Increased Susceptibility of Breast Cancer Cells to Stress Mediated Inhibition of Protein Synthesis

Shehla Pervin, An H. Tran, Shaghayegh Zekavati, Jon M. Fukuto, Rajan Singh, and Gautam Chaudhuri

Abstract

Protein synthesis is a tightly regulated process, and its deregulation plays an important role in tumorigenesis. Protein synthesis remains poorly understood with very few well-identified validated targets for therapeutic purposes. In this study, we use nitric oxide (NO), which suppresses protein synthesis by inactivating eukaryotic initiation factor 2-α (eIF2-α), to examine the mechanism by which low and high oxidative stress inhibits protein synthesis. In breast cancer cells, low NO stress induced heme-regulated inhibitor (HRI) activation, which facilitated gradual decline in short half-life proteins. High NO stress induced HRI and protein kinase R (PKR) activation, leading to a sharp decline in protein synthesis as accessed by a decline in short and long half-life proteins and dramatic morphologic changes. In contrast, human mammary epithelial (HME) and Ras transfected untransformed HME (MCF-10A1 neo N) cells were less susceptible to NO-induced inhibition of protein synthesis and cytostasis. Our results suggest that NO-induced cytostasis in breast cancer cells was due to PKR activation and increased phosphorylation of eIF2-α, whereas the reduced susceptibility of normal mammary epithelial cells to NO could be due to the inaccessibility of PKR, which is bound to inhibitor p58.

Introduction

Protein synthesis is a tightly regulated process that plays a critical role in proliferation and differentiation (1, 2). Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK1/2 and phosphorylidyinositol 3-kinase (PI3K)/Akt are the prominent signaling cascades that deregulate mammalian target of rapamycin (mTOR), a master regulator of protein synthesis and its downstream specific translation factors, like eIF4E and p70 S6 kinase (3, 4). Deregulation of proliferation pathways and protein synthesis has been strongly implicated in the pathogenesis of cancer and metastasis (5–7). In spite of intense research, protein synthesis has been strongly implicated in the pathogenesis of cancer and metastasis (5–7). In spite of intense research, protein synthesis has been strongly implicated in the pathogenesis of cancer and metastasis (5–7). In spite of intense research, protein synthesis has been strongly implicated in the pathogenesis of cancer and metastasis (5–7).

Materials and Methods

Materials. DETA-NONOate, SNAP, ODQ, and 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-1-oxyl-3-oxide (cPTIO) were purchased from Cayman Biochemicals. Actinomycin D, hydrogen peroxide, cycloheximide, DTT, leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), hemin, 2-aminopurine, ponceau S solution, and protein A-Sepharose beads were purchased from Sigma. All of the cell culture media were purchased from Mediatech, Inc. Rabbit polyclonal anti-phosphorylated Akt (pAkt; 599029) was from Pharmingen, BD Biosciences. ERK1/2 MAPK (9102) antibody was purchased from Sigma. All of the chemicals. Actinomycin D, hydrogen peroxide, cycloheximide, DTT, leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), hemin, 2-aminopurine, ponceau S solution, and protein A-Sepharose beads were purchased from Sigma. All of the cell culture media were purchased from Mediatech, Inc. Rabbit polyclonal anti-phosphorylated Akt (pAkt; 599029) was from Pharmingen, BD Biosciences. ERK1/2 MAPK (9102) antibody was purchased from Sigma. All of the cell culture media were purchased from Mediatech, Inc. Rabbit polyclonal anti-phosphorylated Akt (pAkt; 599029) was from Pharmingen, BD Biosciences.

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from New England Biolabs. Cyclin D1, Akt, ornithine decarboxylase (ODC), cyclin-dependent kinase 4 (CDK4), cyclin E, eIF2-α, and ERK1/2 antibodies were from Santa Cruz Biotechnology, Inc. Phosphorylated mTOR, p-eIF4E, phosphorylated p70S6K, pAkt, heme-regulated inhibitor (HRI), p-eIF2-α, p58, and phosphorylated ERK1/2 (p-ERK1/2) were from Cell Signaling Technology. Rabbit anti–phosphorylated PKR (pPKR; pT451) was obtained from Invitrogen BioSource. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Chemicon. Tran 35S label was from MP Biomedicals. PKR antibody was purchased from Sigma.

**Cell culture.** Human breast cancer cell lines MDA-MB-231, MDA-MB-468, and MCF-7 were obtained from American Type Culture Collection. MDA-MB-231 and MCF-7 were cultured in DMEM containing sodium pyruvate, 10 mmol/L nonessential amino acids, 2 mmol/L l-glutamine, 1 μg/mL insulin, and 10% fetal bovine serum (FBS). MDA-MB-468 were cultured in DMEM containing 10 mmol/L nonessential amino acids, 2 mmol/L l-glutamine, 1 μg/mL insulin, and 10% FBS without sodium pyruvate. HME was obtained from Cambrex Bio Sciences. MCF-10A(R) cells, a spontaneously immortalized untransformed HME cell line (MCF-10A transformed with H-Ras oncogene), was obtained from Robert J. Pauley (Barbara Ann Karmanos Cancer Institute). HME and MCF-10A(R) (R) cells were cultured in DMEM/Ham's F-12 (1:1) supplemented with 5% equine serum, 10 mmol/L HEPES, 10 μg/mL hydrocortisone. For experimental purposes, cells were grown in 5% FBS, allowed to seed overnight, and treated with drugs for various time periods.

**Cytostasis assay.** Cells (7.5 × 10^6 per well) were seeded in six-well plates. After overnight seeding, the medium was changed and fresh drugs were added. The cells were collected from 0 to 24 h, viability was determined by trypsin blue exclusion method, and cells were counted on a hemocytometer.

**Cell cycle analysis.** Cells suspended in hypotonic DNA staining buffer (0.1% sodium citrate/0.3% Triton X-100/0.01% propidium iodide/0.005% RNase A) were incubated for 15 min at 4°C and subjected to fluorescence-activated cell sorting (FACS) to analyze the percentage of cells in the different phases of the cell cycle (16).

**Measurement of total protein synthesis by metabolic labeling.** MDA-MB-231 cells (2 × 10^6) were plated overnight in media containing DMEM and 5% FBS. Cells treated with or without DETA-NONOate (1 or 2 mmol/L) for 6 or 16 h were starved for 3 h in methionine-cysteine-free medium (Life Technologies-Bethesda Research Laboratories), after which they were labeled with 100 μCi/mL of a mixture of [35S]methionine for 4 h. After removal of the radioactive media, the cells were collected, washed twice with PBS, and lysed on ice for 30 min in 1 mL of lysis buffer [10 mmol/L Tris-HCl (pH 7.4)/150 mmol/L NaCl/1 mmol/L EDTA [pH 8.0]/0.1% (v/v) Triton X-100 containing 0.2 mmol/L PMSE, 1 μmol/L leupeptin A, and 1 μmol/L leupeptin. Cell lysates were clarified by centrifugation at 2,200 × g for 5 min, and the protein concentrations of the supernatant was determined using Protein Assay Dye Reagent Concentrate (Bio-Rad) for analysis of total cellular protein. The cell fractionation was done according to the manufacturer's protocol.

**Results**

Low and high NO stresses affect protein synthesis at different levels. It is well established that the phosphorylation of eIF2-α, a key player in protein synthesis, attenuates the rate of translation of capped cellular mRNAs (8, 18). NO has been found in a few cell types to suppress protein synthesis by eliciting the phosphorylation of eIF2-α, a regulatory protein in the translational machinery to induce a global decline of protein synthesis, cells pretreated with DETA-NONOate (1 and 2 mmol/L) were metabolically labeled with [35S]methionine for 4 hours. Incorporation of

To determine if NO-induced stress inactivated the translational machinery to induce a global decline of protein synthesis, cells pretreated with DETA-NONOate (1 and 2 mmol/L) were metabolically labeled with [35S]methionine for 4 hours. Incorporation of
A, high and low NO stress induced differential increase of p-eIF2-α levels and decrease in cyclin D1 levels, whereas eIF2-α levels remained unaltered. Left, MDA-MB-231 cells were treated with DETA-NONOate (1 or 2 mmol/L) and prepared for immunoblot analysis at 24 h. The membrane was probed with cyclin D1, p-eIF2-α, eIF2-α, and GAPDH antibodies. Results are representative of three different experiments. Right, arbitrary densitometric units (ADU) of A: columns, mean of three different experiments; bars, SE. *, \( P \leq 0.02; **, P \leq 0.001 \) compared with control untreated p-eIF2-α; #, \( P \leq 0.02; ##, P \leq 0.001 \) compared with control untreated cyclin D1 group.

B, high NO stress induced sharper decline in protein synthesis. MDA-MB-231 cells were treated with DETA-NONOate (1 or 2 mmol/L) before exposure to [35S]methionine and [35S]cysteine. The cells were harvested at 6 and 16 h, as described in Materials and Methods. The labeled proteins were measured with a scintillation counter. Results are representative of three different experiments. *, \( P \leq 0.02; **, P \leq 0.005 \) compared with control untreated cells.

C, left, low NO stress induced decline in the levels of short half-life proteins but not long half-life proteins. MDA-MB-231 cells were treated with DETA-NONOate (1 mmol/L) at different time points and prepared for Western blot analysis, as described in Materials and Methods. The membrane was probed with cyclin D1, c-myc, ODC, cyclin E, CDK4, and GAPDH antibodies. Results are representative of three different experiments. Right, high NO stress induced decline in the levels of short and long half-life proteins. MDA-MB-231 cells were treated with DETA-NONOate (2 mmol/L) at different time points and prepared for Western blot analysis. The membrane was probed with cyclin D1, cyclin E, and GAPDH antibodies. Results are representative of three different experiments.

D, high NO stresses induced morphologic changes in MDA-MB-231 cells. Left, MDA-MB-231 cells were treated with and without DETA-NONOate (1 and 2 mmol/L). Cell numbers for 1 and 2 mmol/L NO–treated cells were comparable. After 24 h, cells were photographed with LEICA DM IRBE microscope at 10× magnification. Results are representative of three different experiments. Right, high NO stress induced increase in G1 phase of cell cycle. FACs analysis of control, 1, and 2 mmol/L DETA-NONOate–treated MDA-MB-231 cells at 24 h. The cells were prepared for FACs analysis, as described in Materials and Methods, and analyzed in a FACsCalibur flow cytometer. The number of cells in each phase of the cell cycle was obtained by MODFIT software. Results are expressed as a mean of three different experiments. *, \( P \leq 0.05 \) compared with untreated control without DETA-NONOate treatment.
the labeled methionine in newly synthesized protein was measured by liquid scintillation counter. Low and high NO stress led to a ~40% and 85% decline in labeled proteins, respectively, at 16 hours compared with untreated cells (Fig. 1B). Because the above experiments indicated that NO induced a global decline in protein synthesis, we assessed the levels of cyclin D1, other cyclins, CDKs and proteins with a similar half-life. In MDA-MB-231 cells, low NO stress induced a down-regulation of short half-life proteins, like ODC and C-Myc, whereas levels of longer half-life proteins (cyclin E and CDK4) remained unaltered (Fig. 1C, left). High NO stress (2 mmol/L DETA-NONOate), however, led to a sharp decline in cyclin D1, whereas cyclin E, a long half-life protein was also reduced by 50% (Fig. 1C, right).

We further examined the effect of NO stress on cell proliferation. We found that both low and high NO stress induced cytostasis, as there was no increase in cell number, which was observed as early as 16 hours (data not shown). However, there was a distinct difference in the effect on the cellular morphology between the levels of NO stress. With low levels of NO stress, the cells were cytostatic but retained the flattened morphology of control cell, whereas with high NO stress, the cytostatic cells changed their flattened morphology to become round (Fig. 1D, left). In the cell cycle analysis, we observed that with low and high NO stress, there was a 20% to 22% increase in G1 cell cycle arrest (Fig. 1D, right). Treatment of MCF-7 and MDA-MB-468 with low and high NO stress also increased p-eIF2-α levels and reduced protein synthesis (data not shown).

Low NO stress activated HRI, whereas high NO stress also activated PKR in breast cancer cells. We further examined the kinases that are upstream of eIF2-α to understand the mechanism by which low and high NO stress differentially inactivates eIF2-α. Specific stresses, such as oxidative stress, heme deprivation, or viral infection in mammalian cells, have been found to phosphorylate eIF2-α via four upstream kinases: double-stranded RNA-dependent PKR, HRI, general control nonderepressible-2, and double-stranded RNA-activated protein kinase–like ER kinase (19, 20). Because NO has an affinity for heme, we initially examined whether HRI contributed to NO-induced up-regulation of p-eIF2-α levels and cell cycle arrest in breast cancer cells. HRI is reported to be present in infection in mammalian cells, have been found to phosphorylate eIF2-α via four upstream kinases: double-stranded RNA-dependent PKR, HRI, general control nonderepressible-2, and double-stranded RNA-activated protein kinase–like ER kinase (19, 20). Because NO has an affinity for heme, we initially examined whether HRI contributed to NO-induced up-regulation of p-eIF2-α levels and cell cycle arrest in breast cancer cells. HRI is reported to be present in

Breast cancer cells were more susceptible to high NO stress–induced inhibition of protein synthesis compared with normal cells. To further validate the exclusive role of PKR in mediating the effects of high NO stress on protein synthesis, we used PKR siRNA to specifically reduce PKR levels in breast cancer cells. PKR siRNA induced a ~70% decline in PKR levels after 48 hours, as accessed by immunoblot analysis (Fig. 3A). PKR siRNA-treated cells, when further exposed to high stress, did not increase in p-eIF2-α levels, which contrasts to ~2-fold increase found with random siRNA treatment (Fig. 3A). NO induced a sharp decline in cyclin D1 levels in random siRNA-treated cells, which was found attenuated in PKR siRNA-treated cells (Fig. 3A). Further examination of NO-induced cytostasis in PKR and random siRNA-treated cells showed that NO induced only ~25% decline in proliferation of PKR siRNA-treated cells compared with ~50% decline in random siRNA-treated cells (Fig. 3B, left). Morphologic changes associated with high NO stress were also found attenuated with PKR siRNA treatment (data not shown). We also examined the reversibility of NO-induced cell cycle arrest in random and PKR siRNA-treated cells. Cells were treated with low and high NO stress for 24 hours before the media were changed and cells were allowed to recover for another 24 hours. Whereas recovery of random siRNA-treated cells from low NO stress was reversible, recovery from high NO stress was irreversible even until 48 hours (Fig. 3B, right). In PKR siRNA cells, however, recoveries from low and high NO stress was reversible (Fig. 3B,
right). These results suggest that NO stress–induced PKR activation led to a stronger and less reversible effect on protein synthesis.

It has been reported that p58 is an inhibitor of PKR and remains tightly associated with PKR in cells that overexpress this inhibitor. The levels of inhibitor p58 were assessed in HME, as well as MDA-MB-231 and MDA-MB-468 cells. We found higher levels of inhibitor p58 in HME cells compared with MDA-MB-231 and MDA-MB-468 cells, whereas HRI levels were comparable in both cell types (Fig. 3C, left). Although the levels of HRI was comparable, we found that high NO stress induced lower levels of p-eIF2-α (1-fold to 1.5-fold) in HME cells compared with the higher levels of p-eIF2-α (2-fold to 2.5-fold) observed in breast cancer cells (Fig. 3C, left). Due to low free PKR and p-eIF2-α levels in HME and MCF-10A1(R) (data not shown) cells, we examined the effect of low and high NO stress on the total cellular protein concentration and also cell proliferation. In mammary epithelial cells, there was no significant decline of total cellular proteins when exposed to low and high NO stress (Fig. 3C, right). This is in sharp contrast to breast cancer cell line MDA-MB-231, where the decline of total cellular protein was prominent with both low and high NO stress (Fig. 3C, right). We also compared the effect of low and high NO stress on cell proliferation in mammary epithelial and breast
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cancer proliferation. In response to low and high NO stress, the decline in cell proliferation was more prominent in breast cancer than mammary epithelials (Fig. 3D, left). We also investigated the effect of high and low NO stress on cyclin D1 and cyclin E levels in MCF-10A1(R) cells. We observed that, while the decline in cyclin D1 in MCF-10A1(R) cells was comparable with cancer cells, there was no significant decline in cyclin E (Fig. 3D, right). These results suggested that activated PKR in breast cancer cells led to a prominent increase of p-eIF2-α and a strong inhibition of protein synthesis.

NO stress potentiated PKR activation after serum starvation or hydrogen peroxide treatment. Although PKR is overexpressed in breast cancer cells, we observed that high NO stress was required for its activation. Because tumors are subjected to numerous stresses in vivo, we wanted to assess whether low NO stress in the presence of another cellular stress could potentiate PKR activation. To determine NO-induced potentiation of PKR activation in the presence of another stress, cells were exposed to 300 μmol/L hydrogen peroxide (H2O2), an oxidative stress, and lower NO stress (DETA-NONOate; 300 or 500 μmol/L). We found that, whereas H2O2 or lower NO stress did not induce PKR activation alone, simultaneous exposure of the cells to lower NO and H2O2 dramatically increased PKR activation when analyzed by native gel electrophoresis (Fig. 4A). Similar potentiation of PKR activation was observed in serum-starved cells exposed to lower NO stress (Fig. 4A). We further examined the effect of these stresses, alone or in combination, on eIF2-α activation. Increased levels of p-eIF2-α was observed in cells with a combination of NO and another stress (Fig. 4B, left). We examined the effect of simultaneous exposure of NO and H2O2 on cell proliferation and observed that the two stresses led to dramatic decline in cell number compared with either stress alone (Fig. 4B, right). We also assessed the effect of these stresses had on protein synthesis by monitoring the levels of total cellular protein, cyclin D1, and cyclin E in these cells. We observed that NO exposure to H2O2 or serum-starved cells had a lower total protein concentration (data not shown) and lower levels of cyclin D1 and cyclin E than either stress alone. Cell cycle analysis was also performed in cells where NO potentiated PKR activation. The potentiation of PKR activation with NO treatment was also found in cells pretreated with either tumor necrosis factor-α (TNF-α) or wortmannin (PI3K inhibitor) compared with stress alone (data not shown).

Most chemotherapeutic agents, like paclitaxel and tamoxifen, when given in high concentrations, target cellular DNA replicating machinery to induce cell cycle arrest and induce apoptosis (30, 31). High concentrations of these agents could also induce toxicity to normal cells (30, 31). We investigated whether NO could potentiate PKR activation in the presence of actinomycin D, which targets cellular DNA. While actinomycin D or NO itself could up-regulate the monomeric forms of PKR, simultaneous exposure of these stresses to cells dramatically increased PKR activation (Fig. 4C). This increase was also reflected in the levels of p-eIF2-α, which increased phosphorylation was observed in cells exposed to NO and actinomycin D (Fig. 4D). We, therefore, concluded that lower or low NO stress in the presence of another stress potentiated PKR activation in breast cancer cells.

NO stress–induced inactivation of eIF2-α was independent of p-ERK, p-AKT or cyclic guanosine 3’,5’-monophosphate levels in breast cancer cells. In this study, we found that, in breast cancer cells, low NO stress increases monomeric cytosolic forms of PKR whereas active forms of PKR was up-regulated by high NO stress. To further assess the mechanism by which NO induced up-regulation of PKR, we examined the possible involvement of p-ERK1/2 and pAkt, which have been implicated to be responsive to NO and other stimuli (32–34). We have reported in MDA-MB-231 cells that, with NO treatment, there was decline in cyclin D1 levels, retinoblastoma was hypophosphorylated, and cells underwent cytostasis (16). In the present study, we observed that, with low and high NO stress (data not shown), the levels of p-ERK1/2 remain elevated whereas ERK remains unchanged (Fig. 5A, top). To further access the role of p-ERK1/2 in NO-induced PKR activation, cells were treated with PD 98059, which inhibits MEK1/2, the upstream kinase of ERK1/2. NO-induced PKR activation occurred even in the absence of ERK activation, suggesting no role of ERK1/2 in NO-induced PKR activation (data not shown). We also examined the levels of pAkt, which was found high with NO treatment, whereas levels of Akt remain unaltered. We examined the effect of NO on the downstream targets of Akt, such as mTOR, eIF4E, and p70s6k, which are critical for the translation of cap-dependent mRNA. We found that in NO-treated MDA-MB-231 cells, key players mediating the translation of cyclin D1, like p-mTOR, eIF4E, and p-p70s6k, were increased and p70s6k retained highly activated despite the decline in cyclin D1 levels (Fig. 5A, top).

We also examined another cell line, MDA-MB-468, where we have reported that NO treatment induces decline in the levels of p-ERK1/2 due to the induction of MAPK phosphatase-1 (32). NO treatment of MDA-MB-468 cells activated PKR although

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**Figure 2.** A, left, NO induced increase in the levels of HIF1. MDA-MB-231 cells were treated with DETA-NONOate (1 or 2 mmol/L) and harvested at 24 h. The cells were prepared for Western blot analysis as described in Materials and Methods. The membrane was probed with HIF1 and GAPHDH antibodies. Results are representative of three different experiments. Right, CO attenuated NO-induced increase of p-eIF2-α levels and decrease of cyclin D1 levels. MDA-MB-231 cells were treated as follows: lane 1, untreated cells; lane 2, cells treated with DETA-NONOate (1 mmol/L); lane 3, cells treated with CORM A1 (500 μmol/L); lane 4, cells pretreated DETA-NONOate (1 mmol/L) for 1 h before exposure to DETA-NONOate (1 mmol/L). The cells were harvested at 24 h and prepared for Western blot analysis. The membranes were probed with p-eIF2-α, cyclin D1, and GAPHDH antibodies. Results are representative of three different experiments. B, arbitrary densitometric units of A (right). Columns, mean of three different experiments; bars, SE. *P < 0.02 compared with control p-eIF2-α group without CORM A1 and DETA-NONOate treatment; ¶, P < 0.01 compared with control cyclin D1 group without CORM A1 and DETA-NONOate treatment. C, high NO stress induced significant increase of dimeric and monomeric forms of PKR. MDA-MB-231, MDA-MB-468, and MCF-10A1 cells were treated as follows: 0 h, cells untreated; 16 h, cells treated with DETA-NONOate (1 or 2 mmol/L) for 16 h as indicated. The cells were harvested at their respective time points and prepared for Western blot analysis, as described in Materials and Methods. The membranes were probed with PKR antibodies and Ponceau-S stained to confirm equal loading of sample in the gel. D, lower panel shows Western blots probed with pPKR antibody. Results are representative of three different experiments. D, left, high NO stress sharply increased PKR association with eIF2-α. MDA-MB-231 cells were treated with 1 and 2 mmol/L DETA-NONOate for 16 h. The cells were prepared for immunoblot analysis as described in Materials and Methods. Immunoprecipitated PKR was analyzed for eIF2-α association. Results are representative of three different experiments. Right, high NO stress–mediated up-regulation of eIF2-α levels was attenuated with 2-aminopurine. Treatment of 2-aminopurine, a PKR inhibitor, did not affect the increase of p-eIF2-α levels with low NO stress (1 mmol/L) but did attenuate the increase of p-eIF2-α levels with high NO stress (2 mmol/L). MDA-MB-231 cells were treated as follows: lane 1, untreated cells; lanes 2 and 3, cells treated with DETA-NONOate (1 and 2 mmol/L) respectively; lanes 4 and 5, cells pretreated with 2-aminopurine (2 mmol/L) for 45 min before exposure to DETA-NONOate (1 and 2 mmol/L). The cells were harvested at 24 h and prepared for Western blot analysis, as described in Materials and Methods. The membranes were probed with p-eIF2-α and GAPHDH antibodies. The results are representative of three different experiments.


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p-ERK was down-regulated, suggesting that activation of PKR was independent of p-ERK levels (Fig. 5A, bottom). The levels of pAkt were also down-regulated in this cell line, whereas Akt remain unchanged. Thus, we conclude that NO-induced PKR activation is independent of the levels of p-ERK and pAkt in breast cancer cells. Because cyclic guanosine 3',5'-monophosphate (cGMP) is a well-characterized second messenger of NO-mediated signaling, we examined whether it had a role in NO-induced PKR activation. Treatment of cells with ODQ, an inhibitor of NO-mediated cGMP generation, did not significantly affect NO-induced up-regulation of PKR (Fig. 5B). These results showed that NO-induced PKR activation and up-regulation of p-εIF2-α was cGMP independent and did not involve p-ERK or pAKT/mTOR/εIF4E pathways.

We assessed PKR stability in a number of breast cancer cell lines MCF-7, MDA-MB-468, and MDA-MB-231 and also in MCF-10A1(R) cells using cycloheximide. There was a sustained increase in cytosolic PKR, which was high even at 36 hours in the breast cancer cells (Fig. 5C) with cycloheximide treatment. In MCF-10A1(R) cells, cycloheximide treatment led to a slight decline in PKR levels as early as 3 to 6 hours. This PKR decline in MCF-10A1(R) cells was a sharp contrast to the stability of PKR in breast cancer cells (Fig. 5C). The cycloheximide-induced increase of PKR in MDA-MB-231 cells was also attenuated by MG-132 treatment.
(chemical inhibitor of the 26S proteasome), suggesting a possible role of the ubiquitin proteosomal machinery in PKR up-regulation (data not shown).

Because there was a sharp contrast in PKR stability between breast cancer and normal mammary epithelial cells, we examined the nuclear and cytosolic localization of PKR in these cells. In cancer cell lines MDA-MB-231 and MDA-MB-468, PKR was predominant in the cytosolic fraction, whereas in MCF-10A1(R) cells, PKR was equally distributed in the cytosolic and nuclear fractions (Fig. 5D).

Because NO-induced increase in PKR activation was independent of the protein synthesis, total cellular levels of PKR in control and after DETA-NONOate treatment was examined. This was accomplished by lysis of the cells in RIPA buffer, which solubilizes all the cellular membranes, and analysis of PKR in SDS PAGE gels. We did not observe any change in PKR levels in control and DETA-NONOate–treated cells, suggesting that total cellular PKR levels remain unaltered (data not shown).

**Discussion**

In this study, we found that breast cancer cells overexpressing PKR were more susceptible to NO stress–induced inhibition of protein synthesis than HME and MCF-10A1(R) cells. PKR is a component of the signal transduction pathways mediating cell growth and responses to stress. It consists of two functionally distinct domains: an NH2 terminal double-stranded RNA (dsRNA) binding regulatory domain, which consists of two dsRNA binding motifs and a COOH terminal kinase catalytic domain. PKR exists in a latent monomeric state, in which dsRNA binding domains autoinhibit the kinase domain. Binding of dsRNA ligand induces conformational changes in PKR to promote its dimerization and autophosphorylation (28, 35). PKR can also be activated by cytokines and stress signaling pathways that likely operate independent of dsRNA. The most well-characterized role of PKR is phosphorylation of eIF2-α at Ser51, which inhibits translation of all capped mRNA (27, 28). PKR has been found to be a potent negative regulator of cell growth and proliferation in yeast and mammalian cells (36).

Our model system using DETA-NONOate, in breast cancer cells, enabled us to expose cells to different levels of stress. Adjustable concentrations of the NO donor and use of pharmacologic inhibitors (2-aminopurine, CO, and hemin) helped us elucidate the sequential steps in low and high NO stress–induced inhibition of protein synthesis, where HRI activation preceded PKR activation (Fig. 6C). We found that NO stress in breast cancer cells up-regulated the levels of cytosolic monomeric and dimeric PKR. This activation of PKR led to high levels of p-eIF2-α, sharp decline in protein synthesis, changed cellular morphology, and irreversible cytostasis. Reducing the levels of PKR in these cells by PKR siRNA treatment, however, led to only diminished effects of NO stress on protein synthesis and reversibility of the cytostatic process. HRI consists of two heme-binding domains: a stable heme-binding site and a reversible heme-binding site. During activation, HRI is phosphorylated at multiple sites by autophosphorylation and exists as an active dimer held by noncovalent interactions (21–25). It is reported that CO and NO compete for the stable heme-binding site on HRI (22, 23). We found that treatment with a CO donor, CORM-A1, attenuated the activation of HRI by low NO stress. High and low NO stress activated HRI in breast cancer cells, inducing small up-regulation of p-eIF2-α and inhibition of protein synthesis with a decline in only short half-life proteins (Fig. 6C).

The effect of low and high NO stress levels mediating HRI activity is likely the result of a direct interaction with its heme group. This is a first-order process involving direct ligation of NO to the heme moiety. However, the somewhat selective effect of high NO stress to mediated PKR activity is likely due to the generation of nitrogen oxide-mediated thiol nitrosation, leading to PKR activation. N2O3 is a predominant nitrosating species derived from NO. Because the generation of N2O3 exhibits higher order kinetics (second order in NO), this chemistry is relevant only at high NO levels. Moreover, because this process is dependent on NO concentration to the second power, even a seemingly minor increase in NO (2-fold in this study) can result in an exponential (and not linear) increase in the generation of N2O3. This likely explains the large differences in activity between low (~1 μmol/L) and high (~2 μmol/L), although there is only a 2-fold difference in the concentration of NO.

Over the past years, there have been efforts to develop methods of using dsRNA to activate PKR selectively in cancer cells to efficiently kill them. One of the strategies took advantage of the many chromosomal rearrangements, truncations, and alternative

**Figure 3.** A, NO exposure to PKR siRNA transfected cells compared with control had lower levels of p-eIF2-α. NO-treated control random siRNA transfected cells up-regulated PKR and p-eIF2-α levels and down-regulated cyclin D1 levels. MDA-MB-231 cells were transfected with random siRNA or PKR siRNA using solution V from the AMAXA nucleofector kit. At 24 h after transfection, cells were treated with DETA-NONOate (1 or 2 mmol/L) and harvested after another 24 h. The cells were prepared for Western blot analysis. The membrane was probed with PKR, p-eIF2-α, cyclin D1, and GAPDH antibodies. Results are representative of three different experiments. B, left, NO-induced decline in proliferation was more dramatic in cells treated with random siRNA compared with PKR siRNA. MDA-MB-231 cells transfected with PKR siRNA or random siRNA were treated with DETA-NONOate (1 or 2 mmol/L). The cells were harvested at 24 h and counted using the trypan blue method. Columns, mean of three experiments; bars, SE. *, P = 0.02 compared with PKR siRNA group treated with 1 mmol/L DETA-NONOate; #, P = 0.05 compared with PKR siRNA group treated with 2 mmol/L DETA-NONOate. B, right, cells with PKR siRNA had complete recovery of cyclin D1 levels after removal of NO. MDA-MB-231 cells were treated as follows: lane 1, untreated control cells; PKR siRNA and random siRNA transfected cells were treated with DETA-NONOate (1 mmol/L) for lanes 2, 3, 6, and 7. PKR siRNA and random siRNA transfected cells were treated with DETA-NONOate (2 mmol/L) for lanes 4, 5, 8, and 9, after 24 h of DETA-NONOate treatment, cells were exposed to NO-free media for another 48 h in lanes 3, 5, 7, and 9. All cells were harvested and prepared for Western blot analysis. The membrane was probed with cyclin D1 and GAPDH antibodies. Results are representative of three different experiments. C, left, HME cells had higher levels of p58, a PKR inhibitor, and low levels of p-eIF2-α compared with breast cancer cell lines. With NO stress, breast cancer cell lines had a higher increase in PKR, HRI, and p58 levels compared with the levels of PKR, HRI, and p58 in HME cells. HME, MDA-MB-231 (231), and MDA-MB-468 (468) cell lines were treated with DETA-NONOate (1 mmol/L) and prepared for Western blot analysis at 24 h. The membranes were probed with p58, PKR, HRI, p-eIF2-α, and GAPDH antibodies. Results are representative of three different experiments. C, right, NO induced sharper decline in protein concentration in breast cancer cells compared with MCF-10A1(R) cells. MCF-10A1(R) and MDA-MB-231 cells were treated with or without DETA-NONOate (1 or 2 mmol/L). After 24 h, cells were harvested and lysed. Protein concentrations were measured using Bradford reagent. Columns, mean of three experiments; bars, SE. *, P = 0.05; **, P = 0.001 compared with MDA-MB-231 control group without DETA-NONOate treatment. D, left, NO stress induced sharper decline in cyclin E levels in MDA-MB-231 cells, but not MCF-10A1(R) cells. MCF-10A1(R) and MDA-MB-231 cells were treated with or without DETA-NONOate (1 or 2 mmol/L) and prepared for Western blot analysis. The membrane was probed with cyclin D1, cyclin E, and GAPDH antibodies. Results are representative of three different experiments.
Figure 4. A, lower NO stress (0.5 mmol/L) with either hydrogen peroxide or serum starvation stress sharply increased PKR dimerization. MDA-MB-231 cells were treated as follows: lanes 1 and 2, cells treated with DETA-NONOate (0.3 or 0.5 mmol/L); lanes 3 and 4, cells coexposed to hydrogen peroxide (50 μmol/L) and DETA-NONOate (0.3 or 0.5 mmol/L) exposure; lane 5, cells treated with hydrogen peroxide (50 μmol/L); lanes 6 and 7, cells were serum starved for 24 h before DETA-NONOate (0.3 or 0.5 mmol/L) exposure; lane 8, cells were serum starved. The cells were harvested at 24 h and prepared for Western blot analysis. The membrane was probed with PKR antibody and Ponceau-S stained to confirm equal loading of sample in the gel. Results are representative of three different experiments.

B, left, low NO stress with either hydrogen peroxide or serum starvation stress sharply increased p-eIF2-α levels and decreased cyclin D1 and cyclin E levels. MDA-MB-231 cells were treated as follows: lane 1, cells treated with hydrogen peroxide (50 μmol/L); lane 2, cells were serum starved; lane 3, cells treated with DETA-NONOate (0.5 mmol/L); lane 4, cells co-exposed to hydrogen peroxide (50 μmol/L) and NO (0.5 mmol/L); lane 5, cells were serum starved for 24 h before NO (0.5 mmol/L) exposure. The cells were harvested at 24 h and prepared for Western blot analysis. The membrane was probed with p-eIF2-α, cyclin D1, cyclin E, and GAPDH antibodies. Results are representative of three different experiments.

B, right, hydrogen peroxide with low NO stress (1 mmol/L) compared with either stress alone induced a greater decrease in cell proliferation. MDA-MB-231 cells were treated with hydrogen peroxide (50 μmol/L) or DETA-NONOate (1 mmol/L) alone or with both stresses. The cells were harvested and counted using the trypan blue method. Columns, mean of three different experiments; bars, SE. H, H2O2; D1, 1 mmol/L DETA-NONOate. *, P < 0.02 compared with control untreated cells. C, low NO stress with actinomycin D induced a sharper increase of PKR dimerization compared with NO or actinomycin D stress alone. MDA-MB-231 cells were treated as follows: lanes 1, 2, and 3, cells were treated with 1, 0.5, and 0.3 mmol/L of DETA-NONOate, respectively; lanes 4, 5, and 6, cells were pretreated with actinomycin D (10 μmol/L) before exposure to 1, 0.5, and 0.3 mmol/L of DETA-NONOate, respectively; lane 7, cells treated with actinomycin D (10 μmol/L). The cells were harvested at 24 h and analyzed on a native PAGE. The membrane was probed with anti-PKR antibody. Results are representative of three different experiments.

C, low NO stress with actinomycin D sharply up-regulated p-eIF2-α levels compared with either stress alone. NO induced PKR dimerization and phosphorylation of eIF2-α was dependent on NO concentration. MDA-MB-231 cells were treated as follows: lane 1, cells untreated; lanes 2 and 3, cells treated with DETA-NONOate (1 and 0.5 mmol/L); lanes 4 and 5, cells treated with actinomycin D (10 μmol/L); lanes 6, 7, 8, and 9, cells pretreated with actinomycin D (10 μmol/L) before exposure to DETA-NONOate (0.5 or 1 mmol/L). Cells were harvested at 24 h and prepared for Western blot analysis. The membrane was probed with anti-p-eIF2-α and anti-GAPDH antibodies. Results are representative of three different experiments.
splicing of pre-mRNA that produced mRNA species unique to the cancer cells. Upon hybridization with mRNA, dsRNA molecules were generated that caused selective death of cells through PKR phosphorylation (37, 38). In another study, this strategy has proved effective in inhibiting glioblastoma growth within mouse brain (38). Activation of PKR has been found to also play a role in esophageal cancer cell apoptosis induced by adenoviral vectors expressing TNF-α gene (39). Breast cancer cells overexpressing PKR seem to be a strong candidate for the dsRNA therapeutic approach. Here, we report, for the first time, a novel strategy for PKR activation. We show that high NO stress by itself or co-exposure of low NO stress simultaneously with another stress (H2O2 or serum deprivation) can activate PKR selectively in breast cancer cells, whereas HME and MCF-10A1(R) remained largely unaffected. This strategy also

Figure 5. A, DETA-NONOate-induced up-regulation of PKR is independent of ERK/AKT pathways. Top, MDA-MB-231 cells were treated with DETA NONOate (1 mmol/L) at different time points and prepared for Western blot analysis. The membrane was probed with anti-PKR, p-ERK, pAkt, p-mTOR, Akt, ERK1/2, and p70S6K antibodies. Results are representative of three different experiments. Bottom, MDA-MB-468 cells were treated with DETA-NONOate (1 mmol/L) at different time points and harvested at 24 h. The cells were prepared for Western blot analysis, and the membrane was probed with anti-PKR, p-ERK, ERK, p-Akt, and Akt antibodies. Results are representative of three different experiments. B, NO-induced decline of PKR is independent of cGMP pathway. MDA-MB-231 cells were used as follows: lane 1, untreated cells; lane 2, cells treated with DETA-NONOate (1 mmol/L); lane 3, cells treated with ODQ (50 μmol/L); lane 4, cells pretreated with ODQ (50 μmol/L) for 45 min before exposure to NO (1 mmol/L). Cells were harvested at 24 h and prepared for Western blot analysis. The membrane was probed with anti-cyclin D1 and anti-GAPDH antibodies. Results are representative of three different experiments. C, cycloheximide-mediated increase of PKR monomers in human breast cancer cells. The cells were treated with cycloheximide (200 μg/mL) and harvested at different time points. The cells were prepared for Western blot analysis, and the membrane was probed with anti-PKR antibody. Results are representative of three different experiments. D, PKR monomers are equally distributed in cytosolic and nuclear fractions of MCF-10A1(R) cells compared with the predominantly cytosolic PKR monomers of breast cancer cells. The cell fractionation was done according to the manufacturer’s protocol. The cells were treated with or without DETA-NONOate (1 mmol/L) and harvested at 0 and 3 h. The cells were prepared for Western blot analysis, and the membranes probed with anti-PKR antibody. Results are representative of three different experiments. N, nuclear fraction; C, cytoplasmic fraction.
seems highly promising as a nontoxic modality for breast cancer treatment, because PKR in normal mammary epithelials remains bound to p58 inhibitor. Although we assessed only the p58 inhibitor of PKR in normal mammary and cancer cells, there are other intracellular regulators, some of which are heat shock proteins, mRNA of p23/TCTP, ribosome, p67, and protein phosphatase 1 (40–44). These intracellular regulators could also contribute to the decreased susceptibility of normal mammary epithelials to NO. In ovarian cancer cells, it has been reported that NO is capable of enhancing H2O2-mediated processes by inhibiting its catalytic degradation (45).

We also find that PKR is very stable in breast cancer cells, having a constant level even after 36 hours of cycloheximide treatment. In examining PKR cellular localization, we find that PKR is predominantly cytosolic in breast cancer cells, whereas in HME cells, they are equally distributed between nuclear and cytosolic fractions. Mammalian ribosomes have been proposed to be a reservoir of inactive PKR monomers in which PKR is prevented from binding to dsRNA. PKR must be displaced from the ribosomes by dsRNA to become activated (42). We think that NO induced a redistribution of compartmentalized PKR, leading to an increase of the cytosolic monomers, which facilitated the formation of dimers. Studies have shown that NO and its derivatives could influence cellular signal transduction and modify main classes of proteins through S-nitrosylation, the coupling of an NO moiety to a reactive cysteine thiol (46, 47). S-nitrosylation could also promote or inhibit disulfide linkages within or between proteins depending on thiol proximity (47, 48). Cysteine thiols with acidic (Asp, Glu) or basic side chains (Arg, His, Lys) nearby could readily undergo S-nitrosylation through a concerted acid-base catalysis (47).

Because the PKR protein has two arginine and cysteine residues in close proximity to each other (within five amino acids), we have preliminary data where NO binds directly to PKR via S-nitrosylation to induce PKR dimerization. In our study, we immunoprecipitated PKR from control cell lysates and treated it with DTT, a reducing agent which abolished DETA-NONOate–induced dimerization (Fig. 6A). It has been reported that H2O2 induced dimerization via disulfide linkages in Yap1p (49). To assess whether H2O2 induced disulfide linkages in PKR, MDA-MB-231 cell lines were treated with H2O2 (0.3, 0.5, and 1 mmol/L) for 16 h. The cells were harvested at respective time points and probed for Western blot analysis on native PAGE as described in Materials and Methods. The membrane was probed with anti-PKR antibodies. Results are representative of three different experiments. B, hydrogen peroxide treatment induced an increase in PKR dimerization in MDA-MB-231 cells in a concentration-dependent manner. MDA-MB-231 cells were treated with various concentrations of hydrogen peroxide (0.3, 0.5, and 1 mmol/L) for 16 h. The cells were harvested at respective time points and prepared for Western blot analysis on native PAGE as described in Materials and Methods. The membrane was probed with anti-PKR antibodies. C, flow charts of differential NO stress leading to inhibition of protein synthesis and cytostasis. Low NO concentrations (1 mmol/L, DETA-NONOate) mediate HRI activation, leading to low levels of p-eIF2α. Phosphorylated eIF2α induced decline in shorter half-life proteins and caused partial cytostasis. High NO concentration (2 mmol/L, DETA-NONOate) mediates the activation of both PKR and HRI, leading to high levels of p-eIF2α. Elevated p-eIF2α level induced sharper decline in long and short half-life proteins, leading to cytostasis.
consolidate these observations. Although one study has tied HRI to NO induced cytostasis via eIF2-α in neuroepithelial and myoblast cells (22), this is the first report of NO inducing cytostasis via PKR activation.

Most therapeutic strategies against cancer use cytotoxic drugs or gene therapies that are directed at the DNA (30, 31). However, an increasing body of data is emerging about the involvement of tumor cell pathways, where breast cancer cells are dependent on cyclin D1 (32). Eukaryotic translation initiation factor 4E induced cytostasis and cell cycle arrest of a human breast cancer cell line (MDA-MB-231) potential role of cyclin D1. Proc Natl Acad Sci U S A 2000;97:11341–47.

We have previously reported interactions between the protein synthesis machinery and the proliferative tumor progression and the regulation of protein synthesis in reticulocyte lysates. J Biol Chem 1992;267:20519–24.


References
22. Uma S, Yun BG, Matts RL. The heme-regulated eukaryotic initiation factor 2α kinase by nitric oxide is induced by the formation of a five-coordinate NO-heme complex. J Biol Chem 2004;279:15751–61.
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