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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 H_2O_2, have been suggested to occur in vitro but also in vivo. In this study, we address whether autoxidation-derived H_2O_2 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 H_2O_2 (IC_{50} 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 H_2O_2 clearance, improved DNA repair, and reduced intracellular ROS generation. Cumulative versus bolus EGCG and H_2O_2 treatment and H_2O_2 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 H_2O_2 and EGCG. In summary, our data suggest that autoxidative generation of low-dose H_2O_2 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.

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 antioxidant 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 H_2O_2, under cell culture conditions [12–14]. Recently, EGCG-induced ROS generation has been verified in an in vivo system [1], which now gives new impulse to the ongoing discussion of whether ROS, apart from the proven H_2O_2 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 H_2O_2 [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 H_2O_2 acts destructively or functions as a cellular signaling molecule with diverse physiological functions [23–25]. A repetitive low-level H_2O_2 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].

Abbreviations: DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; EGCG, (−)-epigallocatechin-3-gallate; $EGCG_{fp}$, freshly prepared EGCG; $EGCG_a$, aged EGCG; PPAR, peroxisome proliferator-activated nuclear receptor; ROS, reactive oxygen species; TB, trypan blue.

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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 H_2O_2 treatment followed by subsequent challenges with H_2O_2 or EGCG or cumulative H_2O_2 treatments compared to bolus. All H_2O_2 treatment designs were performed concurrent with EGCG. Furthermore, EGCG was used as a freshly prepared solution ($EGCG_{fp}$) continuously generating H_2O_2 and as an aged solution ($EGCG_a$) already containing a stable level of H_2O_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 H_2O_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′-dichlorodihydrofluorescein 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 99.7%), 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 epidermal 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 autoxidative 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 H_2O_2 [29], all treatments were performed in six-well plates (Falcon; BD Biosciences, San Jose, CA, USA) at the density of 7×10^5 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 (1.8×10^5 cells/well) to result at the time of bolus at about 7×10^5 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 $EGCG_a$ 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 (Axioscope, 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 followed by 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

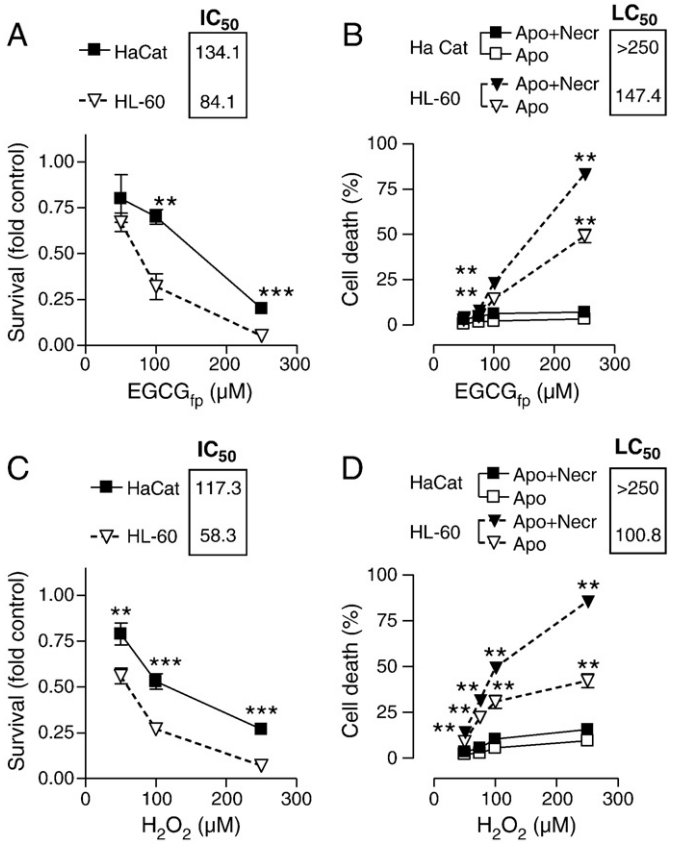

Fig. 1. Cytotoxicity after EGCG and H_2O_2 treatment of HaCat and HL-60 cells. Cells were treated for 24 h with $EGCG_{fp}$ and H_2O_2 (see Materials and methods). Cell death was determined by the trypan blue method (necrosis) and analysis of Hoechst 33258-stained cytospin preparations (apoptosis). (A 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 H_2O_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.

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_2O_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_2O_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_2O_2. Levels of H_2O_2 were determined after addition of EGCG or H_2O_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_2O_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_2O_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 oxidization 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_2O_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 FACSCalibur 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_2O_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_2O_2. Furthermore, HaCat cells were less sensitive to both agents. HL-60 compared to

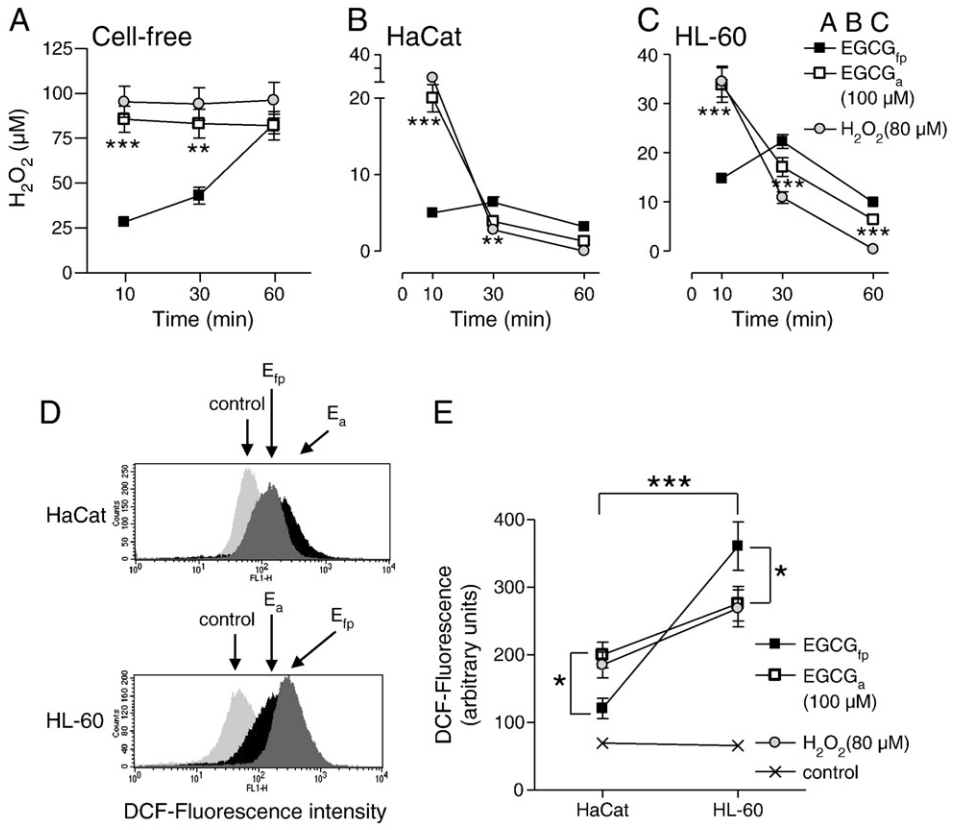

Fig. 2. Generation of H_2O_2 and intracellular ROS by EGCG and H_2O_2 treatment of HaCat and HL-60 cells. (A–C) H_2O_2 levels were determined at various time intervals after treatment in the (A) absence and (B, C) presence of cells using the FOX assay. $EGCG_{fp}$ (freshly prepared), $EGCG_a$ (aged), and H_2O_2 (at the concentration corresponding to the cell-free level generated after 60 min by EGCG) were used. Data are means ± SD; $n=6$; $^{**}p<0.01$, $^{***}p<0.001$, $EGCG_a$ vs $EGCG_{fp}$, Student's *t* test. (D, E) Intracellular ROS generation was measured after 1 h treatment using the DCFH-DA probe and analysis by flow cytometry. Agents and concentrations are indicated. Presented are representative FACS histograms (FL1, DCF fluorescence vs total counts) and the extent of DCF fluorescence. Data are means ± SD; $n=3$; $EGCG_{fp}$ vs $EGCG_a$, $^{*}p<0.05$ for HaCat and HL-60 cells; HaCat vs HL-60 cells, $^{*}p<0.05$, $^{***}p<0.001$ regarding $EGCG_{fp}$ and $EGCG_a$, ANOVA, Bonferroni.

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_2O_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_2O_2 concentrations, which reached a plateau level of 80 μM at 100 μM EGCG after 60 min. The $EGCG_a$ solution contained the 80 μM H_2O_2 concentration right from the start (Fig. 2A). These differences in the H_2O_2 levels at treatment onset also influenced the cell-mediated H_2O_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_2O_2 declined time-dependently (10, 30, 60 min) to 25, 5, and 1 μM, respectively (Fig. 2B), whereas in the presence of HL-60 cells the remaining H_2O_2 concentrations were up to 10-fold higher (35, 20, and 10 μM; Fig. 2C). Expectedly, $EGCG_{fp}$-generated H_2O_2 ranged below $EGCG_a$-generated H_2O_2 after 10 min treatment. The following H_2O_2 increase after 30 min was abolished after 60 min in the presence of HaCat cells. However, in the presence of HL-60 cells, $EGCG_{fp}$-generated H_2O_2 remained elevated at that time point (Fig. 2C).

The different potencies of HaCat and HL-60 cells regarding H_2O_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_2O_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_2O_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_2O_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_2O_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.

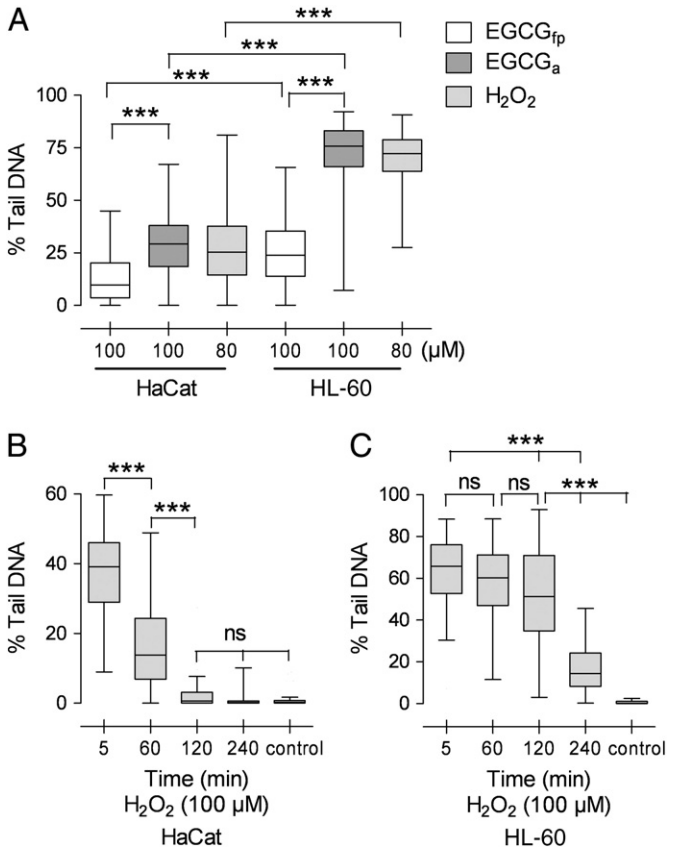

Fig. 3. DNA damage after EGCG and H_2O_2 treatment in HaCat and HL-60 cells. DNA damage was analyzed by using the comet assay (see Materials and methods). (A) Cells were treated with $EGCG_{fp}$, $EGCG_a$, and H_2O_2 for 5 min. Data boxplots of %tail DNA from three tubes/experimental point; $n = 150$; $^{***}p<0.001$, $EGCG_a$ vs $EGCG_{fp}$ for HaCat and HL-60 cells, respectively, and HaCat vs HL-60 cells regarding $EGCG_{fp}$, $EGCG_a$, and H_2O_2, Mann–Whitney). (B, C) Repair of DNA damage at various time points after H_2O_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.

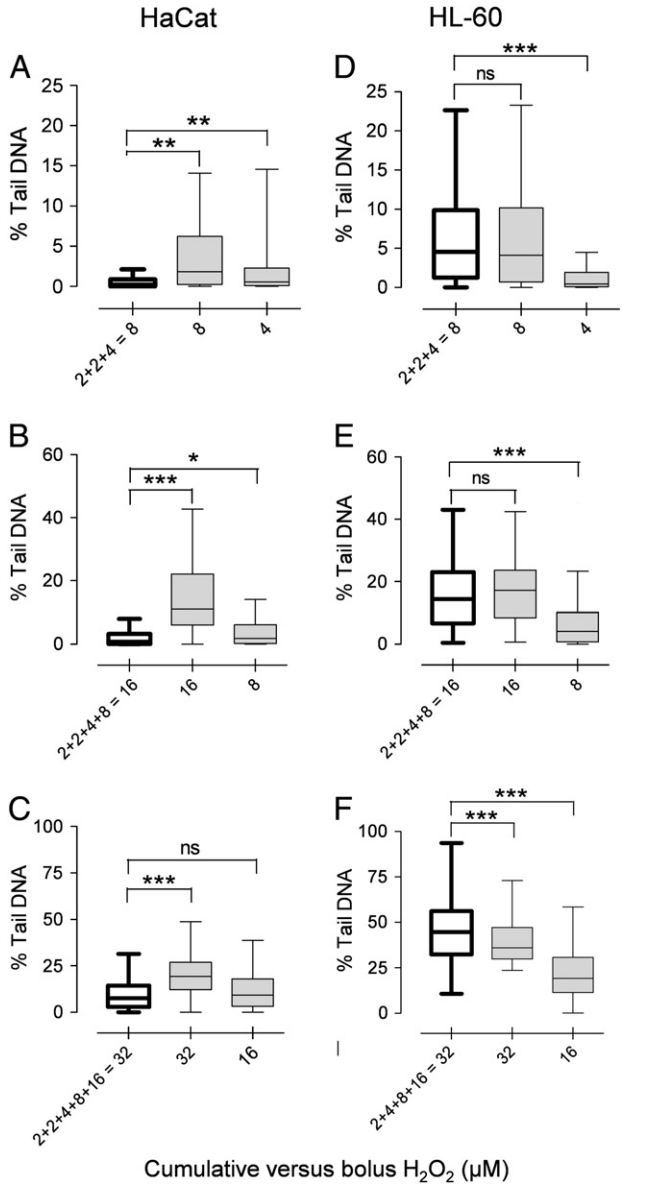

Fig. 4. DNA damage after cumulative and bolus treatment with H_2O_2 in HaCat and HL-60 cells. H_2O_2 treatment was performed in three versions: as a bolus and as three to five cumulative aliquots applied within a maximum of 10 min at 2-min intervals. Concentrations are indicated. Comet assay was performed 5 min after bolus and cumulative low-dose treatment. Data boxplots of %tail DNA from three tubes/experimental point; $n = 150$. (A–C) $^{**}p<0.01$, $^{***}p<0.001$, cumulative vs bolus; $^{*}p<0.05$, $^{**}p<0.01$, 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 $EGCG_a$ in both cell lines. The effects of $EGCG_a$ were similar to those induced by the respective H_2O_2 concentration (Fig. 3A).

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

Time response of H_2O_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_2O_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 rejoining of H_2O_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 $EGCG_a$ and $EGCG_{fp}$.

Cumulative H_2O_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_2O_2 in freshly prepared EGCG solution (Fig. 2A) may be responsible for the consistently lower DNA damage levels by $EGCG_{fp}$ than by $EGCG_a$ treatment in HaCat cells. To resolve this question, cells were treated with either H_2O_2 as a bolus (corresponding to $EGCG_a$) 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_2O_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_2O_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_2O_2 concentrations induce protective effects within minutes in HaCat but not HL-60 cells.

Low-dose H_2O_2 treatment before EGCG and EGCG before H_2O_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_2O_2 concentrations below 10 μM applied before a 50 μM EGCG challenge reduced the DNA-damaging effect significantly (Fig. 5A). H_2O_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_2O_2 challenge (Fig. 5C). Addition of catalase during H_2O_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_2O_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_2O_2 as well as $EGCG_{fp}$ in gradually increasing portions improved the

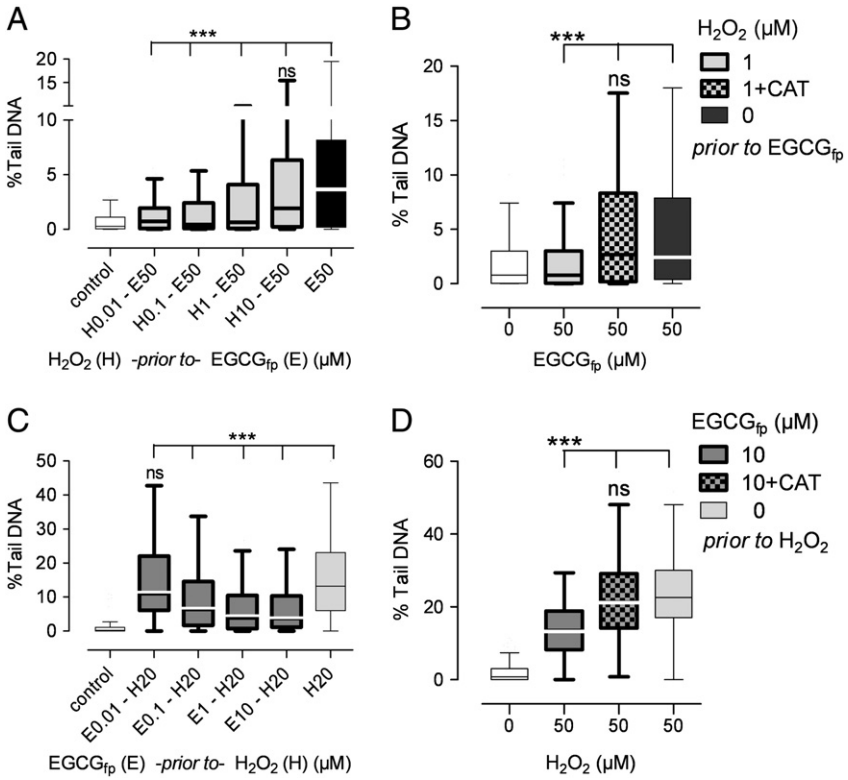

Fig. 5. DNA damage after H_2O_2 pretreatment before EGCG challenge and EGCG before H_2O_2 bolus with and without catalase. HaCat cells were pretreated 30 min before the challenge and comet analysis was performed 5 min after exposure. (A) Cells were challenged with $EGCG_{fp}$ after pretreatment with H_2O_2 at the indicated concentrations. Data boxplots of %tail DNA from three tubes/experimental point, $n=150$; $^{***}p<0.001$, ns, not significant, pretreatment vs bolus, Kruskal–Wallis. (B) Catalase (100 U/ml) was included during the pretreatment time. Data boxplots of %tail DNA from three tubes/experimental point; $n=150$; $^{***}p<0.001$, pretreatment vs bolus; ns, not significant, pretreatment with the addition of catalase vs bolus, Kruskal–Wallis. (C) Cells were challenged with H_2O_2 after pretreatment with $EGCG_{fp}$ at the indicated concentrations. Data boxplots of %tail DNA from three tubes/experimental point, $n=150$; $^{***}p<0.001$, pretreatment vs bolus, Kruskal–Wallis. (D) Catalase (100 U/ml) was included during the $EGCG_{fp}$ pretreatment time. Data boxplots of %tail DNA from three tubes/experimental point; $n=150$; $^{***}p<0.001$, pretreatment vs bolus; ns, not significant, pretreatment with the addition of catalase vs bolus, Kruskal–Wallis).

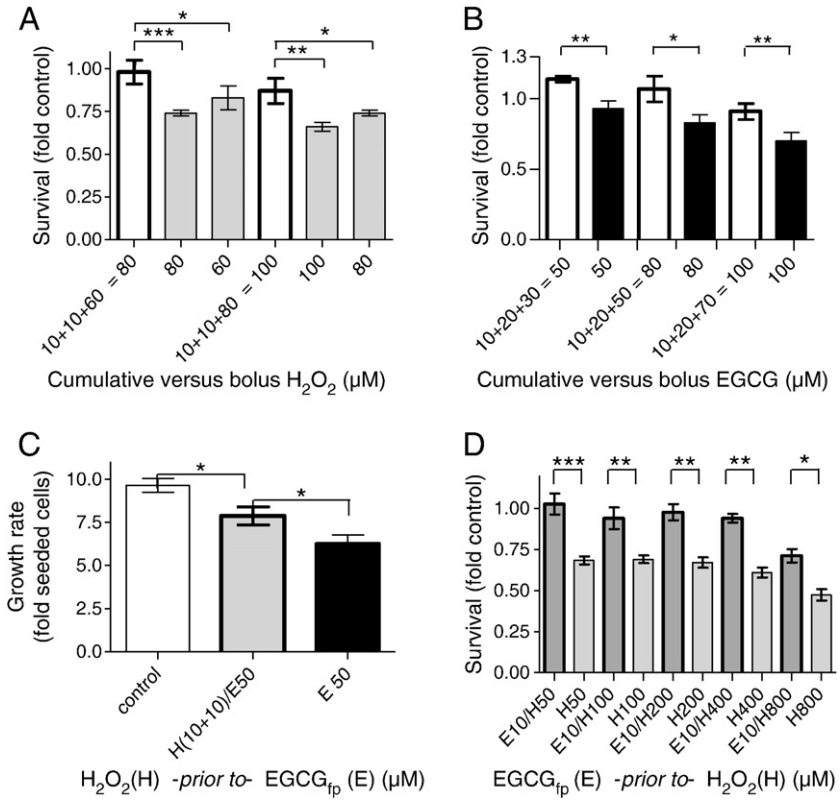

Fig. 6. Cytotoxicity in cumulative and pretreatment experiments. (A) H_2O_2 treatment was performed in three cumulative aliquots (one aliquot/day) 24 h after cell seeding. Cell survival was determined by the trypan blue exclusion method 24 h after the final aliquot or bolus. Data are means ± SD; $n = 3$; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, 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_2O_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_2O_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.

viability of cells compared to bolus or even last-aliquot bolus (Figs. 6A and B). In addition, low-concentration H_2O_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_2O_2 reduced the growth-inhibitory effect of $EGCG_{fp}$ (Fig. 6C). Pretreatment with EGCG (10 μM) was performed twice for 2.30 h each and cell viability was measured 2 h after H_2O_2 bolus at concentrations ranging from 50 to 800 μM (Fig. 6D). Corresponding to previous studies [40], EGCG pretreatment reduced H_2O_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_2O_2 like that generated by EGCG autoxidation can mediate cell-type-dependent cytoprotection.

Discussion

In this study we showed that cumulative H_2O_2 treatments compared to bolus and H_2O_2 treatment before EGCG or H_2O_2 challenge protected HaCat cells by reducing the DNA damage and cytotoxicity. Similar results were achieved when H_2O_2 was replaced by EGCG. Accordingly, freshly prepared EGCG continuously generating H_2O_2 was less toxic than aged EGCG containing the final level of H_2O_2. To our knowledge, this is the first demonstration that cell protection by EGCG is based on its autoxidative potency.

EGCG-generated H_2O_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_2O_2 production [18] in various cell culture media, as well as the differences in the potency of media to catalyze the generated H_2O_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_2O_2 [11] produced in vivo by various mechanisms [47]. Physiological intracellular H_2O_2 concentrations are in the submicromolar range, whereas in exposed tissues such as the eye lens H_2O_2 can reach up to 25 μM [48]. In blood plasma H_2O_2 concentrations between 0.25 and 5 μM were found [49], corresponding to values that were demonstrated in this study as protective against further ROS challenges.

H_2O_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_2O_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_2O_2 in response to apoptotic stimuli in retina-derived 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 pro-oxidant or antioxidant effects in endothelial cells [56]. These data suggest a tuned interaction of polyphenol-generated H_2O_2 with the cellular redox homeostasis either through cytotoxic actions or as a potent mechanism to modulate intracellular signaling [9,25]. A so-called hormesis dose–response model [55,57] is further encouraged by the repetitive H_2O_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 stressors 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_2O_2/ROS as stressors and/or physiological signal molecules

In this study, we report that H_2O_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 are 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_2O_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_2O_2 concentrations may be achieved in vivo that correspond to concentrations of H_2O_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_2O_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_2O_2 [69] involved in various physiological functions including defense mechanisms [24,25,70]. H_2O_2 crosses cell membranes readily [71] and represents a vital cellular compound continuously generated in the cell during respiration and the activity of enzymes including the NADPH oxidase system. Cells are accustomed to detoxifying H_2O_2, depending on their innate and specific constitution; an imbalance between cellular production and elimination of free radical species initiates cellular oxidative stress. Otherwise ROS generation is essential in the regulation of gene expression and modulation of enzyme activities maintaining cellular homeostasis [14,47,72].

Cell-type dependence

Various cancer subtypes and cells with malignant versus nonmalignant phenotypes are differently susceptible to dietary compounds [68,73]. In this study we have included two cell lines, HaCat and HL-60, which were highly different in cyto- and genotoxic sensitivities to H_2O_2 and EGCG. The two cell models were demonstrated to differ in their potency of H_2O_2 clearance, intracellular ROS generation, and DNA repair capacity.

These differences might reflect their cellular origin from skin and the hematopoietic system, respectively. In contrast to promyelocytic leukemia cells [74], keratinocytes, from which HaCat cells were derived, are equipped with highly efficient oxidative defense and repair mechanisms as a consequence of their exposed position and the concomitant frequent encounters with stressors [75]. Accordingly, H_2O_2 and EGCG IC_{50} for HL-60 were 40 to 50% and the LC_{50} were several magnitudes lower than the values for HaCat cells. Notably, substantial apoptotic cell death was found only in HL-60. The activation of the PPAR-β transcription factor through stress-associated kinase pathways [76] has been shown to be critical in enabling HaCat keratinocytes to resist cell death [77]. PPAR-β, involved in the control of cell growth and differentiation of keratinocytes in the response to inflammation [78], has also been shown to become activated by low-grade H_2O_2 stress-induced cell protection [27]. Comparable protective features in THP1 acute monocytic leukemia cells (data not shown) exclude the possibility that the pro-oxidative induction of cytoprotection is a specific characteristic of keratinocyte-derived cells only.

Conclusion

Our data provide strong evidence that autoxidation-derived H_2O_2 plays a role in EGCG-mediated antioxidant protection. The recently published proof of in vivo pro-oxidant activities of EGCG [1] suggests that with daily green tea consumption cells may be modified to better cope with various oxidative stressors. Nevertheless, bolus experiments suggest caution to avoid EGCG overdosing as preparations marketed as dietary supplements and functional foods that contain EGCG or other phytochemicals are usually used beyond medical control.

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