MKP-1-Induced Dephosphorylation of Extracellular Signal-Regulated Kinase Is Essential for Triggering Nitric Oxide-Induced Apoptosis in Human Breast Cancer Cell Lines: Implications in Breast Cancer

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ABSTRACT

Apoptosis is regulated by a series of biochemical events that commits a cell to death. We are interested in understanding and have been investigating the mechanisms by which nitric oxide (NO) induces apoptosis in human breast cancer cell lines. In this study, we investigated the possible interplay of extracellular signal-regulated kinase (ERK) and Akt pathways in NO-induced apoptosis. MKP-1 transcripts were induced in these cells as early as 4 h, peaking at 8 h leading to inactivation of ERK1/2 at 16–24 h after exposure to NO. We also found 50% decrease in the levels of pAkt at 24 h of DETA-NONOate treatment. The inactivation of ERK1/2 preceded the dephosphorylation of Akt and apoptosis. NO was not able to inactivate ERK1/2 or Akt or to induce apoptosis in the presence of a phosphatase inhibitor, sodium orthovanadate, or antisense oligonucleotides, suggesting a cross-talk between the two pathways. NO also upregulated MKP-1 in another breast cancer cell line, ZR 75-30, which led to inactivation of ERK1/2 and induced apoptosis. In MDA-MB-231, NO did not induce MKP-1, and there was no ERK inactivation or apoptosis. Our results indicate that expression of MKP-1 by NO leading to dephosphorylation of ERK1/2 is the initial essential event that commits the cells to the apoptotic pathway in breast cancer cells.

INTRODUCTION

Apoptosis or programmed cell death is an essential process for eliminating unwanted or damaged cells by a series of biochemical events. Death receptor-independent apoptotic stimuli target various intracellular components, which transduces the death signal via specific sensors to the caspase machinery. BH3-only proteins like Bik, Blk, Bim, Bid, Bad, NOXA, and PUMA have been suggested as sensors and immediate targets of apoptotic stimuli, which are kept inert by transcriptional and translational mechanisms. Sensor proteins when activated by apoptotic stimuli transmit signals to the Bcl-2 family of proteins, which are central regulators of apoptosis, leading to alteration of permeability transition of the mitochondrial membrane, the release of cytochrome c and other apoptogenic proteins into the cytoplasm, activation of effector caspases, and apoptosis. The role of BH3-only proteins, which are essential sensors and trigger apoptosis, is not clearly understood. It will also be necessary to determine which BH3 protein is activated by a particular apoptotic stimulus and how their activations are controlled on the transcriptional and posttranslational levels. We have recently reported that Bid, a BH3-only protein, was activated by cleavage to tBid in breast cancer cell line MDA-MB-468 cells after exposure to NO-hydroxy-l-arginine and induced apoptosis by releasing cytochrome c from the mitochondria.

The mitogen-activated protein kinases (MAPKs) are signaling pathways critical for the conversion of diverse extracellular signals to biological responses, which include cell survival, proliferation, differentiation, cytostasis, and apoptosis. It will also be necessary to determine the interplay of extracellular signal-regulated kinase and propagates the cell survival signals of growth.
factors (15, 16). The pleckstrin homology domain on the Akt molecule is essential for activating Akt at the translational levels. Binding of lipid second messenger phosphatidylinositol 3,4,5-triphosphate to the pleckstrin homology domain results in the translocation of the Akt molecule to the vicinity of the membrane and its subsequent phosphorylation by phosphatidylinositol 3,4,5-triphosphate-dependent protein kinase PDK1 and PDK2 (17). Phosphorylation of Akt is required for its function and is positively regulated by phosphatidylinositol 3′-kinase, which phosphorylates phosphatidylinositol 4,5-bisphosphate to produce phosphatidylinositol 3,4,5-triphosphate. Upon activation, Akt translocates from the membrane to the nucleus where it regulates the transcription of genes required for cell survival. Akt prevents cells from undergoing apoptosis by inhibiting proapoptotic factors Bad and caspase-9 as well as nuclear translocation of Forkhead transcription factors by phosphorylation (17). There are reports that decreases in Akt levels initiate Bax integration in the mitochondria and induce apoptosis (18, 19). It is not known how Akt regulates Bax levels in the cytoplasm and controls apoptosis. The mechanism by which apoptotic stimuli decrease Akt levels to initiate apoptosis is also not well characterized.

NO, a highly reactive free radical molecule, is produced by activated macrophages and plays an important role in modulating the host defense mechanism against tumor cells (20, 21). Several in vitro studies have also demonstrated that NO donors are cytotoxic to tumor cells leading to apoptosis, mainly involving changes in mitochondrial permeability transition and release of cytochrome c from the mitochondria (22). Although there are reports of the involvement of subtypes of MAPK in NO-induced apoptosis, the signaling pathways through these MAPKs are poorly understood in most cell types. It has been reported that NO-induced apoptosis in neuronal progenitor cells is mediated by p38 kinase, whereas ERK and JNK but not p38 kinase are involved in NO-induced apoptosis in cardiomyocytes (23). We and others have reported that NO-induced apoptosis in human breast cancer cells was due to mitochondrial permeability transition and cytochrome c release from the mitochondria (24, 25). We additionally demonstrated that exposure of cells MDA-MB-468 cells to NO led to Bax migration and integration in the mitochondrial membrane and commitment of cells to apoptosis (26). NO-induced Bax integration to the mitochondrial membrane leading to apoptosis has also been shown in various other cell lines by other investigators (27, 28). However, very little is known about signaling pathways that precede Bax integration into the mitochondrial membrane, thereby triggering the cells to the apoptotic pathway in vitro as well as in vivo.

In this study, we investigated the targets upstream to Bax migration and the possible involvement of ERK and Akt pathways in NO-induced apoptosis. We initially used human breast cancer cell line MDA-MB-468, which does not contain any detectable NO synthase (24) to assess the effects produced by exogenous NO without any confounding effect of endogenous NO. We used DETA-NONOate, a NO donor with a relatively long half-life (20 h at 37°C), which at a concentration of 1 mM releases a steady state of NO in the range produced by activated macrophages (29). We report here that exposure of MDA-MB-468 cells to DETA-NONOate led to induction of MKP-1 at 4–8 h and dephosphorylation of ERK at 16–24 h. There was 50% inactivation of Akt at 24 h of exposure to DETA-NONOate. Suppression of MKP-1 simultaneously suppressed ERK and Akt inactivation, suggesting a cross-talk between the two pathways. We examined two other breast cancer cell lines and found NO-induced apoptosis in only those cell lines in which there was an up-regulation of MKP-1 and inactivation of ERK.

### Materials and Methods

**Chemicals.** DETA-NONOate was purchased from Cayman Biochemicals (Ann Arbor, MI), Actinomycin D, cycloheximide, and sodium orthovanadate were purchased from Sigma. U0126, Ro-31-8220, PD 98059, and caspase-3 inhibitors were purchased from Calbiochem. Caspase-3 substrate Ac-DEVD-AMC was purchased from BD PharMingen. All of the cell culture media were purchased from Life Technologies, Inc. (Gaithersburg, MD).

**Cell Culture.** Human breast cancer cell lines MDA-MB-468, ZR 75-30, and MDA-MB-231 were obtained from American Type Culture Collection. MDA-MB-468 was grown in DMEM containing 10 mM nonessential amino acids, 2 mM L-glutamine, 1 μg/ml insulin, and 5% fetal bovine serum (FBS). ZR 75-30 was grown in RPMI 1640 with 1 mM sodium pyruvate in 5% FBS. MDA-MB-231 was grown in DMEM containing 5% FBS and 10 μg/ml insulin. For experimental purposes, cells were allowed to seed overnight and treated with drugs for various time points.

**Western Analysis.** Cytosolic protein was extracted from treated or untreated cells, and concentrations were calculated. Thirty μg of cell lysates were electrophoresed and transferred onto polyvinylidene difluoride membranes. Equal loading of samples was confirmed by Ponceau-S staining of membranes. The following primary antibodies (1:1000 dilutions) were used: pRaf (9421S), pMEK (9211S), pERK1/2 (9121L; Cell Signaling), ERK1/2 (New England Biolabs), Akt (Sc-1619), Bax (Sc-493), MKP-1 (Sc-8599; Santa Cruz Biotechnology), and pAkt (559029; PharMingen). Immunoreactivity was detected by using enhanced chemiluminescence (Amersham Biosciences). The bands were quantified using Phospho-Image System (Molecular Dynamics).

**Microarray.** Total RNA was extracted from control and 16-h DETA-NONOate-treated cells using RNAeasy kit (Qiagen) according to the manufacturer’s instructions. The extracted RNA was purified by phenol-chloroform and Phase Lock Gel Light (Eppendorf) treatment. In brief, 10 μg of RNA were reverse transcribed using a T7-(dT)24 primer and Superscript Reverse transcriptase (Invitrogen-Life Technologies, Inc. Carlsbad, CA) at 42°C for 1 h. After synthesis of second strand, in vitro transcription was performed as per the manufacturer’s instructions (Enzo Diagnostics, Farmingdale, NY) using biotin-labeled UTP and CTP. Amplified cRNA was fragmented in a reaction containing 100 mM potassium acetate, 35 mM magnesium acetate, and 40 mM Tris acetate buffer (pH 8.1). Hybridization to 12,000 spotted microarrays on Affymetrix U95A v2 chips was performed at 65°C for 16 h in a buffer containing 15 μg of fragmented cRNA, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, 100 mM MES, 20 mM EDTA, 1 mM NaCl, and 0.01% Tween 20. Laser scanning was performed in the Affymetrix scanner and normalized to a target intensity of 2500. Data analysis was performed in the Microarray Suite 5 and Data Mining Tool, v3 (Affymetrix).

**Semiquantitative Reverse Transcription-PCR.** Total RNA from DETA-NONOate-treated cells was reverse transcribed, and the resulting cDNA was amplified as described previously (24) with the primers (Life Technologies, Inc.): MKP-1 sense, 5′-GCTGTGCAGCAAACAGTCGA-3′, and antisense, 5′-CGATTAGTCCCTCAAAGGTA-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5′-GTGAAAGCCTGTTCAACGAGTT-3′, and antisense, 5′-CACAGTCTTCTGAGTGGCAGTGAT-3′. The amplification was confirmed by automated DNA sequencing (data not shown). The GenBank accession number for Homo sapiens MKP-1 is X68277.

**Northern Analysis.** Cells were treated with DETA-NONOate for various time points, and total cellular RNA was extracted using RNAeasy (Qiagen) according to the manufacturer’s instructions. Equal amounts (15 μg/lane) of RNA were resolved by electrophoresis on a 1.2% formaldehyde-agarose gel, blotted onto nylon membrane, and hybridized with 32P-radiolabeled reverse transcription-PCR product for MKP-1 and GAPDH according to standard protocols. The membranes were analyzed by using Phospho-Image System (Molecular Dynamics).

**Caspase-3 Assay.** Control and treated cells were lysed in insect cell lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 mM NaCl, 2 mM EDTA, 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid, 10% sucrose, 5 mM DTT, and 1× protease inhibitor) for 30 min at 4°C. The protease inhibitors were obtained from Sigma. The lysates were incubated in assay buffer with 20 μg of caspase-3 substrate (acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin) as described previously (4). Active

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caspase-3 activity was monitored by production of fluorescent AMC, which was quantified using a fluorometer with excitation at 380 nm and emission at 440 nm.

**Cell Fractionation.** Cells (6 × 10^6) were harvested and washed with PBS. The cells were suspended in buffer A (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 250 mM sucrose containing [× protease inhibitor mixture], and homogenized by Dounce homogenizer; unbroken cells and nuclei were removed by centrifugation at 1,000 × g for 10 min at 4°C. The supernatant was centrifuged further at 10,000 × g for 20 min, and the resulting supernatant was saved as a cytosolic fraction while the precipitate was suspended in buffer A containing 0.5% (v/v) NP40 and was used as the mitochondrial fraction. For analysis of proteins that were integrated into the mitochondria, the mitochondrial fraction was suspended in 0.1 M Na₂CO₃ (pH 11.5) at 4°C for 30 min and subjected to ultracentrifugation at 100,000 × g for 1 h. The mitochondrial fractions were analyzed by Western blot with anti-bax antibody.

**Antisense Treatment.** Antisense oligonucleotides directed against the coding region of human Bax gene (GenBank accession no. AF339054) were custom synthesized from Life Technologies, Inc. Phosphorothioate-modified 20-mer Bax antisense 5'-H11032-3', and scrambled mismatched control 5'-H11032-3', were also used in this study. GAAAGCGAAGTCG-3', and H11032-TGCTCCCCGGACCCGTCCAT-3' were used in the study. Phosphorothioate-modified 20-mer MEP-1 antisense 5'-H11032-GGAACCTCAGTGGGAACCTCAGG-3' and scrambled mismatched control 5'-H11032-AGGTCTCGAAAGCGGAAGTCG-3', were used in this study. Twenty h after seedling, oligonucleotides were delivered at a 1:1 complex with oligonucleotides (30) were also used in this study. Twenty hours after seedling, oligonucleotides were delivered at a 1:1 complex with the lipofectin transfection reagent to subconfluent MDA-MB-468 cells. Lipofectin-oligonucleotide complexes, prepared to provide final oligonucleotide concentrations of 1 and 3 μM, were added for 16 h in serum and antibiotic-free medium and incubated at 37°C in a chamber with 5% CO₂. Subsequently, the cells were treated with DETA-NONOate for 24 h before harvesting.

**Statistical Analysis.** All values are expressed as mean ± SE. Each value is the mean of at least three separate experiments in each group. The differences in the effects of drug treatment when compared with control values were analyzed by one- or two-way ANOVA as appropriate. P values equal to or less than 0.05 were considered significant.

**RESULTS**

**DETA-NONOate Treatment Induced Dephosphorylation of ERK1/2.** To investigate events upstream to NO-induced Bak integration in the mitochondria, we initially examined the ERK1/2 pathway. Cells treated with DETA-NONOate for various time points were Western blotted with antibodies that specifically recognize phosphorylated Raf, MEK, and ERK1/2. No changes were detected in the levels of pRaf and pMEK; but pERK1/2 was dephosphorylated at 16–24 h of DETA-NONOate treatment (Fig. 1A). To examine whether the protein levels of ERK1/2 were changed after DETA-NONOate treatment, the membranes were stripped and probed with anti-ERK1/2 Abs. No change in the protein levels of ERK1/2 was detected at 36 h of exposure to DETA-NONOate (Fig. 1A). Densitometric scan showed 80–90% dephosphorylated ERK at 24 h of DETA-NONOate treatment (Fig. 1B). We examined further whether dephosphorylation of ERK1/2 led to inactivation of its downstream target, c-myc. Western analysis with phospho-c-myc Ab revealed dephosphorylation of c-myc at 16–24 h of DETA-NONOate treatment (data not shown). To confirm whether it was the NO released from DETA-NONOate that led to inactivation of pERK1/2, the cells were treated with DETA-NONOate in the presence and absence of 50 μM c-PTIO, a NO quencher (29). The DETA-NONOate-induced inactivation of pERK1/2 was abrogated in the presence of c-PTIO, indicating that inactivation was due to the NO released (Fig. 1C). We also observed that oxidized DETA-NONOate (DETA-NONOate solution kept at 37°C for 5 days) did not induce inactivation of pERK1/2 (Fig. 1C).

**DETA-NONOate Led to the Induction of a Phosphatase.** We next examined whether the effect of NO-induced inactivation of ERK 1/2 required de novo protein synthesis. To confirm this, we studied the effect of NO-induced inactivation of ERK 1/2 in the presence and absence of protein synthesis inhibitor cycloheximide (100 μg/ml). The cells after various treatments were prepared for Western analysis, and the membranes were probed with anti-pERK1/2 Abs. We observed that NO-induced dephosphorylation of ERK1/2 was abolished in the presence of cycloheximide (Fig. 2A). This confirmed the need for new protein synthesis in NO-mediated inactivation of pERK1/2.

To investigate whether the newly synthesized protein required for inactivation of pERK1/2 was a phosphatase, we performed in vitro dephosphorylation experiments by mixing lysates from DETA-NONOate-treated cells with those of control cells. Cells treated with or without DETA-NONOate for 16 h were harvested and lysed with insect cell lysis buffer, and equal amounts of control cell lysates (100 μg/ml) and DETA-NONOate-treated cell lysates were incubated either alone or together at 37°C for 30 min and then Western blotted for pERK1/2. The activated pERK1/2 from control cells was inactivated after incubation with lysates from DETA-NONOate-treated cells (Fig. 2B). Sodium orthovanadate, a nonspecific inhibitor of protein tyrosine phosphatases, when added to the above mixture abrogated the dephosphorylation of ERK1/2, identifying the dephosphorylating enzyme as a protein tyrosine phosphatase (Fig. 2B).

We next investigated whether NO led to induction of a phosphatase or activation of an existing inactive phosphatase. The de novo transcription in MDA-MB-468 cells was blocked by treatment with actinomycin D. The inactivation of pERK 1/2 that was observed after DETA-NONOate treatment was not observed in the presence of 5 μM actinomycin D (Fig. 2C), suggesting that NO led to induction of a protein tyrosine phosphatase.

**DETA-NONOate Induced MKP-1 Expression.** In a search for the specific tyrosine phosphatase up-regulated by NO in MDA-MB-
468 cells, we performed microarray analysis to identify the phosphatase gene. Total RNA was extracted from control and DETA-NONOate (16 h)-treated cells, reverse transcribed to cDNA, labeled, fragmented, and processed as described in “Materials and Methods.” The microarray data revealed a 7-fold up-regulation of MKP-1 in DETA-NONOate-treated cells compared with untreated cells. We performed semiquantitative reverse transcription-PCR analysis of RNA samples obtained after DETA-NONOate treatment of the cells. The reverse transcription-PCR product was sequence analyzed, confirmed, and used as a probe for Northern blot analysis to reconfirm the microarray data. We found undetectable levels of MKP-1 transcripts at 0 h, which appeared by 4 h, reached a maximum at 8 h, and thereafter subsequently declined (Fig. 3A). Densitometric scan showed a 7-fold up-regulation of MKP-1 at 8 h of DETA-NONOate treatment (Fig. 3C). A Western analysis was performed with lysates after DETA-NONOate treatment and probed with antibody to MPK-1. Although no MKP-1 protein was detected until 4 h, increased expression was seen at later time points of DETA-NONOate treatment (Fig. 3, B and D).

Inactivation of ERK1/2 Preceded the Akt Inactivation. While investigating for targets upstream to Bax integration, we also observed dephosphorylation of cytosolic Akt levels by 50% at 24 h of DETA-NONOate treatment (Fig. 4, A and C). We next wanted to examine the time course of dephosphorylation of ERK and Akt after treatment of the cells with DETA-NONOate. In all of the above experiments in which the cells were grown in 5% FBS, at 16 h, we found 60–70% of ERK dephosphorylated, whereas there was no significant dephosphorylation of Akt at 16 h of DETA-NONOate treatment (Fig. 4, A–C). Fig. 4, B and C, are densitometric scans of Western from Fig. 4A, and the band intensities of ERK and Akt were compared with GAPDH. To confirm further that ERK inactivation preceded that of Akt and whether these events affected Bax integration into the mitochondria, we varied the levels of activated ERK1/2 in the cells by growing them in either DMEM or 10% FBS. Cells grown in either DMEM or 10% FBS were exposed to DETA-NONOate in 5% FBS for 24 and 48 h, after which sensitivity of the cells to NO-induced apoptosis and Bax integration in the mitochondria were assessed. To examine the Bax integrated in the mitochondria, the cells after various treatments were subjected to cell fractionation, alkaline extraction, and ultracentrifugation as described in “Materials and Methods.” Western analyses of the mitochondrial pellet for Bax and the cytosolic fraction for pERK1/2 and pAkt and GAPDH were carried out. We observed that the sensitivity of MDA-MB-468 cells to NO-induced apoptosis decreased with increasing concentrations of FBS. Casa-
suppress the Bax levels in these cells. Bax suppressed cells treated with DETA-NONOate inactivated the ERK1/2 and Akt (Fig. 4F), but there was no caspase-3 activation or induction of apoptosis (data not shown). This experiment additionally suggested that inactivation of ERK1/2 and Akt are upstream events that occur before Bax integration and commitment of the cell to apoptosis.

Cross-Talk between ERK and Akt Pathways. We next wanted to examine the status of pAkt in the cells in which the up-regulation of MKP-1 by NO was suppressed by antisense MKP-1 oligonucleotides. MKP-1 phosphothioate oligonucleotides were added in 1 and 3 μM concentrations for 16 h before treating the cells with DETA-NONOate for 24 h. By Western blot analysis, we observed that DETA-NONOate-treated cells in presence of MKP-1 antisense oligonucleotides had 50% more pERK and 30–40% more pAkt when compared with DETA-NONOate treatment alone (Fig. 5, A–C). To confirm further the regulation of activated ERK1/2 on pAkt, we treated the cells with Ro-31-8220 (1 μM), which blocks up-regulation of MKP-1 (31). Ro-31-8220 suppressed the NO-induced inactivation of ERK1/2 and Akt when compared with untreated cells. We treated the cells additionally with sodium orthovanadate, a nonspecific phosphatase inhibitor, to inactivate MKP-1 and then exposed them to DETA-NONOate.

Caspase-3 assay was done to confirm the induction of apoptosis in the cells. Cells grown only in DMEM showed maximum caspase-3 activity at 24 h of DETA-NONOate treatment (Fig. 4E), at which point maximum Bax was found integrated in the mitochondria, ERK was completely inactivated, and pAkt levels were reduced by 50% (Fig. 4, D and E). The above membranes had been stripped and probed with GAPDH, and the densitometric scans of pERK/GAPDH and pAkt/GAPDH were done (data not shown). Cells grown in 10% FBS showed very little caspase-3 activity or Bax integration at 24 h of DETA-NONOate treatment, at which time point little or no ERK1/2 was found inactivated and no changes in the levels of pAkt compared with GAPDH were detected (Fig. 4, D and E). It was only at 48 h of treatment with DETA-NONOate that caspase-3 activity increased 6–7-fold (Fig. 4E), there was increased integration of Bax in the mitochondria, ERK1/2 was found to be completely dephosphorylated, and Akt levels were decreased (Fig. 4D). It therefore appears that ERK1/2 dephosphorylation preceded that of pAkt, and both events were found to be closely associated to the induction of apoptosis.

We examined further whether inactivation of ERK1/2 and Akt are events upstream to the migration and integration of Bax into the mitochondrial membrane. Cells were treated with antisense to Bax to

Fig. 4. DETA-NONOate-induced dephosphorylation of ERK1/2 precedes Akt dephosphorylation. A, cells grown in DMEM containing 5% FBS were treated with DETA-NONOate for various time points, harvested, and prepared for Western analysis using anti-pERK1/2 and pAkt Abs. B, densitometric units of pERK/GAPDH from A. Results are expressed as mean of three different experiments ± SE. * indicates the values are significantly different than the control P < 0.01. C, densitometric units of pAkt/GAPDH from A. Results are expressed as mean of three different experiments ± SE. * indicates the values are significantly different than the control P < 0.01. D, cells were grown in DMEM with (10%) or without FBS for 48 h. After 48 h, the medium was changed to 5% FBS, and the cells were treated with DETA-NONOate (1 mM) for various time points. Western analysis was performed using anti-pERK, pAkt, and GAPDH Abs. For the bottom panel, the cells after the previous treatments were fractionated, and mitochondrial fractions were treated as described in “Materials and Methods” to examine the mitochondrial integration of Bax. The mitochondrial fraction was analyzed by Western analysis using anti-Bax Ab. The cells after the above treatments were prepared for caspase-3 assay as described in “Materials and Methods.” F, cells were treated as follows: Lane 1, control; Lane 2, antisense bax oligonucleotide (1 μM) + DETA-NONOate; Lane 3, antisense bax oligonucleotides (3 μM) + DETA-NONOate. The cells were analyzed by Western analysis using anti-pERK 1/2 and pAkt Abs.

Fig. 5. Cells were treated as follows for 24 h: A, Lane 1, control; Lane 2, (DETA-NONOate); Lanes 3 and 4, antisense oligonucleotide to MKP-1 at 1 and 3 μM followed by DETA-NONOate; Lane 5, Ro-31-8220 + DETA-NONOate; and Lane 6, sodium orthovanadate + DETA-NONOate. Cells were analyzed by Western analysis after various treatments using anti-pERK1/2, pAkt, and GAPDH Abs. B, densitometric units of pERK1/2 from A. Results are expressed as mean of three different experiments ± SE. * indicates the values are significantly different than the control P < 0.01. C, densitometric units of pAkt from A. Results are expressed as mean of three different experiments ± SE. * indicates the values are significantly different than the control P < 0.01. D, cells after the above treatments were prepared for caspase-3 assay as described in “Materials and Methods.” Results are expressed as mean of three different experiments ± SE. * indicates the values are significantly different than the control P < 0.01.
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Fig. 6. Inactivation of ERK1/2 was sufficient to induce apoptosis in MDA-MB-468 cells: A, cells were treated as follows: Lane 1, control; Lane 2, DETA-NONOate for 24 h; Lane 3, 10 μg of PD 98059 for 3 days; Lane 4, 30 μg of PD 98059 for 3 days; Lane 5, 1 μg of U0 126 for 3 days; and Lane 6, 3 μg of U0 126 for 3 days. Cells after the above treatments were harvested and prepared for Western analysis, and the membrane was probed with anti-pERK1/2 and GAPDH Abs. B, densitometric units of pERK from A. Results are expressed as mean of three different experiments ± SE. * indicates the values are significantly different than the control. C, cells after the above treatments were prepared for caspase-3 assay as described in “Materials and Methods.” Results are expressed as mean of three different experiments ± SE. * indicates the values are significantly different than the control (P < 0.01).

The levels of pERK (70%) and pAkt (50%) were higher in cells treated with DETA-NONOate in the presence of sodium orthovanadate when compared with cells treated with DETA-NONOate alone. We also investigated whether the suppression of MKP-1 by the antisense oligonucleotides or the inhibitors had any effect on DETA-NONOate-induced apoptosis. After the above treatments for 48 h, caspase-3 assay was performed with cell lysates. Although in DETA-NONOate-treated cells, there was 7.5-fold increase in caspase-3 activity compared with untreated cells, in presence of MKP-1 antisense oligonucleotides, DETA-NONOate could only induce a 2-fold increase of caspase-3 activity (Fig. 6D). Similar reduction in caspase-3 activity was seen with other inhibitors. We also observed that the apoptotic morphology was retarded in cells treated with antisense oligos or inhibitors in the presence of DETA-NONOate (data not shown).

Inactivation of ERK1/2 Is Sufficient to Induce Apoptosis in MDA-MB-468 Cells. We next investigated whether inactivation of ERK1/2 in MDA-MB-468 cells was sufficient to induce apoptosis. The cells were treated for 3 days with MEK inhibitors (PD 98059 or UO126), which inhibit ERK1/2, after which they were prepared for Western analysis to examine the levels of pERK (Fig. 6A). Caspase-3 activity was also measured with the cell lysates after the above treatments. By Western and densitometric scans we observed only 50% decrease in pERK after 3 days of treatment with inhibitor PD 98059, whereas with inhibitor UO126, there was 95% decrease (Fig. 6, A and B). We observed that these inhibitors led to inactivation of ERK1/2, accompanied by activation of caspase-3 (Fig. 6C) and apoptosis induction. To examine further whether inactivation of ERK1/2 by these inhibitors also inactivated pAkt, the membrane was stripped and reprobed with pAkt antibody. We also observed a decrease in pAkt in cells treated with these inhibitors (data not shown).

Induction of DETA-NONOate-Induced Apoptosis Only in Cells in Which MKP-1 Was Induced and ERK1/2 Was Inactivated. We next wanted to assess whether expression of MKP-1 and dephosphorylation of ERK were essential prerequisites for NO-induced apoptosis in breast cancer cells. We used two other breast cancer cell lines, MDA-MB-231 and ZR 75-30, which were treated with DETA-NONOate for various time points. The cells were prepared for Western analysis to examine the levels of pERK, and the lysates were assayed for caspase-3. After the above treatments of these cell lines, RNA was also extracted, and semiquantitative reverse transcription-PCR for MKP-1 was done. NO was found to up-regulate MKP-1 in MDA-MB-468 and ZR 75-30 (Fig. 7B), ERK was found dephosphorylated (Fig. 7A), and caspase-3 was activated (Fig. 7C), indicating the onset of apoptosis. In MDA-MB-231, no MKP-1 induction was found after exposure to DETA-NONOate for 48 h. There was also no ERK inactivation or caspase-3 activity detected after treatment of these cells with DETA-NONOate (Fig. 7, A–C).

DISCUSSION

Despite significant clinical advances, the etiology and pathogenesis of breast carcinoma remains unclear, and breast cancer continues to be a common malignancy among women. The regulation of cell proliferation and cell survival in breast cancer is a complex interplay between steroid hormones, growth factors, and their receptors (32). Increased understanding of the signaling pathways is important to identify predictive factors controlling tumor aggressiveness and development of resistance to therapy. Over-expression of epidermal growth factor receptor HER-2/neu or Erb B2 in breast cancer uses MAPK and phosphatidylinositol 3’-kinase/Akt signaling pathways for mitogenic stimulation and has been shown to have prognostic significance (33). MAPK expression and activity are up-regulated in human breast tumors compared with surrounding normal tissues (34). Elevated ERK kinase activity has been found to be a critical event in the initiation and progression of human breast carcinoma as well as...
mediating invasion and metastasis (35). Akt has also been found to be highly expressed in human breast cancer cells and to actively participate in coordinating events regulating tumor development, progression, and invasion (36, 37).

The primary objective of this study was to elucidate the involvement of ERK and Akt pathways in NO-induced apoptosis. We found dephosphorylation of ERK1/2 at 16–24 h of exposure to DETA-NONOate, whereas no change in pRaf or pMEK was detected (Fig. 1A). This DETA-NONOate-induced dephosphorylation of ERK was due to NO released from the donor, because quenching of NO by cPTIO or oxidized DETA-NONOate could not dephosphorylate ERK (Fig. 1C). Dephosphorylation of ERK required cellular transcriptional and translational machinery, because NO was not able to inactivate ERK1/2 in the presence of actinomycin D or cycloheximide (Fig. 2, A and C). We confirmed the involvement of a phosphatase because NO-treated cell lysate dephosphorylated ERK1/2 from untreated cell lysates when the two were incubated together at 37°C for 30 min (Fig. 2B). Specificity of the protein tyrosine phosphatase to be MKP-1 or CL-100 was confirmed by microarray and Northern analysis. The MKP-transcript was not expressed in control cells but peaked at 4–8 h after exposure to NO, which was followed by increased expression of MKP-1 protein (Fig. 3, A and B). MKP-1 induction by NO has been reported in human embryonic lung fibroblasts (38) and vascular smooth muscle cells (39). MKP-1 could also be induced in a number of cell types, by a variety of stress stimuli like UV irradiation, heat shock, oxidative stress, or DNA-damaging agents, as immediate early gene products (40). MKP-1 has also been found up-regulated in some hypoxic tumors in which it antagonizes transient activation of JNK by enhancing its dephosphorylation (41). The functional importance of NO-induced MKP-1 leading to ERK1/2 inactivation and apoptosis had not been previously investigated in any cell type.

We found the induction of MKP-1 by NO to be an essential prerequisite for induction of apoptosis in breast cancer cells. This was clear from experiments in which NO-induced apoptosis was significantly reduced in cells transfected with antisense oligonucleotides to MKP-1, sodium orthovanadate, or Ro-31-8220 phosphatase inhibitors (Fig. 5D). In the above-treated cells in which NO-induced apoptosis was suppressed, ERK was found phosphorylated (Fig. 5A). We also observed that in MDA-MB-468 cells, inactivation of ERK pathway by PD98059 or U0126 inhibitors was sufficient to induce apoptosis (Fig. 6, A and C). We also examined the requirement of NO-induced ERK inactivation in other breast cancer cell lines. NO induced MKP-1 in MDA-MB-468 and ZR 75-30 cell lines, ERK was found dephosphorylated, and apoptosis was induced (Fig. 7, A–C). In MDA-MB-231 cell line, MKP-1 was not induced by NO. ERK remained phosphorylated, and there was no induction of apoptosis. DETA-NONOate could not induce MKP-1 in MDA-MB-231 cell line, although there is a report that lactacystin, a proteosome inhibitor, induced MKP-1 in this cell line (42). Inactivation of ERK1/2-mediated apoptosis in breast cancer cell lines has also been reported by BRACA 1 overexpression and with proteosome inhibitors (42, 43).

In this study we also observed a 50% decrease in phosphorylated Akt at 24 h of exposure to NO. Interestingly, the dephosphorylation of NO-induced ERK preceded the decrease in Akt levels in MDA-MB-468 cells. The inactivation of ERK and Akt pathways were upstream events independent of the Bax levels or occurrence of downstream apoptotic events in these cells. We also examined the possibility of cross-talk between the ERK and the Akt pathways during NO-induced apoptosis. Our results indicated that the phosphorylation state of ERK modulated the phosphorylation state of Akt. Numerous investigators have reported phosphatidylinositol 3’-kinase/Akt to inactivate MEK/ERK signaling cascade by Raf phosphorylation (44, 45). Only recently MEK/ERK-dependent phosphorylation of Akt has been reported in some ovarian cancer cell lines (46). It is becoming increasingly clear that different cell types interpret some signaling mechanisms differently. Coordination of the ERK and Akt pathways in a single cellular response has pleiotropic effects depending on cell line, stimulus specificity, and the stage of differentiation of the cells. It has been suggested that MEK/ERK signaling may be involved directly or indirectly in the regulation of action of PDK1, thereby leading to phosphorylation of Akt (46).

Caspase-dependent cleavage of Akt has been found in some cell lines during apoptosis (47). We have previously demonstrated that NO mediated inactivation of Akt in MDA-MB-468 cells in the presence of caspase-3 inhibitors, ruling out the involvement of activated caspase-3 (26). Additional work is also in progress to elucidate the precise mechanism(s) by which dephosphorylation of ERK inactivates Akt. Probable targets appear to be some key transcription factors like c-Myc and Elk, which are activated by ERK. NO-induced ERK inactivation also led to dephosphorylation of c-Myc. Some of these transcription factors control expression of genes essential for cell survival, and their inactivation leads to decrease in survival factors, whereas there is increase in the levels of apoptosis inducing factors.

Our results suggests that NO may potentially have therapeutic implications in the treatment of breast cancer. The proliferation of malignant cells is regulated by a variety of intracellular signaling pathways that cross-talk with each other. Combination therapies using two or more drugs have been found to be more effective for treatment of cancer compared with single-drug therapy (48, 49). We have previously reported potential of NO-induced apoptosis in MDA-MB-468 cells by farnesyl transferase inhibitor (50). In this work, we have found that exposure of breast cancer cells to concentrations of NO that is released by macrophages inactivate the two most important pathways that are involved in tumor progression. Drugs modulating these pathways may also be helpful in potentiating effects of agents currently being investigated.

In conclusion, our studies indicate that the initial event that triggers apoptosis by NO in human breast cancer cell lines is an increase in the expression of MKP-1 followed by dephosphorylation of ERK which then leads to dephosphorylation of Akt. This is a novel mechanism by which NO induces apoptosis in human breast cancer cells. In the breast cancer cell line in which NO was not able to induce the expression of MKP-1, the cells did not follow the apoptotic pathway. Additional studies are in progress to evaluate whether this phenomenon is unique to breast cancer cell lines or whether it is a more generalized.

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4. Singh, R., Pervin, S., and Chaudhuri, G. Caspase-dependent cleavage of Akt has been found in some cell lines during apoptosis (47). We have previously demonstrated that NO mediated inactivation of Akt in MDA-MB-468 cells in the presence of caspase-3 inhibitors, ruling out the involvement of activated caspase-3 (26). Additional work is also in progress to elucidate the precise mechanism(s) by which dephosphorylation of ERK inactivates Akt. Probable targets appear to be some key transcription factors like c-Myc and Elk, which are activated by ERK. NO-induced ERK inactivation also led to dephosphorylation of c-Myc. Some of these transcription factors control expression of genes essential for cell survival, and their inactivation leads to decrease in survival factors, whereas there is increase in the levels of apoptosis inducing factors.

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