Chromosomal Location, Structure, and Temporal Expression of the Platelet-Activating Factor Receptor (PAFr) Gene in Porcine Endometrium and Embryos Relative to Estrogen Receptor α Gene Expression

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ABSTRACT Although platelet-activating factor receptor (PAFr) gene was well characterized in the human, little was known about it in domestic animals. Porcine PAFr gene was mapped using fluorescence in situ hybridization (FISH). The structure of this gene was investigated using a 5′ rapid amplification of cDNA ends (RACE) technique. Temporal expression of PAFr and estrogen receptor α genes (ER), and distribution of the PAFr transcripts in porcine endometrial and embryonic tissues on days 0, 10, 12, 14, 16, and 18 were analyzed using DNA competitors and reverse transcription and polymerase chain reaction (RT-PCR). The porcine PAFr gene was mapped to SSC6q26-27. Alternative splicing of primary transcripts of the PAFr gene produced two different transcripts. Transcript 1 was expressed in all tissues and cells, and transcript 2 was detected in all tissues but white blood cells. The temporal expression of the PAFr gene in endometrial (P > 0.05) and embryonic (P < 0.05) tissues of pregnant sows increased from day 10 to 16. The temporal expression of ER genes in endometrial tissues of pregnant sows decreased from day 10 to 18 (P < 0.05). In addition, ER expression was detectable in 20–60% of embryonic tissue samples, which generally decreased. In combination with previously obtained data on PAF and estradiol-17β (E2) concentrations in pregnant uterine luminal fluids (pULF), endometrial and embryonic tissues, the present results indicated that the increasing PAFr transcripts were positively associated with increasing levels of PAF. Both ER transcripts and E2 found in pULF decreased correspondingly from day 13 to 16. These results indicate that via PAFr, PAF could play a dominant role in peri-implantation development in pigs as compared to E2.

Key Words: chromosomal location; alternative transcripts; differential gene expression; quantitative RT-PCR; porcine; PAF; PAFr

INTRODUCTION Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycerol-3-phosphocholine) participates in many mammalian reproductive processes, stimulates ovarian follicular rupture (Tsafriri and Reich, 1999), improves fertilization through greater sperm capacitation (Wu et al., 2001), and enhances sperm motility (Wild and Roudebush, 2001). PAF actively up-regulates pre-implantation embryo development by increasing embryo intracellular calcium (Logan and Roudebush, 2000), acts as a survival factor during early conceptus and blastocyst development (O’Neill, 1998), and stimulates endothelial cell migration and angiogenesis during embryogenesis (Montrucchio et al., 2000). At implantation PAF stimulates uterine prostaglandin production mediated via nitric oxide (Chaud et al., 1998), and at parturition, PAF induces uterine contraction (Zhu et al., 1992), and promotes cervical ripening (Sugano et al., 2001).
Complementary DNA (cDNA) transfection experiments and pharmacological studies showed that intercellular actions of PAF were mediated through a G-protein-linked receptor (PAFr) on the plasma membrane of a variety of cell types. Therefore, PAFr is a member of the super-family of G protein-coupled receptors, characterized by seven transmembrane domains and an extracellular N-terminus. Its intracellular C-terminus is necessary for receptor desensitization. Two types of G-proteins downstream of PAFr signal phosphatidylinositol turnover and increase intracellular calcium (Ali et al., 1994; Honda et al., 1994). Two alternative transcripts of PAFr were found (Fig. 1C, Mutoh et al., 1993). Transcript 1 was ubiquitously expressed, but transcript-2 was not detected in leukocytes and brain. Transcript 2 was up regulated by E$_2$

A. Structure of Porcine $PAFr$ Gene Expressing Transcript 1

B. Structure of Porcine $PAFr$ Gene Expressing Transcript 2

C. Structure of Transcripts 1 & 2 of Human $PAFr$ Gene [adapted from the report by Mutoh et al.(1993)]

**Fig. 1.** Organization of platelet-activating factor receptor ($PAFr$) gene expressing two different transcripts in pig and human. Boxes indicate exons while single lines represent promoter and intron regions. The length of intron is yet to be determined. All introns have the consensus nucleotide sequences of intron–exon junctions ag at their 3’ end and gt at their 5’ end. The position of the first nucleotide immediately upstream of ATG is −1. A: Exon 1, preceded by promoter 1, contains three steroid hormone receptor binding sites (not shown) and has transcription start sites (TSSs) a, b, and c at positions −656, −231, and −135, respectively. Exon 4, intervened by an intron, acts as a common splice acceptor and contains part of 5’-UTR, complete coding sequence (cds) and part of 3’-UTR. B: Exons 2, 3, and 4 are separated by two introns. In exon 2, TSSs d and e at positions −230 and −211 are given, respectively. No steroid–hormone receptor binding sites were found in the promoter region. C: In human, the structure of $PAFr$ gene expressing transcripts 1 and 2 is similar to that in pig. The gene that expresses transcript 1 contains the promoter region, exon 1, an intron and exon 3. TSSs h, i, and k at positions −400, −327, and −137, respectively are indicated in exon 1. Transcript 2 contains exons 2 and 3. In exon 2, TSSs l and m at positions −309 and −249 are indicated, respectively. In promoter 2, two identical halves of the estrogen response elements (ERE) are found (not shown).
through an estrogen response element (ERE), by retinoic acid and thyroid hormone through three cis-acting elements and by phorbol esters through AP-1 sites (Mutoh et al., 1994a,b, 1996). Down-regulation of transcript 2 is exerted by TGF-β 1 through TGF-β 1 inhibitory element (TIE). Transcript-1 was positively regulated through consensus binding sites for NF-κ B by PAF and phorbol esters in promoter 1 (Mutoh et al., 1994a,b). Consequently, promoter 1 responds to inflammatory stimuli, whereas promoter 2 is regulated by different signals.

PAF is found in pULF and nonpregnant uterine luminal fluids (nULF) of pigs; the high content on day 13 in pULF suggests a role in signaling maternal recognition of pregnancy (O’Neill, 1987; Mook et al., 1998). PAF concentrations in endometrial and embryonic tissues were detectable on days 12, 14, 16, and 18. The highest PAF content was found on day 16 in pregnant endometrial tissues, and on day 18 in embryos. PAF mRNA was detected in porcine embryos and endometrium (Diehl et al., 1998). Estrogen via the ER plays a pivotal role in embryo development in addition to in maternal recognition of pregnancy (Flint et al., 1978; Ying et al., 2000). Changes in ER expression in endometrial and embryonic tissues could indirectly reflect demands for estrogen by endometrium and embryos. Even though there were reports about the ER expression in endometrial tissues (Geisert et al., 1993), the ER expression pattern in peri-implantation embryos is yet to be determined (Ying et al., 2000). Changes in ER expression in endometrial and embryonic tissues could indirectly reflect demands for estrogen by endometrium and embryos. Even though there were reports about the ER expression in endometrial tissues (Geisert et al., 1993), the ER expression pattern in peri-implantation embryos is yet to be determined (Ying et al., 2000). Changes in ER expression in endometrial and embryonic tissues could indirectly reflect demands for estrogen by endometrium and embryos. Even though there were reports about the ER expression in endometrial tissues (Geisert et al., 1993), the ER expression pattern in peri-implantation embryos is yet to be determined (Ying et al., 2000).

**MATERIALS AND METHODS**

**Animals**

Endometrial and embryonic tissues were collected from sows of a four-breed composite of Yorkshire, Swedish Landrace, Chester White, and Large White. All sows had produced one litter of pigs, and all had one estrus after their piglets were weaned. Thus, they were just over 1 year of age. Sows were artificially inseminated twice during estrus prior to recovery of pregnant tissue, or were not bred for nonpregnant tissue recovery. Endometrial and embryonic tissues were collected from pregnant females, called pregnant tissues, on days 10, 12, 14, 16, and 18. The endometrial tissues were collected from nonpregnant females, called nonpregnant endometrium, on days 0, 10, 12, 14, 16, and 18. Three to five sows were utilized on each day/pregnancy status combination.

**RNA Extraction and Purification**

The tissues used for RNA extraction were stored in 5 volumes of RNALater (Ambion, Austin, TX), then placed in a −20 or −80°C freezer. RNA was extracted using TRIzol Reagent (Life Technologies, GIBCO BRL, Grand Island, NY) per manufacturer’s instructions. The RNA samples were treated with DNase I to remove traces of DNA contamination. The purified RNA samples were stored at −80°C until analyzed.

**DNA Competitor Construction for PAFr Transcript Quantification**

Porcine PAFr competitor. The sequence of the DNA competitor was a portion of a target gene sequence made by deliberately deleting 10–35% of the target DNA sequence at 3′ or 5′ end. Both the DNA competitor and the target DNA template had the same priming sites but different size. After amplification, they were separated using 3.0% agarose gel and visualized on a UV light box following ethidium bromide staining. According to the porcine PAFr gene coding sequence (cds) (GenBank accession no. AF124054), set A primers were designed to produce the target DNA template and called porcine 1 (forward) and porcine 2 (reverse); porcine 1, 5′-GTG GAC TCT GAG TTC CGA TAC AC-3′, and porcine 2, 5′-GAG CAG CGT GCC GTG CAT GAT GAC-3′. The sequence of primer porcine 3 was 5′-GTG GAC TCT GAG TTC CGA TAC ACC TCC AAG AAG TTG AAC GAT GAT AAG-3′. Primer porcine 3 consisted of primer porcine 1 at its 5′ end and 25 bp at its 3′ end that had a priming site 110 bp downstream of the priming site for porcine 1. Primers porcine 3 (forward) and porcine 2 (reverse) produced a DNA competitor that has a 14% deletion of a target DNA template by amplifying the target via PCR. In order to obtain the PAFr DNA competitor, porcine genomic DNA or porcine bacterial artificial chromosome (BAC) DNA containing the PAFr gene was amplified using PCR in a 25-μl volume. The reaction contained 2.5 μl of 10 × PCR buffer, 2.5 μl of 2 mM dNTPs, 0.5 μl each of set A primers (10 pmol/μl), 3 U Klen Taq DNA polymerase (Ab Peptides, Inc., St. Louis, MO), and 100 ng DNA template. PCR was performed in a DNA thermal cycler 480 (Perkin Elmer, Branchburg, NJ) for 30 cycles at 94°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min, initialized with denaturation at 94°C for 2 min, then followed by a final extension for 10 min at 72°C. A 618-bp PCR DNA product was produced and gel-purified using a QIAquick Gel Extraction kit (Qiagen, Valencia, CA). This 618-bp DNA fragment was the target or endogenous template since it was sequenced and determined to be a portion of porcine PAFr cds. The second round of PCR was carried out using the same conditions as the first round of PCR except that the 618-bp DNA was used as template, and porcine 2 and porcine 3 as primers. A 531-bp DNA fragment was produced as the porcine PAFr DNA competitor. The DNA competitor was re-amplified with set A primers. PCR products were purified using a QIAquik PCR Purification kit (Qiagen). The concentration (copies/μl) of the DNA competitor was calculated using this formula, \((A_{260} \times 50 \text{ ng/μl} \times 6.0 \times 10^{23} \text{ copies/mole})/(bpb \times 660 \text{ g/mole bp} \times 10^{23} \text{ ng/g})\). The DNA competitor was sequenced to confirm that it was the expected product.

**Porcine estrogen receptor α (ER) DNA competitor.** The DNA competitor for porcine ER gene was designed based on the porcine ER α mRNA sequence...
PORCINE PLATELET-ACTIVATING FACTOR RECEPTOR (PAFr) GENE

(GBank accession no. Z37167). In this study, the DNA competitor for porcine ER gene was designed to be 453 bp long, but the endogenous porcine ER DNA was 709 bp in length. The primers for amplification of competitor and target template were ER-F1 (forward), 5'-GTC CCT GAC AGC CGA CCA GAT GAG CTC CTG TTT GCT CCT AAC TTG-3'; and ER-R1 (reverse), 5'-GTC CCT GAC AGC CGA CCA GAT GAG CTC CTG TTT GCT CCT AAC TTG-3'. Another primer, ER-3, 45 bp long, contained the whole sequence of ER-F1 at its 5' end, but also included 22 bp at its 3' end, which bound to a priming site 256 bp downstream of the ER-F1 priming site. Its sequence was 5'-GTC CCT GAC AGC CGA CCA GAT GAC AGC CAG GAA GGA C-3'. Due to intervening sequences found in porcine ER alpha gene, ER 709-bp endogenous template was amplified from ER cDNA (described below). PCR conditions were the same as that for production of the PAFr DNA competitor except for primers and templates. ER-F1, ER-R1, and ER cDNA were used for the first round of PCR to produce a 709-bp PCR product, a portion of porcine ER cds. ER-3, ER-R1, and 709-bp DNA fragments were combined in the second round of PCR to amplify a 453-bp DNA fragment. ER-F1, ER-R1, and 451-bp DNA fragments were mixed for re-amplification. An annealing condition was set to 68°C for 15 sec. The 453-bp DNA fragment was used as the porcine ER DNA competitor. Its concentration and sequence were determined and confirmed.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR) to Quantify Gene Expression Levels

Two micrograms of DNaseI-treated total RNA were used for a reverse transcription reaction. A mix containing 2 μg RNA, 2.5 μl primers (10 pmol/μl), 2.5 μl dNTPs (10 mM) was heated at 80°C for 3 min and chilled on ice for 2 min. Then 10 μl 5 × buffer, 1 μl 0.1 M DTT, 2.5 μl SUPERase. In, an RNase inhibitor (Ambion), and 2.5 μl M-MLV reverse transcriptase (GIBCO BRL) were added and mixed gently. This RT reaction was performed in an air incubator at 37°C for 1.5 hr, followed by heating at 70°C for 15 min to inactivate the reaction. The primer used in RT for porcine PAFr cDNA was a short gene-specific primer-1 (SGSP-1), and its sequence is 5'-ACC ATC CAG AGT-3'; for porcine ER cDNA was an ER short gene-specific primer-1 (ERSGSP-1), and its sequence was 5'-TGC GAT GGA GTT GA-3'. The reaction was diluted by adding 50 μl of water. Therefore, the concentration of total RNA in RT is 20 ng/μl.

A PCR cocktail was made containing 14 μl of H2O, 2.5 μl of 10 × PC2, 2.5 μl of dNTPs (2 mM), 0.5 μl of each primers (10 pmol/μl), 3 U of Klen Taq DNA polymerase in a 20-μl volume. Then 2.5 μl of RT and 2.5 μl of one DNA competitor dilution were added to 20 μl of PCR master mix. For each sample, 4 to 6 DNA competitor dilutions were made to try to ensure that a ratio of target to competitor of 1 to 1 could be visualized. For the PAFr gene, PCR was conducted under the same conditions as production of the PAFr DNA competitor; for porcine ER gene, PCR was performed using the same conditions as obtaining porcine ER DNA competitor. For each sample, there were 4 to 6 PCR replications, from which 10 μl were taken and separated on 3% agarose gel. The gel was stained in 1 × TAE buffer containing 0.5% ethidium bromide for 30 min, then visualized using an UV trans-illuminator (Figs. 2 and 3).

Investigation of 5' Un-Translated Region (UTR) of PAFr

An RT reaction was performed using total RNA from porcine lung or heart tissues per instructions of a GeneRacer kit (Invitrogen, Carlsbad, CA). A porcine PAFr gene-specific primer-1 (GSP1), 5'-GTT GTA AGT GAT GAC AGC CGA GGA GAG C-3', and a porcine nested primer-1 (NPN-1), 5'-AGA GTG ACC AGG AAC AGC AGG T-3' were used to amplify 5'UTR. The UTR sequences were determined by sequencing gel-purified 5'-UTR DNA fragments and cloned plasmid DNA. Based on the 5'-UTR nucleotide sequences, primers were designed and promoter region sequences, sites for intron and intron sequences were obtained by sequencing porcine BAC DNA containing PAFr gene (Yang et al., 2001). All sequences were done bidirectionally and submitted to GenBank.

PAFr Transcription Distribution in Different Tissues

Total RNA was extracted and purified from porcine tissues including heart, lung, skeletal muscle, kidney, liver, spleen, small intestine, white blood cells, and endometrium, and 5 μg of total RNA was used for RT to produce cDNA in a 50-μl reaction volume as described

Fig. 2. Estimation of PAFr gene expression in porcine tissues. The upper band was 618 bp long amplified from endogenous PAFr cds, and the lower band was 531 bp long amplified from DNA competitor. Lanes 1 and 12: phi174 DNA/Hae III markers, with a band, 603 bp long; lane 2, negative control without any template; lane 3, positive control with 33 ng porcine genomic DNA as template; lane 4, 2.5 μl of RT as template; lane 5, 2.5 μl of DNA competitor (7.0 × 10^3 copies/μl) as template; lanes 6–11, 2.5 μl of RT and 2.5 μl of DNA competitor at a concentration of 0.2, 0.6, 1.0, 3.0, 5.0, and 7.0 × 10^3 copies/μl in this order as template. Lane 9 had two bands with same intensity so the PAFr cDNA level in this sample was 3.0 × 10^3 copies/20 ng of total RNA.

Fig. 3. Estimation of porcine ER expression levels in tissues. The upper band was 709 bp long amplified from endogenous ER cds, and the lower band was 453 bp long amplified from DNA competitor. Lanes 1–5 were the results of gel-separated PCR products. DNA competitor was used at one concentration of 1.0, 2.5, 5.0, 7.5 and 10.0 × 10^3 copies/μl in this order. The ER level in this sample was 3.8 × 10^3 copies/20 ng of total RNA estimated by averaging DNA competitor concentrations on lane 2 and lane 3.
before. An aliquot of 2.5 μl of RT reaction was taken to amplify a transcript-1 cDNA in a 25-μl volume by PCR using two primers, T-1 (forward), 5'-CAG GGG CTG CGG ACA CAG AAA CTC-3' (forward), and TR (reverse), 5'-GGG CAA AGA CCC ACA GCA CGT AAC-3'. For amplification of transcript-2 cDNA, the forward primer was T-2, 5'-GCC AGG AGA GAG CAG TGG GAT TTA G-3', and the reverse one was TR. The PCR products were analyzed as described above.

**PAFr Gene Mapping Using a Fluorescence-In Situ-hybridization (FISH) Technique**

A porcine BAC library constructed and characterized elsewhere (Rogel-Gaillard et al., 1999) was used to screen PAFr gene-containing BAC for FISH mapping. The PCR-based screening was performed in a total of 50 μl with either 150 ng of superpool DNA or 30 ng of single pool DNA along with 150 ng of porcine genomic DNA and 150 ng of AB1380 yeast DNA as positive and negative controls.

Metaphase chromosome spreads were obtained from peripheral blood lymphocyte cultures by standard procedures. Chromosomes were G-banded before hybridization with the GTG technique. FISH was performed according to the modified procedure described previously (Yerle et al., 1994). Briefly, 100 ng of BAC DNA was labeled by incorporation of biotinylated 16-dUTP (Boehringer Mannheim, Indianapolis, IN) using random priming. A prehybridization was performed for 3 hr at 37°C in the presence of sonicated porcine genomic competitor DNA (80- to 100-fold excess) and salmon sperm DNA (1,000-fold excess) followed by a hybridization step at 37°C overnight. Hybridization signals were detected by incubation with biotinylated goat anti-avidin followed by a final incubation with avidin conjugated to fluorescein (FITC). The chromosomes were counterstained with propidium iodide and the slides were mounted in antifade solution. Measurements of the relative position of the fluorescent spots were taken of 20 metaphases by using the Cytovision software measuring module (Applied Imaging Corporation, Santa Clara, CA).

**Statistical Analysis**

In the present study, factors affecting PAFr or ER expression level included day, status and source. The differences among day (days 0, 10, 12, 14, 16, and 18), status (pregnant or nonpregnant) and source (endometrium or embryo) for PAFr and ER transcript levels were analyzed with an ANOVA according to day versus type [(nonpregnant × endometrium), (pregnant × endometrium), and (pregnant × embryo)]. Then the difference of the transcription levels in each type including nonpregnant endometrium, pregnant endometrium, and embryos was analyzed, respectively, on different days by the ANOVA followed by t-tests. The correlation analysis between PAFr transcript levels and concentrations of PAF or estradiol-17β was performed and a χ² test was used to analyze ER transcript availability in embryos at different developmental stages. The calculations were performed using the GLM procedure of Statistical Analysis System version 8.0.

**RESULTS**

**PAFr Expression Levels in Endometrial and Embryonic Tissues**

Using quantitative RT-PCR, the temporal expression of the PAFr gene in porcine reproductive tissues was estimated (Table 1). No interaction between day and type was observed (P = 0.2001). No significant difference was found in effects of day on PAFr transcription levels (PTL, P = 0.1301) but not in effects of type on PTL (P < 0.0001). t-Test results indicated that PTL were significantly greater in pregnant endometrium than nonpregnant endometrium (P < 0.05), and embryos (P < 0.05) by comparing PTL from different types. In nonpregnant endometrial tissues, PTL were higher on days 0 and 18 than days 12, 14 and 16 (P < 0.05). In pregnant endometrial tissues, PTL were not statistically different from day 10 to 18 (P > 0.05), however, numerically increased from day 10 to 16 then little decreased. In embryonic tissues, PTL increased to the highest point from day 10 to 16 (P < 0.05), then decreased (P < 0.05, Table 1).

**Estrogen Receptor α Expression Levels in Endometrial and Embryonic Tissues**

Even though the level of ER was significantly higher in nonpregnant endometrial tissues than in pregnant endometrial tissues (P < 0.05), but an interaction between days and types was indicated (P = 0.0002), so the difference of ER levels in each type on different days was analyzed. Compared to days 10, 12, 14, and 16, the ER level in nonpregnant endometrial tissues was higher on day 0 and 18 (P < 0.05) (Table 3). However, ER levels in pregnant endometrial tissues continuously decreased from day 10 to 18 (P < 0.05). The transcription level of ER was detectable in 20–60% of embryos (Table 4). The higher percentage of detectable ER levels was found on day 14. The ER transcript level in embryos generally decreased from day 10 to 18.

**TABLE 1. Platelet-Activating Factor Receptor (PAFr) Expression Levels in Porcine Endometrial and Embryonic Tissues**

<table>
<thead>
<tr>
<th>Day</th>
<th>nPE (× 10⁵)</th>
<th>PE (× 10⁵)</th>
<th>Em (× 10⁵)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>a13.75 ± 2.50</td>
<td>b0.80 ± 0.63</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
<td>b10.50 ± 5.20</td>
<td>a8.00 ± 1.41</td>
<td>b0.23 ± 0.23</td>
</tr>
<tr>
<td>12</td>
<td>c7.33 ± 1.53</td>
<td>b8.75 ± 4.27</td>
<td>b0.88 ± 0.48</td>
</tr>
<tr>
<td>14</td>
<td>a6.00 ± 0.82</td>
<td>a12.40 ± 7.96</td>
<td>a2.00 ± 0.60</td>
</tr>
<tr>
<td>16</td>
<td>a17.00 ± 8.54</td>
<td>b21.50 ± 19.47</td>
<td>a3.00 ± 1.15</td>
</tr>
<tr>
<td>18</td>
<td>a19.50 ± 9.88</td>
<td>b0.55 ± 0.10</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Same superscript letters in each column are not significantly different (P > 0.05, n = 3–5). Differences in PTL among nPE, PE, and Em are significant (P < 0.05), nPE, nonpregnant endometrium; PE, pregnant endometrium; Em, embryos. N/A, not available.
Transcripts of the PAFr Gene and Their Expression in Different Tissues

The full-length of 5'-UTR was obtained from cDNA, with different transcription start sites (TSSs). By comparing cDNA and genomic DNA sequences of 5'-UTR, locations of introns were determined. Two groups of 5' noncoding exons (exon 1 and exons 2 and 3) were alternatively spliced to a common splice acceptor site on exon 4 to produce two different transcripts (Fig. 1). Two TSSs were identified in transcript 2, which consisted of exon 2, exon 3, and exon 4. Two sites between exon 1 and exon 4 were for an intron to intervene. In promoter 1 directing the expression of transcript 1, three nuclear hormone receptor binding sites were suggested using GEMS Launcher 3.2 (Fig. 1A). (Genomatix Software GmbH, 1998–2002). (Genomatix Software GmbH, 1998–2002).

Structure of PAFr gene expressing white blood cell (WBC)-type transcript-1. Three TSSs were found in transcript 1 of the PAFr gene. Transcript 1 was composed of exon 1 and exon 4, and exon 4 included the full-length cds of PAFr gene. One site between exon 1 and exon 4 was for an intron to intervene. In promoter 1 directing the expression of transcript 1, three nuclear hormone (estrogen, androgen, and progesterone) receptor-binding sites were suggested using GEMS Launcher 3.2 (Fig. 1A). (Genomatix Software GmbH, 1998–2002) as described in the website of http://www.genomatix.de/mat_fam.

Structure of PAFr gene expressing tissue-type transcript 2. Two TSSs were identified in transcript 2, which consisted of exon 2, exon 3, and exon 4. Two sites interrupted by introns were located between exons 2 and 3, or exons 3 and 4. Two TSSs were observed in exon 2. No nuclear hormone receptor binding sites were found in promoter 2 directing expression of transcript 2 using GEMS Launcher 3.2 (Fig. 1B). (Genomatix Software GmbH, 1998–2002).

Table 2. Correlation Between Receptor and Factor Concentrations in Tissues and ULF

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Receptor</th>
<th>E2 (mean ± SD)</th>
<th>PAF (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nPE</td>
<td>PAF</td>
<td>0.71 ± 0.96</td>
<td>0.96 ± 0.78</td>
</tr>
<tr>
<td>PE</td>
<td>PAF</td>
<td>0.67 ± 0.90</td>
<td>0.78 ± 0.80</td>
</tr>
<tr>
<td>Em</td>
<td>PAF</td>
<td>-0.78 ± 0.80</td>
<td>-0.78 ± 0.80</td>
</tr>
</tbody>
</table>

Concentrations of E2 and PAF were obtained from other studies, so R-values (Pearson coefficient) are given based on the statistical analysis of mean concentrations of factors and receptors.

Table 3. Porcine ER Expression Levels in Endometrial Tissues

<table>
<thead>
<tr>
<th>Day</th>
<th>nPE (× 10^2)</th>
<th>PE (× 10^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>^100.00 ± 0.00</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
<td>^62.67 ± 12.50</td>
<td>^90.00 ± 0.00</td>
</tr>
<tr>
<td>12</td>
<td>^38.67 ± 1.15</td>
<td>^28.67 ± 10.07</td>
</tr>
<tr>
<td>14</td>
<td>^55.33 ± 30.02</td>
<td>^24.67 ± 11.55</td>
</tr>
<tr>
<td>16</td>
<td>^56.67 ± 20.82</td>
<td>^15.33 ± 4.62</td>
</tr>
<tr>
<td>18</td>
<td>^84.33 ± 19.14</td>
<td>^15.33 ± 4.62</td>
</tr>
</tbody>
</table>

Same superscript letters are not significantly different in each column (P > 0.05, n = 3).

Table 4. Porcine ER Expression Detectable in Peri-Implantation Embryos

<table>
<thead>
<tr>
<th>Day</th>
<th>Copies/50 ng RNA</th>
<th>% Embryo samples with detectable ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>7.0 ± 0.00</td>
<td>^33 (1/3)</td>
</tr>
<tr>
<td>12</td>
<td>4.58 ± 1.91</td>
<td>^33 (3/9)</td>
</tr>
<tr>
<td>14</td>
<td>7.0 ± 9.12</td>
<td>^60 (3/5)</td>
</tr>
<tr>
<td>16</td>
<td>1.0 ± 0.00</td>
<td>^25 (1/4)</td>
</tr>
<tr>
<td>18</td>
<td>3.0 ± 0.00</td>
<td>^20 (1/5)</td>
</tr>
</tbody>
</table>

Same superscript letters are not significantly different in each column (P > 0.05).

Sequence data were submitted to GenBank including promoters 1 and 2, exons 1, 2, and 3 and partial introns (GenBank accession number AF460978, AF460979, and AF460980).

Distribution of two different transcripts in porcine tissues. Transcript 1 was detected in all tissues examined including liver, spleen, skeletal muscle, heart, lung, white blood cells, small intestine, endometrium, and kidney. The levels of transcript 1 were high in all tissues and cells examined (Fig. 4). Transcript 2 was also observed in all tissues but white blood cells. Transcript-2 level was very high in skeletal muscle and heart, but very low in spleen (Fig. 4).

Chromosomal Location of PAFr Gene

A specific signal appearing as bright double spots on both chromatids at a specific chromosomal region on porcine chromosome 6 was obtained in at least 90% of the metaphase plates scored. The measurements of the relative position of the fluorescent spots on this chromosome indicated that PAFr is located in the q26-q27 region (Fig. 5).

Discussion

An ER was found in promoter 1 (Kumar and Chambon, 1988). Androgen and progesterone receptor binding sites were also found in promoter 1 (Nelson et al., 1999). No nuclear hormone receptor binding sites were suggested in human PAFr gene promoter 1 (Mutoh et al., 1994a). Human PAFr transcript 2 rather than transcript 1 was up regulated by estrogen via an ERE found in promoter 2 (Mutoh et al., 1993, 1994b). Transcript 1 of porcine PAFr gene appeared to be more closely related to porcine reproduction than transcript 2, although transcript 1 was also detected in various tissues. Regulation

Fig. 4. Detection of porcine PAFr transcripts 1 and 2 in different tissues by RT-PCR. Lane M: phi174 DNA/Hae III markers; lane 1, negative control without any template; lanes 2–10, amplified PAFr transcript-1 cDNA, 228 bp long and transcript 2 cDNA, 210 bp long from tissues including liver, spleen, skeletal muscle, heart, lung, white blood cells, small intestine, endometrium, and kidney.
of the \( \text{PAFr} \) gene by steroid hormones may be species-specific.

A universal motif was observed starting with the first TSS in transcript 1, which is 5'-ACTTCTGCAATT-3'. This element was also found in transcript 1 in the human (Mutoh et al., 1993), cow, dog, and sheep (data not shown), indicating that this motif is a highly conserved element in transcript 1. Its function remains to be determined. Three TSSs were found in porcine transcript 1, as with human PAFr transcript 1 (Mutoh et al., 1993). No universal motif was found in the 5'-UTR of transcript 2 when porcine and human TSSs were compared. Porcine PAFr transcript 2 was highly expressed in heart and skeletal muscle. The total expression level of PAFr in skeletal muscle was very low compared to that in other tissues (Yang et al., 2002). So transcript 2 may be a major expression form of PAFr gene in skeletal muscle. In addition, transcript 2 may also play an important role by mediating PAF responsible for electrophysiological actions on cardiomyocytes or cardiac dysfunctions (Montrucchio et al., 2000).

The PAFr gene was localized on chromosome 1p35-p34.4 in the human (Seyfried et al., 1992; Chase et al., 1996), and on the R-positive D2.2 band of chromosome 4 in the mouse (Ishii et al., 1996). This gene in pigs was mapped to chromosome 6q26-q27 using FISH analysis. We previously found this gene was located on chromosome 6q22-q23 using a cytogenetic assay (Yang et al., 2002). The apparent discrepancy was deemed due to the use of different mapping techniques. The cytogenetic technique is considered by some researchers to be less accurate than FISH. The cytogenetic contents of each hybrid clone have been determined by reverse painting after pPRS-PCR amplification (Yerle et al., 1996). The painted regions were measured "manually" (as the Cytovision analysis system was not available at that time). Consequently, it could be that some inaccuracies were due to the measurement technique of the painted regions. However, there is general agreement in results of both procedures.

Based on previously obtained concentrations of PAF and \( E_2 \) in ULF, endometrial and embryonic tissues, the correlation between PAF or \( E_2 \) with PTL or ER was summarized (Table 2).

The pattern of the temporal expression of PAFr in nPE was apparently associated with the pattern of changes in \( E_2 \) levels in nULF (\( R = 0.71 \)) and PAF concentrations in nPE (\( R = 0.96 \)). \( E_2 \) was reported to up-regulate PAFr expression levels in cultured human uterine endometrial cells (Sato et al., 1996), and the highest expression level of PAFr transcripts was detected in human endometrium in the mid-late proliferative and late secretory phases of the menstrual cycle (Ahmed et al., 1998). \( E_2 \) and PAF promoted PAFr expression (Mutoh et al., 1994a,b). PAFr expression levels in nPE and PE was on the order of 10^3 copies/20 ng total RNA, Table 1 shows there are differences among days by pregnancy status, thus confirming that the role of PAFr in nPE mediating PAF is also important. The PAFr protein was detected in bovine nonpregnant endometrium (Tiemann et al., 2001). PAF in nonpregnant endometrial tissues acted as an endogenous PGF2\alpha pulse-generator to maintain normal ovine estrous cyclicity by causing luteolysis (Chami et al., 1999). Therefore, \( E_2 \), PAF, or progesterone may regulate PAFr transcript 1 expression to mediate PAF so that via corresponding levels of PAFr, PAF can play a role in maintenance of porcine estrous cycle.

The pattern of changes in PTL in PE was positively associated with PAF content in PE (\( R = 0.90 \)). After day 13, PAF concentrations in pULF also rapidly decreased by day 18 (Mook et al., 1998). High PAFr transcript levels may result in more receptor molecules on cellular membranes to mediate PAF signaling. This gives rise to an increase in intracellular calcium mobilization by IP3 (Ahmed and Smith, 1992), which leads to inhibition of release of PAF into pULF (Billah et al., 1985). Higher concentrations of PAF and high levels of PAFr in PE may play an important role in embryo implantation.

PTL in embryos increased from day 10 to 16 (\( P < 0.05 \)) and significantly decreased by day 18 (\( P < 0.05 \)). In embryos PAF concentrations were much higher on day 18 compared to days 12, 14, and 16 (Diehl, unpublished data), however, a negative correlation existed between PAFr and PAF in embryonic tissues (Table 2, \( R = -0.78 \)). It seems likely then that embryos from day 12 to 16 decreased PAF synthesis since porcine blastocysts significantly reduced PAF synthesis in vitro compared to earlier stage embryos (Diehl, unpublished data). Per-implantation embryos may employ a certain mechanism to increase transcriptional activities and maintain low PAF synthesis activity to most efficiently meet the rapid cell division demands. The presence of PAFr gene in 2-cell mouse embryos was previously reported (Roudebush et al., 1997). PAF antibody markedly
reduced mouse embryo development (Roudebush et al., 1994). This suggested an important role of PAF mediated via PAF receptors in embryo development. Embryo-derived PAF via PAFr in an autocrine/paracrine fashion promoted embryo development.

The transcript level of ER in nPE was significantly higher on days 0 and 18 (~P < 0.05), appearing positively associated with temporal changes in E2 in nULF by stage of the estrous cycle, in particular, follicular growth (Table 2, R = 0.67). The ovine uterus did not respond to PAF if it was not estrogen and/or progesterone-primed (Chami et al., 1999) and E2 up-regulated the PAFr expression (Sato et al., 1996). It thus suggested that E2 and PAF might cooperate to maintain the estrous cycle via corresponding levels of respective receptors in an interactive manner.

The highest level of ER transcripts on day 10 (~P < 0.05) in PE was a pre-requisite for ongoing production of a high level of ER proteins. The high level of nuclear ER proteins along with the high level of E2 in pULF on day 12 reflected the conceptus-maternal communication resulting in a signal to establish pregnancy. This result is consistent with reports regarding the important role of E2 in maternal recognition of pregnancy (Geisert et al., 1990, 1993). After day 12, the E2 levels in pULF decreased by day 16 (Diehl, unpublished data). During this period ER transcript levels also decreased in the endometrium. In cultured human endometrial cells, E2-induced up-regulation of ER expression was prevented by progesterone (Prange-Kiel et al., 2001). Progesterone was higher in pregnant porcine serum (25–35 ng/ml, Diehl, unpublished data; Stone and Seamark, 1985). This suggested that the decrease in E2 in pULF and the increase in progesterone in pregnant serum may be responsible for the decrease in the ER level in PE. The ER transcript was detected in embryos on days 10, 12, 14, 16 and 18, but not all embryos on each day had a detectable level of ER transcripts. The transcript level on each day was estimated from ER-transcript-detectable embryos. A higher percentage of embryos with detectable ER was found on day 14 (~80%, ~P > 0.05), suggesting that the high E2 concentration in pULF on day 12 may up-regulate embryos to express more ER. ER was detected in porcine preimplantation embryos (Ying et al., 2000), indicating that E2 is important to pre-implantation embryo development. ER transcription levels in peri-implantation embryos, however, decreased to undetectable levels in most embryos after day 14. Along with advancing peri-implantation stage, due to decreases of ER transcript levels in PE and embryos, and E2 in pULF, PAF may become a dominant factor to enhance porcine peri-implantation development.

The organization and mapping of the porcine PAFr gene and the temporal expression of PAFr and ER in porcine endometrial and embryonic tissues were studied. Combining the data previously obtained in our lab, results suggested that PAF and E2, mediated via differentially expressed PAFr and ER, played a synergistic role in maintaining normal estrous cycle, establishing pregnancy and continuing embryo development and implantation.

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REFERENCES


