Constitutive Overexpression of Nrf2-dependent Heme Oxygenase-1 in A549 Cells Contributes to Resistance to Apoptosis Induced by EGCG

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Epigallocatechin-3-gallate (EGCG), the major polyphenol found in green tea exerts antiproliferative and proapoptotic effects in many cancer cells. However, we found that among many cancer cells human lung adenocarcinoma A549 cells are markedly resistant to apoptosis induction by EGCG (even at 100 μM, 72 h). Heme oxygenase-1 (HO-1) induced by stress stimuli represents a prime cellular defense mechanism, but it may be associated with enhanced cell proliferation and chemoresistance in some cancer cells. Since we found that A549 cells constitutively overexpress HO-1 and its associated transcription factor, Nrf2, we tested a hypothesis that EGCG-resistance in these cells may be linked with Nrf2-mediated HO-1 overexpression. HO-1 inhibition with tin-protoporphyrin IX and silencing with RNA interference rendered cells more sensitive to apoptosis induction by EGCG and classical prooxidants. Interestingly, EGCG at high concentration (>200 μM) induced apoptosis by suppressing expression of HO-1 protein and mRNA, and this effect correlated with a decrease in both Nrf2-ARE binding and HO-1-ARE-luciferase activity, suggesting Nrf2-driven transcriptional activation of ho-1. Since we observed notably high level of phosphorylated protein kinase C alpha (p-PKCα) and its suppression by EGCG and deferoxamine (an iron chelator, a possible mechanism involving p-PKCα and iron in Nrf2-HO-1 activation was further investigated. Collectively, our findings suggest that Nrf2-mediated HO-1 overexpression confers resistance to apoptosis induction by EGCG and therefore, its inactivation may be a target for overcoming the resistance to chemoprevention and chemotherapy.

Epigallocatechin-3-gallate (EGCG), the major polyphenol found in green tea is a widely studied cancer chemopreventive agent with potential anticancer activity. The major mechanism of EGCG-mediated anticancer effects is considered to be related to induction of apoptosis (1, 2).

Studies have shown differential sensitivity among different tumor cells or tumor cells versus normal cells to EGCG (1, 2). In particular, in many cancer cells EGCG has been shown to modulate multiple and often different signal transduction pathways. The reason for these observed differences is not clear but may be because of the differential oxidative status imposed by EGCG in various cell types or cell type-specific expression of endogenous antioxidant defense enzymes.

Heme oxygenase-1 (HO-1) is known to be highly induced by a variety of stress stimuli and many cancer-chemopreventive agents, and represents a prime cellular defense mechanism against oxidative stress via antioxidant function of its catalytic products like bilirubin and carbon monoxide (CO) with concomitant induction of iron sequestering ferritin (3, 54). On the contrary, its overexpression in human cancers may offer cancer cells a growth advantage and cellular resistance against chemotherapy and photodynamic therapy (4, 5). Because the growth of most tumors depends on HO-1 (6), it is also considered as a target for cancer therapy in humans. In this context, HO-1 induction by stress-related agents in several types of human cancer cells has been reported to play a role in chemoresistance to apoptosis (5, 7, 8). Similarly, inhibition of HO-1 has been shown to lead to
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There is accumulating evidence to show that Nrf2 is a key transcriptional factor that activates antioxidant reactive element (ARE) and in turn regulates the expression of antioxidant phase II detoxifying enzymes. It is interesting to note that the promoter region of ho-1 gene contains ARE sequence (11-14). The mode of transcriptional activation of Nrf2 is not fully understood but available evidences point to two mechanisms. In the first, a sulfhydryl modification of its cytosolic sequestering protein Keap1 by chemical inducers, leads to Nrf2 dissociation from Keap1 and subsequent translocation into the nucleus, thereby activating ARE (11). In the second pathway, it is considered that several upstream signaling kinases including protein kinase C (PKC), phosphoinositol-3-kinase (PI3K), and mitogen-activated protein kinases (MAPKs: p38, ERK1/2, and JNK) regulate Nrf2/ARE activity (12-14). However, it is still unclear which kinase acts as an upstream mediator of Nrf2.

Histologically, non-small cell lung cancer (NSCLC) constitutes approximately 85% of all lung cancers and often shows intrinsic multidrug resistance, which is a major problem in its chemotherapy (15). Expression level of several factors such as p53, p-AKT, apoptosis inducing factor, and multi drug resistance protein have been recently proposed to determine the chemoresistance against conventional treatment protocols (16, 17). While comparing EGCG-mediated cytotoxicity in various cancer cells, we observed that human lung adenocarcinoma A549 cells, which belong to NSCLC, were significantly resistant to the induction of apoptosis by EGCG. We then found that these cells express high levels of constitutive HO-1 and Nrf2, as compared to other human cancer cells.

It has been shown that HO-1 expression can be elevated in the lung in response to oxidative stress associated with infection, hyperoxia, and inflammatory diseases or acute respiratory distress syndrome (18, 19). Although Nrf2 plays a critical role in protection against pulmonary fibrosis, presumably through enhancement of cellular antioxidant capacity (53), HO-1/Nrf2 activation as a defense mechanism in carcinoma cells during lung carcinogenesis may lead to their resistance to chemopreventive and chemotherapeutic regimens.

Here, we first show that constitutively overexpressed HO-1 in A549 lung cancer cells is strongly associated with resistance to apoptosis induction by EGCG. We further demonstrate that this overexpression is regulated by phosphorylation of PKCα and subsequent activation of Nrf2. We suggest that PKCα-Nrf2-HO-1 pathway may be exploited for targeted therapy of lung cancer.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—A purified preparation of EGCG (>98% pure) was procured from Sigma (St. Louis, MO) and aliquots of stock solution (20 mM) prepared in DMSO were stored at -80°C for further use. Ro-317549, PD98059, LY294002, and Triciribine were obtained from Calbiochem (La Jolla, CA). Tin-protoporphyrin-IX (SnPPIX) was obtained from Porphyrin Products (Logan, UT) and L-buthionine-(S,R)-sulfoximine (BSO), N-acetylcysteine (NAC), and deferoxamine (DFO) were purchased from Sigma.

**Cell treatment and viability**—The A549, LNCaP, DU145, PC-3, AsPC-1, HCC1419, SKOV-3, and HT-29, H196 and H441 human cancer cells were obtained from American Type Culture Collection (Manassas, VA) and cultured according to the manufacturer’s protocol. Normal human bronchial epithelial cells (NHBEs) were procured from Cambrex Bio Science (Walkersville, MD) and cultured as per manufacturer’s instructions. For measurement of cell viability, cells (2-5×10^4 cells per well for 24-well plate) were seeded into plate and cultured overnight in complete medium, and then treated with EGCG (0-100 μM) or other agents for 24, 48, and 72 h. After appropriate time periods, cells were subjected to MTT assay as described earlier (20) to determine cell viability.

**DNA fragmentation assay**—Following treatment of cells as described above, the cells were washed twice with PBS (pH 7.4), incubated with DNA lysis buffer (10 mM Tris, pH 7.5, 400 mM NaCl, 1 mM EDTA and 1% Triton X-100) for 30 min on ice and then centrifuged. The supernatant obtained was incubated with RNase (0.2 mg/ml) at room temperature and then with Proteinase K (0.1 mg/ml) for 2 h at 37°C. DNA was extracted and resolved as previously described (21).
**Immunoblot analysis**—Western blotting was performed as described previously (20). Primary antibodies against HO-1 and HO-2 (dilution 1:200 and 1:1000, Stressgen, Victoria, Canada), Nrf2, Keap1, GCLC, ferritinH, p53, SOD-1, PCNA, AIF, Trx, p38, and procaspase-3 (dilution 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), p-PKCa Ser657, HIF-1α, p-AKT Ser473, Gpx, p-NF-kB, c-Fos, Bax, and PI3Kp85 (dilution 1:1000-2000, Upstate, Charlottesville, VA), p21Waf1/Cip1, p-PKCa Thr505, PKCe, PKCδ, PARP, p-ERK44/42, p-p38, and p-SAPK/JNK, and SAPK/JNK (dilution 1:1000-2000) cell signaling. Beverly, MA), and β-actin (1:2000, Sigma, St. Louis, MO) were used to detect their corresponding antigens.

**Electrophoretic mobility shift assay**—EMSA for Nrf2-ARE binding was performed as described previously (22). Briefly, synthetic double strand oligonucleotide corresponding to a human HO-1ARE was biotinylated using the Biotin 3′ end labeling kit (Pierce, Rockford, IL). Biotin end-labeled target DNA was incubated with anti-human Nrf2 antibody (1 μl) for 30 min to detect supershifted Nrf2 and subjected to native gel electrophoresis and transferred onto a nylon membrane. ARE-Nrf2 binding was detected using streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate followed by autoradiography. Binding specificity was tested by adding to nuclear extracts a 100-fold molar excess of cold ARE oligonucleotide.

**Transient transfection of HO-1-ARE-reporter vector and luciferase activity**—HO-1-ARE-reporter construct (hpHO-pTi-Luc) provided by Dr. Jeffrey A. Johnson (University of Wisconsin, Madison) was made by inserting 34 base pair DNA sequence (GAT CCT CTA GAG TCA CAG TGA CTT GGC AAA ATC AGA GAT CTC ACT GAA CCG TTT TAG TCT AG) located in 5′-flanking ARE region of human HO-1 gene into pTi-luciferase vector (23). Cells were plated on 24-well plates at a density of 5 × 10^4 cells/well and transfected with ARE-luciferase reporter construct (0.25 μg/well) and β-gal plasmid (0.1 μg/well) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Luciferase activity was measured according to the manufacturer’s instructions (Promega, Madison, WI) and β-galactosidase activity was determined to normalize transfection efficiency.

**RNA interference**—The small interfering RNA (siRNA) against ho-1 was procured from Dharmacon (Lafayette, CO). Transfection was performed using Dharmafect Transfection kit (Dharmacon, Lafayette, CO) and following the manufacturer’s protocol (24) with ho-1 siRNA, duplex D-0063732-05-0005, human HMOX1 (sense: GCCAGAGGGUGAUAGAGAUU and antisense: PUCUUCUAUCCACCCUCUGCUU). The targeting site was pooled from ORF region of human ho-1 (NCBI NM002133). Cells were also transfected with nontargeting, negative control siRNA (Dharmacon) which allows assessing target specificity and any non-specific gene silencing effects. Briefly, A549 cells were transfected with 25-100 nmol/L of siRNAs directed against the ho-1 (ho-1siRNA) for 6-48 hours. The cells were harvested and processed for preparation of cell lysate or RNA for biochemical assays.

**Flow cytometric analysis**—Flow cytometry for apoptosis analysis was performed as described (20). The cells transfected with siHO-1 RNA for 6, 12, 24, and 48 h were grown at a density of 1 × 10^6 cells in 100 mm culture dishes and treated with EGCG (80 μM) for 24 h or 0.5 mM H₂O₂ and 1 mM t-BHP for 6 h. The cells were trypsinized, processed for labeling with fluorescein-tagged dUTP nucleotide and PI by the use of an Apo-Direct apoptosis kit (Phoenix Flow Systems, San Diego, CA) and analyzed by flow cytometry.

**Soft agar colony formation assay**—Following 24 and 48 h post-transfection with 25 nM HO-1 siRNA, cells were trypsinized and counted. A total of 5,000 cells in 0.7% Agarose containing 20% FBS and 2X RPMI medium were mixed with either EGCG solution (final concentration: 40 μM) for 24 h or 0.5 mM H₂O₂ and 1 mM t-BHP for 6 h. The cells were poured onto 0.5% solidified agar in a 6-well plate and the plates were incubated at 37°C in a CO₂ humidified incubator for 14 days. The resulting plates were stained with 0.5 ml of 0.005% Crystal Violet for 2 h and observed under the microscope.

**Annexin V-fluos-PI staining**—Annexin V-fluos-PI staining for confocal microscopy was performed according to the manufacturer’s instructions (Roche, Indianapolis, IN) and as described previously (24). Briefly, A549 cells were grown to about 60% confluency on cell-culture slides and then treated with EGCG (0-300 μM) for 48 h. Apoptosis and necrosis were
detected by using a Zeiss Axiovert 100 microscope (Carl Zeiss, Inc., Thornwood, NY).

**Immunocytochemistry of Nrf2** – A549 cells grown on culture slides and treated with EGCG as indicated were fixed in 100% methanol and incubated with monoclonal rabbit anti-Nrf2 antibody (2.5 μg/ml) in phosphate-buffered saline containing 1.5% bovine serum albumin for 60 min at room temperature. The cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (Santa Cruz, CA) for an additional 45 min in the dark. Stained cells were washed, mounted in an antifade mounting medium (Molecular Probes, Eugene, OR), and examined with a fluorescence confocal microscope, Zeiss LSM 510 (Thornwood, NY).

**RT-PCR and Northern blotting** – mRNA levels and expression were determined by RT-PCR and Northern blot as described (21). Specific primers for human HO-1(size of PCR product: 775 bp: sense 5'-CAA GGA GGT GCA CAC GG-3', antisense 5'-GCT GGA TGT TGA GCA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control (size of PCR product: 523 bp: sense 5'-GAG TCG NCG CGG TTT CCT GC-3', antisense: 5'-CTG CTC CAA GCA CAG CTC AC-3') were used for the first cDNA synthesis. For Northern blotting, 10 μg of total RNA was electrophoresed in 1% formaldehyde/agarose gel and transferred to a nylon membrane. Human HO-1 cDNA was labeled with [α-32P] dCTP (PerkinElmer, Shelton, CT) by the random hexamer priming system using a Rediprime™ II kit (Amersham Pharmacia Biotech, Piscataway, NJ).

**RESULTS**

**Human lung adenocarcinoma A549 cells are highly resistant to EGCG-cytotoxicity** – Initially we compared in-vitro cytotoxicity of EGCG on eight kinds of human epithelial cancer cells (see Fig. 1B). Treatment of all cells with EGCG (0-100 μM; 24, 48, and 72 h) generated varying degrees of dose- and time-dependent cytotoxic response with least effect in A549 cells (Fig. 1A). The treatment of all but A549 cancer cells with EGCG (>40 μM) for 24 h showed initial toxicity; A549 cells were highly resistant even up to 100 μM EGCG treatment for 24-48 h. At 40 μM of treatment for 48 h significant cytotoxicity (p<0.01-0.001) was noted for SKOV-3, HT-29, HCC1419, and PC-3 cells whereas moderate toxicity at the same concentration of EGCG was observed for AsPC-1 and LNCaP cells. All cells excepting A549 were found to be sensitive to 10 μM EGCG at 72-h exposure, where a significant cytotoxicity (42-76% cell viability, p<0.01-0.001) was observed. Among all cells tested A549 cells were highly resistant in as much as 85% cell viability was sustained for 72-h at 40 μM EGCG (Fig. 1A). The IC50 at 72 h-exposure to EGCG was estimated to be in the following order: A549 > AsPC-1> HCC1419 > LNCaP > DU145 > PC-3 > SKOV-3 > HT-29 (Fig. 1B).

**Expression of redox-related antioxidant enzymes and transcription factors in cancer cells: elevated expression of HO-1, GCL, and Nrf2 in A549 cell** – Because EGCG has been known to generate (i) extracellular H2O2 in cell culture medium, (ii) intracellular ROS by the Fenton reaction upon cell entry, and (iii) subsequently to induce oxidative stress-mediated apoptosis (25, 26), we next determined the expression of HO-1, which is an inducible ROS-scavenging antioxidant, along with its constitutive isoform HO-2, to investigate its relevance to EGCG sensitivity and cellular antioxidant status. Strikingly, HO-1 was observed to be constitutively overexpressed in A549 cells as compared to other cancer cells tested (Fig. 2A). However, the expression level of HO-2 was almost similar in all cell lines.

Since HO-1 is a representative Nrf2-mediated phase II detoxifying defense enzyme against oxidative stress, we also determined the expression level of other antioxidant phase II defense proteins. A549 cells were found to express high levels of glutamate cysteine ligase (GCL) and thioredoxin (Trx) but not ferritin and glutathione peroxidase (Gpx) as compared to other cells (Fig. 2B). In addition, wide variation in the expression level of other primary antioxidant enzymes such as superoxide dismutase (SOD) and catalase was observed in most human epithelial cancer cells tested, which did not show any significant correlation to EGCG-sensitivity (Fig. 2B). We further found that A549 cells, among all other cancer cells express the highest amount of total Nrf2 along with relatively low level of its cytosolic anchor Keap1 and repressor Bach 1 (Fig. 2C). On the contrary, noticeable differences in the level of other redox-related transcription...
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factors such as NFκB, AP-1 or HIF-1, were not observed between A549 cells and other cancer cells. We therefore hypothesized that the constitutive high expression of HO-1 and its associated transcription factor Nrf2 in A549 cells may be associated with the observed high resistance to EGCG-caused cytotoxicity.

To determine whether HO-1 overexpression is a general property of lung cancer cells or unique to NSCLC represented by A549 cells, and if EGCG-resistance is specific to lung-derived cancer cells, we explored this possibility with additional lung cancer cells (NSCLC H441 cells and SCLC H196 cells) and primary normal human bronchial epithelial cells (NHBEs). A549 cells were found to be most resistant to EGCG treatment even than NHBEs cells (data not shown). Significant overexpression of HO-1 as well as higher expression levels of Nrf2 and GCL were observed only in A549 cells (Fig. 2D), indicating that EGCG-resistance, HO-1 and Nrf2 overexpression are unique to A549 cells. But it was interesting to observe unexpectedly low expression level of ferritin in A549 cells (Fig. 2B and 2D), which is known to be concomitantly upregulated with HO-1 induction (3, 54).

Effect of SnPPIX and BSO on resistance to EGCG-induced apoptosis — To test our hypothesis, we first compared the effect of EGCG treatment (50, 100 μM; 48 h) on the expression level of HO-1 and GCL in A549 cells and three kinds of EGCG-sensitive cells, SKOV-3, HCC1419, and HT-29 (Fig. 1B). Additionally, we performed DNA fragmentation analysis to confirm the apoptotic death caused by EGCG treatment. Although the levels of HO-1 and GCL in A549 cells were found to be decreased by EGCG treatment, this decrease was small when compared to other EGCG-sensitive cells (Fig. 3A). Expectedly, DNA fragmentation, which is an indicator of apoptosis, was not evident in A549 cells treated with EGCG (Fig. 3A). In sharp contrast, DNA fragmentation was clearly evident in SKOV3, HCC1419, and HT29 cells treated with EGCG even at 50 μM of concentration. These results suggested that the high expression of HO-1 and GCL in A549 cells might be related to their resistance to EGCG-induced apoptosis.

To verify the anti-apoptotic role of HO-1 and GCL, we performed a combinatorial study of EGCG with specific inhibitors of HO-1 and GCL, tin-protoporphyrin IX (SnPPIX) (9, 21) and L-buthionine-(S,R)-sulfoximine (BSO), respectively (20, 27). Cells were pretreated with SnPPIX (10 μM) and BSO (1 μM) for 2 hr (nontoxic maximum effective concentrations), followed by treatment with EGCG (50-100 μM; 48 hr). Both inhibitors rendered A549 cells more susceptible to EGCG treatment as assessed by the MTT (Fig. 3B) and DNA fragmentation assay (Fig. 3C), and this effect was found to be more pronounced (p<0.01-0.001) by preincubation with SnPPIX than BSO (Fig. 3B and 3C), suggesting a pivotal role of HO-1 in EGCG-resistance of A549 cells.

Effect of ho-1 silencing on cell growth and chemoresistance of A549 cell — To more directly assess involvement of constitutive overexpression of HO-1 in EGCG-resistance, we performed siRNA-mediated silencing of ho-1 gene. As shown in Figure 4, the expression of HO-1 protein (Fig. 4A) and mRNA (Fig. 4B) were effectively inhibited with all concentrations (25, 50, and, 100 nM; 48 h) of ho-1 siRNA used. Non-silencing siRNA did not exhibit any effect on HO-1 protein and mRNA. We next transfected cells with ho-1 siRNA (25 nM) for 6, 12, 24, and 48 h, respectively, to achieve varying degree of ho-1 silencing effect, and then challenged with EGCG (Fig. 4C). As shown in Fig. 4D, targeted silencing of ho-1 induced a time-dependent decrease in number of viable cells and subsequent treatment with EGCG significantly enhanced loss in cell viability. Further, TUNEL assay by flow cytometry suggested enhancement of EGCG-induced apoptosis in ho-1 silenced cells and strongly validated our hypothesis. Furthermore, as assessed by colony formation assay, knockdown of ho-1 resulted in inhibition of cell proliferation and EGCG treatment further augmented this effect (Fig. 4F).

We interestingly found that the protein expression of p53 in ho-1-silenced A549 cells is markedly increased along with its downstream target proteins, p21 and Bax (Fig. 4G). EGCG treatment in ho-1-silenced cells resulted in significant release of mitochondrial cytochrome C, activation of caspase 3, and poly-ADP-ribose-polymerase (PARP) cleavage (Fig. 4G) which are hallmarks of apoptosis (22, 24). These results may be partly supported by previous studies that HO-1
catalytic activity-derived CO mediates antipapoptotic effect by suppressing p53 expression and mitochondrial cytochrome C (45). Also, p53 is activated under oxidative stress (28) and can cause either cell cycle arrest or apoptosis (29). Furthermore, the silencing of ho-1 expression rendered cells more sensitive to apoptosis by other classical prooxidants such as t-BHP and H2O2 as observed by comparison of sub G1 phase in cell cycle analysis (Fig. 4H).

High concentration of EGCG blocks overexpression of HO-1 via suppressing Nrf2 transcriptional activation and causes apoptosis — We next found that high concentrations of EGCG (200-300 μM, 48 h) treatment to A549 cells were able to induce apoptosis as observed by Annexin V-fluous-PI staining (Fig. 5D) and PARP cleavage (Fig. 5E) that was consistent with downregulation of HO-1 expression (Fig. 5A). Similarly, the protein expressions of HO-1-related antioxidant ferritin and other phase II enzymes, GCL and Gpx, decreased in a dose-dependent manner (Fig. 5B). The expression levels of Nrf2 and Keap1 were also found to be slightly decreased (Fig. 5C), whereas levels of other primary antioxidants including SOD and Trx (Fig. 5B) were not affected.

It has been suggested that the primary control of Nrf2 transcriptional activation of phase II gene induction relies on subcellular distribution rather than induction of this transcription factor through de novo synthesis (30), and also overexpression of Nrf2 results in direct localization of Nrf2 in the nucleus (13). Thus, we first determined nuclear and cytosolic levels of Nrf2 in control and EGCG-treated cells to investigate whether the constitutive overexpression of HO-1 in A549 cells is mediated by Nrf2. As shown by immunoblot analysis (Fig. 6A) and immunocytochemistry (Fig. 6B), Nrf2 is that is localized primarily in the nucleus, is gradually decreased and translocated to cytosol following treatment of cells with EGCG in a dose and time-dependent manner, whereas Nrf2 in total cell lysate did not significantly change during the treatment time (12 h) (data not shown).

Nrf2 activates transcription of its genes through binding specifically to ARE found in the gene promoters (11-13). In agreement with nuclear localization of Nrf2, Nrf2-ARE binding (Fig. 6C) as well as HO-1-ARE-promoter activity (Fig. 6D) were observed in basal cells, and EGCG (200 μM, 3-12 h) was found to inhibit both activities in a time-dependent manner. Moreover, these results were consistent with downregulation of HO-1 mRNA and protein by treatment with EGCG (Fig. 6E), indicating that the overexpression of HO-1 is driven by Nrf2 transcriptional activation.

Highly activated PKCa in A549 cells is associated with Nrf2-dependent HO-1 overexpression, which is inhibited by EGCG and DFO — To get more insights into the mechanism of Nrf2-mediated HO-1 overexpression, we next compared levels of phosphorylated-PKCα/PKCδ, PI3K/AKT, and MAPKs including p38, ERK1/2, and JNK in all cancer cells because upstream activation of these kinases has been suggested to be involved in the activation of Nrf2 (12-14, 31, 32). Specifically, p-PKCα was found to be strongly expressed in A549 cells than in other cancer cell lines (Fig. 7A) and relatively high levels of PI3K, p-AKT, and p-ERK1/2 along with their total forms were observed in A549 cells (Fig. 7B and 7C). We therefore tested whether EGCG treatment which was found to inhibit Nrf2-dependent HO-1 expression, also inhibited phosphorylation of these kinases. As shown in Fig. 7D, expression levels of p-PKC and p-ERK1/2 in A549 cells were observed to be decreased by EGCG treatment, which was a relatively early event occurring at 0.5-3 h, whereas there were no obvious changes in expression of p-AKT and PI3K, indicating that EGCG-mediated blockade of HO-1 expression may require inhibition of PKC and ERK.

We next found that a specific inhibitor of PKCα, Ro-317549, among other inhibitors of the kinases significantly suppressed Nrf2 nuclear translocation (Fig. 8A) and Nrf2-ARE binding (Fig. 8B) along with inhibitory effect on the expression of HO-1 protein and mRNA (Fig. 8C). Ro-317549 also markedly inhibited HO-1-ARE-promoter activity in basal cells (Fig. 8D). ERK inhibitor, PD98059, but not other inhibitors, also exhibited these inhibitory effects in a similar manner but it was lower than Ro-317549. Moreover, Ro-317549 was shown to enhance EGCG-sensitivity in a dose-dependent manner (Fig. 8E). These results indicated that activation of PKCα and ERK is involved in the Nrf2-mediated
transcriptional activation of HO-1, but PKCα primarily plays a pivotal role in the process.

Iron overload has been reported to upregulate HO-1 in the lung more rapidly than in other tissues (52), and EGCG is known to have an iron chelating potential (33, 34). Since we observed unexpectedly low levels of ferritin in contrast to overexpression of HO-1 (Fig. 2B and 2D), (ii) we next focused on the possibility that the occurrence of highly activated PKCα may be associated with an accumulation of free iron (48, 51) due to failure of iron sequestering by ferritin which is concomitantly upregulated with HO-1 induction (3, 54). To verify this hypothesis, we tested the effect of an iron chelator, deferoxamine (DFO) (26), along with an ROS scavenging agent, N-acetylcysteine (NAC) (20, 21) during EGCG treatment of A549 cells. DFO significantly augmented EGCG-induced inhibition of PKCα phosphorylation, HO-1 expression, as well as EGCG-induced apoptosis as evidenced by PARP cleavage, whereas NAC did not show any significant effect (Fig. 9). In addition, DFO itself was found to inhibit PKCα phosphorylation and HO-1 expression in a manner similar to EGCG, indicating that intracellular iron rather than ROS may regulate PKCα and its downstream Nrf2/HO-1 pathway. On the basis of these results, we conclude that an accumulated iron-activated PKCα, at least in part, is involved in Nrf2-mediated HO-1 overexpression in the A549 cells, and iron chelation may halt the Nrf2-HO-1 pathway by suppressing PKCα activation (Fig. 10).

DISCUSSION

The most important finding of this study is the demonstration of differential sensitivity of human epithelial cancer cells and the exceptional high resistance of A549 cells to EGCG and establishing the mechanism of this resistance. Overexpression or deletion of antioxidant enzymes can alter the sensitivity of cancer cells to various cytotoxic insults, which substantiates the notion that ROS or their reactive derivatives are critical components of signal transduction pathways involved in cell proliferation and apoptosis (35). Although the mechanism of intracellular induction of oxidative stress by EGCG in malignant cells in vivo needs to be elucidated, high antioxidant capacity of the tumor cells can be hypothesized to lead to high resistance to apoptosis induced by EGCG, chemotherapeutic drugs or radiation, which are known to exert their antitumor effects by inducing the production of ROS (25, 36, 37).

In this study, we show that constitutive overexpression of HO-1 is not common to all lung cancer cells but specific to lung adenocarcinoma A549 cells, and also provide evidence that this overexpression leads to resistance against apoptosis induced by EGCG as well as ROS-producing tBHP and H2O2. Consistent with our findings, overexpression of HO-1 genes has been reported to augment pancreatic cancer aggressiveness, by increasing tumor growth, angiogenesis and metastasis (38). In addition, the observation that inhibition of HO-1 in pancreatic cancer cells having high constitutive HO-1 levels enhances their chemo- and radio-sensitivity (39) also supports our results. However, little is known about the chemoresistant and poor prognostic function of elevated HO-1 in lung cancer.

Several studies (40, 41) have described the cytoprotective effect of HO-1 in A549 cells, where HO-1 was induced by oxidant chemicals or transfection; however most of them have not focused on the highly induced basal levels of HO-1 (42). Our finding suggests significantly increased vulnerability to apoptosis by silencing of basal ho-1 gene (Fig. 4) and provides convincing evidence that the constitutive expression of HO-1 plays a primary role in enhancing chemoresistance in these cells. The significantly increased upregulation of p53 and its downstream p21 and Bax by ho-1 silencing (Fig. 4G) supports the possibility that HO-1-derived CO, generates an antiapoptotic effect by inhibiting both the expression of p53 and release of mitochondrial cytochrome c (43, 45). The proapoptotic tumor suppressor p53 also has been known to be influenced by the redox state of the cells (28) and a shift in oxidative status due to HO-1 knockdown might increase p53 and subsequent EGCG-sensitivity. This explanation agrees with previous other studies (40, 44). Moreover, we found that pure oxidative stress (induced by t-BHP and H2O2) is significantly more effective in killing lung cancer cells after knockdown of ho-1 (Fig. 4H). It is reasonable to postulate that ROS scavenging is likely to play a crucial role in antiapoptotic effect of HO-1.

Although Nrf2-mediated phase II gene induction, which provides protection against
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carcinogens, has been a primary mechanism of many cancer chemopreventive agents (12, 13), it can be speculated that the observed high expression level and transcriptional activity of Nrf2 in A549 cells may render pre-neoplastic lung epithelial cells more resistant to apoptosis. To the best of our knowledge, this is the first report showing that constitutive overexpression of HO-1 in A549 cells is driven by constitutively activated Nrf2-ARE (Fig. 6A-6E), which is linked with the cellular resistance to EGCG and other oxidative stress. This is supported by a recent study that shows Nrf2-controlled constitutive expression of ARE-driven genes (55). It has been suggested that EGCG induces HO-1 and regulates Nrf2-dependent genes in some normal cells and mouse models (46), however it is of interest that EGCG treatment to these cells resulted in inhibition of Nrf2-mediated HO-1 overexpression, suggesting cell type specific regulation of this gene. It is interesting that Bach1 expression was significantly decreased by EGCG treatment and correlated with HO-1 expression (Fig. 5A and C). Therefore, it is unlikely that free heme-driven de-repression of Bach1 mediates overexpression of HO-1 in these cells.

Nuclear factor-kB (NF-kB) is an oxidative stress-sensitive transcription factor that plays a critical role in the regulation of a variety of genes important in cellular homeostasis and cell death (2). The HO-1 pathway of apoptosis resistance in human papillary thyroid carcinoma cells (47) and gastric cancer cells (44) has been reported to be associated with an increase in the levels of p21, involves a p38 MAPK and ERK-mediated pathway and is suppressed by inhibiting NF-kB. We have also observed downregulation of NFkB p65 by EGCG (data not shown), however, the level of HO-1 was found to be negatively associated with p21 and p53 (Fig. 4G), suggesting a different mechanism in the expression of HO-1. The inhibition of Nrf2-mediated HO-1 expression by specific inhibitors of PKCa and ERK, Ro-31-7549 (56) and PD98059 (57) (Fig. 8), suggested possible involvement of PKCa linked with its downstream ERK. Based on our findings and other previous studies (48-50), it is reasonable to conclude that elevated p-PKCa in A549 cells may play a role in the constitutively activated Nrf2 and HO-1, which leads to their antiapoptotic role.

In addition, it appears that the interestingly low expression level of ferritin as compared to HO-1 overexpression (Fig. 2A, 2B and 2D) sustains high concentration of intracellular iron produced by HO-1 catalytic activity that may result in phosphorylation of PKCa and subsequent activation of Nrf2 (48, 51). In this scenario, we speculated that EGCG, as an iron chelator (26, 33) and not as a ROS generator (36, 37) may exert its inhibitory effects on phosphorylation of PKCa (Fig. 7D) followed by inhibition of Nrf2-signaling (Fig. 6). This suggestion is strengthened by the observation that iron deprivation by DFO reduces p-PKCa and HO-1 as well as augments EGCG-mediated downregulation of the two proteins and PARP cleavage (Fig. 9).

In summary, we identified A549 lung cancer cells, among cancer cells, as being resistant to EGCG-induced apoptosis. We further observed upregulation of PKCa-Nrf2-HO-1 pathway as a mechanism for this resistance. Higher concentration of EGCG was able to overcome this resistance by inhibiting iron-involved PKCa-Nrf2-HO-1 pathway as a potential iron chelator (Fig. 10). However, more in-depth studies covering these scenarios as well as further studies using human lung tumor samples will be necessary to illustrate the potential prognostic and diagnostic role of HO-1 in lung carcinoma. Overall, our study provides new insights into the mechanism of resistance and offers new targets for therapy of lung cancer.

REFERENCES


**Acknowledgements**—This study was supported by USPHS grants RO1-CA78809 and RO1-CA101039 and O’Brien Centre Grant P50-DK065303-01.

**Abbreviations**—EGCG: (-)-epigallocatechin-3-gallate; HO-1: heme oxygenase-1; Nrf2: nuclear factor erythroid 2-related factor 2; ARE: antioxidant responsive element; PKC: protein kinase C; MAPKs: mitogen activated protein kinases; ERK: extracellular regulated kinase; SnPPiX: tin protoporphyrin IX; BSO: L-buthionine-(S,R)-sulfoximine; GCL: glutamate cysteine ligase; GCLC: the catalytic subunit of GCL; ROS: reactive oxygen species; DFO: deferoxamine; NAC: N-acetylcysteine; Trx: thioredoxin;
HO-1 confers resistance to apoptosis induction by EGCG

SOD: superoxide dismutase; Gpx: glutathione peroxidase; siRNA: small interfering RNA; NSCLC: non small cell lung cancer; t-BHP: tertiary-butylhydroperoxide; PARP: poly(ADP-ribose) polymerase

FIGURE LEGENDS

Figure 1. Differential sensitivity of human epithelial cancer cells to EGCG treatment. (A) Cell viability was determined by MTT assay. Cells (2-5 × 10⁴ cells per well for 24-well plate) were seeded into plate and cultured overnight in the complete medium, and then treated with EGCG (0-100 μM) for 24, 48, and 72 h, respectively. Each concentration of EGCG was repeated in 4 wells. Data is presented as percent viable cells, where vehicle-treated cells were regarded as 100% viable. Points represent mean±S.E.M of three independent experiments. (B) Fifty percent growth inhibition concentration (IC₅₀) of EGCG for each cancer cell line was obtained from data in Fig. 1A.

Figure 2. Constitutive overexpression of HO-1, GCL, and Nrf2 in A549 cells. (A) Immunoblot analysis of HO-1 and HO-2 in eight kinds of human epithelial cancer cells. (B) Immunoblot analysis of primary and phase II antioxidant enzymes. (C) Immunoblot analysis of redox-associated transcription factors in cancer cells. (D) Immunoblot analysis of targets of interest, HO-1, GCL, and Nrf2 in lung-derived normal cells and cancer cells. Equal loading of protein (30-40 μg) was confirmed by stripping immunoblots and reprobing them for β-actin. Representative immunoblots of two to three independent experiments with similar results. Details are described in Materials and Methods. HO-2, heme oxygenase-2; GCLC, catalytic subunit of glutamate cysteine ligase; Trx, thioredoxin; Gpx, glutathione peroxidase; SOD, superoxide dismutase

Figure 3. Effect of SnPPIX and BSO on resistance to EGCG-induced apoptosis in A549 cells. (A) Effect of EGCG treatment on protein expression of HO-1 and GCL, and genomic DNA. Treatment of cells with EGCG (50 and 100 μM; 48 hr) resulted in DNA laddering in HCC1419, HT-29 and SKOV3 cells but not in A549 cells that displays high level of HO-1 and GCL protein expression. Detached and adherent cells were subjected to DNA isolation and equal amount (5 μg DNA) was resolved on a 1.5% agarose gel. Details are described in Experimental Procedures. (B) Effect of EGCG treatment to A549 cells pretreated with SnPPIX. Cells were pretreated with nontoxic maximum concentrations of SnPPIX (10 μM) and BSO (1 μM) for 2 hr, followed by treatment with EGCG (50, 100 μM; 48 hr) and subsequently determining cell viability by MTT assay. Data are presented as means±S.E.M of four independent experiments. *p < 0.01; **p < 0.001 compared with EGCG only. (C) Effect of SnPPIX or BSO on the EGCG-induced DNA fragmentation in A549 cells. After preincubation of A549 cells with SnPPIX (10 μM) and BSO (1 μM) for 2 hr, cells were washed with PBS and treated with EGCG (100 μM) for 48 h. DNA derived from different groups was resolved on an agarose gel.

Figure 4. Effect of siRNA-mediated silencing of ho-1 on apoptosis induction by EGCG. Dose-dependent ho-1 siRNA transfection effect on the expression of (A) HO-1 protein and (B) ho-1 mRNA. After cells (60-70% confluence) were transfected with different concentration of ho-1 siRNA (25-100 nM) and a non-specific siRNA (100 nM) for 24 h, the efficiency of ho-1 silencing was determined by Western and Northern blot analysis with, β-actin and rRNAs as loading controls respectively. NT: non-transfected control cells; NS-siRNA: non-silencing negative control siRNA. (C) Time-dependent ho-1 silencing as accessed by Western blotting. (D) Effect of ho-1 silencing on the number of viable cells in the absence or presence of EGCG. Of the two sets of ho-1 siRNA-transfected A549 cells (25 nM for 6-48 h) or non-transfected cells (0 h) in 24 well plates (0 h), one set of cells was harvested and stained with 0.4% Trypan blue and the other set of cells was washed with PBS and then treated with EGCG (40 μM for 24 h) followed by counting of viable cells. *p < 0.05; **p < 0.01; ***p <0.001 compared with nontransfected control without EGCG, †p < 0.05; ‡p < 0.01; ‡‡p <0.001 compared with nontransfected control with EGCG. (E) TUNEL-flowcytometry analysis of A549 cells transfected with ho-1 siRNA (25 nM; 6-48 h) or without siRNA (48 h) and subsequently treated with EGCG (40 μM, 24 h). Vehicle cells
were transfected with nonsilencing negative control siRNA for 48 h. Harvested cells containing both detached and attached cells or positive apoptotic cells supplied by the manufacturer were labeled with FITC-dUTP and PI at 37°C for 1 hr and analyzed on a flow/laser scanning cytometer equipped with a 488 nm Argon Laser as the light source. The percent cells in the region drawn from the apoptotic positive cells were considered as apoptotic. Cells silenced with ho-1 siRNA (48 h) without EGCG treatment showed a non-significant apoptotic effect of 1.2% (data not included). (F) Soft agar colony formation assay of A549 cells transfected with ho-1 siRNA (25 nM; 24 and 48 h) and followed by treatment with EGCG (40 μM, 24 h). Details are described in Experimental Procedures. (G) Effect of ho-1 silencing on protein expression of p53 and its targeted proteins, and expression of apoptotic hallmarks by post-treatment with EGCG (40 μM, 24 h). (H) Enhancement in t-BHP and H2O2-sensitivity after ho-1 silencing determined by sub G1 analysis. Cells transfected with ho-1 siRNA (25 nM; 24 h) and subsequently treated with 0.5 mM tBHP and 0.5 mM H2O2 for 6 h were subjected to cell cycle analysis.

Figure 5. **High concentration of EGCG blocks overexpression of HO-1 and induces apoptosis.** Effect of EGCG treatment (0-300 μM for 24 h) on the protein expression of (A) HO-1 and HO-2, (B) ferritin, GCL, Gpx, Trx, and SOD, and (C) Nrf2, Keap1, and Bach 1. High concentration of EGCG (48 h) induces apoptosis in A549 cells as assessed by (D) Annexin-V-fluous-PI staining with confocal microscopy and (E) immunoblotting for PARP cleavage. Cells were grown in a two-well chambered slide covered glass (Falcon; Flanklin Lakes, NJ) and treated with vehicle alone or specified concentrations of EGCG for 48 hours. The Annexin-V-fluous apoptosis detection kit was used for the detection of apoptotic death. The cells were dual-stained with annexin and PI. Stained cells were washed and mounted with an antifade mounting medium (Molecular Probes, Eugene, OR) and examined under a fluorescence confocal microscope for intracellular generation of ROS. Images were merged and processed using IPLab Spectrum and Adobe Photoshop (Adobe Systems) software. Cell populations that potentially may be detected are as follows: viable cells will be non-fluorescent; cells in the metabolically active stages of apoptosis will stain with annexin (green fluorescence) but not with PI (red fluorescence) and necrotic cells with PI staining. In addition, cells undergoing late stage apoptosis bind both annexin and PI.

Figure 6. **Constitutive nuclear localization of Nrf2 and Nrf2/ARE-driven transcriptional activation of HO-1.** Constitutive nuclear localization of Nrf2 and its cytosolic translocation by EGCG in a dose- and time-dependent manner as determined by (A) Western blotting of nuclear and cytosolic lysates and (B) by immunocytochemistry. After treatment with EGCG for 3-12 h, A549 cells grown on a two-well chambered culture slide were fixed in 100% methanol followed by blocking with 10% bovine serum for 2 h. The cells were incubated with monoclonal rabbit anti-Nrf2 antibody (2.5 μg/ml) in 1.5% bovine serum albumin for 60 min and then with fluorescein isothiocyanate-conjugated secondary antibody (Santa Cruz). Stained cells were washed, mounted with an antifade mounting medium (Molecular Probes) and examined under microscope. (C) Treatment of A549 cells with EGCG (200 μM; 1-12 hrs) inhibits basal level of Nrf2-ARE binding as assessed by EMSA. Binding specificity was tested by adding a 100-fold molar excess of cold ARE oligonucleotide. (D) ARE-HO-1 promoter activity as determined by ARE-luciferase activity. (E) HO-1 mRNA and protein as determined by Northern blotting, RT-PCR, and Western blotting. ARE oligonucleotide (5′-TTT TCT GCT GAC TCA AGG TCC G-3′; 3′-AAA AGA GCA GTC AGT TCC AGG C-5′) was biotin-labeled using the Biotin endlabeling kit (Pierce, Rockford, IL). Each binding reaction contained 1 X binding buffer, 50 ng/ml poly (dIdC), 0.05% NP-40, 5 μg of nuclear extract, and 20–50 pM of biotin end-labeled target DNA was incubated with anti-human Nrf2 antibody (2 μg; Santa Cruz) for 30 min to detect Nrf2 supershift and subjected to native gel electrophoresis and transferred to a nylon membrane. The biotin endlabeled DNA was detected using streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate. Ss-Nrf2: supershifted Nrf2; FP: free probe. For promoter assay, cells in 24-well plates were transfected with 0.5 μg of HO-1-ARE-luciferase reporter construct and 0.1 μg of β-gal plasmid for 24 h. Transfected cells were washed, treated with 200 μM EGCG for 3-12 hrs, and harvested for measuring luciferase activity. The fold
induction of luciferase activity was normalized to β-gal activity. **p < 0.01; ***p < 0.001 compared with control. Other details are described in Experimental Procedures.

Figure 7. A549 cells display markedly activated-PKCα that is inhibited by EGCG. Comparison of expression levels of (A) phosphorylated and total forms of PKCα and PKCδ (B) PI3K/p-AKT, and (C) MAPKs including ERK1/2, JNK/SAPK, and p38 in human epithelial cancer cells. (D) Effect of EGCG treatment (200 μM; 0.5-6 hrs) on the expression of p-PKCα, p-ERK1/2, and p-AKT in A549 cells. Equal loading of protein (30-40 μg) was confirmed by stripping immunoblots and reprobing them for β-actin or non-phosphorylated kinases. Representative immunoblots of two to three independent experiments with similar results. Details are described in Experimental Procedures.

Figure 8. PKCα inhibitor Ro-317549 among small molecule inhibitors of protein kinases significantly abrogated Nrf2-dependent HO-1 overexpression and EGCG-resistance. (A) Immunoblotting for nuclear and total Nrf2 expression and (B) Nrf2-ARE binding as assessed by EMSA, (C) Expression of HO-1 protein and mRNA as determined by Western and Northern blotting, (D) HO-1-ARE promoter activity. Nontoxic maximum concentrations of the inhibitors were employed for these experiments; PKCα inhibitor Ro-317549 (5 μM, 3 h), ERK inhibitor PD98059 (20 μM; 3 hrs), LY294002 (10 μM, 3 h); Triciribine (20 μM, 3 h). (E) Effect of PKCα inhibitor Ro-317549 on EGCG-sensitivity. A549 cells were treated with EGCG (80 μM; 24 hrs) in the absence or presence of Ro-31-7549 (2.5-10 μM) and subjected to MTT assay. **p < 0.01; ***p < 0.001 compared with control.

Figure 9. DFO but not NAC augmented EGCG-mediated inhibition of p-PKCα expression and subsequent HO-1 expression and PARP cleavage. For assessing these effect, different treatment time periods were employed; p-PKCα expression (3 h), HO-1 expression (24 h), and PARP (48 h). Three sets of A549 cells were treated with EGCG (80 μM) in presence of DFO (50 μM) or NAC (200 μM), and at each time point whole cell lysates were subjected to Western blotting.

Figure 10. Schematic illustration of a proposed model of HO-1 overexpression and EGCG resistance in human lung adenocarcinoma A549 cells.
Table 1: IC50 values (μM) of EGCG towards human cancerous cell lines.

<table>
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<tr>
<th>Cell line</th>
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<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
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<td>PC-3</td>
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<td>AsPC-1</td>
<td>Pancreatic epithelial adenocarcinoma</td>
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* Fifty percent growth inhibition concentration (IC50) of EGCG towards human cancerous cell lines.
Fig. 6

A

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<tr>
<th></th>
<th>EGCG (6h)</th>
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<td>0 3 6 12 h</td>
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Nuclear

Cytosolic

B

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<td>0  50 100 200</td>
<td>0 3 6 12</td>
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C

<table>
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</table>

Nuclear Cytosolic

D

Relative ARE-Luciferase activity

E

200 μM EGCG

- Relative ARE-Luciferase activity

- TA-Luc

- ARE-Luc

- 200 μM EGCG

- HO-1

- GAPDH

- β-Actin

- Northern

- RT-PCR

- Western
Fig. 9

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<th>Time</th>
<th>p-PKCα</th>
<th>β-Actin</th>
<th>HO-1</th>
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<th>PARP 85</th>
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<td>48 h</td>
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</tbody>
</table>

- EGCG 80 μM
  - 3 h: +
  - 24 h: -
  - 48 h: -

- NAC 200 μM
  - 3 h: -
  - 24 h: +
  - 48 h: -

- DFO 100 μM
  - 3 h: -
  - 24 h: +
  - 48 h: -
Fig. 10

- EGCG
- PKCa
- Keap1
- Nrf2
- MAP
- ARE
- bzip
- Fermitin
- A549 cells
- HO-1

Growth stimulation
Resistant to apoptosis

- DFO
- Fe³⁺
- ERK
- UB
Constitutive overexpression of Nrf2-dependent heme oxygenase-1 in A549 cells contributes to resistance to apoptosis induced by EGCG
Mee-Hyang Kweon, Vaqar Mustafa Adhami, Jeong-Sang Lee and Hasan Mukhtar

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