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Biochemical Pharmacology 67 (2004) 1285–1295
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Interference of the polyphenol epicatechin with the biological chemistry of nitric oxide- and peroxynitrite-mediated reactions

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Received 23 September 2003; accepted 13 November 2003

Abstract

The formation of reactive nitrogen species in mammalians has both beneficial and undesirable effects. Nitric oxide (NO) production in endothelial cells leads to vascular smooth muscle relaxation, but if reactive nitrogen species are generated in high amounts by cells under inflammatory conditions they are toxic. Flavonoids like (−)-epicatechin show an inverse association of their intake with diseases thought to be associated with overproduction of reactive nitrogen species. We found that the formation of cyclic GMP in cultured porcine aortic endothelial cells was not affected by up to 1 mM (−)-epicatechin. Half maximal inhibition of interferon-γ/lipopolysaccharide induced nitrite accumulation in murine macrophages required about 0.5 mM of the flavonoid. In contrast, nitration of free tyrosine triggered by 0.1 and 1 mM authentic peroxynitrite was inhibited by (−)-epicatechin with IC_{50} values of 6.6 and 28.0 μM, respectively. The presence of 15 mM sodium bicarbonate had no significant effect. Nitration of protein-bound tyrosine in phorbol 12-myristate 13-acetate treated HL-60 cells in the presence of nitrite was inhibited by (−)-epicatechin at a similar concentration range (IC_{50} = 10–100 μM). Myeloperoxidase activity of phorbol 12-myristate 13-acetate stimulated HL-60 cells was inhibited by (−)-epicatechin with an IC_{50} value of 77.4 μM. Epicatechin inhibited dihydrorhodamine oxidation by 50 μM authentic peroxynitrite and 1 mM 3-morpholino-sydnonimine with IC_{50} values of 11.8 and 0.63 μM, respectively. Our data suggest that at up to 0.1 mM (−)-epicatechin preferentially inhibits NO-related nitration and oxidation reactions without affecting NO synthesis and cyclic GMP signaling.

Keywords: Epicatechin; Tyrosine nitration; Nitric oxide; Nitric oxide synthase; HL-60 cells; Myeloperoxidase

1. Introduction

During the last years evidence has accumulated that inflammation is involved in many of the diseases that afflict modern Western population, including atherosclerosis and coronary artery disease. Inflammation is accompanied by an increased number of cells mediating non-specific immune response. Phagocytic white blood cells are important in host defense and may inflict oxidative damage during inflammatory processes [1]. Besides the oxidation of cellular biomolecules, nitration of protein tyrosine residues is of particular interest in human pathology of inflammation. The product 3-nitrotyrosine has been identified in numerous infectious and inflammatory diseases [2]. The functional consequences of protein nitration have not been fully clarified, but in some diseases 3-nitrotyrosine formation may be causally linked to pathogenesis through modulation of signaling cascades [3,4], interference with enzyme function [5,6], or structural protein assembly [7,8].

Two main pathways of tyrosine nitration have been described. According to the earlier proposal, peroxynitrite, which is formed in a fast reaction of NO with superoxide anion radicals ($O_2^{\bullet -}$), triggers nitration via nitrogen dioxide radicals ($NO_2^{\bullet}$) or a CO_2 adduct [9–13]. In an alternative mechanism MPO (EC 1.11.1.7) is thought to be the major trigger of tyrosine nitration, either via oxidation of nitrite, the stable endproduct of NO autooxidation to $NO_2^{\bullet}$ by compounds I and II [14] or via reaction of nitrite with the main MPO product, hypochlorous acid to form NO_2Cl as nitrating species [15–18]. Phagocytic white blood cell

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Abbreviations: cGMP, cyclic GMP; HPLC, high performance liquid chromatography; IFN, interferon; LPS, lipopolysaccharide; MPO, myeloperoxidase; NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NO synthase (type III); iNOS, inducible NO synthase (type II); PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; SIN-1, 3-morpholinosydnonimine.

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doi:10.1016/j.bcp.2003.11.017

activation can principally boost both peroxynitrite and MPO-mediated nitration. The generation of oxidants by neutrophils results from activation NADPH oxidase which reduces molecular oxygen to $O_2^{\bullet -}$. Dismutation of $O_2^{\bullet -}$ yields hydrogen peroxide (H_2O_2), but both $O_2^{\bullet -}$ and H_2O_2 are relatively non-toxic to bacteria. Activated neutrophils also secrete MPO, which is a heme protein that localizes to azurophilic granules and is present in these cells at up to 5% of their dry weight [1]. MPO utilizes H_2O_2 and chloride as substrates to form hypochlorous acid (HOCl) according to Eq. (1).

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O \quad (1)$$

Activation of phagocytic white blood cell is also associated with the induction iNOS and sustained release of NO [19]. NO itself is not particularly toxic to cells, but may form secondary oxidants that are responsible for cytotoxicity. An important pathway that enhances NO cytotoxicity is its rapid reaction with $O_2^{\bullet -}$ to yield peroxynitrite ($ONOO^-$). While peroxynitrite may serve as a beneficial molecule in the defense against invading microorganisms, overproduction may have a wide range of pathophysiological consequences, including oxidation and nitration of biomolecules [20].

The flavonoid (–)-epicatechin (Fig. 1), present in high amounts in dietary sources, particularly green tea, certain chocolates, and red wine has been shown to be a potent scavenger of peroxynitrite [21,22]. Recently it has been shown that (–)-epicatechin is cell permeable [23]. Suggested health benefits of these polyphenolic compounds include protection against cancer [24] and inhibition of inflammatory cytokine production [25]. These compounds are also reported to function as potent antioxidants due to their reducing properties [26]. The Rotterdam study [27] showed an inverse association of flavonoid intake with incident of myocardial infarction. In addition, some flavonoids can act as scavengers of NO [28] and $O_2^{\bullet -}$ [29], but the underlying mechanisms are still unclear. Furthermore, it has been proposed that flavonoids like (−)-epicatechin act as potent scavengers of $NO_2^{\bullet}$ [30]. Experimental evidence for the formation of $NO_2^{\bullet}$ produced by the MPO/nitrite system was recently reported [31].

In the present study, we investigated the potential of the flavonoid (−)-epicatechin on various reactions that involve NO and related species. Three different cell lines were used: (i) cultured porcine aortic endothelial cells were used to study the potential interference of (−)-epicatechin with NO/cGMP-mediated signaling. Intracellular accumulation of cGMP was measured upon Ca^{2+} stimulation to see whether or not this compound affects any of the components of endothelial NO/cGMP signaling; (ii) murine macrophages were used to determine the interference of (−)-epicatechin with INF-γ/LPS-induced iNOS expression and iNOS activity of these cells; (iii) human leukocytes, which contain up to 5% MPO, were used to study the effect of (−)-epicatechin on the nitration of protein-bound tyrosine. These cells were also used to find out whether (−)-epicatechin is a direct inhibitor of MPO activity. In addition, the effect of (−)-epicatechin on eNOS activity and peroxynitrite-mediated nitration and oxidation reactions was studied.

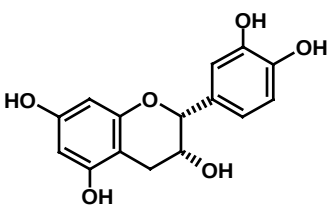

Fig. 1. Chemical structure of (−)-epicatechin.

2. Material and methods

2.1. Materials

All solvents used were of HPLC grade. Dihydrorhodamine 123 (DHR), 3-nitrotyrosine were from Fluka-Sigma. Recombinant mouse IFN-γ and Pronase from *Streptomyces griseus* were from Roche Diagnostics GmbH, anti-iNOS antibody was obtained from BD Transduction Laboratories. Penicillin/streptomycin, amphotericin B, and fetal calf serum were from PAA Laboratories GmbH. L-[2,3,4,5-^{3}H]Arginine hydrochloride (57 Ci mmol^{-1}) was from American Radiolabeled Chemicals Inc., purchased through Humos Diagnostica GmbH. Tetrahydrobiopterin was obtained from Dr. B. Schircks Laboratories. NADPH was purchased from Pharma Waldhof GmbH. Recombinant human eNOS (maximal enzyme activity of 100–150 nmol of L-citrulline mg^{-1} min^{-1}) was expressed in the yeast *Pichia pastoris* as described in detail elsewhere [32]. Centrifuge tube filters (0.22 μm cellulose acetate) were from Szabo (Vienna, Austria). N^G-Nitro-L-arginine, LPS from *Salmonella typhosa*, and all other chemicals were from Sigma.

2.2. Solutions

All solutions were prepared freshly each day. Water was ultrafiltered type I (resistance ≤18 MΩ cm^{-1}) from a Milli-Q Water System. PBS is 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4. NO solutions were prepared as described [33]. Stock solutions of (−)-epicatechin (100 mM) in H_2O/DMSO (v/v) were further diluted with the buffer solution used in the assays (either PBS or as indicated in the legends of the figures). DHR was dissolved in acetonitrile to 10 mM and kept in the dark until use. SIN-1 was dissolved to 10 mM at pH 5.0. Alkaline solutions of peroxynitrite were prepared from acidified NO_2^- and H_2O_2 as described [34,35]. Stock solutions were diluted with H_2O to 10 mM (pH∼12.8)

immediately before the experiments and added to 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4, to obtain final peroxynitrite concentrations as indicated in the text. Changes of buffer pH were <0.1 unit.

2.3. Culture of HL-60 Cells

HL-60 promyelocytic leukemia cells (passage 20–60) were obtained form the American Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 unit mL^{-1} penicillin/streptomycin, 1.25 μg mL^{-1} amphotericin B, and 2 g L^{-1} $NaHCO_3$ in 50 mL flasks at 37° in a 5% CO_2 humidified atmosphere. Cultures were passaged and fed two to three times per week to maintain log phase growth. Once a week cells were centrifuged and resuspended in fresh RPMI. Cell density for the experiments was 1×10^6 cells mL^{-1} (or as indicated in the text). Cells were counted with a haemacytometer (Neubauer improved). The cells were assayed for: formation of protein-bound 3-nitrotyrosine and MPO activity. For the determination of protein-bound 3-nitrotyrosine the cells were incubated for 2 hr with 200 nM PMA and 1 mM NO_2^- in the presence of various concentrations of (−)-epicatechin. MPO activity was determined after treatment of the cells with 200 nM PMA for 1 hr as described below.

2.4. Culture of endothelial cells and determination of intracellular cGMP

Porcine aortic endothelial cells were cultured as previously described [36]. Endothelial cells were isolated by enzymatic treatment (0.1% collagenase in PBS) and cultured up to three passages in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 100 units mL^{-1} penicillin/streptomycin, 1.25 μg mL^{-1} amphotericin B. Prior to experiments, endothelial cells were subcultured in 24-well plastic plates and grown to confluence ($\sim 2 \times 10^5$ cells per well). The culture medium was removed, and the cells were washed once and equilibrated in incubation buffer (isotonic 50 mM Tris buffer, pH 7.4, containing 3 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine, and 10 μM indomethacin) in the absence or presence various concentrations of (−)-epicatechin, or 0.1 mM N^G-nitro-L-arginine. After 15 min, Ca^{2+} ionophore A23187 was added to give initial final concentrations of 1 μM. Reactions were terminated 4 min later by removal of the incubation buffer and treatment for 1 hr with 1 mL of 0.01 N HCl. Intracellular cGMP was measured in the supernatants of the lysed cells by radioimmunoassay.

2.5. Culture and activation of macrophages

RAW 264.7 macrophages (form American Type Culture Collection) were cultured in Petri dishes (diameter 90 mm) at 37° and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 unit mL^{-1} penicillin/streptomycin, 1.25 μg mL^{-1} amphotericin B, and 3.7 g L^{-1} $NaHCO_3$. Cells were grown to confluence ($\sim 5 \times 10^7$ cells per dish) and incubated for 16 hr in the presence of IFN-γ (50 unit mL^{-1}) and LPS (0.5 unit mL^{-1}) in fresh phenol red-free Dulbecco's MEM. The cells were assayed for nitrite accumulation and iNOS expression (see below).

2.6. Determination of nitrite accumulation

Cell culture supernatants of RAW 264.7 macrophages (~300 μL) were centrifuged (20,000 *g*), and the concentration of NO_2^- was determined photometrically with the Griess assay [37]. Aliquots (100 μL) were mixed with 100 μL of freshly prepared Griess reagent (20 mg *N*-(1-naphthyl)-ethylenediamine and 0.2 g sulfanilamide dissolved in 20 mL of 5% (w/v) phosphoric acid), and the absorbance at 546 nm was measured with a microplate reader. Calibration curves were established with $NaNO_2$ dissolved in the cell culture medium.

2.7. SDS–polyacrylamide gel electrophoresis and Western blotting

RAW 264.7 macrophages were washed with PBS, harvested, and centrifuged for 5 min at 200 g. After resuspension cells were washed followed by sonication 3× approximately 10 s at 50 W. SDS–polyacrylamide gel electrophoresis was performed using 8% polyacrylamide gels with electrophoresis at 180 V for 45 min in a Bio-Rad miniprotean chamber as described in [38]. The separated proteins were transferred to nitrocellulose membranes by electroblotting for 90 min at 240 mA, followed by immunodetection with an anti-iNOS (1:2500 dilution) antibody, using horseradish peroxidase-conjugated anti-mouse IgG. For detection of the protein, the ECL system from Amersham Pharmacia Biotech was used. Densitometric analysis was performed using a Herolab E.A.S.Y Win32 HiRes densitometric system additionally equipped with an UV Transilluminator UVT-28 ME. Captured images were analyzed using E.A.S.Y Win32 image analysis software (from Herolab GmbH).

2.8. Determination of eNOS activity

The activity of eNOS was determined as formation of L-[2,3,4,5-^{3}H]citrulline from L-[2,3,4,5-^{3}H]arginine [32,39]. Incubations were for 10 min at 37° in 0.1 mL of 50 mM triethanolamine–HCl buffer, pH 7.4, containing 0.1–0.2 μg of eNOS, 0.1 mM L-[2,3,4,5-^{3}H]arginine (~80,000 counts per min), 0.5 mM $CaCl_2$, 10 μg mL^{-1} calmodulin, 0.2 mM NADPH, 10 μM tetrahydrobiopterin, 5 μM FAD, 5 μM FMN, and 0.2 mM 3-[(3-cholamidopropy-l) dimethylammonio]-1-propanesulfonic acid (CHAPS). L-[^{3}H]Citrulline

was separated from L-[^{3}H]arginine by cation exchange chromatography (Dowex 50 W, 8% cross-linkage, 200–400 mesh size, H^+-form, from Sigma).

2.9. Determination of MPO activity in HL-60 cells

The activity of MPO was determined as formation of hypochlorous acid by taurine chloramine assay as described [40]. HL-60 cells (1×10^6 cells mL^{-1}) were stimulated with PMA (200 nM final) and incubated at 37° in 0.5 mL of 10 mM Na_2HPO_4/KH_2PO_4 buffer, pH 7.4, containing 1 mg mL^{-1} of glucose, 138 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM taurine, and varying concentrations of (−)-epicatechin as indicated. Reactions were terminated after 60 min with 20 μg mL^{-1} catalase and putting the reaction mixture on melting ice for 10 min. Cells were pelleted at 10,000 *g* for 2 min. The concentration of accumulated taurine chloramine, the reaction product of taurine and hypochlorous acid, present in the supernatants was determined as oxidation of 5-thio-2-nitrobenzoic acid (100 μM final) to 5,5′-dithiobis (2-nitrobenzoic acid) by monitoring the decrease in 5-thio-2-nitrobenzoic acid absorbance at 412 nm [40]. Blank values were measured separately for every (−)-epicatechin concentration.

2.10. Tyrosine nitration and HPLC analysis of 3-nitrotyrosine in buffer

To study tyrosine nitration by authentic peroxynitrite, alkaline stock solutions of peroxynitrite (final concentration as indicated in the text) were added dropwise under vigorous vortexing to tyrosine (1 mM) in 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4, containing various (−)-epicatechin concentrations, followed by incubation for 1 hr. Samples were kept on ice until HPLC analysis (typically 1–2 hr). Samples (50 μL) were injected onto a 250 mm × 4 mm C_{18} reversed phase column equipped with a 4 mm × 4 mm C_{18} guard column (LiChrospher 100 RP-18, 5 μm particle size, Merck). Elution was with 50 mM KH_2PO_4/H_3PO_4 buffer (pH 3) containing 10% (v/v) methanol at a flow rate of 1.0 mL min^{-1}. 3-Nitrotyrosine was detected by its absorbance at 274 nm with a Merck Hitachi L-4250 UV-Vis detector. Identification and quantification of 3-nitrotyrosine was based on calibration curves established with the authentic compound (1–100 μM) under the same conditions.

2.11. Determination of protein-bound 3-nitrotyrosine and tyrosine in cell lysates

Protein-bound 3-nitrotyrosine was determined by HPLC and electrochemical detection after derivatization to *N*-acetyl 3-aminotyrosine as described [41], but with a few modifications. HL-60 cells from two flasks (corresponding to 100 mL cell suspension) were used for single determinations. After incubation, cells were centrifuged three times at 1300 *g* for 5 min and resuspended in PBS. Then the cell pellets were resuspended in 250 μL 0.1 M K_2HPO_4/KH_2PO_4 buffer, pH 7.4, giving protein concentrations of 10–20 mg mL^{-1} according to the Bradford method with bovine serum albumin as a standard [42]. Protein was precipitated with 0.8 mL acetone (cooled to −20°), the samples were thoroughly vortexed, kept on ice for 10 min and centrifuged (8000 *g*, 5 min), followed by resuspension of the precipitate in 250 μL of 0.1 M phosphate buffer, pH 7.4, and sonication for 2× approximately 10 s at 50 W. This procedure was repeated three times to remove non-protein material and nitrite. The final suspension (in 250 μL buffer) was incubated for 15–18 hr at 37° with 2 mg pronase and 0.5 mM $CaCl_2$. Samples (∼300 μL) were centrifuged (20,000 *g*) and an equal volume of 3 M potassium phosphate buffer, pH 9.6, was added to the supernatant, followed by the addition of acetic anhydride (30 μL). After 15 min of incubation at ambient temperature, ethyl acetate (1 mL) and formic acid (125 μL) were added. The samples were vortexed for 30 s and then centrifuged at 20,000 *g* for 1 min. The ethyl acetate phase was concentrated to dryness under a stream of N_2 at 45°. For deacetylation of the phenolic acetate group, samples were resuspended in 60 μL 1 N NaOH. After 30 min of incubation at 37°, 120 μL of 1 M K_2HPO_4/KH_2PO_4 buffer (pH 6.5) was added, followed by addition of 10 μL 0.1 M sodium dithionite to reduce the nitro substituent to the corresponding amine. The samples were incubated for 10 min at ambient temperature, acidified by the addition of concentrated HCl (20 μL) and centrifuged at 20,000 *g* for 10 min in centrifuge tube filters. One hundred and forty microliters samples (cooled to 4° by a Varian ProStar 420 autosampler) were injected onto a 250 mm × 4 mm C_{18} reversed phase column equipped with a 4 mm × 4 mm C_{18} guard column (LiChrospher 100 RP-18, 5 μm particle size) which was held at 22° by Varian column oven ProStar 510. Elution was with 10 mM H_3PO_4, containing 2% (v/v) methanol (solvent A) and 50% (v/v) methanol/50% (v/v) 10 mM H_3PO_4 (solvent B) at 0.7 mL min^{-1} with a gradient program (0–18 min 100% solvent A, 18–24 min linear ramp to 100% solvent B, 24–39 min 100% solvent B, 39–43 min linear ramp to 100% solvent A and 43–75 min 100% solvent A). *N*-Acetyl 3-aminotyrosine was detected electrochemically with an ESA Coulochem II detector equipped with a 5011 analytical cell. The potentials of the two electrodes were adjusted to −70 and +70 mV, respectively. Standards of *N*-acetyl 3-aminotyrosine were prepared as described [41], and calibration curves were recorded daily (5–500 nM). Tyrosine was detected as *N*-acetyltyrosine (prepared as *N*-acetyl 3-aminotyrosine) at 280 nm (standards 50–1000 μM) with a Varian ProStar 310 UV-Vis detector. Blank values of non-stimulated cells were subtracted for every experiment.

2.12. Determination of DHR oxidation

Oxidation of DHR by 50 mM authentic peroxynitrite and was monitored at 500 nm with a Hewlett-Packard 8452A Diode Array spectrophotometer at ambient temperature in a total volume of 0.2 mL of a 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4 containing 0.1 mM DHR and 0.1 mM of the metal chelator diethylenetriamine pentaacetic acid, as described [43,44].

With 1 mM SIN-1 as the source of peroxynitrite, the oxidation of DHR was monitored for 10 min in the presence of various concentrations of (−)-epicatechin. Aerobic decay of SIN-1 is a two-step reaction with intermediate formation of SIN-1A, which reacts with O_2 to yield SIN-1C, $O_2^{\bullet -}$ and NO. Consequently, the time course of formation of SIN-1C (and of NO/$O_2^{\bullet -}$) exhibits a lag phase of several minutes. Since subsequent formation of peroxynitrite and reaction with DHR are much faster, DHR oxidation will follow the same time course as SIN-1C formation, exhibiting a similar lag phase. To take this into account, we fitted the time course of DHR oxidation to an equation (Eq. (2)) describing the time course of formation of SIN-1C, using previously measured rate constants for SIN-1A and SIN-1C formation [45].

$$A_{500\,nm} = a(1 + 0.939\,e^{-0.064t} - 1.939\,e^{-0.031t}) + b \qquad (2)$$

The fitting parameters a and b represent the observed absorbance change and the initial absorbance, respectively. The obtained values of a, which are proportional to the effective concentration of the oxidant reacting with DHR, were replotted against the concentration of (−)-epicatechin as apparent relative reactivities, with the value in the absence of (−)-epicatechin taken as 100%, and fitted to the Hill equation.

2.13. Decomposition of peroxynitrite

Peroxynitrite decomposition was studied by stopped-flow absorbance spectroscopy at 37° (Bio-Sequential SX-17MV stopped-flow ASVD spectrofluorimeter, Applied Photophysics, Leatherhead, UK) with the wavelength set at 302 nm ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$) according to a published procedure [34]. Reservoir 1 contained 0.2 mM peroxynitrite in 0.01 M NaOH, and reservoir 2 contained the varying concentrations of (−)-epicatechin (0–10 mM) in 1 M K_2HPO_4/KH_2PO_4 buffer, pH 7.4.

2.14. Determination of the decomposition products of peroxynitrite (NO_2^- and NO_3^-)

Peroxynitrite (1 mM) was decomposed by incubation for 1 hr at 37° in 0.1 M K_2HPO_4/KH_2PO_4 buffer, pH 7.4 in the presence or absence of 1 mM (−)-epicatechin as described [34]. NO_2^- was determined by the Griess assay as described above. For determination of $NO_2^- + NO_3^-$, samples were coincubated for 45 min with saturated solutions of VCl_3 and the Griess reagent as described [46]. The nitrite and total NO_x concentrations in samples were subtracted to give the nitrate concentrations. The amount of NO_2^- present in stock solutions of conventionally prepared peroxynitrite was subtracted from the measured values [34].

2.15. Statistics and data evaluation

Unless otherwise indicated, data are given as means ± SE of three independent experiments. The concentration producing 50% inhibition of the maximal response (IC_{50}) was calculated from individual concentration–response curves which were calculated by non-linear regression analysis. The computations were performed with an IBM-compatible personal computer using SigmaPlot 2002 for Windows Version 8.0.

3. Results

3.1. Interference of (−)-epicatechin with NO/cGMP-mediated signaling, eNOS activity, iNOS expression and accumulation of nitrite

Interference of (−)-epicatechin with NO/cGMP-mediated signaling was studied in cultured porcine aortic endothelial cells. As shown in Fig. 2A, (−)-epicatechin had no effect on cGMP accumulation under basal conditions. Stimulation of the cells with Ca^{2+} ionophore A23187 led to a pronounced accumulation of intracellular cGMP (51.5 ± 4.9 pmol per 10^6 cells) which was completely inhibited by N^G-nitro-L-arginine (0.1 mM). This rise in cGMP was not affected by up to 1 mM (−)-epicatechin, indicating that the polyphenol does neither interfere with Ca^{2+}-triggered endothelial NO synthesis nor with the activation of soluble guanylyl cyclase by NO. Under standard assay conditions (−)-epicatechin was a very poor inhibitor of purified eNOS with an IC_{50} value of about 0.5 mM (Fig. 2B). Activation of RAW 264.7 macrophages with IFN-γ/LPS led to iNOS expression apparent as formation of ~40 μM nitrite. Addition of 1 mM (−)-epicatechin decreased iNOS expression to ~40% of control (data not shown). As shown in Fig. 2C, presence of up to 100 μM (−)-epicatechin did not affect nitrite accumulation in the supernatant of induced RAW cells ($IC_{50} > 1$ mM).

3.2. Effect of (−)-epicatechin on tyrosine nitration

As shown in Fig. 3A, nitration of 1 mM free tyrosine triggered by 0.1 mM authentic peroxynitrite led to the formation of 8.3 ± 0.64 μM 3-nitrotyrosine that was inhibited by (−)-epicatechin with an IC_{50} value of 6.6 ± 1.06 μM (mean value ± SE; N = 3). A 10-fold higher concentration of peroxynitrite (Fig. 3B) led to formation of 66.8 ± 6.26 μM 3-nitrotyrosine that was

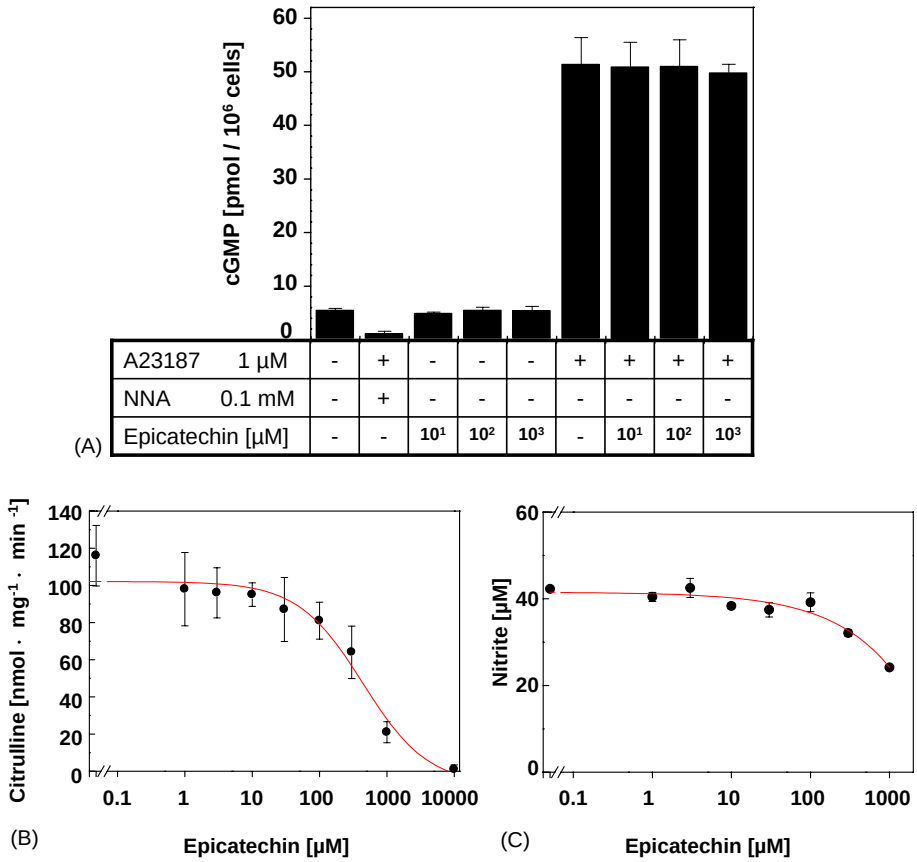

Fig. 2. Interference of (−)-epicatechin with NO/cGMP-mediated signaling, eNOS activity and nitrite formation. (A) NO/cGMP-mediated signaling: porcine aortic endothelial cells were subcultured in 24-well plastic plates, grown to confluence (~2×10^5 cells per well), washed and equilibrated (as described in Section 2) in the absence or presence of various concentrations of (−)-epicatechin, or 0.1 mM N^G-nitro-L-arginine. After 15 min, Ca^{2+} ionophore A23187 was added to give initial final concentrations of 1 µM. Reactions were terminated 4 min later by removal of the incubation buffer and treatment for 1 hr with 1 mL of 0.01 N HCl. Intracellular cGMP was measured in the supernatants of the lysed cells by radioimmunoassay. The data are mean values ± SE of four independent experiments. (B) The activity of purified eNOS was determined as formation of L-[2,3,4,5-^{3}H]citrulline from L-[2,3,4,5-^{3}H]arginine (as described in Section 2). The enzyme was preincubated with (−)-epicatechin for 10 min at 37°. The data are mean values of two experiments performed in duplicate. (C) Nitrite formation by cytokine-activated murine macrophages: RAW 264.7 macrophages were grown to confluence (~5×10^7 cells per dish) and incubated for 16 hr in the presence of IFN-γ (50 unit mL^{-1}) and LPS (0.5 µg mL^{-1}) with the indicated concentrations of (−)-epicatechin. The supernatants of the activated cells were assayed photometrically for nitrite accumulation (as described in Section 2) as a measure of iNOS expression and activity. The data are mean values ± SE of three independent experiments performed in duplicate.

inhibited by (−)-epicatechin with an IC_{50} value of 28.0 ± 2.33 µM (mean value ± SE; N = 3). 3-Nitrotyrosine formation was increased in the presence of 15 mM sodium bicarbonate (81.0 ± 2.63 µM with 1 mM peroxynitrite), but the IC_{50} value for (−)-epicatechin was not significantly increased (IC_{50} = 45.8 ± 5.44 µM; mean value ± SE; N = 3; $P = 0.07$; Fig. 3C).

Stimulation of HL-60 cells with 200 nM PMA in the presence of 1 mM nitrite for 2 hr led to the formation of 0.68 ± 0.18 pmol mg^{-1} protein-bound 3-nitrotyrosine (average blank value of non-stimulated cells was 0.16 ± 0.03 pmol mg^{-1} protein-bound 3-nitrotyrosine). HL-60 cells contained 0.21 ± 0.01 µmol mg^{-1} protein-bound tyrosine, indicating nitration of about three out of one million tyrosine molecules. As shown in, nitration was inhibited by (−)-epicatechin with an IC_{50} value of 10–100 µM. Thus, (−)-epicatechin inhibited nitration of free and protein-bound tyrosine with similar potency. Interestingly, HL-60 cells exhibited (−)-epicatechin-insensitive nitration activity of approximately 25% (Fig. 4).

3.3. Effect of (−)-epicatechin on MPO activity in HL-60 cells

MPO activity in PMA-stimulated HL-60 cells was inhibited by (−)-epicatechin with an IC_{50} value of 77.4 ± 16.19 µM (mean value ± SE; N = 3). Total HOCl production was 17.9 ± 2.31 nmol per 10^6 cells. Note that addition of 1 mM (−)-epicatechin led to complete enzyme inhibition (Fig. 5).

3.4. Effect of (−)-epicatechin on dihydrorhodamine (DHR)-oxidation

Epicatechin inhibited DHR oxidation by 50 µM authentic (Fig. 6A) with an IC_{50} value of 11.8 ± 1.29 µM (mean

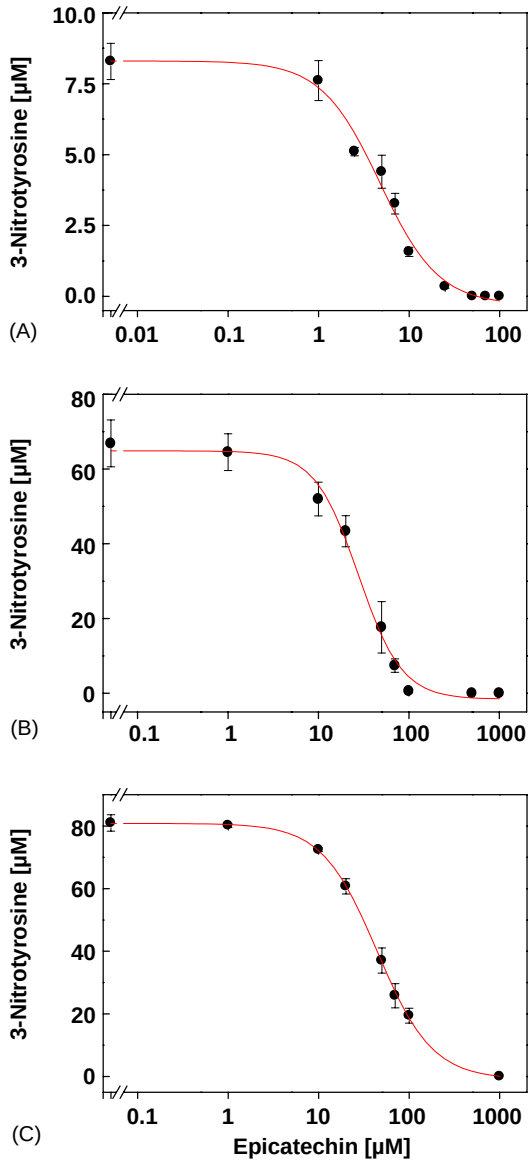

Fig. 3. Effect of (−)-epicatechin on formation of 3-nitrotyrosine. Nitration of free tyrosine in the presence of various concentrations of (−)-epicatechin triggered by (A) 0.1 mM authentic peroxynitrite, (B) 1 mM authentic peroxynitrite, (C) 1 mM authentic peroxynitrite in the presence of 15 mM $NaHCO_3$. UV-Vis detection of 3-nitrotyrosine was performed as described in Section 2. The data are the mean values ± SE of three independent experiments performed in duplicate.

value ± SE; N = 3). The effect of (−)-epicatechin on the oxidation of DHR by the $NO/O_2^{\bullet -}$ donor SIN-1 is illustrated in Fig. 6B. In the presence of 1 mM SIN-1, (−)-epicatechin exhibited an IC_{50} value of 0.64 ± 0.08 µM and a Hill coefficient of 0.90 ± 0.05. The inhibition curve shown in Fig. 6B was obtained by taking into account the non-linear nature of $NO/O_2^{\bullet -}$ formation by SIN-1 (see Section 2). The less sophisticated procedure of plotting the concentration of oxidized DHR obtained at the end of the 10 min incubation against the concentration of (−)-epicatechin yielded identical results (IC_{50} = 0.67 ± 0.10 µM; Hill coefficient = 0.91 ± 0.06, not shown).

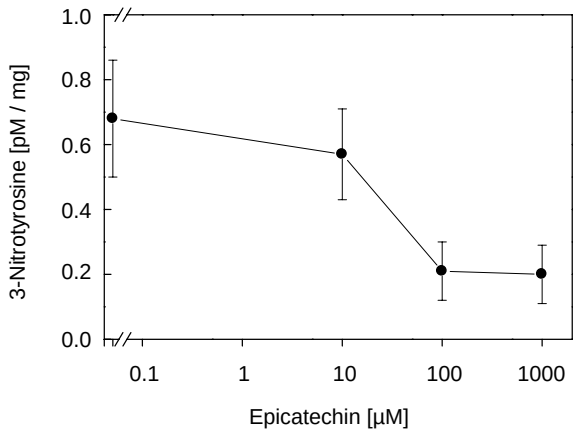

Fig. 4. Effect of (−)-epicatechin on formation of protein-bound 3-nitrotyrosine in HL-60 cells. Cells were incubated for 2 hr with 200 nM PMA and 1 mM NO_2^- in the presence of various concentrations of (−)-epicatechin. 3-Nitrotyrosine was determined electrochemically after HPLC separation as *N*-acetyl 3-aminotyrosine as described in Section 2. The data are the mean values ± SE of three independent experiments performed in duplicate.

3.5. Effect of (−)-epicatechin on the decomposition of peroxynitrite

Decomposition of peroxynitrite was monitored as decrease in absorbance at 302 nm at 37°. All traces could be fitted to single exponentials. The observed pseudo-first order rate constant (1.18 ± 0.03 s^{-1}) was independent of the concentration of (−)-epicatechin (0–10 mM), which puts an upper limit of ∼20 M^{-1} s^{-1} to the rate constant for a reaction between peroxynitrite and (−)-epicatechin. Thus, the potent scavenging activity of (−)-epicatechin cannot be explained by a direct reaction with peroxynitrite.

We consistently observed that the decomposition of peroxynitrite, which was expected to result in formation of about 70% NO_3^- and 30% NO_2^- at pH 7.4 and 37° [34], was not affected by 1 mM (−)-epicatechin. Decomposition

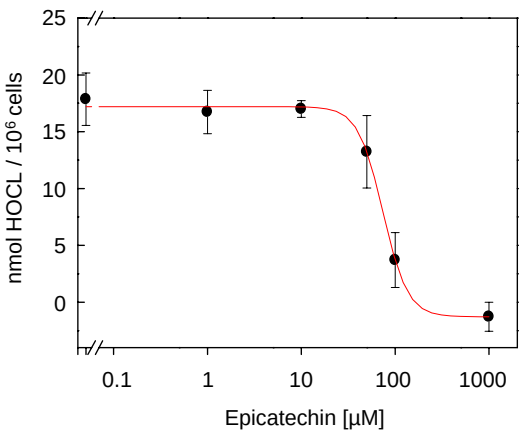

Fig. 5. Effect of (−)-epicatechin on MPO activity. HL-60 were incubated for 1 hr with 200 nM PMA in the presence of various concentrations of (−)-epicatechin. The formed hypochlorous acid was measured with the taurine chloramine assay as described in Section 2. The data are mean values ± SE of three independent experiments performed in duplicate.

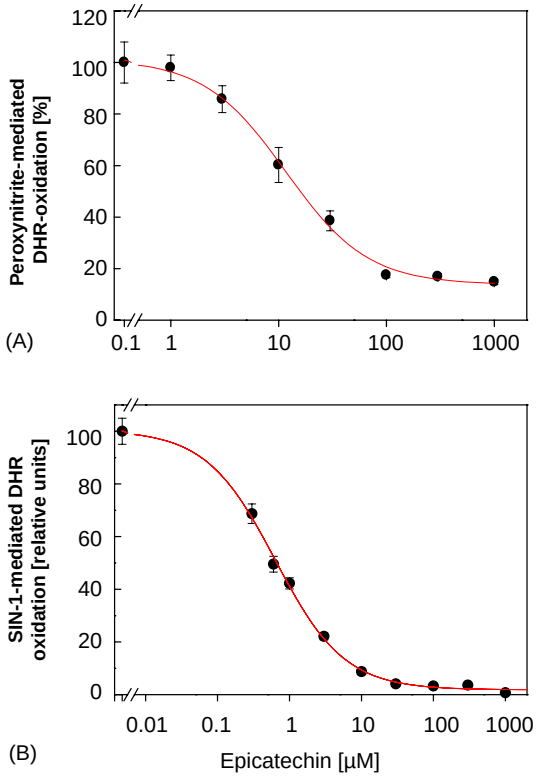

Fig. 6. Effect of (−)-epicatechin on DHR oxidation. DHR oxidation was assayed as described in Section 2 with (A) 50 μM authentic peroxynitrite and (B) 1 mM SIN-1 (the apparent reactivities plotted along the vertical axis were derived as detailed in Section 2). The data are mean values ± SE of three independent experiments performed in duplicate.

without and with (−)-epicatechin yielded 30.2 ± 0.59% and 30.9 ± 1.47% NO_2^-, respectively. The total amount of $NO_2^- + NO_3^-$ was not significantly decreased (0.89 ± 0.025 mM and 0.80 ± 0.035 mM in the absence and presence of 1 mM (−)-epicatechin, respectively; mean values ± SE; N = 3; $P > 0.1$).

4. Discussion

4.1. Interference of (−)-epicatechin with NO/cGMP-mediated signaling and NOS activity

Inflammatory diseases are associated with sustained synthesis and release of free radicals, in particular $O_2^{\bullet -}$, hydroxyl radicals, hydrogen peroxide, and NO [47]. Secondary reactions of these molecules yield highly reactive intermediates, such as peroxynitrite, $NO_2^{\bullet}$ and carbonate anion radical, which cause deleterious protein and DNA modifications [48]. Therefore, free radical scavengers are potentially useful drugs to prevent inflammatory tissue injury associated with oxidative stress [49]. However, some of these compounds exhibit manifold biological activities limiting their clinical application [50,51]. Epicatechin and related polyphenolic flavonoids of plant origin were shown to reduce the risk for myocardial infarction [27] and to inhibit the synthesis of pro-inflammatory cytokines [25]. The protective effects of these compounds may at least partially be due to increased bioavailability and/or synthesis of endothelium-derived nitric oxide [52]. While (−)-epicatechin was proposed to cause eNOS activation *via* increased intracellular Ca^{2+} [53], related red wine polyphenols were reported to enhance eNOS expression in cultured endothelial cells [54,55]. Our data provided no evidence for increased bioavailability of endothelial NO in the presence of (−)-epicatechin (measured as accumulation of cGMP in the absence of Ca^{2+} stimulation; Fig. 6A), but we cannot exclude long-term transcriptional or translational effects of the flavonoid. More importantly with respect to the potential *in vivo* application of (−)-epicatechin as radical scavenger, even very high concentrations (up to 1 mM) of the flavonoid did not interfere with Ca^{2+}-triggered NO/cGMP synthesis in endothelial cells. Since the marginal inhibition of purified eNOS by ≥0.1 mM (−)-epicatechin was not apparent in intact cells, the cellular environment appears to provide protection against potential non-specific interaction of the flavonoid with the eNOS protein and/or redox active cofactors of the enzyme. A previous study reported on similarly low potency of the related compound epigallocatechin-3-gallate to inhibit purified nNOS activity [56]. Interestingly, this compound was identified as relatively potent inhibitor of iNOS mRNA expression ($IC_{50} < 5$ μM) and iNOS activity ($IC_{50} \sim 150$ μM) in IFN-γ/LPS-activated murine peritoneal macrophages. Since we found that (−)-epicatechin did not affect IFN-γ/LPS-induced nitrite synthesis by RAW 264.7 macrophages at up to 100 μM, these earlier reports suggest a critical role of the gallate moiety [56,57].

4.2. Effect of (−)-epicatechin on nitration of free tyrosine by peroxynitrite

Protein tyrosine nitration, caused by peroxynitrite and/or MPO-catalyzed oxidation of nitrite, may have deleterious effects on cellular function and viability due to alterations of protein structure and function, like disassembly of structural proteins, inhibition of tyrosine phosphorylation and decreased catalytic activity of enzymes. It has been shown previously that (−)-epicatechin inhibits peroxynitrite-triggered nitration of free tyrosine and protein-bound tyrosine [22,58]. In accordance with earlier observations [22], we found that the potency of the flavonoid to inhibit nitration decreased with increasing concentrations of peroxynitrite. The IC_{50} values of 6.6 and 28 μM at 0.1 and 1.0 mM peroxynitrite correspond to IC_{50}/[peroxynitrite] ratios of 0.07 and 0.03, respectively, indicating that one equivalent of (−)-epicatechin prevents nitration by 15–30 equivalents of peroxynitrite. Accordingly, the effect of (−)-epicatechin is apparently not due to stoichiometric reaction with peroxynitrite but may result from scavenging of a reactive decomposition product. This conclusion is supported by the virtually identical potency of the flavo-

noid in the presence of millimolar concentrations of bicarbonate and our observation that the compound did not affect the kinetics and the concentration of end products of peroxynitrite decomposition. There is general agreement that formation of 3-nitrotyrosine results from reaction of tyrosyl radical with $NO_2^{\bullet}$, with the tyrosyl radical originating from oxidative attack of tyrosine by $NO_2^{\bullet}$ and carbonate anion radical in the absence and presence of bicarbonate, respectively [59,60]. A recent pulse radiolysis study reported that (−)-epicatechin scavenged $NO_2^{\bullet}$ and carbonate radicals with second-order rate constants of $9 \times 10^7\ M^{-1}\ s^{-1}$ and $5.6 \times 10^8\ M^{-1}\ s^{-1}$ [61], indicating that inhibition of tyrosine nitration is due to quenching of these radicals. The similar potency of (−)-epicatechin to prevent nitration and dimerization of tyrosine suggests that the polyphenol may also react with tyrosyl radicals [62].

4.3. Effect of (−)-epicatechin on nitration of protein-bound tyrosine in HL-60 cells

At submicromolar steady-state concentrations, peroxynitrite does not cause considerable nitration of free or protein-bound tyrosine due to predominant dimerization of tyrosyl radicals to yield dityrosine [63]. Accordingly, NO and $O_2^{\bullet-}$, co-generated endogenously from physiological sources (iNOS and NADPH oxidase, respectively) or from exogenous donor systems, do not cause significant nitration of cellular proteins [64,65], calling into question the pathophysiological significance of nitration by peroxynitrite. To account for the pivotal role of MPO and other heme peroxidases in tyrosine nitration under inflammatory conditions [66–69], we used phorbolester-stimulated HL-60 cells expressing large amounts of active MPO to study the effect of (−)-epicatechin on peroxidase-catalyzed nitration of cellular proteins. The potency of the flavonoid to inhibit tyrosine nitration in the presence of nitrite (but in the absence of detectable NOS activity; data not shown) was identical to that found for nitration of free tyrosine in buffer, strongly suggesting the involvement of the same reactive intermediate in peroxynitrite- and MPO-triggered nitration. Previous suggestions have invoked nitryl chloride (NO_2Cl), formed by the reaction of the MPO product hypochlorous acid with nitrite, or $NO_2^{\bullet}$, formed by MPO-catalyzed oxidation of nitrite, as alternative nitrating species [66,67]. The similar sensitivity of (−)-epicatechin to peroxynitrite- and MPO-mediated nitration favors the $NO_2^{\bullet}$ pathway, a conclusion which is also supported by a recent paper demonstrating lack of tyrosine nitration by nitryl chloride [18]. In contrast to peroxynitrite-triggered nitration of free tyrosine in buffer, there was a significant (−)-epicatechin-insensitive component of protein tyrosine nitration in HL-60 cells. Since MPO was completely inhibited by high concentrations of the flavonoid ($IC_{50} \sim 80\ \mu M$), the residual nitrating activity appears to be unrelated to MPO activity.

4.4. Effect of (−)-epicatechin on peroxynitrite-mediated oxidation reactions

In accordance to previous reports [70,71], we found that (−)-epicatechin inhibited oxidation of DHR by authentic peroxynitrite (50 μM) and the $NO/O_2^{\bullet-}$ donor SIN-1 (IC_{50} values of 12 and 0.6 μM, respectively). Since peroxynitrite is the primary oxidant formed from SIN-1 decomposition [44], the higher potency of (−)-epicatechin towards SIN-1-mediated oxidation appears to be a consequence of the low steady-state concentrations of peroxynitrite rather than scavenging of $O_2^{\bullet-}$. This conclusion is supported by a recent report showing that the pyrogallol moiety is essential for efficient $O_2^{\bullet-}$ scavenging by flavonoids [72] and our findings that 1 mM (−)-epicatechin (unlike superoxide dismutase) did not cause detectable formation of free NO from SIN-1 (data not shown). It is unclear how (−)-epicatechin inhibits DHR oxidation by peroxynitrite. There is general agreement that DHR becomes oxidized by a direct reaction with peroxynitrite anion [44], which does not react with (−)-epicatechin. However, the precise mechanism of DHR oxidation is not completely understood. Based on the unexpected observation that the peroxynitrite-triggered reaction is inhibited by NO, it has been proposed that one-electron oxidation of DHR may generate a radical intermediate which could be quenched by radical–radical addition of NO prior to loss of a second electron yielding rhodamine [43]. Even though attempts to isolate this putative nitroso-DHR adduct have proven unsuccessful [44], one-electron oxidation may be a crucial step of DHR oxidation, and (−)-epicatechin could inhibit the reaction by scavenging a DHR radical intermediate.

In summary, our data identify (−)-epicatechin as selective inhibitor of NO-related oxidation and nitration reactions that are thought to mediate the detrimental outcome of NO overproduction in inflammatory diseases. Together with the reported increased expression and/or activity of eNOS upon long-term exposure to (−)-epicatechin and related polyphenols, our results provide a molecular basis for the well documented protective cardiovascular effects of red wine and green tea.

Acknowledgments

This work was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung in Österreich and by a grant from the Human Frontier Science Program (HFSP): RGP0026/2001-M.

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