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Some Phenolic Compounds Increase the Nitric Oxide Level in Endothelial Cells in Vitro

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The vasorelaxing properties of chocolate and wine might relate to the presence of phenolic compounds. One of the potential mechanisms involved is stimulation of endothelial nitric oxide (NO) production, as NO is a major regulator of vasodilatation. This study aimed to develop an in vitro assay using the hybrid human endothelial cell line EA.hy926 to rapidly screen phenolic compounds for their NO-stimulating potential. The assay was optimized, and a selection of 33 phenolics, namely, procyanidins, monomeric flavan-3-ols, flavonols, a flavone, a flavanone, a chalcone, a stilbene, and phenolic acids, was tested for their ability to enhance endothelial NO level. Resveratrol, a well-known enhancer of NO level, was included as a positive control. Of the 33 phenolics tested, only resveratrol (285% increase in NO level), quercetin (110% increase), epicatechingallate (ECg) (85% increase), and epigallocatechingallate (EGCg) (60% increase) were significant ($P \leq 0.05$) enhancers. Procyanidins showed a nonsignificant tendency to elevate NO level. Concentration-dependent correlations between enhanced NO level and endothelial nitric oxide synthase (eNOS) expression were demonstrated for the three polyphenols tested (resveratrol, ECg, and EGCg). Thus, an easy screening tool for change in cellular NO level was developed. Use of this assay showed that only a limited number of phenolic compounds might enhance NO level with an increased amount of eNOS enzyme as a possible contributing mechanism.

KEYWORDS: Nitric oxide; polyphenols; vasodilation; cardiovascular diseases; EA.hy926 cells

INTRODUCTION

The endothelial lining of blood vessels plays an important role in the regulation of blood pressure and blood distribution to different tissues and in obesity-related cardiovascular diseases (*1*). Nitric oxide (NO) is one of the main mediators of vasodilatation, and decreased NO levels play a central role in endothelial dysfunction (*1*). In mammalians, endothelial NO is produced by the enzyme endothelial nitric oxide synthase (eNOS), which converts L-arginine in the presence of O_2 and NADPH into L-citruline and NO (*2*). Several studies have shown that products rich in phenolics, such as red wine and cocoa, favorably affect endothelial function (*3*, *4*). Taubert (*5*) reviewed intervention studies with cocoa products, which consistently showed reductions in blood pressure. Furthermore, cocoa drinks rich in flavan-3-ols improved the flow-mediated dilation, a measure of endothelial function, in human subjects. This coincided with an increase of NO in plasma (*6*). All together, these studies suggest that phenolic compounds could play a beneficial role in endothelial function.

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Cocoa and wine contain a multitude of phenolic compounds, and it would be impossible to test all candidates in intervention studies. Therefore, several in vitro and ex vivo systems have been developed to screen the vasoactive potency of compounds. Most systems involve isolated arteries and aortas to study vasorelaxing properties (*7*−*11*), and relaxation of porcine coronary arteries was shown to correlate strongly with NO levels (*9*). The use of fresh tissue to screen many compounds would require a lot of animals. Cultured human umbilical vein endothelial cells (HUVEC) could provide a simpler alternative to screen for vasoactive compounds. However, HUVECs have to be freshly isolated and lose their ability to produce NO already after a limited number of passages (*12*). Edgell (*13*) developed the hybrid cell line EA.hy926 by fusing HUVEC cells with the permanent cell line A549. This hybrid cell line stably produces NO, even after a large number of cell divisions, and thus may be used as an efficient screening tool. EA.hy926 cells have been used before by Wallerath et al. (*14*) to measure eNOS expression after exposure to only a limited number of phenolic compounds without measuring NO level except for resveratrol (*15*).

Because NO is directly linked to endothelial function, we used it as a parameter to measure the vasorelaxing potential of

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polyphenols. Data on the ability of polyphenols to enhance NO level are available for only a limited number of polyphenols. Therefore, we used EA.hy926 cells to screen 33 phenolic compounds for their potential to enhance the NO level. Polyphenols, known to be present in chocolate and wine, were tested for their vasoactive potential including (epi)catechin and proanthocyanidins. Furthermore, microbial metabolites of proanthocyanidins and other flavonoids, phenolic acids (*16*, *17*), were tested as the bioavailability of proanthocyanidins is limited. The selection was extended with a number of monomeric flavan-3-ols, flavonols, flavones, flavanones, and one chalcone.

MATERIALS AND METHODS

Materials. All organic solvents used for HPLC analysis were of HPLC grade. Phenolic compounds were obtained as indicated: hippuric acid (Aldrich); 4-hydroxyhippuric acid (Bracher); 2-hydroxyhippuric acid (kindly provided by P. Kroon, IFR, Norwich, U.K.); 4-hydroxyphenylpropionic acid, phenylpropionic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, vanillic acid, *p*-coumaric acid, caffeic acid, benzoic acid, kaempferol (Fluka); 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid, procyanidin dimers B1 until B4 (Apin Chemicals); and phloretin (Extrasynthèse). All other phenolics were obtained from Sigma. Mixtures composed of procyanidin dimers or tetramers were isolated from both peanut skin (kindly provided by Imko-The Nut Co. BV, Doetinchem, The Netherlands) as well as a commercially available grape seed extract (vitaflavan DRT, Levita Chemical International NV, Antwerpen, Belgium) as described elsewhere (*18*). Vitaflavan, mainly composed of B-type dimers, trimers, and tetramers with gallic acid side chains, will be further referred to as B-type DP2−4+ gallic acid (DP, degree of polymerization). Chemicals and enzymes used were obtained from Sigma unless stated otherwise. Epicatechinglucuronide was prepared as described by Vaidyanathan et al. and subsequently purified with HPLC and freeze-dried.

Cell Culture. EA.hy926 cells, kindly provided by Dr. Edgell, were cultured according to their instructions (*13*). In short, cells were grown in DMEM (Gibco) containing 10% fetal bovine serum (FBS) (Gibco), 25 mM HEPES, and 2% penicillin/streptomycin. Cells were seeded in a 24-well plate at a density of 1.75 × 10E5 cells/well and incubated at 37 °C and 5% CO_2 (CO_2 Medical, Hoek-Loos, The Netherlands). After 24 h, the cells were confluent, and after 48 h, they were used to screen for potential bioactive phenolic compounds.

The CO_2 was purified with a sulfur trap (Valco Instruments Co. Inc., Switzerland) to remove NO traces before it entered the incubator. Water in the reservoir of the incubator was refreshed before every experiment. Without refreshment, NO accumulated from 0 to 17000 nM in 3 weeks.

In Vitro Assay for Changes in NO Level. Differences between inner and outer wells of 24-well plates can amount to 1000 nM NO due to contamination with NO from ambient air. Therefore, only the eight inner wells were used to screen the phenolic compounds. The outer wells contained 1 mL of water each to trap NO. Phenolic compounds were dissolved in DMSO (100 mM) and diluted in DMEM containing 25 mM HEPES, 150 U/mL SOD, and 300 U/mL catalase, to a final concentration of 0.1% DMSO. Cells were exposed to 300 μL of cell culture medium, containing the phenolic compound (100 μM), for 24 h; supernatant was then removed and stored at −80 °C to measure the stability of the phenolic compounds. Subsequently, fresh cell culture medium (300 μL) containing calcium ionophore A23187 (5 μM) was added to the cells to increase the sensitivity of the assay (*19*) (addition of 5 μM A23187 for 1 h significantly increased the NO level 6-fold by cells exposed to 100 μM resveratrol for 24 h). After 1 h of incubation with A23187, medium was removed and stored in tightly closed cryovials (Simport Plastics Ltd., Beloeil, Canada) at −80 °C until NO measurement. Each exposure was tested in triplicate (three wells) and repeated on at least three different days. Thus, each exposure generated 3 × 3 = 9 values. In each series, 100 μM resveratrol was taken as a positive control (average SD of 34 nM NO between the three wells). A control was included composed of 0.1% DMSO in DMEM and identically treated as the samples (average SD of 18 nM NO between the three wells). Measurements were corrected for NO produced by cells exposed to only DMEM. As a negative control, cells were exposed for 24 h to resveratrol as described above and subsequently incubated with fresh culture medium containing calcium ionophore and the eNOS inhibitor N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) (10 μM).

NO was measured as the total of NO_2 and NO_2^-, including nitrosated and nitrosylated NO as described by Feelisch et al. (*20*) with a few alterations. In short, a water-jacketed reaction vessel, kept at 60 °C, was filled with 20 mL of freshly prepared Brown's solution. Brown's solution, stored in the dark on ice until use, was made as follows: 45 mM KI and 10 mM I_2 in Milli-Q water (shaken for 5 min at 250 rpm) was subsequently mixed with glacial acetic acid ($1{:}12^1/_3$) followed by ultrasonic treatment for 5 min. Samples were injected into the reaction vessel in triplicate (50 μL) through a septum that was replaced after each series of measurements. Inside the reaction vessel Brown's solution reduced NO_2, NO_2^-, and nitrosated and nitrosylated species to NO(g). NO(g) was transported by helium through a condenser (3 °C) followed by a scrubbing bottle with 1 M NaOH (0 °C) to remove traces of acids. Subsequently, the NO(g) passed a 0.22 μm filter before it entered a chemiluminiscence detector (CLD 88 et., Eco Physics, Duernten, Switzerland). The detection range was set at 0−50 ppb. The whole system was kept at a constant overpressure of 1−1.05 bar throughout the measurements. Brown's solution was refreshed when peak broadening appeared or large bubbles were generated in the reaction vessel. Calibration curves were made with potassium nitrite (0−1000 nM) dissolved in a physiologic saline solution [0.9% (w/v) NaCl] ($R^2 > 0.99$).

To prevent NO_x contamination, tubes and equipment that were used were carefully screened and exposure of samples to air was minimized. NO_x measurements were only performed when air NO_x concentrations (expressed as NO_2) in the Wageningen region were below 40 μg/m^3 [measured by the Dutch National Institute for Public Health and the Environment (*21*)] . A plasma sample, which was stored in small batches at −80 °C, was used to determine the reproducibility of the NO measurements. This control sample had an average NO content of 145 nM (14 measurements of duplicates). The CV was calculated to be 6.9% within days (14 duplicates) and 12.4% between days (n = 14). Results of a series of analysis were rejected when the value obtained in this control sample exceeded $\pm 2 \times SD_{between}$ from the average level.

The mean increase in NO level caused by a compound was calculated as the difference between the mean of the sample values of at least three exposures (≥9 values) and the mean of the DMSO blank values measured within the same exposures and expressed as a percentage of the DMSO blank.

Stability of the Phenolic Compounds during Exposure. The cell culture media, which were collected after 24 h, were filtered through a 0.45 μm filter and analyzed by HPLC. For the procyanidin fractions a Thermo Spectra system was used containing a P 4000 pump, an AS 300 autosampler, and an UV 3000 detector (Thermo Separation Products). Analysis was performed on an XterraRP dC18, 4.6 mm i.d. × 150 mm, 3.5 μm column (Waters) at room temperature. The mobile phase was composed of (A) water + 0.1% (v/v) acetic acid and (B) acetonitrile + 0.1% (v/v) acetic acid. The flow rate was 0.7 mL/min, and detection was performed at 280 nm. For the fractions isolated from peanut skin the elution gradient was as follows: first 5 min, isocratic on 10% B; 5−35 min, B linearly from 10 to 30%; 35−40 min, B linearly from 30 to 90% followed by reconditioning of the column. Another elution gradient was used to analyze the fractions isolated from grape seeds: 15 min isocratic on 10% B; 15−35 min, B linearly from 10 to 50%; 35−40 min, B linearly from 50 to 95% followed by reconditioning of the column. For each sample 20 μL was injected.

Phenolic acids were analyzed on a system composed of L-6200 and L-6000A pumps (Hitachi), a 234 autoinjector (Gilson), a Kratos Spectroflow 783 UV detector (Kratos Analytical Instruments), and a Spark Mistral column oven set at 30 °C (Separations Analytical Instruments B.V.). Analysis was performed on an XBridge C18, 3.0 mm i.d. × 250 mm, 5 μm column (Waters). The mobile phase was composed of (A) 2% (v/v) acetonitrile and (B) 40% (v/v) acetonitrile in sodium phosphate buffer (0.01 M, pH 1.5). The flow rate was 0.42 mL/min, and detection was performed at 220 nm. The elution gradient was as follows: 0−20 min, B linearly from 3 to 100%; 20−24 min, isocratic on 100% B; 24−25 min, B linearly from 100 to 3% followed by reconditioning of the column. For each sample 10 μL was injected.

Epicatechin, catechin, epicatechingallate (ECg), epigallocatechin (EGC), and epigallocatechingallate (EGCg) were analyzed on a Hitachi

Table 1. Primers Used for Quantitative RT-PCR

gene symbol	sequence ID	forward primer (5′−3′)	reverse primer (5′−3′)	product length (bp)
eNOS	NM_000603	GAGACTTCCGAATCTGGAACAG	GCTCGGTGATCTCCACGTT	102
RP L32	NM_000994.3	GCTGGAAGTGCTGCTGATGTG	CGATGGCTTTGCGGTTCTTGG	83
β-actin	NM_001101.2	CCACCCCACTTCTCTCTAAGGAG	GCATTACATAATTTACACGAAAGCAATG	94

system composed of L-2100 pumps, an L-2200 autoinjector a CoulArray detector (ESA, Inc., Chelmsford, MA), and a Spark Mistral column oven set at 30 °C. Analysis was performed on an Inertsil ODS-3, 4.6 mm i.d. × 150 mm, 5 μm column (GL Sciences). The mobile phase was composed of (A) 10% (v/v) acetonitrile in sodium phosphate buffer (25 mM, pH 2.4) and (B) 30% (v/v) acetonitrile in sodium phosphate buffer (25 mM, pH 2.4). The flow rate was 1 mL/min, and detection was performed at −70, −10, 70, and 150 mV. The elution gradient was as follows: 0−20 min, B linearly from 0 to 80%; 20−23 min, B linearly from 80 to 100%; 23−25 min, isocratic on 100% B; 25−26 min, B linearly from 100 to 0% followed by reconditioning of the column. For each sample 10 μL was injected. Resveratrol was analyzed with the same conditions except detection was performed with UV detection. Apigenin, naringenin, quercetin, kaempferol, and phloretin were analyzed on the same system as described for phenolic acids and an Inertsil-ODS3 column as described for the flavan-3-ols. An isocratic elution was performed with 31% (v/v) acetonitrile in sodium phosphate buffer (25 mM, pH 2.4). Recoveries were calculated on the basis of peak areas compared to the original DMSO stocks that were diluted with water instead of medium.

Assessment of eNOS Expression by Quantitative Real-Time PCR Analysis. Total RNA from EA.hy926 cells, exposed for 24 h to resveratrol, EGCg, or ECg (0−200 μM) with or without 10 μM L-NAME, was isolated to assess eNOS expression levels by quantitative RT-PCR ($\Delta\Delta C_T$ method) (*22*). All exposures were performed in triplicate on three different days, and for each condition cells from four wells were pooled. After 24 h of exposure as described above, the medium was removed and the cells were washed with 1 mL of ice-cold PBS. Subsequently, the cells were incubated for 15 min in 0.5 mL of TRIzol (Invitrogen), resuspended, pooled, and stored at −80 °C. After thawing, RNA was extracted with 1.2 mL of chloroform and precipitated with 1.2 mL of isopropanol according Invitrogen's instruction. RNA quality and quantity were verified on a Bio-Rad Experion and Nanodrop spectrophotometer (Nanodrop Technologies) and accepted when $OD_{260/280} > 1.8$.

For each sample, cDNA was synthesized (iScript cDNA Synthesis kit, Bio-Rad) and eNOS expression levels were assessed in triplicate on a MyIQ5 single-color real-time cycler (Bio-Rad) using iQ SYBR-Green Super mix (Bio-Rad) and eNOS specific primers (**Table 1**). Quantitative RT-PCR data were analyzed with iQ5 optical system software (version 2), normalized to ribosomal protein L32 and β-actin reference genes, and results were accepted when the standard curve (of all analyzed genes) of serial dilutions from pooled cDNA samples showed good efficiencies and linear amplification ($R^2 > 0.99$).

Statistical Analysis. Cochran's test (ISO 5725-2, 1994) was used to determine if the highest within-day variation that was measured for each phenolic compound on at least three different days could be considered as an outlier. If the test statistic exceeded the 5% critical value, the measurement was excluded from further statistical analyses. The ability of each phenolic to significantly enhance NO level or eNOS expression compared to the DMSO controls that were measured on the same days was tested by Student's t test. A probability of < 0.05 was considered to be significant. Error bars are depicted as mean ± SEM of at least three exposures. To determine if enhanced NO levels correlated with eNOS fold changes, the NO level was expressed as the concentration of NO detected in exposed cells (0−100 μM phenolic compound) divided by the concentration of NO detected in nonexposed cells (0.1% DMSO).

RESULTS

Vasoactive Potency of a Large Set of Phenolic Compounds. In total, 33 phenolic compounds were tested for their ability to increase the NO level (**Figure 1**). Resveratrol had the largest significant effect (285% increase in NO level), followed by quercetin (110% increase), ECg (85% increase), and EGCg (60% increase). A number of other phenolic compounds also seemed to increase the NO level, although this did not reach statistical significance. As opposed to their galloylated forms, epicatechin and catechin did not increase the NO level. Like epicatechin, epicatechinglucuronide, a human metabolite of epicatechin, did not increase the NO level either. Procyanidins showed a tendency to increase the NO level, which seemed to be related to their degree of polymerization. However, a selection of their colonic metabolites, phenolic acids, had no effects.

After exposure to 100 μM resveratrol, the addition of L-NAME, a competitive inhibitor of eNOS, decreased the NO level (**Figure 1**). Similar results were obtained for epicatechin, ECg, and EGCg (data not shown).

Dose-Dependent Increase in both NO Level and eNOS Expression. Exposure of EA.hy926 cells to resveratrol, EGCg, and ECg resulted in a significant dose-dependent increase in NO levels (**Figure 2**). For resveratrol and EGCg, production of eNOS mRNA was significantly enhanced and paralleled NO levels. The increase in eNOS mRNA level after exposure to ECg did not reach statistical significance (**Figure 2C**). NO levels expressed as the ratio between exposed (0−100 μM, phenolic compound) and nonexposed (0.1% DMSO) cells correlated linearly in a dose-dependent manner with the changes in eNOS mRNA expression levels. Addition of L-NAME did not affect eNOS expression but only decreased NO levels (**Figure 3**). Epicatechin (100 μM) did not elevate eNOS mRNA levels (data not shown), consistent with its failure to increase the NO level (**Figure 1**).

Stability of the Phenolic Compounds. The stability of the phenolic compounds in the medium was analyzed after 24 h by HPLC. Addition of HEPES, to stabilize the pH, and the enzymes SOD and catalase, to prevent oxidative reactions, improved the stability of compounds such as 3,4-dihydroxyphenylpropionic acid, ECg, and EGCg. These additions did not affect the NO level in the DMSO control sample and were included in the protocol. All phenolic acids showed good recoveries ($> 80\%$) with the exception of 3,4-dihydroxyphenylpropionic acid (69%) and caffeic acid (56%). Catechin and epicatechin had recoveries of about 50%. Epicatechin was partly epimerized (∼25%) toward catechin. Catechin did not epimerize. EGC, ECg, EGCg, all procyanidins (grape DP2−4, A- and B-type dimers/tetramers, and the dimers B1−B4), quercetin, and kaempferol showed low recoveries ($< 50\%$). The low recoveries (27−40%) obtained for procyanidins might be caused by their complexation with proteins and peptides from the medium (*23*). Therefore, both A- and B-type tetramers were tested in medium with and without FBS. The stability of both A- and B-type tetramers decreased dramatically in the absence of FBS with recoveries of only 5 and 18%, respectively, already shortly after exposure.

DISCUSSION

Our in vitro assay offers a simple tool to screen phenolic compounds for their ability to increase the NO level. Of 33 phenolic compounds tested at a level of 100 μM, only resveratrol, quercetin, EGCg, and ECg significantly increased the NO level. Dose-dependent correlations between NO stimulation and eNOS up-regulation were found for all three polyphenols tested, resveratrol, EGCg, and ECg, suggesting that stimulation of eNOS expression contributes to the increased NO levels.

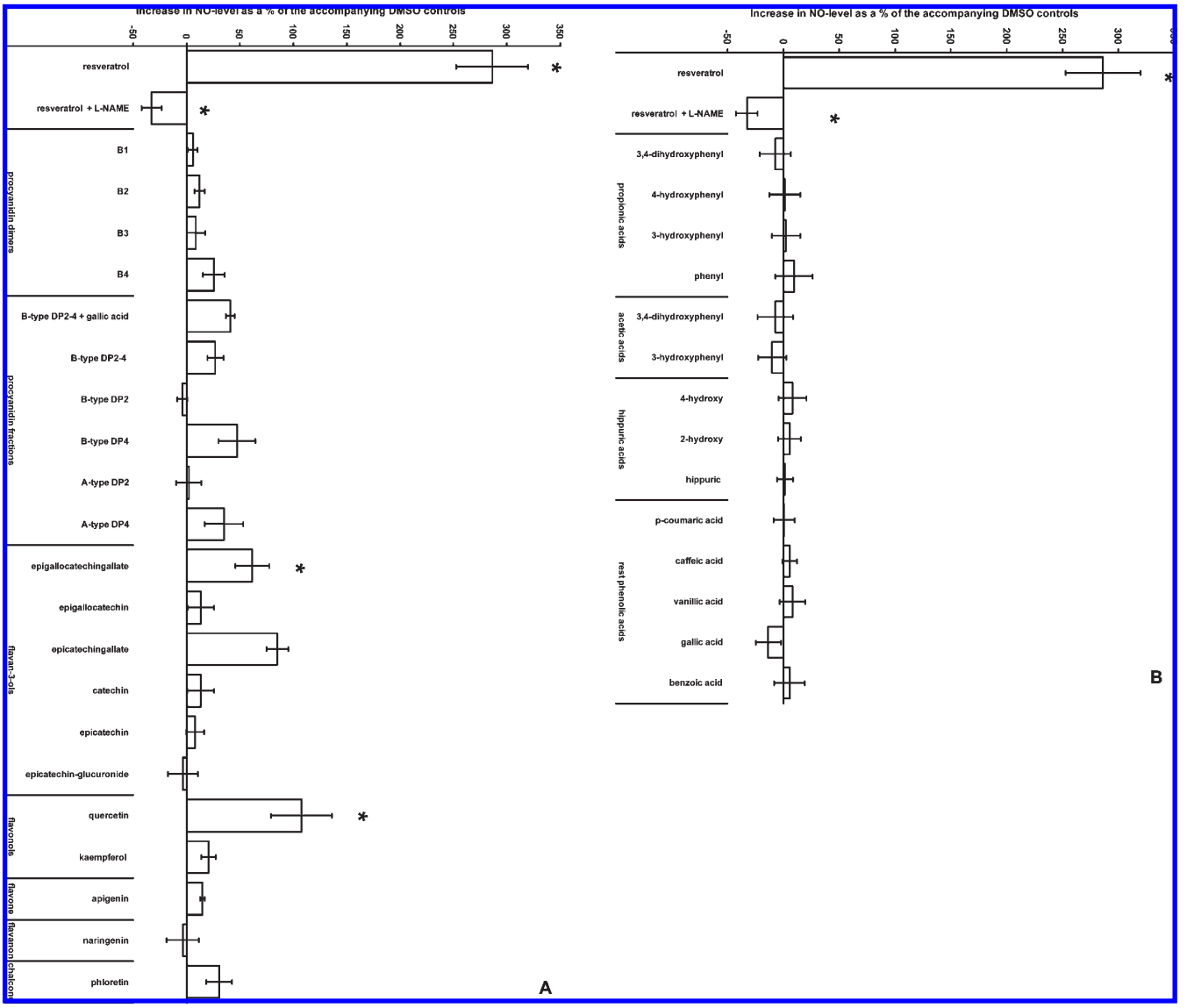

Figure 1. Average increase in the NO level as a percentage of the DMSO control after exposure of EA.hy926 cells to 100 μM flavonoids (**A**) or phenolic acids (**B**) for 24 h. *trans*-Resveratrol served as a positive control. Data represent mean ± SEM of three or more independent exposure experiments. DMSO control levels were on average 233 ± 21 nM. Significant differences were calculated with a Student's *t* test by comparing the NO concentrations (nM) after exposure to a phenolic compound with the NO concentrations after exposure to the control (0.1% DMSO) measured on the same days.

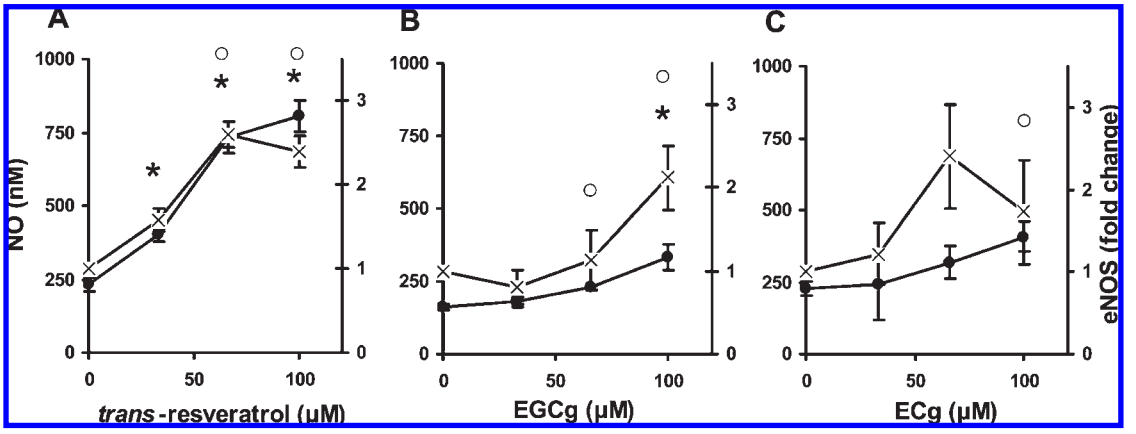

Figure 2. Dose-dependent increase in the NO level (●) and eNOS expression (×) after exposure of EA.hy926 cells for 24 h to (**A**) *trans*-resveratrol, (**B**) EGCg, and (**C**) ECg. Each exposure consisted of three triplicates repeated on at least three different days. Error bars represent ± SEM and significant differences are indicated by ○ for NO level and ∗ for eNOS expression ($P < 0.05$) analyzed by Student's *t* test.

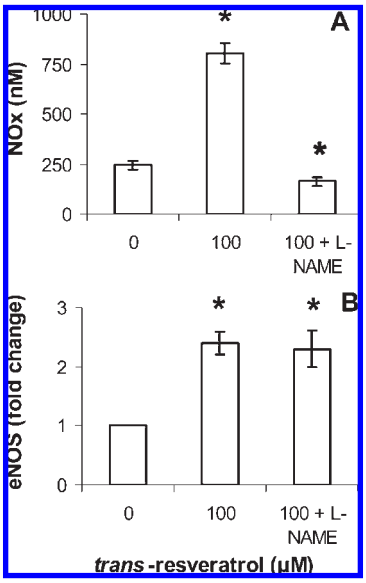

Figure 3. Effect on NO level (**A**) and eNOS expression (**B**) of exposure of L-NAME together with *trans*-resveratrol to EA.hy926 cells for 24 h. Error bars represent ± SEM of $n \geq 14$ for NO measurements and $n = 3$ for expression data. Significant differences compared to the exposure without *trans*-resveratrol were calculated with Student's *t* test ($P < 0.05$) and are indicated by an asterisk (*).

Vasoactive Potential within Several Phenolic Classes. Representatives of different structural classes of phenolics were tested: procyanidins, monomeric flavan-3-ols, flavonols, a flavone, a flavanone, a stilbene, a chalcone, and phenolic acids. Resveratrol, a stilbene, had by far the highest activity, which fits with the results of Wallerath, who showed a comparable enhancement in both NO level and eNOS expression (*14*, *15*) in EA.hy926 cells (1−100 μM). Of the flavonols, only quercetin significantly increased the NO level, whereas kaempferol was inactive. Possibly, multiple hydroxyl groups at the B-ring are favorable as suggested by Taubert (*9*). However, our data on the monomeric flavan-3-ols do not support this hypothesis. ECg and EGCg were the only monomeric flavan-3-ols that significantly increased the NO level in our assay. EGCg has been classified as a potent vasoactive compound in various systems such as bovine aortic endothelial cells and rat vasculature (*24*, *25*). Thus, the galloyl moiety seemed to be critical for the activity of ECg and EGCg. Gallic acid itself was not active at all, which is in accordance with other studies performed with EA.hy926 cells (*14*) and arteries (*9*, *26*). Results for epicatechin do not agree with those obtained in HUVEC (*27*). In these cells epicatechin increased the NO level, and it was shown that this effect was caused by methylated epicatechin that in contrast with epicatechin inhibited NADPH oxidase, thus preventing oxidation of NO. This might suggest that epicatechin is not methylated in EA.hy926.

Effects of isolated proanthocyanidins on the NO level have never been studied before. Except for dimers, procyanidins with a higher degree of polymerization showed a tendency to increase the NO level, but no significance was reached. A range of monomers up to decamers were tested with rabbit aorta and, similarly, only tetramers and higher oligomers caused relaxation (*8*). The interflavanic linkage between the flavan-3-ol units (A- or B-type) seemed to have no effect in our study, in contrast to previous observations when only A-type dimers and trimers showed significant relaxation of rabbit aorta rings (*8*, *28*).

Because procyanidins are very poorly absorbed, the vasodilating properties of their colonic metabolites, phenolics acids, are of interest. However, these phenolics did not enhance the NO level. Wallerath (*14*) showed that some phenolic acids such as benzoic acid, vanillic acid, *p*-coumaric acid, and caffeic acid increased eNOS mRNA production. However, no data on NO levels were presented. The same phenolic acids (up to 2 mM) were not able to cause relaxation of rat aortas (*26*).

In our study apigenin, naringenin, and phloretin, representing a flavone, a flavanone, and a chalcone, respectively, did not increase the NO level. In porcine arteries, conflicting effects for apigenin were reported (*9*, *29*). In agreement with our results, naringenin (12−100 μM) was not able to enhance the NO level and eNOS expression in HUVEC cells (*30*). However, results on porcine arteries are conflicting, because naringenin has been classified as a moderate (*29*) as well as a weak/nonactive (*9*) vasoactive compound. Phloretin has shown moderate relaxation in porcine coronary arteries (30−100 μM) (*29*), but to our knowledge nothing is known about its ability to affect the NO level.

Correlation between NO Level and eNOS Expression. The dose-dependent correlations between the NO level and eNOS expression as found for resveratrol, ECg, and EGCg suggested that NO production was enhanced via a larger amount of eNOS enzyme. This was also observed by Wallerath (*15*) in EA.hy926 cells exposed to up to 100 μM resveratrol and, in addition, the amount of eNOS protein increased. However, other mechanisms could play a role such as increase of eNOS activity or protection of NO produced (*27*).

Stability of the Phenolic Compounds. Many phenolic compounds are unstable at the pH usually required in the test systems used. Until now, stability has not been taken into account. In this study it was shown that several phenolic compounds were unstable during the 24 h of incubation. To prevent oxidation of phenolic compounds by superoxide and hydrogen peroxide (H_2O_2) released by autoxidation, the enzymes superoxide dismutase (SOD) and catalase were added. In addition, H_2O_2 can either increase eNOS activity (*31*) or decrease NO bioactivity (*32*). Generation of H_2O_2 might partly explain differences in the vasoactive potency found for several phenolics within different test systems. Our data confirm the beneficial effects of SOD/catalase on the stability of a number of phenolics. Furthermore, proteins in the medium or on the cell surface (*33*) might interfere during the assay when procyanidins are tested, as was shown by Aldini and co-workers (*11*). Incubations without proteins in the medium could diminish procyanidin−protein interactions. However, in the absence of 10% FBS in the medium, which represents a less physiological condition, recoveries were even lower, suggesting a protective effect of the proteins. The influence of such interactions on the bioactivity of phenolic compounds in vivo remains to be investigated.

Physiological Relevance of the Assay. Physiological concentrations in humans of the phenolics tested are usually well below 10 μM (*34*). Even for the strongest enhancer, resveratrol, we could detect a significant effect only above 30 μM. However, conditions in vivo are quite different from those in the cell culture. The mere fact that NO had to be measured in a relatively large volume of medium against a high background permitted only relatively large changes (~60 nM) in NO to be detected. However, Taubert (*9*) showed that an increase of only 8.5 nM NO already induced vasorelaxation in porcine coronary arteries. Still, results of our screening assay generally fit with this assay: quercetin, but not kaempferol, increased the NO level in vitro as well as vasorelaxation; naringenin, apigenin, catechin, epicatechin, and gallic and coumaric acid were not able to enhance the NO level in vitro and also failed to increase in vivo NO levels or relaxation in the porcine arteries (*9*). In contrast with our study, ECg and resveratrol were not active in that study. However, resveratrol (*35*, *36*),

EGCg (*24*, *25*), and quercetin (*29*, *37*) induced vasorelaxation in various other artery models. Thus, results obtained with our in vitro assay correspond with those of the artery models.

Metabolism of phenolic compounds inside the body, resulting in methylation and conjugation of phenolic hydroxyl groups with glucuronic acid and sulfate, could also affect their activity. We showed that glucuronidation of epicatechin did not change its effect (inactive) in this assay. Modulation by glucuronidation, sulfatation, and/or methylation on the NO level in vitro of phenolics still needs to be studied. To improve the value of this in vitro assay, conjugated and methylated phenolics should become commercially available for testing.

Predictive Relevance. Loke et al. (*38*) showed that quercetin and epicatechin increased the plasma pool of NO in humans by 35%, whereas EGCg had no effect. In a pilot study with three volunteers, epicatechin increased endothelial function (FMD) and microvascular function (PAT index) (*39*). In hypertensive subjects, chronic supplementation of quercetin reduced systolic blood pressure by 7 mmHg and diastolic blood pressure by 5 mmHg (*40*). Thus, our screening results should be cautiously interpreted. Metabolism and bioavailability are important factors to consider in predicting human effects.

In conclusion, this assay proved to be an easy tool to screen large sets of phenolic compounds for their potency to enhance the endothelial NO level. Only 4 of 33 phenolics, resveratrol, quercetin, ECg, and EGCg, were able to enhance the NO level significantly. No clear relationship between their structure and activity was apparent.

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LITERATURE CITED

(1) Cannon, R. O. Role of nitric oxide in cardiovascular disease: focus on the endothelium. *Clin. Chem.* **1998**, *44* (8 Part 2), 1809–1819.

(2) Geller, D. A.; Billiar, T. R. Molecular biology of nitric oxide synthases. *Cancer Metast. Rev.* **1998**, *17* (1), 7–23.

(3) Folts, J. D. Potential health benefits from the flavonoids in grape products on vascular disease. *Adv. Exp. Med. Biol.* **2002**, *505*, 95–111.

(4) Stoclet, J. C.; Chataigneau, T.; Ndiaye, M.; Oak, M. H.; El Bedoui, J.; Chataigneau, M.; Schini-Kerth, V. B. Vascular protection by dietary polyphenols. *Eur. J. Pharmacol.* **2004**, *500* (1−3), 299–313.

(5) Taubert, D.; Roesen, R.; Schomig, E. Effect of cocoa and tea intake on blood pressure: a meta-analysis. *Arch. Intern. Med.* **2007**, *167* (7), 626–634.

(6) Heiss, C.; Dejam, A.; Kleinbongard, P.; Schewe, T.; Sies, H.; Kelm, M. Vascular effects of cocoa rich in flavan-3-ols. *JAMA−J. Am. Med. Assoc.* **2003**, *290* (8), 1030–1031.

(7) Fitzpatrick, D. F.; Hirschfield, S. L.; Coffey, R. G. Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am. J. Physiol.* **1993**, *265* (2 Part 2), H774–H778.

(8) Karim, M.; McCormick, K.; Kappagoda, C. T. Effects of cocoa extracts on endothelium-dependent relaxation. *J. Nutr.* **2000**, *130* (8S Suppl.), 2105S–2108S.

(9) Taubert, D.; Berkels, R.; Klaus, W.; Roesen, R. Nitric oxide formation and corresponding relaxation of porcine coronary arteries induced by plant phenols: essential structural features. *J. Cardiovasc. Pharmacol.* **2002**, *40* (5), 701–713.

(10) Ajay, M.; Gilani, A. U. A.; Mustafa, M. R. Effects of flavonoids on vascular smooth muscle of the isolated rat thoracic aorta. *Life Sci.* **2003**, *74* (5), 603–612.

(11) Aldini, G.; Carini, M.; Piccoli, A.; Rossoni, G.; Facino, R. M. Procyanidins from grape seeds protect endothelial cells from peroxynitrite damage and enhance endothelium-dependent relaxation in human artery: new evidences for cardio-protection. *Life Sci.* **2003**, *73* (22), 2883–2898.

(12) Bouïs, D.; Hospers, G. A. P.; Meijer, C.; Molema, G.; Mulder, N. H. Endothelium *in vitro*: a review of human vascular endothelial cell lines for blood vessel-related research. *Angiogenesis* **2001**, *4*, 91–102.

(13) Edgell, C. J.; McDonald, C. C.; Graham, J. B. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80* (12), 3734–3737.

(14) Wallerath, T.; Li, H.; Godtel-Ambrust, U.; Schwarz, P. M.; Forstermann, U. A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase. *Nitric Oxide* **2005**, *12* (2), 97–104.

(15) Wallerath, T.; Deckert, G.; Ternes, T.; Anderson, H.; Li, H.; Witte, K.; Forstermann, U. Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. *Circulation* **2002**, *106* (13), 1652–1658.

(16) Deprez, S.; Brezillon, C.; Rabot, S.; Philippe, C.; Mila, I.; Lapierre, C.; Scalbert, A. Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids. *J. Nutr.* **2000**, *130* (11), 2733–2738.

(17) Olthof, M. R.; Hollman, P. C. H.; Buijsman, M. N. C. P.; van Amelsfoort, J. M.; Katan, M. B. Chlorogenic acid, quercetin-3-rutinoside, and black tea phenols are extensively metabolized in humans. *J. Nutr.* **2003**, *133* (6), 1806–1814.

(18) Appeldoorn, M. M.; Sanders, M.; Vincken, J. P.; Cheynier, V.; le Guernevé, C.; Hollman, P. C. H.; Gruppen, H. Efficient isolation of major procyanidin A-type dimers from peanut skins and B-type dimers from grape seeds. *Food Chem.* **2009**, *117*, 713−720.

(19) Babcock, D. F.; First, N. L.; Lardy, H. A. Action of ionophore A23187 at cellular level—separation of effects at plasma and mitochondrial-membranes. *J. Biol. Chem.* **1976**, *251* (13), 3881–3886.

(20) Feelisch, M.; Rassaf, T.; Mnaimneh, S.; Singh, N.; Bryan, N. S.; Jourd'Heuil, D.; Kelm, M. Concomitant S-, N-, and heme-nitros-(yl)ation in biological tissues and fluids: Implications for the fate of NO in vivo. *FASEB J.* **2002**, *16* (13), 1775–1785.

(21) RIVM, [homepage on the Internet].Bilthoven:RIVM;c1999−2008 [updated daily]. Air NOx measurements of the Netherlands; [1 screen]; available from http://www.lml.rivm.nl/data/kaart/actueel.html.

(22) de Boer, V. C.; van Schothorst, E. M.; Dihal, A. A.; van der Woude, H.; Arts, I. C.; Rietjens, I. M.; Hollman, P. C.; Keijer, J. Chronic quercetin exposure affects fatty acid catabolism in rat lung. *Cell. Mol. Life Sci.* **2006**, *63* (23), 2847–2858.

(23) Moini, H.; Guo, Q.; Packer, L. Protein binding of procyanidins: studies using polyacrylamide gel electrophoresis and French maritime pine bark extract. In *Flavonoids and Other Polyphenols*; Academic Press: San Diego, CA, 2001; Vol. *335*, pp 333−337.

(24) Lorenz, M.; Wessler, S.; Follmann, E.; Michaelis, W.; Dusterhoft, T.; Baumann, G.; Stangl, K.; Stangl, V. A constituent of green tea, epigallocatechin-3-gallate, activates endothelial nitric oxide synthase by a phosphatidylinositol-3-OH-kinase-, cAMP-dependent protein kinase-, and Akt-dependent pathway and leads to endothelial-dependent vasorelaxation. *J. Biol. Chem.* **2004**, *279* (7), 6190–6195.

(25) Kim, J. A.; Formoso, G.; Li, Y. H.; Potenza, M. A.; Marasciulo, F. L.; Montagnani, M.; Quon, M. J. Epigallocatechin gallate, a green tea polyphenol, mediates NO-dependent vasodilation using signaling pathways in vascular endothelium requiring reactive oxygen species and Fyn. *J. Biol. Chem.* **2007**, *282* (18), 13736–13745.

(26) Andriambeloson, E.; Magnier, C.; Haan-Archipoff, G.; Lobstein, A.; Anton, R.; Beretz, A.; Stoclet, J. C.; Andriantsitohaina, R. Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. *J. Nutr.* **1998**, *128* (12), 2324–2333.

(27) Steffen, Y.; Schewe, T.; Sies, H. (−)-Epicatechin elevates nitric oxide in endothelial cells via inhibition of NADPH oxidase. *Biochem. Biophys. Res. Commun.* **2007**, *359* (3), 828–833.

(28) Holt, R. R.; Karim, M.; Orozco, T. J.; Kwik-Uribe, C.; Schmitz, H. H.; Keen, C. L. A-type procyanidins from peanut skins induce endothelium-dependent relaxation. *FASEB J.* **2004**, *18* (4), A523–A523.

(29) Xu, Y. C.; Leung, S. W.; Yeung, D. K.; Hu, L. H.; Chen, G. H.; Che, C. M.; Man, R. Y. Structure−activity relationships of flavonoids for vascular relaxation in porcine coronary artery. *Phytochemistry* **2007**, *68* (8), 1179–1188.
(30) Liu, L.; Xu, D. M.; Cheng, Y. Y. Distinct effects of naringenin and hesperetin on nitric oxide production from endothelial cells. *J. Agric. Food Chem.* **2008**, *56*, 824–829.
(31) Thomas, S.; Kotamraju, S.; Zielonka, J.; Harder, D. R.; Kalyanaraman, B. Hydrogen peroxide induces nitric oxide and proteosome activity in endothelial cells: a bell-shaped signaling response. *Free Radical Biol. Med.* **2007**, *42* (7), 1049–1061.
(32) Thomas, S. R.; Schulz, E.; Keaney, J. F.Jr. Hydrogen peroxide restrains endothelium-derived nitric oxide bioactivity—role for iron-dependent oxidative stress. *Free Radical Biol. Med.* **2006**, *41* (4), 681–688.
(33) Govers, R.; Bevers, L.; de Bree, P.; Rabelink, T. J. Endothelial nitric oxide synthase activity is linked to its presence at cell-cell contacts. *Biochem. J.* **2002**, *361* (Part 2), 193–201.
(34) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81* (1 Suppl.), 230S–242S.
(35) Rakici, O.; Kiziltepe, U.; Coskun, B.; Aslamaci, S.; Akar, F. Effects of resveratrol on vascular tone and endothelial function of human saphenous vein and internal mammary artery. *Int. J. Cardiol.* **2005**, *105* (2), 209–215.
(36) Nagaoka, T.; Hein, T. W.; Yoshida, A.; Kuo, L. Resveratrol, a component of red wine, elicits dilation of isolated porcine retinal arterioles: role of nitric oxide and potassium channels. *Invest. Ophthalmol. Vis. Sci.* **2007**, *48* (9), 4232–4239.
(37) Woodman, O. L.; Chan, E. Vascular and anti-oxidant actions of flavonols and flavones. *Clin. Exp. Pharmacol. Physiol.* **2004**, *31* (11), 786–790.
(38) Loke, W. M.; Hodgson, J. M.; Proudfoot, J. M.; McKinley, A. J.; Puddey, I. B.; Croft, K. D. Pure dietary flavonoids quercetin and (−)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men. *Am. J. Clin. Nutr.* **2008**, *88* (4), 1018–1025.
(39) Schroeter, H.; Heiss, C.; Balzer, J.; Kleinbongard, P.; Keen, C. L.; Hollenberg, N. K.; Sies, H.; Kwik-Uribe, C.; Schmitz, H. H.; Kelm, M. (−)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (4), 1024–1029.
(40) Edwards, R. L.; Lyon, T.; Litwin, S. E.; Rabovsky, A.; Symons, J. D.; Jalili, T. Quercetin reduces blood pressure in hypertensive subjects. *J. Nutr.* **2007**, *137* (11), 2405–2411.

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