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FAST TRACK

Inactivated Tumor Suppressor Rb by Nitric Oxide Promotes Mitosis in Human Breast Cancer Cells

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Abstract Inactivation of tumor suppressor protein retinoblastoma (Rb) is important mechanism for the G_1/S transition during cell cycle progression. Human breast cancer cells T47D release great amount of nitric oxide (NO), but its relation to tumor suppressor Rb is unknown. In this study, it is shown that NO induces phosphorylation and inactivation of Rb tumor suppressor protein, increasing G_2/M phase and cell proliferation of breast cancer cells T47D. NO did not induce changes in p53 ser-15 phosphorylation, the most phosphorylated site of p53 during its activation. These data indicate that NO induces cell proliferation through the Rb pathway. NO phosphorylates and inactivates tumor suppressor protein Rb inducing mitosis by the p53 independent pathway in breast cancer cell. J. Cell. Biochem. 92: 1–5, 2004.

Key words: breast cancer; Rb; p53; signaling; mitosis; nitric oxide

The retinoblastoma tumor suppressor (Rb) protein controls the G_1/S transition [Weinberg, 1995]. The Rb regulates proliferation of mammalian cells by maintaining the integrity of the G_1/S checkpoint. In majority of human malignancies aberrancies occur in Rb-pathway [Senderowicz, 2000]. In the middle-to-late G_1 phase to progression to S phase of the cell cycle upon mitogenic stimulation, Rb protein is phosphorylated and inactivated [Hatakeyama and Weinberg, 1995]. All human tumors have inactivated either the Rb or p53 pathway. The p53 tumor suppressor protein is latent and highly labile transcriptional factor and activated by wide range of cellular stresses including DNA damage [Levine, 1997]. The protective role of p53 in normal cells depends on its ability to function as transcriptional factor, activated in response to DNA damage [Alarcon et al., 1999]. Activated p53 moves to the nucleus, binds to DNA and coordinates a change in the balance of gene expression leading to growth arrest or apoptosis, event that prevent the growth or survival of damaged cells [Oren, 1999]. The phosphorylation of p53 ser-15 is strongly associated with the response to DNA damage [Kapoor et al., 2000]. Breast cancer cells have great production of NO that contributes to the cancer growth and spread [Vakkala et al., 2000]. In this report, it was shown that NO phosphorilates and inactivates Rb tumor suppressor protein inducing increase in mitosis.

Abbreviations used: Rb, retinoblastoma; NO, nitric oxide; NOS, nitric oxide synthase; L-arg, L-arginine; L-NMMA, N^G-monomethyl-L-arginine mono acetate salt; FBS, fetal bovine serum; BSA, bovine serum albumin; PI3K, phosphoinositol-3-OH kinase; HIF-1α, hypoxia inducible factor.

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MATERIALS AND METHODS

Materials

In this study, the materials were used: L-Arginine (L-Arg) hydrochloride crystalline (Sigma, St. Louis, MO), N^G-monomethyl-L-arginine mono acetate salt (L-NMMA), an inhibitor of NO synthase (CalBiochem, La Jolla, CA), polyclonal rabbit antibodies Rb and phoshorylated-Rb (ser-795), p53 and phosphorylated-p53 (ser-15) from Cell Signaling (Beverly, MA).

Cell Culture

Human breast cancer cell lines T47D were obtained from American Type Culture Collections. The T47D cells were maintained in RPMI-1640 (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (FBS), 5% penicillin/streptomycin, 5% glutamine, bovine insulin at 0.2 U/mL (GIBCO, Grand Island, NY). Cell line was grown at 37°C with 5% CO_2.

Experimental Procedure

Experiments were performed as described previously [Radisavljevic, 2003]. Briefly, cancer cell lines T47D were on starvation for 1 day with 0.1% FBS and then cells were treated with 100 μM of L-arginine in presence of 0.1% FBS/RPMI-1640 or with 50 μM of L-NMMA in 60 mm tissue culture dish (Corning, NY). Cells were washed with PBS (Cellgro) and lysed with SDS sample buffer (125 mM Tris-HCl, pH = 6.8, 4% SDS, 20% glycerol, 1 mM orthovanadate and with 0.02% bromphenol blue) for protein analysis, or cells were harvested for flow cytometry analysis. Protein concentration of the whole cell extract was determined by the Bratford assay (BioRad, Hercules, CA).

Cell Proliferation Assay

Cell proliferation in the presence or absence of the L-arginine was determined using the Cell Proliferation Reagent WST-1 (tetrazolium salt) [Scudiero et al., 1988; Ji et al., 2002]. Briefly, cells were cultured in 0.1% FBS/RPMI-1640 (100 μl) in flat bottom 96-well tissue culture plates at density of 5×10^4/well at 37°C with 5% CO_2 for 16 h. At the indicated times, the WST-1 reagent (10 μl) was added to each well and incubated in a humidified atmosphere for 1 h. The absorbance of the stable formazan product in viable cells (tetrazolium salt, WST-1 was cleaved by mitochondrial dehydrogenases) was measured at 450 nm using a colorimetric microplate reader.

Flow Cytometry

Human breast cancer cells T47D were treated for 16 h and then were washed with PBS and harvested with trypsin 0.05% in concentration of 1×10^6/ml and fixed with 70% alcohol. Cells then were washed in 30% ethanol, pelleted and resuspended in 0.05% BSA/PBS and then DNA was stained with propidium iodide 50 μg/ml with RNAse-A at 40 μg/ml for 30 min at room temperature, and analysed by using a Becton Dickinson flow cytometer for relative DNA content based on red fluorescence levels, and the distribution of cells in different phases of cell cycle was calculated using Cell FIT software (Becton Dickinson, San Jose, CA).

Western Blotting

Equal amounts of protein lysate extracted from breast cancer cell lines T47D (100 μg) were resolved by 8% SDS polyacrylamide gel electrophoresis and electrotransfered for 2 h into polyvinylidene difluoride membrane (PVDF) (BioRad). Blots were blocked with 5% non-fat dry milk in PBS with 0.1% Tween-20 for at least 2 h at 4°C. Blots were then incubated overnight with primary antibody at 4°C (1:1000 in 5% non fat milk and PBS with 0.1% Tween-20 followed by three washes with PBS and 0.1% Tween-20 at room temperature 10 min each. HRP-conjugated secondary antibody (1:20,000) was added to the blots and incubated for 1 h at room temperature. Blots were washed three times with PBS and 0.1% Tween-20 and reactivity recorded by enhanced chemiluminiscence (Enhanced Luminal Reagent NEN, Life Science Products, Inc., Boston, MA), according to the manufacturer's instruction.

RESULTS

Endogenously produced NO (from substrate L-arginine), induced phosphorylation and inactivation of tumor suppressor Rb. Phosphorylation of Rb ser-795 protein was followed during cell cycle progression of breast cancer cells T47D. Phosphorylation of Rb at position ser-795 in control T47D cells occurs at 11 h which represents late S phase and dephosphorylated in G_1 and early S phase (Fig. 1A, upper panel). However, NO significantly increased Rb phosphorylation after first hour of stimulation and continue hyper phosphorylation of Rb through G_1 and S phase through G_2/M phase of cell cycle (Fig. 1A, low panel). Changes of protein phosphorylation of tumor suppressor p53 ser-15, were not observer during NO stimulation (Fig. 1B). NO induced significant ($P < 0.001$) increase in G_2/M phase and cell proliferation ($P < 0.001$) of breast cancer cells T47D during 16 h of cell cycle progression as result of inactivated Rb tumor suppressor protein by NO (Fig. 2A,B). This proliferative effect was abolished by L-NMMA, a specific inhibitor of NOS.

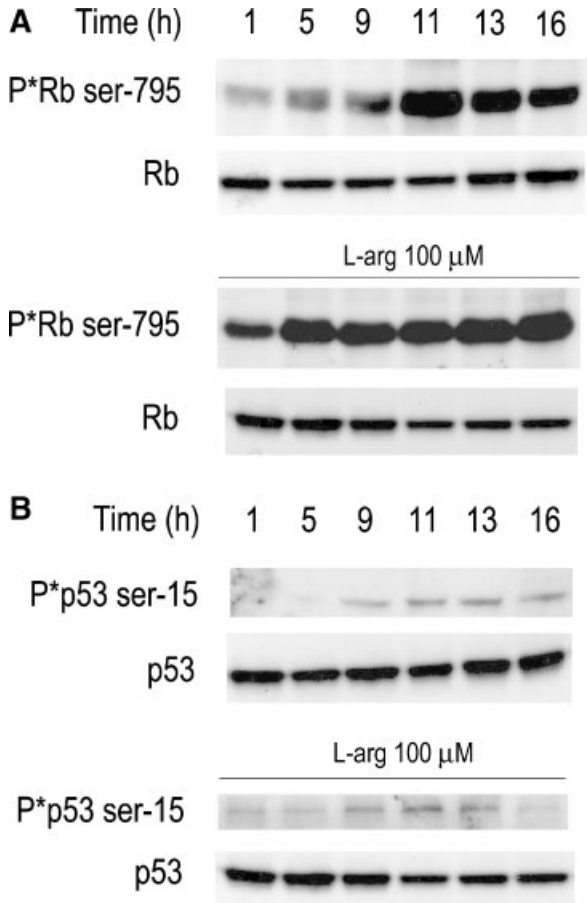

Fig. 1. NO phosphorylates and inactivates Rb. **A**: NO induced phosphorylation (P*) of Rb tumor suppressor protein at position ser-795 and activates cell cycle progression in human breast cancer cells T47D. **B**: NO had no effect on phosphorylation (P*) of p53 tumor suppressor protein at position ser-15, most phosphorylated site of p53 during its activation. The data shown in this figure is representative of three experiments.

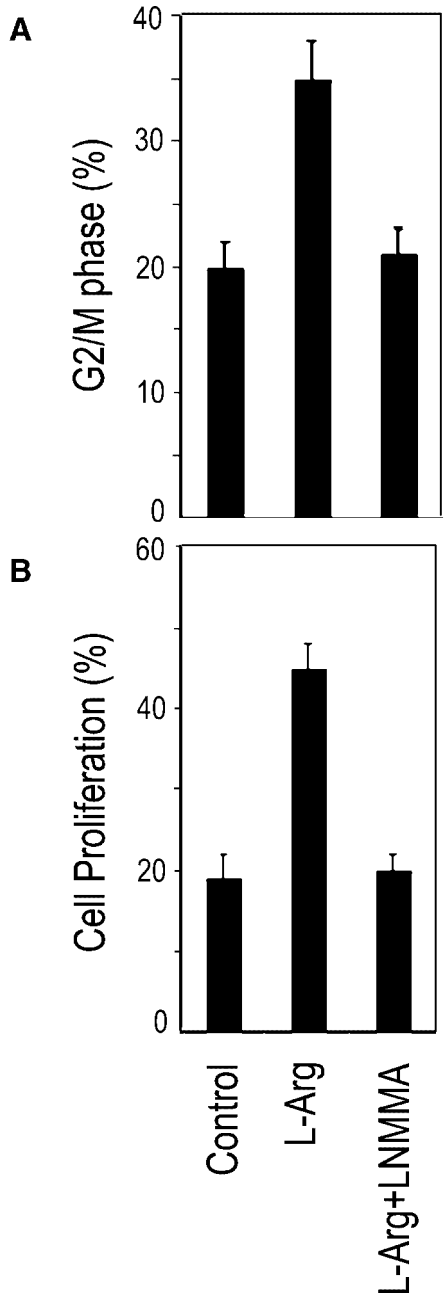

Fig. 2. NO induces mitosis of breast cancer cells. Human breast cancer cells were treated for 16 h with L-arginine (L-Arg, 100 μM) and assessed for **A**: G_2/M phase of cell cycle progression by flow cytometry, and (**B**) cell proliferation by proliferation assay. This effect was abolished by L-NMMA 50 μM, a specific inhibitor of NOS. Results are expressed as the mean ± SD of six independent experiments.

These data provide insight into the pathway that links NO with Rb tumor suppressor phosphorylation and inactivation. Also, this finding elucidate role of NO in inducing mitosis of breast cancer cells. These results suggest that NO is negative regulator of tumor suppressor Rb activity in breast cancer cells.

DISCUSSION

In this study, it was demonstrated that tumor suppressor protein Rb was phosphorylated and inactivated by NO without involvement of p53 tumor suppressor. This inactivation of Rb supports progression in cell cycle leading to increased G_2/M phase and cell proliferation of breast cancer cells. It is known that Rb is phosphorylated and inactivated by a family of serine/threonine kinases such as cyclin-dependent kinases (Cdk) [Morgan, 1997]. Cdk2 in combination with cyclin E is responsible for progression from G_1 into S phase and Cdk2/cyclin A is required for progression through S phase and cdc2/cyclin B is necessary for M phase of cell

cycle [Sherr, 1996]. Recently, it was shown that Rb inactivation was mediated by p38 kinase which is independent of Cdk activity [Nath et al., 2003]. Loss in Rb function results from the phosphorylation of this tumor suppressor protein, however, activation of Rb protein through dephosphorylation arises in cells upon exit from M phase and in response to DNA damage [Senderowicz, 2000]. Phosphorylated Rb is unable to bind and inactivate its downstream target the transcriptional factor E2F, leading to transactivation of E2F-dependent genes and progression to S phases [Dyson, 1998]. Increased proliferation of breast cancer cells was observed with high cyclin D1 and elevated Rb phosphorylation with its inactivation or with high cyclin E protein and defective p53 [Loden et al., 2002].

Mutations in the p53 tumor suppressor gene occur in approximately 50% of all human tumors, making it the most frequent target for genetic alterations in cancer, however, activated p53 leading to cell cycle arrest and apoptosis [Oren, 1999]. Phosphorylation of p53 ser-15 affects the p53-MDM2 interaction [Dumaz and Meek, 1999]. MDM2 regulates the activity of p53 when bound to the N-terminal transactivation domain and inhibits the ability of p53 to interact with transcriptional factor TATA-binding protein [Thut et al., 1997]. In this report, p53 was not affected by NO-induced mitosis through Rb pathway.

The human breast carcinoma cell line T47D has high NOS activity [Harris et al., 2002] and produces a great amount of NO [Kampa et al., 2001]. The T47D cells express both iNOS and eNOS [Harris et al., 2002]. Physiological level of NO trigger entry into S phase and facilitate mitosis [Plachta et al., 2003]. Advanced-stage of human breast invasive carcinoma is correlated with a higher expression of iNOS and high level of NO in cancer cells which contributes to the promotion of apoptosis and angiogenesis in breast carcinoma patients [Mortensen et al., 1999; Reveneau et al., 1999; Vakkala et al., 2000]. In contrast, NO suppression induces apoptosis through the FOXO3a/ROCK kinase pathway (PI3K/Akt/caspase-3 independent pathway) in breast cancer cells mediated by FOXO3a (FKHRL1), a downstream protein from ROCK kinase where NO is negative regulator of ROCK kinase which phosphorylates and activates FOXO3a protein [Radisavljevic, 2003]. NO suppression also blocks cell migration induced by angiogenic promoters [Radisavljevic et al., 2000]. On the other hand, NO decreases ubiquitination and degradation of hypoxia inducible factor-1α (HIF-1α), a regulator of metabolic adaptation to hypoxia, involved in angiogenesis and highly expressed in hypoxic area in tumor distinct from microvessels [Metzen et al., 2003]. Previously, I have reported that NO alone does not increase phosphorylation of Akt, a signaling pathway involved in cell survival and migration [Radisavljevic et al., 2000; Radisavljevic, 2003], and in this report is shown that NO induced cell proliferation though the Rb pathway and NO inhibitor prevented cancer cell proliferation.

These results elucidate signaling mechanism in cell proliferation of human breast cancer cells mediated by tumor suppressor Rb protein which is phosphorylated and inactivated by NO promoting mitosis in breast cancer cells. This finding may have clinical applications in chemotherapeutic approach of breast carcinoma and supports examining NO inhibitor as potential chemotherapeutic agent.

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