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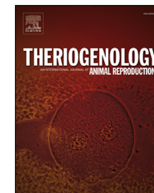

The effect of lysophosphatidic acid together with interferon tau on the global transcriptomic profile in bovine endometrial cells

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A B S T R A C T

In cows, lysophosphatidic acid (LPA), which acts in an auto/paracrine manner, serves as a luteotropic factor during early pregnancy by stimulating progesterone and prostaglandin E_2 secretion, thus protecting the bovine corpus luteum and early embryo development. Our hypothesis was that LPA exerted some local effects on the bovine endometrium prior to early embryo-maternal interactions and that interferon tau (IFNτ), the pregnancy recognition signal, modulated this action. In the present study, we applied an *in vitro* model involving whole-transcriptomic profiling to examine the effects of LPA on gene expression in bovine endometrial cells. Microarray analyses revealed 36, 269 and 284 differentially expressed transcripts in bovine endometrial cells in the control vs. LPA, control vs. LPA + IFNτ and LPA vs. LPA + IFNτ groups, respectively. The expression of matrix metalloproteinase 13 (MMP13) and radical S-adenosyl methionine domain containing 2 (RSAD2) was increased in the LPA-treated endometrial cells. Among the transcripts differentially regulated by LPA together with IFNτ, many of the genes were classical- or novel-type I IFN-stimulated genes (ISGs). The results indicated that 10 of the 16 analyzed genes showed a positive correlation with their corresponding microarray data upon real-time PCR validation, indicating a considerable consistency between both techniques. In summary, these transcriptional profiling studies identified a number of genes that were regulated by LPA alone and LPA together with IFNτ in endometrial cells from the bovine uterus. Available studies support the idea that LPA, which acts in an auto/paracrine manner on the endometrium, alters the expression of genes that are probably important for uterine receptivity, maternal immune tolerance to the embryo and conceptus growth and development during early pregnancy. Moreover, the differentially expressed genes (DEGs) that increased in the LPA + IFNτ-treated endometrial cells are largely in response to IFNτ actions and are possibly associated with crucial biological processes during the peri-implantation period of pregnancy.

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1. Introduction

Lysophosphatidic acid (LPA), which exerts a variety of physiological and pathological actions in various animal species [1-6], has been documented to regulate reproductive performance in cows [7,8]. We have recently found that LPA can be locally produced and released from the bovine endometrium [9]. We have also documented that LPA concentrations and gene expression for its active receptor LPAR1 in the bovine endometrium were significantly higher during early pregnancy than during the estrous cycle [9]. Moreover, LPA stimulated progesterone (P4) and prostaglandin (PG) E_2 secretion *in vivo*, and mRNA expression of LPAR1 was positively correlated with the expression of the enzyme responsible for luteotropic PGE_2 production (PGES) in the bovine endometrium during the estrous cycle and early pregnancy [9,10]. Bovine embryos at different stages of development are also able to synthesize and secrete LPA; additionally, LPA-mediated cell signaling during early embryonic development may be relevant in early embryo-maternal interactions that lead to the embryonic survival [11]. These data indicate that LPA may play autocrine and/or paracrine roles in the uterus and serve as an important factor in the maintenance of early pregnancy not only in mouse [3,12], pig [5] and sheep [6] but also in cow [10].

There is much evidence showing that after fertilization, between days 10 and 21 to 25 in ruminants, the conceptus starts to produce interferon tau (IFNτ), which is the pregnancy recognition signal that prevents development of the endometrial luteolytic

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mechanism [13,14]. The maximal production of IFNτ falls on days 14–18 [15]. However, Kimura et al. [16] reported that IFNτ production during culture of *in vivo*-derived embryos increased dramatically (150,000-fold) from day 9–14. The signals from the conceptus occurring after fertilization are not only local, but spread throughout the entire female as measured in the systemic blood a few days before implantation [17,18]. This peri-implantation period during early pregnancy represents the time when the blastocyst is hatching; from this time, it becomes a free-floating embryo within the lumen of the uterus that is totally dependent on the uterine environment for survival [15]. At this time, early embryo survival mostly depends on the appropriate function of corpus luteum (CL)-adequate P4 synthesis, as well as on IFNτ-dependent inhibition of the development of the endometrial luteolytic mechanism [13,14]. In contrast, LPA also serves as a luteotropic factor during early pregnancy by stimulating P4 and PGE_2 secretion, thus protecting the bovine CL and early embryo development [9].

Studies to date on the endometrial response to various early pregnancy modulating factors, including LPA, have been based on a candidate gene approach. Therefore, a better understanding is needed of all the internal signaling pathways involved in the potential modulation of LPA action by IFNτ. This study will provide a deeper understanding of uterine functions at the time of early pregnancy recognition in cow and can directly influence further investigations.

2. Materials and methods

2.1. Bovine endometrial cell culture

All of the animal procedures were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (Agreement No. 79/2008/N). For all experiments, normally cycling Holstein/Polish Black and White (75/25%, respectively) cows (n = 9) were chosen. Bovine uteri were obtained at a local slaughterhouse within 20 min of exsanguinations and were transported on ice to the laboratory within 40 min. The animals were slaughtered on days 2–5 of the estrous cycle to isolate the endometrial cells as previously described [19,20]. Estimation of the estrous cycle stages was additionally confirmed by macroscopic observation of the ovaries and uterus [21]. The epithelial and stromal cells from bovine endometria were enzymatically separated using solutions I (sterile Hank's Balanced Salt Solution (HBBS) containing 2.4 U/mL dispase-Gibco, Grand Island, NY, USA; 0.005% DNase IV-Sigma Aldrich, Madison, USA; and 0.05% collagenase I-Sigma Aldrich) and II (HBBS with 0.005% DNase IV and 0.05% collagenase I). The final pellets of both the stromal and epithelial cells were suspended in Dulbecco-modified Eagle's medium (DMEM, Sigma Aldrich) containing 10% fetal calf serum (Sigma Aldrich), 10 000 U/mL penicillin G, 10 mg/mL streptomycin and 25 μg/mL amphotericin B (antibiotic antimycotic solution, Sigma Aldrich). The cells were collectively seeded to co-culture at a density of 1×10^6 viable cells/mL at a 3 to 1 (stromal to epithelial cells) ratio in 6-well plates (Costar®, Corning® CellBIND® Surface, Corning B.V. Life Sciences, Amsterdam, The Netherlands) and cultured at 38.5 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.2. Experimental design

As the epithelial and stromal cells attached 24–48 h after plating, the medium in the culture was replaced with fresh DMEM supplemented with 0.1% BSA (Sigma Aldrich) and antibiotic antimycotic solution (Sigma Aldrich). When the cells reached 80–90% confluency (6–7 days after the start of the culture), the cells were then used to establish the following experimental groups: (1) control, cells exposed to vehicle (phosphate-buffered saline (PBS)), (2) LPA, cells stimulated with an LPA agonist (1-oleoyl-sn-glycerol 3-phosphate sodium salt, LPA; 10^{-6} M, Sigma Aldrich), and (3) LPA together with IFNτ (10^3 AVU; donated by Dr. FW Bazer of Texas A&M University, College Station, TX, USA). The concentration of the stimulators and time of incubation were determined in preliminary experiments. After 6 h incubation, the cells were disrupted and stored at −80 °C until they were processed for RNA isolation.

2.3. Isolation and quantitative and qualitative RNA analysis

Total RNA extraction from the endometrial cells was carried out using the TRI Reagent (Sigma Aldrich) followed by the Total RNA Mini Kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. Isolated RNA was quantified using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA), with each sample displaying a concentration of 250 ng/μl or greater and ratios of absorbance at 260 and 280 nm within the range from 1.9 to 2.0. RNA quality was monitored with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA integrity numbers for all of the samples were nine or higher.

2.4. Microarray hybridization and analysis

Whole-transcriptomic gene expression for each sample was measured with an Affymetrix Bovine Genome Array (Affymetrix, Santa Clara, CA, USA; National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) accession GPL 2112). Microarray hybridization and scanning were performed according to the manufacturer's recommendations. Three replicates with 100 ng of total RNA from each of the experimental conditions (control, LPA and LPA + IFNτ (3 different animals per condition)) were used for aRNA synthesis. Amplification, labeling and fragmentation of aRNA were conducted according to the Affymetrix GeneChip 3′ IVT Express kit protocol. Following fragmentation, 12 μg of biotin-labeled aRNA were hybridized to the GeneChips Bovine Genome Array for 16 h at 45 °C in a rotating Affymetrix GeneChip Hybridization Oven 640. The arrays were washed and stained in an Affymetrix GeneChip Fluidics Station 450 and were then scanned using an Affymetrix GeneChip Scanner 3000 7G. The signal intensity for each probe on each chip was calculated from scanned images using the Affymetrix GeneChip® Command Console® (AGCC) software. Pearson's analysis was applied to estimate the correlation between linear variables, which examined the homogeneity of the samples. Signal intensities were analyzed using the Partek Genomic Suite v 6.6 software (Partek Incorporated, St. Louis, USA). Probe intensities were background-corrected, normalized, and summarized using the GC Robust Multichip Average method. Background correction was applied based on the global distribution of the Perfect Match probe intensities and the affinity of each probe was calculated based on its sequence data. Quantile normalization and median-polish summarization was applied for each probe set. Principal Component Analysis was performed to identify outliers and artifacts on the microarray. After a performing a quality check on the data with one-way analysis of variance (ANOVA), lists of significantly and differentially expressed genes (DEGs) between the experimental variants (control, LPA and LPA + IFNτ) were created. Benjamini-Hochberg multiple testing correction for false discovery rate (FDR) was also applied. Probe sets (control vs. LPA and control vs. LPA + IFNτ) were considered differentially expressed when the signed fold change was ≥2, the adjusted P-value was <0.05 and the FDR was <0.05. To compare the LPA vs. LPA + IFNτ groups, only a signed fold change ≥2 and a P-value cut-off < 0.05 were used. The selected lists were subjected to cluster analysis to identify genes and samples with similar profiles

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