

Roles of Catalase and Hydrogen Peroxide in Green Tea Polyphenol-Induced Chemopreventive Effects

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ABSTRACT

The green tea polyphenol (–)-epigallocatechin-3-gallate (EGCG) possesses promising anticancer potential. Although in vivo studies unveiled the metabolic routes and pharmacokinetics of EGCG and showed no adverse effects, in vitro studies at high concentrations demonstrated oxidative stress. EGCG causes differential oxidative environments in tumor versus normal epithelial cells, but the roles that EGCG, hydrogen peroxide (H₂O₂), and intracellular catalase play in the epithelial system are largely unknown. The current study employed enzyme activity assays, reactive oxygen species quantification, and immunoblotting to investigate whether EGCG-induced differential effects correlate with levels of key antioxidant enzymes and H₂O₂. It was found that normal human keratinocytes with high catalase activity are least susceptible to

H₂O₂, whereas H₂O₂ caused significant cytotoxicity in oral carcinoma cell lines. However, the EGCG-induced differential effects could not be duplicated by H₂O₂ alone. The addition of exogenous catalase failed to completely prevent the EGCG-induced cytotoxicity and rescue the EGCG-induced growth arrest in the tumor cells. The antioxidant *N*-acetyl-L-cysteine rescued the tumor cells from H₂O₂-induced damage only, but not from EGCG-induced mitochondrial damage. Finally, alterations in catalase or superoxide dismutase activities were not observed upon EGCG exposure. In conclusion, although endogenous catalase may play a role in response to H₂O₂-induced cytotoxicity, the EGCG-induced cytotoxic effects on tumor cells mainly result from sources other than H₂O₂.

Green tea polyphenols (GTPP), particularly (–)-epigallocatechin-3-gallate (EGCG), are strong antioxidants (Tanaka, 2000; Higdon and Frei, 2003). The ability of these compounds to scavenge reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) and superoxide radicals depends on their phenolic chemical structures (Wei et al., 1999; Zhu et al., 2000). GTPP, especially EGCG, may help to protect various cells from chemical or physical damage that leads to carcinogenesis (Wei et al., 1999; Tanaka, 2000; Katiyar and Elmets, 2001; Chen et al., 2002; Lee et al., 2003). Conversely, GTPP and EGCG induce cytotoxicity and apoptosis in many types of

tumor cells (Lin et al., 1999; Roy et al., 2003). The EGCG-induced apoptosis has been reported to be associated with oxidative stress imposed on tumor cells, especially by H₂O₂ (Long et al., 1999; Yang et al., 2000; Zhu et al., 2000). EGCG-induced production of H₂O₂ was recently observed under in vitro conditions with or without the presence of cells (Long et al., 1999; Hong et al., 2002). The EGCG-induced oxidative stress triggers an apoptotic pathway that is distinct from chemical or Fas-mediated pathways and acts through activation of mitogen-activated protein kinases, c-Jun N-terminal kinase and p38, and the caspase cascade (Kong et al., 1998; Yang et al., 2000; Balasubramanian et al., 2002; Saeki et al., 2002; Chen et al., 2003a). This apoptotic pathway also involves activator protein-1 (AP-1) inactivation (Dong, 1997; Barthelman et al., 1998; Balasubramanian et al., 2002). Apoptosis induced by EGCG in certain in vitro cell models was reversed by exogenous catalase, suggesting that H₂O₂ was

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ABBREVIATIONS: GTPP, green tea polyphenols; EGCG, (–)-epigallocatechin-3-gallate; ROS, reactive oxygen species; AP-1, activator protein-1; NHEK, normal human primary epidermal keratinocytes; SOD, superoxide dismutase; NAC, *N*-acetyl-L-cysteine; 3-AT, 3-amino-1,2,4-triazole; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; DFDA, dihydrofluorescein diacetate; HPS, Hallam's physiological saline; PBS, phosphate-buffered saline.

the main cause of apoptotic pathway activation (Nakagawa et al., 2002; Chai et al., 2003). It was also noted that whereas at low concentrations EGCG (<10 μM) functions as a ROS scavenger, at high concentrations it functions as a ROS producer and can cause DNA damage (100 μM and above) (Saeki et al., 2002). These observations led to a hypothesis that GTPP/EGCG-induced apoptosis under in vitro conditions is artifactual, especially when the GTPP or EGCG concentration is higher than the C_{max} in the plasma (10 μM), since high levels of H_2O_2 cannot be achieved in vivo (Halliwell, 2003). However, it is not clear whether 1) EGCG-induced apoptosis in tumor cells is indeed due to H_2O_2 generated in the culture medium (Halliwell, 2003), or 2) H_2O_2 is irrelevant to EGCG-induced responses when the EGCG concentration is at physiological levels (Dashwood et al., 2002). Thus, it is important to determine whether H_2O_2 generated under in vitro experimental conditions by EGCG at concentrations greater than 10 μM could be the driving force for tumor cell apoptosis (Hong et al., 2002). We hypothesized that EGCG-induced intracellular signaling (and the subsequent effects on the cell) depends upon the combination of many factors, such as the concentration of EGCG, the origin of the cells, the culture medium used, and the intracellular antioxidant enzymatic activity/quantity of the cell population.

We previously observed that GTPP/EGCG activate different pathways depending on the cell type (Hsu et al., 2001). EGCG at concentrations significantly higher than the C_{max} found in the serum activates the survival pathway associated with terminal differentiation in normal epidermal keratinocytes, and the apoptotic pathway in oral carcinoma cells (Hsu et al., 2002a, 2003a). Recently, we showed that EGCG in the 15 to 200 μM range reduced ROS/ H_2O_2 to background levels in normal human primary epidermal keratinocytes (NHEK) and immortalized normal human salivary gland cells, whereas intracellular ROS/ H_2O_2 levels were significantly elevated in oral carcinoma cells (Yamamoto et al., 2003). This evidence suggests that high concentrations of EGCG could still be considered physiologically and clinically relevant for certain cells/tissues since the digestive tract and the epidermis can be exposed to significant levels of GTPP from the environment. Whether the key intracellular ROS scavenging enzymes catalase and superoxide dismutase (SOD) are differentially regulated by EGCG in normal versus tumor cells, or whether EGCG-induced cytotoxicity and growth arrest in tumor cells can be reversed by catalase or antioxidant is not clear. The current study was designed to address these questions and to compare the effects of EGCG with H_2O_2 in normal versus tumor cells.

Materials and Methods

Cell Lines. NHEK were obtained from Cambrex Bio Science Baltimore, Inc. (Baltimore, MD) and maintained in KGM-2 medium (Cambrex Bio Science Baltimore, Inc.). The OSC-2 and OSC-4 cell lines, which were isolated from cervical metastatic lymph nodes of patients with oral squamous cell carcinoma (Yamamoto et al., 2003), were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 50/50 MIX medium (Cellgro, Kansas City, MO) and supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 5 $\mu\text{g}/\text{ml}$ hydrocortisone.

Reagents. Catalase, diamide, EGCG, H_2O_2 , *N*-acetyl-L-cysteine (NAC), 3-amino-1,2,4-triazole (3-AT), and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from

Sigma-Aldrich (St. Louis, MO). Dihydrofluorescein diacetate (DFDA) and SOD were obtained from Molecular Probes Inc. (Eugene, OR) and ICN Biomedicals Inc. (Aurora, OH), respectively.

MTT Assay. This method quantitatively measures the viability of cells when stress is introduced in cell cultures through chemical or physical means. In a 96-well microplate, 1.5×10^4 cells were seeded in each well. After 24 h of treatment of EGCG at indicated doses, culture medium was removed and replaced with 100 μl of 2% MTT in a solution of 0.05 M Tris, 0.5 mM MgCl_2 , 2.5 mM CoCl_2 , and 0.25 M disodium succinate as substrate (Sigma-Aldrich), and the plate was incubated at 37°C for 30 min. Then 100 μl of 0.2 M Tris-HCl (pH 7.7) containing 4% (v/v) formalin was added to each well, and the microplate was incubated for 5 min at room temperature. After the incubation, the contents in each well were aspirated, and each well was rinsed with 200 μl of H_2O followed by the addition of 100 μl of dimethyl sulfoxide containing 6.25% (v/v) 0.1 N NaOH. Solubilized colored formazan product was measured using a Thermo MAX microplate reader (Molecular Devices Corp., Sunnyvale, CA) at a wavelength of 562 nm.

Measurement of Intracellular ROS Levels. The ROS assay (DFDA assay) measures the accumulation of intracellular ROS levels. The non-fluorescent dye DFDA passively diffuses into cells, in which the acetates are cleaved by intracellular esterases. The metabolites are trapped within the cells and oxidized by ROS, mainly H_2O_2 , to the fluorescent form 2',7'-dichlorofluorescein, which can be measured by a fluorescence plate reader to reflect levels of intracellular ROS (mainly H_2O_2). Thus, values of the fluorescence in the cell cultures are constantly rising in this assay due to the accumulation of ROS. Cells (1.5×10^4 cells/well) were incubated with Hallam's physiological saline (HPS) containing DFDA (10 μM) in a 96-well microplate for 30 min at 37°C. After the incubation, cells were washed three times with HPS and then incubated with HPS containing EGCG (50–200 μM) or diamide (5 mM) for the indicated times. The intracellular ROS levels were measured by using a fluorescence plate reader (BIO-TEK FL600; Bio-Tek Instruments, Inc., Winooski, VT) at an excitation wavelength of 485 nm and emission wavelength of 530 nm.

Caspase-3 Activity Assay. The caspase-3 apoptosis detection kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to measure caspase-3 activity. Cells (10^5 cells/well) were plated in triplicate in a 24-well tissue culture plate. After 24-h treatments with EGCG, the cells in each well were washed with 1 ml of PBS and incubated with 100 μl of cell lysis buffer on ice for 10 min. To each well, 100 μl of 2 \times reaction buffer was added with 10 mM dithiothreitol. Finally, 5 μl of DEVD-AFC substrate was added to each well containing cell lysates. The reaction mixtures were incubated for 1 h at 37°C, and caspase-3 activity in each well was measured using a fluorescence microplate reader (SPECTRAFLUOR Plus; Tecan US, Durham, NC) at a wavelength of 405 nm for excitation and 505 nm for emission.

Western Blotting. After EGCG treatments, cells were washed in ice-cold PBS and lysed for 10 min in 1 \times PBS containing 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 $\mu\text{g}/\text{ml}$ leupeptin, 3 $\mu\text{g}/\text{ml}$ aprotinin, and 100 mM phenylmethylsulfonyl fluoride. Samples of lysates containing 25 μg of protein were loaded in each lane and electrophoretically separated on a 7.5% SDS polyacrylamide gel. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Trans-Blot Transfer medium; Bio-Rad, Hercules, CA). The membrane was blocked for 1 h with 5% (w/v) nonfat dry milk powder in PBST (0.1% Tween-20 in PBS) and then incubated for 1 h with anti-catalase rabbit polyclonal antibody (Abcam Ltd., Cambridge, UK), anti-Mn-SOD rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY), and anti-actin goat polyclonal antibody (Santa Cruz Biotechnology, Inc.). The membrane was washed three times with PBST and incubated with peroxidase-conjugated, affinity-purified anti-rabbit or anti-goat IgG (Santa Cruz Biotechnology, Inc.) for 1 h. Following extensive washing, the reaction was developed by enhanced chemiluminescent staining using

ECL Western blotting detection reagents (Amersham Biosciences Inc.).

Assays for SOD and Catalase Activities. Cells (10^5 cells/well) were incubated with or without EGCG ($50 \mu\text{M}$) in 24-well culture plates for the indicated time periods at 37°C . After the incubation, cells were harvested and disrupted in $100 \mu\text{l}$ of 10 mM Tris-HCl (pH 7.4) containing 0.1% (v/v) Triton X-100, $10 \mu\text{g/ml}$ leupeptin, $10 \mu\text{g/ml}$ pepstatin A, and 100 mM phenylmethylsulfonyl fluoride by three cycles of freezing/thawing. After centrifugation at $17,000g$ for 20 min at 4°C , the supernatants were used for SOD and catalase assays using the SOD assay kit-WST (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) and the Amplex Red catalase assay kit (Molecular Probes, Eugene, OR), respectively. The activities of SOD and catalase were calibrated using a standard curve prepared with purified human SOD and catalase. The activities of SOD and catalase were expressed as units per milligram protein.

Statistical Analysis. All data are reported as mean \pm S.D. A one-way analysis of variance and unpaired Student's *t* tests were used to analyze statistical significance. Differences were considered statistically significant at $p < 0.05$.

Results

Susceptibility of NHEK and OSC Cell Lines to EGCG and H_2O_2 . After a 24 h of incubation with EGCG at various

concentrations, cell viability of NHEK was not altered (Fig. 1a). However, both OSC-2 and OSC-4 cells exhibited cytotoxicity. OSC-2 was the more sensitive cell line, with cell viability declining to less than 50% of untreated control levels after incubation with $200 \mu\text{M}$ EGCG (Fig. 1a). Unlike EGCG, H_2O_2 induced cytotoxicity in all cell types, with noticeable differences among the cell types. For instance, cell viability of all cell types gradually declined when H_2O_2 concentrations increased from 100 to $500 \mu\text{M}$. At H_2O_2 concentrations higher than $500 \mu\text{M}$, cell viability in the OSC cell lines decreased more rapidly than in NHEK. Treatment with 1 mM H_2O_2 caused a 25% reduction of cell viability in NHEK, but only $250 \mu\text{M}$ was needed to cause the same reduction in OSC-2 and OSC-4 cells. When these cells were exposed to 1 mM H_2O_2 for 24 h, the cell viability was reduced to less than 20% in both cell lines (Fig. 1b).

Generation of Intracellular ROS by EGCG Compared with Exogenous H_2O_2 in OSC Cell Lines. We previously showed that EGCG caused differential oxidative environments in normal versus tumor cells. EGCG at concentrations of 15 to $200 \mu\text{M}$ lowered ROS to background levels in NHEK (Yamamoto et al., 2003). In contrast, the current study showed that, following a 60-min exposure to

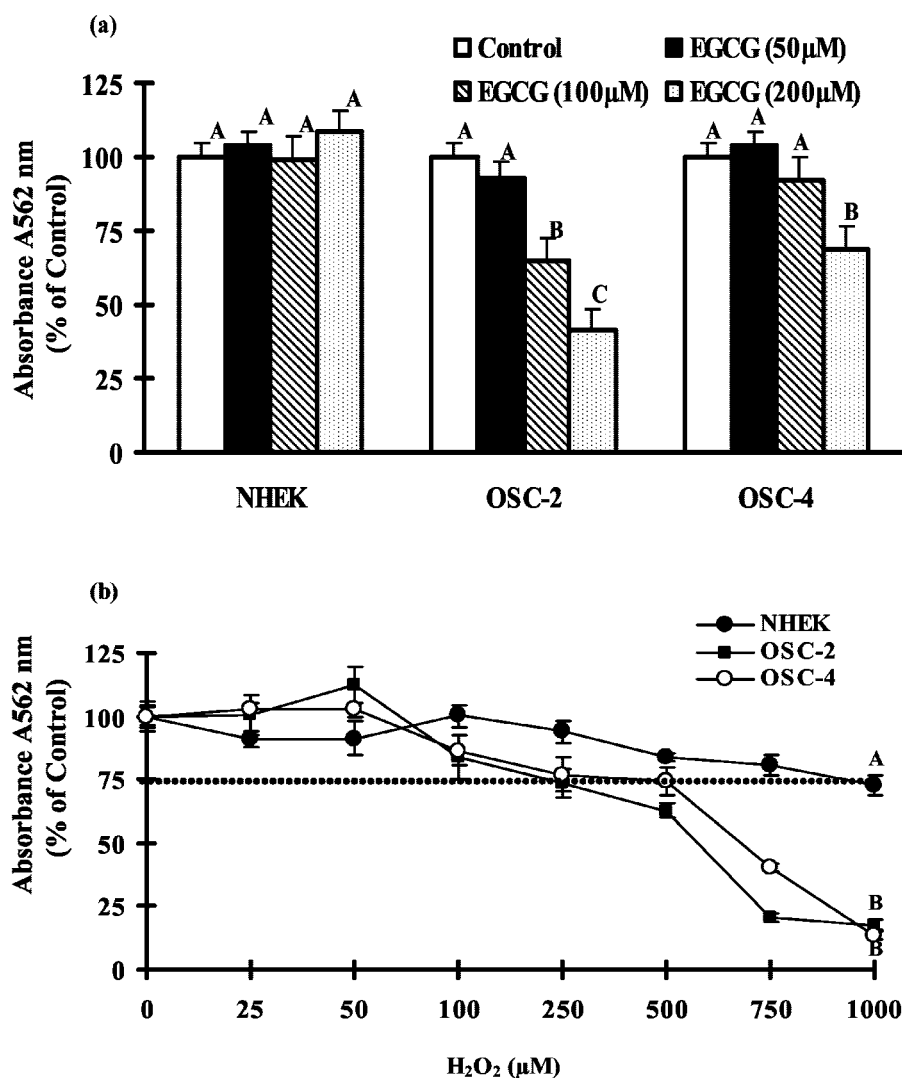


Fig. 1. MTT assay results from NHEK, OSC-2, and OSC-4 cells treated with EGCG or H_2O_2 . Cells were incubated with the indicated concentrations of EGCG (a) or H_2O_2 (b) for 24 h, followed by MTT assay. Each concentration was assayed in triplicate within an experiment. Data are expressed as percentage of untreated cells, and error bars represent one standard deviation of the mean. These figures are representative of three independent replications of the experiments, all with similar results. Different capital letters indicate statistically significant differences among cell types (ANOVA, Tukey post hoc test, $\alpha = 0.05$, $n = 3$).

either exogenous H_2O_2 or EGCG, both OSC-2 and OSC-4 cell lines exhibited a dose-dependent accumulation of intracellular ROS, as detected by DFDA (Fig. 2). Under identical conditions, 5 mM diamide-induced ROS in OSC-2 cells was double that found in OSC-4 cells and H_2O_2 at 100 or 200 μM (Fig. 2).

Comparison of the Effect of EGCG with NAC in OSC Cell Lines. Two-hour pretreatment with 10 mM NAC significantly inhibited the cytotoxic effect of H_2O_2 at 250 and 500 μM in OSC-2 and OSC-4 cell lines (Fig. 3a). EGCG at 200 μM significantly reduced cell viability in both OSC-2 and OSC-4 cell lines (Fig. 1a and Fig. 3b). However, NAC not only failed to rescue both cell lines from EGCG-induced cytotoxicity, it also enhanced the cytotoxicity measured by MTT assays seen at higher EGCG levels (Fig. 3b). Treatment with exogenous catalase or 3-AT, a catalase inhibitor, had no effect on this reduction (data not shown). Moreover, NHEK did not become susceptible to EGCG cytotoxicity after pretreatment with 3-AT (data not shown).

Impact of Catalase on EGCG-Induced Activation of Caspase-3. Exogenous catalase partially inhibited EGCG-induced caspase-3 activation in OSC-2 and OSC-4 cells during a 24-h period (Fig. 4).

Levels of Activity and Quantity of Endogenous Catalase and SOD in Response to EGCG Exposure. When enzymatic activities were compared among these cells, NHEK were shown to have the highest levels of catalase activity—twice that found in OSC-4 and three times that found in OSC-2 cells (Fig. 5a). However, OSC-2 cells exhibited the highest levels of total SOD activity, twice those found in either NHEK or OSC-4 cells (Fig. 5a). EGCG had no effect on the enzymatic activity levels during the 24-h treatment period, except for the catalase activity in OSC-4 cells, which showed a slight decrease (Fig. 5a). Of the three cell types, OSC-2 cells possessed the lowest amount of endogenous catalase protein compared with NHEK and OSC-4 cells, and—consistent with the activity levels—the highest levels of Mn-SOD protein levels. Significant alteration in the protein levels of these enzymes was not observed during the 24-h period following EGCG treatment (Fig. 5b). When exposed to EGCG, NHEK showed a slight decrease in catalase protein level and an increase in Mn-SOD protein at the 24-h time point (Fig. 5b).

Discussion

Previous *in vitro* studies demonstrated that EGCG induces differential effects in normal versus tumor cells. Effects of EGCG on tumor cells include generation of intracellular ROS, induction of growth arrest, inhibition of cell invasiveness, inhibition of AP-1, nuclear factor κB , activation of caspase cascade, and induction of apoptosis through regulation of mitogen-activated protein kinase pathway (Chung et al., 1999; Liang et al., 1999; Ahmad et al., 2000; Chen et al., 2000; Dong, 2000; Gupta et al., 2000, 2003; Kong et al., 2000; Liberto and Cobrinik, 2000; Yang et al., 2000; Jung and Ellis, 2001; Hsu et al., 2002b, 2003b; Saeki et al., 2002). In normal epithelial cells, the effects of EGCG involve reduction of intracellular ROS, increase in cell viability, induction of cell differentiation associated with activation of AP-1, p57, and caspase 14 (a terminal differentiation marker for epidermal keratinocytes; Hsu et al., manuscript under review) (Hsu et al., 2001, 2002a, 2003a; Balasubramanian et al., 2002). Based on these observations, the roles of H_2O_2 and endogenous antioxidant enzymes in EGCG-induced effects are unlikely to be identical among different cell types from various origins. For example, EGCG elevated ROS levels (especially H_2O_2) in tumor cells, but not in NHEK or immortalized normal salivary gland cells. This correlated with apoptotic pathways in the tumor cells and survival pathways in the normal cells (Yamamoto et al., 2003). In addition, elimination of H_2O_2 by addition of catalase could not prevent EGCG-induced inhibition of AP-1 and activation of c-Jun N-terminal kinase and extracellular signal-regulated kinase, suggesting that EGCG signaling might not solely rely on oxidative stress (Chung et al., 1999).

The current study further confirmed that high concentrations of EGCG damaged only tumor cells (OSC-2 and OSC-4), but not normal cells (NHEK) (Fig. 1a). This EGCG-induced differential effect was observed in coculture of OSC-2 cells and NHEK in a 50:50 mix of KGM-2 and Dulbecco's modified Eagle's medium/Ham's F-12, therefore eliminating the medium as the source of the difference (data not shown). Importantly, the EGCG-induced differential effect in normal versus tumor cells could not be reproduced entirely by H_2O_2 alone (Fig. 1b). OSC cell lines showed a significant decline in cell viability at H_2O_2 concentrations of 250 μM or more, and the viable cells were reduced to less than 25% of control levels

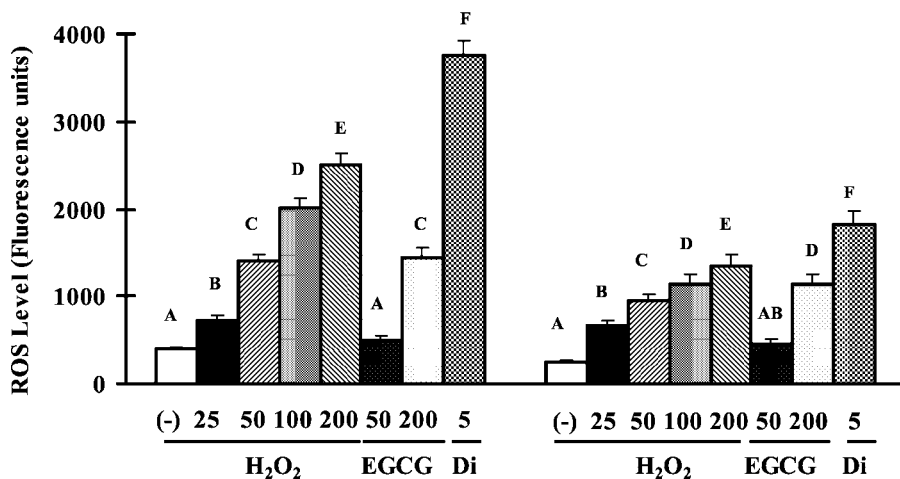


Fig. 2. Intracellular ROS formation in OSC-2 and OSC-4 cells exposed to H_2O_2 (25–200 μM), EGCG (50 and 200 μM), and diamide (Di, 5 mM). Cells were incubated with or without these agents for 60 min, and then intracellular ROS levels were determined by the ROS (DFDA) assay. ROS levels are represented by relative fluorescence units. Each concentration was assayed in triplicate within an experiment. Error bars represent one standard deviation of the mean. These figures are representative of three independent replications of the experiments, all with similar results. Different capital letters indicate statistically significant differences among conditions (ANOVA, Tukey post hoc test, $\alpha = 0.05$, $n = 3$).

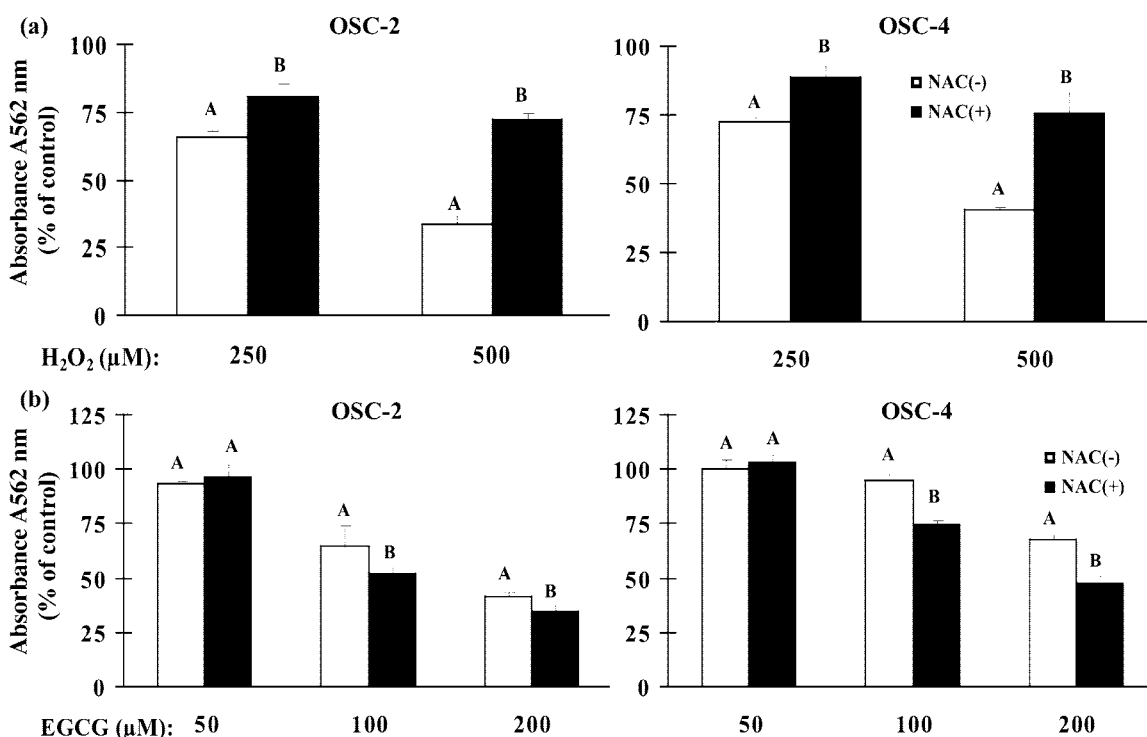


Fig. 3. MTT assay result of OSC-2 and OSC-4 cells pretreated with NAC followed by incubation with either H₂O₂ or EGCG. OSC-2 and OSC-4 cells were pretreated with or without 10 mM NAC for 2 h prior to incubation with the indicated concentrations of H₂O₂ (a) or EGCG (b) followed by MTT assay. Each concentration was assayed in quadruplicate within an experiment. Data are expressed as percentage of untreated cells, and error bars represent one standard deviation of the mean. These figures are representative of three independent replications of the experiments, all with similar results. Different capital letters indicate statistically significant differences among conditions (ANOVA, Tukey post hoc test, $\alpha = 0.05$, $n = 4$).

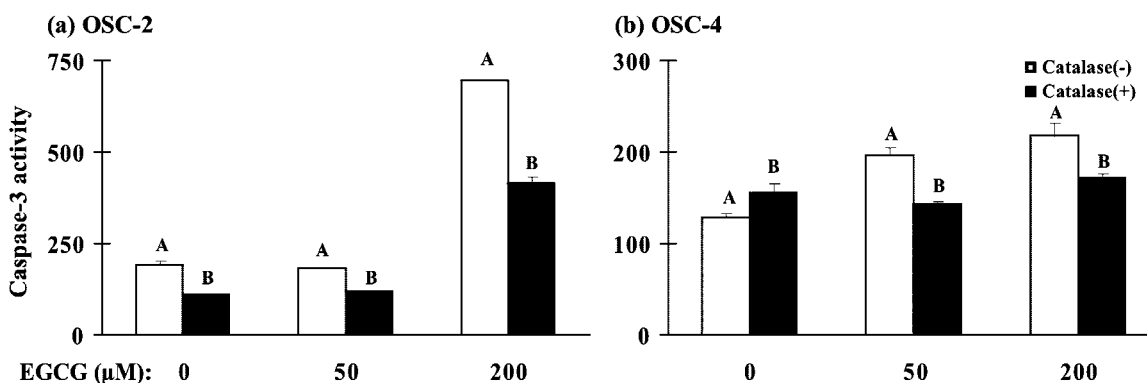


Fig. 4. Caspase-3 activity in OSC-2 and OSC-4 cells pretreated with catalase and incubated with EGCG. Cells were pretreated with 200 U/ml exogenous catalase for 5 min prior to addition of EGCG at concentrations indicated. Caspase-3 activity assay was performed immediately after a 24-h incubation with EGCG. Error bars indicate one standard deviation of the mean. Different capital letters indicate statistically significant differences among conditions (ANOVA, Tukey post hoc test, $\alpha = 0.05$, $n = 4$).

when the H₂O₂ concentration was increased to 1 mM (Fig. 1b). In comparison, 75% of viable NHEK cells remained when treated with 1 mM H₂O₂ for 24 h (Fig. 1B). These results demonstrated that NHEK possess a stronger ability to resist the oxidative stress from H₂O₂, whereas OSC cells are more sensitive to H₂O₂-induced cytotoxicity. In contrast, EGCG at various concentrations did not induce cytotoxicity in NHEK but caused significant cytotoxicity in OSC cells, suggesting that H₂O₂-induced effects among these cell types are quantitative, whereas EGCG-induced effects are qualitative.

Between the tumor cell lines, OSC-2 cells appeared to be more sensitive to H₂O₂-induced cytotoxicity than OSC-4 cells, as measured by the MTT assay (Fig. 1b). Consistent with this observation, when OSC-2 and OSC-4 cells were

incubated with relatively high concentrations of H₂O₂ or diamide, OSC-2 cells accumulated significantly higher (approximately 2×) ROS than OSC-4 cells, indicating that OSC-2 cells possess weaker defenses against H₂O₂ (Fig. 2). In OSC-2 cells, incubation with 200 μM EGCG produced ROS equivalent to that from 50 μM H₂O₂ during the first hour (Fig. 2). The cell viability was reduced to 40% of untreated control after 24 h (Fig. 1a). In contrast, a 24-h treatment with 50 μM H₂O₂ had no effect on cell viability (Fig. 1b). Similarly, incubation of OSC-4 cells with 200 μM EGCG produced ROS equivalent to that from 100 μM H₂O₂ during the first hour (Fig. 2), and the cell viability was reduced to less than 75% of untreated control after 24 h (Fig. 1A), but 100 μM H₂O₂ had no significant effect on cell viability (Fig. 1b). Further discor-

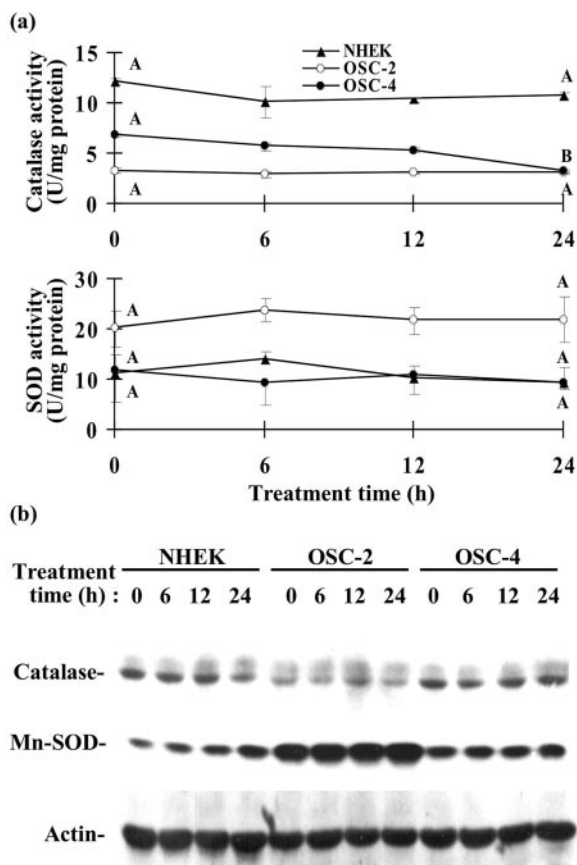


Fig. 5. Enzymatic activity and quantity determination of endogenous catalase and SOD in NHEK, OSC-2, and OSC-4 cells incubated with EGCG. The enzymatic activities of catalase and total SOD were assayed after cells were incubated with 100 μM EGCG for 0, 6, 12, and 24 h (a). Error bars indicate one standard deviation of the mean. Different capital letters indicate statistically significant differences among conditions (ANOVA, Tukey post hoc test, $\alpha = 0.05$, $n = 4$). Protein levels of catalase, Mn-SOD, and actin were determined by Western blot in cells treated with 100 μM EGCG for 0, 6, 12, and 24 h (b). The figure is a representative experiment repeated three times with similar results.

dance between the effects of H_2O_2 and EGCG was seen using reagents that directly or indirectly affect H_2O_2 concentration. Neither exogenous catalase nor the addition of catalase inhibitor 3-AT had any major effect on the EGCG-induced cytotoxicity in OSC cells (data not shown). In addition, the strong antioxidant NAC not only failed to rescue the OSC cells from EGCG-induced cytotoxicity, it enhanced it (especially in OSC-4 cells), whereas NAC significantly rescued the OSC cells from H_2O_2 -induced cytotoxicity (Fig. 3). Therefore, the cytotoxicity induced by EGCG in these tumor cells, as measured by MTT assay, did not correlate with the ability of EGCG to produce ROS. In addition, EGCG-induced growth arrest also appeared to be independent of ROS production (data not shown). This is consistent with a previous report suggesting that EGCG-induced growth arrest involves the gallate structure (Yang et al., 2000).

In contrast to the above observations regarding cytotoxicity and growth arrest, EGCG-derived ROS do appear to have a role in caspase-3 activation. Exogenous catalase partially rescued OSC-2 cells and substantially rescued OSC-4 cells from EGCG-induced caspase-3 activation during a 24-h period (Fig. 4). Furthermore, the levels of endogenous catalase activity are inversely correlated with sensitivity to EGCG,

H_2O_2 , and diamide (Fig. 1, Fig. 2, and Fig. 5a). SOD is unlikely to be involved since there is no correlation between endogenous total SOD activity and cell sensitivity to EGCG, H_2O_2 , or diamide (Fig. 1, Fig. 2, and Fig. 5b). In fact, OSC-2 cells, which showed high sensitivity to EGCG, H_2O_2 , and diamide, have the highest levels of Mn-SOD expression (Fig. 5b) and total SOD activity (Fig. 5a). The above observations are unlikely to be the result of an effect of EGCG on enzymes involved in ROS breakdown in OSC cells. EGCG did not appear to markedly regulate either catalase or SOD enzymatic activities or protein levels over a 24-h period (Fig. 5).

In conclusion, EGCG-induced ROS formation is not simply concentration-dependent, but is also cell type-dependent. Identical concentrations of EGCG (as high as 200 μM) may cause severe damage in one tumor cell line (OSC-2), less severe damage in another tumor cell line (OSC-4), but reduce ROS levels in normal epithelial cells (NHEK). These data support our hypothesis that cells in potentially frequent contact with plant-derived polyphenols, such as cells found in the epidermis, oral mucosa, and digestive tract, have developed mechanism(s) to mitigate cytotoxicity otherwise caused by the polyphenols and benefit from these compounds. However, when applied in high doses, EGCG is cytotoxic to other human cells that lack this tolerance and to cancer cells that have lost these protective mechanisms. Thus, whether an EGCG concentration is physiologically or clinically relevant is organ/tissue-dependent. In NHEK, EGCG induces a survival pathway associated with differentiation that does not appear to involve ROS. In OSC cells, EGCG induces different pathways that lead to cell death. Caspase-3 activation appears to involve EGCG-induced ROS formation, whereas cytotoxicity and growth arrest do not. Endogenous catalase plays a role in the response of cells to EGCG; cells without adequate catalase are more sensitive to EGCG-induced H_2O_2 formation, as shown in the current study and previous reports (Yang et al., 1998; Sakagami et al., 2001; Chai et al., 2003). However, H_2O_2 alone cannot reproduce the EGCG effects in other cell lines or cell types. Thus, applications of high concentrations of EGCG on epithelial tissues, especially the epidermal and digestive tract tissues, could deliver cytotoxic effects for chemoprevention purposes involving growth arrest/apoptosis signaling and oxidative stress that are clinically relevant, whereas normal epidermal cells are guided to safety by a cell differentiation pathway.

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References

- Ahmad N, Gupta S, and Mukhtar H (2000) Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor kappaB in cancer cells versus normal cells. *Arch Biochem Biophys* **376**:338–346.
- Balasubramanian S, Efimova T, and Eckert RL (2002) Green tea polyphenol stimulates a Ras, MEKK1, MEK3, and p38 cascade to increase activator protein 1 factor-dependent involucrin gene expression in normal human keratinocytes. *J Biol Chem* **277**:1828–1836.
- Barthelman M, Bair WB, 3rd, Stickland KK, Chen W, Timmermann BN, Valcic S, Dong Z and Bowden GT (1998) (-)-Epigallocatechin-3-gallate inhibition of ultraviolet B-induced AP-1 activity. *Carcinogenesis* **19**:2201–2204.
- Chai PC, Long LH, and Halliwell B (2003) Contribution of hydrogen peroxide to the cytotoxicity of green tea and red wines. *Biochem Biophys Res Commun* **304**:650–654.
- Chen C, Shen G, Hebbar V, Hu R, Owuor ED, and Kong AN (2003) Epigallocatechin-3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells. *Carcinogenesis* **8**:1369–78.
- Chen C, Yu R, Owuor ED, and Kong AN (2000) Activation of antioxidant-response element (ARE), mitogen-activated protein kinases (MAPKs) and caspases by major

- green tea polyphenol components during cell survival and death. *Arch Pharm Res* **23**:605–612.
- Chen L, Yang X, Jiao H, and Zhao B (2002) Tea catechins protect against lead-induced cytotoxicity, lipid peroxidation, and membrane fluidity in HepG2 cells. *Toxicol Sci* **69**:149–156.
- Chung JY, Huang C, Meng X, Dong Z, and Yang CS (1999) Inhibition of activator protein 1 activity and cell growth by purified green tea and black tea polyphenols in H-ras-transformed cells: structure-activity relationship and mechanisms involved. *Cancer Res* **59**:4610–4617.
- Dashwood WM, Orner GA, and Dashwood RH (2002) Inhibition of beta-catenin/Tcf activity by white tea, green tea, and epigallocatechin-3-gallate (EGCG): minor contribution of H(2)O(2) at physiologically relevant EGCG concentrations. *Biochem Biophys Res Commun* **296**:584–588.
- Dong Z, Ma W, Huang C, and Yang CS (1997) Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (-)-epigallocatechin gallate, and theaflavins. *Cancer Res* **57**:4414–4419.
- Dong Z (2000) Effects of food factors on signal transduction pathways. *Biofactors* **12**:17–28.
- Gupta S, Ahmad N, Nieminen AL, and Mukhtar H (2000) Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent (-)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells. *Toxicol Appl Pharmacol* **164**:82–90.
- Gupta S, Hussain T, and Mukhtar H (2003) Molecular pathway for (-)-epigallocatechin-3-gallate-induced cell cycle arrest and apoptosis of human prostate carcinoma cells. *Arch Biochem Biophys* **410**:177–185.
- Halliwell B (2003) Oxidative stress in cell culture: an under-appreciated problem? *FEBS Lett* **540**:3–6.
- Higdon JV and Frei B (2003) Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit Rev Food Sci Nutr* **43**:89–143.
- Hong J, Lu H, Meng X, Ryu JH, Hara Y, and Yang CS (2002) Stability, cellular uptake, biotransformation and efflux of tea polyphenol (-)-epigallocatechin-3-gallate in HT-29 human colon adenocarcinoma cells. *Cancer Res* **62**:7241–7246.
- Hsu S, Bollag WB, Lewis J, Huang Q, Singh B, Sharawy M, Yamamoto T, and Schuster G (2003a) Green tea polyphenols induce differentiation and proliferation in epidermal keratinocytes. *J Pharmacol Exp Ther* **306**:29–34.
- Hsu S, Lewis J, Singh B, Schoenlein P, Osaki T, Athar M, Porter AG, and Schuster G (2003b) Green tea polyphenol targets the mitochondria in tumor cells inducing caspase 3-dependent apoptosis. *Anticancer Res* **23**:1533–1539.
- Hsu S, Lewis JB, Borke JL, Singh B, Dickinson DP, Caughman GB, Athar M, Drake L, Aiken AC, Huynh CT et al. (2001) Chemopreventive effects of green tea polyphenols correlate with reversible induction of p57 expression. *Anticancer Res* **21**:3743–3748.
- Hsu S, Yu FS, Lewis J, Singh B, Borke J, Osaki T, Athar M, and Schuster G (2002a) Induction of p57 is required for cell survival when exposed to green tea polyphenols. *Anticancer Res* **22**:4115–4120.
- Hsu SD, Singh BB, Lewis JB, Borke JL, Dickinson DP, Drake L, Caughman GB, and Schuster GS (2002b) Chemoprevention of oral cancer by green tea. *Gen Dent* **50**:140–146.
- Jung YD and Ellis LM (2001) Inhibition of tumour invasion and angiogenesis by epigallocatechin gallate (EGCG), a major component of green tea. *Int J Exp Pathol* **82**:309–316.
- Katiyar SK and Elmets CA (2001) Green tea polyphenolic antioxidants and skin photoprotection (Review). *Int J Oncol* **18**:1307–1313.
- Kong AN, Yu R, Chen C, Mandelkar S, and Primiano T (2000) Signal transduction events elicited by natural products: role of MAPK and caspase pathways in homeostatic response and induction of apoptosis. *Arch Pharm Res* **23**:1–16.
- Kong AN, Yu R, Lei W, Mandelkar S, Tan TH, and Ucker DS (1998) Differential activation of MAPK and ICE/Ced-3 protease in chemical-induced apoptosis: the role of oxidative stress in the regulation of mitogen-activated protein kinases (MAPKs) leading to gene expression and survival or activation of caspases leading to apoptosis. *Restor Neurol Neurosci* **12**:63–70.
- Lee SR, Im KJ, Suh SI, and Jung JG (2003) Protective effect of green tea polyphenol (-)-epigallocatechin gallate and other antioxidants on lipid peroxidation in gerbil brain homogenates. *Phytother Res* **17**:206–209.
- Liang YC, Lin-Shiau SY, Chen CF, and Lin JK (1999) Inhibition of cyclin-dependent kinases 2 and 4 activities as well as induction of Cdk inhibitors p21 and p27 during growth arrest of human breast carcinoma cells by (-)-epigallocatechin-3-gallate. *J Cell Biochem* **75**:1–12.
- Liberto M and Cobrinik D (2000) Growth factor-dependent induction of p21(CIP1) by the green tea polyphenol, epigallocatechin gallate. *Cancer Lett* **154**:151–161.
- Lin JK, Liang YC, and Lin-Shiau SY (1999) Cancer chemoprevention by tea polyphenols through mitotic signal transduction blockade. *Biochem Pharmacol* **58**:911–915.
- Long LH, Lan AN, Hsuan FT, and Halliwell B (1999) Generation of hydrogen peroxide by “antioxidant” beverages and the effect of milk addition. Is cocoa the best beverage? *Free Radic Res Commun* **31**:67–71.
- Nakagawa H, Wachi M, Woo JT, Kato M, Kasai S, Takahashi F, Lee IS, and Nagai K (2002) Fenton reaction is primarily involved in a mechanism of (-)-epigallocatechin-3-gallate to induce osteoclastic cell death. *Biochem Biophys Res Commun* **292**:94–101.
- Roy M, Chakrabarty S, Sinha D, Bhattacharya RK, and Siddiqi M (2003) Anticlastogenic, antigenotoxic and apoptotic activity of epigallocatechin gallate: a green tea polyphenol. *Mutat Res* **523–524**:33–41.
- Saeki K, Kobayashi N, Inazawa Y, Zhang H, Nishitoh H, Ichijo H, Isemura M, and Yuo A (2002) Oxidation-triggered c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase pathways for apoptosis in human leukaemic cells stimulated by epigallocatechin-3-gallate (EGCG): a distinct pathway from those of chemically induced and receptor-mediated apoptosis. *Biochem J* **368**:705–720.
- Sakagami H, Arakawa H, Maeda M, Satoh K, Kadofuku T, Fukuchi K, and Gomi K (2001) Production of hydrogen peroxide and methionine sulfoxide by epigallocatechin gallate and antioxidants. *Anticancer Res* **21**:2633–2641.
- Tanaka R (2000) Protective effects of (-)-epigallocatechin gallate and (+)-catechin on paraquat-induced genotoxicity in cultured cells. *J Toxicol Sci* **25**:199–204.
- Wei H, Zhang X, Zhao JF, Wang ZY, Bickers D, and Leibold M (1999) Scavenging of hydrogen peroxide and inhibition of ultraviolet light-induced oxidative DNA damage by aqueous extracts from green and black teas. *Free Radic Biol Med* **26**:1427–1435.
- Yamamoto T, Hsu S, Lewis J, Wataha J, Ueta E, Osaki T, Luckwood P, Singh B, Dickinson D, and Schuster G (2003) Green tea polyphenol creates differential oxidative environments in normal vs. malignant cells. *J Pharmacol Exp Ther*, in press.
- Yang GY, Liao J, Kim K, Yurkow EJ, and Yang CS (1998) Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis* **19**:611–616.
- Yang GY, Liao J, Li C, Chung J, Yurkow EJ, Ho CT, and Yang CS (2000) Effect of black and green tea polyphenols on c-jun phosphorylation and H(2)O(2) production in transformed and non-transformed human bronchial cell lines: possible mechanisms of cell growth inhibition and apoptosis induction. *Carcinogenesis* **21**:2035–2039.
- Zhu N, Huang TC, Yu Y, LaVoie EJ, Yang CS, and Ho CT (2000) Identification of oxidation products of (-)-epigallocatechin gallate and (-)-epigallocatechin with H(2)O(2). *J Agric Food Chem* **48**:979–981.

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