Original Contribution

Hydrogen peroxide mediates EGCG-induced antioxidant protection in human keratinocytes

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ABSTRACT
The beneficial health effects of (−)-epigallocatechin-3-gallate (EGCG), the main catechin of green tea, have been attributed to complex interactions with a focus on antioxidative properties. Susceptibility to autoxidation and production of cytotoxic reactive oxygen species (ROS), mostly H2O2, have been suggested to occur in vitro but also in vivo. In this study, we address whether autoxidation-derived H2O2 may be involved in the cytoprotective effects of EGCG. To that end we investigated keratinocyte-derived HaCat and HL-60 promyelocytic leukemia cells with significantly different sensitivities to H2O2 (IC50 117.3 versus 58.3 μM, respectively) and EGCG (134.1 versus 84.1 μM). HaCat cells significantly resisted cytotoxicity and DNA damage based on enhanced H2O2 clearance, improved DNA repair, and reduced intracellular ROS generation. Cumulative versus bolus EGCG and H2O2 treatment and H2O2 pretreatment before subsequent high-dose EGCG and vice versa significantly reduced DNA damage and cytotoxicity in HaCat cells only. Addition of catalase abolished the protective activities of low-dose H2O2 and EGCG. In summary, our data suggest that autoxidative generation of low-dose H2O2 is a significant player in the cell-type-specific cytoprotection mediated by EGCG and support the hypothesis that regular green tea consumption can contribute as a pro-oxidant to increased resistance against high-dose oxidative stressors.

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Habitual green tea consumption has long been associated with health benefits and cancer prevention, although the overall clinical evidence is inconclusive [2]. Among the bioactive chemicals of green tea leaves, (−)-epigallocatechin-3-gallate (EGCG), the predominant polyphenolic catechin constituent, has been postulated to be the main effective agent [3]. Consequently, many efforts to develop health-promoting dietary supplements and nutraceuticals focus on EGCG.

Chemoprevention attributed to EGCG includes antioxidiant properties [4] of direct scavenging of reactive oxygen/nitrogen species and chelation of reactive metal ions, as well as interactions with signal transduction pathways [5,6] and binding to target proteins [7,8] resulting in cell cycle arrest and/or apoptosis. EGCG and other dietary chemopreventive compounds have also been shown to activate the redox-sensitive Nrf2 signaling pathway [9,10], which regulates phase II detoxification and antioxidant enzymes in response to environmental and oxidative stressors [11]. Green tea catechins, undergoing extensive metabolic biotransformation in vivo [12,13], have been shown to polymerize oxidatively with cogeneration of reactive oxygen species (ROS), including H2O2, under cell culture conditions [12–14]. Recently, EGCG-induced ROS generation has been verified in an in vivo system [1], which now gives new impetus to the ongoing discussion of whether ROS, apart from the proven H2O2 generation within the oral cavity [15], may also affect inner organs [16].

Several cell-damaging effects of EGCG, classified as in vitro artifacts, have been linked to the generated H2O2 [13,17,18] and oxidative degradation products of EGCG [19,20]. Nevertheless, in vivo liver toxicity has been reported after high-dose green tea exposure following consumption of dietary supplements in humans [21] and administration of tea extracts in animal studies. Potential pro-oxidant activities of EGCG have been implicated in this high-dose-induced hepatotoxicity [22]. Beyond doubt, it is a matter of dosage whether H2O2 acts destructively or functions as a cellular signaling molecule with diverse physiological functions [23–25]. A repetitive low-level H2O2 stress is capable of protecting against apoptosis induced by subsequent acute stress by initiating upregulation of antioxidant enzymes [26] and the enhanced expression and activity of the peroxisome proliferator-activated nuclear receptor (PPAR)-β [27].
Consequently, it might be hypothesized that green tea consumption causes a continuous low-level stress, which increases resistance to acute stressors. To investigate this in depth, we analyzed DNA damage and cytotoxicity by either $\mathrm{H}_2\mathrm{O}_2$ treatment followed by subsequent challenges with $\mathrm{H}_2\mathrm{O}_2$ or EGCG or cumulative $\mathrm{H}_2\mathrm{O}_2$ treatments compared to bolus. All $\mathrm{H}_2\mathrm{O}_2$ treatment designs were performed concurrent with EGCG. Furthermore, EGCG was used as a freshly prepared solution (EGCG$_{fp}$) continuously generating $\mathrm{H}_2\mathrm{O}_2$ and as an aged solution (EGCG$_{aa}$) already containing a stable level of $\mathrm{H}_2\mathrm{O}_2$. Experiments were performed with two human cell lines, keratinocyte-derived HaCat and HL-60 promyelocytic leukemia cells, differing distinctly in their sensitivity to EGCG and $\mathrm{H}_2\mathrm{O}_2$.

**Materials and methods**

**Reagents**

The cell culture medium RPMI 1640 was obtained from Invitrogen (Paisley, UK) and was supplemented with 10% heat-inactivated fetal calf serum (FCS; PAA, Linz, Austria). The medium contained neither vitamin C nor vitamin E. EGCG with 99.7% purity (ALX-270-263) was purchased from Alexis Corp. (Lausen, Switzerland). The dyes trypan blue (TB), Hoechst 33258, and 2′,7′-diamino-2-fluorofluorescein diacetate (DCFH-DA) were purchased from Fluka (Buchs, Switzerland), high- and low-melting agarose was from Invitrogen. All other reagents were from Sigma–Aldrich (St. Louis, MO, USA). Solutions including the EGCG stock (10 mM in 4 °C phosphate-buffered saline (PBS)) were freshly prepared except for the DCFH-DA stock (33.4 mM in dimethyl sulfoxide (DMSO), liquid, Hybri-Max, minimum 99.5%), which was stored at −80 °C. The final concentration of DMSO in the assay was below 0.5%.

**Cell culture and treatment**

HaCat, a human spontaneously immortalized nontumorigenic epithelial keratinocyte cell line [28], and the human promyelocytic leukemia cell line HL-60 (American Type Culture Collection, Manassas, VA, USA) were both maintained and treated in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine. Use of the identical medium is essential for comparative analysis, as type and supplementation of medium directly influence the dimension of antioxidative ROS generation [13,19]. Cells were grown at 37 °C in a humidified 5% CO$_2$ atmosphere. Absence of *Mycoplasma* contamination was verified regularly by means of Hoechst 33258 staining. To avoid an impact of cell density differences (cell number/working volume) as demonstrated for $\mathrm{H}_2\mathrm{O}_2$ [29], all treatments were performed in six-well plates (Falcon; BD Biosciences, San Jose, CA, USA) at the density of $7 \times 10^5$ cells/2 mL/well for both cell lines. With regard to toxicity studies, when cells were pre- or cumulatively treated over several days before the final challenge, the cells were seeded at an adapted density ($1.8 \times 10^5$ cells/well) to result at the time of bolus at about $7 \times 10^5$ cells/well. During the 24-h recovery period proliferative activity was negligible. Adherent HaCat cells displayed about 50% confluence at the time of treatment.

EGCG treatment was performed with two different solutions: EGCG$_{fp}$ was freshly prepared from a 10 mM stock in cold (4 °C) PBS and EGCG$_{aa}$ represents an aged solution used after incubation of the prepared solution for 60 min at 37 °C (CO$_2$ incubator).

**Cytotoxicity**

Cell death was monitored by TB staining. For HaCat, both detached and attached cells were collected. After the cells were mixed (1:1) with 0.2% TB solution, they were left at room temperature for 5 min and counted in a Neubauer chamber under a Leitz Diavert microscope. Viable and dead cells are indicated by dye exclusion and staining, respectively.

Apoptosis was determined by microscopic evaluation of the nuclear morphology (chromatin condensation and fragmentation) of cells collected simultaneously and prepared by cytospin (Shandon Southern Products, Cheshire, UK). Cells were fixed with formaldehyde (2% v/v) and stained with Hoechst 33258 (8 μg/mL). At least 1000 cells per sample were scored under a fluorescence microscope (Axioskop, Zeiss, Oberkochen, Germany). Experiments were set up in duplicate and repeated at least three times.

**DNA damage and repair**

The alkaline version ([pH > 13]) of the single-cell gel electrophoresis (comet) assay was used to determine DNA damage [30,31] and the cellular repair capacity [32]. To determine the ability of cells to remove induced damage, cells were treated for 5 min following a washing step with PBS and further incubation for various time intervals in full culture medium. For comet assay performance, cells were collected after two PBS washing steps, whereby, for HaCat, detached and attached cells were harvested. The comet assay was performed with single-cell suspensions according to the guidelines of Hartmann et al. [33]. After DNA staining with 2 μg/mL ethidium bromide, coded slides were examined with a fluorescence microscope (Nikon Eclipse E600) equipped with an automated digital imaging system (Comet Assay IV, Perceptive Instruments, Haverhill, Suffolk, UK). The extent of DNA migration is indicative of DNA damage and expressed as percentage DNA in tail (%tail DNA). Experiments were set up in triplicate and repeated three times. Data were collected from 150 cells per experiment in triplicate (50 cells/slide/culture). To obtain valid results, concurrent experiments were performed in triplicate and repeated at least three times.

![Fig. 1. Cytotoxicity after EGCG and $\mathrm{H}_2\mathrm{O}_2$ treatment of HaCat and HL-60 cells. Cells were treated for 24 h with EGCG$_{fp}$ and $\mathrm{H}_2\mathrm{O}_2$ (see Materials and methods). Cell death was determined by the trypan blue method (necrosis) and analysis of Hoechst 33258-stained cytospin preparations (apoptosis). (A and C) Survival relative to control was calculated by considering the total numbers of dead cells (necrosis + apoptosis). (B and D) Percentages of total dead cells (Apo + Necr) and apoptotic cells (Apo) at the indicated EGCG and $\mathrm{H}_2\mathrm{O}_2$ concentrations are presented. For both cell lines the 50% cell survival inhibitory concentration (IC$_{50}$) and the 50% lethal concentration (LC$_{50}$) are indicated. Data are means ± SD; n = 6; **p < 0.01, ***p < 0.001, HaCat vs HL-60 cells, Student’s t test.](Image)
assessment of cell death was performed using TB exclusion. Cell viability at the time of assay was >90% in all experiments.

**FOX assay for hydrogen peroxide measurement**

H$_2$O$_2$ measurements were carried out by ferrous oxidation of xylenol orange (FOX assay) following the FOX-1 protocol of the PeroXOquant Quantitative Peroxide Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Because any other oxidizing agent can oxidize reagent ferrous ion to ferric ion, the H$_2$O$_2$ authenticity was confirmed by the addition of catalase (100 U/ml) [34], which almost completely inhibited the formation of a colored complex by solutions of EGCG and H$_2$O$_2$. Levels of H$_2$O$_2$ were determined after addition of EGCG or H$_2$O$_2$ in the absence or presence of HaCat or HL-60 cells after various time points (30, 60, and 120 min). For that purpose, medium samples were transferred to a 96-well microplate (Falcon; BD Biosciences) and the H$_2$O$_2$ concentration was measured according to the manufacturer’s protocol. Absorbance was read at 595 nm. Experiments were repeated three times.

**Fluorescence measurement of intracellular ROS**

Measurement of the overall cellular oxidative stress after EGCG and H$_2$O$_2$ treatment was performed using the cell-permeative and ROS-sensitive probe DCFH-DA [35], which generates the fluorescent 2’,7’-dichlorofluorescein (DCF) upon enzymatic reduction and subsequent oxidation by various ROS [36]. Cells labeled with DCFH-DA (10 μM) in Hanks’ balanced salt solution (HBSS) with 1% FCS (30 min) and treated with EGCG or H$_2$O$_2$ for 60 min were harvested, washed once in HBSS with 1% FCS, put on ice, and immediately forwarded to analysis by flow cytometry performed with the BD FACS Calibur system (Becton–Dickinson, Rutherford, NJ, USA). DCF fluorescence was analyzed with excitation and emission settings of 480 and 525 nm, respectively. Background fluorescence was determined from DCFH-DA-labeled cells without treatment. Experiments were set up in duplicate and repeated three times.

**Statistical analysis**

Comet assay results are not normally distributed. Therefore, as suggested by Tice et al. [30] and Collins [31], we used the nonparametric Mann–Whitney U test and Kruskal–Wallis test, which calculate differences between two and more than two unpaired groups, respectively. All other comparisons for differences among two and more than two data sets (means±SD) were performed by applying Student’s t test and ANOVA Bonferroni test, respectively. Statistical analyses were performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA, USA). Significance was established at values of p<0.05.

**Results**

**HaCat cells are less sensitive than HL-60 to EGCG- and H$_2$O$_2$-induced cytotoxicity**

In both cell lines, the effect of a 24-h EGCG$_{fp}$ treatment was generally less pronounced than that of equimolar H$_2$O$_2$. Furthermore, HaCat cells were less sensitive to both agents. HL-60 compared to...
HaCat cells displayed a significantly reduced survival (Figs. 1A and C) as well as increased cell death, including a substantial proportion of apoptosis (Figs. 1B and D).

**Freshly prepared versus aged EGCG: H$_2$O$_2$ clearance and intracellular ROS**

In a number of experiments, HaCat and HL-60 cells were treated simultaneously with EGCG$_{fp}$ and EGCG$_{a}$. In cell-free solutions, EGCG$_{fp}$ generated increasing H$_2$O$_2$ concentrations, which reached a plateau level of 80 $\mu$M at 100 $\mu$M EGCG after 60 min. The EGCG$_{a}$ solution contained the 80 $\mu$M H$_2$O$_2$ concentration right from the start (Fig. 2A). These differences in the H$_2$O$_2$ levels at treatment onset also influenced the cell-mediated H$_2$O$_2$ clearance, which generally was managed more efficiently by HaCat than by HL-60 cells. In the presence of HaCat cells, EGCG$_{a}$-formed H$_2$O$_2$ declined time-dependently (10, 30, 60 min) to 25, 5, and 1 $\mu$M, respectively (Fig. 2B), whereas in the presence of HL-60 cells the remaining H$_2$O$_2$ concentrations were up to 10-fold higher (35, 20, and 10 $\mu$M; Fig. 2C). Expectedly, EGCG$_{a}$-generated H$_2$O$_2$ ranged below EGCG$_{a}$-generated H$_2$O$_2$ after 10 min treatment. The following H$_2$O$_2$ increase after 30 min was abolished after 60 min in the presence of HaCat cells. However, in the presence of HL-60 cells, EGCG$_{a}$-generated H$_2$O$_2$ remained elevated at that time point (Fig. 2C).

The different potencies of HaCat and HL-60 cells regarding H$_2$O$_2$ clearance were also reflected by the significantly different intracellular ROS generation and cellular oxidative stress after treatment with EGCG$_{fp}$, EGCG$_{a}$, or H$_2$O$_2$ (Figs. 2D and E). The pro-oxidant potencies of various treatments were significantly lower in HaCat cells. The effects of EGCG$_{a}$ were similar to those induced by H$_2$O$_2$ at the cell-free concentration generated after 60 min.

These results demonstrate that the oxidative stress response in cells depends on the efficacy of H$_2$O$_2$ clearance, the age of the EGCG solution, and the cell type.

**Freshly prepared versus aged EGCG: DNA damage**

EGCG$_{fp}$-, EGCG$_{a}$-, and H$_2$O$_2$ (at the cell-free concentration generated after 60 min)-induced DNA damage in HaCat and HL-60 cells was determined by performing comet assays after 5 min treatment.

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**Fig. 3. DNA damage after EGCG and H$_2$O$_2$ treatment in HaCat and HL-60 cells. DNA damage was analyzed by using the comet assay (see Materials and methods).** (A) Cells were treated with EGCG$_{fp}$, EGCG$_{a}$, and H$_2$O$_2$ for 5 min. Data boxplots of %Tail DNA from three tubes/experimental point; $n = 150$; ***$p < 0.001$, EGCG$_{a}$ vs EGCG$_{fp}$ for HaCat and HL-60 cells, respectively, and HaCat vs HL-60 cells regarding EGCG$_{fp}$, EGCG$_{a}$, and H$_2$O$_2$; Mann-Whitney). (B, C) Repair of DNA damage at various time points after H$_2$O$_2$ treatment. Data boxplots of %Tail DNA from three tubes/experimental point; $n = 150$. (B) ***$p < 0.001$, 30 min vs 5 min, 60 min vs 30 min; ns, not significant, control vs 60 min; (C) ns, not significant, 30 min vs 5 min, 60 min vs 30 min, ***$p < 0.001$, 60 min vs 5 min and control vs 60 min; Mann-Whitney.

**Fig. 4. DNA damage after cumulative and bolus treatment with H$_2$O$_2$ in HaCat and HL-60 cells.** H$_2$O$_2$ treatment was performed in three versions: as a bolus and as three to five cumulative aliquots applied within a maximum of 10 min at 2-min intervals. Concentrations are indicated. Comet assay was performed 5 min after bolus and cumulative low-dose treatment. Data boxplots of %Tail DNA from three tubes/experimental point; $n = 150$. (A–C) ***$p < 0.001$, cumulative vs bolus; ns, not significant, last aliquot bolus vs cumulative aliquots; (D–F) ns, not significant, ***$p < 0.001$, cumulative vs bolus and last aliquot vs cumulative aliquots; Kruskal-Wallis.
Generally, HaCat cells were less susceptible than HL-60 cells. Exposure to EGCG_{FP} induced significantly less DNA damage (expressed as %tail DNA) compared to EGCG_{SP} in both cell lines. The effects of EGCG_{SP} were similar to those induced by the respective H_{2}O_{2} concentration (Fig. 3A).

These results demonstrate that aging of EGCG solutions has a strong impact on DNA-damaging effects.

**Time response of H_{2}O_{2}-induced DNA damage: HaCat versus HL-60 cells**

Comet formation was assessed after various treatment times (5, 60, 120, and 240 min) with H_{2}O_{2}. In HaCat cells, the extent of DNA damage (%) showed a rapid and significant decline of comet formation (removal of nearly 60% within 60 min and almost 100% by 120 min; Fig. 3B), in accordance with published data showing that rejoicing of H_{2}O_{2}-induced DNA strand breaks is a rapid process [37]. Although baseline levels were reached after 120 min in HaCat cells, DNA damage in HL-60 cells remained significantly increased during the total time interval (Fig. 3C).

These results demonstrate that the DNA repair capacity of cells may be a decisive parameter for the different sensitivities to EGCG_{SP} and EGCG_{FP}.

Cumulative H_{2}O_{2} is less damaging to DNA than bolus treatment in HaCat but not HL-60 cells

We wondered whether an immediate adaptive response to the gradual generation of H_{2}O_{2} in freshly prepared EGCG solution (Fig. 2A) may be responsible for the consistently lower DNA damage levels by EGCG_{FP} than by EGCG_{SP} treatment in HaCat cells. To resolve this question, cells were treated with either H_{2}O_{2} as a bolus (corresponding to EGCG_{SP}) or the same dose applied in three to five aliquots added within 10 min (corresponding to EGCG_{FP}). Comet assays were performed 5 min after bolus treatment or after addition of the last aliquot. Both cell lines differed remarkably with respect to their responses. Only HaCat cells benefited from the cumulatively applied H_{2}O_{2}. Bolus treatments induced significantly higher DNA damage than multiple aliquots (Figs. 4A–C) as well as concentrations below 10 μM applied as the last aliquot (Figs. 4A and B). In HL-60 cells, however, cumulative treatments did not reduce but rather increased H_{2}O_{2} genotoxicity (Figs. 4D–F). A similar response pattern was found after cumulative and bolus EGCG treatments (40 and 80 μM; not shown). These data indicate that cumulative increasing H_{2}O_{2} concentrations induce protective effects within minutes in HaCat but not HL-60 cells.

**Low-dose H_{2}O_{2} treatment before EGCG and EGCG before H_{2}O_{2} protect against DNA damage and cytotoxicity in HaCat but not in HL-60 cells**

Pretreatments were performed for 30 min and comet assays were carried out 5 min after the final challenge. H_{2}O_{2} concentrations below 10 μM applied before a 50 μM EGCG challenge reduced the DNA-damaging effect significantly (Fig. 5A). H_{2}O_{2} analyzed at 5 μM was still significantly protective (not shown). Consistent with previous results [38,39], EGCG_{FP} pretreatment at physiological doses (0.01–10 μM) also protected against a subsequent 20 μM H_{2}O_{2} challenge (Fig. 5C). Addition of catalase during H_{2}O_{2} (Fig. 5B) and also EGCG (Fig. 5D) pretreatment abolished the DNA-protective potential.

Comparable long-term experiments were performed using the cell survival readout. Treatments with cumulative H_{2}O_{2} (Fig. 6A) and cumulative EGCG_{FP} administration (Fig. 6B) compared to the same agent as bolus treatment were performed within 3 days. Addition of H_{2}O_{2} as well as EGCG_{FP} in gradually increasing portions improved the...
viability of cells compared to bolus or even last-aliquot bolus (Figs. 6A and B). In addition, low-concentration H$_2$O$_2$ pretreatment before a cytotoxic EGCG challenge (Fig. 6C) was performed within 2 days. Survival of HaCat cells was analyzed 24 h after bolus treatment. Pretreatment with H$_2$O$_2$ reduced the growth-inhibitory effect of EGCG$_{fp}$ (Fig. 6C). Pretreatment with EGCG (10 $\mu$M) was performed twice for 2.30 h each and cell viability was measured 2 h after H$_2$O$_2$ bolus at concentrations ranging from 50 to 800 $\mu$M (Fig. 6D). Corresponding to previous studies [40], EGCG pretreatment reduced H$_2$O$_2$ toxicity significantly, resulting in around 20–30% enhanced cell survival at all concentrations tested. Corresponding to the DNA damage data (compare Fig. 5D), the presence of catalase during pretreatment abolished this protective effect, resulting in survival ratios of 0.91 and 1.05 pretreated/nonpretreated samples. No protection by EGCG pretreatment but rather increased cytotoxicity was found in HL-60 cells (not shown), consistent with previous results [41]. These data show that low-dose H$_2$O$_2$ like that generated by EGCG autoxidation can mediate cell-type-dependent cytoprotection.

**Discussion**

In this study we showed that cumulative H$_2$O$_2$ treatments compared to bolus and H$_2$O$_2$ treatment before EGCG or H$_2$O$_2$ challenge protected HaCat cells by reducing the DNA damage and cytotoxicity. Similar results were achieved when H$_2$O$_2$ was replaced by EGCG. Accordingly, freshly prepared EGCG continuously generating H$_2$O$_2$ was less toxic than aged EGCG containing the final level of H$_2$O$_2$. To our knowledge, this is the first demonstration that cell protection by EGCG is based on its antioxidative potency.

**EGCG-generated H$_2$O$_2$ as cell protector**

The autoxidative ROS formation and cellular toxicity have been considered an in vitro artifact of EGCG in cell culture media [13,14]. Several cytotoxic and genotoxic effects of EGCG on cells in culture have been connected causatively with the extent and rate of H$_2$O$_2$ production [18] in various cell culture media, as well as the differences in the potency of media to catalyze the generated H$_2$O$_2$ [42], as proven by the substantial abrogation of these effects in presence of catalase [13,18]. However, pro-oxidant activities of dietary polyphenols in vivo, so far the subject of discussion [13,16,43], have recently been confirmed by verifying the generation of intracellular and mitochondrial ROS in tumor xenografts after EGCG treatment [1].

Dietary antioxidants have been shown to directly interact with multiple cellular targets including receptors for mitogenic signals [3] and to activate the redox-sensitive Keap1–Nrf2–ARE signaling pathway [10], which represents a major mechanism in the cellular defense against oxidants, inflammatory states, and chemical carcinogens [10,44]. Activation of the Nrf2 transcriptional factor, which recently was demonstrated to occur within minutes [45], regulates phase II detoxification enzymes and antioxidant proteins and significantly reduces the susceptibility of cells to toxic damage. Potent inducers of Nrf2 are oxidative stressors including exogenous [46] and endogenous H$_2$O$_2$ [11] produced in vivo by various mechanisms [47]. Physiological intracellular H$_2$O$_2$ concentrations are in the submicromolar range, whereas in exposed tissues such as the eye lens H$_2$O$_2$ can reach up to 25 $\mu$M [48]. In blood plasma H$_2$O$_2$ concentrations between 0.25 and 5 $\mu$M were found [49], corresponding to values that were demonstrated in this study as protective against further ROS challenges.

![Fig. 6. Cytotoxicity in cumulative and pretreatment experiments. (A) H$_2$O$_2$ treatment was performed in three cumulative aliquots (one aliquot/day) 24 h after cell seeding. Cell survival was determined by the trypan blue exclusion method 24 h after the final aliquot or bolus. Data are means±SD; n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, cumulative aliquots vs bolus and last aliquot, respectively, ANOVA, Bonferroni. (B) Cumulative vs bolus EGCG$_{fp}$ treatment was performed as described for (A). Data are means±SD; n = 3; *p < 0.05, **p < 0.01, cumulative aliquots vs bolus, ANOVA, Bonferroni. (C) H$_2$O$_2$ pretreatment was performed in two steps (one/day) 24 h after cell seeding, and growth rate (cell proliferation since seeding) was determined 24 h after the EGCG$_{fp}$ challenge. Data are means±SD; n = 3; *p < 0.05, pretreatment vs bolus respective control, ANOVA, Bonferroni. (D) EGCG$_{fp}$ pretreatment was performed in two steps (one/2.30 h) performed 24 h after cell seeding, and cell survival was determined 2 h after H$_2$O$_2$ bolus at the concentrations as indicated. Data are means±SD; n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, pretreatment vs bolus, Student’s t-test.](image-url)
H$_2$O$_2$ has traditionally been seen only as a cell-damaging molecule when oxidative stress and subsequent cell damage occur because of an imbalance between cellular ROS and antioxidant capacity. Currently H$_2$O$_2$ is additionally recognized as a second messenger in a physiologically relevant manner [47], contributing dose-dependently to cell proliferation, migration, and survival [50]. ROS as unique players under physiological conditions have been demonstrated recently by the fact that NADPH oxidase-deficient and ROS-impaired mouse keratinocytes are sensitized to apoptotic stimuli [51] and by the prosurvival activity of H$_2$O$_2$ in response to apoptotic stimuli in retinaderived cells [52].

The involvement of polyphenols in the regulation of inflammation and redox signaling and the induction of several stress-response-related cytoprotective enzymes [10,53–55] implies that polyphenols activate responses commonly initiated by stress conditions. Natural antioxidants have recently been shown to induce dose-dependently either prooxidant or antioxidant effects in endothelial cells [56]. These data have recently been shown to induce dose-dependently either prooxidant or antioxidant effects in endothelial cells [56]. These data have recently been shown to induce dose-dependently either prooxidant or antioxidant effects in endothelial cells [56]. These data have recently been shown to induce dose-dependently either prooxidant or antioxidant effects in endothelial cells [56]. These data have recently been shown to induce dose-dependently either prooxidant or antioxidant effects in endothelial cells [56]. These data have recently been shown to induce dose-dependently either prooxidant or antioxidant effects in endothelial cells [56]. These data have recently been shown to induce dose-dependently either prooxidant or antioxidant effects in endothelial cells [56]. These data have recently been shown to induce dose-dependently either prooxidant or antioxidant effects in endothelial cells [56]. These data have recently been shown to induce dose-dependently either prooxidant or antioxidant effects in endothelial cells [56]. These data have recently been shown to induce dose-dependently either prooxidant or antioxidant effects in endothelial cells [56]. These data have recently been shown to induce dose-dependently either prooxidant or antioxidant effects in endothelial cells [56].

Hormesis refers to low-dose trigger of responses that are beneficial. A so-called hormesis dose–response model [55,57] is further encouraged by the repetitiveness of H$_2$O$_2$-caused adaptive stress responses showing alterations in the expression of several cellular genes involved in defense pathways [26,27,58,59], including increases in total glutathione, antioxidant enzyme activity, heme oxygenase activity, stress protein gene expression, DNA repair pathways [59], and elevation in catalase protein and mRNA levels [26] and catalase gene amplification [59]. Hormesis refers to low-level stresses that protect against a subsequent challenge with higher doses of the same or a similar agent by activating stress resistance and increasing growth rates [60]. This concept of a physiological beneficial effect is corroborated by reports of stronger protective effects after frequent tea consumption [61,62] and a higher antioxidant potential after daily and not acute fruit intake [63]. Moreover, it is also consistent with our findings that cumulative treatments and treatment before subsequent challenges were cytoprotective.

**H$_2$O$_2$/ROS as stressors and/or physiological signal molecules**

In this study, we report that H$_2$O$_2$ (0.01–5 μM) and EGCG (0.1–10 μM) had the potential to induce stress resistance. These concentrations also stimulated the growth rate after 24 h treatment (not shown) before cell survival was reduced at increasing concentrations. Concordantly, a low-dose trigger of responses that is beneficial has been shown for several dietary components [57]. Data from human studies support that low-dose intake of EGCG (2 mg/kg po, for calculation: 1 μg EGCG/ml = 2.18 μM) did not exert serious adverse effects [64]. Green tea generates over time up to millimolar H$_2$O$_2$ concentrations in the mouth [15], and plasma levels of polyphenols range at the micromolar level, which implies a much greater concentration in stomach and intestinal lumen [48]. Theoretically EGCG-derived H$_2$O$_2$ concentrations may be achieved in vivo that correspond to concentrations of H$_2$O$_2$ shown to affect cell signaling pathways that activate adaptive responses to further stressors.

Feeding experiments in animals gave pharmacological evidence of a widespread tissue distribution of EGCG into nearly all organs [65] with highest levels in the intestine, followed by kidney and liver, from where EGCG is mainly excreted through bile [53]. This biodistribution might explain ROS-induced liver toxicity after intake of high-dose green-tea-based supplements (10–29 mg/kg/day po) [21] and bolus application of EGCG (750–1500 mg/kg, ig) in mice [22], as well as morbidity and/or mortality in fasted dogs fed with 150–500 mg EGCG/kg/day [66].

After regular tea consumption, which is generally regarded as safe, EGCG (up to 90% in a free form in plasma [53]) rarely exceeds 1 μM concentrations in blood or urine [67,68], which corresponds to EGCG concentrations that we have shown to inhibit H$_2$O$_2$-induced DNA damage. It may be hypothesized that in vivo ROS formation mediated by regular green tea consumption reaches physiological threshold concentrations of H$_2$O$_2$ [69] involved in various physiological functions including defense mechanisms [24,25,70].

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