Epicatechin and its *in vivo* metabolite, 3′-*O*-methyl epicatechin, protect human fibroblasts from oxidative-stress-induced cell death involving caspase-3 activation

Jeremy P. E. SPENCER*[1], Hagen SCHROETER*[1], Gunter KUHNLE†, S. Kaila S. SRAI‡, Rex M. TYRRELL§, Ulrich HAHN† and Catherine RICE-EVANS*[2]

*Wolfson Centre for Age-Related Diseases, Guy's, King's and St Thomas' School of Biomedical Sciences, King's College, Hodgkin Building, Guy's Campus, London SE1 9RT, U.K., †Institute of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, University of Leipzig, Leipzig 04103, Germany, ‡Department of Biochemistry and Molecular Biology, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, U.K., and §Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U.K.

There is considerable current interest in the cytoprotective effects of natural antioxidants against oxidative stress. In particular, epicatechin, a major member of the flavanol family of polyphenols with powerful antioxidant properties *in vitro*, has been investigated to determine its ability to attenuate oxidative-stress-induced cell damage and to understand the mechanism of its protective action. We have induced oxidative stress in cultured human fibroblasts using hydrogen peroxide and examined the cellular responses in the form of mitochondrial function, cell-membrane damage, annexin-V binding and caspase-3 activation. Since one of the major metabolites of epicatechin *in vivo* is 3′-*O*-methyl epicatechin, we have compared its protective effects with that of epicatechin. The results provide the first evidence that 3′-*O*-methyl epicatechin inhibits cell death induced by hydrogen peroxide and that the mechanism involves suppression of caspase-3 activity as a marker for apoptosis. Furthermore, the protection elicited by 3′-*O*-methyl epicatechin is not significantly different from that of epicatechin, suggesting that hydrogen-donating antioxidant activity is not the primary mechanism of protection.

Key words: antioxidant, apoptosis, catechol O-methyltransferase, flavanol, oxidation.

INTRODUCTION

Many studies have investigated the antioxidant effects of flavonoids and phenolic compounds in the context of a variety of cell functions. These include modulation of cell signalling [1,2], suppression of tumour necrosis factor α expression [3], the down-regulation of the expression of adhesion molecules [4–6], the improvement of vascular dysfunction induced by oxidative stress [7] and the inhibition of DNA damage [8,9]. Flavonoids are powerful antioxidants *in vitro* by virtue of the reducing properties of their phenolic hydroxy groups, defined by their redox potentials [9], in combination with the stability of the flavonoid phenoxyl radicals [10]. The antioxidant properties of flavonoids are structure-dependent and the major contributing factor is the presence of a 3′,4′-dihydroxycatechol structure in the B-ring (Figure 1) [10–12].

The cytoprotective effects of flavonoids have been attributed to their antioxidant properties either through their reducing capacities [13] or through their influences on intracellular redox status [14]. However, the flavonoids applied to those systems are not, in general, the forms circulating *in vivo* after absorption. Flavonoids are generally modified on absorption *in vivo*, and studies of uptake [15] and investigations of isolated intestinal models [16,17] and human intestinal cells [18] have shown that they are extensively metabolized, initially during transfer across the small intestine and secondly by the liver, to a variety of conjugates and metabolites. For example, absorption through the small intestine promoted deglycosylation, glucuronidation as well as O-methylation of catechol-containing phenolics [16,17,19]. Recent work reported that both the O-methylated form and glucuronidated conjugates are detectable in rat urine after oral administration of epicatechin [20]. Furthermore, in humans, increases in 3′-*O*-methyl catechin sulphate and glucuronide metabolites were observed in plasma after consumption of either catechin-rich red wine or de-alcoholized red wine [15]. As well as small-intestinal transformations, metabolism in the colon can result in extensive modifications including hydrolysis, oxidation and ring cleavage, forming secondary phenolic metabolites [21].

The purpose of this research was to address two issues: whether O-methylation of epicatechin, as is known to occur during small-intestinal and liver metabolism, modified its cytoprotective effects and whether the resulting modulation of the reducing properties might give insights into the possibility of an antioxidant mechanism. Therefore we have examined the effects of epicatechin in protecting fibroblasts from H_2O_2-induced mitochondrial dysfunction, cell-membrane damage and caspase-3 activation. In addition, we have investigated whether the pattern of effects of one of its major *in vivo* metabolites, 3′-*O*-

Abbreviations used: ABTS, 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid); $ABTS^{\cdot+}$, ABTS radical cation; LDL, low-density lipoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; LDH, lactate dehydrogenase; COMT, catechol O-methyltransferase; SAM, *S*-adenosyl-L-methionine; CID, collision-induced dissociation; TEAC, Trolox equivalent antioxidant capacity; nanoES-MS, nano-electrospray MS; HBM, Hepes-buffered incubation medium; LOOH, lipid hydroperoxides.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed (e-mail catherine.rice-evans@kcl.ac.uk).

methyl epicatechin, was affected by the influence of methylation on its antioxidant activity.

EXPERIMENTAL

Materials

Specialized chemicals used were obtained from Sigma (Poole, Dorset, U.K.): (−)-epicatechin, catechin, trifluoroacetic acid, catechol O-methyltransferase (COMT; from porcine liver, EC 2.1.1.6, S-adenosyl-L-methionine:catechol O-methyltransferase), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), β-NADH as pyruvate substrate®, 2,4-dinitrophenylhydrazine (20 % in 1 M HCl, colour substrate®), the colorimetric caspase-3 assay kit (CASP-3-C) and the apoptosis-detection system Annexin V-CY3®. Ionomycin was obtained from Calbiochem. HPLC specialized solvents, acetonitrile and methanol, were purchased from Rathburn (Walkerburn, Scotland, U.K.) and HPLC columns were from Waters (Watford, Herts., U.K.). Elgastat UHP double-distilled water (18.2 MΩ grade) was used throughout the study. All other reagents used were of analytical grade and obtained from Sigma. Tissue-culture reagents, including growth media, growth factors and materials for the passage of cells were from PromoCell® (Heidelburg, Germany).

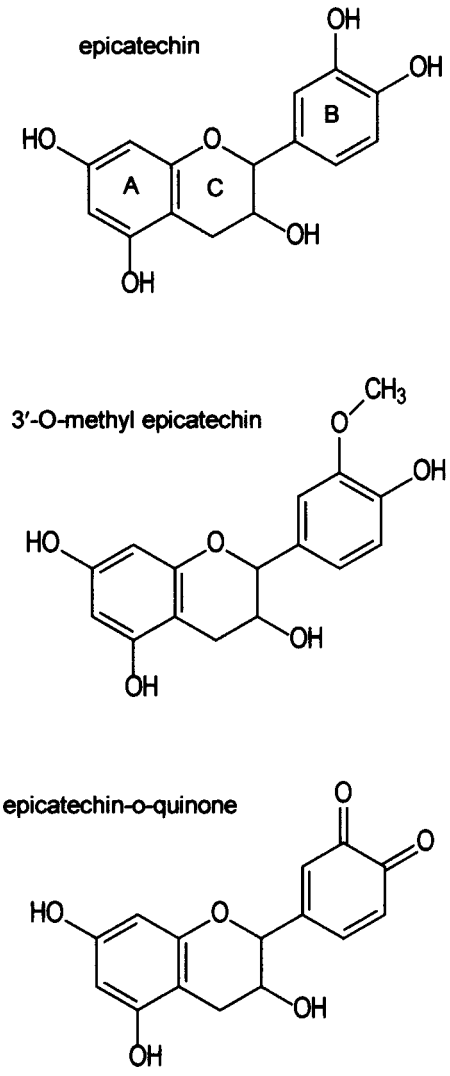

Figure 1 Structures of epicatechin, 3′-*O*-methyl epicatechin and epicatechin-*o*-quinone

Synthesis of 3′-*O*-methyl epicatechin

Synthesis of 3′-*O*-methyl and 4′-*O*-methyl epicatechin was undertaken using a method based on that of Ball et al. [22] utilizing COMT and the methyl group donor *S*-adenosyl-L-methionine (SAM). COMT (500 units/ml) was pre-incubated in 10 ml of buffer containing 20 mM cysteine, 2 mM $MgCl_2$ and 10 mM NaH_2PO_4, pH 7.5, at 37 °C for 10 min. After incubation, the buffer was bubbled with nitrogen gas to lower the concentration of oxygen in solution and SAM and epicatechin were added to final concentrations of 16.3 and 2.0 mM, respectively. The mixture was stirred continuously and incubated under nitrogen at 37 °C for 6 h. Further additions of SAM (30 mg) took place every 2 h. The reaction was stopped by addition of methanol and the reaction mixture lyophilized. Epicatechin and its O-methylated forms were extracted using 50 % methanol. The 3′-*O*-methyl and 4′-*O*-methyl epicatechin were isolated by semi-preparative HPLC using a Prodigy 10 μm ODS preparative column (250 × 10 mm; Phenomenex®) on a Waters HPLC system. The mobile phase consisted of solvent A (10 % aqueous methanol in 0.1 % HCl) and solvent B (acetonitrile/methanol, 1:1) and was pumped through the column at 5.0 ml·min^{-1} using the following gradient system: 0 min, 0 % solvent B; 30 min, 50 % solvent B; 60 min, 5 % solvent B. Injections (200 μl) were made on to the column (30 °C) and collected peaks were pooled, lyophilized and finally reconstituted in 50 % methanol/water (v/v) before MS analysis.

Nano-electrospray MS (nanoES-MS) analysis of 3′-*O*-methyl epicatechin

Samples of synthesized 3′-*O*-methyl epicatechin were purified by semi-preparative HPLC and then analysed by nanoES-MS/MS to enable characterization of a pure standard. NanoES-MS/MS was performed using a Perkin-Elmer API 365 LC/MS/MS mass spectrometer (PE Sciex, Concord, Canada) equipped with a specially developed nanoES source [23] and metal-coated capillaries (New Objective, Cambridge, MA, U.S.A.). To increase sensitivity, trifluoroacetic acid (final concentration 1 %) was added to the samples in 50 % methanol. Collision-induced dissociation (CID) was performed using nitrogen as the collision gas and all spectra were recorded in positive-ion mode.

Determination of antioxidant activity

The Trolox equivalent antioxidant capacity (TEAC) assay was performed as described by Re et al. [24] to determine the H-donating potential of epicatechin and the O-methylated form. Solutions of 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS; 7 mM) and potassium persulphate (2.45 mM) were mixed and allowed to stand for 16 h to generate the ABTS radical cation (ABTS$^{\bullet+}$). For experiments, the ABTS$^{\bullet+}$ stock solution was diluted with PBS (pH 7.4) and equilibrated at 30 °C to an absorbance of 0.7 at 734 nm. Each compound under investigation, epicatechin, 3′-*O*-methyl epicatechin and Trolox, was added to the ABTS$^{\bullet+}$ solution to achieve final concentrations of 1.25, 2.5 and 5 μM, the solutions were mixed (30 s) and the

decline in absorbance (734 nm) was followed for 10 min. Appropriate solvent blanks were run for each addition. Calculation of the TEAC value was as detailed in Re et al. [24].

Cell culture

Normal human dermal fibroblasts derived from foreskin (FEK4) were used between passages 8 and 12 [25]. The cells were cultured routinely in fibroblast growth medium supplemented with insulin (5 μg/ml), basic fibroblast growth factor (1 ng/ml), amphotericin (50 ng/ml) and gentamicin (50 μg/ml) at 37 °C in a humidified atmosphere of 95 % air/5 % CO_2. Stock cultures were maintained in sterile T75 flasks and medium changes took place every 24 h until cells were ready for experimental use (75–95 % confluent).

Low-density lipoprotein (LDL) isolation and modification

LDL isolation and oxidation were carried out as described previously by Chung et al. [26]. Conditions for LDL oxidation were: 62.5 μg/ml LDL in PBS containing a final concentration of 5 μM $CuSO_4$ incubated at 37 °C. The extent of oxidation was monitored as a function of time through the formation of conjugated dienes at 234 nm (absorption coefficient, 2.8×10^4 $M^{-1} \cdot cm^{-1}$) [27] and the generation of lipid hydroperoxides (LOOH) using the FOX (ferrous-oxidation Xylenol Orange) assay [28], applying a standardized time point of 45 min [29]. At 45 min the average concentrations of LOOH and conjugated dienes corresponded to 474 ± 45 nmol/mg of LDL protein ($n = 3$) and 211 ± 33 nmol/mg of LDL protein ($n = 3$), respectively. Under these conditions only lipid peroxidation was detected with no modification of the surface charge on the apolipoprotein B100, using the Beckman Paragon gel-electrophoresis system, as described in [29].

Cell exposure

Fibroblasts were sub-cultured on to 24-well plates at a seeding density of 2.5×10^4 cells/well and grown for 48 h (4×10^4 cells/well) before use. Exposure to oxidized LDL and to H_2O_2 took place in Hepes-buffered incubation medium (HBM), pH 7.4 (5 mM Hepes/154 mM NaCl/4.6 mM KCl/2.3 mM $CaCl_2$/33 mM glucose/5 mM $NaHCO_3$/1.1 mM $MgCl_2$/1.2 mM Na_2HPO_4). The duration of exposure to the oxidants was 5 h (MTT assay) and 18 h [lactate dehydrogenase (LDH) release]. To evaluate the potential protective effects of the two compounds, cells were pre-treated with epicatechin (up to 30 μM) or 3′-*O*-methyl epicatechin (up to 30 μM) in medium for 18 h and washed twice with PBS (to prevent direct extracellular interactions between the compounds and the oxidants) prior to addition of H_2O_2 (25 and 50 μM) or oxidized LDL (as above). The 30 μM concentration of compounds used was based on a concentration of epicatechin known to prevent cell death in striatal neurons induced by oxidized LDL [29]. Cells were also pre-treated with a range of concentrations of epicatechin (1–30 μM) and 3′-*O*-methyl epicatechin (10–30 μM, final concentration) prior to exposure to 50 μM hydrogen peroxide.

Assessment of mitochondrial function and cell integrity

Mitochondrial function was evaluated after the treatments by a colorimetric assay using MTT. MTT (0.5 mg/ml) was dissolved in HBM. Following exposure for 5 h, cells were washed twice with sterile PBS before addition of the MTT solution (0.5 ml/well) and incubated for 150 min at 37 °C. After incubation, MTT solutions were removed, 0.5 ml of DMSO was added and the absorbance was measured using a SPECTRAmax® 190 microplate photometer (Molecular Devices) at a wavelength of 505 nm. The absorbance was always measured within 30 min of adding the DMSO. To assess the effects on membrane integrity 18 h after exposure to hydrogen peroxide, LDH release was assayed. The absorbance data were transformed into percentage of LDH release using Triton X-100-treated control cultures as 100 % enzyme release. Visual observation using a microscope (eclipse TS100, Nikon) was also used.

Markers of cell death

Possible mechanisms of cell death were assessed by measuring annexin-V binding [30,31] and caspase-3 activity [32,33]. The binding of annexin V-CY3.18 was investigated using the apoptosis-detection system Annexin V-CY3®. The fibroblasts were sub-cultured on to 24-well black Krystal Microplates® (Porviar, Middx, U.K.) suitable for fluorescence microscopy and grown for 48 h as detailed above. Cells were pre-treated with epicatechin or methylated epicatechin (30 μM, final concentration), washed and hydrogen peroxide (50 μM) introduced in HBM. After 5 h of incubation the fibroblasts were treated according to the manufacturer's protocol (Sigma). Briefly, cells were washed with HBM and incubated for 10 min at room temperature with double-label staining solution containing annexin V-CY3.18 (1 μg/ml) and 6-carboxyfluorescein diacetate (100 μM) in binding buffer. Following incubation, the cultures were washed five times with binding buffer and observed using a fluorescence microscope equipped with a standard rhodamine and fluorescein filter set (annexin V-CY3.18 wavelength maxima, excitation at 552 nm and emission at 572 nm; 6-carboxyfluorescein wavelength maxima, excitation at 500 nm and emission at 530 nm). Annexin V-CY3.18 binds to phosphatidylserine present in the outer leaflet of the plasma membrane of cells undergoing early stages of apoptotic cell death. The non-fluorescent 6-carboxyfluorescein diacetate enters cells and is subsequently hydrolysed to its fluorescent form by esterases present and active in living cells. Thus cells that are alive will only stain green for 6-carboxyfluorescein, necrotic cells will only stain red for annexin V-CY3.18 and cells undergoing early stages of apoptotic processes will stain both red and green. Fibroblasts incubated for 30 min using the calcium ionophore ionomycin (final concentration, 10 μM) were used as a positive control for necrotic cell death. Identical areas of the fibroblast cultures were then photographed twice using the rhodamine and the fluorescein filter set to observe possible co-localization of the annexin V-CY3.18 and 6-carboxyfluorescein label.

To determine the activity of caspase-3-like proteases in cells following exposure to hydrogen peroxide the fibroblasts were sub-cultured on to 6-well plates at a density of 1×10^5 cells/well and grown for 48 h ($\approx 2 \times 10^5$ cells) before pre-treatment. Fibroblasts were pre-treated with epicatechin (30 μM, final concentration) or 3′-*O*-methyl epicatechin (30 μM) for 18 h, and subsequently washed as described above, prior to addition of H_2O_2 (50 μM). Cultures were incubated for 3 h at 37 °C. Cells were then washed with ice-cold sterile PBS and lysed to allow measurement of caspase-3-like protease activity. Briefly, the reaction mixture contained the cell lysate and the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide in assay buffer. To account for non-specific hydrolysis of the substrate a control reaction mixture contained cell lysate, substrate and the specific caspase-3 inhibitor acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide in assay buffer. Both mixtures were incubated for 90 min at 37 °C in a 96-well plate and the absorbance was read at 405 nm using a

SPECTRAmax® 190 microplate photometer. Absorbance data obtained using the caspase-3 inhibitor were subtracted from the absorbance data obtained without capase-3 inhibitor to correct for any non-specific hydrolysis. Vehicle controls and blanks were incorporated and caspase-3 protein was used as a positive control.

Statistical analysis

Data were expressed as means ± S.D. Statistical comparisons were made using an unpaired, two-tailed Student's t test with a confidence level of 95 %. The significance level was set at $P < 0.05$.

RESULTS

3′-*O*-Methyl epicatechin was synthesized using COMT and purified using semi-preparative HPLC. HPLC analysis revealed three peaks with catechin-like spectra (retention time, 29.8 min, epicatechin; retention time, 34.2 and 37.4 min, 3′-*O*-methyl and 4′-*O*-methyl epicatechin, with a 4:1 ratio). Characterization of the major peak was achieved by the use of nanoES-MS/MS, allowing the identification of O-methylated epicatechin by the presence of an abundant signal at m/z 305, which was confirmed using CID (Figure 2). This resulted in fragmentation to form a major peak at m/z 139, which corresponds to an A-ring fragment formed by a retro-Diels–Alder reaction (RDA) [34], and the ion at m/z 137 corresponding to the O-methylated B-ring (Figure 2).

The effects on cellular function of exposure of fibroblasts to oxidative stress, in the form of hydrogen peroxide or oxidized LDL, were investigated by assessing mitochondrial function (MTT reduction by succinate dehydrogenase) and membrane integrity (LDH release). Exposure of fibroblasts to H_2O_2 (25 and 50 μM) for 5 h led to a concentration-dependent loss of mitochondrial function (Figure 3A), MTT reduction decreasing to 65.3 ± 7.6 and 45.5 ± 5.7 %, respectively (untreated cells with 0 μM H_2O_2 = 100 %). Pre-treatment with epicatechin (30 μM) or 3′-*O*-methyl epicatechin (30 μM) for 18 h prior to the addition of H_2O_2 (50 μM) significantly attenuated the loss of MTT reduction to approximately the same extent for both compounds, 81.8 ± 5.9 and 88.5 ± 8.2 % respectively. Importantly, the cells were washed after the pre-treatment to avoid compound and oxidant reacting in the medium. It should be noted that at both concentrations of H_2O_2 there was no significant difference in protection by epicatechin and 3′-*O*-methyl epicatechin (25 μM, $P = 0.12$; 50 μM, $P = 0.27$). The attenuation of hydrogen peroxide-mediated decline in MTT reduction by epicatechin and O-methylated epicatechin is dose-dependent (Figure 3B).

Exposure of cells to oxidized LDL (12.5 μg of LDL protein/ml; LOOH, 474 ± 45 nmol/mg of LDL protein; conjugated dienes, 211 ± 33 nmol/mg of LDL protein) resulted in a decrease in MTT reduction (29.9 ± 4.9 %) that was attenuated by a pre-exposure to both epicatechin (63.4 ± 4.1 %) and 3′-*O*-methyl epicatechin (62.9 ± 6.5 %) to the same extent (no significant difference, $P = 0.2$; Figure 4).

Because it was not clear whether hydrogen peroxide- or oxidized-LDL-mediated loss of mitochondrial function, under these conditions, reflected a loss of viability or cell-cycle arrest, we also studied the effects of the oxidants and the epicatechin compounds on membrane integrity and LDH release after 18 h. Exposure of cells to H_2O_2 (50 μM) for 18 h induced an LDH release of 51.4 ± 6.1 % in cells, relative to the 100 % LDH release produced by Triton X-100. LDH release was attenuated to the same extent (no significant difference, $P = 0.24$) by epicatechin

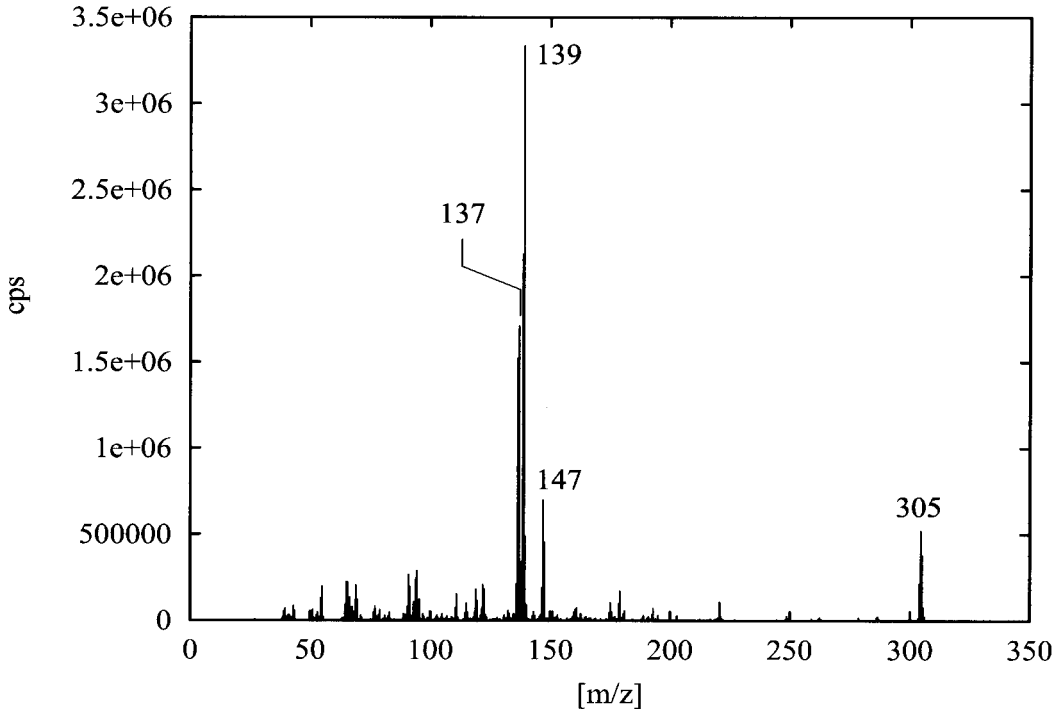

Figure 2 Structural confirmation of 3′-*O*-methyl epicatechin using nanoES-MS/MS with CID

The product ion scan of the peak at m/z 305 corresponds to *O*-methyl epicatechin $[M+H]^+$. The abundant signal at m/z 139 represents the retro-Diels–Alder product and derives from the A-ring. The ion with a m/z value of 137 corresponds to the methylated B-ring. The mass analysis was carried out as detailed in the Experimental section.

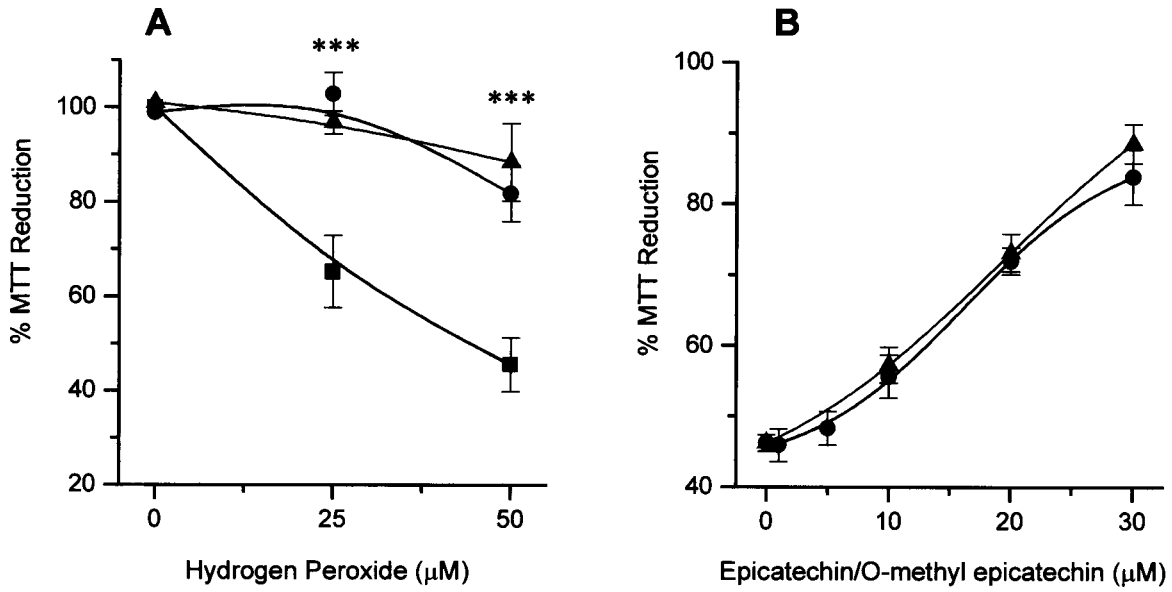

Figure 3 Effects of epicatechin and its methylated metabolite on oxidative-stress-induced loss of mitochondrial function in fibroblasts

(**A**) Mitochondrial function after exposure to hydrogen peroxide (25 and 50 μM, 5 h) with or without pre-treatment with epicatechin (30 μM) or 3′-*O*-methyl epicatechin (30 μM), as assessed by the MTT assay. ■, No pre-treatment; ●, pre-treatment with epicatechin; ▲, pre-treatment with 3′-*O*-methyl epicatechin. (**B**) Attenuation of the hydrogen peroxide-induced loss of mitochondrial function by epicatechin (●) and 3′-*O*-methyl epicatechin (▲) is dose-dependent. Fibroblasts were pre-treated with epicatechin (0–30 μM) prior to the treatment with hydrogen peroxide (50 μM) and the mitochondrial function was assessed by the MTT assay. Data are presented as means ± S.D. from four independent expreiments. ***$P < 0.01$ compared with respective non-pretreated cells (two-tailed Student's *t* test).

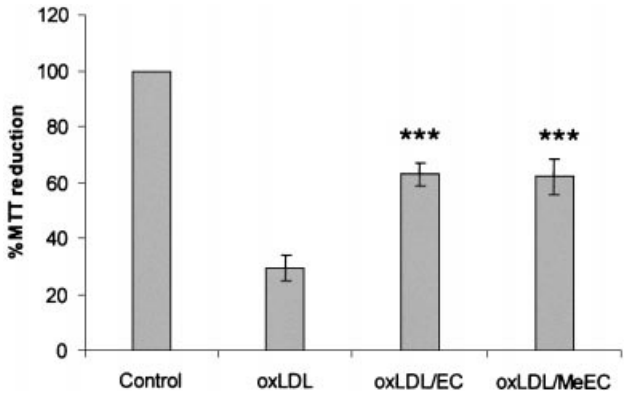

Figure 4 Effects of epicatechin and its methylated metabolite on oxidized-LDL-induced loss of mitochondrial function in fibroblasts

Fibroblasts treated with oxidized LDL (oxLDL; 12.5 μg of LDL protein/ml) exhibited a loss of mitochondrial function as measured using the MTT assay. Pre-treatment with epicatechin (EC) and 3′-*O*-methyl epicatechin (MeEC; both 30 μM) inhibited the effect of oxidized LDL. Data are presented as means ± S.D. from four independent expreiments. ***$P < 0.001$ compared with the control (two-tailed Student's *t* test).

(13.4 ± 2.4 %) and 3′-*O*-methyl epicatechin (14.1 ± 3.5 %). This suggests that the measured decline in mitochondrial function is associated with loss of cell viability rather than processes involving cell-cycle arrest.

Visual observations by light microscopy (results not shown) indicated changes in the morphological appearance of the fibroblasts as compared with untreated cultures following exposure to H_2O_2 and oxidized LDL. Fibroblasts exhibited distortion of the cell body and contained condensed nuclei after exposure to the oxidants. To investigate the possible mechanism of cell death induced by hydrogen peroxide, annexin-V binding and caspase-3 activity were also investigated. Fibroblasts treated with hydrogen peroxide (50 μM) for 5 h were stained using the apoptosis-detection system Annexin V-CY3® to demonstrate annexin V-CY3.18 binding and 6-carboxy-fluorescein diacetate hydrolysis. Live cells stain only green for 6-carboxyfluorescein, necrotic cells stain only red for annexin V-CY3.18 and cells undergoing early stages of apoptotic processes will stain both red and green. Figure 5(A) shows the untreated control cultures only staining for 6-carboxyfluorescein, whereas Figure 5(B) illustrates a co-localized staining for annexin V-CY3.18 and 6-carboxy-fluorescein after treatment of the cells with hydrogen peroxide (50 μM). For further clarity the staining pattern of a single cell after treatment with hydrogen peroxide is also demonstrated (Figure 5C). The positive control for necrotic cell death of fibroblasts treated with ionomycin is depicted in Figure 5(D), a strong red staining with only marginal green labelling. Fibroblasts pre-treated with epicatechin and methylated epicatechin prior to oxidative stress were not significantly different from each other, exhibiting green staining and strongly reduced red staining compared with the stressed cells without pre-treatment (results not shown).

Caspases are known to be markers of apoptosis [32,33] and have been observed to be activated in fibroblasts exposed to low-micromolar doses of H_2O_2 [35,36]. Exposure of fibroblasts to H_2O_2 (50 μM) for 3 h resulted in enhanced caspase-3 activity, which was apparent as an increase in absorbance to 0.21 at 405 nm (untreated A_{405}, 0.05; Figure 6), which reflects the increased cleavage of the caspase-3-specific substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide. Cells pre-treated with epicatechin (30 μM) or 3′-*O*-methyl epicatechin (30 μM) for 18 h prior to H_2O_2 addition exhibited caspase-3 activity that was not significantly different from untreated cells.

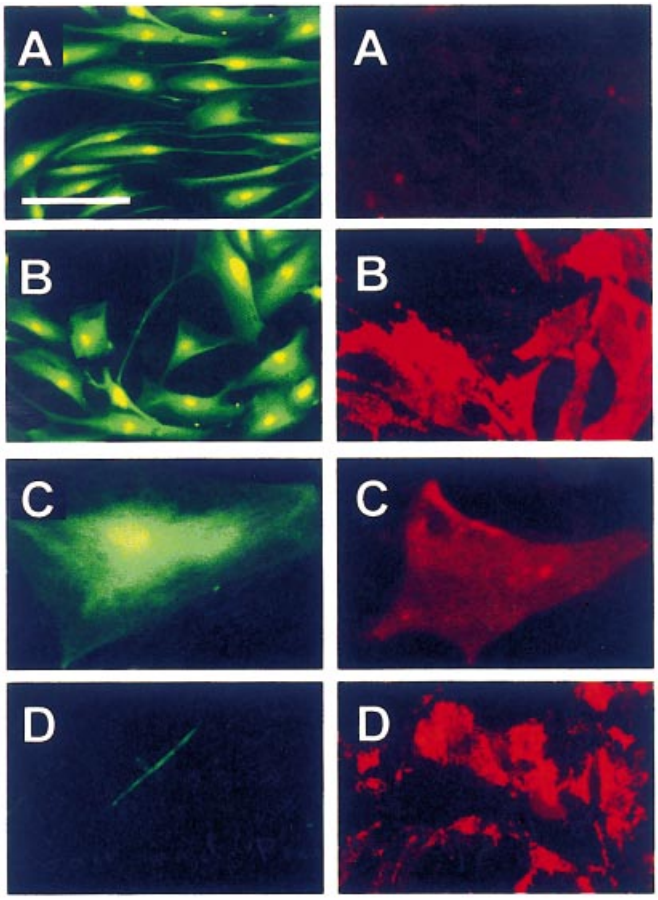

Figure 5 Hydrogen peroxide-mediated apoptotic processes

Fibroblasts treated with hydrogen peroxide (50 μM) for 5 h were stained using the apoptosis-detection system Annexin V-CY3® to demonstrate annexin V-CY3.18 binding (right-hand panels) and 6-carboxyfluorescein diacetate hydrolysis (left-hand panels). Live cells only stain green (for 6-carboxyfluorescein), necrotic cells only stain red (for annexin V-CY3.18), and cells undergoing early stages of apoptotic processes will stain both red and green. (**A**) Untreated control cultures; (**B**) cluster of fibroblasts treated with hydrogen peroxide; (**C**) single cells treated with hydrogen peroxide; (**D**) cluster of fibroblasts treated with ionomycin as a positive control for necrotic cell death. Scale bar, 15 μm.

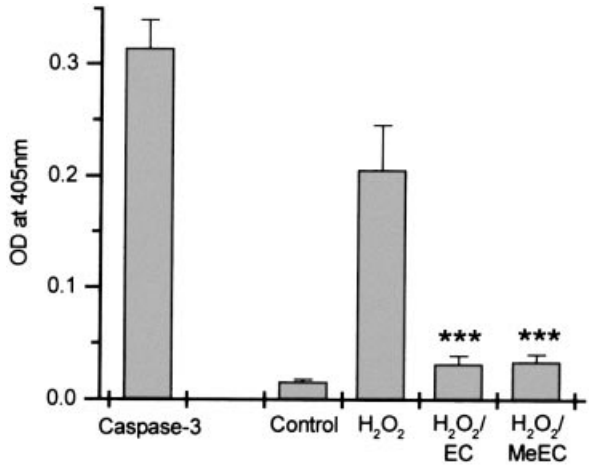

Figure 6 Increased activity of caspase-3-like proteases induced by hydrogen peroxide (50 μM) and the inhibition by pre-treatment with epicatechin and 3′-*O*-methyl epicatechin

After incubation with fibroblast cultures for 3 h, the cells were lysed and the activity of caspase-3-like proteases was measured by a spectrophotometric method using the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide. Hydrogen peroxide induced a strong increase in caspase-3-like activities, which was inhibited by pre-treatment with either epicatechin (EC) or 3′-*O*-methyl epicatechin (MeEC). Data are presented as means $\pm$ S.D. from three independent experiments. $^{***}P < 0.001$ compared with H_2O_2-treated cells (two-tailed Student's *t* test). OD, absorbance.

Because the catechol-containing flavonoid structures are well known to be powerful H-donating antioxidants, the influence of methylation of the catechol group on the antioxidant activity of epicatechin, in the form of 3′-*O*-methyl epicatechin, was determined using the TEAC assay. Such assays are designed for chemical assessment of the reducing properties of antioxidants through their hydrogen-donating abilities. The TEAC values of epicatechin and 3′-*O*-methyl epicatechin (relative to Trolox) were 4.8 and 1.8, respectively, indicating that the H-donating ability is greatly reduced on methylation of the B-ring.

DISCUSSION

The treatment of fibroblasts with hydrogen peroxide (50 μM) for 5 h resulted in a loss of mitochondrial function, and increases in the activity of caspase-3-like proteases and the binding of annexin V-CY3.18 to the cell membrane. Measurements of LDH 18 h after the exposure of fibroblasts to hydrogen peroxide (50 μM) showed enhanced activities of this enzyme in the culture medium compared with control cultures, demonstrating a decline in membrane integrity. Taken together, these findings suggest that hydrogen peroxide treatment of fibroblasts leads to cell death and that the mechanism of this cell death involves apoptotic processes during the 5 h time frame. These findings are consistent with those of other investigators, showing an involvement of apoptotic processes in hydrogen peroxide-induced cell death in fibroblasts and other cells [35,37,38].

There is considerable current interest in the cytoprotective effects of natural antioxidants against oxidative stress and whether the mode of action involves their reducing properties or some other mechanism independent of their antioxidant activities. This study demonstrates that the epicatechin metabolite, 3′-*O*-methyl epicatechin, up to 30 μM, has the ability to inhibit cell death induced by hydrogen peroxide, as assessed by the effects on mitochondrial function, membrane integrity, caspase-3 activity and annexin-V binding. A similar response is also obtained with oxidized LDL as the oxidative stress. Interestingly, the level of protection elicited by 3′-*O*-methyl epicatechin was not significantly different from that of epicatechin itself. Under the conditions of the experiments described here, protection was observed in the range 5–30 μM of the flavanols. Although little is known about the uptake of these compounds into cells, reported levels in plasma for humans and animals suggest that regular consumption of dietary agents containing epicatechin/catechin might lead to low-micromolar levels of accumulated metabolites in the circulation.

The major structural feature underlying the antioxidant activities or reducing properties of flavonoids is the *o*-dihydroxy catechol structure in the B-ring, which is mono-substituted in 3′-*O*-methyl epicatechin (Figure 1) [10,11]. This indicates that although the H-donating potentials of the two compounds are very different, as determined by the TEAC assay, the protection against the oxidant-induced cell death is identical. This is the first evidence that 3′-*O*-methyl epicatechin, a form that is found *in vivo*, may function to protect cells against cell death induced by

oxidants by a mechanism independent of its antioxidant properties.

The metabolism of catechin and epicatechin has been investigated by several groups by monitoring blood, bile and urine. Glucuronide and sulphate conjugates, and O-methylated forms of both catechin and epicatechin have been detected *in vivo* [39–42]. Recently, we have detected glucuronidated epicatechin, 3′-*O*-methyl epicatechin, 4′-*O*-methyl epicatechin and 3′-*O*-methyl epicatechin-glucuronide on the serosal side of enterocytes after perfusion of rat small intestine with epicatechin [17]. Such studies indicate that the bioavailability of the native flavanol is limited and that O-methylated and conjugated derivatives are the forms most likely to be in the circulation. The findings support the notion that studies investigating the action of flavanols, and other flavonoids, should be undertaken with the *in vivo*-conjugated and/or metabolized forms of the compounds, as well as native forms, in order to assess potential mechanisms of action in cell protection.

Our data suggest a non-redox mechanism of action of flavonoids *in vivo*. Although the native flavonoid is efficacious in scavenging reactive oxygen and reactive nitrogen species *in vitro*, on absorption *in vivo* it is subject to extensive metabolism and conjugation, both in the small intestine and in the liver, which may affect the redox potential and therefore the ability to act as classical H-donating antioxidants. 3′-O-Methylation of epicatechin greatly reduces the H-donating ability of the compound, but does not affect its ability to protect cells from oxidative insults leading to cell death. Since the major factor underlying the antioxidant activity and thus the reduction potential of flavonoids is the 3′,4′-catechol structure of the B-ring, O-methylation not only limits the reducing properties but also the predisposition to quinone formation (Figure 1), which might arise on autoxidation of epicatechin, inducing potential cytotoxicity.

The mechanism of protection by both native compound and the O-methylated metabolite remains unclear. 3′-*O*-Methyl epicatechin would be expected to enter cells more freely than epicatechin itself due to its higher lipophilicity and higher partition coefficient. However, the possibility cannot be excluded that demethylation might occur intracellularly by the action of cytochrome P450 or etherases cleaving the O-methylated metabolite to epicatechin. In conclusion, these findings suggest that the ability of flavonoids such as epicatechin to provide health benefits may not necessarily be dependent on the ability of the native compound to act as a scavenger of free radicals or reactive oxygen and nitrogen species, but by the ability of their metabolites to interact with cell-signalling cascades, to influence the cell at a transcriptional level and to down-regulate pathways leading to cell death. Consequently, the redox potential *per se* may not be the fundamental feature when determining the ability of specific phenolics to protect against either oxidative or other cell insults.

This research was supported by a European Union Fifth Framework RTD Programme Grant (grant no. QLK4-1999-01590) in which C.R.-E. and R.M.T. are contracting partners. Support by the Deutsche Forshungsgemeinschaft and the German Fonds der Chemischen Industrie to U.H. is gratefully acknowledged.

REFERENCES

1 Musconda, C. A. and Chipman, J. K. (1998) Quercetin inhibits hydrogen peroxide-induced NFκB DNA binding activity and DNA damage in HepG2 cells. Carcinogenesis **19**, 1583–1589

2 Tsai, S. H., Lin-Shiau, S. and Lin, J. K. (1999) Suppression of nitric oxide synthase and the down-regulation of the activation of NFκB in macrophages by resveratrol. Br. J. Pharmacol. **126**, 673–680

3 Manthey, J. A., Grohmann, K., Montanari, A., Ash, K. and Manthey, C. L. (1999) Polymethoxylated flavones derived from citrus suppress tumour necrosis factor-alpha expression in human monocytes. J. Nat. Prod. **62**, 441–444

4 Gerritsen, M. E., Carley, W. W., Ranges, G. E., Shen, C. P., Phan, S. A., Ligon, G. F. and Perry, C. A. (1995) Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. Am. J. Pathol. **147**, 278–292

5 Panes, J., Gerritsen, M. E., Anderson, D. C., Miyasaka, M. and Granger, D. M. (1996) Apigenin inhibits tumour necrosis factor-induced intercellular adhesion molecule-1 upregulation *in vivo*. Microcirculation **3**, 279–286

6 Soriani, M., Rice-Evans, C. A. and Tyrrell, R. M. (1998) Modulation of the UVA activation of haem oxygenase, collagenase and cyclooxygenase gene expression by epigallocatecgin in human skin cells. FEBS Lett. **439**, 253–257

7 Youdim, K. A., Martin, A. and Joseph, J. A. (2000) Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. Free Radicals Biol. Med. **29**, 51–60

8 Sestili, P., Guidarelli, A., Dacha, M. and Cantoni, O. (1998) Quercetin prevents DNA single strand breakage and cytotoxicity caused by tert-butylhydroperoxide: free radical scavenging versus iron chelating mechanism. Free Radicals Biol. Med. **25**, 196–200

9 Duthie, S. J., Johnson, W. and Dobson, V. L. (1997) The effects of dietary flavonoids on DNA damage (strand breaks and oxidised pyrimidines) and growth in human cells. Mutat. Res. **390**, 141–151

10 Jovanovic, S. V., Steenken, S., Simic, M. G. and Hara, Y. (1998) Antioxidant properties of flavonoids: reduction potentials and electron transfer reactions of flavonoid radicals. In Flavonoids in Health and Disease (Rice-Evans, C. and Packer, L., eds.), pp. 137–161, Marcel Dekker, New York

11 Bors, W., Heller, W., Michel, C. and Saran, M. (1990) Flavonoids as antioxidants: determination of radical scavenging efficiencies. Methods Enzymol. **186**, 343–355

12 Rice-Evans, C., Miller, N. and Paganga, G. (1996) Structure-antioxidant activity relationships of flavonoids and phenolic compounds. Free Radicals Biol. Med. **20**, 933–956

13 Wang, H. and Joseph, J. A. (1999) Structure-activity relationships of quercetin in antagonising hydrogen peroxide-induced calcium dysregulation in PC12 cells. Free Radicals Biol. Med. **27**, 683–694

14 Skaper, S. D., Fabris, M., Ferrari, V., Carbonare, M. and Leon, A. (1997) Quercetin protects cutaneous tissue-associated cell types including sensory neurons from oxidative stressinduced by glutathione depletion: cooperative effects of ascorbic acid. Free Radicals Biol. Med. **22**, 669–678

15 Donovan, J. L., Bell, J. R., Kasim-Karakas, S., German, J. B., Walzem, R. L., Hansen, R. J. and Waterhouse, A. L. (1999) Catechin is present as metabolites in human plasma after consumption of red wine. J. Nutr. **129**, 1662–1668

16 Spencer, J. P. E., Chowrimootoo, G., Choudhury, R., Debnam, E. S., Srai, S. K. and Rice-Evans, C. (1999) The small intestine can both absorb and glucuronidate luminal flavonoids. FEBS Lett. **458**, 224–230

17 Kuhnle, G., Spencer, J. P. E., Schroeter, H., Shenoy, B., Debnam, E. S., Srai, S. K., Rice-Evans, C. and Hahn, O. (2000) Epicatechin and catechin are O-methylated and glucuronidated in the small intestine. Biochem. Biophys. Res. Commun. **227**, 507–512

18 Walle, U.K., Galijatovic, A. and Walle, T. (1999) Transport of the flavonoid chysin and its conjugated metabolites by the human intestinal cell line Caco-2. Biochem. Phamacol. **58**, 431–438

19 Day, A. J., DuPont, M. S., Ridley, S., Rhodes, M. J. C., Morgan, M. R. A. and Williamson, G. (1998) Deglycosylation of the flavonoid and its isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity. FEBS Lett. **436**, 71–75

20 Okushio, K., Suzuki, M., Matsumoto, N., Nanjo, F. and Hara, Y. (1999) Identification of (−)-epicatechin metabolites and their metabolic fate in the rat. Drug Metab. Dispos. **27**, 309–316

21 Scheline, R. R. (1991) CRC Handbook of Mammalian Metabolism of Plant Compounds, pp. 267–290, CRC Press, Boca Raton

22 Ball, P., Knuppen, R., Haupt, M. and Breuer, H. (1972) Kinetic properties of a soluble catechol O-methyltransferase of human liver. Eur. J. Biochem. **25**, 560–569

23 Kuhnle, G. G., Haferburg, D., Grunow, M., Hirsch, D. and Hahn, U. (2000) Nano-electrospray mass spectrometry with a modified commercial IonSpray source. Rapid Commun. Mass Spec. **14**, 1307–1308

24 Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. **26**, 1231–1237

25 Tyrrell, R. M. and Pidoux, M. (1986) Quantitative differences in host cell reactivation of ultraviolet damaged virus in human skin fibroblasts and epidermal keratinocytes cultured from the same foreskin biopsy. Cancer Res. **46**, 2665–2669

26 Chung, B. H., Wilkinson, T., Geer, J. C. and Segrest, J. P. (1980) Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. J. Lipid Res. **21**, 284–291

27 Pryor, W. A. and Castle, B. C. (1984) Chemical methods for the detection of lipid hydroperoxides. Methods Enzymol. **105**, 293–299
28 Jiang, Z.-Y., Hunt, J. V. and Wolff, S. P. (1992) Ferrous ion oxidation in the presence of xylenol orange for the detection of lipid hydroperoxide in low density lipoprotein. Anal. Biochem. **202**, 384–389
29 Schroeter, H., Williams, R. J., Matin, R., Iversen, L. and Rice-Evans, C. A. (2000) Phenolic antioxidants attenuate neuronal cell death following uptake of oxidized low density lipoprotein. Free Radicals Biol. Med. **29**, 1222–1233
30 Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M. and Green, D. R. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J. Exp. Med. **182**, 1545–1556
31 Aupeix, K., Toti, F., Satta, N., Bischoff, P. and Freysinnet, J. M. (1996) Oxysterols induce membrane coagulant activity in monocytic THP-1 cells. Biochem. J. **314**, 1027–1033
32 Davis, R. (2000) Signal transduction by the JNK group of MAP kinases. Cell **103**, 239–252
33 Yuan, J. and Yankner, B. (2000) Apoptosis in the nervous system. Nature (London) **407**, 802–809
34 Miketova, P., Schram, K. H., Whitney, J. L., Kerns, E. H., Valcic, S., Timmermann, B. N. and Volk, K. J. (1998) Mass spectrometry of selected components of biological interest in green tea extracts. J. Nat. Prod. **61**, 461–467
35 Sugano, N., Ito, K. and Murai, S. (1999) Cyclosporin inhibits hydrogen peroxide-induced apoptosis of human fibroblasts. FEBS Lett. **447**, 274–276
36 Chen, Q. M., Lui, J. and Merrett, J. B. (2000) Apoptosis or senescence-like growth arrest: influence of cell-cycle position, p53, p21 and bax in hydrogen peroxide response of normal human fibroblasts. Biochem. J. **347**, 543–551
37 Lee, S. D., Lee, B. D., Han, J. M., Kim, J. H., Kim, Y., Suh, P. G. and Ryu, S. H. (2000) Phospholipase D2 activity suppresses hydrogen peroxide-induced apoptosis. J. Neurochem. **75**, 1053–1059
38 Teramoto, S., Tomita, T., Matsui, H., Ohga, E., Matuse, T. and Ouchi, Y. (1999) Hydrogen peroxide-induced apoptosis and necrosis in human lung fibroblasts: protective roles of glutathione. Jpn. J. Pharmacol. **79**, 33–40
39 Das, N. P. and Griffiths, L. A. (1969) Studies on flavonoid metabolism. Metabolism of (+)-[^{14}C]catechin in the rat and guinea pig. Biochem. J. **115**, 831–836
40 Harada, M., Kan, Y., Naoki, H., Fukui, Y., Kageyama, N., Nakai, M., Miki, W. and Kiso, Y. (1999) Identification of the major antioxidative metabolites in biological fluids of the rat with ingested (+)-catechin and (−)-epicatechin. Biosci. Biotechnol. Biochem. **63**, 973–977
41 Piskula, M. K. and Terao, J. (1998) Accumulation of (−)-epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues. J. Nutr. **128**, 1173–1178
42 Shaw, I. C., Hackett, A. M. and Griffiths, L. A. (1982) Metabolism and excretion of the liver-protective agent (+)-catechin in experimental hepatitis. Xenobiotica **12**, 405–416

Received 30 October 2000/28 November 2000; accepted 16 January 2001