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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

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TL using the Image J software (NIH, USA) [10]. Densitometric analysis of the bands is expressed as rOD to the expression of GAPDH.

2.6. Drugs

All drugs were purchased from Sigma Chemical Co. (St. Louis, MI, USA) unless otherwise stated. Solutions were prepared fresh daily.

2.7. Statistical analysis

Results were expressed as mean ± S.E.M. of at least four experiments. A GraphPad Prism computer program one-way ANOVA analysis for paired samples was used to determine the significance of differences between mean values in all control and test samples. The analysis was complemented by using a Tukey test to compare among mean values. Differences between means were considered significant if $p < 0.05$.

3. Results

3.1. Cytotoxic action of carbachol plus paclitaxel on MCF-7 cells via muscarinic activation

We had reported that carbachol was able to induce cell death in two different murine mammary cell lines that express mAChRs. In this work, we analyzed the effect of the same cholinergic agonist on MCF-7 cells derived from a human mammary adenocarcinoma that also express mAChRs. Carbachol induced cytotoxicity of MCF-7 cells at a narrow range of concentrations (10^{-10} M and 10^{-9} M) (Fig. 1A), while it was ineffective on the non-tumorigenic cell line MCF-10A, that lacks mAChRs (Fig. 1B). We confirmed that tumor cell viability was reduced in a concentration-dependent manner by the cytotoxic agent paclitaxel, which is usually used in breast cancer treatment, but it was also effective on normal mammary cells, MCF-10A (Fig. 1C and D). Taking into account that the latter constitutes an undesirable effect induced by chemotherapy at usual doses, we analyzed the ability of a combination

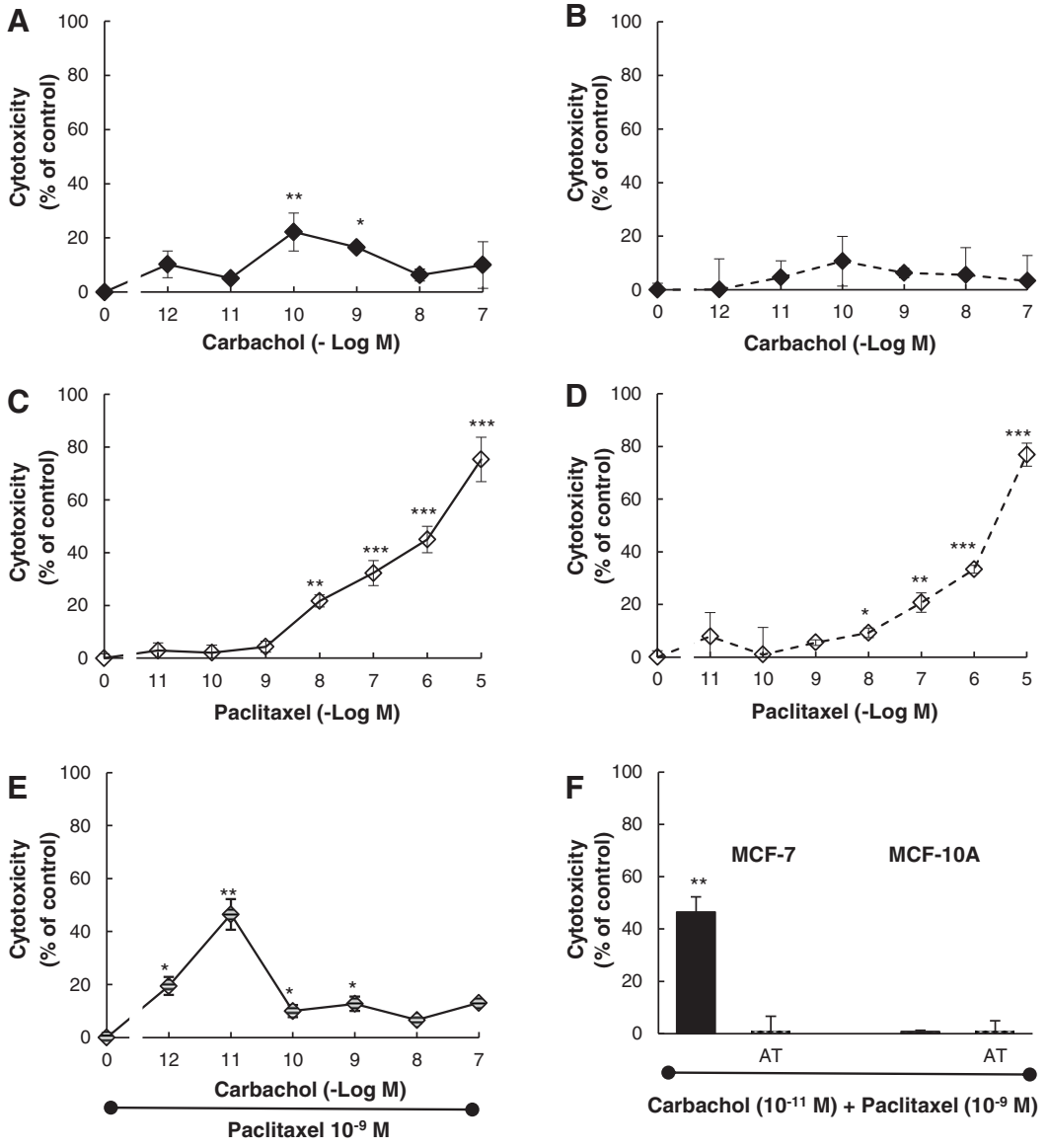

Fig. 1. Cytotoxic effect of carbachol and paclitaxel on mammary cells. Increasing concentrations of carbachol were added for 40 h to A) MCF-7 cells or to B) MCF-10A cells. Concentration–response curves of paclitaxel added for 40 h to C) MCF-7 cells or to D) MCF-10A cells. E) Concentration–response curve of carbachol in the presence of paclitaxel (10^{-9} M) added for 40 h to MCF-7 cells. Cytotoxic effect of the combination of carbachol (10^{-11} M) with paclitaxel (10^{-9} M) on MCF-7 and MCF-10A cells in the F) absence or E) presence of atropine (AT) (10^{-8} M). Results were expressed as percent of change with respect to control (cells without treatment). Values are mean ± S.E.M. of 4 experiments performed in triplicate. *p<0.05; **p<0.01; ***p<0.001 vs. control.

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of carbachol with paclitaxel at subthreshold doses (10^{-11} M and 10^{-9} M respectively), to induce cytotoxicity on tumor cells. Fig. 1E shows that this combination increased the cytotoxicity of tumor cells by 45.6±5.8% ($p<0.001$ vs. untreated cells) meanwhile MCF-10A cells were not sensible to this treatment (Fig. 1E and F). The action of carbachol plus paclitaxel was reduced in the presence of the muscarinic antagonist atropine (10^{-8} M) (Fig. 1F).

3.2. Participation of nitric oxide synthases 1 and 3 and arginase II in the cytotoxic effect of carbachol plus paclitaxel in MCF-7 cells

We have previously described that calcium-dependent NOS is expressed in MCF-7 cells, and that NOS-derived NO is produced after mAChR activation in these cells. The pre-incubation of tumor cells with A5727 (5×10^{-6} M) or I134 (10^{-5} M), selective inhibitors of NOS1 and NOS3 respectively, significantly reduced the lytic action of carbachol plus paclitaxel ($p < 0.05$ and $p < 0.01$ respectively). The NOS2 inhibitor, aminoguanidine, did not modify the cytotoxicity produced by the combination (Fig. 2A). In addition, the levels of NO measured as nitrites were significantly increased by carbachol plus paclitaxel (13.2±0.9 μM) in comparison to untreated cells (7.2±0.4 μM) (Fig. 2B). This action was reduced in the presence of atropine, revealing the participation of mAChRs in this effect. In addition, A5727 or I134 also reduced nitrite formation induced by carbachol plus paclitaxel, confirming that NO production is derived from both isoforms, NOS1 and NOS3, which are expressed in tumor cells (Fig. 2B). The latter was accompanied by a down-regulation of arginase activity (9.9± 0.2 μmol/mg prot) in comparison with the untreated cells' (13.4± 0.5 μmol/mg prot) effect that was reverted in the presence of atropine (10^{-8} M) or NOHA (10^{-4} M) (Fig. 2C).

3.3. Regulation of nitric oxide synthase 3 expression by carbachol plus paclitaxel in MCF-7 cells

In addition, carbachol plus paclitaxel increased NOS3 protein expression as it was demonstrated by Western blot assays (Fig. 3A) without affecting the expression of arginase II, which is the only isoform expressed in MCF-7 cells (Fig. 3B). By Western blot we demonstrated that MCF-10A cells just express NOS1, NOS3 and arginase II, but as they do not express mAChRs, the expression and activity of these enzymes were not affected by carbachol plus paclitaxel (data no shown).

4. Discussion

We have previously reported that MCF-7 cells express mAChR subtypes 3 and 4 [18]. The activation of these receptors with carbachol during short periods of time promotes MCF-7 cell proliferation. On the other hand, we observed that long term stimulation (more than 12 h) with the same agonist increased cell death in two different murine mammary adenocarcinomas favoring apoptosis and necrosis [19]. Here we obtained similar effects in human breast adenocarcinoma cells, MCF-7, that opens the possibility of using muscarinic agonists as cytotoxic drugs in cancer as it has been reported by other authors. Similarly, substantial cell death was observed in HEK293 cells transfected with M_1 receptor within 24 h after carbachol application [20]. Differences in the susceptibility to cytotoxicity reported in HEK293 cells could be due to differences in the ectopic and non-constitutive expression of an M_1 receptor subtype (MCF-7 expresses M_3 and M_4 subtypes constitutively), the concentrations of carbachol tested and/or the treatment time period. Moreover, the treatment of human cell lines and primary cultures obtained from different human biopsies of glioblastomas, which constitutively express the M_2 receptor with arecaidine, an M_2 selective agonist also promotes cell death [21].

Surgery and chemotherapy are the most common treatments in cancer. Regarding the latter, paclitaxel is one of the cytotoxic agents administered to patients with different types of tumors like those of the breast.

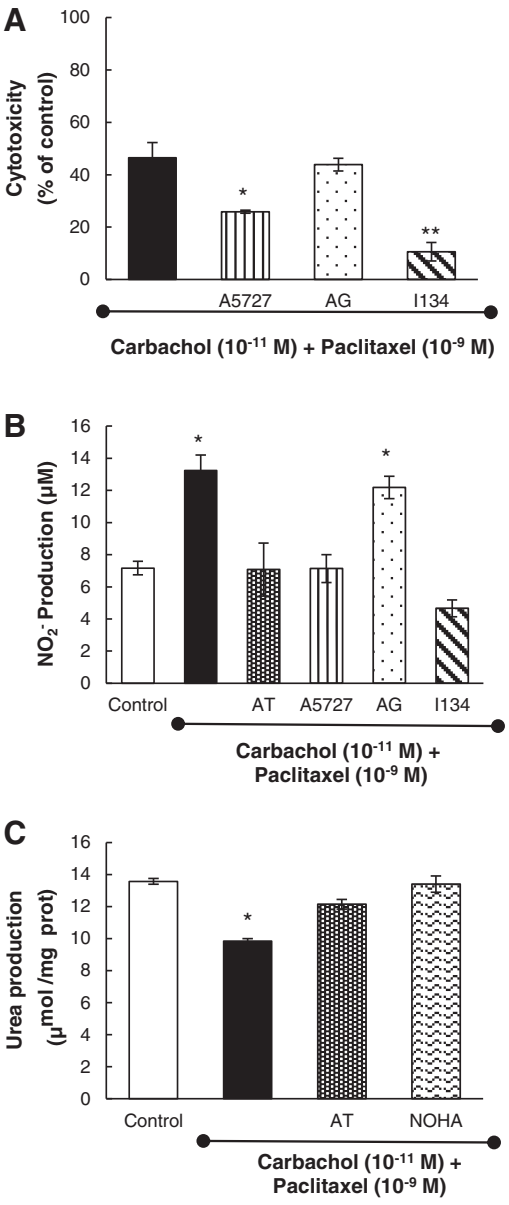

Fig. 2. Participation of nitric oxide synthase and arginase. MCF-7 cells were treated with the combination of carbachol (10^{-11} M) with paclitaxel (10^{-9} M) for 40 h in the absence or presence of atropine (AT) (10^{-8} M), or selective enzymatic inhibitors A5727 (5×10^{-6} M), aminoguanidine (AG) (10^{-6} M) or I134 (10^{-5} M) for nitric oxide synthase 1, 2 or 3 respectively or N^G hydroxy-L-arginine (NOHA) (10^{-4} M) for arginase. A) Cytotoxicity on MCF-7 cells. Results were expressed as percent of change with respect to control (cells without treatment). *$p < 0.05$; **$p < 0.01$ vs. carbachol plus paclitaxel. B) Nitrite (NO_2^-) production was expressed as micromolar (μM) concentration in culture supernatants. *$p < 0.05$ vs. control. C) Arginase activity was measured as urea production and expressed as micromoles per milligram of protein (μmol/mg prot). *$p<0.05$. Values are mean ± S.E.M. of 4 experiments performed in duplicate.

Paclitaxel belongs to the family of taxanes, which stabilizes tubulin polymerization, in order to inhibit cell duplication [22]. Usually this drug, like other cytotoxic drugs are administered at high doses, named maximun tolerated dose, every 3 weeks, and besides its effect on tumor cells, it produces serious side effects (damages in the intestinal mucosa, heart and kidney, nausea and mucositis). In order to diminish these undesirable effects, and to improve therapeutic results, a novel schedule of treatment, named metronomic therapy has been introduced [23]. It consists in the administration of repetitive, low doses of cytotoxic drugs. Metronomic therapy exerts an effect not only on tumor cells, but also on their microenvironment. In particular, the low-dose schedule compromises the repairing process of endothelial cells, leading to

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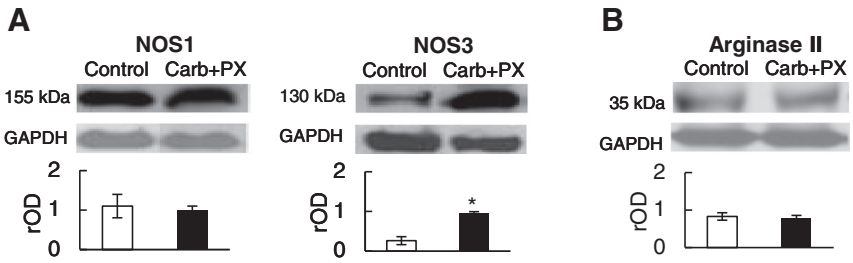

Fig. 3. Western blot assays. The expression of A) nitric oxide synthase (NOS) and B) arginase was analyzed in MCF-7 cells. Tumor cells were treated with the combination of carbachol (10^{-11} M) with paclitaxel (10^{-9} M) for 40 h and then cell lysates were prepared as it is stated in Materials and methods. Molecular weights are indicated on the left. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as loading control. Densitometric analysis of the bands is expressed as relative optical density units (rOD) in relation to the expression of GAPDH. One representative experiment of 3 is shown. *$p < 0.05$ vs. control (cells without treatment).

an anti-angiogenic effect, immunological benefits and low toxicity. Our results support these previous findings, since the combination of subthreshold doses of carbachol with paclitaxel, that did not exert any effect on normal MCF-10A cells, increased MCF-7 cell lysis. In line with our results, Hossain et al. [24] demonstrated that the combination of low concentrations of paclitaxel with curcumine produced a cytotoxic effect on human glioblastoma cells, LN18 and U138MG.

Interestingly, we observed that the action of the combination was reduced by atropine, revealing that not only carbachol acts by interacting with mAChRs but also paclitaxel. Previous results from our group demonstrated that paclitaxel shifted in a dose-dependent manner the binding of the non-selective muscarinic antagonist quinuclidinyl benzilate to murine mammary adenocarcinoma cells [19]. Similar interactions could be occurring between mAChRs and paclitaxel in MCF-7 cells.

In order to investigate the mechanism involved in the cytotoxic action exerted by carbachol plus paclitaxel, we analyzed the participation of NOS considering it as an effector enzyme in the signal transduction pathway of mAChRs that synthesizes NO, a central mediator in breast tumor progression [10,18]. NO liberation due to NOS1 and NOS3 activities is mediating the lytic effect produced by carbachol plus paclitaxel. But since I134 was more effective than A5727 to reduce the action of the combination on MCF-7 cell cytotoxicity, NOS3 could be mainly involved in this effect. In addition, we observed that the levels of NOS3 protein were up-regulated by carbachol plus paclitaxel treatment. The regulation of NOS expression and activity by cytotoxic agents is a little known area but despite this, Zeibek et al. [25] reported that the treatment of MCF-7 cells with a combination of two cytotoxic drugs, gemcitabine and vinorelbine, increased cell apoptosis with an increment in NOS3 immunolabeling.

Our results indicate that the increment in NO production triggered by the combination is accompanied by a reduction in arginase II activity, which could be useful to reduce the ability of cancer cells to grow. Meanwhile, MCF-10A cells that express the same enzyme were resistant to drug treatment because of the absence of mAChRs.

In conclusion our work demonstrates that mAChRs expressed in breast tumor cells could be a useful target for metronomic therapy in cancer treatment.

Author contributions

Conceived and designed the experiments: AJE, AS, DR, and MES. Contributed the reagents/materials and analytic tools: AS and DR. Performed the experiments: AJE, AS and DR. Analyzed the data: AJE, AS, DR and MES. AJE and MES contributed to the writing and editing of the manuscript.

Conflict of interest

There is no conflict of interest related to this study.

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