Low Concentrations of Diindolylmethane, a Metabolite of Indole-3-Carbinol, Protect against Oxidative Stress in a BRCA1-Dependent Manner

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Abstract

The indole-3-carbinol (I3C) metabolite 3,3′-diindolylmethane (DIM) is a proposed cancer prevention agent for various tumor types, including breast cancer. Here, we show that DIM up-regulates expression of the tumor suppressor protein BRCA1 in carcinoma and normal cell types. Up-regulation of BRCA1 was dose and time dependent, and it was observed at physiologically relevant micromolar and submicromolar DIM concentrations when cells were exposed for 72 hours. Treatment with the parent compound (I3C) or DIM (1 μmol/L) protected against cell killing due to H2O2 and other oxidants, and the protection was abrogated by knockdown of BRCA1. DIM stimulated signaling by the antioxidant transcription factor NFE2L2 (NRF2) through the antioxidant response element in a BRCA1-dependent manner. We further showed that DIM rapidly stimulated phosphorylation of BRCA1 on Ser 1387 and Ser 1524 and that these phosphorylations are required for protection against oxidative stress. DIM-induced phosphorylation of BRCA1 on Ser 1387 was dependent on ataxia-telangiectasia mutated. Finally, in our assay systems, H2O2-induced cell death was not due to apoptosis. However, a significant component of cell death was attributable to autophagy, and both DIM and BRCA1 inhibited H2O2-induced autophagy. Our findings suggest that low concentrations of DIM protect cells against oxidative stress via the tumor suppressor protein BRCA1 by several distinct mechanisms. [Cancer Res 2009;69(15):6083–91]

Introduction

Indole-3-carbinol (I3C), a phytochemical from cruciferous vegetables, is of interest because a diet rich in cruciferous vegetables is associated with a reduced risk of several tumor types, such as breast cancer (1, 2), and because dietary supplementation with I3C can prevent estrogen-dependent cancers (breast, cervix, and endometrium) in animals (3–5). In the acid environment of the stomach, I3C undergoes hydrolysis to a number of products, including a dimeric product, 3,3′-diindolylmethane (DIM), its major active metabolite (6). DIM is acid stable and is detected in the bloodstream after oral intake of I3C or DIM (7, 8). Most studies on DIM have used supraphysiologic concentrations (15–50 μmol/L). These studies indicate that DIM can inhibit invasion, angiogenesis, and cell proliferation and induce apoptosis by modulating signaling pathways involving Akt, nuclear factor-κB, and FOXO3 (9–12). It can also modulate basal and estrogen-inducible gene expression (13, 14), and it can induce an endoplasmic reticulum stress response (15). Like I3C, DIM can regulate estrogen metabolism by shifting the metabolism from potentially carcinogenic 16α-hydroxy derivatives to inert 2-hydroxy derivatives (16). DIM is also an androgen receptor antagonist in prostate cancer cells (17). Previous studies suggest that I3C and DIM can function as ligands for the aryl hydrocarbon receptor (18, 19) and can inhibit estrogen receptor activity independently of any effect on estrogen metabolism (20).

There is limited available information on plasma levels of DIM that can be achieved through oral ingestion of I3C or DIM. In humans, after single oral doses of I3C of 400 to 1200 mg, the peak plasma concentrations (observed after 2–3 hours) ranged from 61 to 607 ng/mL of DIM (about 0.25–2.5 μmol/L; ref. 8). Oral administration of DIM might yield higher plasma DIM levels because oral I3C is converted to various acid condensation products in the stomach (21). The percent conversion of I3C to its hydrolysis products and absorption is unclear, but in the study cited, no I3C was detected in plasma.

Previously, we reported that I3C up-regulates expression of the breast cancer susceptibility genes BRCA1 and BRCA2 due, in part, to an endoplasmic reticulum stress response (22). Here, we report that DIM stimulates BRCA1 signaling and expression at low concentrations and protects cells against oxidative stress in a BRCA1-dependent manner.

Materials and Methods

Cells and Culture

All cell lines, except normal human mammary epithelial cells (HMEC) and Brca1-deficient mouse embryonic fibroblasts (MEF), were obtained from the American Type Culture Collection. MEFs homozygous for a deletion of Brca1 exon 11 and wild-type MEFs were generously provided by Dr. Chuxia Deng (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD; ref. 23). All cell types, except HMECs, were cultured using standard techniques (22, 24). HMECs were obtained from Clonetics and grown in a defined medium containing mammary epithelial cell basal medium (ScienCell Research Laboratories) supplemented with 4 μg/mL bovine pituitary extract (BD Biosciences), 5 μg/mL insulin, 10 ng/mL epidermal growth factor (Sigma Chemical Co.), 0.5 μg/mL hydrocortisone (Sigma), and 10−5 mol/L isoproterenol (Sigma).

Reagents

I3C was obtained from Sigma and dissolved in DMSO before dilution in cell culture medium. A bioavailable formulation of DIM (BR-DIM) was generously provided by Dr. Michael Zeligs (Bioresponse, Boulder, CO). It is herein referred to as "DIM." DIM was also dissolved in DMSO. H2O2,
Expression Vectors
The wild-type BRCA1 vector (wtBRCA1) was described earlier (24). Expression vectors encoding full-length BRCA1 with point mutations at different serine residues (S1387A, S1423A, S1457A, and S1524A) were created by site-directed mutagenesis of wtBRCA1 within the pcDNA3 vector (Invitrogen) using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The mutations were confirmed by sequencing. A human beclin 1 cDNA (provided by Dr. G. Kroemer, Institut Gustave Roussy, Villejuif, France) and a human NFE2L2/NRF2 cDNA (Invitrogen) were inserted into the pcDNA3 vector. The green fluorescent protein-light chain 3 (GFP-LC3) vector was provided by Dr. Gabriel Lopez-Berestein (Mount Sinai School of Medicine, New York, NY; ref. 25).

Transient Transfections
Subconfluent proliferating cells were transfected overnight with the indicated vector (15 μg DNA/100-mm dish or 5 μg/well in six-well dishes) using Lipofectamine 2000 (Invitrogen), washed, and allowed to recover for several hours. The cells were then harvested using trypsin, seeded into 48- or 96-well dishes, allowed to attach overnight, and subjected to the indicated treatments before MTT assays.

Small Interfering RNA Treatments
Proliferating cells in 12-well dishes were treated with gene-specific small interfering RNA (siRNA) or control-siRNA using siRNA Transfection Reagent (Santa Cruz Biotechnology). BRCA1-siRNA, ataxia-telangiectasia mutated (ATM)-siRNA, ATM and Rad3-related (ATR)-siRNA, beclin 1-siRNA, and control-siRNA were purchased from Santa Cruz Biotechnology. The cells were exposed to the indicated siRNA (50 nmol/L) for at least 48 h. For experiments lasting >72 h, the cells were refed with fresh siRNA of the same type (50 nmol/L) on the 3rd day. The efficacy of the knockdown was confirmed by Western blotting.

Reporter Assays
The NOQ1-ARE-Luc reporter contains the antioxidant response element (ARE) of NAD(P)H dehydrogenase quinone 1 (NOQ1) driving a minimal promoter upstream of luciferase (26). GST-α1-Luc contains the luciferase gene under the control of a promoter segment containing 940 bp upstream of the transcription start site of mouse (g protein) were separated using a 12% gel DNA/100-mm dish or 5 μg/well in six-well dishes) using Lipofectamine 2000. They were then washed, allowed to recover for several hours, treated as indicated, and harvested for luciferase measurements. Luciferase values were normalized to the control conditions and expressed as mean ± SE of quadruplicate wells. Transfection efficiency was monitored using the Galacto-Star Mammalian Reporter Gene Assay System (Applied Biosystems). Each experiment was performed at least twice to assure that the findings were reproducible.

Treatment with Oxidants
Subconfluent proliferating cells in 48- or 96-well dishes were treated with different doses of H2O2, paraquat, or nickel acetate for 24 h (unless otherwise stated) in the presence of DIM or vehicle (DMSO) and assayed for MTT dye reduction, a measure of mitochondrial viability (28).

MTT Assays
After the indicated treatment, cells in 48- or 96-well dishes were tested for MTT dye conversion. Cell viability was calculated as the amount of dye conversion relative to sham-treated control cells and expressed as mean ± SE of 10 replicate wells. At least two independent experiments were performed to assure reproducibility of the findings.

Autophagy Assays
Monodansylcadaverine staining. Autophagic vacuoles were detected based on monodansylcadaverine (MDC) staining (29). After the indicated treatments, cells cultured on glass coverslips were incubated with 0.05 mmol/L MDC for 60 min at 37°C, fixed in 4% paraformaldehyde (15 min), and washed twice with PBS. The glass coverslips were mounted onto slides using Geltof as mounting medium. Quantitative determination of MDC-positive cells was performed immediately after preparation using a Nikon Mikrophot-FXA with a 356-nm excitation filter and a 545-nm barrier filter. Cells were manually scored as positive if they contained significant staining for autophagic vacuoles compared with the large majority of untreated control cells. The percentage of MDC-positive cells was determined by counting of 1,000 cells per culture and plotted as mean ± SE based on three experiments.

Western Blotting
Western blotting was performed as described earlier (22, 24). Briefly, aliquots of whole-cell lysate (100 μg protein) were separated using a 12% paraquat, nickel acetate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma.

Figure 1. DIM up-regulates BRCA1 expression. A, subconfluent proliferating cells were incubated with 10 μmol/L DIM (or DMSO) for 24 h and Western blotted for BRCA1 and α-actin (loading control). B to D, cells were treated with DIM for the indicated times using the indicated concentrations and harvested for Western blotting for BRCA1, BRCA2, and α-actin.
SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane, which was incubated with primary antibody overnight at 4°C followed by the addition of horseradish peroxidase–linked secondary antibody (Santa Cruz Biotechnology) at 1:2,000. Protein bands were visualized using the enhanced chemiluminescence detection system (Amersham). The primary antibodies were rabbit polyclonal anti-BRCA1 (1:500; Santa Cruz Biotechnology), mouse monoclonal anti-BRCA2 (1:500; Santa Cruz Biotechnology), mouse monoclonal α-actin (1:500; Santa Cruz Biotechnology), anti–phospho-BRCA1 (S1387; 1:1,000; Millipore), anti–phospho-BRCA1 (S1423; 1:500; Millipore), anti–phospho-BRCA1 (S1457; 1:500; Millipore), anti–phospho-BRCA1 (S1524; 1:500; Cell Signaling Technology), mouse monoclonal anti-ATM (Santa Cruz Biotechnology), rabbit polyclonal anti-ATR (Santa Cruz Biotechnology), rabbit polyclonal anti–beclin 1 (Sigma), and rabbit polyclonal anti-LC3 (1:200; Novus Biologicals). Equal protein loading was confirmed by immunoblotting for α-actin. Colored markers (Bio-Rad) were used as molecular size standards.

**Semiquantitative RT-PCR**

Semiquantitative RT-PCR was performed as described before (22, 24). The cycle number was adjusted so that all reactions fell into the linear range of amplification. The forward and reverse primers (product sizes) were as follows: BRCA1, 5′-TTGCGGGAGGAAAATGGGTAGTTA-3′ and 3′-TGTGCCAAGGGTGTAATGGAAG-5′ (285 bp); MGST1, 5′-ACTGCCGTGGCTTGGGAAAG-3′ and 3′-AGATCCGAGCACCTACAAAG-5′ (200 bp); h-actin, 5′-TGTGTTACAATGGGACGATA-3′ and 3′-TGTGTTACAATGGGACGATA-5′ (764 bp); and h2-macroglobulin, 5′-CTCGCCTACTCTCTCTTTCT-3′ and 5′-TGTCGGATGGATGAAACCCAG-3′ (136 bp).

**Cell Cycle Analysis**

Cells were harvested using trypsin, washed with PBS, and fixed in cold 70% ethanol. The samples were then treated with RNase A, stained with propidium iodide (100 mg/mL), and analyzed by FACSort (Becton Dickinson) using the ModFit software (Verity Software House). At least 20,000 events were collected and analyzed.

**Statistical Methods**

Where appropriate, comparisons were made using two-tailed Student’s t tests.

**Results**

**DIM stimulates BRCA1 expression.** We found that DIM (10 μmol/L × 24 hours) stimulated BRCA1 protein expression in various carcinoma cell types, including prostate (DU-145) and breast (BT549; MCF-7, and MDA-MB-231; Fig. 1A). Similar results were observed in two additional breast cancer cell lines (BT474 and MDA-MB-453), a lung cancer cell line (A549), and a cervical cancer cell line (HeLa; Supplementary Fig. S1A). It seemed that cell lines with low BRCA1 expression exhibited more stimulation than those with high basal expression. In DU-145 and MDA-MB-231 cells, stimulation of BRCA1 protein expression was dose dependent (24-hour exposure) and time dependent (10 μmol/L DIM; Supplementary Fig. S1B–E). At 10 μmol/L DIM, increased BRCA1 protein levels were first observed after 8 hours. Densitometry corresponding to these Western blots is provided in Supplementary Fig. S2.

In MDA-MB-231 cells exposed to a low dose of DIM (1 μmol/L), BRCA1 (and BRCA2) protein levels continued to increase for 4 days (Fig. 1B), and in cells exposed to DIM for 72 hours, increased BRCA1/2 levels were detected at ≤0.5 μmol/L DIM (Fig. 1C). Other breast cancer cell lines (T47D, MCF-7, and MDA-MB-468) also showed increases in BRCA1 protein after a 72-hour exposure to 1 μmol/L DIM (Fig. 1D). MDA-MB-231 cells treated with DIM for 72 hours showed dose-dependent increases in BRCA1 mRNA at ≥0.5 μmol/L DIM (Supplementary Fig. S1F). These findings suggest that low, physiologically relevant concentrations of DIM stimulate BRCA1 expression.

**Figure 2.** I3C and DIM protect breast carcinoma cells against oxidative stress. A and B, MCF-7 or T47D cells were pretreated with BRCA1-siRNA, control-siRNA (50 nmol/L) or vehicle (DMSO) plus H2O2 for 24 h; and harvested for MTT assays. Points, mean cell viability values of 10 wells; bars, SE. C and D, MCF-7 or T47D cells were pretreated with siRNAs, incubated with DIM (25 μmol/L) plus the indicated dose of H2O2 for 24 h, and assayed for cell viability.

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**Figure 2.** I3C and DIM protect breast carcinoma cells against oxidative stress. A and B, MCF-7 or T47D cells were pretreated with BRCA1-siRNA, control-siRNA (50 nmol/L × 48 h), or no siRNA; incubated with I3C (25 μmol/L) or vehicle (DMSO) plus H2O2 for 24 h; and harvested for MTT assays. Points, mean cell viability values of 10 wells; bars, SE. C and D, MCF-7 or T47D cells were pretreated with siRNAs, incubated with DIM (25 μmol/L) plus the indicated dose of H2O2 for 24 h, and assayed for cell viability.
I3C and DIM protect breast cancer cells against oxidative stress. MCF-7 and T47D cells were incubated with I3C (25 μmol/L) or vehicle (DMSO) plus H2O2 for 24 hours and tested for cell viability using MTT assays. Without H2O2, 25 μmol/L I3C has no effect on cell viability using MTT assays (22). I3C enhanced the survival of MCF-7 and T47D cells at all but the lowest dose of H2O2 (P < 0.001–0.01; Fig. 2A and B). To test the role of BRCA1 in I3C cell protection, cells were pretreated with BRCA1-siRNA or control-siRNA for 48 hours before incubation with I3C/H2O2, BRCA1-siRNA (but not control-siRNA) abrogated I3C-mediated cell protection (P < 0.01). We next tested the effect of DIM (1 μmol/L) on sensitivity to H2O2. Like I3C, DIM protected both cell lines against H2O2 (P < 0.001–0.01), and BRCA1-siRNA (but not control-siRNA) abrogated DIM-mediated cell protection (P < 0.01; Fig. 2C and D). The efficacy of BRCA1 knockdowns is illustrated in Supplementary Fig. S3.

DIM also protected DU-145 cells against H2O2, and it protected MDA-MB-231 cells against nickel acetate, an agent whose toxicity is mostly due to oxidative stress (Supplementary Fig. S4A and B; ref. 30). DIM protected MDA-MB-231 cells against paraquat, a herbicide that causes toxicity via generation of superoxide ions (Supplementary Fig. S4C and D; ref. 31), and it protected MDA-MB-231 cells against H2O2 in a BRCA1-dependent manner (P < 0.001; Supplementary Fig. S4E).

DIM stimulates ARE-dependent signaling. In response to oxidative stress, the transcription factor NFE2L2 (NRF2) dissociates from its cytoplasmic inhibitor, translocates to the nucleus, and activates transcription of genes containing an ARE in their promoters (32). We used a luciferase reporter driven by the NQO1-ARE (26) to determine if DIM regulates antioxidant signaling. Because DIM stimulates BRCA1 expression in a time-dependent manner, we tested exposure times from 24 to 96 hours. DIM (1 μmol/L) stimulated NQO1-ARE-Luc activity in MDA-MB-231 at all time points (P < 0.01; Fig. 3A). The fold stimulation relative to cells incubated without DIM was ~3-fold at 72 to 96 hours in this experiment, although it was up to 7-fold in other experiments. In cells cotransfected with an NRF2 expression vector, NRF2 stimulated NQO1-ARE-Luc by 10-fold, and DIM enhanced NRF2-stimulated reporter activity by another 4-fold (P < 0.001; Fig. 3B).

We tested the role of BRCA1 in DIM-stimulated NRF2 activity by pretreating MDA-MB-231 cells with BRCA1-siRNA and determining the effect of DIM (1 μmol/L × 72 hours) on NRF2-stimulated NQO1-ARE-Luc activity. BRCA1-siRNA (but not control-siRNA) inhibited NQO1-ARE-Luc activity due to NRF2 alone (P < 0.001) and reduced the DIM + NRF2-stimulated reporter to less than that of NRF2 alone (P < 0.001; Fig. 3C). BRCA1-siRNA also reduced the basal reporter activity and blocked the DIM-stimulated activity (P < 0.001; Fig. 3D).

We tested the ability of DIM to stimulate two additional ARE-containing reporters: GST-α-1-Luc and c-CT-Luc (27). NRF2 stimulated GST-α-1-Luc activity by about 6- to 7.5-fold in MDA-MB-231 and T47D cells, and DIM (1 μmol/L) gave an additional 5-fold stimulation (P < 0.001; Supplementary Fig. S5A). The DIM stimulation of NRF2 activity was abrogated by BRCA1-siRNA (P < 0.001). The ability of NRF2 to stimulate GST-α-1-Luc activity without DIM was also attenuated by BRCA1-siRNA (P < 0.001). Similar results were obtained using the c-CT-Luc reporter, and DIM caused dose-dependent increases in the mRNA for MGST1, another ARE-regulated gene (Supplementary Fig. S5B and C; ref. 33), suggesting that DIM stimulates ARE signaling in a BRCA1-dependent manner.

To further examine the role of endogenous BRCA1 in NRF2 signaling, we tested wild-type (Brca1+/−) versus Brca1-deficient (Brca1−/−) MEFs (23). In wild-type cells, NRF2 and DIM (1 μmol/L × 72 hours) robustly stimulated NQO1-ARE-Luc activity, and the combination of DIM + NRF2 gave much greater stimulation than...
either agent alone ($P < 0.001$; Supplementary Fig. S5D). In contrast, Brca1$^{-/-}$ cells showed no DIM-induced stimulation of reporter activity, and the stimulation by NRF2 was less than that observed in Brca1$^{+/+}$ cells.

**DIM stimulates BRCA1 signaling.** Although DIM (1 $\mu$mol/L) may take $>24$ hours to increase BRCA1 protein levels, a 24-hour exposure conferred BRCA1-dependent protection against H$_2$O$_2$, suggesting that some DIM effects occur before BRCA1 levels increase. We tested the effect of DIM on BRCA1 phosphorylation by Western blotting using phospho-specific antibodies in MDA-MB-231 and T47D cells. A 30-minute exposure to DIM caused increased phosphorylation of BRCA1 on S1387 and S1524 but not on S1423 or S1457 (Fig. 4A and B). The DIM-induced phosphorylation of BRCA1 on S1387 was time dependent (Fig. 4C and D), with high levels of phosphorylation observed at short times (10–20 minutes) and reduction in phosphorylation from 2 to 24 hours. ATM and ATR are phosphatidylinositol 3-kinase family proteins that phosphorylate BRCA1 and other substrates in response to DNA damage (see Discussion). We tested the effect of knocking down these proteins on phospho-BRCA1 (S1387) levels. ATM-siRNA had little effect on basal phosphorylation but blocked DIM-induced phosphorylation in MDA-MB-231 and T47D cells (Supplementary Fig. S6), whereas ATR-siRNA had no effect on DIM-induced phosphorylation (data not shown).

We tested the significance of these findings using a cell line with no detectable endogenous BRCA1 (U87MG glioma cells). The cells were transfected with wtBRCA1 or expression vectors for BRCA1 proteins with alanine mutations of different serine residues and tested for sensitivity to H$_2$O$_2$ (50 nmol/L for 24 hours) ± DIM. Expression of the mutant BRCA1 proteins was confirmed by Western blotting. Consistent with the absence of endogenous BRCA1, DIM did not significantly protect untransfected cells or cells transfected with empty pcDNA3 vector. wtBRCA1 alone conferred significant protection, as did the BRCA1 mutants S1423A and S1457A ($P < 0.001$; Fig. 5A). However, S1387A and S1524A conferred little or no protection. DIM conferred additional protection of cells transfected with wtBRCA1, S1423A, S1457A, and S1524A but little protection of cells transfected with S1387A. These findings suggest that DIM-induced phosphorylation of S1387 and, to some extent, S1524A contributes to cell protection.

**DIM does not protect BRCA1 mutant breast cancer cells against H$_2$O$_2$.** We similarly studied HCC1937, a breast cancer cell line homozygous for mutant BRCA1 (5382insC; ref. 34). DIM failed to protect HCC1937 cells against H$_2$O$_2$, but wtBRCA1 conferred significant protection ($P < 0.001$; Fig. 5B). Here, DIM + wtBRCA1 did not confer additional protection beyond that of wtBRCA1 alone. The S1387A and S1524A mutations abrogated BRCA1 protection against H$_2$O$_2$, whereas the S1423A and S1457A mutants retained full protection activity ($P < 0.001$). These results are similar to those obtained in U87MG, except that none of the BRCA1 proteins allowed additional protection by DIM. Explanations for this difference between U87MG and HCC1937 are considered in Discussion.

**DIM stimulates BRCA1 signaling and expression in normal mammary epithelial cells.** We tested the effects of DIM on normal HMECs and MCF-10A, an immortal line of nontumor HMECs (35). DIM caused phosphorylation of BRCA1 on S1387, with a peak effect by 20 minutes (Supplementary Fig. S7A and B). Over the time period studied (0–4 hours), total BRCA1 protein levels were unchanged. At longer times, DIM caused increases in total BRCA1 protein levels that first occurred at 8 to 16 hours and continued over 24 to 48 hours (Supplementary Fig. S7C and D). Finally, DIM protected both cell types against H$_2$O$_2$ ($P < 0.01$; Supplementary Fig. S7E and F).

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**Figure 4.** DIM stimulates BRCA1 signaling. A to D, cells were treated with DIM (1 $\mu$mol/L) for the indicated time and harvested to detect total or phosphorylated BRCA1 by Western blotting.
DIM blocks autophagy caused by H$_2$O$_2$. Based on flow cytometric detection of cells with sub-G$_1$ DNA content, H$_2$O$_2$ caused little or no apoptosis in MDA-MB-231 or T47D cells at a dose that should kill a large majority of cells (200 nmol/L; Supplementary Fig. S8). As a positive control, the DNA-damaging agent Adriamycin yielded a large peak of cells with apoptotic sub-G$_1$ DNA content. We quantified the autophagy rate by staining the cells with MDC, a dye that stains autophagic vacuoles. MDA-MB-231 cells were treated with H$_2$O$_2$ (100 nmol/L) for 24 hours and assayed for the presence of MDC-positive autophagic vacuoles. H$_2$O$_2$ caused an increase in the percentage of cells exhibiting autophagy, from <2% to ~50%, and most of this increase was blocked by DIM ($P < 0.001$; Fig. 6A). H$_2$O$_2$ also caused conversion of a portion of the cytoplasmic LC3 (LC3-I) to membrane-associated LC3 (LC3-II; Fig. 6B), a marker of autophagy (36). The H$_2$O$_2$-induced conversion of LC3-I into LC3-II was reduced by DIM treatment. wtBRCA1 also inhibited H$_2$O$_2$-induced autophagy ($P < 0.001$; Fig. 6C) and attenuated the conversion of LC3-I to LC3-II (Fig. 6D). Photomicrographs of MDC-stained cells corresponding to Fig. 6A and C are provided in Supplementary Figs. S9 and S10. We measured autophagy by a second method in which MDA-MB-231 cells were transfected with a GFP-LC3 expression vector and examined for formation of puncta (autophagosomes; ref. 37). These assays gave results similar to those in Fig. 6A and C (data not shown).

To further test the contribution of autophagy to H$_2$O$_2$-mediated cell killing, we knocked down or overexpressed the essential autophagy gene beclin 1. In MDA-MB-231 cells, beclin 1-siRNA increased cell survival by ~2-fold at each dose of H$_2$O$_2$ ($P < 0.001$), whereas a wt-beclin 1 expression vector reduced cell survival (Supplementary Fig. S11A), suggesting that autophagy contributed to H$_2$O$_2$-induced cell death in our assays. Finally, we showed that
DIM causes dose-dependent inhibition of beclin 1 expression in MDA-MB-231 cells (Supplementary Fig. S11B). wtBRCA1 similarly inhibited beclin 1 expression and blocked the H$_2$O$_2$-induced stimulation of beclin 1 expression (Supplementary Fig. S11C). DIM also inhibited beclin 1 expression in T47D cells (Supplementary Fig. S11D), and wtBRCA1 blocked the H$_2$O$_2$-induced increase in beclin 1 protein (Supplementary Fig. S11E). Densitometry showing the effect of DIM and wtBRCA1 on beclin 1 levels is provided in Supplementary Fig. S12. Thus, DIM and BRCA1 may render cells resistant to autophagy, in part, through beclin 1.

**Discussion**

We showed that low doses of DIM (1 μmol/L) stimulate BRCA1 expression and protect cells against oxidative stress, in part, through BRCA1. Protection against oxidative stress by DIM (and I3C) is consistent with the previous finding that BRCA1 protects against oxidative stress (24). The BRCA1 protection was attributed, in part, to stimulation of an antioxidant response via NRF2, a redox-sensitive transcription factor that stimulates expression of phase II detoxifying enzymes and antioxidant genes through the ARE (32, 38). We identified several mechanisms by which DIM protects against oxidative stress, including the BRCA1-dependent stimulation of NRF2 activity. DIM stimulated several NRF2-regulated promoters, including NQO1 (an oxidoreductase), GST-α1 (a glutathione S-transferase), and x-CT (cystine/glutamate transporter). NRF2 seems to be a target for some dietary agents proposed for cancer prevention (38), although this was not shown previously for DIM or I3C.

Interestingly, DIM stimulated BRCA1 phosphorylation on S1387 and S1524 before any increase in BRCA1 protein levels. DIM-induced phosphorylation of S1387 was observed after 10 minutes and showed a time course suggestive of a signaling event. In cell lines lacking functional BRCA1, S1387 and S1524 were required for protection against oxidative stress by exogenous BRCA1. Although the significance of these phosphorylations is not clear, prior studies suggest that these sites are phosphorylated by ATM and/or ATR in response to ionizing or UV radiation (39, 40). ATM was required for DIM-induced phosphorylation of BRCA1 on S1387, suggesting that DIM signals through ATM.

In U87MG cells (which do not express BRCA1), DIM conferred additional protection against H$_2$O$_2$ beyond that afforded by exogenous BRCA1, but BRCA1 with an S1387A mutation failed to support DIM-mediated protection. In contrast, HCC1937 cells, which express mutant BRCA1, showed no additional protection from DIM beyond that due to exogenous BRCA1. It is possible that the endogenous mutant BRCA1 interferes with the transfected BRCA1 proteins, that the relevant phosphorylations cannot be induced or enhanced by DIM in this cell type, or that the protection by exogenous BRCA1 is already maximal and cannot increase further.

Although the mechanism by which DIM induces BRCA1 phosphorylation is unclear, DIM and I3C can each activate an endoplasmic reticulum stress response (unfolded protein response) that is BRCA1 dependent, and this response is required for I3C stimulation of BRCA1 expression (15, 22). We speculate that DIM induces low-grade stress signaling that directly or indirectly leads to rapid phosphorylation of BRCA1. One possibility is that DIM interacts with one or more stress-responsive proteins and the signal is transmitted to BRCA1. Although DIM and/or I3C can bind to several proteins (e.g., aryl hydrocarbon receptor, estrogen receptor, and androgen receptor; refs. 17–20), relatively little is known about how DIM interacts with cell proteins or other macromolecules.

Another interesting finding was that H$_2$O$_2$ caused little or no apoptosis. Reactive oxygen species, including H$_2$O$_2$, can cause different combinations of autophagy, apoptosis, and/or necrosis in...
a cell line–dependent and stimulus-dependent manner (41–43). Autophagy is a process by which cells degrade macromolecules and organelles that can lead to cell survival (e.g., under austere conditions in tumors) or cell death in different contexts. In our assays, H2O2-induced autophagy was cytotoxic and DIM/BRCA1 inhibition of autophagy was cytoprotective, as evidenced by the following: (a) inhibition of autophagy by knockdown of beclin 1 (44) increased cell survival, (b) DIM and wtBRCA1 inhibited H2O2-induced autophagy and at the same time protected cells against cytotoxicity, and (c) we detected little or no H2O2-induced apoptosis. Because the effects of oxidative stress are variable in induced autophagy and at the same time protected cells against starvation, we cannot say whether DIM/BRCA1 inhibition of autophagy would be cytoprotective in a different context (e.g., starvation rather than oxidative stress).

H2O2 caused a large increase in the percentage of cells undergoing autophagy that was inhibited by DIM or wtBRCA1. Consistent with this finding, H2O2 caused conversion of LC3-I to LC3-II, a marker of autophagy (36), and the conversion of LC3-I to LC3-II was attenuated by DIM or wtBRCA1. Consistent with the idea that the protective effect of DIM is mediated by BRCA1, wtBRCA1 blocked the H2O2-induced up-regulation of beclin 1. These findings link DIM and BRCA1 to regulation of autophagy.

Low doses of DIM also stimulated BRCA1 signaling and expression and protected against oxidative stress in normal or nontumor-derived HMECs. These findings are important because normal cell types are presumably the main target for cancer prevention. Because we did not test whether H2O2 causes and DIM blocks autophagy in these cell types, we cannot say whether the observed protection reflects autophagy inhibition.

Our findings have implications for understanding chemoprevention and its limitations. Because the protective effects of DIM occurred at physiologic concentrations, it is reasonable to speculate that DIM blocks carcinogenesis, in part, by enabling normal cells to mount a more effective antioxidant response. This antioxidant response may include an increased ability of cells to repair oxidative DNA damage because BRCA1 contributes to various DNA repair processes (45). Tumor cells often exhibit oxidative stress due to impaired antioxidant defenses. Thus, the presence of preexisting cancer cells that are protected by antioxidants is a possible explanation for the mixed results obtained in clinical studies using antioxidants to prevent cancer (46–48).

Here, the ability of DIM to promote survival of oxidatively stressed tumor cells could limit its activity as a chemoprevention agent.

Autophagy is regarded as a double-edged sword. On the one hand, autophagy is a tumor suppressor mechanism that can serve as a second type of programmed cell death replacing apoptosis. On the other hand, it can be used as a survival mechanism by tumor cells in nutrient limiting conditions (49). As noted above, we do not know whether DIM inhibition of autophagy will extend to other cell types and other causes of autophagy (e.g., starvation). Nor do we know the extent to which autophagy inhibition by DIM is the consequence of a reduced stimulus for autophagy due to an increased cellular antioxidant response. Thus, at present, it is difficult to assess the contribution of autophagy as a target for DIM-mediated chemoprevention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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