

Induction of apoptosis by 5,7-dihydroxy-8-nitrochrysin in breast cancer cells: The role of reactive oxygen species and Akt

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Abstract. 5,7-dihydroxy-8-nitrochrysin (NOC), a novel synthetic chrysin analogue, induces apoptosis in human cancer cells. We previously demonstrated that NOC possesses stronger cytotoxicity towards human colon carcinoma and human gastric carcinoma cells than chrysin. Herein, we demonstrate the mechanism by which NOC preferentially suppresses the viability of the MDA-MB-453 human breast cancer cell line (ER negative, Her2 overexpressing) and moderately suppresses the viability of the MCF-7 cell line (ER positive, Her2 low), but has little effect on the immortalized non-cancerous HBL-100 breast cell line (ER positive, Her2 low). Moreover, the results of our studies, for the first time, provide mechanistic evidence that NOC induces apoptosis by the generation of reactive oxygen species and Akt dephosphorylation. Our findings highlight a new mechanism responsible for NOC-induced apoptosis, and raise the possibility that NOC might be promising as a candidate for human breast cancer therapy.

Introduction

The incidence of breast cancer is lower in Asia than in Western countries (1). This may be attributable to the Asian dietary regimen that is rich in flavonoid-containing plants, which are thought to be anti-tumorigenic. Chrysin (5,7-dihydroxy-flavone, ChR), a natural flavonoid present in our daily diet, has recently been shown to be a potent inhibitor of the enzyme aromatase (2). ChR also has anti-inflammatory (3,4), and anti-oxidant (5) effects, and has been found to possess the ability to inhibit growth and induce apoptosis in a variety of human cancer cells, including cervical cancer (6), leukemia (7,8), colon carcinoma (9), esophageal adenocarcinoma (10) and lung adenocarcinoma cells (11). Nevertheless, poor oral bio-

availability has been a major limitation for the successful use of dietary flavonoids as cancer chemotherapeutic agents (12,13).

In order to improve the biological activities of ChR, a number of its derivatives had been synthesized and investigated. Park *et al* (14) indicated that 6,8-disubstituted chrysin derivatives, which were obtained from naturally occurring ChR by halogenation, showed as strong an inhibitory activation of PGE2 production from LPS-induced RAW 264.7 cells as wogonin, a well-known natural flavone that has strong and selective COX-2 inhibitory activity. Comte *et al* (15) reported that through alkylation, the hydrophobic degree of ChR is increased, the KD value is decreased and the binding affinity toward P-glycoprotein (P-gp) in the cell is enhanced. We previously showed that 5,7-dihydroxy-8-nitrochrysin (NOC) was obtained through synthesis (16). NOC showed stronger cytotoxicity to human colon carcinoma cells, human gastric carcinoma cells, and human hepatocellular carcinoma cells than ChR (16-18). However, whether NOC induced apoptosis in the human breast cancer cells and its molecular mechanisms remained to be elucidated.

Overexpression of HER2/*neu* is found in ~30% of human breast cancers and is associated with more aggressive tumors and more resistance to antitumor agents (19). By binding and activating HER/*neu*, tyrosine-phosphorylated HER3 is able to directly couple to PI3K (phosphatidylinositol 3-kinase), which is involved in the proliferation, survival, adhesion and motility of cancer cells (20,21). Activation of PI3K is necessary for the activation of Akt/PKB, a downstream mediator of PI3K signaling, through the phosphorylation of Thr-308 and Ser-473 by PDK1 and PDK2 kinase (22). The mechanisms by which Akt/PKB regulates cell survival involve the phosphorylation and inactivation of the apoptotic mediators BAD (23), caspase-9 (24) and FKHRL1 (25).

Dysfunctions in apoptosis can result in the development of cancer, and resistance to anticancer treatment may be caused by insensitivity to induction of apoptosis. Therefore, apoptosis is a mechanism that needs to be exploited when developing new chemotherapeutic drugs for cancer. Reactive oxygen species (ROS) play an important role in the intrinsic pathway of apoptosis. The accumulation of intracellular ROS led to the loss of mitochondrial transmembrane potential (MTP, $\Delta\Psi$ m), release of cytochrome c, followed by activation of the caspase cascade, and ultimately to apoptosis (26,27).

The present study was performed to examine whether and how NOC induces apoptotic cell death in human breast

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cancer cells. Here, we have demonstrated that NOC-induced apoptosis of human breast cancer cells was associated with NOC-promoted generation of ROS and inhibited Akt phosphorylation.

Materials and methods

Cell line and cell culture. The human breast cancer cell lines used in this study were MDA-MB-453 (ER positive, Her2 low) and MCF-7 (ER positive, Her2 low). We also used the HBL-100 cell line, which is derived from normal human breast tissue that has been transformed by the SV40 large T antigen. All of the cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 U/ml streptomycin in humidified atmosphere with 5% CO₂ at 37°C.

Drugs and chemical reagents. NOC was synthesized at the Institute of Pharmacy and Pharmacology, University of South China as previously described (16). NOC has a molecular weight of 299 ku, appears as yellow crystals and has a purity of 99.0%. NOC was dissolved in dimethyl sulfoxide (DMSO), and was prepared as a 10 mmol/l stock solution. ChR, adriamycin (ADR), L-glutamyl-L-cysteinylglycine (GSH), propidium iodide (PI), MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], N-acetylcysteine (NAC), LY294002, GW9662 and dichlorodihydrofluorescein diacetate (H₂DCFDA) were obtained from Sigma (St. Louis, MO). Molecular weight markers were obtained from Bio-Rad (Hercules, CA). The Apoptotic DNA Ladder Detection Kit was from the Bodataike Company, Beijing. RNase was from Promega Corporation (Madison, WI). The caspase-3 substrate N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA), and the caspase-3 specific inhibitor Z-Asp-Glu-Val-Asp-CH₂F (Z-DEVD-fmk), were obtained from Calbiochem (La Jolla, CA). anti-phospho-PDK1 (Ser241) antibody, anti-phospho-Akt (Ser473) antibody, were from Cell Signaling (Danvers, MA). Antibodies against Akt, PDK1 and β -actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from Cell Signaling Technology. All other reagents and compounds were analytical grades.

MTT assay. Cells were seeded in a 96-well plate at a density of 0.5×10^4 cells/well and treated with serum-free medium for 24 h, followed by treatment with various concentrations of experimental agents that were added to each well and cultured for 48 h, followed by incubation with media containing 0.5 mg/ml MTT for 4 h. The supernatant was removed following centrifugation. Finally, 100 μ l DMSO was added and the absorbance at the 570-nm wavelength (A₅₇₀) was measured by means of an enzyme-labeling instrument (ELX-800 type). Relative cell viability inhibition rate (IR) = (1 - average A₅₇₀ of the experimental group/average A₅₇₀ of the control group) \times 100%.

Flow cytometry (FCM) with PI staining. Cells were treated with serum-free medium for 24 h, followed by treatment with media containing various concentrations of experimental

agents for 48 h, respectively. Cells were collected and washed twice with cold PBS, fixed with 700 ml/l alcohol at 4°C for 24 h, stained with propidium iodide (PI) and cell apoptosis was detected using FCM (American BD Company, FACS 420).

DNA agarose gel electrophoresis. The cells were treated with serum-free medium for 24 h, followed by treatment with media containing various concentrations of experimental agents for 48 h. Cells were washed twice with PBS and DNA was extracted with the Apoptotic DNA Ladder Detection Kit according to manufacturer's instructions. The extracted DNA was kept at 4°C overnight. Then 8.5 μ l of DNA sample was mixed with 1.5 μ l of 6X buffer solution, electrophoresed on a 20 g/l agarose gel containing ethidium bromide at 40 V, and observed using a DBT-08 gel image analysis system.

Caspase-3 activity assay. To evaluate caspase-3 activity, cell lysates were prepared after their respective treatment with experimental agents. Assays were performed in 96-well plates by incubating 20 μ g cell lysates in 100 μ l reaction buffer [1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 10% glycerol] containing a 5- μ M caspase-3 substrate (Ac-DEVD-pNA). Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with an enzyme-labeling instrument (ELX-800 type). In the caspase inhibitors assay, cells were pretreated with a caspase-3 specific inhibitor (20 μ M, Z-DEVD-fmk) for 1 h prior to the addition of test agents.

Determination of ROS. Intracellular ROS accumulation was measured by flow cytometry using the fluorescent probe H₂DCFDA. Cells were incubated briefly with 10 μ mol/l H₂DCFDA for 30 min at 37°C in the darkroom after treatment with various concentrations of test agents for three hours. After incubation, the cells were washed with PBS and analyzed within 30 min using an FCM (American BD Company, FACS 420) equipped with an air-cooled argon laser tuned to 488 nm. The specific fluorescence signals corresponding to H₂DCFDA were collected with a 525-nm band pass filter. As a rule, 10,000 cells were counted in each determination.

Western blot analysis. Cells were collected after treatment with various concentrations of test agents, washed 3 times with PBS, lysed in cell lysate containing 0.1 mol/l NaCl, 0.01 mol/l Tris-Cl (pH 7.6), 0.001 mol/l EDTA (pH 8.0), 1 μ g/ml aprotinin, 100 μ g/ml PMSF, and then centrifuged at 12000 \times g for 10 min at 4°C. Extracted protein sample (25 μ g total protein/lane) was added to the same volume of sample buffer solution and subjected to denaturation at 100°C for 10 min, then electrophoresed on 100 or 60 g/l SDS-PAGE, and finally transferred onto a polyvinylidene fluoride membrane (PVDF) (Millipore, Shanghai, China). The PVDF membrane was treated with TBST containing 50 g/l skimmed milk at room temperature for 2 h, followed by incubation with the primary antibodies anti-phospho PDK1 (Ser241), anti-phospho-Akt (Ser473), anti-PDK1, anti-Akt and anti- β -actin (1:1000 dilution), respectively, at 37°C for 2 h. After being washed with TBST for 30 min, the corresponding secondary antibody was added and incubated at room temperature for 1 h.

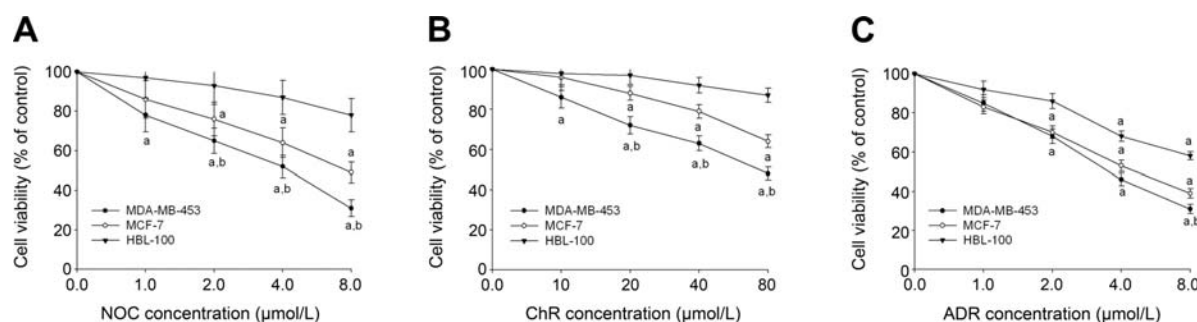


Figure 1. Effect of NOC on the viability of human breast cancer cells. After incubation with different concentrations of NOC (A), ChR (B) or ADR (C) at 37°C for 48 h, the effect on cell viability was examined using an MTT assay. The number of viable cells after treatment is expressed as a percentage of viable cells from the vehicle control. The data represent the mean values obtained from three independent experiments and the bars represent the standard deviation. ^aP<0.05 vs. treatment with the vehicle control group. ^bP<0.05 vs. MCF-7 or HBL-100 cell line by treatment using the same concentration.

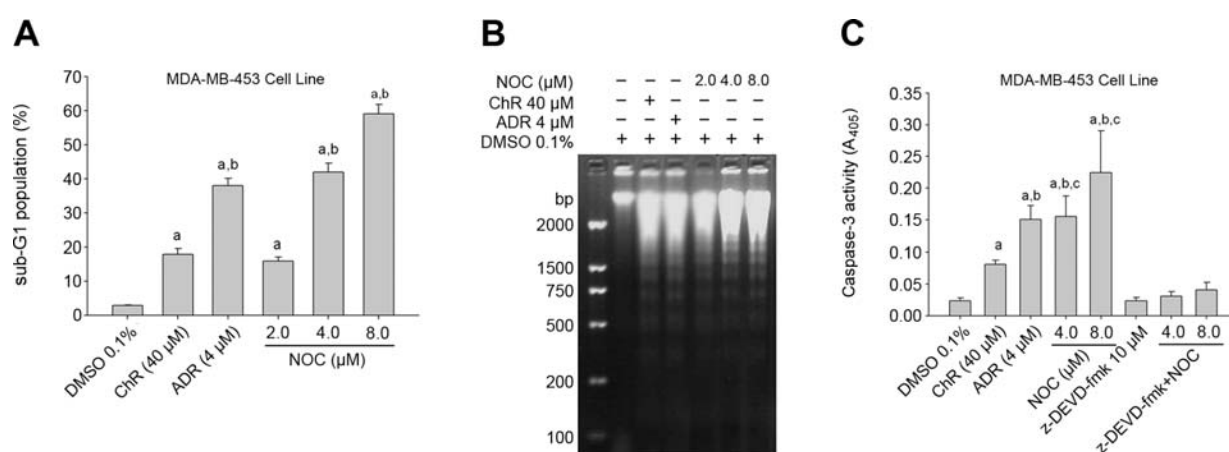


Figure 2. NOC-induced apoptosis in MDA-MB-453 cells. (A) MDA-MB-453 cells were treated with the indicated concentration of NOC for 48 h and analyzed by flow cytometry. (B) DNA ladder formation in the MDA-MB-453 cells incubated with the indicated concentration of NOC for 48 h using DNA agarose gel electrophoresis. (C) Caspase-3 activation in MDA-MB-453 cells incubated with the indicated concentration of NOC for 48 h using an ELISA assay. The data are the mean values obtained from three independent experiments; the bars represent the standard deviation. ^aP<0.05 vs. treatment with the vehicle control group. ^bP<0.05 vs. treatment with ChR. ^cP<0.05 vs. treatment with z-DEVD-fmk+NOC.

The signal intensity was then measured using a chemiluminescent detection system (Pierce, US). Images were scanned, followed by densitometry analysis with Un-Scan-It software (Silk Scientific). The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of β -actin.

Statistical analysis. The database was set up with the SPSS 15.0 software package (SPSS Inc, Chicago, IL) for analysis. Data are represented as the mean \pm SD. The means of multiple groups were compared using one-way ANOVA, after the check for equal variance, and the comparison of two means was performed using the LSD method. Statistical comparison was also performed using the two-tailed t-test when appropriate. P<0.05 was considered statistically significant.

Results

NOC preferentially inhibited the viability of MDA-MB-453 cells. The results of the MTT assay showed that the viability of the tested cell lines was inhibited by NOC in a dose-concentration manner but to varying extents (Fig. 1A). At

4.0 μ mol/l NOC inhibited 48 \pm 4.3% of the viability in MDA-MB-453 cells for 48 h. MDA-MB-453 cells overexpressed the Her2, FGF and AR receptors, were mutant for p53, and were ER negative. However, under the same conditions, NOC inhibited only 32 \pm 2.5% of the viability in MCF-7 cells. MCF-7 cells were ER positive and had lower Her2 expression. NOC had little effect on the immortalized non-cancerous HBL-100 breast cell line even at 8.0 μ mol/l. The potency of NOC is higher than the lead compound ChR (Fig. 1B). However, adriamycin inhibited cell viability of the tested cell lines in the same pattern, which selectivity was lower than NOC and ChR (Fig. 1C). These results suggest that NOC and its lead compound preferentially suppress the viability of the MDA-MB-453 cell line (ER negative, Her2 overexpressing). For these reasons, we subsequently examined the MDA-MB-453 cell line.

NOC induces apoptosis in MDA-MB-453 cells. The NOC-treated MDA-MB-453 cell line underwent apoptosis in a dose-dependent manner, as measured by flow cytometry using propidium-iodide staining (Fig. 2A). A significant number of the cells (59 \pm 2.8%) started to undergo apoptosis after treatment with 8.0 μ mol/l NOC for 48 h. A lower

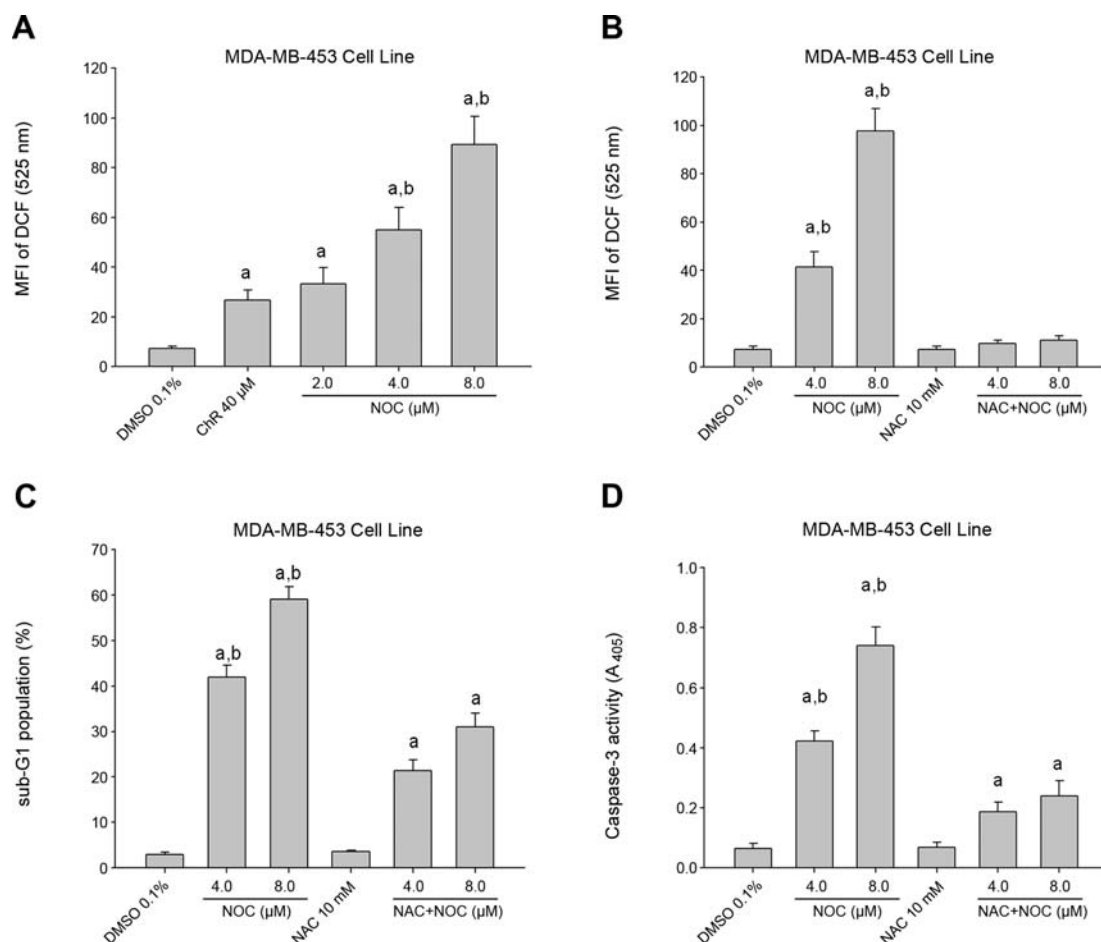


Figure 3. Increase of intracellular ROS is required for apoptosis in MDA-MB-453 cells treated with NOC. (A) Elevation of intracellular ROS levels after NOC treatment. The MDA-MB-453 cells were stimulated with NOC (2.0, 4.0, 8.0 μ mol/l) and ChR (40.0 μ mol/l) for 3 h. DCF fluorescence was measured using flow cytometry after incubation with the fluorescent probe H₂DCFDA. The data are the mean values obtained from three independent experiments and the bars represent the standard deviation. ^aP<0.05 vs. treatment with the vehicle control group. ^bP<0.05 vs. treatment with the ChR group. (B) NAC (10 mM) blocked the ROS generation induced by NOC. (C) The ROS-dependent accumulation of sub-G1 population after NOC treatment. The cells were treated with NOC (4.0 or 8.0 μ mol/l) in the presence or absence of NAC (10 mmol/l). The number of sub-G1 cells was measured using flow cytometry. (D) ROS-dependent activation of caspase-3 after NOC treatment. The cells were treated with NOC (4.0 or 8.0 μ mol/l) in the presence or absence of NAC (10 mmol/l). Caspase-3 activation was measured using an ELISA assay. The data are the mean values obtained from three independent experiments and the bars represent the standard deviation. ^aP<0.05 vs. treatment with the vehicle control group. ^bP<0.05 vs. treatment with the NAC + NOC groups.

concentration of NOC (2.0 μ mol/l) resulted in fewer cells undergoing apoptosis ($16 \pm 1.2\%$) at 48 h. As shown in Fig. 2B, in comparison with the vehicle control, the NOC treatment (4.0 and 8.0 μ mol/l for 48 h) resulted in DNA fragmentations in MDA-MB-453 cells. Parallel to effect of cell lethality and enhanced caspase-3 activity, the treatment of MDA-MB-453 cells with NOC (4.0 and 8.0 μ mol/l) for 48 h increased the levels of active caspase-3 (Fig. 1C). The requirement of caspase activation for NOC-induced apoptosis was examined by using the caspase-3-specific inhibitor z-DEVD-fmk. The data showed that z-DEVD-fmk was able to prevent caspase-3 activation (Fig. 1C). These results indicate that NOC-induced apoptotic cell death occurs in a caspase-dependent fashion.

Effects of NOC on ROS generation in MDA-MB-453 cells. Because oxidative damage plays an important role in the anticancer effect of ChR (11,28), we subsequently examined the level of intracellular ROS in MDA-MB-453 cells after treatment with NOC using an oxidation-sensitive fluorescent probe H₂DCFDA, which was oxidized to 2', 7'-dichlorofluo-

rescein (DCF) in the presence of ROS. Fig. 3A shows that treatment of cells with NOC for 3 h led to an increase of the mean fluorescence intensity (MFI) of DCF in a dose-dependent manner. Pretreatment with NAC at 10 mM can abolish NOC-induced ROS generation (Fig. 3B). Next, we investigated whether the generation of ROS induced by NOC is accompanied by apoptotic cell death. To determine a link between the elevation of intracellular ROS levels and apoptotic cell death in NOC-treated cells, MDA-MB-453 cells were pre-incubated with the thiol-containing antioxidant NAC before treatment with NOC. NOC treatment failed to induce cell death and caspase-3 activation in cells pretreated with NAC (Fig. 3C and D). These observations suggest that an increase in intracellular ROS levels after NOC treatment is required for activation of the cell death pathway accompanied by caspase-3 activation.

The effects of NOC on Akt phosphorylation in MDA-MB-453 cells. Activated Akt is considered the focal point of a survival pathway known to protect cells from apoptosis by several

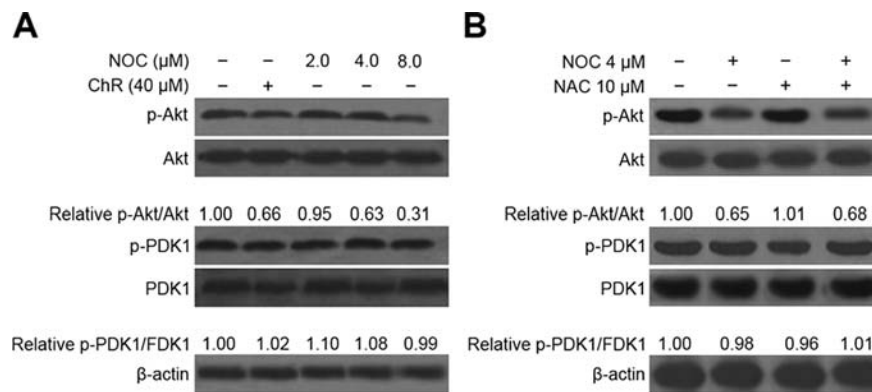


Figure 4. The effect of NOC on the phosphorylated protein level of Akt and PDK1 in MDA-MB-453 cells. The MDA-MB-453 cells were incubated with DMSO or NOC (2.0, 4.0 and 8.0 $\mu\text{mol/l}$) or ChR (40.0 $\mu\text{mol/l}$) at 37°C for 24 h. Immunoblotting was used to measure the phosphorylated levels of Akt and PDK1. The relative p-Akt/Akt or p-PDK1/PDK1 shows the average results from three independent experiments. (B) Cells were treated with NOC (4.0 $\mu\text{mol/l}$) in the presence or absence of NAC (10 mmol/l). The phosphorylation levels of Akt and PDK1 were analyzed using immunoblotting. The relative p-Akt/Akt or p-PDK1/PDK1 shows the average results from three independent experiments.

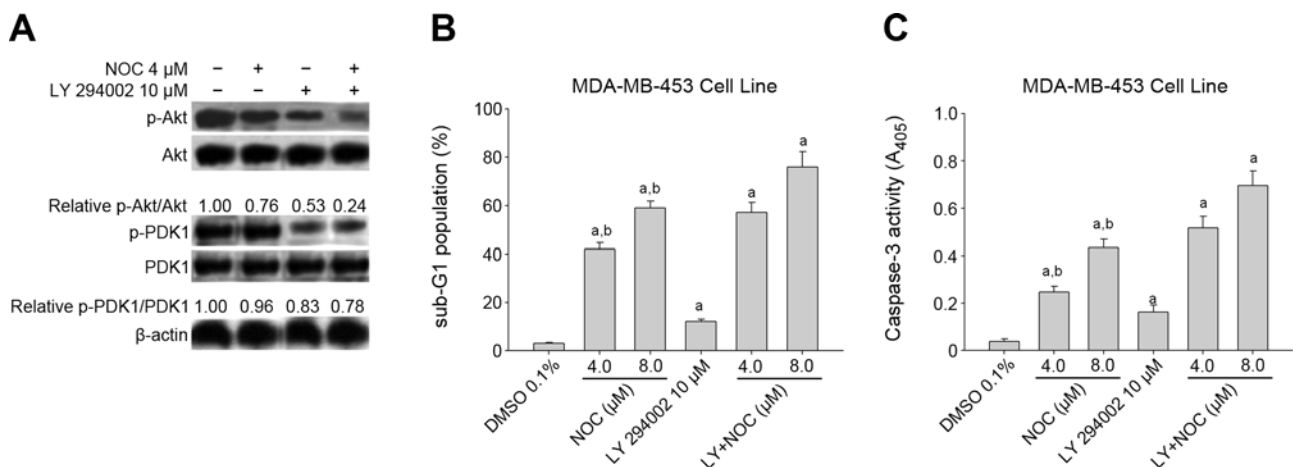


Figure 5. Combining LY294002 with NOC synergistically inhibited Akt phosphorylation and induced apoptosis in MDA-MB-453 cells. (A) MDA-MB-453 cells were treated with NOC (4.0 $\mu\text{mol/l}$) in the presence or absence of LY294002 (10 $\mu\text{mol/l}$). The phosphorylation levels of Akt and PDK1 were analyzed using immunoblotting. Relative p-Akt/Akt or p-PDK1/PDK1 shows the average results from three independent experiments. (B) Cells were treated with NOC (4.0 or 8.0 $\mu\text{mol/l}$) in the presence or absence of LY294002 (10 $\mu\text{mol/l}$). The number of sub-G1 cells was measured using flow cytometry. (C) Cells were treated with NOC (4.0 or 8.0 $\mu\text{mol/l}$) in the presence or absence of LY294002 (10 $\mu\text{mol/l}$). The activity of caspase-3 was measured using an ELISA assay. The data are the mean values obtained from three independent experiments and the bars represent the standard deviation. ^aP<0.05 vs. treatment with the vehicle control group. ^bP<0.05 vs. treatment with the LY+NOC groups.

stimuli, whereas in a recent report, ChR displayed potent inhibitory effects on Akt activity (8). As shown in Fig. 4, in the MDA-MB-453 cells, treatment with NOC had no effect on the phosphorylation levels of PDK1. However, phosphorylated Akt, its downstream effector, was inhibited in a dose-dependent manner (Fig. 4A). Preincubation with the thiol-containing antioxidant NAC (10 mmol/l) did not affect the inhibition of Akt phosphorylation by NOC (Fig. 4B).

The effects of LY294002 on NOC inhibited Akt phosphorylation and induced apoptosis in MDA-MB-453 cells. To further certify that NOC inhibited the activation of Akt but did not affect the activity of the upstream regulation factor PI3K, LY294002 (an irreversible PI3K inhibitor) was used. The MDA-MB-453 cells were treated with 10 $\mu\text{mol/l}$ preincubated LY294002 for 1 h followed by 10 $\mu\text{mol/l}$ NOC 4.0 for

24 h, which almost completely inhibited Akt phosphorylation (Fig. 5A). In addition, cotreatment of NOC and LY294002 synergistically induced apoptosis and activated caspase-3 in the MDA-MB-453 cells (Fig. 5B and C).

The effects of GW9662 on NOC inhibited Akt phosphorylation and induced apoptosis in MDA-MB-453 cells. It has been reported that chrysin derivatives induce apoptosis in human hepatocellular carcinoma and human gastric cancer cells by activating PPAR γ (17,18). For this reason, we examined the effects of a PPAR γ blocker, GW9662, on NOC-inhibited Akt phosphorylation and induced apoptosis and caspase-3 activation in MDA-MB-453 cells. Fig. 6 shows that GW9662 slightly attenuated the inhibition of Akt phosphorylation, but had no effect on the induction of apoptosis and caspase-3 activation by NOC.

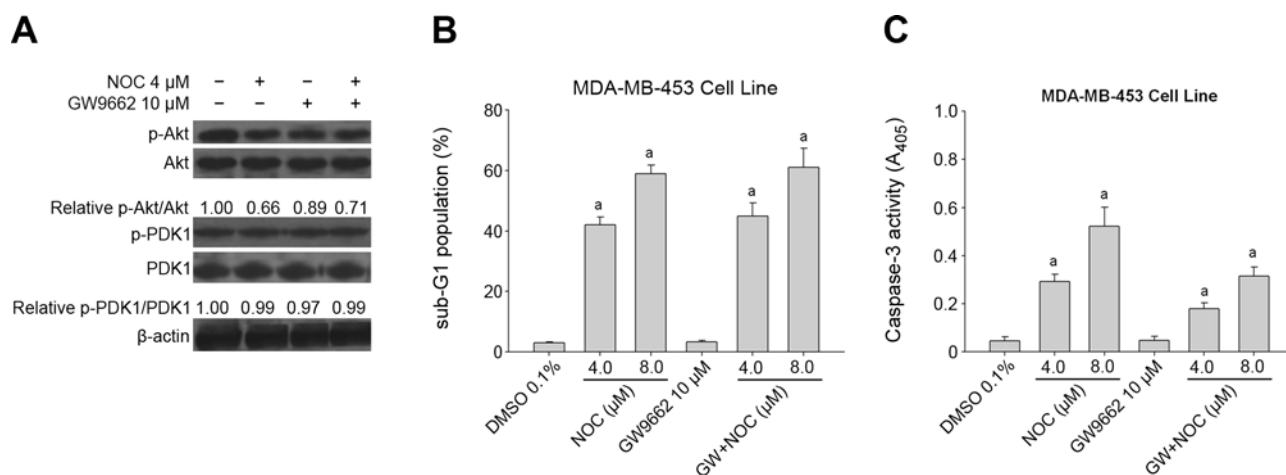


Figure 6. The effect of PPAR γ inhibition on NOC inhibited Akt phosphorylation and induced apoptosis in MDA-MB-453 cells. (A) MDA-MB-453 cells were treated with NOC (4.0 μ mol/l) in the presence or absence of GW9662 (10 μ mol/l). The phosphorylation levels of Akt and PDK1 were analyzed using immunoblotting. Relative p-Akt/Akt or p-PDK1/PDK1 shows the average results from three independent experiments. (B) Cells were treated with NOC (4.0 or 8.0 μ mol/l) in the presence or absence of GW9662 (10 μ mol/l). The number of sub-G1 cells was measured using flow cytometry. (C) Cells were treated with NOC (4.0 or 8.0 μ mol/l) in the presence or absence of GW9662 (10 μ mol/l). The activity of caspase-3 was measured using an ELISA assay. The data are the mean values obtained from three independent experiments and the bars represent the standard deviation. ^aP<0.05 vs. treatment with the vehicle control group.

Discussion

Our previous study showed that the effect of NOC on the inhibition of proliferation and induction of apoptosis in the colon cancer cell line HT-29 and the gastric cancer cell line SGC-7901 was stronger than that of ChR (16-18). In this study, we first showed that NOC and its lead compound ChR preferentially inhibit the growth of MDA-MB-453 cells (ER negative, Her2 overexpressing) and moderately suppress the viability of MCF-7 cells (ER positive, Her2 low), but have little effect on HBL-100 cells (ER positive, Her2 low) (Fig. 1) (29). Our results suggest that NOC preferentially suppresses the growth of the HER2/*neu*-overexpressing breast cancer cell lines, and has relative selectivity for breast cancer cells. Here, we further showed that NOC also induced apoptosis in HER2/*neu*-overexpressing MDA-MB-453 cells (Fig. 2). Therefore, we demonstrated for the first time that NOC induces cell growth inhibition of MDA-MB-453 cells, the HER2/*neu*-overexpressing breast cancer cell line, accompanied by the induction of apoptosis.

ROS is associated with carcinogenesis but also, paradoxically, with mitochondrial-mediated cell death in cancer cells. The overproduction of ROS as a central event in mitochondrial-mediated apoptosis is now well-documented (30-33). The antioxidant properties of flavonoids were associated with their cardioprotective and neuroprotective properties. In the case of flavonoids, however, their chemopreventive properties may rather rely on eliminating precancerous cells due to their pro-oxidant properties *in vivo*. This is likely the case of apigenin and ChR, in which their cytotoxicity may result from a combination of interference with the mitochondrial respiratory chain and MRP-mediated GSH depletion (34-36). Intracellular ROS mediates multiple cellular responses, including protein kinase activation (37), cell cycle progression (38), myeloid cell differentiation (39,40) and apoptosis (41). It has been documented in several studies that depletion

of intracellular GSH plays a critical role in initiating apoptosis by ChR (11,28). In the present study, we first found that the ChR derivative, NOC, promoted the accumulation of ROS products in a concentration-dependent manner in MDA-MB-453 cells (Fig. 3A). Moreover, we used NAC as an antioxidant to investigate the ROS generation induced by NOC. NAC treatment not only reduced ROS generation but also attenuated the induction of apoptosis in MDA-MB-453 cells (Fig. 3B and C). Taken together, the data indicate that induction of ROS generation contributes to NOC-induced apoptosis of HER2/*neu*-overexpressing MDA-MB-453 cells.

Studies using breast cancer cell lines and human tumors are accumulating and demonstrate that the constitutive phosphorylation of HER2/*neu* is associated with resistance to systemic therapies and local radiation therapies. The PI3K-Akt pathway is one of the signaling pathways activated by HER2/*neu*. The PI3-kinase/Akt pathway contributes to tumor formation by elevating the anti-apoptotic cell death activity of Akt. Akt inhibits apoptosis through the phosphorylation of Bad, GSK3 and caspase-9 and through the activation of transcription factors such as Forkhead (FOXO1) and NF- κ B (42,43). The present study showed an increase in Akt phosphorylation in HER2/*neu*-overexpressing MDA-MB-453 cells and NOC-inhibited Akt phosphorylation. Phosphorylation of Akt is routinely used as readout for Akt activation. The inhibition of Akt phosphorylation by NOC is also an important mechanism of action in the MDA-MB-453 cells. Similar results were observed using treatment with ChR in U937 leukemia cells (8). Moreover, we found that treatment of the MDA-MB-453 cells with NOC had no effect on the phosphorylation levels of PDK1, whereas phosphorylated Akt, its downstream effector, was inhibited in a dose-dependent manner (Fig. 4A). NAC did not affect the inhibition of Akt phosphorylation by NOC (Fig. 4B). These findings suggest that NOC repressed the phosphorylation levels of Akt and did not directly inhibit PI3K activity of MDA-MB-453 cells.

due to ROS generation. This study has demonstrated that the inhibition of PI3-kinase by LY294002 results in sensitization to NOC-induced apoptosis, suggesting that PI3K activation prevents the efficient induction of apoptosis by NOC.

A possible candidate for mediating the effects of NOC-induced inhibition of Akt signaling is proximosome proliferator-activated receptor γ (PPAR γ), a member of the nuclear hormone receptor superfamily. Recently, it has been found that PPAR γ is overexpressed in various types of tumor cells and PPAR γ agonists can induce apoptosis (44,45). It has been reported that ChR derivatives induce apoptosis of human hepatocellular carcinoma and human gastric cancer cells by activating PPAR γ (17,18). However, we found that GW9662, a selective antagonist of PPAR γ , slightly attenuated inhibition of Akt phosphorylation, but had little effect on the induction of apoptosis and caspase-3 activation by NOC (Fig. 6). The rational explanation for these results may be that ChR and ChR derivatives activate PPAR γ only in specific cell types.

In conclusion, the results of our studies provide mechanistic evidence, for the first time, that NOC induces apoptosis by ROS generation and Akt dephosphorylation. The apoptosis-inducing ability of NOC, in conjunction with its ineffectiveness on HBL-100 cells (non-cancer cells), raises the possibility that NOC might be a promising candidate for human breast cancer therapy. However, additional *in vivo* studies are needed to establish the role of NOC as a chemopreventive and/or therapeutic agent for cancer.

Acknowledgements

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