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Journal

Pharmacological Reports, 71(2)

ISSN

1734-1140

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Publication Date

2019-04-01

DOI

10.1016/j.pharep.2019.01.001

Peer reviewed

HHS Public Access

Author manuscript

Pharmacol Rep. Author manuscript; available in PMC 2019 May 28.

Published in final edited form as:

Pharmacol Rep. 2019 April ; 71(2): 289–298. doi:10.1016/j.pharep.2019.01.001.

Anticancer effect of nor-wogonin (5, 7, 8-trihydroxyflavone) on human triple-negative breast cancer cells via downregulation of TAK1, NF-κB, and STAT3

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Conflict of Interests

All authors declare that they have no conflicts of interest.

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Abstract

Background: Nor-wogonin, a polyhydroxy flavone, has been shown to possess antitumor activity. However, the mechanisms responsible for its antitumor activity are poorly studied. Herein, we investigated the mechanisms of nor-wogonin actions in triple-negative breast cancer (TNBC) cells.

Methods: Effects of nor-wogonin on cell proliferation and viability of four TNBC cell lines (MDA-MB-231, BT-549, HCC70, and HCC1806) and two non-tumorigenic breast cell lines (MCF-10A and AG11132) were assessed by BrdU incorporation assays and trypan blue dye exclusion tests. Cell cycle and apoptosis analyses were carried out by flow cytometry. Protein expression was analyzed by immunoblotting.

Results: Nor-wogonin significantly inhibited the growth and decreased the viability of TNBC cells; however, it exhibited no or minimal effects in non-tumorigenic breast cells. Nor-wogonin (40 μM) was a more potent anti-proliferative and cytotoxic agent than wogonin (100 μM) and wogonoside (100 μM), which are structurally related to nor-wogonin. The antitumor effects of nor-wogonin can be attributed to cell cycle arrest *via* reduction of the expression of cyclin D1, cyclin B1, and CDK1. Furthermore, nor-wogonin induced mitochondrial apoptosis, (as evidenced by the increase in % of cells that are apoptotic), decreases in the mitochondrial membrane potential (ΔΨm), increases in Bax/Bcl-2 ratio, and caspase-3 cleavage. Moreover, nor-wogonin attenuated the expression of the nuclear factor kappa-B and activation of signal transducer and activator of transcription 3 pathways, which can be correlated with suppression of transforming growth factor-β-activated kinase 1 in TNBC cells.

Conclusion: These results showed that nor-wogonin might be a potential multi-target agent for TNBC treatment.

Keywords

Breast cancer; nor-wogonin; apoptosis; cell cycle arrest; multi-target flavone

Introduction

Triple-negative breast cancer (TNBC) is a subtype of breast cancer that lacks estrogen receptors, progesterone receptors, and human epidermal growth factor 2 overexpression [1]. It comprises 15–20 % of all breast cancer cases and is considered a clinical challenge because this cancer does not respond to available targeted agents, mandating the search for new agents for treatment of TNBC [2].

Transforming growth factor (TGF)-β-activated kinase 1 (TAK1) is a serine/threonine kinase, which is frequently involved in human cancer development [3]. Activation of TAK1 in cancer cells results in the activation of nuclear factor kappa-B (NF-κB) and activator protein-1 (AP-1), which are key transcription factors that regulate the expression of many genes involved in cancer progression [3, 4]. Moreover, signal transducer and activator of

transcription 3 (STAT3) plays pivotal roles in tumor cell proliferation, survival, and invasion [5]. STAT3 must be activated by its phosphorylation in different sites [6]. Ohkawara *et al.* showed that the TAK1/Nemo-like kinase (NLK) pathway is responsible for the phosphorylation of STAT3 at Ser727 [7]. Collectively, since TAK1, NF-κB, and STAT3 were found to be activated in TNBC and play critical roles in TNBC progression and chemoresistance, new chemotherapeutic agents that can inhibit TAK1, NF-κB, and STAT3 signaling have the potential to improve the outcome of patients with TNBC [8–10].

In this context, several natural products have been shown to possess potent antitumor activities [11, 12]. Wogonin (5,7-dihydroxy-8-methoxyflavone) and nor-wogonin (5,7,8-trihydroxyflavone) are bioactive polyhydroxy flavones isolated from *Scutellaria baicalensis* Georgi [13, 14]. The antitumor activity and mechanisms of action of wogonin have been studied in several cancers, including breast, leukemia, and colorectal cancers [15]. Nor-wogonin is a flavone that is structurally related to wogonin; they differ in the presence of OH group at the C-8 position in nor-wogonin instead of methoxy (OMe) group in wogonin (Fig.1A). The anticancer activity and mechanisms of action of nor-wogonin are poorly studied. Chow et al., (2008) reported that nor-wogonin was more effective than wogonin in inducing apoptosis in HL-60 leukemia cells [16]. However, the molecular mechanisms underlying the antitumor effects of nor-wogonin have been poorly investigated. The structural similarity between wogonin and nor-wogonin prompted us to elucidate the mechanisms of nor-wogonin actions in TNBC cells.

Materials and Methods

Cell cultures and reagents

Human TNBC cell lines (MDA-MB-231, BT-549, HCC70, and HCC1806) and non-tumorigenic breast cell line (MCF-10A) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). A normal breast cell line (AG11132) was obtained from Coriell Institute for Medical Research (Camden, NJ, USA). TNBC cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium while non-tumorigenic breast cells (MCF-10A and AG11132) were cultured in Dulbecco's modified Eagle's medium (DMEM) or Mammary Epithelial Cell Growth Medium (MEGM), respectively. The media contained 10 % fetal calf serum (FCS) and were cultured in a humidified atmosphere with 5 % CO_2 at 37 °C. Cells between the 3rd and 10th passages were used for this study. Nor-wogonin was purchased from Chem Faces (Wuhan, Hubei, China) whereas wogonin and wogonoside were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Proliferation and viability assays

Cell proliferation was quantified in terms of bromodeoxyuridine (BrdU) incorporation using a colorimetric cell proliferation BrdU ELISA kit (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions. TNBC cells (MDA-MB-231, BT-549, HCC70, and HCC1806) and non-tumorigenic breast cells (MCF-10A and AG11132) were seeded at 5×10^3 cells/well and cultured overnight in a 96-well plate. The cells were treated with nor-wogonin, wogonin, wogonoside, or dimethyl sulfoxide (DMSO; vehicle) for 24 h.

BrdU was added at a final concentration of 10 μM and cells were cultured for 2 h. BrdU incorporation was quantified by measuring the optical density (OD) at 450 nm.

Trypan blue exclusion assay was performed to determine cell viability after treatment with nor-wogonin, wogonin, or wogonoside. TNBC cells (MDA-MB-231, BT-549, HCC70, and HCC1806) and non-tumorigenic breast cells (MCF-10A and AG11132) cells/well) were plated (5×10^3/well) in 96-well plates and treated with nor-wogonin, wogonin, wogonoside, or DMSO for 24 h. The cultured cells were harvested and resuspended in 100 μl of RPMI 1640 medium, DMEM, or MEGM and the cell suspension was thoroughly mixed with an equal volume of 0.4 % trypan blue solution (Gibco, Grand Island, NY, USA). The numbers of viable and dead cells were counted using a hemocytometer, and the cell viability percentage was determined.

Cell cycle analyses

Cell cycle arrest of MDA-MB-231 cells was analyzed by a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) using the BD Pharmingen™ FITC-BrdU flow kit (BD Biosciences), according to the manufacturer's instructions. Briefly, MDA-MB-231 cells were treated with different concentrations of nor-wogonin (10, 20, or 40 μM), or DMSO for 24 h. For the time course experiments, MDA-MB-231 cells were treated with nor-wogonin (30 μM) or DMSO for 24, 48, and 72 h. After treatment, cells were labeled by incubating overnight with BrdU solution at a final concentration of 10 μM in cell culture medium. After labeling, cells were fixed, permeabilized using BD Cytofix/Cytoperm Buffer and incubated with BD Cytoperm Permeabilization Buffer Plus for 10 minutes on ice and washed by 1X BD Perm/Wash Buffer. Then, cells were fixed again using BD Cytofix/Cytoperm Buffer and incubated with DNase for 1 h at 37°C to expose incorporated BrdU. Cells were then washed with BD Perm/Wash Buffer and resuspended in 50 μl of BD Perm/Wash Buffer containing diluted fluorescein isothiocyanate (FITC)-labeled anti-BrdU antibody and incubated for 20 minutes at room temperature. Cells were then washed with 1 ml of BD Perm/Wash Buffer and resuspended in 20 μl of the 7-aminoactinomycin D (7-AAD) solution. Finally, cells were resuspended in 1 ml of staining buffer and analyzed using a FACS Calibur flow cytometer. Quantitative analysis of the FACS data was carried out using the FlowJo software (FlowJo, Ashland, OR, USA)

Apoptosis analyses

Induction of apoptosis of MDA-MB-231 cells by nor-wogonin was investigated by the FACS Calibur flow cytometer (BD Biosciences) using the annexin V/propidium iodide (PI) staining kit (BioLegend, San Diego, CA, USA), according to the manufacturer's instructions. MDA-MB-231 cells were treated with nor-wogonin, nor-wogonin combined with 30 μM pan-caspase inhibitor (Z-VAD-FMK), or DMSO for 24 h. Cells were then harvested, washed twice with PBS, resuspended in annexin V binding buffer, and stained with annexin V-FITC and PI at room temperature in the dark for 30 min. The MDA-MB-231 cells were then analyzed by flow cytometry using quadrant statistics for apoptotic and necrotic cell populations. The fluorescence intensity in X-axis and Y-axis were detected in the FL1-A and FL2-A channels respectively. Annexin V was used to detect both the early

and late stages of apoptotic cells while PI was used to detect late apoptotic and necrotic cells. Quantitative analysis of the FACS data was performed using the FlowJo software.

Mitochondrial membrane potential—Alteration of mitochondrial membrane potential (ΔΨm) was measured using a fluorescent probe JC-1 dye (tetraethylbenzimidazolylcarbocyanine iodide). The MDA-MB-231 cells were seeded in 6-well plates at a density of 2×10^5 cells/well and incubated for 24 h. Cells were treated with DMSO, nor-wogonin (10, 20 or 40 μM), or 25 μM FCCP (positive control) for 24 h. and incubated in a CO2 incubator at 37 °C. The cells were then stained with JC-1 reagent (100 μl) for 30 min. at 37 °C. Mitochondria with normal function (normal ΔΨm) emits red fluorescence whereas the depolarized mitochondria emits green fluorescence. Red fluorescence (excitation 550 nm, emission at 600 nm) and green fluorescence (excitation 485 nm, emission at 535 nm) intensity were measured using fluorescence plate reader. The ratio of fluorescent intensity of red j-aggregates to fluorescent intensity of green j-monomers was used as indicator of the loss of ΔΨm. The lower the value of this ratio, the lower the mitochondrial membrane potential.

Western blot analyses

MDA-MB-231 cells were incubated with nor-wogonin, nor-wogonin combined with 30 μM pan-caspase inhibitor (Z-VAD-FMK), or DMSO for 24 h. For the time course experiment, MDA-MB-231 cells were incubated with nor-wogonin (30 μM) for 24, 48, or 72 h. MCF-10A cells were incubated with nor-wogonin (40 μM) or DMSO for 24 h. To generate whole cell lysate (WCL), cells were harvested, washed twice with chilled PBS, and lysed with an ice-cold lysis buffer containing 0.1 % sodium dodecyl sulfate (SDS), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 % Triton X-100, 2 μg/mL aprotinin, 5 μg/mL 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Pefabloc SC; a protease inhibitor cocktail), 1 % phosphatase inhibitor cocktail, and 50 mM Tris-HCl (pH 7.4). The cell lysates were kept on ice for 30 min, collected, and centrifuged at 14,000 ×*g* for 15 min at 4 °C. To generate nuclear protein extract for p65 assessment, cells were suspended in 420 μl of buffer A (10 mm HEPES, pH 7.9, 10 mm KCl, 0.1 mm EDTA, 0.1 mm EGTA, 1 mm dithiothreitol, 1 mm phenylmethylsulfonyl fluoride, 20 mm β-glycerophosphate, 0.1 mm sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and chilled on ice for 15 min. Then, 25 μl of 10% Nonidet P-40 was added, and the suspension was vortexed, and centrifuged at 15,000 rpm for 5 min. The nuclear pellets were washed with 200 μl of buffer A and suspended in 50–100 μl of buffer B (20 mm HEPES, pH 7.9, 0.4 m NaCl, 1 mm EDTA, 1 mm EGTA, 1 mm dithiothreitol, 1 mm phenylmethylsulfonyl fluoride, 20 mm β-glycerophosphate, 1 mm sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The mixture was kept on ice for 15 min with frequent agitation. Nuclear extracts were prepared by centrifugation at 15,000 rpm for 5 min and stored at −80 °C. The protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. For western blot analysis, 30 μg of protein was loaded onto a 10% or 15% SDS-PAGE gel according to the molecular weight of the tested protein. Proteins separated on the SDS-PAGE gel were transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was incubated in a blocking buffer containing 3 % non-fat milk powder, 1 % BSA (Sigma-Aldrich), and 0.5 %

Tween-20 in PBS for 1 h. Subsequently, the PVDF membrane was incubated with the suitable and validated primary antibody (Cell Signaling Technology, Danvers, MA, USA) overnight, followed by horseradish peroxidase (HRP)-conjugated IgG (Cell Signaling Technology, Beverly, MA, USA) for 1 h with gentle agitation at room temperature. Signals were detected by enhanced chemiluminescence (ECL) prime (GE Healthcare, Little Chalfont, UK) and autoradiography using an X-ray film (Konica Minolta Medical Imaging, Wayne, NJ, USA)

Statistical analyses

Data are represented as mean ± SD. Non-repeated ANOVA with post hoc Dunnett's test was performed to determine the statistical significance compared to a corresponding negative control. Statistical significance was defined as $P < 0.05$. The IC_{50} of nor-wogonin was calculated using GraphPad Prism 5 (Version 5.01, GraphPad Software, San Diego, CA, USA) from the results of the trypan blue exclusion assay. Data are representative of three independent experiments.

Results

Nor-wogonin has superior antiproliferative and cytotoxic activities in human TNBC cells, compared to its structurally related compounds

Cell proliferation of four TNBC cell lines (MDA-MB-231, BT-549, HCC70, and HCC1806) and two non-tumorigenic breast cell lines (MCF-10A and AG11132) were assessed using BrdU incorporation assays after treatment with different polyhydroxy flavones, including nor-wogonin, wogonin, and wogonoside (Fig. 1A). We found that nor-wogonin inhibited the proliferation of TNBC cells in a dose-dependent manner; however, it had little or no impact on the growth of the non-tumorigenic breast cells (Fig. 1B). A head-to-head comparison of anti-proliferative effect against TNBC cell lines showed that nor-wogonin (40 μM) was more potent than wogonin (100 μM) or wogonoside (100 μM) in all lines tested. Unlike nor-wogonin, wogonin and wogonoside significantly reduced the growth of the non-tumorigenic breast cells (Fig. 1B). These findings were further confirmed using an alternate assay to monitor cell viability, as determined using the widely-accepted trypan blue exclusion assay. Nor-wogonin (80 μM) reduced the percent viability of MDA-MB-231, BT-549, HCC70, and HCC1806 cells to 32, 31.8, 40.5, and 35.3 %, respectively. Nor-wogonin did not significantly reduce the viability of MCF-10A and AG11132 cells. The IC_{50s} of nor-wogonin in MDA-MB-231, BT-549, HCC70, and HCC1806 were 32.24, 56.2, 39.05 and 37.3 μM, respectively, while the IC_{50s} of nor-wogonin for non-tumorigenic breast cells (MCF-10A and AG11132) were more than 100 μM. Therefore, the effects of nor-wogonin on proliferation and viability is more evident in cancer cells when compared to non-tumorigenic cells.

Nor-wogonin induces cell cycle arrest in TNBC cells that can be correlated with modulation of the expression regulators of cell cycle progression

To elucidate the mechanism of action underlying the antiproliferative effects of nor-wogonin in TNBC cells, next we tested whether nor-wogonin affected cell cycle progression of MDA-MB-231 cells. As shown in Figure 2A and 2B, treatment of MDA-MB-231 cells with nor-

wogonin resulted in a dose-dependent increase in the percentage of cells in the G_1 phase with a concomitant decrease in the percentage of those in the S phase, and a dose- and time-dependent increase in the G_2/M phases. These results suggest that nor-wogonin induces cell cycle arrest at both the G_1 and G_2/M phases, although the induction of G_2/M arrest was more significant. To determine the molecular mechanism by which nor-wogonin induced cell cycle arrest, we examined the effects of nor-wogonin on the expression of a few well-known cell cycle regulatory proteins, e.g., the cyclin dependent kinase inhibitor p21, cyclin dependent kinases (CDK1 and CDK4), and cyclins (cyclin B1 and cyclin D1). We observed that nor-wogonin upregulated p21 protein expression and downregulated cyclin D1, cyclin B1, and CDK1 protein expressions in a dose-and time-dependent manner (Fig. 2C, 2D, 2E, and 2F). Moreover, nor-wogonin downregulated CDK4 in a dose-dependent manner only (Fig. 2C and 2E). These results show that nor-wogonin induced cell cycle arrest in TNBC that can be correlated with upregulation of p21 and downregulation of expression of cyclins and CDKs that regulate cell cycle progression.

Nor-wogonin induces apoptosis in TNBC cells via a caspase-dependent mitochondrial mechanism

The cytotoxic effects of nor-wogonin (Fig. 1C), as well as nor-wogonin-induced increase in the percentage of cells in the sub G_1 phase (Fig. 2A and 2B) suggested that nor-wogonin might induce apoptosis. To test this hypothesis, we further characterized the proapoptotic effects of nor-wogonin in MDA-MB-231 cells. Results from flow cytometry experiments indicated that nor-wogonin increased the percentage of both early and late apoptotic cells in a dose-dependent manner (annexin-V positive cells; Fig. 3A). Mitochondrial changes, including loss of mitochondrial membrane potential ($\Delta\Psi m$), are considered as key events in phytochemical agents-induced apoptosis in cancer cells. Thus, the effects of nor-wogonin on the mitochondria, in particular the changes in $\Delta\Psi m$, were examined using the lipophilic dye JC-1. Results presented in Fig. 3B indicate that nor-wogonin induced decreases in $\Delta\Psi m$ in a dose-dependent manner in MDA-MB-231 cells. To further investigate whether nor-wogonin induced apoptosis by triggering the mitochondrial apoptosis pathway, we measured the expression levels of the antiapoptotic protein (Bcl-2) and proapoptotic proteins (Bax and caspase-3), which are involved in the mitochondrial apoptosis pathway. Western blot analysis results showed that nor-wogonin downregulated the expression of Bcl-2 and upregulated the expression of Bax, resulting in an increase in Bax/Bcl-2 ratio (Fig. 3C and 3D). Moreover, nor-wogonin induced caspase-3 activation (Fig. 3C and 3E). To test whether caspase-3 pathway was essential for nor-wogonin-induced apoptosis, the pan-caspase inhibitor, Z-VAD-FMK was used. We found that Z-VAD-FMK reduced nor-wogonin-induced caspase-3 cleavage and percentage of apoptotic cells (Fig. 3F and 3G). These results indicated that nor-wogonin might induce apoptosis *via* a caspase-3 dependent mitochondrial pathway.

Nor-wogonin suppressed the activation of NF-κB and STAT3 pathways that can be correlated with suppression of TAK1 expression in TNBC cells

NF-κB and STAT3 are transcription factors involved in cancer progression. Activation of both NF-κB and STAT3 can contribute to TNBC cell proliferation and resistance to apoptosis [8, 10]. To further understand the molecular mechanisms by which nor-wogonin

induced cell cycle arrest and apoptosis, we next investigated whether nor-wogonin affected the NF-κB and STAT3 pathways in MDA-MB-231 cells using Western blot analysis. As shown in Figure 4A and 4B, nor-wogonin treatment resulted in a dose-dependent decrease in NF-κB (p65), in the nuclear fraction as well as the whole cell lysate (WCL), and phospho-IκBα protein levels and increase in that of IκBα. Nor-wogonin inhibited the phosphorylation of STAT3 at Ser727 in a dose-dependent manner, resulting in a decrease in pSTAT3/STAT3 ratio (Fig. 4C and 4D). Both NF-κB and STAT3 are downstream targets for TAK1. Therefore, to understand of the molecular basis for the inhibitory effects of nor-wogonin on NF-κB and STAT3 expression, the effects of nor-wogonin on the expression of TAK1 was explored. We found that nor-wogonin reduced the expression of TAK1 in MDA-MB-231 cells, compared to that in the control (Fig. 4E and 4F). To test whether or not nor-wogonin-inhibited NF-κB, STAT3, and TAK1 pathways is selective for TNBC cells when compared to non-tumorigenic cells, we tested the effect of nor-wogonin on the expression of NF-κB (p65), p-STAT3, STAT3, and TAK1in MCF-10A non-tumorigenic breast cells. Results in Fig. 5A and 5B showed that nor-wogonin (40 μM) had no effect on the expression of NF-κB (p65), p-STAT3, STAT3, and TAK1in MCF-10A non-tumorigenic breast cells. These results showed that nor-wogonin inhibited the activation of the NF-κB and STAT3 pathways in TNBC cells that can be correlated with suppression of TAK1 expression in TNBC cells.

Discussion

Flavonoids constitute common components in human diet that exhibit many health benefits. Among them, polyhydroxy flavones, such as nor-wogonin and wogonin have been reported to exhibit many pharmacological activities, and includes anxiolytic, antimicrobial, and anticancer activities [13–16]. Previously, nor-wogonin showed more potent anti-leukemic effects than wogonin due to potent proapoptotic activity [16]. However, unlike wogonin, the anticancer effects and mechanisms of actions of nor-wogonin were poorly understood. Here we sought to investigate the anticancer activity and mechanisms of action of nor-wogonin in human TNBC cells. Our findings showed that nor-wogonin is both potent and specific anti-cancer agent; it selectively inhibited the proliferation and induced cytotoxicity in four human TNBC cell lines, MDA-MB-231, BT-549, HCC70, and HCC1806, (IC_{50}: 32.24–56.2 μM), whereas its effects in non-tumorigenic breast cells (MCF-10A and AG11132) were minimal (IC_{50} > 100 μM). Furthermore, nor-wogonin is a more potent antiproliferative and cytotoxic agent in TNBC cells when compared to structurally related anticancer compounds, such as wogonin (50–200 μM) and wogonoside (50–400 μM) [17, 18]. From a structure-activity relationship (SAR) point of view, methoxylated flavones are less active *in vitro* as antiproliferative compounds, in comparison with the hydroxylated flavones. For example, Chow *et al.*, analyzed the effect of six structurally related compounds, including 5-OH, 7-OH, 5,7-diOH, 5,7-diOCH(3), 7,8-diOCH(3), and 7-OCH(3)-8-OH flavones on HL-60 cells [16]. Their results suggest that the hydroxyl group at C5 and C7 is essential for the anticancer activity of flavones [16]. Moreover, Androutsopoulos *et al.*, reported that hydroxylation of eupatorin in MDA-MB-468 human breast cancer cells as a result of CYP1-family enzymes-mediated metabolism can enhance its anti-proliferative effect. Our results are consistent with the above findings with regards to the importance of the hydroxyl groups

in nor-wogonin, when compared to the methoxy (in wogonin) or the glycosides (in wogonoside) groups for anti-cancer activities [19].

The antiproliferative effects of polyhydroxy flavone derivatives have been previously shown to involve cell cycle arrest at the G_1 and G_2/M phases [20,21]. We found that nor-wogonin inhibited the growth of MDA-MB-231 cells *via* cell cycle arrest at both the G_1 and G_2/M phases, although the induction of G_2/M arrest was more significant. The mammalian cell cycle is controlled by cyclins and CDKs. Progression through the G_1 phase is activated by the binding of cyclin D family (D1, D2, and D3) to CDK4 or its homolog, CDK6 (CDK4/CDK6). Progression through the G_2/M phase is activated by the binding of cyclin B to CDK1 [22–24]. The CDK inhibitor p21, also known as $p21^{waf1/cip1}$, is a well-known inhibitor of cell cycle and can arrest cell cycle progression at the G_1/S and G_2/M transitions by inhibiting CDK4,6/cyclin-D and CDK1/cyclin-B respectively [25]. In our study, we showed that nor-wogonin upregulated the expression of p21 in a dose- and time-dependent manner. Moreover, nor-wogonin downregulated the expression of the G_1 and G_2/M regulatory proteins in a dose-dependent manner. While cyclin D1 is typically associated with G_1/S phase transition, cyclin D1 levels are also increased during the G_2 phase [26,27]. Together our results suggest nor-wogonin-induced cell cycle arrest in MDA-MB-231 cells, which may be attributed to direct upregulation of p21 and downregulation of other regulators of the cell cycle including cyclin D1, cyclin B1, and CDK1.

Apoptosis is another possible mechanism that can contribute to the cytotoxicity of polyhydroxy flavones in cancer cells [16]. For example, approximately 50% of TNBC cells underwent apoptosis after treatment with 100 μM wogonin [17] while around 60% of osteosarcoma cells underwent apoptosis after treatment with 75 μM of wogonoside [28]. Herein, approximately 60% of TNBC cells underwent apoptosis after treatment with 40 μM of nor-wogonin which indicates that nor-wogonin is a more potent pro-apoptotic agent than its structurally related compounds, wogonin and wogonoside. Mechanistically, apoptosis can be induced through two different pathways that are known as the death receptor (extrinsic) and mitochondrial (intrinsic) pathways [29]. Changes in the mitochondrial membrane potential ($\Delta\Psi m$) have been considered to be early events in the mitochondrial pathway [30, 31]. Along this line, we observed that nor-wogonin induced loss of $\Delta\Psi m$. which may support the ability of these phytochemicals to induce apoptosis via an intrinsic mitochondrial pathway. Some pro-apoptotic members of Bcl-2 family such as Bax and Bak can form membrane channels, resulting in the loss of $\Delta\psi m$ [32]. On the contrary, anti-apoptotic molecules such as Bcl-2 can prevent the conformational change and oligomerization of Bax and Bak [33, 34]. Furthermore, induction of apoptosis is correlated with an increase in the ratio of proapoptotic/antiapoptotic Bax/Bcl-2 [35, 36]. Our results showed that nor-wogonin treatment downregulated Bcl-2 protein expression and upregulated Bax protein expression, resulting in an increase in Bax/Bcl-2 ratio, which could be a possible mechanism by which nor-wogonin induced a loss in the $\Delta\psi m$. An increase in the mitochondrial membrane permeability results in the release of some apoptotic proteins and eventually caspase-3 activation [37]. Our data showed that nor-wogonin activated caspase-3 in TNBC cells. In addition, nor-wogonin-induced increase in the percent of annexin V positive cells was significantly attenuated in the presence of the pan caspase inhibitor, Z-

VAD-FMK, suggesting that nor-wogonin-induced apoptosis through a caspase-dependent mitochondrial pathway.

NF-κB, is a transcription factor that is present as an inactive complex bound to its endogenous inhibitor, IkB in the cytoplasm [38]. Phosphorylation of IkB induces its degradation and release of NF-κB from the complex, allowing NF-κB to translocate to the nucleus [39]. Nuclear translocation of NF-κB activates genes involved in cell proliferation (cyclins/CDKs), thereby promoting cell growth [40]. Furthermore, there is a positive correlation between the activation of NF-κB and increase in the expression of antiapoptotic Bcl-2 family members, resulting in resistance to apoptosis [41]. Many polyhydroxy flavones have been shown to block NF-κB activation leading to tumor growth inhibition. For example, wogonoside (50–150 μM) inhibited the activation of NF-κB signaling *via* suppression of tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2) and TRAF4 expression [42]. In addition, wogonin (15–60 μM) blocked the NF-κB pathway *via* inhibition of the phosphorylation of IkBα and inhibitor-kappa B kinase alpha (IKKα) in B16-F10 cells [43]. In the present study, we observed that nor-wogonin treatment (20–40 μM) significantly downregulated expression of NF-Kb/p65 that can be correlated with a reduction in the level of phospho-IkBα. Since nor-wogonin treatment reduced the expression of Bcl-2 and cyclin/CDK proteins, which are downstream targets of NF-κB, nor-wogonin-induced apoptosis and cell cycle arrest may be partially attributed to the inhibition of the NF-κB pathway.

STAT3 pathway is a major pathway that plays critical roles in the pathogenesis of many cancers, including breast cancer [44]. Constitutively active STAT3 induces gene expression changes and molecular events that result in dysregulated cell growth, resistance to apoptosis, and promotion of metastasis [45]. The present study showed that nor-wogonin (10–40 μM) inhibited STAT3 activation in MDA-MB-231 cells *via* inhibition of STAT3 phosphorylation at Ser727. Interestingly, wogonin showed similar effects in $CD4^+$ T cells, but at a slightly higher concentration (50 μM) [46]. Overall, inhibition of STAT3 and NF-κB activation could be the primary underlying mechanisms for nor-wogonin-induced cell cycle arrest and apoptosis in MDA-MB-231 cells. Furthermore, we investigated the effects of nor-wogonin on an upstream regulator of STAT3 and NF-κB in breast cancer cells, TAK1. TAK1 is a mitogen-activated protein (MAP) kinase that plays important roles in the activation of NF-κB and STAT3. TAK1-mediated NF-κB activation involves direct phosphorylation of IKK complex [47]. Moreover, Ohkawara *et al.* showed that the TAK1-NLK pathway plays a critical role in the phosphorylation of STAT3 at Ser727 [7]. In the present study, we found that nor-wogonin-induced inhibition of the NF-κB and STAT3 pathways in MDA-MB-231 cells can be correlated with a decrease in TAK1 expression (Fig. 4E and 4F). Moreover, the inhibitory actions of nor-wogonin on NF-κB, STAT3, and TAK1 pathways were not evident in a non-tumorigenic cell line (Fig. 5A and 5B), which is consistent with selective antiproliferative and cytotoxic effects on TNBC cells when compared to non-tumorigenic cells.

In summary, our studies provided critical insights into the mechanistic basis for the antitumor activities of nor-wogonin in TNBC cells. Nor-wogonin inhibited the growth of TNBC cells by inducing cell cycle arrest and apoptosis. Furthermore, nor-wogonin inhibited

TAK1 expression, which can be correlated with downregulation of NF-κB and STAT3 pathways. Therefore, nor-wogonin might be an attractive multi-target candidate drug for treatment of TNBC.

Financial support

This research did not receive any funding.

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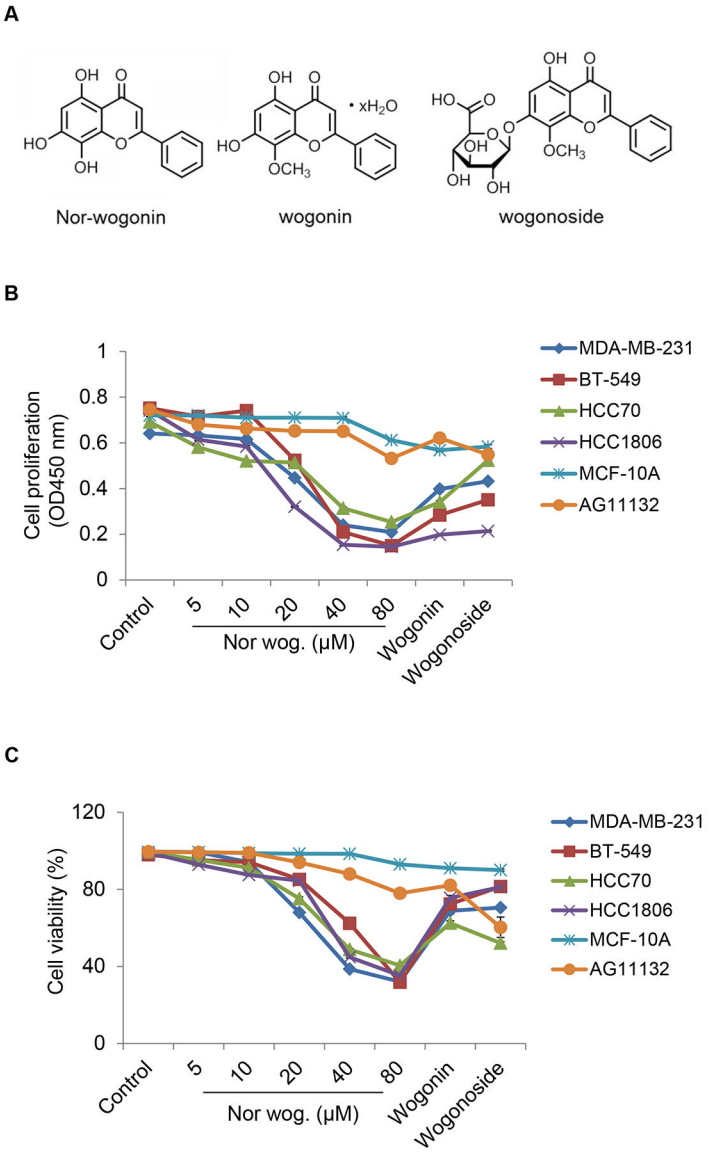

Fig. 1.

Proliferation and viability of TNBC cells and non-tumorigenic breast cells after treatment with nor-wogonin and structurally related compounds. **A**. Chemical structures of nor-wogonin, wogonin, and wogonoside. **B**. Effects of nor-wogonin (5–80 μM), wogonin (100 μM), and wogonoside (100 μM) on the proliferation of TNBC cells (MDA-MB-231, BT-549, HCC70, and HCC1806) and non-tumorigenic breast cells (MCF-10A and AG11132) were determined by BrdU incorporation assays. Dimethyl sulfoxide (DMSO vehicle) was used as a negative control. **C**. The cytotoxic effects of nor-wogonin (5–80 μM), wogonin (100 μM), and wogonoside (100 μM) on TNBC cells (MDA-MB-231, BT-549, HCC70, and HCC1806) and non-tumorigenic breast cells (MCF-10A and AG11132) were determined by trypan blue exclusion assays. DMSO was used as a vehicle control. Data are expressed as the means ± SD based on three independent experiments.

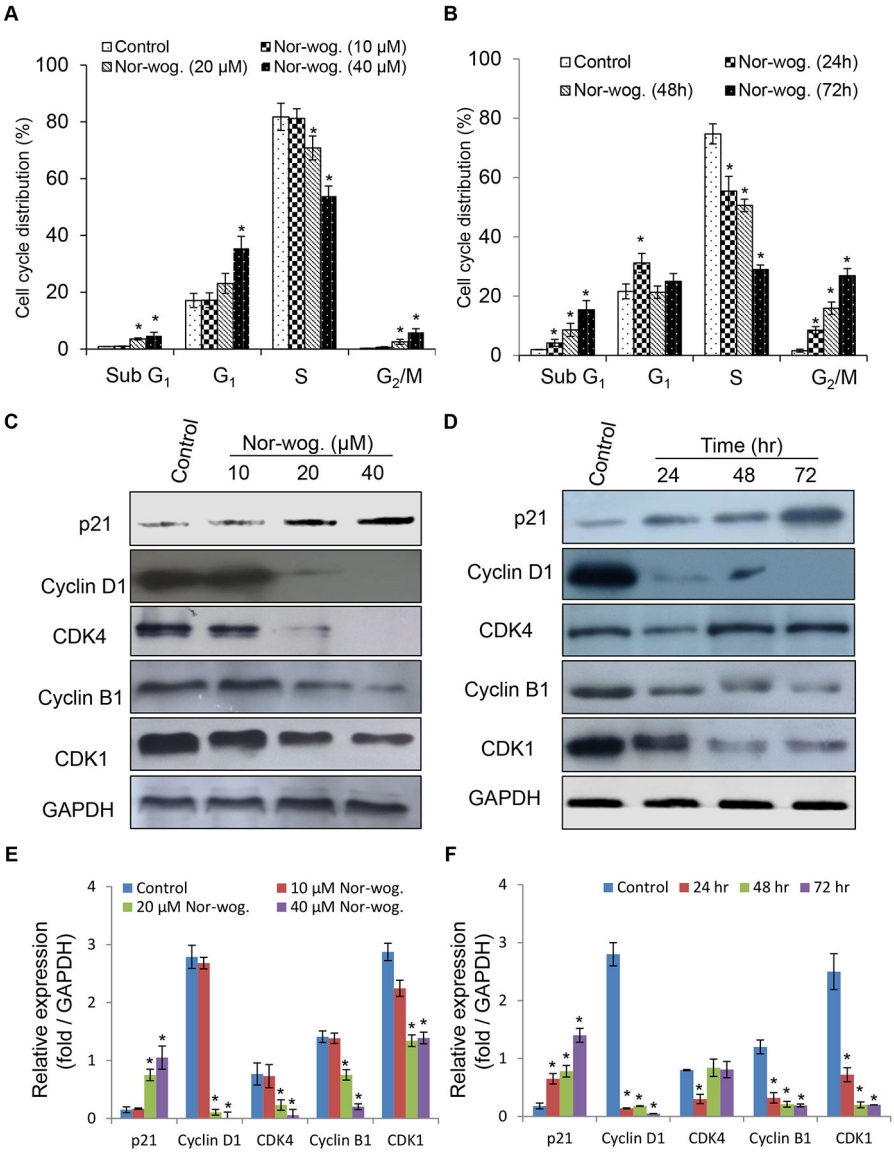

Fig. 2.

Effects of nor-wogonin on cell cycle distribution and cell cycle regulatory proteins in MDA-MB-231 cells. **A**. Percentage of cells in the sub G_1, G_1, S, and G_2/M phases after 24-h incubation with DMSO or 10, 20, or 40 μM nor-wogonin. **B**. Percentage of cells in the sub G_1, G_1, S, and G_2/M phases after 24, 48, and 72-h incubation with DMSO or 30 μM nor-wogonin. **C**. Western blot analyses for determination of the expression of cell cycle regulatory proteins (p21, cyclin D1, CDK4, cyclin B1, and CDK1) following 24-h treatment with nor-wogonin (10–40 μM), compared to the negative control (DMSO) **D**. Western blot analyses for determination of the expression of cell cycle regulatory proteins (p21, cyclin D1, CDK4, cyclin B1, and CDK1) following 24, 48, or 72-h treatment with nor-wogonin (30 μM), compared to the negative control (DMSO). **E**. The protein expression levels of p21, cyclin D1, CDK4, cyclin B1, and CDK1 relative to GAPDH, following 24 h treatment with nor-wogonin (10–40 μM) were quantified using ImageJ software. **F**. The protein expression

levels of p21, cyclin D1, CDK4, cyclin B1, and CDK1 relative to GAPDH, following 24, 48, and 72-h treatment with nor-wogonin (30 μM) were quantified using ImageJ software Values represent the means ± SD of three independent experiments. *$p < 0.05$ indicate significant differences, compared to the DMSO-treated control.

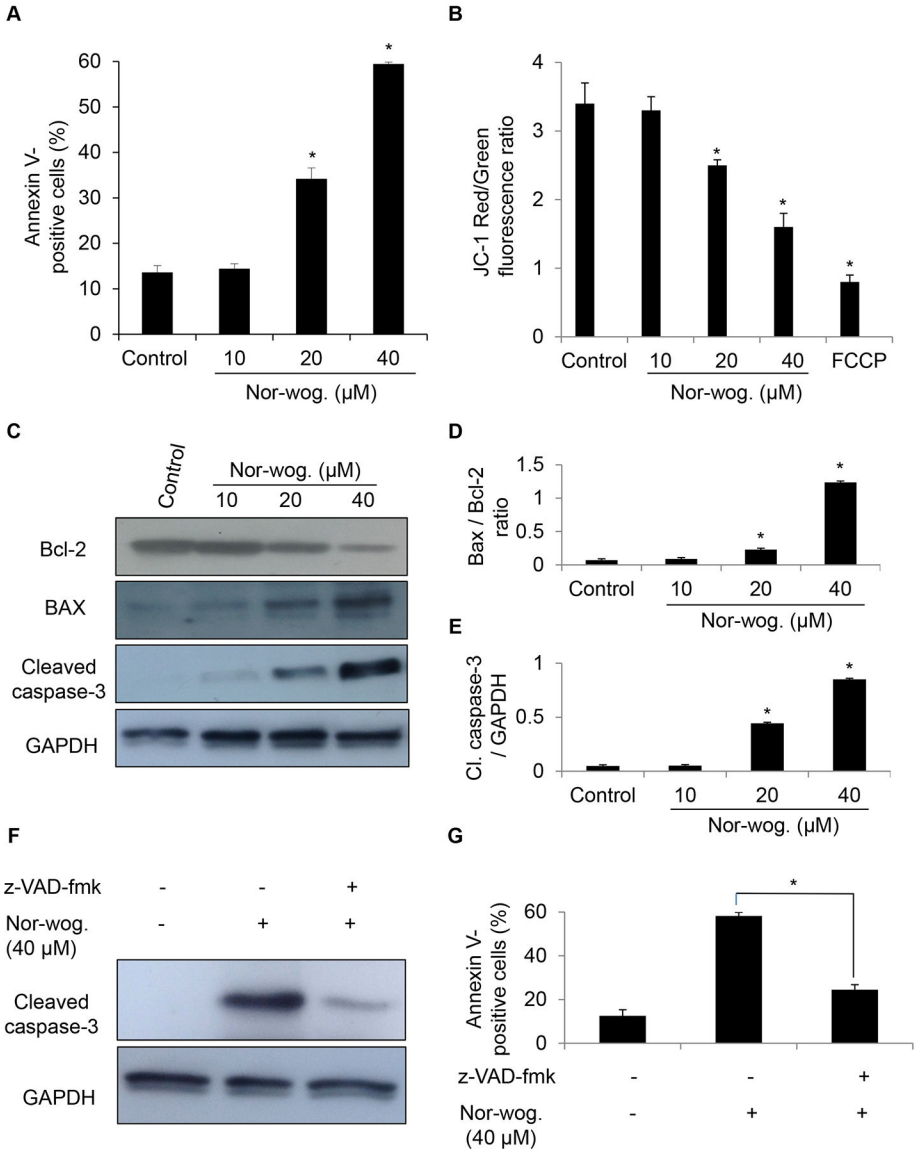

Fig. 3.

Proapoptotic actions of nor-wogonin in MDA-MB-231 cells. **A**. Quantification of annexin V-positive apoptotic cells after treatment with nor-wogonin **B**. The average JC-1 red/green fluorescence ratio in MDA-MB-231 cells **C**. The expression of Bcl-2, Bax, and cleaved caspase-3 were determined by western blot analyses. GAPDH served as a loading control. **D**. Bax/Bcl-2 ratio was quantified by ImageJ software. **E**. The protein expression levels of cleaved caspase-3 relative to GAPDH, were quantified by ImageJ software. **F**. The expression levels of cleaved caspase-3 in vehicle or nor-wogonin (40 μM)-treated cells in the absence or presence of the pan-caspase inhibitor, Z-VAD-FMK (30 μM) were determined by western blot analysis. DMSO and GAPDH were used as negative and loading controls, respectively. **G**. Proportion of annexin-V positive cells after 24-h incubation with vehicle or nor-wogonin (40 μM)-treated cells in the absence or presence of the pan-caspase inhibitor, Z-VAD-FMK (30 μM). Values represent the means ± SD of three independent experiments.

*$p < 0.05$ indicate significant differences, compared to the control or nor-wogonin-treated cells.

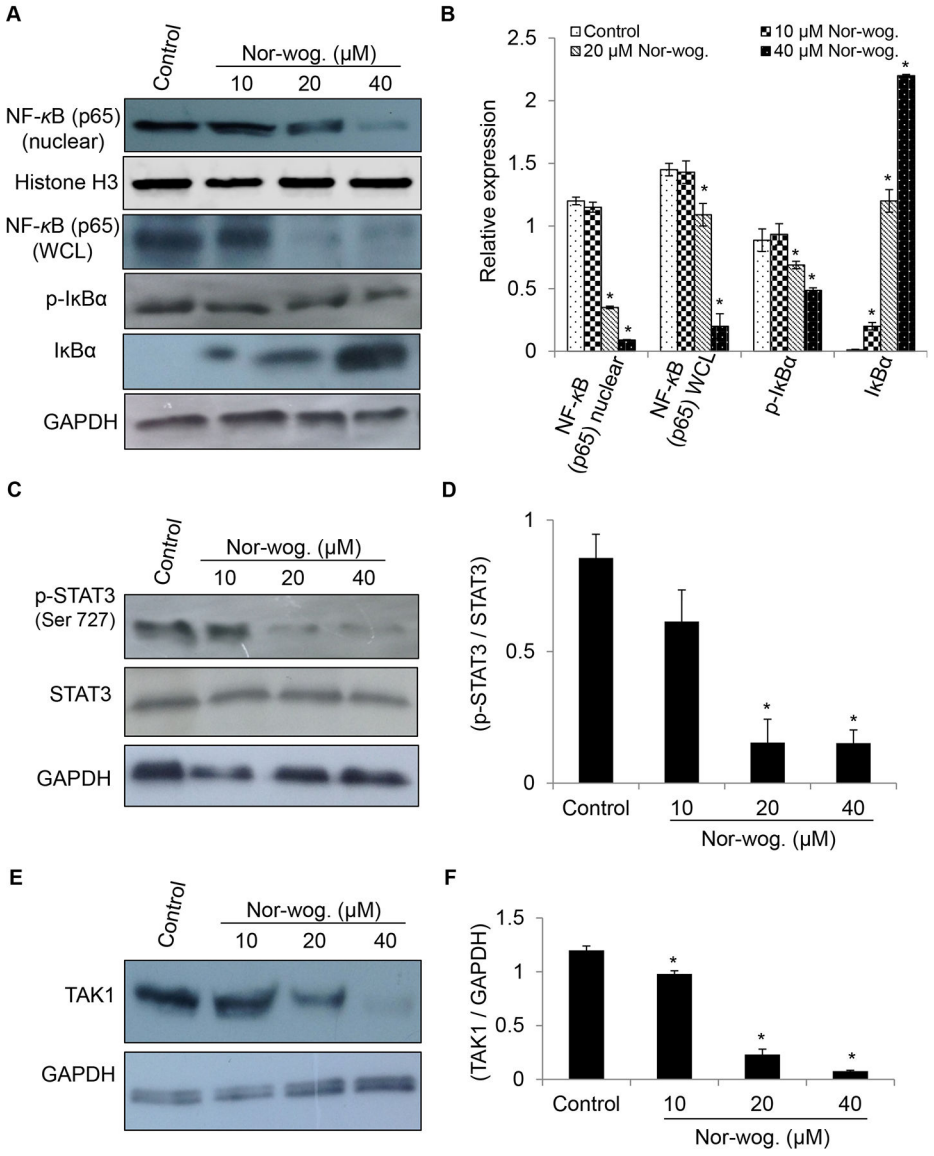

Fig. 4.

Effects of nor-wogonin on expression of NF-κB, STAT3, and TAK1 in MDA-MB-231 cells. **A**. The expression levels of NF-κB (p65), phospho-IKBα, and IKBα were determined by western blot analyses. **B**. The protein expression level of NF-κB (p65; nuclear fraction) was quantified relative to Histone H3, while the protein expression levels of NF-κB (p65; whole cell lysate), phospho-IKBα, and IκBα were quantified relative to GAPDH. **C**. The expression of p-STAT3 (Ser727) and STAT3 were determined by western blot analyses. **D**. The protein expression levels of p-STAT3 relative to STAT3 were quantified. **E**. The expression of TAK1 was measured by western blot analyses. **F**. The protein expression levels of TAK1 were quantified relative to GAPDH. Values represent the means ± SD of three independent experiments. *$p < 0.05$ indicate significant differences, compared to the negative control.

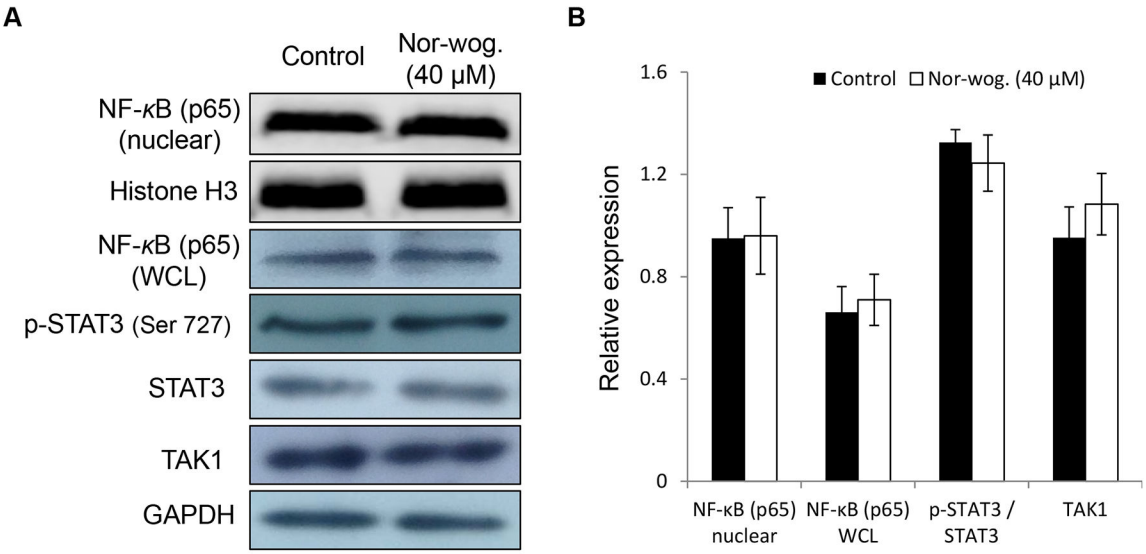

Fig. 5.

Effects of nor-wogonin on expression of NF-κB, STAT3, and TAK1 in MCF-10A cells. **A**. The expression levels of NF-κB (p65), p-STAT3 (Ser727), STAT3, and TAK1 were determined by western blot analyses. **B**. The protein expression level of NF-κB (p65; nuclear fraction) was quantified relative to Histone H3, while the protein expression levels of NF-κB (p65; whole cell lysate), p-STAT3 (Ser727), STAT3, and TAK1 were quantified relative to GAPDH. Values represent the means ± SD of three independent experiments.