CHAPTER 8
β1-ADRENOCEPTOR AGONIST DOBUTAMINE SELECTIVELY MODULATES BREAST CANCER CELL LINE PROLIFERATION DEPENDING ON THE ESTROGEN-RECEPTOR STATUS

Specific Objective 2B: Involvement of Cellular Mechanisms in β1-AR-induced effects on human breast cancer cells.

8.1 Rationale:
Dysregulation in neuroendocrine-immune functions, increased sympathetic activity in stress associated with chronic diseases, the presence of multiple adrenergic receptor subtypes on cancer cells and the scope of how altered hormone sensitivity may influence downstream signaling pathways triggered by adrenergic agonists have not been investigated. Previously, we have reported that reproductive aging is characterized by alterations in hypothalamic catecholaminergic activity, decline in sympathetic noradrenergic innervation in the lymphoid organs and associated immunosuppression which may contribute to development of age-related diseases and cancer.

Presence of β1-adrenoceptors on mammary cells and breast cancer cell lines have been characterized. However, not much is known about the role of these receptors in vivo. In order to delineate the receptor-specific effects of β1-AR on breast cancer cell lines the specific agonists were added to ER (+) MCF-7 and ER (-) MDA MB-231 cells in vitro in the presence and absence of specific inhibitor metoprolol to assess its effects on proliferation, expression of pro-angiogenic molecules (VEGF A, VEGF C, nitric oxide) and expression of cell survival signaling molecules (p-Akt, p-ERK and p-CREB).
8.2 Methods:

8.2.1 Culture:

8.2.1.1 MCF-7 cells

MCF-7 human breast cancer cell line was obtained from NCCS, Pune and maintained in DMEM medium, supplemented with 2mM L-Glutamine, 100 units/ml Penicillin, 100 µg/ml Streptomycin, 1.5 g/l sodium bicarbonate and 10% Fetal Bovine Serum and incubated at 37°C in a humidified atmosphere with 5% CO₂.

8.2.1.2 MDA MB-231 cells

MDA MB-231 human breast cancer cell lines were obtained from NCCS, Pune and maintained in L15 medium, supplemented with 2mM L-Glutamine, 100 units/ml Penicillin, 100 µg/ml Streptomycin, 1.5 g/l sodium bicarbonate and 10% Fetal Bovine Serum and incubated at 37°C in a humidified atmosphere without CO₂.

Cells were cultured until they reach 70% confluence and such sub confluent flasks were tripsinised and seeded in 96 well plates (5000 cells/well) and incubated for 24 hours until the monolayer is formed. (Cells were stained using trypan blue, live cells were counted by the exclusion method and the desired cell number was achieved for plating onto 96 well plates.)

8.2.2 Treatment

Cells were incubated with $10^{-3}$ M, $10^{-6}$ M and $10^{-9}$ M dobutamine (beta 1 adrenergic agonist) with and without $10^{-5}$ M metoprolol ($\beta_1$-AR-specific antagonist) for 2, 4 and 6 days.

8.3 Results

8.3.1 $\beta_1$ adrenergic agonist and antagonist did not alter proliferation of MCF 7 cell line:

$\beta_1$–AR agonist dobutamine ($10^{-9}$ M, $10^{-6}$ M and $10^{-3}$ M) treatment did not alter the proliferation of ER (+) MCF-7 cells after 2 days (Fig. 8.1A), 4 days (Fig. 8.1B) and 6 days (Fig. 8.1C). Co-incubation of dobutamine-treated MCF-7 cells with $\beta_1$–AR antagonist metoprolol also did not significantly alter proliferation after 2, 4 and 6 days.
Fig. 8.1  *In vitro effects of β₁-AR agonist on MCF-7 cell proliferation.* β₁-AR agonist dobutamine did not affect proliferation of ER (+) MCF-7 cells after 2 days (A), 4 days (B) and 6 days of treatment in the presence or absence of β₁-AR-specific antagonist metoprolol.

8.3.2  β₁ adrenergic agonist enhanced proliferation of MDA MB-231 cell line:

The proliferation of ER (-) MDA MB-231 cells was significantly enhanced by β₁-AR agonist dobutamine after 2 days (10⁻⁹ M; Fig 8.2A), 4 days (10⁻⁶ M and 10⁻³ M; Fig.
2B) and 6 days (10^{-6} \text{ M} and 10^{-3} \text{ M}; Fig. 2C) of incubation. However, co-treatment with \( \beta_1 \)-AR antagonist metoprolol (10^{-5} \text{ M}) significantly decreased the proliferation of dobutamine-treated MDA MB-231 cells after 2 days (10^{-9} \text{ M}) and 6 days (10^{-3} \text{ M} and 10^{-6} \text{ M}). Treatment of ER (-) MDA MB-231 cells with \( \beta_1 \)-AR antagonist metoprolol alone did not significantly alter proliferation after 2, 4 and 6 days.

Fig. 8.2  Effect of \( \beta_1 \) agonist and antagonist on proliferation of MDAMB-231 cell line. There was a significant increase in the proliferation of MDA MB-231 cell line after treatment with agonist for 2 days (A), 4 days (B) and 6 days (C) which was reversed by
co-treatment with AR β₁ antagonist metoprolol. *p<0.05 compared to control.  #p<0.05 compared to agonist-treated group.

8.3.3 Effects of AR β₁ agonists and antagonists on VEGF A production by MCF 7 cells:

There was no significant effect of β₁AR agonist dobutamine on VEGF A production by ER (+) MCF-7 cells after 2 (Fig. 8.3A), 4 (Fig. 8.3B) and 6 (Fig. 8.3C) days of treatment. Co-treatment of dobutamine (10⁻⁶ M)-treated cells with β₁-AR antagonist metoprolol (10⁻⁵ M) significantly decreased VEGF A production after 2 days alone.

8.3.4 Effects of AR β₁ agonists and antagonists on VEGF A production by MDA MB-231 cells:

Similar to ER (+) cells dobutamine treatment did not alter VEGF A production by ER (-) MDA MB-231 cell line after treatment with agonist for 2 (Fig. 8.4A), 4 (Fig. 8.4B) and 6 (Fig. 8.4C) days. However, co-incubation of dobutamine (10⁻⁹ M and 10⁻⁶ M)-treated cells with β₁-AR antagonist significantly decreased the production of VEGF A by MDA MB-231 cells after 2 days and 4 days. Also, treatment of MDA MB-231 cells with metoprolol alone significantly decreased VEGF A production after 2 days.

8.3.5 Effects of AR β₁ agonists and antagonists on VEGF C production by MCF 7 cells:

There was no significant change in the VEGF C production by MCF 7 cell line after treatment with β₁-AR agonist dobutamine for 2 days (Fig. 8.5A), 4 days (Fig. 8.5B) and 6 days (Fig. 8.5C). Co-treatment with β₁-AR antagonist metoprolol (10⁻⁵ M) however, significantly decreased the VEGF C production by MCF 7 cells after 2 days (10⁻⁶M), 4 days (10⁻³ M, 10⁻⁶M and 10⁻⁹ M) and 6 days (10⁻³M).
Fig. 8.3  

$\beta_1$-AR agonist and antagonist did not affect VEGF A production by MCF7 cells. In vitro treatment of MCF-7 cells with dobutamine did not alter VEGF A production after 2 days (A), 4 days (B) and 6 days (C) although co-treatment with specific antagonist metoprolol decreased VEGF A production after 2 days alone. 

#p<0.05 compared to agonist-treated group.
Fig. 8.4  In vitro effects of β₁ agonist and antagonist on VEGF A production of MDAMB231 cell line: Although β₁-AR agonist dobutamine did not alter the production of VEGF A by MDA MB-231 cell line after 2 days (Fig. 8.4A), 4 days (Fig. 8.4B) and 6 days (Fig. 8.4C), co-treatment with specific antagonist significantly decreased it. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
Fig. 8.5  $\beta_1$-AR agonist and antagonist on VEGF C production of MCF-7 cells. VEGF C production by MCF-7 cells was not altered after 2 days (Fig. 8.5A), 4 days (Fig. 8.5B) and 6 days (Fig. 8.5C) of incubation with dobutamine. Co-treatment with AR $\beta_1$ antagonist significantly decreased the VEGF C production by MCF 7 cells. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
8.3.6 Effects of AR β₁ agonists and antagonists on VEGF C production by MDA MB-231 cells:

Co-incubation of β₁–AR agonist dobutamine with ER (-) MDA MB-231 cells did not significantly alter VEGF C production after 2 days (Fig. 8.6A). However, after prolonged incubation there was a significant decrease in the production of VEGF C by MDA MB-231 cell line after treatment with agonist for 4 days (10⁻⁶M and 10⁻⁹M; Fig. 8.6B) and 6 days (10⁻³M and 10⁻⁶M; Fig. 8.6C).

Co-treatment with AR β₁ antagonist did not reverse the agonist mediated effects after 2, 4 and 6 days. Interestingly treatment of MDA MB-231 cells with β₁–AR-specific antagonist metoprolol (10⁻⁵ M) also significantly decreased VEGF C production after 4 and 6 days.

8.3.7 Effects of AR β₁ agonists and antagonists on nitric oxide production by MCF-7 cells:

Nitric oxide production by ER (+) MCF 7 cells was significantly enhanced after 2 days (10⁻³ M; Fig. 8.7A), 4 days (10⁻⁶ M; Fig. 8.7B) and 6 days (10⁻³ M; Fig. 8.7C) of incubation with dobutamine.

Co-treatment with AR β₁ antagonist did not reverse the agonist-mediated increase in nitric oxide production.

8.3.8 Effects of AR β₁ agonists and antagonists on nitric oxide production by MDA MB-231 cells:

AR β₁ agonist dobutamine did not significantly alter nitric oxide production by MDA MB-231 cell line after 2 days (Fig. 8.8A), 4 days (Fig. 8.8B) and 6 days (Fig. 8.8C).

However, co-treatment of dobutamine (10⁻⁹M)-treated cells with AR β₁ antagonist metoprolol (10⁻⁵ M) significantly increased the nitric oxide production by MDA MB-231 cells after 4 days and 6 days.
Fig. 8.6  \( \beta_1 \)-AR agonist and antagonist on VEGF C production of MDAMB-231 cell line: Although VEGF C production by dobutamine-treated MDA MB-231 cells was not altered after 2 days (A), there was a significant decrease after 4 days (B) and 6 days (C) which was not reversed by co-treatment with \( \beta_1 \)-AR antagonist metoprolol. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
**Fig. 8.7** β₁–AR agonist enhances nitric oxide production by MCF7 cell line: There was a significant increase in the nitric oxide production by MCF7 cell line after treatment with agonist for 2 days (A), 4 days (B) and 6 days (C) although it was not reversed by co-treatment with AR β₁ antagonist. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
Fig. 8.8  $\beta_1$–AR agonist did not alter nitric oxide production by MDA MB-231 cells. Although there was no significant effect of dobutamine treatment on nitric oxide production by MDA MB-231 cells after 2 days (A), 4 days (B) and 6 days (C), co-treatment with metoprolol enhanced it. #p<0.05 compared to agonist-treated group.
8.3.9 High dose of β₁-AR agonist suppresses p-ERK ½ expression by MCF-7 cells:

Treatment with AR β₁ agonist dobutamine (10⁻³ M) significantly decreased the p-ERK expression in MCF 7 cells after 2 days (Fig. 8.9A), 4 days (Fig. 8.9B) and 6 days (Fig. 8.9C) of incubation and the effect was not reversed by co-treatment with antagonist metoprolol. Interestingly treatment of MCF-7 cells with metoprolol alone also significantly decreased p-ERK expression after 2 days.

**Fig. 8.9** *In vitro treatment of β₁–AR agonist on p-ERK expression of MCF-7 cells.* There was a significant decrease in the p-ERK expression by MCF 7 cells treated with dobutamine after 2 days (A), 4 days (B) and 6 days (C) and the effect was not reversed by treatment with antagonist (Metoprolol).*p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
8.3.10 Lower doses of β₁-AR agonist decreases p-ERK expression in MDA MB-231 cells:

β₁-AR agonist dobutamine treatment significantly decreased the p-ERK expression in MDA MB-231 cells after 2 days (10⁻³ M, 10⁻⁶M and 10⁻⁹M; Fig. 8.10A), 4 days (10⁻⁶M and 10⁻⁹M) and 6 days (10⁻⁹M) of incubation and the effect was not reversed by treatment with antagonist metoprolol.

Interestingly, treatment of MDA MB-231 cells with metoprolol alone also significantly decreased p-ERK expression after 2 and 6 days.

8.3.11 β₁-AR agonists and antagonists decrease CREB expression by MCF-7 cells:

Treatment with AR β₁ agonist (10⁻³ M, 10⁻⁶M and 10⁻⁹ M) significantly decreased the p-CREB expression in MCF 7 cells after 2 days (Fig. 8.11A) of incubation and the effect was not reversed by treatment with antagonist (Metoprolol).

Upon prolonged incubation with the agonist, there was no significant effect on p-CREB expression.

8.3.12 Co-incubation with β₁–AR agonist and antagonist decreased CREB expression by MDA MB-231 cells:

There was no significant change in the CREB expression of dobutamine-treated ER (-) MDA MB-231 cells after 2 days (Fig. 8.12A), 4 days (Fig. 8.12B) and 6 days (Fig. 8.12C).

However, co-treatment of dobutamine (10⁻³ M, 10⁻⁶ M and 10⁻⁹ M)-treated cells with β₁–AR-specific antagonist metoprolol significantly decreased the p-CREB expression in MCF 7 cells after 2, 4 and 6 days.

8.3.13 β₁–AR agonist treatment enhanced p-Akt expression by MCF-7 cells:

β₁–AR agonist dobutamine (10⁻³ M, 10⁻⁶ M and 10⁻⁹ M) treatment significantly increased the p-AKT expression in ER (+) MCF 7 cells after 2 days (Fig. 8.13A), 4 days (Fig. 8.13B) and 6 days (Fig. 8.13C) of incubation and the effect was reversed by treatment with specific antagonist metoprolol after 4 and 6 days alone.
In vitro treatment of β₁ agonist on p-ERK expression of MDA MB-231 cells. Treatment with AR β₁ agonist dobutamine decreased p-ERK expression of MDA MB-231 cells after 2 days (A), 4 days (B) and 6 days (C) of incubation and the effect was not reversed by treatment with antagonist metoprolol. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
Fig. 8.11  In vitro treatment of β₁ agonist on p-CREB expression of MCF-7 cells. Treatment with AR β₁ agonist dobutamine decreased p-CREB expression of MCF-7 cells after 2 days (A). The effect was not reversed by co-treatment with antagonist metoprolol. Also, there was no significant effect after 4 days (B) and 6 days (C) of incubation. *p<0.05 compared to control.
Fig. 8.12  
In vitro treatment of β1 agonist on p-CREB expression of MDA MB-231 cells. Co-treatment with β1-AR agonist dobutamine significantly decreased p-CREB expression of MDA MB-231 cells after 2 days (A), 4 days (B) and 6 days (C). #p<0.05 compared to agonist-treated cells.
Fig. 8.13 *In vitro effects of β₁ agonist and antagonist on p-Akt expression of MCF-7 cells*:
There was a significant increase in p-AKT expression of dobutamine-treated cells after 2 (A), 4 (B) and 6 (C) days of incubation and the effect was reversed by co-incubation with the antagonist. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
8.3.14 β₁–AR agonist enhances p-Akt expression by MDA MB-231 cells:

Treatment with β₁–AR agonist dobutamine (10⁻³ M, 10⁻⁶ M and 10⁻⁹ M) significantly increased the p-AKT expression in MCF 7 cells after 2 days (Fig. 8.13A), 4 days (Fig. 8.13B) and 6 days (Fig. 8.13C) of incubation and the effect was reversed by treatment with antagonist (Metoprolol). Interestingly, treatment of MDA MB-231 cells with metoprolol alone also significantly enhance p-Akt expression after 2, 4 and 6 days.

Fig. 8.14  *In vitro effects of AR β₁ agonists and antagonists on p-AKT expression by MDA MB-231 cells:* p-AKT expression was significantly enhanced in dobutamine-treated MDA MB-231 cells after 2 (A), 4(B) and 6 (C) days. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
8.4 **Key Findings**

β1-AR agonist dobutamine did not affect proliferation of ER (+) MCF-7 cells in the presence or absence of β1-AR-specific antagonist metoprolol. However, there was a significant increase in the proliferation of MDA MB-231 cell line after dobutamine treatment which was reversed by co-treatment with AR β1 antagonist metoprolol. There was no significant effect of β1 AR agonist dobutamine on VEGF A and VEGF C production by ER (+) MCF-7 cells, while a significant decrease was observed in VEGF A and VEGF C production by ER (-) MDA MB-231 cells. Nitric oxide production by ER (+) MCF-7 cells alone was significantly increased by dobutamine treatment and reversed by co-incubation with metoprolol.

There was a significant decrease in the p-ERK expression by ER (+) MCF 7 cells and ER (-) MDA MB-231 cells treated with dobutamine and the effect was not reversed by treatment with antagonist (Metoprolol) in both cell lines. Treatment with AR β1 agonist dobutamine decreased p-CREB expression of ER (+) MCF-7 and ER (-) MDA MB-231 cells and the effect was not reversed by co-treatment with antagonist metoprolol. p-AKT expression of dobutamine-treated ER (+) MCF-7 cells and ER (-) MDA MB-231 cells was significantly enhanced and the effect was reversed by co-incubation with the antagonist in MCF-7 cells alone.

Taken together these results show that β1-AR agonist did not alter proliferation, VEGF A and VEGF C production in ER (+) MCF 7 cells although it decreased p-ERK and p-CREB expression and enhanced nitric oxide production and p-Akt expression alone. In ER (-) cells, β1-AR stimulation enhanced proliferation and p-Akt expression while down regulating p-ERK and p-CREB expression although it did not alter VEGF A and VEGF C production.