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# Hypoxia Suppresses Cysteine Deprivation-induced Cell Death *Via* ATF4 Regulation in MDA-MB-231 Breast Cancer Cells

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**Abstract.** *Background/Aim: Cancer cells are frequently exposed to microenvironmental stresses, including amino acid deprivation and hypoxia, which are often targeted for cancer therapy. Here, we examined the effect of hypoxia in cysteine-deprived breast cancer cells and the mechanism to counteract the hypoxia effect. Materials and Methods: Cell death was determined by annexin V-FITC and propidium iodide staining. Expression of mRNAs and proteins was determined by reverse transcription polymerase chain reaction and western blot analysis, respectively. Results: Cysteine deprivation or sulfasalazine, a potent inhibitor of cysteine/glutamate transporter, induced cell death by activating transcription factor 4 (ATF4) up-regulation. Hypoxia significantly suppressed cell death and ATF4 up-regulation induced by cysteine deprived conditions. In addition, tumor necrosis factor-related apoptosis-inducing ligand reversed the effect of hypoxia on cysteine deprived conditions. Conclusion: Prevention of hypoxia may be a means for augmenting the effect of amino acid deprivation as a strategy for cancer therapy.*

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*Key Words:* Activating transcription factor 4, breast cancer, cysteine, hypoxia, sulfasalazine, tumor necrosis factor-related apoptosis-inducing ligand.

Cancer cells have a high nutrient demand to sustain rapid growth and proliferation (1-3). Therefore, cancer cells overexpress various transporters to increase the uptake of nutrients, including amino acids (1-3). Recently, it has been reported that drugs that can cause glutamine, asparagine, and arginine deprivation are strategies for cancer therapy (4). Cysteine is critical for cellular homeostasis and growth in cancer cells (5). Cystine/glutamate transporter (xCT) plays an important role in the uptake of cysteine (6). Furthermore, xCT is overexpressed in various types of cancers, such as breast cancer, lung cancer and liver cancer and elevated xCT expression in cancer is associated with poor prognosis (7-10). Sulfasalazine, an inhibitor of xCT, has been shown to induce cancer cell death (11, 12) and is being tested in clinical trials for breast cancer. Therefore, cysteine deprivation and targeting cysteine transporters in cancer cells are emerging as anticancer therapeutic strategies.

Hypoxia is a common phenomenon in various solid malignant tumors and leads to a poor prognosis in cancer patients (13-15). The transcription factor hypoxia-inducible factor (HIF) responds to oxygen levels. HIF1 is a heterodimer of α- and β-subunits. The α-subunit (HIF1α) is sensitive to oxygen, while β-subunit (HIF1β) is ubiquitously expressed (16). Hypoxia causes uncontrollable cell proliferation and dysfunctional vascularization, resulting in cancer cell invasion and metastasis (17). Furthermore, tumor hypoxia leads to altered cancer cell metabolism and contributes to resistance to chemotherapy or radiotherapy (17). In our previous study, hypoxia rendered resistance to cell death induced by metabolism targeting drugs, metformin and dichloroacetic acid (18). Therefore, hypoxia may be considered when targeting amino acid metabolism as a strategy for cancer therapy. The aim of this study was to investigate the effect of hypoxia in cysteine-deprived breast cancer cells and to find the mechanism to counteract the effect of hypoxia.

## Materials and Methods

*Cell cultures and reagents.* MDA-MB-231 breast cancer cells were obtained from the ATCC (Manassas, VA, USA) and maintained in DMEM medium (#LM001-05; Welgene, Gyeongsangbuk-do, Republic of Korea) supplemented with 10% fetal bovine serum (FBS) (Corning, Corning, NY, USA). For culture under hypoxic conditions, cells were incubated in a hypoxic chamber (Forma Anaerobic System; Thermo Fisher Scientific, Waltham, MA, USA) with 5% $CO_2$/1.0% $O_2$ and 94.0% $N_2$ (all v/v). Sulfasalazine, cobalt chloride ($CoCl_2$) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), and the recombinant human TRAIL/Apo2 ligand was obtained from Alexis Biochemicals (Enzo Life Science, Farmingdale, NY, USA). Cysteine deprivation was accomplished by washing the cells with DPBS and then incubating them in cysteine-free DMEM or complete DMEM containing 10% dialyzed fetal bovine serum (dFBS) (Gibco, Thermo Fisher Scientific).

*Measurement of cell viability.* MDA-MB-231 cells were seeded in a 24-well plate and grown overnight until they reached about 50% confluence. The cells were cultured in media deprived of various amino acids for 24 h. The medium was removed, and serum-free medium containing the MTT reagent (0.5 mg/ml) was added. After 1 h at 37˚C, the medium was removed and the formazan crystals in the cells were dissolved in dimethyl sulfoxide. The absorbance was determined by a microplate photometer (Molecular Devices, San Jose, CA, USA) at 595 nm.

*Evaluation of cell death.* MDA-MB-231 cells were seeded in a 6-well plate. After an overnight incubation, the cells were treated with cysteine deprivation medium, siRNAs, or 500 μM sulfasalazine or 200 μM $CoCl_2$ under normoxia or hypoxia conditions (1% $O_2$) for 24 h. The cells were stained with annexin V-FITC and propidium iodide (Biovision, Milpitas, CA, USA) and analyzed by a FACScan flow cytometer (BD Biosciences, Bergen, NJ, USA).

*Transient transfection and small interfering RNA (siRNA).* DR4 (sequence: CAGGAACUUUCCGGAAUGACAdTdT), *DR5* (sequence: AAGACCCUUGUGCUCGUUGUCdTdT) (19) *HIF-1α* (sequence: GGGAUUAACUCAGUUUGAACUAACUdTdT) (20) and control (sequence: CCUACGCCACCAAUUUCGUdTdT) small interfering RNA (siRNA)s were synthesized by Bioneer (Daejeon, Republic of Korea). Negative control (sc-37007) and *ATF4* (sc-35112) siRNAs were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Transfections with siRNAs in MDA-MB-231 cells were performed using Lipofectamine RNAiMAX, according to the manufacturer's instructions (Invitrogen; Thermo Scientific). Eight h after transfection, the cells were exposed to cysteine-free medium, cultured in hypoxic conditions (1% $O_2$), or treated with 500 μM sulfasalazine or 200 μM $CoCl_2$. After 24 h of incubation, the cells were harvested for cell death analysis, reverse transcription-polymerase chain reaction (RT-PCR) and western blot.

*RNA isolation and RT-PCR analysis.* Total RNA was isolated from MDA-MB-231 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific). cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (Invitrogen; Thermo Fisher Scientific). The following specific primers were used: DR4 (5'-CTGAGCAACGC AGACTCGCTGTCCAC-3' and 5'- TCCAAGGACACGGCAGA GCCTGTGCCAT-3') (21); DR5 (5'-CGCTGCACC AGGTGT GATTC-3' and 5'- GTCTCCTCCACAGCTGGGAC-3') (22); and β-actin, (5'-GGATTCCTATGTGGGCGACGA-30 and 5'-GAGTCCATC ACGATGCCAGTG-3') (23). Real-time PCR protocol involved an initial 3-min denaturing step at 95˚C, followed by 40 cycles of 95˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec. Amplification of a single product was confirmed by a melting curve (from 65 to 95˚C) analysis. Relative quantification of *DR4* and *DR5* expression levels was determined by the $2^{-\Delta\Delta Ct}$ method (24).

*Western blot analysis.* Proteins from cell lysates were separated by 8~10% sodium dodecyl sulphate-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was then immunoblotted with specific primary and horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized with SuperSignal West Pico chemiluminescent substrate (Pierce; Thermo Fisher Scientific). The following antibodies were used: anti-ATF4 (#sc-200) obtained from Santa Cruz Biotechnology, anti-HIF-1α (#610958) from BD Biosciences, and anti-β-actin (#A5361) from Sigma-Aldrich (Merck KGaA).

*Statistical analysis.* The data are presented as the means±standard deviations (SD). Statistical differences were made by Student's *t*-test of two groups or a one-way ANOVA for multiple groups, followed by Tukey's test, using GraphPad Prism software (version 5.0, San Diego, CA, USA); $p<0.05$ was considered to indicate statistically significant differences.

## Results

*Cysteine deprivation induces cell death via activating transcription factor 4 (ATF4).* To identify which amino acids are important in the survival of MDA-MB-231 human breast cancer cells, we investigated the viability of cells cultured in media deprived of various single amino acids. Cell viability was reduced the most by cysteine deprivation compared to deprivation from other amino acids (Figure 1A). The cells deprived of cysteine exhibited greater than 80% cell death (Figure 1B). ATF4 has been reported to be involved in the death of breast cancer cells by cysteine deprivation (25). We also observed that ATF4 protein expression was increased in cells that have been cysteine deprived (Figure 1C). Furthermore, knock-down of ATF4 markedly reduced cell death induced by cysteine deprivation (Figure 1D and E). These results suggest that cysteine deprivation-induced cell death is mediated by ATF4 up-regulation.

*Hypoxia inhibits cysteine deprivation-induced cell death and ATF4 expression.* Since most solid tumors are exposed to hypoxic environments, we investigated the effect of hypoxia on cysteine deprivation-induced cell death. Interestingly, hypoxia (1% $O_2$) reduced cysteine deprivation-induced cell death (Figure 2A). Cobalt chloride ($CoCl_2$), a hypoxia mimetic agent, also reduced cysteine deprivation-induced cell death (Figure 2B). As shown in Figure 2C, ATF4 protein

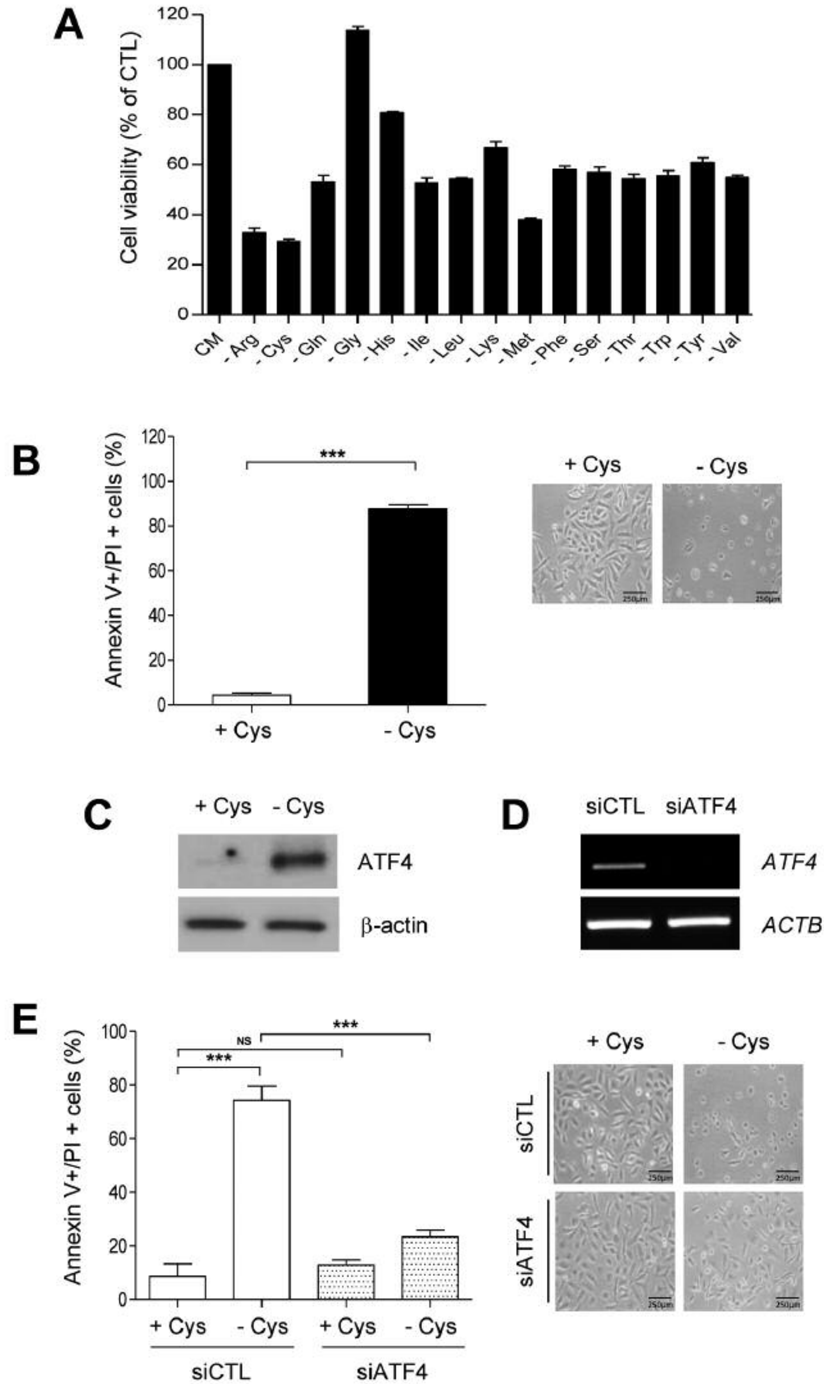


Figure 1. *Cysteine deprivation induces cell death by up-regulating ATF4 in MDA-MB-231 cells. A: MDA-MB-231 cells were washed with DPBS and treated with media deprived of the indicated amino acids for 24 h. Measurement of cell viability was performed using the MTT colorimetric assay. B: MDA-MB-231 cells were cultured in cysteine deprivation medium for 24 h. Cell death is presented as the percentage of cells stained with annexin V-FITC and/or propidium iodide. The data are presented as the mean±SD (n=3). Cellular morphology was observed using an inverted microscope (magnification=100×, scale bar=250 μm). C: MDA-MB-231 cells were cultured in cysteine deprivation medium for 24 h. Protein levels of ATF4 and β-actin were examined by western blot. The blot is representative of three independent experiments. D: MDA-MB-231 cells were transfected with negative control siRNA or ATF4 siRNA for 24 h. mRNA levels of ATF4 and ACTB were determined by reverse transcription polymerase chain reaction. E: MDA-MB-231 cells were transfected with negative control siRNA or ATF4 siRNA for 8 h and then exposed to cysteine deprivation medium for 24 h. Cell death is presented as the percentage of cells stained with annexin V-FITC and/or propidium iodide. The data are presented as the mean±SD (n=3). Cellular morphology was observed using an inverted microscope (magnification=100×, scale bar=250 μm). The blot is representative of three independent experiments. Significantly different at ***p<0.001. ns: Not significant; Cys: cysteine; CTL: control.*

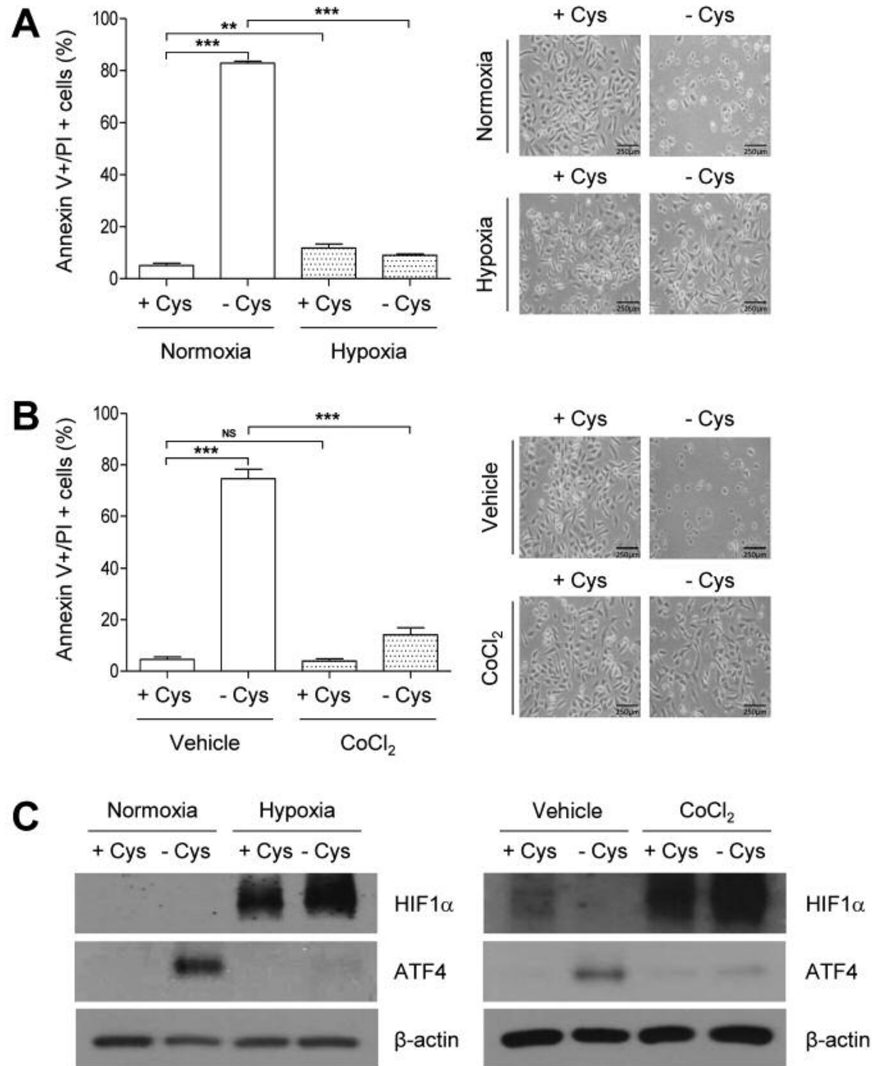


Figure 2. *Hypoxia suppresses cell death induced by cysteine deprivation. A: MDA-MB-231 cells were cultured in complete medium or cysteine deprivation medium in hypoxic (1% $O_2$) or normoxic (21% $O_2$) conditions for 24 h. Cell death is presented as the percentage of cells stained with annexin V-FITC and/or propidium iodide. The data are presented as the mean±SD (n=3). Cellular morphology was observed using an inverted microscope (magnification=100×, scale bar=250 μm). B: MDA-MB-231 cells were cultured in complete medium or cysteine deprivation medium with or without 200 μM $CoCl_2$ for 24 h. Cell death is presented as the percentage of cells stained with annexin V-FITC and/or propidium iodide. The data are presented as the mean±SD (n=3). Cellular morphology was observed using an inverted microscope (magnification=100×, scale bar=250 μm). C: MDA-MB-231 cells were cultured in complete medium or cysteine deprivation medium in hypoxic (1% $O_2$) or normoxic (21% $O_2$) conditions for 24 h. MDA-MB-231 cells were placed in complete medium or cysteine deprivation medium with or without 200 μM $CoCl_2$ for 24 h. HIF1α, ATF4 and β-actin protein expression were examined by western blot. The blot is representative of two independent experiments. Significantly different at **p<0.01, ***p<0.001. ns: Not significant; Cys: cysteine.*

expression induced by cysteine deprivation was reduced under hypoxic conditions (1% $O_2$ or $CoCl_2$). These results suggest that hypoxic conditions suppress cysteine deprivation-induced cell death and ATF4 expression.

*Hypoxia suppresses sulfasalazine-induced cell death and ATF4 expression.* It has been shown that xCT is overexpressed in breast cancer cells and that its overexpression is associated with poor outcomes (7, 9). Sulfasalazine is a potent inhibitor of xCT that inhibits the cysteine supply in cells. Similar to the results in cells deprived of cysteine, sulfasalazine induced cell death and up-regulated ATF4 protein expression (Figure 3A and B). Next, we investigated the effect of hypoxia on sulfasalazine-

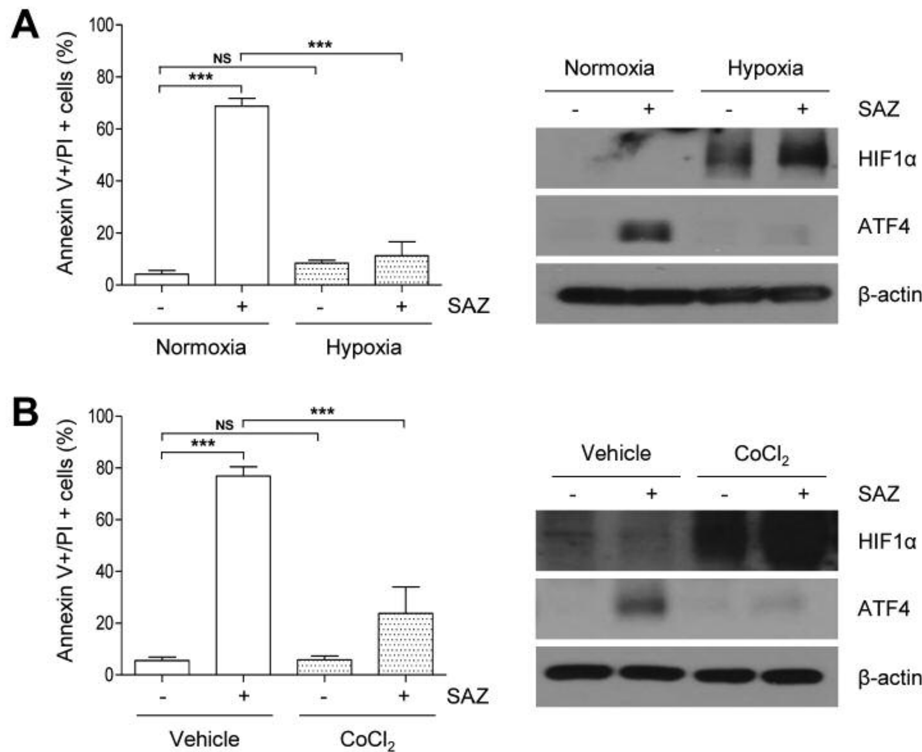


Figure 3. *Hypoxia inhibits sulfasalazine-induced cell death. A: MDA-MB-231 cells were treated with 0.5 mM sulfasalazine under hypoxic (1% $O_2$) or normoxic (21% $O_2$) conditions for 24 h. Cell death is presented as the percentage of cells stained with annexin V-FITC and/or propidium iodide. The data are presented as the mean±SD (n=3). HIF1α, ATF4 and β-actin protein expressions were examined by western blot. The blot is representative of two independent experiments. B: MDA-MB-231 cells were pretreated with or without 200 μM $CoCl_2$ for 30 min and then treated with or without 0.5 mM sulfasalazine for 24 h. Cell death is presented as the percentage of cells stained with annexin V-FITC and/or propidium iodide. The data are presented as the mean±SD (n=3). HIF1α, ATF4 and β-actin protein expressions were examined by western blot. The blot is representative of two independent experiments. Significantly different at ***$p<0.001$. ns: Not significant; SAZ: sulfasalazine.*

induced cell death. As expected, hypoxic conditions (1% $O_2$ or $CoCl_2$) resulted in reduction in cell death and ATF4 protein expression induced by sulfasalazine (Figure 3A and B). These results suggest that hypoxic conditions suppress sulfasalazine-induced cell death and ATF4 expression.

TRAIL enhances cell sensitivity to cysteine deprivation in hypoxia. In our previous reports, TRAIL potentiated cell death by metabolism targeting drugs, DCA and metformin, even under hypoxic conditions (26). Therefore, we investigated the effect of TRAIL in hypoxic cells treated with cysteine deprivation. As shown in Figure 4A, TRAIL enhanced cell death induced by cysteine deprivation, and increased susceptibility to cell death by cysteine deprivation, even in hypoxic conditions ($CoCl_2$). In addition, TRAIL also overcame the effect of hypoxia on sulfasalazine-mediated cell death (Figure 4B). We investigated whether ATF4 expression affects death receptor (DR) 4 and 5 expression in cells deprived of cysteine in normoxic or hypoxic conditions. *DR4/5* mRNA expression was up-regulated by cysteine deprivation (Figure 4C). ATF4 siRNA decreased *DR5* mRNA expression induced by cysteine deprivation, but did not affect the expression of *DR4* mRNA (Figure 4C). Exposure to hypoxia reduced expression of ATF4 protein (Figure 2C), but interestingly, it significantly enhanced *DR4/5* mRNA expression induced by cysteine deprivation (Figure 4C). These data suggest that induced *DR4/5* expression in cysteine-deprived cells under hypoxia may be independent of ATF4 expression. Next, we investigated whether DR4/5 plays a role in the TRAIL-induced cell death caused by cysteine deprivation under hypoxic conditions. *DR5* knock-down reduced cell death induced by the combined treatment of cysteine deprivation and TRAIL under hypoxic conditions, but knockdown of DR4 did not affect cell death (Figure 4D), suggesting that DR5 induction by cysteine deprivation under hypoxia enhances the cell's sensitivity to TRAIL.

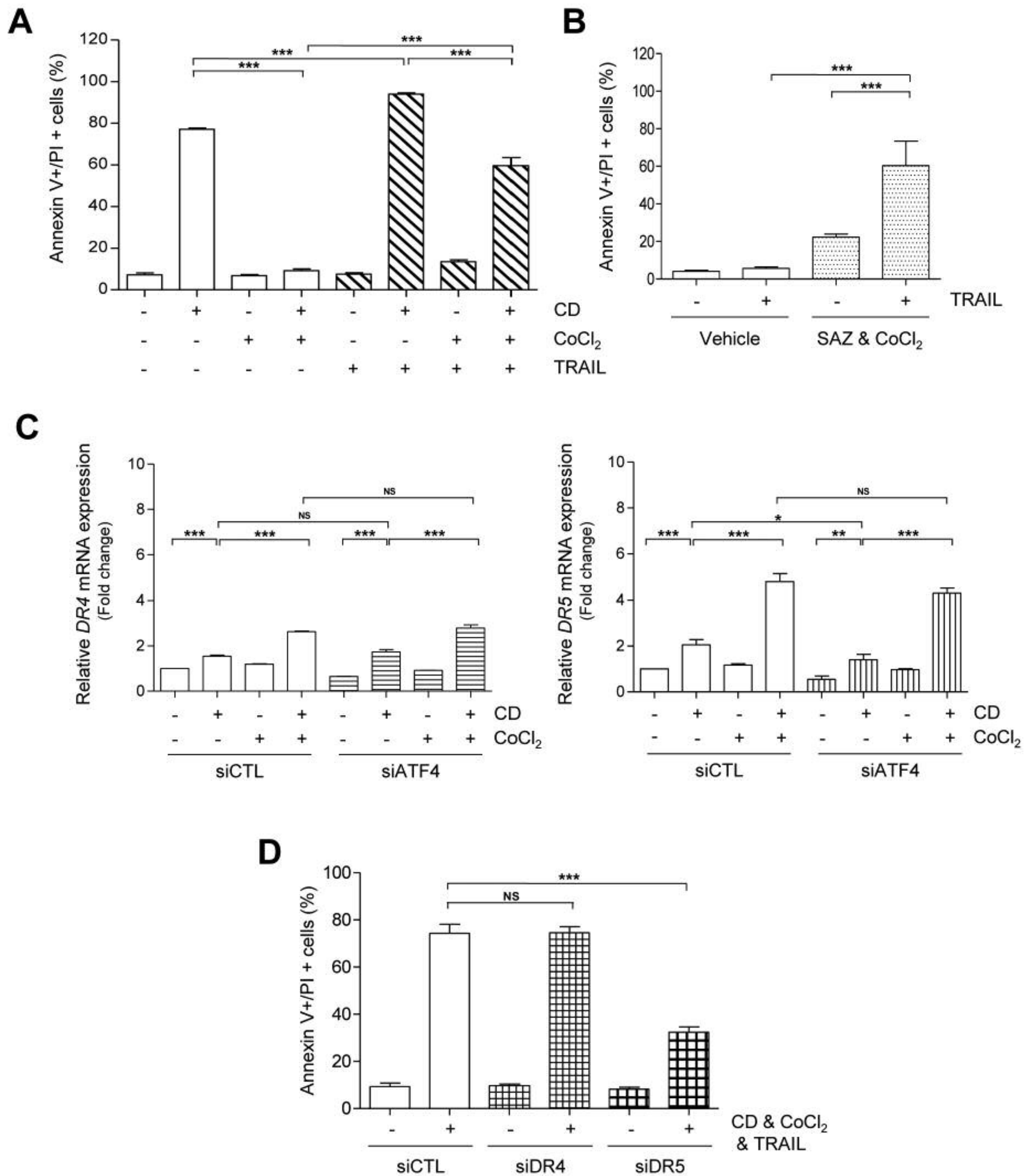


Figure 4. *TRAIL overcomes the effects of hypoxia on cell death induced by cysteine deprivation and sulfasalazine. A: MDA-MB-231 cells were pretreated with 200 μM $CoCl_2$ for 30 min and then treated with TRAIL (50 ng/ml) in complete or cysteine deprivation medium for 24 h. Cell death is presented as the percentage of cells stained with annexin V-FITC and/or propidium iodide. The data are presented as the mean±SD (n=3). B: MDA-MB-231 cells were pretreated with 200 μM $CoCl_2$ for 30 min and then treated with 0.5 mM sulfasalazine and/or TRAIL (50 ng/ml) for 24 h. Cell death is presented as the percentage of cells stained with annexin V-FITC and/or propidium iodide. The data are presented as the mean±SD (n=3). C: MDA-MB-231 cells were transfected with negative control siRNA or ATF4 siRNA and treated with or without 200 μM $CoCl_2$ under cysteine deprivation conditions for 24 h. The indicated mRNA levels were measured using real-time PCR. The data are presented as the mean±SD (n=3). D: MDA-MB-231 cells were transfected with negative control siRNA, DR4 siRNA or DR5 siRNA, and then treated with 200 μM $CoCl_2$ and TRAIL (50 ng/ml) under cysteine deprivation conditions. Cell death is presented as the percentage of cells stained with annexin V-FITC and/or propidium iodide. The data are presented as the mean±SD (n=3). Significantly different at ***p<0.001, **p<0.01, *p<0.05. ns: Not significant; CD: cysteine deprivation; SAZ: sulfasalazine.*

## Discussion

Cancer cells are frequently exposed to microenvironmental stresses, including amino acid deprivation and hypoxia, which are often targeted for cancer therapy. Recently, compounds or enzymes targeting glutamine, asparagine, and arginine metabolism either alone or in combination with antitumor drugs have been introduced in clinical trials (4, 27, 28). Drugs that inhibit cysteine transport have recently drawn considerable attention for cancer treatment (29, 30). Here, we examined the effect of hypoxia in cysteine-deprived breast cancer cells. The results are as follows: 1) cysteine deprivation leads to the induction of cell death in MDA-MB-231 cells *via* ATF4 up-regulation; 2) hypoxia inhibits cysteine deprivation-induced cell death and ATF4 expression; and 3) TRAIL enhances cell death induced by cysteine deprivation and overcomes the effect of hypoxia on cell death induced by cysteine deprivation.

ATF4, a member of the ATF/CREB family, is a transcription factor that regulates the expression of downstream genes in response to a variety of cellular stresses (31). In our study, silencing of ATF4 by siRNA markedly reduced cell death induced by cysteine deprivation (Figure 1E), suggesting that up-regulation of ATF4 is involved in cysteine deprivation-induced cell death. Interestingly, hypoxic conditions (1% $O_2$ or $CoCl_2$) suppressed cysteine deprivation-induced cell death and ATF4 up-regulation. The mechanism by which hypoxia inhibits increased ATF4 expression and cell death in cells deprived of cysteine has not been elucidated. However, a recent study has reported that HIF-1α directly binds and represses the expression of ATF4 in hypoxic fetal cardiomyocytes (32). HIF1α protein expression induced by hypoxia was further increased in cells deprived of cysteine (Figure 2C) and silencing of HIF1α resulted in activation of ATF4 when cells were deprived of cysteine under hypoxic conditions (data not shown). These data suggest the possibility that HIF1α may reduce the expression of ATF4 protein in cells deprived of cysteine under hypoxia.

TRAIL enhanced cell death induced by cysteine deprivation and overcame the effect of hypoxia on cell death induced by cysteine deprivation or treatment with sulfasalazine (Figure 4A and B). As shown in Figure 4D, DR5 knock-down reduced the cell death induced by combined treatment with cysteine deprivation and TRAIL under hypoxic conditions. These data suggest that increased expression of DR5 in cells deprived of cysteine under hypoxia may enhance the cell's sensitivity to TRAIL.

In summary, cysteine deprivation induces cell death *via* ATF4 up-regulation. Interestingly, hypoxic conditions block cell death and the up-regulation of ATF4 induced by cysteine deprivation. TRAIL reversed the effect(s) of hypoxia which reduced cell death induced by cysteine deprivation or sulfasalazine. Taken together, our findings suggest that preventing hypoxia is essential for increasing cellular sensitivity to cysteine deprivation-induced cell death.

## Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

## Authors' Contributions

Hyeon-Ok Jin and In-Chul Park developed the concept and designed the study. Sung-Eun Hong, Mi-Ri Kim and Se-Kyeong Jang carried out the experiments. Min-Ki Seong, Hyun-Ah Kim and Woo Chul Noh provided technical support and conceptual advice. Sung-Eun Hong, Mi-Ri Kim, Hyeon-Ok Jin and In-Chul Park wrote the article. All Authors read and approved the final article.

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