Role of NOX1 and NOX5 in protein kinase C/reactive oxygen species-mediated MMP-9 activation and invasion in MCF-7 breast cancer cells

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Abstract. NADPH oxidases (NOXs) are a family of membrane proteins responsible for intracellular reactive oxygen species (ROS) generation by facilitating electron transfer across biological membranes. Despite the established activation of NOXs by protein kinase C (PKC), the precise mechanism through which PKC triggers NOX activation during breast cancer invasion remains unclear. The present study aimed to investigate the role of NOX1 and NOX5 in the invasion of MCF-7 human breast cancer cells. The expression and activity of NOXs and matrix metalloprotease (MMP)-9 were assessed by reverse transcription-quantitative PCR and western blotting, and the activity of MMP-9 was monitored using zymography. Cellular invasion was assessed using the Matrigel invasion assay, whereas ROS levels were quantified using a FACSCalibur flow cytometer. The findings suggested that NOX1 and NOX5 serve crucial roles in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced MMP-9 expression and invasion of MCF-7 cells. Furthermore, a connection was established between PKC and the NOX1 and 5/ROS signaling pathways in mediating TPA-induced MMP-9 expression and cellular invasion. Notably, NOX inhibitors (diphenyleneiodonium chloride and apocynin) significantly attenuated TPA-induced MMP-9 expression and invasion in MCF-7 cells. NOX1- and NOX5-specific small interfering RNAs attenuated TPA-induced MMP-9 expression and cellular invasion. In addition, knockdown of NOX1 and NOX5 suppressed TPA-induced ROS levels. Furthermore, a PKC inhibitor (GF109203X) suppressed TPA-induced intracellular ROS levels, MMP-9 expression and NOX activity in MCF-7 cells. Therefore, NOX1 and NOX5 may serve crucial roles in TPA-induced MMP-9 expression and invasion of MCF-7 breast cancer cells. Furthermore, the present study indicated that TPA-induced MMP-9 expression and cellular invasion were mediated through PKC, thus linking the NOX1 and 5/ROS signaling pathways. These findings offer novel insights into the potential mechanisms underlying their anti-invasive effects in breast cancer.

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Introduction

Breast cancer, characterized by malignant solid tumors originating from mammary gland epithelium, is a major cause of mortality among women worldwide (1). Notably, the 5-year overall survival rate remains poor, primarily owing to the metastasis of cancer cells to vital organs such as the lungs, brain, and bones (2-4). Therefore, elucidating the molecular mechanisms underlying breast cancer metastasis is imperative.

The metastatic cascade involves a complex array of biological processes, including extracellular matrix (ECM) degradation; ECM, a crucial noncellular tissue component, provides structural and biochemical support essential for cellular functions (5,6). Degradation of ECM requires the involvement of several extracellular proteinases, among which matrix metalloproteases (MMPs), a family of zinc-dependent proteinases, play pivotal roles in pathological processes, including breast cancer (7). Notably, MMP-9 is a key player in cancer cell invasion and metastasis. MMP-9 expression is induced through the activation of various intracellular signaling proteins through the stimulation of probol esters,

inflammatory cytokines, epidermal growth factors (EGFs), or phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) (8-10).

TPA, a selective activator of protein kinase C (PKC), orchestrates a cascade of events leading to the production of reactive oxygen species (ROS) either upstream or downstream (11-13). Consequently, ROS modulate several intracellular signaling pathways, including protein kinase B (AKT) and mitogen-activated protein kinases (MAPKs) (14-16), thereby influencing the activation of transcription factors such as activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB), which are intricately associated with MMP expression during tumor invasion and metastasis (15-19).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) are a family of membrane proteins involved in intracellular ROS production and facilitate electron transfer across biological membranes. NOXs catalyze superoxide generation at the plasma membrane, subsequently releasing it into the extracellular space, where it is converted into ROS, such as hydrogen peroxide and superoxide anions (12,13). NOXs are closely associated with phosphatidylinositol 3-OH kinase (PI3K) signaling; PKC, a downstream molecule of PI3K, is essential for superoxide generation via NOXs (14,20,21). PKC mediates the activation of NOXs (22); however, the mechanism underlying NOX activation via PKC during breast cancer invasion remains unclear.

In this study, we investigated whether PKC regulates ROS production through NOXs during cell invasion of MCF-7 cells, a human breast cancer cell. Furthermore, we confirm the significance of NOXs in TPA-induced MMP-9 expression and cell invasion in these cells. We believe that our findings would help broaden our understanding of the molecular mechanisms underlying breast cancer metastasis.

Materials and methods

Cells and reagents. MCF-7 cells (cat. no. HTB-22) were procured from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum (FBS) and 1% antibiotics. The cell culture was maintained in a controlled environment with a temperature of 37˚C in a 5% CO_2 atmosphere in an incubator. β-actin antibody (cat. no. A5441), bovine serum albumin (BSA), skim milk, TPA, diphenyleneiodonium chloride (DPI), and apocynin (APO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MMP-9 (cat. no. SC-12759) and goat anti-mouse IgG-HRP (cat. no. sc-2005) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). GF109203X (PKC inhibitor; GF) was purchased from Abcam (Cambridge, MA, USA). Phosphate-buffered saline (PBS), FBS, and DMEM were purchased from Gibco-BRL (Gaithersburg, MD, USA).

RNA interference. NOX1-5-specific and control siRNAs were purchased from BIONEER (Daejeon, Korea). The siRNA sets used for amplification were as follows: NOX1, sense: 5'-GAGCAUGAAUGAGAGUCAU-3', antisense: 5'-AUGACUCUCAUUCAUGCUC-3'; NOX2, sense: 5'-GUAAUGUCAGUGGAAGUUA-3', antisense: 5'-UAACUUCCACUGACAUUAC-3'; NOX3, sense: 5'-CACCAUGUUUUCAUCGUCU-3', antisense: 5'-AGACGAUGAAAACAUGGUG-3'; NOX4, sense: 5'-CAGAGUUUACCCAGCACAA-3', antisense: 5'-UUGUGCUGGGUAAACUCUG-3'; NOX5, sense: 5'-GUGACUACUUGUAUCUGAA-3', antisense: 5'-UUCAGAUACAAGUAGUCAC-3'; control siRNA, sense: 5'-UUCUCCGAACGUGUCACGU-3', antisense: 5'-ACGUGACACGUUCGGAGAA-3'. The cells were transfected with NOX1 (30 pmol) and NOX3 (30 pmol) siRNAs for 24 h and with NOX2 (30 pmol), NOX4 (100 pmol), and NOX5 (100 pmol) siRNAs for 48 h. Control siRNA transfections were also conducted for each respective time point. All siRNA transfections in MCF-7 cells were performed using Lipofectamine™ RNAiMAX (Invitrogen, San Diego, CA, USA), in accordance with the manufacturer's forward transfection protocol.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from cells using TRIzol® (Life Technologies, Grand Island, NY, USA) following the manufacturer's protocol. RNA concentration and purity were calculated using BioSpec-nano (Shimadzu, Kyoto, Japan). cDNA was synthesized using 1 μg total RNA using a PrimeScript™ RT reagent Kit (cat. no. RR047A; TaKaRa, Shiga, Japan). The RT-qPCR cycling protocol comprised an initial denaturation step at 95˚C for 10 min, followed by 40 cycles of amplification consisting of denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 1 min. Subsequently, a melting curve analysis was conducted with temperature ramping from 95˚C for 15 sec, followed by annealing/extension at 60˚C for 1 min, and concluding with a final denaturation step at 95˚C for 15 sec. The mRNA expression levels of MMP-9, NOX1, NOX5, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were analyzed using the ABI PRISM 7900 sequence detection system and SYBR Green (Applied Biosystems, Foster City, CA, USA). Relative quantitation was performed using the comparative $2^{-\Delta\Delta Cq}$ method (23). The primer sets used for amplification were as follows: MMP-9 (NM 004994), forward primer: 5'-CCTGGAGACCTGAGAACCAATCT-3', reverse primer: 5'-CCACCCGAGTGTAACCATAGC-3'; GAPDH (NM 002046), forward primer: 5'-ATGGAAATCCCATCACCATCTT-3', reverse primer: 5'-CGCCCCACTTGATTTTGG-3'; Primers for NOX1 (NM_007052, cat. no. PPH06068A) and NOX5 (NM_024505, cat. no. PPH17569A) were obtained from QIAGEN (Hilden, Germany). To account for variations in mRNA concentration, the expression levels of MMP9, NOX1, and NOX5 were normalized to that of the housekeeping gene, GAPDH. Relative quantification was analyzed using the comparative $2^{-\Delta\Delta Cq}$ method, following the manufacturer's instructions.

Quantification of intracellular ROS. The intracellular ROS was detected using an oxidation-sensitive fluorescent probe dye (CM-H_2DCFDA; Invitrogen). MCF-7 cells were stimulated with 20 nM TPA for 24 h, after which the cells were incubated with 10 μM CM-H_2DCFDA at 37˚C for 30 min. CM-H_2DCFDA was oxidized to the green fluorescent dichlorofluorescein (DCF) using hydrogen peroxide. The DCF fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA). ROS production was expressed as the mean fluorescence intensity and analyzed using the CellQuest software (BD Biosciences).

Western blot analysis. The cells ($7x10^5$) were incubated with 20 nM TPA for 24 h at 37˚C. Following the treatments, cells were lysed with ice-cold radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Rockford, IL, USA) for 30 min on ice, and the protein concentration in the resulting lysates was determined using a Bio Spec-nano (Shimadzu). Subsequently, 20 μg of protein samples were resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond™ polyvinylidene fluoride (PVDF) membranes (GE Healthcare Life Sciences, Buckinghamshire, UK) through western blotting. Following the blocking with 5% BSA or skim milk for 2 h at 4˚C, the membranes were cropped around 70 kDa. Subsequently, the upper and lower portions of the cropped membranes were incubated overnight at 4˚C with the primary antibody (1:2,500) targeting MMP-9 (92 kDa) and β-actin (45 kDa), respectively, HRP-conjugated IgG (1:2,500) served as the secondary antibody for 1 h at 4˚C. Protein expression levels were determined through signal analysis using a MINI HD6 image analyzer (UVITEC, Cambridge, UK).

Matrigel invasion assay. The invasion of MCF-7 cells was assessed using a 24-well cell culture insert (8-μm pore size) coated with rehydrated 20 μl Matrigel™ (Corning Life Sciences, Corning, NY, USA) in culture medium for 30 min. Suspended cells ($4x10^5$) and chemical attractant, 20 nM of TPA, in 0.5 ml culture medium (supplemented with 10% FBS and 1% antibiotics) were transferred into the upper and lower chambers. Following 24 h incubation in a 5% CO_2 incubator at 37˚C, the cells on the upper side of the membrane were gently removed using cotton swabs. The cells that invaded the lower chamber through the membrane were fixed with a 10% formalin solution for 30 min at room temperature. Following fixation, they were stained with 0.2% crystal violet for an additional 30 min at room temperature. Subsequently, the invading cells were counted in five random areas of the membrane under a light microscope.

Gelatin zymography assay. MCF-7 cells were pre-treated with DPI (5 μM) or APO (300 μM) for 1 h and then stimulated with TPA (20 nM) for 24 h at 37˚C in serum-free DMEM medium. The collected culture medium was suspended in a zymography sample buffer. Electrophoresis was performed under non-reducing conditions using 10% sodium dodecyl sulfate-polyacrylamide gel containing 0.1% (w/v) gelatin. Following electrophoresis, the gel was washed with 2.5% Triton X-100 for 30 min under gentle agitation. Subsequently, the gel was incubated overnight at 37˚C in a developing solution (50 mM Tris-HCl, 5 mM $CaCl_2$, 100 mM NaCl, 0.02% Brij-35; pH 7.5). After incubation, the gel was stained with 0.25% Coomassie blue R-250 at room temperature for 30 min and washed with a destaining solution (40% methanol and 7% acetic acid) until the bands were visible. The proteolytic activity of MMP-9 was measured by comparing the transparent bands resulting from the decomposition of gelatin. Bands were visualized using a MINI HD6 image analyzer (UVITEC).

NOX activity assay. Cells were washed twice in ice-cold PBS and then scraped from the plate using the same solution. Subsequently, the cells were centrifuged at 4,000 rpm at 4˚C for 10 min, and the resulting pellet was suspended in a buffer containing 20 mM KH_2PO_4, 1 mM EGTA, 150 mM sucrose, and a protease inhibitor mixture. The cell suspension was lysed on ice for 30 min. For the assay, 20 μl of lysate was mixed with 180 μl of an assay buffer containing 250 mM HEPES (pH 7.4), 250 μM lucigenin, 1.2 mM $MgSO_4$ ($7H_2O$), 120 mM NaCl, 1.75 mM $CaCl_2$ ($2H_2O$), 11 mM glucose, 0.5 mM EDTA, 5.9 mM KCl, and 200 μM NADPH for a duration of 10 sec. Photoemission in terms of RLU was measured using the GloMax®-Multi Jr Detection System (Promega, Madison, Wisconsin, USA) every minute for 15 min.

Membrane fractionation. MCF-7 cells ($5x10^7$) were pre-treated with GF for 1 h followed by incubation with TPA for an additional hour at 37˚C. Subsequently, the cells were suspended in homogenization buffer (composed of 20 mM Tris-HCl, 2 mM EDTA, 5 mM EGTA, 5 mM DTT, and protease inhibitor; pH 7.5) and homogenized using a sonicator (5 times of 10 sec each at 10% amplitude), followed by a 30-min incubation on ice. The resulting cell lysate was then subjected to centrifugation at 16,000 x g for 15 min at 4˚C to separate the soluble (cytosolic) fraction from the pellet (membrane) fraction. The pellet fraction was further treated with solubilization buffer (homogenization buffer supplemented with 1% NP-40) for 30 min on ice, followed by another centrifugation step at 16,000 x g for 15 min at 4˚C.

Statistical analyses. Statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). Data are presented as the mean and standard error of the mean. An unpaired Student's t-test was used to compare two groups, and one-way analysis of variance with Tukey's post hoc test was used to compare independent multiple groups. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed at least in triplicate.

Results

Inhibition of NOX suppresses TPA-induced cell invasion and MMP-9 expression in MCF-7 cells. In our previous study, we reported that stimulation of MCF-7 cells with 20 nM TPA induces the expression of MMP-9 and cell invasion, which occurs through various intracellular signaling pathways (24,25). In addition, according to previous reports, breast cancer cells are treated with DPI at concentrations from 1 to 10 μM and APO at concentrations from 100 to 500 μM. Therefore, in our study, we used intermediate concentrations of 5 μM DPI and 300 μM APO (26-30). To investigate the impact of NOXs on TPA-induced MMP-9 expression and cell invasion, MCF-7 cells were pre-treated with 5 μM DPI and 300 μM APO for 1 h, followed by stimulation with 20 nM TPA for 24 h. Western blotting and zymography revealed that NOX inhibitor treatment effectively inhibited the upregulation of TPA-induced MMP-9 protein expression and exocytosis in MCF-7 cells (Fig. 1A). RT-qPCR revealed that TPA increased MMP9 levels in MCF-7 cells, whereas NOX inhibitors mitigated TPA-induced MMP9 mRNA upregulation (Fig. 1B). Additionally, the Matrigel invasion assay confirmed that pretreatment with NOX inhibitors mitigated the increase in

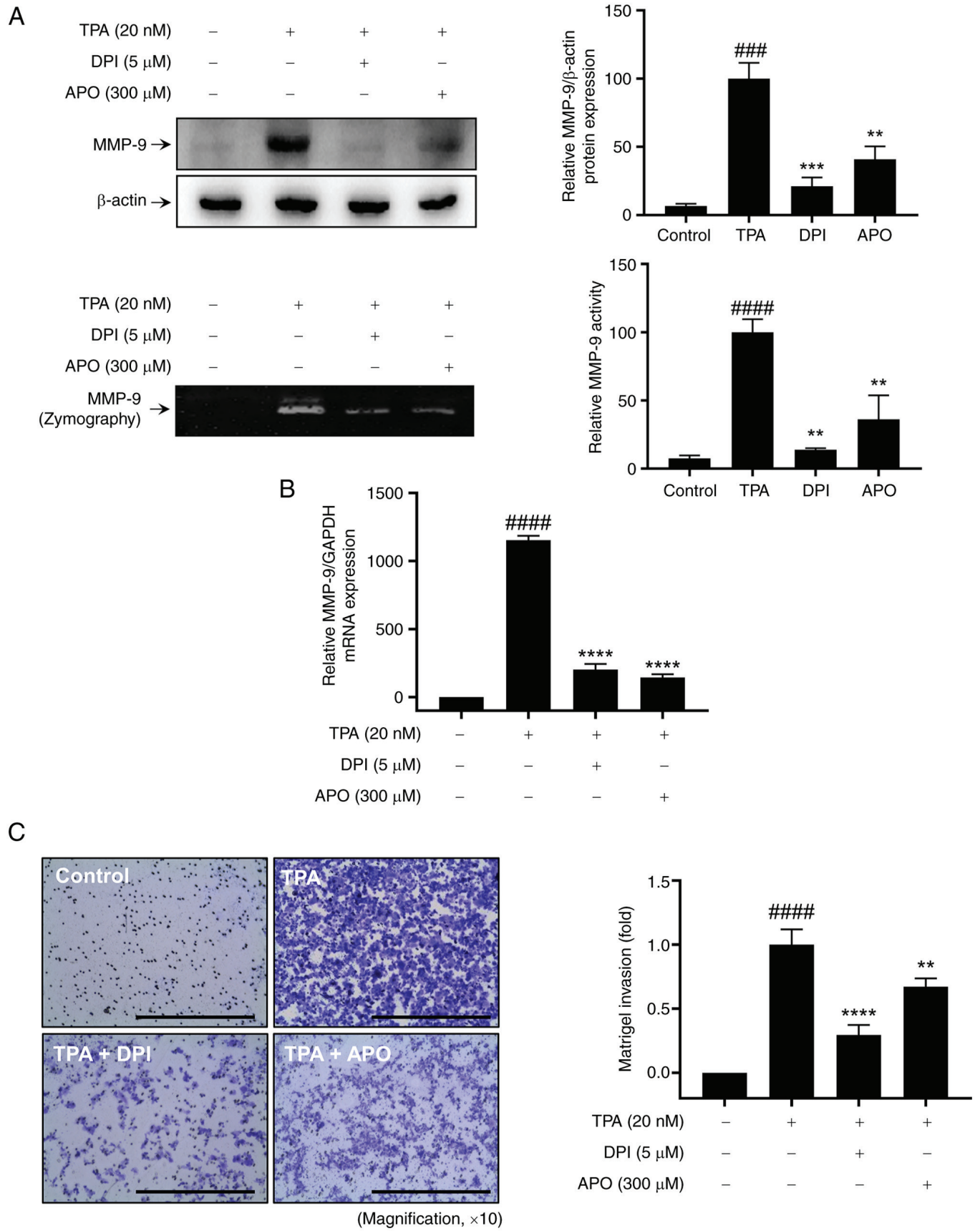

Figure 1. Effect of NOX inhibitors on TPA-induced MMP-9 expression and Matrigel invasion of MCF-7 cells. (A) Western blot analysis (upper panel) and gelatin zymography assay (lower panel) were performed to assess MMP-9 expression and activity, respectively. (B) Reverse transcription-quantitative PCR was used to analyze *MMP9* mRNA expression. (C) Matrigel invasion assay was conducted to evaluate cell invasion, with DPI or APO added to the lower chamber along with 20 nM TPA for 24 h. Scale bar, 100 μm. Results represent mean ± SEM of three independent experiments. $^{###}$P<0.0005, $^{####}$P<0.0001 vs. control; **P<0.005, ***P<0.0005, ****P<0.0001 vs. TPA. TPA, 12-O-tetradecanoylphorbol-13-acetate; MMP, matrix metalloprotease; DPI, diphenyleneiodonium chloride; APO, apocynin; SEM, standard error of the mean.

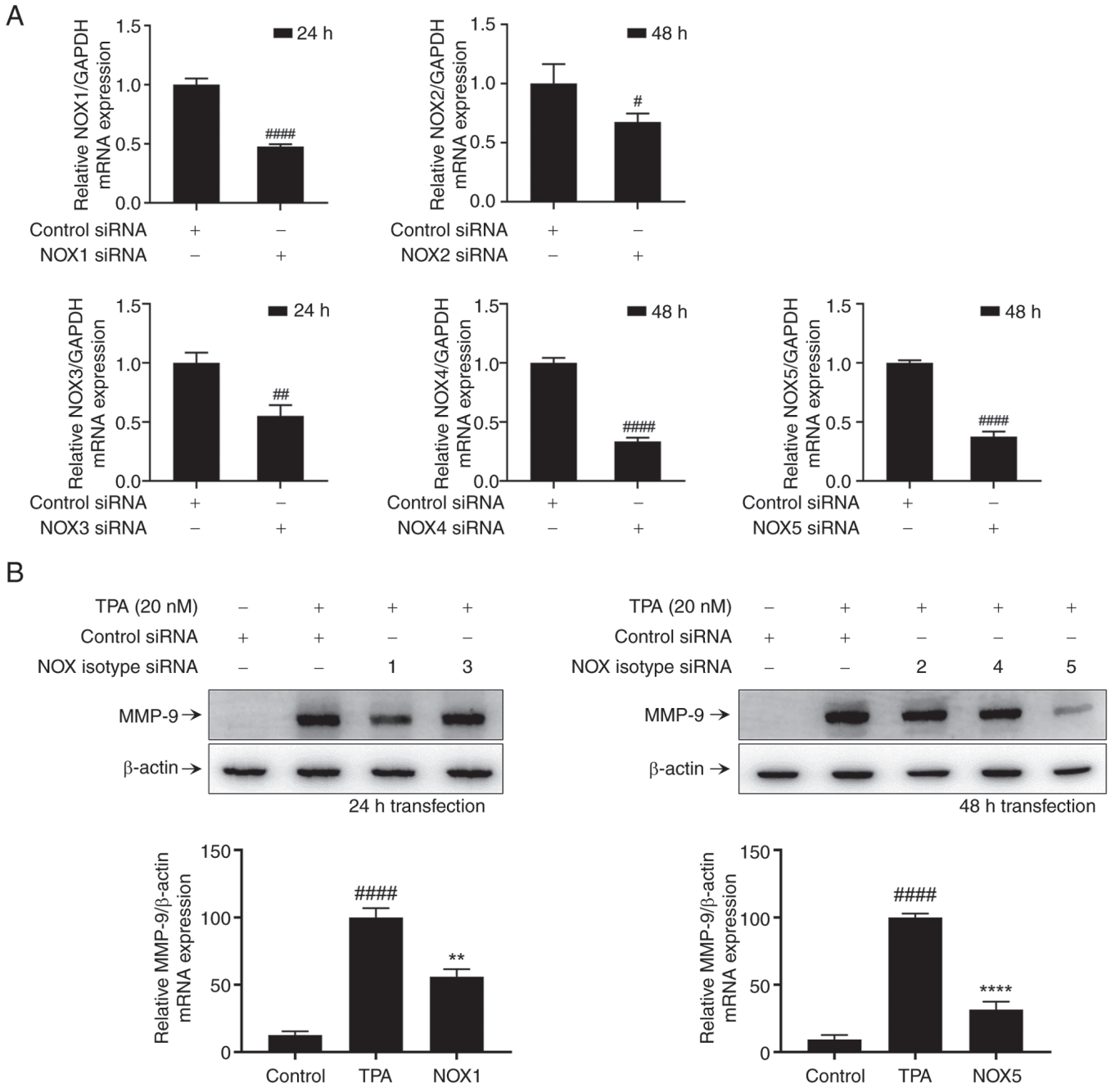

Figure 2. Effect of NOX knockdown on TPA-induced MMP-9 expression in MCF-7 cells. (A) Cells were transfected with NOX-specific siRNAs to reduce mRNA expression levels. (B) After transfection under each condition, cells were stimulated with 20 nM TPA for 24 h. Followed by western blot analysis to assess MMP-9 protein expression; β-actin was used as loading control. Results represent mean ± SEM of three independent experiments. $^{\#}$P<0.05, $^{\#\#}$P<0.005, $^{\#\#\#\#}$P<0.0001 vs. control; **P<0.005, ****P<0.0001 vs. TPA. TPA, 12-O-tetradecanoylphorbol-13-acetate; MMP, matrix metalloprotease; NOX, NADPH oxidase; siRNA, small intefering RNA; SEM, standard error of the mean.

TPA-induced cell invasion (Fig. 1C). Therefore, the inhibition of NOX expression suppresses TPA-induced breast cancer cell invasion by inhibiting MMP-9 expression.

Role of NOX isotypes in TPA-induced expression in MCF-7 cells. NOXs contribute to breast cancer cell invasion by modulating MMP-9 expression and activity (Fig. 1). Among the members of the NOX family, we investigated which isotypes (NOX1-5) were implicated in TPA-induced MMP-9 protein expression in MCF-7 cells. For accurate comparison, we identified conditions wherein the expression of NOX mRNA decreased by approximately 50% following cell transfection with NOX-specific small interfering RNA (siRNA) (Fig. 2A). We also identified a decrease in protein expression by western blot analysis (Fig. S1). However, we could not detect the NOX3 band under our conditions, so we conducted the experiment based on mRNA expression levels. To assess the contribution of NOX isotypes in TPA-induced MMP-9 expression, MCF-7 cells were stimulated with TPA for 24 h following each condition of NOX isotype transfection. Western blot analysis revealed that transfection with NOX1 and NOX5 siRNA attenuated TPA-induced MMP-9 expression in MCF-7

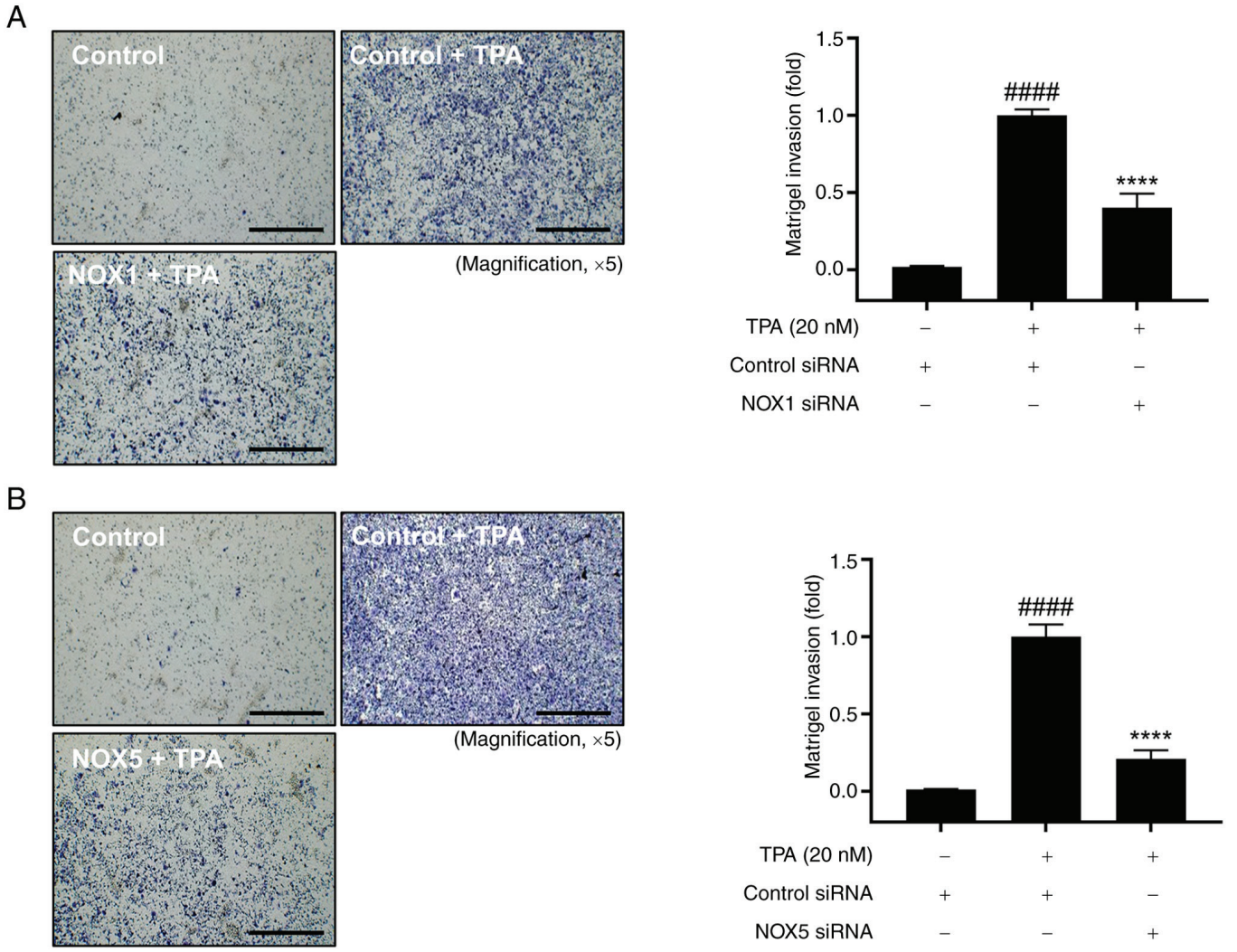

Figure 3. Effect of NOX1 and NOX5 knockdown on TPA-induced Matrigel invasion of MCF-7 cells. Matrigel invasion assay was conducted after transfection with (A) NOX1- and (B) NOX5-specific siRNA. Scale bar, 100 μm. Results represent mean ± SEM of three independent experiments. $^{\#\#\#\#}$P<0.0001 vs. control; ****P<0.0001 vs. TPA. TPA, 12-O-tetradecanoylphorbol-13-acetate; NOX, NADPH oxidase; siRNA, small interfering RNA; SEM, standard error of the mean.

cells (Fig. 2B). Therefore, TPA-induced MMP-9 expression in MCF-7 cells is mediated by NOX1 and NOX5.

Validation of NOX1 and NOX5 roles in cell invasion and ROS regulation in TPA-induced MCF-7 cells. We further validated the roles of NOX1 and NOX5 in cell invasion. Cell invasion was significantly increased in TPA-treated cells compared with that in control cells, and this increase was significantly reduced by the knockdown of NOX1 and NOX5 (Fig. 3). Furthermore, we investigated whether NOX1 and NOX5 knockdown mediates ROS regulation. The 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) fluorescence assay helped confirm that TPA treatment (20 nM) induced ROS production in MCF-7 cells, this effect was mitigated by knockdown of NOX1 and NOX5 (Fig. 4). Therefore, NOX1 and NOX5 inhibit TPA-induced cell invasion by regulating ROS production.

PKC modulation of NOX activity and MMP-9 expression in TPA-induced MCF-7 cells. TPA selectively activates PKC, acting as an upstream or downstream regulator to generate ROS (11-13). Therefore, we used GF109203X (GF), a widely used PKC inhibitor, to confirm the involvement of PKC in TPA-induced ROS generation, MMP-9 expression, and MCF-7 cell activation. It is known that the activation of PKC by TPA involves the translocation of PKC isoforms from the cytosol to the plasma membrane (31). Pretreatment with GF was found to inhibit the membrane translocation of the PKC isoforms α, β, and δ, which was induced by TPA stimulation (Fig. S2). DPI was used as a positive control in this experiment as a representative NOX inhibitor (32). We observed that GF effectively inhibited TPA-induced ROS production (Fig. 5A) and MMP-9 expression and activation (Fig. 5B) in MCF-7 cells. Additionally, GF treatment attenuated the increase in TPA-induced NOX activity in MCF-7 cells (Fig. 5C). Therefore, NOX activity is mediated by PKC, which plays a pivotal role in regulating TPA-induced MMP-9 expression and invasion in MCF-7 cells.

Discussion

Breast cancer is a malignant tumor that is the leading cause of mortality among women (33). Metastasis to diverse organs, including bones, lungs, liver, brain, and kidneys, accounts for

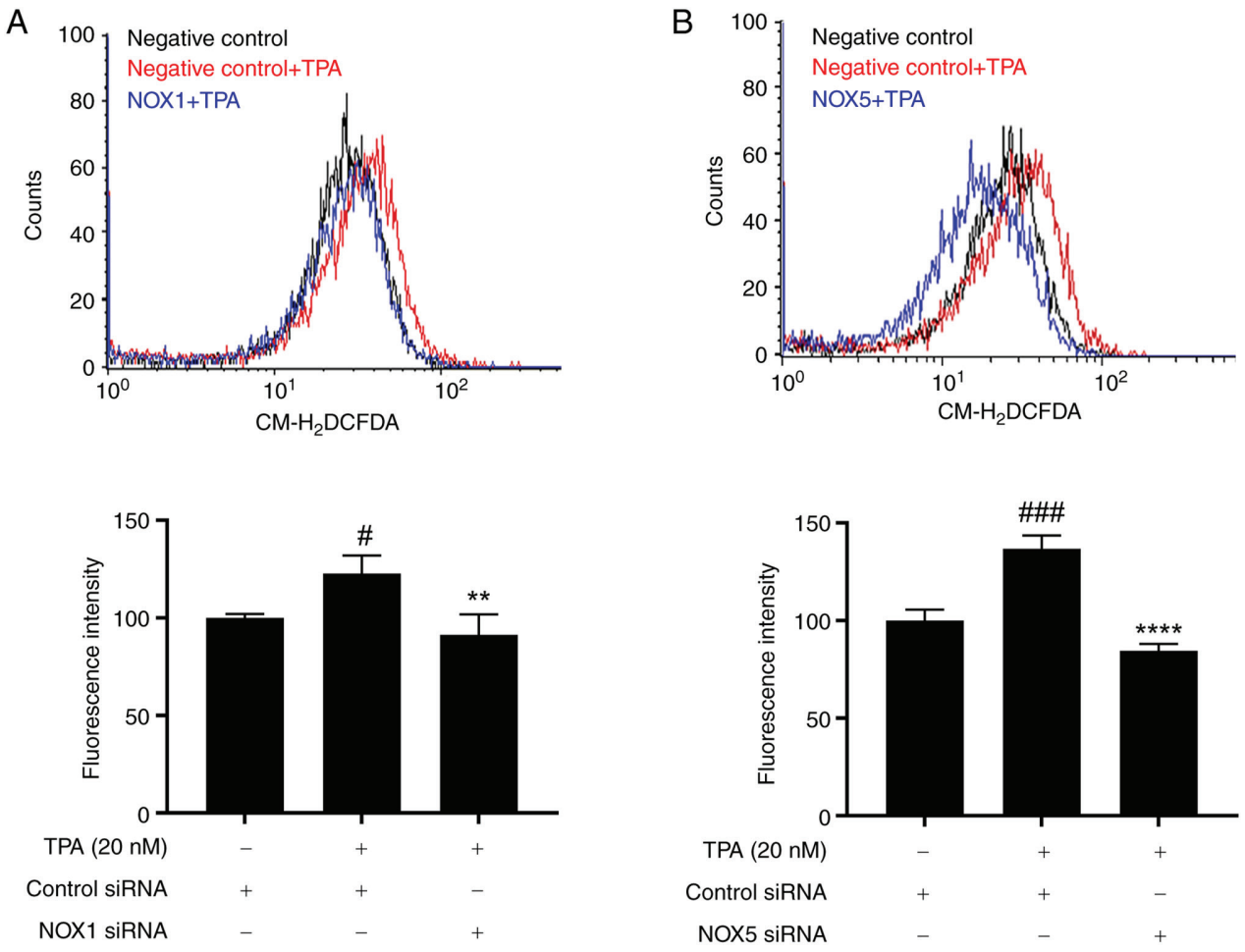

Figure 4. Effects of NOX1 and NOX5 knockdown on TPA-induced intracellular ROS level in MCF-7 cells. ROS levels were assessed by measuring CM-H_2DCFDA fluorescence following 24 h stimulation with 20 nM TPA subsequent to the knockdown of (A) NOX1 and (B) NOX5. Results represent mean ± SEM of three independent experiments. $^{\#}$P<0.05, $^{\#\#\#}$P<0.0005 vs. control; **P<0.005, ****P<0.0001 vs. TPA. TPA, 12-O-tetradecanoylphorbol-13-acetate; NOX, NADPH oxidase; siRNA, small interfering RNA; ROS, reactive oxygen species; SEM, standard error of the mean.

most breast cancer-related deaths (34). The initial event in cancer cell invasion and migration involves a decrease in the ECM, which poses biochemical and mechanical barriers to cell movement (6,35). The most important factors in ECM degradation are MMP expression and activity, which play pivotal roles in breast cancer (6,36). MMP is a family of proteases that play important roles in the development and progression of cancer; among these, MMP-9 is vital in tumor invasion and metastasis owing to its collagenase activity in ECM degradation (37). Breast cancer cells, stimulated by various factors, including TPA, increase MMP-9 expression by activating several intracellular signaling pathways (36,38). Following MMP-9 expression and activation, ECM loss in blood vessels or lymphatic walls facilitates cancer cell invasion into these systems, leading to metastasis to other organs. Therefore, regulation of MMP-9 expression is pivotal in controlling cancer metastasis.

Mitochondria and the NOX family (NOX1-5 and DUOX1/2) constitute two important sources of ROS production in cancer cells (39). NOX, a protein facilitating electron transport across biological membranes, generates superoxide in the plasma membrane, which is converted into hydrogen peroxide; this facilitates its entry into the cell in the form of superoxide or hydrogen peroxide, thus impacting various intracellular signaling mechanisms (40,41). NOX family members play crucial roles in various human cancer tissues (42,43). However, the role of NOX isotypes (NOX1-5) in TPA-induced breast cancer cell invasion remains unexplored. NOX1 is overexpressed in various human solid tumors, including colon cancer, prostate cancer, and melanoma (44,45), and it contributes to the regulation of cell invasion by regulating MMP-9 production and cell migration (46). NOX5 expression is increased in tumor tissues of patients with breast cancer, and it promotes breast cancer cell proliferation and metastasis (47,48). However, we lack reports on the importance of NOX1 and NOX5 in breast cancer invasion. We confirmed that the previously known NOX inhibitors, DPI and APO, inhibit MMP-9 expression and cell invasion in MCF-7 cells (Fig. 1). Furthermore, we confirmed that the inhibition of NOX1 and NOX5 among the NOXs is involved in MMP-9 expression and cell invasion in MCF-7 cells (Figs. 2 and 3).

TPA induces multiple signaling pathways in a PKC-dependent manner (49); PKC activation promotes tumor development and is associated with special cell functions, such as adhesion, invasion, and metastasis (50). PKC activation in

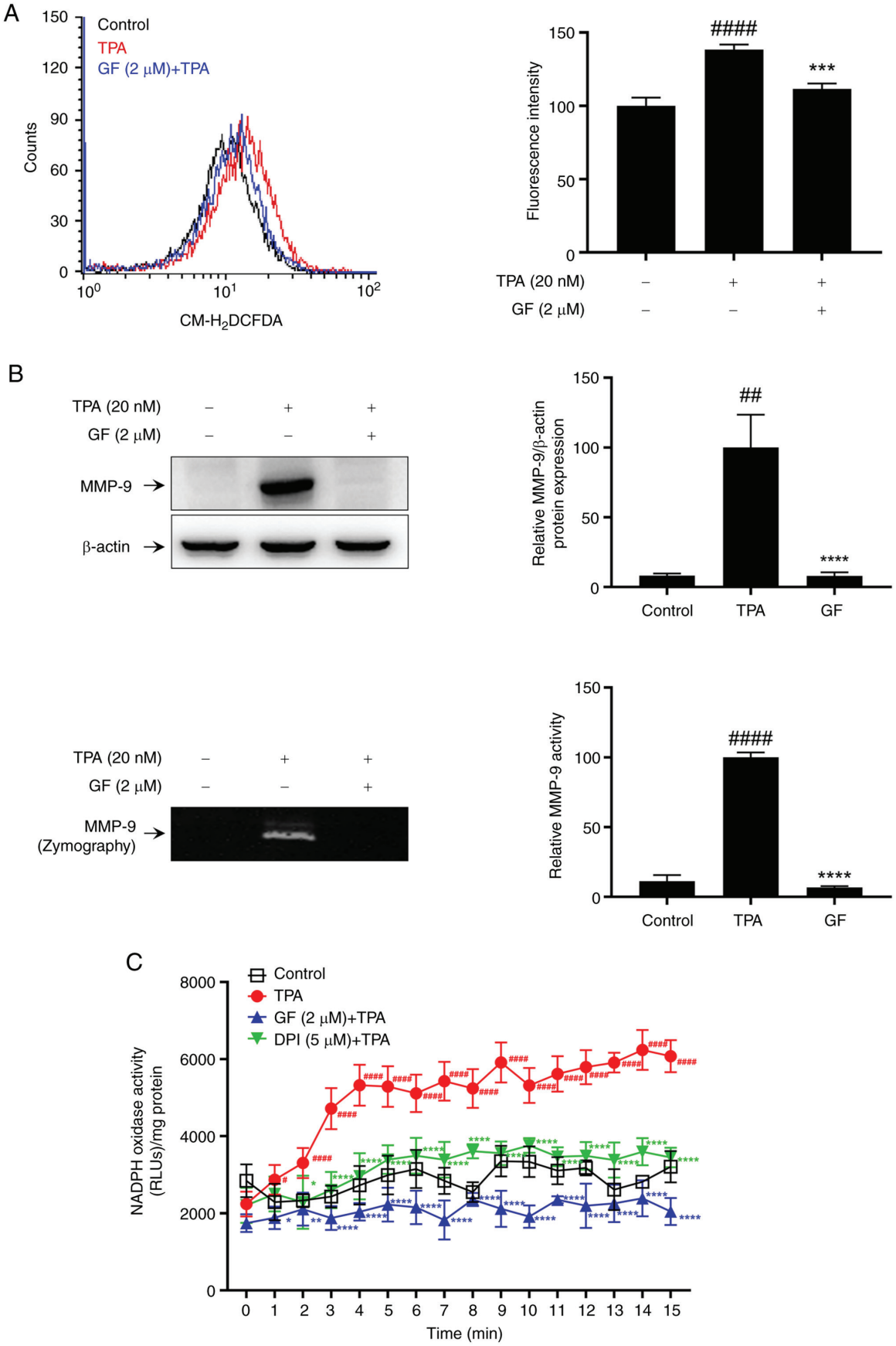

Figure 5. Effect of PKC inhibitors on TPA-induced intracellular ROS levels, MMP-9 expression, and NOX activity in MCF-7 cells. (A) DCF fluorescence was measured to assess ROS levels after treatment with a 2 μM PKC inhibitor (GF) and 20 nM TPA stimulation. (B) Cells pre-treated with GF for 1 h and stimulated with TPA for 24 h. MMP-9 expression was analyzed as western blotting. Secreted MMP-9 activity was confirmed by gelatin zymography assay. (C) NAPDH oxidase activity was evaluated using a luminometer after GF treatment and 20 nM TPA stimulation. Results represent mean ± SEM of three independent experiments. $^{\#}$P<0.05, $^{\#\#}$P<0.005, $^{\#\#\#\#}$P<0.0001 vs. control; *P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001 vs. TPA. TPA, 12-O-tetradecanoylphorbol-13-acetate; MMP, matrix metalloprotease; PKC, protein kinase C; ROS, reactive oxygen species; GF, GF109203X; SEM, standard error of the mean.

breast cancer is strongly associated with increased invasion through the production and secretion of MMP-9 (24,25,51). Furthermore, ROS generation induces TPA-mediated migration and invasion (52). PKC activates NADPH oxidases, leading to the production of ROS (22,53-57). Therefore, we confirmed the inhibitory effects of NOX1 and NOX5 suppression on TPA-induced ROS generation in MCF-7 cells (Fig. 4). Finally, we confirmed the inhibitory effects of PKC inhibitors on TPA-induced ROS production, MMP-9 expression, and NOX activation in MCF-7 cells (Fig. 5).

However, the current study has limitations as it lacks evidence from animal experiments and does not explore the role of DUOX1 and DUOX2 in breast cancer metastasis. Therefore, future studies incorporating animal models and investigating the involvement of additional NOX isoforms could provide a more comprehensive understanding of the mechanisms underlying breast cancer invasion.

In conclusion, our findings confirm that NOX1 and NOX5 mediate TPA-induced invasion of MCF-7 cells by regulating MMP-9 expression and activation; this is achieved mainly by modulating ROS generation via PKC. To the best of our knowledge, this is the first study to demonstrate that TPA-induced PKC-dependent-MCF-7 cell invasion is modulated by NOXs. Despite some limitations in our study, our findings highlight potential strategies for treating breast cancer metastasis via NOX1 and NOX5 regulation.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YRL and HJY designed and conceptualized the experiments. HKS, EMN and JMK performed the experiments and data collection. HKS wrote the original draft and revised the manuscript. JMK analyzed and generated the figures. YRL and HJY confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publications

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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