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SHORT COMMUNICATION

Direct evidence that (−)-epicatechin increases nitric oxide levels in human endothelial cells

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Abstract
Background The dietary flavanol (−)-epicatechin has been suggested to mediate its vasodilatory effect by increasing nitric oxide levels in endothelial cells.
Aim of the study To directly prove the formation of nitric oxide (NO) in human endothelial cells (HUVEC) in vitro by trapping NO to yield a fluorescent nitrosamine.
Methods HUVEC were treated with (−)-epicatechin; nitrite and NO formation were determined by reductive chemiluminescence detection and the NO-sensitive fluorophore 5-methoxy-2-(1*H*-naphthol[2,3-d]imidazol-2-yl)-phenol copper complex (MNIP-Cu), respectively. MNIP was synthesized in a rapid and convenient one-step microwave reaction. Endothelial nitric oxide synthase (eNOS) mRNA levels and mRNA stability were measured.
Results Incubation with (−)-epicatechin (0.3–10 μM) led to elevated NO levels in HUVEC measured via reductive chemiluminescence detection and visualized as the fluorescent NO derivative of MNIP. Expression of eNOS mRNA and mRNA stability were not affected by (−)-epicatechin treatment within the time frame studied.
Conclusion (−)-Epicatechin augments the level of NO in endothelial cells, a process suggested to be responsible for the vasodilatory properties of the compound.

Keywords (−)-Epicatechin · eNOS · HUVEC · Chemiluminescence · Fluorescence microscopy · NO

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Introduction

(−)-Epicatechin is a flavan-3-ol, a secondary plant constituent found in berries, grapes, apples, red wine, tea, or cocoa [1]. There is evidence from epidemiological studies that an increased intake of dietary flavanols or flavonoid-rich products is associated with a lower risk for cardiovascular diseases [2]. Vasodilatory effects of plant constituents likely play a role in the mechanism of protection, and a number of natural products and plant extracts have been shown to influence endothelial NO levels [3]. However, information on their mechanisms of action is sparse. NO is a major mediator of vasodilation. Defects in generation of NO are associated with increased risk for cardiovascular disease [4, 5]. Cardioprotective effects of cocoa, improvement of endothelial function, and lowering of blood pressure are attributed to flavanol-mediated elevation of the NO level [6, 7]. Vasodilatory activity was also observed after oral intake of isolated (−)-epicatechin similar to the response observed upon consumption of high-flavanol cocoa [8]. Thus, (−)-epicatechin appears to be a major bioactive constituent of cocoa. The observed improvement of endothelial function by dietary (−)-epicatechin attributed to the enhancement of the NO level is reflected by an L-NMMA-sensitive augmentation in

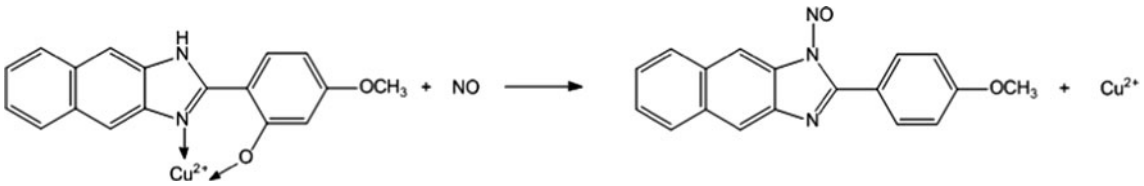

Fig. 1 Reaction of the non-fluorescent MNIP-Cu to the fluorescent NO derivative

dilation of arterial vessels [8]. Increased levels of nitrite as indirect proof of NO synthesis have been measured after flavanol exposure in cell culture with human umbilical vein endothelial cells (HUVEC) and in blood samples from human intervention studies [9]. However, proof of an elevation of NO levels after exposure to (−)-epicatechin is missing. In the present paper, we used a NO-sensitive fluorescent dye to directly visualize NO production within HUVEC upon exposure to (−)-epicatechin.

Methods

Chemicals and reagents

(−)-Epicatechin was obtained from Sigma–Aldrich (Steinheim, Germany) and used as 10 mM stock solution. Iodine, copper sulfate pentahydrate and actinomycin D, 17β-estradiol were purchased from Sigma–Aldrich (Steinheim, Germany). Dimethylsulfoxide (DMSO) was obtained from Roth (Karlsruhe, Germany). Diaminonaphthalene, 4-methoxysalicylic acid was from Acros Organics, Belgium. Potassium iodide was purchased from Fluka (Buchs, Switzerland) and acetic acid from Merck (Darmstadt, Germany). Recombinant human cytokines were purchased from Strathmann (Hannover, Germany).

Synthesis of MNIP and MNIP-Cu

5-methoxy-2-(1*H*-naphtho[2,3-d]imidazol-2-yl)phenol (MNIP) was synthesized in a rapid and convenient one-step microwave reaction modified according to [10, 11]. An amount of 158 mg (1.0 mmol) of 2,3-diaminonaphthalene and 168 mg (1.0 mmol) of 4-methoxysalicylic acid are dissolved in 5 mL dry pyridine. A volume of 0.35 mL (1.23 mmol) triphenyl phosphite is added, and the reaction vessel is sealed. The reaction mixture is heated to 220 °C for 10 min in a microwave reactor (CEM Discover, Kamp-Lintfort, Germany). After cooling, 100 mL water is added, and the mixture extracted three times with ethyl acetate (3 × 50 mL). After drying with sodium sulfate, the solvent is removed. Column chromatography was performed on silica gel with hexane/ethyl acetate (2/1) as the eluent. The product is a yellow solid (66.0 mg/0.227 mmol/23%) of blue-greenish color in solution.

^{1}H-NMR (Bruker Advance DRX 200 MHz, DMSO-d6); in accordance with [10]: δ = 13.80–12.70 (2H), 8.15–7.95 (5H), 7.45–7.35 (2H), 6.70–6.60 (2H), 3.84 (3H) ppm.

The MNIP copper complex is prepared directly before use. Equimolar amounts of MNIP (10 mM in DMSO) and $CuSO_4$ (50 mM in H_2O) are mixed and vortexed for 5 min excluding light. The mixture is used without further purification.

The secondary amine group of MNIP reacts with NO to form a stable fluorescent nitrosamine and releases copper from the molecule (Fig. 1), which has been demonstrated in model systems and in cell culture [10].

Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from Promocell (Heidelberg, Germany) and used at passages 2–5. HUVEC were cultured in endothelial cell growth medium (PromoCell) supplemented with 2% fetal calf serum, 4% endothelial cell growth supplement/heparin, 0.1 ng/mL human epidermal growth factor, 1 ng/mL human basic fibroblast growth factor, and 1 µg/mL hydrocortisone. Cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2. For experiments, HUVEC were grown in six-well plates to a confluence of 80–90%. Growth medium was then removed, and a customer-formulated, nitrite- and nitrate-free as well as serum-free medium (PromoCell) was added. Appropriate amounts of (−)-epicatechin from a stock solution (10 mM in PBS/ethanol 1/1) were added immediately.

Nitrite determination

HUVEC were treated with (−)-epicatechin for 2 h at 37 °C as described earlier. After incubation, the concentration of nitrite was determined in cell culture supernatant employing the nitric oxide analyser CLD 88 (Eco Physics, München, Germany). One hundred microliters of the supernatant was injected into a solution of acidified tri-iodide in order to reduce nitrite to NO gas, which was determined in a chemiluminescence reaction with ozone. The tri-iodide solution was adjusted to a final concentration of 0.05 M potassium iodide and 0.01 M iodine in acetic

acid and placed in the purge vessel into which samples were injected at 60 °C. Nitrite concentrations were normalized to protein content, measured by Bio-Rad D_c Protein Assay (Bio-Rad, München, Germany).

Direct detection of NO in HUVEC

HUVEC were grown on coverslip-bottomed 30-mm Petri dishes as described elsewhere. Cells were then incubated with Hank's balanced salt solution supplemented with 1 or 10 μM (−)-epicatechin or 0.1 μM 17-β-estradiol at 37 °C, while 10 μM MNIP-Cu was applied simultaneously. Fluorescence was monitored after 2 and 4 h with fluorescence microscopy (Axiovert 100 TV, Zeiss, Jena, Germany) at excitation and emission wavelengths of 395 and 490 nm, respectively.

eNOS expression and mRNA stability

HUVEC were incubated with (−)-epicatechin for 24 h at 37 °C as described elsewhere. Cells incubated with a proinflammatory cytokine mix (TNF-α, IL-1β, each 500 U/mL) instead of (−)-epicatechin were used as a positive control. After incubation, total mRNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed using Omniscript RT Kit (Qiagen). The mRNA expression level of eNOS was then determined by real-time PCR using LightCycler FastStart DNA Master SYBR-Green I and the Roche LightCycler® 2.0 (Roche Diagnostics, Mannheim, Germany). PCR reactions were performed in a total volume of 20 μL containing 40 ng cDNA, 1× FastStart Reaction Mix SYBR-Green I, 2 mM $MgCl_2$, and 0.5 μM of both, forward primer (5′-CTAGCCAAAGTCACCATCGT-3′) and reverse primer (5′-CGGGGACAGGAAATAGTTG-3′). The amplification program consisted of 1 cycle at 95 °C followed by 50 cycles at 95 °C, an optimal annealing temperature of 61 °C for 20 s, and finally 20 s at 72 °C for PCR product elongation. Samples were quantified (LightCycler® analysis software, version 3.5) using the housekeeping gene GAPDH as standard and the untreated control probe as calibrator.

For determination of eNOS mRNA stability, HUVEC were treated with 0.5 μg/mL actinomycin D alone and in combination with 3 μM (−)-epicatechin for 12, 24, 36, and 48 h. The relative mRNA content after actinomycin D incubation compared to untreated control cells was established using real-time PCR. The PCR reactions and conditions were the same as described elsewhere.

Results

In endothelial cells, NO is formed by the endothelial-derived NO synthase (eNOS). Nitrite is a major oxidation product of NO and frequently is quantified to estimate NO production. Nitrite in cell culture medium was determined using the reductive chemiluminescence method. Compared to untreated HUVEC, exposure to 0.3 and 1 μM (−)-epicatechin significantly increased nitrite levels after 2 h of incubation by 35 ± 3% and 45 ± 11%, respectively (Fig. 2). At higher concentrations of 3 and 10 μM, this effect was abolished.

Intracellular NO formation was followed with the NO-sensitive fluorophore 5-methoxy-2-(1*H*-naphthol[2,3-d]imidazol-2-yl)-phenol (MNIP). Cells are incubated with the non-fluorescent MNIP-Cu complex. The secondary amine group of MNIP reacts with cellular NO to form a stable fluorescence nitrosamine and releases copper from the molecule.

Figure 3 shows fluorescence images of HUVEC after incubation with 1 and 10 μM (−)-epicatechin for 2 h. Compared to solvent control, NO-associated fluorescence was increased, while no effect was observed at lower levels of the flavanol. Fluorescence intensity was comparable to that of the positive control 0.1 μM 17β-estradiol. After 4 h of incubation, fluorescence in cells exposed to (−)-epicatechin or 17β-estradiol was further elevated whereas only a small effect was observed in the solvent control.

Applying two different methods, the present data provide evidence that (−)-epicatechin increases levels of NO. Expression of eNOS mRNA as well as mRNA stability was investigated (Fig. 4a). For this purpose, HUVEC were incubated with (−)-epicatechin as described above. (−)-Epicatechin did not affect eNOS mRNA expression neither after 2 nor 24 h of incubation. The slight decrease after 2 h of incubation observed with 3 and

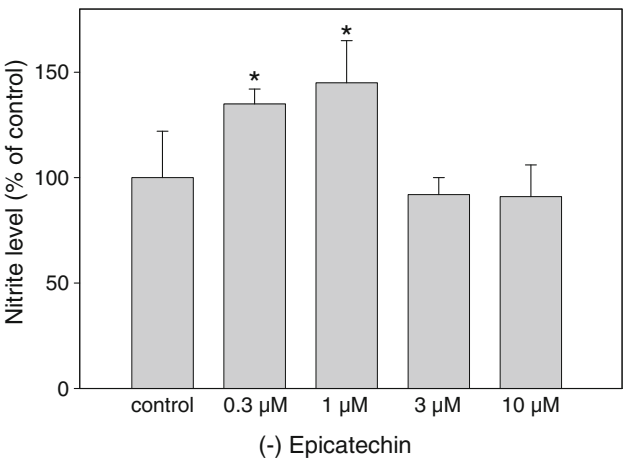

Fig. 2 Effect of (−)-epicatechin on nitrite level in the medium of human endothelial cells. HUVEC were cultured in the presence of (−)-epicatechin for 2 h. Nitrite concentration in cell culture supernatant was measured by reductive chemiluminescent detection. Data represent the means of three individual experiments ± SD; *$p < 0.05$ versus solvent control

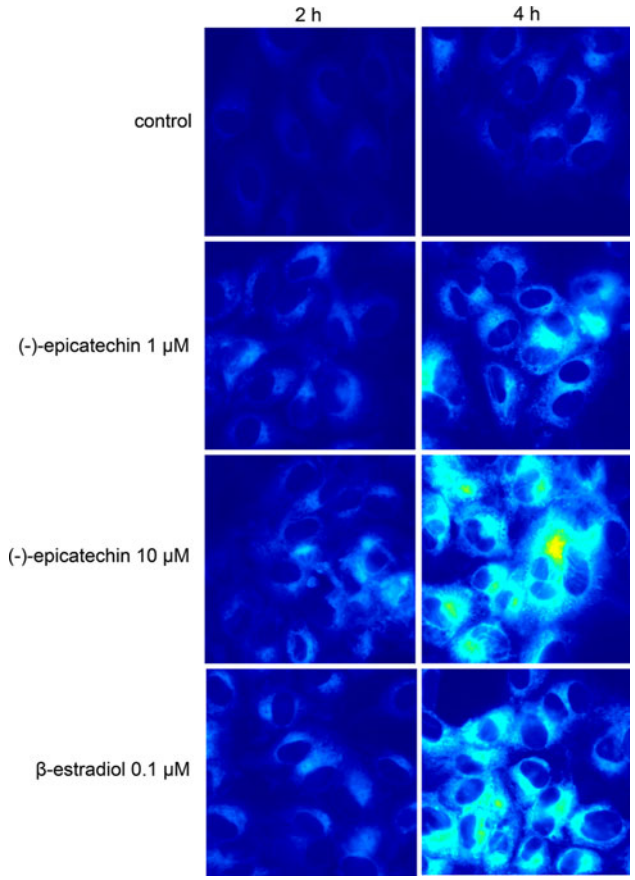

Fig. 3 NO levels in HUVEC visualized by the fluorescent MNIP NO derivative. Cells were treated with (−)-epicatechin or 17β-estradiol (an activator of eNOS), and 10 μM MNIP-Cu was applied simultaneously. Control cells were only exposed to 10 μM MNIP-Cu. After 2- and 4-h fluorescence, images were taken (excitation 395 nm, emission 490 nm)

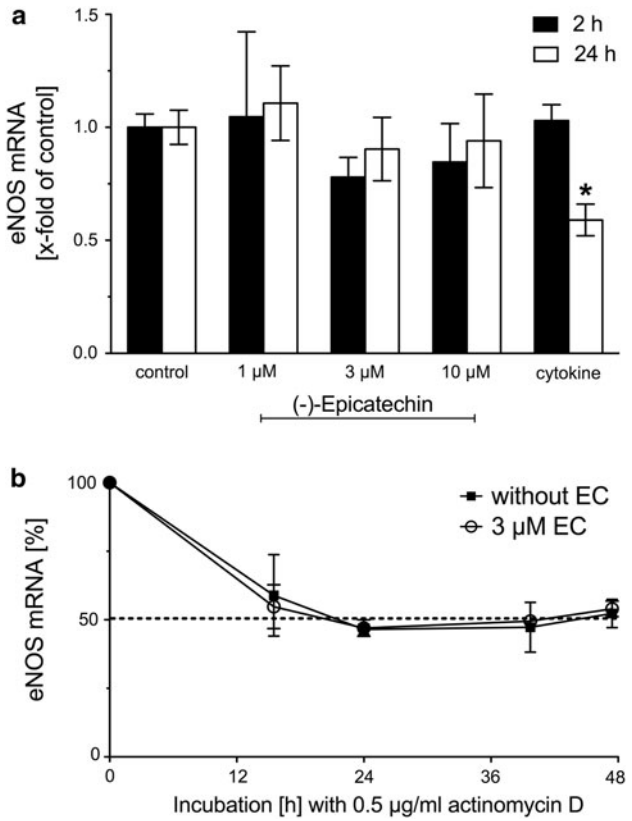

Fig. 4 Effect of (−)-epicatechin on eNOS mRNA expression and mRNA stability. **a** HUVEC were incubated with (−)-epicatechin for 2 and 24 h. mRNA was extracted, reversely transcribed, and amplified by real-time PCR. Relative expression was calculated and normalized using the housekeeping gene GAPDH. A cytokine mix (TNF-α + IL-1β, each 500 U/mL) was used as a positive control. **b** HUVEC were incubated with 0.5 μg/mL actinomycin D in the presence or absence of 3 μM (−)-epicatechin. Data represent the means of three individual experiments ± SD; *$p < 0.05$ versus solvent control

10 μM (−)-epicatechin correlates with the nitrite levels (Fig. 1) but was statistically not significant. For control, a mixture of proinflammatory cytokines was applied known to decrease eNOS mRNA levels [12].

In order to determine eNOS mRNA stability, HUVEC were treated with the transcription inhibitor actinomycin D, in the presence or absence of (−)-epicatechin. mRNA was quantified by real-time PCR and compared to untreated control (Fig. 4b). The data demonstrate that the eNOS mRNA stability is not influenced by (−)-epicatechin. (−)-Epicatechin has no effect on eNOS mRNA level in human endothelial cells.

Discussion

Vasodilatory effects of flavanol-rich cocoa have already been demonstrated in humans measuring pulse wave amplitude or flow-mediated dilation of the brachial artery [6, 13]. Following a single intake, vasodilation is observed a few hours after consumption, thus representing acute effects. Here, the vascular response was tightly associated in time with the changes in plasma levels of polyphenols and their metabolites [8]. In clear distinction from the acute effects, there are longer-term effects as a sustained increase in FMD baseline levels with high-flavanol cocoa [7, 14]. In human skin, blood flow was increased after ingestion of a high-flavanol cocoa beverage [15]. Effects were correlated with increased blood levels of (−)-epicatechin, the major flavonoid in cocoa, and evidence has been provided that vasodilation is mediated via NO-dependent mechanisms [8]. In a randomized placebo-controlled human study, treatment with (−)-epicatechin increased plasma S-nitrosothiols, plasma nitrite, and urinary nitrate concentrations [16]. After 24 h of incubation with (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate, a dose-dependent increase in nitrite was

determined in HUVEC [9]. Here, we provide direct evidence that (−)-epicatechin increases NO levels in HUVEC trapping NO in a direct reaction to yield a fluorescent nitrosamine. The method is highly sensitive; the resulting fluorescent compound is stable and accumulates within cells over time. As shown in Fig. 3, fluorescence after 4 h of incubation is more pronounced than at 2 h. Flavonoids are efficient chelators [17], and (−)-epicatechin may complex the Cu^{2+} ion from the non-fluorescent MNIP-Cu. However, such a reaction does not induce the formation of a fluorescent molecule, thus not interfering with specificity for NO (data not shown).

The biochemical mechanism underlying the regulation of NO synthesis by (−)-epicatechin and related compounds is not completely understood. Interference with NO scavenging pathways by inhibition of NADPH oxidase [18], modulation of competing arginine-dependent enzymes [19], or direct effects on eNOS expression have been proposed [20]. In vitro, data provide evidence for NO-preserving activity of (−)-epicatechin, based on the inhibition of endothelial NADPH oxidase by *O*-methyl ethers of the parent flavanol. Structural similarity suggests an apocynin-like mechanism [21].

At present, there is no evidence that (−)-epicatechin directly influences the transcription of eNOS (Fig. 4). In an in vitro study with human coronary artery endothelial cells, it was shown that epicatechin activates eNOS via serine 633 and serine 1177 phosphorylation and threonine 495 dephosphorylation [22]. Phosphatidylinositol 3-kinase-dependent pathways are apparently involved in posttranslational eNOS activation.

Applying the present method for direct detection of NO at least in cell culture will provide further insight into the mechanisms underlying the regulation of NO-dependent vasodilatory effects. It is obvious that dietary components have impact on NO signaling, which may be related to the effects of these compounds in the protection against cardiovascular disease.

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References

1. Gu L, Kelm MA, Hammerstone JF, Beecher G, Holden J, Haytowitz D, Gebhardt S, Prior RL (2004) Concentrations of proanthocyanidins in common foods and estimations of normal consumption. J Nutr 134:613–617
2. Hollman PC, Geelen A, Kromhout D (2010) Dietary flavonol intake may lower stroke risk in men and women. J Nutr 140:600–604
3. Schmitt CA, Dirsch VM (2009) Modulation of endothelial nitric oxide by plant-derived products. Nitric Oxide 21:77–91
4. Forstermann U, Munzel T (2006) Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation 113:1708–1714
5. Ignarro LJ (2002) Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. J Physiol Pharmacol 53:503–514
6. Heiss C, Dejam A, Kleinbongard P, Schewe T, Sies H, Kelm M (2003) Vascular effects of cocoa rich in flavan-3-ols. JAMA 290:1030–1031
7. Heiss C, Finis D, Kleinbongard P, Hoffmann A, Rassaf T, Kelm M, Sies H (2007) Sustained increase in flow-mediated dilation after daily intake of high-flavanol cocoa drink over 1 week. J Cardiovasc Pharmacol 49:74–80
8. Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, Sies H, Kwik-Uribe C, Schmitz HH, Kelm M (2006) (−)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. Proc Natl Acad Sci USA 103:1024–1029
9. Persson IA, Josefsson M, Persson K, Andersson RG (2006) Tea flavanols inhibit angiotensin-converting enzyme activity and increase nitric oxide production in human endothelial cells. J Pharm Pharmacol 58:1139–1144
10. Ouyang J, Hong H, Shen C, Zhao Y, Ouyang C, Dong L, Zhu J, Guo Z, Zeng K, Chen J, Zhang C, Zhang J (2008) A novel fluorescent probe for the detection of nitric oxide in vitro and in vivo. Free Radic Biol Med 45:1426–1436
11. Lin SY, Isome Y, Stewart E, Liu JF, Yohannes D, Yu L (2006) Microwave-assisted one step high troughput synthesis of benzimidazoles.Tetrahedron Lett 47:2883–2886
12. Tai SC, Robb GB, Marsden PA (2004) Endothelial nitric oxide synthase: a new paradigm for gene regulation in the injured blood vessel. Arterioscler Thromb Vasc Biol 24:405–412
13. Corti R, Flammer AJ, Hollenberg NK, Luscher TF (2009) Cocoa and cardiovascular health. Circulation 119:1433–1441
14. Sies H (2010) Polyphenols and health: update and perspectives. Arch Biochem Biophys 501:2–5
15. Neukam K, Stahl W, Tronnier H, Sies H, Heinrich U (2007) Consumption of flavanol-rich cocoa acutely increases microcirculation in human skin. Eur J Nutr 46:53–56
16. Loke WM, Hodgson JM, Proudfoot JM, McKinley AJ, Puddey IB, Croft KD (2008) Pure dietary flavonoids quercetin and (−)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men. Am J Clin Nutr 88:1018–1025
17. Flora SJ (2009) Structural, chemical and biological aspects of antioxidants for strategies against metal and metalloid exposure. Oxid Med Cell Longev 2:191–206
18. Steffen Y, Gruber C, Schewe T, Sies H (2008) Mono-O-methylated flavanols and other flavonoids as inhibitors of endothelial NADPH oxidase. Arch Biochem Biophys 469:209–219
19. Schnorr O, Brossette T, Momma TY, Kleinbongard P, Keen CL, Schroeter H, Sies H (2008) Cocoa flavanols lower vascular arginase activity in human endothelial cells in vitro and in erythrocytes in vivo. Arch Biochem Biophys 476:211–215
20. Appeldoorn MM, Venema DP, Peters TH, Koenen ME, Arts IC, Vincken JP, Gruppen H, Keijer J, Hollman PC (2009) Some phenolic compounds increase the nitric oxide level in endothelial cells in vitro. J Agric Food Chem 57:7693–7699
21. Steffen Y, Schewe T, Sies H (2007) (−)-Epicatechin elevates nitric oxide in endothelial cells via inhibition of NADPH oxidase. Biochem Biophys Res Commun 359:828–833
22. Ramirez-Sanchez I, Maya L, Ceballos G, Villarreal F (2010) (−)-Epicatechin activation of endothelial cell endothelial nitric oxide synthase, nitric oxide, and related signaling pathways. Hypertension 55:1398–1405