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(−)-Epigallocatechin-3-gallate decreases thrombin/paclitaxel-induced endothelial tissue factor expression via the inhibition of c-Jun terminal NH2 kinase phosphorylation

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ABSTRACT

Patients with paclitaxel-eluting stents are concerned with stent thrombosis caused by premature discontinuation of dual antiplatelet therapy or clopidogrel resistance. This study investigates the effect of (−)-epigallocatechin-3-gallate (EGCG) on the expression of thrombin/paclitaxel-induced endothelial tissue factor (TF) expressions in human aortic endothelial cells (HAECs). EGCG was nontoxic to HAECs at 6 h up to a concentration of 25 μmol/L. At a concentration of 25 μmol/L, EGCG pretreatment potently inhibited both thrombin-stimulated and thrombin/paclitaxel-stimulated endothelial TF protein expression. Thrombin and thrombin/paclitaxel-induced 2.6-fold and 2.9-fold increases in TF activity compared with the control. EGCG pretreatment caused a 29% and 38% decrease in TF activity on thrombin and thrombin/paclitaxel treatment, respectively. Real-time polymerase chain reaction (PCR) showed that thrombin and thrombin/paclitaxel-induced 3.0-fold and 4.6-fold TF mRNA expressions compared with the control. EGCG pretreatment caused an 82% and 72% decrease in TF mRNA expression on thrombin and thrombin/paclitaxel treatment, respectively. The c-Jun terminal NH2 kinase (JNK) inhibitor SP600125 reduced thrombin/paclitaxel-induced TF expression. Furthermore, EGCG significantly inhibited the phosphorylation of JNK to 49% of thrombin/paclitaxel-stimulated HAECs at 60 min. Immunofluorescence assay did not show an inhibitory effect of EGCG on P65 NF-κB nuclear translocation in the thrombin/paclitaxel-stimulated endothelial cells. In conclusion, EGCG can inhibit TF expression in thrombin/paclitaxel-stimulated endothelial cells via the inhibition of JNK phosphorylation. The unique property of EGCG may be used to develop a new drug-eluting stent by co-coating EGCG and paclitaxel.

Introduction

Since their introduction, drug-eluting stents (DESs) have revolutionized the field of interventional cardiology. Despite their rapid acceptance, the major limitation of DESs is the risk of stent thrombosis, which is often caused by premature discontinuation of dual antiplatelet therapy that especially involves clopidogrel [1]. In the clinical scenario, prolonged dual antiplatelet therapy can increase bleeding episodes and cause patients to discontinue clopidogrel prematurely, predisposing them to catastrophic events [2]. Furthermore, those patients that did not respond to aspirin and clopidogrel and those that had a reduced function CYP2C19 allele with resultant lower levels of the active metabolite of clopidogrel are associated with a higher risk of DES thrombosis or death [3,4].

One of the first-generation DESs was coated with paclitaxel, which is a microtubule-stabilizing drug causing the inhibition of smooth muscle cell proliferation and migration. Its cytotoxic effect was used to reduce the restenosis rate among patients receiving paclitaxel-eluting stents (PESs; Boston Scientific, Natick, MA). However, PES implantation to treat coronary artery diseases can result in significant local inflammation in the coronary vascular wall and apoptosis in the endothelial cells [5]. A large meta-analysis revealed that the risk of stent thrombosis increases with PESs compared to that with bare-metal stents or rapamycin-eluting stents [6].

Tissue factor (TF) is an initiator of the extrinsic pathway of blood coagulation, which is the predominant clotting pathway

Abbreviations: AP1, activator protein 1; DES, drug-eluting stent; DMSO, dimethyl sulfoxide; EGCG, (−)-epigallocatechin-3-gallate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAEC, human aortic endothelial cell; JNK, c-Jun terminal NH2 kinase; MTS, methoxyphenyl tetrazolium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PES, paclitaxel-eluting stent; TF, tissue factor.

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for thrombin generation *in vivo* [7]. Experimental results have revealed important contributions of TF to the pathogenesis of stent thrombosis [8,9]. Stähli et al. [10] and Wang et al. [11] have recently reported that paclitaxel can enhance thrombin-induced endothelial TF expression, which can explain PES-associated thrombotic risks. Because of the inherent thrombogenic risk of DESs, co-coating a chemotherapeutic solvent (i.e., dimethyl sulfoxide) to paclitaxel DES has been suggested as a promising direction for further DES design [12]. However, dimethyl sulfoxide has no known beneficial effect on human health.

Some naturally occurring substances in a normal diet provide a new insight in cardiovascular therapy. Emerging data suggested that green tea, particularly its major polyphenolic constituent, (−)-epigallocatechin-3-gallate (EGCG), possesses remarkable cancer therapeutic potential as well as cardioprotective effects [13,14]. Epidemiological studies revealed that the habitual consumption of green tea is associated with lower cardiovascular mortality [15]. The beneficial cardiovascular effects of EGCG may be the result of its pleiotropic effects, which include its antioxidant, antithrombogenic, and anti-inflammatory effects [16–20]. The pleiotropic effects of EGCG suggested that it may be effective in reducing TF expression in thrombin/paclitaxel-treated endothelial cells.

This study investigates whether EGCG can modulate the effect of thrombin/paclitaxel on TF expression in human aortic endothelial cells (HAECs).

Materials and methods

Cell culture. HAECs were purchased from Cell Applications, Inc. (San Diego, CA) and cultured in endothelial cell growth medium (Cell Applications, Inc.) according to the manufacturer's recommendations. The cells were grown to near confluence in 10-cm culture dishes before the experiment. The HAECs were grown overnight under serum-starved conditions in M-199 medium supplemented with 1% fetal bovine serum (HyClone, Logan, UT) before stimulation with 1 U/mL human thrombin (Sigma–Aldrich, St. Louis, MO). To block c-Jun terminal NH2 kinase (JNK), HAECs were treated with SP600125 (Calbiochem, Merck4Biosciences, Darmstadt) for 90 min before thrombin stimulation. Paclitaxel and EGCG (Sigma–Aldrich) were added to the HAECs 1 h before thrombin stimulation.

Methoxyphenyl tetrazolium inner salt cell viability assay. HAECs were seeded in a 96-well plate to a final concentration of 1×10^4 cells/well. After serum starvation overnight, HAECs were treated with dimethyl sulfoxide (DMSO, 0.1%, vehicle for 10 μmol/L paclitaxel; treatment for 6 h) and different concentrations of EGCG (1 μmol/L, 10 μmol/L, 25 μmol/L, 50 μmol/L, and 100 μmol/L; 6 h). Twenty microliters of methoxyphenyl tetrazolium (MTS, 0.5 mg/mL; Promega, Madison, WI) was added to each well, and the cells were cultivated at 37 °C for 2 h. The absorbance was then recorded at 490 nm.

Western blot analysis. The cells were lysed in 50 mM Tris buffer, and 35 μg of the samples was loaded and separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were transferred onto a polyvinylidene fluoride membrane by a semidry transfer method at 5 V for 100 min. Antibodies against TF (American Diagnostica, Stamford, CT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA) were used at concentrations of 1:2000 and 1:5000, respectively. Antibodies against phosphorylated JNK and total JNK (both from Cell Signaling, Danvers, MA) were used at concentrations of 1:1000 and 1:2000, respectively. All blots were normalized to the blot for GAPDH expression and were probed for the whole cell lysates.

TF activity assay. The TF activity in the HAECs was analyzed with an Actichrome® TF assay (American Diagnostica), as previously described [11]. Briefly, the extracted TF was placed in a 96-well microplate and incubated with human Factor VIIa and human Factor X for 15 min at 37 °C. The FVIIa/FXa complex thus formed could cleave a chromogenic substrate Spectrozyme FXa, which was added to each well and incubated for 20 min at 37 °C. This chromogenic reaction was stopped by adding glacial acetic acid after the 20-min incubation period. The data were recorded as the absorbance of the reaction mixtures at 405 nm. A standard curve of different concentrations of lipidated human TF was prepared to ensure that the measurements were recorded in the linear range of detection.

Real-time polymerase chain reaction analysis. Endothelial cells were harvested by trypsinization and obtained as a pellet after centrifugation. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Shanghai) according to the manufacturer's instructions. All polymerase chain reactions (PCRs) were performed using real-time fluorescence detection and LightCycler System (Roche Diagnostics, Mannheim) with a FirstStart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals, Indianapolis, IN). A reaction mixture containing the following components at the indicated end concentration was prepared according to the manufacturer's instructions: 0.2 μL of forward primer (20 μmol), 0.2 μL of reverse primer (20 μmol), and 5 μL Master mix. Fifty nanograms of reverse transcribed total RNA in a volume of 4.4 μL was added as the PCR template. The GAPDH gene was selected for normalization of data. A negative control without a cDNA template was performed to assess overall specificity. PCR cycle conditions were as follows: initial denaturation for 10 min at 95 °C followed by 45 cycles of amplification at 95 °C for denaturation, 60 °C for annealing, and 72 °C for extension. After amplification, the temperature was slowly elevated above the melting temperature of the PCR product to measure the fluorescence and thereby determine the melting curve. These real-time PCR data were calculated by the $2^{-\Delta\Delta C_t}$ method for RNA quantification. The primer sequences were as follows: TF: forward primer: 5′-caggaatgtggagagcac-3′; reverse primer: 5′-ggctgtccgaggtttgtc-3′ and GAPDH: forward primer: 5′-ctctgctcctcctgttcgac-3′; reverse primer: 5′-acgaccaaatccgttgactc-3′.

Immunofluorescence assay. HAECs were plated onto coverslips in a 6-well plate at a density of 1×10^5 cells/slide. After serum starvation, HEACs were treated with DMSO (0.1% for 2 h), paclitaxel (10 μmol/L for 2 h) + thrombin (1 U/mL for 1 h), and paclitaxel (10 μmol/L for 2 h) + thrombin (1 U/mL for 1 h) + EGCG (25 μmol/L for 2 h). They were then fixed with 4% paraformaldehyde at 4 °C for 10 min and permeabilized with 0.5% saponin in phosphate-buffered saline (PBS) for 20 min. Subsequently, the HEACs were incubated with anti-P65 antibody (Santa Cruz Biotechnology) for 1 h. After extensive washing with PBS, a fluorophore-conjugated secondary antibody (Invitrogen, Carlsbad, CA) was added at room temperature for 1 h, and the nuclei were stained with 0.5 mg/mL 4′,6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich) for 5 min. The slides were mounted using a SlowFade Antifade Kit (Molecular Probes, Eugene, OR) and examined using a Nikon Eclipse TE2000-S microscope.

Statistical analysis. All data are presented as mean ± SEM. The significance was determined by the unpaired *t*-test. *P* values less than 0.05 were considered statistically significant.

Results

EGCG was nontoxic to endothelial cells up to a concentration of 25 μM

An MTS cell viability assay revealed that the treatments with different concentrations of EGCG (1 μmol/L, 10 μmol/L, 25 μmol/L, 50 μmol/L, and 100 μmol/L) for 6 h caused significant cytotoxicity at drug concentrations of 50–100 μmol/L (Fig. 1A). However,

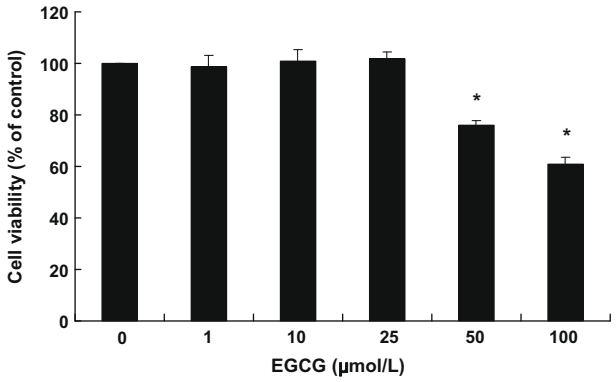

Fig. 1A. EGCG is nontoxic to endothelial cells up to a concentration of 25 μmol/L at 6 h. HAEC cell viability was determined by MTS assay. After serum starvation, HAECs were treated with DMSO (0.1% for 6 h) and EGCG (1 μmol/L, 10 μmol/L, 25 μmol/L, 50 μmol/L, and 100 μmol/L, all 6 h). EGCG was not toxic to the HAECs at a concentration equal to or lesser than 25 μmol/L. However, EGCG caused significant cytotoxicity at drug concentrations greater than 50 μmol/L. The data shown are the mean ± SEM of six independent experiments. $^{*}P < 0.001$ for EGCG (50 μmol/L and 100 μmol/L) compared with the control.

EGCG concentrations equal to or less than 25 μmol/L were not toxic to HAECs. Further experiments used the 25 μmol/L EGCG treatment.

EGCG downregulated TF protein expression

In the Western blotting analysis, EGCG (25 μmol/L for 6 h) downregulated the expression of TF protein in both thrombin (1 U/mL, 5 h) stimulated and thrombin (1 U/mL, 5 h)/paclitaxel (10^{-5} mol/L, 6 h) co-stimulated HAECs (Fig. 1B). EGCG alone did not increase TF expression.

EGCG downregulated TF activity

HAECs were treated with paclitaxel (10 μmol/L) and EGCG (25 μmol/L) for 6 h. Thrombin (1 U/mL) was added 1 h after paclitaxel treatment. The TF activity was determined by a colorimetric assay. Consistent with the above experiments, although thrombin alone caused a 2.6-fold increase in TF activity compared with the control level, EGCG treatment caused a 29% decrease in TF activity. Similarly, thrombin/paclitaxel treatment caused a 2.9-fold enhancement in TF activity, and EGCG treatment caused a 38% decrease in TF activity (Fig. 2A).

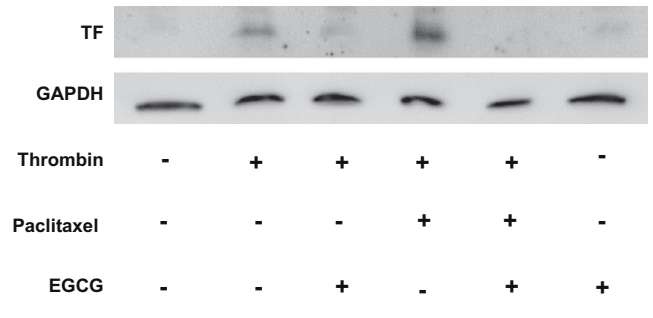

Fig. 1B. EGCG decreases TF protein expression. TF protein expression was determined by Western blot analysis. After serum starvation, HAECs were treated with thrombin (1 U/mL, 5 h) or thrombin (1 U/mL, 5 h)/paclitaxel (10 μmol/L, 6 h). Pretreatment with EGCG (25 μmol/L, 6 h) downregulated the expression of TF in both thrombin-stimulated and thrombin/paclitaxel-stimulated HAECs. The blot represents results from four independent experiments.

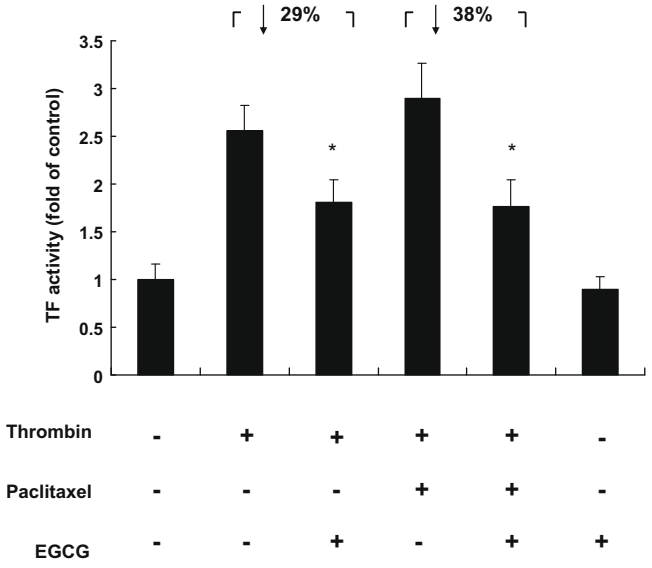

Fig. 2A. EGCG induces downregulation of TF activity. TF activity was determined by a colorimetric assay. After serum starvation, HAECs were treated with thrombin (1 U/mL, 5 h) or thrombin (1 U/mL, 5 h)/paclitaxel (10 μmol/L, 6 h). EGCG (25 μmol/L, 6 h) pretreatment downregulated the expression of TF activity in both thrombin-stimulated and thrombin/paclitaxel-stimulated HAECs. Although thrombin treatment caused a 2.6-fold increase in TF activity compared to the control, EGCG pretreatment caused a 29% decrease in TF activity. Similarly, thrombin/paclitaxel treatment caused a 2.9-fold increase in TF activity, and EGCG pretreatment caused a 38% decrease in TF activity. Bars represent the means ± SEM from seven experiments. $^{*}P < 0.05$ for thrombin compared with thrombin/EGCG and for thrombin/paclitaxel compared with thrombin/paclitaxel/EGCG.

EGCG downregulated TF mRNA expression

In the real-time PCR analysis, thrombin (1 U/mL for 3 h) caused a 3.0-fold increase in TF mRNA expression compared with the con-

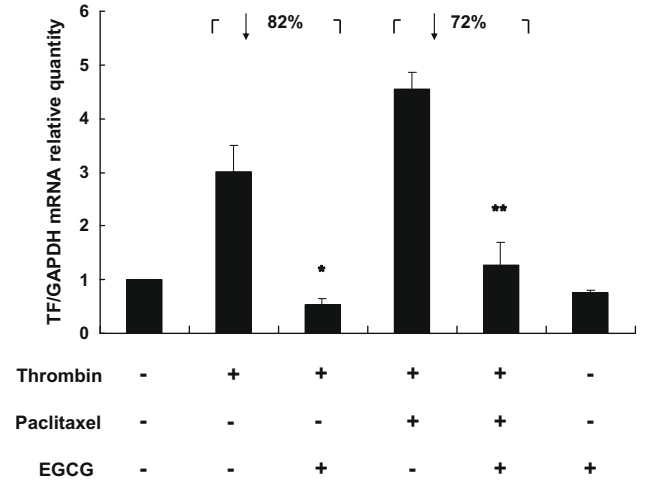

Fig. 2B. EGCG induces TF mRNA downregulation. The relative quantity of TF mRNA was determined by real-time PCR. After serum starvation, HAECs were treated with thrombin (1 U/mL, 3 h) or thrombin (1 U/mL, 3 h)/paclitaxel (10 μmol/L, 4 h). EGCG pretreatment (25 μmol/L, 4 h) downregulated the expression of TF mRNA in both thrombin-stimulated and thrombin/paclitaxel-stimulated HAECs. Although thrombin treatment induced a 3.0-fold increase in TF mRNA expression compared to the control, EGCG pretreatment caused an 82% decrease in TF mRNA expression. Similarly, thrombin/paclitaxel treatment induced a 4.6-fold increase in TF mRNA expression, and EGCG pretreatment caused a 72% decrease in TF mRNA expression (Fig. 2B). The bars represent the mean ± SEM from six experiments. $^{*}P < 0.005$ for thrombin compared with thrombin/EGCG; $^{**}P < 0.001$ for thrombin/paclitaxel compared with thrombin/paclitaxel/EGCG.

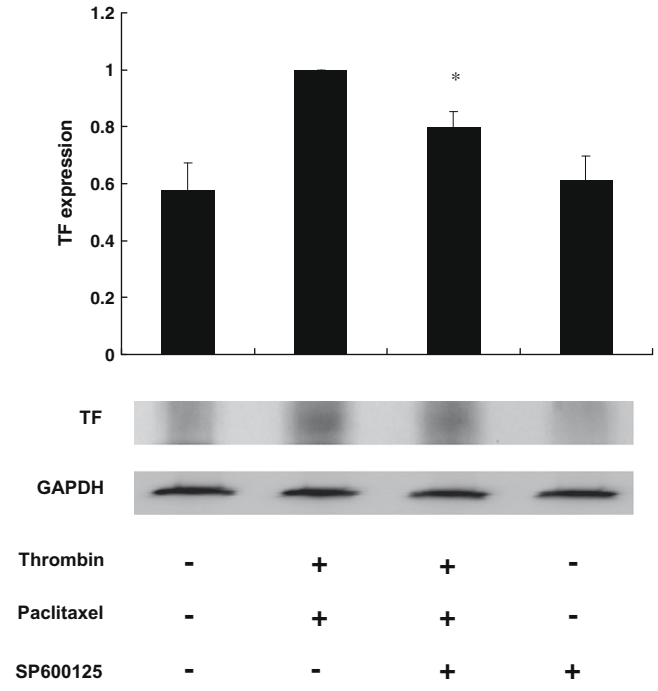

Fig. 3A. JNK inhibitor downregulated TF expression in thrombin/paclitaxel-stimulated HAECs. SP600125 (10 μmol/L, 6.5 h), a specific JNK inhibitor, was used to pretreat the HAECs before thrombin (1 U/ml, 5 h)/paclitaxel (10 μmol/L, 6 h) stimulation. SP600125 caused TF downregulation in thrombin/paclitaxel-stimulated HAEC. The bars represent the means ± SEM from four experiments. $^{*}P < 0.05$ for thrombin/paclitaxel compared with thrombin/paclitaxel/SP600125.

trol. EGCG pretreatment (25 μmol/L for 4 h) caused an 82% decrease in TF mRNA expression. Similarly, thrombin (1 U/mL for 3 h)/paclitaxel (10 μmol/L for 4 h) co-treatment caused a 4.6-fold increase in TF mRNA expression, and EGCG treatment (25 μmol/L for 4 h) caused a 72% decrease in TF mRNA expression (Fig. 2B).

JNK inhibitor downregulated TF expression in thrombin/paclitaxel-stimulated HAECs

To determine whether JNK activation is involved in thrombin/paclitaxel-induced TF expression, the effect of the JNK inhibitor was examined in HAECs. Pretreatment with JNK inhibitor SP600125 (10 μmol/L, 6.5 h) in paclitaxel (10 μmol/L, 6 h)/thrombin (1 U/mL, 5 h) stimulated HAECs caused TF downregulation (Fig. 3A).

EGCG inhibited JNK phosphorylation

To determine whether EGCG affects JNK activation, HAECs were examined at different time points after thrombin stimulation (1 U/mL). Paclitaxel (10 μmol/L) and EGCG (25 μmol/L) were added 1 h before thrombin stimulation. Maximal phosphorylation of JNK in thrombin/paclitaxel HAECs occurred at 60 min. EGCG potently inhibited phosphorylation of JNK to 49% of thrombin/paclitaxel-stimulated HAECs at 60 min (Fig. 3B).

EGCG did not inhibit P65 nuclear translocation in thrombin/paclitaxel-stimulated HEACs

Immunofluorescence assay showed that the P65 subunit of NF-κB was mainly localized within the cytoplasm of the DMSO-treated (0.1% for 2 h) HAECs. In the thrombin (1 U/mL for 1 h)/paclitaxel (10^{-5} μmol/L for 2 h) treated HAECs, the P65 subunit showed intense localization in the nuclei. EGCG treatment to the thrombin/paclitaxel-stimulated HAECs did not alter the P65 subunit of NF-κB nuclear translocation (Fig. 4).

Discussion

This study demonstrated that EGCG can downregulate thrombin and thrombin/paclitaxel-induced TF activity, protein expression, and mRNA levels in HAECs. The effect of EGCG on the inhibition of thrombin/paclitaxel-induced TF expression is medi-

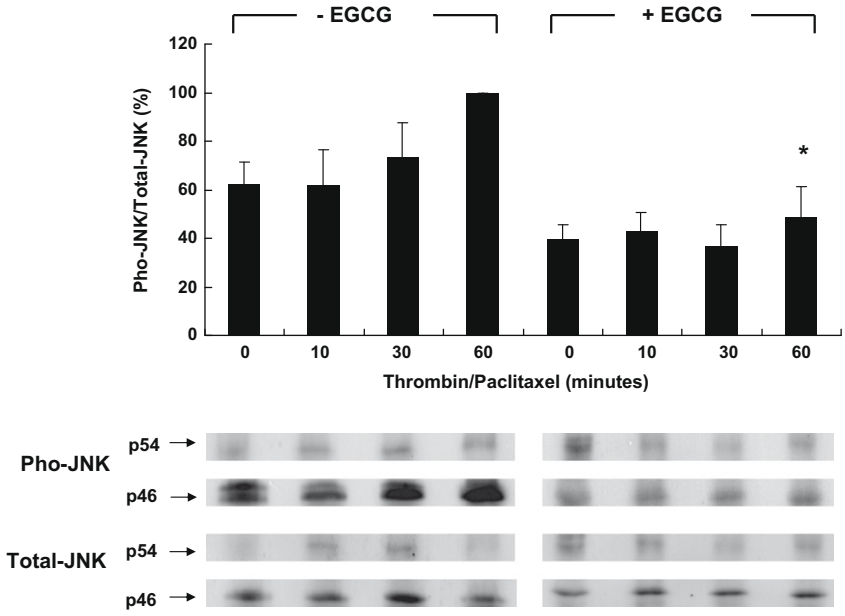

Fig. 3B. EGCG inhibits JNK phosphorylation. HAECs were examined at different time points (0 min, 10 min, 30 min, and 60 min) after thrombin stimulation (1 U/mL). Paclitaxel (10 μmol/L) and EGCG (25 μmol/L) were added 1 h before thrombin stimulation. Maximal phosphorylation of JNK occurred at 60 min after thrombin/paclitaxel treatment. Notably, EGCG pretreatment potently inhibited phosphorylation of JNK to 49% of the maximal JNK phosphorylation level at 60 min. All the blots were normalized to the blot for total JNK. The bars represent the means ± SEM from four experiments. $^{*}P < 0.05$ for thrombin/paclitaxel compared with thrombin/paclitaxel/EGCG.

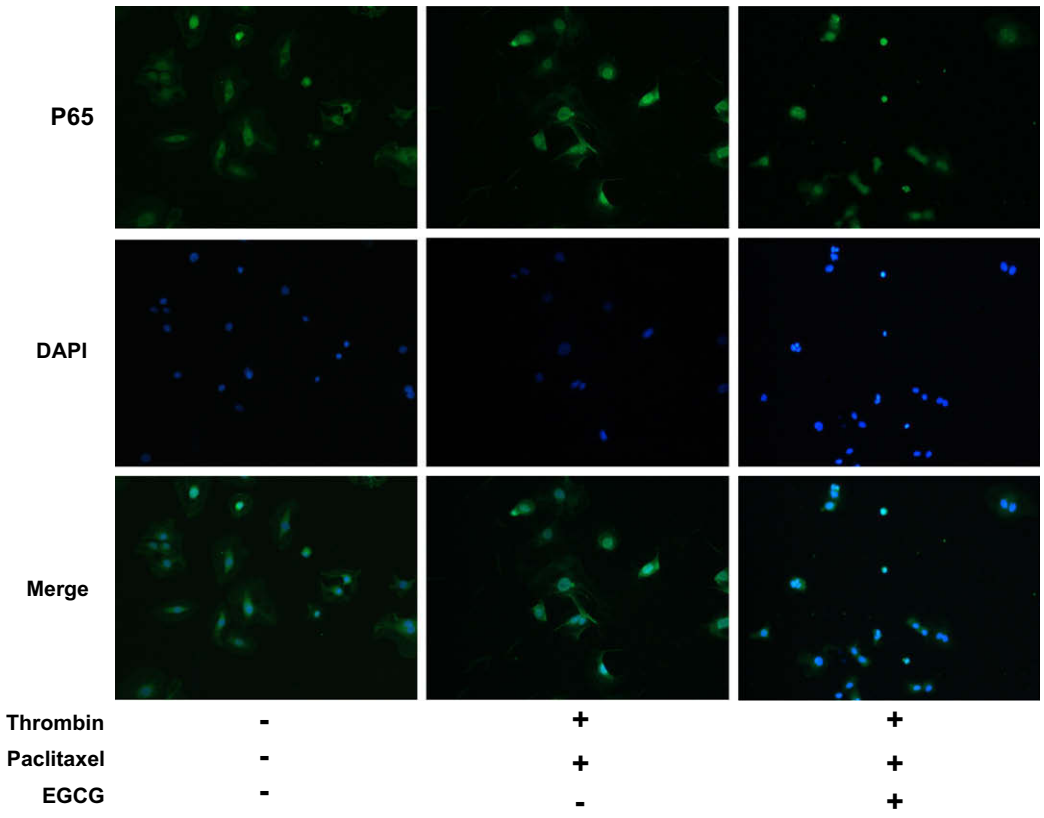

Fig. 4. EGCG does not cause nuclear translocation of the P65 subunit of NF-κB in thrombin/paclitaxel-stimulated HAECs. The nuclear translocation of P65 NF-κB was determined by an immunofluorescence assay. HAECs were treated with DMSO (0.1% for 2 h), thrombin (1 U/mL for 1 h)/paclitaxel (10 μmol/L for 2 h), and thrombin (1 U/mL for 1 h)/paclitaxel (10 μmol/L for 2 h)/EGCG (25 μmol/L for 2 h). In the thrombin/paclitaxel-treated HAECs, P65 NF-κB showed unbalanced localization in the nuclei. EGCG pretreatment did not inhibit the P65 subunit of NF-κB nuclear translocation in the thrombin/paclitaxel HAECs. The images represent results from three different experiments.

ated at the transcriptional level via inhibition of JNK phosphorylation. Our findings showed that EGCG can exert an antithrombotic effect in endothelial cells upon thrombin or thrombin/paclitaxel stimulation, and such properties may be used for further DES design.

Kang et al. [17] showed that EGCG could inhibit adenosine diphosphate- and collagen-induced rat platelet aggregation *ex vivo* and reduced mice death caused by pulmonary embolism thrombosis. However, the addition of EGCG to human platelet-poor plasma did not alter the coagulation parameters such as activated partial thromboplastin time, prothrombin time, and thrombin time. They concluded that EGCG exerted an antithrombotic effect via its antiplatelet activities. In contrast, Stampfuss et al. [18] showed that EGCG could exert anticoagulation activities via concentration-dependent inhibition of TF-mediated thrombin generation because of its galloyl group in the 3′ position. Our result provides additional evidence that EGCG can exert an anticoagulation effect by reducing TF induction upon thrombin or thrombin/paclitaxel stimulation.

The beneficial cardiovascular effect of green tea has been attributed to its polyphenolic flavonoids [16]. Several studies have shown that other polyphenolic constituents are able to reduce TF activity in stimulated endothelial cells. Most notably, Pendurthi et al. [21] and Pendurthi and Rao [22] reported that the resveratrol found in red wine and the quercetin found in some vegetables and fruits such as onions, apples, and berries are effective in reducing IL-1β stimulated TF activity in endothelial cells. However, not all phenolic components are equally effective. For example, catechin and epicatechin are more abundant phenolic components than resveratrol in red wine, but both compounds lack TF inhibitory ability [22]. Our study revealed that EGCG can inhibit TF expression in endothelial cells under two strong stimulation conditions (i.e., thrombin and thrombin/paclitaxel), which suggested that the antithrombotic effect of EGCG is similar to that of resveratrol or quercetin.

Recently, EGCG has been reported to act at multiple signaling cascades [13,23]. Choi et al. [24] reported that EGCG can diminish oxidized LDL-induced JNK phosphorylation and apoptosis in human umbilical vein endothelial cells. In a mouse epidermal cell line, JNK activity was also inhibited by EGCG [25]. TF gene expression in the endothelial cells is dependent on two binding sites, i.e., the activator protein 1 (AP1) binding site and a nonconsensus NF-κB-like binding site; their transcription factors are Fos/Jun and c-Rel/P65, respectively [26]. Activation of AP1 activity occurs partly through mitogen- and cellular stress-induced phosphorylation of JNKs. The activated JNKs then translocate into the nucleus, where they phosphorylate Jun at residues Ser 63 and Ser 73, and thereby, enhance its transactivation potential [27]. In our study, we demonstrated that EGCG can downregulate the phosphorylation of JNK induced by thrombin/paclitaxel and inhibit TF expression.

The inhibitory activity of EGCG on NF-κB signaling pathways are well documented in different cell lines [28,29]. Notably, EGCG was reported to inhibit the NF-κB P65 subunit nuclear translocation in human bronchial epithelial cells stimulated by cigarette smoke condensate and HEK293 stimulated by TNF-α [29,30]. Our data have shown that paclitaxel/thrombin can cause a predominant NF-κB P65 translocation into the nuclei in thrombin/paclitaxel-stimulated HAECs, which could explain why the TF expression was induced in the thrombin/paclitaxel-treated HAECs [10,11]. However, we did not observe the inhibitory effect of EGCG

on the nuclear translocation of the NF-κB P65 subunit in the HAECs. Interestingly, EGCG has also failed to alter the NF-κB P65 binding activity in the TNF-α stimulated endothelial cells [31].

Conclusion

In summary, this study demonstrates that EGCG can inhibit endothelial TF expression upon thrombin/paclitaxel stimulation by reducing the phosphorylation of JNK. This finding provides an insight into developing a co-coating stent with an anti-inflammatory agent (i.e., EGCG) and cytotoxic agent (i.e., paclitaxel). Further efforts to develop a safe DES should consider the influence of different eluting drugs on the thrombogenicity of the underlying endothelium.

Acknowledgments

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