Guggulsterone induces heme oxygenase-1 expression through activation of Nrf2 in human mammaery epithelial cells: PTEN as a putative target

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Abbreviations: ARE, antioxidant response element; DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GS, guggulsterone; HO-1, heme oxygenase-1; MAPK, mitogen-activated protein kinase; NAC, N-acetyl-L-cysteine; NF-E2-related factor 2 (Nrf2) to the ARE sequences (4). In resting conditions, Nrf2 is sequestered in the cytoplasm as an inactive complex with its repressor protein Keap1, which allows Nrf2 to undergo proteasomal degradation. Under oxidative stress conditions, Nrf2 dissociates from Keap1, escapes from the proteasomal degradation and translocates into the nucleus where it heterodimerizes with other transcription factors such as small Maf and binds to the ARE/electrophile response element (5).

Many chemopreventive phytochemicals, especially those containing an α,β-unsaturated carbonyl moiety, are known to activate Nrf2 signaling, thereby inducing the expression of cytoprotective enzymes (6). Guggulsterone (GS) [4,17(20)-pregnadiene-3,16-dione] is a phytosterol found in the gum resin of the Commiphora mukul. GS exists naturally in two stereoisomers: E-GS (cis-GS) and Z-GS (trans-GS). In this study, the effects of both isomers on the expression of the cytoprotective enzyme heme oxygenase-1 (HO-1) were evaluated in human mammary epithelial (MCF10A) cells. NF-E2-related factor 2 (Nrf2) is considered a master regulator in activating antioxidant response element (ARE)-driven expression of HO-1 and many other antioxidant/cytoprotective proteins. cis-GS induced the transcription and protein expression of HO-1 to a greater extent than did trans-GS, cis-GS-mediated enhanced nuclear translocation and ARE-binding activity of Nrf2. MCF10A cells transfected with an ARE luciferase construct exhibited significantly elevated Nrf2 transcriptional activity upon cis-GS treatment compared with cells transfected with the control vector. In addition, silencing of the Nrf2 gene abrogated cis-GS-induced expression of HO-1. Incubation of MCF10A cells with cis-GS increased phosphorylation of Akt. The pharmacological inhibition of phosphoinositide-3-kinase (PI3K), an upstream kinase responsible for Akt phosphorylation, abrogated cis-GS-induced Nrf2 nuclear translocation. Pretreatment with the thiol-reducing agents attenuated Akt phosphorylation, Nrf2 activation and HO-1 expression, suggesting that cis-GS may cause thiol modification of an upstream signaling modulator. Phosphatase and Tensin Homologue Deleted on Chromosome 10 (PTEN), Akt and phospho-Akt were measured in human mammary epithelial (MCF10A) cells. Materials and methods

Materials

cis-GS and trans-GS were purchased from Steraloids (Newport, RI). Dulbecco’s modified Eagle’s medium/F-12, heat-inactivated horse serum, t-glutamic acid, penicillin, streptomycin and Trizol salt solution was purchased from Meditech (Herndon, VA). Bicinchoninic acid and the human-specific are obtained from Zymed Laboratories (San Francisco, CA). The ECL chemiluminescent detection kit and [γ-32P] ATP were purchased from Amersham Pharma Biotech (Buckinghamshire, UK), LYS94002, U0126, SB203580 and SP600125 were purchased from TOCRIS (Ellisville, MO). The human-specific Nrf2-siRNA (sense 5′-AGAGAUAGACGGAGAAACTT-3′, antisense 5′-GUUUUCAGGACCUACCU-CU-3′), Stealth™ RNAi negative control duplexes and 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) were provided by Invitrogen (Carlsbad, CA). Hank’s balanced salt solution was purchased from Meditech (Herndon, VA). Bicinchoninic acid protein assay reagent was a product of Pierce Biotechnology (Rockford, IL). Polyvinylidene difluoride membranes were supplied from Gelman Laboratory.

Introduction

Cellular redox status is a critical determinant of cell fate, such as apoptosis, differentiation and proliferation. Oxidative stress characterized by excessive generation of reactive oxygen species (ROS) can lead to various chronic diseases including cancer (1). Cells are equipped with a battery of cytoprotective enzymes, which can protect against ROS-mediated damage of critical macromolecules, such as DNA, proteins and lipids, and hence function as the first-line of defense against oxidative stress. These cytoprotective enzymes include reduced nicotinamide adenine dinucleotide phosphate:quione oxidoreductase-1, superoxide dismutase, catalase, glutathione peroxidase, thioredoxin reductase, heme oxygenase-1 (HO-1), etc. (2). HO-1 is the enzyme that catalyzes the rate-limiting step in converting heme into bilirubin, carbon monoxide and iron. Heme is the byproduct of protein degradation and is highly cytotoxic if accumulated in excess in the cell (3).

The promoter regions of genes encoding cytoprotective enzymes contain a specific consensus sequence known as antioxidant response element (ARE) or alternatively called electrophile response element. The transcriptional activation of these genes is largely mediated through binding of the redox-regulated transcription factor NF-E2-related factor2 (Nrf2) to the ARE sequences (4). In resting conditions, Nrf2 is sequestered in the cytoplasm as an inactive complex with its repressor protein Keap1, which allows Nrf2 to undergo proteasomal degradation. Under oxidative stress conditions, Nrf2 dissociates from Keap1, escapes from the proteasomal degradation and translocates into the nucleus where it heterodimerizes with other transcription factors such as small Maf and binds to the ARE/electrophile response element (5).

Many chemopreventive phytochemicals, especially those containing an α,β-unsaturated carbonyl moiety, are known to activate Nrf2 signaling, thereby inducing the expression of cytoprotective enzymes. Guggulsterone (GS) [4,17(20)-pregnadiene-3,16-dione] is a phytosterol found naturally in the gum resin of the guggul plant. This phytochemical has been used in the Ayurvedic medicine to treat many human ailments. GS exists naturally in two stereoisomeric forms (Figure 1), cis-GS and trans-GS (7).

Since HO-1 confers protection against oxidative stress and inflammation that are implicated in the majority of carcinogenesis, induction of HO-1 expression can be a rational chemopreventive strategy. As part of our research program to assess the chemopreventive activity of GS, we investigated its ability to induce HO-1 expression and the underlying molecular mechanisms with special focus on Nrf2 signaling in the human mammary epithelial (MCF10A) cells.

Cells

MCF10A cells were purchased from Steraloids (Newport, RI). Dulbecco’s modified Eagle’s medium/F-12, heat-inactivated horse serum, t-glutamic acid, penicillin, streptomycin and Trizol reagent were purchased from GibCO BRL (Grand Island, NY). Dithiothreitol (DTT), insulin (bovine), cholesta toxin, hydrocortisone, recombinant epidermal growth factor, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, N-acetyl-L-cysteine (NAC), pyrroline dione uridin and primary antibodies for lamin B and actin were purchased from the Sigma Chemical Co. (St Louis, MO). Rabbit polyclonal antibody was purchased from Stresses (Ann Arbor, MI). Primary antibody against each of Nrf-2, Keap1 and phospho-Akt was purchased from Cell Signaling Technology (Beverly, MA). Secondary antibodies were obtained from Zymed Laboratories (San Francisco, CA). The ECL chemiluminescent detection kit and [γ-32P] ATP were purchased from Amersham Pharma Biotech (Buckinghamshire, UK), LYS94002, U0126, SB203580 and SP600125 were purchased from TOCRIS (Ellisville, MO). The human-specific Nrf2-siRNA (sense 5′-AGAGAUAGACGGAGAAACTT-3′, antisense 5′-GUUUUCAGGACCUACCU-CU-3′), Stealth™ RNAi negative control duplexes and 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) were provided by Invitrogen (Carlsbad, CA). Hank’s balanced salt solution was purchased from Meditech (Herndon, VA). Bicinchoninic acid protein assay reagent was a product of Pierce Biotechnology (Rockford, IL). Polyvinylidene difluoride membranes were supplied from Gelman Laboratory.

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cDNA were used for the complementary DNA synthesis using random primers. Reverse transcriptase-PCR was performed following standard procedures. PCR conditions for HO-1 and the house keeping gene GAPDH were as follows: HO-1, 26 cycles of 94°C for 1 min and 72°C for 1 min. The primer pairs and the size of the expected products were as follows: HO-1, 5’-CAGG-GAGAAATGCTAGTCT-3’ and 5’-GATGTTGACG-GAAGCAAT-3’; 55 bp and GAPDH, 5’-TGAAGGTCCGGTCAACGATTTGTCG-3’ and 5’-CATGTAGGCAATGAGGTCAAAC-3’. Amplification products were resolved by 1.0% agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light. Primers were purchased from Bionics (Seoul, South Korea).

GS-induced HO-1 expression via Nrf2 activation

Preparation of cytosolic and nuclear extracts

After treatment with cis-GS, cells were washed two times with ice-cold PBS, scraped in 1 ml PBS and centrifuged at 13 000 rpm for 15 min at 4°C. Pellets were suspended in 100 μl of hypotonic buffer A [10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (pH 7.8), 1.5 mM MgCl₂], 10 mM KCl, 0.5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride] for 30 min on ice, and afterward, 1 μl of 10% Nonidet P-40 solution was added for 5 min. The mixture was centrifuged at 12 000 rpm for 15 min. Supernatant was collected as cytosolic extract. The pellets were washed with hypotonic buffer and resuspended in hypertonic buffer C [20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (pH 7.8), 20% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM ethylendiaminetetraacetic acid (EDTA), 0.5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride] for 30 min on ice and centrifuged at 12 000 rpm for 15 min. The supernatant containing nuclear proteins was collected and stored at −70°C after determination of the protein concentration. The protein concentration of the nuclear extracts was determined using the Bio-Rad protein assay dye (Bradford) Reagent (Bio-Rad Laboratories, Hercules, CA).

Electrophoretic mobility gel shift assay

Synthetic double strand oligonucleotide containing the ARE, the binding site of Nrf2, was labeled with [γ-32P] adenosine triphosphate by T4 polynucleotide kinase and purified using a DNA microcolumn (Amersham Pharmacia Biotech). The nuclear protein (10 μg) was mixed with 5× incubation buffer [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol and 0.1 mg/ml sonicated salmon sperm DNA] and the hypertonc buffer was added to make the final volume 20 μl. After preincubation at room temperature for 15 min, the labeled oligonucleotide (100 000 c.p.m) was added and the incubation continued for additional 50 min at room temperature. To

[Fig. 1. The chemical structures of GS isomers.]

(Ann Arbor, MI). Protease inhibitor cocktail were provided from Boehringer Mannheim (Mannheim, Germany). All other chemicals used were of analytical or the highest purity grade available. The nrf2 knock out mice were generously supplied by Dr Jeffrey Johnson, University of Wisconsin–Madison.

Cell culture

MCF10A cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 5% heat-inactivated horse serum, 10 mg/ml insulin and 100 ng/ml cholera toxin, 0.5 mM GTP, 10% heat-inactivated horse serum, 10 mg/ml insulin, 20 ng/ml recombinant human epidermal growth factor, 2 mML-glutamate and 100 units/ml penicillin or streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Plasmids

The pSG5L HA PTEN expressing (wild type), PTEN with C124S mutation (mutant type) and PTEN mock plasmids were purchased from addgene (Addgene, South Korea). Site-directed mutagenesis was performed by polymerase chain reaction (PCR) assembly using pSG5L-HA-PTEN (Addgene) plasmid as a template. The following oligonucleotides and their complementary were used to design the mutations in PTEN and Cys71 → Ser71: 5’-CAAGATATATA-CAATCCAGTGCTGAAAGACA-3’. The PCR fragments were annealed and 18 cycles of PCR were performed. The PCR fragments are then subcloned into the pSG5L-expressing vector. The presence of site-directed mutations was confirmed by complete sequencing of the PTEN gene. MCF10A cells were transfected with indicated plasmid constructs using WelFect-MTM Gold transfection reagent (WelGENE, Seoul, Korea) according to the manufacturer’s instructions. Western blot analysis

After treatment, the cells were washed twice with phosphate-buffered saline (PBS) 1× and then mixed with 1× lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 25 mM NaF, 20 mM ethyleneglycol-bis(aminoethyl ether)-tetraacetic acid, 1 mM DTT, 1 mM NaVO₃, 0.5% Triton X-100 and protease inhibitor cocktail tablets] with 1% phenylmethylsulfonyl fluoride for 1 h on ice followed by centrifugation at 17 000g for 15 min. The protein concentration of the supernatant was measured by using the bicinchoninic acid protein assay reagent. The protein samples were solubilized with 5× sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis sample loading buffer and boiled for 5 min. Proteins (30–60 μg) underwent electrophoresis on 10 or 7% SDS–polyacrylamide gel and transferred to polyvinylidene difluoride membrane. The blots were then blocked with 5% fat-free dry milk-PBST (Tris-buffered saline containing 0.1% Tween-20) buffer for 1 h at room temperature. The blots were incubated with primary antibodies in 3% fat-free dry milk in PBS. After three times of washing with PBS, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies in 3% fat-free dry milk-PBS for 1 h at room temperature. The blots were rinsed again three times with PBS, and the transferred proteins were incubated with the Enhanced Peroxidase Detection Western blot Detection Kit (ELPIS-BIOTECH, Daejon, Korea) for 1 min or ECL Plus Western Blotting Reagent Pack for 1 min at room temperature. The blots were then blocked with 5% fat-free dry milk-PBST (Tris-buffered saline containing 0.1% Tween-20) buffer for 1 h at room temperature. The blots were rinsed again three times with PBS, and the transferred proteins were incubated with the Enhanced Peroxidase Detection Western blot Detection Kit (ELPIS-BIOTECH, Daejon, Korea) for 1 min or ECL Plus Western Blotting Reagent Pack for 5 min (GE Healthcare, Piscataway, NJ) according to the manufacturer’s instructions or/and visualized with an X-ray film or the imagequantTM LAS 4000 (Fujifilm Life Science).

Reverse transcriptase-PCR

Total RNA was isolated from MCF10A cells using TRizol (Invitrogen, Carlsbad, CA). One microgram of total RNA was used for the complementary DNA synthesis using random primers. Reverse transcriptase-PCR was performed following standard procedures. PCR conditions for HO-1 and the house keeping gene GAPDH were as follows: HO-1, 26 cycles of 94°C for 1 min; 60°C for 1 min and 72°C for 1 min. The primer pairs and the size of the expected products were as follows: forward and reverse, respectively: HO-1, 5’-CAGG-GAGAAATGCTAGTCTGTTGTC-3’ and 5’-GATGTTGACG-GAAGCAAT-3’; 55 bp and GAPDH, 5’-TGAAGGTCCGGTCAACGATTTGTCG-3’ and 5’-CATGTAGGCAATGAGGTCAAAC-3’. Amplification products were resolved by 1.0% agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light. Primers were purchased from Bionics (Seoul, South Korea).

Nrf2-siRNA transient transfection

MCF10A cells were transfected with Nrf2-siRNA or Nrf2-negative control siRNA for 48 h upon plating cells (1 × 10⁵/ml) in 60 mm dishes. Transfected cells were then treated with cis-GS for additional 6 h followed by treatment with lysis buffer for western blot analysis.

ARE luciferase assay

MCF10A cells were co-transfected with 2 μg of the luciferase reporter gene fusion construct (pTi-luciferase), wild-type ARE and 0.5 μg of pCMV-β-galactosidase control vector with WelFect-MTM Gold transfection reagent (WelGENE) according to the manufacturer’s instructions. After 24 h transfection, the cells were treated with cis-GS for additional 12 h, and cell lysis was carried out with the 1× reporter lysis buffer (Promega, Madison, WI). After mixing the cell extract with a luciferase substrate (Promega), the luciferase activity was measured by the luminometer (Anto.Lumat LB 953; EGA/G Berthold, Bad Wildbad, Germany). The β-galactosidase assay was done according to the supplier’s instructions (Promega β-galactosidase Enzyme Assay System) for normalizing the luciferase activity. The sequences of ARE luciferase construct were as follows: 5’-CTCACGCTTCCAATCGTAGTCACTGACGAGAATC-3’.
insure the specificity of the binding, a competition assay was carried out with the excess unlabeled oligonucleotide. After incubation, 2 l of 0.1% bromophenol blue was added, and the samples were electrophoresed on a 6% non-denaturing polyacrylamide gel at 150 V for 90 min. Finally, the gel was dried and exposed to an X-ray film.

Immunocytochemical analysis of Nrf2
To demonstrate the nuclear translocation of Nrf2, immunocytochemistry was performed. MCF10A cells were seeded on the chamber slide and treated with GS or vehicle alone. After fixation with 10% neutral-buffered formalin solution for 30 min at room temperature, samples were incubated with blocking agents (0.1% Tween-20 in PBS containing 5% bovine serum albumin) for 2 h at room temperature, washed with PBS and then incubated with a diluted (1:100) primary antibody in 1% bovine serum albumin/PBST 0.1% overnight at 4°C. After washing with PBS, samples were incubated with a diluted (1:1000) fluorescein isothiocyanate-goat anti-rabbit IgG secondary antibody for 1 h at room temperature. After cell washing, cells were incubated with (1:5000) PI for 5 min and examined under confocal microscope (Leica, Wetzlar, Germany).

Chromatin immunoprecipitation assay
MCF10A cells grown on 10 mm dishes were treated with 25 μM cis-GS for 3 h. DNA and proteins of the treated cells were cross-linked by incubating cells in 37% formaldehyde for 10 min at room temperature. Cells were washed twice with ice-cold PBS containing protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany), scraped in 1 ml PBS and centrifuged at 2000 g for 4 min. Pellets were suspended in 200 l SDS lysis buffer [1% SDS, 10 mM EDTA and 50 mM Tris (pH 8.1)] with protease inhibitor cocktail. Lysates were incubated for 10 min on ice and sonicated to get fragments of 200–500 bp in length. Insoluble material was removed by centrifugation at 13 000 g for 10 min and each sample was separated for input positive control. Immunoprecipitating with specific Nrf2 antibody or non-specific negative control IgG. Chromatin immunoprecipitation dilution buffer containing protease inhibitor Cocktail tablets was added into each tube containing 100 μl of chromatin. Samples were pre-immunoprecipitated with Protein G agarose beads to preclear the chromatin for 1 h at 4°C. After centrifugation at 7000 g for 1 min, supernatant was transferred into new eppendorf tubes. Each sample was immunoprecipitated with 5 μg of specific Nrf2 antibody or normal mouse IgG overnight at 4°C. Immune complexes were

Fig. 2. Effect of GS on HO-1 expression in MCF10A cells. (A) MCF10A cells were treated with GS isomers (5, 10 and 25 μM) for 6 h and then harvested for HO-1 expression by western blot analysis. Actin was included to ensure equal protein loading. (B) MCF10A cells were treated with cis-GS (25 μM) for the indicated times and then collected for western blot analysis of HO-1 expression. (C) Treatment of MCF10A cells with cis-GS (25 μM) for the same periods was done, and the cells were harvested for RNA preparation. RNA samples were analyzed by reverse transcriptase-PCR to determine the levels of HO-1 messenger RNA as described in Materials and methods. GAPDH levels were included as a control. (D) HaCaT, JB6 and CCD841CoN cells were treated with cis-GS (5, 10 and 25 μM) for 6 h and then harvested to determine levels of HO-1 and Nrf2 by western blot analysis as described in Materials and methods. All samples were prepared in triplicates. Columns and bars represent means and standard deviation, respectively. The criterion for statistical significance was *P < 0.05, **P < 0.01 and ***P < 0.005.
precipitated with Protein G agarose beads for 4 h at 4°C with rotation. Pelleted Protein G agarose beads were washed once with Low Salt Immune Complex Wash Buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1) and 150 mM NaCl], once with High Salt Immune Complex Wash Buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1) and 500 mM NaCl], once with LiCl Immune complex Wash buffer [0.25 M LiCl, 0.5% NP40, 1% deoxycholic acid, 1 mM EDTA and 10 mM Tris–HCl (pH 8.1)] and twice with Tris and EDTA buffer [10 mM Tris–HCl (pH 8.1) and 1 mM EDTA]. DNA–protein complexes were eluted from Protein G agarose beads with an elution buffer (0.1 M NaHCO3 and 1% SDS). Cross-linking was reversed at 65°C overnight and DNA was extracted using AccuPrep Genomic DNA Extraction Kit (Bioneer) according to the manufacturer’s protocol for cultured cells. PCR was performed against the distal E2 (∼9.0 kb region) ARE region of the HO-1 (primers 5’-CCCTGCTGAGTAATCCTTTCCGA-3’ and 5’-ATGTCCCGACTCCAGCTC-CA-3’) promoter, in reaction buffer. Initial denaturation (5 min at 95°C) was followed by 40 cycles for 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. PCR was completed by 10 min at 72°C and products were separated on 2.5% agarose gel.

Accumulation of ROS in MCF10A cells treated with cis-GS was monitored using the fluorescence-generating probe DCF-DA. Treated cells (1 × 10^5/ml) were rinsed with PBS and loaded with 10 μM DCF-DA. After 30 min incubation at 37°C, cells were examined under a confocal fluorescence microscope set at 488 nm for excitation and 530 nm for emission. Treated cells (1 × 10^5/ml) were washed twice with Hank’s balanced salt solution buffer and loaded with 10 mM DCF-DA for 30 min at 37°C to assess ROS-mediated oxidation of DCF-DA to the fluorescent compound DCF. Fluorescence of oxidized DCF was measured at an excitation wavelength of 480 nm and an emission wavelength of 525 nm using a flow cytometer.

Statistical analysis
When necessary, data were expressed as means ± SD of at least three independent experiments, and statistical analysis for single comparison was performed using the Student’s t-test. The criterion for statistical significance was *P < 0.05, **P < 0.01 and ***P < 0.005.
cis-GS resulted in modest increase in the intracellular accumulation of ROS in MCF10A cells as assessed by DCF-DA fluorescence staining (Figure 5A) and fluorescence activated cell sorting (Figure 5B) analysis. Pretreatment with NAC (5 mM) abolished cis-GS-induced ROS generation as analyzed by the DCF-DA assay (Figure 5C) and FACS analysis (Figure 5D). NAC is not only a ROS scavenger but also can act as a thiol-reducing agent. Pretreatment of MCF10A cells with NAC and other thiol-reducing agents, DTT (0.5 mM) and pyridoline dithiocarbamate (25 μM), abrogated cis-GS-induced upregulation of Nrf2 and/or HO-1 (Figure 5E and F). The removal of thiol-reducing agents from the culture media prior to the addition cis-GS abrogated their inhibitory effects on HO-1 induction, further supporting the direct cysteine thiol modification in the target protein by cis-GS. However, a non-thiol antioxidant trolox failed to inhibit the cis-GS-induced HO-1 induction (data not shown). These findings, taken together, suggest that cis-GS may directly modify critical cysteine thiols present in the proteins responsible for Nrf2-mediated upregulation of HO-1 expression rather than acting as a mild prooxidant.

Discussion
Oxidative stress and inflammation are two major culprits in various chronic diseases, including cancer (12). Being alarmed by mild oxidative stress, cells are stimulated to express a variety of antioxidant and phase II detoxification enzymes, collectively known as cytoprotective enzymes (13). Recent studies have demonstrated that HO-1, a representative cytoprotective enzyme, confers protection against oxidative stress and inflammation (14). Many phytochemicals derived from medicinal plants as well as dietary sources have been shown to induce HO-1 as one of the underlying mechanisms of their antioxidative, anti-inflammatory and chemopreventive properties (15). The herbal...
formulations containing diverse classes of phytochemicals from medicinal plants have long been used for the treatment of different human ailments. GS is a medicinal phytosterol present in the gum resin of guggul plants, such as Commiphora mukul and Commiphora myrrha. These plants are members of Burseraceae family and found mainly in India, Kenya, China, Bangladesh, Pakistan and Arabia. They have been used to treat obesity, rheumatism and atherosclerosis (16,17). GS exists in nature as two stereoisomers (7). Several studies have reported the antioxidant (18), anti-inflammatory (19,20) and anticarcinogenic activities (21,22) of GS. In the present study, we found that cis-GS induces activation of Nrf2 signaling and the subsequent HO-1 expression in human mammary epithelial MCF10A cells. This provides the MCF10A

![Fig. 5. cis-GS-induced HO-1 expression and Nrf2 activation in MCF10A is mediated by ROS generation. (A) MCF10A cells were treated with cis-GS (25 μM) for the intervals indicated and then examined for the intracellular accumulation of ROS under the confocal microscope using the DCF-DA fluorescence staining method. (B) Cells were treated with cis-GS (25 μM) for 3 h and ROS generation was measured by FACS as described in Materials and methods. (C and D) Cells were pretreated with NAC (5 mM) for 1 h before treatment with cis-GS (25 μM) for 3 h. ROS levels were examined after DCF-DA staining or measured by FACS. (E) Cells were pretreated with NAC (5 mM) or DTT (0.5 mM) for 1 h and then cis-GS (25 μM) for 1 h or 6 h, respectively, to determine Nrf2 or HO-1 levels by western blot analysis. (F) Cells were pretreated for 1 h with pyrrolidine dithiocarbamate (25 μM) and then treated with cis-GS for 6 h. Cells were harvested for western blot analysis of HO-1 levels. Data are means ± SD (N = 3). The criterion for statistical significance is same as defined in Figure 2.]
cells with the necessary cytoprotection against the exogenous and endogenous oxidative and other stressful insults that can lead to carcinogenesis.

It is well known nowadays that phytochemicals possessing antioxidant and/or anti-inflammatory properties induce HO-1 expression via the Nrf2 signaling (23,24). It is plausible that cellular exposure to electrophilic phytochemicals especially those containing the α,β-unsaturated carbonyl moiety (called Michael acceptor) stimulate cellular proliferation by inducing a mild oxidative or electrophilic stress that can activate Nrf2/ARE signaling (25,26). Our results demonstrate clearly that GS-induced HO-1 expression is mediated by Nrf2 nuclear translocation and subsequent binding to the ARE–DNA. Silencing of Nrf2 using siRNA transfection as well as using nrf2 knocked out in MEFs abrogated cis-GS-induced HO-1 expression as well as Nrf2 activation. GS possesses two electrophilic carbon centers, one in the cyclopentanone ring and the other in the cyclohexenone ring (Figure 1), which may account for its capability of activating Nrf2 signaling. GS possesses two electrophilic carbon centers, one in the cyclopentanone ring and the other in the cyclohexenone ring (Figure 1), which may account for its capability of activating Nrf2 signaling. GS possesses two electrophilic carbon centers, one in the cyclopentanone ring and the other in the cyclohexenone ring (Figure 1), which may account for its capability of activating Nrf2 signaling. GS possesses two electrophilic carbon centers, one in the cyclopentanone ring and the other in the cyclohexenone ring (Figure 1), which may account for its capability of activating Nrf2 signaling. GS possesses two electrophilic carbon centers, one in the cyclopentanone ring and the other in the cyclohexenone ring (Figure 1), which may account for its capability of activating Nrf2 signaling.

We found that both isomers of GS induced the transcription and protein expression of HO-1. Interestingly, cis-GS induced HO-1 expression to a greater extent than trans-GS. This might be due to the stereoisomeric difference between GS isomers. The carbonyl group on the cyclopentanone ring is more likely to be involved in the Michael addition reactions than the one located on the cyclohexenone ring due to the rigidity of the later moiety. The conformational differences between the two isomers in the direction of the methyl group may influence the spatial orientation of each molecule, with cis-GS maintaining a structure more favorable in Michael addition with nucleophiles. The Michael acceptors are strong electrophiles that can attack or oxidize the nucleophilic cysteine thiols located within some proteins by undergoing the nucleophilic substitution reaction (30). One potential molecule with which cis-GS is likely to form a Michael addition adduct is Keap1, an inhibitory protein that sequesters Nrf2 in the cytosol. The electrophilic center of cis-GS can modify the critical cysteine thiols of Keap1 indirectly by oxidation, or directly binding to it, allowing Nrf2 to escape from Keap1-dependent proteasomal degradation and to translocate into the nucleus.

Alternatively, Nrf2 can be activated through phosphorylation of aspartate and threonine residues found in its Neh2 domain by kinases,
such as PI3K (31) and MAPKs (32–34). On the other hand, it was reported that phosphorylation of Nrf2 by MAPKs is not a mandatory event to regulate the expression of antioxidant phase II enzymes (35). Our data also suggest that MAPKs do not appear to play a major role in Nrf2 activation by cis-GS (data not shown). It has been reported that HO-1 expression can be regulated by activation of tyrosine kinase/Akt signaling (36). Thus, we investigated whether cis-GS-induced Nrf2 activation was associated with Akt phosphorylation and found that cis-GS induced the phosphorylation of Akt prior to activation of Nrf2. The use of the specific inhibitor of PI3K/Akt (LY294002) further support that cis-GS-induced HO-1 expression as well as Nrf2 nuclear translocation is Akt-dependent. These findings suggest that Akt is likely to be involved in cis-GS-induced expression of HO-1 via the Nrf2 signaling. PI3K/Akt activity is negatively regulated by PTEN (37,38). PTEN inhibits basal activation of Akt unless it is inactivated by exogenous stimuli, such as growth factors (37,39). In addition, inhibition of PTEN activity by mutation or gene knockdown stimulates Akt activation (40). The human PTEN has essential cysteine residues at 71, 83, 105, 124 and 136 positions in its catalytic domain. The crystal structure of PTEN reveals that Cys71 and Cys124 are adjacent and prone to oxidation by ROS to form a disulfide bond (11). Thus, hydrogen peroxide can oxidize both Cys71 and Cys124 of PTEN, causing loss of its phosphatase function (11,41,42). However, proteins isolated from MCF10A cells challenged with cis-GS did not exhibit any detectable amounts of the oxidized form of PTEN, whereas hydrogen peroxide treatment generated oxidized PTEN that migrates faster on the non-reducing gel (I.A.- GS-induced expression of HO-1 via the Nrf2 signaling. GS-induced HO-1 expression via Nrf2 pathway in MCF10A cells. GS-induced expression of HO-1 via the Nrf2 signaling. GS-induced HO-1 expression via Nrf2 pathway in MCF10A cells.

References

35. Sun, Z. et al. (2009) Phosphorylation of Nrf2 at multiple sites by MAP kinases has a limited contribution in modulating the Nrf2-dependent antioxidant response. PLoS One, 4, e6588.

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