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Basic Study

Nicotinic receptors modulate antitumor therapy response in triple negative breast cancer cells

Nicotine as a modulator of chemotherapy

Abstract

BACKGROUND

Triple negative breast cancer is more aggressive than other breast cancer subtypes and constitutes a public health problem worldwide since it has high morbidity and mortality due to the lack of defined therapeutic targets. Resistance to chemotherapy complicates the course of patients' treatment. Several authors have highlighted the participation of nicotinic acetylcholine receptors (nAChR) in the modulation of conventional chemotherapy treatment in cancers of the airways. However, in breast cancer, less is known about the effect of nAChR activation by nicotine on chemotherapy treatment in smoking patients.

AIM

To investigate the effect of nicotine on paclitaxel treatment and the signaling pathways involved in human breast MDA-MB-231 tumor cells.

METHODS

Cells were treated with paclitaxel alone or in combination with nicotine, administered for one or three 48-h cycles. The effect of the addition of nicotine (at a concentration similar to that found in passive smokers' blood) on the treatment with paclitaxel (at a

therapeutic concentration) was determined by using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The signaling mediators involved in this effect were determined by using selective inhibitors. We also investigated nAChR expression, and ATP "binding cassette" G2 drug transporter (ABCG2) expression and its modulation by the different treatments by Western blot. The effect of the treatments on apoptosis induction was determined by flow cytometry using annexin-V and 7AAD markers.

RESULTS

Our results confirmed that the treatment with paclitaxel reduced MDA-MB-231 cell viability in a concentration-dependent manner and that the presence of nicotine reversed the cytotoxic effect induced by paclitaxel by involving the expression of functional $\alpha 7$ and $\alpha 9$ nAChRs in these cells. The action of nicotine on paclitaxel treatment was linked to the modulation of the protein kinase C, mitogen-activated protein kinase, extracellular signal-regulated kinase, and NF- κ B signaling pathways, and to an up-regulation of ABCG2 protein expression. We also detected that nicotine significantly reduced the increase in cell apoptosis induced by paclitaxel treatment. Moreover, the presence of nicotine reduced the efficacy of paclitaxel treatment administered in three cycles to MDA-MB-231 tumor cells.

CONCLUSION

Our findings point to nAChR as responsible for the decrease in the chemotherapeutic effect of paclitaxel in triple negative tumors. Thus, nAChR should be considered as targets in smoking patients.

Key Words: Breast cancer; Paclitaxel; Nicotinic acetylcholine receptors; Drug therapy; Signal transduction; Drug transporter

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Core Tip: Smokers with lung tumors are more likely to generate resistance to chemotherapy than non-smokers. However, little is known about the effect of nicotinic activation during the treatment of breast cancer, a cancer which arises close to the lung. In triple negative human breast cells, nicotine reduces the chemotherapeutic effect of paclitaxel through the participation of several kinases, as well as by modulating ATP “binding cassette” G2 drug transporter expression and inducing resistance to treatment. These results indicate that nicotinic acetylcholine receptors are a new possible target in antitumor therapy for this subtype of breast cancer.

INTRODUCTION

Cigarette smoke contains many harmful components for human health^[1]. Among them, nicotine (NIC), which presents addictive properties^[2], exerts its effects by activating nicotinic acetylcholine receptors (nAChR)^[3]. These receptors belong to the Cys-loop family of pentameric ionic channels activated by ligands^[4]. nAChR can be made up of 17 different subunits, whose assembly creates several homopentameric and heteropentameric channel subtypes in the cell membrane^[5].

Originally, nAChRs were found in the nervous system. However, their expression has been described in several organs such as the lungs^[6-7], kidney^[8-9], intestine^[10-11] and breast, where mainly the $\alpha 7$ and $\alpha 9$ subunits are expressed^[12-13]. The activation of nAChRs can induce an increase in the levels of intracellular calcium ^[14], which has been related to tumorigenesis in the lungs^[15], liver^[16], pancreas^[17] and brain^[18]. This increase in intracellular calcium can in turn activate kinase signaling pathways^[19-20], which regulate different parameters of tumor biology such as proliferation, migration and invasion^[21-23].

Previous evidences have demonstrated that the activation of these receptors can decrease the effectiveness of different antitumor agents in various tumor types such as those from the oral and nasal cavity^[14; 24], pancreas^[25], head and neck^[26] and lungs^[27-29]. In the lungs, many authors have described that NIC may exert its modulatory effect on the actions of chemotherapeutic agents through the activation of signaling pathways involving protein kinases^[20-33]. However, little is known about the effect of nicotinic activation during the treatment of breast cancer, a cancer that arises close to the lungs.

Breast cancer is characterized by a high incidence that causes a high number of deaths in women worldwide^[34]. According to their genetic profile, breast tumors are classified in different subtypes, a fact that allows doctors to choose the most effective antitumor therapy. Tumors can be categorized according to the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki-67 protein by immunohistochemistry. This allows defining four subtypes of breast tumors: luminal A, luminal B, HER2+ and normal-like or triple negative (TN)^[35-36]. TN tumors lack the expression of ER, PR and HER2, but present high expression of Ki-67 protein and are usually very invasive and aggressive^[37]. Since these tumors do not exhibit a defined therapeutic target, there is not a specific treatment, and they respond poorly to taxanes like paclitaxel (PX), usually administered as conventional chemotherapy to other breast cancer patients.

PX is an effective drug used not only in the treatment of breast cancer, but also in non-small cell lung cancer, prostate cancer, and head and neck cancer^[38]. It is a cytostatic compound that causes hyper-stabilization of polymerized microtubules, inhibiting the mitotic spindle and arresting cells in G₂/M phases^[39]. The persistence of cell arrest eventually produces cell death by apoptosis^[40]. Thus, a low level of apoptosis could be an important factor in the development of resistance to treatment.

In this work, we evaluated the ability of NIC to interfere in the treatment of human TN breast cancer MDA-MB-231 cells with PX and the signaling pathway involved in this action.

MATERIALS AND METHODS

Cell culture

The human breast adenocarcinoma cell lines MDA-MB-231 (TN, CRM-HTB-26), MDA-MB-468 (TN, HTB-132) and MCF-7 (luminal A, HTB-22) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Inc., Carlsbad, CA, USA) supplemented with 100 mL/L heat inactivated fetal bovine serum (FBS) (Internegocios SA, Mercedes, Buenos Aires, Argentina), L-glutamine (0.3 mg/L) and gentamicin (80 mg/L). The cultures were maintained at 37°C in humidified air with 50 mL/L CO₂ and the medium was replaced three times a week. Cells were detached with 250 mg/L trypsin in Ca²⁺- and Mg²⁺-free PBS containing 20 mg/L EDTA. Cell viability was determined by the Trypan blue exclusion test and the absence of mycoplasma was observed by Hoechst staining^[41].

Cell viability assay

Cell viability after treatment was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining method (Life Technologies, Eugene, OR, USA). A suspension containing 4x10³ cells/well was added to each well of a 96-well plate in culture medium supplemented with 50 mL/L FBS and cells were then left to attach overnight. When cells reached 60–70% of confluence, they were deprived of FBS for 24 h to induce the synchronization of cultures. Then, cells were treated with PX (Bristol-Myers Squibb, Buenos Aires, Argentina) and/or NIC (which is a non-selective nAChR agonist) in medium supplemented with 20 mL/L FBS, for 48 h in triplicate. To inhibit the action of the nicotinic agonist, cells were previously treated with the antagonists mecamylamine (MM), methyllycaconitine (MLA) or luteolin (Lut) at 10⁻⁶M; these three are respectively nAChR non-selective, α7 nAChR selective and α9 nAChR selective antagonists. To determine the participation of kinases in the effects of PX, cells were previously treated with inhibitors of: protein kinase C (PKC) [staurosporine (Stau), 10⁻⁸M], mitogen-activated protein kinase kinase (MEK) [PD098059 (PD), 10⁻⁵M],

Ras [S-trans,trans-farnesylthiosalicylic acid (FTS), 10^{-6} M], extracellular signal-regulated kinases (ERK1/2) (U126, 10^{-5} M), p38 mitogen-activated protein kinases (p38MAPK) [SB203580 (SB), 10^{-5} M] or the mediator of the activation of the NF- κ B pathway I κ B kinase (IKK β) [IMD354 (IMD), 5×10^{-8} M].

To determine the modulation of the sensitivity of MDA-MB-231 cells to chemotherapy, cells were treated with PX in the absence or presence of NIC for three 48-h cycles with 24 h intercycles without treatment. Then, surviving cells were treated with a new cycle of PX for 48 h. After treatment, the medium was removed and 100 μ L of MTT solution (500 mg/L medium free of phenol red and FBS) was added. Plates were incubated for 4 h at 37°C and the production of formazan was measured by analyzing the absorbance at 540 nm with an ELISA reader (BioTek, Winooski, VT, USA). Values are indicated as mean \pm standard deviation (S.D.) and expressed as the percentage of cell viability in comparison to cells without treatment considered 100%.

A diagram of the administration schedule for the determination of cell viability or cell sensitivity to chemotherapy is shown in figure 1.

Calculation of the effective concentration 50

Dose-response data were transformed, changed to percentage and fitted to a sigmoidal curve, following a maximal effective concentration (Emax) model with at least six data points, using the GraphPad Prism 6 software. This allowed calculating the effective concentration 50 (EC50) and Emax values. Only data with a coefficient of variation lower than 20% were considered for the EC50 values.

Detection of nicotinic receptors by Western blot

MDA-MB-231 cell proteins were extracted by washing them with a buffer containing 6 g/L Tris-HCl, 3 g/L NaCl, 210 mg/L NaF, 480 mg/L MgCl₂, 300 mg/L EDTA, 380 mg/L EGTA, 870 mg/L phenylmethanesulfonyl fluoride, 10 mL/L Triton X-100 and 10 mg/L trypsin inhibitor, aprotinin and leupeptin, at pH 7.4. Samples were incubated on ice for 1 h and centrifuged at 800 G for 20 min at 4°C, afterwards supernatants were

collected and saved at -80°C . Protein concentrations were determined by the Bradford assay^[42].

Samples (80 μg protein per lane) were separated by 10% SDS-PAGE minigel electrophoresis and then transferred to nitrocellulose membranes. They were then blocked with 50 g/L skim milk and incubated overnight with rat anti-human- $\alpha 7$ nAChRs monoclonal antibody or mouse anti-human- $\alpha 9$ nAChRs monoclonal antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA), both diluted 1:200. Then, strips were incubated with anti-rat or anti-mouse IgG coupled to horseradish peroxidase diluted 1:10000 in buffer containing 2.4 g/L Tris-HCl buffer, 9 g/L NaCl and 500 mg/L Tween 20 (TBS-T) at 37°C for 1 h. $\alpha 7$ nAChR and $\alpha 9$ nAChR bands were detected by chemiluminescence and quantified by densitometric analysis using the Image J software (NIH). The results are expressed as optical density (O.D.) units relative to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology Inc., Dallas, TX, USA), which was used as loading control^[43].

⁶ *Trypan blue exclusion assay*

Cell viability was also determined by the trypan blue dye exclusion test. Briefly, cells were treated with the corresponding drugs for 48 h in 48-well plates at a density of 10^4 cells/well. Cells were collected and centrifuged at 900 r/min for 10 min. Then, pellets were resuspended in DMEM and the trypan blue solution was added in a 1:1 ratio. The number of viable cells, identified as non-stained cells, was counted using a hemocytometer under an inverted microscope at 10X magnification and the percentage of these cells with respect to the total cell number was calculated.

Apoptosis determination by flow cytometry

For apoptosis determination, MDA-MB-231 cells were grown in six-well plates and treated for 48 h with PX, NIC or their combination, in the presence or absence of selective and non-selective nicotinic antagonists and kinase inhibitors. Then, cells were harvested and resuspended in binding buffer, and 2 μL of AnnexinV-FITC was added

to each sample. Cells were then incubated for 15 min at room temperature in the dark. After that, 2 μ L of 7-aminoactinomycin D (7AAD) was added and the samples were immediately analyzed by the BD Accuri C6 Plus Flow Cytometer. Data were analyzed by the BD Accuri C6 Plus software.

ATP binding cassette transporter G2 detection by Western blot

For the detection of ATP binding cassette transporter G2 (ABCG2), cells (2×10^6) were treated for 48 h with the different drugs and samples were prepared as indicated to detect nicotinic receptors. Then, samples (80 μ g protein per lane) were separated by 12% SDS-PAGE minigel electrophoresis, transferred to nitrocellulose membranes, blocked with 50 g/L skim milk and incubated overnight with a rabbit anti-human ABCG2 polyclonal antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) diluted 1:200. Then, strips were incubated with horseradish peroxidase-linked anti-rabbit IgG, diluted 1:10000 in TBS-T at 37°C for 1 h. Bands were detected by chemiluminescence and then quantified by densitometric analysis using the Image J program (NIH) and expressed as O.D. units in comparison to the expression of GAPDH, which was used as loading control^[44].

⁸ *Statistical analysis*

Results are expressed as mean \pm S.D. and statistical analysis was performed using the GraphPad Prism6 software. To determine differences between mean values, one-way ANOVA was performed with Tukey's post-hoc analysis. ⁴ $P < 0.05$ was accepted as statistically significant. The data and statistical analysis complied with the recommendations on experimental design and analysis in pharmacology^[45].

RESULTS

Effect of nicotine on MDA-MB-231 cell viability

First, we analyzed the effect of NIC administered for 48 h to MDA-MB-231 cells in culture. The addition of different concentrations of NIC increased cell viability from 10-

10^{-10} M, with an Emax of $189.3 \pm 5.2\%$ (Figure 2A). This effect was reduced by the pre-treatment of cells with different nicotinic antagonists: MM (non-selective) (Emax: $118.4 \pm 3.36\%$), MLA ($\alpha 7$ nAChR selective) (Emax: $143.6 \pm 1.38\%$), or Lut ($\alpha 9$ nAChR selective) (Emax: $135.6 \pm 5.69\%$), all of them added at 10^{-6} M (all $P < 0.001$ vs. NIC treatment) (Figure 2A). The nicotinic antagonists alone did not modify cell viability (data not shown). In addition, expression of $\alpha 7$ and $\alpha 9$ nAChRs in MDA-MB-231 cells was detected by Western blot assay (Figure 2B).

Paclitaxel treatment of MDA-MB-231 cells in the presence of nicotine

In the next set of experiments, we analyzed the action of PX on tumor cells in the presence of NIC. First, we confirmed that PX reduced MDA-MB-231 cell viability in a concentration-dependent manner and that PX effect was significant at concentrations equal to or higher than 10^{-8} M (EC50: 1.0×10^{-7} M) (Figure 3). On the other hand, the presence of the first effective concentration of NIC (10^{-10} M) shifted the concentration-response curve to the right, increasing the EC50 value in more than one order (EC50: 1.2×10^{-6} M) (Figure 3).

After three cycles of PX (48 h each) in the presence of NIC, followed by 24 h without drugs, surviving cells were less sensitive to a subsequent PX cycle in comparison to the same treatment in the absence of NIC. The latter was evidenced by an increase in the EC50 value obtained from the concentration-response-curve (PX+NIC EC50: 3.6×10^{-6} M; PX EC50: 4.8×10^{-7} M) (Figure 4).

It has been documented that 10^{-10} M can be considered a concentration of NIC similar to that present in the blood stream of passive smoking patients^[46]. To analyze the ability of NIC to interfere with the action of therapeutic concentrations of PX, we treated MDA-MB-231 cells with a combination of 10^{-10} M NIC and 10^{-7} M PX for 48 h. We determined that NIC reduced PX effectiveness by increasing tumor cell viability to $110.8 \pm 1.9\%$ in comparison to PX treatment in the absence of NIC ($51.1 \pm 3.9\%$, $P < 0.01$). This effect of NIC on PX action was partially reduced by the pre-treatment of cells with nicotinic antagonists (MM, MLA or Lut) added at 10^{-6} M ($P < 0.001$ vs PX+NIC) (Figure 5A) in a

manner similar to that of NIC treatment alone (NIC: 138.16 ± 6.08 ; NIC+MM: 116.52 ± 0.12 ; NIC+Lut: 126.16 ± 5.96 ; NIC+MLA: 119.69 ± 4.26 ; $P < 0.001$; $P < 0.05$ and $P < 0.001$ vs NIC respectively). We confirmed that PX effect was independent of nAChR activation since pre-treatment with nicotinic antagonists did not modify the effect of PX treatment alone (PX+MM: 52.12 ± 6.65 ; PX+Lut: 49.97 ± 5.88 ; PX+MLA: 54.6 ± 4.01). To confirm the action of drugs on cell viability, we next analyzed the ratio of living cells by the Trypan blue exclusion test after the different treatments. The results plotted in figure 5B (control: 100 ± 3.3 ; NIC: 129.3 ± 1.1 , $P < 0.001$; PX: 64.1 ± 4.3 , $P < 0.001$; PX+NIC: 92.3 ± 2.5 , $P = \text{ns}$; PX+NIC+MM: 56.3 ± 3.4 , $P < 0.001$; PX+NIC+MLA: 66.7 ± 5.4 , $P < 0.001$; PX+NIC+Lut: 52.3 ± 6.0 , $P < 0.001$; all being % or significance respect to control) show values similar to those obtained by the MTT assay (Figure 5A).

To confirm that nicotinic agonists can modulate the action of PX in reducing viability in other breast cancer cells, we tested the effect of PX (10^{-7}M) in the absence or presence of NIC (10^{-10}M) on MDA-MB-468 and MCF-7 cell viability. We determined that the presence of NIC reduced the effect of PX in MDA-MB-468 and MCF-7 cells, and that these effects were prevented by pre-incubating cells with 10^{-6}M of MM (Table 1).

Signal transduction pathways and mechanism involved in the effect of paclitaxel on nicotine-treated tumor cells

Previous reports have indicated that the activation of protein kinases is essential to modulate cell viability by triggering pro/anti-apoptotic gene transcription^[47-48]. Additionally, the induction of the expression of these proteins could implicate the activation of the NF- κ B pathway. In the present study, we observed that the effect of PX on MDA-MB-231 cells is mediated, at least in part, by the Ras, p38MAPK ($P < 0.01$ vs. PX) and NF- κ B pathways ($P < 0.05$ vs. PX) since the addition of their specific inhibitors FTS, SB or IMD respectively modified PX action (Figure 6A). On the other hand, the presence of NIC during PX treatment not only involved the previously mentioned molecules, but also the PKC ($P < 0.05$ vs. PX), MEK ($P < 0.01$ vs. PX) and ERK1/2 ($P < 0.01$ vs. PX) pathways, as revealed by the action of Stau, PD and U126 respectively, which

were added to the cultures (Figure 6B). We confirmed that the inhibitors alone did not modify cell viability (data not shown).

Apoptosis is a cell death mechanism that could improve antitumor actions of chemotherapy, and the activation of protein kinases is frequently associated with the activation of pro/anti-apoptotic signaling pathways. As expected, we found a reduction in cell viability due to the apoptosis induced by PX (PX: $16.7 \pm 1.4\%$; control: $6.1 \pm 0.7\%$, $P < 0.001$). The presence of NIC reduced the effect of PX. The percentage of apoptotic cells was 9.3 ± 0.6 ($P < 0.05$ *vs* control or $P < 0.001$ *vs* PX) (Figure 7). We also observed that the effect of NIC on PX-treated cells was totally reversed in the presence of nicotinic antagonists ($P > 0.05$ *vs* PX) (Figure 7).

An important aspect in cancer chemotherapy is the development of resistance. This phenomenon has been linked to several proteins, including ABCG2, which is a drug extrusion pump that decreases the effectiveness of antitumor drugs. Thus, we next analyzed ABCG2 expression by Western blot. As shown in figure 8, MDA-MB-231 cells expressed this protein and the addition of PX caused a significant increase in its expression ($P < 0.05$). The presence of NIC during PX treatment potently increased ABCG2 Levels in tumor cells ($P < 0.001$ *vs* PX). The pretreatment of cells with nicotinic antagonists reduced NIC action on ABCG2 expression ($P > 0.05$ *vs* PX+NIC) (Figure 8).

DISCUSSION

Our results reveal the mechanisms by which NIC decreases the cytotoxic effects of PX on human TN breast cancer MDA-MB-231 cells. Several authors have described the effect of nAChR activation on cell proliferation. Regarding the latter, NIC from tobacco smoke stimulates nAChRs expressed in the oral cavity^[49], esophagus^[50], stomach^[51], intestine^[52] and lungs^[53-55]. NIC can also trigger malignant transformation in smoking patients and increases the risk of developing lung cancer^[56-57]. Less is known about the effects of NIC on developing tumors or promoting malignant growth in other organs near the lung that also express nAChRs, such as is the case of the breast. Previous studies have shown that human breast tumors express the $\alpha 7$ and $\alpha 9$ nAChR subtypes

[58-59]. In the present study, we confirmed that these receptors are functional in MDA-MB-231 tumor cells since the treatment with NIC increased cell viability in a concentration-dependent manner. This effect was reversed by the pre-treatment of cells with selective nicotinic antagonists.

Previous reports have indicated that the presence of NIC (because of smoking) reduces the effectiveness of chemotherapy in lung cancer patients^[60]. Less evidence is available about the effect of NIC on breast cancer patients during chemotherapy administration. PX is a first-choice drug in breast cancer treatment due to its antimitotic ability and its effect inhibiting different tumor progression pathways^[61]. Our results confirm that PX acts on MDA-MB-231 tumor cells by reducing their viability in a dose-dependent manner. The presence of NIC at a concentration similar to that present in the blood of passive smokers ^[62-63] reduced the potency of PX in more than one order of magnitude. It is also important to highlight that the administration of NIC is effective in reducing PX effect in other TN tumor cells such as MDA-MB-468, and in luminal A MCF-7 tumor cells. In a human gastric cancer model, Tu *et al.*^[64] observed a reduction in the effect of PX on cell viability due to NIC through the activation of $\alpha 7$ nAChRs. Similarly, here we demonstrated that both the $\alpha 7$ and $\alpha 9$ nAChR subtypes are involved in this effect, a fact also evident when PX chemotherapy was administered in cycles to TN breast tumor cells.

Several authors have demonstrated that chemotherapeutic drugs control tumor tissue growth by reducing cell viability ^[65-66; 28] and activating distinct signaling transduction pathways that involve PKC^[67], MEK^[68], ERK1/2^[69], Ras^[70], p38MAPK^[71] and NF- κ B^[72]. Particularly, PX exerts its effects *via* the activation of different kinase signaling pathways depending on the cell type analyzed ^[73-75]. In our model, PX reduced cell viability through the activation of the Ras, p38MAPK and NF- κ B pathways. These results are in line with those obtained by Lu *et al.*^[76], who described that the treatment with PX decreases ovarian carcinoma cell viability by activating the p38MAPK pathway, as well as with those of Okano *et al.*^[77], who observed that the treatment of

human esophageal squamous cancer cells with PX increases cell death through the activation of Ras.

In the present study, when we analyzed signaling pathways involved in PX treatment in the presence of NIC, we determined that PKC, MEK and ERK1/2 also participate in the reduction of breast tumor cell viability. These results are similar to those of Chernyavsky *et al.*^[78], who described that, in human corneal epithelial cells, the stimulation of $\alpha 7$ nAChRs can activate the PKC-MEK-ERK1/2 signaling pathway through an increase in intracellular calcium, which induces an up-regulation of E cadherin expression related with corneal re-epithelization. In the same line of evidence, Wan *et al.*^[79] described that the activation of $\alpha 7$ nAChRs can mediate the proliferation of a hepatocellular carcinoma through a TRAF6/NF- κ B-dependent mechanism similar to that here observed. Our results and those of others would thus indicate the presence of antagonistic effects between PX and NIC on cell viability.

PX reduces breast cell viability, at least in part, through the induction of apoptosis^[71]. We confirmed that this effect also occurs in our model since an increase in MDA-MB-231 cell apoptosis was observed after 48 h of treatment. The effect of PX on cellular apoptosis was attenuated by the activation of $\alpha 7$ and $\alpha 9$ nAChRs with NIC.

A frequent undesirable effect of antitumor therapy is innate or acquired resistance. In particular, PX can induce acquired resistance^[80-81] by the transactivation of signaling pathways^[82] or by triggering the overexpression of several proteins^[83-84]. Considering the latter, the ABCG2 transporter plays an important role in the generation of resistance to PX treatment in breast adenocarcinomas^[85]. In the present study, PX induced an increase in ABCG2 expression and this effect was potentiated by $\alpha 7$ and $\alpha 9$ nAChR activation by NIC. The increase in ABCG2 expression should lead to a higher rate of PX extrusion, partly explaining the reduction in the cytostatic effect of PX in the presence of NIC, as well as the decrease in the sensitivity to PX when administered in cycles in the presence of NIC. Similar results have been obtained by Mukherjee *et al.*^[86] and Amawi *et al.*^[87], who described that PX treatment increases the activation and expression of ABCG2 in different breast cancer cell lines and that this factor could mediate the

resistance to treatment^[88]. Moreover, Nimmakayala *et al.*^[89] and An *et al.*^[90] observed that the exposure of pancreatic and lung tumor cells to cigarette smoke caused an increase in the expression of this pump.

A possible mechanism of action of PX treatment in the presence of NIC in a TN tumor-bearer smoker patient is proposed in figure 9.

CONCLUSION

Our results demonstrate that NIC at a concentration similar to that present in passive smokers' plasma can negatively modulate the cytotoxic/apoptotic effect of PX in TN breast tumors. The reduction in the sensitivity to PX could be due to an increase in the expression of the ABCG2 transporter in malignant cells. Additionally, our findings evidenced the participation of different kinases and the NF- κ B pathway, which would modulate cell viability in this effect. This information could allow developing better strategies to improve TN breast cancer therapy, such as blocking nAChRs together with PX during chemotherapy administration to passive smoking patients.

ARTICLE HIGHLIGHTS

Research background

Triple negative is the subtype of breast cancer with the worst prognosis, showing an increase in resistance to chemotherapy in smoking patients, who present high levels of nicotine in their blood. In lung cancer, it has been proposed that the activation of nicotinic acetylcholine receptors could be responsible for the modulation of several parameters of tumor biology and the loss of effectiveness of chemotherapeutic treatment, but it is not known what occurs in a nearby organ such as the breast.

Research motivation

Given that breast tumor-bearing patients present a low efficiency to antitumor therapy, knowledge of the signaling pathways involved in this phenomenon is important to generate new therapeutic targets that improve sensitivity to treatment.

Research objectives

This research aimed to determine the signaling pathways involved in the nicotinic modulation of the cytostatic effect of paclitaxel in human triple negative breast cancer cells.

Research methods

The modulatory effect of nicotine on paclitaxel treatment was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Trypan blue exclusion assay was used to evaluate viable cells in response to different treatments. Protein expressions were evaluated by Western blot assays and apoptosis was determined using immunofluorescence assays with annexin V and 7AAD.

Research results

Nicotine decreased paclitaxel's inhibition of viability and apoptosis on MDA-MB-231 breast cancer cells. This modulation on viability is mediated by the activation of $\alpha 7$ and $\alpha 9$ nicotinic acetylcholine receptors and protein kinases PKC, Ras, MEK, ERK, p38MAPK and the NF- κ B pathway. Cells surviving paclitaxel treatment in the presence of nicotine are less sensitive to another cycle with the chemotherapeutic agent probably due an increase in the protein expression of ATP binding cassette transporter G2.

Research conclusions

Nicotine modulates the cytotoxic/apoptotic effects of paclitaxel and the knowledge of its signaling pathway mediators could allow the development of better strategies to improve triple negative breast cancer therapy, such as the nicotinic acetylcholine receptors blockage together with paclitaxel during chemotherapy administration to smoking patients.

Research perspectives

The knowledge of the mediators that participate in the nicotinic modulation of paclitaxel's effect will allow the development of new antitumor strategies that could be applied not only to other subtypes of mammary tumors, but also to tumors in other organs of smoking patients.

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