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Green tea polyphenols inhibit human vascular smooth muscle cell proliferation stimulated by native low-density lipoprotein

Rudolf Locher, Luca Emmanuele, Paolo M. Suter, Wilhelm Vetter, Matthias Barton *

Department of Internal Medicine, Medical Policlinic and Clinical Atherosclerosis Research Laboratory, University Hospital Zürich, Rämistrasse 100, CH-8091 Zürich, Switzerland

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Abstract

This study investigated whether human vascular smooth muscle cell proliferation induced by native low-density lipoprotein (LDL) is affected by green tea catechins. Furthermore, the effects of native LDL on extracellular signal-regulated kinase (ERK) 1/2 activity were determined. Cell proliferation stimulated by native LDL was concentration-dependently inhibited by epigallocatechin, epigallocatechin-3-gallate, green tea polyphenon, and the nonspecific antioxidant *N*-acetylcysteine ($P < 0.05$). Combined treatment of green tea polyphenon and *N*-acetylcysteine markedly potentiated the effect of each drug on vascular smooth muscle cell proliferation. ERK1/2 activity was only partly inhibited by green tea catechins alone or in combination with *N*-acetylcysteine ($P < 0.05$). These data suggest that green tea constituents inhibit proliferation of human vascular smooth muscle cells exposed to high levels of native LDL. Green tea constituents and antioxidants may exert vascular protection by inhibiting human vascular smooth muscle cell growth associated with hypercholesterolemia. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Antioxidant; Atherosclerosis; Cell proliferation; Green tea catechin; LDL (low-density lipoprotein); Mitogen-activated kinase; Risk factor; Smooth muscle cell, vascular

1. Introduction

Elevated plasma levels of low-density lipoprotein (LDL) have been implicated in the pathogenesis of atherosclerotic vascular disease (Gotto, 1992). Native LDL stimulates cell proliferation and intracellular Ca^{2+} turnover of vascular smooth muscle cells in vitro (Scott-Burden et al., 1989; Locher et al., 1997), even in the absence of LDL receptors (Metzler et al., 2000; Metzler et al., 1999; Sachinidis et al., 1990). Other effects of native LDL include stimulation of DNA synthesis (Björkerud and Björkerud, 1994; Locher et al., 1997) and upregulation of extracellular signal-regulated kinase (ERK) protein (Metzler et al., 1999).

Reactive oxygen species have been implicated in vascular smooth muscle cell growth and extracellular signal regulated (ERK) mitogen-activated protein kinase (MAP) activation (Baas and Berk, 1995; Greene et al., 2000; Irani, 2000; Liao et al., 2000). This is further supported by the observation that treatment with antioxidants interferes with vascular smooth muscle cell proliferation, at least in certain species (Greene et al., 2000; Ruef et al., 1998; Sundaresan et al., 1995). In rat aortic vascular smooth muscle cells, DNA synthesis stimulated by platelet-derived growth factor or serum is inhibited by green tea constituents (Ahn et al., 1999). Whether proliferation of human vascular smooth muscle cells is affected by green tea constituents is unknown. Moreover, no information is available whether such effects are present in cells stimulated with native LDL, and whether activity of ERK1/2 mitogen-activated kinase, which is increased in atherosclerosis (Hu et al., 2000) is affected by native LDL.

In the present study, we therefore determined the effects of native LDL, different green tea constituents, and the antioxidant *N*-acetylcysteine, on cell proliferation and ERK1/2 activity in human vascular smooth muscle cells.

* Corresponding author. Tel.: +41-1-255-5663; fax: +41-1-255-8747.
E-mail address: bartonm@swissonline.ch (M. Barton).
URL: http://www.dim-unizh.eh/earl.

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2. Materials and methods

2.1. Human vascular smooth muscle cell culture

Experimental procedures were in accordance with the institutional guidelines. Human umbilical cords were obtained at the Department of Obstetrics and Gynecology, University Hospital Zürich. Umbilical vein smooth muscle cells were isolated using the explant technique as described (MacLeod et al., 1994) and cultured in 75 cm^2 culture flasks (Falcon™), using smooth muscle cell growth medium including 5% fetal calf serum. Cells were passaged by treatment with 0.05% trypsin/0.02% EDTA in phosphate-buffered saline. Subconfluent cells of passages 3–6 were used for all experiments. Cells were analyzed for smooth muscle cell-specific α-actin by immunofluorescence (Skalli, 1986), yielding >98% of α-actin positive cells.

2.2. Isolation of low-density lipoprotein

Human low-density lipoprotein was isolated from pooled EDTA plasma. Freshly drawn EDTA blood from normolipemic donors taking no medication was obtained from the bloodbank of our hospital. Plasma obtained from 125 healthy individuals was used for LDL isolation. Plasma was subjected to ultracentrifugation in the presence of 1 mM EDTA in a KBr gradient (Redgrave et al., 1975) at 200,000 × *g* for 14 h at 4 °C. The LDL fraction was decanted and dialyzed at 4 °C for 24 h against three changes of 1500 ml of 150 mM of NaCl and 0.25 mM EDTA at pH 7.4. After dialysis, LDL was concentrated to 2 mg/ml LDL protein using membrane separation (Centrisart 1 tubes; Sartorius, Göttingen, Germany) with a cutoff at 20 kDa. LDL was sterilized using 0.22 μm Millipore® filters and stored in the dark at 4 °C. Protein was measured using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) (Smith et al., 1985). Concentrations of LDL are given as μg LDL protein.

To determine whether LDL is oxidized by vascular smooth muscle cells, native LDL (100 μg/ml) was incubated either in Dulbecco's modified Eagle medium or incubation medium (Dulbecco's modified Eagle medium plus Ham's F-10 medium, 1:1, vol/vol, containing 0.1% fetal calf serum) in the presence or absence of vascular smooth muscle cells at 37 °C for 24 h. The reaction was terminated with 5 mM EDTA and 5 μg/ml of 2,6-di-*tert*-butyl-*p*-cresol. Three microliters of sample (0.3 μg LDL) was subjected to electrophoresis using the Titan Gel Electrophoresis Kit (Helena BioSciences) (Yang and Koo, 2000). Incubation of LDL in Dulbecco's modified Eagle medium containing Cu^{2+} (10 μM), yielding oxidized LDL (Augé et al., 1995) was used as positive control.

2.3. Measurement of cell proliferation

Vascular smooth muscle cells were starved for 24 h with incubation medium (0.1% fetal calf serum). Cell proliferation was stimulated with LDL (100 μg/ml) in the absence or presence of inhibitors. Cell proliferation was measured by [^{3}H]-thymidine incorporation (Locher et al., 1997). After 19 h of incubation, 3 μCi of [methyl-^{3}H] thymidine (1.5 μM) was added. Cell proliferation was determined after 24 h by aspirating the medium and subjecting the cells to DNA extraction as described (Nemecek et al., 1986).

2.4. Measurement of extracellular signal-regulated 1/2 kinase activity

ERK1/2 activity was measured by Western blotting with a commercially available kit using a specific antibody for phospho-elk-1, the phosphorylated product of activated ERK1/2 (De Cesaris et al., 1998; Marais et al., 1993). After 24 h of starvation, cells were stimulated for 10 min using native LDL in absence or presence of inhibitors, which were added 2 h before LDL administration. The experiment was terminated by rinsing cells with phosphate-buffered saline (4 °C). After lysis of the cells, extraction of phosphorylated (''activated'') ERK1/2 and the subsequent in vitro phosphorylation of the transcription factor elk-1 were performed according to the manufacturer's instructions. Aliquots (20 μl) of the immunoprecipitates containing phosphorylated elk-1 protein were separated by 10% sodium dodecyl sulfate/polyacryl amide gel electrophoresis and transferred to cellulose nitrate membrane. Membranes were blocked in 5% skim milk powder in Tris-buffered saline/0.1% Tween-20 (TBS-T) for 1 h at 22 °C and then incubated with anti-phospho-elk-1 polyclonal antibody (1:2000) in 5% bovine serum albumin in TBS/T for 14 h at 4 °C. After three washes, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) second antibody (1:2000) in blocking buffer for 1 h at 22 °C. The labeled proteins were visualized using the Phototope® Western blot detection kit (New England BioLabs). Densitometric analysis of bands was performed on a Macintosh computer using the NIH Image® program (version 1.6.1, National Institute of Health, Bethesda, MD, USA).

2.5. Azobis-amidinopropane-induced oxidation of human serum

Antioxidant capacity of green tea polyphenon was determined in human serum subjected to oxidation by 2,2′ azobis(2-amidinopropane)·HCl (AAPH). Experiments were performed according to previously published protocols (Esterbauer and Jürgens, 1993; Kontush and Beisiegel, 1999). Human serum was obtained from healthy donors taking no medication. Serum (20 μl) was added to 1 ml of buffer (in mM; NaCl 160, KH_2PO_4 1.54, $Na_2HPO_4 \cdot 7\ H_2O$ 2.7), pH 7.4, containing citrate (0.72 mM), AAPH (0. 5 mM), and increasing concentrations of polyphenon. Oxidation was measured as the increase in absorbance at 245 nm at 37 °C using an Ultrospec® 300 photometer (Pharmacia,

Switzerland) and calculated as the difference of absorbance between 10 h and time zero.

2.6. Determination of cytotoxicity

To determine cellular integrity after treatments, cytotoxicity was evaluated by measuring lactic acid dehydrogenase activity released into the culture medium at the end of the experiments using an enzymatic method (Hitachi 747 autoanalyzer) (Wacker et al., 1956). In addition, Trypan blue exclusion tests were performed (Johns and Riehl, 1982).

2.7. Materials

Smooth muscle cell growth medium containing 5% fetal calf serum (growth medium), Dulbecco's modified Eagle medium, Ham-F10, Dulbecco's phosphate-buffered saline and the ERK1/2 activity kit (New England BioLabs) were from PromoCell (BioConcept, Allschwil, Switzerland). Monoclonal antibodies against smooth muscle cell specific α-actin and fluorescein isothiocyanate-conjugated anti-mouse IgG second antibody, epigallocatechin, epigallocatechin-3-gallate, polyphenon 100 (81.3% catechins), were from Sigma, (Buchs, Switzerland). AAPH (2,2′ azobis(2-amidinopropane)·HCl) was from Polysciences (Eppelheim, Germany). The Titan® gel electrophoresis kit (Helena Biosciences, Sunderland, UK) was obtained from Morwell Diagnostics, (Egg, Switzerland).

2.8. Statistical analysis

Data were analyzed by analysis of variance or unpaired Student's *t*-test and expressed as mean ± S.E.M., *n* equals the number of experiments. When appropriate, data were analyzed by using the alternate Welch *t*-test (InStat, Graphpad™). Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Effects of native low-density lipoprotein and green tea catechins on vascular smooth muscle cell proliferation

Exposure of native low-density lipoprotein (LDL) to vascular smooth muscle cells for 24 h had no effect on

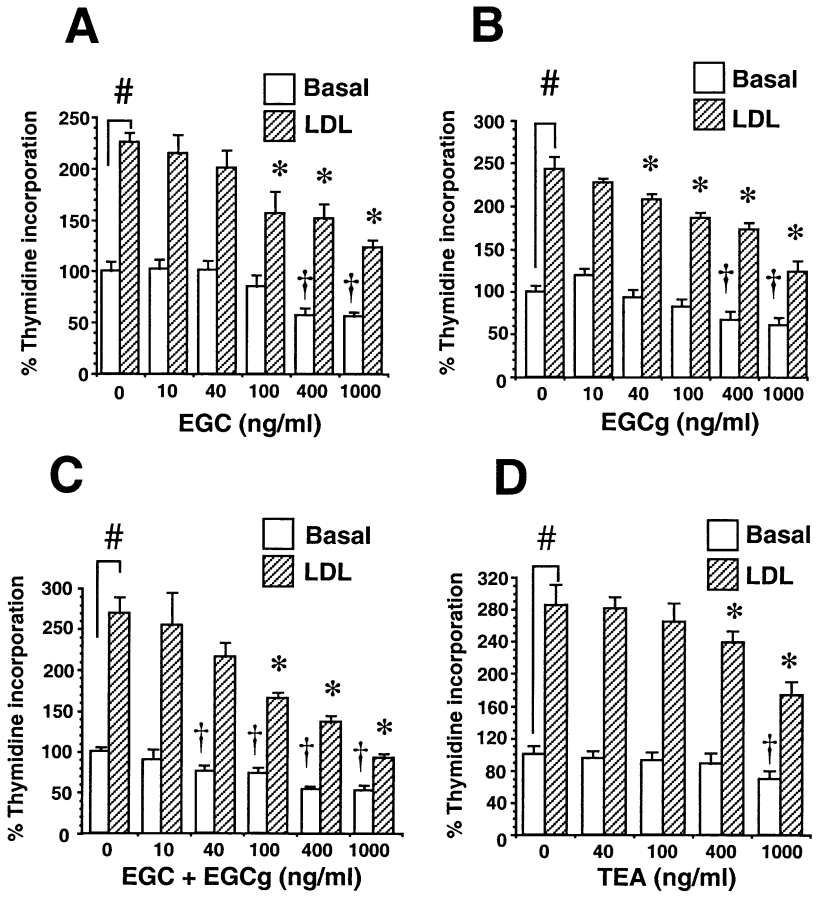

Fig. 1. Effects of epigallocatechin (A) and epigallocatechin-3-gallate (B) alone or in combination (C), and tea polyphenon (D) on basal (open bars) and LDL-stimulated DNA synthesis (hatched bars). Data are means ± S.E.M. and given as percent of controls. EGC, epigallocatechin; EGCg, epigallocatechin gallate; †$P < 0.05$ vs. basal; *$P < 0.05$ vs. LDL.

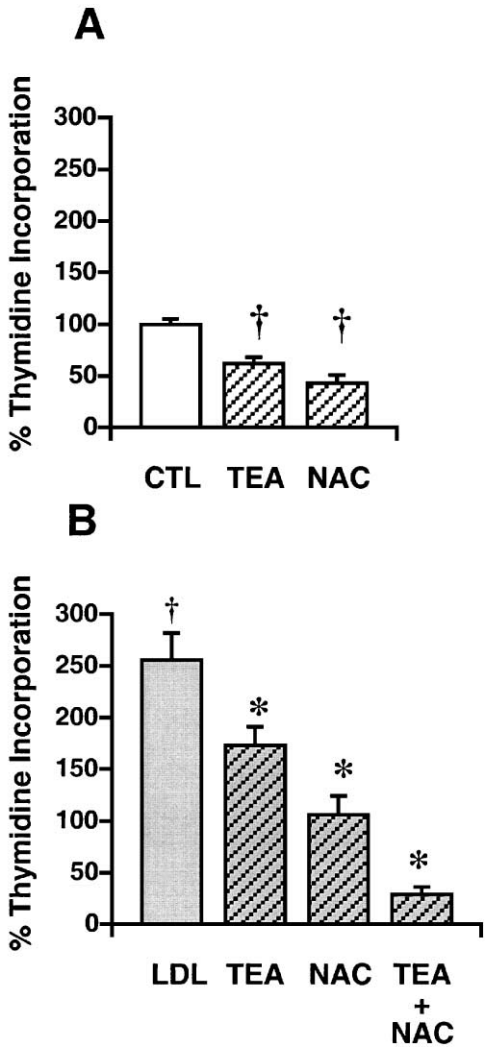

Fig. 2. Effects of tea polyphenon (TEA, 1 μg/ml) and *N*-acetylcysteine (NAC, 20 mM) on basal (A) and LDL-stimulated DNA synthesis (B) in human VSMC. Data are means ± S.E.M. and given as percent of control, * $P<0.05$ vs. LDL; † $P<0.05$ vs. CTL.

electrophoretic mobility of LDL compared to medium (data not shown). Incubation of human vascular smooth muscle cells with native LDL (100 μg/ml) for 24 h increased DNA synthesis 2.6-fold ($n=20$, $P<0.05$ vs. basal). Epigallocatechin ($n=5$, Fig. 1A) and epigallocatechin-3-gallate ($n=5$, Fig. 1B) at concentrations from 10 to 1000 ng/ml concentration-dependently inhibited both LDL-stimulated and basal cell proliferation (both $P<0.05$ vs. LDL). Combined administration of both catechins showed additive inhibitory effects ($n=5$, Fig. 1C), while green tea polyphenon was less effective than epigallocatechin or epigallocatechin-3-gallate alone or in combination (Figs. 1D and 2, lower panel).

The antioxidant *N*-acetylcysteine (20 mM) reduced LDL-induced cell proliferation ($P<0.05$, Fig. 2, lower panel). In combination with green tea polyphenon, the inhibitory effect of *N*-acetylcysteine was markedly potentiated (Fig. 2, lower panel). LDL alone or in the presence of *N*-acetylcysteine had no effect on cytotoxicity as assessed by lactic acid dehydrogenase release and Trypan blue exclusion tests (data not shown).

3.2. Effects of green tea catechins on extracellular signal-regulated 1/2 kinase activity

Exposure of cells to LDL for 10 min increased extracellular signal-regulated 1/2 kinase activity threefold as measured by in vitro phosphorylation of elk-1 protein ($n=5$, $P<0.05$ vs. control, Fig. 3). ERK1/2 activity was inhibited by the green tea constituents epigallocatechin (400 ng/ml, inhibition by 43 ± 3%, $P<0.05$, $n=4$), or epigallocatechin-3-gallate (400 ng/ml, inhibition 66 ± 17%, $n=4$, $P<0.05$). In contrast, pretreatment of cells with green tea polyphenon inhibited ERK1/2 kinase activity only by 21 ± 2% ($n=4$, $P<0.05$), and the combination of tea polyphenon (1 μg/ml) and *N*-acetylcysteine (20 mM) had no additional effect (inhibition by 30 ± 5%, $n=4$, $P<0.05$, Fig. 3).

3.3. Effect of green tea polyphenon on oxidation of human serum

2,2′ Azobis(2-amidinopropane)·HCl (AAPH) caused a 10-fold increase of serum oxidation (Fig. 4, $P<0.05$). Green

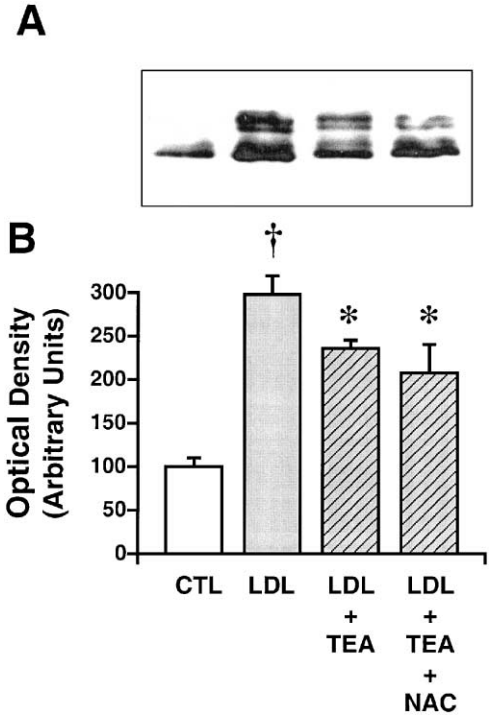

Fig. 3. Effect of native LDL, tea polyphenon (TEA) alone or in combination with *N*-acetylcysteine (NAC) on extracellular signal-regulated (ERK)1/2 kinase activity measured by elk-1 phosphorylation. Human vascular smooth muscle cells were pretreated with tea polyphenon alone (1 μg /ml, TEA) or in combination with *N*-acetylcysteine (20 mM), TEA + NAC) before cells were stimulated with LDL (100 μg/ml) for 10 min. (A) Representative Western blot; (B) pooled data from four independent experiments. † $P<0.05$ vs. control; * $P<0.05$ vs. LDL.

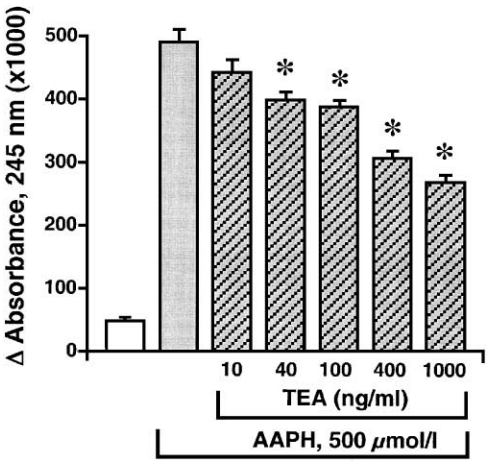

Fig. 4. Effects of tea polyphenon on 2,2′ azobis (2-amidinopropane)·HCl (AAPH)-induced oxidation of human serum in vitro. Diluted serum (1:50) was incubated in the presence of 0.5 mol/l AAPH (grey bar) and the indicated polyphenon concentrations for 10 h at 37 °C (hatched bars). The open bar indicates auto-oxidation of serum in the absence of AAPH. Values are given as means ± S.E.M.; * $P < 0.05$.

tea polyphenon at concentrations similar to those found in human plasma after ingestion of green tea (1–1000 ng/ml) concentration-dependently inhibited AAPH-induced serum oxidation. The maximal inhibitory effect of green tea polyphenon at 1000 ng/ml was 51 ± 3% ($n = 6$, $P < 0.05$, Fig. 4).

4. Discussion

This study provides evidence that in cultured human vascular smooth muscle cells proliferation stimulated with native low-density lipoprotein (LDL) is inhibited by green tea constituents and the antioxidant *N*-acetylcysteine and that green tea catechins inhibit oxidation of human serum. Native LDL also potently increases activity of ERK1/2 kinase, this activity is differently inhibited by green tea catechins alone or in combination with *N*-acetylcysteine.

We show that both native LDL-stimulated and basal cell proliferation of human vascular smooth muscle cells are inhibited in a dose-dependent fashion by several green tea constituents. These effects were observed at concentrations found in plasma in humans after ingestion of green tea (Van het Hof et al., 1999; Yang et al., 1998). Cell proliferation was inhibited to a different degree by different catechins. Interestingly, the combination of epigallocatechin and epigallocatechin-3-gallate was more potent to inhibit cell proliferation than either drug alone. In contrast, using green tea polyphenon, a mixture of several green tea components, including epicatechin, epicatechin-3-gallate epigallocatechin, and epigallocatechin-3-gallate, we observed that the inhibitory effect on LDL-induced and basal cell proliferation was less pronounced than with epigallocatechin or with epigallocatechin-3-gallate alone. Differences in the relative antioxidant capacity or the relative proportion of individual catechin components in the green tea mixture could account for the reduced efficacy to inhibit LDL-induced cell proliferation. The mechanisms responsible for the relative inhibitory effect of each constituent are not known, but could be due, at least in part, to different antioxidant properties of each compound (Ishikawa et al., 1997; Yang and Koo, 2000).

Native LDL stimulates protein expression of ERK1/2 MAP kinase in rat vascular smooth muscle cells (Sachinidis et al., 1997), whereas the effects of native LDL on activity of ERK MAP kinase and particularly its effects in human vascular smooth muscle cells have not yet been defined. We here show that native LDL stimulates ERK1/2 activity in human vascular smooth muscle cells and that green tea catechins differently inhibit ERK1/2 activity which is in line with the cell proliferation experiments (Fig. 3). Thus, our data support a role of ERK1/2 activity in LDL-induced human vascular smooth muscle cell growth and that green tea catechins can interfere with ERK1/2 activity. Recent work from our laboratory demonstrated that the MEK-1 inhibitor PD98059 completely prevents induction of ERK1/2 activity by native LDL, and that proliferation in response to native LDL is inhibited to a great extent by PD98059 (Locher et al., 2001). Based on these studies and the results presented in the present study, it can be suggested that redox-dependent regulation of ERK1/2 plays a significant role for cell proliferation induced by native LDL in human vascular smooth muscle cells.

We also observed that cell proliferation was inhibited by the antioxidant *N*-acetylcysteine, suggesting that inhibition of cell proliferation by green tea constituents was at least partly due to their antioxidant activity. Indeed, previous studies have demonstrated antioxidant activity of green tea constituents (Ruidavets et al., 2000; Van het Hof et al., 1999); however, the concentrations used in these previous studies were well above those found in human plasma after ingestion of green tea. Our data demonstrate for the first time that green tea constituents do exert antioxidant and antiproliferative effects at ‘‘physiological’’ concentrations (Ruidavets et al., 2000; Van het Hof et al., 1999). The antioxidant effect in our experiments was more pronounced than the antioxidant effect on plasma of healthy volunteers after ingestion of green tea (Van het Hof et al., 1999). Possibly the differences between the antioxidant effects of plasma obtained after ingestion of green tea of healthy volunteers and plasma oxidized in vitro are related to the presence of other antioxidant systems in the vascular wall (superoxide dismutases, catalase, glutathion peroxidase). These antioxidant systems may mask the antioxidative capacity of green tea constituents in plasma in vivo and were not present in our experiments.

The effects of green tea constituents reported here may be of potential therapeutic relevance. As noted, the concentrations of epigallocatechin or epigallocatechin-3-gallate used in our experiments are comparable to those found in plasma following the ingestion of green tea (Yang et al., 1998). Inhibitory effects of green tea constituents on LDL-

induced cell proliferation appear to be independent of oxidation of low-density lipoprotein (Van het Hof et al., 1999), which is further supported by electrophoretic mobility experiments in our study. This suggests direct actions of native LDL on the vascular smooth muscle cells unlike in endothelial cells, which rapidly oxidize LDL (Yang and Koo, 2000). The effects of native LDL on human vascular smooth muscle cells proliferation provide a target for antioxidants including green tea polyphenols, which have been shown to inhibit the progression of experimental atherosclerosis (Miura et al., 2001). As shown by the additive inhibitory effects of tea catechin and *N*-acetylcysteine, these data also suggest that there may be therapeutic potential for the combination of antioxidants, as has been suggested for other antioxidative substances (Hwang et al., 2000).

In summary, we have demonstrated that native LDL induces cell proliferation and ERK1/2 activity in human vascular smooth muscle cells. As native LDL-induced cell proliferation is potently inhibited by green tea constituents, atherogenic effects of native LDL may provide a direct target for antioxidant therapy, such as catechin-rich diets (Ruidavets et al., 2000).

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