Complex effects of different green tea catechins on human platelets

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Abstract Epigallocatechin gallate (EGCG), a major component of green tea, has been previously shown to inhibit platelet aggregation. The effects of other green tea catechins on platelet function are not known. Pre-incubation with EGCG concentration-dependently inhibited thrombin-induced aggregation and phosphorylation of p38 mitogen-activated protein kinase and extracellular signal-regulated kinases-1/2. In contrast EGCG stimulated tyrosine phosphorylation of platelet proteins, including Syk and SLP-76 but inhibited phosphorylation of focal adhesion kinase. Other catechins did not inhibit platelet aggregation. Interestingly, when EGCG was added to stirred platelets, a tyrosine kinase-dependent stimulation of platelet aggregation was observed. The two other catechins containing a galloyl group in the 3′ position (catechin gallate, epicatechin gallate) also stimulated platelet aggregation, while catechins without a galloyl group (catechin, epicatechin) or the catechin with a galloyl group in the 2′ position (epigallocatechin) did not.

Key words: Green tea; Catechin; Epigallocatechin gallate; Platelet; Aggregation

1. Introduction

Flavonoids are polyphenolic compounds of plant origin [1]. Catechins (catechin, C; epicatechin, EC; epigallocatechin, EGC; catechin gallate, CG; epicatechin gallate, ECG; epigallocatechin gallate, EGCG) are the main flavonoid components of green tea [2]. Several epidemiological studies have suggested that regular intake of flavonoids reduces the risk of coronary artery disease [3,4]. These beneficial effects can be partially explained by the antioxidative actions of the compounds [5–10]. Flavonoids have also been shown to reduce elevated blood pressure [11], to stimulate endothelium-dependent vasodilatation [12], and to inhibit tyrosine phosphorylation of the platelet-derived growth factor (PDGF) β receptor, resulting in inhibition of vascular smooth muscle cell proliferation [13–15]. In addition, inhibition of platelet function by flavonoids may contribute to their anti-atherosclerotic effects [16]. For example, EGCG has been shown to inhibit platelet aggregation [17,18]. These effects were explained by an inhibition of cytoplasmic calcium increase [18]. However, high (millimolar) concentrations of EGCG were used and no systematic investigation of the effects of various green tea catechins on platelet function is available.

The present study provides evidence for complex (inhibitory and stimulatory) effects of catechins on platelet function. A marked inhibition of thrombin-induced platelet aggregation was only observed with EGCG and may involve an inhibition of platelet activation. Interestingly, proaggregatory activating effects of catechins containing a galloyl group in the 3′ position were also observed that may involve a stimulation of tyrosine phosphorylation.

2. Materials and methods

2.1. Preparation of washed platelets and platelet aggregation

Platelet-rich plasma was prepared from buffy coats by centrifugation at 250×*g* and room temperature for 10 min. Platelets were washed as previously described [20] and were resuspended in HEPES-buffered Tyrode buffer (pH 7.4) containing 2 mmol/l Ca^{2+}. Catechins were pre-incubated for 30 min at 37°C if not otherwise indicated. Platelet aggregation was measured turbidimetrically as previously described [21]. Aggregation values are given as the maximum change in light transmission (100% was the difference between washed platelets and buffer).

2.2. Flow cytometry

Expression of CD62P, CD63 and CD40L was measured by flow cytometry as previously described [22].

2.3. Immunoprecipitation and Western blotting

For immunoprecipitation, platelets were lysed in 2× RIPA buffer (1×: 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.4% deoxycholate, 1% Nonidet P-40, pH 8.0) supplemented with protease inhibitors (mammalian protease inhibitor cocktail, Sigma, Deisenhofen, Germany, 1% phenylmethylsulfonyl fluoride of a saturated solution in ethanol). After a pre-clear with 50 μl of a 50/50 (v/v) slurry of protein A agarose (Amersham Pharmacia Biotech, Freiburg, Germany) in phosphate-buffered saline (PBS) for 2 h at 4°C, proteins were incubated with antibodies (5 μg) on a rotating device overnight at 4°C and precipitated with 50 μl of a 50/50 (v/v) slurry of protein A agarose in PBS on a rotating device for 2 h at 4°C. For Western blotting, platelets were lysed in 2× lysis buffer (1×: 62.5 mmol/l Tris–HCl, pH 6.8, 10% glycerol, 2% w/v sodium dodecyl sulfate (SDS), 0.1% w/v bromophenol blue). Proteins were resolved by SDS polyacrylamide (10%) gel electrophoresis, blotted and probed with antibodies as previously described [23]. Densitometric analysis was performed using a calibrated scanner densitometer (GS-800, Bio-Rad, Munich, Germany).

2.4. Platelet membrane fluidity measurements and cytotoxicity assays

The effects of EGCG (100 μmol/l) on platelet membrane fluidity were determined by measuring fluorescence anisotropy of the lipid soluble fluorophore diphenylhexatriene (DPH) using a variable temperature fluorescence polarization system (Beacon 2000, Panvera, Madison, MI, USA) as previously described [20]. The effects of EGCG (100 μmol/l) on the release of pre-loaded (30 min, 37°C)

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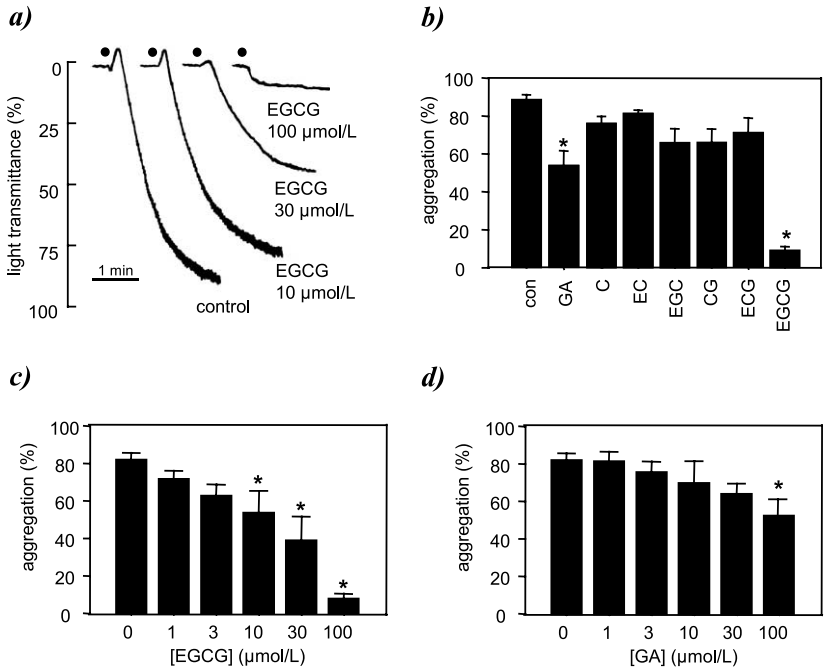

Fig. 1. Effects of various green tea catechins on thrombin (0.1 U/ml)-induced aggregation of washed human platelets. a: Original tracings, demonstrating the concentration-dependent inhibition of platelet aggregation and shape change by EGCG. b: Effects of various catechins (100 µmol/l) on thrombin-induced platelet aggregation (means ± S.E.M., $n = 5$, $^*P < 0.05$ versus control). c: Concentration-dependent inhibition of platelet aggregation by EGCG (means ± S.E.M., $n = 5$, $^*P < 0.05$ versus control). d: Concentration-dependent inhibition of platelet aggregation by GA (means ± S.E.M., $n = 5$, $^*P < 0.05$ versus control).

calcein-AM (3 µM) from platelets was measured by flow cytometry [22].

2.5. Materials

Phospho-specific antibodies against p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases (ERK)-1 and -2 were from Cell Signaling Technology, Beverly, MA, USA. Antibodies against phosphotyrosine (PY99), Syk (LR), LSP-76 (H-300), and phospho-focal adhesion kinase (FAK) (sc-11765-R) were from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Genistein, herbimycin A, and PP2 were from Calbiochem-Novabiochem, San Diego, CA, USA. α-Thrombin was a gift from Dr. J. Stürzebecher (Erfurt, Germany). All other reagents were from Sigma, Deisenhofen, Germany.

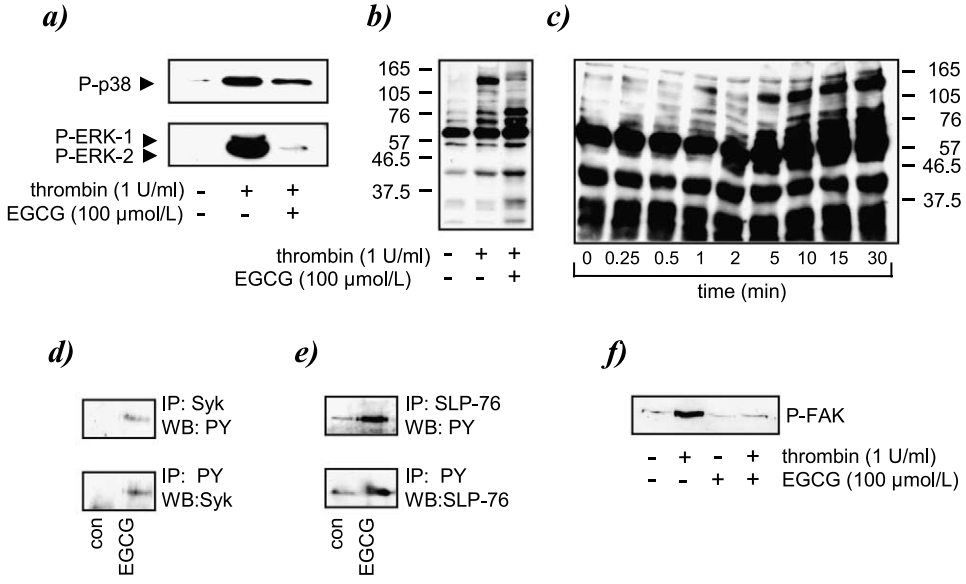

Fig. 2. Effects of EGCG (100 µmol/l) on platelet signaling. a: Inhibition of thrombin (1 U/ml, 5 min)-induced phosphorylation of p38 MAPK and ERK-1 and -2. b: Effects of EGCG on thrombin (1 U/ml, 5 min)-induced tyrosine phosphorylation of platelet proteins. c: Time course of EGCG (100 µmol/l)-induced tyrosine phosphorylation. d: Effects of EGCG (100 µmol/l, 30 min) on tyrosine phosphorylation of Syk (IP, immunoprecipitation; WB, Western blot). e: Effects of EGCG (100 µmol/l) on tyrosine phosphorylation of SLP-76 (IP, immunoprecipitation; WB, Western blot). f: Effects of EGCG (100 µmol/l) on (1 U/ml, 5 min) tyrosine phosphorylation of FAK. The blots shown are representative of $n = 3$–4 experiments.

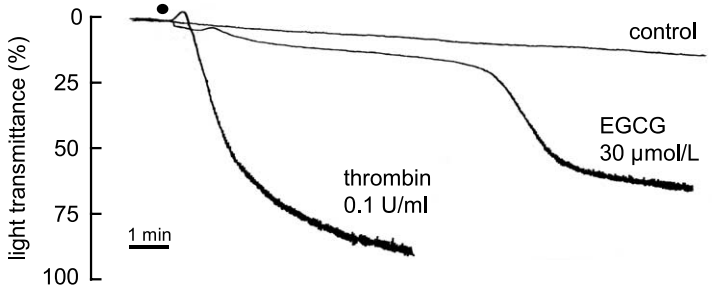

Fig. 3. Original tracings demonstrating the proaggregatory effects of EGCG (30 µmol/l) as compared to thrombin (0.1 U/ml).

2.6. Statistics

Data are mean ± S.E.M. of n independent experiments. Statistical analysis was performed by one-way analysis of variance followed by Bonferroni Multiple Comparisons test using GraphPad InStat version 3.01 for Windows 95 (GraphPad Software, San Diego, CA, USA). P levels of < 0.05 were considered significant.

3. Results

3.1. Inhibitory effects of green tea catechins on thrombin-induced platelet aggregation

EGCG concentration-dependently inhibited thrombin (0.1 U/ml)-induced platelet aggregation and shape change (Fig. 1a). A complete inhibition was seen at 100 µmol/l EGCG (Fig. 1c). These inhibitory effects were time-dependent. Maximum inhibition was observed after 30 min of incubation time (37°C, unstirred conditions) (data not shown). EGCG also concentration-dependently inhibited thrombin-induced externalization of CD62P, CD63 and CD40L as measured by flow cytometry (data not shown). Other green tea catechins (C, EC, EGC, CG, ECG, 100 µmol/l) did not inhibit platelet aggregation (Fig. 1b). Some inhibition of thrombin-induced platelet aggregation was observed with gallic acid (100 µmol/l) (Fig. 1b,d).

3.2. Effects of EGCG on platelet signaling

EGCG (100 µmol/l) largely inhibited thrombin (1 U/ml)-induced phosphorylation of p38 MAPK and ERK-1 and -2 (Fig. 2a). In contrast, EGCG had complex effects on thrombin (1 U/ml)-induced tyrosine phosphorylation of platelet proteins. Thrombin-induced tyrosine phosphorylation of some proteins (e.g. p115) was inhibited by EGCG. Surprisingly, thrombin-induced tyrosine phosphorylation of other proteins (e.g. p70, p76) was even higher after pre-incubation with EGCG (Fig. 2b). Thus, the hypothesis was tested that EGCG might directly stimulate tyrosine phosphorylation in platelets. In fact, a stimulation of tyrosine phosphorylation by EGCG was observed (Fig. 2c). These effects consistently occurred with 30 µmol/l EGCG. In some experiments, however, EGCG concentrations as low as 3 µmol/l were effective. In contrast to thrombin, which very rapidly stimulates tyrosine phosphorylation, the effects of EGCG were slow with maximum phosphorylation at 10 min incubation time. Using immunoprecipitation, the tyrosine kinase Syk and the adapter protein SLP-76 were identified to be tyrosine-phosphorylated upon stimulation with EGCG (Fig. 2d,e). Using phospho-specific antibodies, phosphorylation of FAK was shown to be inhibited by EGCG in thrombin-stimulated platelets (Fig. 2f).

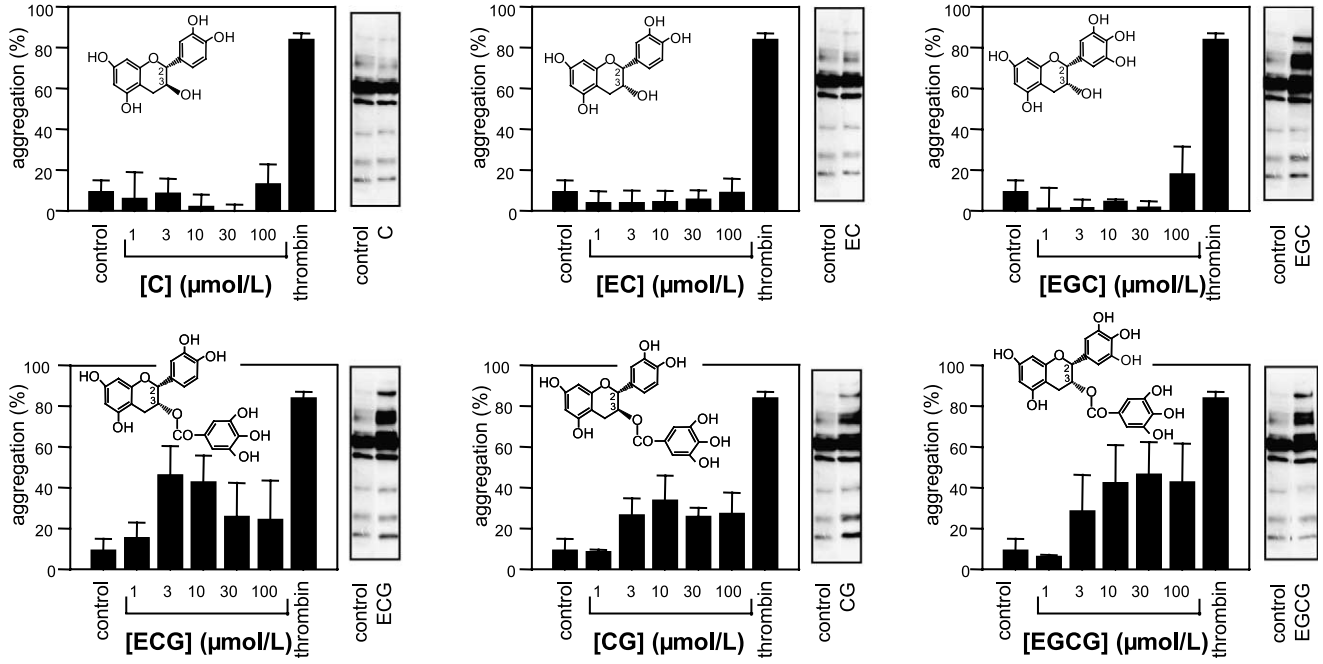

Fig. 4. Direct effects of various green tea catechins (100 µM) on platelet aggregation (left panels, means ± S.E.M., $n = 5$) and tyrosine phosphorylation (right panels, representative blots of $n = 5$ experiments). Tyrosine phosphorylation was measured 15 min after addition of the catechins.

3.3. Stimulatory effects of green tea catechins on platelet function

In initial experiments, where platelet aggregation was recorded for 4 min, no direct effects of green tea catechins on platelet aggregation were seen (data not shown). However, because of the slow effects of EGCG on tyrosine phosphorylation, additional aggregation experiments were carried out with longer recording times. When EGCG was added to stirred platelets, a stimulation of platelet aggregation was consistently observed after about 10 min (Fig. 3). Although the amplitude of the aggregation response varied, a clear aggregation wave occurred in each single experiment. These activating effects were observed with ≥ 3 μmol/l EGCG without a clear concentration-dependency (Fig. 4). Interestingly, the two other catechins containing a galloyl group in the 3′ position (CG, ECG) also stimulated platelet aggregation, while catechins without a galloyl group (C, EC) or the catechin with a galloyl group in the 2′ position (EGC) did not. Gallic acid was not effective either (data not shown). In accordance with these findings, the three catechins that stimulated platelet aggregation (ECG, CG, EGCG) also stimulated tyrosine phosphorylation of platelet proteins (Fig. 4). EGC did stimulate tyrosine phosphorylation but did not induce platelet aggregation. Nevertheless, we hypothesized that tyrosine phosphorylation might be involved in the proaggregatory effects. In fact, the src family protein tyrosine kinase inhibitors genistein (10 μmol/l), herbimycin A (3 μmol/l), and PP2 (10 μmol/l) inhibited EGCG (30 μmol/l)- but not thrombin (0.1 U/ml)-induced platelet aggregation (Fig. 5).

3.4. Possible non-specific effects of EGCG

EGCG (100 μmol/l) did not change platelet membrane fluidity as measured by DPH fluorescence anisotropy. The measurement of cytotoxicity by the MTT assay, as proposed by Kang et al. [18], was not possible because EGCG resulted in a strong color reaction even in the absence of cells. The same applied to the XTT assay. However, EGCG did not affect the leakage of pre-loaded calcein, indicating the absence of cytotoxic effects. More importantly, the inhibitory effects of EGCG were completely reversible when EGCG-treated platelets were pelleted and resuspended in EGCG-free buffer. The data are summarized in Fig. 6.

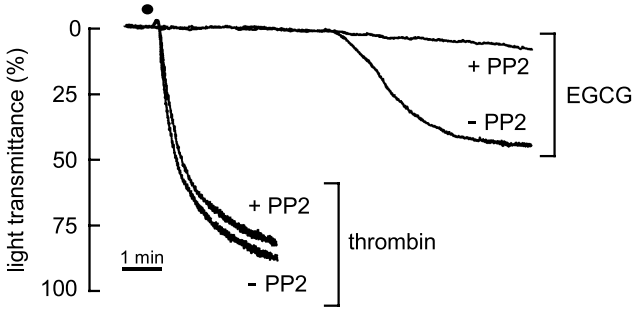

Fig. 5. Original tracings, demonstrating the effects of the Src family tyrosine kinase inhibitor PP2 (10 μM) on thrombin (1 U/ml)- and EGCG (100 μmol/l)-induced platelet aggregation. Similar findings were observed with other tyrosine kinase inhibitors, including herbimycin A (3 μmol/l) or genistein (10 μmol/l).

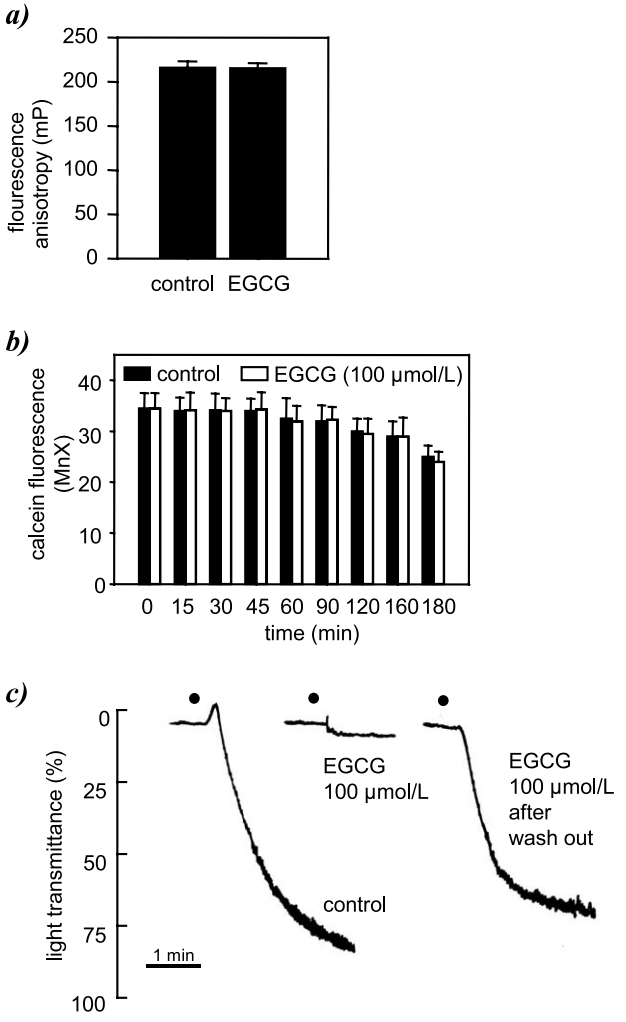

Fig. 6. Possible non-specific effects of EGCG. a: Effects of EGCG (100 μmol/l) on platelet membrane fluidity as determined by measuring fluorescence anisotropy (mP) of the lipid soluble fluorophore DPH (means ± S.E.M., $n = 3$). b: Effects of EGCG (100 μmol/l) on the release of pre-loaded (30 min, 37°C) calcein-AM (3 μM) from platelets (means ± S.E.M., $n = 3$). c: Original tracings, demonstrating the reversibility of the inhibitory effects of EGCG (100 μmol/l) on thrombin (0.1 U/ml)-induced platelet aggregation.

4. Discussion

The present study provides evidence for complex (inhibitory and stimulatory) effects of catechins on platelet function. Platelet inhibitory effects of EGCG have already been described [17,18]. These effects were explained by an inhibition of cytoplasmic calcium increase [19]. In our study, ECGC was shown to interrupt other signaling transduction pathways such as phosphorylation of p38 MAPK or of ERK-1/2. Interestingly, none of the other major green tea catechins inhibited platelet function and only small inhibitory effects were observed with high concentrations of gallic acid. Thus, the present study is the first to demonstrate that only EGCG appears to represent the active principle of platelet inhibitory effects of green tea catechins.

The effects of EGCG were time-dependent with maximum inhibition after 30 min of incubation time. This may explain why in previous studies much higher concentrations of EGCG were required to achieve an inhibitory effect. The time-depen-

dency of the anti-aggregatory effects of EGCG might be explained by a time-dependent incorporation of the compound into the platelet. Thus, one might speculate that other catechins, such as CG or ECG, might also exhibit some inhibitory effects upon prolonged incubation. However, it was not possible to study this hypothesis because washed platelets have only a limited viability. Importantly, the inhibitory effects of EGCG were not due to cytotoxicity because a full reversibility of the aggregation response was observed upon removal of the compound. In addition, no non-specific effects (e.g. alteration of membrane fluidity) of EGCG were observed.

Some catechins from green tea have recently been shown to inhibit tyrosine phosphorylation in vascular smooth muscle cells [15]. Very similar findings have recently been reported for catechins from red wine [24]. Thus, the hypothesis was tested that this mechanism may explain the anti-aggregatory effects of EGCG. However, EGCG had complex effects on thrombin-induced tyrosine phosphorylation of platelet proteins. Thrombin-induced tyrosine phosphorylation of some proteins (e.g. p115) was inhibited by EGCG. Using phospho-specific antibodies, tyrosine phosphorylation of FAK was demonstrated to be inhibited by EGCG. Since FAK is involved in outside-in signaling [25], the inhibition of FAK phosphorylation may contribute to the anti-aggregatory effects of this flavonoid.

Interestingly, thrombin-induced tyrosine phosphorylation of some other proteins (e.g. p70, p76) was potentiated by the compound. Therefore, possible direct stimulation of tyrosine phosphorylation by EGCG was studied. These experiments clearly demonstrated that EGCG is able to directly stimulate tyrosine phosphorylation of several proteins. Using immunoprecipitation, the tyrosine kinase Syk and the adapter protein SLP-76 were identified to be tyrosine-phosphorylated upon stimulation with EGCG. Activation of Syk is necessary for tyrosine phosphorylation of the adapter protein SLP-76. This pathway is crucial for the activation of phospholipase Cγ2, which regulates $[Ca^{2+}]_i$ elevation and protein kinase C activation [26–28].

The demonstration of stimulation of tyrosine phosphorylation of platelet proteins involved in platelet aggregation by EGCG was a striking and unexpected finding. Interestingly, the stimulation of tyrosine phosphorylation by EGCG was much slower as compared to other platelet stimuli, such as thrombin. Therefore, in initial experiments, where platelet aggregation was recorded for 4 min, no direct effects of green tea catechins on platelet aggregation were seen. However, when the aggregation experiments were carried out with longer recording times, EGCG consistently stimulated platelet aggregation after about 10 min. Interestingly, the two other catechins containing a galloyl group in the 3′ position (CG, ECG) also stimulated platelet aggregation, while catechins without a galloyl group (C, EC) or the catechin with a galloyl group in the 2′ position (EGC) did not. Thus, although a different effect was measured in our study, the same structure–activity relationship was observed as described for the inhibition of the PDGF receptor phosphorylation by green tea catechins [15].

Interestingly, the three catechins that stimulated platelet aggregation (ECG, CG, EGCG) also stimulated tyrosine phosphorylation of platelet proteins. However, EGC did also stimulate tyrosine phosphorylation but did not induce platelet aggregation. Nevertheless, we hypothesized that tyrosine phosphorylation might be involved in the proaggregatory effects of catechins. In fact, tyrosine kinase inhibitors inhibited EGCG- but not thrombin-induced platelet aggregation. It is difficult to explain why the observed stimulation of tyrosine phosphorylation by EGC does not translate to platelet aggregation. One might speculate about additional inhibitory effects of EGC that may interfere with possible stimulatory actions of the compound. In this context, it is noteworthy that EGC was also active in inhibiting PDGF-induced smooth muscle cell proliferation but did not inhibit PDGF receptor phosphorylation [15], further supporting the view that additional actions of EGC actions might have prevented platelet aggregation despite stimulation of tyrosine phosphorylation. Similarly, the dual action of EGCG is difficult to understand. When incubated with unstirred platelets for 30 min, this catechin inhibits thrombin-induced platelet signaling and aggregation but exhibits stimulatory effects when it is added to platelets under stirring conditions. EGCG differs from other catechins in that this compound contains two galloyl groups. Because high concentrations of gallic acid also inhibited platelet aggregation to some extent, this structure may be involved in the inhibitory effects of EGCG.

Taken together, all catechins containing a galloyl group (EGC, ECG, CG, EGCG) did stimulate tyrosine phosphorylation of platelet proteins but only catechins containing the galloyl group in the 3′ position (ECG, CG, EGCG) stimulated platelet aggregation. EGCG, a catechin containing two galloyl groups, inhibited platelet function.

In summary, the present study demonstrated that green tea catechins exhibit a variety of actions on human platelets. Many mechanisms may contribute to the beneficial effects of catechins in atherosclerosis. The data presented in this study do not exclude the possibility that inhibition of platelet function is one of these mechanisms. However, the demonstration of stimulatory effects on platelet aggregation and tyrosine phosphorylation raises some concerns. Thus, further studies are clearly needed before green tea or isolated catechins from green tea can be generally recommended for prevention and/or therapy of atherosclerosis.

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