CHAPTER 9

ESTROGEN RECEPTOR STATUS OF BREAST CANCER CELL LINES DIFFERENTIALLY DETERMINES DOWNSTREAM SIGNALING MECHANISMS INVOLVED IN AR-β2MEDIATED PROLIFERATION

9.0 Specific Objective 2 Involvement of Cellular Mechanisms in β2-AR-induced effects on human breast cancer cells.

9.1 Rationale:

Sympathetic noradrenergic stimuli are transduced at the systemic and cellular level through multiple adrenergic receptor subtypes, predominant of which is the β2-adrenoceptor. In female-specific hormone-sensitive cancers like breast cancer, the effects of adrenergic stimulation and altered hormone sensitivity on cancer cell survival signals have not been investigated.

The present study investigates the role of β2-adrenoceptors (β2-AR) in modulating proliferation and expression of pro-angiogenic factors, and signaling molecules in ER (+) and ER (-) breast cancer cell lines. ER (+) cell line MCF-7 and ER (-) cell line MDA MB-231 were cultured in vitro and treated with various concentrations (10⁻³M to 10⁻⁹M) of β2-AR specific agonist, terbutaline and β-AR specific antagonist, propranolol (10⁻⁵M) to examine its effects on proliferation and expression of pro-angiogenic and signaling
molecules. Proliferation, VEGF C and NO production were enhanced in ER (+) MCF-7 cells and decreased in ER (-) MDA MB-231 cells treated with β2- AR agonist. VEGF A was decreased and NO was enhanced in both ER (+) MCF-7 and ER (-) MDA MB-231 cells on β2- AR stimulation. β2-AR agonist enhanced the expression of p-ERK, p-CREB and p-Akt in ER (+) MCF-7 cells; decreased p-ERK expression and enhanced p-CREB and p-Akt expression in MDA MB-231 cells. Differential regulation of β2-AR agonist may involve cross-talk with the estrogen receptors involving p-ERK signaling pathways.

9.2 Methods:

9.2.1 Culture:

9.2.1.1 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.

9.2.1.2 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 tripinsonised 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.)

9.2.2 Treatment

9.2.2.1 Experiment 1 MCF-7 and MDA MB-231 cells were incubated with 10⁻³ M, 10⁻⁶ M and 10⁻⁹ M terbutaline (β2–AR agonist) with and without 10⁻⁵ M propranolol (β-AR-specific antagonist) for 2, 4 and 6 days. Pellets and supernatants were collected after 2, 4 and 6 days and frozen at -80°C for further assays.
9.2.2.2 Experiment 2 Cells were incubated with $10^{-3}$ M, $10^{-6}$ M and $10^{-9}$ M terbutaline ($\beta_2$-AR agonist) with and without 10 $\mu$M PKA and PKC inhibitor, H89, or 5 $\mu$M ERK inhibitor, PD98059, or 10$\mu$MAkt inhibitor A6730 for 2, 4 and 6 days. Pellets and supernatants were collected after 2, 4 and 6 days and frozen at -80° C for further assays.

9.3 Results

9.3.1 Effects of $\beta_2$ adrenergic agonist/antagonist on MCF 7 cell line:

There was a significant increase in the proliferation of MCF 7 cells after treatment with AR $\beta_2$ agonist terbutaline for 2 days ($10^{-3}$ M; Fig. 9.1A), 4 days ($10^{-6}$M and $10^{-3}$M; Fig. 9.1B) and 6 days ($10^{-6}$M and $10^{-3}$M; Fig. 9.1C). Co-treatment with AR $\beta_2$ antagonist Propranolol significantly decreased the proliferation of MCF 7 cells after 2 days($10^{-6}$M), 4 days ($10^{-6}$M and $10^{-3}$M) and 6 days ($10^{-6}$M and $10^{-3}$M).

9.3.2 Effects of $\beta_2$ adrenergic agonist/antagonist on MDA MB-231 cell line:

The proliferation of MDA MB-231 cells was not altered after treatment with AR $\beta_2$ agonist terbutaline for 2 days (Fig. 9.2A). Prolonged incubation with terbutaline however significantly decreased proliferation of ER (-) MDA MB-231 cells after 4 ($10^{-9}$ M and $10^{-6}$ M; Fig. 9.2B) and 6 ($10^{-9}$ M and $10^{-6}$ M; Fig. 9.2C) days. Co-treatment with AR $\beta_2$ antagonist Propranolol significantly enhanced the proliferation of MDA MB-231 cells after 2 ($10^{-9}$ M) and 6 ($10^{-9}$ M and $10^{-6}$ M) days.

9.3.3 Effects of AR $\beta_2$ agonists and antagonists on VEGF A production by MCF 7 cells:

Treatment with AR $\beta_2$ agonist terbutaline did not significantly affect VEGF A production by ER (+) MCF-7 cells after 2 days (Fig. 9.3A) and 4 days (Fig. 9.3B) although there was a significant decrease after 6 days (Fig. 9.3C) which was not reversed by co-incubation with the antagonist. Interestingly, there was a significant decrease in the production of VEGF A by MCF 7 cells after treatment with AR $\beta_2$ antagonist propranolol ($10^{-5}$M) for 4 and 6 days.
Fig. 9.1  

*In vitro* effects of β2-AR on proliferation of ER (+) MCF-7 cells.

Proliferation of MCF-7 cells was significantly enhanced after 2 days (A), 4 days (B) and 6 days (C) of treatment with terbutaline. Co-incubation with propranolol significantly decreased proliferation after 2, 4 and 6 days. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
Fig. 9.2  *In vitro* effects of β2-AR on proliferation of ER (-) MDA MB-231 cells. Terbutaline did not alter proliferation of MDA MB-231 cells after 2 days (A) although prolonged treatment significantly decreased proliferation after 4 days (B) and 6 days (C). Co-incubation of terbutaline-treated cells with propranolol enhanced proliferation after 2 and 6 days. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
Fig. 9.3  *In vitro* effects of β2-AR on VEGF A production by ER (+) MCF-7 cells. VEGF A production by MCF-7 cells was not altered after 2 (A) and 4 (B) days and significantly decreased after 6 days (C) of treatment with terbutaline. Co-incubation with propranolol alone also decreased VEGF A production after 4 and 6 days. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
9.3.4 Effects of AR β₂ agonists and antagonists on VEGF A production by MDA MB-231 cells: AR β₂ agonist terbutaline significantly decreased the production of VEGF A by ER (-) MDA MB-231 cells after 2 (10⁻⁹ M; Fig. 9.4A), 4 (10⁻³ M; Fig. 9.4B) and 6 days (10⁻⁹ M, 10⁻⁶ M and 10⁻⁹ M; Fig. 9.4C) of incubation. Co-treatment with AR β antagonist propranolol significantly increased the production of VEGF A by MDA MB-231 cells after 4 days (10⁻³ M) and 6 days (10⁻⁹ M).

**Fig. 9.4** *In vitro* effects of β₂-AR on VEGF A production by ER (-) MDA MB-231 cells. Terbutaline did not alter proliferation of MDA MB-231 cells after 2 days (A) although prolonged treatment significantly decreased proliferation after 4 days (B) and 6 days (C). Co-incubation of terbutaline-treated cells with propranolol enhanced proliferation after 2 and 6 days. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
9.3.5 Effects of AR β2 agonists and antagonists on VEGF C production by MCF 7 cells:

There was no significant effect of AR β2 agonist terbutaline treatment on VEGF C production by MCF 7 cell line after 2 days (Fig. 9.5A), although there was a significant increase after 4 days ($10^3$M; Fig. 9.5B) and 6 days ($10^9$M, $10^6$M, $10^3$M; Fig. 9.5C) of incubation were studied. There was a significant decrease in the VEGF C production by MCF 7 cells after treatment with AR β2 agonist ($10^3$M) for 4 days and the effect was reversed by the antagonist. Co-treatment with AR β2 antagonist Propranolol significantly increased the VEGF C production by MCF 7 cells after 4 days ($10^3$M) and 6 days ($10^3$M).
**Fig. 9.5 In vitro effects of β2-AR on VEGF C production by ER (+) MCF-7 cells.** VEGF C production by MCF-7 cells was unaltered after 2 days (A), but significantly enhanced after 4 days (B) and 6 days (C) of treatment with terbutaline. Co-incubation with propranolol significantly decreased VEGF C production 4 and 6 days.

*p<0.05 compared to control.  #p<0.05 compared to agonist-treated group.

### 9.3.6 AR β2 agonists and antagonists on VEGF C production by MDA MB-231 cells:

Treatment with AR β2 agonist terbutaline significantly decreased the production of VEGF C by MDA MB-231 cell line after 2 days (10^{-9} M, 10^{-6} M, 10^{-3} M; Fig. 9.6A), 4 days (10^{-9} M, 10^{-6} M, 10^{-3} M; Fig. 9.6B) and 6 days (10^{-9} M, 10^{-6} M, 10^{-3} M; Fig. 9.6C) of incubation were studied. The effects were reversed by co-treatment with the antagonist after 2 (10^{-3} M), 4 (10^{-3} M) and 6 (10^{-6} M, 10^{-3} M) days. Interestingly, treatment of MDA MB-231 cells with propranolol alone also significantly decreased VEGF C expression after 2 days.

### 9.3.7 Effects of AR β2 agonists and antagonists on nitric oxide production by MCF-7 cells:

There was a significant increase in the nitric oxide production by MCF 7 cells after treatment with AR β2 agonist for 2 (10^{-9} M, 10^{-6} M, 10^{-3} M; Fig. 9.7A) and 4 (10^{-6} M; Fig. 9.7B) days. However, treatment with AR β2 antagonist propranolol significantly decreased the nitric oxide production by MCF 7 cells after 2 days alone (10^{-9} M, 10^{-6} M, 10^{-3} M).

### 9.3.8 Effects of AR β2 agonists and antagonists on nitric oxide production by MDA MB-231 cells:

AR β2 agonist terbutaline significantly enhanced nitric oxide production by MDA MB-231 cell line after 2 days (10^{-9} M, 10^{-6} M, 10^{-3} M; Fig. 9.8A), 4 days (10^{-9} M, 10^{-6} M, 10^{-3} M; Fig. 9.8B) and 6 days (10^{-9} M; Fig. 9.8C) of incubation. Co-treatment with AR β2 agonist significantly decreased nitric oxide production after 2 days (10^{-9} M, 10^{-6} M and 10^{-3} M), 4 days (10^{-9} M, 10^{-6} M) and 6 days (10^{-9} M).
Fig. 9.6  *In vitro* effects of β2-AR on VEGF C expression of ER (-) MDA MB-231 cells. VEGF C production by MDA MB-231 cells was significantly decreased after 2 days (A), 4 days (B) and 6 days (C) of treatment with terbutaline and reversed on co-treatment with propranolol. *p<0.05 compared to control.  #p<0.05 compared to agonist-treated group.
**Fig. 9.7**  *In vitro* effects of β2-AR on nitric oxide production by ER (+) MCF-7 cells. Nitric oxide production by MCF-7 cells was significantly enhanced after 2 (A) and 4 days (B) and remained unaltered after 6 days (C) of treatment with terbutaline. Co-incubation with propranolol significantly decreased nitric oxide production after 2 days alone. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
Fig. 9.8 In vitro effects of β2-AR on nitric oxide production by ER (-) MDA MB-231 cells. Nitric oxide production by MCF-7 cells was significantly enhanced after 2 days (A), 4 days (B) and 6 days (C) of treatment with terbutaline. Co-incubation with propranolol significantly decreased nitric oxide production by MDA MB-231 cells after 2, 4 and 6 days. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
9.3.9 Effects of AR β<sub>2</sub> agonists and antagonists on p-ERK ½ expression by MCF-7 cells:

Treatment with AR β<sub>2</sub> agonist terbutaline significantly enhanced p-ERK expression after 2 days (10<sup>-3</sup> M; Fig. 9.9A), 4 days (10<sup>-3</sup> M, 10<sup>-6</sup> M and 10<sup>-9</sup> M; Fig. 9.9B) and 6 days (10<sup>-9</sup> M, 10<sup>-6</sup> M; Fig. 9.9C). Co-incubation with AR β-specific antagonist propranolol significantly decreased p-ERK expression after 2 days (10<sup>-3</sup> M), 4 days (10<sup>-9</sup> M) and 6 days (10<sup>-9</sup> M, 10<sup>-6</sup> M).

9.3.10 Effects of AR β<sub>2</sub> agonists and antagonists on p-ERK ½ expression by MDA MB-231 cells:

There was no significant change in the p-ERK expression of MDAMB-231 cells after 2 days of treatment with terbutaline (Fig. 9.10A). Prolonged incubation with terbutaline however significantly decreased p-ERK expression after 4 days(10<sup>-9</sup> M, 10<sup>-6</sup> M; Fig. 9.10B) and 6 days (10<sup>-3</sup> M; Fig. 9.10C). Co-treatment with propranolol significantly reversed the agonist-mediated decrease after 6 days (10<sup>-6</sup> M, 10<sup>-3</sup> M).

9.3.11 Effects of AR β<sub>2</sub> agonists and antagonists on CREB expression by MCF-7 cells:

The AR β<sub>2</sub> agonist terbutaline significantly enhanced p-CREB expression inER (+) MCF 7 cell line after 2 days (10<sup>-9</sup> M; Fig. 9.11A), 4 days (10<sup>-9</sup> M, 10<sup>-6</sup> M, 10<sup>-3</sup> M; Fig. 9.11B) and 6 days (10<sup>-9</sup> M, 10<sup>-6</sup> M, 10<sup>-3</sup> M; Fig. 9.11C). Co-incubation of terbutaline-treated cells with propranolol significantly decreased p-CREB expression after 2 days (10<sup>-9</sup> M), 4 days (10<sup>-6</sup> M, 10<sup>-3</sup> M) and 6 days (10<sup>-9</sup> M, 10<sup>-6</sup> M, 10<sup>-3</sup> M).

9.3.12 Effects of AR β<sub>2</sub> agonists and antagonists on CREB expression by MDA MB-231 cells:

There was a significant increase in the p-CREB expression of terbutaline-treated MDA MB-231 cells after 2 days (10<sup>-9</sup> M, 10<sup>-6</sup> M, 10<sup>-3</sup> M; Fig. 9.12A), 4 days (10<sup>-9</sup> M, 10<sup>-6</sup> M, 10<sup>-3</sup> M; Fig. 9.12B) and 6 days (10<sup>-9</sup> M, 10<sup>-6</sup> M, 10<sup>-3</sup> M; Fig. 9.12C). Prolonged incubation with terbutaline for 6 days did not alter p-CREB expression (Fig. 9.12C). Co-treatment with AR βantagonist propranolol significantly decreased p-CREB expression after 2 days (10<sup>-6</sup> M, 10<sup>-3</sup> M) and 4 days (10<sup>-9</sup> M, 10<sup>-6</sup> M).
**Fig. 9.9** *In vitro* effects of β2-AR on p-ERK expression of ER (+) MCF-7 cells. p-ERK expression of MCF-7 cells was significantly enhanced after 2 days (A), 4 days (B) and 6 days (C) of treatment with terbutaline. Co-incubation with propranolol significantly decreased p-ERK expression after 2, 4 and 6 days. *p*<0.05 compared to control. #p<0.05 compared to agonist-treated group.
**Fig. 9.10**  *In vitro* effects of β2-AR on p-ERK expression of ER (-) MDA MB-231 cells. p-ERK expression of MDA MB-231 cells was significantly decreased after 2 days (A), 4 days (B) and 6 days (C) of treatment with terbutaline. Co-incubation with propranolol significantly increased p-ERK expression after 6 days. *p<0.05 compared to control.  #p<0.05 compared to agonist-treated group.
Fig. 9.11  *In vitro* effects of β2-AR on p-CREB expression of ER (+) MCF-7 cells. p-CREB expression of MCF-7 cells was significantly enhanced after 2 days (A), 4 days (B) and 6 days (C) of treatment with terbutaline. Co-incubation with propranolol significantly decreased p-CREB expression after 2, 4 and 6 days. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
Fig. 9.12  *In vitro* effects of β2-AR on p-CREB expression of ER (-) MDA MB-231 cells. p-CREB expression of MDA MB-231 cells was significantly enhanced after 2 days (A) and 4 days (B) and unaltered after 6 days (C) of treatment with terbutaline. Co-incubation with propranolol significantly decreased p-CREB expression after 2 and 4 days. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
9.3.13 Effects of AR β₂ agonists and antagonists on AKT expression by MCF-7 cells:

There was no significant change in p-Akt expression of terbutaline-treated MCF-7 cells after 2 days (Fig. 9.13A). However, after 4 days there was a significant increase in p-Akt expression by terbutaline (10⁻⁹ M, 10⁻⁶ M, 10⁻³ M)-treated MCF-7 cells which was reversed upon co-treatment with the β-AR antagonist propranolol; Fig. 9.13B). Prolonged incubation with terbutaline for 6 days did not alter p-Akt expression (Fig. 9.13C) although co-treatment with propranolol significantly decreased p-Akt expression (10⁻³ M) after 6 days.

9.3.14 Effects of AR β₂ agonists and antagonists on AKT expression by MDA MB-231 cells:

There was a significant increase in p-Akt expression of terbutaline-treated MDA MB-231 cells after 2 days (10⁻⁶ M, 10⁻³ M; Fig. 9.14A) and 4 days (10⁻⁶ M, 10⁻³ M; Fig. 9.14B). Prolonged incubation with terbutaline for 6 days did not alter p-Akt expression (Fig. 9.14C). Co-treatment with propranolol significantly decreased p-Akt expression after 2 and 4 days (10⁻⁶ M, 10⁻³ M).

9.3.15 Inhibition of PKA, PKC, Akt and ERK using specific inhibitors reverses terbutaline mediated MCF 7 proliferation

Proliferation of MCF-7 cells was significantly increased by AR β₂-agonist terbutaline after 2 days (10⁻³ M; Fig. 9.15A), 4 days (10⁻⁶ M, 10⁻³ M; Fig. 9.15B) and 6 days (10⁻⁶ M, 10⁻³ M; Fig. 9.15C).

Co-treatment with PKA and PKC inhibitor H89, and the ERK inhibitor PD98059 significantly reversed terbutaline-mediated increase after 2 days (10⁻³ M), 4 days (10⁻⁹ M, 10⁻⁶ M, 10⁻³ M); and 6 days (10⁻⁹ M, 10⁻⁶ M, 10⁻³ M).

Co-incubation with Akt inhibitor A6730 also significantly reversed the terbutaline-mediated increase in MCF-7 cell proliferation after 4 days and 6 days (10⁻⁹ M, 10⁻⁶ M, 10⁻³ M).
**Fig. 9.13**  *In vitro* effects of β2-AR on proliferation of ER (+) MCF-7 cells. p-Akt expression by MCF-7 cells was not altered after 2 days (A) and 6 days (C) of treatment with terbutaline while there was a significant increase after 4 days (B). Co-incubation with propranolol significantly decreased p-Akt expression after 4 and 6 days. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
Fig. 9.14  

*In vitro* effects of β2-AR on p-Akt expression of ER (-) MDA MB-231 cells.

The expression of p-Akt was significantly enhanced in MDAMB-231 cells treated with terbutaline after 2 days (A) and 4 days (B) although there was no change after 6 days (C). Co-incubation with propranolol significantly decreased p-Akt expression after 2 and 4 days. *p<0.05 compared to control.  #p<0.05 compared to agonist-treated group.
A

B

C

Proliferation of MCF-7 cells

(-) Inhibitor  (+) 5 µM H89  (+) 10 µM PD98059  (+) 10 µM A6730

Proliferation of MCF7 cells

(-) Inhibitors  (+) 5 µM H89  (+) 10 µM PD98059  (+) 10 µM A6730

Proliferation of MCF 7 cells

(-) Inhibitors  (+) 5 µM H89  (+) 10 µM PD98059  (+) 10 µM A6730

Control
Ter 10⁻⁹ M
Ter 10⁻⁶ M
Ter 10⁻³ M
Fig. 9.15 In vitro effects of pathway inhibitors on β2-AR mediated proliferation of ER (+) MCF-7 cells. Proliferation of MCF-7 cells was significantly enhanced after 2 days (A), 4 days (B) and 6 days (C) of treatment with terbutaline. PKA/PKC inhibition with H89 or ERK inhibition using PD98059 or Akt inhibition with A6730 significantly reversed the increase in proliferation after 2, 4 and 6 days. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.

9.3.16 Inhibition of PKA, PKC, Akt and ERK using specific inhibitors reverses terbutaline mediated MDA MB-231 proliferation

Proliferation of MDA MB-231 cells was significantly decreased by AR β2-agonist terbutaline after 4 days ($10^{-9}$ M, $10^{-6}$ M; Fig. 9.16B) and 6 days ($10^{-9}$ M; Fig. 9.16B) of treatment. Co-treatment with PKA and PKC inhibitor H89, ERK inhibitor PD98059 and the Akt inhibitor A6730 did not reverse the terbutaline-mediated decrease in MDA MB-231 cell proliferation.

9.3.17 Inhibition of PKA, PKC, Akt and ERK using specific inhibitors reverses terbutaline mediated VEGF C production by MCF-7 cells

VEGF C production was significantly enhanced by terbutaline in MCF-7 cells after 4 days ($10^{-3}$ M; Fig. 9.17B) and 6 days ($10^{-9}$ M, $10^{-6}$ M, $10^{-3}$ M; Fig. 9.17C) of incubation. PKA and PKC blockade using H89 ($10^{-9}$ M, $10^{-6}$ M, $10^{-3}$ M), ERK blockade using PD98059 ($10^{-9}$ M, $10^{-6}$ M, $10^{-3}$ M) and Akt blockade using A6730 ($10^{-9}$ M, $10^{-6}$ M, $10^{-3}$ M) significantly reversed the terbutaline-mediated increase after prolonged incubation for 6 days.
Proliferation of MDA MB-231 cells

A

B

C

(-) Inhibitor  (+) 5 µM H89  (+) 10 µM PD98059  (+) 10 µM A6730

Control  Ter 10^{-9} M  Ter 10^{-6} M  Ter 10^{-3} M

Control  Ter 10^{-9} M  Ter 10^{-6} M  Ter 10^{-3} M

Control  Ter 10^{-9} M  Ter 10^{-6} M  Ter 10^{-3} M
Fig. 9.16  *In vitro* effects of PKA/ PKC, ERK or Akt inhibition using specific inhibitors β2-AR on proliferation of ER (-) MDA MB-231 cells. There was a significant decrease in the proliferation of MDA MB-231 cells after 4 days (B) and 6 days (C) of treatment. PKA and PKC blockade using H89, ERK blockade using PD98059 and Akt inhibition using A6730 did not reverse the agonist-mediated decrease. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.
Fig. 9.17  *In vitro* effects of PKA/ PKC, ERK or Akt inhibition using specific inhibitors β2-AR on VEGF C expression by ER (+) MCF-7 cells.

There was a significant increase in VEGF C production by terbutaline-treated MCF-7 cells after 4 and 6 days of incubation which was reversed by prolonged incubation with PKA and PKC inhibitor H89, ERK inhibitor PD98059 and Akt inhibitor A6730 for 6 days. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.

9.3.18 Inhibition of PKA, PKC, Akt and ERK using specific inhibitors enhances VEGF C expression by MDA MB-231 cells

VEGF C production was significantly decreased by terbutaline in MDA MB-231 cells after 2 days (10⁻⁹ M, 10⁻⁶ M, 10⁻³ M; Fig. 9.18A), 4 days (10⁻⁹ M, 10⁻⁶ M, 10⁻³ M; Fig. 9.18B) and 6 days (10⁻⁹ M, 10⁻⁶ M, 10⁻³ M; Fig. 9.18C) of incubation.

Akt blockade using A6730 alone significantly reversed the terbutaline-mediated increase after 2 days (10⁻³ M), 4 days (10⁻³ M) and 6 days (10⁻⁶ M, 10⁻³ M). Prolonged incubation with PKA and PKC inhibitor, H89 (10⁻⁶ M, 10⁻³ M) and ERK inhibitor PD98059 (10⁻⁹ M, 10⁻⁶ M) significantly reversed the terbutaline-mediated increase after prolonged incubation for 6 days.
Figure A

VEGF C production (ng/ml) for MDA MB-231 cells in the absence and presence of (-) and (+) various concentrations of inhibitors: 10 µM H89, 5 µM PD98059, and 10 µM A6730. The bars with # indicate statistically significant differences compared to the control group.

Figure B

VEGF C production (ng/ml) for another cell line with similar treatment conditions as in Figure A. The bars with # indicate statistically significant differences compared to the control group.

Figure C

VEGF C production (ng/ml) for the third cell line with the same treatment conditions as in Figures A and B. The bars with # indicate statistically significant differences compared to the control group.
9.18 *In vitro* effects of PKA/ PKC, ERK or Akt inhibition using specific inhibitors β2-AR on VEGF C expression by ER (-) MDA MB-231 cells.

There was a significant decrease in VEGF C production by terbutaline-treated MDA MB-231 cells after 4 and 6 days of incubation which was reversed by incubation with A6730 alone. Prolonged incubation with PKA and PKC inhibitor H89, ERK inhibitor PD98059 and Akt inhibitor A6730 for 6 days also reversed the agonist-mediated decline. *p<0.05 compared to control. #p<0.05 compared to agonist-treated group.

9.4 Key Findings

Proliferation of ER (+) MCF-7 cells was significantly increased by treatment with β2-AR agonist terbutaline and co-treatment with propranolol significantly reversed the terbutaline mediated increase. On the other hand ER (-) MDA MB-231 cells showed significantly decreased proliferation when treated with terbutaline which was reversed by co-incubation with propranolol. VEGF C expression was significantly enhanced by terbutaline in ER(+) MCF-7 cells while VEGF C expression by ER (-) MDA MB-231 cells was significantly decreased by terbutaline Terbutaline enhanced NO and VEGF C expression by both ER (+) MCF-7 and ER(-) MDA MB-231 cells.

p-ERK expression of ER (+) MCF-7 cells was significantly enhanced after 2 days, 4 days and 6 days of treatment with terbutaline and co-incubation with propranolol significantly decreased p-ERK expression after 2, 4 and 6 days. p-ERK expression of ER (-) MDA MB-231 cells was significantly decreased after 2 days, 4 days and 6 days of treatment with terbutaline. Co-incubation with propranolol significantly increased p-ERK expression after 6 days. Terbutaline enhanced p-CREB and p-Akt expression by both MCF-7 and MDA MB-231 cells.

Inhibition of PKA, PKC, Akt and ERK using specific inhibitors reverses terbutaline mediated ER (+) MCF 7 proliferation. On the other hand there was no significant effect of inhibition of PKA/PKC, Akt and ERK using specific inhibitors on the proliferation of ER (-) MDA MB-231 cells. Inhibition of PKA, PKC, Akt and ERK using specific inhibitors reverses the terbutaline mediated increase in VEGF C production by
MCF-7 cells. In ER (-) MDA MB-231 cells, inhibition of PKA, PKC, Akt and ERK using specific inhibitors enhances VEGF C, thereby reversing the terbutaline-mediated suppression.

Taken together, proliferation and pro-angiogenic signals (VEGF C), signaling molecule expression (p-ERK) are differentially modulated by β2-AR agonists in ER (+) MCF-7 and ER (-) MDA MB-231 breast cancer cell lines. Further, selective blockade of β-AR signaling using propranolol reverses the agonist-mediated effects in ER (+) MCF-7 and ER (-) MDA MB-231 breast cancer cell lines. Thus, differential regulation of β2-AR agonists may involve cross-talk with estrogen receptors involving p-ERK signaling pathway.