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Participation of non-neuronal muscarinic receptors in the effect of carbachol with paclitaxel on human breast adenocarcinoma cells. Roles of nitric oxide synthase and arginase

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ABSTRACT

Breast cancer is the most common type of cancer in women and represents a major issue in public health. The most frequent methods to treat these tumors are surgery and/or chemotherapy. The latter can exert not only beneficial effects by reducing tumor growth and metastasis, but also toxic actions on normal tissues. Metronomic therapy involves the use of low doses of cytotoxic drugs alone or in combination to improve efficacy and to reduce adverse effects. We have previously reported that breast tumors highly express functional muscarinic acetylcholine receptors (mAChRs) that regulate tumor progression. For this reason, mAChRs could be considered as therapeutic targets in breast cancer. In this paper, we investigated the ability of a combination of the cytotoxic drug paclitaxel plus carbachol, a cholinergic agonist, at low doses, to induce death in breast tumor MCF-7 cells, via mAChR activation, and the role of nitric oxide synthase (NOS) and arginase in this effect. We observed that the combination of carbachol plus paclitaxel at subthreshold doses significantly increased cytotoxicity in tumor cells without affecting MCF-10A cells, derived from human normal mammary gland. This effect was reduced in the presence of the muscarinic antagonist atropine. The combination also increased nitric oxide production by NOS1 and NOS3 via mAChR activation, concomitantly with an up-regulation of NOS3 expression. The latter effects were accompanied by a reduction in arginase II activity. In conclusion, our work demonstrates that mAChRs expressed in breast tumor cells could be considered as candidates to become targets for metronomic therapy in cancer treatment.

1. Introduction

Breast cancer continues to be the most common cancer in women and represents a major issue of public health [1]. The incidence and mortality rates of breast cancer in developing countries are increasing in the last decade [2]. Standard treatment modalities have improved the overall outlook and quality of life for women with breast cancer; however, the fact that 40% still succumb to this disease highlights the need for new therapeutic approaches and identification of new therapeutic targets. We have previously reported that muscarinic acetylcholine receptors (mAChRs) are involved in breast cancer progression [3]. mAChRs belong to the G protein-coupled receptor family which constitutes the largest family of cell surface receptors involved in signal transduction. In the last decade, it has been reported that mAChRs, acetylcholine, the enzymes that synthesize and degrade it (choline acetyltransferase and acetylcholinesterase, respectively) and also nicotinic receptors are expressed in non-neuronal cells, and constitute the non-neuronal cholinergic system [4]. Five subtypes of mAChRs have been identified by molecular cloning: M_1-M_5 [5]. mAChR signaling has been extensively reviewed and large amounts of knowledge have been accumulated concerning their distribution and function [5]. M_1, M_3, and M_5 subtypes are generally coupled to $G_{q/11}$ protein and activate A_2, C, or D phospholipases and/or calcium influx. The latter is responsible of calcium dependent nitric oxide synthase (NOS) activation that releases nitric oxide (NO), one of the most important mediators in tumorigenesis [6–8]. NO is generated from arginine by the action of NOS, but arginine is also metabolized by arginase, which yield urea and ornithine. These metabolites are precursors in the synthesis of polyamines, which are necessary for cell division. There are two isoforms of the enzyme named arginase I and arginase II [9].

We have previously described that cholinergic short-time stimulation with the synthetic agonist carbachol promotes tumor progression in LM2, LM3 and LMM3 murine mammary tumors. Meanwhile, long term activation stimulates cell death in those tumor cells [10,11]. Paclitaxel is one of the cytotoxic agents most widely used for treatment of breast cancer. Although, this drug's efficacy has been extensively proved in different tumors, including those of the breast, these tumors have also developed resistance to its usage [12]. For this reason, combinations of low doses of paclitaxel with other chemotherapeutic agents that

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increase anti-tumor efficacy and reduce side effects, have been tested [12–14]. MCF-7 cell line constitutes one of the most studied models in breast cancer because it is derived from a luminal, estrogen dependent human mammary adenocarcinoma, which is the most common tumor in women. In this paper, we investigated the ability of a combination of paclitaxel with carbachol at low doses, to induce cell death in MCF-7 cells via mAChR activation and the role of NOS and arginase in this effect.

2. Materials and methods

2.1. Cell culture

The human breast adenocarcinoma cell line MCF-7 was obtained from the American Type Culture Collection (ATCC; Manassas, VI, USA), and cultured in Dulbeccos modified Eagle´s medium and F12 medium (DMEM:F12; 1:1; Invitrogen Inc., Carlsbad, CA, USA) with 2 mM L-glutamine and 80 μg/ml gentamycin, supplemented with 10% heat inactivated fetal bovine serum (FBS) (PAA laboratories GmbH, Haidmannweg, Austria) at 37°C in a humidified 5% CO_2 air. MCF-10A cells were also purchased by ATCC and constitute a non-tumorigenic cell line derived from human mammary tissue. Cells were grown on tissue culture plastic dishes, in DMEM:F12 medium supplemented with 10% FBS, hydrocortisone (0.5 μg/ml), insulin (10 μg/ml), and epidermal growth factor (20 ng/ml). Both cell lines were detached using the following buffer: 0.25% trypsin and 0.02% EDTA in Ca^{2+} and Mg^{2+}-free phosphate buffer saline (PBS) from confluent monolayers. The medium was replaced three times a week. Cell viability was assayed by Trypan blue exclusion test, and the absence of mycoplasma was confirmed by Hoechst staining.

2.2. Cell cytotoxicity assay

Cells were seeded in 96-well plates at a density of 10^4 cells per well in triplicate in F12 medium supplemented with 10% FBS and were left to adhere overnight. When subconfluent conditions, about 60%–70% were reached, cells were deprived of FBS 24 h previous to the assay, to induce quiescence. Cells were treated with increasing concentrations of the synthetic cholinergic agonist carbachol (10^{-11} M) or paclitaxel (10^{-9} M) alone or in combination for 40 h. The effect of carbachol plus paclitaxel was tested in the absence or presence of the non-selective muscarinic antagonist, atropine (10^{-8} M) or different enzymatic inhibitors: N-[(4S)-4-amino-5-[(2-aminoethyl](amino]pentyl]-N′-nitroguanidine, Tris (A5727) (5×10^{-6} M) for NOS1, aminoguanidine (10^{-3} M) for NOS2, L-N5-(1-iminoethyl) ornithine hydrochloride (I134) (10^{-5} M) for NOS3 and N^G hydroxy-L-arginine (NOHA) (10^{-4} M) to inhibit arginase. After treatment, the medium was replaced by fresh medium free of FBS and viable cells were detected by using the soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) colorimetric assay (Cell Titer 96TM Aqueous Non-Radioactive Cell Proliferation Assay) (Promega, Madison, WI). MTT couples to phenazine methosulfate and is reduced to formazan. 20 μl of MTT:PMS (20:1) were added to each well, and the production of formazan was evaluated by measuring the absorbance at 540 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad Laboratories Inc., Oakland, CA) after 4 h at 37°C. Values are mean ± S.E.M. of 4 experiments, and results were expressed as percentage of cytotoxicity in relation to control (cells without treatment).

2.3. Nitric oxide production

NO production was determined by measuring nitrite (NO_2^-) accumulation in culture supernatants. Cells (10^4 per well) were seeded in triplicate in a 96-well plate with 100 μl medium supplemented with 10% FBS. Then, the medium was replaced with fresh medium without FBS and cells were treated with the combination of carbachol (10^{-11} M) plus paclitaxel (10^{-9} M) in the absence or presence of atropine or different NOS inhibitors as it was described in Section 2.2. Nitrite accumulation was evaluated after 40 h in culture supernatants by Griess reagent [1% sulphanylamine in 30% acetic acid with 0.1% N-(1-naphtyl) ethylenediamine in 60% acetic acid)] [15]. Absorbance was measured at 540 nm with an ELISA reader (Bio-Rad Laboratories Inc., Oakland, CA, USA). Nitrite concentration was determined using a standard curve of $NaNO_2$ diluted in culture medium. Values are mean ± S.E.M. of 4 experiments, and results were expressed in micromolar concentration of nitrite in culture supernatants.

2.4. Arginase activity assay

Arginase activity was determined in cell lysates according to a method previously described [16]. Cells were seeded in 48 well plates (5×10^4 per well) in 500 μl of culture medium DMEM:F12 with 5% FBS for 24 h. They were treated with the combination of carbachol plus paclitaxel for 40 h in the absence or presence of atropine or the arginase inhibitor NOHA (10^{-4} M). After treatment, cells were lysed with 0.5 ml of 25 mM Tris–HCl, 0.1% Triton X-100 and 5 mM $MnCl_2$, pH 7.4. Lysates (25 μl) were activated at 56°C for 10 min and then incubated with an equal volume of 0.5 M arginine, pH 9.7, at 37°C for 1 h. The reaction was stopped in acid medium. Urea production was measured at 540 nm using an ELISA reader (Bio-Rad Laboratories Inc., Oakland, CA, USA). Results were expressed as micromoles of urea per milligram of protein of 4 experiments.

2.5. Western blot

2.5.1. Detection of nitric oxide synthase isoforms

After treatment cells (2×10^6) were washed twice with PBS and lysed in 1 ml of buffer: 50 mM Tris–HCl, 150 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM PMSF, 1% Triton X-100, 4 μg/ml trypsin inhibitor, 5 μg/ml aprotinin and 5 μg/ml leupeptin, pH 7.4. After 1 h in ice bath, lysates were centrifuged at 8000 ×*g* for 20 min at 4°C. The supernatants obtained were stored at −80°C and protein concentration was determined by the method of Bradford [17]. Samples (80 μg protein/lane) were subjected to 7.5% SDS-PAGE minigel electrophoresis. Then, proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories Inc., Oakland, CA, USA) and washed with TBS plus Tween 0.05% (TBS-T). The nitrocellulose strips were blocked in TBS-T with 5% skim milk for 1 h at 25°C and subsequently incubated overnight with polyclonal antibodies raised in rabbit: anti-NOS1 or anti-NOS3, or goat anti-NOS2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), all diluted 1:100 in TBS-T. After several rinses with TBS-T, strips were incubated with the secondary antibody: horseradish peroxidase-linked anti-rabbit IgG, diluted 1:10,000 or horseradish peroxidase-linked anti-goat IgG, diluted 1:20,000 in TBS-T at 37 °C for 1 h. Bands were visualized by chemiluminescence in a ImageQuant TL using the Image J software (NIH, USA) [10]. Densitometric analysis of the bands is expressed as optical density units relative (rOD) to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.5.2. Detection of arginase isoforms

Cell lysates were prepared following the same procedure stated for NOS immunoblotting. Then, proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories Inc., Oakland, CA, USA) and washed with TBS-T. The nitrocellulose strips were blocked in TBS-T with 5% skim milk for 1 h at 25°C and subsequently incubated overnight with polyclonal antibodies raised in mouse: anti-arginase I (Cayman Chemical Co), or rabbit anti-arginase II (Dr. Masataka Mori, University of Kumamoto, Japan), all diluted 1:100 in TBS-T. After several rinses with TBS-T, strips were incubated with the secondary antibody: horseradish peroxidase-linked anti-rabbit IgG, diluted 1:10,000 or horseradish peroxidase-linked anti-goat IgG, diluted 1:20,000 in TBS-T at 37 °C for 1 h. Bands were visualized by chemiluminescence in a ImageQuant