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Molecular insights into the differences in anti-inflammatory activities of green tea catechins on IL-1 β signaling in rheumatoid arthritis synovial fibroblasts

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Abstract

In this study, we found that catechins found in green tea (EGCG, EGC, and EC) differentially interfere with the IL-1 β signaling pathway which regulates the expression of pro-inflammatory mediators (IL-6 and IL-8) and Cox-2 in primary human rheumatoid arthritis synovial fibroblasts (RASFs). EGCG and EGC inhibited IL-6, IL-8, and MMP-2 production and selectively inhibited Cox-2 expression. EC did not exhibit any inhibitory effects. When we looked at the expression of key signaling proteins in the IL-1 β signaling pathway, we found all the tested catechins could inhibit TAK-1 activity. Therefore, the consumption of green tea offers an overall anti-inflammatory effect. Molecular docking analysis confirms that EGCG, EGC, and EC all occupy the active site of the TAK1 kinase domain. However, EGCG occupies the majority of the TAK1 active site. In addition to TAK1 inhibition, EGCG can also inhibit P38 and nuclear NF- κ B expression whereas EC and EGC were not effective inhibitors. Our findings suggest one of the main health benefits associated with the consumption of green tea are due to the activity of EGCG and EGC which are both present at higher amounts. Although EGCG is the most effective catechin at inhibiting downstream inflammatory signaling, its effectiveness could be hindered by the presence of EC. Therefore, varying EC content in green tea may reduce the anti-inflammatory effects of other potential catechins in green tea.

Keywords

Green tea; Catechins; Inflammation; Rheumatoid arthritis; EGCG; EGC

1. Introduction

There are over 80 known autoimmune diseases where the immune system fails to recognize itself resulting in chronic inflammation and tissue damage. In many cases of auto immunity,

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Conflicts of interest

No conflict of interest related to this work.

there are not any cures for them. In addition to no available cures, the population of people living with an autoimmune disease has been steadily increasing worldwide (Lerner et al., 2015). One such autoimmune disease is rheumatoid arthritis (RA), which is characterized by synovial inflammation and tissue destruction in the articular joints. In RA, activated synovial fibroblasts (RASFs) cause the joint synovium lining to thicken due to increased rate of proliferation. Infiltrating monocytes and macrophages produce pro-inflammatory cytokine interleukin-1 β (IL-1 β) that activates synovial fibroblasts to produce inflammatory mediators such as IL-6 and IL-8 (Ahmed et al., 2008). In addition to producing inflammatory mediators, RASFs become resistant to apoptosis resulting in synovial hyperplasia (Flannery and Bowie, 2010; Boraschi and Tagliabue, 2013). High levels of IL-6 drives systemic and synovial inflammation and as a result RASFs release matrix-degrading enzymes such as MMP-2 to degrade adjacent joint cartilage and bone leading to an overall joint destruction.

Treatments that alleviate symptoms target the inflammatory aspect of RA. Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, ketoprofen, and naproxen sodium are typically prescribed to reduce prostaglandin production (Crofford, 2013). Although these treatments are effective at relieving the symptoms of RA, they have reported to have some side effects such as gastrointestinal and cardiovascular toxicities (Marsico et al., 2016). Thus, many patients seek alternative therapies to complement their prescribed medication. For example, the addition of dietary supplements such as fish oil (omega-3 fatty acids), ginger, and green tea have shown to improve pathological condition of RA (James et al., 2010; Ribel-Madsen et al., 2012; Ahmed, 2010).

Green tea (*Camellia sinensis*) is one of the most commonly consumed beverages worldwide. The active compounds in green tea are catechins, which are phytochemical compounds classified as flavanols/flavonoids. The most abundant catechin in green tea is epigallocatechin-3-gallate (EGCG) which makes up to 59% of green tea catechins in dry weight (Singh et al., 2010). Green tea also contains considerable amounts of epicatechin (EC) and epigallocatechin (EGC) which makes up to 6.4% and 19% of total catechins in green tea respectively (Singh et al., 2010). Many of the health benefits associated with the consumption of green tea are attributed to EGCG. These health benefits include antioxidant, anti-diabetic, neuroprotective, and anti-cancer effects (Chowdhury et al., 2016). Because green tea is usually taken orally, the bioavailability of EGCG is taken into account when considering its effects in vivo. A study evaluating the plasma levels of catechins in Sprague-Dawley rats showed their levels peaked between 1.1 and 1.8 h after administration indicating fast absorption. When the C_{max} and AUC are considered, they found EGC was present in plasma in the highest amount followed by EC then EGCG. Although EGCG was found in the lowest amount, the half-life of EGCG was the longest being in the range of 5.9–10 h whereas EGC and EC's half-life was 2.7–4.8 h suggesting higher EGCG bioavailability (Huo et al., 2016).

Earlier we have shown EGCG has anti-inflammatory properties by abrogating IL-6 and IL-8 production in RASFs (Ahmed et al., 2006; Ahmed et al., 2008). We showed the mechanism of EGCG inhibition is by binding and inhibiting the active site of an upstream signaling protein kinase TGF- β activated MAP kinase (TAK1) (Singh et al., 2016). Until now, TAK1 inhibition has been accomplished with small molecule 5Z-7-oxozeaenol (Wu et

al., 2012; Ninomiya-Tsuji et al., 2003). One major disadvantage of 5Z-7-oxozeanol as a therapeutic is that it irreversibly inhibits TAK1. Because TAK1 is important in several signaling pathways, irreversible inhibition would be result in major toxicity in patients. Although EGCG binds to TAK1 in the same region as 5Z-7-oxozeanol, EGCG only forms hydrogen bonds making TAK1 inhibition a reversible process thereby being potentially less toxic and more therapeutic in its action. Since green tea is rich in EGCG, it may be the most effective catechin in reducing inflammation caused by RA. However, there is very little information on the properties of other green tea catechins like EGC and EC and if they provide additive anti-inflammatory effects in RASFs. Therefore, we tested EGCG, EGC, and EC alone and in combination to study the impact on anti-inflammatory outcome in human RASFs and the underlying molecular mechanisms.

2. Materials and methods

2.1. Antibodies and reagents

EGCG (95% purity HPLC), EGC (95% HPLC), and EC (90% HPLC) compounds were purchased from Sigma (St. Louis, MO; cat# E4143, E3768, E7153). Gelatin from bovine skin was purchased from Sigma (G6650). Cyclooxygenase (Cox)-1 and Cox-2 antibodies were purchased from Cayman Chemical (Ann Arbor, MI; cat# aa160110 and aa 570–598). P-P38, p-JNK, and p-ERK, p-TAK1 (Thr184/187), p-cJun(Ser73), and p65-NF- κ B were purchased from Cell Signaling Technologies (Danvers, MA; Cat# 4511, 9251,4695,4508,3270, and 3033). β -Actin and Lamin A/C loading controls were purchased from Santa Cruz (Santa Cruz, CA; sc-47,778; sc-6215).

2.2. Culturing of human RASFs

De-identified human RA synovium tissues were obtained from Co-operative Human Tissue Network (CTHN; Columbus, OH) and National Disease Research Interchange (NDR1; Philadelphia, PA). RA tissue received were taken from the donor's knee or hip during total joint replacement surgery or synovectomy according to an Institutional Review Board (IRB) approved protocol in compliance with the Helsinki Declaration. Donor population contained both Caucasian males and females diagnosed with rheumatoid arthritis. Disease RA tissue was digested in Dipase, collagenase, and DNAase before being seeded in 72 cm² flasks. Cells were grown in RPM11640 medium supplemented with 10% fetal bovine serum (FBS), 5000 U/ml penicillin, 5 mg/ml streptomycin, and 10 μ g/ml gentamicin. Upon confluency (>85%) cells were passaged with brief trypsinization. Experiments were done using cells that were passed at least 4 to 5 times to ensure pure fibroblast population. For experimental purpose, RASFs between passages 5–10 were used. All treatments were done in serum free media. The experiments were performed on three cell lines established from different RA donors for this study.

2.3. Preparation of EGCG, EGC, and EC solution

Stock solutions were prepared as described previously (Ahmed et al., 2006).

2.4. Treatment of RASFs

RASFs were seeded in 6-well plates and grown to > 85% confluency. Cells were pretreated with 5–20 μ M of EC, EGC, or EGCG in serum free media overnight prior to IL-1 β (10 ng/ml) stimulation. Stimulation duration was for 30 min for signaling studies or 24 h to evaluate the production of IL-6 and IL-8, the expression of Cox-1, and Cox-2. The activation of MMP-2 was evaluated with gelatin zymography.

2.5 MTT viability assay

RASFs were seeded in 96-well format and grown to > 85% confluency. Cells were serum-starved before being treated with various concentrations of EGCG, EC, or EGC. Catechins were serially diluted in serum free media (RPM1) from 2.5–20 μ M. 2 h prior to termination, 10 μ l of MTT dye (5 mg/ml) was added to each well. At the time of termination, the conditioned media was aspirated and cells were washed with PBS before DMSO was added to solubilize MTT dye and absorbance was read at 570 nm.

2.6. Assay for IL-6 and IL-8 production

The conditioned media was collected from 24 h IL-1 β stimulated samples with or without catechins, spun down at 10,000 rpm for 10 min at 4 °C to remove particulate matter, and collected in fresh Eppendorf tubes. The collected supernatants were analyzed for human IL-6 (DY206) and IL-8 (DY208) levels using colorimetric sandwich ELISA kits (R&D Systems, Minneapolis, MN) as per manufacturer's instructions.

2.7. Gelatin zymography

MMP-2 activity was determined using gelatin zymography as previously described (Ahmed et al., 2006). In summary, 20 μ l of supernatant was added to 20 μ l of 2 $\times$ non-reducing sample buffer and resolved under non-reducing conditions on 7.5% SDS-polyacrylamide gels polymerized with 1 mg/ml gelatin (type B from bovine skin; Sigma) as a substrate and electrophoresed at 120 V. Following electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 min with gentle shaking, then gels were washed for 30 min in developing buffer (50 mM Tris HCl [pH 8.0], 5 mM CaCl₂, and 0.02% NaN₃). Gels were incubated overnight in fresh developing buffer at 37 °C, stained in Coomassie brilliant blue R250, and then destained using a solution of 7% glacial acetic acid and 5% methanol. Gels were imaged using BioRad XRX plus GelDoc 1 imaging system to observe digested regions of MMP activity.

2.8. Western immunoblotting

Whole cell extract was prepared using RIPA buffer (50 mM Tris pH 7.6, 150 mM CaCl₂, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease and phosphatase inhibitors (Roche Basel, Switzerland). Cytosolic fractions were prepared using buffer containing HEPES (10 mM pH 7.9), EDTA (0.1 mM), EGTA (0.1 mM), DTT (1 mM), and PMSF (0.5 mM). Cells were incubated in buffer for 30 min before collection and the addition of 10% Triton-X 100. Supernatant was then collected as cytosolic fraction. Another buffer containing HEPES (10 mM), NaCl (0.4 M) and 1 mM of EDTA, EGTA, DTT, and PMSF were added to the remaining cell pellet and vortexed at

4 °C for overnight. The supernatant was collected after centrifugation at 10,000 rpm for 15 min as nuclear fraction.

Protein was measured using BCA method (Thermo Fisher, Waltham, MA). Equal amount of protein (25 µg) for each sample was loaded and separated on a 10% acrylamide gel and transferred onto PVDF membrane (EMD Millipore, Billerica, MA). Blots were then blocked in TBST containing 5% nonfat dry milk for 2 h prior to overnight incubation with respective primary antibody (p38, JNK, ERK, TAK1, NF-κBp65, and p-c-Jun) with dilution according to manufacturer. Protein bands were visualized using chemiluminescence and analyzed using Image Lab software (Bio Rad) for band intensity. Blots were probed with β-actin to ensure equal loading.

2.9 Molecular modelling

We have taken the prepared TAK1 protein structure from our previous study (Singh et al., 2016). The ligand epigallocatechin ((-)-cis-2-(3,4,5-Trihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol, (—)-cis-3,3',4',5,5',7-Hexahydroxyflavane) and epicatechin ((-)-cis-3,3',4',5,7-Pentahydroxyflavane, (2*R*,3*R*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol) were prepared using ligprep 3.4 module of Schrodinger Suite 15.2 (Schrödinger Release, 2015a,b). The prepared ligands were docked in the active site of TAK1 using standard precision (SP) followed by extra-precision (XP) mode of Glide 6.7. The best docked pose of XP docking of both the protein-ligand complex has been used as an initial structure for the molecular dynamics (MD) simulation. The complex was solvated in the orthorhombic box of 10 Å from the protein surface using TIP3P water model. The solvated systems have been first relaxed using the default protocol of Desmond 4.2 using OPLS2005 force field, then final production run of 100 ns have been submitted with NPT ensemble using Nose-Hoover chain thermostat and Martyna-Tobias-Klein algorithm with isotropic coupling at constant temperature 300 K and pressure of 1 atm. Energy and Trajectory were saved at every 10 ps time interval. The RMSD based clustering of frames of 100 ns trajectory has been done using inbuilt script of desmond.

2.10. In vitro TAK1 kinase assay

In vitro TAK1 kinase assay was performed according to the manufacturer's instructions (Promega, Madison, WI; catalog V4088). EGCG, EGC, and EC were used at 10 µM concentration. 5*z*-7-oxozeaenol was used at 5 µM concentration which has already been shown to inhibit TAK1 activity (Singh et al., 2016). Experiment was carried out in 96-well plate format and 25 µl total reaction volume. The inhibitors were incubated with TAK-TAB fusion protein and substrate mix for 10 min. 500 µM of ATP was then added to initiate kinase activity and the reaction went for 60 min at room temperature. After 60 min, ADP-Glo Assay kit (Promega catalog V6930) was used. 25 µl of ADP-Glo was added and incubated for 40 min prior to the addition of 50 µl kinase detection reagent. Luciferase was read 30 min after kinase reagent was added.

2.11. Statistical analysis

Statistical analysis was using one-way ANOVA to evaluate differences of means between EGCG, EGC, and EC treatment groups in 1L-6 and 1L-8 ELISA, and densitometric analysis

of proteins and zymograms. Due to large variability in protein expression profiles for signaling pathway analysis, data was normalized to positive control (IL-1 β) before Student's *t*-test with Bonferroni correction was performed. All tests assumed normal distribution where $\alpha = 0.05$ was considered significant.

3. Results

3.1. Green tea catechins EGCG, EC, or EGC do not exhibit cytotoxicity in human RASFs

We first determined the effects of EGCG, EGC, and EC on cell viability. Human RASFs were treated with 5–20 μ M of each catechin for 24 or 36 h in serum free media. The result of the MTT assay for different treatment groups showed that there was no significant effect of EGCG, EGC, or EC on the viability of human RASFs (Fig. 1; $p < 0.05$).

3.2. EGCG inhibits IL-1 β -induced production of IL-6 and IL-8 more effectively than EGC or EC in human RASFs

Next, we tested the efficacy of selected catechins in inhibiting pro-inflammatory cytokine production. RASFs were pre-treated with 5–20 μ M of each catechin overnight, followed by stimulation with IL-1 β (10 ng/ml) for 24 h. Concentrations range used in the experiments were based on our previous findings with EGCG (Ahmed et al., 2006; Ahmed et al., 2008). Condition media was collected and analyzed for production of IL-6 (Fig. 2A) and IL-8 (Fig. 2B) using ELISA assay. Our results showed that EGCG was the most effective catechin at inhibiting IL-6 and IL-8 production by 59% and 57%, respectively ($p < 0.05$). We also observed that EGC significantly inhibited the production of IL-6 by 48% ($p < 0.05$) and that of IL-8 by 35% in human RASFs, however this reduction did not reach statistical-significance. Surprisingly, EC displayed no inhibitory effect on IL-1 β -induced IL-6 and IL-8 production, suggesting its limited role in the anti-inflammatory effects of catechins present in green tea extract.

3.3. EGC and EGCG inhibit IL-1 β induced metalloproteinase-2 (MMP-2) activity in human RASFs

We have shown that EGCG is a potent inhibitor of tissue invasion in human RASFs by the virtue of downregulating IL-1 β induced MMP-2 activity (Ahmed et al., 2006). Inflammation is directly driven by IL-1 β which results in upregulation of inflammatory cytokines like IL-6 that mediates the activation of MMP-2 activity (Ahmed et al., 2008). We extended our studies to evaluate the differences in potency of EGCG, EGC, and EC in inhibiting IL-1 β -induced MMP-2 activity (Fig. 3). Our results of gelatin zymography showed that IL-1 β significantly induced MMP-2 activity in human RASFs (Fig. 3; $p < 0.05$). Pretreatment with EGCG (5–20 μ M) showed a significant reduction in MMP-2 activity by 85% ($p < 0.05$ for all concentrations). While we observed that EGC (5–20 μ M) also inhibited IL-1 μ -induced MMP-2 activity by 84%, EC at a similar concentration range had no apparent inhibitory effect.

3.4. EGCG, EGC, and EC differ on the selectivity of Cox-2 inhibition in human RASFs

To further gain insights into the differences in the anti-inflammatory activities of green tea catechins, cell lysates prepared from RASFs activated with IL-1 β with or without EGCG,

EGC, or EC pretreatment (5–20 μ M) were utilized for the determination of Cox-1 and Cox-2 expression (Fig. 4). Densitometric analysis of the immuno-noblots showed that both EGCG and EGC were equally effective in inhibiting Cox-2 expression by 86% and 82% respectively, while EC again showed no inhibitory action on IL-1 β -induced Cox-2 expression in RASFs (Fig. 4A). To further decipher the mechanistic differences in EGCG and EGC inhibition, we also probed the same cell lysates for Cox-1, a constitutive isoform, expression (Fig. 4B). Our results showed that none of the catechins at any given dose had no effect on constitutive Cox-1 expression in RASFs.

3.5. EGCG, EGC, and EC inhibit p-TAK1 expression

For signaling studies, RASFs were pre-treated with 10 μ M of catechin either alone or in combination overnight prior to IL-1 β (10 ng/ml) stimulation for 30 min. We decided to opt for 10 μ M of catechin since this concentration of EGCG is the minimum required to inhibit p-TAK1 activity (Singh et al., 2016) and it will provide the direct comparison among other catechins. Expression of p-TAK1 (Thr^{184/187}) was analyzed using western immunoblotting and densitometric analysis was performed (Fig. 5A). EGCG alone inhibited 50% of p-TAK1(Thr^{184/187}). EC alone inhibited 26% of p-TAK1 (Thr^{184/187}) expression whereas EGC alone inhibited by 35%. When EC, EGC, and EGCG are given altogether, p-TAK1 (Thr^{184/187}) expression is only 31% reduced.

TAK1-TAB1 in vitro kinase assay results shows a similar trend. 5z-7-oxozeaenol, a TAK inhibitor which binds covalently and blocks the active site permanently, inhibited TAK kinase activity by 77% compared to the no-inhibitor control. EGCG (10 μ M) inhibited TAK-TAB activity by 66%. Surprisingly, EC inhibited 14% of TAK activity and EGC showed similar inhibition to EC (Fig. 5B).

3.6. EGC and EC both bind to TAK1

We have shown recently that EGCG inhibits TAK1 kinase activity by occupying C174 site with H-bonds that results in reversible, yet stable inhibition of TAK1 kinase activity (Singh et al., 2016). To further understand why EC and EGC differentially inhibited TAK1 kinase activity, we performed molecular docking of EC and EGC to the active site of TAK1. The residue D175 plays an important role and crucial for the binding of both the ligands as it is forming two or three H-bonds with the ligands. EGC docking shows an H-bond with the C174 of TAK1 which is considered to be an important residue for TAK1 inhibition (Fig. 5C). This interaction is absent in case of EC since EC lacks the one hydroxyl group thereby, it is unable to bind to the active site as efficiently as EGC (Fig. 5D). The bridging water molecules also play an important role in the binding of these ligands. Although these two ligands are sharing common binding site, EGC shows a better binding interaction network than the EC.

3.7. EGCG, EGC, and EC inhibit p-P38 expression

Using the same conditions as before for p-TAK1 analysis, we also looked at MAPK inhibition (Fig. 6A). The expression of IL-1 β -induced p-JNK and p-ERK1/2 were not significantly inhibited by any catechin alone or in combination (Fig. 6C). However, IL-1 β -induced p-P38 expression was inhibited by 35% with EGCG alone. When EGCG is given in

combination with EC the inhibition decreases to 32%. The addition of EGC to EGCG/EC results in a 39% inhibition of p-P38, suggesting that EGCG is able to maintain its inhibitory potential in the presence of other catechins as well. A summary table of the effects of each catechin (EC, EGC, EGCG) at inhibiting different proteins involved in the IL-1 β signaling cascade has been provided in Table 1. An average inhibition (Mean $\pm$ SEM) is represented as a percent of IL-1 β positive control.

3.8. EGCG selectively inhibits nuclear localization of NF- κ B and cJun

Finally, we looked at nuclear translocation and activation of NF- κ B and AP-1 (using p-c-Jun expression) in human RASFs (Fig. 6B). EGCG alone inhibited NF- κ Bp65 and p-c-Jun(Ser⁷³) effectively. EGC was somewhat effective, and EC showed no activity. Surprisingly EGCG's effectiveness was reduced when given in combination with and EC.

4. Discussion

In the present study, we provided molecular basis for the differences in the mechanism of anti-inflammatory effects of catechins EGCG, EGC, and EC in human RASFs. Our findings highlighted differential effects of these green tea catechins on the signaling proteins in IL-1 β signal transduction pathways to elicit their responses. We observed that EGCG and EGC exhibited anti-inflammatory effects in human RASFs by inhibiting IL-1 β -induced soluble mediators (IL-6 and IL-8), cellular proteins (Cox-1/2), and matrix degrading enzymes such as MMP-2, which may serve the basis for anti-inflammatory effects found in green tea extracts. Furthermore, we observed that equimolar concentrations of EC have no anti-inflammatory properties in regulating IL-1 β signaling, suggesting that green tea extracts with high EGCG and EGC content with concomitantly low EC content may have better anti-inflammatory activity. However, further studies are warranted to compare different green tea compositions and their biological effects in vitro, in order to rationalize the approaches for their use as dietary supplement.

Cytokine and MMP production in RASFs directly correlate with increased levels of prostaglandins in response to IL-1 β (Egg, 1984). In addition, Cox-2^{-/-} mice have been shown to be less susceptible to collagen-induced arthritis. Therefore, Cox-2 inhibition has its own therapeutic advantage for RA therapy (Myers et al., 2000). While we have previously shown that EGCG selectively inhibited IL-1 β -induced Cox-2 expression in human osteoarthritis chondrocytes in vitro (Ahmed et al., 2002) another study showed the effect of EGCG in down-regulating IL-1 β -induced Cox-2 expression in human RASFs (Huang et al., 2010). For the first time, we compared different green tea catechins to find that both EGCG and EGC are equally potent, with no effect of EC, in inhibiting IL-1 β -induced Cox-2 expression in human RASFs. Interestingly, compounds that selectively inhibit Cox-2 are of therapeutic value for diseases characterized by inflammation (Hawkey, 1999). In our assays, EGCG and EGC are superior in this regard as it did not inhibit Cox-1 expression at any given concentration in vitro, strongly suggesting that EGCG is a better compound among other catechins and green tea extracts with higher EGCG content may elicit higher ability for treating inflammation.

Because green tea contains a combination of EC, EGC, and EGCG, and that EC showed no protection in our studies, we tested the effectiveness of combinations of catechins at inhibiting expression of key signaling molecules important in IL-1 β pathway. TAK1 plays a central role in inflammatory signaling pathways such as IL-1 β (Fechtner et al., 2016). Interestingly, EC and EGC alone showed some degree of p-TAK1 inhibition. Molecular docking shows EC forms an H-bond with Val42 and Val50 residue within the TAK1 binding pocket (Singh et al., 2016). Therefore, EC may have some inhibitory effect by preventing the ability of ATP to bind to TAK1. EGC makes an H-bond with C174 residue in addition to binding with nucleotide binding pocket which may explain why EGC is a better TAK1 inhibitor compared to EC. C174 is one of the major residues involved in the autocatalysis of Ser 184 and 187 which activates the TAK1-TAB1 complex. Therefore, EGC mimics the binding pattern of EGCG. As previously shown by us, EGCG is effective at abrogating TAK1 activity because of its ability to completely occupy C174 residue in the ATP-binding pocket through H bond formation and stabilize its position by forming additional hydrophobic links to distal V42, V50, and L163 residues in the TAK1 nucleotide-binding regions, and establishing π - π interactions at Y113 (Ahmed et al., 2008; Singh et al., 2016). Interestingly, EC given in equimolar combination with EGCG reduced the p-P38 and p-TAK1 inhibitory effect observed with EGCG alone which suggests some competition to occupy the TAK1 binding pocket. Since these catechins may compete with each other for binding, thereby, changing the anti-inflammatory effects of green tea based on its composition where the more EC could result in less anti-inflammatory effect.

To further understand what happens when green tea is consumed as a whole, we performed signaling studies with all three catechins present in equal concentrations. When all three catechins are given in combination, we observed no change in the inhibition of IL-1 β -induced p-P38 activation, but a modest decrease in the inhibition of p-TAK1 compared to EGCG alone group. This suggests EGCG may be the most effect catechin at inhibiting p-P38 activation in human RASFs. Also, EGCG was found to completely abrogate NF- κ B and p-c-Jun nuclear translocation in response to IL-1 β activation. Therefore, our overall findings from RASFs signaling mechanisms suggest that EGCG is a better anti-inflammatory catechin than EGC and EC. Most of the catechins are reversible inhibitors for TAK1-TAB1 complex whereas 7-z-oxozeaenol binds to protein covalently and permanently inhibits the TAK-TAB1 kinase activity. This reversibility makes green tea catechins a better choice for a TAK1 inhibitor activity, especially when some host-defense mechanisms govern by these cytokines also rely on TAK1-mediated signaling events.

Structurally, EGCG and EGC differ by an addition galloyl moiety at the P3 position whereas EGCG and EGC have a hydroxyl group that EC lacks. Previous studies have confirmed the importance of the galloyl group in EGCG's anti-proliferative effects. In breast epithelial cells, EGCG was more effective than EGC in inhibiting HGF/Met signaling, partly due to presence of the galloyl and R2 hydroxyl groups in EGCG to mediate this effect (Bigelow and Cardelli, 2006). In colorectal cancer cells, the galloyl moiety in EGCG improved anti-proliferative effects (Du et al., 2012). Additionally, in vascular smooth muscle cells, the galloyl group in EGCG is suggested to be important for inhibiting platelet-derived growth factor (PDGF)-induced proliferation as well as inhibiting PDGF-Rb signaling (Sachinidis et al., 2002). All of these studies indicate that the structural differences between EGCG and

EGC, specifically the galloyl moiety, are what makes EGCG a more unique and effective anti-inflammatory catechin.

The pharmacokinetics of catechins have been studied in humans in a study done by Chow et al. (2005). The study consisted of thirty healthy participants that were asked to take Polyphenon E capsules (200 mg epigallocatechin gallate, 48.5 mg epigallocatechin, 34.2 mg epicatechin, 20 mg epicatechin gallate, and other tea catechins) that would equate to 400, 800, and 1200 mg of EGCG content. Pharmacokinetic analysis from the study showed that the average plasma AUC, the maximum concentration, and the half-life increased with an increase in the dosages. Interestingly, when capsules are taken under fasting conditions these values increased which may be in part due to reduced conversion by glucuronidation process. EGC nor EGCG were not detected in urine right after administration, but did appear 24 h later. The amount of EC and EGC found in urine 24 h after Polyphenon E were reduced under fasting conditions when compared to the fed state (Chow et al., 2005).

While the present study was carried out to decipher molecular mechanisms of these catechins using equimolar concentrations, the contents of these catechin in different green tea brands may not be the same. For example, Henning et al. (2003), extensively studied the catechin content of eight green tea brands to find the total catechin content in green tea. Based on the findings, the mean $\pm$ SD catechin content in 100 ml of brewed green tea leaves comes out to be 122.04 ± 4.51 mg. As described in that study, the average EGCG, EGC, and EC content from eight different brand was 55.65 mg, 42.90 mg, and 7.64 mg, respectively, which constituted approximately 46%, 34.8%, and 5.6% of total catechin content (Khokhar and Magnusdottir, 2002). The catechin content observed in this study was also reflective of the values obtained in another study on catechin quantitative analysis in different green tea brands. These findings suggest that EGCG:EC (8.2:1) and EGC:EC (6.2:1) ratio in average green tea preparations is much higher than used in this study. However, our mechanistic insights provide rationale that proportionally lower content of EC may benefit in the overall anti-inflammatory activity of any green tea supplement in diet. However, further rigorous and unbiased studies are warranted in this regard that address some of the current challenges that green tea consumption as a dietary supplement in inflammatory conditions such as RA face, which include catechins and drug interactions, dose-optimization for consumption to achieve optimum bioavailability (higher benefit-to-risk ratio), and the potential method of intake (drinking 3–4 cups or taking a formulated capsules).

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Fig.1.

Green tea catechins do not affect RASFs viability. RASFs were treated for 24 or 36 h in serum free media with 20 μ M, 10 μ M, and 5 μ M of EGCG, EGC, and EC. 2 h prior to termination, MTT dye was added. Absorbance was read at 570 nm after DMSO was added. Values are expressed as mean $\pm$ SEM of three cell lines from different RA donors. Structures of EC, EGC and EGCG are also shown for reference.

Fig. 2.

EGCG and EGC inhibit IL-6 and IL-8 production. RASFs were treated overnight in serum free media with 20 μ M, 10 μ M, and 5 μ M of EGCG, EGC, and EC. IL-1 β (10 ng/ μ l) was added the next morning and cells were stimulated for 24 h before collecting supernatant. IL-6 (A) and IL-8 (B) production was quantitated with ELISA assay. Values are expressed as mean $\pm$ SEM of three cell lines from different RA donors. NS = non-stimulated *NS vs. IL-1 β $p < 0.05$ #IL-1 β vs. EGC $p < 0.05$ one-way ANOVA.

Fig. 3.

EGCG and EGC inhibit MMP-2 expression. RASFs were treated overnight in serum free media with 20 μ M, 10 μ M, and 5 μ M of EGCG, EGC, EC. IL-1 β (10 ng/ml) was added the next morning for 24 h before collecting supernatant. MMP-2 production was analyzed using gelatin zymography. Values are expressed as mean $\pm$ SEM of three cell lines from different RA donors. NS = non-stimulated *NS vs. IL-1 β $p < 0.05$; #IL-1 β vs. EGCG $p < 0.05$; ##IL-1 β vs. EGC $p < 0.05$ one-way ANOVA.

Fig. 4.

EGCG and EGC inhibit Cox-2 expression without effecting Cox-1. RASFs were treated overnight in serum free media with 20 μM, 10 μM, and 5 μM of EGCG, EGC, EC. IL-1 β (10 ng/ml) was added the next morning for 24 h before collecting whole cell lysate. Cox-2 (4A) and Cox-1 (4B) expression was analyzed using western immunoblotting (4C). Values are expressed as mean ± SEM of three cell lines from different RA donors. NS = non-stimulated *NS vs. IL-1β $p < 0.05$; #IL-1β vs. EGCG $p < 0.05$; ##IL-1β vs. EGC $p < 0.05$ one-way ANOVA.

Fig. 5.

Catechins inhibit TAK1 Activity. (A) EGCG, EGC and EC (10 μ M) were incubated overnight prior to IL-1 β (10 ng/ml) stimulation for 30 min. Cells were lysed and analyzed for MAPKs p-TAK1 (Thr184/187) using western immunoblotting densitometric analysis is shown. Values are expressed as mean $\pm$ SEM of three cells lines from different RA donors. *IL-1 β vs treatment one-way ANOVA $p < 0.05$. (B) A TAK-TAB fusion protein kinase assay was performed according to manufacturer's recommendations. EGCG, EGC and EC were used at 20 μ M and 5z-7-oxozeaenol was used at 5 μ M. Inhibitors were incubated for 10 min prior to 60-minute incubation with 500 μ M of ATP. Luciferase was measured with ADP-Glo assay kit at 30 min. Average intensity $\pm$ SEM of three independent experiments is shown. * $p < 0.05$ treatment vs no inhibitor one-way ANOVA. Average representative structure of biggest cluster from the trajectory of 100 ns molecular dynamics simulation of (C) TAK1-EGC complex and (D) TAK1-EC complex. The H-bonds between ligand and protein are represented in black dotted lines and water has been labelled in cyan colour. An image of EGCG docked to TAK1 can be seen in (Singh et al., 2016).

Fig.6.

Catechins inhibit MAPKs in IL-1 β Signaling Pathway. EGCG, EGC and EC (10 μ M) were incubated overnight prior to IL-1 β (10 ng/ml) for 30 min. Cells were lysed and analyzed for MAPKs p-P38, p-JNK, p-ERK in the cytosol (A) and p65 NF- κ B, and p-cJun (Ser73) in the nucleus (B). Densitometric analysis shown for P38 (C). JNK and ERK1/2 were not statistically significant. Values are expressed as mean $\pm$ SEM of three cell lines from different RA donors. *IL-1 β vs treatment one-way ANOVA $p < 0.05$.

Table 1

A summary table of inhibition of IL-1 β induced expression of key inflammatory mediators in RA.

Catechin	Protein	Percent inhibition of IL-1 β control (Mean $\pm$ SEM)	Catechin	Protein	Percent inhibition of IL-1 β control (Mean $\pm$ SEM)	Catechin	Protein	Percent inhibition of IL-1 β control (Mean $\pm$ SEM)
EC	p-TAK1 ^{Thr184/187}	26 $\pm$ 4 *	EGCG	p-TAK1 ^{Thr184/187}	36 $\pm$ 12 *	EGCG	p-TAK1 ^{Thr184/187}	51 $\pm$ 8 *
	p-P38	0 $\pm$ 19		p-P38	0.3 $\pm$ 18		p-P38	36 $\pm$ 7 *
	p-JNK	0 $\pm$ 31		p-JNK	0 $\pm$ 34		p-JNK	22 $\pm$ 33
	p-ERK	0 $\pm$ 11		p-ERK	0 $\pm$ 9		p-ERK	8 $\pm$ 10
	P65-NF- κ B	1 $\pm$ 30		P65-NF- κ B	29 $\pm$ 14		P65-NF- κ B	67 $\pm$ 9 *
	p ^(Ser73) -cJun	0 $\pm$ 20		p ^(Ser73) -cJun	0 $\pm$ 13		p ^(Ser73) -cJun	32 $\pm$ 5 *
	Cox-2	0 $\pm$ 6		Cox-2	82 $\pm$ 3 *		Cox-2	86 $\pm$ 3 *
	MMP-2	0 $\pm$ 3		MMP-2	84 $\pm$ 3 *		MMP-2	85 $\pm$ 10 *
	IL-6	0 $\pm$ 26		IL-6	42 $\pm$ 4 *		IL-6	67 $\pm$ 14 *
	IL-8	0 $\pm$ 17		IL-8	46 $\pm$ 14		IL-8	96 $\pm$ 7 *

Inhibition shown as percent of IL-1 β control $\pm$ SEM.

* Indicates statistical significance were $p < 0.05$.