ANTIOXIDANTS & REDOX SIGNALING
Volume 11, Number 5, 2009

DOI: 10.1089/ars.2008.2321

# Forum Original Research Communication

# Different Redox States in Malignant and Nonmalignant Esophageal Epithelial Cells and Differential Cytotoxic Responses to Bile Acid and Honokiol

Gang Chen,[1] Julie Izzo,[2] Yusuke Demizu,[1] Feng Wang,[1] Sushovan Guha,[3] Xifeng Wu,[4] Mein-Chie Hung,[5] Jaffer A. Ajani,[6] and Peng Huang[1]

## Abstract

Esophageal adenocarcinoma (EAC) is a highly lethal cancer in western countries. EAC cells are believed to develop from esophageal epithelial cells through complex transformation processes involving inflammation and oxidative stress. The purpose of this study was to compare the redox status of malignant and nonmalignant esophageal epithelial cells and to test their responses to bile acid–induced oxidative stress and to treatment with honokiol (HNK), a natural product with anticancer activity. We demonstrated that esophageal adenocarcinoma cells express significantly higher levels of antioxidant molecules and were resistant to reactive oxygen species (ROS) stress induced by bile acid, but were sensitive to the cytotoxic action of HNK. Mechanistic study showed that HNK caused cancer cell death by disruption of mitochondrial transmembrane potential and was correlated with cyclophilin D (CypD) expression. Inhibition of CypD by cyclosporin A or abrogation of its expression by siRNA significantly suppressed the cytotoxicity of HNK, suggesting that CypD may be a key molecule that mediates the cytotoxicity. Our study suggests that the high antioxidant capacity in EAC cells confers on them the ability to survive the oxidative microenvironment in the reflux esophagus, and that HNK is a promising compound to kill the transformed cells preferentially. *Antioxid. Redox Signal.* 11, 1083–1095.

## Introduction

Esophageal adenocarcinoma (EAC) is a highly lethal cancer, and its incidence has been increasing during the past 2 decades (13, 18, 46). Chronic gastroesophageal reflux is an important factor that contributes to the development of Barrett esophagus (BE) (3, 7). BE is the premalignant condition with the replacement of squamous esophageal lining by a layer of columnar epithelium (3, 15, 31). Esophageal adenocarcinoma cells are believed to develop from esophageal epithelial cells through complex transformation processes in a tissue microenvironment involving inflammation and oxidative stress (9, 40). Prolonged exposure to bile acids is believed to be an important cause of local damage to esophageal epithelial cells, leading to chronic inflammation and generation of ROS, which may cause genetic alterations and promote the carcinogenesis processes. Both BE and EAC are inflammatory conditions, and the oxidative stress and free-radical generation associated with inflammation could provide a link between BE and EAC (9, 14, 40). Bile acid/bile salts and/or low pH in the refluxed substances can induce ROS generation and consume antioxidants such as glutathione. This may in turn lead to a compensative upregulation of the expression of oxidative stress–responsive genes (51), which will then affect cellular survival and drug response. Thus, examination of the redox states in malignant and nonmalignant esophageal epithelial cells will be important both for understanding the role of ROS in the development of EAC from BE and for designing rational strategies to prevent and treat esophageal cancers effectively.

Mitochondria play an important role in ATP generation, ROS production, and apoptosis regulation. Metabolic alterations and dysfunction of mitochondria have been observed in many types of cancer cells (10). Although several biologic

[1]Department of Molecular Pathology; [2]Department of Experimental Therapeutics; [3]Department of Gastroenterology, Hepatology and Nutrition; [4]Department of Epidemiology; [5]Department of Molecular and Cellular Oncology; and [6]Department GI Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas.

processes are responsible for the production of ROS, mitochondria seem quantitatively to be the major source of ROS in the cells (55). Previous investigators reported that chronic inflammation in BE and EAC cause the ROS level to increase. Dysfunction of mitochondria may be evolved in the tumor-transformation processes because of ROS generation and possibly compromised ability to cope with oxidative stress. Furthermore, mitochondria play a central role in drug-induced cell death through regulation of apoptosis (22, 32). Aberrant formation of the mitochondrial permeability transition pore (MPTP) may lead to mitochondrial swelling, uncoupling of oxidative phosphorylation, and subsequent ATP depletion, releasing of apoptotic factors such as cytochrome *c*, apoptosis-including factor (AIF), and Diablo/SMAC (8, 42). It has been proposed that the components of MPTP may include the adenosine nucleotide transporter (ANT) associated with the inner membrane, the voltage-dependent anion channel (VDAC) associated with the outer membrane, and cyclophilin D (CypD) in the matrix (2, 23, 53). The association of these components and their interaction with other cellular proteins such as hexokinase II and Bcl-2 family members affects the permeability of this protein complex. The components of the MPTP can be regarded as potential targets for pharmacologic intervention of resistant cancer therapies. Distinctly different properties of mitochondria are found between normal and cancer cells, which may offer a possibility to target preferentially the altered mitochondria in cancer cells as a therapeutic strategy (22, 45).

Honokiol, a natural product found in the cones and bark of *Magnolia officinalis*, has antioxidant/antiinflammatory properties and has been used in traditional medicine to treat a number of conditions including anxiety, thrombosis, and emesis (16, 21, 30, 48). In addition to these effects, new pharmacologic effects including anticancer activity and biochemical activities of HNK have been reported during the last decade. HNK was found to have a remarkable inhibitory effect on the proliferation of human leukemic HL-60 cells (27). It was suggested that this compound may function as a potent scavenger of hydroxyl radicals (35), which may be attributed to the allyl groups (36). HNK has been shown to have both antiangiogenesis and antitumor effects through undefined mechanisms, which may include activation of caspase 3, 7, 8 and 9, cleavage of PARP or Mcl-1 (6, 24, 28); and inhibition of the NF-$\kappa$B pathway (1, 52).

In this study, we compared the redox states of malignant and nonmalignant esophageal epithelial cells and their responses to oxidative stress induced by primary bile acid CDCA. The potential differential effect of HNK on these two cell types also was examined. We demonstrated that esophageal adenocarcinoma cells express high levels of antioxidant molecules and are resistant to bile acid–induced oxidative stress but were highly sensitive to the cytotoxic action of HNK in a dose- and time-dependent manner. This compound exhibited low toxicity to nonmalignant esophageal cells. The potential underlying mechanisms also were investigated.

## Materials and Methods

### Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), Rhodamine 123, CM-H2DCF-DA, hydroethidine (HEt), MitoTracker Green, and MitoTracker Red were purchased from Invitrogen/Molecular Probes (Carlsbad, CA). Honokiol was a kind gift from Zhongxin Innova Laboratories (Tianjin, China). *N*-acetyl-L-cysteine (NAC), bovine catalase, bongkrekic acid, and cyclosporin A were purchased from Sigma-Aldrich (St. Louis, MO). Cyclophilin D siRNA was purchased from Dharmacon, Inc. (Lafayette, CO). The nucleotide sequences of the siRNA are as follows: sense, 5-UUU GAC GUG ACC GAA CAC AAC AUGC-3; antisense, 5-GCA UGU UGU GUU CGG UCA CGU CAAA-3. Lipofectamine 2000 transfection reagent was purchased form Invitrogen, Inc. (Carlsbad, CA).

### Cell lines and cell culture

Immortalized human Barrett esophageal epithelial cell lines (CP-A and CP-C) were cultured in MEBM (Lonza) medium containing 5% fetal bovine serum and the MEGM singlequots (Lonza) growth factors cocktail. CP-A and CP-C cells were originally derived from Barrett esophagus premalignant epithelium with hyperplasia and dysplasia, respectively, and were transfected with the human telomerase to extend the cell life span (43). Human esophageal adenocarcinoma cell lines (BE-3, SK-4, OE-21, and OE-23) were cultured in DMEM/F12 (Invitrogen) medium supplemented with 10% fetal bovine serum. All cell lines were maintained in a tissue-culture incubator at 37°C in humidified air with 5% $CO_2$. To determine the effect of CDCA and HNK in Barrett esophageal epithelial and esophageal adenocarcinoma cells, the cells were incubated with various concentrations of each compound for various times as indicated, followed by assays of cytotoxicity (MTT and flow-cytometry analysis) or ROS measurement as described later.

### Assays for cytotoxicity

Cell death was determined with flow cytometry after cells were double-stained with annexin-V-FITC and propidium iodide (PI), by using an assay kit from BD PharMingen (San Diego, CA) as described previously (44). To determine the drug effect on cell viability and long-term proliferation, we used the MTT assay by seeding cells in 96-well plates for drug treatment. After drug incubation for 72 h, cells were then incubated with MTT reagent for 4 h. After removal of the culture medium, the cells were lysed with DMSO, and optical density at 570 nm was quantified by a plate reader.

### Analyses of cellular ROS and mitochondria properties

Cellular $H_2O_2$ contents were measured by incubating the control or drug-treated cells (CP-A, CP-C, BE-3, OE-21, -23, and SK-4 cells) with 3 $\mu M$ CM-$H_2$DCF-DA for 30 min, followed by flow-cytometry analysis with an FACSCalibur equipped with the CellQuestPro software. Cellular $O_2^-$ was measured with flow cytometry by using HEt staining (100 ng/ml) as described (44). Mitochondria mass was measured with flow cytometry by using MitoTracker Green (200 ng/ml) and MitoTracker Red (200 ng/ml) as potential-independent and potential-dependent dye, respectively. Mitochondrial transmembrane potential was also quantified with flow-cytometry analysis by using Rhodamine 123 (100 n$M$) as fluorescent dye.

### Determination of cellular glutathione

A glutathione assay kit (Cayman Chemical Co., Ann Arbor, MI) was used to measure total cellular glutathione. Cell ex-

tracts were prepared with sonication and deproteination by using the conditions recommended by the manufacturer. Samples were transferred to 96-well plates, and total glutathione was detected by measuring the product of glutathionylated DTNB, which was quantified by optical density at 405 nm by using a plate reader. The cellular glutathione contents were calculated by using the standard curve generated in parallel experiments. Cellular protein concentrations were determined by BCA assay (Pierce, Rockford, IL) after sonication. The GSH assay-reading results were normalized by samples of protein concentration.

### *Determination of cellular ATP*

The CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI) was used to measure cellular ATP levels. In brief, cells were seeded in 96-well cell-culture plates overnight and then treated with honokiol for the desired incubation times. The assay reagents were reconstructed and added to the cell samples in 96-well plates according to the manufacturer's protocol. The samples were agitated on a shaker for 5 min to lyse the cells. The lysates were transferred to clean 96-well plates for determination of luminescent signal by using a plate reader.

### *Measurement of cellular respiration activity*

Oxygen consumption in intact cells was measured as an indication of mitochondrial respiration activity. The control or drug-treated cells were suspended in 1 ml of culture medium preequilibrated with 21% oxygen and then were placed in a sealed respiration chamber to monitor oxygen consumption, by using the Oxytherm system (Hansatech Instrument, Norfolk, England), as previously described (44).

### *Confocal microscopy*

Cells were cultured on sterilized glass-slide covers until they reached 70–80% confluence. Bile acid or honokiol was added at different time points, and 200 n*M* MTtracker Red was given 1 h before harvesting. Cells were washed with fresh warm medium for 5 min twice and fixed with 3.7% paraformaldehyde in medium at 37°C for 15 min. After washing 3 times, cells were stained with 400 ng/ml DAPI in PBS for 5 min at room temperature. The slide covers were mounted to glass slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) to preserve fluorescence. Images were acquired by using a NIKON Eclipse TE2000 confocal microscope and analyzed by using the Nikon EZ-C1 software.

### *Statistical analysis*

The statistical significance of the difference between two sample sets in their cytotoxicity, cellular ATP levels, and mitochondrial mass was evaluated by using Student's $t$ test. A $p$ value of $<0.05$ was considered statistically significant.

## Results

### *Different redox states in malignant and nonmalignant esophageal epithelial cells and their differential responses to ROS stress induced by bile acid exposure*

It has been known for some time that bile acids can cause ROS generation and oxidative stress to esophageal epithelial cells because of bile reflux (17, 50). However, it is not clear how the transformed esophageal epithelial cells could sustain the repeated oxidative stress induced by bile acids and eventually proliferate and emerge as tumors. To investigate the possible underlying mechanisms, we first compared the basal cellular ROS contents in two malignant esophageal adenocarcinoma cell lines (BE-3 and SK-4) and two nonmalignant esophageal epithelial lines (CP-A, CP-C), and then tested their responses to treatment with primary bile acid CDCA. As shown in Fig. 1A, the basal superoxide ($O_2^-$) contents of all four cell lines were similar, as revealed by flow-cytometry analysis with hydroethidine (HEt) as a chemical probe for $O_2^-$. Exposure of the cells to CDCA did not cause any significant change in cellular $O_2^-$. In contrast, the cellular contents of hydrogen peroxide ($H_2O_2$), as measured by CM-DCFDA, were heterogeneous among the cell lines (Fig. 1A). Exposure of the cells to CDCA for 30 min caused a significant increase of $H_2O_2$ in each cell line (approximately 10-fold increase; Fig 1A). This rapid increase of ROS suggests that induction of $H_2O_2$ accumulation was an early biochemical event triggered by CDCA. Despite this similar increase in bile acid–induced ROS generation, all esophageal carcinoma cells (BE-3, OE-23, SK-4) were resistant to bile acid–induced cell death, with only $\sim$20% cell death even at a high concentration (200 $\mu M$) of CDCA. In contrast, the nontumorigenic esophageal epithelial cells (CP-A and CP-C) were much more sensitive to the oxidative damage, with >90% cell death when CP-A cells were exposed to 50 $\mu M$ CDCA for 72 h (Fig. 1B). The cytotoxic effect of CDCD was confirmed by using flow-cytometry analysis after the cells were double-stained with annexin-V/PI (data not shown). Interestingly, CP-C cells, which were originally derived from high-grade dysplasia Barrett esophagus, were less sensitive than CP-A cells to CDCA treatment. It appeared that the extent of CDCA-induced cell death was inversely correlated with the degree of malignancy.

Consistent with these observations, CDCA exerted a significant inhibitory effect on cellular respiration in CP-A cells, evidenced by a substantial decrease in oxygen consumption (Fig. 1C). Incubation of CP-A cells with 100 $\mu M$ CDCA for 3 and 6 h led to a decrease of the oxygen-consumption rate by 38 and 82%, respectively. In contrast, the same bile acid caused a minimal change (10% decrease) in respiration in the carcinoma cells (SK-4 and BE-3 lines) even at a higher CDCA concentration (200 $\mu M$) (Fig. 1D).

To examine the biochemical basis responsible for the cancer cell resistance to ROS stress induced by bile acid, we used Western blot and biochemical assays to determine the expression of major antioxidant molecules and cellular glutathione levels in the esophageal carcinoma cells in comparison with nonmalignant CP-A and CP-C cells. As shown in Fig. 2A, among the major antioxidant molecules, the protein expression of superoxide dismutase-2 (SOD2 or MnSOD) and thioredoxin-2 (Trx-2) was elevated substantially in all three esophageal carcinoma cell lines; a moderate increase of catalase and Trx-1 also was noted. Strikingly, the cellular glutathione levels increased by 400% in all cancer cell lines ($n = 3$; $p < 0.05$). This increase in expression of antioxidant molecules and glutathione levels likely played an important role in the cancer cell resistance to ROS stress induced by CDCA. Because both MnSOD and Trx-2 are mitochondria-specific antioxidant molecules, we compared the mitochondrial mass

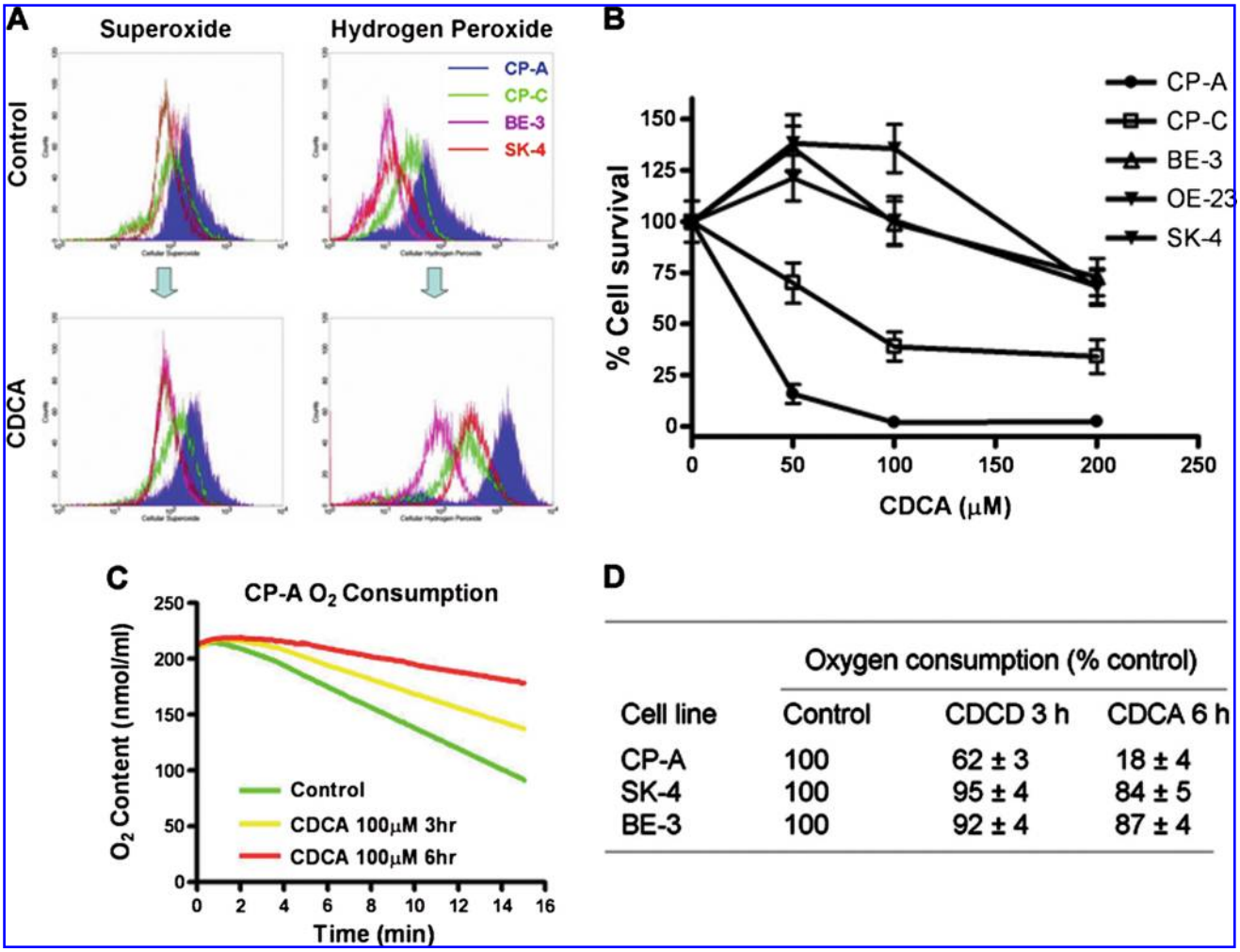



**FIG. 1. Malignant and nonmalignant esophageal epithelial cells exhibited significant difference in their redox states and responses to ROS stress induced by bile acid.** (**A**) The nonmalignant (CP-A, CP-C) and malignant BE-3, SK-4) esophageal cells showed various levels of basal cellular $H_2O_2$ contents, which increased dramatically on exposure to bile acid CDCA (100 $\mu M$, 30 min). Cellular $O_2^-$ levels were not significantly affected by the CDCA under the same conditions. (**B**) Comparison of cytotoxic effect of CDCA in nonmalignant and malignant esophageal cells. Cytotoxicity was measured by MTT assay (72 h; $n = 3$). *Bars* indicate SD. (**C**) Effect of CDCA on mitochondrial respiration in CP-A cells, measured by oxygen consumption. (**D**) Comparison of CDCA effect on oxygen consumption in nonmalignant (CP-A) and malignant esophageal cells (BE-3, SK-4). Cells were incubated with 100 $\mu M$ for 3–6 h as indicated. Data are expressed as the mean ± SEM of three independent experiments.

and the expression of several other important mitochondrial molecules in malignant and nonmalignant esophageal epithelial cells. As shown in Fig. 2C, the cancer cells contain significantly more mitochondrial mass than did the nonmalignant cells. The expression of cyclophilin D (CypD), ANT, hexokinase II (HXK II), and HSP60 proteins also increased in the cancer cells (Fig. 2D). Because CypD, ANT, and HXK II are involved in the mitochondrial permeability transition pore during apoptosis, the increase of these components might affect the apoptotic response.

### *Selective cytotoxic effect of honokiol against esophageal adenocarcinoma cells*

The differences between malignant and nonmaligant esophageal epithelial cells in their redox states and response to bile acid exposure prompted us to seek compounds that might preferentially affect the cancer cells with low toxicity to nonmalignant cells. A natural product, honokiol (HNK), exhibited promising activity. As shown in Fig. 3A, when HNK (10 $\mu$g/ml) was added to the CP-A, BE-3, and SK-4 cell culture and incubated for 12 or 24 h, a massive death of the cancer cells occurred, whereas minimal cell death was detected in the nonmalignant cells with flow-cytometry analysis. For instance, 10 $\mu$g/ml HNK induced <10% cell death in CP-A (nonmalignant) cells during a 24-h incubation, whereas under the same drug-incubation conditions, 79 and 83% cell deaths were found in the malignant BE-3 and SK-4 cells, respectively. This preferential cytotoxic activity of HNK against esophageal cancer cells was further confirmed by using a different assay (MTT) method (Fig. 3B). Interestingly, although MnSOD and Trx-2 were highly expressed in the esophageal carcinoma cells (Fig. 2B), the expression levels of these proteins did not change after treatment with HNK (data not shown).

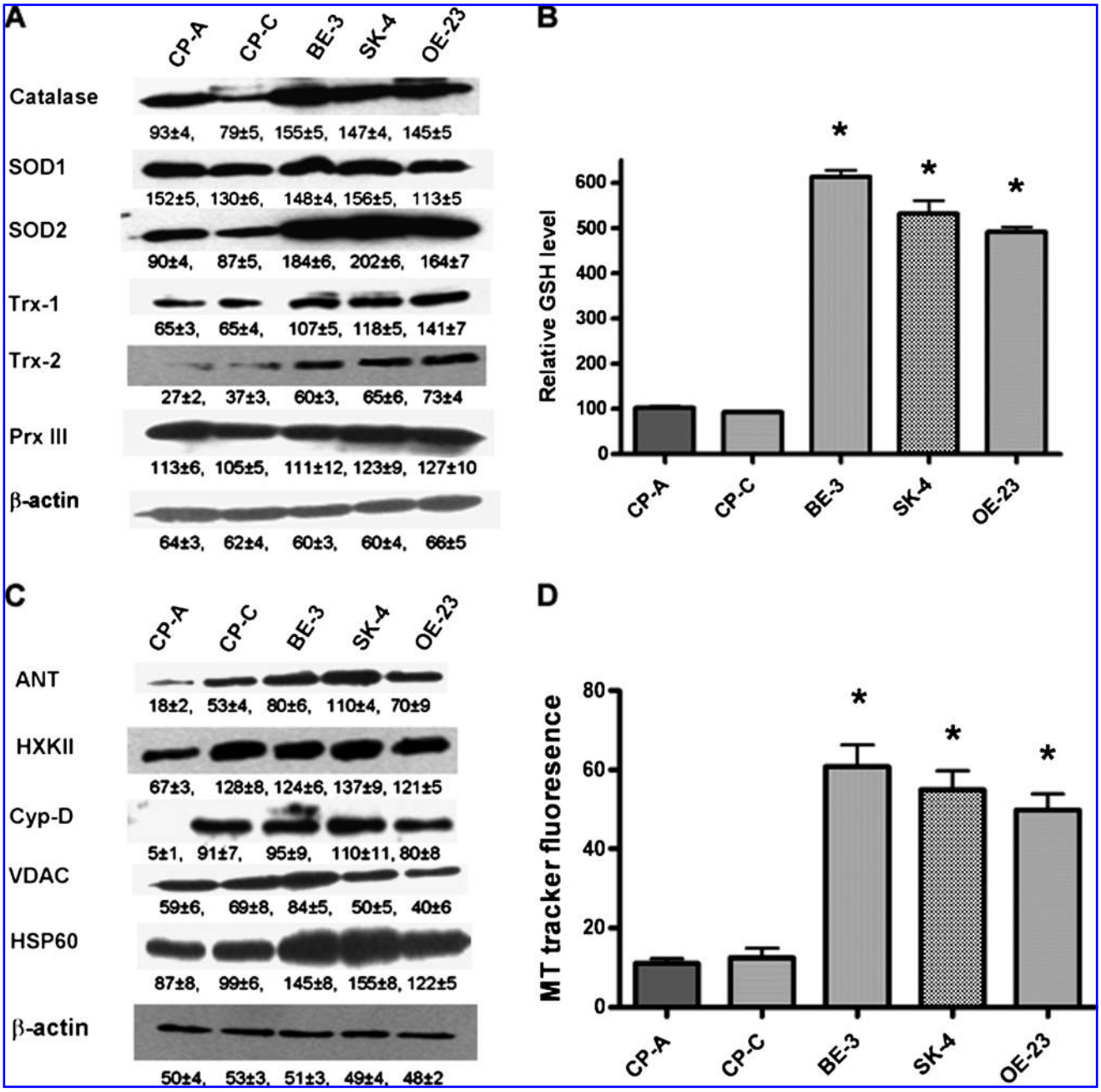
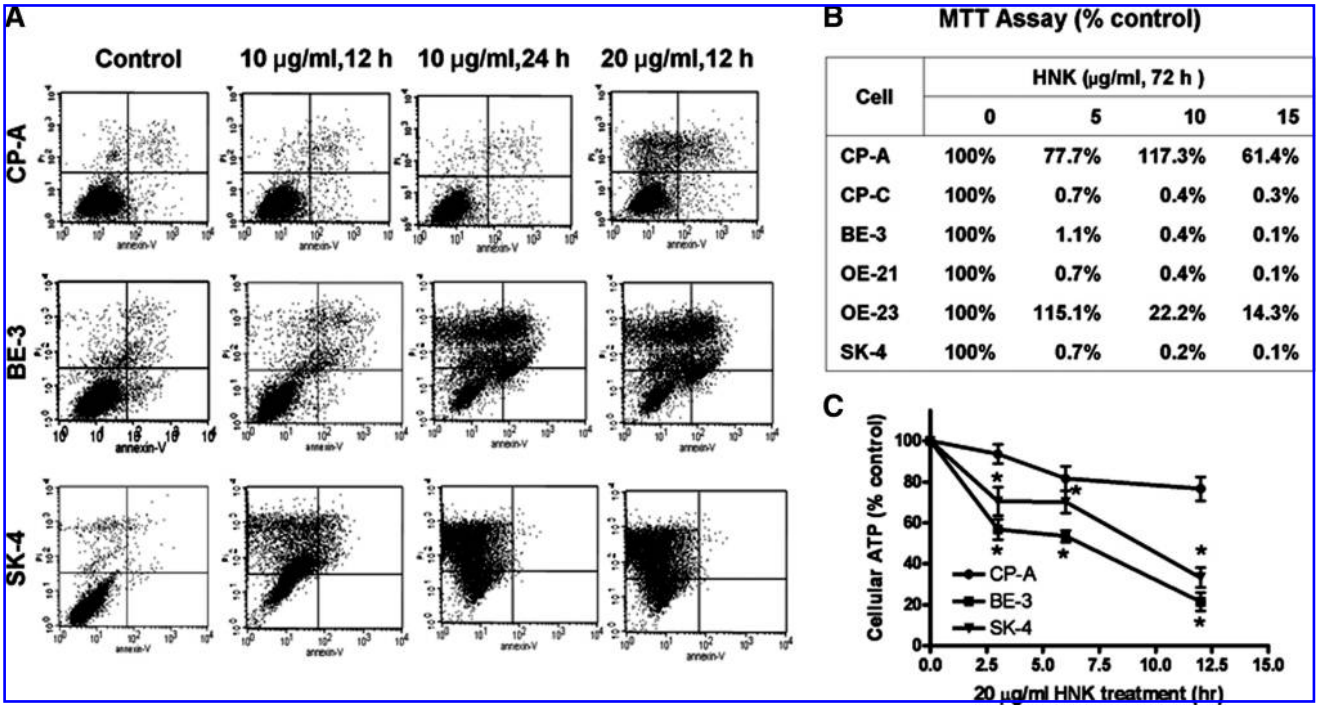



**FIG. 2. Comparison of antioxidant expression, mitochondrial mass, and relevant molecules in nonmalignant and malignant esophageal cell lines.** (**A**) Western-blot analysis of the indicated major antioxidant molecules in two nonmalignant (CP-A, CP-C) and three malignant esophageal cell lines (BE-3, SK-4, OE-23). $\beta$-Actin also was blotted as the loading control. The numbers under the blots indicate the densitometry mean $\pm$ SD ($n = 3$). (**B**) Comparison of the cellular GSH levels in nonmalignant and malignant esophageal cell lines. Data are expressed as the mean $\pm$ SEM of three independent experiments. *$p < 0.05$, comparing nonmalignant and malignant cells. (**C**) Western-blot analysis of several mitochondrial proteins relevant to MPTP in nonmalignant and malignant esophageal cell lines. The numbers under the blots indicate the values of densitometry quantitation (mean $\pm$ SD, $n = 3$). (**D**) Comparison of mitochondria mass in nonmalignant to malignant cells, measured with flow-cytometry analysis by using MitoTracker green as a fluorescent probe. Data are expressed as the mean $\pm$ SEM of three independent experiments. *$p < 0.05$ comparing nonmalignant and malignant cells.




| Cell | HNK (μg/ml, 72 h ) | | | |
|---|---|---|---|---|
| | 0 | 5 | 10 | 15 |
| CP-A | 100% | 77.7% | 117.3% | 61.4% |
| CP-C | 100% | 0.7% | 0.4% | 0.3% |
| BE-3 | 100% | 1.1% | 0.4% | 0.1% |
| OE-21 | 100% | 0.7% | 0.4% | 0.1% |
| OE-23 | 100% | 115.1% | 22.2% | 14.3% |
| SK-4 | 100% | 0.7% | 0.2% | 0.1% |

**FIG. 3. Preferential killing of malignant esophageal carcinoma cells by the natural compound honokiol.** (**A**) Annexin-PI cytotoxicity assay of cytotoxic effect in HNK in CP-A, BE-3, and SK-4 cells. (**B**) Comparison of HNK effect on nonmalignant and malignant esophageal epithelial cells. MTT assay was used to measure cytotoxic effect (72-h drug incubation). (**C**) Differential effect of HNK on cellular ATP in nonmalignant (CP-A) and malignant esophageal epithelial cells (BE-3, SK-4). Cellular ATP was measured in triplicate as described in Methods. $^*p < 0.01$ comparing nonmalignant and malignant cells.

MTT assay revealed that the high-grade dysplasia Barrett cells (CP-C cells) were highly sensitive to HNK.

ATP analysis showed that HNK induced a rapid depletion of cellular ATP in the cancer cells, whereas the ATP decrease in CP-A cells was only modest (Fig. 3C). For instance, incubation with 20 $\mu$g/ml HNK for 12 h caused a dramatic decrease of ATP to 40 and 22% in BE-3 and SK-4 cells, respectively. The same drug incubation caused a decrease of ATP to only 81% of the control in CP-A cells.

### *HNK killed esophageal cancer cells by disrupting the CsA-sensitive MPTP, leading to the collapse of mitochondria*

To investigate the mechanism by which HNK killed esophageal cancer cells, we first examined the effect of this compound on the integrity of mitochondria and its impact on the cellular redox state. As shown in Fig. 4A, incubation of BE-3 and SK-4 esophageal cancer cells with HNK (20 $\mu$g/ml, 12 h) caused a substantial decrease of mitochondrial transmembrane potential, as revealed by staining of the cells with a potential-sensitive dye MitoTracker Red and analyzed with the confocal-microscopy technique. The same HNK incubation conditions did not cause a significant change in the nonmalignant CP-A cells, consistent with the selective activity of this compound. The differential effect of HNK on mitochondrial transmembrane potential was further confirmed with flow cytometry analysis, by using rhodamine-123 as another transmembrane potential–sensitive dye (Fig. 4B). Interestingly, the ability of HNK to induce collapse of transmembrane potential could be largely prevented by a 2-h pretreatment with 5 $\mu M$ cyclosporin A (CsA; Fig. 4B). Because CsA is a compound that specifically interacts with the MPTP component cyclophilin D and affects the pore permeability, these data suggest a possibility that cyclophilin D may be a target of HNK. The decrease in transmembrane potential induced by HNK was associated with a significant suppression of mitochondrial respiration, as evidenced by a substantial reduction of cellular oxygen consumption (Fig. 4C).

Because the mitochondrial respiratory chain is the major site of ROS generation and HNK exhibited a major impact on mitochondrial transmembrane potential and respiration, we then evaluated the effect of this compound on ROS generation in two esophageal cancer cell lines in the presence and absence of CsA. As illustrated in Fig. 4D, incubation with HNK caused a more than 20-fold increase of ROS ($H_2O_2$) generation in both cancer cell lines (BE-3 and SK-4). This ROS increase could be partially oversuppressed by pretreatment with 5 $\mu M$ CsA, suggesting that the increase of ROS generation might be a consequence of HNK-induced collapse of mitochondrial potential, leading to respiratory dysfunction and electron leakage from the respiratory chain.

### *Cyclophilin D played a key role in mediating the cytotoxic action of HNK*

Because HNK caused a dramatic increase of ROS, which can be prevented by the cyclophilin D–targeted agent CsA, we further evaluated the role of ROS generation and the significance of cyclophilin D in mediating cytotoxic action of HNK in

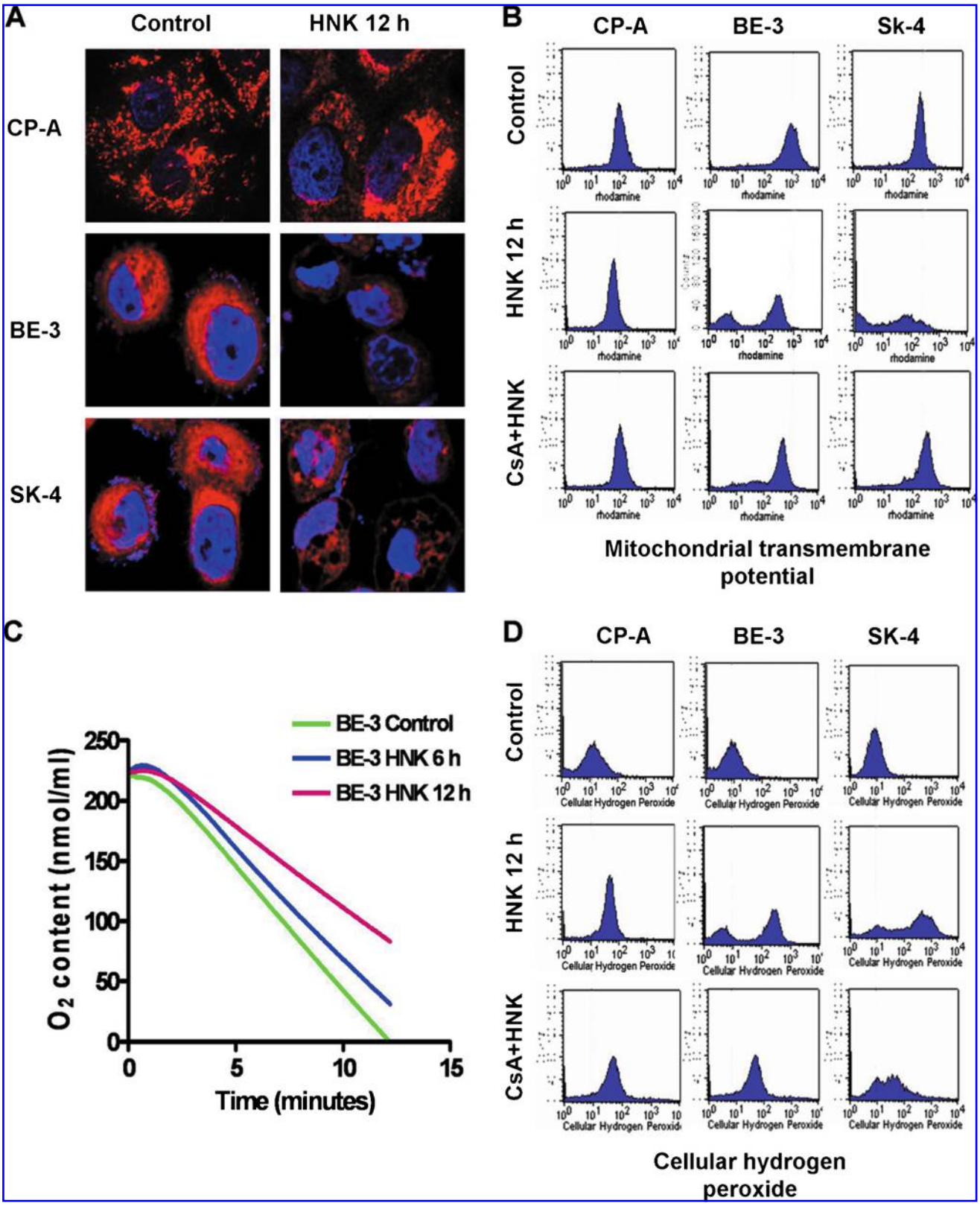



**FIG. 4. Effect of HNK on mitochondrial transmembrane potential, respiration, and ROS generation in nonmalignant and malignant esophageal cell lines.** (**A**) Esophageal cells were treated with 20 $\mu$g/ml HNK for 12 h and then stained with 200 ng/ml MT tracker Red for 30 min. After fixation, cell images were acquired by using confocal microscopy. (**B**) Quantitative comparison of mitochondrial transmembrane potential in CP-A, EB-3, and SK-4 cells treated with 20 $\mu$g/ml HNK in the presence or absence of 5 $\mu M$ cyclosporin A (CsA). (**C**) Time-dependent inhibition of mitochondrial respiration by HNK in malignant esophageal BE-3 cells. Oxygen consumption was measured as an indicator of respiration, as described in Methods. (**D**) Differential effect of HNK and CsA on cellular ROS in nonmalignant and malignant esophageal epithelial cells. Cellular ROS contents were measured with flow-cytometry analysis by using CM-$H_2$DCF-DA as a fluorescent dye.

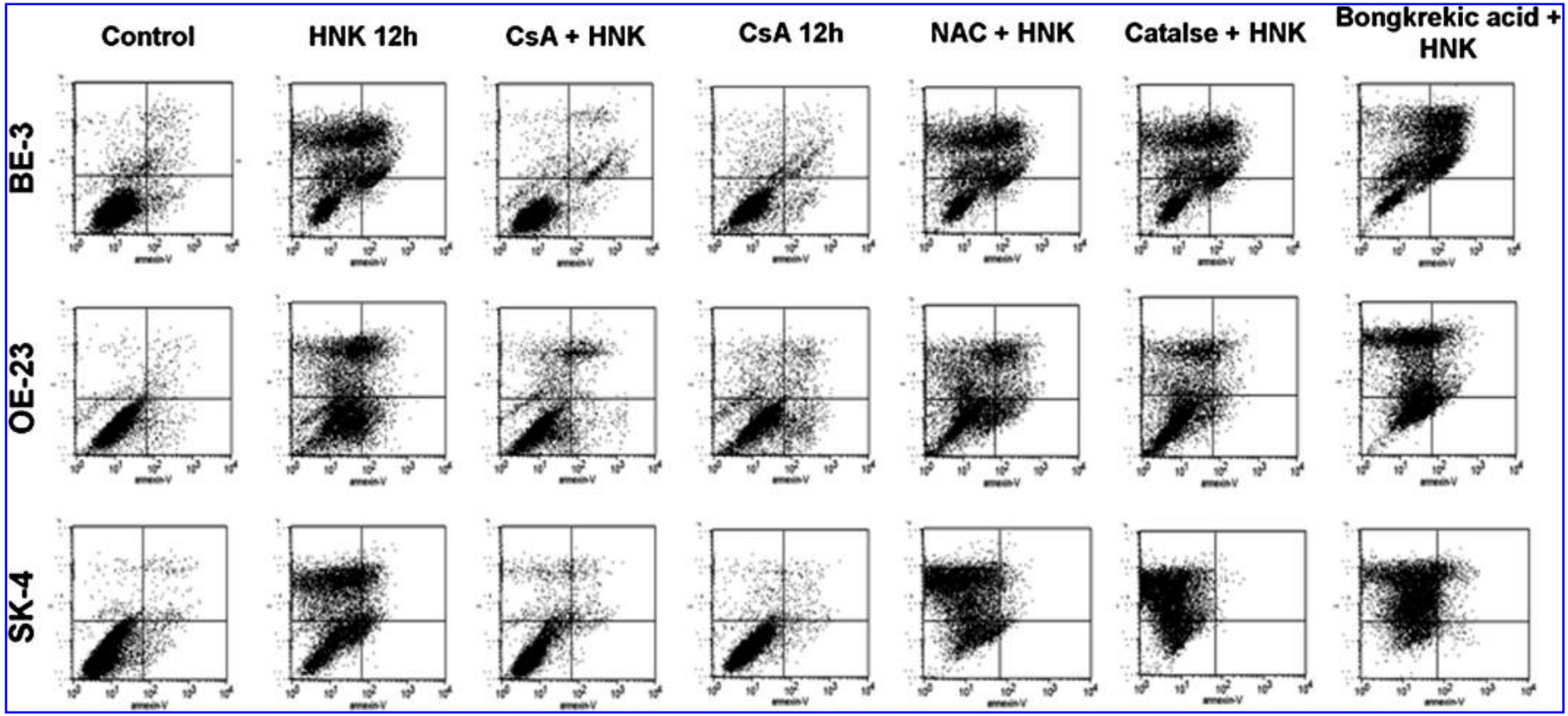



**FIG. 5. Cytotoxic action of HNK against malignant esophageal carcinoma cells through a CsA-dependent mechanism.** Malignant esophageal cells (BE-3, OE-23, and SK-4) were incubated with HNK (20 $\mu$g/ml, 12 h) in the presence or absence of a 2-h pretreatment with 5 $\mu M$ CsA, 1 m$M$ NAC, 1,000 unit/ml catalase, or 5 $\mu M$ bongkrekic acid, as indicated. Cytotoxicity was measured with flow-cytometry analysis by using annexin-PI double staining.

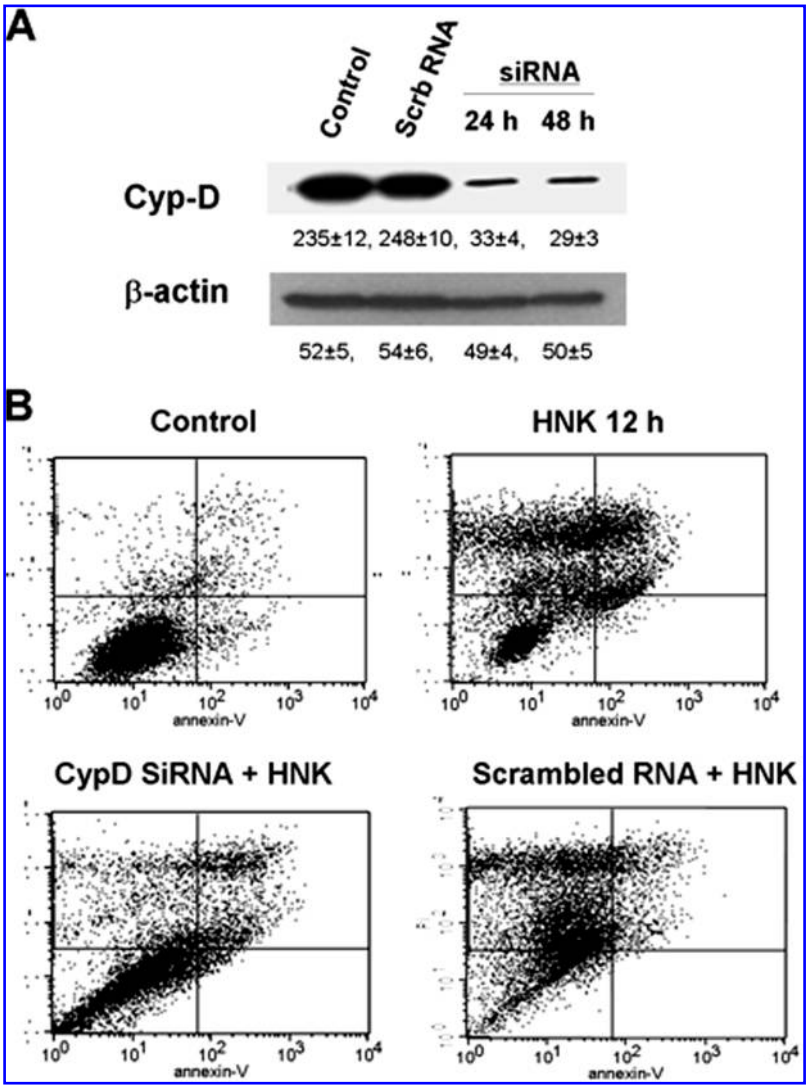



**FIG. 6. Attenuation of HNK-induced cytotoxicity by siRNA knockdown of cyclophilin D (CypD).** (**A**) Western-blot analysis of CypD protein expression in BE-3 cells transfected with siRNA against CypD or scrambled RNA. $\beta$-Actin also was blotted as the protein-loading control. The numbers under the blots indicate the values of densitometry quantitation (mean $\pm$ SD, $n = 3$). (**B**) Attenuation of HNK cytotoxicity by suppression of CypD expression with siRNA. BE-3 cells were transfected with either control scrambled RNA or siRNA against CypD for 24 h and then incubated with 20 $\mu$g/ml HNK for another 12 h. Cytotoxicity was measured with flow-cytometry analysis by using annexin-PI double staining. The overall death cells (apoptosis + necrosis) were 8.7%, 72.6%, 17.7%, and 75.9% for the control, HNK-treated, HNK + CypDsiRNA, and HNK + scrambled RNA samples, respectively.

esophageal adenocarcinoma cells. As expected, treatment with 20 $\mu$g/ml HNK resulted in a massive death of cancer cells in all three esophageal cancer lines (BE-3, OE-23, and SK-4 cells), and pretreatment of cells with 5 $\mu M$ CsA for 2 h largely suppressed HNK-induced cell death (Fig. 5). Interestingly, addition of antioxidants NAC (1 m$M$) or catalase (1,000 unit/ml) did not have a significant effect on HNK-induced cell death (Fig. 5), although cellular $H_2O_2$ was reduced by the antioxidants (data not shown). These data suggest that the observed increase in ROS generation was not a critical event for HNK-induced cell death and might be merely an indication of HNK-induced mitochondrial dysfunction. The loss of mitochondrial membrane integrity itself might be sufficient to trigger cell death. Furthermore, preincubation of cells with bongkrekic acid, an inhibitor of adenosine nucleotide translocase (ANT, also a component of MPTP), did not prevent HNK-induced cell death (Fig. 5). These data together suggest that cyclophilin D is likely a key target that mediated HNK cytotoxic action.

To test further the critical role of cyclophilin D, we used siRNA technique to suppress the expression of this molecule and then evaluated its effect of HNK cytotoxicity. Figure 6A shows that transfection of BE-3 cells with siRNA led to a significant reduction of cyclophilin D protein levels, whereas the scrambled RNA did not alter CypD expression. Importantly, the partial knockdown of CypD expression by siRNA resulted in a partial suppression of HNK-induced cell death, further confirming the important role of this molecule in mediating the cytotoxic action of HNK.

## Discussion

The development of Barrett esophagus (BE) and its progression to esophageal adenocarcinoma (EAC) represent a unique carcinogenesis process in which alterations in tissue microenvironment, due in part to gastroesophageal reflux and chronic inflammation, interacts with cellular genetic components and signaling pathways leading to cancer (34). Bile acids are believed to play an important role in the transformation of BE to EAC, owing to their ability to cause tissue inflammation and generation of ROS (26, 29), which can, in turn, cause oxidative damage to DNA, leading to genetic mutations (12, 41). However, high levels of oxidative stress or prolonged exposure to ROS may also cause cell death, which would suppress the emergence of cancer cells. Thus, for the cancer cells to

survive in the microenvironment with ROS stress in the reflux esophagus, the transformed cells must be equipped with mechanisms to counteract the cytotoxic effect of ROS effectively. All esophageal carcinoma cells tested in this study showed a significant increase in antioxidant capacity, which included an increased expression of MnSOD, Trx-2, and catalase. The total cellular glutathione levels were also substantially increased in these cancer cells (Fig. 2). This may explain why the basal hydrogen peroxide levels were lower in the cancer cells compared with those in the CP-A cells (Fig. 1A), and why the cancer cells were resistant to bile acid–induced cell death (Fig. 1B). The mechanisms by which the malignant esophageal cells acquired high antioxidant capacity remain to be investigated. Adaptation to prolonged or repeated ROS stress and selection by the oxidative tissue environment are possible mechanisms. It is likely that the increase of MnSOD protein expression and high GSH levels in esophageal cancer cells were due to the adaptive response to intrinsic ROS stress in the cancer cells with high basal ROS output due to active metabolism and possibly mitochondrial dysfunction. The overexpression of antioxidant molecules in turn reduces the overall ROS levels. In contrast, the nonmalignant CP-A cells exhibited low levels of SOD and GSH.

Among the increased antioxidant molecules, MnSOD and Trx-2 are localized mainly within the mitochondria, whereas glutathione and catalase can be found both in mitochondria and in cytosol. The substantial increase of MnSOD and Trx-2 observed in all esophageal cancer cells tested suggests that a prompt elimination of ROS in the mitochondria might be critical for cell survival. Mitochondria contain several important apoptotic molecules, including cytochrome c, AIF, and SMAC/DIABLO, which would trigger cell death once they are released from the mitochondria to cytosol. The oxidation of mitochondrial membranes by ROS would cause the loss of membrane integrity and leakage of these apoptotic factors. Thus, it is not surprising that the cancer cells that have survived oxidative stress possess high levels of antioxidant molecules in the mitochondria.

It is known that a high level of ROS can inhibit mitochondrial respiration because certain electron-transport chain components are sensitive to ROS damage (11, 47). Our study showed that the primary bile acid CDCA caused a substantial increase in $H_2O_2$ in both malignant and nonmalignant esophageal epithelial cells, and yet caused only suppression of mitochondrial respiration and cell death in the nonmalignant cells but not in the malignant cells. These results support the idea that oxidative damage to the mitochondria may play a critical role in CDCA-induced cell death. Because the malignant cells (BE-3, SK-4, OE-23) all express high levels of mitochondrial antioxidants, the increased ROS generation induced by CDCA in the cytosol and near the plasma membranes may not have significant impact on mitochondria in these cells. In contrast, high levels of ROS induced by CDCA in nonmalignant cells (CP-A) caused a significant inhibition of mitochondrial respiration which led to cell death (Fig. 1C). Of note, bile acids are known to induce ROS generation through stimulation of the NAD(P)H oxidase (NOX) enzyme complex (20, 49, 50), which are mainly localized to the cell membranes and cytosol. These observations together are consistent with the important role of mitochondrial antioxidants in mediating resistance to CDCA in esophageal cancer cells. Superoxide is the major form of ROS produced during the mitochondrial electron transport and is also a major product of NADPH oxidase (NOX). We mainly observed a significant increase in $H_2O_2$ but not superoxide in cells treated with CDCA, probably because of the powerful action of CuZnSOD and MnSOD, which effectively convert superoxide to $H_2O_2$. Thus, the final dynamic result would be an accumulation of $H_2O_2$.

It is interesting to note that although CP-A and CP-C cells showed similar low levels of GSH, CP-C cells were more resistant to CDCA toxicity. It is unclear why CP-C cells were less sensitive to the bile acid. One possible mechanism would be the high expression of the anti-apoptotic molecules HXKII and ANT, which might provide a protective effect to CP-C cells. It should be noted, however, that CP-C cells were still more sensitive to CDCA when compared with all three esophageal cancer lines (Fig. 1B), which all exhibited high GSH levels (Fig. 2B). Thus, GSH is likely to play an important role in protecting cells from the toxic effect of bile acids.

In contrast to the effect of CDCA in causing oxidative damage mainly in nonmalignant cells but not in esophageal cancer cells, the natural product HNK preferentially killed the malignant cells (Fig. 3). HNK is a biphenolic compound found in the cones, bark, and leaves of *Magnolia grandifloris,* which has been used in the traditional oriental medicine as an anxiolytic, antithrombotic, antidepressant, and antiemetic agent (16, 21, 48). Recently, HNK was reported to have antitumorigenic activities with undefined mechanisms of action (4, 25, 37).

Our study suggests that the mechanism by which HNK exerts its cytotoxic effect on esophageal cancer cells appeared not directly through ROS-mediated damage, but likely through activating the mitochondrial permeability transition pore, leading to mitochondrial dysfunction and cell death.

Several lines of evidence support this conclusion. First, pretreatment of cells with antioxidants led to a decrease in cellular ROS but did not prevent HNK-induced cell death (Fig. 5), suggesting ROS may not play a critical role in HNK cytotoxicity. Second, incubation of esophageal cancer cells with HNK resulted in a substantial decrease of mitochondrial transmembrane potential, an inhibition of mitochondrial respiration, and a rapid depletion of cellular ATP (Figs. 3 and 4). These mitochondrial alterations and cell death can be prevented by pre-incubation with CsA, a compound that binds to cyclophilin D and prevents the opening of the mitochondrial permeability transition pore (5, 38, 39). The third evidence supporting the critical role of CypD in mediating the cytotoxic action of HNK was the observation that a partial knockdown of CypD expression of CypD by siRNA partially abrogated the cytotoxicity of HNK (Fig. 6). These data are consistent with the observations by others (33, 35).

A recent study suggests that HNK may function as a potent scavenger of superoxide and peroxyl radicals both in a cell-free system and in melanoma cells, revealed by electron spin resonance (ESR) and the spin-probe CMH methods (16). In our study, HNK caused not a significant change in cellular superoxide but a significant increase in hydrogen peroxide in esophageal cells. The exact reason for these differences remains unclear. Nevertheless, both studies seem to suggest that HNK does not cause cell death through an ROS-mediated mechanism.

Interestingly, although HNK showed low toxicity to nonmalignant CP-A cells, this compound was toxic to CP-C cells, which were originally derived from primary esopha-

geal epithelial cells of Barrett esophagus with high-grade dysplasia. Of note, CP-C cells also expressed a high level of CypD, similar to the high expression in esophageal carcinoma cells (Fig. 2D). These observations further suggest that the cytotoxic action of HNK may be correlated with the expression levels of CypD, which seemed to play an important role in HNK-induced cell death. A current model of mitochondrial permeability transition (MPT) suggests that this protein complex contains three major components: VDAC located on the outer membrane, ANT associated with the inner membrane, and CypD in the matrix (2, 23, 53). The observation that the ANT inhibitor bongkrekic acid failed to prevent HNK-induced cell death further suggests that CypD might be specific for the cytotoxic action of HNK. This is consistent with the results in a previous study using different cell lines (33).

It should be pointed out that our data, together with those reported in the literature, only suggest that a functional CypD is important in mediating the cell-killing effect of HNK, but have not demonstrated that CypD is the target of HNK. Further studies are required to determine whether CypD is a direct target of HNK and the underlying molecular basis. It would also be important in future studies to test whether such a mechanism of action could be demonstrated *in vivo* by using proper animal models. It is still unclear why esophageal adenocarcinoma cells and epithelial cells with high-grade dysplasia express high levels of CypD. It is possible that this may be associated with the adaptation process in response to prolonged ROS stress in reflux esophagus. Our study revealed that the malignant cells became insensitive to bile acid–induced ROS stress owing to increased expression of antioxidants and prosurvival molecules, especially those located in the mitochondria (Fig. 2). It would be of interest to test whether tacrolimus (FK-506), an immunosuppressive drug with a mechanism of action similar to that of cyclosporin A, would also attenuate the cytotoxic activity of HNK in esophageal cancer cells. Furthermore, because mutant p53 seems to interact physically with cyclophilin in certain chaperone protein complexes (54), it would be important to examine whether the p53 status in esophageal cancer cells might affect their sensitivity to honokiol.

In summary, our study suggests that upregulation of antioxidant molecules in esophageal carcinoma cells may be an important adaptive mechanism that confers resistance to ROS stress induced by bile acids and chronic inflammation in the microenvironment of esophageal reflux. These alterations in redox states seem to be associated with changes in expression of mitochondrial molecules, including overexpression of CypD. We further demonstrated that CypD is an important target of HNK and likely mediated the cytotoxic action of this compound by triggering the opening of the mitochondrial permeability transition pore. Thus, HNK is a promising natural compound capable of selectively killing esophageal cancer cells with low toxicity to normal cells, in part because of their difference in expression of CypD. Furthermore, because esophageal epithelial cells with high-grade dysplasia also express high level of CypD and are highly sensitive to HNK, this compound may be used to kill preferentially the premalignant cells and prevent the transformed cells from progression to cancer. Thus, HNK warrants further evaluation for potential use in prevention and treatment of esophageal adenocarcinoma.

## Acknowledgments

We thank Zhongxin Pharmaceutics (Tianjin, China) for providing honokiol for this study. This work was supported in part by grants CA085563, CA100428, and CA109041 from the National Institutes of Health, and by the Multidisciplinary Research Grant from UT MD Anderson Cancer Center and the Cantu, Dallas, Park, and Caporella families.

## Abbreviations

ANT, adenine nucleotide translocase; BE, Barrett esophagus; CDCA, chenodeoxycholic acid; CsA, cyclosporin A; CypD, cyclophilin D; EAC, esophageal adenocarcinoma; GSH, glutathione; HNK, honokiol; HSP60, heat-shock protein 60; HXKII, hexokinase II; MMP, mitochondria membrane potential; MPT, mitochondrial permeability transition; MPTP, mitochondrial permeability transition pore; NAC, *N*-acetyl cysteine; PPIases, peptidylprolyl isomerases; PrxIII, peroxiredoxin III; redox, reduction and oxygenation; ROS, reactive oxygen species; SOD1, superoxide dismutase-1 (CuZnSOD); SOD2, superoxide dismutase-2 (MnSOD); Trx-1, thioredoxin-1; Trx-2, thioredoxin-2; VDAC, voltage-dependent anion channel.

## Disclosure Statement

No competing financial interests exist.

## References

1. Ahn KS, Sethi G, Shishodia S, Sung B, Arbiser JL, and Aggarwal BB. Honokiol potentiates apoptosis, suppresses osteoclastogenesis, and inhibits invasion through modulation of nuclear factor-kappaB activation pathway. *Mol Cancer Res* 4: 621–633, 2006.
2. Armstrong JS. Mitochondria: a target for cancer therapy. *Br J Pharmacol* 147: 239–248, 2006.
3. Atherfold PA and Jankowski JA. Molecular biology of Barrett's cancer. *Best Pract Res Clin Gastroenterol* 20: 813–827, 2006.
4. Bai X, Cerimele F, Ushio-Fukai M, Waqas M, Campbell PM, Govindarajan B, Der CJ, Battle T, Frank DA, Ye K, Murad E, Dubiel W, Soff G, and Arbiser JL. Honokiol, a small molecular weight natural product, inhibits angiogenesis *in vitro* and tumor growth *in vivo*. *J Biol Chem* 278: 35501–35507, 2003.
5. Basso E, Fante L, Fowlkes J, Petronilli V, Forte MA, and Bernardi P. Properties of the permeability transition pore in mitochondria devoid of cyclophilin D. *J Biol Chem* 280: 18558–18561, 2005.
6. Battle TE, Arbiser J, and Frank DA. The natural product honokiol induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia (B-CLL) cells. *Blood* 106: 690–697, 2005.
7. Bernstein H, Bernstein C, Payne CM, Dvorakova K, and Garewal H. Bile acids as carcinogens in human gastrointestinal cancers. *Mutat Res* 589: 47–65, 2005.
8. Burri L, Strahm Y, Hawkins CJ, Gentle IE, Puryer MA, Verhagen A, Callus B, Vaux D, and Lithgow T. Mature DIABLO/Smac is produced by the IMP protease complex on the mitochondrial inner membrane. *Mol Biol Cell* 16: 2926–2933, 2005.
9. Chen X and Yang CS. Esophageal adenocarcinoma: a review and perspectives on the mechanism of carcinogenesis and chemoprevention. *Carcinogenesis* 22: 1119–1129, 2001.

10. Chen Z, Lu W, Garcia-Prieto C and Huang P. The Warburg effect and its cancer therapeutic implications. *J Bioenerg Biomembr* 39: 267–274, 2007.
11. Choksi KB, and Papaconstantinou J. Age-related alterations in oxidatively damaged proteins of mouse heart mitochondrial electron transport chain complexes. *Free Radic Biol Med* 44: 1795–1805, 2008.
12. D'Amico TA. Molecular biologic staging of esophageal cancer. *Thorac Surg Clin* 16: 317–327, 2006.
13. de Jonge PJ, Steyerberg EW, Kuipers EJ, Honkoop P, Wolters LM, Kerkhof M, van Dekken H, and Siersema PD. Risk factors for the development of esophageal adenocarcinoma in Barrett's esophagus. *Am J Gastroenterol* 101: 1421–1429, 2006.
14. Diakowska D, Lewandowski A, Kopeć W, Diakowski W, and Chrzanowska T. Oxidative DNA damage and total antioxidant status in serum of patients with esophageal squamous cell carcinoma. *Hepatogastroenterology* 54: 1701–1704, 2007.
15. Dias Pereira A, Suspiro A, and Chaves P. Cancer risk in Barrett's oesophagus. *Eur J Gastroenterol Hepatol* 19: 915–918, 2007.
16. Dikalov S, Losik T, and Arbiser JL. Honokiol is a potent scavenger of superoxide and peroxyl radicals. *Biochem Pharmacol* 76: 589–596, 2008.
17. Dvorak K, Payne CM, Chavarria M, Ramsey L, Dvorakova B, Bernstein H, Holubec H, Sampliner RE, Guy N, Condon A, Bernstein C, Green SB, Prasad A, and Garewal HS. Bile acids in combination with low pH induce oxidative stress and oxidative DNA damage: relevance to the pathogenesis of Barrett's oesophagus. *Gut* 56: 763–771, 2007.
18. el-Serag HB. The epidemic of esophageal adenocarcinoma. *Gastroenterol Clin North Am* 31: 421–440, 2002.
19. Feagins LA, Zhang HY, Zhang X, Hormi-Carver K, Thomas T, Terada LS, Spechler SJ, and Souza RF. Mechanisms of oxidant production in esophageal squamous cell and Barrett's cell lines. *Am J Physiol Gastrointest Liver Physiol* 294: G411–G417, 2008.
20. Fu X, Beer DG, Behar J, Wands J, Lambeth D, and Cao W. cAMP-response element-binding protein mediates acid-induced NADPH oxidase NOX5-S expression in Barrett esophageal adenocarcinoma cells. *J Biol Chem* 281: 20368–20382, 2006.
21. Garcia A, Zheng Y, Zhao C, Toschi A, Fan J, Shraibman N, Brown HA, Bar-Sagi D, Foster DA, and Arbiser JL. Honokiol suppresses survival signals mediated by Ras-dependent phospholipase D activity in human cancer cells. *Clin Cancer Res* 14: 4267–4274, 2008.
22. Gogvadze V and Zhivotovsky B. Alteration of mitochondrial function and cell sensitization to death. *J Bioenerg Biomembr* 39: 23–30, 2007.
23. Grimm S and Brdiczka D. The permeability transition pore in cell death. *Apoptosis* 12: 841–855, 2007.
24. Hahm ER, Arlotti JA, Marynowski SW, and Singh SV. Honokiol, a constituent of oriental medicinal herb *Magnolia officinalis*, inhibits growth of PC-3 xenografts *in vivo* in association with apoptosis induction. *Clin Cancer Res* 14: 1248–1257, 2008.
25. Hahm ER and Singh SV. Honokiol causes G0-G1 phase cell cycle arrest in human prostate cancer cells in association with suppression of retinoblastoma protein level/phosphorylation and inhibition of E2F1 transcriptional activity. *Mol Cancer Ther* 6: 2686–2695, 2007.
26. Hao Y, Sood S, Triadafilopoulos G, Kim JH, Wang Z, Sahbaie P, Omary MB, and Lowe AW. Gene expression changes associated with Barrett's esophagus and Barrett's-associated adenocarcinoma cell lines after acid or bile salt exposure. *BMC Gastroenterol* 7: 24, 2007.
27. Hirano T, Gotoh M, and Oka K. Natural flavonoids and lignans are potent cytostatic agents against human leukemic HL-60 cells. *Life Sci* 55: 1061–1069, 1994.
28. Ishitsuka K, Hideshima T, Hamasaki M, Raje N, Kumar S, Hideshima H, Shiraishi N, Yasui H, Roccaro AM, Richardson P, Podar K, Le Gouill S, Chauhan D, Tamura K, Arbiser J, and Anderson KC. Honokiol overcomes conventional drug resistance in human multiple myeloma by induction of caspase-dependent and -independent apoptosis. *Blood* 106: 1794–800, 2005.
29. Jenkins GJ, D'Souza FR, Suzen SH, Eltahir ZS, James SA, Parry JM, Griffiths PA, and Baxter JN. Deoxycholic acid at neutral and acid pH, is genotoxic to oesophageal cells through the induction of ROS: the potential role of antioxidants in Barrett's oesophagus. *Carcinogenesis* 28: 136–142, 2007.
30. Kim BH, and Cho JY. Anti-inflammatory effect of honokiol is mediated by PI3K/Akt pathway suppression. *Acta Pharmacol Sin* 29: 113–122, 2008.
31. Krishnadath KK. Novel findings in the pathogenesis of esophageal columnar metaplasia or Barrett's esophagus. *Curr Opin Gastroenterol* 23: 440–445, 2007.
32. Kroemer G and Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. *Cancer Cell* 13: 472–482, 2008.
33. Li L, Han W, Gu Y, Qiu S, Lu Q, Jin J, Luo J, and Hu X. Honokiol induces a necrotic cell death through the mitochondrial permeability transition pore. *Cancer Res* 67: 4894–4903, 2007.
34. Li Y and Martin RC 2nd. Reflux injury of esophageal mucosa: experimental studies in animal models of esophagitis, Barrett's esophagus and esophageal adenocarcinoma. *Dis Esophagus* 20: 372–378, 2007.
35. Lin YR, Chen HH, Ko CH, and Chan MH. Neuroprotective activity of honokiol and magnolol in cerebellar granule cell damage. *Eur J Pharmacol* 537: 64–69, 2006.
36. Liou KT, Shen YC, Chen CF, Tsao CM, and Tsai SK. The anti-inflammatory effect of honokiol on neutrophils: mechanisms in the inhibition of reactive oxygen species production. *Eur J Pharmacol* 475: 19–27, 2003.
37. Liu H, Zang C, Emde A, Planas-Silva MD, Rosche M, Kühnl A, Schulz CO, Elstner E, Possinger K, and Eucker J. Antitumor effect of honokiol alone and in combination with other anti-cancer agents in breast cancer. *Eur J Pharmacol* 591: 43–51, 2008.
38. Naga KK, Sullivan PG, and Geddes JW. High cyclophilin D content of synaptic mitochondria results in increased vulnerability to permeability transition. *J Neurosci* 27: 7469–7475, 2007.
39. Nathan M, Friehs I, Choi YH, Cowan DB, Cao-Danh H, McGowan FX, and del Nido PJ. Cyclosporin A but not FK-506 protects against dopamine-induced apoptosis in the stunned heart. *Ann Thorac Surg* 79: 1620–1626, 2005.
40. Oh TY, Lee JS, Ahn BO, Cho H, Kim WB, Kim YB, Surh YJ, Cho SW, and Hahm KB. Oxidative damages are critical in pathogenesis of reflux esophagitis: implication of antioxidants in its treatment. *Free Radic Biol Med* 30: 905–915, 2001.
41. Olliver JR, Hardie LJ, Dexter S, Chalmers D, and Wild CP. DNA damage levels are raised in Barrett's oesophageal mucosa relative to the squamous epithelium of the oesophagus. *Biomarkers* 8: 509–521, 2003.
42. Otera H, Ohsakaya S, Nagaura Z, Ishihara N, and Mihara K. Export of mitochondrial AIF in response to proapoptotic

stimuli depends on processing at the intermembrane space. *EMBO J* 24: 1375–1386, 2005.
43. Palanca-Wessels MC, Klingelhutz A, Reid BJ, Norwood TH, Opheim KE, Paulson TG, Feng Z and Rabinovitch PS. Extended lifespan of Barrett's esophagus epithelium transduced with the human telomerase catalytic subunit: a useful *in vitro* model. *Carcinogenesis* 24: 1183–1190, 2003.
44. Pelicano H, Xu RH, Du M, Feng L, Sasaki R, Carew JS, Hu Y, Ramdas L, Hu L, Keating MJ, Zhang W, Plunkett W, and Huang P. Mitochondrial respiration defects in cancer cells cause activation of Akt survival pathway through a redox-mediated mechanism. *J Cell Biol* 175: 913–923, 2006.
45. Rasola A and Bernardi P. The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis. *Apoptosis* 12: 815–833, 2007.
46. Sayana H, Wani S, and Sharma P. Esophageal adenocarcinoma and Barrett's esophagus. *Minerva Gastroenterol Dietol* 53: 157–169, 2007.
47. Semenza GL. Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor 1. *Biochem J* 405: 1–9, 2007.
48. Sheu ML, Chiang CK, Tsai KS, Ho FM, Weng TI, Wu HY, and Liu SH. Inhibition of NADPH oxidase-related oxidative stress-triggered signaling by honokiol suppresses high glucose-induced human endothelial cell apoptosis. *Free Radic Biol Med* 44: 2043–2050, 2008.
49. Si J, Behar J, Wands J, Beer DG, Lambeth D, Chin YE, and Cao W. STAT5 mediates PAF-induced NADPH oxidase NOX5-S expression in Barrett's esophageal adenocarcinoma cells. *Am J Physiol Gastrointest Liver Physiol* 294: G174–G183, 2008.
50. Si J, Fu X, Behar J, Wands J, Beer DG, Souza RF, Spechler SJ, Lambeth D, and Cao W. NADPH oxidase NOX5-S mediates acid-induced cyclooxygenase-2 expression via activation of NF-kappaB in Barrett's esophageal adenocarcinoma cells. *J Biol Chem* 282: 16244–16255, 2007.
51. Stoner GD, Dombkowski AA, Reen RK, Cukovic D, Salagrama S, Wang LS, and Lechner JF. Carcinogen-altered genes in rat esophagus positively modulated to normal levels of expression by both black raspberries and phenylethyl isothiocyanate. *Cancer Res* 68: 6460–6467, 2008.
52. Tse AK, Wan CK, Shen XL, Yang M, and Fong WF. Honokiol inhibits TNF-alpha-stimulated NF-kappaB activation and NF-kappaB-regulated gene expression through suppression of IKK activation. *Biochem Pharmacol* 70: 1443–1457, 2005.
53. Waldmeier PC, Zimmermann K, Qian T, Tintelnot-Blomley M, and Lemasters JJ. Cyclophilin D as a drug target. *Curr Med Chem* 10: 1485–506, 2003.
54. Whitesell L, Sutphin PD, Pulcini EJ, Martinez JD, Cook PH. The physical association of multiple molecular chaperone proteins with mutant p53 is altered by geldanamycin, an hsp90-binding agent. *Mol Cell Biol* 18: 1517–1524, 1998.
55. Zhang DX and Gutterman DD. Mitochondrial reactive oxygen species-mediated signaling in endothelial cells. *Am J Physiol Heart Circ Physiol* 292: H2023–H2031, 2007.

Address reprint requests to:
*Peng Huang*
*Department of Molecular Pathology, Unit 951*
*The University of Texas M.D. Anderson Cancer Center*
*Houston, Texas 77030*

*E-mail:* phuang@mdanderson.org

Date of first submission to ARS Central, October 3, 2008; date of final revised submission, January 9, 2009; date of acceptance, January 11, 2009.

**This article has been cited by:**


1. W. Chen, L. Feng, H. Nie, X. Zheng. 2012. Andrographolide induces autophagic cell death in human liver cancer cells through cyclophilin D-mediated mitochondrial permeability transition pore. *Carcinogenesis* . [CrossRef]
2. K. R. McQuaid, L. Laine, M. B. Fennerty, R. Souza, S. J. Spechler. 2011. Systematic review: the role of bile acids in the pathogenesis of gastro-oesophageal reflux disease and related neoplasia. *Alimentary Pharmacology & Therapeutics* **34**:2, 146-165. [CrossRef]
3. Dennis Ma, Phillip Tremblay, Kevinjeet Mahngar, Pardis Akbari-Asl, Jonathan Collins, Tomas Hudlicky, James McNulty, Siyaram Pandey. 2011. A novel synthetic C-1 analogue of 7-deoxypancratistatin induces apoptosis in p53 positive and negative human colorectal cancer cells by targeting the mitochondria: enhancement of activity by tamoxifen. *Investigational New Drugs* . [CrossRef]
4. Xiajing Tang, Ke Yao, Li Zhang, Yunmei Yang, Hangping Yao. 2011. Honokiol inhibits H2O2-induced apoptosis in human lens epithelial cells via inhibition of the mitogen-activated protein kinase and Akt pathways. *European Journal of Pharmacology* **650**:1, 72-78. [CrossRef]
5. Patrick Steinmann, Denise K. Walters, Matthias J. E. Arlt, Ingo J. Banke, Urs Ziegler, Bettina Langsam, Jack Arbiser, Roman Muff, Walter Born, Bruno Fuchs. 2011. Antimetastatic activity of honokiol in osteosarcoma. *Cancer* n/a-n/a. [CrossRef]
6. Feng Wang, Marcia A. Ogasawara, Peng Huang. 2010. Small mitochondria-targeting molecules as anti-cancer agents. *Molecular Aspects of Medicine* **31**:1, 75-92. [CrossRef]