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Progesterone signals through membrane progesterone receptors (mPRs) in MDA-MB-468 and mPR-transfected MDA-MB-231 breast cancer cells which lack full-length and N-terminally truncated isoforms of the nuclear progesterone receptor

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ABSTRACT

The functional characteristics of membrane progesterone receptors (mPRs) have been investigated using recombinant mPR proteins over-expressed in MDA-MB-231 breast cancer cells. Although these cells do not express the full-length progesterone receptor (PR), it is not known whether they express N-terminally truncated PR isoforms which could possibly account for some progesterone receptor functions attributed to mPRs. In the present study, the presence of N-terminally truncated PR isoforms was investigated in untransfected and mPR-transfected MDA-MB-231 cells, and in MDA-MB-468 breast cancer cells. PCR products were detected in PR-positive T47D Yb breast cancer cells using two sets of C-terminus PR primers, but not in untransfected and mPR-transfected MDA-MB-231 cells, nor in MDA-MB-468 cells. Western blot analysis using a C-terminal PR antibody, 2C11F1, showed the same distribution pattern for PR in these cell lines. Another C-terminal PR antibody, C-19, detected immunoreactive bands in all the cell lines, but also recognized α-actinin, indicating that the antibody is not specific for PR. High affinity progesterone receptor binding was identified on plasma membranes of MDA-MB-468 cells which was significantly decreased after treatment with siRNAs for mPRα and mPRβ. Plasma membranes of MDA-MB-468 cells showed very low binding affinity for the PR agonist, R5020, ≤1% that of progesterone, which is characteristic of mPRs. Progesterone treatment caused G protein activation and decreased production of cAMP in MDA-MB-468 cells, which is also characteristic of mPRs. The results indicate that the progestin receptor functions in these cell lines are mediated through mPRs and do not involve any N-terminally truncated PR isoforms.

1. Introduction

In addition to the classic intracellular genomic mechanism of steroid action mediated by nuclear steroid receptors [1], there is extensive evidence that steroids also activate specific receptors on the surface of cells resulting in rapid induction of intracellular signaling transduction pathways and hormonal responses that are often nongenomic [2,3]. However, despite extensive research over the last decade, the identities of the steroid membrane receptors that act as intermediaries for many of these nonclassical steroid actions remain unresolved and controversial. For example, nuclear progesterone receptors (PRs) have been implicated in progesterone's rapid activation of second messengers in several cell models [4,5], whereas the novel membrane progesterone receptors (mPRs) appear to mediate the nonclassical actions of progesterone in others [6,7]. The mPRs are 7-transmembrane 40 kDa proteins that are unrelated to the nuclear steroid receptor and G protein coupled receptor superfamilies, but instead belong to the newly described progestin and adipoQ receptor (PAQR) family [8,9].

The mPRs were discovered in spotted seatrout ovaries where an mPR subtype, named mPRalpha (mPRα), was shown to function as a progesterone membrane receptor and act as an intermediary in the progestin induction of oocyte maturation by a nongenomic mechanism [6]. Subsequently mPRα and two related proteins, mPRβ and mPRγ, were identified in other vertebrates, including humans, and were also shown to have the binding

Abbreviations: mPRα, membrane progesterone receptor alpha; mPRβ, membrane progesterone receptor beta; PR-A, nuclear progesterone receptor A; PR-B, nuclear progesterone receptor B; PR-C, nuclear progesterone receptor C; PR-M, nuclear progesterone receptor M.

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characteristics of progesterone membrane receptors [10]. The functional characteristics of mPRs, especially mPRα, have been extensively studied in various cell models since their discovery in 2003 [7]. Recombinant human, spotted seatrout and goldfish mPRα proteins expressed on PR-negative MDA-MB-231 breast cancer cell membranes display high-affinity, limited-capacity, specific progestin binding typical of membrane progestin receptors, with highest binding affinities for their endogenous progestin hormones, progesterone, 17,20β,21-trihydroxy-4-pregnen-3-one, and 17,20β-dihydroxy-4-pregnen-3-one, respectively [9,11]. The mPRαs have very different progestin binding affinities from those of the PRs which have been exploited to investigate their specific functions in cells which express both types of progesterone receptors [12,13]. The recombinant mPRαs are coupled to inhibitory G proteins (G_i) in MDA-MB-231 cell membranes and down-regulate adenylyl cyclase activity resulting in decreased cAMP levels [9]. Similar functional characteristics to those of the recombinant mPRα proteins have been reported for endogenous mPRα and mPRβ in human myometrial cells [13], human T lymphocytes and Jurkat cells [14], human SKBR3 breast cancer cells [15], a rodent GnRH neuronal cell line [16], in fish oocytes [17] and in fish granulosa/theca cells [18]. Taken together, these results suggest that the progestin binding and signaling characteristics of mPRs are fundamental functions of these proteins in vertebrate cells.

The progesterone receptor characteristics of mPRs need to be confirmed in vertebrate cells lacking any other progesterone receptors in order to provide definitive proof that these functions are solely attributable to mPRs. The MDA-MB-231 breast cancer cell line was selected for investigating the functions of recombinant mPRs because it lacks the full-length PR [19]. However, N-terminally truncated PR isoforms have been identified in breast cancer tissues and cell lines [20–22] as well as in other tissues [23,24], which raises the possibility that they are also present in breast cancer cells lacking the full-length PR, but would have not been detected using the commonly used PR primers and antibodies directed against the N-terminus of the receptor. An N-terminally truncated variant of the estrogen receptor (ER), named ERα-36, has recently been detected by Kang and coworkers in SKBR3 breast cancer cells [25] which lack the full-length ER, but express the 7-transmembrane membrane estrogen receptor, GPR30 [26]. They also detected ERα-36 in HEK-293 cells [25] which have been used to investigate the estrogen receptor functions of recombinant GPR30 [27]. On the basis of their results showing that membrane ERα-36 expression was up-regulated in the HEK293 cells after transfection with human GPR30, Kang et al. proposed that the increased estrogen receptor functions in the GPR30-transfected cells were associated with the increase in ERα-36 protein levels and not with the increase in GPR30 levels [25]. While these conclusions are inconsistent with our findings with endogenous fish GPR30s [28], and are not supported by the results of our recent studies on human GPR30, they do indicate a need to investigate the potential role of truncated forms of nuclear steroid receptors in nonclassical steroid signaling proposed to be mediated through novel steroid membrane receptors.

The occurrence of N-terminally truncated forms of the PR in MDA-MB-231 cells and in MDA-MB-231 cells transfected with mPRα or mPRβ was investigated in the present study. Progesterone membrane receptor binding and G protein activation through endogenous mPRs have not been investigated in a full-length PR-negative breast cancer cell line, such as MDA-MB-468 cells. Therefore, progesterone binding and signaling through endogenous mPRs and the effects of mPR knockdowns were investigated in MDA-MB-468 cells, as well as the presence of N-terminally truncated PR isoforms in these breast cancer cells.

2. Materials and methods

2.1. Expression of recombinant mPRs and cell culture

Human mPRα or mPRβ cDNA was amplified, ligated into a pBK-CMV expression vector and transfected into MDA-MB-231 human breast cancer cells with Lipofectamine as described previously [9]. Control MDA-MB-231 cells were transfected with an empty pBK-CMV vector at the same time. Stably transfected mPRα, mPRβ, or empty vector cell lines were established by continued selection with geneticin (G-418, Invitrogen, Carlsbad, CA) over several weeks. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) as described previously [9]. T47D Yb and MDA-MB-468 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in L-15 medium supplemented with 10% FBS and antibiotics.

2.2. Preparation of plasma membranes and cell lysates

Cells were grown in 15 cm culture dishes to 80% confluence, washed 3 times with ice-cold PBS and then harvested with a cell scraper in HAED (25 nM HEPES, 10 mM NaCl, 1 mM dithioerythritol, 1 mM EDTA, pH 7.6) buffer [9]. Cells were homogenized using a handheld glass tissue grinder (Wheaton Tenbroek, Fisher Scientific, Pittsburgh, PA) and centrifuged at $1000 \times g$ for 7 min to remove nuclei. The supernatant was centrifuged at $20{,}000 \times g$ for 20 min to pellet the plasma membranes. The pellets were reconstituted in HAED buffer and the membranes were enriched by layering them on a 1.2 M sucrose pad and centrifugation for 40 min at $9600 \times g$. The enriched plasma membrane layers were pelleted by centrifugation at $20{,}000 \times g$ for 20 min and reconstituted in HAED for subsequent analysis. Cells were lysed in the culture dish by incubation with RIPA buffer (Pierce, Rockford, IL) for 30 min at 4 °C. The cell lysate sample was transferred to a tube, shaken for 30 min at 4 °C, centrifuged to pellet the cellular debris, and the supernatant was used for Western blot analysis.

2.3. RT-PCR

Primers were designed according to the sequence of human progesterone receptor (GenBank access number M15716) within the region of 2233–2977 base pairs, which corresponds to the ligand binding domain (LBD, amino acids 687–933, Fig. 1A) in the C-terminus of PR; sense1: 5′-GAG CTT AAT GGT GTT TGG TC-3′ (2443); antisense1: 5′-GTT TGA CTT CGT AGC CCT T-3′ (2692); sense2: 5′-GAA GGG CTA CGA AGT CAA A-3′ (2672); antisense2: 5′GCA GCA ATA ACT TCA GAC ATC-3′ (2919). Total RNA was extracted from the cell lines using Tri-reagent (Sigma–Aldrich) following the manufacturer's instructions. Reverse transcription (RT) of 5 μg of total RNA from each cell line was conducted with SuperScript III transcriptase (Invitrogen) at 50 °C for 1 h. The RT products (0.5 μl/cell line) were amplified through PCR in 20 μl Mastermix (Promega, Madison WI) containing 200 nM of each sense and antisense primer. The PCR amplification protocol consisted of 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by a 72 °C, 10 min extension step. The PCR products were separated by 1% agarose electrophoresis and then visualized by ethidium bromide staining.

2.4. Western blot analysis

Western blot analyses were performed as described previously [9]. Briefly, plasma membranes or cell lysates were solubilized by