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Effects of Varied EGCG and (+)-Catechin Concentrations on Proinflammatory Cytokines mRNA Expression in ConA-Stimulated Primary White Blood Cell Cultures

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EGCG [(−)-epigallocatechin gallate] and (+)-catechin hydrate are flavanoids, which are known as anticancer and healthy drugs. To test the immune modulatory effects of EGCG and catechin, various concentrations were tested on primary white blood cells (WBC) in cell cultures stimulated with the T-cell mitogen concanavalin A (ConA). WBC from dairy cows (1×10^6 cells/mL) were cultivated using RPMI medium with FCS and gentamycin. First, WBC were stimulated with ConA, and 6 h later the flavanoid treatment was started. Cultivated WBC were treated with various physiological flavanol concentrations (0−100 μM) in cross-combination with various ConA concentrations (0−1 μg/mL). After 24 h, cells were harvested, cell viability was verified, and total RNA was isolated. Relative mRNA expression levels of proinflammatory cytokines TNFα, IL1β, IL6, and transcription factor cFos and of nucleosome component histon H3 were quantified with real-time qRT-PCR. High EGCG and catechin concentrations had inhibitory effects on total RNA expression. Low EGCG concentration can induce total RNA expression in WBC. EGCG reduced cFos mRNA expression, which can be abolished by high ConA concentrations in a reverse dose-dependent manner. TNFα showed a flavanoid-specific expression pattern. EGCG acts in blood physiological concentrations (micromolar range), and catechin acts in higher gut-relevant concentrations (millimolar range) and has the potential to influence the proinflammatory TNFα expression. Higher flavanoid concentration had more pronounced effects than lower, whereas EGCG showed a more potent suppression of gene expression than catechin (toward TNFα). EGCG and catechin had no significant effects in primary WBC on the expression pattern of the proinflammatory cytokines IL1β and IL6 and on the expression of the housekeeping genes GAPDH and histon H3. It is presumed that both flavanoids have the potential to regulate total RNA expression and gene-specific expression in WBC.

KEYWORDS: Catechin; EGCG; real-time RT-PCR; proinflammatory cytokines; ConA; GAPDH; TNFα; IL-1β; IL-6; cFos; histon H3

INTRODUCTION

Over the past decades, there has been an increasing awareness of the potential health benefits of phytochemicals in foods and beverages, particularly in tea. After water, tea is the world's most consumed beverage, existing in three major forms (green, black, and oolong tea) with different chemical compositions. Green tea is widely consumed and contains polyphenolic compounds, which account for up to 30−42% of the dry weight of leaves (*1*, *2*). Also, food onions and apples contain large amounts of flavonoids (*3*). A subclass of flavonoids are the flavanoids. Typical representatives are catechin, epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin-3 gallate (EGCG). The physiological concentration of flavanoids in plasma is found to be up to 1 μM (*1*, *4*); in the gut it is much higher (~3 mM; *5*).

Green tea components have shown remarkable anti-inflammatory and cancer chemopreventive effects in many animal tumor bioassays, cell culture systems, and epidemiological studies. Animal models observed that green tea has preventive activity against cancer of the oral cavity, esophagus, stomach, intestine, colon, liver, lung, prostate, skin, and others (*1*). Many of these biological effects of green tea are mediated by EGCG, the major flavanol present therein. Further flavonoids exhibit antimicrobial, antioxidative, antithrombosis, immune-modulated, anti-inflammatory, blood pressure dropping, and blood-glucose-

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level dropping effects (*6*). However, the EGCG treatment effects are complex in different tissues, for example, in cancer cells as compared to healthy primary cells (*7*). In several cancer cell lines cancer apoptosis is increased, whereas in healthy alive cells it is not (*7*). The fundamental mechanism of differential response to EGCG is not yet known in detail. Up to now, mainly studies in cell lines, carcinoma cell lines, or immortalized cell lines are shown in the literature, having all cancer or artificial attributes.

In this study primary white blood cells (WBC) cultured with various EGCG or catechin concentrations were used, taking physiological treatment concentrations into account. Primary leukocytes demonstrate an appropriate model to study the effects of flavanoids on the regulation of inflammatory cytokines in the immune system.

MATERIALS AND METHODS

White Blood Cell Isolation and Culture. Blood was taken from the jugular vein of a healthy dairy cow. Coagulation was prevented by the addition of 0.5 mL of EDTA per 25 mL of total blood. For WBC separation 25 mL of sampled blood was mixed with 25 mL of lysis buffer (0.83 g of NH_4Cl, 3.7 mg of Na-EDTA, and 1 g of KCl in 100 mL, pH 7.4) and centrifuged for 10 min at 2000 rpm. The cell pellet was resuspended in 25 mL of lysis buffer and centrifuged; this was repeated three times. After centrifugation, remaining WBC were suspended in RPMI medium (Sigma, Steinheim, Germany) with 10% FCS (Gibco, Carlsbad, CA) and 0.1% gentamycin (Selectavet, Weyam-Holzolling, Germany). A viability test with trypan blue was made before cultivation. The cell concentration was adjusted to 1×10^6 cells/mL in 12-well plates in 3 mL of medium per well. The cells were maintained at 37 °C and 5% CO_2 in a humid environment for 2 days.

Treatment. Two days after seeding in 12-well plates, WBC were first stimulated with different concentrations of ConA (0, 0.01, 0.1, and 1 μg/mL of medium). After a further 6 h, the WBC were treated with (−)−EGCG (95% purified and extracted from green tea; Sigma-Aldrich) or catechin (Sigma-Aldrich). The six different concentrations of EGCG or (+)-catechin were 0 (control), 0.1, 1, 10, 30, and 100 μM in the medium. Each flavanol concentration was tested with each ConA in five repeats, in total, 240 cell culture wells. After 24 h of ConA treatment, the cells were harvested and total RNA was isolated.

RNA Isolation and cDNA Synthesis. Total RNA of leukocytes was isolated using TriFast (Roche Diagnostics, Basle, Switzerland) according to the manufacturer's instructions. To quantify the extracted RNA concentration, the optical density was determined in triplicates at three different dilutions of the final total RNA preparations at 260 nm. RNA integrity was verified by the optical density $OD_{260\ nm}/OD_{280\ nm}$ absorption ratio >1.70. Additionally, the RNA quantity and quality were checked in the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Synthesis of cDNA was performed with reverse transcriptase (MMLV H minus RT, Promega, Madison, WI) and random hexamer primers (MBI Fermentas, St Leon-Rot, Germany) according to the manufacturer's instructions with 1000 ng of total RNA in 40 μL of RT reaction buffer.

Relative Quantification by Real-Time PCR. Relative quantification of cDNA (respectively, mRNA) was carried out with the LightCycler (Roche Diagnostics). Reverse transcribed 25 ng of cDNA in a 1 μL volume was used as PCR template. Further reaction components for the qPCR reactions were 1.2 μL of $MgCl_2$ (4 mM), 0.2 μL of forward or reverse primer (0.4 μM; **Table 1**) synthesized by MWG (Ebersberg, Germany), 1.0 μL of LightCycler DNA Master SYBR Green I (Roche Diagnostics), and water up to a final volume of 10 μL. Before amplification, an initial denaturation step (10 min at 95 °C) was performed to ensure complete denaturation of the cDNA. Quantitative PCR was performed with 40 cycles and a product-specific annealing temperature, as shown in **Table 1**. The crossing points (CP) were acquired with the "second derivate maximum" method (LightCycler Software, Roche Diagnostics). Amplification PCR products underwent a melting curve analysis after the last cycle to specify the integrity of amplification, and finally a cooling step was performed. A relative quantification was performed, using GAPDH as a housekeeping gene and the following target genes (**Table 1**). The CP of target gene expression were standardized by the CP derived from GAPDH mRNA expression, according to the ΔΔCP method (*8*, *9*), described in the following equation:

Table 1. Annealing Temperature of the Used Forward and Reverse Primers

factor	annealing temp (°C)	primer
GAPDH	60	forward: GTC TTC ACT ACC ATG GAG AAG G
		reverse: TCA TGG ATG ACC TTG GCC AG
cFos	59	forward: ACT CCA GGC GGA GAC AGA
		reverse: GGT GAA GGC CTC CTC AGA
TNFα	62	forward: TAA CAA GCC GGT AGC CCA CG
		reverse: GCA AGG GCT CTT GAT GGC AGA
IL1β	60	forward: TTC TCT CCA GCC AAC CTT CAT T
		reverse: ATC TGC AGC TGG ATG TTT CCA T
IL6	60	forward: ATG AAT CCC GCT TCA CAA GCG C
		reverse: CCG AAT AGC TCT CAG GCT GAA CTG
histon H3	60	forward: ACT GGC TAC AAA AGC CGC TC
		reverse: ACT TGC CTC CTG CAA AGC AC

$$\Delta CP_{(GAPDH-target\ gene)} = CP_{(GAPDH)} - CP_{(target\ gene)}$$

Each ΔCP data point was standardized within one target gene by the aid of the mean expression CP of the control group ($CP_{(control)}$ = nontreated with ConA and nontreated with catechin or EGCG):

$$\Delta\Delta CP = CP_{(control)} - \Delta CP_{(GAPDH-target\ gene)}$$

Positive ΔΔCP values represent an up-regulation and negative ΔΔCP values a down-regulation of the described mRNA gene expression, compared to the control group.

Statistical Evaluations. Statistical analysis was made with Sigma Stat 2.03 (*10*). Means were compared with two-way ANOVA (polyphenol concentration and ConA concentrations). Values of p <0.05 were considered to be significant. The significance of EGCG or catechin treatment groups was compared to the untreated control within the corresponding ConA treatment group.

RESULTS

The viability of the freshly isolated and the 24 h cultured WBC, determined by trypan blue staining, ranged between 88 and 96% (n = 240), and the variation was constant over all treatment groups. Extracted total RNA concentrations were not influenced by ConA treatment [in the presence of catechin (p = 0.069) or EGCG (p = 0.246)]. The two polyphenol treatments with EGCG and catechin decreased the total RNA concentrations in the WBC per well, regardless of various ConA stimulations as shown in regression analysis. However, the two polyphenol treatments, EGCG (p < 0.001) and catechin (p < 0.005), decreased the RNA concentrations in the WBC regardless of the various ConA stimulations (**Figure 1**). The slope of linear regression showed a higher and more significant decrease of extracted total RNA with EGCG-treated WBC than with catechin-treated WBC (in **Figure 1**, x-axis is shown in log-scale):

$$\text{total RNA concn}_{[\text{EGCG treatment}]} = 5.48 - 0.029 \times x\ \mu\text{M EGCG [mg of RNA}/10^6\text{ cells] } (p < 0.001)$$

$$\text{total RNA concn}_{[\text{catechin treatment}]} = 4.88 - 0.013 \times x\ \mu\text{M catechin [mg of RNA}/10^6\text{ cells] } (p < 0.005)$$

However, analyzing only the flavanol concentrations up to 1 μM (dotted regression lines in **Figure 1**), for catechin the

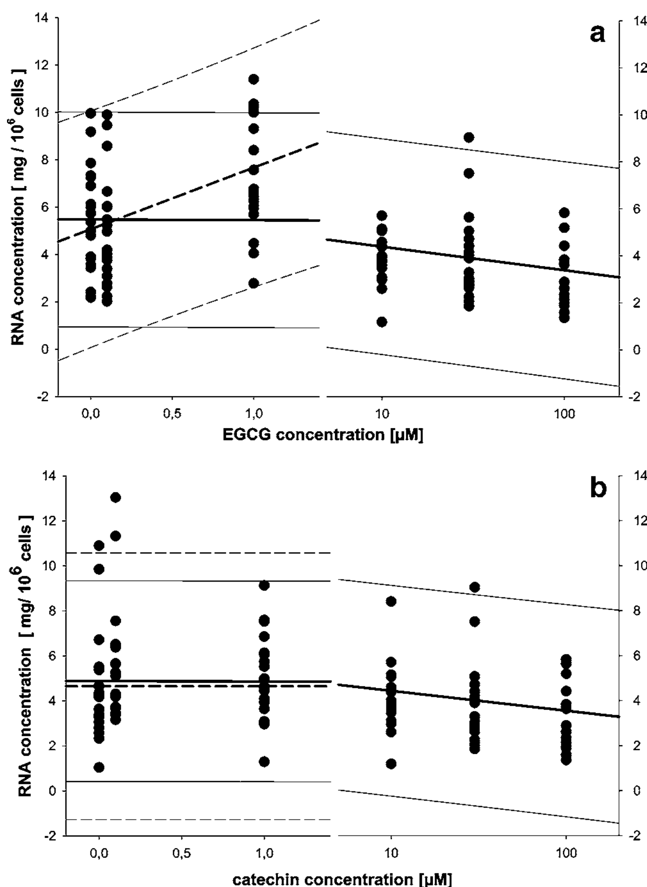

Figure 1. Total RNA concentration in mg/10^6 WBC stimulated with ConA and with six different EGCG (**a**) or catechin (**b**) concentrations (each group $n = 20$, presented in log-scale). Linear regression with 95% prediction interval is shown. The linear regression analysis of all six investigated flavanol concentrations (solid regression lines) indicates a reduction of the total RNA content both for EGCG ($p < 0.001$) and catechin ($p < 0.005$). The linear regression analysis of the three low flavanol concentrations (dotted regression lines) remained unchanged for catechin, but for EGCG an increase in total RNA concentrations could be shown ($p < 0.01$).

decreasing trend remained unchanged, but for EGCG a new significant trend could be seen. For low EGCG concentrations an induction of total RNA gene expression was observed ($p < 0.01$).

Furthermore, the RNA quality was verified by a capillary electrophoresis with the Bioanalyzer 2100 on a microchip lab-on-chip system (Agilent Technologies, Waldbronn, Germany). The RNA integrity number (RIN) recently developed by Müller et al. (*11*) and determined by the Bioanalyzer software (Agilent Technologies) showed average RINs between 7.5 and 9.0 (RIN range between 1 and 10; RIN 1 for degraded RNA, RIN 10 for a perfect and intact total RNA). An electropherogram is shown in **Figure 2** with a characteristic total RNA profile, isolated from WBC, representing an internal reference peak (22 s), a small 5S RNA peak (27 s), a dominant 18S RNA peak (41 s), and a dominant 28S RNA peak (47 s). Thus, the extracted and analyzed total RNA from WBC was fully intact and of high quality.

All tested genes were abundant and showed single peaks in melting curve analysis (LightCyler software) and a single band in high-resolution 4% agarose gel electrophoresis (gels not shown). The housekeeping gene GAPDH mRNA expression remained constant during the entire study and was not affected by either the ConA ($p = 0.130$) or the flavanoid treatments ($p = 0.280$).

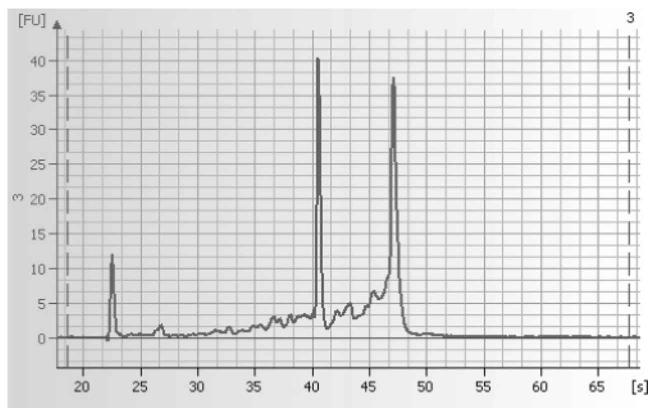

Figure 2. Electropherogram of total RNA (typical for these probes) (178 ng/μL total RNA; RIN = 7.9; ratio 28 s/18 s = 1.4). The characteristic total RNA profile represents an internal reference peak (22 s), a small 5S RNA peak (27 s), a dominant 18S RNA peak (41 s), and a dominant 28S RNA peak (43 s). ([FU] = fluorescence unit, s = seconds, 3 stands for the third probe on the chip.)

The expression pattern of cFos mRNA was similar in all treatment groups (**Figure 3**). However, high EGCG concentrations caused a down-regulation of cFos mRNA expression ($p < 0.001$), which was more pronounced in low ConA concentrations than in high ones. High ConA concentrations up to 1 μg of ConA/mL of medium diminished dose-dependently the cFos down-regulation of EGCG. Thus, ConA treatment leads to a dose-dependent gene expression. EGCG treatment showed high-reduced cFos mRNA expression in the ranges of 30 and 100 μM. The catechin treatment caused a slight down-regulation of cFos mRNA expression ($p < 0.05$).

TNFα showed a flavanoid-specific expression pattern (**Table 2**). Catechin showed an up-regulated effect on the TNFα mRNA expression ($p < 0.05$), especially in low ConA treatment groups. EGCG enhanced TNFα expression only in the blood physiological concentrations up to 1 μM; beyond, it depended on the ConA stimulation. The effect was more pronounced in groups with lower ConA stimuli. The high ConA concentrations overlay the effects derived from EGCG or catechin.

The IL6 and IL1β expressions level were not influenced by the flavanoid treatments (data not shown). The expression profiles remained unchanged. ConA had here only minor effects on the induction or repression of the gene expression in investigated primary bovine WBC culture.

The histon H3 mRNA level was very stably expressed and independent of flavanol or ConA treatment (data not shown). In this study the mRNA level of histon H3 and that of GAPDH were the most stably expressed genes and are obviously appropriate candidates for housekeeping genes.

DISCUSSION

Flavanoids in tea are known as anticancer, immune modulatory, and anti-inflammatory drugs (*6*). The ingredients of red wine (the "French paradox") and of green tea moved more and more into the focus of research. Leukocytes play a central role in the immune system; therefore, the used primary WBC culture is an appropriate model to study the effects on the immune system and the regulation of mRNA expression of proinflammatory cytokines. Here, the immune modulatory effects of EGCG and catechin were tested in various physiological (in plasma, up to 1 μM; *1*, *4*) and higher concentrations. To mimic an immune stimulus, WBC cultures were stimulated with the superantigen and T-cell antigen ConA. In earlier studies

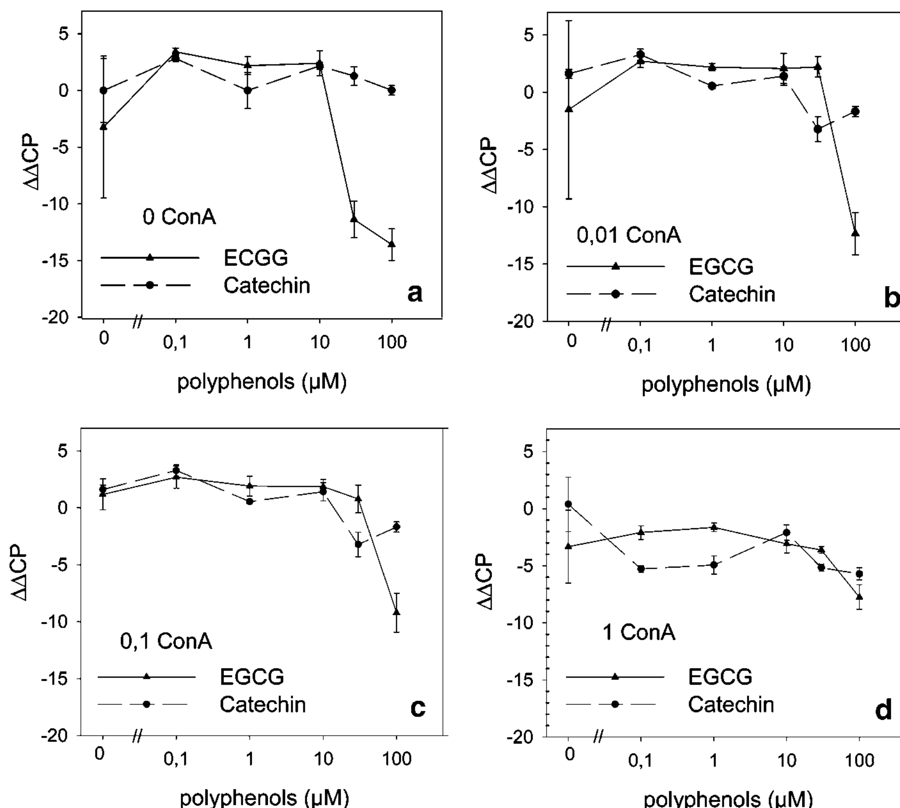

Figure 3. Effects of EGCG and catechin stimulation on cFos mRNA expression. WBC were preincubated for 6 h with 0 (**a**), 0.01 (**b**), 0.1 (**c**), or 1 (**d**) μg of ConA/mL of medium and then treated with 0.1, 1, 10, 30, and 100 μM EGCG or catechin. The mRNA expression was quantified with RT-PCR and standardized with the housekeeping GAPDH and with the mean of the group without ConA and without catechin ($n = 6$; mean ± sem). The *x*-axes are log-scaled.

Table 2. Effects of EGCG and Catechin Stimulation on TNFα mRNA Expression, Shown in ΔΔCP Values[a]

ΔΔCP (μM)	0 μg/mL ConA		0.01 μg/mL ConA		0.1 μg/mL ConA		1 μg/mL ConA	
	catechin	EGCG	catechin	EGCG	catechin	EGCG	catechin	EGCG
0	0.00 ± 0.44	1.42 ± 1.82	0.91 ± 0.85	1.77 ± 1.77	0.76 ± 1.14	1.89 ± 0.80	2.00 ± 1.37	2.60 ± 1.45
0.1	0.46 ± 0.34	3.56 ± 0.31**	0.99 ± 0.54	3.69 ± 0.68**	1.22 ± 0.57	3.06 ± 0.39**	2.16 ± 0.36	1.09 ± 0.30*
1	2.76 ± 1.56***	−0.17 ± 0.36*	1.65 ± 0.52	0.37 ± 0.71	2.14 ± 1.33	−0.38 ± 0.72***	2.23 ± 0.41	1.88 ± 0.37
10	2.24 ± 0.34**	2.11 ± 0.53	1.64 ± 0.85	2.21 ± 1.02	3.33 ± 0.39***	1.85 ± 0.44	2.52 ± 0.17	2.33 ± 0.60
30	2.48 ± 1.06***	1.04 ± 0.42	2.73 ± 0.34**	3.41 ± 0.68*	2.21 ± 1.06	3.30 ± 0.89	2.32 ± 0.56	3.08 ± 0.62
100	3.27 ± 0.54***	1.08 ± 0.38	4.03 ± 0.57***	0.55 ± 0.30	2.03 ± 0.55	1.66 ± 0.63	3.18 ± 0.59	3.89 ± 0.46

[a] Positive values indicate an up-regulation and negative values a down-regulation. WBC were preincubated for 6 h with 0.01, 0.1, and 1 μg of ConA/mL of medium and then treated with 0.1, 1, 10, 30, and 100 μM EGCG or catechin ($n = 5 \pm$ SEM). An asterisk indicates significance between polyphenols treatment and control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

catechins showed stimulatory effects in cell culture and in vivo experiments (*7*). One general question is whether catechins induce or inhibit gene expression and, therefore, prevent or inhibit cells in their proliferation rate and growth. EGCG and a few flavanoids have been shown to inhibit the growth and induce cell cycle arrest and apoptosis in different cancer cell lines (*12*).

Flavanoids such as EGCG and catechin extracted from green tea stimulated WBC cultures from cattle in different ways. Higher applied EGCG and catechin concentrations decreased the total RNA expression level with high significance. The inhibitory effect is more pronounced with EGCG than with catechin (**Figure 1**, solid regression lines). Low EGCG concentrations (<1 μM) as found in the blood had a stimulatory effect on RNA expression, whereas catechins did not influence the total RNA expression levels (dotted regression line). Therefore, it is a matter of EGCG concentration how the total gene expression is regulated. These findings are a hint that high flavanoid concentrations, as occurring in gut epithelial cells (millimolar range) and in immediate neighbor cells may have inhibitory effects on the total RNA synthesis rate and consequently on epithelial cell turnover. That can be an explanation that catechins have the described negative effects on cell growth via decreased cell proliferation.

Furthermore, the cFos expression levels were down-regulated at high EGCG treatment concentrations, whereas a catechin treatment hardly affected the cell culture gene expression. Additionally, ConA exposure induced the cFos mRNA expression in a dose-dependent manner. cFos is an early response gene and a potent transcription factor and is responsible for the induction of many inflammatory genes. The ConA treatment can abolish the reduced cFos expression through EGCG. In a recent study using a human keratinocyte cell line, EGCG reduced the cFos mRNA transcription as well (*13*). At high EGCG concentrations, these results correlate with the decreased total RNA cell expression, the lower cell turnover, and the anticancer effects.

TNFα showed a flavanoid-specific expression pattern. Expression was induced by high catechin concentrations from 10

to 100 μM. EGCG enhances the TNFα expression in blood physiological ranges in the lower range (up to 0.1 μM). Both effects were overlaid by the varied ConA concentrations, which could enhance the proinflammatory TNFα expression (*14*, *15*). High flavanoid concentrations in the upper micromolar range may be found in the gut content, and therefore they might act in the gastrointestinal tract on cells exposed to it. In the literature, the effects of EGCG on TNFα mRNA expression are controversially discussed (*16*−*19*). TNFα enhances the phagocytosis and the cytotoxity of monocytes and macrophages. In a previous study by Crouvezier and co-workers (*19*) primary human leukocytes were treated with ECG, EGC, and EGCG, resulting in no significant effects on TNFα expression, whereas stable cell lines showed inhibition of TNFα expression with EGCG or catechin treatment (*18*, *20*, *21*).

In primary WBC cultures EGCG and catechin had no significant effects on the expression pattern of the proinflammatory cytokines IL1β and IL6. Ahmad et al. (*7*) describe different effects of flavanoids on IL1β gene expression on stable cell lines and primary cell cultures. In further studies ECG, EGC, and EGCG decreased the production of IL1β in human leukocytes (*19*). Both proinflammatory genes IL1β and TNFα reacted similarly in the studies (*7*, *19*).

Histon genes were hardly investigated in flavanoid studies. In this study, we found unchanged and very stable histon H3 mRNA expression levels. It is still open whether EGCG might have an influence on histon H3 gene activity because EGCG binds in vitro to histon proteins, that is histon sulfate (*22*).

In conclusion, high EGCG and catechin concentrations had inhibitory effects on total RNA expression. Low EGCG concentrations can induce cellular total RNA content and, therefore, gene expression in general. EGCG reduced cFos mRNA expression, which can be abolished by high ConA concentrations in a reverse dose-dependent manner. TNFα showed a flavanoid-specific expression pattern. EGCG leads to a stimulation in blood physiological concentrations, and both flavanoids lead to an inhibition in higher gut-relevant concentrations and have the potential to influence the proinflammatory TNFα expression in WBC cultures. Higher flavanoid concentrations had more pronounced effects than lower, whereas EGCG showed at higher concentrations a more potent suppression of gene expression than catechin. EGCG and catechin had no significant effects in primary WBC on the expression pattern of the proinflammatory cytokines IL1β and IL6 and on the expression of the housekeeping genes GAPDH and histon H3. We presume that both flavanoids have the potential to regulate total RNA expression and gene-specific expression in WBC. That might be an explanation for why catechins have the described negative effects on cell growth via decreased cell proliferation.

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