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Alpha$_2$-adrenoceptor agonists trigger prolactin signaling in breast cancer cells

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ABSTRACT

Breast cancer is the most frequent malignancy among women worldwide. We have described the expression of α_2-adrenoceptors in breast cancer cell lines, associated with increased cell proliferation and tumor growth. A mitogenic autocrine/paracrine loop of prolactin (Prl) has been described in breast cancer cells. We hypothesized that the α_2-adrenergic enhancement of proliferation could be mediated, at least in part, by this Prl loop.
In both T47D and MCF-7 cell lines, the incubation with the α_2-adrenergic agonist dexmedetomidine significantly increased Prl release into the culture medium (measured by the Nb2 bioassay), this effect being reversed by the α_2-adrenergic antagonist rauwolscine. No change in Prl receptors (PrlR) was observed by RT-qPCR in these cell lines. In IBH-6 cells a decrease in Prl secretion was observed at the lower dexmedetomidine concentration.
The signaling pathways involved in ovine Prl (oPrl) and dexmedetomidine action were also assessed. Both compounds significantly activated STAT5 and ERK in all three cell lines. In T47D and MCF-7 cell lines also AKT was activated by both Prl and dexmedetomidine. We therefore describe the STAT5 phosphorylation by an α_2-adrenergic agonist, dexmedetomidine.
In T47D cells, the α_2-adrenergic stimulation of cell proliferation is probably mediated, at least in part, by the Prl autocrine/paracrine loop, because this effect is abrogated by the specific PrlR antagonist Δ1–9-G129R-hPrl. The implication of Prl loop describes a novel mechanism of action of this GPCR.

1. Introduction

Breast cancer is the most frequent malignancy among women worldwide, with 1.67 million of new cancer cases diagnosed (25.2% of all cancers in women) and 522,000 deaths (14.7% of all deaths by cancer) as assessed by GLOBOCAN 2012. This disease is the most common cancer and is still the most frequent cause of cancer death in women in less developed regions while it is now the second cause in some developed regions [1,2]. Breast cancer is a heterogeneous disease, as highlighted by the description of different molecular subtypes based on their gene expression profile. Both MCF-7 [3] and T47D [4] are paradigmatic luminal cell lines (estrogen receptor-α positive, progesterone receptor positive, and no overexpression of HER-2) [5]. IBH-6 cells, developed in our laboratory from a primary breast cancer sample, also belonging to this subtype [6].

α_2-Adrenoceptors (α_2-AR, subdivided in α_{2A}, α_{2B}, and α_{2C}-AR) are G protein–coupled receptors (GPCR) which classically couple to Gi inhibiting adenylyl cyclase activity and therefore intracellular cAMP levels. G protein activation causes its dissociation, releasing the βγ-subunit that may promote an alternative signaling through activation of mitogen-activated protein kinases (MAPKs) and ion channels. Adrenoceptors also signal through regulatory proteins (mainly G protein–coupled receptor kinases (GRKs)) and scaffolding proteins (β-arrestins), in a G protein–independent manner [7]. Recently, it has been described that the activation of ERK1/2 pathway by the α_2-AR agonists like dexmedetomidine and clonidine promotes the proliferation and migration capacity of triple negative MDA-MB-231 breast cancer cells [8]. Our group has described the expression of α_2-adrenoceptors in several benign and malignant cell lines. Their stimulation with specific agonists is associated with increased cell proliferation [9] and tumor growth in experimental models of breast cancer. Moreover, the α_2-adrenergic antagonist rauwolscine behaves as an inverse agonist,

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inhibiting cell proliferation and tumor growth below control levels [10,11,12].

The synthesis and secretion of prolactin (Prl) as well as the expression of Prl receptors (PrlR) have been described in various breast cancer cells, suggesting the existence of a stimulatory autocrine/paracrine Prl loop [13]. Although the latter was initially described to promote breast cancer cell proliferation [14], this conclusion was not confirmed by others [15]. However, Prl stimulation of breast cell proliferation has been confirmed by many authors, as reviewed [16]. Recently, it was suggested that Prl levels measured <10 years before diagnosis are associated with postmenopausal breast cancer risk, especially for luminal tumors and metastatic disease [17]. Moreover, the PrlR has been implicated in resistance to chemotherapy [18]. Otherwise, the levels of PrlR expression have been shown to be an independent favorable prognostic marker in breast cancer [19], while Prl stimulation prevented the growth of triple negative MDA-MB-231 breast cancer cells [20]. On the other hand, in a group of patients with PrlR-positive metastatic breast cancer or metastatic castration-resistant prostate cancer, no effect was found when a humanized monoclonal antibody that binds to and inhibits the Prl was administered [21]. Nevertheless, this was a phase I study with a limited number of patients [22].

Prl interacts with a specific, single-pass transmembrane PrlR which exists as different isoforms (named long, intermediate and short) as a result of alternative splicing or posttranslational modifications, as reviewed [23]. Prolactin binding to preformed PrlR homodimers triggers conformational changes within the receptor cytoplasmic tail leading to activation (phosphorylation) of the receptor-associated JAK-2 tyrosine kinase. Activated JAK-2 phosphorylates several tyrosine residues in the intracellular domain of the long and the intermediate PrlR isoforms which subsequently serve as docking sites for signaling proteins including the transcription factor STAT5. Once phosphorylated by JAK-2, STAT5 dimers translocate to the nucleus to activate transcription of PrlR target genes. Phosphorylated tyrosines in the PrlR also serve as docking sites for adaptor proteins like Src homology 2 (Shc), growth factor receptor-bound protein 2 (Grb2) and Son of Sevenless (SOS) that couple the receptor to the mitogen activated protein kinase (MAPK) cascade [24]. c-Src mediated activation of ERK1/2 and AKT by Prl has been described in T47D and MCF-7 human breast cancer cells, with a close correlation with cell proliferation and invasion [25,26].

In the present work, we investigated whether the α_2-adrenergic enhancement of cell proliferation could be mediated, at least in part, by the autocrine/paracrine loop of Prl in breast cancer cells. To asses this hypothesis both components of the loop were analyzed in three luminal breast cancer cell lines T47D, MCF-7 and IBH-6. The luminal breast cancer subtype was chosen because the two former cell lines are paradigmatic in the study of the paracrine/autocrine Prl loop. Our data show that in some luminal cell lines, the α_2-adrenergic stimulation of cell proliferation is mediated, at least in part, by the Prl autocrine/paracrine loop.

2. Materials and methods

2.1. Reagents

Fetal calf serum (FCS), horse serum (HS), culture media, antibiotics and trypsin were purchased from Invitrogen Thermo Fisher Scientific (Waltham, MA, USA). Dexmedetomidine (Precedex) was from Abbott Laboratories. Rauwolscine-HCl was purchased from Sigma-Aldrich (St. Louis, MO, USA). NIDDK ovine prolactin (oPrl) was from NIDDK-NIH, Bethesda, USA. Methyl [^{3}H]-thymidine (NET 027E; specific activity: 20 Ci/mmol) was from Dupont-New England Nuclear (Boston, MA, USA). Liquid scintillation cocktail was Optiphase 'Hisafe' 3 (PerkinElmer Health Sciences, Groningen The Netherlands). Antibodies against pERK (Tyr 204, sc-7383), ERK (sc-94), p-STAT5 (Tyr 694, sc-101806), and STAT5 (sc-835) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while anti-p-AKT (Ser473, #4058) and anti-AKT (#4685) were from Cell Signaling. For immunofluorescence the antibodies used were the anti-α_{2A} (A-271) antibody from Sigma-Aldrich, a rabbit polyclonal antibody raised against amino acids 218-235 of human, mouse, rat, and pig α_{2A} adrenergic receptor localized within the third intracellular loop; anti-α_{2B} (H-96, sc-10723) from Santa Cruz Biotechnology, a polyclonal rabbit IgG raised against an epitope corresponding to amino acids 202-297 mapping to an internal region of α_{2B}-AR of human origin and antihuman α_{2C} (C-20, sc-1480) goat polyclonal antibody also from Santa Cruz Biotechnology, an affinity-purified goat polyclonal antibody raised against a peptide mapping at the COOH terminus of the α_{2C}-adrenergic receptor of human origin and cross-reacts with rat and mouse (per data sheets from the providers). Secondary antibodies were from Amersham (GE Healthcare Argentina S.A., Buenos Aires, Argentina). Vectashield H-1000 was from Vector Laboratories (Burlingame, CA, USA). TRI reagent was from Molecular Research Centre, Inc. (Cincinnati, OH, USA), oligo-dT primers, M-MLV reverse transcriptase were acquired from Promega (Madison, WI, USA) and FastStart SYBR Green Master Mix was from Roche (Mannheim, Germany). All the other reagents, including glutamine, DNAse, luminol and p-coumaric acid were from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

The breast cancer MCF-7 and T47D (recently acquired at the ATCC) and IBH-6 cell lines (developed in our laboratory) [6], were cultured as already described [9]. Cells were maintained in DMEM:F12 (1:1) medium supplemented with heat-inactivated 10% FCS, 2 mM glutamine, 2 μg/ml insulin, 100 UI/ml penicillin, 100 μg/ml streptomycin and 15 mM HEPES. Cells were incubated at 37 °C in 5% CO_2 and sub-cultured at 80% confluence using 0.25% trypsin–0.025% EDTA.

The lactogen-dependent rat T-cell lymphoma Nb2 cell line was maintained in culture with DMEM:F12 (1:1) medium supplemented with heat-inactivated 10% FCS, 10% horse serum (HS), 2 mM glutamine, 2 μg/ml bovine insulin, 100 UI/ml penicillin, 100 μg/ml streptomycin and 15 mM HEPES. These non-adherent cells were incubated at 37 °C in 5% CO_2 and passed at high density.

2.3. Breast cancer cell proliferation assays

The human T47D cells were seeded at 10,000 cells per well in 2% charcoal-stripped FCS and maintained for 24 h. Phenol red-free medium was used to avoid pro-estrogenic actions, [27]. Then the cells were treated (with daily medium changes) with the α_2- adrenergic agonist dexmedetomidine (2 μM), with 2 μg/ml (87 nM) ovine (oPrl) and/or the PrlR antagonist Δ1–9-G129R-hPrl in 2 log molar excess (10 μM) for 72 h. [^{3}H]-Thymidine at 0.2 μCi/well was added with the last change of medium. After 24 h, cells were harvested in a Nunc Cell Harvester 8 (Nunc, Rochester, NY, USA), and filters were counted in a Tri-Carb 2800TR PerkinElmer liquid scintillation Analyzer.

2.4. Nb2 bioassay

The Nb2 bioassay was originally developed and validated for serum Prl and GH determinations [28,29,30,31]. The conditions were modified to measure Prl in conditioned medium (CM). To obtain the CM, breast cancer cells were incubated in 10% FCS medium up to 80% confluence. The medium was changed to FCS- and insulin-free medium and then the cells were treated with or without the adrenergic compounds for 48 h. After centrifugation, the CM was frozen at −20 °C until used.

The Nb2 cells were arrested in culture medium DMEM/F12, without FCS but with 10% HS, 24 h prior to the bioassay and then 10,000 cells/well were seeded in 96 well plates. The bioassay was performed with 40 μl of CM in 200 μl final volume of medium with 0% FCS, 10% HS in quadruplicates with a standard curve for each assay (oPrl concentration: 100 fg/ml-1 μg/ml). The incubation lasted 72 h, with 0.20 μCi/well [^{3}H]-thymidine during the last 24 h. The cells were harvested in a Nunc Cell Harvester 8 (Nunc, Rochester, NY, USA), and filters were