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Steroid hormones induce *in vitro* human first trimester trophoblast tubulogenesis by the lysophosphatidic acid pathway

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ABSTRACT

Successful implantation and placentation requires that extravillous cytotrophoblast acquires an endovascular phenotype and remodels uterine spiral arteries. Progesterone (P4) and estradiol (E2) control many of the placental functions, but their role in vascular remodeling remains controversial. Here, we investigated whether P4 and E2 regulate the acquisition of the human first trimester trophoblast endovascular phenotype, and the participation of the lysophosphatidic acid pathway. For this purpose, human first trimester HTR-8/SVneo cells were seeded on Geltrex and assayed for capillary-like tube formation. P4 and E2 increased HTR-8/SVneo tube formation in a concentration-dependent manner and this effect is mediated by the LPA3 receptor. Moreover, sex steroids increased the mRNA levels of the main enzyme that produce lysophosphatidic acid (lysophospholipase-D) but did not regulate LPA3 mRNA levels. Overall, we demonstrate that steroid hormones regulate HTR-8/SVneo trophoblast capillary-like structures formation and we propose that this process could be modulated directly or indirectly by mechanisms associated to the LPA/LPA3 pathway.

1. Introduction

Spiral artery remodeling at the maternal-fetal interface is crucial and involves extravillous trophoblast differentiation into an endovascular trophoblast. In this mechanism, the endovascular trophoblast invades maternal spiral arteries, disrupts the endothelium-myometrium interactions and replaces endothelial and myometrial cells. These adaptations of the maternal vessels ensure an adequate blood flow in response to the increasing metabolic demands of the embryo (Demir et al., 2010). Failures in this process are correlated with severe obstetric complications such as implantation failure and preeclampsia (Zhou et al., 1997; Plaisier et al., 2009).

Sex steroids, P4 and E2, are the master hormones that orchestrate most of the reproductive events during implantation and placentation in mammals. P4 and E2 intervene to achieve appropriate maternal vascular adaptations and placental vasculature during the early stages of pregnancy (Clark et al., 2017). Although steroid hormones modulate uterine blood flow following remodeling of uterine arteries, placental angiogenesis and vasculogenesis (Chen et al., 2012; Maliqueo et al., 2016), their specific role in vascular remodeling at the maternal-fetal interface remains controversial.

Lysophosphatidic acid (LPA) is a phosphorylated lipid mediator that regulates several female reproductive functions through G protein-coupled receptors (Ye et al., 2005; Hama et al., 2007). LPA modulates blood vessel development and is involved in vascular pathologies in different biological systems (Mueller et al., 2015). In this sense, we have previously shown that LPA augments the production of vascular mediators in the rat uterus during implantation (Sordelli et al., 2012; Beltrame et al., 2013). Recently, we reported that LPA binding to LPA3 receptor participates in angiogenesis at the implantation sites in the rat (Sordelli et al., 2017) and promotes the acquisition of the endovascular phenotype by the human first trimester trophoblast (Beltrame et al., 2018). Interestingly, patients displaying recurrent implantation failure and endometriosis show reduced levels of LPA3 in the endometrium (Achache et al., 2010; Wei et al., 2009). Furthermore, it has been shown that P4 and E2 modulate uterine receptivity by LPA3-mediated signaling in mice during early pregnancy (Diao et al., 2015).

Based on these antecedents, we decided to investigate whether P4 and E2 regulate human first trimester trophoblast tube formation, and if LPA pathway participates in this process.

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2. Methods

2.1. Cell culture

The immortalized human first trimester trophoblast cell line HTR-8/SVneo (H8, a kind gift from Dr. Udo Markert, Plancenta Lab, Department of Obstetrics, Jena University Hospital, Jena, Germany) was maintained as previously described (Beltrame et al., 2018). H8 trophoblast cells were obtained from explant cultures of human first trimester placenta (8–10 weeks of gestation) and immortalized by transfection with a cDNA construct that encodes the SV40 large T antigen (Graham et al., 1993). These cells are non-tumourigenic, non-metastatic and highly invasive *in vitro*. However, they are not tumorigenic when injected into nude mice. H8 cells exhibit various properties of extravillous cytotrophoblast including the expression of cytokeratins 7, 8, and 18, placental alkaline phosphatase, uPAR, human leukocyte antigen framework antigen W6/32, IGF-II mRNA and protein, as well as an integrin profile characteristic of invasive cytotrophoblast (Graham et al., 1993; Irving et al., 1995). These cells have been shown to secrete variable levels of hCG (Armant et al., 2006) and to express cytoplasmic and membrane-associated HLAG (Kilburn et al., 2000; Kalkunte et al., 2008).

2.2. Tube formation assay

H8 cell line and tube formation assay were used to model the acquisition of the trophoblast endovascular phenotype at the maternal-fetal interface (Beltrame et al., 2018). Briefly, 96 well plates were coated with 50 µL/well of Geltrex (Gibco, Invitrogen, Argentina) and incubated at 37 °C for 30 min to promote solidification. H8 were seeded on the top of the gel (15000/well) and incubated at 37 °C with 5% CO_2 in DMEM/F12 medium without fetal bovine serum. Cells were treated with P4 (medroxyprogesterone 17-acetate, Sigma Aldrich Co., Argentina), E2 (17β-estradiol, Sigma Aldrich Co., Argentina), BMT (BMT-183172-01-002, LPA1 antagonist, Bristol Myers Squibb, Pennington, USA), DGPP (diacylglycerol pyrophosphate 8:0, LPA3 antagonist, Sigma Aldrich Co., Argentina) or BrP-LPA (1-bromo-3(S)-hydroxy-4-(palmitoyloxy)-butyl-phosphonate, LPA1 to LPA4 antagonist, Echelon Biosciences, Inc., Pennsylvania, USA). After 6 h, tubules were observed in an inverted light microscope (10x, IMT2 Olympus) and photographed with a digital camera (Olympus C-5060). Five different fields per well were analyzed and extreme edges were excluded due to gel meniscus formation. Image J (open source) software package was used to quantify tubule length of the capillary network formation. The length of each tubule was determined by drawing a line over each tubule and the mean length of the lines (pixels) drawn in each image was calculated. The number of capillary interconnections or branch points between cells was counted manually.

2.3. RNA isolation and polymerase chain reaction analysis (PCR)

H8 cells were plated in a 6 well plate (400000/well) and were treated with P4 10^{-7} M + E2 10^{-5} M. Total RNA isolation, cDNA synthesis and real time PCR were performed as previously described (Sordelli et al., 2011; Beltrame et al., 2013). First strand cDNA was synthesized from total RNA (3 µg). The PCR conditions in all cases started with a denaturation step at 95 °C for 5 min and followed by up to 40 cycles of denaturation, annealing and primer extension (lysophospholipase-D, LPA3 and GADPH: 94 °C 5 min, 59 °C 30 seg, 72 °C 1 min). PCR primers are detailed for lysophospholipase-D (NM_006209.4, Forward 5′-GGCACACACTCTCCCTACAT-3′, Reverse 5′-GTTCCAGCTTTCACCCCTTG-3′, product: 233 bp), LPA3 (NM_012152.2, Forward 5′-CTTAGGGGCGTTTGTGGTAT-3′, Reverse 5′-GTGCCATACATGTCCTCGTC-3′, product: 177 bp) and GAPDH (NM_000572.2, Forward 5′-CACATCGCTGAGACACCATG-3′, Reverse 5′-GATGACAAGCTTCCCGTTCTC-3′, product: 224 bp). A melting curve analysis was performed to confirm the amplification specificity. Lysophospholipase-D and LPA3 mRNA levels were normalized against levels of human GAPDH using the $2^{-\Delta\Delta Ct}$ method. GAPDH was chosen as the housekeeping gene because its expression did not change under the present experimental conditions.

2.4. Statistical analyses

All values represent mean ± S.E.M. Data was normally distributed according to the Shapiro-Wilk normality test. Comparisons between values of different groups were performed using analysis of variance (ANOVA) and significance was determined using Bonferroni or Tukey *post hoc* tests. A number of three replicates were used per treatment and each experiment was repeated 4–6 times. Differences between means were considered significant when $p < 0.05$. Statistical analysis was performed using the InfoStat Program (Córdoba, Argentina).

3. Results

3.1. A fine balance of steroid hormones is necessary to induce H8 tubulogenesis

First, we investigated the effect of P4 and E2 on trophoblast tubulogenesis. H8 cells were incubated with increasing concentrations of P4 (10^{-8} - 10^{-4} M) or E2 (10^{-10} - 10^{-4} M) for 6 h. We observed that P4 and E2 stimulated the formation of tubules in a concentration-dependent manner. The incubation with P4 10^{-7} M or 10^{-6} M increased the length of the tubules and the number of branches compared to the control (Fig. 1A). In contrast, the incubation with P4 10^{-8} M or 10^{-5} M did not show differences when compared to the basal tube formation. In the case of E2, we observed that the incubation with E2 10^{-8} M stimulated the formation of the tubular network (Fig. 1B). In contrast, E2 10^{-10}, 10^{-9}, 10^{-7}, 10^{-6} or 10^{-5} M showed no effect. Finally, the treatment with P4 10^{-4} M or E2 10^{-4} M inhibited tubulogenesis below the control.

In the uterine microenvironment, P4 and E2 are present simultaneously regulating the processes triggered before and after blastocyst implantation. Therefore, we evaluated the effect of the co-incubation of P4 + E2 on trophoblast tubulogenesis. For this purpose, H8 cells were incubated with P4 10^{-7} M and increasing concentrations of E2 (10^{-10} - 10^{-4} M) or with E2 10^{-8} M and increasing concentrations of P4 (10^{-8} - 10^{-4} M). Although P4 10^{-7} M and E2 10^{-8} M increased H8 tubule network when incubated separately, the combination of P4 10^{-7} M + E2 10^{-8} M did not change H8 tubulogenesis compared to the control (Fig. 2). We found that only the incubation of H8 cells with P4 10^{-7} M + E2 10^{-5} M stimulated trophoblast tube formation. The incubation with P4 10^{-4} M + E2 10^{-8} M suppressed the formation of tubules below control levels (Fig. 2). However, the treatment with these concentrations of P4 and E2 separately did not modify the tubule network (Fig. 1A and B). The effect of P4 10^{-4} M + E2 10^{-8} M was due to an inhibition in the process of tubulogenesis and not to deleterious actions of steroid hormones on cell viability or survival (data not shown). However, P4 10^{-3} M + E2 10^{-8} M showed a negative action on the survival of H8 cells (Fig. 2), as this combination of steroid hormones exert a toxic effect in H8 cell line (data not shown).

3.2. LPA mediates the induction of P4 + E2 on trophoblast tube formation

We have previously reported that LPA promotes the acquisition of the human first trimester trophoblast endovascular phenotype (Beltrame et al., 2018). Therefore, we decided to evaluate the participation of LPA in the effect of P4 + E2 on trophoblast tubulogenesis. H8 cells were incubated with P4 10^{-7} + E2 10^{-5} M alone or in the presence of DGPP 100 µM (a selective LPA3 antagonist), BrP-LPA 5 µM (a broad LPA1 to LPA4 antagonist) or BMT 10 µM (a selective LPA1 antagonist) for 6 h. The concentrations of the antagonists were selected