Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 307 (2003) 69–73
BBRC
www.elsevier.com/locate/ybbrc

Amphiphilic properties of (−)-epicatechin and their significance for protection of cells against peroxynitrite

Peter Schroeder, Lars-Oliver Klotz, and Helmut Sies*

Institut für Biochemie und Molekularbiologie I, Heinrich-Heine-Universität Düsseldorf, Postfach 101007, Gebäude 22.03 E02.R31, Universitätsstraße 1, D-40225 Düsseldorf, Germany

Received 4 June 2003

Abstract

The dietary flavanol (−)-epicatechin protects against nitration and oxidation reactions of the inflammatory mediator peroxynitrite in hydrophilic and hydrophobic environments. Bioavailability and cellular uptake of (−)-epicatechin are not yet fully characterized. Here, the octanol/buffer partition coefficient of (−)-epicatechin is observed to be 1.5, indicating that the flavanol is soluble in aqueous as well as lipophilic cellular phases, thus capable of permeating the cell membrane. In line with this, the ability of murine aortic endothelial cells (MAECs) to remove (−)-epicatechin from cell culture media is demonstrated. Epicatechin accumulates in cells, likely due to epicatechin binding to cellular proteins. Even after repeated washing, (−)-epicatechin accumulated by MAEC affords protection of the cells against peroxynitrite-induced nitration of protein tyrosyl residues and against oxidation of intracellular dichlorodihydrofluorescein.

The flavanol (−)-epicatechin has been shown to efficiently protect biomolecules against oxidation and nitration by peroxynitrite, with protection against tyrosine nitration being much more efficient than against oxidation [1–3]. Peroxynitrite is formed in the diffusion-controlled reaction ($k = 0.5$–$1.9 \times 10^{10}\,M^{-1}\,s^{-1}$) between superoxide and nitrogen monoxide during inflammatory processes (for reviews, see [4,5]), and its excessive generation not only triggers the activation of various cellular stress-responsive signaling pathways (for review, see [6]) but may also result in cell death. Peroxynitrite is capable of crossing lipid bilayers [7–9] and oxidizes and nitrates various hydrophilic and hydrophobic molecules [10], which (−)-epicatechin has been shown to inhibit: nitration and dimerization of tyrosine or of the tyrosine analogue *N-t*-BOC L-tyrosine *tert*-butyl ester by peroxynitrite in hydrophilic and hydrophobic environments were efficiently prevented by the flavanol [11].

The bioavailability of (−)-epicatechin after consumption of dietary sources containing the monomer or its oligomers and the metabolic fate of the polyphenol are currently the subject of investigation [12–16]. The fact that (−)-epicatechin is found in plasma after consumption of flavonoid-rich food and beverages, e.g., chocolate, red wine, or green tea (for reviews, see [17,18]) and that the flavonoid and its metabolites may appear in plasma in concentrations up to >60 μM after a single intragastric dose of 100 mg epicatechin per kilogram body weight in rat studies [15] prompted us to investigate its distribution between hydrophilic and hydrophobic phases and to study the cellular uptake of (−)-epicatechin in murine aortic endothelial cells (MAECs). We here demonstrate that (−)-epicatechin is indeed accumulated by MAEC, resulting in significant protection of the cells against peroxynitrite-induced damage.

Materials and methods

Reagents. All chemicals were purchased from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany) unless stated otherwise. α-Glucosyl-rutin was a gift from Dr. Franz Stäb, Beiersdorf AG, Hamburg, Germany. Cell culture materials were from Greiner (Frickenhausen, Germany).

Stock solutions of (−)-epicatechin (30–100 mM) were prepared in methanol and stored at −80 °C in the dark. Working solutions were

* Corresponding author. Fax: +49-211-8113029.
E-mail address: sies@uni-duesseldorf.de (H. Sies).

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)01132-X

prepared in PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.4) or serum-free cell culture media. Methanol was used instead of epicatechin in the respective control experiments.

Peroxynitrite was synthesized from sodium nitrite and H_2O_2 using a quenched-flow reactor [19], and residual H_2O_2 was eliminated by passage of the peroxynitrite solution over MnO_2 powder. The peroxynitrite concentration was determined spectrophotometrically at 302 nm ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$). All peroxynitrite dilutions were in 0.1% (w/v) KOH.

Partition coefficients. Octanol/buffer partition coefficients P were determined after vigorous mixing of flavonoid solutions (500 μM) in PBS with the same volume of *n*-octanol and maintaining the mixture in the dark at room temperature for 20 h to ensure that partition equilibrium was reached. Flavonoid concentrations in the aqueous phase were determined by HPLC and partition coefficients were calculated from the respective flavonoid (F) concentrations in the aqueous and octanol phases, respectively, according to $P = [F]_{octanol}/[F]_{buffer}$ (with $[F]_{octanol} = [F]_{buffer\ after\ incubation\ without\ octanol} - [F]_{buffer\ after\ incubation\ with\ octanol}$). All flavonoids tested were soluble up to 500 μM in both phases, except for α-glucosyl-rutin, the solubility limit of which in octanol was 100 μM.

Dialysis experiments. Epicatechin solutions in PBS (2 mL) were dialysed against 20 mL PBS containing varying concentrations of BSA (0.5–50 mg/mL) under constant shaking in the dark at room temperature for 20 h. The used dialysis tubes (Spectra/Por, Spectrum Laboratories, Rancho Dominguez, CA, USA) had a molecular weight cutoff of 12–14 kDa.

Cell culture. Primary murine aortic endothelial cells (MAECs) were a gift from Prof. V. Kolb-Bachofen, Düsseldorf, and were grown in RPMI-1640 medium containing fetal calf serum [FCS, Greiner; 20% (v/v)] and endothelial cell growth factor (ECGF, Boehringer, Mannheim; 1.8 μg/mL) and kept in a humidified atmosphere containing 5% (v/v) CO_2. Having grown to near confluence, cells were kept in RPMI-1640 medium containing 20% FCS without ECGF for at least 20 h before treatment. In experiments with epicatechin preincubation, cells were washed with PBS once and epicatechin was added to the serum-free medium for 60 min, followed by washing the cells with PBS at least twice. If desired, PSG buffer (100 mM potassium phosphate, 10 mM NaCl, and 5 mM glucose, pH 7.4) was then added to the cells and peroxynitrite was added as a bolus from a 100-fold concentrated working solution in 0.1% (w/v) KOH. Predecomposed peroxynitrite in PSG was added in the respective control experiments. Following a 5 min incubation in PSG, cells were either lysed directly or after incubation in fresh culture medium for a desired time by the addition of SDS–PAGE sample buffer (250 mM Tris/HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 20 mM DTT, and 0.01% (w/v) bromophenol blue), followed by brief sonication. For the preparation of cell lysate used for the measurement of epicatechin content, confluent MAECs were harvested in PBS using a cell scraper. Cells were lysed by sonication and freeze-thawing. Lysates were stored at −80 °C. Protein concentrations were estimated spectrophotometrically according to [20].

HPLC analysis. Analysis of flavonoid-containing samples was done using a C18 reversed phase column (Merck, Darmstadt) with a mobile phase of 15% acetonitrile and 85% of 10 mM $H_3PO_4/H_2PO_4^-$ buffer (pH 2.4) for α-glucosyl-rutin, catechin, epigallocatechin-3-*O*-gallate, epicatechin, rutin, and taxifolin or of 20% acetonitrile and 80% of 10 mM $H_3PO_4/H_2PO_4^-$ buffer (pH 2.4) for morin. Elution profiles were monitored at 280 nm, quantitation was done by integrating the elution peak. Before analysis of flavonoid contents in cell extracts or culture supernatants, samples were centrifuged at 4000*g* for 5 min at room temperature to remove cellular debris.

Dot blot. For dot blotting, lysates in SDS–PAGE sample buffer were applied onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After drying, the membrane was blocked using 5% (w/v) non-fat dried milk (Bio-Rad, Hercules, CA, USA) in TBST (20 mM Tris, 137 mM NaCl, and 0.1% (v/v) Tween 20, pH 7.4). Immunodetection of tyrosine nitration was performed with a monoclonal anti-3-nitrotyrosine antibody (Upstate Biotech, Charlottesville, VA, USA) and a peroxidase-coupled anti-mouse secondary antibody (Amersham, Buckinghamshire, UK), followed by chemiluminescent detection [21].

Oxidation of dichlorodihydrofluorescein. Cells were incubated with 100 μM of 2′,7′-dichlorodihydrofluorescein diacetate (Sigma) in RPMI-1640 medium in the presence of varying (−)-epicatechin concentrations for 1 h in 24-well plates (Greiner, Frickenhausen, Germany). After washing twice with PBS, cells were exposed to peroxynitrite. 2′,7′-Dichlorofluorescein fluorescence (excitation at 485 nm and emission at 535 nm) was measured in a Victor 1420 Multilabel Counter (Wallac, Freiburg, Germany) [22].

Calculation of (−)-epicatechin concentration in cellular water. (−)-Epicatechin content in cell lysate was determined via HPLC as described above and cellular water was estimated based on the protein content of cell lysate (2.37 μL cellular water/mg protein for rat cardiomyocytes) according to [23].

Statistics. ANOVA followed by Dunn's test was used for determination of statistical significance of differences between treatment groups. A P value less than 0.05 was selected before the study as the level of significance.

Results and discussion

Partition coefficient of (−)-epicatechin and comparison with other flavonoids

The octanol/buffer partition coefficient of (−)-epicatechin was shown to be 1.45, demonstrating its amphiphilic properties and qualifying the flavonoid to passively enter cells. It was further examined whether similar P-values can be obtained with flavonoids of structures similar to epicatechin (Table 1).

The fact that P-values for catechin, epicatechin, morin, and taxifolin are in the same range implies that neither the addition of a C-ring carbonyl (as in taxifolin and morin) nor a C2–C3 double bond (morin) significantly affects partition behaviour. Rather, the addition of hydrophilic moieties, such as gallic acid or glycosyl residues, should impair solubility in lipid phases, which is indeed reflected by the partition coefficients (Table 1), e.g., $P_{(epigallocatechin\ gallate)} < P_{(epicatechin)}$.

Table 1
Octanol/buffer partition coefficients P of selected flavonoids: 500 μM flavonoid in PBS was incubated with an equal volume of *n*-octanol for 20 h at room temperature in the dark

Family	Compound	P[a]
Flavanols	(−)-Epicatechin	1.45 ± 0.07
	(+)-Catechin	2.92 ± 0.35
	Epigallocatechin-3-*O*-gallate	0.86 ± 0.03
Flavonols	Morin	2.53 ± 0.09
	Rutin	0.25 ± 0.02
	α-Glucosyl-rutin	0.04 ± 0.00
Flavanone	Taxifolin	2.02 ± 0.02

[a] Means ± SD ($n \geqslant 4$); $P = [flavonoid]_{octanol}/[flavonoid]_{buffer}$.

Interestingly, there is a significant difference between *P*-values of (−)-epicatechin and its diastereomer, (+)-catechin, that appears to be brought about by the mere change in stereochemistry at C3. A possible explanation is the changed interaction between the 3-OH group and the B-ring, resulting in changed hydrophobicity.

Cellular uptake of (−)-epicatechin

In order to investigate consequences of the amphiphilic properties of (−)-epicatechin, the interaction between cultured cells and the flavonoid dissolved in cell culture media was analysed. The concentration of (−)-epicatechin in cell culture medium (no cells) did not change significantly during 180 min (Fig. 1, open squares), while in the presence of cells there was a significant decrease in the (−)-epicatechin concentration already after 30 min of incubation (Fig. 1, solid squares). In parallel, a significant increase of the (−)-epicatechin cellular content was detectable, highest at 10 and 30 min of exposure. It is known that epicatechin is metabolized efficiently, which is a possible explanation for the decrease in cellular (−)-epicatechin content at later time points (for review on flavonoid metabolites, see [17]). In Fig. 1, cellular content of epicatechin is expressed per mg protein and, in addition, per liter of cellular water.

The fact that the estimated epicatechin concentrations in cell lysate transiently exceed that in the surrounding cell culture medium cannot be explained by uptake into cellular water and lipid fractions alone and points to a contribution of binding to protein. Hence, the capability of a model protein to bind epicatechin was further investigated. Dialysis of epicatechin solutions against solutions of bovine serum albumin (BSA) of varying concentrations (0.5–50 mg/mL; Fig. 2) followed by HPLC analysis of epicatechin concentrations showed that epicatechin binds to BSA in a concentration-dependent manner, pointing to protein binding of epicatechin as major reason for the apparent accumulation of epicatechin in cellular fractions.

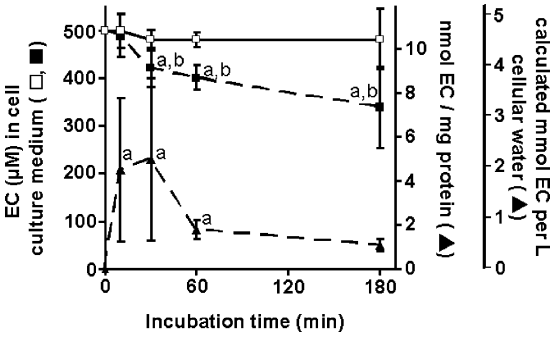

Fig. 1. Changes in (−)-epicatechin (EC) content of cell culture media and murine aortic endothelial cells (MAECs) during incubation. Serum-free cell culture medium (RPMI 1640) containing EC (starting concentration 500 μM) was incubated at 37 °C in the presence (solid squares) or absence (open squares) of confluent MAEC for the given period of time. EC concentrations in media (squares) and cell lysate (solid triangles) were analysed by HPLC. Content of EC is given per mg protein or in mmol/L of cellular water. Data are means ± SD ($n = 3$–4). (a) Significantly different from respective control ($t = 0$ min). (b) Significantly different from EC concentration in cell culture media incubated without cells at the same time point.

Cells loaded with (−)-epicatechin are protected against peroxynitrite

As epicatechin is known to efficiently protect against peroxynitrite-induced oxidation and nitration of hydrophilic and hydrophobic target molecules when added to the buffer shortly before peroxynitrite treatment [11] and incubation of MAEC in cell culture media containing (−)-epicatechin leads to an increased (−)-epicatechin concentration in the cells (Fig. 1), we further investigated whether the accumulation of epicatechin results in any cytoprotective effect. In order to test whether the apparent uptake of (−)-epicatechin by cells results in protection against peroxynitrite, and whether the loading of cells with (−)-epicatechin prior to exposure to peroxynitrite renders the cells more resistant, MAECs were incubated for 60 min in the presence of varying concentrations of (−)-epicatechin (0.0001–2 mM), washed twice with PBS, and subsequently exposed to a 500 μM peroxynitrite bolus. The loading of cells with (−)-epicatechin dose-dependently afforded protection against peroxynitrite (Fig. 3), the values for half-maximal inhibition of the peroxynitrite effect (normalized over peroxynitrite concentration, IC_{50}/[PN]) being 0.01 and 1.7 for protection against protein tyrosine nitration and 2′,7′-dichlorodihydrofluorescein oxidation, respectively. These IC_{50}-values closely resemble those determined for protection provided by (−)-epicatechin when present in the buffer during exposure to peroxynitrite rather than washed

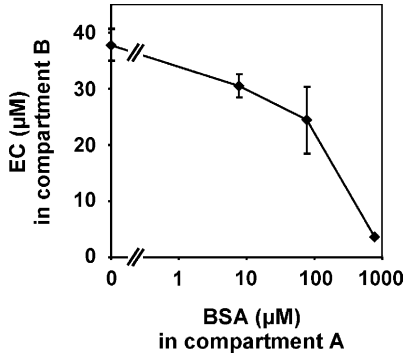

Fig. 2. Epicatechin (EC) binding to bovine serum albumin (BSA). EC in PBS (compartment B) was dialysed against various concentrations of BSA in PBS (compartment A) as described in Materials and methods. EC concentrations after dialysis were analysed by HPLC; data are means ± SD of an experiment performed in triplicate.

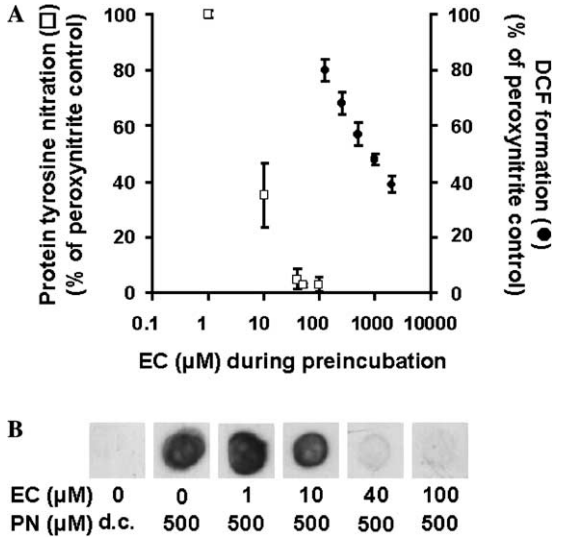

Fig. 3. Protection against formation of dichlorofluorescein (DCF; A, solid circles) and nitration of protein tyrosyl residues (A, open squares; and B) by cell-associated (−)-epicatechin (EC) after preincubation. Murine aortic endothelial cells (MAECs), grown to near confluence, were loaded with EC by incubation with cell culture media containing varying concentrations of EC for 60 min. After extensive washing, cells were subsequently exposed to 500 μM peroxynitrite (PN) by bolus addition. Results are means ± SEM ($n = 4$) (A) or representatives of at least four independent experiments (B). d.c., peroxynitrite decomposition control.

out after loading of cells as in Fig. 3 (0.02 concerning protection against protein tyrosine nitration and 1.1 for protection against 2′,7′-dichlorodihydrofluorescein oxidation [3]).

Conclusions

It is demonstrated here that (−)-epicatechin has amphiphilic properties, rendering the flavonoid an efficient protectant against peroxynitrite-induced nitration and oxidation in both hydrophilic and hydrophobic environments. Due to the amphiphilic nature of (−)-epicatechin, it is removed from cell culture media in the presence of cells which, in turn, are loaded with the flavonoid. Cellular protein appears to play an important role in the accumulation of epicatechin. This loading of cells results in protection against peroxynitrite-induced nitration and oxidation, implying that under physiological conditions protection by epicatechin, via accumulation in blood cells or endothelial cells, may outlast the presence of (−)-epicatechin in plasma.

Acknowledgments

This work was supported by Deutsche Forschungsgemeinschaft, SFB 503/B1. H.S. is a Fellow of the National Foundation for Cancer Research (NFCR), Bethesda, MD.

References

[1] A.S. Pannala, C.A. Rice-Evans, B. Halliwell, S. Singh, Inhibition of peroxynitrite-mediated tyrosine nitration by catechin polyphenols, Biochem. Biophys. Res. Commun. 232 (1997) 164–168.
[2] G.R.M.M. Haenen, J.B.G. Paquay, R.E.M. Korthouwer, A. Bast, Peroxynitrite scavenging by flavonoids, Biochem. Biophys. Res. Commun. 236 (1997) 591–593.
[3] P. Schroeder, L.O. Klotz, D.P. Buchczyk, C.D. Sadik, T. Schewe, H. Sies, Epicatechin selectively prevents nitration but not oxidation reactions of peroxynitrite, Biochem. Biophys. Res. Commun. 285 (2001) 782–787.
[4] J.S. Beckman, The physiological and pathophysiological chemistry of nitric oxide, in: J. Lancanster (Ed.), Nitric Oxide: Principles and Actions, Academic Press, San Diego, CA, 1996, pp. 1–82.
[5] R. Radi, G. Peluffo, M.N. Alvarez, M. Naviliat, A. Cayota, Unraveling peroxynitrite formation in biological systems, Free Radic. Biol. Med. 30 (2001) 463–488.
[6] L.O. Klotz, P. Schroeder, H. Sies, Peroxynitrite signaling: receptor tyrosine kinases and activation of stress-responsive pathways, Free Radic. Biol. Med. 33 (2002) 737–743.
[7] R.F. Khairutdinov, J.W. Coddington, J.K. Hurst, Permeation of phospholipid membranes by peroxynitrite, Biochemistry 39 (2000) 14238–14249.
[8] A.J. Macfadyen, C. Reiter, Y. Zhuang, J.S. Beckman, A novel superoxide dismutase-based trap for peroxynitrite used to detect entry of peroxynitrite into erythrocyte ghosts, Chem. Res. Toxicol. 12 (1999) 223–229.
[9] S.S. Marla, J. Lee, J.T. Groves, Peroxynitrite rapidly permeates phospholipid membranes, Proc. Natl. Acad. Sci. USA 94 (1997) 14243–14248.
[10] H. Zhang, J. Joseph, J. Feix, N. Hogg, B. Kalyanaraman, Nitration and oxidation of a hydrophobic tyrosine probe by peroxynitrite in membranes: comparison with nitration and oxidation of tyrosine by peroxynitrite in aqueous solution, Biochemistry 40 (2001) 7675–7686.
[11] P. Schroeder, H. Zhang, L.O. Klotz, B. Kalyanaraman, H. Sies, (−)-Epicatechin inhibits nitration and dimerization of tyrosine in hydrophilic as well as hydrophobic environments, Biochem. Biophys. Res. Commun. 289 (2001) 1334–1338.
[12] A.R. Rechner, G. Kuhnle, P. Bremner, G.P. Hubbard, K.P. Moore, C.A. Rice-Evans, The metabolic fate of dietary polyphenols in humans, Free Radic. Biol. Med. 33 (2002) 220–235.
[13] L.Y. Rios, R.N. Bennett, S.A. Lazarus, C. Remesy, A. Scalbert, G. Williamson, Cocoa procyanidins are stable during gastric transit in humans, Am. J. Clin. Nutr. 76 (2002) 1106–1110.
[14] C. Rice-Evans, J.P. Spencer, H. Schroeter, A.R. Rechner, Bioavailability of flavonoids and potential bioactive forms in vivo, Drug Metabol. Drug Interact. 17 (2000) 291–310.
[15] M.M. Abd El Mohsen, G. Kuhnle, A.R. Rechner, H. Schroeter, S. Rose, P. Jenner, C. Rice-Evans, Uptake and metabolism of epicatechin and its access to the brain after oral ingestion, Free Radic. Biol. Med. 33 (2002) 1693–1702.
[16] J.P. Spencer, F. Chaudry, A.S. Pannala, S.K. Srai, E. Debnam, C. Rice-Evans, Decomposition of cocoa procyanidins in the gastric milieu, Biochem. Biophys. Res. Commun. 272 (2000) 236–241.
[17] A. Scalbert, G. Williamson, Dietary intake and bioavailability of polyphenols, J. Nutr. 130 (2000) S2073–S2085.
[18] J.F. Wang, D.D. Schramm, R.R. Holt, J.L. Ensunsa, C.G. Fraga, H.H. Schmitz, C.L. Keen, A dose–response effect from chocolate

consumption on plasma epicatechin and oxidative damage, J. Nutr. 130 (2000) S2115–S2119.
[19] W.H. Koppenol, R. Kissner, J.S. Beckman, Syntheses of peroxynitrite: to go with the flow or on solid grounds?, Methods Enzymol. 269 (1996) 296–302.
[20] J.R. Whitaker, P.E. Granum, An absolute method for protein determination based on difference in absorbance at 235 and 280 nm, Anal. Biochem. 109 (1980) 156–159.
[21] K. Briviba, L.O. Klotz, H. Sies, Defenses against peroxynitrite, Methods Enzymol. 301 (1999) 301–310.
[22] H. Wang, J.A. Joseph, Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader, Free Radic. Biol. Med. 27 (1999) 612–616.
[23] P.B. Garlick, S. Soboll, G.R. Bullock, Evidence that mitochondrial phosphate is visible in ^{31}P NMR spectra of isolated perfused rat hearts, NMR Biomed. 5 (1992) 29–36.