Red Wine Polyphenols and Vascular Function

Ilse P.G. Botden

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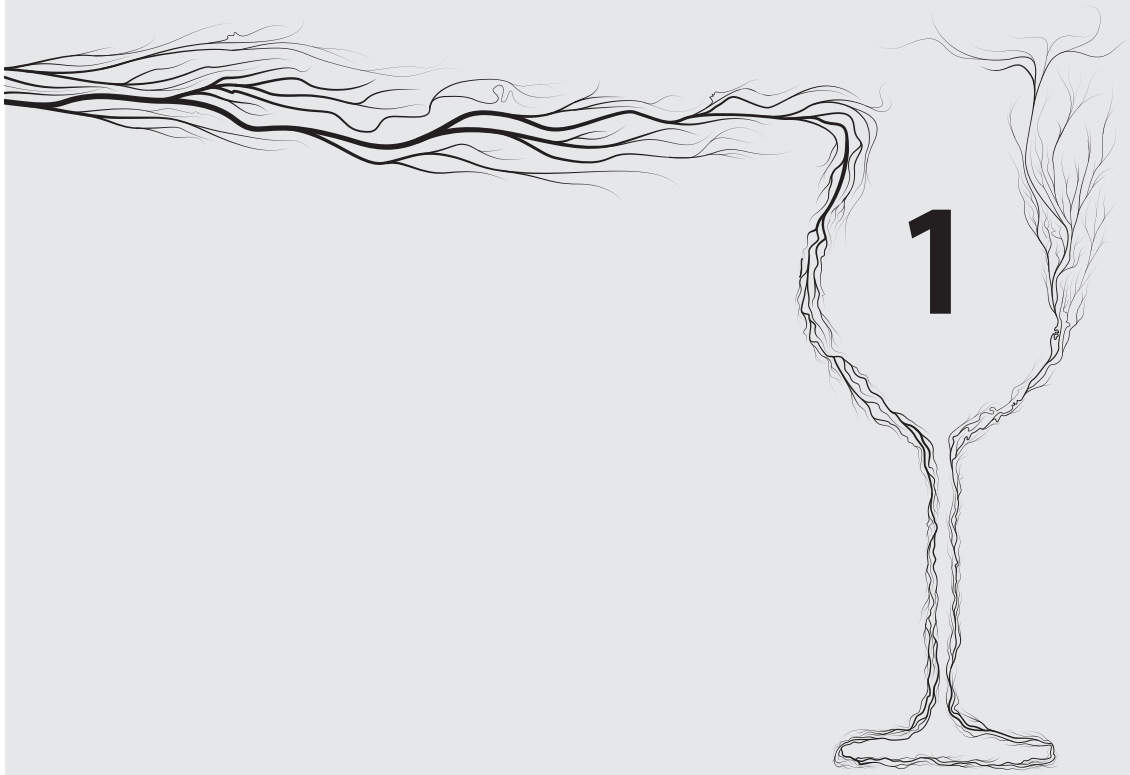

INTRODUCTION

Hippocrates, the father of modern medicine, said many centuries ago: *"let food be your medicine."* Today, this quote still shows its value, amongst others by the finding that red wine consumption attributes to a healthy life style, reducing the risk to develop cardiovascular diseases. Cardiovascular diseases are one of the leading causes of death in many economically developed countries as well as in emerging economies.[1] It has become a global epidemic problem, with type 2 diabetes, hypertension and, obviously, aging as one of the major risk factors. Red wine might interfere with one or more of these factors, and thus contribute in the prevention of cardiovascular diseases.

Aging

Aging is an independent and important risk factor for cardiovascular disease.[2] Red wine might influence this risk factor, since one of its polyphenols, resveratrol, showed to increase life span in mice.[3] Cellular aging (cellular senescence) of the vascular wall may be on the causal paths to age-related endothelial dysfunction and atherogenesis, as it stimulates inflammation, decreases vasodilator function and promotes thrombosis.[4, 5] Cellular senescence is a non-proliferative state, characterized by cell cycle arrest in the G0 or G1 phase, and loss of accurate cellular function. Senescent endothelial cells are characterized by nuclear abnormalities, and positive staining for senescence-associated β-galactosidase activity at pH 6.0.[6] DNA damage through excessive production of reactive oxygen species is an important mechanism underlying endothelial senescence.[7] Currently, a large number of studies is ongoing that address the question if diet or pharmacological intervention can inhibit this process.

Hypertension

Hypertension is a highly prevalent risk factor for cardiovascular disease worldwide and responsible for 12.8% (7.5 million) of total deaths worldwide.[8] Red wine components might be promising therapeutical interventions, as they were shown to reduce blood pressure in hypertensive animals.[9-11] In humans, peripheral brachial blood pressure is a predictor of cardiovascular outcome, and 24 h ambulatory blood pressure measurement predicts the cardiovascular risk over and above the prediction provided by office values.[12, 13] However, central blood pressure may be even more strongly related to cardiac hypertrophy, the extent of atherosclerosis, and cardiovascular events than peripheral brachial blood pressure, and therefore may be a better outcome parameter in blood pressure-lowering intervention trials than peripheral blood pressure measurements.[14]

Type 2 diabetes mellitus

Type 2 diabetes mellitus is characterized by hyperglycemia, insulin resistance, and a relative impairment in insulin secretion and is a major risk factor for cardiovascular disease. The Framingham Heart Study showed in 2006 that the incidence of type 2 diabetes was doubled over the last 30 years.[15] Although we know that subjects with impaired fasting glucose, an impaired glucose tolerance and obesity are at high risk to develop diabetes[16], our ability to predict en prevent type 2 diabetes is still limited. A greater maternal weight during pregnancy is associated with greater offspring adiposty and adverse cardiovascular factors.[17] On the other hand, fetal malnutrition due to reduced food intake by the mother (e.g. during the WOII) may also predispose to type 2 diabetes.[18, 19] This may be caused by fetal programming, which offers an organism the ability to develop while adapting to environmental and nutritional signals in early life. However, this may have consequences in adulthood, such as the development of type 2 diabetes.

Red wine and cardiovascular disease: epidemiological evidence

St Leger was in 1979[20] the first who showed an inverse correlation between mortality from coronary heart disease and wine consumption. This finding was later called "The French Paradox", because French people have the lowest mortality and a low risk of cardiovascular disease despite a high consumption of saturated fat and prevalence of other risk factors.[21] Although many epidemiological studies confirmed this inverse relation between red wine consumption and cardiovascular disease,[22-25] other studies reported this relation for all types of alcoholic drinks.[26, 27] Alcohol might protect against cardiovascular disease by increasing levels of high-density lipoprotein[28] and by lowering platelet activity[21], but, on the other hand, it has been shown to increase blood pressure.[29] A unifying concept is that alcohol consumption, from whatever source, results in a J-shaped curve, whereby a modest intake is beneficial and either no intake or an increased intake is harmful.

Red wine and vascular function: *in vivo* studies

Whether there is a causal relationship between red wine consumption and cardiovascular disease is far from clear and almost impossible to investigate, since many confounding, social and dietary, factors are simultaneously involved. In addition, the duration of clinical trials should be at least several years for such research, which is unfeasible. A number of studies have been performed to investigate the *in-vivo* effect of short term red wine consumption on endothelial function.[30-33] One of the first studies that investigated the acute effect of red wine consumption on flow-mediated dilation, as a

parameter of vascular function, was performed by Agewall et al. in 2000.[32] He showed in a small study that the intake of one dose of dealcoholized red wine acutely increased flow-mediated dilation in healthy persons, whereas red wine itself had no effect. Concurrently, another study demonstrated that both dealcoholized and alcohol-containing red wine increased flow-mediated dilation two hours after intake.[33] Besides the effect of red wine polyphenols on vascular function, blood pressure was not influenced by grape polyphenols in normotensive subjects,[34-36] but decreased in subjects with the metabolic syndrome.[37] Whether subjects with increased cardiovascular risk should be advised to regularly use red wine polyphenols in order to reduce blood pressure remains however uncertain. Nevertheless, in animal studies alcohol-free red wine extract and red wine has been shown to reduce blood pressure, improve endothelial function, reduce atherosclerosis, and prevent age-induced endothelial dysfunction.[9-11, 38-41] Thus, the issue whether drinking of red wine is beneficial for the healthy human population is still a matter of debate.

Polyphenols in red wine

Polyphenols, characterized by the presence of phenol structural units, are assumed to be responsible for the beneficial effects of red wine. Polyphenols vary extremely in red wine due to differences in variety and grape sources as well as the grape processing. Red wine contains a higher concentration of polyphenols than white wine, because red wine is maturated for weeks with the skin and seeds during the wine-making process and these contain most of the polyphenols.[42, 43] In addition, because grape polyphenols have been shown to represent only one-half of the polyphenol content of a 2-y-old red wine polyphenol extract,[44] it is suggested that new polyphenols are formed during maturation and conservation. Red wine polyphenols can be categorized into flavonoid compounds, such as quercetin, catechin, epicatechin and anthocyanidins, and non-flavanoid compounds, such as resveratrol.[45] Yet, since red wine contains at least 200 different polyphenols[45], and because it may not be a single constituent that confers the protective effect, it is desirable to initially investigate all compounds in red wine together.

Resveratrol and SIRT1

About 10 years ago, researchers found that the red wine polyphenol resveratrol activates the silent information regulator 2 (Sir2 or sirtuin), a versatile protein deacetylase known from longevity studies.[46, 47] Humans have seven sirtuins (SIRT1-7*)*, of which SIRT1 has been studied most extensively.[48] This new intriguing finding encouraged many researchers to further investigate SIRT1 and resveratrol as possible novel therapeutic

targets. Resveratrol improved survival of mice on a high fat diet, and it protected against metabolic disease and diabetes through activation of SIRT1.[3, 49] Even in humans, resveratrol induced beneficial metabolic changes via SIRT1, as very recently reported.[50] SIRT1 is known to lead to epigenetic alterations by direct deacetylation of histones causing repression of transcription.[51] Furthermore, it controls cell metabolism by deacetylation of non-histone targets. For example, SIRT1 has been implicated in endothelial cell aging by inhibition of p53 (via its deacetylation), a key mediator in senescence.[52] SIRT1 also deacetylated endothelial nitric oxide (NO) synthase (eNOS), and this resulted in eNOS activation and an improved vasodilator function.[53] SIRT1 activity is regulated by changes in NAD^+/NADH ratio and can be influenced by dietary factors. Caloric restriction can modulate this ratio and thereby SIRT1 activity.[54] Because SIRT1 activation might protect against diabetes and is upregulated by caloric restriction, it might theoretically interact with fetal malnutrition to influence type 2 diabetes risk. This potential interaction has not been studied so far. Very recently however, a number of extensive studies disputed the promising findings of SIRT1 on longevity and its activation by resveratrol.[55, 56] SIRT1 activators including resveratrol directly interacted with fluorophore-containing peptide substrates in the SIRT1 assay, but did not lead to apparent activation of SIRT1 with native peptides.[55] Furthermore, *sir-2.1* overexpression in *C. Elegans* did not increase longevity after standardization for background, in contrast to what was previously mentioned.[56,47] Therefore, it is currently far from clear if the reported beneficial effects of red wine and resveratrol are indeed mediated by SIRT1.

Red wine extract and NO-containing components as endothelium-derived relaxing and hyperpolarizing factor: *in vitro* studies

Vascular NO originates from *de novo* synthesis by eNOS and/or storage forms of NO. The latter comprise nitrite, nitrate, and *S*-nitrosothiols.[57] Their presence is not limited to endothelial cells. *S*-nitrosothiols activate endothelial intermediate-conductance and small-conductance Ca^{2+}-activated K^+-channels, and via soluble guanylyl cyclase, smooth muscle Na^+-K^+-ATPase.[58-60] Bradykinin dilates porcine coronary arteries, at least in part, by stimulating the release of *S*-nitrosothiols from endothelial cells,[58-60] and *S*-nitrosothiols may thus act as endothelium-derived hyperpolarizing factors (EDHF). Importantly, *S*-nitrosothiols, by reacting with other thiols at physiological pH, yield nitroxyl (HNO), a recently discovered EDHF.[61] Light-induced vasorelaxation ('photorelaxation') also depends on *S*-nitrosothiols, which decompose to a disulfide and NO.[57, 60] Additionally, nitrite may undergo photolysis to NO.[62, 63] Fitzpatrick et al.[64] was the first who demonstrated the potential of red wine and grape juice to promote endothelium-dependent vasodilation of isolated vessels. From later studies it became evident that red wine polyphenol induced-vasorelaxation is mediated by NO and EDHF.[65] Red wine extract increased eNOS

expression and subsequent endothelial NO release.[66] In addition, red wine polyphenols reduced the synthesis of the vasoconstrictor endothelin-1 in bovine aortic endothelial cells.[67] Despite these data, the exact mechanism of red wine polyphenols-induced vaso-relaxation is currently not fully clear. Furthermore, whether HNO and/or nitrite as 'new EDHF' could mediate red wine polyphenol-, bradykinin- and light-mediated relaxation is not known yet.

AIMS AND SCOPE OF THIS THESIS

Although much research has been performed in the past few decades on the value of red wine consumption and its derived compounds in the prevention of cardiovascular disease, many issues remain unresolved. A better understanding of the underlying mechanisms, including the role of SIRT1, might lead to novel therapeutic targets, and would allow a well-founded advice to patients (and healthy subjects) on the protective/deleterious effects of daily red wine consumption. Therefore, the aim of the present thesis is to investigate the effect of red wine consumption and its compounds on vascular functions, e.g. vascular dilatation and blood pressure in humans, and to delineate the molecular mechanisms underlying this effect, focussing in particular on SIRT1 and EDHF.

In chapter 2 and 3, we performed *in vivo* studies investigating the effect of red wine consumption and its de-alcoholised composition on vascular function, respectively by forearm blood flow and blood pressure measurement as indices of vascular function. In chapter 2, we investigated whether daily red wine consumption for three weeks improves *in-vivo* vascular function by reducing endothelin-1. To study additional pathways mediating this effect, we quantified the *in-vitro* effects of red wine extract and resveratrol on vascular function, using porcine coronary arteries. In chapter 3, we evaluated the effect of intake of an alcohol-free red wine extract on blood pressure in hypertensive patients. We conducted a placebo-controlled, randomized cross-over study and assessed both office and 24-h ambulatory blood pressure measurements, as well as central hemodynamic measurements.

Chapter 4 investigates in an *in vitro* study how red wine extract reduces endothelial cellular aging (cellular senescence). Senescence was induced by exposing human endothelial cells to *tert*-butylhydroperoxide, and quantified by senescence-associated β-galactosidase staining. Although only present in very small amounts in red wine extract, we also explored a possible role for the most studied red wine polyphenol resveratrol, and we evaluated if the observed effect was mediated by SIRT1, by use of the SIRT1 inhibitor sirtinol.

To discriminate between late modulations by red wine, i.e. the effect on aging, in chapter 4 and very early, i.e. fetal, exposure to a red wine polyphenol-induced factor, we focussed in chapter 5 on the interaction between *SIRT1* genetic variants and fetal malnutrition influencing the risk of type 2 diabetes, based on the role of SIRT1 in epigenetic and glucose regulation and its sensitivity to dietary factors. We addressed this question in the Dutch Famine Birth Cohort, which is composed of individuals born as term singletons in Amsterdam around the famine in the Netherlands during World War II. This unique study population contains information on both exposure to famine during gestation and the incidence of type 2 diabetes in adulthood.

In Chapter 6, we studied the effect of red wine extract on photorelaxation and HNO-induced relaxation to determine if nitrite and HNO are involved in the known RWE-induced vasorelaxing effect. In addition, we present the mechanism of nitrite-dependent photorelaxation and the possibility that HNO, rather than *S*-nitrosothiols, acts as EDHF in bradykinin-induced vasorelaxation. Since the relative contribution of EDHF is related to vessel size (EDHF is more prominent in resistance vessels), the experiments were performed in both coronary arteries and coronary microarteries of the pig.

Finally, chapter 7 discusses the main findings of this thesis, taking into consideration the limitations of the performed studies, the implications of our findings for clinical practice and potential directions for further research.

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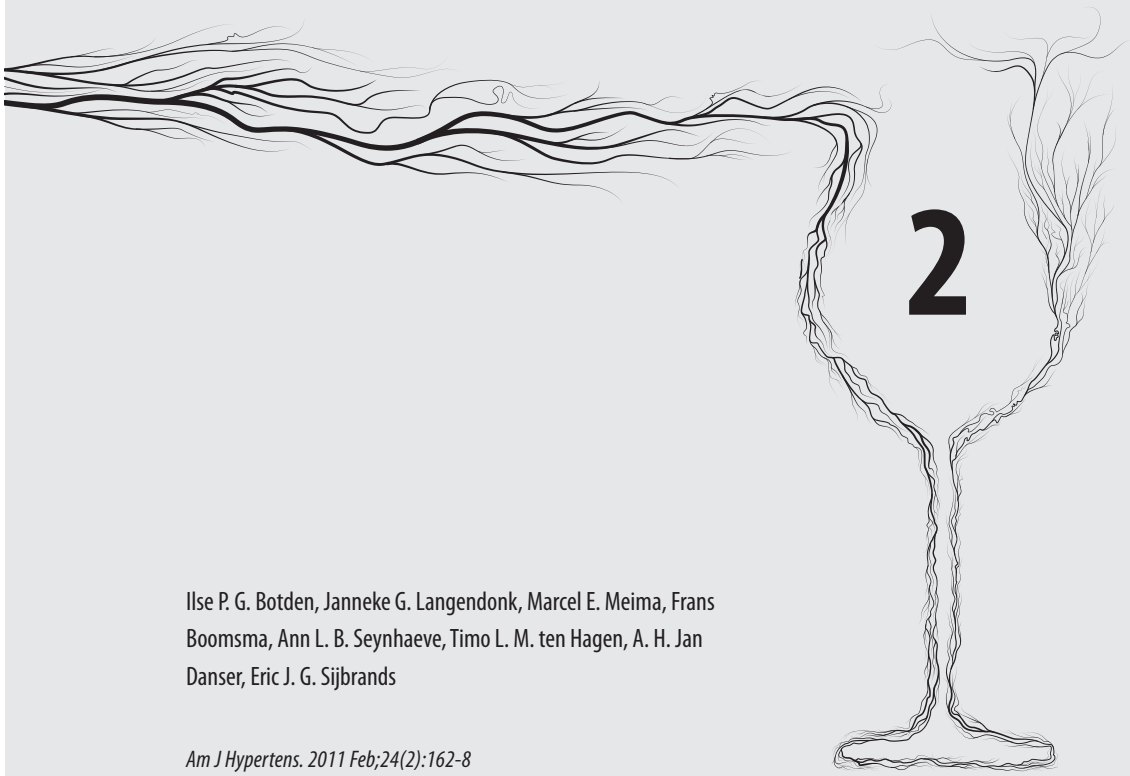
Daily red wine consumption improves vascular function by a soluble guanylyl cyclase-dependent pathway



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Ilse P. G. Botden, Janneke G. Langendonk, Marcel E. Meima, Frans Boomsma, Ann L. B. Seynhaeve, Timo L. M. ten Hagen, A. H. Jan Danser, Eric J. G. Sijbrands

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ABSTRACT

Background. Polyphenols in red wine are supposed to improve endothelial function. We investigated whether daily red wine consumption improves *in-vivo* vascular function by reducing endothelin-1 (ET-1). Additional pathways mediating this effect were studied using porcine coronary arteries (PCAs).

Methods. Eighteen young healthy women drank red wine daily for three weeks. Vascular function was evaluated by determining forearm blood flow (FBF) responses to endothelium-dependent (acetylcholine) and endothelium–independent (sodium nitroprusside) vasodilators. PCAs were suspended in organ baths and exposed to the endothelium-dependent vasodilator bradykinin, the NO donor SNAP and/or red wine extract (RWE).

Results. Acetylcholine-induced and sodium nitroprusside-induced FBF increases were equally enhanced after three weeks of red wine consumption, but an immediate enhancement (i.e. after drinking the first glass) was not observed. Vice versa, plasma ET-1 levels were not decreased after three weeks, but we observed an acute drop after drinking one glass of wine. RWE relaxed preconstricted PCAs in an endothelium-, NO-, and soluble guanylyl cyclase (sGC)/cGMP-dependent manner. Short RWE exposure reduced the response to bradykinin and SNAP by inactivating sGC. This effect disappeared upon prolonged RWE exposure.

Conclusions. The enhanced FBF response following three weeks of red wine consumption, but not after one glass, reflects a change in smooth muscle sensitivity. Alterations in sGC responsiveness/activity, rather than changes in ET-1, appear to underlie this phenomenon.

INTRODUCTION

Polyphenols, which are abundantly present in red wine, are assumed to improve endothelial function, thus leading to cardioprotection.[1] They enhance the production of vasodilating factors, like nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF).[2] In addition, red wine polyphenols reduce the synthesis of the vasoconstrictor endothelin-1 (ET-1) in bovine aortic endothelial cells,[3] but their effect in humans after daily red wine consumption is unknown. Resveratrol, a red wine polyphenol that activates the protein deacetylase SIRT1 known from longevity studies, has attracted particular interest as a cardioprotective compound.[4]

A number of studies have been performed to investigate the *in-vivo* effect of red wine on endothelial function,[5-8] but the issue whether drinking of red wine is beneficial for the healthy population is still a matter of debate.

The purpose of the present study was to investigate the effect of daily red wine consumption on forearm vasodilation in healthy volunteers by venous occlusion plethysmography. Endothelium-dependent and endothelium-independent functions were evaluated by infusing acetylcholine (ACh) and sodium nitroprusside (SNP), respectively. In addition, we measured plasma ET-1 levels before and during red wine consumption and investigated the effect of red wine extract (RWE) on ET-1 release in cultured human endothelial cells. To determine through which pathways red wine constituents influence vascular function, we quantified the *in vitro* effects of RWE and resveratrol on vascular function, using porcine coronary arteries (PCAs).

METHODS

Subjects

Nineteen young, healthy, non-smoking women, whose age was below 30 years, were enrolled in the study. The inclusion criteria were: moderate consumption of alcoholic beverages, defined as at least three consumptions per week and less than two daily, and use of a hormonal contraceptive. The study complies with the Declaration of Helsinki. The Institutional Review Board of the Erasmus MC, Rotterdam, approved the study protocol. All subjects gave written informed consent before the start of the study.

Protocol of the in-vivo study

The subjects were asked to fully abstain from alcohol in the three weeks preceding the basal evaluation of vascular function. Subjects started with 112 ml of red wine (corresponding with 10 g of alcohol) in the morning at the study center and thereafter, with daily ingestion of 224 ml of red wine with each dinner at home. After one week, a fasting blood sample was taken and the daily red wine consumption was increased to 336 ml (30 g alcohol) for another two weeks. Additional alcoholic beverages, red grape juice and red grapes were not allowed during the study. The wine used was Toques et Clochers, Cabernet Sauvignon, Vin de Pays d'Oc, 2002, France. The total phenol content of this wine was 2 g/l, determined as gallic acid equivalents using Folin Ciocalteu reagent.[9]

Forearm blood flow (FBF) was studied at baseline, one hour after the intake of the first 112 ml of red wine, and after three weeks of daily red wine consumption, following an overnight fast. FBF was measured with venous occlusion plethysmography (Periflow, Janssen Scientific Instruments, Beerse, Belgium). This technique has been described previously.[10] All experiments were performed in a quiet, air-conditioned room (22 - 24°C). The brachial artery of the non-dominant arm was cannulated with a catheter, followed by a 60-minute rest. A mercury-in silastic strain gauge was fixed at the widest circumference of both forearms.

Venous occlusion was achieved with blood pressure cuffs around the upper arms with rapid cuff inflating to 40 mmHg. Bilateral wrist cuffs were inflated to above-systolic blood pressure to exclude hand circulation. Intra-arterial blood pressure and heart rate were monitored continuously. Intra-arterial blood pressure and heart rate were monitored continuously. The FBF response was measured to intra-arterially cumulative doses of the endothelium-dependent vasodilator acetylcholine (ACh; 10, 20, 30 µg/min) and the endothelium-independent vasodilator sodium nitroprusside (SNP; 2, 4, 10 µg/min). The flow applied to the brachial artery was kept constant. The different infusion blocks were separated by a 30-minute rest period. FBF was calculated from the rate of increase in forearm volume, whereas venous return from the forearm was prevented by inflating the upper arm cuff. FBF was calculated from the rate of increase in forearm volume, whereas venous return from the forearm was prevented by inflating the upper arm cuff. FBF was expressed in ml per minute per 100 ml of forearm tissue volume. Measurements during 2 minutes (steady state) were averaged to determine FBF.

Blood sampling

Blood samples were obtained after a 12-hour overnight fast at baseline, and after one and three weeks of daily wine ingestion. Additional blood samples were drawn one hour after the first glass of red wine. After centrifugation, plasma was collected and stored at -80°C until ET-1 and high-density lipoprotein (HDL) were assessed.

Cell culture studies

Alcohol-free red wine extract (RWE, Provinols, Seppic, France) was used for the cell culture studies and organ bath studies. This RWE contained 632 mg polyphenols/g, determined as gallic acid equivalents using Folin Ciocalteu reagent.[9]

Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion as described by Jaffe et al.[11] HUVECs (passage 5) were cultured in HUVEC medium containing human endothelial-serum free medium (Invitrogen, Breda, The Netherlands), 20% heat inactivated newborn calf serum (Lonza, Verviers, Belgium), 10% heat-inactivated human serum (Lonza), 20 ng/mL human recombinant basic fibroblast growth factor (Peprotech, London, UK), and 100 ng/mL human recombinant epidermal growth factor (Peprotech) in a humidified incubator at 37°C and 5% CO_2.

HUVECs were plated at a density of 6 x 10^4 cells/ml. After 24 hours of culturing, medium was replaced by serum-free DMEM to secure low basal stimulation for an additional 24 hours. Subsequently, the cells were incubated in RWE in different concentrations (0, 6.25, 12.50, 25, and 50 µg/ml), dissolved in serum-free DMEM, for 6 hours. The supernatant was removed and immediately stored at -80°C until the ET-1 assay was performed.

Growth of endothelial cells of the 48-well multiplates was measured using the sulforhodamine B (SRB) protein stain assay, according to the method of Skehan.[12]

ET-1 and HDL measurements

ET-1 was measured in plasma samples and HUVEC supernatant using a chemiluminescent immunoassay ELISA (QuantiGlo, R&D Systems, Abingdon, United Kingdom) according to the manufacturer's protocol. At mean levels of 1.8 pg/ml the intra-assay CV was 3.4% and inter-assay CV was 8.9%. All plasma samples were measured within one assay plate.

HDL cholesterol was measured by a direct enzymatic HDL cholesterol method (Roche modular P800).

Organ bath studies and guanosine-3′,5′-cyclic monophosphate (cGMP) measurement

Methods for organ bath studies and cGMP measurement were set up as previously described.[13] Briefly, porcine coronary arteries (PCAs) were obtained from 45 slaughterhouse pigs. The PCAs were removed, cut into segments, and then stored in the absence or presence of RWE (30 µg/ml) for 16 hours in cold, oxygenated Krebs bicarbonate solution. In some segments, the endothelium was removed. The segments were suspended in 15 ml-organ baths containing Krebs bicarbonate solution, aerated with 95% O_2/5% CO_2 and maintained 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 allowed to equilibrate in fresh organ bath fluid for 30 minutes or 2 hours in the absence or presence of one or more of the following compounds: the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich; 100 µmol/l), the guanylyl cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ, Sigma-Aldrich; 10 µmol/l), or RWE (30 µg/ml). Vessels were then preconstricted with 9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin $F_{2\alpha}$ (U46619, Sigma-Aldrich, 0.1 to 1 µmol/l) to ~80% of the maximal constriction, and concentration-response curves were constructed to bradykinin, S-nitroso-N-acetyl-l,l-penicillamine (SNAP), resveratrol (all from Sigma-Aldrich) or RWE (0.01 to 100 µg/l).

To quantify cGMP production, vessel segments were exposed to bradykinin (1 µmol/l) or RWE (100 µg/ml) for 10 min in 15-ml oxygenated Krebs bicarbonate solution at 37°C in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 100 µmol/l). The response to bradykinin was also quantified after incubating the vessel segments for 120 min with 30 µg/ml RWE. Tissues were immediately frozen in liquid nitrogen and stored at -80°C until cGMP was determined by ELISA following acetylation (ITK Diagnostics, Uithoorn, The Netherlands). Results were analysed as pmol/mg protein and are expressed as content cGMP, fold over baseline. The lower limit of detection was 0.1 pmol/mg protein.

Statistical analysis

All data are expressed as mean ± SEM. A probability value of $P<0.05$ was considered statistically significant.

Statistical analysis of FBF measurements for individual subjects was performed by two-way ANOVA for repeated measures. One subject did not complete the study. We performed analyses with and without her FBF data. We did not find different results with

these two methods and decided to present the FBF data of the 18 participants whose measurements were fully available.

ET-1 levels in cell culture supernatant were corrected for cell growth. Statistically significant differences compared with basal levels were determined by one-way ANOVA with Dunnett's post hoc correction for multiple comparisons.

Peak relaxant responses of the organ bath studies are expressed as percentage of the concentration to U46619. Statistical analysis was obtained by two-way ANOVA with Bonferroni's post hoc comparisons or by one-way ANOVA of the mean pEC_{50} and maximum relaxation values, where appropriate. cGMP data were analysed by one-way ANOVA with Bonferroni's post hoc comparisons.

RESULTS

The average age of the eighteen females was 22.4 years, ranging from 19 to 28 years. Their average BMI was 22.7 kg/m^2, ranging from 19 to 28 kg/m^2 (Table 1). Systolic and diastolic blood pressure did not decrease significantly after three weeks of daily red wine consumption, nor directly after one unit of red wine, and no significant changes in HDL levels were observed (Table 1). We studied the effects one hour after drinking one unit of red wine to enable comparison with previous studies.[5, 6, 8] Resting FBF at that time had increased from 3.3±0.4 to 5.6±0.7 ml/min per 100 ml of FBF (P<0.01). We corrected for this increase in FBF by subtracting the resting FBF values. Figure 1 then shows that the ACh- and SNP-induced increases in FBF after drinking 1 unit of red wine were identical to those before wine drinking.

After three weeks of daily red wine consumption, when resting FBF had returned to pre-wine drinking level (3.5±0.5 ml/min per 100 ml of FBF), the mean delta FBF responses

Table 1. Parameters of the subjects, at baseline, and after red wine consumption

	Baseline	After 1 glass	After 3 weeks
Age (years)	22.4 ± 0.5		
BMI (kg/m^2)	22.7 ± 0.6		
Systolic blood pressure (mmHg)	121.6 ± 2.5	118.2 ± 2.3	118.7 ± 2.6
Diastolic blood pressure (mmHg)	69.3 ± 1.3	67.3 ± 1.4	67.8 ± 1.6
Plasma HDL (mmol/L)	1.31 ± 0.07		1.34 ± 0.06

Parameters at baseline, after one glass of red wine, and after 3 weeks of daily red wine consumption.
N = 18. Values represent mean ± SEM.

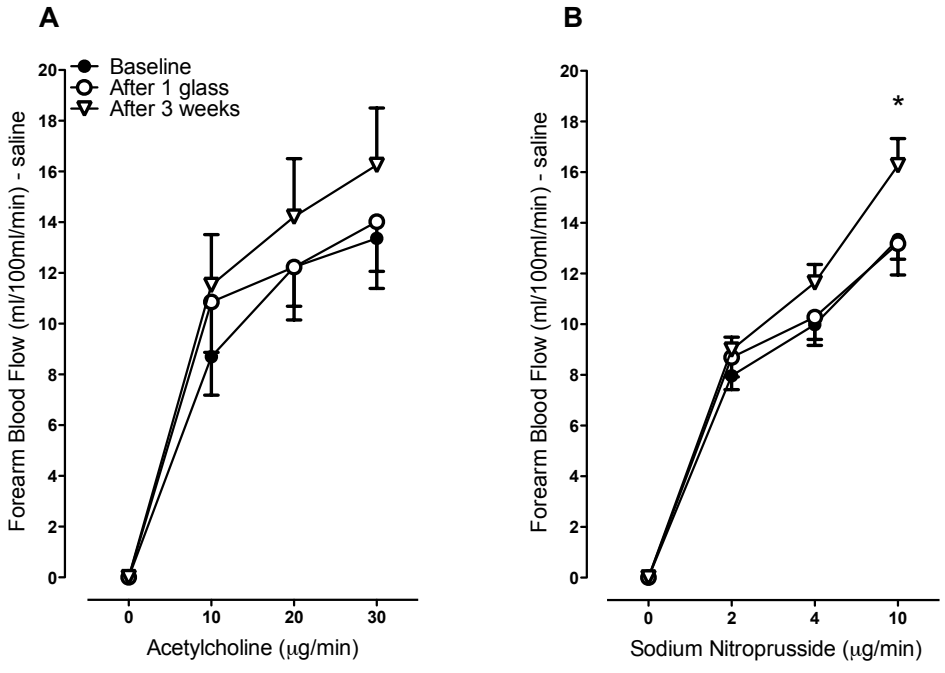

Figure 1. Forearm blood flow responses to acetylcholine infusion (a) and sodium nitroprusside infusion (b) at baseline, after one glass of red wine, and after 3 weeks of daily red wine consumption. Data (mean ± SEM of 18 subjects) are corrected for the response to saline. *P<0.05 vs. baseline.

to ACh and SNP were equally increased (by 2.6±1.3 (Fig. 1a, P=0.06) and 1.9±0.6 (Fig. 1b, P<0.01) ml/min per 100 ml of FBF, respectively).

Plasma ET-1 levels tended to decrease after one unit of red wine, from 1.7±0.1 to 1.4±0.7 pg/ml (P=0.08). After one week of daily red wine consumption ET-1 levels decreased further to 1.1±0.1 pg/ml (P<0.01 vs. baseline). After three weeks of daily red wine consumption (n=19), ET-1 levels were back to 1.5±0.1 pg/ml (P=0.25 vs. baseline; data not shown).

In vitro, incubating endothelial cells in RWE resulted in a concentration-dependent decrease (P<0.05 vs. basal) of ET-1 release (Fig 2; n=6).

To study the pathways via which red wine consumption might cause vasorelaxation, the direct effect of addition of increasing concentrations of resveratrol or RWE to U46619-preconstricted PCAs was studied. Resveratrol, up to a concentration of 100 μmol/l was without effect (n=15, data not shown). In contrast, RWE relaxed preconstricted vessels by maximally 73±7.3% (n=16). L-NAME and ODQ greatly reduced this effect (P<0.001), whereas endothelium denudation totally abolished it (Fig. 3). This indicates that the

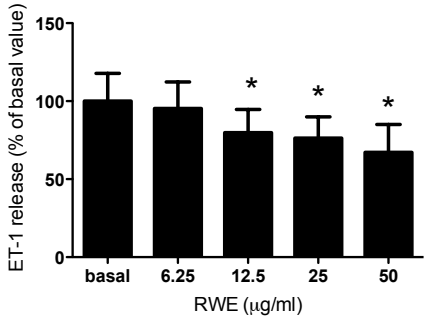

Figure 2. Effect of red wine extract (RWE) on endothelin-1 (ET-1) release from human umbilical vein endothelial cells (HUVECs) after a 6-h incubation period. Data are mean ± SEM of 6 experiments, performed in duplicate. *P<0.05 vs. basal.

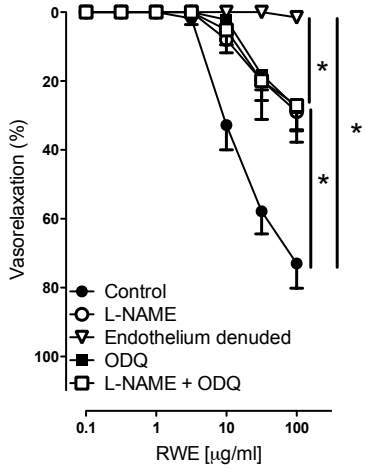

Figure 3. Concentration-response curves of U46619-precontracted porcine coronary arteries to cumulative doses of red wine extract (RWE), in the absence and presence of 100 µmol/L L-NAME and/or 10 µmol/l ODQ. In some vessels, the endothelium was denuded before the concentration-response curve was generated. Data (mean ± SEM of 5-17 experiments) are expressed as a percentage of the contraction induced by U46619. *P<0.05 vs. RWE or RWE + endothelium denuded.

effect of RWE predominantly depends on de novo formation of NO by eNOS and subsequent sGC activation. In agreement with this concept, RWE, like bradykinin, increased the vascular cGMP content (Fig. 4).

The endothelium-dependent vasodilator bradykinin relaxed PCAs by maximally 83±5.9%. Pre-incubation of the vessels with RWE (30 µg/ml) for 0.5 or 2 hours greatly

diminished the vasorelaxing effect of bradykinin ($P<0.05$; Fig. 5), and abolished the bradykinin-induced increase in cGMP content (Fig. 4). When prolonging the incubation with RWE to 16 hours, the relaxant effect of bradykinin tended to return to normal. In agreement with previous studies[14], L-NAME modestly reduced the effect of bradykinin ($P<0.05$; Fig. 5). L-NAME, however, did not prevent the short-term effect of RWE on bradykinin-induced relaxation. Yet, with L-NAME, the effect of RWE after 16 hours was no longer significant. These data indicate that the blocking effect of RWE towards bradykinin involves both an eNOS-dependent and an eNOS-independent phenomenon.

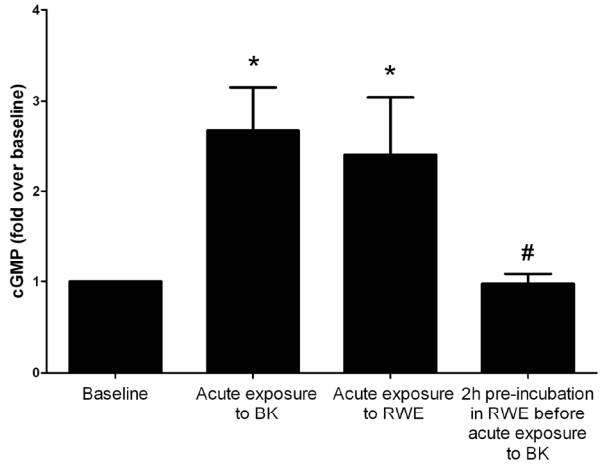

Figure 4. Porcine coronary artery cGMP content following exposure to 1 µmol/l bradykinin (BK) for 1 min, or 100 µg/ml red wine extract (RWE) for 10 min. Experiments were also performed after pre-incubation of the vessel segments for 2 hours in 30 µg/ml RWE. All experiments were performed in the presence of IMBX (100 µmol/l). Data are mean ± SEM of 2-11 experiments. *P<0.05 vs. baseline, #P<0.05 vs. bradykinin.

To investigate the latter, the endothelium-independent vasodilator SNAP was used. SNAP relaxed PCAs by maximally 94±2.7% (Fig. 6). Short, but not long pre-incubation of the vessels with RWE diminished this effect, as evidenced by a significant ($P<0.001$) rightward shift of the concentration-response curve. ODQ largely blocked the effect of SNAP ($P<0.01$) and in the presence of this sGC inhibitor, the inhibiting effect of RWE did no longer occur (Fig 6). This indicates that the inhibiting effect of RWE towards SNAP involves sGC.

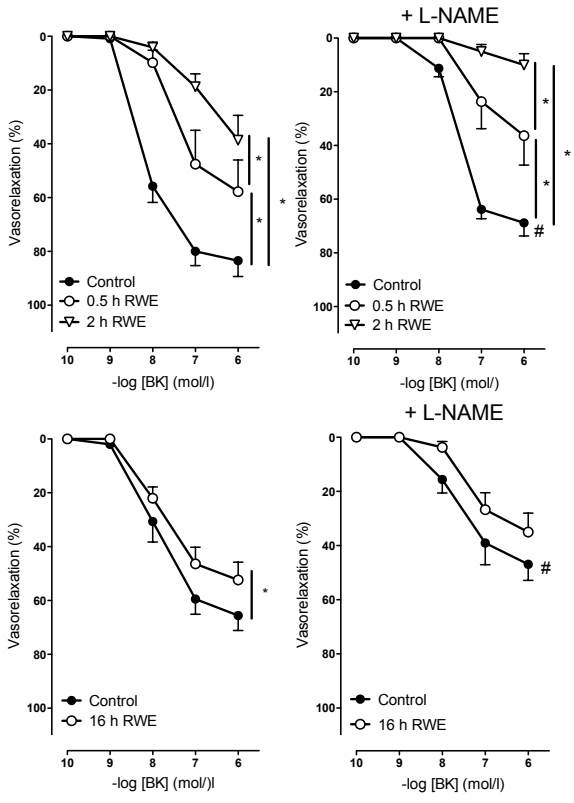

Figure 5. Concentration-response curves of U46619-precontracted porcine coronary arteries to cumulative doses of bradykinin (BK) in the absence or presence of 100 µmol/l L-NAME after incubation with red wine extract (RWE, 30 µg/ml) for the indicated time periods. Data (mean ± SEM of 6-12 experiments) are expressed as a percentage of the contraction induced by U46619. *P<0.05 vs. control or 2h RWE, #P<0.05 vs. no L-NAME.

DISCUSSION

We found that daily red wine consumption during three weeks improved vascular function of young healthy women. FBF increased in response to ACh but also to SNP, and both increases were of similar magnitude. This suggests an increased sensitivity of the vascular smooth muscle (VSM) to NO instead of an amelioration of endothelial function. Drinking a single unit of red wine reduced plasma ET-1. In agreement, RWE reduced endothelial ET-1 release *in vitro*. Clearly, this effect was not long lasting *in vivo*, since the ET-1 plasma levels returned to normal after three weeks of red wine consumption. Finally, RWE relaxed PCAs in an endothelium-dependent manner, involving NO generation by eNOS and subsequent sGC activation and cGMP formation, whereas resveratrol

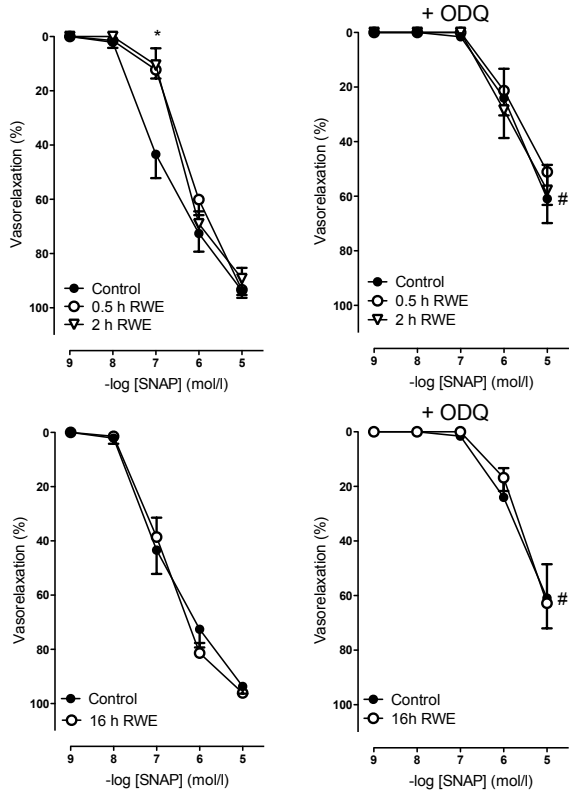

Figure 6. Concentration-response curves of U46619-precontracted porcine coronary arteries to cumulative doses of SNAP in the absence or presence of 10 µmol/l ODQ after incubation in red wine extract (RWE, 30 µg/ml) for the indicated time periods. Data (mean ± SEM of 7-8 experiments) are expressed as a percentage of the contraction induced by U46619. *P<0.05 vs. control or 2h RWE, #P<0.05 vs. no ODQ.

exerted no such effect. Simultaneously however, pre-incubation in RWE diminished the bradykinin- and SNAP-induced relaxations.

This study is among the few that investigated both the endothelium-dependent and endothelium-independent effects of daily red wine consumption during a prolonged period (3 weeks) by healthy subjects, using the robust and precise method of plethysmography.[15] It therefore differs from studies that investigated the endothelium-dependent component only.[16] Individuals with proven endothelial dysfunction seem to profit from polyphenols of grape products without alcohol, both acutely and during follow-up.[6, 17-19] Red wine consumption during a maximum of two months however, did not affect vascular reactivity in patients with type 2 diabetes or after an acute coronary syndrome.[7, 20] This suggests that either the alcohol has diminished the beneficial effect

of the polyphenols, or that the endothelial dysfunction in patients with type 2 diabetes and acute coronary syndrome was in an irreversible state. We assessed the effect of red wine in healthy, young women to enable a study of the normal physiology, which is essential for understanding the preventive potential of red wine.

Our *in-vivo* study has a number of limitations. First, the number of participants was relatively small. However, the technique used to measure vascular function is very sensitive and reproducible[15, 21] and each subject was used as its own control. Venous occlusion plethysmography allows us to evaluate the response to stepwise increased infusion of vasoactive substances, which is not common in flow-mediated dilation (FMD), the technique that has been used in a number of other studies.[6, 16-20] Secondly, it could be argued that a period of three weeks is too short to investigate the effect of daily red wine consumption, although our follow-up was longer than most other studies. Furthermore, only women were studied. This was done to exclude known hormonal differences between men and women, especially since red wine polyphenols are known to stimulate the estrogen-α receptor in vascular endothelial cells.[22] It is unlikely that hormonal status influenced our results, because we restricted our studies during the periods on hormonal contraceptives, and these do not affect endothelial function in the forearm microcirculation of young healthy women, as investigated before.[23]

We assessed the vascular function before and after three weeks of red wine consumption, consumed during dinner. In addition, we tested the participants after drinking a single unit of red wine, to enable comparison with a number of studies in which the direct effect on healthy endothelium of red wine consumption had been analyzed.[5, 8, 16] Although baseline FBF had increased at that time, the delta FBF induced by either ACh or SNP was identical to the delta FBF induced by these agonists before the start of wine drinking. An acute effect of red wine on resting (saline) FBF has been observed before,[5, 16] and could relate to the direct effects of alcohol. Indeed, Bau et al.[24] showed an increased diameter of the brachial artery with FMD immediately after intake of alcohol, but this was absent after 13 hours. FMD and FBF cannot be compared directly, but adjustment for the resting values would reduce the endothelium-dependent effect in the FMD study to a similar extent as the resting response did in our setting. The effect after three weeks in our study, determined after an overnight fasting, may therefore not be caused by the alcohol content of red wine. The unaltered resting FBF levels combined with the increased responses to ACh and SNP at 3 weeks suggest that compensatory mechanisms (e.g. a decrease in the baseline levels of various endothelium-dependent agonists, including ACh) had occurred to overcome the consequences of the increased smooth muscle responsiveness. Under such circumstances, only the infusion of ACh or a NO donor allowed us to observe the enhanced sGC responsiveness.

In order to delineate the mechanisms that might underlie the *in-vivo* observations, *in-vitro* studies in porcine coronary arteries and human endothelial cells were performed. Obviously, this approach cannot entirely mimic the *in-vivo* conditions, as such studies cannot last for several weeks, and rely on the use of a RWE that does not necessarily yield the same polyphenol concentrations reached in blood following the intake of red wine. It is important to note that previous findings from our laboratory regarding the (endothelium-dependent) relaxant effects of bradykinin and the constrictor effects of angiotensin II in porcine coronary arteries[14, 25, 26] fully resembled those in human coronary arteries.[25, 27, 28] Since RWE contained 632 mg polyphenols/g, the RWE concentration of 30 µg/ml that we used in most organ bath experiments will yield polyphenol concentrations in the range expected in blood after drinking 336 ml red wine/day for 2 weeks.[29]

In vitro, RWE caused endothelium-dependent, NO-mediated and sGC/cGMP-mediated relaxations of coronary arteries, in full agreement with previous studies.[30-32] L-NAME and ODQ, alone or in combination, did not fully prevent the vasorelaxant effects of RWE. Thus, the relaxant response to RWE may also involve a non-NO, 'EDHF'-like response. The lack of effect of resveratrol *in vitro*, as opposed to RWE, suggests that the relaxant, NO-mediated effect of RWE does not involve this polyphenol, despite earlier studies showing that resveratrol increases eNOS activity through deacetylation by SIRT1.[33] This latter phenomenon, however, has recently been disputed.[34]

A short pre-incubation (0.5-2 hours) in RWE diminished the responses to both the endothelium-dependent vasodilator bradykinin and the endothelium-independent vasodilator SNAP, an effect that tended to disappear upon prolonged (16 hours) pre-incubation. The effect of RWE on bradykinin was greatly diminished upon prolonged (16 hours) pre-incubation, whereas its effect on SNAP was no longer present after a 16-hour pre-incubation. The eNOS inhibitor L-NAME fully prevented the (modest) prolonged effect of RWE towards bradykinin, but not its effects after short pre-incubation, while the sGC inhibitor ODQ fully prevented the effects of short RWE pre-incubation towards SNAP. These data indicate that the blocking effect of RWE involves sGC inactivation and NO depletion. The inability of bradykinin to increase cGMP following RWE exposure supports this view. The reduced sGC responsiveness following short RWE exposure resembles the cross-tolerance known to occur following nitroglycerin treatment.[35] This reduced responsiveness to NO seems to be a temporary effect, since it had almost disappeared after a 16-hour pre-incubation with RWE. Apparently a restoration of sGC responsiveness had occurred during longer exposure to RWE, e.g. via overexpression of sGC in VSMC.

With regard to the effect of red wine on ET-1, we showed an acute reduction of ET-1 release after treating human endothelial cells with different concentrations of RWE. These *in vitro* results are in line with Corder et al.,[3] who showed an inhibition of ET-1 release in bovine aortic endothelial cells over 6 hours during incubation in RWE. *In vivo,* red wine reduced plasma ET-1 levels directly after the intake of one glass, in line with a recently published study.[36] Unfortunately, the effect of longer term red wine intake on ET-1 plasma levels was not determined in the latter study. In our study, we observed that the ET-1 reducing effect disappeared following longer exposure to red wine.

In conclusion, our study demonstrates a positive *in-vivo* vasodilatory effect of prolonged red wine consumption in young, healthy women. Constituents of red wine other than alcohol and resveratrol increased the vascular NO sensitivity. In the presented *in-vitro* work, we identified a number of mechanisms underlying these observations in young, healthy volunteers. The lack of an acute effect of red wine may be attributed to a reduced responsiveness of sGC to NO, whereas the improved effect after prolonged wine exposure may reflect sGC upregulation. These changes are unlikely to be mediated by ET-1. Red wine consumption can contribute to primary cardiovascular disease prevention by the above pathways. Further *in-vivo* and e*x-vivo* research, regarding the vascular and metabolic effects of daily red wine consumption on healthy endothelium, but clearly also on vascular smooth muscle, are needed to obtain a full understanding of the protective vascular effects of red wine.

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Red wine polyphenols do not lower peripheral or central blood pressure in high normal blood pressure and hypertension

3

Ilse P.G. Botden, Richard Draijer, Berend E. Westerhof,
Joost H.W. Rutten, Janneke G. Langendonk, Eric J.G. Sijbrands,
A.H. Jan Danser, Peter L. Zock, Anton H. van den Meiracker

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ABSTRACT

Background. Epidemiological data suggest that modest red wine consumption may reduce cardiovascular-disease risk. Red wine polyphenols improved human endothelial vascular function and reduced blood pressure (BP) in animal studies, but the results of human intervention studies investigating the effect of red wine polyphenols on BP are inconsistent. The objective was to investigate whether polyphenols extracted from red wine reduce peripheral and central BP in subjects with high normal BP or grade 1 hypertension.

Methods. In a double-blind, placebo-controlled 3-period crossover trial we assigned 61 subjects (mean age 61.4±8.4 years) with office systolic BP 135±9 mmHg and diastolic BP 82±8 mmHg to dairy drinks containing either placebo, 280 mg red wine polyphenols, or 560 mg red wine polyphenols. After each 4-week intervention period, office and 24-h ambulatory BP measurements, and central hemodynamic measurements derived from continuous finger BP recordings were assessed.

Results. Polyphenol treatment did not significantly affect 24-h BP: systolic/diastolic BP was 143±2/84±1 mmHg after placebo, 143±2/84±1 mmHg after 280 mg/d of red wine polyphenols, and 142±2/83±1 mmHg after 560 mg/d. Neither dose of polyphenol treatment changed office or central BP, aortic augmentation index or pulse wave reflection index.

Conclusions. Intake of red wine polyphenols in two different dosages for 4 weeks did not decrease peripheral or central BP in subjects with a high normal or grade 1 hypertension. Our findings do not support the hypothesis that polyphenols account for the suggested cardiovascular benefits of red wine consumption by lowering BP.

INTRODUCTION

There is epidemiological evidence that modest red wine consumption is associated with a reduced risk of cardiovascular diseases.[1, 2] Polyphenols, which are abundantly present in red wine, improve endothelial function by enhancing the production of nitric oxide (NO) and endothelium-derived hyperpolarizing factor.[3] We and others have shown that red wine consumption improves vascular function *in vivo*, probably via an NO-soluble guanylyl cyclase-dependent pathway.[4, 5]

A reduction in blood pressure (BP) from intake of red wine polyphenols has been observed in normotensive and hypertensive rats.[6-11] Hence, red wine consumption may exert a beneficial effect on cardiovascular disease in part by lowering BP. However, the results of studies investigating the effect of grape polyphenols on BP in humans are inconsistent.[12-17] This might be due to different dosages of grape polyphenols applied, the period of exposure, the characteristics of subjects included, and the type of study design. Although no favorable effects were observed in normotensive subjects,[13, 17, 18] grape polyphenols decreased BP in subjects with the metabolic syndrome.[16] Whether subjects with a high normal BP or mild hypertension should be advised to regularly use red wine polyphenols in order to reduce their BP remains however uncertain.

Based on the promising BP-reducing effects of red wine polyphenols in animal studies, we hypothesized that the intake of red wine polyphenols decreases blood pressure in hypertensive patients. To address this point, we conducted a placebo-controlled, randomized cross-over study to investigate whether daily intake of two different dosages of red wine polyphenols in the form of dairy drink decreases BP in subjects with high-normal BP or grade 1 hypertension, who were not treated with antihypertensive drugs.

METHODS

We investigated the effect of a dairy drink containing red wine extract on BP in subjects with high-normal BP or grade 1 hypertension. Because the intention of this study was to seek for a BP-lowering product for persons in whom antihypertensive medication was not indicated, subjects that used any BP-lowering medication or medication acting on similar pathways as red wine polyphenols such as nitric oxide were excluded.

The study was approved by the Medical Ethical Committee of the Wageningen University, The Netherlands, and conducted in accordance with the ICH-GCP guidelines. All participants gave their written informed consent.

Study participants

Subjects were recruited through advertisements in local newspapers within one month. Male and female subjects aged between 35 and 70 years with (mildly) elevated BP and normal values of general health markers were invited to participate. To minimize the misclassification of a person's BP, BP was measured on two separate screening visits 1 week apart. Three consecutive BP measurements were performed at 2 min intervals by a physician using an automatic oscillometric device with the subject in a sitting position for 15 minutes. Subjects with a high normal BP or grade 1 hypertension on the first screening visit (average of last 2 measurements) were invited for the second visit. BP criteria for inclusion were between 130 to 179 mmHg systolic, and less than 100 mmHg diastolic. During the second visit BP was measured again. If the BP fulfilled the inclusion criteria as mentioned above again, height and body weight were measured and blood and urine samples were taken. Subjects with a 10-year risk of cardiovascular disease of 10% or higher according to the Framiningham risk score[19] were excluded. Also subjects were excluded with a history of metabolic diseases, chronic gastrointestinal disorders, cardiovascular or renal disease, medically prescribed diet or slimming, performing intense sporting activities, using oral medication potentially affecting BP or bioavailability of the red wine polyphenols, using systemic antibiotics in three months prior to the study, intolerance or allergy to dairy products, lactating, pregnancy or wishing to become pregnant during the study, weight change of 10% or more 6 months prior to the study, consuming meat and/or fish less than twice a week, smoking cigarettes during the year prior to the study or an irregular pulse or pulse below 50 or above 100 beats per minute (bpm).

Study design

The study had a double-blind, placebo-controlled randomized full-crossover design with 3 treatments periods (Figure 1). After baseline measurements, participants were randomly assigned according to their office systolic BP value at the second screening visit to a treatment sequence of a dairy drink to be consumed with or directly after breakfast and with or directly after dinner containing the following: (A) placebo, with no added red wine polyphenols (two times daily); (B) 280 mg of red wine polyphenols (280 mg around breakfast and a placebo around dinner); or (C) 560 mg of red wine polyphenols (280 mg around breakfast and 280 mg around dinner). We chose doses of 280 mg and 560 mg polyphenols to be in the order of usual polyphenol intake from moderate wine consumption; 560 mg red wine polyphenols is the equivalent of approximately 250 mL (2-3 glasses) of red wine.[20] The placebo and polyphenol products were identical with respect to taste and appearance and only differed in coding on the bottles. During

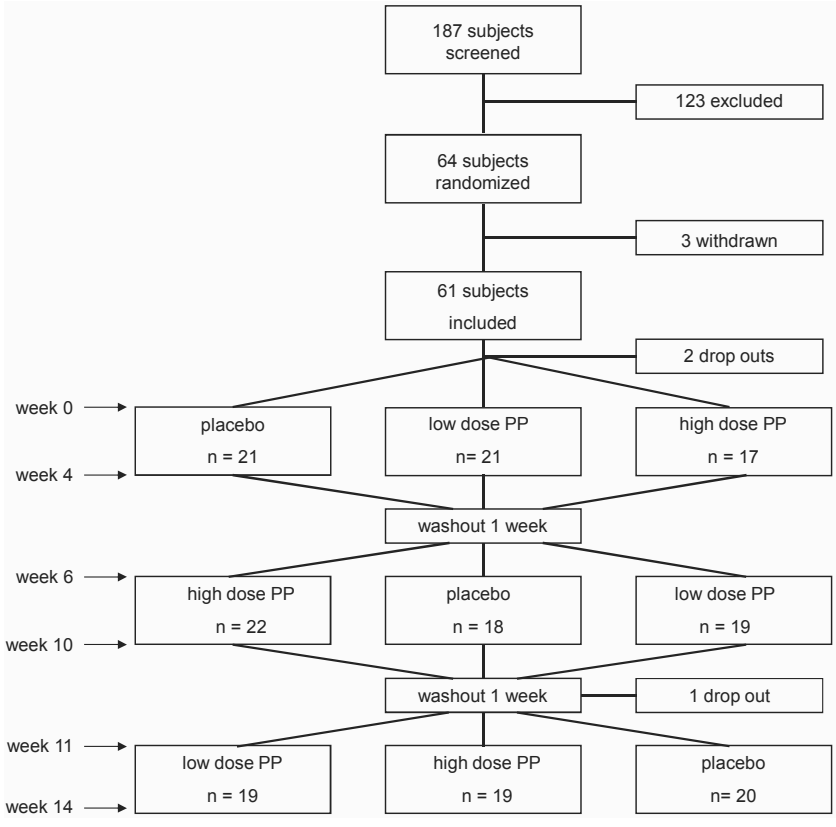

Figure 1. Flow diagram of participants through the study. PP indicates red wine polyphenols.

the study, the treatment code of the products was blinded and only known by a person not directly involved in the study.

The total study duration was 14 weeks: three 4-week intervention periods separated by two 1-week washout periods each (Figure 1). The active ingredient was a red wine extract, Provinols, which was manufactured at the Societé Française de Distelleries and supplied by Seppic S.A. France. The red wine extract was dissolved in a dairy drink, made by Unilever, to mask the bitterness of the red wine polyphenols. During the study, participants were asked to maintain their normal diet and lifestyle. They were not allowed to use supplements 4 weeks prior to the study and during the study, or to drink more than two units of alcohol beverages per day. One day prior to the measurement days, the participants were not allowed to drink red grape juice or alcoholic beverages or to engage in strenuous exercise. On the first visit day, subjects carefully recorded the content of their breakfast. The breakfast on the next measurement days was standardized

to the breakfast of the first visit day with a maximum of one cup of caffeine-containing beverage. Breakfast was consumed 2.5 to 3.5 hours prior to the office BP measurement.

Hemodynamic measurements

24-Hour ambulatory BP was measured at the start of the study and at the end of each intervention period with a portable BP monitor (Spacelabs monitor type 90217), fitted to the non-dominant arm. BP was measured every 20 minutes during the day and every hour during the night for at least 24 hours. The BP monitoring was repeated if more than 30% of the data was missing. Office BP was measured on two consecutive days, both at the beginning and at the end of each intervention period, approximately 3 hours after consumption of the test product to reduce variability due to morning surge, lunch, and circadian rhythm in general. Participants had to sit for 15 minutes after which 6 BP measurements were taken using an automatic BP monitor (Omron HEM 907). The average of the last 4 measurements was taken for analyses. The 24-hour ambulatory BP as well as the office BP measurements were performed on the same day and the same time of the week using the same BP monitor (office and ambulatory) for each individual. Central hemodynamic measurements were assessed at the start of the study and at the end of each intervention period. For this purpose finger BP was recorded continuously for 15 minutes with a Finometer (Finapres Medical Systems, Amsterdam, The Netherlands). Mean arterial pressure was measured by taking the true integral of the arterial pressure wave over 1 beat divided by the corresponding beat interval, and pressure wave forms were recorded. Brachial BPs were reconstructed from the finger arterial pressure, and used for generation of the corresponding central aortic waveform by a generalized transfer function.[21-24] Central systolic and diastolic BP, augmentation index and reflection index were calculated by analysis of the central waveform. Aortic augmentation index (AIx) was defined as the augmentation of the pressure following the inflection point divided by the total pressure amplitude, and was corrected for a heart rate of 75 bpm. Reflection index was defined as the amplitude of the backward pressure wave divided by the sum of the amplitudes of both the forward and backward pressure waves. Measurements took place at the Centre for Nutrition Intervention Trials, Vlaardingen, The Netherlands.

Laboratory analyses

Blood was collected after an overnight fast, centrifuged and separated into plasma and cells within 30 min after collection. From the 24-hour urine collections portions of 10 ml urine were taken and these were, together with the blood samples, stored immediately at -20°C and within 24 hours at -80°C. Glucose and lipids were measured in the blood

samples, and sodium, potassium, creatinin, and albumin were measured in the urine samples, using standard clinical analytic equipment.

Study outcomes

The primary outcome was the difference in BP between placebo and red wine-polyphenol intake after 4 weeks of treatment as assessed by 24-hour ambulatory and office BP. Secondary outcomes were differences between placebo and red wine-polyphenols intake in central hemodynamic, heart rate, plasma lipids and glucose, urinary albumin, and sodium/potassium ratio excretion after 4 weeks of treatment.

Sample size and statistical analysis

Power calculation based on office BP showed that 58 participants were needed to detect a difference of 3 mmHg between placebo and red wine-polyphenol treatment with a power of 90% and a significance level of 0.05. Taking 6 drop outs into account, 64 subjects were randomized into the study.

Baseline data are expressed as mean plus standard deviation for continuous variables and as n (%) for categorical variables. The absolute values of 24-hour ambulatory and office systolic BP and diastolic BP were statistically analyzed by using a linear mixed-effects model with compound symmetry repeated covariance with treatment and period as fixed effects. This model allowed correction for correlation patterns between the different periods within one subject. For ambulatory measurements, statistical analyses were performed on 24-hour daytime (9:00 hr - 21:00 hr), and night-time values (0:00 hr - 6:00 hr). Analyses of covariance taking the subjects' baseline values into account were also performed. These yielded similar results and are therefore not shown. Analyses were based on the intention-to-treat principle. A $P<0.05$ was considered significant. Data were analyzed using SPSS software (SPSS Inc, version 17.0).

RESULTS

Baseline characteristics

187 subjects were screened to find 64 eligible participants. Fifty-eight of these participants completed the study. Three withdrew just after randomization and before the start of the first intervention period because of personal reasons. Therefore, 61 participants were included in the intention-to-treat analysis (Figure 1). Three participants (4.9%)

Table 1. Baseline Characteristics of Participants

Characteristics	Mean
n	61
Age, y	61±8
Male, n (%)	46 (78%)
Weight, kg	81±11
BMI, kg/m^2	27±3
Office SBP, mmHg	135±9
Office DBP, mmHg	82±8
Fasting glucose, mg/dl	92±7
Total cholesterol, mg/dl	217±35
LDL, mg/dl	135±30
HDL, mg/dl	50±15
Triglycerides, mg/dl	115±44

Data are mean ± SD.
BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein

dropped out during the study; one because of diarrhea, one because of posttraumatic dystrophia, and one because of grade 2 hypertension. The baseline characteristics of the 61 participants are presented in Table 1. They were middle-aged (mean age 61±8 years), slightly overweight (body mass index 27±3 kg/m^2), and 78% was male. The average office BP at baseline was 135±9/82±8 mmHg, the average 24-h BP was 145±12/86±8 mmHg, and plasma glucose and lipid levels were within the normal reference values.

Peripheral BP

Results of the office and 24-hour ambulatory BP measurements are given in Table 2. Office BP was not significantly affected by either dosage of red wine polyphenols. Nor was there an effect of 280 mg polyphenols on 24-h BP (systolic BP/diastolic BP was 143±2/84±1 mmHg after placebo, 143±2/84±1 mmHg after 280 mg/d, and 142±2/83±1 mmHg after 560 mg/d of polyphenols; Figure 2A). 24-hour and daytime heart rate showed a borderline significant difference between the three treatment periods (P=0.04), but neither the 280 mg dose nor the 560 mg dose of red wine polyphenols was different from placebo (Figure 2B). Overall, ambulatory daytime BP was higher than office BP (average of 4 measurements) obtained after 15 min of rest (145/85 mmHg versus 133/80 mmHg, P<0.01).

Table 2. Office and 24-hour ambulatory blood pressure measurements at the end of the three intervention periods

	Placebo	Red wine polyphenols 280 mg	Red wine polyphenols 560 mg	*P*
Blood pressure				
Office SBP,mmHg	133±1	132±1	132±1	0.55
Office DBP, mmHg	80±1	80±1	80±1	0.92
24-hour SBP, mmHg	143±2	143±2	142±2	0.76
Awake SBP, mmHg	146±2	145±2	145±2	0.79
Asleep SBP, mmHg	127±2	126±2	127±2	0.81
24-hour DBP, mmHg	83±1	84±1	83±1	0.71
Awake DBP, mmHg	85±1	85±1	85±1	0.78
Asleep DBP, mmHg	74±1	74±1	73±1	0.68
Heart rate				
24-hour HR, bpm	72±1	73±1	71±1	0.04
Awake HR, bpm	76±1	77±1	74±1	0.04
Asleep HR, bpm	60±1	61±1	60±1	0.14

Data are mean ± SEM.
SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate.

Central hemodynamic

The results of the central hemodynamic measurements are shown in Table 3. AIx was about 33%, AIx corrected for a heart rate of 75 bpm was about 25%, and reflection index about 41%. There were no significant differences between the three treatments in central BP, aortic AIx or reflection index (Table 3).

Plasma and urinary parameters

Intake of red wine polyphenols had no effect on plasma glucose or plasma levels of total, HDL or LDL cholesterol or triglycerides. Also, 24-h urinary excretion of sodium, potassium, sodium/potassium ratio, albumin, and creatinin were not significantly different among treatments (data not shown).

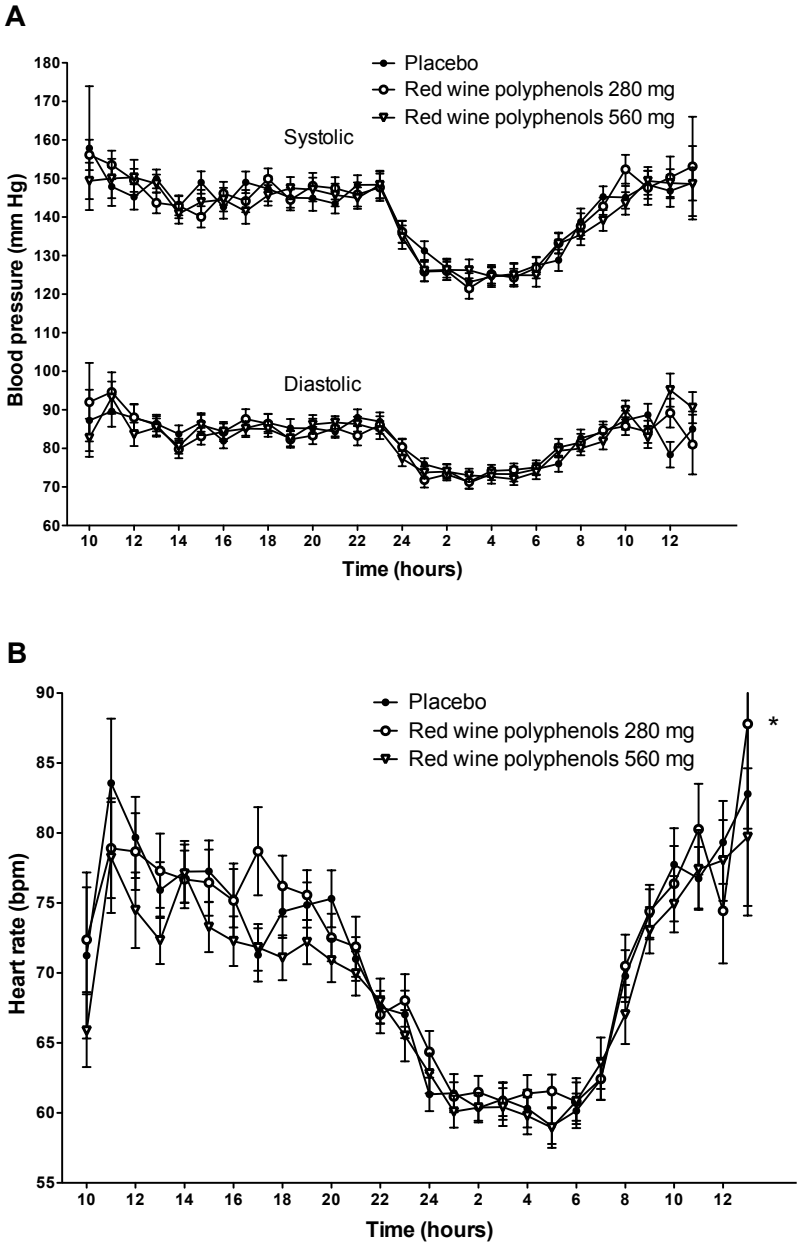

Figure 2. 24-Hour systolic and diastolic blood pressure profiles after intake of test drink (A) and 24-hour heart rate profile after intake of test drink (B). Data shown are mean±SEM. *P<0.05 versus red wine polyphenols 560 mg.

Table 3. Central hemodynamic measurements after intake of test drink at the end of the three intervention periods

	Placebo	Red wine polyphenols 280 mg	Red wine polyphenols 560 mg	*P*
Central SBP, mmHg	136±2	136±2	137±2	0.76
Central DBP, mmHg	80±1	80±1	80±1	0.91
Central MAP, mmHg	104±1	104±1	104±1	0.94
Heart rate, bpm	62±1	64±1	62±1	0.27
Augmentation index, %	32±1	33±1	33±1	0.55
Augmentation index (75), %	25±1	25±1	24±1	0.60
Reflection index, %	41±1	41±1	41±1	0.88

Data are mean ± SEM.
SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure, Augmentation index (75), augmentation index corrected for heart rate of 75 bpm.

DISCUSSION

This double-blind, placebo-controlled, randomized full-crossover study shows no effect of two different dosages of red wine polyphenols on 24-hour ambulatory and office BP compared with placebo after 4 weeks of treatment in subjects with high normal BP or grade 1 hypertension. Similarly, no effect was found on central hemodynamic parameters.

The hypothesis that the red wine extract in the present study lowers BP derives from several experimental studies.[6-8, 11] Two studies in normotensive rats showed that that oral administration of red wine polyphenolic compounds decreased systolic BP.[6, 11] In two other rat studies, red wine polyphenol-treatment prevented the increase in systolic BP induced by DOCA-salt[8] or angiotensin II administration.[7] To the best of our knowledge, our study is the first human intervention study that investigated the BP-lowering effect of this particular red wine extract on ambulatory BP. Two doses of red wine polyphenols were investigated, and no difference in BP was observed between the treatment groups in our adequately powered study.

A number of other human studies have evaluated the effects on BP of either other red wine polyphenol supplements than tested here or grape polyphenols intake.[13, 14, 16-18, 25] An open label randomized, cross-over study performed in 24 normotensive men showed no difference in 24-hour systolic and diastolic BP between a 4 week control-abstinence period and a 4-week period of consumption of de-alcoholized red wine which contained about 759 mg of polyphenols.[18] Also no change in office BP was observed in 25 normotensive

men who consumed capsules with 800 mg of wine polyphenols and grape polyphenols for two weeks in a placebo-controlled crossover study.[13] Furthermore, 8-week intake of juice made of Concord grapes which contained 965 mg of polyphenols did not lower 24-hour BP in 64 male and female subjects with high normal BP and stage 1 hypertension.[14] Although this latter study applied similar inclusion criteria for baseline BP of subjects as our study, their average baseline 24-hour BP was considerably lower (average BP 124/77 mmHg versus 145/86 mmHg in our group). Nevertheless, in one placebo-controlled trial, 4-week daily intake of 150 mg grape seed extract did lower BP from 134/83 to 123/77 mmHg in 9 subjects with metabolic syndrome.[16] Furthermore, quercetin, a red wine polyphenol, decreased systolic BP by 3 mmHg in 60 subjects with metabolic syndrome and apolipoprotein E genotype ε3/ε3.[26] It might therefore be that grape polyphenols only affect BP favorably in subjects with already marked endothelial dysfunction.

Consistent with the office and ambulatory BP results, no effect of red wine-polyphenol intake was observed on central aortic BP or AIx. Central BP measurement is more strongly related to cardiac hypertrophy, the extent of atherosclerosis, and cardiovascular events than peripheral BP, and therefore may be a better outcome parameter for BP lowering intervention trials than peripheral BP measurements.[27] In addition, BP lowering agents and other interventions may affect central and peripheral BP differently.[28, 29] For example, although a recent study showed no effect of theobromine-enriched cocoa consumption on brachial BP, central BP was significantly lower.[29] We also computed reflection index. This index is the amplitude of the backward pressure wave divided by the sum of the amplitudes of both the forward and backward pressure waves, derived from aortic flow triangulation taken from finger arterial pressure measurements by using a transfer function.[30] Unlike AIx the reflection index is independent of the timing of the reflected wave and may therefore be a better index of pressure wave reflection.[30, 31] Intake of red wine polyphenols also had no effect on the reflection index.

The effect of grape polyphenols on central hemodynamics has been reported previously with contradictory results.[32-34] A study with 8 subjects suggested that ingestion of red wine, but not of de-alcoholized red wine, acutely reduces aortic BP and AIx as well as aortic femoral pulse-wave velocity.[33] In contrast to that study is the observation that the acute BP rise due to smoking of a single cigarette, was prevented by both red wine and de-alcoholized red wine.[34] These two products also decreased pulse wave reflection.[34] Karatzi et al.[32] investigated the acute effects of red wine and de-alcoholized red wine on aortic BP and arterial stiffness in patients with coronary artery disease. They found that both products had favorable effects on wave reflection and central systolic BP, which they ascribed to the polyphenols in red wine. In a relatively large study, we investigated the chronic effects of red wine-polyphenol intake without any ethanol for

four weeks, but observed no effect on central BP and augmentation index. Therefore, as there were no smokers or coronary heart disease patients in our cohort, the favorable effects of red wine polyphenols on central hemodynamics may only appear in cardiovascular compromised subjects. Furthermore, the acute beneficial effects of red wine polyphenols on central hemodynamics may fade away after chronic intake, possibly by counter-regulatory mechanisms.

Some limitations of our study should be addressed. First, although our subjects were exposed to red wine polyphenols for an extended period, 4 weeks might still have been too short to see an effect of daily red wine-polyphenol intake on BP. Second, we can not extrapolate our results to other stages of hypertension, since only individuals with high normal BP or grade 1/2 hypertension were investigated. However, we did not find a difference in BP response between subjects with a BP above or below the median (data not shown). Finally, the bioavailability of the red wine polyphenols in our subjects was not assessed. However, in a separate bioavailability study, concentrations of urinary hippuric acid and plasma resveratrol were measured in 35 subjects after ingestion of a comparable, though not similar wine grape extract, containing in total 870 mg polyphenols of which 560 mg derived from the Provinols wine extract used in the present study. The extract was incorporated in dairy drink (containing 3.4% protein) or gelatin capsules (R. Draijer, PhD, unpublished data, 2007). Hippuric acid is an indicator of gut microbiota-mediated degradation of dietary polyphenols[35] and resveratrol has been suggested to be one of the vasoactive components in red wine.[36] This study showed that the bioavailability of phenolic compounds was not significantly affected when incorporated into a dairy matrix compared to gelatin capsules. Although we cannot extrapolate these results to our study with a different polyphenol composition, it seems that dairy products have no or only a marginal influence on the bioavailability of the various polyphenols present in red wine extract.

In summary, we have shown in a randomized placebo-controlled, well-powered study that a four-weeks intake of red wine polyphenols did not reduce peripheral or central BP in subjects with high normal BP or grade 1 hypertension. The reported cardiovascular benefits of red wine consumption may still be present, but our findings suggest that this occurs in a blood pressure-independent manner. Consumption of red wine extract with the objective to reduce blood pressure is not supported by our findings and therefore should not be advised.

Acknowledgements

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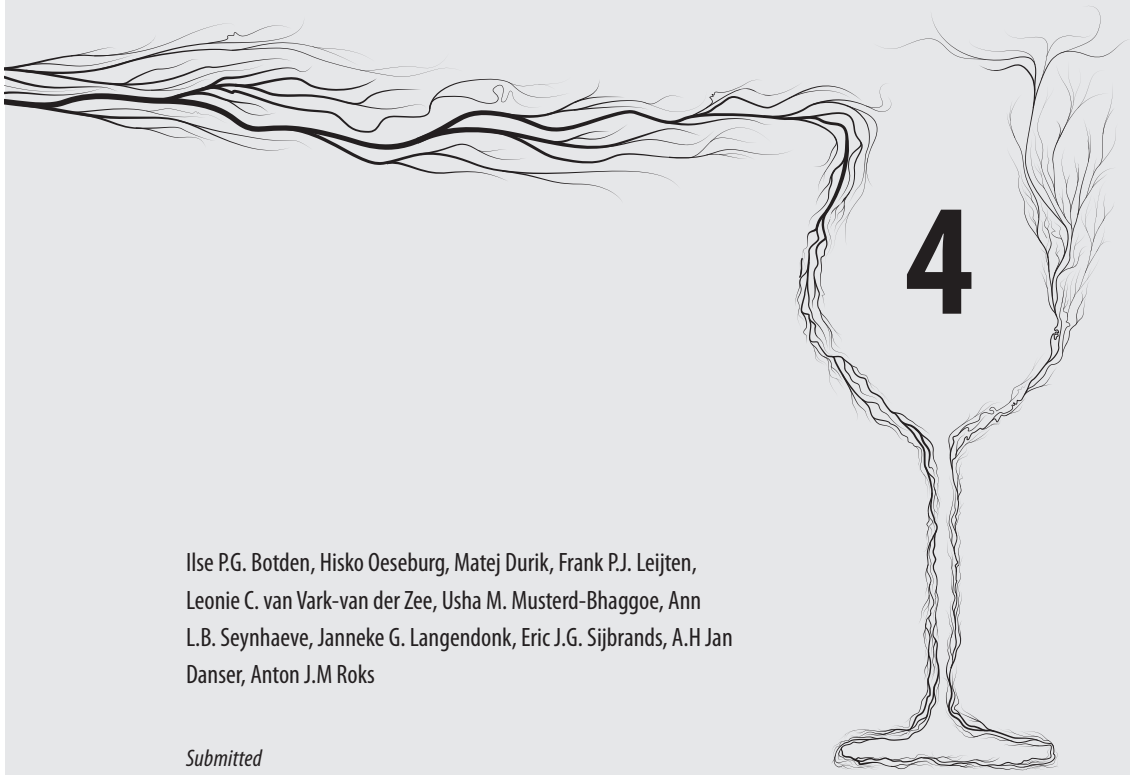
Red wine extract protects against oxidative stress-induced endothelial senescence through nitric oxide and prostaglandins



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Ilse P.G. Botden, Hisko Oeseburg, Matej Durik, Frank P.J. Leijten, Leonie C. van Vark-van der Zee, Usha M. Musterd-Bhaggoe, Ann L.B. Seynhaeve, Janneke G. Langendonk, Eric J.G. Sijbrands, A.H Jan Danser, Anton J.M Roks

Submitted

ABSTRACT

Objective. Red wine polyphenols may preserve endothelial function during aging. Endothelial cell senescence enhances age-related endothelial dysfunction. We investigated whether red wine extract (RWE) prevents oxidative stress-induced senescence in human umbilical vein endothelial cells (HUVECs).

Methods and Results. Senescence was induced by exposing HUVECs to *tert*-butylhydroperoxide (*t*BHP), 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 *t*BHP-induced DNA damage of endothelial cells, and induced relaxation of porcine coronary arteries. Inhibition of SIRT1 by sirtinol did not reverse the effect of RWE on *t*BHP-induced senescence, whereas both the nitric oxide (NO) synthase inhibitor L-NMMA and the cyclo-oxygenase (COX) inhibitor indomethacin totally abolished the senescence-protective effect of RWE. Furthermore, incubation of HUVECs with RWE increased eNOS and COX-2 mRNA levels as well as phosphorylation of eNOS at Ser1177.

Conclusions. RWE protects endothelial cells from *t*BHP-induced senescence. NO and prostaglandins, rather than 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 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 blood pressure and promotes thrombosis.[2, 3] DNA damage through excessive production of reactive oxygen species (ROS) is an important mechanism underlying endothelial senescence.[4] Currently, a large number of studies are ongoing that address the question if lifestyle or pharmacological intervention can inhibit this process.

Moderate consumption of red wine since long has been postulated to be part of a healthy life style.[5] Under controlled conditions in animal studies, red wine extract (RWE) 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, a versatile deacetylase that has been implicated in endothelial cell aging.[7-12] Recently, we have also shown that the release of nitric oxide (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 signaling 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 we used is Provinols (Seppic, France). This RWE is derived from red wine produced in the Languedoc-Roussillon regions in the South-East of France. RWE contained 632 mg polyphenols/g, determined as gallic acid equivalents using Folin Ciocalteu reagent. The specific polyphenol contents of this RWE has been described elsewhere as RWPC2.[15] The specific polyphenol contents in the red wine extract as used here were assessed by HPLC analysis and this revealed that 550mg of wine solids contained 18.8 mg anthocyanins, 6.9 mg phenolic acids, 4.0 mg catechins, 0.4 mg flavonols and 0.1 mg stilbenes.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion as described by Jaffe et al.[16] HUVECs were cultured on 0.2% gelatin coated plates in HUVEC culture medium containing human endothelial-serum free medium and Dulbecco's modified Eagle's medium (Invitrogen), 10% heat–inactivated newborn calf serum, 5% heat-inactivated human serum (Lonza), 10 ng/mL human recombinant basic fibroblast growth factor, and 50 ng/mL human recombinant epidermal growth factor (Peprotech) in a humidified incubator at 37 °C and 5% CO_2. Experiments were conducted on cells with a passage number between 3 and 9.

Design of the pharmacological studies

HUVECs were seeded at a density of 5000 cells/cm^2. After 24 hours, cells were starved with DMEM + 0.5% fetal calf serum for at least 6 hours. 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 cyclooxygenase (COX) inhibitor indomethacin, the NO synthase inhibitor N^G-Methyl-L-arginine acetate salt (L-NMMA; Sigma-Aldrich), or the SIRT1 inhibitor sirtinol (Calbiochem). After one hour, tert-butylhydroperoxide (*t*BHP; Sigma-Aldrich) was added to the medium for 2 hours to induce senescence. Subsequently, the medium was replaced with HUVEC culture medium with or without RWE, indomethacin, L-NMMA or sirtinol.

Apoptosis

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

Evaluation of the number of senescent cells

When cells reached confluency, they were fixated in 2% formaldehyde / 0.2% glutaraldehyde for 10 minutes and the number of senescent cells was determined by senescence-associated SA-β-gal staining (150 mmol/L of NaCl, 2 mmol/L of $MgCl_2$, 5 mmol/L of $K_3Fe(CN)_6$, 5 mmol/L of $K_4[Fe(CN)_6]$, 140 mmol/L of Na_2HPO_4, 40 mmol/L citric acid, and 1 mg/mL of 5-bromo-4-chloro-3indolyl-β-*D*- galactoside, pH 6.0 for 18 hours at 37°C).[17] Cells were counterstained with 4′,6-diamidino-phenylindole (DAPI; 2 µg/mL) to

allow total cell number counting. Light 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, 4 random fields were evaluated.

DNA damage assay

DNA damage was determined by single nuclei electrophoresis, also called comet assay.[18] Cells were harvested and approximately 700 cells were placed on a Trevigen Cometslide in 0.7% low melting agarose (Serva). Cells were lysed for one hour in Trevigen lysis solution, followed by 30 minutes of denaturation by 300 mmol/L alkaline solution and 1 mmol/L EDTA at pH>13 which was followed by 30 minutes of electrophoresis at 1 volt / cm in 300 mmol/L alkaline solution and 1 mmol/L EDTA at pH>13. DNA was stained with 1x SYBR Green (Invitrogen) and photos were taken with a 10x objective (Zeiss Axiovert 200M). Olive tail moment (percentage of DNA in the tail x distance to center of gravity of tail) was determined with CASP 1.2.2 software.[19] Experiments were repeated 3 times and per experiment more than 100 comets were analysed per treatment group.

Western blot analyses

Cultured cells were lysed with 50 mmol/L TrisHCl, pH 7.4, 150 mmol/L NaCl, 10 mmol/L Igepal CA-630, 5 mmol/L deoxycholic acid, and 1 mmol/L sodium dodecyl sulfate, in the presence of protease inhibitor cocktail (Roche) and serine-threonine phosphatase inhibitor cocktail 3 (Sigma-Aldrich). Lysates were analyzed by standard Western blotting techniques under denaturating conditions. The following antibodies were used: anti-p21 (12D1, Cell Signalling), anti-p53 (DO-1, Sigma-Aldrich), anti acetylated p53 (Lys 382, Cell Signalling), anti-eNOS (C-20, Santa Cruz), anti phosphorylated eNOS (Ser1177, Santa Cruz), and anti-actin (C4, Millipore) for normalization of the protein levels. Signals were detected by enhanced chemiluminescence detection method and quantified by densitometry.

Real-time quantitative 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). Four nanograms of cDNA was amplified by real-time polymerase chain reaction (qPCR) and normalized to 36B4 as an endogenous control. Each reaction was performed in duplo with SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used: SIRT1 forward 5'-AGGCCACGGATAGGTCCATAT-3, reverse 5'-CCAATCATAAGATGTTGCT-

GAAC-3; eNOS forward 5′-CTTCCGCTACCAGCCAGAC-3′, reverse 5′-TCTCGGAGCCATA-CAGGATT-3′; COX-2 forward 5′-CCCAGCACTTCACGCATCAG-3′, reverse 5′-AGACCAG-GCACCAGACCAAAGACC-3′.

Vascular reactivity studies

Methods were set up as previously described.[20] Briefly, porcine coronary arteries (PCAs) were obtained from 8 slaughterhouse pigs. The arteries were cut into segments and suspended in 15-ml organ baths containing Krebs bicarbonate solution, aerated with 95% O_2 / 5% CO_2 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 Nω-nitro-L-arginine methyl ester (L-NAME; 100 µmol/L), the intermediate- and small-conductance Ca^{2+}-dependent K^+-

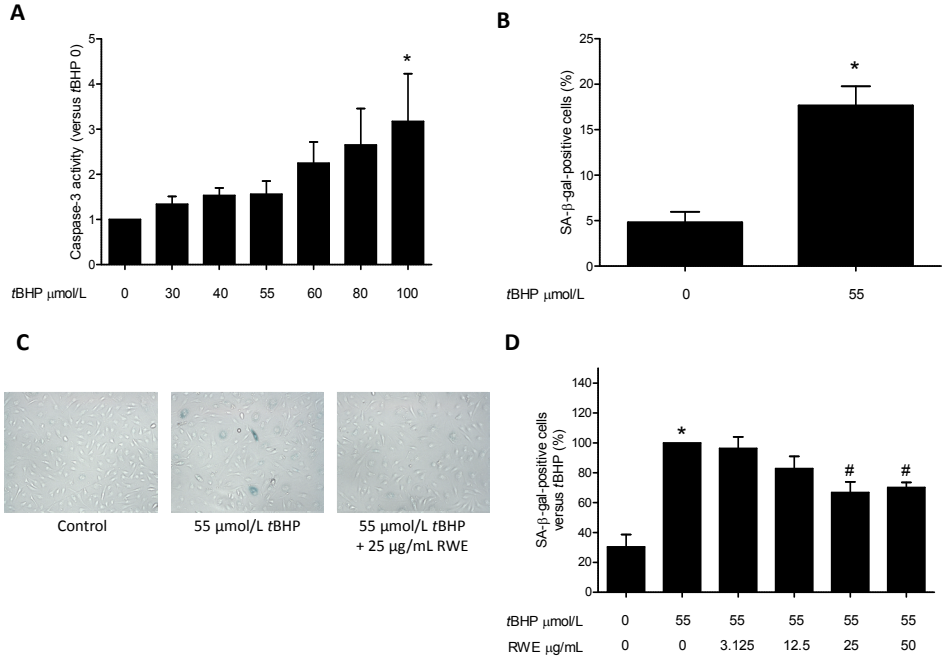

Figure 1. A, The effect of different concentrations of *t*BHP on apoptosis in HUVECs as analyzed by caspase-3 activity at 24 hours after addition of *t*BHP (*P<0.05 versus control; n=3-5). B, The effect of 55 µmol/L *t*BHP for 2 hours on endothelial senescence in HUVECs as judged by SA-β-gal staining at 48 hours after addition of *t*BHP (*P<0.05; n=6). C and D, Examples of SA-β-gal staining (C) and the effect of increasing concentrations of RWE (0-50 µg/mL) on *t*BHP-induced senescence in HUVECs as judged by SA-β-gal staining at 48 hours after addition of *t*BHP. D, Percentage SA-β-gal positive cells expressed relative to the 55 µmol/L *t*BHP group (*P<0.05 versus control, #P<0.05 versus 55 µmol/L *t*BHP; n=5-6).

channels inhibitors TRAM34 (10 µmol/L) and apamin (100 nmol/L), or indomethacin (10 µmol/L). Vessels were then preconstricted with 9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin F2α (U46619; 0.1–1µmol/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 mean values ± SEM in the text and figures. Differences between the groups of the cell culture experiments, in which treatments were always performed in parallel, were analyzed using two-tailed paired Student *t* tests or 1-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons, unless indicated otherwise. Differences between groups in vascular reactivity studies were analyzed using the general linear model for repeated measures. Probability values 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 *t*BHP for 2 hours. To ensure that apoptosis did not bias the results, we measured caspase-3 activity (an indicator of apoptosis) after *t*BHP exposure. Caspase-3 increased at *t*BHP 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 *t*BHP.

Exposure of HUVECs to 55 µmol/L *t*BHP increased the percentage of senescent cells by 3.6 fold (Figure 1B). Treatment with RWE concentration-dependently decreased endothelial senescence by maximally 33±7.1% (Figure 1C and D). Removing RWE immediately before the application of *t*BHP yielded a similar effect (n=5; data 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).

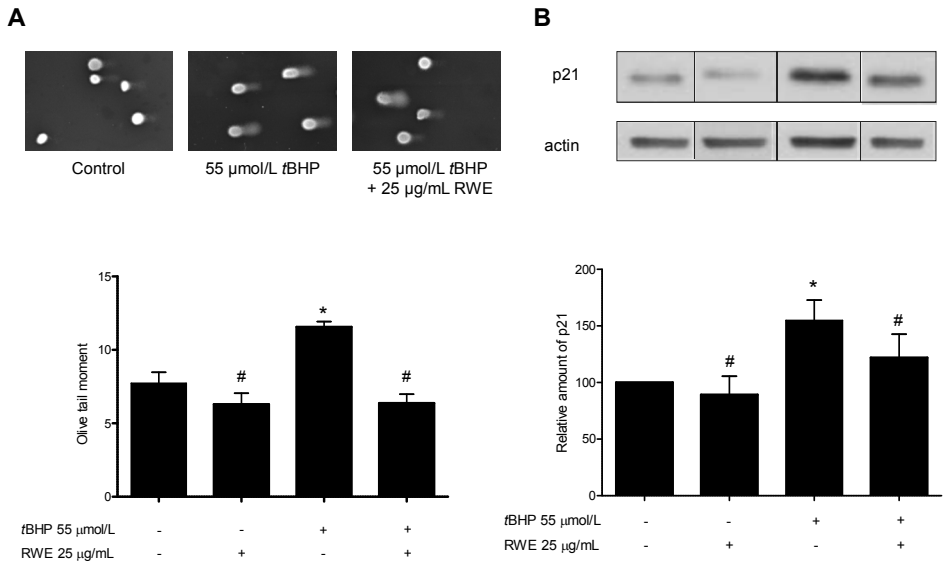

Figure 2. A, Effect of RWE on DNA damage induced by *t*BHP, as assessed by comet assay. Top panel shows examples of single cell electrophoresis 24 hours after no treatment (control), 55 µmol/L *t*BHP or 55 µmol/L *t*BHP + 25 µg/mL RWE (*P<0.05 versus control, #P<0.05 versus *t*BHP; n=3). B, Expression of p21 protein levels 24 hours after no treatment, 55 µmol/L *t*BHP, 55 µmol/L *t*BHP + 25 µg/mL RWE or 25 µg/mL RWE, as measured by western blot. P21 protein levels are corrected for actin protein levels (*P<0.05 versus control, #P<0.05 versus *t*BHP; n=6-7).

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

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

RWE increased SIRT1 gene expression (by 14±10% at 10 minutes of exposure, n=8, P<0.05; data not shown). However, the SIRT1 activator resveratrol did not prevent *t*BHP-induced senescence (Figure 3A), although it did cause the typical morphological elongations described earlier.[21] In fact, resveratrol in a concentration of 50 µmol/L even increased senescence. The SIRT1 inhibitor sirtinol, like *t*BHP, increased the number of senescent cells, although the effects were not additive (Figure 3B). Yet, it did not reverse the effect of RWE on *t*BHP-induced senescence. Sirtinol increased acetylation of the senescence-associated tumor suppressor protein p53, and decreased total p53 by »50% (Figure 3C and D). RWE blocked the effect of sirtinol on acetylation without altering total p53.

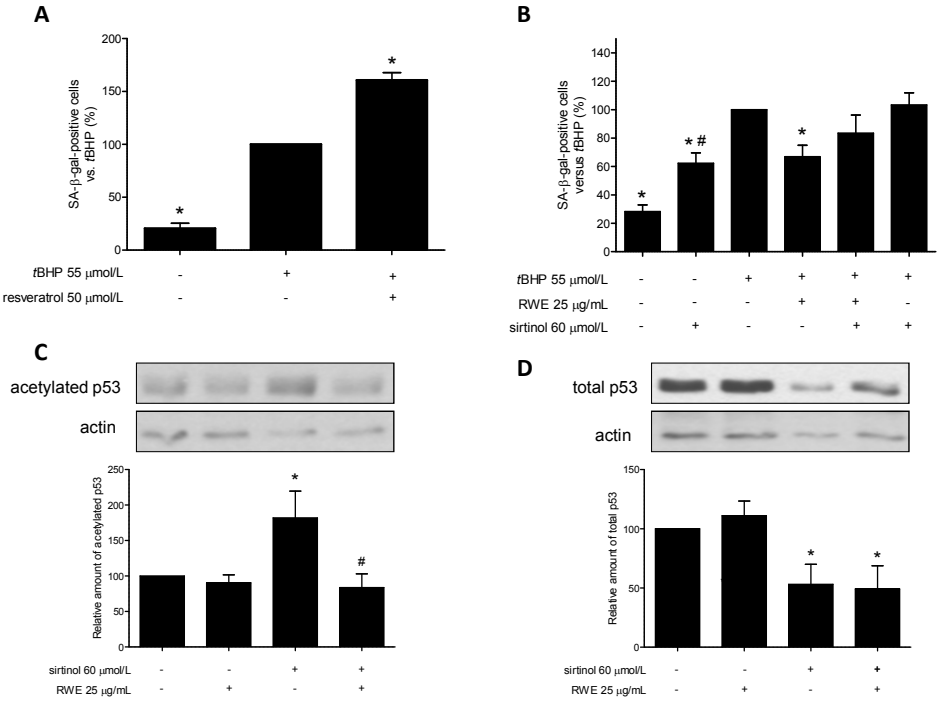

Figure 3. A, The effect of resveratrol (50 µmol/L) on *t*BHP-induced senescence in HUVECs according to SA-β-gal staining at 48 hours after addition of *t*BHP. Percentage SA-β-gal positive cells are expressed relative to 55 µmol/L *t*BHP group (*P<0.05 versus *t*BHP; n=3). B, The effect of sirtinol (60 µmol/L) and RWE (25 µg/mL) on *t*BHP-induced senescence in HUVECs according to SA-β-gal staining at 48 hours after addition of *t*BHP. The percentage of SA-β-gal positive cells are expressed relative to 55 umol/L *t*BHP group (*P<0.05 versus BHP, #P<0.05 versus control; n=5-6). C and D, levels of p53 acetylation (C) and total p53 (D) protein levels 24 hours 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 blot. Both acetylated and total p53 levels are corrected for actin levels (*P<0.05 versus control, #P<0.05 versus sirtinol; n=4-8).

RWE and oxidative stress-induced endothelial senescence according to the activation of eNOS and prostaglandins

RWE upregulated eNOS and COX-2 in HUVECs (Figure 4A), and both L-NMMA (Figure 4B) and indomethacin (Figure 4C) abolished the protective effect of RWE in *t*BHP-induced senescence, whereas these drugs were without effect when given alone. RWE increased levels of Ser1177-phosphorylated eNOS, and addition of *t*BHP did not significantly decrease this effect (Figure 4D).

Finally, RWE relaxed preconstricted PCAs in an NO- and endothelium-derived hyperpolarizing factor (EDHF)-dependent manner, as evidenced by the blockade obtained with

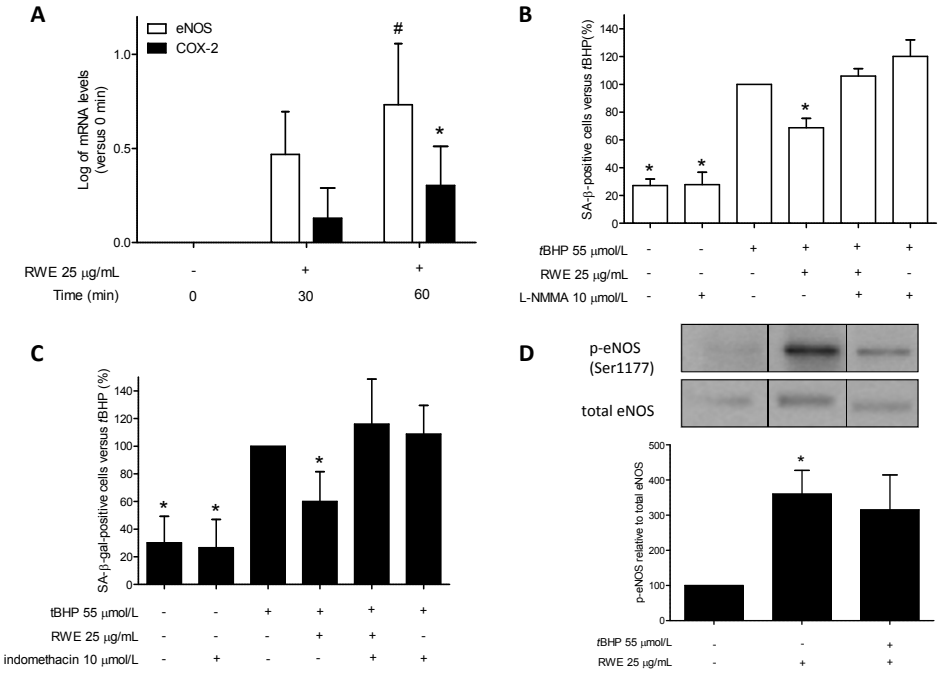

Figure 4. A, eNOS and COX-2 mRNA levels in HUVECs after treatment with 25 µg/mL RWE for different time periods, as analyzed by real-time polymerase chain reaction. RNA is normalized to the internal control 36B4 (mean values are expressed as logarithmic values; *P<0.05 versus control, #P=0.058 versus control; n=5-8). B, The effect of L-NMMA (10 µmol/L) and RWE (25 µg/mL) on *t*BHP-induced senescence in HUVECs as judged by SA-β-gal staining at 48 hours after addition of *t*BHP. Percentage SA-β-gal positive cells are expressed relative to 55 µmol/L *t*BHP group (*P<0.05 versus *t*BHP; n=6-7). C, The effect of indomethacin (10 µmol/L) and RWE (25 µg/mL) on *t*BHP-induced senescence in HUVECs according to SA-β-gal staining at 48 hours after addition of *t*BHP. Percentage SA-β-gal positive cells are expressed relative to 55 µmol/L *t*BHP group (*P<0.05 versus *t*BHP; n=6-7). D, Levels of phosphorylated eNOS (p-eNOS) at Ser^{1177} 2 hours after no treatment, 25 µg/mL RWE, or 55 µmol/L *t*BHP + 25 µg/ml RWE, as measured by Western blot. P-eNOS levels are corrected for total eNOS levels (*P<0.05 versus control; n=4-5).

L-NAME and/or TRAM34 + apamin (Figure 5). Indomethacin did not block vasorelaxation, but rather seemed to increase the RWE-induced vasodilation.

DISCUSSION

Our results show that RWE protects endothelial cells from *t*BHP-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 was depen-

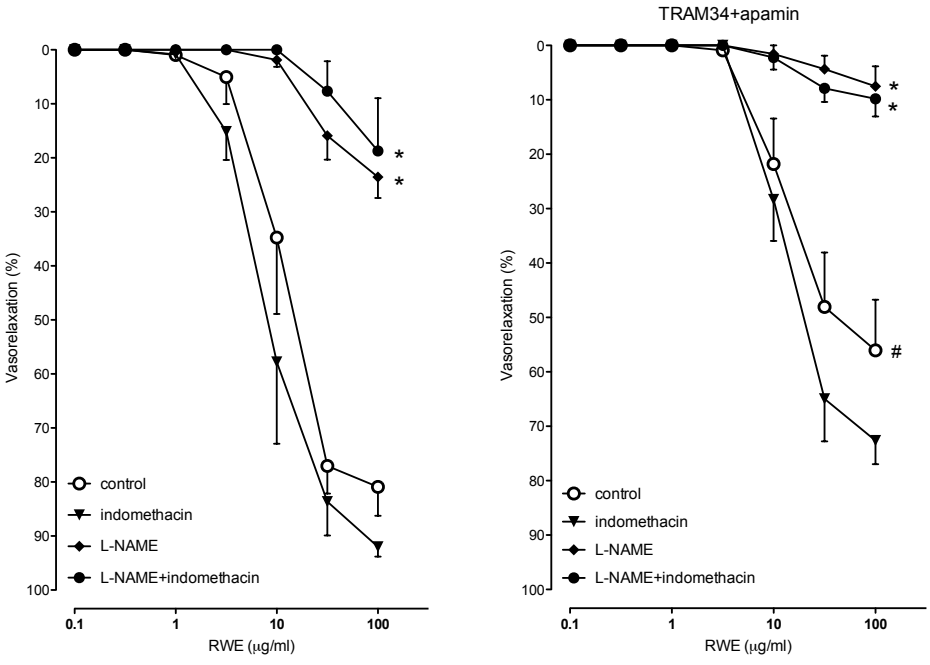

Figure 5. Concentration–response curves of U46619-preconstricted PCAs to cumulative doses of RWE, in the absence (A) and presence (B) of 10 μmol/L TRAM34 in combination with 100 nmol/L apamin (TRAM34+apamin), with or without 100 μmol/L L-NAME and 10 μmol/L indomethacin. Data are expressed as percentage of the contraction induced by U46619 (*P< 0.05 vs. control, #P<0.05 vs. control without TRAM34+apa; n=6-8).

dent on NO and prostaglandin release, and associated with upregulation of eNOS and COX-2. SIRT1 did not play a critical role.

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 introduce a novel protective effect of RWE that might be closely associated with this previous finding in aged rats.

Based on 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[22] and by promoting eNOS activity.[23] Moreover, resveratrol-containing red wine decreases the levels of assymetric dimethylarginine, an endogenous inhibitor of NO,

in a SIRT1-dependent manner.[24] In line with these observations, we observed that the SIRT1 inhibitor sirtinol increased endothelial senescence and acetylation of p53, and RWE prevented the latter. In addition, RWE modestly upregulated SIRT1 expression. Yet, sirtinol did not reverse the effect of RWE on *t*BHP-induced senescence. Combined with our observation that resveratrol increased *t*BHP-induced senescence, these data suggest that RWE did not act through SIRT1. Furthermore, deacetylation of p53 by SIRT1 is not necessarily involved in the protective effect of RWE on senescence. The observation that SIRT1 and resveratrol were not involved in stress-induced premature senescence is complementary to recent studies[25, 26] disputing the claim that resveratrol activates SIRT1 and thereby increases longevity.[10, 27] Moreover, 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, Rennes, France), 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,[28] which may explain the effect on *t*BHP-induced senescence in our experiments. We wondered whether the release of the signal factors NO and prostaglandins are involved in the protective effect of red wine against *t*BHP-induced senescence. We found that blockade of either NOS or COX reversed the senescence-inhibitory effect of RWE. This demonstrates that RWE prevents the onset of cellular senescence via pathways involving both NO and prostaglandins. Red wine has been shown to upregulate eNOS mRNA and protein expression in endothelial cells,[29] and RWE increases endothelial NO production.[9] Consistent with these findings, we found that RWE increased eNOS mRNA levels and augmented phosphorylation of eNOS within one hour.

Prostacyclin production decreases during *in vitro* aging of endothelial cells, and this may also stimulate senescence.[30] The RWE-induced upregulation of COX-2 and the inhibitory effect of the non-selective COX-2 inhibitor indomethacin support this view. Given the identical blockade obtained with L-NMMA and indomethacin towards the effect of RWE on senescence, it seems reasonable to assume that NO and prostaglandins are sequentially involved in the protective effect of RWE. To test this hypothesis for signaling of endothelial cells, releasing NO and prostacyclin towards smooth muscle cells, we made use of PCAs which are known to display an NO-mediated relaxant effect to RWE.[7] Our data show that this relaxant effect additionally involves intermediate- and small-conductance Ca^{2+}-dependent K^{+}-channels. Yet, under no condition did indomethacin block the relaxant effect of RWE - if anything, it tended to increase the relaxant effect of RWE, thereby arguing against the concept that NO and prostaglandins sequentially

mediate vasorelaxation. Clearly, RWE-induced relaxation and inhibition of senescence did not share the same pathways.

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.[31] 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,[32] 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 prostaglandins, rather than activation of 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 cardiovascular diseases.

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Variants in the *SIRT1* gene may affect diabetes risk in interaction with prenatal exposure to famine

5

Based on:
Ilse P.G. Botden, M. Carola Zillikens, Susanne R. de Rooij, Janneke G. Langendonk, A.H. Jan Danser, Eric J.G. Sijbrands, Tessa J. Roseboom

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ABSTRACT

Objective. Fetal malnutrition predisposes to type 2 diabetes. SIRT1, a nutrient-sensing histone deacetylase, is involved in glucose and insulin metabolism by regulating expression of various transcription factors in different tissues. We hypothesized that *SIRT1* genetic variants might interact with fetal malnutrition influencing the risk of type 2 diabetes.

Research Design and Methods. In 793 individuals of the Dutch Famine Birth Cohort Study (*n*=337 exposed, *n*=456 unexposed), we analyzed the interaction between three *SIRT1* tagging single nucleotide polymorphisms (SNPs) and prenatal exposure to famine on type 2 diabetes risk later in life.

Results. In the total population (exposed and unexposed), *SIRT1* variants were not associated with type 2 diabetes. A significant interaction was found between two *SIRT1* SNPs and exposure to famine in utero on type 2 diabetes risk (p=0.03 for rs7895833; p=0.01 for rs1467568, adjusted for gender and BMI). Minor alleles of these SNPs were associated with lower prevalence of type 2 diabetes only in individuals who had been exposed to famine prenatally (OR for rs7895833 0.50, p=0.06; OR for rs1467568 0.48, p=0.02). No interactions were found for glucose and insulin levels, nor BMI. However, BMI was significantly higher in carriers of these two genetic *SIRT1* variants compared to non-carriers after exposure to famine prenatally.

Conclusions. When exposed to famine in utero, carriers of common variants in the *SIRT1* gene had 50% lower risk of developing diabetes. SIRT1 may be an important genetic factor involved in fetal programming during malnutrition, influencing type 2 diabetes risk.

INTRODUCTION

Fetal malnutrition or low birth weight may predispose to type 2 diabetes.[1-3] Fetal programming offers an organism the ability to develop while adapting to environmental and nutritional signals in early life. However, this may have consequences in adulthood.[4-5] An unfavorable environment in utero, e.g. restricted nutrient supply, may alter gene expression profiles in metabolic and growth pathways by epigenetic mechanisms.[5] Both the genome and epigenome influence the mature phenotype and determine the sensitivity to develop diseases like cardiovascular diseases and type 2 diabetes later in life.[4]

The silent information regulator 2 (Sir2 or sirtuin; an NAD^+-dependent histone deacetylase) has been associated with longevity in lower organisms.[6] Humans have seven sirtuins (SIRT1-7), of which SIRT1 has been studied most extensively.[7] SIRT1 activation leads to epigenetic alterations by direct deacetylation of histones as well as by promoting methylation of DNA, both leading to repression of transcription.[8] Furthermore, it controls cell metabolism by deacetylation of non-histone targets. SIRT1 activity is regulated by changes in NAD^+/NADH ratio and can be influenced by dietary factors. Fasting can modulate this ratio and thereby SIRT1 activity.[9]

Based on the role of SIRT1 in epigenetic and glucose regulation and its sensitivity to dietary factors, we hypothesized that genetic variants in *SIRT1* may interact with fetal malnutrition influencing type 2 diabetes risk later in life. We addressed this question in the Dutch Famine Birth Cohort, since it contains information on both exposure to famine during gestation and incidence of type 2 diabetes in adulthood. We also analyzed plasma glucose and insulin levels and body mass index (BMI) to investigate potential underlying mechanisms.

RESEARCH DESIGN AND METHODS

The Dutch Famine Birth Cohort is composed of individuals born as term singletons in Amsterdam around the famine in the Netherlands during World War II, as described in detail earlier.[10] 2,414 singletons were born between 1 November 1943 and 28 February 1947. 810 out of 1,423 invited persons agreed to participate in the cohort study in 2002. The study was approved by the local Medical Ethics Committee. All participants gave written informed consent. Exposure to famine was defined according to official daily food rations for the general population aged ≥21 years. Prenatal exposure to famine was

defined as a daily food ration for the pregnant mother of less than 1,000 calories during any 13-week period of gestation.

Study parameters

Information on medical history, lifestyle, and medication was derived from standardized interviews with participants at the age of about 58 years. Information on each mother, her pregnancy, and her baby's size at birth was derived from medical birth records.

An oral glucose tolerance test (OGTT) was performed in all participants, except in pre-existent diabetic patients, who were defined as using any antidiabetes medication. Type 2 diabetes was defined as pre-existent diabetes or fasting glucose levels >7.0 mmol/l or 120-min glucose level >11.0 mmol/l in the OGTT.

The primary outcome measure of the current study was type 2 diabetes. Secondary outcome measures were glucose-metabolism parameters (120-min glucose and insulin levels, area under the curve (AUC) for glucose and insulin), and BMI.

Genotyping

DNA was available of 793 subjects for Taqman allelic discrimination assays. Three tagging single-nucleotide polymorphisms (SNPs) were selected covering most of the common variants (minor allele frequency >10%) of the *SIRT1* gene in Caucasians: rs7895833, rs1467568, and rs497849, as described earlier [11]. Minor allele frequencies of the SNPs were 19%, 36%, and 22%, respectively. The SNPs were in Hardy-Weinberg equilibrium ($X^2 < 3.1$; 1 df; $P > 0.07$). Correlation between SNPs was measured as correlation coefficient (r^2), which was 0.41 between rs7895833 and rs1467568, 0.07 between rs7895833 and rs497849, and 0.16 between rs1467568 and rs497849.

Haplotyping

We used the Phase program to select four common multimarker haplotypes with a frequency >10% from the three SNPs. Haplotype alleles were numbered in order of decreasing frequency in the population. The frequencies of the most common haplotypes were 40.3% for haplotype 1, 22.7% for haplotype 2, 19.6% for haplotype 3, and 17.2% for haplotype 4. Haplotype 1 contains the major alleles of the three SNPs. Since rs497849 fully tags haplotype 2, and rs7895833 fully tags haplotype 3, only haplotype 1 and 4 were analyzed in addition to the SNPs. Subjects were grouped according to genotype.

Groups were defined as non-carriers (no allele copy number) or carriers (1 or 2 allele copy numbers) of haplotype alleles.

Statistical methods

Continuous variables are expressed as means±SD. Comparisons between groups were analyzed with *t* tests for continuous variables, and with X^2 tests for dichotomous variables.

We investigated associations of genetic variants in *SIRT1* and type 2 diabetes risk with binary logistic regression. Associations of *SIRT1* genetic variants and BMI, AUC, and 120-min levels of glucose and insulin were investigated with linear regression.

Genotype by prenatal famine exposure interactions were tested by creating interaction terms for each genetic variant with the exposure group. The genetic variant was coded 0 for non-carriers of SNP minor alleles or haplotypes. The genetic variant was coded 1 for SNP minor allele carriers or haplotype carriers. The exposure group was coded 0 for unexposed subjects and coded 1 for exposed subjects. Unexposed subjects were subjects born before or conceived after the Dutch famine. A $P<0.05$ was considered significant.

RESULTS

General characteristics

Table 1 shows birth characteristics, maternal characteristics and general characteristics of the individuals at the age of 58 years according to exposure to famine. Birth weight of the children who had been exposed to famine in in utero was on average 161 gram lower than birth weight of the children born before or conceived after the famine ($P<0.001$). In adulthood, BMI and waist-hip ratio were slightly lower in subjects who had been exposed to famine.

Differences in diabetes risk between exposed and unexposed

337 subjects were exposed to famine in utero, and 456 subjects were born before or after the famine. In adulthood, BMI and type 2 diabetes prevalence were not different between subjects exposed and non-exposed to famine. 117 of the 791 individuals had developed diabetes in adulthood (15.8% in the exposed and 14.1% in the unexposed group). Plasma glucose levels at 120-min of the OGTT were 0.37 mmol/l ($P=0.03$) higher

Table 1. Birth characteristics, maternal characteristics, and general characteristic of the individuals at the age of 58 years among subjects exposed and unexposed to the Dutch famine

	No exposure to famine in utero	Exposure to famine in utero	*P*	n
n	456	337		-
Birth characteristics				
Gestational age (days)	284.7±11.0	285.2±11.5	0.55	677
Birth weight (g)	3427.5±465.4	3266.5±460.1	<0.001	793
Maternal characteristics				
Age at delivery (years)	28.6±6.3	29.4±6.5	0.08	793
Primiparous (%)	37.3	29.1	0.02	793
Weight gain in third trimester (kg)	3.1±2.5	2.5±3.5	0.03	554
Weight at last antenatal visit (kg)	67.9±8.6	64.5±8.5	<0.001	699
General characteristics at age of 58 years				
% men	48.9	42.1	0.06	793
Current smoking (%)	21.9	28.0	0.05	789
BMI (kg/m^2)	28.8±5.0	28.1±4.6	0.05	788
Waist (cm)	97.5±13.5	95.6±13.1	0.05	791
Waist-hip ratio	93.2±8.8	91.9±9.0	0.03	786

Data are means ± SD, except where given as numbers and percentages. BMI, body mass index.

for those exposed to famine in utero. Plasma insulin levels at 120-min of the OGTT also tended to be higher in the exposed group (difference 23.29 pmol/l, *P*=0.14).

Differences in diabetes risk, BMI, and glucose/insulin levels between SIRT1 genetic variants

Table 2 shows associations between *SIRT1* genetic variants (three SNPs and two haplotypes) and type 2 diabetes in the total population (i.e. exposed and unexposed individuals together), depicted as odds ratios. The table also shows comparisons for carriers of SNP minor alleles or haplotypes with non-carriers for BMI and logarithm of 120-min glucose and insulin levels during OGTT. This is depicted as ß-coefficients (95% CI) derived from linear regression analyses.

Carriers of the minor allele of rs7895833 (AG and GG) had a higher BMI than non-carriers (AA; ß: 0.82, 95% CI: 0.09 to 1.56; *P* =0.028). Carriers of the minor allele of rs1467568 (AA and GA) also had a higher BMI than non-carriers (GG; ß: 0.96, 95% CI: 0.25 to 1.67; *P* = 0.008). There was no association between any of the genetic *SIRT1* variants and type 2 diabetes risk nor between any of the variants and AUCs of glucose and insulin levels

Table 2. Associations with diabetes, BMI, plasma glucose levels, and plasma insulin levels by *SIRT1* genotype in the total population

SNP/ Haplotype	Major/ minor allele	OR DM (95% CI)	ß BMI (kg/m^2) (95% CI)	ß log glucose 120 min (mmol/l) (95% CI)	ß log insulin 120 min (pmol/l) (95% CI)
rs7895833	A/G	0.85 (0.54-1.33)	0.82(0.09 to 1.56)*	-0.02(-0.08 to 0.04)	-0.05(-0.17 to 0.07)
rs1467568	G/A	0.81(0.53 -1.25)	0.96 (0.25 to 1.67)*	-0.05(-0.10 to 0.01)	-0.08(-0.20 to 0.03)
rs497849	G/A	0.86(0.55 - 1.36)	-0.21(-0.95 to 0.54)	-0.06(-0.11 to 0.00)*	-0.08(-0.21 to 0.04)
Haplotype 1	N.A.	1.12(0.70 - 1.79)	-0.57(-1.34 to 0.20)	0.07(0.01 to 0.13)*	0.08(-0.05 to 0.21)
Haplotype 4	N.A.	0.71(0.43 - 1.18)	0.03(-0.77 to 0.82)	-0.05(-0.11 to 0.01)	-0.01(-0.14 to 0.12)

All models were adjusted for gender and BMI. *P<0.05. OR, odds ratio; DM, type 2 diabetes; BMI, body mass index.

during OGTT (data not shown). None of the SNPs or haplotypes were associated with birth weight or famine exposure (data not shown).

Interactions between genetic SIRT1 variants and prenatal exposure to famine on type 2 diabetes risk and BMI

Next, we investigated interactions of the *SIRT1* SNPs and haplotypes with prenatal exposure to famine on type 2 diabetes risk and BMI at the age of 58 years. An interaction between prenatal exposure to famine and rs7895833 (OR: 0.35, 95% CI: 0.14 – 0.89; P = 0.03), and between prenatal exposure to famine and rs1467568 (OR: 0.32, 95%CI: 0.14 – 0.78; P = 0.01) significantly influenced diabetes risk but not BMI. We further analysed these interactions by performing stratified analyses in exposed and unexposed subjects. In subjects who had not been exposed prenatally to famine there was no significant association between rs7895833 and type 2 diabetes (OR: 1.31, 95% CI: 0.72 – 2.37; P = 0.37). In subjects who had been exposed there was a borderline significant association between rs7895833 and type 2 diabetes (OR: 0.50, 95% CI: 0.24 – 1.03; P = 0.058). For rs1467568, the association with diabetes was also not significant in subjects who had not been prenatally exposed (OR: 1.33, 95% CI: 0.72 – 2.46; P = 0.36), but significant for those who had been exposed (OR: 0.48, 95% CI: 0.25 – 0.91; P = 0.02). Figure 1 shows the prevalence of type 2 diabetes stratified for prenatal exposure to famine for these SNPs. Interactions were not significant between prenatal exposure to famine and the other genetic variants (data not shown). There was no significant interaction between any of the *SIRT1* genetic variants and prenatal exposure to famine that influenced glucose and insulin values (data not shown).

There were also no significant interactions between *SIRT1* SNPs or haplotypes and prenatal exposure to famine on BMI at the age of 58 years (data not shown). However, because both rs7895833 and rs1467568 were associated with BMI in the total popula-

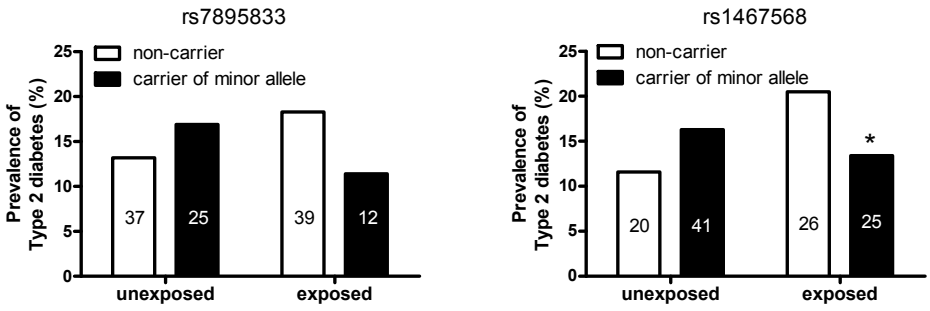

Figure 1. The relationship of the rs7895833 and rs1467568 *SIRT1* SNPs with the prevalence of diabetes according to prenatal exposure to famine. The number of subjects per group (i.e., diabetic patients) are shown inside each bar. *P<0.05 vs. non-carriers.

tion (exposed and unexposed), we decided to further analyse these associations by stratifying for exposure groups. For both these SNPs, BMI was significantly higher in carriers of minor alleles who had been prenatally exposed to famine compared to non-carriers (ß for rs7895833: 1.26, 95% CI: 0.20 to 2.31; $P = 0.02$; ß for rs1467568: 1.24, 95% CI: 0.21 to 2.28; $P = 0.018$). In subjects who had not been exposed to famine prenatally, no significant association was found, although the direction of the effect was the same (ß for rs7895833: 0.49, 95% CI: -0.52 to 1.49; $P = 0.34$; ß for rs1467568: 0.75, 95% CI: -0.22 to 1.73; $P = 0.13$).

DISCUSSION

Our results show that an interaction between two SNPs in *SIRT1* (rs7895833 and rs1467568) and in-utero exposure to malnutrition significantly influences type 2 diabetes risk in adulthood. Minor-allele carriers of these two genetic *SIRT1* variants who had been exposed to famine in utero had 50% lower risk of developing diabetes than non-carriers, but, surprisingly, a higher BMI. There was no interaction between SIRT1 genetic variants and prenatal malnutrition on plasma glucose or insulin levels.

This is the first study suggesting that diabetes risk in adulthood is influenced by an interaction between *SIRT1* genetic variants and prenatal exposure to famine. While it is not yet clear what accounts for this interaction, there are two possible mechanisms.

First, SIRT1 could influence type 2 diabetes risk by epigenetic regulation, as it can alter chromatin function by direct deacetylation of histones as well as by promoting methylation of histones and DNA, thus potentially repressing gene transcription. The gene-regulatory activity of SIRT1 is controlled by cellular NAD^+/NADH ratio.[8] Epigenetic

regulation is active mainly during fetal development, causing stable changes in the epigenome that persists throughout life. Its influence by maternal diet in pancreatic islets was investigated recently by Sandovici et al.[12] He showed that suboptimal nutrition in rats during early development led to epigenetic silencing and consequently to reduction in the expression of hepatocyte nuclear factor 4-α (Hnf4a), a transcription factor required for pancreatic β-cell differentiation and glucose homeostasis. Sandovici's study illustrates that transcriptional activity is responsive to environmental factors through changes in the epigenome. The authors suggested that histone-modifying enzymes, such as histon-deacetylases which are are particularly susceptible to intracellular fluctuations in NAD^+/NADH ratio, are a link between environmental components and gene function. Since SIRT1 was shown to deacetylate Hnf4a in cultured hepatocytes,[13] SIRT1 might provide the link between maternal diet and these epigenetic effects.

The second possible mechanism is that SIRT1 may influence type 2 diabetes risk by affecting ß-cell apoptosis induced by malnutrition in utero, which may also represent an epigenetic effect of SIRT1. In mice offspring Valat et al.[14] investigated how food restriction during gestation affects pancreas function later in life. As adult mice, the offspring had impaired glucose tolerance, decreased ß-cell mass, and reduced islet expression of most genes involved in ß-cell function. Previous research in the Dutch Famine Birth Cohort showed that individuals who had been exposed to famine in utero, had higher plasma glucose levels 2h after a standard oral glucose tolerance test (OGTT) at the age of 50 and 58 years than individuals born before or after the famine.[10] Two separate studies have shown a link between SIRT1 and ß-cell apoptosis. The first, by Tang et al,[15] demonstrated that overexpression of SIRT1 in diabetes-induced mice is able to prevent ß-cell death and glucose intolerance. The second, by Lafontaine-Lacasse et al,[16] showed that reduction of SIRT1 levels by glucosamine, an amino sugar, contributes to ß-cell apoptosis *in vitro*. Taken together, these studies suggest that SIRT1 may affect the decrease in ß-cell mass in adulthood induced by maternal food restriction and may consequently decrease the risk of developing type 2 diabetes.

We previously reported an interaction between variants of the PPAR-γ2 gene and the IGF2BP2 gene and exposure to famine in utero on the prevalence of impaired glucose tolerance and type 2 diabetes.[17-18] Whether SIRT1 interacts with these genes on pathways influencing diabetes risk, should be elucidated by further studies.

Unexpectedly, associations between two *SIRT1* SNPs and BMI in subjects who had been exposed to famine were in the opposite direction of those described in the literature. Three studies that previously investigated the relation between *SIRT1* genetic variants and BMI all had mutually consistent results.[19-21] Zillikens et al.[19] found that the SNPs

rs7895833, rs1467568, and haplotype 1 were associated with BMI in two large Dutch population-based cohorts. Although we found no significant interaction on BMI between two SNPs and exposure to famine in utero, BMI differed significantly among carriers and noncarriers of minor alleles who had been exposed to famine prenatally, while no such difference was present in those who had not been exposed to famine prenatally. One might thus speculate that our findings diverged from those in the literature because the effect of SIRT1 on BMI is dependent on in-utero conditions but due to limited number of subjects not exposed to famine prenatally, power issues may also play a role in these findings. However, the mechanism whereby SIRT1 genetic variants interacted with exposure to famine in utero on diabetes risk appears not to be mediated by BMI.

Only one other study has published a potential association between *SIRT1* genetic variants and type 2 diabetes risk. A significant association was found between type 2 diabetes and *SIRT1* SNP rs3758391 in 519 Mexican subjects with type 2 diabetes and 547 controls but this finding has so far not been replicated.[22] Moreover, two reports showed a significant interaction between *SIRT1* genetic variants and nutrition, the first involving niacin intake[11] and the second involving vitamin E intake.[23] It is possible that associations between *SIRT1* variants and diabetes risk can be found only when interaction with environmental factors are studied, such as fetal nutrition in our current study.

Our study has a number of limitations. The first is the relatively small number of individuals. We should nevertheless point out that this cohort is unique, as it enables us to study interaction with malnutrition in utero in a way that is hard to perform in any human experiment. While it would be desirable to replicate our findings in a second independent study population, it would be very difficult to find a truly similar cohort. Second, plasma glucose and insulin levels during OGTT were not assessed in participants with previously diagnosed diabetes. Therefore, we cannot exclude that glucose and insulin are related to *SIRT1* genetic variants and famine in utero explaining the relation we observed with diabetes risk. The third limitation is that the SNPs investigated were noncoding. However, they covered most of the common variants and may be linked to a functional variant. Finally, we did not correct for multiple testing. However, even if we had applied the stringent Bonferroni correction for testing five genetic variants (that are not totally independent), the interaction between rs1467568 and prenatal exposure to famine on type 2 diabetes risk would still have been statistically significant ($p=0.01$).

In conclusion, we have shown, although in a small cohort, that, when exposed to famine in utero, carriers of two common variants in the *SIRT1* gene have 50% lower risk of developing diabetes compared to non-carriers. We suggest that SIRT1 may play an important role in the fetal programming of type 2 diabetes through fetal undernutrition.

Our findings may offer opportunities for further research into the influence of SIRT1 on fetal programming and thus on diabetes risk in adulthood.

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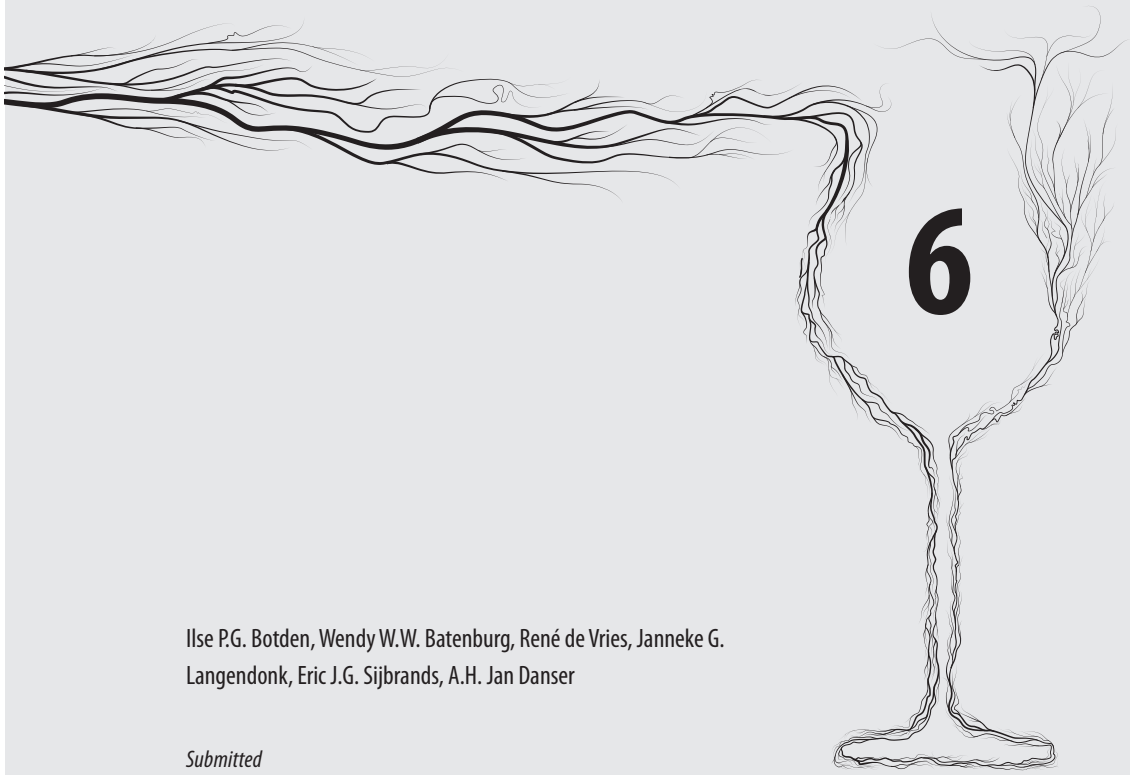
Nitrite- and nitroxyl-induced relaxation in porcine coronary (micro-)arteries: underlying mechanisms and role as endothelium-derived hyperpolarizing factor(s)



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Ilse P.G. Botden, Wendy W.W. Batenburg, René de Vries, Janneke G. Langendonk, Eric J.G. Sijbrands, A.H. Jan Danser

Submitted

ABSTRACT

Objective. To investigate the vasorelaxant efficacy of nitrite and nitroxyl (HNO) in porcine coronary (micro)arteries (PC(M)As), evaluating their role as endothelium-derived hyperpolarizing factors (EDHFs).

Methods. Preconstricted PCAs and PCMAs were exposed to UV light (a well-known inductor of nitrite; wavelength: 350-370 nm), nitrite, the HNO donor Angeli's salt, or bradykinin.

Results. UV light-induced relaxation of PCAs increased identically after endothelium removal and endothelial nitric oxide (NO) synthase (eNOS) blockade. UV light-induced relaxation diminished during Na^+-K^+-ATPase inhibition and *S*-nitrosothiol-depletion, and disappeared during NO scavenging with hydroxocobalamin or soluble guanylyl cyclase (sGC) inhibition with 1*H*-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ). Nitrite-induced relaxation of PCAs required mmolar levels, i.e., >1000 times endogenous vascular nitrite. Angeli's salt relaxed PCMAs more potently than PCAs, and this was due to the fact that HNO directly activated sGC in PCMAs, whereas in PCAs this occurred following its conversion to NO only. sGC activation by NO/HNO resulted in Na^+-K^+-ATPase stimulation and K_v channel activation. The HNO scavenger L-cysteine blocked bradykinin-induced relaxation in PCAs, and potentiated it in PCMAs. The latter did not occur in the presence of hydroxocobalamin, suggesting that it depended on L-cysteine-induced generation of vasorelaxant *S*-nitrosothiols. In all experimental setups, incubation with red wine extract mimicked the effects of ODQ.

Conclusion. Nitrite, via its conversion to NO and *S*-nitrosothiols, and HNO, either directly, or via its conversion to NO, mediate relaxant effects involving the sGC-cGMP pathway, Na^+-K^+-ATPase and/or K_v channels. Red wine extract counteracts these beneficial effects. NO blocks nitrite activation, and HNO, but not nitrite, may act as EDHF in the coronary vascular bed.

INTRODUCTION

Vascular nitric oxide (NO) originates from de novo synthesis by endothelial NO synthase (eNOS) and/or storage forms of NO. The latter comprise nitrite, nitrate, *S*-nitroso compounds, and *N*-nitroso compounds.[1] Their presence is not limited to endothelial cells. *S*-nitrosothiols activate endothelial intermediate-conductance and small-conductance Ca^{2+}-activated K^+-channels (IK_{Ca}, SK_{Ca}), and via soluble guanylyl cyclase (sGC), smooth muscle Na^+-K^+-ATPase.[2-4] Bradykinin dilates porcine coronary arteries (PCAs), at least in part, by stimulating the release of *S*-nitrosothiols from endothelial cells,[2-4] and *S*-nitrosothiols may thus act as endothelium-derived hyperpolarizing factors (EDHF). Importantly, *S*-nitrosothiols, by reacting with other thiols at physiological pH, yield nitroxyl (HNO), a recently discovered EDHF.[5-7] In addition, HNO can be produced by NOS in the absence of tetrahydrobiopterin.[8]

Light-induced vasorelaxation ('photorelaxation') also depends on *S*-nitrosothiols, which decompose to a disulfide and NO.[1, 4] Additionally, nitrite may undergo photolysis to NO.[9, 10] However, its photoactivity is about two orders of magnitude lower than that of *S*-nitrosothiols,[1] and apparent only when exposing vessels to UV light. Two different sources contributing to NO release following light exposure (i.e., *S*-nitrosothiols and nitrite) may explain why visible light and UV light induce relaxant responses that differ in length and intensity.[11] The *S*-nitrosothiol-dependent response is transient and can be demonstrated a second time only when the vessel is allowed to recover in the dark ('repriming').[4, 11] Repriming for the nitrite-dependent response has not yet been investigated in detail. *In vivo*, nitrite is believed to be the largest vascular storage pool of NO. Its reduction to NO depends on the reductase activity of deoxyhemoglobin, which is increased under hypoxic conditions.[12] Interestingly, *S*-nitrosothiols have also been described to be generated from nitrite-released NO.[13, 14]

Red wine consumption is associated with a reduced risk of cardiovascular disease,[15, 16] possibly because the polyphenols in red wine induce relaxation via NO and/or EDHF.[17-19] Yet, acutely red wine extract (RWE) inactivates sGC, most likely as a consequence of massive NO exposure.[18]

It was the aim of the present study to investigate the mechanisms underlying nitrite- and HNO-induced vasorelaxation in coronary arteries, evaluating their potential role as EDHFs. PCAs were exposed to UV light in the presence or absence of suppressors of *S*-nitrosothiols, NO and EDHF, allowing us to delineate the vasodilator actions of nitrite. Secondly, the responses of bradykinin and Angeli's salt (a HNO donor)[5, 6] were compared in PCAs and porcine coronary microarteries (PCMAs), two vessel types where,

respectively, NO and EDHF are the predominant mediators of the bradykinin-induced relaxant response.[20, 21] Finally, among the many inhibitors of EDHF pathways, we tested the consequences of sGC inactivation by RWE.

METHODS

Drugs

N^{ω}-nitro-L-arginine methyl ester HCl (L-NAME), N^{ω}-methyl-L-arginine acetate salt (L-NMMA), *p*-hydroxymercurobenzoic acid (PHMBA), ethacrynic acid, L-cysteine, indomethacin, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1one (ODQ), hydroxocobalamin, 4-aminopyridine, ouabain, glibenclamide, iberiotoxin, apamin, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM34), bradykinin, diethylamine NONOate (DEA-NONOate) and substance P were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Angeli's salt was obtained from Calbiochem (Darmstadt, Germany). RWE (Provinols, Seppic, France) was a kind gift of Unilever, the Netherlands. This RWE contained 632 mg polyphenols/g, determined as gallic acid equivalents using Folin Ciocalteu reagent.[22] Stock solutions of ODQ, ouabain, TRAM34, indomethacin, ethacrynic acid and glibenclamide were made in DMSO. Stock solutions of L-cysteine (300 mmol/l), Angeli's salt (10 mmol/l; dissolved in 0.01 mol/l NaOH) and DEA/NONOate (10 mmol/l) were prepared fresh daily. All subsequent dilutions and other drugs were made in distilled water. Solutions containing Angeli's salt and DEA-NONOate were stored in the dark.

Tissue collection

Pig hearts (n=105) were collected at the slaughterhouse and brought to the laboratory in cold, oxygenated Krebs bicarbonate solution of the following composition (mmol/l): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. The right proximal (internal diameter 2-3 mm; PCAs) and distal (internal diameter 500-600 µm; PCMAs) coronary arteries were obtained from the hearts and stored in cold, oxygenated Krebs bicarbonate solution for 12-36 h. PCAs were then cut into segments of ≈4 mm length suspended on stainless steel hooks in 15 ml-organ baths containing Krebs bicarbonate solution, aerated with 95% O_2/5% CO_2 and maintained at 37°C. To remove the endothelium in some segments, the ring was gently rolled back and forward over physiological saline-loaded filter paper after the tips of a pair of watchmaker forceps had been inserted into the lumen. PCMAs were cut into segments of ≈2 mm length and mounted in a Mulvany myograph with separated 6-ml organ baths, aerated with 95% O_2/5% CO_2 and maintained at 37°C.

Organ bath studies

Nitrite-induced vasorelaxation was investigated by exposing PCAs to UV-light, a well-known nitrite inductor. A comparison was made versus polychromatic light. These experiments were performed in the dark. To study HNO-induced vasorelaxation, as well as its potential role as EDHF, vessels were exposed to Angeli's salt, an HNO donor, or bradykinin. All experiments were repeated in presence of inhibitors of EDHF pathways, including the HNO scavenger L-cysteine and the sGC inactivator RWE.

Vessel segments were allowed to equilibrate for at least 30 min, and the organ bath fluid was refreshed every 15 min during this period. In PCAs, changes in contractile force were recorded with a Harvard isometric transducer (South Natick, Massachusetts, USA). The PCA vessel segments were stretched to a stable force of 15 mN. The tension of the PCMA vessel segments was measured by Powerlab with Labchart Sofware and was normalized to 90% of the estimated diameter at 100 mmHg effective transmural pressure.[23] 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 allowed to equilibrate in fresh organ bath fluid for 30 min in the absence or presence of one or more of the following inhibitors: the NOS inhibitors L-NAME (100 µmol/l) and L-NMMA (100 µmol/l), the *S*-nitrosothiol-depleting agents PHMBA (10 µmol/) and ethacrynic acid (50 µmol/), L-cysteine (3 mmol/l), the cyclooxygenase (COX) inhibitor indomethacin (10 µmol/), the sGC inhibitor ODQ (10 µmol/), the NO scavenger hydroxocobalamin (200 µmol/), RWE (30 µg/ml), K^+ (20 mmol/l KCl), the voltage-gated K^+ (K_v) channel inhibitor 4-aminopyridine (5 mmol/l in the photorelaxation experiments, 0.5 mmol/l in all other experiments), the Na^+-K^+-ATPase inhibitor ouabain (0.5 mmol/l), the ATP-sensitive K^+-channel (K_{ATP}) inhibitor glibenclamide (1 µmol/l), the BK_{Ca} inhibitor iberiotoxin (100 nmol/l), the SK_{Ca} inhibitor apamin (100 nmol/l) or the IK_{Ca} inhibitor TRAM34 (10 µmol/l). After 30 min of incubation with RWE, the organ bath fluid was refreshed.

For the light experiments, PCAs were preconstricted with the thromboxane A_2 analogue U46619 (1 µmol/l) and exposed six times for 5 min to light (wavelength: 350-370 nm) from a UV source (Omnilux UV-Röhe 18W, G13, 600 x 26 mm, T8) or from a halogen dissection lamp omitting polychromatic light. Each exposure was followed by a period in the dark of 2-30 min. After six light exposures, the vessels were subjected to 100 nmol/l bradykinin. In case of sGC inhibition, ODQ was added to the organ bath fluid just before each UV light exposure, since preliminary experiments showed degradation of ODQ due to UV light exposure.

For the experiments not involving photorelaxation, vessels were preconstricted with U46619 (1 µmol/l for PCAs, 0.01-1 µmol/l for PCMAs), and concentration-response curves (CRCs) were constructed to bradykinin (0.1 nmol/l – 1 µmol/l), Angeli's salt (sodium tiroxodinitrate; 0.1 nmol/l – 10 µmol/l), the NO donor DEA-NONOate (0.1 nmol/l – 10 µmol/l), or substance P (0.01 nmol/l – 1 µmol/l).

Data analysis

Data are given as mean ± SEM. No differences were observed between vessel segments that had been stored for 12 h or 36 h, and data from all vessels, were, therefore, combined. Peak relaxant responses are expressed as a percentage of the contraction to U46619. Statistical analysis was obtained by two-way analysis of variance (ANOVA) for the light experiments, followed by post hoc evalution. For all the other experiments, statistical analysis was obtained by one-way ANOVA of the mean pEC_{50} ($-^{10}logEC_{50}$) and maximum relaxation (E_{max}) values, followed by post hoc evaluation or t-tests. pEC_{50} values were not calculated when E_{max} was <20%, and in such cases statistical analysis was performed under the assumption that pEC_{50} equalled the highest concentration used. $P<0.05$ was considered significant.

RESULTS

UV light-induced relaxation

A 5-minute UV light exposure (wavelength: 350-370 nm) relaxed PCAs by maximally 42±2.8% (n=55). The maximum effect was reached within 1 minute and diminished slowly therafter, although relaxation remained apparent during the entire light exposure period. After switching off the light, the vessels immediately returned to their preconstrictive state. Subsequent UV light exposures within 2 minutes after switching off the light resulted in diminished initial maximum relaxant responses, although an identical sustained secondary relaxation response persisted throughout all subsequent light exposures, as described before (Figure 1A).[11] However, when a longer period (10 min, and particularly 30 min) in the dark was allowed, vessels reobtained the rapid initial maximum relaxant response (Figure 1A and B). Therefore, we performed all further experiments with a 30 min recovery period in dark, and for each light exposure the maximum (initial) response was used for further evaluation.

The maximum relaxation obtained with UV light was about half that obtained with polychromatic light (80±3.3%, $P<0.001$; Figure 1C). The responses were visibly different (see

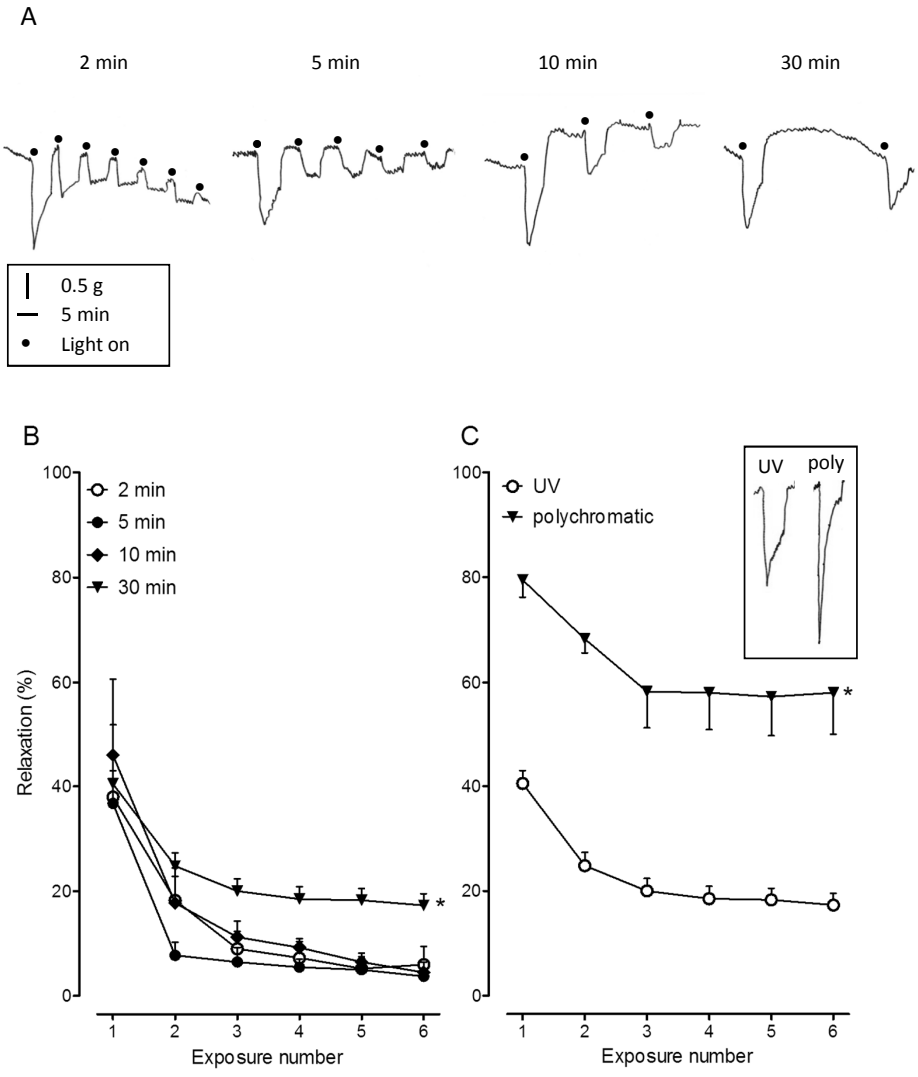

Figure 1. A, Representative tracings of an experiment showing 1-6 exposures to UV light. Porcine coronary arteries (PCAs) were preconstricted with U46619 and then subjected to 5 min of UV light, with in-between recovery periods of 2, 5, 10 or 30 min. B, UV light-induced relaxations of U46619-preconstricted PCAs (n=4; *P<0.05 vs. all other time periods). C, Relaxations of U46619-preconstricted PCAs exposed to UV or polychromatic (poly) light (n=4; *P<0.05).

Figure 1C), with a single, steep and short-lasting relaxation response for polychromatic light and a sustained secondary component as described above for the UV light-induced response only.

Pre-incubation with the NO-synthase inhibitor L-NAME greatly increased the UV light-induced relaxation, to maximally 91±8.7%, and this effect remained unaltered upon subsequent UV light exposures (Figure 2A). Because L-NAME, as an NO_2-containing compound, has been described to release NO upon UV light exposure,[10] these data suggest that the L-NAME effect (at a concentration of 100 µmol/l) may simply reflect its ability to repetitively release NO in micromolar amounts during UV light exposure. Additional experiments were therefore performed using an alternative, non-NO_2-containing NOS inhibitor, L-NMMA. Although L-NMMA also enhanced the relaxant effect of UV light (to maximally 55±2.1%), this effect diminished over time, despite the fact that L-NMMA had been applied to the organ bath at the same concentration as L-NAME (100 µmol/l). The effect of L-NMMA is therefore unlikely to be due to NO release from L-NMMA. Indeed, endothelium-removal, like NOS inhibition, similarly enhanced the relaxant effect of UV light, suggesting that endogenous, endothelium-derived NO counteracts this effect (Figure 2A).

The thiol-donating agent L-cysteine did not alter the response to UV light exposure (Figure 2B), indicating that UV light-induced vasorelaxation does not depend on HNO. The *S*-nitrosothiol-depleting agent ethacrynic acid diminished the UV light-induced relaxation, but a similar effect was not observed with the *S*-nitrosothiol-depleting agent PHMBA (Figure 2B). These data indicate that, at most, there is a modest contribution of *S*-nitrosothiols to the UV light-induced effect.

ODQ, hydroxocobalamin and RWE greatly reduced or abolished the UV light-induced relaxation (Figure 2C). Adding L-NMMA on top of ODQ or hydroxycobalamin did not alter their effect (data not shown; n=4), but when combining L-NMMA with RWE, the UV-light relaxation returned (Figure 2C). This indicates that the relaxant effect of UV light depends on the NO-sGC-cGMP pathway, and that L-NMMA abolishes the consequences of the RWE-induced sGC downregulation.

K^+ and ouabain reduced/abolished the UV light-induced relaxation, whereas aminopyridine and glibenclamide were without effect (Figure 2D). Similarly, indomethacin and iberiotoxine did not affect the UV light-induced relaxation (data not shown, n=4-5). These data indicate that the relaxant effect of UV light depends on Na^+-K^+-ATPase, and is abolished by K^+-induced depolarization.

To allow a direct comparison of the UV light-induced responses with reponses induced by bradykinin, and to verify the efficacy of the various blockers, the vessels were exposed to bradykinin after the six UV light exposures with or without the blockers. All bradykinin responses with or without inhibitors were similar to those reported previously when

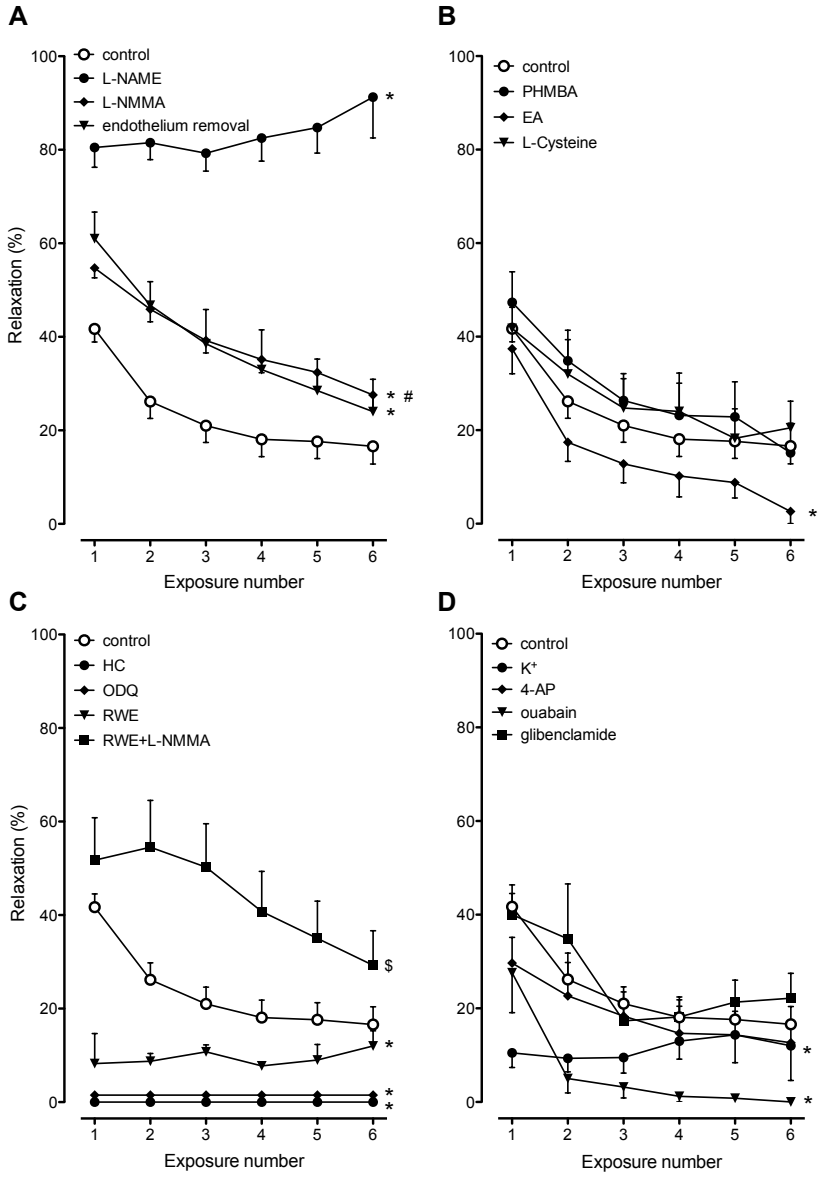

Figure 2. UV light-induced relaxations of U46619-preconstricted intact and endothelium-denuded porcine coronary arteries (PCAs) in the absence (control) or presence of 100 µmol/l L-NAME or 100 µmol/l L-NMMA (A), intact PCAs in the absence or presence of 50 µmol/l ethacrynic acid (EA), 10 µmol/l p-hydroxymercurobenzoic acid (PHMBA) or 3 mmol/l L-cysteine (B), intact PCAs in the absence or presence of 200 µmol/l hydroxocobalamin (HC), 10 µmol/l ODQ, 30 µg/ml red wine extract (RWE) and/or 100 µmol/l L-NMMA (C), and intact PCAs in the absence (control) or presence of 20 mmol/l K^+, 5 mmol/l 4-aminopyridine (4-AP), 0.5 mmol/l ouabain, or 1 µmol/l glibenclamide (D) (n=3-6; *P<0.05 vs. control; #P<0.05 vs. L-NAME, $P<0.05 vs. RWE).

studying bradykinin post-polychromatic light-induced vasorelaxation [4], and these data are therefore not shown here.

Angeli's salt-induced vasorelaxation

Angeli's salt concentration-dependently relaxed preconstricted PCAs and PCMAs (Figure 3). Its maximum effect (E_{max} 82±2.3% vs. 96±0.9%) and potency (pEC_{50} 6.2±0.11 vs. 6.8±0.13) were larger in PCMAs than PCAs (P<0.05 for both). Angeli's salt decomposes to HNO and nitrite. However, although nitrite (added as acidified $NaNO_2$) could relax PCAs to the same degree (E_{max} 72±11%; n=4) as Angeli's salt, its potency was »100 times less (pEC_{50} 4.2±0.17, P<0.05; data not shown). This indicates that the effect of Angeli's salt involves HNO rather than nitrite. Yet, the HNO scavenger L-cysteine blocked the effect of Angeli's salt in PCMAs only (pEC_{50} 6.0±0.08, P<0.05; Figure 3A and D), and in the presence of this drug the potency of Angeli's salt in PCMAs was identical to that in PCAs. Apparently therefore, the direct effects of HNO are limited to PCMAs, and its effects in PCAs occur largely indirectly, most likely following its conversion to NO.

In support of this concept, the NO scavenger hydroxocobalamin and ODQ identically shifted the Angeli's salt CRC to the right in PCAs (pEC_{50} 5.4±0.12 and 5.4±0.14, P<0.001 for both; Figure 3B), indicating that in this vessel type all Angeli's salt effects were mediated via NO. In contrast, in PCMAs the hydroxocobalamin effect (pEC_{50} 6.2±0.12, P<0.01) was only half that of ODQ (pEC_{50} 5.6±0.10, P<0.001; Figure 3E), indicating that in this vessel type sGC stimulation did not occur exclusively by NO, but was also mediated by HNO. Finally, in combination, hydroxocobalamin and ODQ fully blocked the effect of Angeli's salt in both vessel types. RWE marginally (P=NS) blocked the effect of Angeli's salt in PCAs, whereas its effect in PCMAs was significant (pEC_{50} 6.1±0.11, P<0.05). Taken together, these data indicate that the effect of Angeli's salt involves the NO-sGC-GMP pathway, stimulated by either HNO or HNO-derived NO.

TRAM34+apamin and iberiotoxin did not affect the Angeli's salt CRC in either PCAs or PCMAs (Figure 3A and D). K^+ blocked the effect of Angeli's salt in PCMAs (P<0.05) but not PCAs, and the opposite was true for 4-AP (P<0.05 vs. control in PCAs; Figure 3C and F). Ouabain shifted the Angeli's salt CRC »10-fold to the right in both vessel types, and in combination with hydroxocobalamin, almost completely prevented Angeli's salt-induced relaxation. These data suggest a role for Na^+-K^+-ATPase and K_v channels, but not Ca^{2+}-activated K^+ channels in the effect of Angeli's salt.

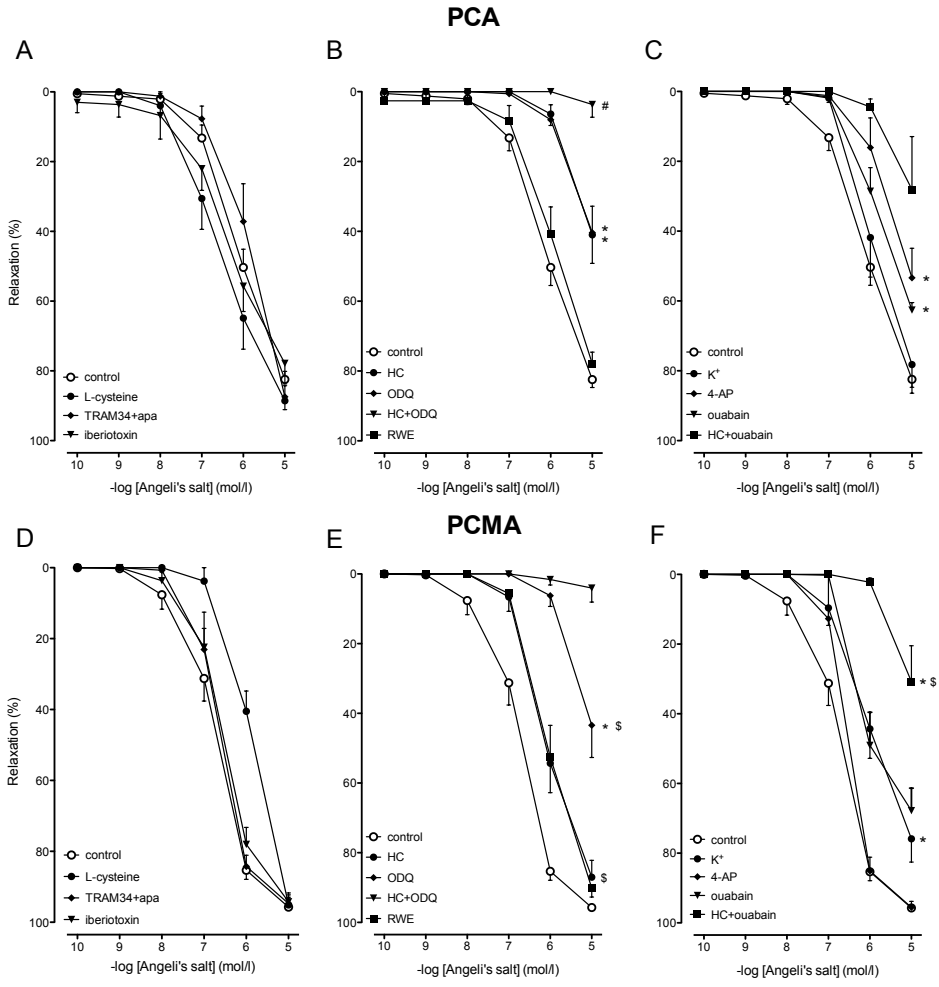

Figure 3. Angeli's salt-induced relaxations of U46619-preconstricted porcine coronary arteries (PCAs; A-C) or porcine coronary microarteries (PCMAs; D-F) in the absence (control) or presence of 3 mmol/l L-cysteine, 10 nmol/l TRAM34 + 100 nmol/l apamin (apa), 0.1 µmol/l iberiotoxin (A, D), 200 µmol/l hydroxocobalamin (HC) and/or 10 µmol/l ODQ, 30 µg/ml red wine extract (RWE; B, E), 20 mmol/l K^+, 0.5 mmol/l 4-aminopyridine (4-AP), 0.5 mmol/l ouabain and/or 200 µmol/l hydroxocobalamin (HC; C, F) (n=4-13; *P<0.05 vs. control, #P<0.05 vs. HC, $P<0.05 vs. HC+ODQ).

Bradykinin-induced vasorelaxation

Bradykinin concentration-dependently relaxed preconstricted PCAs and PCMAs (Figure 4). Its maximum effect (E_{max} 80±2.8% vs. 96±1.6%, P<0.05) and potency (pEC_{50} 7.9±0.11 vs. 8.3±0.13) were slightly larger in PCMAs (Figure 4A and C). In agreement with previous studies, both L-NAME and TRAM34+apamin shifted the bradykinin CRC 5 to 10-fold

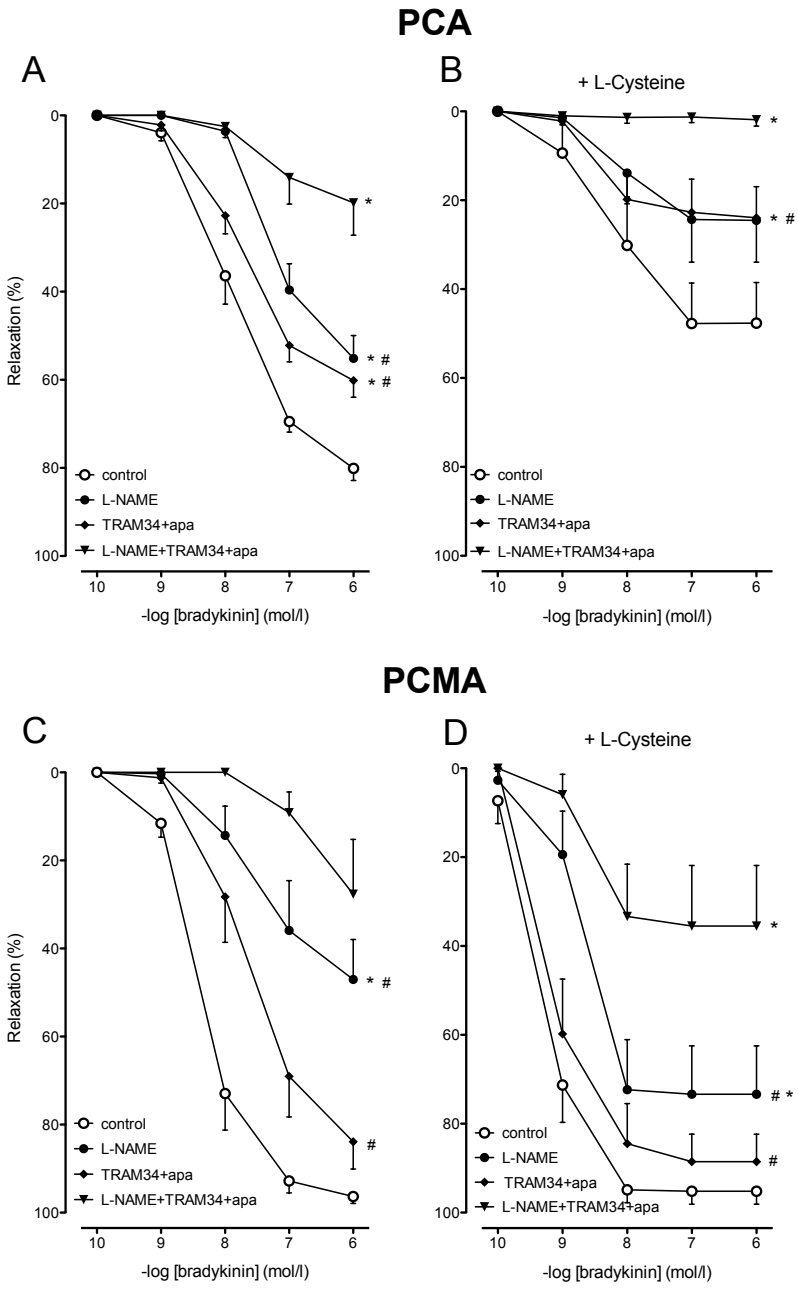

Figure 4. Bradykinin-induced relaxations of U46619-preconstricted porcine coronary arteries (PCAs; A and B) or in porcine coronary microarteries (PCMAs; C and D) with (B, D) or without (A, C) L-cysteine (3 mmol/l) in the absence (control) or presence of 100 µmol/l L-NAME and/or 10 nmol/l TRAM34 + 100 nmol/l apamin (apa) (n=6-15; *P<0.05 vs. control, #P<0.05 vs. L-NAME+TRAM34+apa).

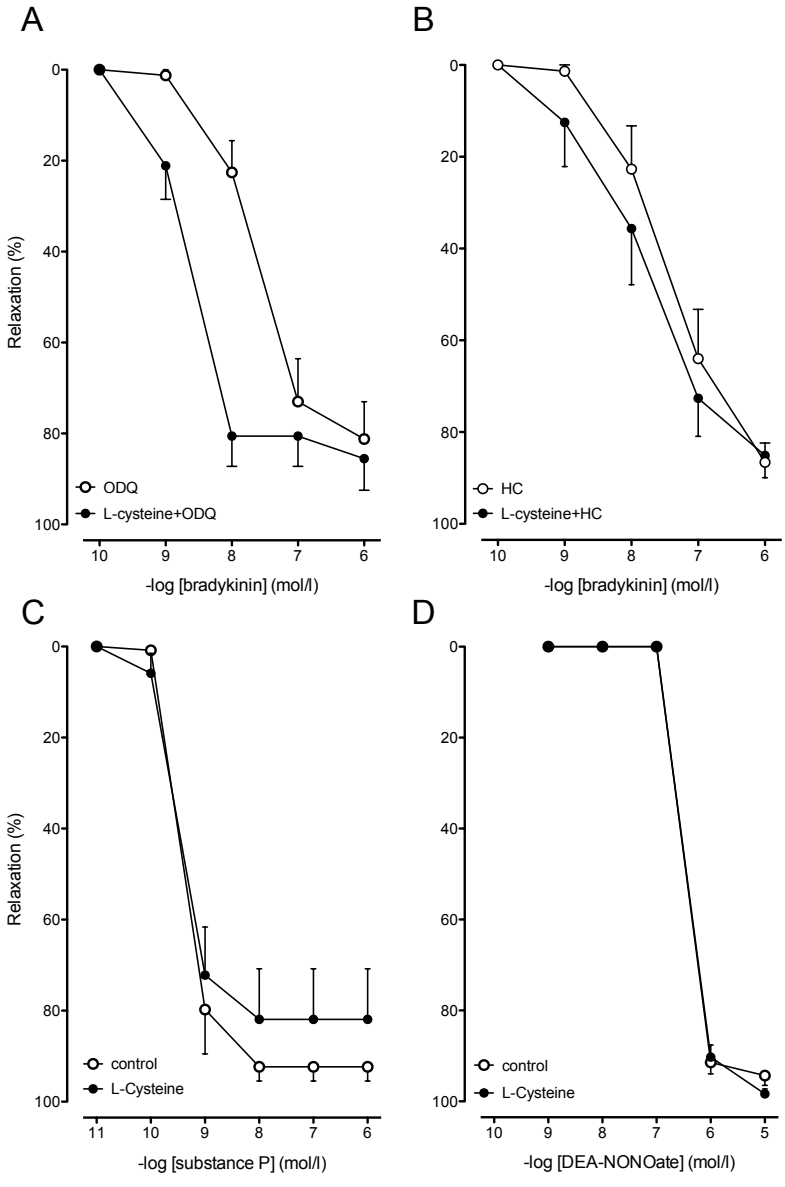

Figure 5. Concentration response curves to bradykinin (A, B), substance P (C), or DEA-NONOate (D) in the absence (control) or presence of 3 mmol/l L-cysteine, 200 µmol/l hydroxocobalamin (HC) or 10 µmol/l ODQ (n=3-6).

to the right in both vessel types, and, when combined, their effects were addititive. L-cysteine blocked the maximum effect of bradykinin in PCAs, and potentiated bradykinin in PCMAs (pEC_{50} 9.2±0.14, P<0.05; Figure 4B and D). L-NAME and TRAM34+apamin, when given on top of L-cysteine, exerted the same effect towards bradykinin as without L-cysteine. As a consequence, in PCAs, but not in PCMAs, the combination of L-NAME, TRAM34+apamin and L-cysteine fully blocked the effect of bradykinin. Taken together, these data suggest that HNO mediates the relaxant effect of bradykinin in PCAs but not necessarily in PCMAs.

In PCMAs, the potentiating effect of L-cysteine towards bradykinin also occurred during sGC blockade with ODQ (pEC_{50} 7.7±0.11 vs. 8.7±0.12, P<0.01; Figure 5A), but not in the presence of the NO/*S*-nitrosothiol scavenging compound hydroxocobalamin (Figure 5B). L-cysteine did not potentiate substance P or DEA-NONOate (Figures 5C and 5D). Combined with the fact that L-cysteine did not relax preconstricted PCMAs in the absence of bradykinin (n=8, data not shown), these data indicate that the potentiating effect of L-cysteine in PCMAs requires the release of NO-containing factors (*S*-nitrosothiols) by bradykinin.

DISCUSSION

This study shows that nitrite, via its conversion to NO and *S*-nitrosothiols, and HNO, either directly, or via its conversion to NO, mediate relaxant effects involving the sGC-cGMP pathway, Na^+-K^+-ATPase and/or K_v channels (Figure 6). RWE counteracts these beneficial effects. NO blocks nitrite activation, and HNO, but not nitrite, may act as EDHF in the coronary vascular bed.

UV light-induced vasorelaxation was found to depend on the previously described photolytic release of NO from nitrite and, to a minor extent, on *S*-nitrosothiols. The maximum relaxant effect induced by UV light occurred rapidly, and was only half that observed after activation of stored *S*-nitrosothiols by polychromatic light. The rapid initial effect was followed by a sustained secondary, modest relaxation. Only the rapid initial effect diminished upon rapid repetitive stimulation, and 'repriming' in the dark allowed its partial return. This resembles the properties of the polychromatic light-induced release of *S*-nitrosothiols. The decrease in response could not be attributed to a diminished responsiveness of sGC, since repetitive NO release from L-NAME by UV light always yielded the same maximum response.

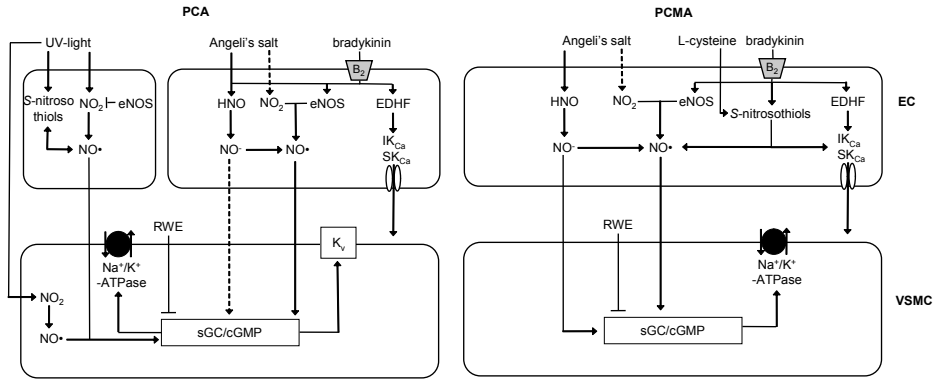

Figure 6. Scheme summarizing the mechanisms underlying the relaxant effects of UV light, Angeli's salt and bradykinin in porcine coronary arteries (PCAs) and porcine coronary microarteries (PCMAs). —> , activation/stimulation; —| , inhibition; --- >, limited activation/stimulation; B2, bradykinin type 2 receptor; cGMP, guanosine-3',5'-cyclic monophosphate; EC, endothelial cell; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; HNO, nitroxyl; IK_{Ca}/SK_{Ca}, intermediate-conductance and small-conductance Ca^{2+}-activated K^+-channels; K_v, voltage-dependent K^+ channel; NO^-, nitroxyl anion; NO•, nitric oxide; NO_2, nitriet; RWE, red wine extract; sGC, soluble guanylylcyclase; VSMC, vascular smooth muscle cell.

A previous study in humans has revealed that whole-body exposure to UV light resulted in NO release from cutaneous nitrite,[14] which subsequently elevated the levels of *S*-nitrosothiols in blood, allowing a pronounced decrease in blood pressure. This was due to the fact that nitrite facilitates the generation of *S*-nitrosothiols in the presence of NO sinks like hemoglobin, albumin and glutathion.[12, 14] Therefore, an explanation of the smaller initial relaxant effect of UV light as compared to polychromatic light might be that it depends on de novo *S*-nitrosothiol formation from nitrite-derived NO rather than on stored *S*-nitrosothiols, as was the case during exposure to polychromatic light (Figure 6).

The modest, sustained secondary relaxant effect has been suggested to be due to direct sGC activation by UV light.[11] However, the complete blockade of this effect by hydroxocobalamin argues against this possibility, and suggests that it fully depends on NO. Taken together therefore, the biphasic UV light induced relaxation involves both NO release from stored nitrite, stored *S*-nitrosothiols and/or *S*-nitrosothiol generation from NO. This explains why the mechanism underlying UV light-induced relaxation resembles that following exposure to polychromatic light, i.e., stimulation of sGC resulting in cGMP formation and Na^+-K^+-ATPase activation. Interestingly, NOS inhibition and endothelium removal identically facilitated the relaxant effect of UV light, suggesting

that endogenous NO suppresses nitrite activation/release, and that vascular nitrite is not necessarily limited to the endothelium.

It is unlikely that nitrite itself is the mediator of the relaxant effects. Although its vascular levels are in the µmolar range,[1] i.e., 3 orders of magnitude above the vascular levels of *S*-nitrosothiols, the nitrite concentrations required to directly induce relaxation in PCAs and PCMAs were found to be in almost the mmolar range, in full agreement with previous studies.[7, 24, 25] Nevertheless, the µmolar levels of nitrite might explain why the effect of UV light persisted during the entire light exposure period. In humans, the hypotension observed following nitrite infusion or oral inorganic nitrate supplementation (indirectly resulting in elevated circulating and tissue nitrite levels) also supports the concept that nitrite functions as an endocrine reservoir of NO, inducing vasodilation by increasing cGMP levels.[26-29] This appeared to be particularly important under hypoxic conditions, due to the fact that nitrite-dependent generation of NO and *S*-nitrosothiols involves the nitrite reductase activity of deoxyhemoglobin.[12]

Secondly, we addressed the putative contribution of HNO in PCAs and PCMAs. The latter vessels were of interest since HNO has been reported to be a new EDHF,[5] and the contribution of EDHF is usually larger in small vessels.[20, 21] Studies with the HNO-blocker L-cysteine revealed that the relaxant effects of bradykinin in PCAs does involve HNO, in addition to NO, IK_{Ca} en SK_{Ca}. Opposite to our expectations, L-cysteine potentiated bradykinin in PCMAs, both in the absence and presence of inhibitors of NO, sGC, IK_{Ca} en SK_{Ca}. This potentiation did not occur in the presence of the NO/*S*-nitrosothiol scavenger hydroxocobalamin, nor did L-cysteine potentiate substance P or the NO donor DEA-NONOate. It appears therefore that potentiation involves the bradykinin-induced release of *S*-nitrosothiols, either because L-cysteine prolongs their half life or because it stimulates their production (in particular that of the *S*-nitrosothiol L-*S*-nitrosocysteine). The potentiating effect of L-cysteine cannot be taken as evidence that HNO plays no role in PCMAs. Indeed, when applying the HNO donor Angeli's salt to PCMAs, vasorelaxation occurred at lower concentrations than in PCAs, and could be blocked by L-cysteine. No such blockade by L-cysteine was observed in PCAs, where the effect of HNO appeared to depend on its conversion to NO. The relaxant effects of Angeli's salt, like those of nitrite, involved sGC and Na^+-K^+-ATPase, in full agreement with its capacity to stimulate sGC, and thus to facilitate both cGMP- and Na^+-K^+-ATPase-dependent pathways. The latter sGC-mediated (cGMP-independent) phenomenon can be mimicked by direct sGC stimulators like BAY 41-2272.[4, 30] Whether Angeli's salt stimulates sGC directly or indirectly, following it conversion to NO (by superoxide dismutase), is still being debated.[5, 6, 31] Our observation that the NO scavenger hydroxocobalamin blocked Angeli's salt-induced vasorelaxation in PCAs to the same degree as ODQ supports the latter. Yet, the larger

blocking effects of ODQ and L-cysteine towards Angeli's salt in PCMAs (combined with its »5-fold greater potency) suggests that, at least in PCMAs, HNO also stimulates sGC directly. That this did not occur in PCAs cannot be taken as evidence against a role for HNO in PCAs, since the generation of endogenous HNO may occur at sites where HNO is not immediately (like Angeli's salt-derived HNO) converted to NO.

Finally, we observed no role for Ca^{2+}-activated K^+ channels in the effect of Angeli's salt, in full agreement with the possibility that its effects are mediated largely, if not completely by NO/HNO-induced sGC activation. We did observe a blocking effect by the K_v channel inhibitor 4-AP in PCAs, in line with studies in rat and mouse mesenteric arteries supporting cGMP-dependent K_v channel activation.[5-7] However, such blockade did not occur in PCMAs, possibly because the K_v channel subtype and/or expression differs in these vessels. Similar observations have been made in the rat coronary vascular bed.[32]

Incubation of PCAs with RWE gives rise to the generation of large amounts of NO (in an NOS-dependent manner), thereby acutely downregulating sGC.[18] As a consequence, RWE, like ODQ, blocked UV light-induced relaxation, and L-NMMA reversed this effect. RWE also mimicked the effect of ODQ towards Angeli's salt in PCMAs, although in PCAs at most a similar trend was seen. Since the RWE concentrations applied here refer to the amount observed in blood following the intake of »3 glasses of red wine, these data imply that red wine will acutely diminish the protective effects of nitrite and HNO.

In conclusion, we have shown that the relaxant pathways occurring following UV light exposure and Angeli's salt application involve sGC-dependent cGMP generation and Na^+-K^+-ATPase activation, largely, if not completely, because both approaches raise NO (Figure 6). UV light liberates NO from nitrite, comparable to nitrite-NO conversion by deoxyhemoglobin under hypoxic conditions in humans.[12] The role of *S*-nitrosothiols, either from storage sites or formed de novo from nitrite-derived NO is modest and finite, and *S*-nitrosothiol repriming only occurs when switching off the UV light. This resembles earlier findings on polychromatic light, which activates stored *S*-nitrosothiols, allowing potent and short-lasting photorelaxations that also returned only upon repriming in the dark.[4] Nitrite itself clearly cannot function as EDHF, given its low, millimolar potency. Angeli's salt-induced relaxation depended on NO generation from HNO, although direct sGC activation by HNO also appeared to occur, particularly in PCMAs (Figure 6). Most likely, the degree of HNO/NO-dependent sGC activation depends on the rapidity of HNO-NO conversion by superoxide dismutase, i.e., on the site where HNO is being formed. Indeed, in PCAs, bradykinin-induced relaxation depended on HNO, activating, among others, K_v channels. Earlier studies have shown that *S*-nitrosothiols mediate bradykinin's effect in PCMAs.[3] As a consequence, the HNO scavenger L-cysteine (which

facilitates *S*-nitrosothiol formation) enhanced rather than blocked the relaxant effects of bradykinin in PCMAs, thus not allowing us to conclude whether HNO also contributes to the effect of bradykinin in PCMAs. However, given the direct HNO effects observed in PCMAs, this is highly probable. Finally, RWE partially or completely prevented the above effects of nitrite and HNO, most likely because RWE acutely downregulates sGC (Figure 6).

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Summary and general discussion

7

SUMMARY

Since the first epidemiological research in the nineties showed an inverse relation between red wine consumption and prevalence of coronary heart disease ("the French paradox"), little progress has been made in unraveling the potential mechanisms behind this paradox. Despite promising findings showing that the red wine polyphenol resveratrol activates the deacetylase SIRT1, knowledge on the exact mechanisms underlying the protective effect of red wine consumption is currently still very limited. Very recently, contradicting results about SIRT1, aging and resveratrol were published. A better understanding of these mechanisms could contribute to well-founded guidelines regarding red wine consumption and, more important, to novel therapeutic targets for prevention of cardiovascular diseases.

In this thesis, *in vivo, in vitro* and genetic studies are presented on the role of red wine consumption and its constituents on endothelial and vascular function. SIRT1 is an important signalling molecule of energy metabolism, which is influenced by components of red wine and caloric restriction. Therefore, we assessed the contribution of SIRT1 to the effect of red wine on vascular function as well as its effect on the development of type 2 diabetes following fetal malnutrition. In the *in vivo* studies, vascular function is determined as forearm blood flow and blood pressure during the intake of respectively red wine consumption and its dealcoholized form. In the *in vitro* studies, the exact mechanisms are investigated behind the beneficial effect of red wine extract (RWE) free of alcohol on endothelial cellular aging (cellular senescence) and vascular function. In addition, we show the importance of nitric oxide (NO)-containing compounds in vasorelaxation in general, as well as in RWE-induced vasorelaxation.

Chapter 1 starts with a general introduction about the relationship between red wine consumption and cardiovascular disease. This relationship has been investigated extensively, motivated by epidemiological studies performed about 20 years ago. A number of *in vivo* studies have been conducted to confirm this relationship, to unravel the mechanisms, and to demonstrate a causal relationship, but the results, so far, are conflicting.

In chapter 2, the effect of red wine consumption on vascular function is shown combining findings of *in vivo* and *in vitro* experiments. Vascular function was studied in young healthy women drinking red wine daily. Young healthy women were chosen to assess the effect in a population without vascular dysfunction. Vascular function improved after three weeks of daily red wine consumption. Drinking a single glass of red wine reduced plasma endothelin-1, and, in agreement, RWE reduced endothelial endothelin-1

release *in vitro*. In addition, we showed that RWE relaxed porcine coronary arteries in an endothelium-dependent manner, involving NO generation by endothelial NO-synthase (eNOS) and subsequent activation of soluble guanylyl cyclase (sGC) and formation of guanosine-3′,5′-cyclic monophosphate (cGMP). Pre-incubation with RWE diminished the relaxations induced by bradykinin and an NO-donor, which could be blocked by respectively the eNOS inhibitor L-NAME and the sGC inhibitor ODQ. This suggests that RWE acutely downregulates sGC. Hence, prolonged red wine consumption improves vascular function in young healthy women, apparently by upregulation of sGC, thereby increasing vascular NO sensitivity.

In chapter 3, the effects of RWE were investigated in a double-blind, placebo-controlled, randomized full-crossover study in middle-aged subjects with high normal blood pressure or grade 1 hypertension. The main endpoint was a difference in office and 24 h blood pressure between placebo- and RWE-treated individuals. Also, central hemodynamic measurements were conducted, since this might more strongly relate to the extent of atherosclerosis and cardiovascular events than peripheral brachial blood pressure. Although we described promising results of red wine consumption on vascular function in chapter 2, we did not observe any effect of four weeks of treatment with two different dosages of RWE on 24-hour ambulatory and office blood pressure as compared with placebo. Similarly, no effect was found on central hemodynamic parameters. We concluded that if cardiovascular benefits of red wine consumption exist, they are blood pressure-independent.

A few years ago, resveratrol was identified as potential cardioprotective compound that mediates its effects through the activation of SIRT1. Furthermore, SIRT1 may influence endothelial cellular aging (cellular senescence) by deacetylation and subsequent inhibition of the important senescence mediator p53. Therefore, we hypothesized in chapter 4 that RWE protects against endothelial cell senescence induced by reactive oxygen species (ROS), and that this is due to SIRT1 activation and release of other vasodilator signalling factors. We indeed found that RWE protects endothelial cells against ROS-induced oxidative senescence. The protective effect of RWE was associated with a reduction in DNA damage and a decrease in p21, a DNA damage-related cyclin-dependent kinase inhibitor. Although this effect was dependent on NO and prostaglandin release, SIRT1 did not play a critical role. We concluded that RWE exhibits a beneficial effect by protecting endothelial cells against ROS-induced senescence, independently of SIRT1.

Nonetheless, SIRT1 may be an important factor in the development of cardiovascular disease and hypertension. Chapter 5 presents the results of an interaction analysis between *SIRT1* genetic variants and fetal malnutrition on the development of type 2 diabetes later in life in the Dutch Famine Birth Cohort. We found that an interaction between two SNPs in *SIRT1* and in-utero exposure to malnutrition significantly influenced type 2 diabetes risk in adulthood. Minor allele-carriers of these genetic *SIRT1* variants, who had been exposed to famine in utero, had a 50% lower risk of developing diabetes than non-carriers, but, surprisingly, had a higher BMI. We concluded that specific *SIRT1* variants may be protected to fetal programming by undernutrition reducing type 2 diabetes.

In Chapter 6, we assessed the effects of the NO-containing compounds nitrite and nitroxyl on photo- and bradykinin induced-relaxation in porcine coronary (micro)arteries and investigated whether RWE-induced relaxation alters these effects.

We also investigated whether nitroxyl and nitrite could exhibit their relaxation via vascular smooth muscle hyperpolarization and thus function as endothelium-derived hyperpolarizing factor (EDHF). We found that the relaxant pathways after UV light exposure and application of Angeli's salt (a nitroxyl donor) involve sGC-dependent cGMP generation and subsequent Na^+-K^+-ATPase activation. Nitrite-derived NO, rather than *S*-nitrosothiols, was important in UV light-mediated photorelaxation. Angeli's salt-induced relaxation depended on NO generation from nitroxyl, although direct sGC activation by nitroxyl also appeared to occur, particularly in microarteries. We found that bradykinin-induced relaxation in porcine coronary arteries depended on nitroxyl, activating, among others, K_v channels. The nitroxyl scavenger L-cysteine enhanced rather than blocked the relaxant effects of bradykinin in the microarteries, most likely because it facilitated the formation of *S*-nitrosothiols. Furthermore, we observed that RWE incubation partially or completely prevented the above effects of nitrite and nitroxyl, as earlier found in chapter 2 for bradykinin, presumably because RWE acutely downregulates sGC. We concluded that nitroxyl, but not nitrite, may act as EDHF in the coronary vascular bed and that RWE counteracts the relaxations induced by these compounds.

DISCUSSION

The effect of red wine and its components on vascular function: *in vivo* studies

Epidemiological studies suggest that modest red wine consumption is associated with a reduced risk of cardiovascular disease.[1-3] A recent study showed that high intake of polyphenols was negatively associated with blood pressure levels and prevalence of

hypertension in an elderly Mediterranean population at high cardiovascular risk.[4] Clinical trials investigating the effect of red wine polyphenol intake on vascular function do not always show a beneficial effect.[5-8] Differences in study design, composition of the red wine product and population characteristics may underlie these inconsistent findings. We performed our study in healthy, young women, allowing an understanding of the preventive potential of red wine under normal physiological conditions. Our results are in full agreement with recently published studies in healthy subjects investigating the effect of daily red wine consumption for three weeks on vascular function.[9, 10] These studies unfortunately did not include the administration of NO-donors to assess the endothelium-independent effect. Far more studies are published about the effect of red wine or grape polyphenols in patients with endothelial dysfunction.[6, 7, 11-14] Although short-term red wine consumption did not affect vascular reactivity in patients with type 2 diabetes or patients with an acute coronary syndrome,[7, 14] higher doses of grape polyphenols without alcohol did improve vascular function in patients with coronary heart disease.[12, 13] This suggests that either the alcohol diminishes the beneficial effect of polyphenols or that higher doses of polyphenols are needed in patients with already diminished endothelial function. Therefore, RWE free of alcohol was used in our next study in hypertensive middle-aged patients, who might suffer from endothelial dysfunction **(chapter 3)**. Another reason for the use of alcoholic-free RWE is that alcohol consumption might increase blood pressure.[15] Although animal studies with this specific RWE had showed a blood pressure lowering effect, no effect of RWE was present on 24 h ambulatory and office blood pressure compared with placebo after four weeks of treatment in this human study population. A few other studies have investigated the effect of grape polyphenols on blood pressure, with different results.[16, 17] In patients with marked endothelial dysfunction, such as the metabolic syndrome, there was however a clear benefit of grape and red wine polyphenols on blood pressure.[18, 19] Possibly, more pronounced endothelial dysfunction is required to visualize an effect of red wine polyphenols on blood pressure. We did not find a difference in blood pressure response between subjects with a blood pressure above or below the median. Apparently, the effects of red wine polyphenols on blood pressure differ from the effects on vascular function and further research is required to elucidate this. A complicating factor of these studies is that a period of two to four weeks might be too short to show an effect, especially considering that standard drug trials last much longer.

How do red wine polyphenols induce vasorelaxation? The mechanisms behind photorelaxation, nitroxyl-induced vasorelaxation and the impact of red wine extract

Vascular tone is controlled by the endothelium. The endothelium releases NO by activation of eNOS and prostaglandins by activation of COX that, together, induce vasorelaxation. In addition, the endothelium releases non-NO/non-prostaglandin factors that are capable of hyperpolarizing (and thus relaxing) the underlying smooth muscle. These factors are now known as 'endothelium-derived hypolarizing factors' or EDHFs, and comprise many candidates.[20] A traditional way of inhibiting EDHF pathways is the combination of TRAM34 and apamin, which block intermediate-conductance and small-conductance Ca^{2+}-activated K^{+}-channels, respectively. EDHFs vary according to vascular bed and vessel size, and in general the contribution of EDHF to vasorelaxation is more prominent in resistance vessels than in conductance vessels.

Red wine polyphenols are rapidly metabolized in humans, resulting in very small amounts of unchanged polyphenols in the circulation.[21] More than 80% of the ingested polyphenols are present in their conjugated forms in plasma and urine.[22] Obviously, it is unfeasible to measure all the different metabolised and unmetabolised polyphenols in the circulation.[23] Nevertheless, using the Fiolin-Ciocalteau method, the intake of 100 ml of red wine increased the plasma concentration of total phenols as gallic acids by »2-3 µg/ml in healthy volunteers.[24] Since RWE contains 632 mg polyphenols/g, the RWE concentrations of 25-30 µg/ml we used in most of our *in vitro* experiments **(chapter 2, 4, 6)** will match serum polyphenol concentrations in the range expected after drinking 336 ml red wine.

Red wine polpyhenols induce endothelium- and NO-dependent vasorelaxation **(chapter 2, 4).**[25] EDHF is also involved in the red wine polyphenol-induced vasorelaxation **(chapter 4)**.[26] Additional studies demonstrated that red wine polyphenols upregulate eNOS mRNA and protein expression in endothelial cells **(chapter 4)**.[27, 28] We **(chapter 4)** and others[29] found that RWE augmented eNOS phosphorylation at Ser^{1177} thereby activating eNOS leading to NO production.[28] Some studies showed that oxygen radicals are formed following RWE application, acting as signalling molecules.[30] Thus, although RWE is a well-known anti-oxidant, it may also be considered as a 'pro-oxidant'. Although we observed increased COX-2 gene expression after treatment with RWE in endothelial cells, under no condition was the COX inhibitor indomethacin able to block the relaxant effect of RWE in porcine coronary arteries **(chapter 4)**. This indicates that the pathways through which RWE either mediates vasorelaxation or prevents endothelial senescence are different. COX-2 expression was increased in the endothelium, leading to senes-

cence protection via prostaglandin production. RWE gives rise to the generation of large amounts of NO in an eNOS-dependent manner **(chapter 2, 4)**,[28, 29] thereby acutely downregulating sGC, which then leads to diminished vasorelaxant responses to NO. Yet, prolonged exposure to RWE improves NO-induced vasorelaxation, suggesting sGC upregulation. Measuring sGC activity and/or protein expression is required to assess the latter.

As mentioned before, the relaxant response to RWE also involves a non-NO EDHF response. Precise characterization of the EDHF-mediated response would allow further determination whether new drugable targets can be identified for the treatment of cardiovascular diseases. A candidate is nitroxyl, the reduced form of nitric oxide. It might be a good drugable target, since tolerance was not developed to nitroxyl (which is known from current vasodilators as nitroglycerin) in rat isolated aortae.[31] In support of its role as EDHF, the nitroxyl scavenger L-cysteine blocked bradykinin-induced relaxation in porcine coronary microarteries. Nitrite might also opt as EDHF, as it reduced blood pressure in human healthy volunteers, again without tolerance development.[32] We showed that UV light, as inductor of nitrite, caused relaxation involving both NO release from stored nitrite, stored *S*-nitrosothiols and/or *S*-nitrosothiol generation from NO. This is in line with a human study that showed *de novo S*-nitrosothiol formation from nitrite-derived NO **(chapter 6)**.[33] Nitrite can be reduced back to the bioactive form of NO under, amongst others, hypoxic, acidic or ischemic conditions.[34, 35] However, although nitrite has promising properties for direct vasodilation in clinical setting, it appears unlikely that endogenous nitrite acts as EDHF, since the required nitrite concentrations to directly induce relaxation were much higher than the concentrations which are available in tissue **(chapter 6)**. The relevance of *ex vivo* studies in porcine coronary arteries for the *in vivo* situation in human coronary arteries is supported by previous findings from our laboratory regarding the (endothelium-dependent) relaxant effects of bradykinin and the constrictor effects of angiotensin II in porcine coronary arteries[36-38] that fully resembled those in human coronary arteries.[37, 39, 40]

Red wine polyphenols and aging: is SIRT1 involved?

Aging is believed to be an independent risk factor for atherosclerosis, and epidemiological and *in vivo* data show atherosclerosis to be a characteristic feature of aging.[41] Cellular senescence of the endothelium contributes to the pathogenesis of atherosclerosis, as senescent cells are found in human atherosclerotic plaques and not in healthy vessels.[42] Cell senescence, or cellular aging, is a state in which the cell cycle is arrested. The cell survives this state for a long period, and meanwhile its cytoplasm enlarges and the cell becomes polynuclear. Furthermore, senescent cells display beta-galactosidase activity

at the low pH of 6.0, a feature that is conveniently used to detect cell senescence.[43] DNA damage by excessive production of ROS is an important mechanism underlying endothelial senescence.[44] SIRT1 has been implicated in endothelial cellular senescence as overexpression of SIRT1 prevented cellular senescence through deacetylation of the DNA damage-related cell cycle regulator p53 **(chapter 4)**[45] and through promotion of eNOS activity.[46] Inhibition of SIRT1 by sirtinol can induce senescence in endothelial cells **(chapter 4)**.[47] In contrast to studies in endothelial progenenitor cells,[9] the SIRT1 activator resveratrol did not prevent oxidative stress-induced senescence in endothelial cells **(chapter 4)**. Furthermore, resveratrol, up to a concentration of 100 µmol/l did not exert any vasorelaxation in preconstricted porcine coronary arteries **(chapter 2)**. This therefore suggests that resveratrol has no direct effect on the endothelium. The observation that resveratrol is not involved in stress-induced premature endothelial senescence is complementary to very recent studies[48, 49] disputing the claim that resveratrol activates SIRT1 and induces longevity.[50, 51] However, RWE prevents endothelial senescence **(chapter 4)**. SIRT1 inhibition by sirtinol did not reverse the protective effect of RWE on oxidative stress-induced senescence **(chapter 4)**. The protective effects or RWE on endothelial senescence are therefore unlikely to be related to SIRT1 activation, but, amongst others, do appear to be related to NO and prostaglandins **(chapter 4)**[9] Red wine is well-known for the ROS-scavenging ability of some of its polyphenols.[52] This may explain some of the beneficial effect on oxidative stress-induced senescence in our experiments. On the other hand, the beneficial effects of polyphenol-rich grape seed ingestion by cholesterol-fed hamsters on atherosclerosis was independent of the antioxidant effects.[53] Therefore, it would be very interesting to investigate the contribution of ROS scavenging on our results. This is complex, because the effects of RWE could be anti- as well as pro-oxidant, as mentioned before. Obviously, although it is difficult to extrapolate our findings to a human situation, they could provide further evidence that the intake of red wine polyphenols might influence endothelial cellular senescence and thus endothelial dysfunction, independent or dependent of ROS.

Interaction between *SIRT1* genetic variance and fetal malnutrition influencing type 2 diabetes risk

Although the exact role of SIRT1 in aging is currently under debate due to recent discrepant findings, SIRT1 seems to play an important role in the development of type 2 diabetes. SIRT1 regulates the cell metabolism by deacetylation of non-histon targets, like transcription factors and co-regulators, and is for example involved in glucose metabolism.[54] SIRT1 can also regulate gene expression by epigenetic mechanisms, which are changes in gene function that cannot be explained by changes in genomic DNA sequence.[55] This can be due to modifications in chromatin structure by chemical

modification of DNA and/or modification of associated proteins, such as histones.[54] These epigenetic mechanisms are especially important during fetal development.[56] SIRT1 activity is regulated by changes in NAD^+/NADH ratio and can be influenced by dietary factors. Caloric restriction can modulate this ratio and thereby activate SIRT1 activity.[57] On the other hand, fetal malnutrition (i.e. caloric restriction in utero) or low birth weight predisposes to type 2 diabetes.[58-60] This may be caused by fetal programming. An unfavorable environment in utero, e.g. a restricted nutrient supply, may alter gene expression profiles in metabolic and growth pathways by epigenetic mechanisms. This hypothesis was confirmed in the Dutch Famine Birth Cohort, a unique cohort of individuals born around the time of famine during World War II about whom information on both exposure to famine during gestation and incidence of type 2 diabetes in adulthood is available.[61] Although previous research in this cohort revealed that the effects of exposure to fetal malnutrition may depend on the timing of exposure during gestation,[62] the effects on glucose metabolism were present during all stages of gestation.[61] Lagouge et al.[63] was the first who reported an association between three single-nucleotide polymorphisms (SNPs) in the human *SIRT1* gene and energy homeostasis. Further research was performed to study if *SIRT1* genetic variance was related to longevity in humans, but no strong evidence was found.[64, 65] Only one study of many has published a potential association between *SIRT1* genetic variants and type 2 diabetes risk.[66] We neither found a significant association between *SIRT1* genetic variance and type 2 diabetes risk in our total population of the Dutch Famine Birth Cohort (i.e. exposed and unexposed individuals together) **(chapter 5)**. In this cohort, an interaction between two SNPs in *SIRT1* (rs7895833 and rs1467568) and in-utero exposure to malnutrition significantly lowered type 2 diabetes risk in adulthood **(chapter 5)**. This is in line with two other reports that showed a significant interaction between *SIRT1* genetic variants and nutrition, the first involving niacin intake[67] and the second involving vitamin E intake.[68] It is therefore possible that associations between *SIRT1* variants and diabetes risk can be found only when interaction with environmental factors are studied, such as fetal nutrition in our current study **(chapter 5)**. Interestingly, DNA methylation of the imprinted *IGF2* gene was found to be reduced in exposed individuals of the Dutch Hunger Winter (a cohort similar to the Dutch Famine Birth Cohort), as compared to their unexposed same-sex siblings at the age of 60.[69] It is tempting to speculate that this is mediated by the epigenic mechanisms of SIRT1. In summary, awaiting replications in independent similar cohorts, the findings of our study support the hypothesis that an interaction between *SIRT1* genetic variance and (fetal) malnutrition lowers type 2 diabetes risk. Thus, SIRT1 seems to be involved in the development of diabetes, especially in combination with environmental factors.

CLINICAL IMPLICATIONS

The primary goal of this thesis was to better understand the underlying mechanisms of protective effects of daily red wine consumption, including the role of SIRT1. At the moment, the complete mechanisms and involved pathways affecting vascular function are still not clear. Two complicating factors impede good research on the effects of red wine. First, the rapid and extensive metabolism of red wine polyphenols in many different metabolites hampers the translation of *in vitro* to *in vivo* work. Second, red wine contains more than 200 different polyphenols, apparently all exerting a different effect on vascular function. Since resveratrol is unlikely to be the protective component out of these hundreds, the question remains what is/are the responsible candidate(s). Possibly, it is not one red wine polyphenol, but a combination of several polyphenols, or probably the whole mixture that exerts the beneficial effects, all via slightly different specific mechanisms. Therefore, rational use of the protective effects of red wine polphenols can only be established by isolation of the different single components or different groups containing specific relevant constituent(s). Such a search demands high throughput screening systems. Polyphenol fractions could be separated by HPLC approaches and subsequently explored for endothelial gene or protein expression levels known to be involved in the beneficial effects of RWE. Using this approach, the red wine polyphenol petunidin-*O*-coumaroyl-glucoside was recently identified as potent activator of eNOS.[70]

No trials have been performed yet to determine the specific effects of red wine on the risk of developing cardiovascular disease. Based on the results in chapter 2 and 3, we can conclude that red wine polyphenols improve vascular function although they do not automatically reduce blood pressure. Simultaneously, we have to keep in mind the negative effects of alcohol, especially in risk groups, and it is thus advisable to adhere to the current recommendations by the American Heart Association that men limit their wine intake to no more than two drinks per day, whereas women should limit their intake to no more than one drink per day.[71] Although evidence is still insufficient to encourage people who do not drink to start consuming red wine as part of a strategy to protect against cardiovascular diseases, it is worthwhile to advise alcohol-consuming people to drink red wine.

Furthermore, the studies in this thesis provide a number of mechanistic insights on the effect of red wine extract, in concentrations achievable with moderate red wine consumption, on different pathways regarding vascular function that may have important consequences. In addition, they provide insight into the vasorelaxant efficacy of nitrite and nitroxyl and their possible targets in a clinical setting. Although photorelaxation is commonly used as tool to investigate the bioactivation of NO stores, UVA irradition

might be a therapeutic vasodilator in the treatment of hypertension,[33] whereas direct application of nitrite and nitroxyl might also be used as direct vasodilators. Mechanistic studies were performed to reveal these mechanisms. The first results are promising, as they showed that nitrite[32, 72] as well as the nitroxyl donor Angeli's salt[31] did not develop tolerance in rat isolated aortae.

DIRECTIONS FOR FURTHER RESEARCH

Although much progress had been made in unraveling the effects and underlying mechanisms of red wine polyphenols on vascular function, many unanswered questions remain. Epidemiological studies promise a glorious role for red wine in prevention of cardiovascular disease, but so far, no trials have been performed that determine the effect of red wine consumption nor of RWE nor of isolated polyphenols on cardiovascular disease. Extrapolation of the beneficial effects of red wine components to the wider population, including patients with cardiovascular disease, will require large-scale randomized controlled trials to prove the thesis that red wine components are a potential preventive measure of treatment for cardiovascular diseases. Such randomized controlled trials could compare subjects consuming red wine daily with subjects consuming another alcohol-containing drink daily or with subjects taking a placebo drink daily for at least a few years, with cardiovascular disease as major outcome. However, given the many possible confounders such as lifestyle and diet, such a study is unlikely to ever be performed. A large clinical trial for a longer period (e.g. several years) comparing the effect of placebo versus RWE intake on intermediate end points such as forearm blood flow is more feasible. Even outcomes such as coronary events might be assessed in such a trial.

Dal et al.[73] found that RWE intake by old rats protected against age-dependent deterioration of endothelium-dependent relaxation. Going from this point forward, animal studies, e.g. in aging mice models,[74] could be used to investigate whether this protection is dependent on eNOS and prostaglandin release. A next step could be to confirm these results in humans of different age, and eventually preventive therapeutics might then come available containing one or more red wine polpyhenols.

Additional studies should be conducted to reveal the pathophysiological mechanisms through which *SIRT1* genetic variance influences type 2 diabetes risk. Animal studies revealed that a postnatal low dietary protein intake showed improved insulin sensitivity together with an increased SIRT1 expression,[75] while SIRT1 protein and activity levels were lower in maternal low-protein offspring than in controls.[54]. This suggests that post-

natal caloric restriction could increase SIRT1 activity with consequent beneficial effects, whereas fetal caloric restriction reduces SIRT1 activity with consequent inferior effects. Further research could be conducted in such animal models to investigate the effect of SIRT1 on diabetes later in life, possibly also in genetically manipulated animal models.

Our results suggest an interaction between *SIRT1* genetic variance and fetal malnutrition on the development of type 2 diabetes later in life in the Dutch Famine Birth Cohort. As epigenetic mechanisms are very important in fetal development as well as in the action of SIRT1, it would be of great interest to investigate the effects of famine in utero on SIRT1 deacetylation and methylation. Since new techniques are available to asses gene-specific DNA methylation and histon deacetylation it would be attractive to determine the relationship between genetic variation, epigenetics and type 2 diabetes simultaneously.

CONCLUDING REMARKS

Important risk factors for cardiovascular disease are aging, hypertension and type 2 diabetes. The findings presented in this thesis provide improved insight into the effect of red wine polyphenols on vascular function, the underlying mechanisms and into the effect of genetic variance of *SIRT1*, a possible target of red wine polyphenols on the development of type 2 diabetes. These findings were performed by respectively an *in vivo*, *in vitro* and a genetic approach. These insights may be translated into future interventions providing protection against cardiovascular disease for healthy, aged, hypertensive and diabetic people.

What is already known?

- Red wine consumption reduces mortality from cardiovascular diseases
- Clinical trials investigating the acute effects of red wine consumption on vascular function yield mixed results
- *In vitro,* red wine polyphenols induce endothelium-dependent vasorelaxation through NO and EDHF

What this thesis adds

- Chronic red wine consumption improves vascular function by an increased NO responsiveness at the level of the smooth muscle cell
- Red wine extract consumption does not lead to sub-acute reductions of blood pressure in hypertensive patients
- The beneficial effects of red wine polyphenols on vascular function are mainly caused by eNOS-dependent NO release
- Red wine extracts protect against endothelial senescence, independent of SIRT1
- SIRT1 plays an important role in the fetal programming of type 2 diabetes

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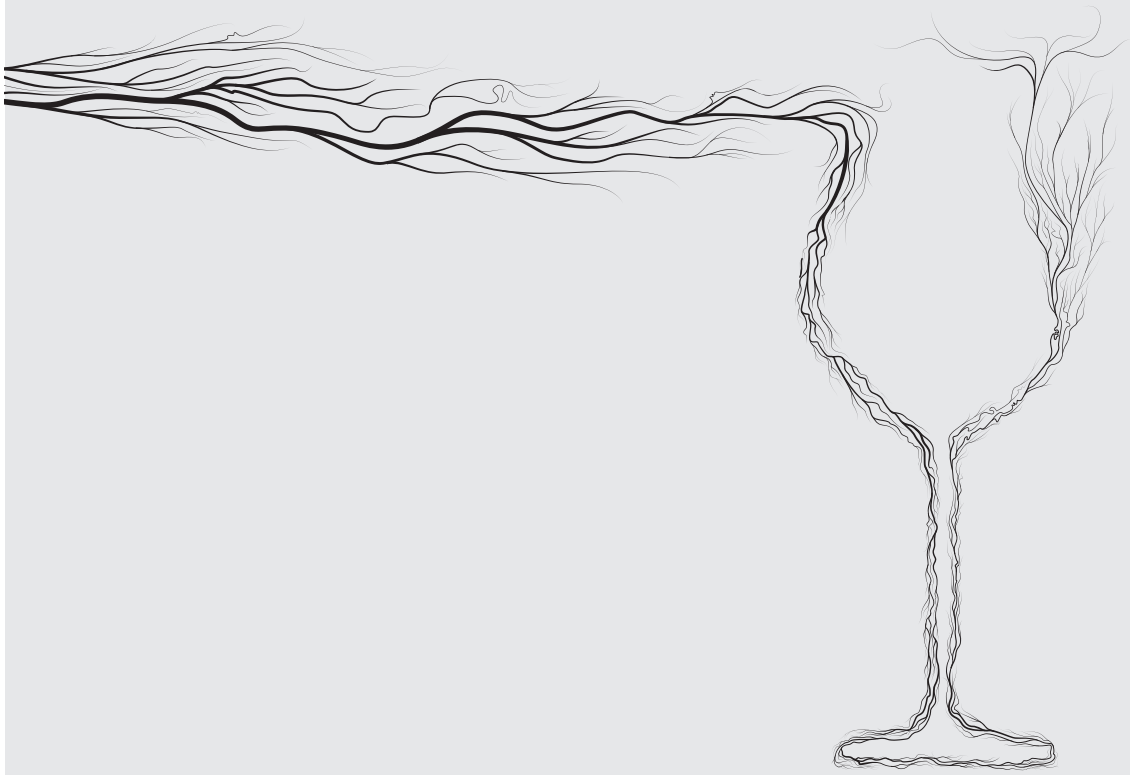
Nederlandse samenvatting



NEDERLANDSE SAMENVATTING

Dagelijkse rode wijn consumptie beschermt mogelijk tegen hart- en vaatziekten. Dit liet St Leger in 1979 als een van de eerste zien; hij toonde een omgekeerd verband aan tussen wijn consumptie en hart- en vaatziekten, waarbij de Fransen de laagste mortaliteit lieten zien. Dit werd de French Paradox genoemd, omdat de Fransen verhoudingsgewijs veel verzadigde vetten innemen en andere risicofactoren voor hart- en vaatziekten hebben. Sindsdien is er veel onderzoek verricht naar de mogelijke mechanismen achter deze paradox. Onderzoeken toonden met veelbelovende resultaten aan dat het rode wijn polyphenol resveratrol het signaaleiwit SIRT1 activeert. Dit eiwit zou beschermen tegen veroudering en diabetes en vaatfunctie verbeteren. Echter, zeer recent werden onderzoeken in hoogstaande bladen gepubliceerd met tegenstrijdige bevindingen over SIRT1, veroudering en de relatie met resveratrol. De huidige kennis rondom de mechanismen van het beschermend effect van rode wijn consumptie is bovendien nog steeds beperkt. Als we deze mechanismen beter zouden begrijpen, zouden we mogelijk een beter advies over rode wijn consumptie kunnen geven voor verschillende populaties en zouden we, nog belangrijker, mogelijk nieuwe medicijnen kunnen ontwikkelen voor het voorkomen van hart- en vaatziekten.

In dit proefschrift worden de resultaten beschreven van onderzoek verricht in mensen, geisoleerde cellen en kransslagaders, waarin de rol van rode wijn consumptie en zijn bestanddelen op vaatfunctie werd onderzocht. De mogelijke rol van SIRT1 werd hierin uitgelicht en specifiek ondezocht. Het effect van SIRT1 zelf in de ontwikkeling van type 2 diabetes werd ook onderzocht in een genetische studie. In de humane studies onderzochten we het effect van inname van rode wijn of zijn gedealcoholiseerde vorm op vaatfunctie, dat wil zeggen respectievelijk bloeddoorstroming in de onderarmsvaten en bloeddruk. Dit effect werd daarna preciezer onderzocht in het laboratorium door gebruik te maken van varkenskransslagaders en cellen van de binnenste laag van het bloedvat welke erg belangrijk zijn voor het doorgeven van signalen, de zogenaamde endotheelcellen.

Daarvoor maakten we gebruik van rode wijnextract (RWE); gedealcoholiseerde rode wijn in poedervorm. Ook hebben we onderzocht of stikstof-(NO) bevattende verbindingen belangrijk zijn in vaatverwijding, ook als de vaatverwijding door RWE wordt geinduceerd. De genetische studie beschrijft de relatie tussen foetale ondervoeding en *SIRT1* genetische variatie welke het ontstaan van type 2 diabetes beinvloedt.

Hoofdstuk 1 start met een algemene introductie over het omgekeerd verband tussen rode wijn consumptie en hart- en vaatziekten (de French paradox) en de mogelijk on-

derliggende mechanismen. Gemotiveerd door de grote populatie studies die de French Paradox een aantal decennia geleden aantoonden, is er al veel en grondig onderzoek verricht naar dit verband. Daarna zijn ook kleine studies verricht om een oorzakelijk verband te kunnen aantonen, maar de resultaten hiervan zijn tot nu toe tegenstrijdig. Het eiwit SIRT1, bekend van de studies naar 'langer leven', zou mogelijk worden geactiveerd door de veelbelovende stof resveratrol, een rode wijn polyphenol.

Hoofdstuk 2 toont de resultaten van een studie naar het effect van rode wijn consumptie op vaatfunctie, waarin de bevindingen van een humane studie en studie in geisoleerde kransslagaders werden gecombineerd. De studie werd verricht in in jonge, gezonde vrouwen om te kunnen onderzoeken of dagelijkse rode wijn consumptie gunstige effecten heeft in een gezonde populatie. Wij zagen dat dagelijkse rode wijn consumptie gedurende drie weken de vaatfunctie verbeterde, terwijl we dit na inname van één glas rode wijn niet zagen. Het drinken van één glas rode wijn verlaagde wel in het bloed de hoeveelheid endotheline-1, een bloedvatvernauwer, en RWE verlaagde ook de hoeveelheid endotheline-1 afgifte uit endotheelcellen. Bovendien zagen we dat RWE leidde tot vaatverwijding in varkensslagaders en dat dit afhankelijk was van NO vorming door het enzym eNOS dat vervolgens soluble guanylyl cyclase (sGC) activeerde dat via guanosine-3',5'-cyclic monophosphate (cGMP) tot vaatverwijding leidde. Resveratrol liet dit niet zien. Tegelijkertijd zagen we echter dat pre-incubatie met RWE vaatverwijding op bradykinine of een NO-donor verminderde, waarschijnlijk door een verminderde respons van sGC op NO. Concluderend vonden wij dat langdurige rode wijn consumptie in jonge, gezonde vrouwen de vaatfunctie verbetert, waarschijnlijk door upregulatie van sGC, en dat bestanddelen in rode wijn, anders dan alcohol en resveratrol, de vasculaire gevoeligheid voor NO verbetert.

Vervolgens hebben we in hoofdstuk 3 het effect van inname van RWE onderzocht in een dubbel-blinde, placebo-gecontroleerde, gerandomiseerde volledige-crossover studie in proefpersonen van middelbare leeftijd met een hoog normale bloeddruk of graad 1 hypertensie. De belangrijkste uitkomstmaat was het verschil tussen placebo en RWE op spreekkamer en 24-uurs bloeddruk. Ook werden centraal hemodynamische metingen verricht, omdat deze mogelijk beter correleren met de mate van atherosclerose en cardiovasculaire ziekten dan de perifeer gemeten bloeddruk aan de arm. Echter, ondanks dat we in hoofdstuk 2 gunstige effecten van rode wijn consumptie op vaatfunctie konden aantonen, zagen we nu geen effect van twee verschillende doseringen RWE op 24-uurs en spreekkamer bloeddruk vergeleken met placebo na 4 weken van behandeling. Ook werd geen verschil gezien op de centrale hemodynamiek. Daarom concludeerden we dat rode wijn consumptie nog steeds de gesuggereerde cardiovascular gunstige effecten kan hebben, maar dat deze in een bloeddruk-onafhankelijke manier plaatsvinden.

Een aantal jaar geleden werd resveratrol geidentificeerd as potentiele hart- en vaatziekten beschermende stof door zijn actieve werking op SIRT1. SIRT1 zou bovendien tegen endotheliale celveroudering (cellulaire senescence) beschermen door deacetylatie en vervolgens remming van de belangrijke senescence factor p53. Daarom veronderstelden we in hoofdstuk 4 dat RWE zou beschermen tegen endotheliale cellulaire senescence, geinduceerd door reactieve zuurfstof radicalen (ROS), and dat dit werd veroorzaakt door activatie van SIRT1 en het vrijkomen van andere vaatverwijdende signaalfactoren. Inderdaad vonden we dat RWE endotheelcellen beschermt tegen ROS-geinduceerde senescence. Dit beschermende effect van RWE was geassocieerd met een vermindering in DNA schade en verlaging van p21 eiwit expressie, een DNA schade-gerelateerd eiwit. Echter, alhoewel dit effect afhankelijk was van NO en prostaglandine afgifte, speelde SIRT1 hier geen belangrijke rol in. RWE heeft dus een gunstig effect op de vaatwand door bescherming van endotheelcellen tegen senescence, maar dit is onafhankelijk van SIRT1.

SIRT1 zelf kan echter nog steeds een belangrijke factor zijn in de ontwikkeling van hart- en vaatziekente en hypertensie. Dit gebeurt dan mogelijk vooral door zijn epigenetische kenmerken. Dit zijn erfelijke veranderingen zonder wijzigingen in het DNA, welke erg belangrijk zijn tijdens de foetale ontwikkeling. Hoofdstuk 5 laat de resultaten zien van een interactie-analyse tussen *SIRT1* genetische variatie en foetale ondervoeding op de ontwikkeling van type 2 diabetes op latere leeftijd in het Dutch Famine Birth Cohort. Dit cohort bestaat uit personen die in Amsterdam tijdens de periode van hongersnood in de 2^{e} Wereldoorlog geboren zijn. We toonden aan dat een interactie tussen twee zogeheten Single Nucleotide Polmorphismen in *SIRT1* en foetale ondervoeding significant het risico op type 2 diabetes op latere leeftijd beinvloedde. Dragers van de minst voorkomende allelen van deze genetische *SIRT1* varianten die blootgesteld waren aan foetale ondervoeding, hadden 50% minder risico om diabetes te ontwikkelen dan niet-dragers, maar ze hadden, onverwacht, ook een hogere BMI. SIRT1 speelt dus waarschijnlijk een belangrijke rol in foetale programmering van type 2 diabetes door foetale ondervoeding.

In hoofdstuk 6 hebben we de effecten onderzocht van de NO-bevattende verbindingen nitriet en nitroxyl op licht- en bradykinine geinduceerde vaatverwijding in grote en kleine varkenskransslagaders. Ook hebben we onderzocht of RWE-geinduceerde vaatverwijding door deze factoren werd beinvloedt. Vaatverwijding door blootstelling aan UV licht en toevoeging van de nitroxyl donor Angelis's salt werd gemedieerd door sGC-afhankelijke cGMP vorming en vervolgens door activatie van Na^{+}-K^{+}-ATPase. NO is belangrijker dan S-nitrosothiolen in UV licht-gemedieerde fotorelaxatie en dit is waarschijnlijk nitriet-gemedieerd. Vaatverwijding door Angeli's salt was afhankelijk van NO

vorming door nitroxyl, alhoewel directe activatie van sGC door nitroxyl ook plaatsvond, vooral in de kleine vaten. Ook zagen we dat in de grote vaten bradykinine-geinduceerde vaatverwijding afhankelijk was van nitroxyl, dat vervolgens onder andere K_v-channels activeerde. L-cysteine, dat nitroxyl wegvangt, vergrootte het vaatverwijdende effect van bradykinine in de kleine vaatjes, waarschijnlijk doordat het de vorming van S-nitrosothiolen bevordert. Incubatie met RWE voorkwam de bovengenoemde effecten van nitriet en nitroxyl, zoals we eerder ook al hadden gezien in hoofdstuk 2 voor bradykinine. Dit komt waarschijnlijk omdat RWE sGC acuut downreguleert. We concludeerden dat nitroxyl, maar niet nitriet, als zogenaamde 'endothelium-derived hyperpolarizing factor' kan functioneren in het coronaire vasculair bed.

De belangrijkste bevindingen van dit proefschrift zijn:

- Langdurige rode wijn consumptie verbetert de vaatfunctie door een verhoogde NO respons op het niveau van de gladde spiercel, m.a.w. door verhoogde upregulatie van sGC, alhoewel dit niet leidt tot een verlaging van bloeddruk in patiënten met hypertensie
- De gunstige effecten van rode wijn polyphenolen op vaatfunctie worden voornamelijk veroorzaakt door eNOS-afhankelijke NO afgifte
- Rode wijn polyphenolen beschermen tegen endotheliale celveroudering, onafhankelijk van SIRT1
- SIRT1, onafhankelijk van enige activator, speelt een belangrijke rol in de foetale programmering van type 2 diabetes

Deze bevindingen werden gevormd door onderzoek in mensen, geisoleerde kransslagaders, endotheelcellen en door middel van genetisch onderzoek. Hopelijk kunnen deze nieuwe inzichten worden vertaald in interventies die meer bescherming geven tegen hart- en vaatziekten in gezonde, oudere, hypertensieve of type 2 diabetes personen.

CURRICULUM VITAE

Ilse Botden werd geboren op 12 juli 1982 in Boxmeer en voltooide daar in 2000 het Gymnasium aan het Elzendaalcollege. In hetzelfde jaar begon zij aan de studie Geneeskunde aan de Universiteit Maastricht. Tijdens haar studie liep zij stage aan het Christian Medical College in Vellore, India. In 2004 behaalde zij haar doctoraal Cum Laude. Haar wetenschapsstage liep zij aan het Children's & Women's Health Centre in British Columbia, Vancouver, Canada en in 2006 behaalde zij haar artsexamen, waarna ze begon als arts-assistent Interne Geneeskunde in het toenmalige Medisch Centrum Rijnmond-Zuid, het huidige Maasstadziekenhuis te Rotterdam. In 2008 startte zij onder begeleiding van dr. J.G. Langendonk, prof. dr. E.J.G. Sijbrands en prof. dr. A.H.J. Danser op de afdeling Inwendige Geneeskunde – sectie Farmacologie en Vasculaire Geneeskunde met wetenschappelijk onderzoek naar de effecten van rode wijn polyphenolen op vaatfunctie, hetgeen de basis zou vormen voor dit proefschrift. Begin 2012 startte zij met de opleiding tot internist (supervisors: prof. dr. J.L.C.M. van Saase, Erasmus MC, and dr. H.E. van der Wiel, IJssellandziekenhuis, Capelle aan den IJssel).

PUBLICATIONS

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PHD PORTFOLIO

Red wine polyphenols and Vascular function

I.P.G. Botden

ErasmusMC Department:	Internal Medicine, Division of Pharmacology and Vascular Medicine
Research School:	Cardiovascular Research School Erasmus University Rotterdam (COEUR)
Promotors:	Prof.dr. A.H.J. Danser Prof.dr. E.J.G. Sijbrands
PhD-period:	2008-2011

PhD training	**year**
General academic skills (4.0 ECTS)	
Biomedical English writing and communication	2009
Organization Committee Wetenschapsdagen Inwendige Geneeskunde	2009-2011
Research skills (16.6 ECTS)	
Classical Methods for Data Analysis	2008
COEUR Courses and research seminars	2008-2011
Course Biomedical Research Techniques	2008
Course Genetics for Dummies	2010
Symposia and conferences (13 ECTS)	
High Blood Pressure Research 2011 Scientific Sessions, Orlando, United States * **	2011
European Meeting on Hypertension and Cardiovascular Research, Milan, Italy **	2011
Cardiovascular Conference, Noordwijkerhout, the Netherlands *	2011
High Blood Pressure Research 2010 Scientific Sessions, Washington DC, United States *	2010
European Meeting on Hypertension and Cardiovascular Research Oslo, Norway *	2010
MIVAB/DEBS, Biezenmortel, the Netherlands **	2010
Wetenschapsdagen Inwendige Geneeskunde, Antwerpen, Belgie *	2009-2011
Dutch Atherosclerosis Society (DAS) Symposium, Ede, the Netherlands *	2009

FIGON Dutch Medicine Days, Lunteren, the Netherlands **	2009
Teaching (1.2 ECTS)	
Junior Med School	2009, 2010
Supervising Medical Students in research and curricular skills	2009-2011
Grants, prizes	
Accomodation Grant - European Meeting on Hypertension and Cardiovascular Research	2010, 2011
Prize for the best poster presentation - Cardiovascular Conference, Noordwijkerhout, the Netherlands	2011

* poster presentation, ** oral presentation

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