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A Novel Mechanism by Which *N*-(4-hydroxyphenyl)retinamide Inhibits Breast Cancer Cell Growth: The Production of Nitric Oxide

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Abstract

***N*-(4-Hydroxyphenyl)retinamide (4-HPR) induces apoptosis in breast cancer cells; however, the molecular basis by which 4-HPR induces apoptosis is not well understood. In breast cancer cells, nitric oxide (NO) is predominantly an apoptotic inducer. Apoptotic agents, such as phorbol ester, tumor necrosis factor-α, and peptide hormones, have been shown to increase NO production in breast cancer cells. Therefore, we hypothesized that the production of NO is vital for 4-HPR to induce apoptosis in breast cancer cells. We found that 4-HPR induced NO production in a dose-dependent manner in all of the breast cancer cell lines tested. The degree of growth inhibition and apoptotic induction by 4-HPR was directly correlated with the amount of NO produced. To prove that NO is essential for 4-HPR to induce apoptosis, breast cancer cells were coincubated with a competitive NO synthase (NOS) inhibitor, N^G-monomethyl-L-arginine (L-NMMA), and 4-HPR. L-NMMA prevented 4-HPR from inducing inhibitory effects, indicating that NO is crucial for 4-HPR to induce its apoptotic effects in breast cancer cells. IFNs and tamoxifen (TAM) have been shown to potentiate 4-HPR effects in breast cancer cells. Both IFN-γ and TAM enhanced the ability of 4-HPR to induce NO production in breast cancer cells, which was correlated with increased apoptosis. Alone, 4-HPR increased expression of both inducible NOS (NOSII) and endothelial NOS (NOSIII). When combined with 4-HPR, IFN-γ and TAM enhanced NOSII expression. Thus, we have identified a novel mechanism by which 4-HPR induces apoptosis in breast cancer cells, *i.e.,* by increasing NOS expression to induce NO production.**

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Introduction

4-HPR,[2] a synthetic derivative of all-*trans* retinoic acid, exhibits apoptotic and anti-invasive effects in breast cancer cells *in vitro* (1–4). 4-HPR also inhibits carcinogen-induced mammary cancer in animal models (5). Furthermore, 4-HPR accumulates selectively in breast tissue, and this may account for its reduced toxicity in comparison to other retinoids (6–8). Clinically, 4-HPR effectively reduces local recurrent and contralateral breast cancer in premenopausal women (9, 10). However, the use of 4-HPR in clinical trials in women with advanced breast cancer has shown only limited success (11). Other agents have been combined with 4-HPR to improve its clinical use in breast cancer chemoprevention and treatment. IFNs have been shown to potentiate retinoid-induced growth inhibition in breast cancer cells *in vitro* (12, 13). TAM has been found to increase 4-HPR-induced growth inhibition *in vitro* and *in vivo* (14–16). Clinical data collected thus far indicate that combined 4-HPR and TAM may have potential chemopreventive effects in ER+ breast cancer patients (17) and therapeutic effects for patients with ER+ metastatic breast cancer but not for those with ER− metastatic disease (18). Yet, the molecular mechanisms by which 4-HPR exerts its apoptotic effects in breast cancer cells as well as the mechanisms by which IFNs and TAM enhance the potency of 4-HPR are not well understood.

Unlike typical retinoids, 4-HPR appears to induce its apoptotic effects via retinoid receptor-independent mechanisms (1, 19–21). 4-HPR has been shown to reduce telomerase activity (22, 23) and insulin growth factor-1 production (24, 25) and to elevate transforming growth factor-β production (3); whether these observations are the mechanisms or the results of 4-HPR-induced apoptosis are not known. Recent reports suggest that 4-HPR-induced apoptosis may be mediated through increases in free radicals (26), reactive oxygen species (27–30), mitochondrial permeability transition (31, 32), ceramide levels (33, 34), and caspase 3 activity (30, 35). Whether 4-HPR uses these or some other mechanisms to induce apoptosis in breast cancer has not been fully investigated.

NO has been shown to inhibit proliferation and induce apoptosis in breast cancer cells (36–40). NO is a free radical synthesized from arginine by three different isoforms of NOS: NOSI, NOSII, and NOSIII. The constitutive isoforms NOSI and NOSIII are calcium and calmodulin dependent, whereas the inducible isoform NOSII is calcium and calmodulin independent. Increased NO production has been reported in

[2] The abbreviations used are: 4-HPR, *N*-(4-hydroxyphenyl)retinamide; TAM, tamoxifen; NO, nitric oxide; NOS, NO synthase; NOSI, neuronal NOS; NOSII, inducible NOS; NOSIII, endothelial NOS; L-NMMA, N^G-monomethyl-L-arginine; ER, estrogen receptor; FBS, fetal bovine serum.

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breast cancer cells treated with various apoptotic agents, such as tumor necrosis factor-α, phorbol ester, and peptide hormones (36, 38, 41). Recently, the antiproliferative effects of all-*trans* retinoic acid have been correlated with increased NO production in a breast cancer cell line (42).

On the basis of this information, we hypothesized that one mechanism by which 4-HPR exerts its growth-inhibitory effects on breast cancer cells is by inducing NO production. In this study, we determined the effects of 4-HPR alone and in combination with IFN-γ and TAM on the growth and the production of NO in breast cancer cells. Data reported here demonstrate that 4-HPR induces NO production in breast cancer cells and that NO production is essential for 4-HPR-induced inhibition. We further demonstrated that one potential mechanism by which IFN-γ and TAM enhance the potency of 4-HPR in breast cancer cells is by increasing NO production.

Materials and Methods

Reagents. 4-HPR, IFN-γ, TAM citrate, and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). NOSII and NOSIII antibodies were purchased from BD Transduction Laboratories (San Diego, CA). Vectastain Elite ABC (avidin-biotin-peroxidase complex) kit, hematoxylin, and 3-amino-9-ethyl-carbazole were purchased from Vector Laboratories (Burlingame, CA). Aqua-Mount was purchased from Lerner Laboratories (Pittsburgh, PA). Stock solutions (10 mM) of 4-HPR and TAM were prepared in DMSO and stored at −20°C. IFN-γ was prepared in PBS at a concentration of 1 μg/ml and stored at −20°C. L-NMMA was purchased from Alexis Biochemicals (San Diego, CA). Stock solutions (10 mM) of L-NMMA were prepared in PBS and stored at 4°C. All of the reagents were diluted in culture medium to the indicated final concentration.

Cell Lines and Culture Conditions. ER+ breast cancer cell lines (MCF-7 and T47D) and ER− breast cancer cell lines (MDA-MB-453 and SKBr-3) were obtained from American Type Cell Culture (Manassas, VA). The cells were cultured in DMEM/F12 medium supplemented with 5% heat-inactivated FBS at 37°C under 5% CO_2 in a humidified incubator.

Cell Growth and NO Assay. Breast cancer cells were plated at 1×10^5 cells/well in 6-well plates in 2 ml of DMEM/F12 medium supplemented with 5% FBS. Twenty-four h later, cells were treated with 4-HPR (1, 2.5 μM), IFN-γ (25, 50, 100 IU/ml), or TAM (0.1, 1 μM). For combination treatments, a 1-μM dose of 4-HPR was used. After 5 days of incubation, cell growth was determined by total live cell counts using trypan blue exclusion. Supernatants were collected from treated cells, and aliquots were stored at −80°C for NO determination. Total NO was determined by quantifying nitrite, the stable end product of NO oxidation. Nitrite accumulation was determined spectrophotometrically using a Colorimetric Non-enzymatic Nitric Oxide Assay kit (Oxford Biomedical Research, Oxford, MI). Briefly, 100-μl samples were incubated with 0.5 g of cadmium beads overnight. Cadmium was used to catalyze the reduction of nitrate to nitrite, thus allowing for the measurement of total NO present in the samples. The samples were reacted with an equal volume of Greiss reagent (1% sulfanilamide and 0.1% naphthylethylenediamine), and the absorbance was measured at 540 nm in a Microplate reader (Molecular Devices, Sunnyvale, CA). Sodium nitrite was used as a standard. Nitrite values were normalized for total cell counts and expressed as μM per 1,000,000 cells. Values are reported as means ± SD for experiments performed in triplicate.

Western Blot. Protein lysates (50 μg) from untreated exponentially growing MCF-7, T47D, SKBr-3, and MDA-MB-453 cells were loaded on a 12% polyacrylamide gel to determine ERα status in these breast cancer cell lines. Proteins were electrophoresed and electrotransferred as described by Tari *et al.* (43). Membranes were incubated with monoclonal ERα mouse antibody (Novacastra Laboratories Ltd., Burlingame, CA). Protein bands were visualized by enhanced chemiluminescence (Kirkgaard and Perry Laboratories, Gaithersburg, MD). Images were scanned using an Alpha Imager application program (Alpha Innotech, San Leandro, CA).

Apoptosis Analysis. The effect of 4-HPR alone and in combination with IFN-γ and TAM on apoptosis was analyzed by flow cytometry. Approximately 1×10^6 T47D cells were trypsinized, collected by centrifugation at 1500 rpm for 5 min, washed in PBS, and resuspended in 1 ml of PBS. The cell suspension was added to 1 ml of cold 70% ethanol and incubated overnight at −20°C. Cells were centrifuged at 1500 rpm for 10 min at 4°C and then washed twice in PBS, and the pellet was left loose. Approximately 0.5–1 ml of PBS containing RNase (20 μg/ml) and propidium iodide (50 μg/ml) was added to each cell pellet, followed by 20 min of incubation at room temperature. Flow cytometric analysis was performed using a Coulter Epics Profile 488 laser. Supernatants from treated cells were collected for subsequent NO determination as described above.

Inhibition of NO Production. To determine the importance of NO production in 4-HPR-induced growth inhibition in breast cancer cells, the competitive NOS inhibitor L-NMMA was used to block NO synthesis. T47D and MCF-7 cells were plated (1×10^5 cells/well) in 6-well plates in 2 ml of DMEM/F12 medium supplemented with 5% FBS. The next day, cells were treated with 4-HPR (1 μM) in the absence and presence of L-NMMA (1, 10, 100 μM) for 5 days. After incubation, cell growth and NO production were determined as described above.

Immunohistochemical Staining. T47D cells (1×10^5 cells) were plated in 6-well plates in DMEM/F12 medium supplemented with 5% FBS and treated with 4-HPR alone (1, 2.5 μM) or in combination with IFN-γ (50 IU/ml) or TAM (1 μM). Cells were incubated with 1 μM 4-HPR alone or in combination with TAM for 5 days. Cells were treated with 2.5 μM 4-HPR or a combination of 4-HPR and IFN-γ for 3 days. Cells were harvested and suspended in PBS (1×10^6 cells/ml). Cytospins for each treatment were prepared by using 100 μl of the appropriate cell suspension. Slides were quick-fixed in −20°C acetone and stored at −20°C until immunostaining. Slides were prepared according to the protocol of Ekmekcioglu *et al.* (44). Briefly, slides were fixed in acetone at −20°C and then incubated in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. The slides were incubated in PBS containing 0.05% Triton X-100

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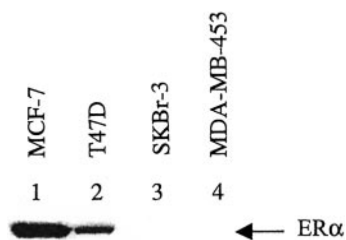

Fig. 1. Expression of ERα in breast cancer cells. Western blot was performed to determine the levels of ERα in MCF-7, T47D, SKBr-3, and MDA-MB-453 breast cancer cells.

to permeabilize the cells. An ABC kit (Vectastain Elite) was then used to detect the primary antibody staining. The immunostaining was developed using 3-amino-9-ethyl-carbazole as a chromagen. Slides were counterstained with hematoxylin and mounted with Aqua-Mount. The slides were analyzed for both percentage and intensity of immunolabeling. The intensity of NOS immunostainings was evaluated by dividing the cytoplasmic staining reaction into four groups: −, none; +, light; ++, moderate; and +++, intense. Percentages of immunostainings were evaluated as follows: −, <5% positive cells; +, 5–25%; ++, 26–50%; +++, 51–75%; ++++, 76–95%; and +++++, >95%.

Statistical Analysis. Synergy was tested using a two-sample, one-tailed, *t* test between two populations. The first population is the one with the mean that is equal to the average NO production when 4-HPR and IFN-γ (or TAM) are present. The second population is the one in which the mean is the sum of the average NO production from 4-HPR and the average NO production from IFN-γ (or TAM). The null hypothesis is that the means of the two populations are the same. The alternative hypothesis is that the mean of the first population is greater than that of the second. The null hypothesis (no synergy) is rejected in favor of the alternative hypothesis (synergy) if the *P* of the *t* test is less than 0.05.

Results

4-HPR-induced Inhibitory Effects Were Directly Correlated with NO Production. To assess the effect of 4-HPR on growth inhibition and NO production in breast cancer cells, ER+ (MCF-7 and T47D) and ER− (SKBr-3 and MDA-MB-453) cells were treated with 4-HPR (1, 2.5 μM). We selected 1 and 2.5 μM 4-HPR because these doses are clinically achievable (8). The ER status of each cell line was verified by Western blot analysis (Fig. 1). 4-HPR induced dose-dependent growth inhibition in all of the cell lines (Table 1A). 4-HPR also induced NO production in a dose-dependent manner in all of the cell lines (Table 1B). 4-HPR-mediated growth inhibition was directly correlated with NO generation. The ability of 4-HPR to induce growth inhibition and NO production was substantially greater in ER+ cells than in ER− cells. The 2.5-μM concentration of 4-HPR increased NO production by 24-fold in ER+ cells and 3.3-fold in ER− cells compared with untreated cells.

Because 4-HPR is known to exert its growth-inhibitory effects on breast cancer cells via apoptotic induction (45), we

Table 1 4-HPR induced growth inhibition and NO production in ER+ and ER− breast cancer cells

4-HPR (μM)	ER-positive		ER-negative	
	MCF-7	T47D	MDA-MB-453	SKBr-3
A. Growth inhibition, total cell count (× 10^4)[a]				
0	264.0 ± 2.98	60.5 ± 2.24	64.5 ± 2.64	55.0 ± 2.37
1	28.0 ± 1.41	18.0 ± 0.50	40.0 ± 1.73	23.0 ± 0.87
2.5	7.3 ± 0.289	6.5 ± 0.50	20.0 ± 0.43	12.5 ± 0.43
B. NO production, μM nitrite/1 × 10^6 cells[b]				
0	6.5 ± 0.68	11.1 ± 0.16	19.6 ± 0.33	22.6 ± 2.41
1	64.4 ± 2.40	80.9 ± 1.61	31.9 ± 1.41	44.2 ± 0.41
2.5	230.7 ± 7.6	227.4 ± 1.29	63.6 ± 6.36	76.3 ± 1.55

[a] Total live cell counts were determined by trypan blue exclusion. The values reported are the means (±SD) of experiments performed in triplicate.
[b] Total NO production was determined by measuring its stable end product nitrite. Nitrite values were normalized to cell number. Values reported are the means (±SD) of experiments performed in triplicate.

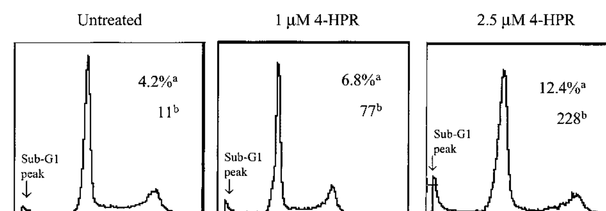

Fig. 2. 4-HPR-induced apoptosis was directly correlated with NO production. T47D cells were plated (1 × 10^5 cells/well) in 6-well plates in DMEM/F12 medium supplemented with 5% FBS. Cells were treated with 4-HPR (1, 2.5 μM) for 5 days. Approximately 1 × 10^6 cells were collected, and apoptosis was evaluated by flow cytometric analysis of propidium iodide staining. NO production was determined by measuring its stable end product nitrite using a Colorimetric Non-enzymatic Nitric Oxide Assay kit. [a], the percentage of apoptotic cells (*i.e.,* cells in the sub-G_1 peak); [b], μM nitrite/10^6 cells.

determined whether NO production was associated with 4-HPR-mediated apoptosis. 4-HPR treatment resulted in a dose-dependent increase in the percentage of apoptotic cells, as evidenced by the rise in the sub-G_1 peak (Fig. 2). 4-HPR-mediated apoptosis was also directly correlated with NO production (Fig. 2).

Inhibition of NO Production Suppressed 4-HPR-induced Inhibition. To determine whether NO is involved in 4-HPR-induced inhibition, we used the NOS competitive inhibitor L-NMMA to inhibit NO production. T47D and MCF-7 cells were treated with 4-HPR (1 μM) in the presence and absence of L-NMMA. L-NMMA at the concentrations used (1, 10, 100 μM) was not cytotoxic to cells. L-NMMA effectively suppressed 4-HPR-induced NO production in a dose-dependent manner in both of the cell lines (Table 2). Inhibition of NO production prevented 4-HPR from inducing growth inhibition. L-NMMA at a concentration of 100 μM was able to return NO levels and cell counts to those of untreated breast cancer cells. These data indicate that NO induction is an essential mechanism by which 4-HPR induces growth inhibition.

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Table 2 Inhibition of NO production suppressed 4-HPR-mediated growth inhibition in breast cancer cells

4-HPR (1 μM)	L-NMMA[a] (μM)	Cell count[b] (× 10^4)	Nitrite[c] (μM/10^6 cells)
T47D cells			
−	−	68.5 ± 1.8	13.7 ± 0.7
+	−	21.5 ± 1.3	63.3 ± 3.8
−	1	67 ± 2.8	12.3 ± 0.5
−	10	60 ± 2.2	12.6 ± 0.9
−	100	74 ± 2.6	12.3 ± 0.6
+	1	23 ± 1.8	43.3 ± 2.7
+	10	50 ± 2.6	19.9 ± 1.0
+	100	72 ± 3.0	13.9 ± 0.6
MCF-7 cells			
−	−	264 ± 5.6	6.5 ± 0.6
+	−	27.5 ± 1.3	65.9 ± 2.1
−	1	263 ± 3.6	6.7 ± 0.5
−	10	264 ± 8.2	7.0 ± 0.4
−	100	260 ± 4.4	6.6 ± 1.1
+	1	30 ± 2.0	63.4 ± 3.2
+	10	112 ± 3.6	28.2 ± 1.6
+	100	228 ± 5.3	8.1 ± 0.7

[a] NOS competitive inhibitor.
[b] Total live cell counts were determined by trypan blue exclusion. Values are means ± SD of experiments performed in triplicate.
[c] Total NO production was determined by measuring its stable end product nitrite. Nitrite values were normalized to cell number. Values are means ± SD of experiments performed in triplicate.

Table 3 4-HPR increased NOS expression in breast cancer cells

	Intensity[a]		Percentage[b]	
	NOSII	NOSIII	NOSII	NOSIII
Untreated	+	−	−	−
4-HPR (1 μM)	+	+++	+	+
4-HPR (2.5 μM)	+++	+	++++	+++
IFN-γ (50 IU/ml)	−	−	−	−
IFN-γ + 4-HPR[c]	+++	−	++++	−
TAM (1 μM)	++	−	+	−
TAM + 4-HPR[d]	++	−	++	−

[a] Intensity of NOS immunostainings: −, none; +, light; ++, moderate; +++, intense.
[b] Percentage of NOS immunostainings: −, <5%; +, 5–25%; ++, 26–50%; +++, 51–75%; ++++, 76–95%; +++++, >95%.
[c] 50 IU/ml IFN-γ and 1 μM 4-HPR were used.
[d] 1 μM TAM and 1 μM 4-HPR were used.

4-HPR Increased the Expression of NOSII and NOSIII in Breast Cancer Cells. Because NO is produced by NOS, we wanted to determine which isoform(s) was responsible for 4-HPR-induced NO production in breast cancer cells. T47D cells were treated with 4-HPR alone (1, 2.5 μM) and subjected to immunohistochemical staining for the different NOS isoforms (NOSII and NOSIII; Table 3). NOSIII expression was not detected in untreated T47D cells, and fewer than 5% of cells exhibited a light NOSII staining. NOSII and NOSIII expression levels were increased in cells treated with 1 μM 4-HPR. A higher intensity of staining and a higher percentage of positive cells for NOSII and NOSIII were observed in cells treated with 1 μM 4-HPR in comparison with untreated cells (Fig. 3). NOSIII had a higher intensity of staining than did NOSII in cells treated with 1 μM 4-HPR; however, a similar percentage of positive cells was observed (Table 3). 4-HPR at the 2.5-μM concentration showed dramatic increases in intensity and percentage of positive cells for NOSII. Although NOSIII intensity was lower for the 2.5-μM concentration of 4-HPR than for the 1-μM concentration, a greater percentage of cells stained positive for NOSIII expression at the 2.5-μM concentration than at the 1-μM concentration (Table 3). NOSII staining was higher in intensity and in percentage of cells than NOSIII staining when cells were treated with 2.5 μM 4-HPR. Thus, 4-HPR mediates increases in NO production by inducing NOSII and NOSIII expression.

IFN-γ and TAM Enhanced 4-HPR-induced Apoptosis in Breast Cancer Cells. IFNs and TAM are known to potentiate retinoid-induced growth inhibition in breast cancer cells (12–14, 16, 46–48). To confirm the ability of IFNs and TAM to enhance 4-HPR-mediated growth inhibition, T47D cells were treated with 4-HPR alone or in combination with IFN-γ or TAM. At the 1-μM concentration, 4-HPR resulted in a slight increase in the percentage of apoptotic cells (Fig. 4). Alone, IFN-γ and TAM at the concentrations used had little effect on apoptosis, but they enhanced 4-HPR-mediated apoptotic activity. The percentage of apoptotic cells was increased from 7.3% to 27.7 and 14.7% when 4-HPR was combined with IFN-γ and TAM, respectively.

IFN-γ and TAM Enhanced 4-HPR-induced NO Production. Fig. 5 shows the effect exerted by 4-HPR in combination with IFN-γ on NO production. Production of NO was not affected by IFN-γ treatment alone in any of the cell lines. IFN-γ enhanced 4-HPR-mediated NO production in a dose-dependent manner in all of the cell lines, although ER+ cells were more sensitive to this combination than were ER− cells. There were 3.6-fold and 2.0-fold increases in NO production over that observed for 4-HPR alone with the 100-IU/ml dose of IFN-γ in combination with 4-HPR in ER+ and ER− cells, respectively. A synergistic effect of IFN-γ and 4-HPR on NO production was observed in MCF-7 and T47D cells at all three doses of IFN-γ used ($P < 0.05$). In SKBr-3 cells, a synergistic effect on NO production was observed when the 50- and 100-IU/ml doses of IFN-γ were used in combination with 4-HPR ($P < 0.05$). In MDA-MB-453 cells, an additive effect was observed when 4-HPR was combined with IFN-γ at a dose of 50 IU/ml, and a synergistic effect was observed when 4-HPR was combined with 100 IU/ml IFN-γ ($P < 0.05$).

TAM increased 4-HPR-induced NO production in ER+ cells (Fig. 6). A 2.2-fold increase in NO production over that observed for 4-HPR alone was obtained with the 1-μM dose of TAM in combination with 4-HPR in ER+ cells. TAM and 4-HPR had a synergistic effect on NO production in MCF-7 cells at both doses of TAM ($P < 0.05$). For T47D cells, a synergistic effect on NO production was observed when 1.0 μM TAM was combined with 1 μM 4-HPR ($P < 0.05$), whereas an additive effect was observed when 0.1 μM TAM was combined with 1 μM 4-HPR. The TAM and 4-HPR combination was not very effective in ER− cells. TAM did not enhance NO production in SKBr-3 cells or MDA-MB-453 cells, except when 4-HPR was combined with the highest dose of TAM.

4-HPR in Combination with IFN-γ or TAM Enhanced NOSII Expression. Table 3 illustrates the intensity and percentage of NOS immunostainings for cells treated with a combination of 4-HPR and IFN-γ or TAM. NOSII and NOSIII

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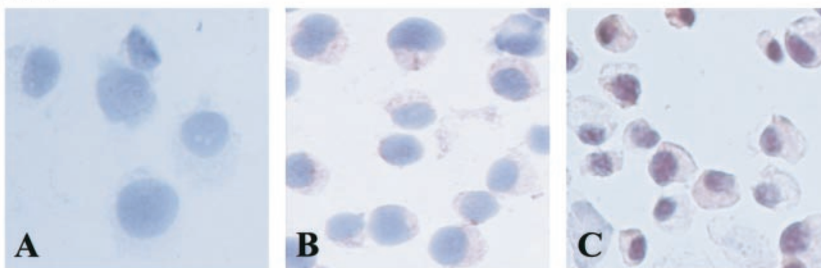

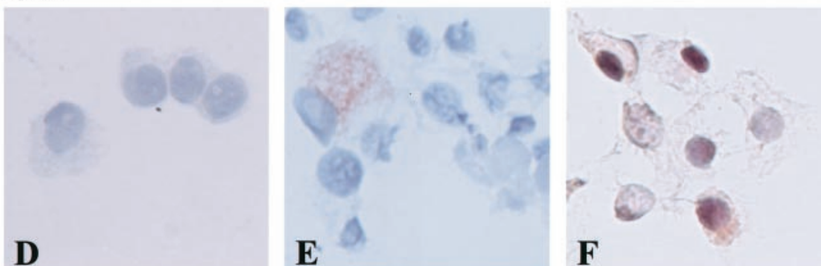

Fig. 3. 4-HPR increases NOSII and NOSIII expression in breast cancer cells. T47D cells were plated (1×10^5 cells/well) in 6-well plates in DMEM/F12 medium supplemented with 5% FBS. The next day, cells were treated with 1 μM 4-HPR for 5 days or 2.5 μM 4-HPR for 3 days. Cells were harvested and immunostained for NOS isoforms. Untreated (*A*, *D*); 1 μM 4-HPR (*B*, *E*); 2.5 μM 4-HPR (*C*, *F*).

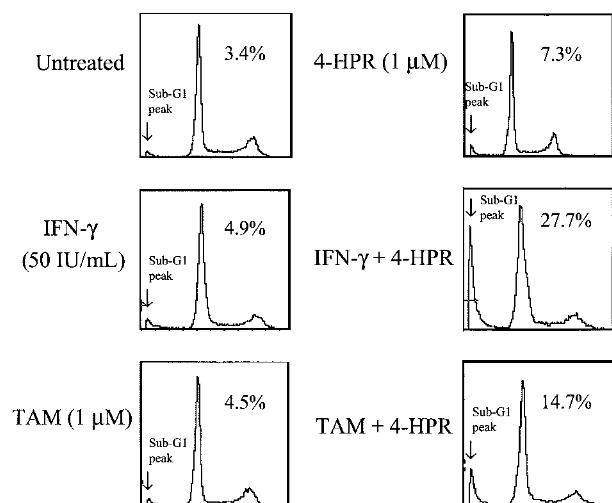

Fig. 4. IFN-γ and TAM enhance 4-HPR-mediated apoptosis in breast cancer cells. T47D cells were plated (1×10^5 cells/well) in 6-well plates in DMEM/F12 medium supplemented with 5% FBS. Cells were treated with 4-HPR (1 μM) alone or in combination with IFN-γ (50 IU/ml) or TAM (1 μM) for 5 days. Approximately 1×10^6 cells were collected, and apoptosis was evaluated by flow cytometric analysis of propidium iodide staining. *Numbers in the right hand corner of each histogram*, the percentage of apoptotic cells.

were not detected in cells treated with IFN-γ alone. NOSIII was also not detected in cells treated with TAM alone; however, both the intensity and percentage of NOSII immunostainings were increased in TAM-treated cells compared with untreated cells. Both IFN-γ and TAM, when combined with 1 μM 4-HPR, enhanced the expression of NOSII in comparison with 4-HPR alone (Fig. 7*A*). In contrast, NOSIII expression was not detected in cells treated with 4-HPR in combination with IFN-γ or TAM (Fig. 7*B*). The intensity and percentage of NOSII positive cells were greater for the 4-HPR and IFN-γ combination than for the 4-HPR and TAM combination, which was correlated with higher apoptotic rates (Fig. 4) and induction of NO production by the 4-HPR and IFN-γ combination (Figs. 5 and 6). Thus, IFN-γ and TAM enhance 4-HPR-mediated NO production by increasing NOSII expression.

Discussion

We have identified NO production as a novel mechanism by which 4-HPR induces apoptosis in breast cancer cells. The production of NO is specific to 4-HPR because the NOS inhibitor, L-NMMA, can selectively abrogate 4-HPR-induced inhibitory effects. Depending on the concentration of NO in the microenvironment, the effects of NO can be tumor promoting or tumor suppressing. Low concentrations of NO may protect some cell types from apoptosis induced by DNA-damaging agents (49) and increase the invasiveness and the metastatic potential of murine tumors (50, 51). But, high concentrations of NO are cytotoxic (52) and can prevent murine tumors from metastasizing (53, 54). Our findings that high concentrations of NO are inhibitory to breast cancer cells agree with previous findings (36–40).

We found that 4-HPR increases the expression of NOSII and NOSIII in breast cancer cells. NOS activity has been detected in human breast cancer (39, 55). The expression of NOSIII is restricted to the vascular endothelial cells within the breast tumors (56). NOSII is detectable in benign breast tumors (56) and breast hyperplasia (57). NOSII has been found predominantly in the stromal cells of breast tumors (55, 56, 58); however, some studies suggest that NOSII is expressed mainly in breast tumor cells (39, 59). The endogenous expression of NOSII within breast tumor cells has been inversely correlated to the tumor's metastatic potential and may have an inhibitory role on breast cancer metastasis (56). On the other hand, high expression of NOSII within the

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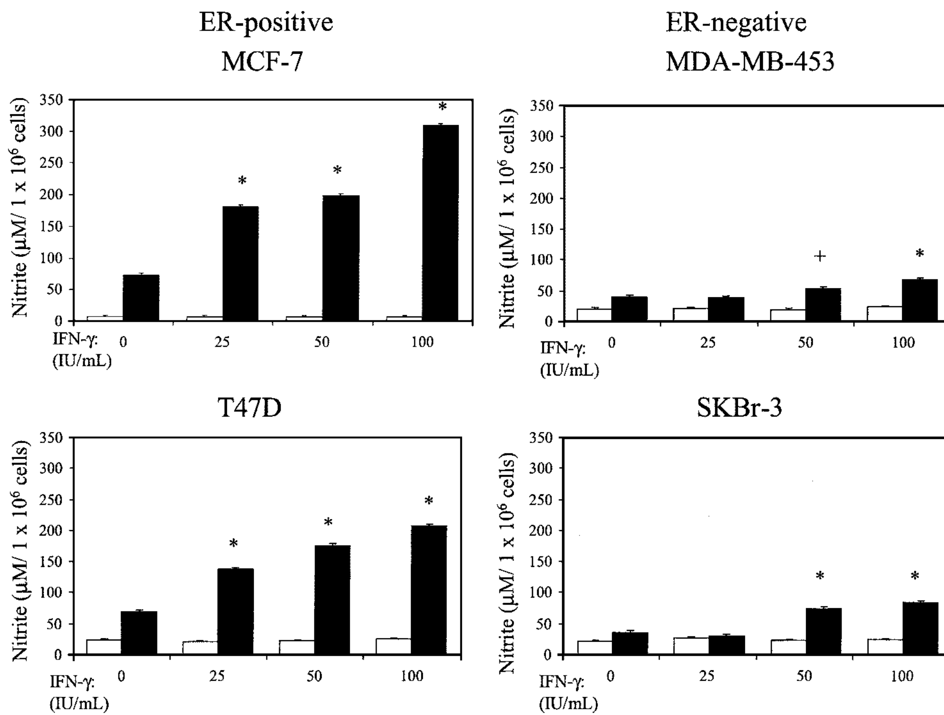

Fig. 5. IFN-γ enhances 4-HPR-mediated NO production in breast cancer cells. ER+ (MCF-7, T47D) and ER− (MDA-MB-453, SKBr-3) breast cancer cells were plated (1 × 10^5 cells/well) in 6-well plates in DMEM/F12 medium supplemented with 5% FBS. The next day, cells were treated with 4-HPR (1 μM) alone or in combination with IFN-γ (25, 50, 100 IU/ml) for 5 days. NO production was determined by measuring its stable end product nitrite using a Colorimetric Non-enzymatic Nitric Oxide Assay kit. Nitrite values were normalized to cell number. Each experimental treatment was performed in triplicate; values shown are means ± SD. □, −4-HPR; ■, +4-HPR; *, synergistic effects; +, additive effects.

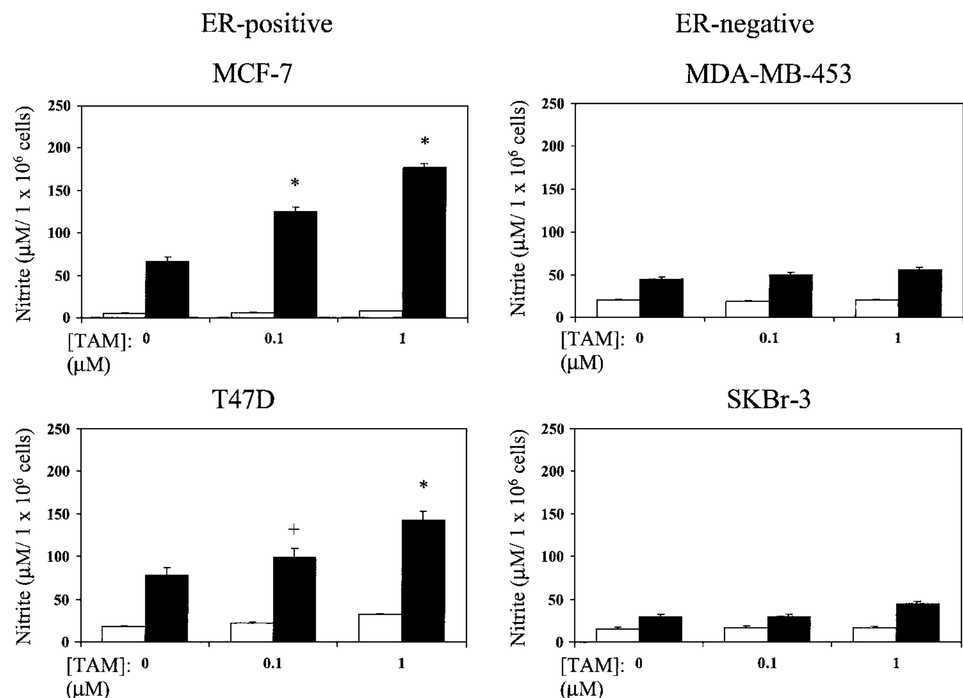

Fig. 6. TAM enhances 4-HPR-mediated NO production in ER+ breast cancer cells. ER+ (MCF-7, T47D) and ER− (MDA-MB-453, SKBr-3) breast cancer cells were plated (1 × 10^5 cells/well) in 6-well plates in DMEM/F12 medium supplemented with 5% FBS. Cells were treated with 4-HPR (1 μM) alone or in combination with TAM (0.1, 1 μM) for 5 days. NO production was determined by measuring its stable end product nitrite using a Colorimetric Non-enzymatic Nitric Oxide Assay kit. Nitrite values were normalized to cell number. Each experimental treatment was performed in triplicate; values shown are means ± SD. □, −4-HPR; ■, +4-HPR; *, synergistic effects; +, additive effects.

stromal cells of breast tumors (55, 58) or within tumors ≥2 cm (59) has been correlated with breast cancer metastasis, possibly because of its association with higher vascular density and increased angiogenesis (58). Our data indicate that 4HPR-induced high concentrations of NO by NOSII are inhibitory to breast cancer cells. The effects of 4HPR in breast stromal cells remain to be investigated.

We demonstrated that 4-HPR induced growth inhibition and NO production in both ER+ and ER− breast cancer cells; however, ER− cells were less responsive than ER+ cells to the effects of 4-HPR. A lower sensitivity of ER− cells to 4-HPR inhibitory effects in comparison with ER+ cells is consistent with the results of previous studies (1). Our present results indicate that 4-HPR induces NO production by

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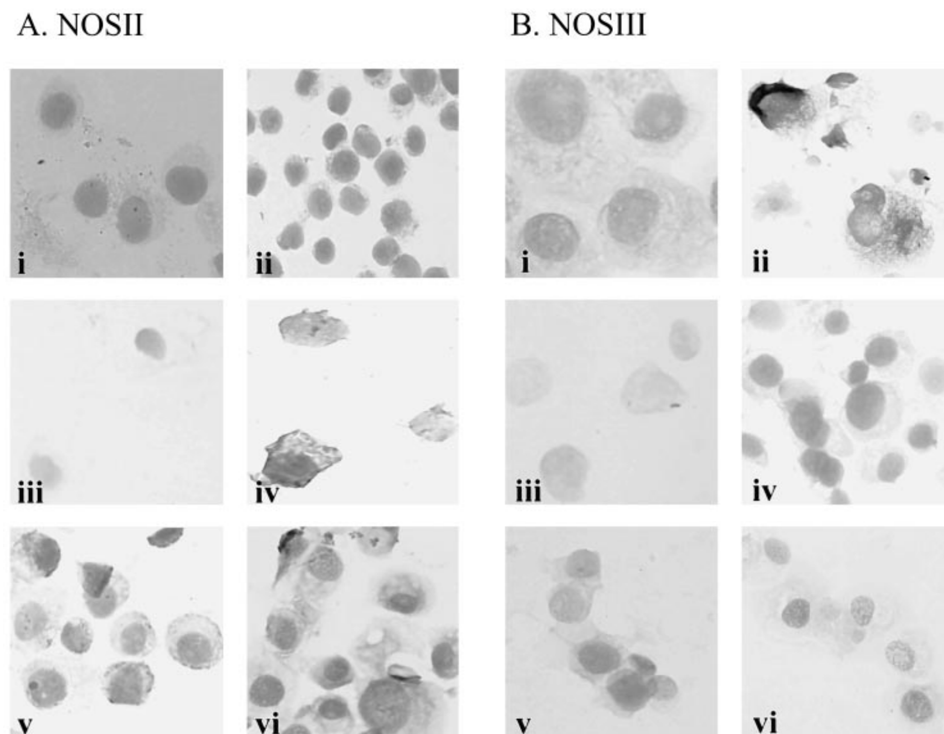

Fig. 7. NOSII expression was increased when 4-HPR was combined with IFN-γ or TAM. T47D cells were plated (1×10^5 cells/well) in 6-well plates in DMEM/F12 medium supplemented with 5% FBS. Cells were treated with 4-HPR (1 μM) alone or in combination with IFN-γ (50 IU/ml) or TAM (1 μM). Cells treated with 4-HPR alone or in combination with TAM were incubated for 5 days, and cells treated with a combination of 4-HPR and IFN-γ were incubated for 4 days. Cells were harvested and immunostained for NOS isoforms. *A*, NOSII immunostainings; *B*, NOSIII immunostainings. Untreated (*i*); 1 μM 4-HPR (*ii*); IFN-γ (*iii*); IFN-γ and 4-HPR (*iv*); TAM (*v*); TAM and 4-HPR (*vi*).

increasing the expression of NOSII and NOSIII. Although NOSII expression has not been correlated with ER expression, the expression of NOSII has been correlated with the expression of progesterone receptors (39), which is an indicator of functional ER (60). NOSIII expression is found mainly in ER+ breast cancer cells (61, 62). Although only four cell lines were used, we speculate that a reduced expression of NOSII and NOSIII may be one potential explanation for the lower sensitivity of ER− cells to 4-HPR. Future studies will be conducted to determine whether 4-HPR induces the transcriptional expression of NOS.

In the present study, the IFN-γ and 4-HPR combination resulted in additive or synergistic effects on NO production in all of the breast cancer cell lines investigated; however, ER− cells were less responsive to this combination. Furthermore, our results show that TAM enhanced 4-HPR-mediated NO production in ER+ but not in ER− breast cancer cells. The mechanisms by which IFN-γ or TAM enhance 4-HPR action are unknown. We have shown that one potential mechanism by which IFN-γ or TAM enhances 4-HPR-mediated growth inhibition is by increasing NO production via NOSII. A reduced ability to express NOSII may possibly be responsible for the lower response of ER− cells to the 4-HPR/IFN-γ and the 4-HPR/TAM combinations. We are aware that our results are in contrast to those of Coradini *et al.* (12), who reported that the effectiveness of the 4-HPR/TAM and 4-HPR/IFN combinations was independent of ER status. These discrepancies may be because we used different ER− cell lines (MDA-MB-453 and SKBr-3 *versus* BT20 and MDA-MB-231) and different IFN (type II, γ, *versus* type I, β) than they did.

4-HPR has potential chemopreventative effects in breast cancer (9, 10) as well as in ovarian cancer (63), bladder cancer (64, 65), and oral leukoplakia (66, 67). It remains to be determined whether the ability of 4-HPR to induce apoptosis via NO production is a mechanism specific to breast cancer cells or whether it represents a common apoptotic pathway in other cancer types as well. Nonetheless, furthering our understanding of the molecular mechanisms of 4-HPR action alone and in combination therapies will benefit the clinical application of 4-HPR in all of these cancers.

To summarize, we demonstrated a critical role of NOS induction and NO production in 4-HPR-mediated inhibition in breast cancer cells. Pharmacologically achievable doses of 4-HPR and TAM (68) were used in the present study. Thus, we believe our present study is highly relevant to the ongoing clinical trials. We speculate that determining the level of ER as well as NOS expression in breast tumor cells may allow us to identify patients who will most likely respond to 4-HPR.

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