

The Synthetic Triterpenoids, CDDO and CDDO-Imidazolide, Are Potent Inducers of Heme Oxygenase-1 and Nrf2/ARE Signaling

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Abstract

The synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and its derivative 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) are multifunctional molecules with potent antiproliferative, differentiating, and anti-inflammatory activities. At nanomolar concentrations, these agents rapidly increase the expression of the cytoprotective heme oxygenase-1 (HO-1) enzyme *in vitro* and *in vivo*. Transfection studies using a series of reporter constructs show that activation of the human HO-1 promoter by the triterpenoids requires an antioxidant response element (ARE), a cyclic AMP response element, and an E Box sequence. Inactivation of one of these response elements alone partially reduces HO-1 induction, but mutations in all three sequences entirely eliminate promoter activity in response to the triterpenoids. Treatment with CDDO-Im also elevates protein levels of Nrf2, a transcription factor previously shown to bind ARE sequences, and increases expression of a number of antioxidant and detoxification genes regulated by Nrf2. The triterpenoids also reduce the formation of reactive oxygen species in cells challenged with *tert*-butyl hydroperoxide, but this cytoprotective activity is absent in Nrf2 deficient cells. These studies are the first to investigate the induction of the HO-1 and Nrf2/ARE pathways by CDDO and CDDO-Im, and our results suggest that further *in vivo* studies are needed to explore the chemopreventive and chemotherapeutic potential of the triterpenoids. (Cancer Res 2005; 65(11): 4789-98)

Introduction

Triterpenoids are natural products that resemble steroids in their biogenesis by cyclization of squalene and their pleiotropic actions. Triterpenoids such as oleanolic acid and ursolic acid have been used for medicinal purposes in many Asian countries and have weak antitumorigenic and anti-inflammatory properties (1–4). To improve the potency of these compounds, we have synthesized and tested over 270 derivatives of oleanolic acid and ursolic acid for potential use as chemopreventive and chemotherapeutic agents (5–8). Two of the most potent synthetic triterpenoids, 2-cyano-3,12-

dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and its derivative 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), inhibit cell proliferation and induce apoptosis in leukemia, myeloma, and carcinoma cell lines (9–12). Moreover, CDDO and CDDO-Im block the production of nitric oxide (NO) and suppress *de novo* synthesis of inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase 2 (COX-2) in mouse macrophages treated with IFN- γ (5, 7–9, 13). These synthetic triterpenoids also induce differentiation of human leukemia cells and 3T3-L1 fibroblasts and suppress tumor growth *in vivo* in B16 melanoma and L1210 leukemia mouse models (13, 14).

Despite the potent activities of CDDO and CDDO-Im, the molecular mechanisms that mediate these biological effects are not known. However, microarray analysis of breast cancer cells treated with synthetic triterpenoids revealed a dramatic up-regulation of heme oxygenase-1 (HO-1) mRNA (15). Heme oxygenase is the rate-limiting enzyme in the catabolism of heme, producing biliverdin, iron, and carbon monoxide. Three heme oxygenase isoforms have been described (16–18); HO-2 and HO-3 [the latter recently described as a pseudogene (19) are constitutively expressed]. HO-1 is induced by a variety of stimuli, including growth factors, cytokines, NO, and oxidants such as heme, hydrogen peroxide, oxidized lipids, and heavy metals (20). HO-1 and its breakdown products possess potent anti-inflammatory and cytoprotective properties (reviewed in refs. 21–23).

The diversity of stimuli that induce HO-1 suggests that the molecular mechanisms that regulate HO-1 are complex. Several studies have described the regulatory sites and transcription factors required for activation of the mouse HO-1 promoter (24–26) and have begun to explore the human HO-1 promoter (27–30), but there are significant differences between the two species (reviewed in ref. 31). In the mouse, phytochemicals such as curcumin (32) and carnosol (26) induce HO-1 by regulating the expression and location of the Nrf2 transcription factor, but the role of Nrf2 in the activation of human HO-1 is still in question. Nrf2 normally is sequestered in the cytoplasm by its inhibitor Keap1. Once activated, Nrf2 translocates to the nucleus and dimerizes with another member of the Cap'n'Collar/basic leucine zipper family of transcription factors (33, 34). Nrf2 then activates transcription by binding to a stress response element (StRE) found in the promoters of a number of antioxidative and anti-inflammatory genes, including HO-1 (35). StREs are regulatory DNA sequences also referred to as antioxidant response elements (ARE) or Maf recognition elements (MARE). Because of the structural and functional similarity among StRE, ARE, and MARE sequences (reviewed in ref. 36), these terms are often used interchangeably but hereafter will be referred to as AREs.

Note: Preliminary results from this investigation were presented at the 95th Annual Meeting of the American Association for Cancer Research in Orlando, FL, 2004.

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Activation of the Nrf2/ARE pathway can suppress oxidative stress and inflammation and thus has important implications for carcinogenesis (37, 38).

In this study, we established that the synthetic triterpenoids CDDO and CDDO-Im dramatically increase HO-1 expression *in vitro* and *in vivo* and explored the molecular mechanisms that mediate this induction. When mutations were simultaneously introduced into the cyclic AMP response element (CRE), ARE, and E Box regulatory sequences in the human HO-1 promoter, activation of HO-1 by the triterpenoids was completely blocked. The triterpenoids also increased expression of a number of genes regulated by the Nrf2 transcription factor, and Nrf2 is required for the triterpenoids to reduce oxidative stress. The potent induction of HO-1 and the Nrf2/ARE cytoprotective pathways by low nanomolar concentrations of CDDO and CDDO-Im suggests that these triterpenoids could be used therapeutically for cancer prevention.

Materials and Methods

Reagents. Details of the synthesis of CDDO and CDDO-Im have been published (5, 7, 8). Triterpenoids were dissolved in DMSO, and controls containing equal concentrations of DMSO ($\leq 0.1\%$) were included in all experiments. Sources of other reagents were as follows: zinc protoporphyrin IX from Frontier Scientific (Logan, UT); SB203580, H89, and Gö6976 from Calbiochem (San Diego, CA); H₂DCFDA from Molecular Probes (Eugene, OR); rabbit polyclonal antibodies against HO-1 and Nrf2 from Santa Cruz Biotechnology (Santa Cruz, CA); AKT, phospho-AKT, CRE binding protein (CREB), and phospho-CREB polyclonal antibodies from Cell Signaling Technology (Beverly, MA). LY294002 and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture. U937 and THP-1 human leukemia cells [American Type Culture Collection (ATCC), Manassas, VA] were maintained in RPMI containing 5% fetal bovine serum (FBS), but for the HO-1 induction experiments, the U937 cells were plated and treated in RPMI + 1% horse serum. CV-1 cells (ATCC), monkey kidney cells routinely used in transfection experiments, were maintained in MEM + 10% FBS. Nrf2^{-/-} and Nrf2^{+/+} mouse embryo fibroblasts (ref. 39; kindly provided by Jeff Chan, University of California Irvine) were grown in DMEM/F12 supplemented with 10% FBS, nonessential amino acids, and 2-mercaptoethanol. HK-2 cells (ATCC), an immortalized human proximal tubule epithelial cell line from normal adult kidney, were grown in collagen-coated tissue culture plates in keratinocyte-serum free medium supplemented with 5 ng/mL recombinant epidermal growth factor and 40 μ g/mL bovine pituitary extract. All tissue culture media, sera, and supplements were from Invitrogen (Carlsbad, CA).

Northern blot analysis. Total RNA was isolated from cells with Trizol (Invitrogen) and prepared for blotting as previously described (40). Probes for human HO-1 or growth hormone (hGH; ref. 28) were radiolabeled with [α -³²P]dCTP using random primers. Membranes were stripped and reprobed with a cDNA probe for glyceraldehyde-3-phosphate dehydrogenase.

Heme oxygenase enzyme assay. U937 cells were treated with CDDO-Im for 12 hours, and the generation of bilirubin was used to assess heme oxygenase activity as previously described (41, 42). Heme oxygenase activity was expressed as pmol of bilirubin per mg protein per hour.

Induction of heme oxygenase-1 *in vivo*. Male CD1 mice were gavaged with 2 μ mol CDDO-Im (three mice per group) dissolved in DMSO or with vehicle alone (two mice per group). After 6 hours, various organs were removed and homogenized in lysis buffer [50 mmol/L Tris (pH 8), 100 mmol/L NaCl, 0.5% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ mol/L leupeptin, and 5 μ g/mL aprotinin]. Lysates (100 μ g per organ, except 20 μ g for spleen) were analyzed by Western blotting (40). A representative sample from each organ is shown for both treated and control animals.

Adenoviral expression of dominant-negative AKT. The recombinant adenoviral construct encoding HA-tagged dominant-negative AKT (43) was provided by Kenneth Walsh (Boston University School of Medicine, Boston, MA). MCF10 cells (Fred Miller, Barbara Ann Karmanos Cancer Institute, Detroit, MI) were infected with adenoviral supernatant (1:10 to 1:50 dilutions) in DMEM/F12 + 5% horse serum for 2 days. During the last 6 hours of infection, cells were treated with 100 nmol/L CDDO-Im, and HO-1 and AKT levels were analyzed by Western blotting.

Plasmids and site-directed mutagenesis. The following human HO-1 reporter plasmids have been described: -11.6 kb (pHOGL3/11.6; ref. 27), enhancer (pHOGL3/4.5/+12.5), -9.1 kb (pHOGL3/9.1), -4.5 kb (pHOGL3/4.5), and -4.0 kb (pHOGL3/4.0; ref. 28). The Nrf2 antisense (pEF/nrf2-AS) plasmid (44) was a gift from Kohsuke Kataoka (Tokyo Institute of Technology, Yokohama, Japan). The dominant-negative expression vectors for CREB (A-CREB; ref. 45) and USF (A-USF; ref. 46) were generously provided by Charles Vinson (National Cancer Institute, Bethesda, MD). For all other HO-1 promoter constructs, the pHOGL3/4.5 or the pHOGL3/4.5 plasmid was used as the parental clone, and mutations or deletions were made using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the following oligonucleotides: (a) pHOGL3/ARE1 forward 5'-CCTTGGGAATGCTGAGTCGTGATTTCCTCATCCCTCG-3' and reverse 5'-CGAGGGGATGAGGAAATCAGACTCAGCATTCCAAGGC-3'; (b) pHOGL3/ARE2 forward 5'-GCCTTGGGAATGCTGAGTCGCGATTTCCTCATCCCTCG-3' and reverse 5'-CGAGGGGATGAGAAAATCGCGACTCAGCATTCCAAGGC-3'; (c) pHOGL3/ARE3 forward 5'-GCCTTGGGAATGCTGAGTCGCGATTTCCTTATCCCTCGTGC-3' and reverse 5'-GCACGAGGGGATAAGGAAATCGCGACTCAGCATTCCAAGGC-3'; (d) pHOGL3/ARE4 forward 5'-GTGCAGCTGCATTTCTGCTGTGCATGTTTGGG-3' and reverse 5'-CCCAAACATGACACAGCAGAAAATGCAGCTGCAC-3'; (e) pHOGL3/ARE5 and pHOGL3/4.5/ARE5 M forward 5'-GCTAGATTTTGCTGAGTTACCAGTGCCTCCTCAGC-3' and reverse 5'-GCTGAGGAGGCACTGGTAACTCAGCAAAATCTAGC-3'; and (f) pHOGL3/4.5/ Δ CRE forward 5'-GTGCAGCTGCATTTCTGCTGTTTGGGAGGGGGG-3' and reverse 5'-CCCCCTCCCAAACAGCAGAAAATGCAGCTGCAC-3'. For the double- and triple-mutant constructs, each mutation was made sequentially. The accuracy of all new HO-1 promoter-reporter constructs was confirmed by sequencing.

Transfection assays. For experiments with the various HO-1 promoter luciferase constructs, CV-1 cells in 24-well plates were transiently transfected with FuGene 6 (Roche Applied Science, Indianapolis, IN), equimolar concentrations of luciferase reporter plasmids, and the CMX- β -gal expression vector. The pG5CAT plasmid (Clontech, Palo Alto, CA) was used to equalize the total amount of plasmid per well. In separate experiments, increasing concentrations of the Nrf2 antisense expression construct were cotransfected with pHOGL3/11.6 and CMX- β -gal, and pHOGL3/4.5 was cotransfected with increasing concentrations of DN-CREB or DN-USF expression vectors and CMX- β -gal. Twenty-four hours after transfection, cells were treated with triterpenoids for an additional 18 to 24 hours. Cells were lysed in 100 μ L reporter lysis buffer (Promega, Madison, WI), and luciferase activity was measured and normalized to β -galactosidase (β -gal) activity. All transfection experiments were repeated at least thrice. For transfections with the ARE1-5 growth hormone reporter constructs, HK-2 cells were transiently transfected with LipofectAMINE (Invitrogen) and equimolar concentrations of ARE1-5 pHOGL3 reporter plasmids using a batch transfection protocol (28, 47). Cells were allowed to recover for 4 to 6 hours after transfection and split into two 10-cm plates. The next day, cells were treated with DMSO or 10 nmol/L CDDO-Im and analyzed by Northern blotting.

Microarray analysis. THP-1 human leukemia cells were treated with vehicle alone (control), with 300 nmol/L CDDO, or with 100 nmol/L CDDO-Im for 4 and 12 hours. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA), and cDNA was synthesized with the Superscript Choice kit (Invitrogen). Biotin-labeled cRNA was synthesized by *in vitro* transcription. The cDNA was then fragmented and hybridized to a human HG-133A chip (Affymetrix, Santa Clara, CA) that contains ~22,000 genes and expressed sequence tags. The chips were washed, stained and scanned using an Affymetrix scanner. Scanned output files analyzed with the

Affymetrix Gene Chip Operating Software (ver 1.1.1.052 GCOS). Signal values were determined by a one-step Tukey's biweight algorithm and normalized to a mean value of 500. To determine significant changes between treatment and control groups, ratios were calculated by GCOS, and genes with a signal level of at least 200 and that were 2-fold higher than the control ($P < 0.003$) were selected.

Detection of reactive oxygen species. Cells were treated with CDDO-Im for 18 to 24 hours and incubated with 10 $\mu\text{mol/L}$ nonfluorescent indicator H_2DCFDA for 30 minutes. Cells were challenged with 250 $\mu\text{mol/L}$ *tert*-butyl hydroperoxide (tBHP) for 15 minutes, and mean fluorescence intensity of 10,000 cells was analyzed by flow cytometry using a 480-nm excitation wavelength and a 525-nm emission wavelength. All reactive oxygen species (ROS) experiments were repeated at least thrice, and representative experiments are shown.

Results

CDDO and CDDO-imidazolide induce heme oxygenase-1 expression and activity *in vitro* and *in vivo*. Preliminary microarray analysis revealed that HO-1 mRNA was significantly up-regulated in cells treated with the synthetic triterpenoids CDDO and CDDO-Im (15). In U937 human leukemia cells treated with 100 nmol/L CDDO-Im, HO-1 mRNA increased in a time-dependent manner (Fig. 1A), as determined by Northern blotting. The HO-1 message was evident after 1 hour of incubation with CDDO-Im, with maximal induction occurring at 4 hours. By 8 hours, HO-1 levels had noticeably declined, and after 24 hours, mRNA levels had decreased to basal levels. Similarly, 30 to

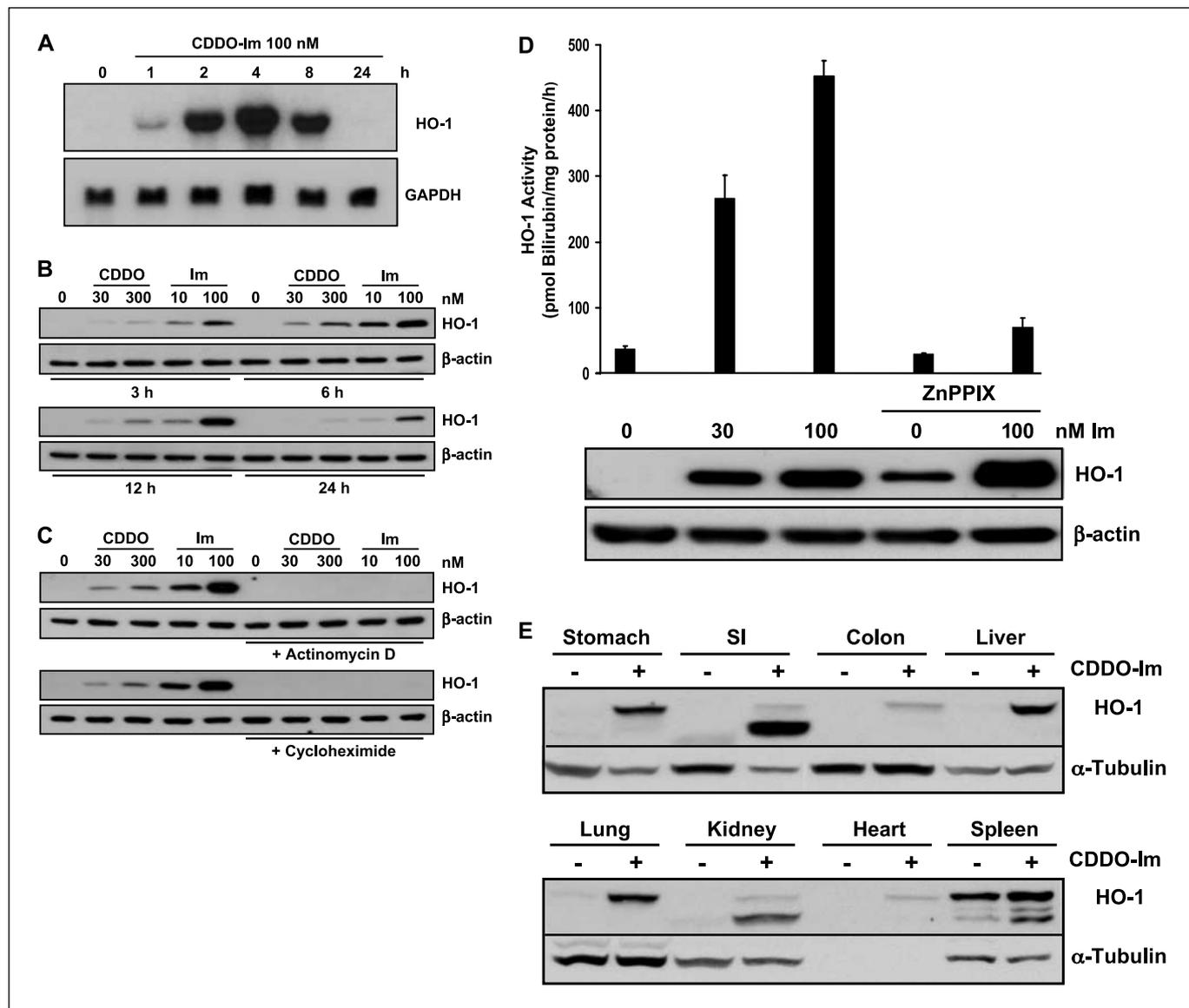


Figure 1. CDDO and CDDO-Im induce HO-1 expression and activity *in vitro* and *in vivo*. **A**, U937 cells were treated with 100 nmol/L CDDO-Im for 0 to 24 hours, and total RNA was isolated and analyzed by Northern blotting. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. **B**, cells were incubated with CDDO or CDDO-Im (*Im*) for 3 to 24 hours. Cell lysates were separated by SDS-PAGE, transferred to a membrane, and probed with antibodies against HO-1 and β -actin. **C**, Actinomycin D (5 $\mu\text{g/mL}$) or cycloheximide (10 $\mu\text{g/mL}$) was added to U937 cells for 1 hour and cells were treated with CDDO or CDDO-Im for an additional 6 hours. HO-1 levels were determined by Western blot analysis. **D**, U937 cells were incubated with 0 to 100 nmol/L CDDO-Im for 12 hours, and bilirubin production was measured to determine HO-1 enzyme activity. The same proteins were also examined by Western blot. *ZnPPiX*, zinc protoporphyrin IX. **E**, CD1 mice were gavage with 2 μmol CDDO-Im. After 6 hours, various organs were harvested and HO-1 induction was analyzed by Western blotting. Equal protein concentrations were observed in the heart samples when reprobed with a β -tubulin antibody.

300 nmol/L CDDO and 10 to 100 nmol/L CDDO-Im induced HO-1 protein in a time- and dose-dependent manner (Fig. 1B). By 24 hours, HO-1 protein levels had declined, and HO-1 protein was undetectable 48 hours after treatment (data not shown). CDDO-Im was a significantly more potent inducer of HO-1 than CDDO, (Fig. 1B, lane 3 versus 5 or lane 8 versus 10). CDDO-Im also markedly elevated HO-1 protein in T-47D and MCF10 breast epithelial cells, THP-1 leukemia cells, and A549 lung carcinoma cells (data not shown).

To explore whether the induction of HO-1 by the triterpenoids requires *de novo* transcription, we pretreated U937 cells with the transcriptional inhibitor Actinomycin D and then exposed the cells to varying concentrations of CDDO or CDDO-Im for 6 hours. Under these conditions no induction of HO-1 protein was observed (Fig. 1C, top), suggesting that *de novo* transcription is required for HO-1 induction. Similarly, the requirement for *de novo* protein synthesis was confirmed by the ability of cycloheximide to block HO-1 protein induction (Fig. 1C, bottom).

The HO-1 protein induced by the triterpenoids was enzymatically active. Bilirubin, a breakdown product of heme catabolism, increased 7- to 12-fold in U937 cells treated for 12 hours with 30 to 100 nmol/L CDDO-Im (Fig. 1D, top). This induced enzyme activity was blocked by the competitive HO-1 inhibitor zinc protoporphyrin IX (1 μ mol/L). The same samples used in the enzyme assay were also analyzed by Western blot. Although the enzyme activity induced by CDDO-Im correlated with induction of HO-1 protein, the zinc protoporphyrin IX inhibitor increased HO-1 protein levels both individually and in combination with CDDO-Im (Fig. 1D, bottom).

CDDO-Im also induced HO-1 *in vivo*. As shown in Fig. 1E, HO-1 protein levels increased in the stomach, small intestine, colon, liver, lung, kidney, and heart of CD-1 mice gavaged with 2 μ mol CDDO-Im; highest induction of HO-1 was observed in the stomach, liver, and small intestine. No endogenous HO-1 expression was detected in any of these tissues. Although HO-1 is constitutively expressed in the spleen of mice, even in this organ, HO-1 levels were further increased in response to CDDO-Im. Originally, organs from three mice per group were analyzed, and the upper HO-1 band was dominant (data not shown). However, when the same samples were used for a final representative blot, additional lower bands were observed in the small intestine, kidney, and spleen.

Kinase inhibitors block heme oxygenase-1 induction in U937 cells treated with CDDO-imidazole. The signaling pathways that regulate HO-1 induction vary depending on the stimulus. To identify potential pathways activated by the triterpenoids, U937 cells were exposed to various kinase inhibitors and subsequently to CDDO-Im. Although the PKA inhibitor H89 (10 μ mol/L) and the p38 inhibitor SB203580 (10 μ mol/L) only slightly reduced the induction of HO-1 by CDDO-Im, both the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (15 μ mol/L) and Gö6976 (0.5 μ mol/L), an inhibitor of the classic Ca^{2+} -dependent PKC isoforms, markedly blocked the expression of HO-1 protein (Fig. 2). We next tested the effects of CDDO-Im on AKT, a known downstream target of PI3K. As shown in Fig. 2B, phospho-AKT was significantly but transiently increased in U937 cells treated with CDDO-Im for 1 hour. The importance of the PI3K pathway in HO-1 induction in response to CDDO-Im was further verified by the use of a dominant-negative AKT (AAA) adenoviral construct. For this experiment, we used MCF10 breast epithelial cells because of the low transduction efficiency of U937 cells. Infection with dominant-negative AKT blocked induction of HO-1 in response to CDDO-Im treatment by ~70% to 80% (Fig. 2C).

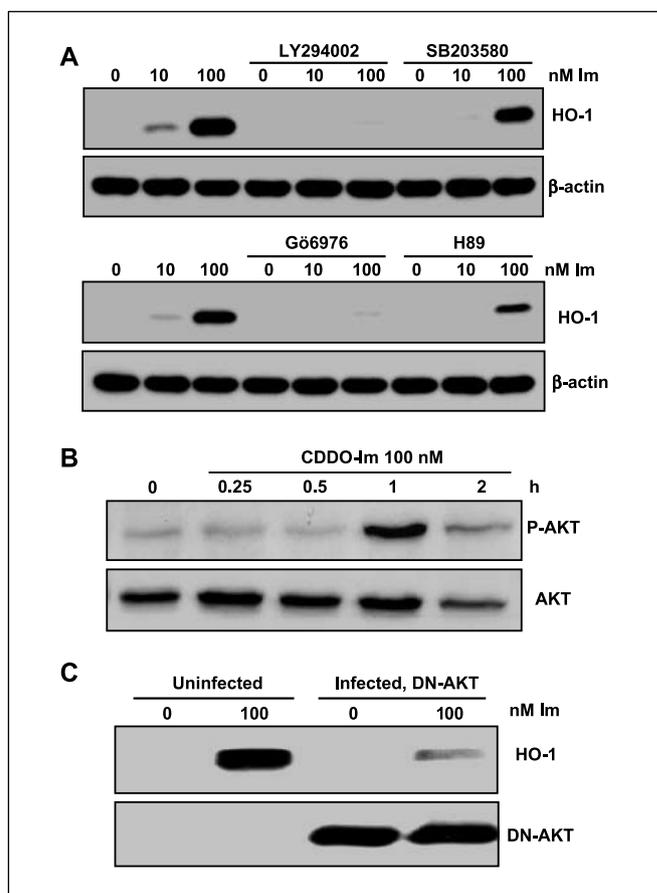


Figure 2. Inhibition of the PI3K and PKC signaling pathways blocks induction of HO-1. A, U937 cells were incubated with various protein kinase inhibitors for 1 hour and treated with 0 or 100 nmol/L CDDO-Im for 8 hours. Total proteins were analyzed by Western blotting for HO-1 and β -actin. B, CDDO-Im increases phosphorylation of AKT. U937 cells were incubated with 100 nmol/L CDDO-Im for 0 to 2 hours, and Western blots were probed for phospho-AKT followed by total AKT. C, a dominant-negative (DN) AKT construct partially blocks induction of HO-1. MCF10 cells were infected with a DN-AKT adenoviral construct for 2 days. During the last 6 hours of infection, 0 or 100 nmol/L CDDO-Im was added to the infected cells and to uninfected controls. Cell lysates were analyzed by Western blotting for HO-1 and DN-AKT.

Region between -4.0 and -4.5 kb of the human heme oxygenase-1 promoter is required for the induction of heme oxygenase-1 by the triterpenoids. The data from the kinase inhibitor experiments (Fig. 2) suggested that several different signaling pathways control the induction of HO-1 in our system; thus, we next sought to identify the specific regulatory elements that mediate the induction of HO-1 in response to the triterpenoids. A series of human HO-1 promoter-reporter constructs, described by Hill-Kapturczak et al. (27, 28), were transiently transfected into CV-1 cells. Luciferase activity for the full-length HO-1 promoter (-11.6 kb) and an internal enhancer were 3- to 5-fold higher in cells treated with 10 and 100 nmol/L CDDO-Im than in cells treated with vehicle alone (Fig. 3A). The -9.1 and -4.5 kb HO-1 promoter constructs also were activated by CDDO-Im. However, transfection of a -4.0 kb HO-1 promoter construct was minimally responsive to 100 nmol/L CDDO-Im. A similar pattern of promoter activation was observed in cells treated with 30 and 300 nmol/L CDDO (data not shown). These data suggest that a region between -4.0 and -4.5 kb of the human HO-1 promoter contains the regulatory element(s) responsible for triterpenoid-mediated induction of HO-1.

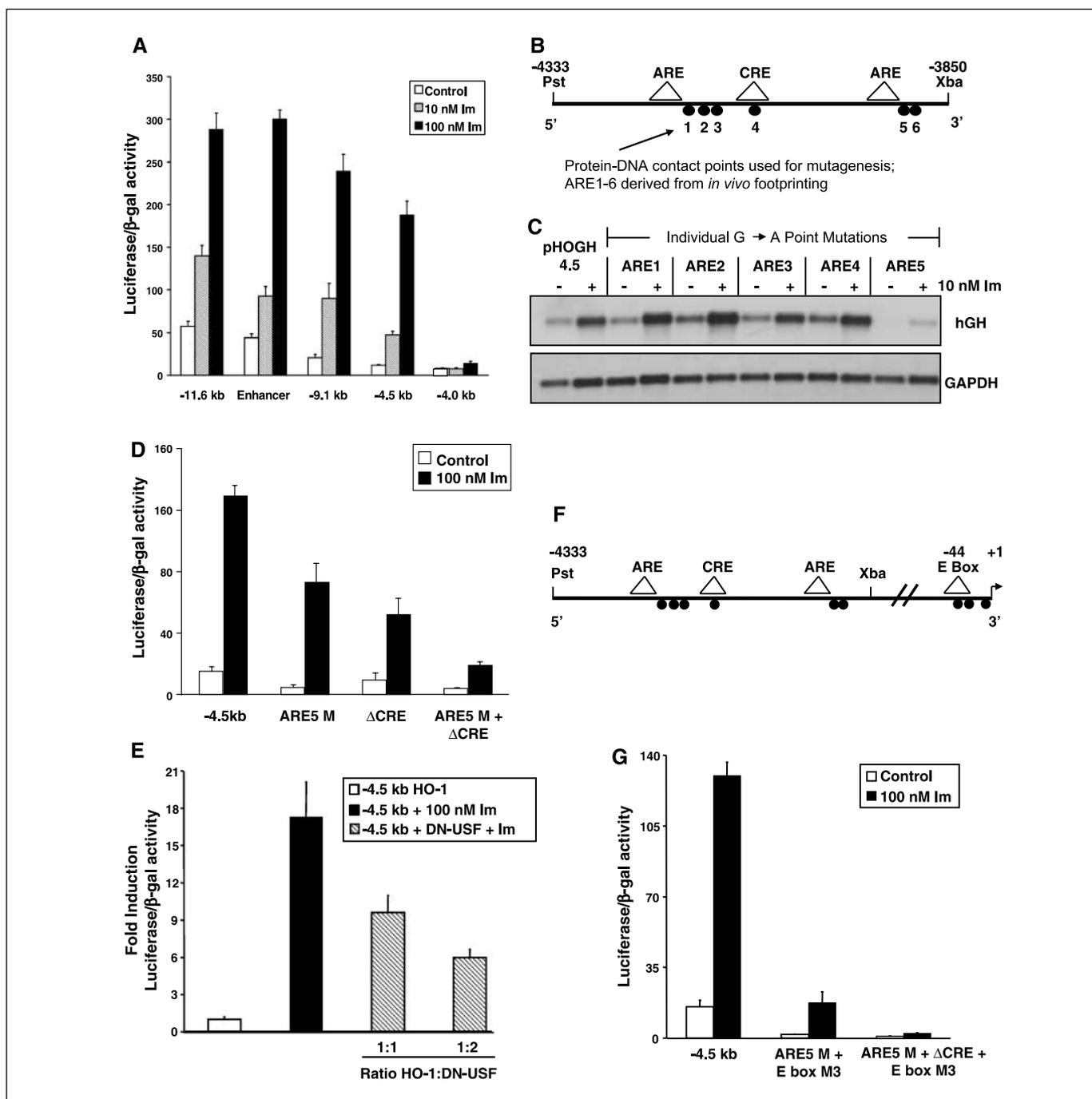


Figure 3. Activation of the human HO-1 promoter by CDDO-Im requires functional CRE, ARE, and E Box sites. *A*, CV-1 cells were transiently transfected with equimolar concentrations of various human HO-1 promoter reporter plasmids and 40 ng pCMX- β -gal for a total of 390 ng plasmid per well. Twenty-four hours later, cells were treated with control media or media containing 10 to 100 nmol/L CDDO-Im. After an additional 24 hours, cells were lysed and luciferase activity was normalized to β -gal activity for each well. *Columns*, means from four replicate wells; *bars*, SD. *B*, known regulatory regions in the proximal HO-1 promoter include two AREs and a CRE. Six protected guanine residues also have been identified (ARE1-6), and their locations are indicated. *C*, HK-2 cells were transfected with pHOGH/4.5 or with plasmids containing point mutations (G to A) within the AREs. Cells were treated with 0 to 10 nmol/L CDDO-Im, and total RNA was extracted and hybridized with 32 P-labeled growth hormone (*GH*), HO-1 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probes. *D*, luciferase reporter constructs with a deletion of the CRE site (Δ CRE) or a point mutation within the ARE5 sequence (ARE5 M) were transfected into CV-1 cells. *E*, CV-1 cells were cotransfected with pHOGL3/4.5 (40 ng per well), increasing concentrations of a DN-USF expression vector (40-80 ng per well), and pCMX- β -gal (40 ng per well) and treated as described above. *F*, point mutations in the E box and proximal ARE5 and deletion of the CRE site were introduced into the pHOGL3/4.5 reporter plasmid. *G*, CV-1 cells were transfected with reporter constructs (200 ng per well) containing mutations in the E box and ARE 5 (E Box M3 + ARE5 M) and with the additional CRE deletion (ARE5 M + Δ CRE + E Box M3).

Cyclic AMP response element and antioxidant response element 5 contribute to activation of the human heme oxygenase-1 promoter by CDDO-imidazole. The promoter region required for HO-1 induction, which is flanked by *Pst*I and

*Xba*I restriction enzyme sites (31), contains two putative AREs and a putative binding site for the CREB, as illustrated in Fig. 3*B*. Using dimethylsulfate *in vivo* footprinting, six protected guanine residues were identified within the -4.0 to -4.5 kb region of the

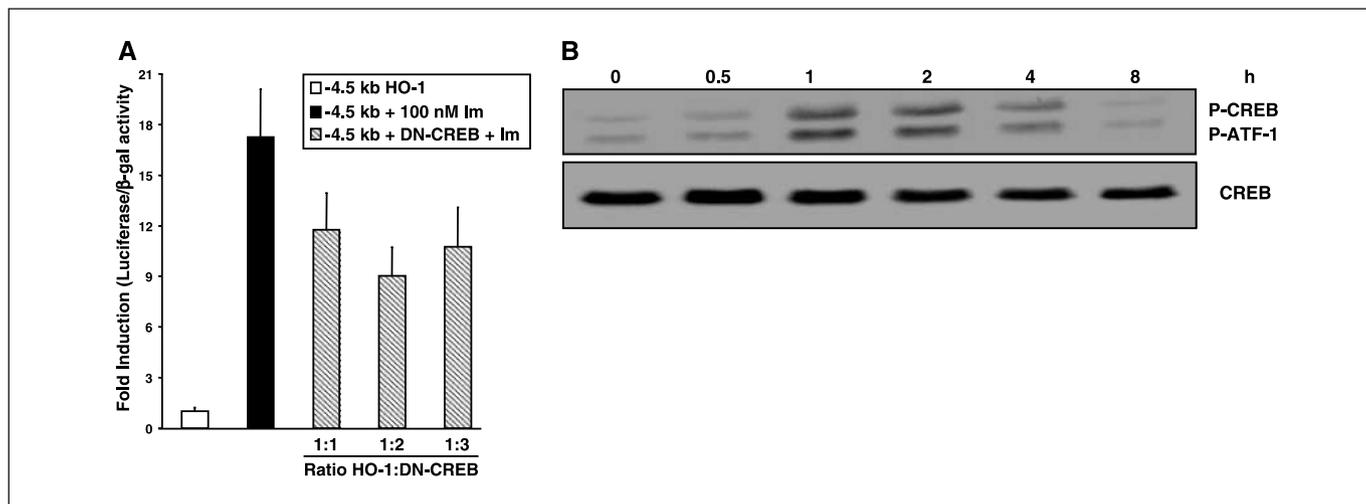


Figure 4. CDDO-Im activates the CRE and increases phosphorylation of CREB. **A**, CV-1 cells were cotransfected with pHOGL3/4.5 (40 ng per well), increasing concentrations of a DN-CREB expression vector (40–120 ng per well), and pCMX- β -gal (40 ng per well). Cells were treated and harvested as described in Fig. 3A. **B**, U937 cells were incubated with 100 nmol/L CDDO-Im for 0 to 8 hours. Cell lysates were analyzed by Western blotting, using a phospho-CREB antibody (P-CREB) and stripped and reprobed with a CREB antibody. The polyclonal phospho-CREB antibody also binds to phospho-ATF-1.

human HO-1 promoter in human renal proximal tubular cells.⁴ These footprints are called ARE1–6, and their relative locations in the human HO-1 promoter are shown in Fig. 3B. ARE1–3 are located within 8 bp of each other, ARE4 resides within a previously reported CRE site (30), and ARE5 and ARE6 are located immediately downstream of the proximal ARE sequence. Reporter constructs containing point mutations within these AREs were generated, in which the protected G residue was changed to an A. In HK-2 cells transiently transfected with the mutated 4.5-kb HO-1 promoter constructs (pHOGH/4.5), the point mutation in ARE5 markedly reduced expression of the hGH transcript in the cells treated with CDDO-Im (Fig. 3C). No significant changes were detected in the expression of hGH mRNA with the mutations in the other AREs (Fig. 3C) including ARE6 (data not shown).

To confirm the findings observed in HK-2 cells, site-directed mutagenesis in the –4.5 kb HO-1 promoter-reporter construct was used to delete the CRE or to introduce a point mutation (G to A) in the ARE5 sequence. When transiently transfected into CV-1 cells, the single nucleotide mutation in the ARE5 site (ARE5 M) reduced luciferase activity by 44% in cells treated with CDDO-Im (Fig. 3D). Although a point mutation within the CRE (ARE4) did not alter induction of the hGH reporter plasmid shown in Fig. 3D, deletion of the entire CRE site (Δ CRE) reduced reporter activity by 60%. Interestingly, the double mutation construct, including the ARE5 point mutation and the CRE deletion, decreased HO-1 promoter activity by 85% but did not completely abolish reporter activity (Fig. 3D).

Inactivation of the cyclic AMP response element, antioxidant response element 5, and E box sequences totally abolishes induction of the heme oxygenase-1 promoter by CDDO-imidazolide. Most of the induction of the HO-1 promoter by the triterpenoids was eliminated with the –4.0-kb reporter construct (Fig. 3A) or with mutations in the CRE and ARE5 sites (Fig. 3D). However, treatment with CDDO-Im induced a small but reproducible increase in luciferase activity with these constructs, which suggested the presence of an additional regulatory site

within the HO-1 promoter. Recently, Hock et al. reported that upstream stimulatory factors (USF), members of the basic helix-loop-helix family of transcription factors, bind to an E Box in the proximal human HO-1 promoter and regulate HO-1 induction (47). When a DN-USF construct was transfected into CV-1 cells treated with CDDO-Im, activation of the –4.5-kb HO-1 promoter-luciferase construct was reduced by 44% to 65% (Fig. 3E). The importance of the E box site was further verified by a point mutation (G to A) at –44 bp from the transcriptional start site of HO-1 (Fig. 3F). Luciferase activity declined by 86% in CV-1 cells transfected with a reporter plasmid containing a double mutation in the E box (E box M3) and the ARE5 site (ARE5 M) of the HO-1 promoter (Fig. 3G), results similar to those with the double mutation of the CRE and ARE5 sites (Fig. 3D). To examine if the E Box was responsible for mediating the induction of the HO-1 promoter observed in the constructs containing the ARE5 and CRE point mutations, cells were transfected with a reporter construct containing a triple mutation (CRE site deletion, ARE5 M, and E box M3 mutation). Luciferase activity decreased by 98%, demonstrating that full induction of the human HO-1 promoter by CDDO-Im requires functional CRE, ARE5, and E Box sites (Fig. 3G).

CDDO-imidazolide increases phosphorylation of cyclic AMP response element binding protein. Although maximum induction of the HO-1 promoter by the triterpenoids requires three unique sites, deletion of the entire CRE site or the point mutation in the ARE5 reduced reporter activity by 44% and 60%, respectively (Fig. 3D); thus, additional experiments focused on these sites. Cotransfection of a DN-CREB construct with the –4.5-kb HO-1 promoter construct decreased luciferase activity by 47% in CV-1 cells treated with CDDO-Im (Fig. 4A). Moreover, 100 nmol/L CDDO-Im increased the phosphorylation of CREB after 1 to 2 hours of treatment in U937 cells (Fig. 4B). At 4 hours after treatment, CREB phosphorylation had declined and by 8 hours, the levels of phosphorylated CREB were the same as the untreated cells. The polyclonal antibody used in these experiments also recognizes the CREB family member activating transcription factor-1 (ATF-1), and CDDO-Im increased ATF-1 phosphorylation in a time-dependent manner.

⁴ Hock and Agarwal, in preparation.

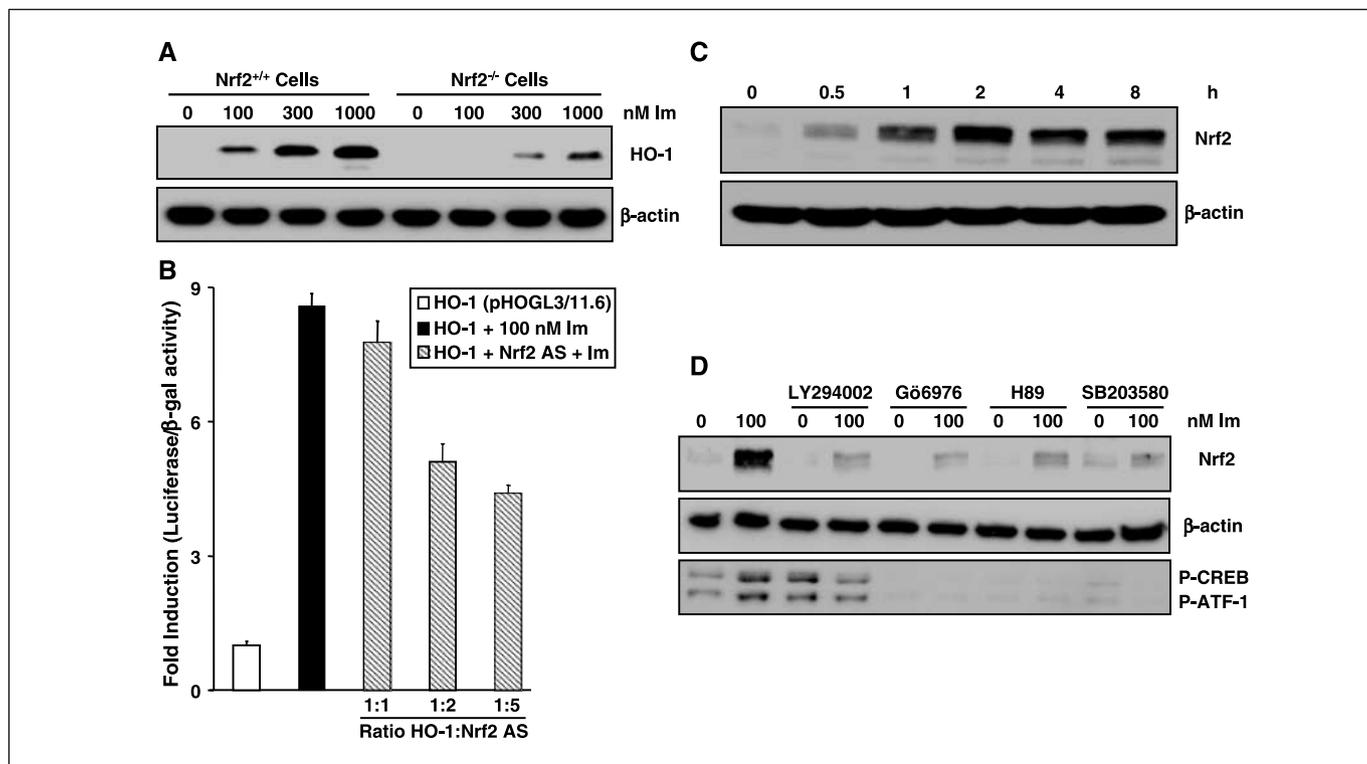


Figure 5. HO-1 induction is reduced in Nrf2 knockout cells and CDDO-Im increases Nrf2 protein levels. **A**, Nrf2^{+/+} or Nrf2^{-/-} fibroblasts were incubated with 0 to 1,000 nmol/L CDDO-Im for 6 hours. Cell lysates were analyzed for HO-1 by Western blotting. **B**, CV-1 cells were cotransfected with 50 ng of the full-length HO-1 promoter construct (pHOGL3/11.6) and increasing concentrations of an Nrf2 antisense (AS) expression vector (50-250 ng per well). Twenty-four hours later, cells were treated with control media or media containing 100 nmol/L CDDO-Im and analyzed for luciferase activity. **C**, U937 cells were treated with 100 nmol/L CDDO-Im for 0 to 8 hours, and Nrf2 protein levels were determined by Western blot analysis. **D**, U937 cells were incubated with kinase inhibitors for 45 minutes and treated with 0 to 100 nmol/L CDDO-Im for an additional 90 minutes. Total proteins were analyzed by Western blotting for Nrf2, phospho-CREB, and β -actin.

Role of Nrf2 in the induction of heme oxygenase-1 by the triterpenoids. Previous studies with the mouse HO-1 promoter have shown that the Nrf2 transcription factor binds to an ARE and activates transcription of HO-1 in response to various stimuli (31).

High concentrations of CDDO-Im (300 and 1,000 nmol/L) caused partial induction of HO-1 in Nrf2 knockout (Nrf2^{-/-}) mouse embryonic fibroblasts, but this was markedly lower than the induction of HO-1 by 100 to 300 nmol/L CDDO-Im in the wild-type

Table 1. CDDO-Im increases expression of genes regulated by Nrf2

Accession no.	Gene	Fold increase			
		CDDO		CDDO-Im	
		4 h	12 h	4 h	12 h
Z82244	<i>Heme oxygenase (decycling) 1</i>	19.70	17.15	90.51	111.43
AA083483	<i>Ferritin, heavy polypeptide 1</i>	2.64	6.06	2.64	6.96
J03934	<i>NAD(P)H dehydrogenase, quinone 1</i>	2.00	4.59	1.87	4.59
L35546	<i>gamma-glutamylcysteine synthetase, regulatory</i>	2.83	4.00	2.30	4.29
BC003567	<i>epoxide hydrolase 1, microsomal (xenobiotic)</i>	1.41	2.64	1.87	4.00
J02888	<i>NAD(P)H dehydrogenase, quinone 2</i>	1.15	1.41	1.52	4.00
D88687	<i>thioredoxin reductase 1</i>	2.46	3.48	2.30	3.48
AF061016	<i>UDP-glucose dehydrogenase</i>	1.23	1.62	1.41	2.30
NM_000637	<i>glutathione reductase</i>	1.41	2.30	1.52	2.14
L13278	<i>crystallin, zeta (quinone reductase)</i>	1.00	1.15	1.32	2.14
M90656	<i>gamma-glutamylcysteine synthetase, catalytic</i>	1.32	1.32	1.52	2.00
BC015523	<i>glutathione S-transferase A4</i>	1.15	1.23	1.62	2.00

NOTE: See Materials and Methods for experimental details. A complete description of the microarray results will be described elsewhere.

(Nrf2^{+/+}) cells (Fig. 5A). Furthermore, activation of the HO-1 promoter was reduced by 49% in CV-1 cells cotransfected with increasing concentrations of an Nrf2 antisense construct and the full-length human HO-1 promoter (Fig. 5B), whereas an Nrf2 sense construct did not reduce HO-1 luciferase activity (data not shown). In U937 cells treated with 100 nmol/L CDDO-Im, total Nrf2 protein levels were elevated within 30 minutes and remained elevated for 8 hours (Fig. 5C). Notably, the same protein kinase inhibitors (LY294002, Gö6976, H89, and SB203580) that partially blocked the induction of HO-1 (Fig. 2) also significantly reduced the increased Nrf2 levels observed when U937 cells were treated with CDDO-Im (Fig. 5D). These kinase inhibitors also significantly inhibited CREB and ATF-1 phosphorylation induced by CDDO-Im, although the PI3K inhibitor LY294002 alone increased CREB and ATF-1 phosphorylation.

Although the contribution of Nrf2 to the activation of the human *HO-1* gene requires further study, microarray studies show that the triterpenoids significantly increase expression of a number of other genes regulated by the Nrf2/ARE pathway (Table 1). In our studies, THP-1 human leukemia cells were treated with 300 nmol/L CDDO, 100 nmol/L CDDO-Im, or vehicle alone for 4 and 12 hours, and total RNA from these cells was hybridized to Affymetrix HG-U133A chips. The expression of a number of genes that mediate antioxidative and cytoprotective activities including HO-1, ferritin, thioredoxin reductase, and glutathione reductase (GSR) was significantly increased by both triterpenoids in a time-dependent manner (Table 1). Notably, HO-1 mRNA levels increased 19 fold above control levels in cells treated with CDDO and 90-fold in cells treated with CDDO-Im for 4 hours, which correlates with the dramatic increases in HO-1 mRNA and protein levels shown in Fig. 1. Detoxification genes such as NAD(P)H quinone oxidoreductase (*NQO1*) and the two subunits of glutamylcysteine synthetase were highly induced by the triterpenoids. All of the genes listed in Table 1 have been shown to be regulated by the Nrf2/ARE pathway (48–50). Moreover, a number of the cytoprotective genes activated by Nrf2 also are regulated by the PI3K pathway. In IMR-32 human neuroblastoma cells treated with *tert*-butylhydroquinone, the PI3K inhibitor LY294002 blocked the induction of *NQO1*, HO-1, GSR, and glutathione transferase mRNA (51).

CDDO-imidazole attenuates oxidative stress through the Nrf2 pathway. A number of the cytoprotective genes regulated by Nrf2, including HO-1, have antioxidative activities (38, 48). To study the effects of CDDO-Im on oxidative stress, U937 cells were incubated with varying concentrations of CDDO-Im for 18 hours. Cells were then loaded with 2',7'-dichlorofluorescein diacetate (H₂DCFDA) for 30 minutes and finally challenged with tBHP for 15 minutes. The H₂DCFDA probe passively diffuses into cells, where it is deacetylated and oxidized to the fluorescent compound 2',7'-dichlorofluorescein (52), which can be analyzed by flow cytometry. As shown in Fig. 6A, CDDO-Im reduced the oxidation of H₂DCFDA induced by tBHP in a dose-dependent manner, with a maximum inhibition of 53% with 100 nmol/L CDDO-Im. Increases in the concentration of CDDO-Im above 100 nmol/L did not provide additional protection against oxidative stress and indeed, as shown in Fig. 6B, at concentrations between 300 and 500 nmol/L, CDDO-Im markedly increased the formation of ROS. Notably, no reduction in ROS was observed when cells were treated with 10 to 100 nmol/L CDDO-Im for <6 hours before the addition of the tBHP (data not shown). However, incubation of U937 cells with 10 to

100 nmol/L CDDO-Im for times ranging from 12 to 48 hours reduced the oxidation of H₂DCFDA by ~50% (data not shown). When U937 cells were incubated with CDDO-Im and the HO-1 enzyme inhibitor zinc protoporphyrin IX (Fig. 1D), ROS levels did not change (Fig. 6C), suggesting that induction of HO-1 alone does not protect against oxidative stress. Indeed, the reduction in ROS formation following treatment with low concentrations of CDDO-Im requires the Nrf2 pathway, because no alterations in ROS levels were observed in Nrf2 knockout cells treated with CDDO-Im (Fig. 6D). In contrast, treatment with CDDO-Im (0.1–100 nmol/L) reduced the formation of ROS in the Nrf2 wild-type cells by 62%. Taken together, these experiments suggest that the antioxidative activity of CDDO-Im requires activation of the Nrf2/ARE system and not just the induction of HO-1.

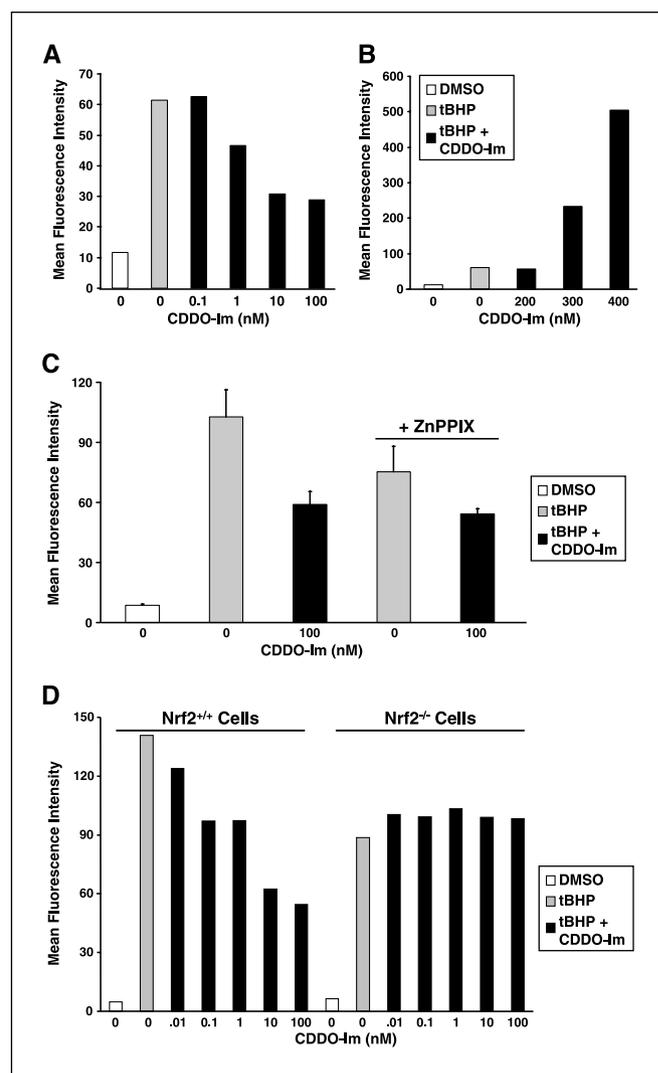


Figure 6. Low concentrations of CDDO-Im reduce the formation of ROS but high concentrations enhance ROS formation. The reduction in oxidative stress by CDDO-Im requires Nrf2. U937 cells (A–C) or Nrf2 wild-type and knockout cells (D) were treated with CDDO-Im for 18 hours. H₂DCFDA was added for 30 minutes, and the cells were challenged with 250 μ M tBHP for 15 minutes to induce ROS. Mean fluorescence intensity of 10,000 cells was detected by flow cytometry for each bar in all four figures. C, U937 cells were preincubated with zinc protoporphyrin IX (ZnPPiX) for 15 minutes before CDDO-Im was added. Representative experiment from three independent experiments (A, B, and D). Columns, means from three replicate wells; bars, SD (C).

Discussion

We have previously shown that the synthetic triterpenoids CDDO and CDDO-Im significantly inhibit the growth of cancer cells and block the expression of proinflammatory molecules such as iNOS and COX-2 (7–9). The results of the present study show that nanomolar concentrations of CDDO and CDDO-Im are potent inducers of HO-1 and the Nrf2/ARE system. Thus, synthetic triterpenoids should be added to the growing number of compounds that induce these cytoprotective molecules; other potentially promising chemopreventive agents on this list include sulforaphane (53), curcumin (32, 54), avicins (55), carnosol (26), resveratrol (56), retinoic acid (57), and aspirin (58). In contrast to these other inducers which are only active at micromolar concentrations, 10 nmol/L CDDO-Im or 30 nmol/L CDDO activates HO-1 *in vitro* and a 2 μ mol dose of CDDO-Im induces HO-1 *in vivo*. Regardless of the stimulus, the induction of HO-1 is usually considered a beneficial and adaptive response that offers protection against oxidative damage and inflammation (22).

Although the metabolic products of the HO-1 reaction are cytoprotective at low levels, excessive HO-1 activity is cytotoxic because of the accumulation of reactive iron (59). We have shown here that the effects of CDDO-Im on oxidative stress are dose dependent; concentrations of CDDO-Im between 0.1 and 100 nmol/L reduced ROS levels (Fig. 6A) whereas higher concentrations (300–400 nmol/L) increased ROS (Fig. 6B). Notably, high concentrations of CDDO-Im (2 μ mol/L) induce apoptosis by increasing ROS levels and disrupting the redox status in cells (60). In our experiments, the antioxidative activity of CDDO-Im required activation of the Nrf2/ARE system, not just HO-1 induction, as no inhibition in the formation of ROS was observed in Nrf2-deficient cells and the HO-1 inhibitor zinc protoporphyrin IX did not alter ROS levels. Although HO-1 induction might also affect cell proliferation (59), no differences in growth inhibition were observed in the Nrf2 wild-type or knockout fibroblasts treated with CDDO or CDDO-Im,⁵ suggesting that the potent growth inhibitory properties of these triterpenoids are not mediated by

the Nrf2/ARE pathway. Instead, CDDO inhibits proliferation of breast cancer cells by regulating expression of cyclin D1, p21, and Bcl-2 (15). Future experiments will examine the molecular mechanisms for the disparate, dose-dependent effects of the triterpenoids on the generation of ROS and the role of HO-1 and the Nrf2/ARE pathway in apoptosis.

Because of the pathogenetic importance of oxidative stress and inflammation in carcinogenesis and other disease processes, the induction of the HO-1 and Nrf2/ARE pathways is being explored as a potential therapeutic strategy (21–23, 37, 38, 48). Our present results indicate that CDDO and CDDO-Im are potent inducers of these cytoprotective molecules, with clinical implications for prevention of diseases as diverse as cancer, diabetes, asthma, acute renal failure, atherosclerosis, and Alzheimer's (21). In a recent study, we have shown that the anti-inflammatory and antioxidative actions, not only of CDDO but also of an entire set of synthetic triterpenoids, are closely correlated and that the Nrf2/ARE system seems to provide a common mechanism for these activities of the triterpenoids (61). Beyond these cytoprotective actions, which are associated with relatively low doses of the triterpenoids, there are known proapoptotic activities of these agents, which seem to require higher concentrations of triterpenoids that enhance formation of ROS (60, 62). Thus, an immediate problem is to understand this bifunctionality of the triterpenoids and to define the specific mechanisms that control either their antioxidative or pro-oxidative functions, to allow optimal application for either prevention or treatment of disease.

Acknowledgments

Received 12/22/2004; revised 3/2/2005; accepted 3/24/2005.

Grant support: National Foundation for Cancer Research and NIH grants RO1 CA78814 (M.B. Sporn), K22 CA99990-02 (N. Suh), RO1 DK59600, and RO1 HL068157 (A. Agarwal); Oscar M. Cohn professorship (M.B. Sporn); and the members of the Dartmouth College Class of 1934 and Reata Discovery, Inc.

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We thank Gadiseti V.R. Chandramouli (NIH) for assistance with the microarray analysis, Jeff Chan for the Nrf2^{-/-} and Nrf2^{+/-} fibroblasts, Charles Vinson for the A-USF and A-CREB expression vectors, Kohsuke Kataoka for the Nrf2 antisense construct, and Kathy Martin and Kenneth Walsh for the dominant-negative AKT constructs.

⁵ Unpublished data.

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