butylhydroperoxide), and quantified by senescence-associated β-galactosidase staining. RWE (0–50 μg/ml) concentration dependently decreased senescence by maximally 33 ± 7.1 %. RWE prevented the senescence-associated increase in p21 protein expression, inhibited tBHP-induced DNA damage of endothelial cells and induced relaxation of PCAs (porcine coronary arteries). Inhibition of SIRT1 (sirtuin 1) by sirtinol partially reversed the effect of RWE on tBHP-induced senescence, whereas both the NOS (nitric oxide synthase) inhibitor L-NMMA (L-Nmonomethyl-L-arginine) and the COX (cyclo-oxygenase) inhibitor indomethacin fully inhibited it. Furthermore, incubation of HUVECs with RWE increased eNOS (endothelial NOS) and COX-2 mRNA levels as well as phosphorylation of eNOS at Ser1177. RWE protects endothelial cells from tBHP-induced senescence. NO and COX-2, in addition to activation of SIRT1, play a critical role in the inhibition of senescence induction in human endothelial cells by RWE.

INTRODUCTION

Aging is an independent and important risk factor for CVD (cardiovascular disease) [1]. Senescence of the vascular wall may be on the causal path to age-related endothelial dysfunction and atherogenesis, as it stimulates inflammation, raises BP (blood pressure) and promotes thrombosis [2,3]. DNA damage through excessive production of ROS (reactive oxygen species) is an important mechanism underlying endothelial senescence [4,5]. Red wine polyphenols may preserve endothelial function during aging. PPAR-γ is involved in the development of cell senescence induced by oxidative stress through interaction with the senescence promoter p53, leading to decreased expression of PPAR-γ, and increased expression of senescence proteins p21 and p16INK4a [6]. The role of PPAR-γ in the inhibition of senescence by RWE is unknown. In the current study, we investigated whether RWE (red wine extract) prevents oxidative-stress-induced senescence in HUVECs (human umbilical-vein endothelial cells). Senescence was induced by exposing HUVECs to tBHP (t-butylhydroperoxide), and quantified by senescence-associated β-galactosidase staining. RWE (0–50 μg/ml) concentration dependently decreased senescence by maximally 33 ± 7.1 %. RWE prevented the senescence-associated increase in p21 protein expression, inhibited tBHP-induced DNA damage of endothelial cells and induced relaxation of PCAs (porcine coronary arteries). Inhibition of SIRT1 (sirtuin 1) by sirtinol partially reversed the effect of RWE on tBHP-induced senescence, whereas both the NOS (nitric oxide synthase) inhibitor L-NMMA (L-Nmonomethyl-L-arginine) and the COX (cyclo-oxygenase) inhibitor indomethacin fully inhibited it. Furthermore, incubation of HUVECs with RWE increased eNOS (endothelial NOS) and COX-2 mRNA levels as well as phosphorylation of eNOS at Ser1177. RWE protects endothelial cells from tBHP-induced senescence. NO and COX-2, in addition to activation of SIRT1, play a critical role in the inhibition of senescence induction in human endothelial cells by RWE.

Key words: cellular senescence, endothelium, nitric oxide, prostaglandin, reactive oxygen species, red wine polyphenol.
Abbreviations: BAEC, bovine aortic endothelial cell; COX, cyclo-oxygenase; CVD, cardiovascular disease; DMEM; Dulbecco’s modified Eagle’s medium; HBSS, Hanks balanced salt solution; HUVEC, human umbilical-vein endothelial cell; t-NMMA, N(G)-monomethyl-L-arginine; t-NAME, N(G)-nitro-L-arginine methyl ester; NOS, NO synthase; eNOS, endothelial NOS; COX, cyclo-oxygenase; PGE2, prostaglandin E2; PGI2, prostacyclin; ROS, reactive oxygen species; RWE, red wine extract; SA-β-gal, senescence-associated β-galactosidase; SIRT1, sirtuin 1; tBHP, t-butylhydroperoxide; TXA2, thromboxane A2.
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Moderate consumption of red wine has for a long time been postulated to be part of a healthy lifestyle [5]. Under controlled conditions in animal studies, RWE (red wine extract) prevented age-induced endothelial dysfunction [6]. We and others have shown that RWE elicits the release of endothelium-derived vasodilating factors and activation of SIRT1 (sirtuin 1), a versatile deacetylase that has been implicated in endothelial cell aging [7–12]. Recently, we have also shown that the release of NO, induced by the vasodilator peptide hormone bradykinin protects against ROS-induced endothelial cell senescence [13]. We hypothesized that RWE protects against ROS-induced endothelial cell senescence and that this is due to the release of vasodilator signalling factors and SIRT1 activation.

In the present study, we investigated how RWE reduces ROS-induced endothelial cell senescence. Although only present in very small amounts, we also explored a possible role for the most studied red wine polyphenol resveratrol, which was also implicated in SIRT1-mediated protection against endothelial senescence [12,14].

MATERIALS AND METHODS

Composition of RWE
The alcohol-free RWE used is Provinols (Seppic). This RWE is derived from red wine produced in the Languedoc–Roussillon region in the south-east of France. RWE contained 632 mg of polyphenols/g, determined as gallic acid equivalents using Folin–Ciocalteu reagent. The specific polyphenol contents in the RWE were assessed by HPLC analysis and this revealed that 550 mg of wine solids contain 18.8 mg of anthocyanins, 6.9 mg of phenolic acids, 4.0 mg of catechins, 0.4 mg of flavonols and 0.1 mg of stilbenes.

Cell culture studies
HUVECs (human umbilical vein endothelial cells) were isolated by collagenase digestion as described by Jaffe et al. [15]. HUVECs were cultured on 0.2 % gelatin-coated plates in HUVEC culture medium containing human endothelial serum-free medium and DMEM (Dulbecco’s modified Eagle’s medium; Invitrogen), 10 % heat-inactivated newborn bovine serum, 5 % heat-inactivated human serum (Lonza), 10 ng/ml human recombinant bFGF (basic fibroblast growth factor) and 50 ng/ml human recombinant EGF (epidermal growth factor; Peprotech) in a humidified incubator at 37 °C and 5 % CO2. Experiments were conducted on cells with a passage number between 3 and 9.

Primary BAECs (bovine aortic endothelial cells) were cultured in DMEM (Invitrogen), supplemented with 10 % fetal bovine serum (Thermo Scientific) in a humidified incubator at 37 °C and 5 % CO2. Experiments were conducted on cells with a passage number between 5 and 9.

Design of the pharmacological studies
HUVECs were seeded at a density of 5000 cells/cm². After 24 h, cells were starved with DMEM + 0.5 % fetal bovine serum for at least 6 h. Next, the medium was replaced by HUVEC culture medium with or without RWE (3.125–50 μg/ml) in the presence or absence of the COX (cyclo-oxygenase) inhibitor indomethacin, the NOS (NO synthase) inhibitor l-2-NMMA (Nα-monomethyl-l-arginine) salt (Sigma–Aldrich) or the SIRT1 inhibitor sirtinol (Calbiochem). After 1 h, rBHP (t-butyldihydroperoxide; Sigma–Aldrich) was added to the medium for 2 h to induce senescence. Subsequently, the medium was replaced with HUVEC culture medium with or without RWE, indomethacin, l-2-NMMA or sirtinol.

Apoptosis
Apoptosis was determined with the Caspase-Glo 3/7 Assay (Promega) 18 h after treatment. In 96-well plates, a 50 μl sample of supernatant was mixed gently for 30 s with 50 μl of Caspase-Glo 3/7 reagent and incubated for 2 h at room temperature (20 °C). Caspase 3 activity was determined by luminescence of the samples measured using a Victor Wallac Multilabel Counter 1420.

ROS production
DCF (2′,7′-dichlorofluorescein), a fluorescent dye, was used to assess endothelial ROS production. BAECs were cultured in a 96-well plate. At least 8 h before the measurements, medium was replaced by DMEM without serum. Subsequently, the medium was replaced by HBSS (Hanks balanced salt solution) with or without RWE (25 μg/ml). After 30 min, cells were loaded with CM-H2DCFDA (Invitrogen) at a concentration of 10 μmol/l at 37 °C in the dark for 30 min. Next, the medium was replaced by HBSS and rBHP (55 μmol/l) was added. Fluorescence (λexcitation, 488 nm; λemission, 515–530 nm) was measured after 1 h using a fluorescence plate reader (Wallac Victor2; PerkinElmer).

Evaluation of the number of senescent cells
When cells reached confluency, they were fixed in 2 % formaldehyde/0.2 % glutaraldehyde for 10 min and the number of senescent cells was determined by SA-β-gal (senescence-associated β-galactosidase) staining [150 mmol/l NaCl, 2 mmol/l MgCl2, 5 mmol/l K3Fe(CN)6, 5 mmol/l K4[Fe(CN)6], 140 mmol/l Na2HPO4, 40 mmol/l citric acid and 1 mg/ml 5-bromo-4-chloroindol-3-yl β-d-galactoside, pH 6.0 for 18 h at 37 °C] [16]. Cells were counterstained with DAPI.
(4′,6-diamidino-2-phenylindole; 2 μg/ml) to allow total cell number counting. Microscopic pictures were taken on an inverted microscope (Zeiss Axiovert 200M) and the absolute number of senescent cells and the total number of cells were counted per microscopic field by ImageJ software. In each well, four random fields were evaluated.

**DNA damage assay**

DNA damage was determined by single nuclei electrophoresis, also called comet assays [17]. Cells were harvested, and approximately 700 cells were placed on a Trevigen Cometslide in 0.7% low-melting-point agarose (Serva). Cells were lysed for 1 h in Trevigen lysis solution, followed by 30 min of denaturation by 300 mmol/l alkaline solution and 1 mmol/l EDTA at pH>13, which was followed by 30 min of electrophoresis at 1 V/cm in 300 mmol/l alkaline solution and 1 mmol/l EDTA at pH>13. DNA was stained with SYBR Green (Invitrogen) and photos were taken with a Zeiss Axiovert 200M. Olive tail moment (percentage at 1 V/cm in 300 mmol/l alkaline solution and 1 mmol/l EDTA at pH>13, which was followed by 30 min of electrophoresis at 1 V/cm in 300 mmol/l alkaline solution and 1 mmol/l EDTA at pH>13. DNA was stained with SYBR Green (Invitrogen) and photos were taken with a ×10 objective (Zeiss Axiovert 200M). Olive tail moment (percentage of DNA in the tail×distance to the centre of gravity of tail) was determined with CASP 1.2.2 software [18]. Experiments were repeated three times and per experiment more than 100 comets were analysed per treatment group.

**Western blot analyses**

Cultured cells were lysed with 50 mmol/l Tris/HCl, pH 7.4, 150 mmol/l NaCl, 10 mmol/l Igepal CA-630, 5 mmol/l deoxycholic acid and 1 mmol/l SDS, in the presence of protease inhibitor cocktail (Roche) and serine–threonine phosphatase inhibitor cocktail 3 (Sigma–Aldrich). Lysates were analysed by standard Western blotting techniques under denaturing conditions. The following antibodies were used: anti-p21 (12D1; Cell Signalling), anti-p53 (DO-1; Sigma–Aldrich), anti-(acetylated p53) (Lys382) (Cell Signaling), anti-eNOS (endothelial NOS; C-20, Santa Cruz Biotechnology) and anti-p-eNOS (phospho-eNOS) (Ser1177) (Santa Cruz Biotechnology). Anti-actin (C4, Millipore) was used for normalization of the protein levels. Signals were detected by the enhanced chemiluminescence detection method and were quantified by densitometry. Ratios of acetylated or phosphorylated proteins against total proteins were calculated as (acetylated or phosphorylated protein/actin)/(total protein/actin) for each separate experiment.

**Real-time quantitative RT–PCR (reverse transcription–PCR)**

Total RNA isolation was performed with the NucleoSpin RNA II kit (Machery-Nagel). RNA was reverse-transcribed by use of the Quantitect Rev. Transcription Kit (Qiagen). cDNA (4 ng) was amplified by real-time PCR (qPCR) and normalized to 36B4 as an endogenous control. Each reaction was performed in duplicate with SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used: SIRT1 forward, 5′-AGGCCACGGATAGGTCCATAT-3′ and reverse, 5′-CCATCATAAGAATGTTGCTGAAC-3′; eNOS forward, 5′-CTTCCGCTACACAGCACAGC-3′ and reverse, 5′-TCTCGGAGGCCATAAGATT-3′; COX-2 forward, 5′-CCAGGACTCTCAGCATCAG-3′ and reverse, 5′-AGACCAGGCACACGACAAAGACC-3′.

**PGE₂ (prostaglandin E₂) and PGI₂ (prostacyclin) release**

HUVECs were cultured in 12- or 24-well plates without serum as described above. Next, RWE (25 μg/ml) or IL-1β (interleukin-1β) (1 ng/ml; positive control) was added to the medium for 6 h. Subsequently, the medium was collected and stored at −80°C until the analysis of PGE₂ (KGE00B; R&D Research) and PGI₂ (ADI-900-025; Enzo Life Sciences) by ELISA.

**Vascular reactivity studies**

Experiments were set up as described previously [19]. Briefly, PCAs (porcine coronary arteries) were obtained from eight slaughterhouse pigs. The arteries were cut into segments and suspended in 15-ml organ baths containing Krebs bicarbonate solution, aerated with 95 % O2/5 % CO₂ at 37 °C. The vessel segments were exposed to 30 mmol/l KCl twice, and subsequently to 100 mmol/l KCl, to determine the maximal contractile response. The segments were then incubated for 30 min in the absence or presence of one or more of the following compounds: the NOS inhibitor l-NAME (N⁵-nitro-L-arginine methyl ester; 10 μmol/l), the intermediate- and small-conductance K⁺ channels inhibitors TRAM34 (10 μmol/l) and apamin (100 nmol/l) or indomethacin (10 μmol/l). Vessels were then pre-constricted with U46619 (9,11-dideoxy-11α,9α-epoxy- methanoprostaglandin F₂₀; 0.1–1 μmol/l) to ≈80 % of the maximal constriction, and RWE concentration–response curves were constructed. Apart from RWE, all compounds were from Sigma–Aldrich.

**Statistical analysis**

Values are presented as means ± S.E.M. Differences between the groups of the cell-culture experiments, in which treatments were always performed in parallel, were analysed using two-tailed paired Student t tests or one-way ANOVA with correction for multiple comparisons, unless indicated otherwise. Differences between groups in vascular reactivity studies were analysed using the general linear model for repeated measures. Probability values of less than 0.05 (or corrected after post-hoc tests) were considered significant.
RESULTS

RWE and oxidative-stress-induced endothelial senescence

To investigate the effect of RWE on endothelial oxidative-stress-induced senescence, we exposed HUVECs to 55 μmol/l tBHP for 2 h. To ensure that apoptosis did not bias the results, we measured caspase 3 activity (an indicator of apoptosis) after tBHP exposure. Caspase 3 increased at tBHP concentrations above 55 μmol/l (Figure 1A), and significance for this effect was reached at 100 μmol/l. Therefore we performed all further experiments with 55 μmol/l tBHP.

Exposure of BAECs to 55 μmol/l tBHP increased ROS production almost 1.5-fold (P < 0.05, n = 4), and RWE prevented this effect (Figure 1B). Exposure of HUVECs to 55 μmol/l tBHP increased the percentage of senescent cells 3.8-fold (from 4.8 ± 1.2 % to 18 ± 2.1 %, P < 0.05, n = 6). Treatment with RWE concentration-dependently decreased endothelial senescence by maximally 33 ± 7.1 % (Figures 1C and 1D). Removing RWE immediately before the application of tBHP yielded a similar effect (n = 5; results not shown). To exclude the possibility that the effect of RWE on the percentage of senescent cells was due to an increase in cell proliferation, we counted the absolute number of cells. RWE had no effect on the total number of cells, but decreased the absolute number of senescent cells from 26 ± 4.5 cells/microscopical field without RWE to respectively 20 ± 4.1 and 15 ± 2.5 cells/microscopical field for 25 and 50 μg/ml RWE (P < 0.05, linear regression for trend, n = 6).

tBHP-induced DNA damage, as shown by the increased olive tail moment of the comet assay (Figure 2A), and up-regulated p21 protein expression (Figure 2B), and RWE clearly reduced these effects. We did not find an effect of RWE on cells not exposed to tBHP.

RWE and oxidative-stress-induced endothelial senescence: role of SIRT1

RWE increased SIRT1 gene expression (by 14 ± 10 % at 10 min of exposure, n = 8, P < 0.05; results not shown). However, the SIRT1 activator resveratrol did not prevent tBHP-induced senescence (Figure 3A), although it did cause the typical morphological elongations described previously [20]. In fact, resveratrol in a concentration of 50 μmol/l even increased senescence (P < 0.05). The SIRT1 inhibitor sirtinol, like tBHP, increased the number of senescent cells, although their effects were not additive (Figure 3B). Sirtinol, at least partly, reversed the effect of RWE on tBHP-induced senescence. This indicates that...
Figure 2  Effect of RWE on DNA damage
(A) Effect of RWE on DNA damage induced by tBHP, as assessed by comet assay. Top panel shows examples of single cell electrophoresis 24 h after no treatment (control), 25 μg/ml RWE, 55 μmol/l tBHP or 55 μmol/l tBHP + 25 μg/ml RWE, with quantification in the bottom panel (*P < 0.05 compared with control; #P < 0.05 compared with tBHP; n = 3). (B) Expression of p21 protein levels 24 h after no treatment (control), 25 μg/ml RWE, 55 μmol/l tBHP or 55 μmol/l tBHP + 25 μg/ml RWE, as measured by Western blotting. p21 protein levels were corrected for actin protein levels, with quantification in the bottom panel (*P < 0.05 compared with control; #P < 0.05 compared with tBHP; n = 6–7).

Figure 3  Interaction of RWE, resveratrol and SIRT1
(A) The effect of resveratrol (50 μmol/l) on tBHP-induced senescence in HUVECs according to SA-β-gal staining at 48 h after addition of tBHP. Percentage SA-β-gal positive cells are expressed relative to 55 μmol/l tBHP group (*P < 0.05 compared with tBHP; n = 3). (B) The effect of sirtinol (60 μmol/l) and RWE (25 μg/ml) on tBHP-induced senescence in HUVECs according to SA-β-gal staining at 48 h after the addition of tBHP. The percentage of SA-β-gal positive cells are expressed relative to 55 μmol/l tBHP group (*P < 0.05 compared with tBHP; #P < 0.05 compared with control; n = 5–6). (C, D) Levels of p53 acetylation (C) and total p53 (D) protein levels 24 h after either no treatment, 60 μmol/l sirtinol, 60 μmol/l sirtinol + 25 μg/ml RWE or 25 μg/ml RWE, as measured by Western blotting. Both acetylated and total p53 levels were corrected for actin levels (*P < 0.05 compared with control; #P < 0.05 compared with sirtinol; n = 4–8).
SIRT1 activation by factors other than resveratrol may underline the protective effect of RWE.

Sirtinol increased the levels of acetylated p53 (Figure 3C), and RWE fully reversed this effect. This finding supports the concept that SIRT1 decreases senescence through p53 acetylation. Yet sirtinol decreased total p53 by \( \approx 50\% \) (\( P < 0.05 \); Figure 3D), and RWE did not alter this decrease. As a consequence, the ratio of acetylated/total p53 increased after sirtinol (from 2.8 \pm 0.6 to 9.7 \pm 2.0; \( P < 0.05 \)). RWE reversed this increase [to 5.2 \pm 1.6; \( P = \text{NS} \) (not significant) compared with control].

**DISCUSSION**

Our present results show that RWE protects endothelial cells from \( \text{tBHP} \)-induced oxidative senescence. The protective effect of RWE was associated with a decrease in p21, which is a DNA-damage-related cyclin-dependent
kinase inhibitor. Consistent with these findings, RWE protected endothelial cells from DNA damage. This protective effect appeared to be dependent on eNOS, COX-2 and SIRT1.

Our data provide first evidence that RWE is able to decrease the number of senescent cells and to reduce DNA damage in endothelium. Although to the best of our knowledge no other study has investigated the effect of RWE on endothelial senescence before, RWE intake by old rats was found to protect against deterioration of endothelium-dependent relaxations [6]. Since endothelial cellular senescence leads to diminished release of vasodilator substances, we here suggest a novel protective effect of RWE that might be closely associated with this previous finding in aged rats.

On the basis of studies with the red wine product resveratrol [12,14], we expected that the protective pathway would involve SIRT1 activation. Overexpression of SIRT1 antagonizes cellular senescence through deacetylation of the DNA-damage-related cell cycle regulator p53 [21] and by promoting eNOS activity [22]. Moreover, resveratrol-containing red wine decreases the levels of asymmetric dimethylarginine, an endogenous inhibitor of NO, in a SIRT1-dependent manner [23]. In line with these observations, we observed that the SIRT1 inhibitor sirtinol increased p53 acetylation and endothelial senescence. In addition, RWE modestly up-regulated SIRT1 expression, and sirtinol, at least partially, reversed the effect of RWE on tBHP-induced senescence. Finally, RWE counteracted the effect of sirtinol on acetylated p53. Yet resveratrol increased tBHP-induced senescence. The latter observation is complementary to recent studies [24,25] disputing the claim that resveratrol activates SIRT1 and thereby increases longevity [10,26]. Taken together, our data indicate that SIRT1 activation by RWE does act protectively, but is not induced by resveratrol. In fact, the actual resveratrol content of our RWE preparation (when applied at a concentration of 25 μg/ml) is estimated to result in a medium concentration of ≈0.2 μmol/l (analysed by Nutrinov Lab), i.e., far below the resveratrol concentrations (10–50 μmol/l) that have been claimed to exert protective effects in vitro [14].

The protective effect of red wine against endothelial dysfunction was shown to be dependent on ROS scavenging [27]. This may also explain the effect on tBHP-induced senescence in our experiments. Indeed, RWE diminished ROS induced by tBHP, and protective effects were even observed when removing RWE shortly prior to the addition of tBHP. Clearly therefore these effects do not depend on direct ROS scavenging by RWE, but rather on ROS inactivation by a secondary factor induced by RWE, possibly NO. In support of the latter, red wine up-regulates eNOS (mRNA and protein) expression [28] and NO production [9] in endothelial cells. Consistent with these findings, we found that RWE increased eNOS mRNA levels and augmented eNOS phosphorylation in HUVECs. Moreover, RWE relaxed PCAs largely in an NO-dependent manner. Importantly, NOS inhibition reversed the senescence-inhibitory effect of RWE. Thus the RWE-induced protection against senescence indeed depends on functional eNOS.

Figure 5  Role of endothelial vasodilating factors in RWE-induced relaxation
Concentration–response curves of U46619-preconstricted PCAs to cumulative doses of RWE, in the absence (A) and presence (B) of 10 μmol/l TRAM34 in combination with 100 nmol/l apamin (TRAM34 + apamin), with or without 100 μmol/l L-NAME and 10 μmol/l indomethacin. Results are expressed as percentage of the contraction induced by U46619 ( * P < 0.05 compared with control, # P < 0.05 compared with control without TRAM34 + apamin; n = 6–8).
Alternatively, given the diminishing production of PGI2 during in vitro aging of endothelial cells, a decreased production of (protective) prostaglandins might underlie senescence [29]. In line with this observation, the non-selective COX inhibitor indomethacin, similar to NOS inhibition, reversed the senescence-inhibitory effect of RWE. In addition, RWE up-regulated COX-2, in full agreement with previous results in rats treated with red wine polyphenols [30]. However, RWE did not increase endothelial PGI2 production, and even tended to decrease the production of PGE2. Furthermore, indomethacin did not block the relaxant effects of RWE in PCAs, which argues against the possibility that RWE stimulates the production of vasodilator PGs like PGE2 and PGI2. Thus, if COX-2-mediated PG production protects against senescence, this does not involve PGE2 and PGI2. It might involve TXA2 (thromboxane A2), since Diebolt et al. [30] demonstrated that red wine polyphenols increase endothelial, COX-2-dependent TXA2 release. In fact, the latter study proposed that the NO/TXA2 balance underlies the beneficial effects of red wine polyphenols. Other possible candidates include PGD2, PGJ2 and EFOX (electrophile oxo-derivative) molecules. The latter are of particular interest since they regulate Nrf2 (nuclear factor erythroid-2 p45 subunit-related factor 2), which confers protection against oxidative stress [31].

Because of the complex composition of RWE, it is difficult to determine the RWE bio-availability in vivo. Using the Fioli–Ciocalteau method, Duthie et al. [32] found that intake of 100 ml of red wine by healthy volunteers increased the plasma concentration of polyphenolic monomers by ≈2–3 μg/ml. Since RWE contains 632 mg of polyphenols/g, the RWE concentrations of 25 μg/ml we used in most experiments will yield polyphenol concentrations in the range expected in blood after drinking 336 ml (about two glasses) of red wine. Given that resveratrol is unlikely to be the protective constituent of our RWE extract, the question remains what is/are the responsible candidate(s). Identification of the specific RWE constituents that protect endothelial cells is important, because wine consumption and RWE may have large variability in composition. Different red wines showed different effects on vascular function [33]. Therefore rational use of RWE protective effects can only be established by isolation of the specific relevant constituent(s). Since RWE contains at least 200 different polyphenols [34], and because it may not be a single constituent that confers the protective effect, such a search demands high-throughput screening systems. Our present study suggests that such screening assays could use eNOS and COX-2 activation as read-out variables.

In summary, we have shown that RWE inhibits oxidative-stress-induced endothelial senescence, and that activation of eNOS and COX-2, in addition to SIRT1, plays a critical role in the inhibition of a senescent phenotype in human endothelial cells. Our results indicate that RWE could exhibit a beneficial effect on the vasculature by protecting endothelial cells against senescence. Identification of the responsible components and testing them in clinical trials may provide novel therapeutic opportunities to counteract oxidative stress and age-associated CVD.

**AUTHOR CONTRIBUTION**

Ilse Botden, Hisko Oeseburg, Janneke Langendonk, Eric Sijbrands, Jan Danser and Anton Roks conceived and designed the research; Ilse Botden, Hisko Oeseburg, Matej Durik, Frank Leijten, Leonie van Vark-van der Zee, Usha Musterd-Bhaggoe, Ingrid Garrelds, and Ann Seynhaeve acquired the data; Ilse Botden, Hisko Oeseburg, Matej Durik and Anton Roks analysed and interpreted the data; Ilse Botden performed the statistical analysis; Ilse Botden, Hisko Oeseburg and Anton Roks drafted the paper; Ann Seynhaeve, Janneke Langendonk, Eric Sijbrands, Jan Danser and Anton Roks made critical revision of the paper for important intellectual content.

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