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SUPPRESSIVE EFFECT OF EPIGALLOCATECHIN-3-GALLATE, A CONSTITUENT OF GREEN TEA, ON PLATELET-DERIVED GROWTH FACTOR-BB SIGNALING PATHWAY IN RAT A7r5 CELLS

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Epigallocatechin-3-gallate (EGCG), a polyphenol constituent of popular beverage green tea, has been recently attracted much attention for the prevention of atherosclerosis, which is a systemic vessel disease and may lead to a variety of diseases including cardiovascular disease. Platelet-derived growth factor (PDGF) plays an important role as cell-proliferating factor in the pathogenic course of atherosclerosis, and increased activity in the PDGF signaling pathway has been implicated as a contributing factor in the progression. In the present study, to elucidate how EGCG affects the PDGF signaling pathway, we investigated the effect of EGCG on the PDGF-BB-induced activation of Ras-MAPK (mitogen-activated protein kinase) pathway, expression of immediate-early genes, and cell proliferation in rat A7r5 cells. EGCG significantly decreased PDGF-BB-induced ERK1/2 (extracellular signal-regulated kinase 1/2) and MEK1 (MAPK/ERK kinase 1) phosphorylation states dose-dependently, and also inhibited PDGF-BB-induced c-***fos*** and c-***jun*** mRNA expressions and cell proliferation. Furthermore, PDGF-BB-induced tyrosine phosphorylation of the cognate receptor was significantly suppressed by EGCG, indicating that EGCG may act at and/or downstream the PDGF β-receptor. These results suggest that EGCG may suppress the cell proliferative signaling pathway through the inhibition of PDGF receptor kinase, MEK/ERK activity and immediate-early gene expressions. Thus, the habitual drinking of green tea may be useful to prevent atherosclerosis, and consequently decrease the mortality of cardiovascular diseases.

Key words: EGCG, green tea, PDGF-BB, cell proliferation, MAPK, immediate-early genes

INTRODUCTION

Polyphenols are naturally occurring compounds in a wide variety of the plants (1). Among them, the polyphenols in red wine and green tea, which are consumed by many peoples in the world, have been recently attracted much attention for the prevention of cardiovascular and inflammatory diseases or cancers (2, 3). Epigallocatechin-3-gallate (EGCG) is most prominent and abundant polyphenol, catechin, in green tea (2). EGCG possesses antioxidant properties, and has been shown to inhibit the development of atherosclerotic lesions in animal models, although the precise molecular mechanisms remain obscure (4, 5).

Atherosclerosis is a systemic vessel disease and may lead to a variety of diseases including cerebral infarction or cardiovascular disease. The progression of atherosclerosis may be based on the migration and proliferation of vascular smooth muscle cells in the intima, and platelet-derived growth factor (PDGF) could strongly promote the growth and migration of the cells (6). PDGF binds to its cognate receptor

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expressed on the cell surface, activating the intrinsic tyrosine kinase within the cellular cytoplasmic domain, followed by the association with many signaling molecules containing SH2 domain. In turn, multiple signaling pathways are initiated, leading to numeral cellular responses including cell proliferation (7, 8).

In the present study, we aimed to clarify the role of EGCG on the proliferative signal transduction pathways by PDGF. Here, we show that EGCG effectively suppressed PDGF-BB-induced phosphorylations of PDGF β-receptor, MEK1, ERK1/2 and Akt, and further, repressed the expressions of immediate-early genes, c-***fos*** and c-***jun***, and cell proliferation.

MATERIALS AND METHODS

Reagents and Cell culture

Epigallocatechin-3-gallate (EGCG, purity: minimum 95%) was obtained from Sigma (St. Louis, USA). Platelet-derived growth factor-BB (PDGF-BB), Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were from GIBCO BRL (Gaithersburg, USA). Polyclonal antibodies against phospho-ERK1/2 (PhosphoPlus p44/p42 MAP kinase antibody) and phospho-MEK1 (PhosphoPlus MEK antibody) were purchased from Cell Signaling Technology, Inc (Beverly, USA). Anti-phosphotyrosine antibody (PY 99) and antibody against PDGF β-receptor (A-2) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

Rat A7r5 vascular smooth muscle cell line (5×10^5 cells) was maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS) in a humidified atmosphere of 5% CO_2 at 37°C. For the experiments, the cells were seeded into 60 mm dishes and grown until 70% confluent. Prior to treatment, the medium was replaced by 0.5% FCS/DMEM, and cultured for 48 h to set the cell stage as G_0. After medium was replaced with a fresh one, the cells were pretreated with or without EGCG, and incubated with 10 ng/ml PDGF-BB for 30 min unless indicated otherwise. Cell viability was checked by cell number and trypan blue dye exclusion tests in all experiments (cell survival rate: more than 95%).

To determine the effect of EGCG on cell proliferation, A7r5 cells (1.0×10^4 cells) grown in DMEM with 1% FCS were incubated with 10-50 μM EGCG in the presence of 10 ng/ml PDGF-BB for 48 h. After incubation, the cells were detached with 0.25% trypsin, and then the cell numbers were counted with hemocytometer.

Total RNA preparation and RT-PCR

Total RNA (1 μg), extracted from the cultured A7r5 cells by the AGPC method (9), was reverse-transcribed with MuLV reverse-transcriptase and random primers. Then, the first strand was subjected to PCR with the following primers: c-***fos*** Fwd, AGCTGACAGATACGCTCCAA; c-***fos*** Rev, TAGGTGAAGACAAAGGAAGACG; c-***jun*** Fwd, CGGCTACAGTAACCCTAAGA; c-***jun*** Rev, CCTGAGACTCCATGTCGATA; GAPDH Fwd, ACCACAGTCCATGCCATCAC; GAPDH Rev, TCCACCACCCTGTTGCTGTA. The amplification cycle was set to reflect the original quantity of each mRNA.

Western blot analysis

Western blot analysis was carried out as described previously (10). Briefly, A7r5 cells incubated with indicated concentrations of EGCG and/or PDGF-BB for 30 min, were lysed with 100 μl of lysis buffer (20 mM Tris/HCl, 7.4, 150 mM NaCl, 1% Triton X-100, and 1 mM sodium orthovanadate), and ultrasonicated. Aliquots (20 μg) of the cell lysates were separated on SDS/PAGE (12.5% polyacrylamide) and transferred to PVDF membranes. The membrane blocking was performed with 5% (w/v) nonfat dry milk in TBS (Tris-buffered saline) for 1 h at room temperature, followed by probing with the specific antibodies. Primary antibodies were used at the dilution of 1:2,000. To reprobe the blots, the membranes were submerged in the stripping buffer (62.5 mM Tris/HCl, pH 6.8, 100 mM β-mercaptoethanol, and 2% SDS) and incubated at 65°C for 30 min with shaking, followed by probing with each antibody against the non-phosphorylated form. The resultants were visualized using the enhanced chemiluminescence (ECL detection system, Amersham Biosciences). For immunoprecipitation assay, the lysates were incubated with anti-PDGF β-receptor antibody at 4°C for overnight, and the immunocomplex was separated by adding Protein A/G Sepharose. The

eluates from the Sepharose were subjected to Western blot analysis as described above.

RESULTS

Effect of EGCG on PDGF-BB-induced phosphorylations of ERK 1/2 and MEK1 in A7r5 cells

PDGF-BB is a potent stimulator of growth and motility of smooth muscle cells, and strongly activates Ras-MAPK pathway (7, 8). We at first examined the effect of EGCG on 10 ng/ml PDGF-BB-induced phosphorylation of ERK1/2. The cells pretreated with 50 μM EGCG for the indicated time, were treated with 10 ng/ml PDGF-BB for 30 min, and the cell lysates were subjected to Western blot analysis using the specific antibody against phospho-ERK1/2. As shown in Fig. 1, PDGF-BB alone significantly increased phospho-ERK1/2 level (lane 1). The pretreatment of 50 μM EGCG for 2 h almost completely blocked PDGF-BB-stimulated phospho-ERK1/2 level (lane 3), although the pretreatment for 0, 6 or 12 h less affected (lanes 2, 4 and 5). Next, we investigated the effect of EGCG concentrations on PDGF-BB-induced phosphorylation of ERK1/2. Pretreatment of EGCG for 2 h dose-dependently suppressed phosphorylation levels of ERK1/2 (lanes 6-10), and 50 μM EGCG completely blocked the phosphorylation (lane 9). In parallel, EGCG also inhibited the PDGF-BB-induced phosphorylation of MEK1, an upstream kinase for ERK1/2 (Fig. 1, lower panel). These results indicate that 50 μM EGCG with pretreatment for 2 h effectively blocked MEK1 and ERK1/2 activations.

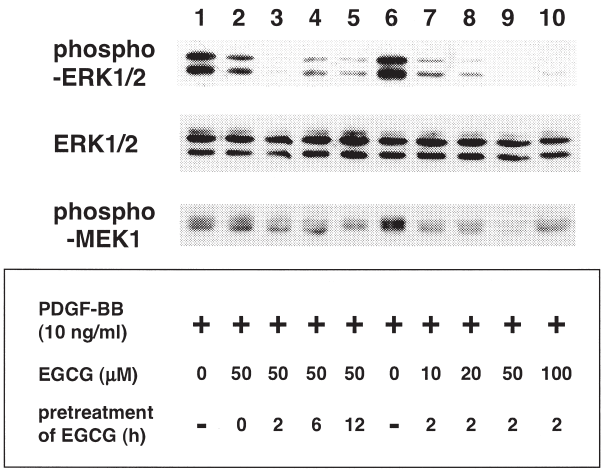

Fig. 1. Effect of EGCG on PDGF-BB-induced phosphorylations of ERK1/2 and MEK1 in A7r5 cells. Quiescent cells were treated with 10 to 100 μM EGCG for the indicated time, and then stimulated with 10 ng/ml PDGF-BB for 30 min. The cell lysates were subjected to Western blot analysis using anti-phospho ERK1/2, anti-ERK1/2 and anti-phospho MEK1 antibodies as described in Materials and methods. Results of the blotting using anti-phospho ERK1/2 (upper panel), anti-ERK1/2 (middle panel) and anti-phospho MEK1 (lower panel) antibodies are shown. The data are the representative of four independent experiments.

Effect of EGCG on PDGF-BB-induced c-fos and c-jun mRNA expressions in A7r5 cells

PDGF-BB is known to stimulate not only c-***fos*** but also c-***jun*** mRNA expressions to form the stable AP-1 heterodimer complex (11, 12). We considered whether EGCG affects the PDGF-BB-induced expressions of immediate-early genes such as c-***fos*** and c-***jun***, which locate downstream of MEK-ERK pathway, and are closely involved in the cell proliferation induced by PDGF-BB. To investigate the effect of EGCG on PDGF-BB-induced c-***fos*** and c-***jun*** mRNA expressions, total RNA obtained from the cells treated with PDGF-BB and/or EGCG were analyzed by RT-PCR. As shown in Fig. 2, 10-50 μM EGCG with pretreatment for 2 h dose-dependently suppressed PDGF-BB-induced c-***fos*** and c-***jun*** mRNA expressions (lanes 3-6). At 50 μM EGCG, the c-***fos***

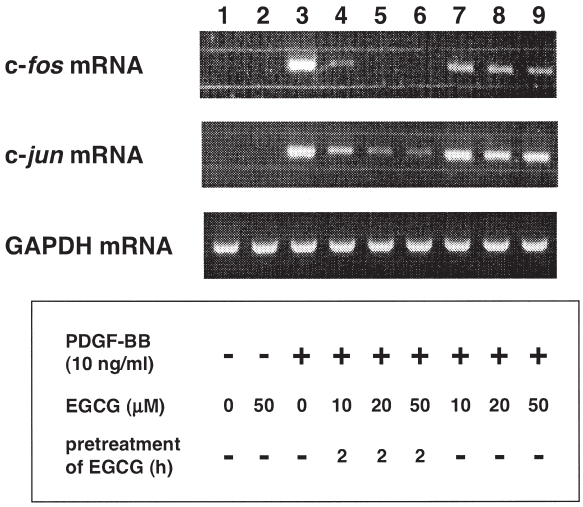

Fig. 2. Effect of EGCG on PDGF-BB-induced c-***fos*** and c-***jun*** mRNA expressions in A7r5 cells. Quiescent cells were treated with 10, 20 and 50 μM EGCG for the indicated time, and then stimulated with 10 ng/ml PDGF-BB for 30 min. Total RNA (1 μg) extracted was subjected to RT-PCR as described in Materials and methods. Expressions of c-***fos*** (upper panel), c-***jun*** (middle panel) and GAPDH (lower panel) mRNA are shown. The data are the representative of four independent experiments.

and c-***jun*** expressions were completely and almost blocked, respectively (Fig. 2, lane 6). When the cells were treated simultaneously with EGCG and PDGF-BB, the suppressive effects on c-***fos*** and c-***jun*** mRNA expressions were lower than those by pretreatment of EGCG for 2 h (lanes 7-9). These results well coincide with the effects of EGCG on ERK1/2 and MEK1 phosphorylations, and EGCG effectively suppressed PDGF-BB-induced c-***fos*** and c-***jun*** mRNA expressions in A7r5 cells.

Effect of EGCG on PDGF-BB-induced PDGF β-receptor tyrosine phosphorylation in A7r5 cells

PDGF-BB-binding to the PDGF β-receptor causes dimerization of the receptor and activates the intrinsic tyrosine kinase to autophosphorylate, and the phosphorylated receptor activates the Ras and MAPK cascade, resulting in c-***fos*** mRNA expression and AP-1 activation (7, 8-11). To see whether EGCG affects this initial step for PDGF signaling pathway, we next investigated whether EGCG affects 10 ng/ml PDGF-BB-induced PDGF β-receptor tyrosine phosphorylation in A7r5 cells. As shown in Fig. 3, 50 μM EGCG alone did not induce PDGF β-receptor tyrosine phosphorylation, though 10 ng/ml PDGF-BB did significantly (lanes 2 and 3). Pretreatment of EGCG for 2 h dose-dependently reduced PDGF-BB-induced receptor tyrosine phosphorylation (lanes 3-6), and 50 μM EGCG completely blocked the phosphorylation (lane 6). When the cells were treated simultaneously with EGCG and PDGF-BB, the suppressive effects on the receptor tyrosine phosphorylation were apparent, but lower than those by pretreatment of EGCG for 2 h (lanes 7-9). These results suggest that EGCG could act at the PDGF β-receptor as well as on the downstream pathway of the PDGF β-receptor to activate MEK1 and ERK1/2, leading to suppression of the immediate-early gene expressions.

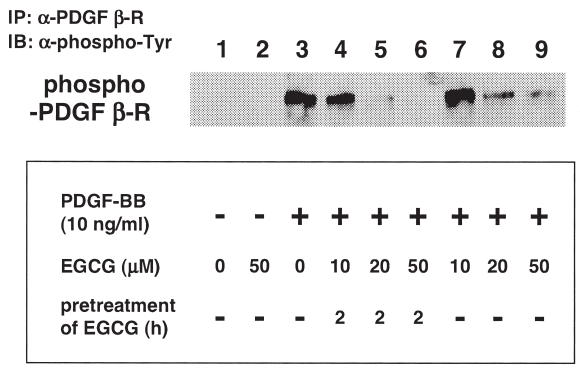

Fig. 3. Effect of EGCG on PDGF-BB-induced PDGF β-receptor tyrosine phosphorylation in A7r5 cells. Quiescent cells were treated with 10, 20 and 50 μM EGCG for the indicated time, and then stimulated with 10 ng/ml PDGF-BB for 30 min. The cell lysates were subjected to immunoprecipitation with anti-PDGF β-receptor antibody followed by Western blot analysis using anti-phosphotyrosine antibody as described in Materials and methods. The data are the representative of three independent experiments.

Effect of EGCG on PDGF-BB-induced phosphorylation of Akt in A7r5 cells

Having shown that EGCG significantly inhibited PDGF-BB-induced phosphorylations of its cognate receptor, MEK1 and ERK1/2 as well as the expressions of c-***fos*** and c-***jun***, we considered whether EGCG affects PI3-K-Akt pathway, another downstream signaling pathway from the PDGF β-receptor (7, 8). Akt is a serine/threonine protein kinase critically involved in cell survival or insulin signaling, and is activated by PDGF β-receptor via PI3-K (13). Thus, to see whether EGCG inhibits PDGF signaling pathway at the receptor level, we next examined the effect of EGCG on PDGF-BB-induced Akt phosphorylation. As shown in Fig. 4, 10 ng/ml PDGF-BB significantly stimulated Akt phosphorylation, though 50 μM EGCG alone did not affect

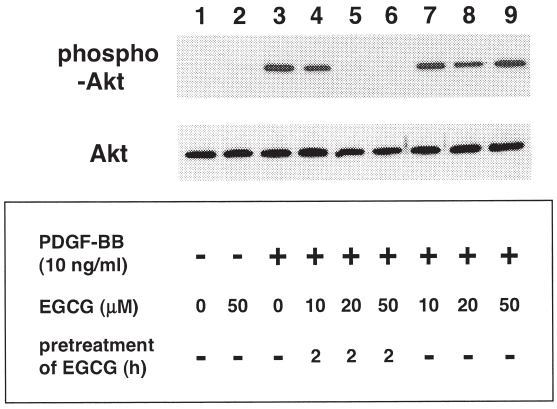

Fig. 4. Effect of EGCG on PDGF-BB-induced phosphorylation of Akt in A7r5 cells. Quiescent cells were treated with 10, 20 and 50 μM EGCG for the indicated time, and then stimulated with 10 ng/ml PDGF-BB for 30 min. The cell lysates were subjected to Western blot analysis using anti-phospho Akt and anti-Akt antibodies as described in Materials and methods. Results of the blotting using anti-phospho Akt (upper panel) and anti-Akt (lower panel) antibodies are shown. The data are the representative of four independent experiments.

phosphorylation level (lanes 2 and 3). Pretreatment of EGCG for 2 h dose-dependently decreased PDGF-BB-induced Akt phosphorylation (lanes 3-6), and 20 and 50 μM EGCG completely blocked the phosphorylation (lanes 5 and 6). When the cells were treated simultaneously with EGCG and PDGF-BB, the suppressive effects on Akt phosphorylation were only slightly observed (lanes 7-9). These results clearly indicate that EGCG suppressed PI3-K-Akt pathway as well as Ras-MAPK pathway possibly through the inhibition of PDGF β-receptor activation.

Effect of EGCG on PDGF-BB-induced cell proliferation in A7r5 cells

As EGCG was shown to suppress the ligand-induced PDGF signaling pathway as described above, next we investigated the effect of EGCG on PDGF-BB-induced cell proliferation. A7r5 cells ($1.5x10^4$ cells) grown in DMEM with 1% FCS were incubated with 10-50 μM EGCG in the presence of 10 ng/ml PDGF-BB for 48 h, then the cell numbers were counted. As shown in Fig. 5, the cells treated with EGCG and 10 ng/ml PDGF-BB were decreased in its cell numbers with increasing concentrations of EGCG, as compared with those treated with PDGF-BB alone. At the concentration of 50 μM EGCG, the cell numbers were reduced to the same level of control or 50 μM EGCG treatment alone. These results indicate that EGCG could act long-term to suppress the cell proliferation, as well as act short-term to block the PDGF signaling pathway.

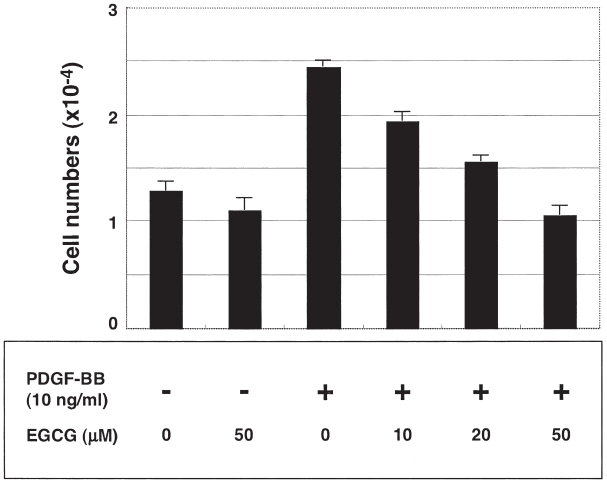

Fig. 5. Effect of EGCG on PDGF-BB-induced cell proliferation in A7r5 cells. A7r5 cells (1×10^4 cells) grown in DMEM with 1%FCS were incubated with 10, 20 and 50 μM EGCG in the presence or absence of 10 ng/ml PDGF-BB for 48 h. After the incubation, the cells were detached with 0.25% trypsin, and the numbers were counted as described in Materials and methods. The data shown are the means ± S.E. of three independent experiments.

DISCUSSION

Epigallocatechin-3-gallate (EGCG) is a most prominent and abundant catechin in green tea, and has been shown to modulate numerous molecular targets in the setting of inflammation, heart disease and cancer (2, 4, 14). PDGF is a potent stimulant of growth and motility of connective tissue cells, such as smooth muscle cells and fibroblasts, and plays a crucial role in the pathogenic course of atherosclerosis as a cell-proliferative factor (6-8). The present study was aimed to elucidate the suppressive mechanisms of EGCG on PDGF signaling pathway, which may cause the progression of atherosclerosis. In rat vascular smooth muscle cell line A7r5 cells, the pretreatment of EGCG for 2 h significantly reduced PDGF-BB-induced phosphorylation levels of PDGF signaling molecules such as ERK1/2, MEK1, PDGF β-receptor and Akt, as compared with simultaneous treatment of EGCG. The delay in the action may be ascribed to the properties of EGCG, including the efficiency or velocity of plasma membrane diffusion and subsequent subcellular distribution. EGCG suppressed not only Ras-MAPK signaling but also PI3-K-Akt signaling, indicating that the target molecule of EGCG would be PFGF-β receptor itself. Recently, it was reported that EGCG directly inhibited ERK1/2 and Akt activities as well as epidermal growth factor (EGF) receptor activation (15). These results suggest that EGCG could suppress at multiple levels on the growth-promoting signaling pathway. Although the precise molecular mechanisms of EGCG still remain obscure, the present study revealed that PDGF β-receptor and its downstream kinases, which are crucial for cell proliferation, could be the targets of EGCG.

In physiological state, drinking of a few cup of green tea would result in the elevation of serum catechin up to 1 μM, and in some cases, maximum 5 μM (1, 14). The maintenance of a high concentration in

plasma thus should require a repeated ingestion of the catechin over time (1). In this study, at least 10 μM EGCG significantly reduced the phosphorylation states of ERK1/2 and MEK1, and immediate early gene expressions as well as cell proliferation. Thus, frequent drinking of green tea may be favorable for the prevention of atherosclerosis or promotion of health.

We previously reported that taurine, semi-essential sulfur-containing β-amino acid, effectively suppressed PDGF-BB-induced proliferative signaling in NIH/3T3 cells (10). Taurine intake would be profitable for the prevention of atherosclerosis and subsequent chronic diseases including heart disease and cerebral infarction, though, 5-10 mM concentration of taurine was required to inhibit the immediate-early gene expressions and ERK1/2 phosphorylation (10). In contrast, at least 10 μM EGCG significantly suppressed PDGF-BB signaling and cell proliferation as shown in the present study. Thus, besides its antioxidant activity, EGCG was proved to be a potent anti-proliferative reagent.

In conclusion, here we provided evidence that EGCG could suppress proliferative signaling induced by PDGF-BB, and ultimately vascular smooth muscle cell proliferation. Thus, consumption of green tea may contribute to many health benefits including the prevention of atherosclerosis and cardiovascular disease. Further study would be performed to explore the profitable function of green tea catechin.

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