Free Radical Research, Vol. 35, pp. 925–931
Reprints available directly from the publisher
Photocopying permitted by license only

Protection by Quercetin and Quercetin 3-*O*-β-D-Glucuronide of Peroxynitrite-induced Antioxidant Consumption in Human Plasma Low-density Lipoprotein

JUNJI TERAO[a,*], SACHIYO YAMAGUCHI[a], MUTSUKO SHIRAI[a], MARIKO MIYOSHI[a], JAE-HAK MOON[a], SYUNJI OSHIMA[b], TAKAHIRO INAKUMA[b], TOJIRO TSUSHIDA[c] and YOJI KATO[d]

[a]*Department of Nutrition, School of Medicine, The University of Tokushima, Tokushima 770-8503, Japan;* [b]*Kagome Research Institute, Kagome Company, Nishinasuno, Tochigi 329-2762, Japan;* [c]*National Food Research Institute, Tsukuba Ibaraki 305-0856, Japan;* [d]*School of Environmental Policy and Technology, Himeji Institute of Technology Hyogo 670-0092, Japan*

Accepted by Professor H. Sies

(Received 15 August 2000; In revised form 29 May 2001)

Effect of quercetin and its conjugated metabolite quercetin 3-*O*-β-D-glucuronide (Q3GA), on peroxynitrite-induced consumption of lipophilic antioxidants in human plasma low-density lipoprotein (LDL) was measured to estimate the role of dietary flavonoids in the defense system against oxidative modification of LDL based on the reaction of nitric oxide and superoxide anion. Synthesized peroxynitrite-induced consumption of endogenous lycopene β-carotene and α-tocopherol was effectively suppressed by adding quercetin aglycone into LDL solution. Q3GA also inhibited the consumption of these antioxidants effectively. These results indicate that dietary quercetin is capable of inhibiting peroxynitrite-induced oxidative modification of LDL in association with lipophilic antioxidants present within this lipoprotein particle.

Keywords: Quercetin; Quercetin glucuronide; Peroxynitrite; Low-density lipoprotein; α-tocopherol; Lycopene

INTRODUCTION

Oxidative modification of low-density lipoprotein (LDL) has been implicated in the initial event of early stages of atherosclerosis.[1] Role of dietary antioxidants in the prevention and attenuation of atherosclerosis is the subject of argument, because they are expected to suppress the oxidative process of LDL in blood stream and intima.[2,3] In particular, lipophilic antioxidants present within LDL, that is, carotenoids and α-tocopherol, are frequently discussed from the viewpoint of anti-atherosclerosis.[4] Nevertheless, precise mechanism in

*Corresponding author.

LDL oxidation *in vivo* remains unclear. Peroxynitrite is produced in vascular system by the reaction of nitric oxide and superoxide generating from endothelial cells, macrophages and neutrophils.[5] This reactive nitrogen species is a potent oxidant and is capable of initiating lipid peroxidation in liposomes[6] and rat brain synaptosomes.[7] Considerable studies[8–13] also reported that human LDL is subjected to oxidative modification by the exposure to synthesized peroxynitrite and peroxynitrite-generating agent. Peroxynitrite is therefore likely to be responsible, at least partly, for *in vivo* oxidative modification of LDL.

Quercetin is a typical flavonol-type flavonoid distributed in plant kingdom. A substantial amount of quercetin is daily consumed from vegetables and fruits[14]. Its antioxidant activity involves scavenging of superoxide anion and peroxyl radicals, inhibition of lipoxygenase, and chelation of metal ions responsible for the generation of reactive oxygen species.[15] Recently, quercetin and its related flavonoids were found to possess strong peroxynitrite-scavenging activity.[16] On the other hand, Manach *et al.*[17] and our group[18] have demonstrated that conjugated quercetin metabolites accumulated exclusively in human plasma by the ingestion of quercetin-rich foods. Recently we identified quercetin 3-*O*-β-D-glucuronide (Q3GA) as a major conjugated metabolite in rat plasma after oral intake of quercetin (Fig. 1).[19] It is therefore of much interest to know the role of quercetin and Q3GA in the protection of LDL from peroxynitrite-induced oxidative modification.

This study was conducted to clarify the protective effect of quercetin and Q3GA on peroxynitrite-induced consumption of major lipophilic antioxidants present in LDL, lycopene, β-carotene and α-tocopherol, by using synthesized peroxynitrite. It will provide information to know whether or not dietary quercetin can affect the antioxidant status of LDL on peroxynitrite-induced oxidative modification.

MATERIALS AND METHODS

Materials

Quercetin (3,3′,4′,5,7-pentahydroxylflavone), β-carotene, trans-β-8′-apocarotenal, D-α-tocopherol and D-δ-tocopherol were obtained from Sigma Chemical (St. Louis, MO, USA). Lycopene was from Wako Pure Chem. (Osaka, Japan). Quercetin 3-*O*-β-D-glucuronide was synthesized chemically and purified by column chromatography as described previously.[19] Peroxynitrite was synthesized from sodium nitrite and H_2O_2[20] and excess H_2O_2 was eliminated by passage of the peroxynitrite solution over the column packed with MnO_2 powder.[21] The concentration of peroxynitrite was calculated by using its molecular coefficient at 302 nm in 1.2 M NaOH ($\varepsilon_{302\,nm} = 1670\,mol^{-1}cm^{-1}$).[21] LDL was isolated from fresh human plasma by discontinuous density-gradient ultracentrifugation according to the method described previously.[22] The protein concentration of the LDL solution was determined by the method of Lowry *et al.*[23] Isolated LDL was stored under nitrogen gas for not longer than one week.

Exposure of LDL to Synthesized Peroxynitrite

Quercetin or Q3GA was added as ethanol solution (20 μl) to 2.0 ml of LDL solution (0.1 mg protein/ml) containing 0.5 mM diethylenetriaminepentaacetic acid (DTPA) in 0.01 M Tris-HCl buffer (pH 7.4) and incubated for 5 min at 37°C. In the case of control experiment, ethanol (20 μl) was added to the LDL solution. Then, peroxynitrite solution (10 μl) was added to the LDL solution and mixed vigorously for 1 min. For the analysis of α-tocopherol and carotenoids in the solution, 2.0 ml of solution was mixed with 10 μl of 1.0 mM aqueous EDTA solution, 20 μl of ethanol containing internal standards (8′-apocarotenal; 0.25 nmol and δ-tocopherol; 0.5 nmol), and 0.5 ml of methanol solution containing 1.0 mM butyl hydroxytoluene (BHT). α-tocopherol and carotenoids were extracted with 0.5 ml hexane by mixing

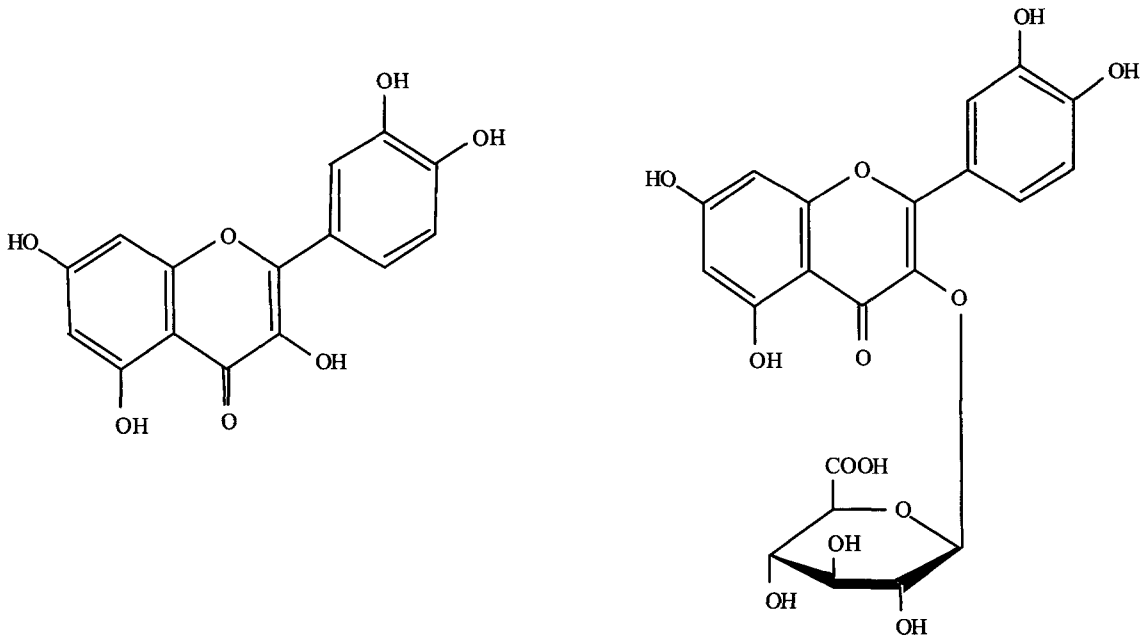

FIGURE 1 Structures of quercetin and Q3GA.

vigorously. Extraction was carried out twice and the hexane layers were combined and evaporated in vacuo. The residue was dissolved in 50 μl chloroform and used as the sample for HPLC assay.

Determination of Lycopene β-Carotene and α-Tocopherol

Lycopene and β-carotene were quantified by HPLC using a column of TSK gel Octyl-80Ts (Tosoh, Japan, 4.6 mm × 250 mm) with mobile phase of methanol/acetonitrile/dicholomethane/water (7:7:2:0.16, v/v/v/v) at a flow rate of 1.4 ml/min. The effluent was monitored at 450 nm using a Shimadzu SPD-10AV spectrophotometric detector (Shimadzu, Kyoto, Japan). For the determination of α-tocopherol, mobile phase of methanol/water (93:7, v/v) was used and the effluent was monitored with spectrofluorometer (Shimadzu RF10A) with extinction at 290 nm and emission at 325 nm.

RESULTS

Figure 2 shows the contents of lipophilic antioxidants present in the lipoprotein particles after the exposure to peroxynitrite at the concentration of 10 ~ 500 μM. Lycopene and α-tocopherol present within the lipoprotein particles were decreased dramatically by the exposure to peroxynitrite at 10 μM. In contrast, β-carotene content was not decreased significantly by the exposure to peroxynitrite at 10 μM. However, β-carotene was also consumed at higher peroxynitrite concentration (50 ~ 500 μM).

Figure 3 shows the effect of quercetin aglycone and Q3GA on the consumption of lycopene β-carotene and α-tocopherol by the exposure of human LDL to peroxynitrite at 50 μM. The same concentration of quercetin aglycone, 50 μM, significantly suppressed the decrease of these three compounds. In particular, its effect was remarkable for β-carotene as compared to the other two lipophilic antioxidants. Q3GA at the

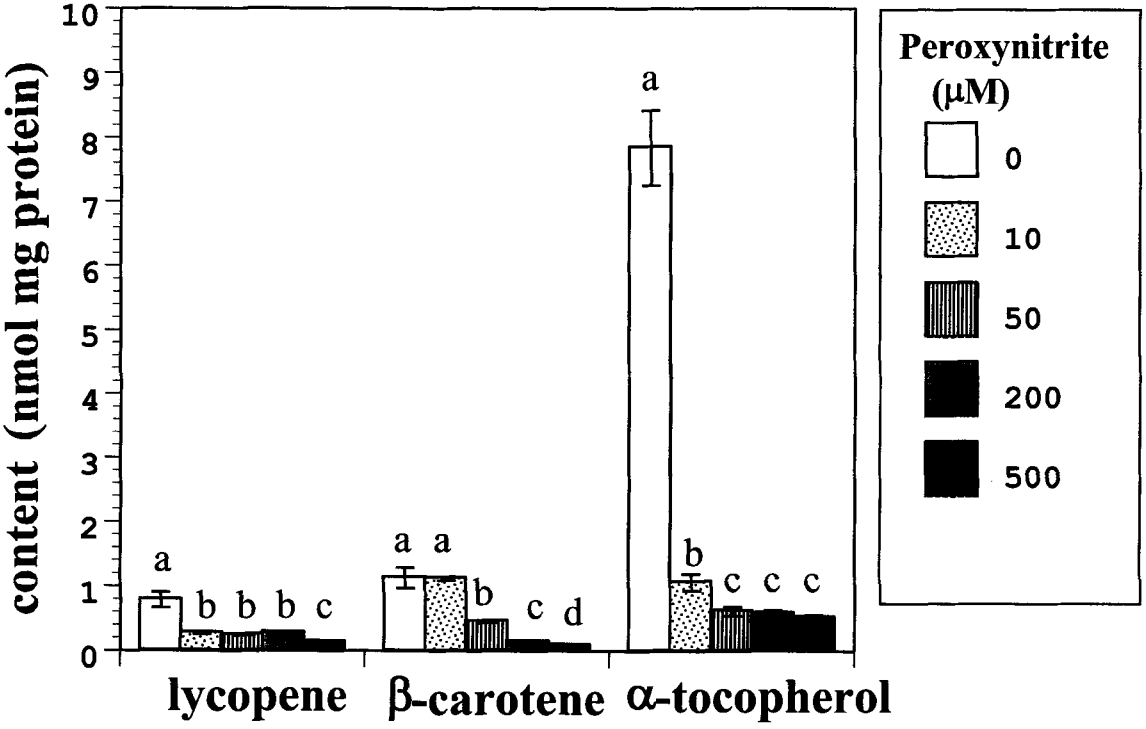

FIGURE 2 Contents of lipophilic antioxidants present in LDL after exposure to peroxynitrite at different concentration (10–500 μM). Contents of the lipophilic antioxidants in LDL before exposure to peroxynitrite were as follows: lycopene, 0.78 ± 0.12 nmol/mg protein; β-carotene, 1.10 ± 0.60 nmol/mg protein, α-tocopherol, 7.84 ± 0.60 nmol/mg protein). Protein concentration of the LDL preparation was 0.1 ml/ml. Values are given as the mean ± S.D. for three experiments. Means not sharing a common letter in the same group (lycopene, β-carotene, α-tocopherol, respectively) are significantly different ($p < 0.05$), as determined by Bonferroni/Dunn's multiple comparison test

same concentration was also found to suppress the consumption of the three antioxidants significantly. Its suppressive effect was comparable to that of quercetin aglycone in lycopene and α-tocopherol consumption, although it was lower in β-carotene consumption. These results clearly demonstrate that Q3GA, as well as quercetin aglycone, is capable to inhibit peroxynitrite-induced consumption of endogenous lipophilic antioxidants in human LDL.

DISCUSSION

Peroxynitrite is formed by the reaction of superoxide anion and nitric oxide at nearly diffusion controlled rate.[24] At neutral pH, peroxynitrite is easily protonated to generate peroxynitrous acid, which seems to be responsible for the initiation of lipid peroxidation.[8] It is plausible that peroxynitrite is generated from endothelial cells or macrophages and this reactive nitrogen species attacks LDL particles leading to oxidative modification. We used synthesized peroxynitrite for this study. Although synthesized peroxynitrite is stable in alkaline condition, it induces tyrosine nitration,[12] oxidation of α-tocopherol to α-tocopherylquinone,[10] and accumulation of cholesteryl ester hydroperoxides (CE-OOH)[13] in human plasma LDL at neutral pH. We also found slight increase in CE-OOH concentration

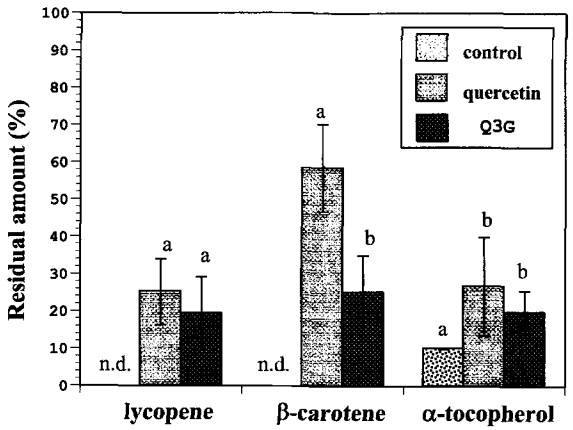

FIGURE 3 Effect of quercetin and Q3GA on peroxynitrite-induced consumption of lipophilic antioxidants present within LDL. Human LDL solution (0.1 mg protein/ml) was exposed to peroxynitrite at 50 μM. Concentrations of quercetin and Q3GA were adjusted to 50 μM. Values are given as the mean ± S.D. for three experiments. n.d., not detected. Means not sharing a common letter in the same group (lycopene, β-carotene, α-tocopherol, respectively) are significantly different ($p < 0.05$), as determined by Bonferroni/Dunn's multiple comparison test

by the exposure to peroxynitrite at 10–500 μM (0.5–1.0 nmol/mg protein; data are not shown here).

Pannala *et al.*[25] claimed that carotenoids are consumed at higher level than α-tocopherol with lycopene being more reactive than β-carotene in peroxynitrite-induced LDL oxidation. Panasenko *et al.*[26] demonstrated that lycopene is the most reactive among all carotenoids detected in LDL, when this lipoprotein particle was exposed to peroxynitrite. It is therefore likely that lycopene serves as an effective peroxynitrite scavenger when LDL is exposed to peroxynitrite. It is recently reported that some flavonoids and polyphenols are efficient peroxynitrite scavengers.[16,27–32] In particular, Haenen *et al.*[16] pointed out that quercetin scavenges peroxynitrite most effectively among seven flavonol-type flavonoids. Our result clearly demonstrates that quercetin is helpful in protecting LDL from peroxynitrite attack by retarding the consumption of lycopene and other lipophilic peroxynitrite-scavengers present within LDL. It is likely that quercetin present in LDL solution scavenges peroxynitrite effectively before this reactive nitrogen species reaches to the site of reaction with lipophilic antioxidants within LDL.

Blood plasma contains a variety of water-soluble antioxidants, which are distributed in aqueous phase of the plasma. Among them, bilirubin is reported as an antioxidant for peroxynitrite induced LDL oxidation.[33] Uric acid is highly reactive to peroxynitrite and produce nitrated uric acid derivative.[34] Quercetin may help the plasma antioxidant defense by coordinating with these water-soluble antioxidants. We recently confirmed that Q3GA accumulated in rat plasma as a major metabolite of orally administered quercetin.[19] This study demonstrated that Q3GA also possesses an inhibitory effect on peroxynitrite-induced consumption of lipophilic antioxidants within LDL. Thus, it is plausible that dietary quercetin retains peroxynitrite-scavenging activity at least partly in the circulation even after metabolic conversion during the absorption process. We already found that G3GA exerted a substantial antioxidant effect on copper-ion induced oxidation of human plasma LDL.[19] It is therefore conceivable that some quercetin metabolites such as Q3GA contribute to the defense system of human plasma LDL against the damage originated from reactive oxygen/nitrogen species involving peroxynitrite.

In conclusion, both quercetin aglycone and Q3GA are effective peroxynitrite scavengers and they are capable of inhibiting peroxynitrite-attack on human plasma LDL in association with lipophilic antioxidants within LDL.

Acknowledgements

This work was supported partly by the Special Coordination Funds for Promoting Science Technology of the Science Technology Agency of the Japanese Government and Promotion of Basic Research Activities for Innovative Biosciences.

References

[1] Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989) "Beyond cholesterol; modification of low-density lipoprotein that increases its atherogenecity", *New England Journal of Medicine* **320**, 915–924.

[2] Parthasarathy, S., Santanam, N., Ramachandran, S. and Meilhac, O. (1999) "Oxidants and antioxidants in atherogenesis: an appraisal", *Journal of Lipid Research* **40**, 2143–2157.

[3] Stocker, R. (1999) "Dietary and pharmacological antioxidants in atherosclerosis", *Current Opinion in Lipidology* **10**, 589–597.

[4] Kaplan, M. and Aviram, M. (1999) "Oxidized low-density lipoprotein: atherogenic and proinflammatory characteristics during macrophase foam cell formation. An inhibitory role for nutritional antioxidants and serum paraoxonase", *Clinical Chemistry and Laboratory Medicine* **37**, 777–787.

[5] Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman, B.A. (1990) "Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide", *Proceedings of the National Academy of Sciences USA* **87**, 1620–1624.

[6] Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. (1991) "Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide", *Archives of Biochemistry and Biophysics* **288**, 481–487.

[7] Shi, H., Noguchi, N., Xu, Y. and Niki, E. (1999) "Formation of phospholipid hydroperoxides and its inhibition by α-tocopherol in rat brain synaptosomes induced by peroxynitrite", *Biochemical and Biophysical Research Communication* **257**, 651–656.

[8] Darley-Usmar, V.M., Hogg, N., O'leary, V.J., Wilson, M.T. and Moncada, S. (1992) "The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low-density lipoprotein", *Free Radical Research Communication* **17**, 9–20.

[9] Graham, A., Hogg, N., Kalyanaraman, B., O'leary, V., Darley-Usmar, V. and Moncada, S. (1993) "Peroxynitrite modification of lower-density lipoprotein leads to recognition by the macrophage scavenger receptor", *FEBS Letters* **330**, 181–185.

[10] Hogg, N., Darley-Usmar, V.M., Wilson, M.T. and Moncada, S. (1993) "The oxidation of α-tocopherol in human low-density lipoprotein by the simultaneous generation of superoxide and nitric oxide", *FEBS Letters* **326**, 199–203.

[11] Patel, R.P. and Darley-Usmar, V.M. (1996) "Using peroxynitrite as oxidant with low-density lipoprotein", *Methods in Enzymology* **269**, 375–384.

[12] Leeuwenburgh, C., Hardy, M.M., Hazen, S.L., Wagner, P., Oh-ishi, S., Steinbrecher, U.P. and Heinecke, J.W. (1997) "Reactive nitrogen intermediates promote low-density lipoprotein oxidation in human athrosclerotic intima", *Journal of Biological Chemistry* **272**, 1433–1436.

[13] Thomas, S.R., Davies, M.J. and Stocker, R. (1998) "Oxidation and antioxidation of human low-density lipoprotein and plasma exposed to 3-morpholinosyld-nonimine and reagent peroxynitrite", *Chemical Research in Toxicology* **11**, 484–494.

[14] Hertog, M.G.L., Hollman, P.C.H. and Katan, M.B. (1992) "Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in The Netherlands", *Journal of Agricultural and Food Chemistry* **40**, 2379–2383.

[15] Rice-Evans, C., Miller, N.J. and Paganga, G. (1996) "Structure–activity relationship of flavonoids and phenolic acids", *Free Radical Biology and Medicine* **20**, 933–956.

[16] Haenen, G.R.M.M., Paquay, J.B.G., Korthouwer, E.M. and Bast, A. (1997) "Peroxynitrite scavenging by flavonoids", *Biochemical and Biophysical Research Communication* **236**, 591–593.

[17] Manach, C., Morand, C., Crespy, V., Demigne, C.M., Texier, O., Regerat, F. and Remesy, C. (1998) "Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties", *FEBS Letters* **426**, 331–336.

[18] Moon, J.-H., Nakata, R., Oshima, S., Inakuma, T. and Terao, J. (2000) "Accumulation of quercetin conjugates in blood plasma after the short-term ingestion of onion by women", *American Journal of Physiology* **279**, R461–R467.

[19] Moon, J.-H., Tsushida, T., Nakahara, K. and Terao, J. (2001) "Identification of quercetin 3-*O*-β-D-glucuronide as an antioxidative metabolite in rat plasma after oral administration of quercetin", *Free Radical Biology and Medicine* **30**, 1274–1285.

[20] Huges, M.W. and Nicklin, H.G. (1970) "The chemistry of peroxynitrite part II. Copper (II)-catalyzed reaction between hydroxylamine and peroxynitrite in alkali", *Journal of Chemical Society A.*, 925–928.

[21] Beckman, J.S., Chen, J., Ischiropoulos, H. and Crow, J.P. (1994) "Oxidative chemistry of peroxynitrite", *Methods in Enzymology* **233**, 229–240.

[22] da Silva, E.L., Tsushida, T. and Terao, J. (1999) "Inhibition of mammalian 15-lipoxygenase-dependent lipid peroxidation in low-density lipoprotein by quercetin and quercetin monoglucosides", *Archives of Biochemistry and Biophysics* **349**, 313–320.

[23] Lowry, O.H., Rosebrough, J.N., Farr, A.L. and Randall, R.J. (1951) "Protein measurement with Folin phenol reagent", *Journal of Biological Chemistry* **193**, 165–175.

[24] Koppenol, W.H. (1998) "The basic chemistry of nitrogen monoxide and peroxynitrite", *Free Radical Biology and Medicine* **25**, 385–391.

[25] Pannala, A.S., Rice-Evans, C., Sampson, J. and Singh, S. (1998) "Interaction of peroxynitrite with carotenoids and tocopherols within low-density lipoprotein", *FEBS Letters* **423**, 297–301.

[26] Panasenko, O.M., Sharow, V.S., Briviba, K. and Sies, H. (2000) "Interaction of peroxynitrite with carotenoids in human low-density lipoproteins", *Archives of Biochemistry and Biophysics* **373**, 302–305.

[27] Pannala, A.S., Rice-Evans, C.A., Halliwell, B. and Singh, S. (1997) "Inhibition of peroxynitrite-mediated tyrosine nitration by catechin polyphenols", *Biochemical and Biophysical Research Communication* **232**, 164–168.

[28] Chung, H.Y., Yokozawa, T., Soung, D.Y., Kye, I.S., No, J.K. and Bark, B.S. (1998) "Peroxynitrite-scavenging activity of green tea tannin", *Journal of Agricultural and Food Chemistry* **46**, 4484–4486.

[29] Arteel, G.E. and Sies, H. (1999) "Protection against peroxynitrite by cocoa polyphenol oligomers", *FEBS Letters* **462**, 167–170.

[30] Kato, Y., Ogino, Y., Aoki, T., Uchida, K., Kawakishi, S. and Osawa, T. (1997) "Phenolic antioxidants prevent peroxynitrite-derived collagen modification *in vitro*", *Journal of Agricultural and Food Chemistry* **45**, 3004–3009.
[31] Tsuda, T., Kato, Y. and Osawa, T. (2000) "Mechanism for the peroxynitrite scavenging activity by antioxidants", *FEBS Letters* **484**, 207–210.
[32] Kerry, N. and Rice-Evans, C.A. (1999) "Inhibition of peroxynitrite-mediated oxidation of dopamine by flavonoid and phenolic antioxidants and their structural relationship", *Journal of Neurochemistry* **73**, 247–253.
[33] Minetti, M., Mallozzi, C., di Stasi, M. and Pietraforte, D. (1998) "Bilirubin is an effective antioxidant of peroxynitrite-mediate protein oxidation in human blood plasma", *Archives of Biochemistry and Biophysics* **352**, 165–174.
[34] Skinner, K.A., White, C.R., Patel, R., Tan, S., Barnes, S., Kirk, M., Darley-Usmar, V. and Parks, D.A. (1998) "Nitrosation of uric acid by peroxynitrite", *Journal of Biological Chemistry* **273**, 24491–24497.