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Original Contribution

Mitochondrial-associated nitric oxide synthase activity inhibits cytochrome *c* oxidase: Implications for breast Cancer

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ABSTRACT

Nitric oxide (NO) is produced and nitric oxide synthase (NOS) activity is expressed in many types of tumor cells, but their precise role in tumor proliferation has not been clearly elucidated. Recently, it has been observed that patients with triple-negative breast tumors expressing NOS have a significantly worse prognosis compared to those that do not express any NOS. We observed that NOS activity was associated with the mitochondria in two breast cancer cell lines, ZR-75-30 and BT-474, compared with another NO-producing benign breast epithelial cell line, MCF-12F, in which no significant mitochondrial-associated NOS activity was detected. The rate of proliferation of the malignant cells expressing mitochondrial-associated NOS was decreased in the presence of an inhibitor of NO synthesis, but it had no effect on the normal breast epithelial cells, MCF-12F, which also expressed NOS, but not associated with mitochondria. The basal rate of proliferation was not affected by ODQ, an inhibitor of soluble guanylate cyclase, indicating that the effects of the endogenous NO produced by the malignant cell lines on proliferation are cGMP independent. Our results indicate that mitochondrial-associated NOS activity exhibited by the cancer cell lines ZR-75-30 and BT-474 inhibited cytochrome *c* oxidase, resulting in increased production of hydrogen peroxide (H_2O_2), which inhibited protein phosphatase 2A activity. This resulted in the maintenance of Akt and ERK1/2 in a phosphorylated state, leading to cell proliferation.

Nitric oxide synthase (NOS) activity (both constitutive and inducible) has been reported in human breast tumors and was found to be higher in invasive tumors compared to normal or benign tissues [1]. NO biosynthesis was significantly greater for grade III compared with grade II breast tumors. Immunohistochemistry revealed that the inducible NO synthase (iNOS) was predominantly associated with tumor-associated macrophages, although stromal cells and tumor cells also accounted for the NOS activity in the tumors [1–4]. More recently, it has been demonstrated that patients with triple-negative breast tumors expressing NOS had a significantly worse prognosis compared to those that did not express NOS [4]. However, the precise mechanism(s) by which the NO produced by the breast tumor modulates its growth has not been fully elucidated.

We had earlier demonstrated the presence of both iNOS and endothelial NOS (eNOS) activity in a panel of human breast cancer (HBC) cells [5]. We had also demonstrated that low concentrations of exogenous NO in the nanomolar range increased thymidine incorporation in HBC cells [6] and this was accompanied by increased cell proliferation [6]. In these studies we used DETA-NONOate, a long-lasting NO donor with a half-life of approximately 20 h to mimic a steady-state production of NO as seen in vivo [6]. We further observed that exogenous NO in the nanomolar range significantly increased both total protein synthesis and cell proliferation of HBC cells, MDA-MB-231 and MCF-7, and this was accompanied by increased translation of cyclin D1 and ornithine decarboxylase along with the activation of mammalian target of rapamycin and its downstream substrates such as eIF4E and p70 S6 kinase [7]. These actions of NO were independent of cyclic GMP (cGMP) [7]. This prompted us to investigate alternate, initial targets of endogenously produced NO in HBC cells that may mediate cell proliferation.

We utilized two malignant HBC cell lines, ZR-75-30 and BT-474, as our model system. These cells produce significant amounts of NO from both eNOS and iNOS expressed in these cells [5,8]. We compared these with MCF-12F cells, which are nonmalignant NO-producing human breast epithelial (HBE) cells [5,8]. Our results indicated that the endogenous NO produced by HBC cells inhibited cytochrome *c* oxidase (CcO) to constitutively generate basal levels of reactive oxygen species (ROS), including H_2O_2. This plays an important role in the proliferation of these

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cells. On the other hand this was not observed in the nonmalignant HBE cells, MCF-12F. We further elucidated the important role of protein phosphatase 2A (PP2A) in the overall mechanism of endogenous NO-driven cell growth in this breast cancer model.

Materials and methods

Materials

2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), 1*H*-[1,2,4]oxadiazolo[4,3a]quinoxalin-1-one (ODQ), and N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) were procured from Cayman Chemical (Ann Arbor, MI, USA). CcO assay kit, CYTOCOX1, and L-arginine were obtained from Sigma (St. Louis, MO, USA). MitoSOX red was purchased from Molecular Probes (Eugene, OR, USA). Antibodies against GAPDH (sc-166545), phospho-p42/44 MAPK (4370), and pAKT (sc9275L) were obtained from Cell Signaling (Danvers, MA, USA). Mitochondrial isolation kit was obtained from Thermo Scientific (Rockford, IL, USA). The NOS assay kit was procured from Oxford Biomedical (Rochester Hills, MI, USA).

Cell culture

Human breast cancer cell lines ZR-75-30 and BT-474 and normal breast epithelial cells MCF-12F were obtained from the ATCC. ZR-75-30 and BT-474 cells were grown in RPMI 1640, supplemented with 2 mM glutamine and 10% fetal bovine serum (FBS). MCF-12F cells were maintained in Dulbecco's modified essential medium with Ham's F-12 nutrient mix containing 5% Chelex-treated horse serum. The tissue culture medium also contained epidermal growth factor (10 mg/500 ml), cholera toxin (50 mg/500 ml), insulin (5 mg/500 ml), and hydrocortisone (250 mg/500 ml). All cells were cultured in CO_2 incubators at 37 °C. All treatments were done under serum-free conditions. For L-NAME treatment, cells were treated with the drug under serum-free conditions for 1 h after which the cells were grown in L-NAME-containing medium with 0.1% serum and harvested at time points as indicated in the figures. L-Arginine and ODQ were added 1 h before L-NAME treatment wherever applicable.

Viable cell count

Cell viability was determined by trypan blue exclusion method using a hemocytometer as well as the Vi-CELL XR cell viability analyzer (Beckman Coulter).

Proliferation studies

Cells (15,000) seeded in each well of a 12-well plate were allowed to grow overnight. To the plates, L-NAME at indicated concentrations was added from 0 to 80 h. Cells were collected at time points indicated in the figures and the viability was determined on a hemocytometer by the trypan blue exclusion method as well as by the Vi-CELL analyzer. After this, the number of viable cells at each concentration and time point was determined. Cells from three wells were collected for each counting event. Initial growth rates were calculated from equations fitting the linear portions of growth curves. Average rates were then calculated from multiple experiments ($n=3$). Proliferation was also assessed by MTS using the CellTitre96 Aqueous assay (Promega, Madison, WI, USA) as per the manufacturer's protocol.

Nitric oxide measurement

NO was measured using electrochemical NO sensors obtained from World Precision Instruments (Sarasota, FL, USA) as described by Zhang [9]. Values for NO (nmol/10^5 cells) were ascertained from standard curves using SNAP as a NO donor.

Nitric oxide synthase assay

NOS activity was determined using a kit from Oxford Biomedical as described by Shen et al. [10]. Briefly, samples containing NOS were incubated in the presence of L-arginine and NADPH. Thereafter Griess reagent was employed to detect nitrite. Nitrate reductase was also employed to convert any nitrate to nitrite before detection by Griess reagent.

Citrate synthase assay

Citrate synthase assay was performed utilizing 10 μg of protein and a kit from Sigma (CS0720) as per the manufacturer's protocol. Briefly, mitochondrial/cell extracts were incubated with oxaloacetate and acetyl-CoA in a suitable buffer at 37 °C to generate CoA–SH, a thiol subsequently detected by a reaction with 5,5′-dithiobis(2-nitrobenzoic acid) to produce absorbance at 412 nm. Activity was normalized against equal amounts of protein.

Mitochondrial isolation

Mitochondria were isolated using a kit from Thermo Scientific as per the manufacturer's protocol and as described by Zhang et al. [11].

Cytochrome c oxidase assay

CcO was assayed from isolated mitochondria using a kit from Sigma as per the manufacturer's protocol and as described by Lemberg [12]. Briefly, a change in absorbance at 550 nm is monitored as ferrocytochrome *c* is oxidized to ferricytochrome *c* by CcO.

Detection of ROS

Intracellular H_2O_2 and mitochondrial ROS were determined by employing DCFH-DA and MitoSOX red as described respectively by Eruslanov and Kusmartsev [13] and Mukhopadhyay et al. [14]. Flow cytometry was used to detect fluorescent signals in the FL-1 and Fl-2 channels for H_2O_2 and mitochondrial ROS, respectively. Signals were obtained after whole cells were treated with relevant drugs (at 2 μM final concentrations) for 30 min at 37 °C. Thereafter cells were washed twice with phosphate-buffered saline (PBS) +0.5% FBS before being subjected to flow cytometry using a FACScan.

Cell cycle analysis

We determined phases of cell cycle using flow cytometry, 48 h after treatment/transfection. Cells were trypsinized, washed with 1 × PBS, fixed, permeabilized with cold 70% ethanol, and finally incubated in the dark for 30 min with 1 ml of propidium iodide (containing NP-40) (Biosure, Grass Valley, CA, USA). The DNA content of these cells was measured based on the presence of propidium iodide (PI) staining. Flow cytometric analysis was done on at least 10,000 cells from each sample, and cell cycle data were analyzed using a FACSCalibur flow cytometer (BD BioSciences,