

Differential Roles of MAPK-Erk1/2 and MAPK-p38 in Insulin or Insulin-Like Growth Factor-I (IGF-I) Signaling Pathways for Progesterone Production in Human Ovarian Cells

Authors

D. Seto-Young¹, D. Avtanski¹, M. Varadinova¹, A. Park², P. Suwandhi¹, A. Leiser², G. Parikh¹, L. Poretsky¹

Affiliations

¹ Division of Endocrinology, Department of Medicine, Beth Israel Medical Center and Albert Einstein College of Medicine, New York, USA

² Department of Obstetrics and Gynecology, Beth Israel Medical Center and Albert Einstein College of Medicine, New York, USA

Key words

- insulin
- IGF-1
- steroid hormones
- mitogen activated protein kinase
- progesterone synthesis
- polycystic ovary syndrome

Abstract

▼ Insulin and insulin like-growth factor-I (IGF-I) participate in the regulation of ovarian steroidogenesis. In insulin resistant states ovaries remain sensitive to insulin because insulin can activate alternative signaling pathways, such as phosphatidylinositol-3-kinase (PI-3 kinase) and mitogen-activated protein-kinase (MAPK) pathways, as well as insulin receptors and type 1 IGF receptors. We investigated the roles of MAPK-Erk1/2 and MAPK-p38 in insulin and IGF-I signaling pathways for progesterone production in human ovarian cells. Human ovarian cells were cultured in tissue culture medium in the presence of varying concentrations of insulin or IGF-I, with or without PD98059, a specific MAPK-Erk1/2 inhibitor, with or without SB203580, a specific MAPK-p38 inhibitor or with or without a specific PI-3-kinase inhibitor LY294002. Progesterone concentrations were measured using radioimmunoassay. PD98059 alone stimulated

progesterone production in a dose-dependent manner by up to 65% ($p < 0.001$). Similarly, LY294002 alone stimulated progesterone production by 13–18% ($p < 0.005$). However, when used together, PD98059 and LY294002 inhibited progesterone production by 17–20% ($p < 0.001$). SB203580 alone inhibited progesterone production by 20–30% ($p < 0.001$). Insulin or IGF-I alone stimulated progesterone production by 40–60% ($p < 0.001$). In insulin studies, PD98059 had no significant effect on progesterone synthesis while SB203580 abolished insulin-induced progesterone production. Either PD98059 or SB203580 abolished IGF-I-induced progesterone production. Both MAPK-Erk1/2 and MAPK-p38 participate in IGF-I-induced signaling pathways for progesterone production, while insulin-induced progesterone production requires MAPK-p38, but not MAPK-Erk1/2. These studies provide further evidence for divergence of insulin and IGF-I signaling pathways for human ovarian cell steroidogenesis.

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Bibliography

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Correspondence

D. Seto-Young, PhD

L. Poretsky, MD

Division of Endocrinology

Beth Israel Medical Center

317 East 17th Street

Fierman Hall, 7th Floor

New York

NY 10003

USA

Tel.: +1/212/420 4666

Fax: +1/212/420 2224

dyoung@chpnet.org

lporetsk@chpnet.org

Introduction

▼ Insulin and insulin like-growth factor-I (IGF-I) participate in the regulation of ovarian function and steroidogenesis. In patients with hyperinsulinemic insulin resistant states, ovarian steroidogenesis can be stimulated by insulin. This preserved ovarian sensitivity to insulin in the face of insulin resistance in classical insulin target tissues (liver, muscle, and adipose) can be explained by the following phenomena: first, insulin can bind to and upregulate ovarian IGF-I receptors [1–3]; and second, insulin can activate PI-3 kinase independent insulin signaling pathways which are not related to glucose transport [1]. Studies of progesterone synthesis in human granulosa-lutein cells and thecal cells suggested that cAMP-dependent activation of MAPK-Erk1/2 by forskolin/LH increases progesterone produc-

tion and steroid acute regulatory protein (StAR) expression. Further, protein kinase-A inhibitor (H89) significantly reduced these effects. However, in the presence of a potent MAPK-Erk1/2 inhibitor, PD98059, LH-induced progesterone production and stimulation of StAR mRNA expression are preserved [4–6]. Thus, the requirement for MAPK-Erk1/2 activation in regulation of progesterone production in the ovary is stimulus-specific.

There are limited data on the role of MAPK-p38 in progesterone production. In one study, SB203580 at a concentration of 10 μ M had no significant effect on progesterone production in human granulosa cells [7]. In another study, SB203580 stimulated FSH-induced progesterone production and StAR mRNA expression, but inhibited estradiol production and aromatase mRNA expression [8].

The role of MAPK-Erk1/2 and MAPK-p38 in insulin or IGF-I signaling pathways for progesterone production in human ovarian cells is not understood. In this report, we used a mixed ovarian cell culture containing granulosa, theca and stromal cells to examine the role of MAPK in progesterone production using 2 different MAPK inhibitors: PD98059, a specific inhibitor of MAPK-Erk1/2, and SB203580, a specific inhibitor of MAPK-p38.

Materials and Methods

All studies described in this report were approved by the Institutional Review Board at Beth Israel Medical Center.

Ovarian cell culture

Ovarian tissue samples were collected from 52 women at the time of indicated oophorectomy. All patients were premenopausal or perimenopausal and their age ranged from 22–50 years. No patients were using hormonal therapy at the time of oophorectomy. Indications for oophorectomy included follicular cyst, inclusion cyst, endometrial cyst, hemorrhagic corpus luteum cyst, teratoma, adenofibroma, tubal-ovarian adhesions, epithelial inclusions with focal mucinous metaplasia associated with calcifications, and normal ovaries with abnormalities in the uterus.

After pathological examination, ovarian tissue was dissected so that only unaffected tissue was used to establish the mixed ovarian cell culture as described by Seto-Young et al. [9–11]. This culture contains granulosa, thecal, and stromal cells, and is responsive to stimulation by gonadotropins, insulin, and IGF-I [9–11]. Cells were incubated in tissue culture medium M199 supplemented with 2% FBS [9–11] with or without 10, 10², 10³ or 10⁴ ng/ml insulin or 1, 2.5, 5, or 10 ng/ml IGF-I, with or without 25–50 μM PD98059, with or without 2.5–5 μM LY294002 and with or without 10–25 μM SB203580. For the studies of IGF-I-induced progesterone production, cells were preincubated with 10 ng/ml of insulin for 24 h in M199 tissue culture medium supplemented with 2% FBS to induce type I IGF receptors [12]. The cells were then incubated with various concentrations of IGF-I for additional 24 h [12].

Cell viability

Ovarian cells were incubated with or without 1–50 μM PD98059 or 1–25 μM SB203580. The treated cells were trypsinized, centrifuged, resuspended in tissue culture medium and counted in the hemocytometer using trypan blue method. PD98059 or SB203580 had no significant effect on cell viability.

Progesterone production

Progesterone concentration in the conditioned tissue culture medium was measured using radioimmunoassay (RIA). The data were normalized per 0.5 × 10⁴ cells/ml.

Assessment of MAPK-Erk1/2 expression

Approximately 1 × 10⁶ cells were cultured in 100 mm Falcon tissue culture dishes under the cell culture conditions previously described by Seto-Young et al. [12]. Cells were incubated with 0, 10, 10², or 10³ ng/ml insulin or 0, 1, 2.5 or 5 ng/ml IGF-I, and in the presence or absence of PD98059 (25–50 μM) or SB203580 (10–25 μM). The immunoprecipitation and immunoblot procedures were performed as previously described [12]. PD98059 or SB203580 were included in all steps because both reagents are

reversible MAPK inhibitors. Rabbit/mouse anti-phospho-MAPK-Erk1/2 and anti-phospho-p38-T180-Y182-MAPK antibodies were used for probing the MAPK-Erk1/2 and MAPK-p38 expression.

Materials

All cell culture supplies, Medium M199, and heat inactivated FBS were obtained from Invitrogen Corporation (Grand Island, NY, USA); PD98059, LY294002 and SB203580 were from EMD Chemicals (Gibbstown, NJ, USA); rabbit/mouse anti-phospho-MAPK-Erk1/2 antibodies, and goat anti-rabbit/mouse IgG conjugated with horseradish peroxidase were from Upstate (Lake Placid, NY, USA); mouse anti-phospho-p38-Y180T182-MAPK antibodies were from Abcam Inc. (Cambridge, MA, USA); Protein A Sepharose-tm-6MB and Chemiluminescence were from Pierce (Rockford, IL, USA); and progesterone RIA was from Diagnostic Systems Laboratories, Inc. (Webster, TX, USA).

Statistical analysis

2-way analysis of variance (ANOVA) was used to compare mean values according to insulin or IGF-I concentrations in the presence or absence of PD98059, LY294002, or SB203580. The statistical interactions between the sets of data obtained with or without PD98059, LY294002, or SB203580 were examined. Pairwise Bonferroni-adjusted contrasts were analyzed to determine statistical significance. Analysis of covariance (ANCOVA) was used to assess statistical significance of mean differences among the sets of data according to insulin or IGF-I concentrations in the presence or absence of PD98059 or SB203580. Adjustments were made for initial inhibition or stimulation of progesterone production induced by the MAPK inhibitors in the absence of insulin or IGF-I.

Results

PD98059 effects on phospho-MAPK-Erk1/2 activity and progesterone production (○ Fig. 1)

PD98059 completely inhibited both insulin-induced and IGF-I-induced phospho-MAPK-Erk1/2 activity (○ Fig. 1a, b).

A. Insulin experiments

PD98059 alone independently stimulated progesterone production in a dose-dependent manner by up to 65% ($p < 0.001$) (○ Fig. 1c). Insulin alone stimulated progesterone production in a dose-dependent manner by 50% ($p < 0.001$) (○ Fig. 1d). In the presence of 25 μM of PD98059, insulin-induced progesterone production was stimulated by 80% ($p < 0.001$) (○ Fig. 1d) and in the presence of 50 μM PD98059, it was stimulated by 100% ($p < 0.001$). However, the effect of PD98059 on insulin-induced stimulation of progesterone production was not significant when the adjustments were made for initial stimulation of progesterone production induced by PD98059 alone (○ Fig. 1d).

B. IGF-I experiments

PD98059 stimulated progesterone production by 110% ($p < 0.001$). This excessive stimulation of progesterone production by PD98059 was attributed to the preincubation with insulin to induce type 1 IGF-I receptors, as described under the section 'Materials and Methods'. IGF-I alone stimulated progesterone production by 60% ($p < 0.001$) (○ Fig. 1e). In the presence of PD98059 (25–50 μM),

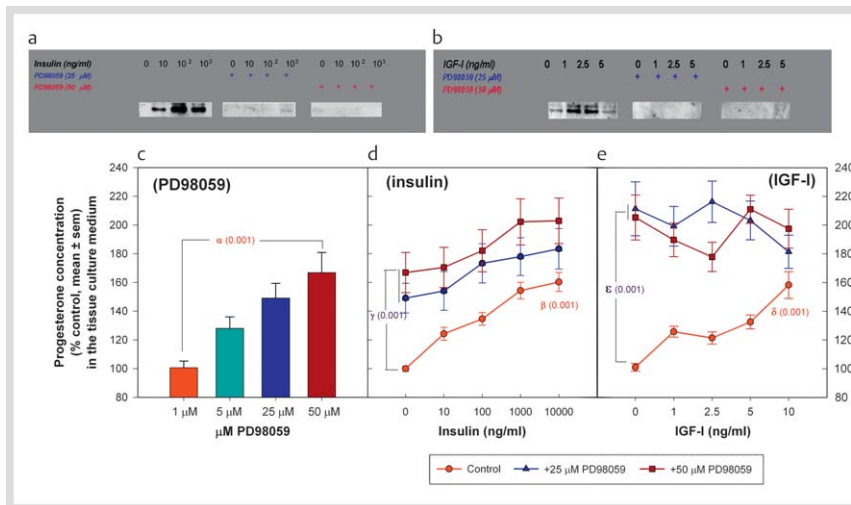


Fig. 1 The effects of PD98059 (MAPK-Erk1/2 inhibitor) on phospho-MAPK-Erk1/2 activity and progesterone production in cultured human ovarian cells. **a:** A representative immunoblot of the effect of 25–50 μ M PD98059 on the activity of phospho-MAPK-Erk1/2 in the absence or in the presence of insulin (0– 10^3 ng/ml); **b:** a representative immuno-blot of the effect of 25–50 μ M PD98059 on the activity of phospho-MAPK-Erk1/2 in the absence or in the presence of IGF-I (0–5 ng/ml); **c:** dose-dependent effect of PD98059 alone on progesterone production; α , $p < 0.001$ ($n = 5$) compared to control (in the absence of PD98059); **d:** the effect of insulin and PD98059 on progesterone production; β and γ , $p < 0.001$ ($n = 5$) compared to control (in the absence of insulin and in the absence of PD98059); **e:** effect of IGF-I and PD98059 on progesterone production; δ and ϵ , $p < 0.001$ ($n = 8$) compared to control (in the absence of IGF-I and in the absence of PD98059).

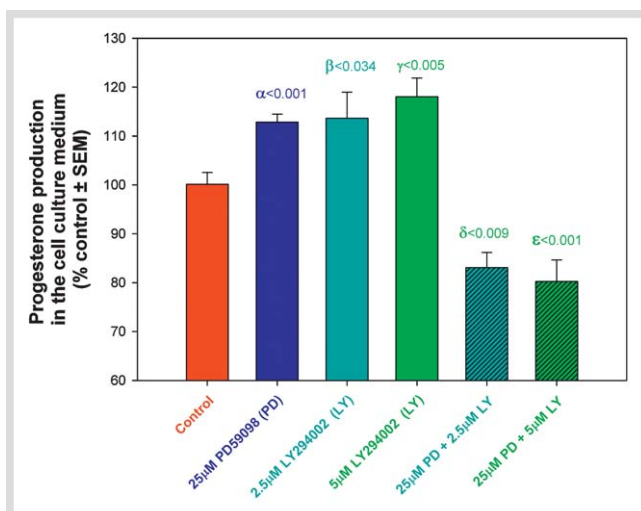


Fig. 2 The effects of PD98059 and LY294002 (specific PI-3-kinase inhibitor) on progesterone production in the absence of insulin or IGF-I in cultured human ovarian cells. The effect of PD98059 (25 μ M), LY294002 (2.5 μ M and 5 μ M), PD98059 (25 μ M) + LY 294002 (2.5 μ M) and PD98059 (25 μ M) + LY 294002 (5 μ M) on progesterone production: α , $p < 0.001$ ($n = 5$) compared to control (in the absence of PD98059); β , $p < 0.034$ ($n = 5$) and γ , $p < 0.005$ compared to control [in the absence of LY294002 (2.5 μ M or 5 μ M)]; δ , $p < 0.009$ ($n = 5$) compared to control [in the absence of PD98059 + LY294002 (2.5 μ M)]; and ϵ , $p < 0.001$ ($n = 5$) compared to control [in the absence of PD98059 + LY294002 (5 μ M)].

IGF-I had no additional stimulatory effect on progesterone production.

To assess the mechanisms involved in PD98059-induced activation of progesterone production, we utilized LY294002, a specific inhibitor of PI-3 kinase. In these experiments (which involved ovarian samples from 2 patients), MAPK inhibitor PD98059 (25 μ M) stimulated progesterone production by 13% ($p < 0.001$) (● Fig. 2).

Amounts equivalent to 2.5 μ M and 5 μ M LY294002 also stimulated progesterone production by 13.6% ($p < 0.034$) and 18.1% ($p < 0.001$), respectively (● Fig. 2). When 25 μ M PD98059 and 2.5 μ M or 5 μ M LY294002 were used together, progesterone production was inhibited by 17% ($p < 0.005$) and 20% ($p < 0.009$), respectively (● Fig. 2).

SB203580 effects on phospho-MAPK-p38 activity and progesterone production (● Fig. 3)

At 0– 10^2 ng/ml insulin, 10 μ M of SB203580 inhibited phospho-MAPK-p38 activity by 20% (● Fig. 3a) and 25 μ M of SB203580 inhibited phospho-MAPK-p38 activity by 90%. At 0–10 ng/ml of IGF-I, 10 μ M and 25 μ M of SB203580 inhibited phospho-MAPK-p38 by 50–80% (● Fig. 3b).

A. Insulin experiments

On its own, 10 μ M of SB203580 had no significant effect on progesterone production, while 25 μ M of SB203580 inhibited progesterone production by 30% ($p < 0.001$) (● Fig. 3c). Insulin alone stimulated progesterone production in a dose-dependent manner by 40% (● Fig. 3c). Amounts equivalent to 10 μ M and 25 μ M of SB203580 completely abolished insulin-induced stimulation of progesterone production (● Fig. 3c).

B. IGF-I experiments

Both 10 μ M and 25 μ M SB203580 alone inhibited progesterone production by 20% ($p < 0.001$). IGF-I alone stimulated progesterone production by 40% ($p < 0.001$) (● Fig. 3d). In the presence of SB203580 (10–25 μ M), IGF-I induced stimulation of progesterone production was completely abolished (● Fig. 3d).

Discussion

Insulin and IGF-I participate in the regulation of ovarian function, including ovulation and steroidogenesis [13]. As mentioned previously, in insulin resistant hyperinsulinemic states the ovary can remain sensitive to insulin while classical target organs (liver, muscle and fat) are insulin resistant [1]. This paradox is explained in part by activation of IGF-I signaling pathways and insulin signaling pathways unrelated to glucose transport [13]. For example, our previous studies demonstrated that activation of PI-3-kinase is not necessary for the ovarian effects of insulin: inhibition of PI-3-kinase by specific inhibitor wortmannin had no effect on insulin-stimulated steroidogenesis [9]. We also demonstrated that activation of MAPK (Erk1/2) is not necessary for the effects of insulin in granulosa cells while IGF-I induced progesterone synthesis in these cells is MAPK-dependent [12]. These studies provided initial evidence for the divergence of insulin signaling pathways and IGF-I signaling pathways for steroidogenesis in the human ovary.

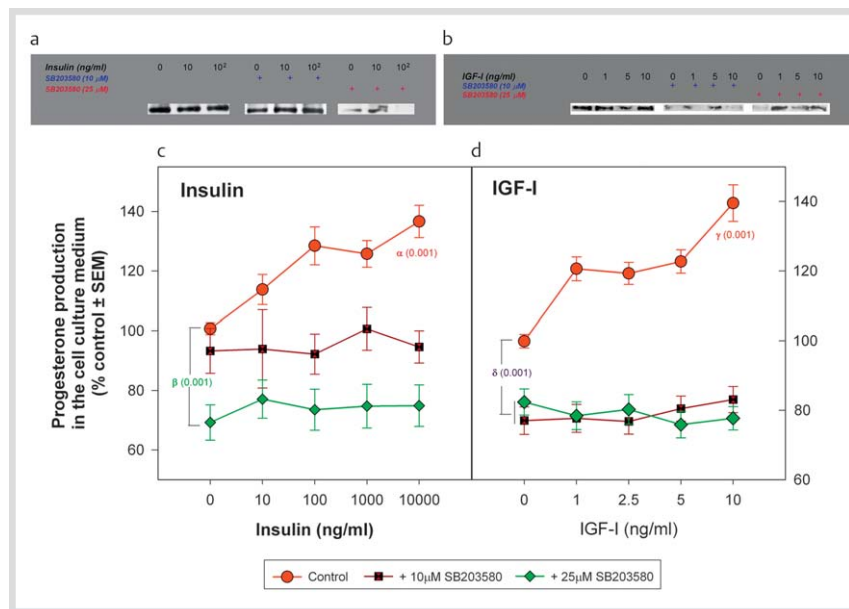


Fig. 3 The effect of SB203580 (MAPK-p38 inhibitor) on phospho-MAPK-p38 activity and progesterone production in cultured human ovarian cells. **a:** A representative immuno-blot of the effect of 10–25 μM SB203580 on the activity of phospho-MAPK-p38 in the absence or in the presence of insulin (0–10³ ng/ml); **b:** a representative immuno-blot of the effect of 10–25 μM SB203580 on the activity of phospho-MAPK-p38 in the absence or in the presence of IGF-I (0–5 ng/ml); **c:** the effect of insulin and SB203580 on progesterone production; α and β, $p < 0.001$ ($n = 10$) compared to control (in the absence of insulin and in the absence of SB203580); **d:** effect of IGF-I and SB203580 on progesterone production; γ and δ, $p < 0.001$ ($n = 8$) compared to control (in the absence of IGF-I and in the absence of SB203580).

In this report, we used a mixed granulosa, theca and stromal cell culture to explore the role of specific types of MAP-kinase, MAPK-Erk1/2 and MAPK-p38, in progesterone synthesis pathways in human ovarian cells. The culture system which we used was previously described in detail [10] and consists of all 3 steroidogenic cell types present in the ovary (granulosa, theca, and stroma). Unlike purified granulosa cells obtained during in vitro fertilization procedures, the cells in the mixed ovarian culture have not been a subject to hyperstimulation by gonadotropins in vivo. The lack of hyperstimulation by the gonadotropins and presence of all steroidogenically active ovarian cell types make this cell culture system more physiological than the purified culture of granulosa cells obtained during in vitro fertilization.

We have now confirmed using the mixed ovarian cell culture that activation of MAPK-Erk1/2 is not necessary for the stimulatory effects of insulin on progesterone production, while the IGF-I-induced progesterone synthesis is MAPK-Erk1/2 dependent. Alternatively, the lack of stimulation of progesterone production by IGF-I in the presence of PD98059 may be due to the maximal stimulation of progesterone production by PD98059. However, in our previous studies utilizing the human granulosa cells, stimulation of progesterone synthesis by PD98059 (1 μM) was much less pronounced, and yet IGF-I-induced progesterone production was completely abolished in the presence of PD98059 [12]. These findings, taken together, suggest that, in contrast to MAPK-Erk1/2, MAPK-p38 is necessary for stimulation of progesterone production by both insulin and IGF-I.

Previously published studies have demonstrated that LH-induced stimulation of progesterone synthesis in granulosa cells may be mediated by 2 signaling pathways: either MAPK-Erk1/2 or cAMP-dependent protein-kinase A (PKA) pathway. In these studies, LH-induced progesterone production and stimulation of StAR mRNA expression were preserved in the presence of specific inhibitor of MAPK-Erk1/2 [4, 5]. Thus, activation of progesterone production by PD98059 alone, observed in our studies, may involve a MAPK-Erk1/2-independent signaling pathway.

In order to explain which signaling pathways are involved in the stimulation of progesterone production by PD98059, we utilized a PI-3 kinase inhibitor LY294002. Our data show that inhibition of either MAPK-Erk1/2 or PI-3-kinase enhanced progesterone

production. When both pathways were blocked, however, progesterone production was inhibited. These results suggest that activation of progesterone production may involve either MAPK or PI-3 kinase pathway, providing support for the hypothesis postulating the existence of alternative progesterone synthesis pathways in the human ovary.

As mentioned in the introduction, in previous studies by Lin et al. [7], 10 μM of SB203580 had no effect on progesterone production in human granulosa cells. We confirmed this finding, but also demonstrated that at higher concentration (25 μM) SB203580 independently inhibited progesterone production by 20–30%.

We examined the effect of PD98059 or SB203580 on the components of steroidogenic signaling cascade. PD98059 and U0126 stimulated both steroidogenic acute regulatory protein (StAR) and 3-β-hydroxysteroid dehydrogenase (3β-HSD) mRNA and protein expression (data not shown), corresponding to a significant effect on progesterone production (stimulation by 65%). However, SB203580 had no significant effect on enzyme mRNA or protein expression (data not shown) and this lack of effect can possibly explain relatively small magnitude of the observed SB203580 effect on progesterone production (inhibition by 20–30%). The mechanisms of the independent effects of LY294002, PD98059 and SB203580 on progesterone production remain to be elucidated.

We conclude that insulin-induced progesterone production in human ovarian cells is dependent on the activation of MAPK-p38, but not of MAPK-Erk1/2. On the other hand, IGF-I induced progesterone production in these cells is both MAPK-Erk1/2 and MAPK-p38-dependent. These data provide further evidence for the divergence of insulin and IGF-I signaling pathways in the human ovary.

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