Supporting Information

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SI Materials and Methods

Quantification of NO and NO Metabolites. The Griess assay was used to measure nitrite, a stable end product of NO. Briefly, culture supernatant was centrifuged to remove cell debris and 100 μL was mixed with 50 μL of 10% (wt/vol) sulfanilamide (Sigma) in 5% (vol/vol) H_3PO_4 and 50 μL of 1% (wt/vol) naphthalene ethlyenediamine (Sigma) in a 96-well plate. The absorbance at 545 nm was measured within 30 min and compared with a freshly prepared nitrite calibration curve.

The NO flux in a 96-well plate was measured to simulate conditions in the xCelligence migration assay. To a 96-well plate, 200 μL of pH 7.4 DPBS was added, and varying concentrations of DETA/NO were allowed to decompose for 1, 3, 6, and 24 h. The entire well volume was then injected into the purge vessel of the GE Sievers 280i Nitric Oxide Analyzer, and NO quantities were calculated by using a nitrite/KI calibration curve. The molar flux of NO was determined from the moles of NO and the volume of the injection.

Western Blotting and Immunoprecipitation. Total protein was isolated (Cell Signaling; 9803 lysis buffer, PMSF) and quantified by using the BCA protein assay (Thermo Scientific). Total protein loaded for NOS2 detection was 40–50 μg. TIMP1 was immunoprecipitated by using a TIMP1 rabbit polyclonal antibody (Abcam) conjugated to agarose beads (Santa Cruz). Proteins were heat denatured and electrophoresed on NuPage 4–12% (wt/vol) Bis-Tris SDS polyacrylamide gels (Life Technologies). The proteins were transferred to iBLOT stack nitrocellulose membranes (Invitrogen) and blocked for 1 h by using 1% milk in TBS-Tween (TBST). Primary antibodies were incubated with the blots at 4 °C in 1% milk or 5% (wt/vol) BSA in TBST. NOS2 (sc-651; Santa Cruz), hypoxanthine phosphoribosyltransferase 1 (HPRT) (sc-2079; Santa Cruz), and the secondary antibodies (Cell Signaling) were used per manufacturer's instructions. The HRP-conjugated secondary antibodies were incubated in 1% milk in TBST, and the blots were exposed to the TMA-6 chemiluminescent substrate (Lumigen). The protein was visualized on an Alpha Innotech FluorChem Imager (Cell Biosciences). The background was corrected by using local averaging, and all proteins were corrected with the loading controls, either HPRT or the stained gel.

RNA Isolation and RT-PCR Analysis. TRIzol was used to extract total RNA per the manufacturer's protocol. For in vivo studies, the brains and tumors of the control and AG-treated mice were homogenized by using a Power Masher II (Funakoshi) and TRIzol. Total RNA concentration was determined by using a NanoDrop (Thermo Scientific) and was reverse transcribed into cDNA by using the RNA to cDNA EcoDry Premix (Clontech). Human primers were designed to cross an exon-exon junction by using the Primer3 primer design engine. Quantitative real-time PCR on *NOS2, TLR4, IL8, IL6, COX2, S100A8, GFP*, and *RPL18* were done by using the ABI 7500 system (Applied Biosystems) and a two-step protocol was used: 95 °C for 15 s and 60 °C for 30 s for a total of 40 cycles. The δ-δ Ct method was used for relative gene expression calculation, and *RPL18* was used as a housekeeping gene.

Reporter Transfections. Cells were seeded in 24-well plates in penicillin/streptomycin-free RPMI 1640 supplemented with FBS (optimal conditions found for MCF-7 at 4×10^4 per well, MDA-MB-231, and MDA-MB-468 at 1×10^5 per well) and transfected by using Lipofectamine LTX with Plus Reagent (Invitrogen) for 18 h. To each well, 500 ng of NOS2-7.0 luciferase reporter and 500 ng of the control pCMV Renilla luciferase plasmid were added. After transfection, the cells were washed with PBS and incubated with various stimulants in serum and phenol-free RMPI 1640. The reporter activity was determined by using the Dual-Luciferase Reporter Assay System (Promega) and a GloMax 96-well microplate luminometer and compared against a Lipofectamine LTX transfection only control.

Confocal Microscopy. MDA-MB-231 cells were seeded in four-well Lab-Tek chamber slides (1×10^5 per well) and grown overnight. The cells were washed in PBS and treated with IFN-γ (500 U/mL) in serum and phenol-free RMPI 1640 for 4 h. The cells were washed with PBS, followed by the addition of fresh serum and phenol-free media containing 1 mM L-arginine. After 48 h, cells were rinsed with PBS and fixed with 4% (wt/vol) paraformaldehyde (Electron Microscopy Sciences) in PBS for 15 min and then washed with PBS. Slides were permeabilized for 10 min in PBS with 0.01% Tween-20 (PBST). The slides were blocked by using 4% BSA for 10 min. The NOS2 rabbit antibody (Santa Cruz; sc-651) was preblocked in 4% BSA for 10 min and diluted at a 1:100 working solution. NOS2 antibody was added, and the cells were incubated overnight at 4 °C in a humidified slide chamber. Slides were gently rinsed with PBST. The slides were blocked with 4% BSA for 10 min before adding the fluorescent secondary antibody. Goat anti-rabbit secondary antibodies conjugated to Alexa-594 (Invitrogen) were diluted in 4% BSA to a final working concentration of 1:200. Diluted secondary antibodies were added to the slides for 1 h at room temperature in a humidified slide chamber in the dark. The slides were gently rinsed in PBST. Nuclei were fluorescently stained with 4',6-diamidino-2-phenylindole in PBS (1 μg/mL) for 5 min. Slides were rinsed in PBS and mounted in Vectashield (Vector Laboratories).

Slides were imaged by confocal microscopy using a Zeiss LSM710 microscope system equipped with a HE/NE laser, Kr/Ar laser, and UV laser. Using Zen 2010 software package, Z-stacks of tumor cells were obtained by using 63× oil-immersion objective. Three-dimensional images were displayed by using Imaris x64 version 7.6.0.

Cell Migration. The xCelligence RTCA DP system (Acea Biosciences) was used to measure MDA-MB-231 cell migration. Serum containing RMPI 1640 was added to the lower chamber (160 μL) of a CIM-16 Plate and serum-free RPMI 1640 containing L-Arg (1 mM) was added to the upper chamber (50 μL). The plate was blanked, and MDA-MB-231 cells were plated at 10,000 cells per well (100 μL) in serum-free RMPI 1640 supplemented with L-Arg (1 mM). The cells were allowed to adhere overnight, and the experiment was started upon addition of AG (1 mM) and DETA/NO (100 and 300 μM). The cells were allowed to migrate from the upper chamber to the lower chamber for 60 h.

Clonogenic Survival. MDA-MB-231 cells were plated in 60-mm plates (1×10^6 cells per plate) and allowed to adhere overnight in RPMI. The cells were washed with PBS, and 2 mL of L-Arg (1 mM) supplemented serum or serum-free RPMI 1640 was added in the presence or absence of AG (1 mM) and DETA/NO (100 μM). The cells were treated for 24 h, and Taxol was added directly to the medium (10 nM in DMSO) and incubated for 18 h. The cells were washed, trypsinized, and plated at 250 cells per 60-mm dish in 5 mL of RMPI and allowed to grow for 14 d. The colonies were then fixed and stained by using Crystal Violet in methanol, and viable colonies were counted.

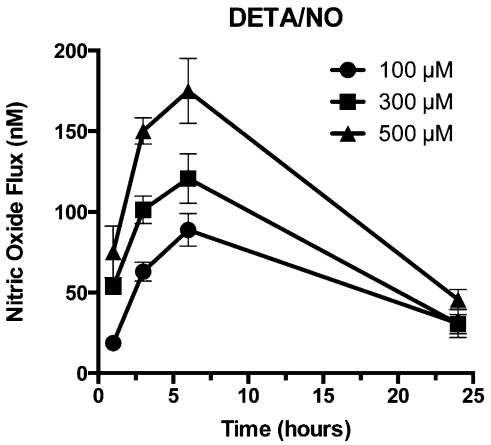

Fig. S1. NO flux from DETA/NO (100, 300, and 500 μM) was determined by using the Sievers Nitric Oxide Analyzer at 1, 3, 6, and 24 h in 200 μL of serum-free RPMI in a 96-well plate.

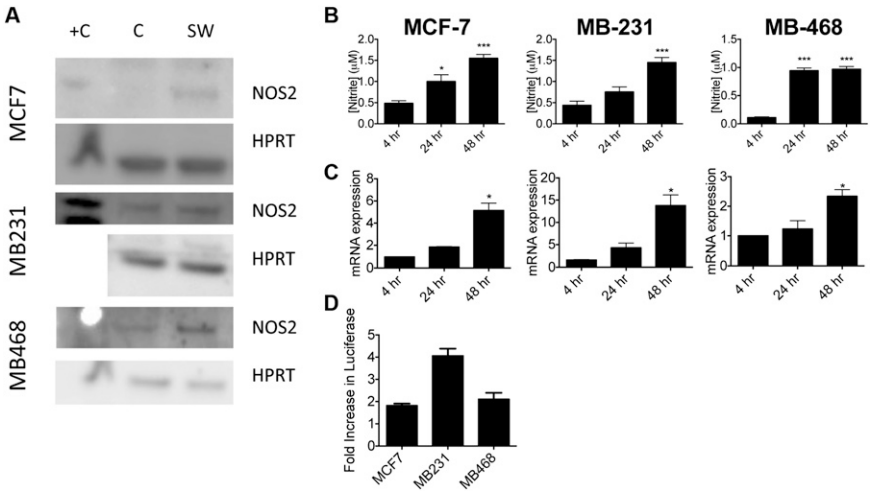

Fig. S2. MCF-7, MDA-MB-231, and MDA-MB-468 cells were serum starved (SW). (*A*) NOS2 protein was assessed after 24 h of SW. Nitrite accumulation (*B*) and *NOS2* mRNA (*C*) were measured temporally at 4, 24, and 48 h. (*D*) The NOS2 7.0-kb luciferase reporter was used to evaluate NOS2 activity after 48 h of SW (±SEM, $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$).

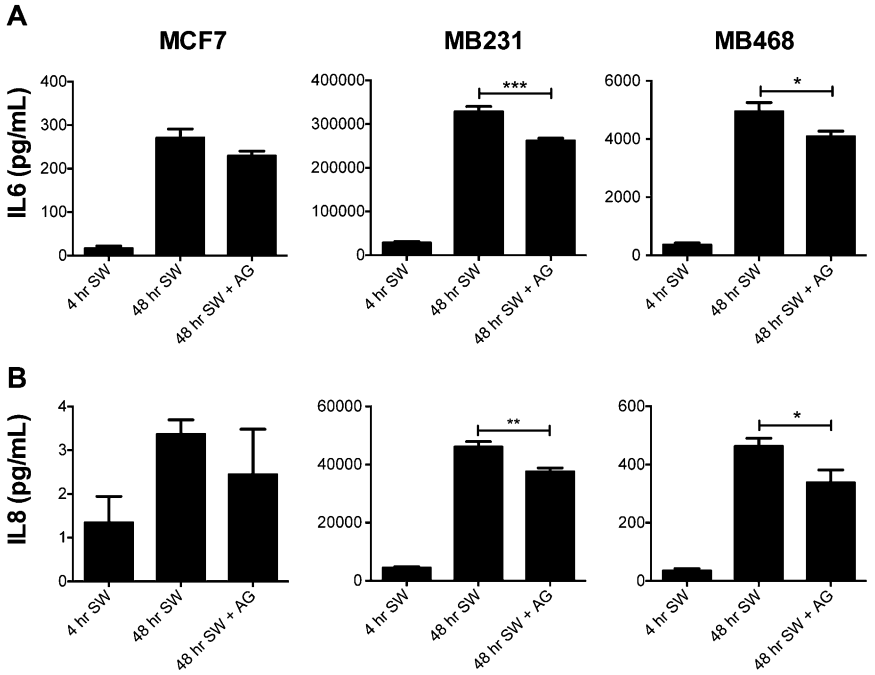

Fig. S3. IL-6 (*A*) and IL-8 (*B*) levels were measured in MCF-7, MDA-MB-231, and MDA-MB-468 cells after 48 h SW with or without AG (±SEM, $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$).

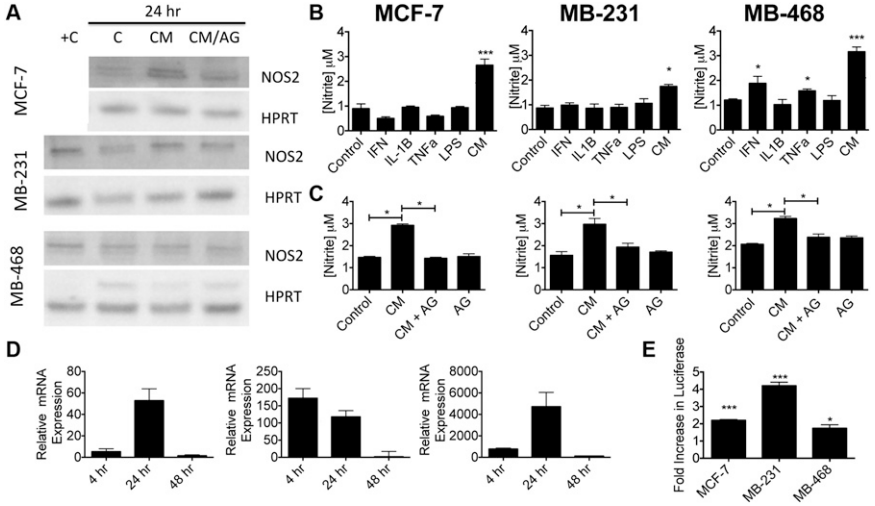

Fig. S4. Cytokine stimulation of MCF-7, MDA-MB-231, and MDA-MB-468 cells was used to stimulate NOS2. (*A*) NOS2 protein levels in each cell line after 24-h cytokine mix (CM) stimulation. (*B*) Nitrite levels were assessed by the Greiss assay after 24-h stimulation by using single cytokines and CM. (*C*) Nitrite after CM stimulation was inhibited by addition of AG. (*D*) *NOS2* mRNA was measured after 4, 24, and 48 h of CM stimulation. (*E*) The NOS2-7.0 kb luciferase reporter was used to measure NOS2 promoter activity after 24-h CM stimulation. (±SEM, $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$).

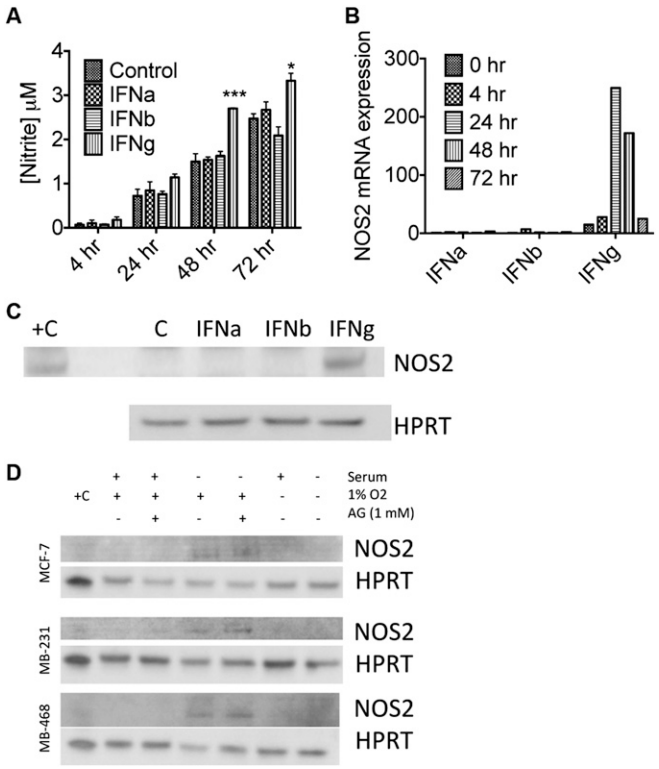

Fig. S5. NOS2 regulation in MDA-MB-231 cells by either Type-1 IFN-α,β, Type 2 IFN-γ, or hypoxia. *NOS2* mRNA (*A*) and nitrite levels (*B*) were evaluated at 0, 4, 24, 48, and 72 h, and NOS2 protein was measured after 48-h IFN treatment (*C*). (*D*) NOS2 protein levels were measured after 24 h hypoxia with or without SW (±SEM, $*P \leq 0.05$ and $***P \leq 0.001$).