Identification of Ligand for Salivary Lipocalin Secreted from the Uterine Endometrium during Early Pregnancy in Pigs

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ABSTRACT

Salivary lipocalin (SAL1) is a member of the lipocalin protein family that has a property to associate with many lipophilic molecules and was identified as pheromone-binding protein in pigs. Our previous study has shown that SAL1 is expressed in the uterine endometrium in a cell type- and implantation stage-specific manner and secreted into the uterine lumen in pigs. However, function of SAL1 in the uterus during pregnancy in pigs is still not known. To understand physiological function of SAL1 in the uterine endometrium during pregnancy in pigs, it needs to elucidate the ligand(s) for SAL1. Thus, to identify the ligand for SAL1 in the porcine uterus, we collected uterine luminal fluid from pigs on day 12 of pregnancy by flushing with PBS. Proteins from the uterine luminal fluid were separated by ion exchange chromatography and gel filtration. Fractions containing SAL1 protein were pooled and concentrated. Immunoblot analysis confirmed successful purification of SAL1. Then, we extracted lipids from the purified SAL1 protein and analyzed the lipids by liquid chromatography-mass spectrometry, and predicted to be steroid hormones and prostaglandins as SAL1 ligands. Results in this study showed that SAL1 protein in the uterine secretions has a small lipophilic molecule as a natural ligand. Further characterization of ligand extracted from purified SAL1 will be useful for understanding physiological function of SAL1 during pregnancy and its application to increase the pregnancy rate in pigs.

(Key words : Pig, Uterus, Endometrium, Implantation, SAL1)

INTRODUCTION

Pheromone-binding proteins (PBPs), including odorant-binding protein (OBP) and major urinary protein (MUP), seem to play important roles in chemical communication between conspecifics (Pelosi, 2001). They are secreted abundantly into biological fluids involved in chemical communication, such as urine, saliva and vaginal secretion, to deliver pheromones. In pigs, a PBP was identified from the boar submaxillary gland that has steroid pheromones, 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol as endogenous ligands (Marcheses *et al*., 1998; Loebel *et al*., 2000). This protein was named as salivary lipocalin (SAL1) and, like other PBPs, belongs to lipocalin protein family that has a common structural β-barrel feature, which allows these proteins to bind lipophilic molecules and primarily acts as transporters in aqueous biological fluids (Flower, 1996). Study to determine tissue distribution of *SAL1* expression showed that the *SAL1* gene is expressed in the boar submaxillary gland, but not in the female tissues studied (Loebel *et al*., 2000), indicating that SAL1 is male-specific PBP in pigs.

Maternal recognition of pregnancy in pigs occurs approximately on day (D) 12 of pregnancy, which is critical for embryo implantation and the establishment of pregnancy. During this period, proper environment in uterine lumen composed of proteins, carbohydrates, lipids, and ions are required for the development of the conceptus (Spencer *et al*, 2004). Interestingly, our previous study to search genes differentially expressed in the endometria on D12 of pregnancy compared to those on D12 of the estrous cycle showed that the *SAL1* gene was expressed in the uterine endometrium with higher levels on D12 of pregnancy compared to those on D12 of the estrous cycle (Ka *et al*., 2009). Further study to determine expression pattern of *SAL1* in the uterine endometrium during the estrous cycle and pregnancy in pigs showed that *SAL1* is expressed in the uterine endometrium in a cell type- and pregnancy stage-specific manner and that SAL1 protein is secreted into uterine

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lumen, suggesting its important role in the establishment of pregnancy (Seo and Ka; unpublished data). However, function of SAL1 in the uterus during pregnancy in pigs is still not known.

Because SAL1 is a member of the lipocalin family that binds to lipid molecules and acts as transporter, we have speculated that SAL1 may be also a transporter of lipid molecules present in uterine lumen to the developing conceptus in pigs. Thus, it is essential to determine the ligand(s) for SAL1 to understand the physiological function of SAL1 in the uterine endometrium during pregnancy in pigs. To identify the ligand for SAL1 in the porcine uterus, we purified SAL1 protein from uterine flushings on D12 of pregnancy, extracted lipids from the purified SAL1 and analyzed the lipids by liquid chromatography-mass spectrometry (LC-MS).

MATERIALS AND METHODS

1. Purification of SAL1 from Uterine Luminal Fluid

Uterine luminal fluids from pigs on D12 of pregnancy were collected by flushing with 25 ml PBS (pH 7.2) per horn followed by centrifugation at 2,000 rpm for 5 min. SAL1 protein purification from uterine luminal fluids was performed according to previous report (Marcheses *et al*., 1998). Four milliliter of uterine flush was chromatographed through a carboxymethyl sepharose (Amersham Pharmacia Biotech, Piscataway, New Jersey) column. Elution was performed using a linear gradient of 0 to 1 M NaCl in 20 mM Tris/HCl (pH 7.4). Each fraction was analyzed by dot blotting using rabbit polyclonal anti-SAL1 antiserum (kindly provided by Dr. P. Pelosi, University of Pisa, Italy). Fractions containing SAL1 was pooled and concentrated using a centrifugal concentrator (Centriplus; Millipore, Bedford, MA). Concentrated SAL1 solution was applied to a gel-filtration using Superose-12 (Amersham Pharmacia Biotech) column and eluted with 50 mM ammonium bicarbonate. Each fraction was analyzed again by dot blotting, and fractions containing SAL1 protein were pooled, concentrated, and analyzed by immunoblotting.

2. Lipid Extraction from Purified SAL1 Protein

Lipid molecules were extracted from purified SAL1 protein according to method devised by Bligh and Dyer (1959). Briefly, chloroform:methanol (1:2 v/v) was added to SAL1 protein sample. Sample was vortexed and centrifuged at 1,550 × g for 10 min to pellet insoluble material. Chloroform and water was added to the supernatant recovered. Sample was again vortexed and centrifuged. From resulting two liquid phases, organic lower phase was recovered and solvent were removed under vacuum in a rotary evaporator. The dried extract was reconstituted with 300 μl methanol.

3. LC/MS Analysis

LC was performed on a Hewlett Packard Series 1100 HPLC (Hewlett-Packard, Germany). Twenty microliters of lipid extract was chromatographed on a Capcell pak C18 column (250 × 4.6 mm inside diameter, 5 μm particle size; Shiseido, Japan). For fractionating the analytes, the phase A was acetonitrile and the phase B was water. The initial composition of mobile phase was 30% phase A that was increased to 70% after 24 min. The flow rate of the mobile phases was 1 ml/min. MS performed on a QUATTRO Triple Quadrupole Tandem Mass Spectrometer (Waters Micromass, U.K.) through electrospray ionization (ESI) source. The source was operated in the negative ionization mode. The capillary voltage was set at 2600 V, source temperature 80℃, desolvation temperature 200℃, and cone voltage 30 V.

RESULTS

1. Purification of SAL1 Protein from Uterine Luminal Fluid

To identify natural ligand of SAL1 protein present in uterine lumen, we purified SAL1 protein from uterine luminal fluid using chromatography, extracted lipid from purified SAL1, and analyzed the lipid by LC-MS (Fig. 1). Uterine flush obtained from pig on D12 of pregnancy was separated by ion-exchange chromatography and gel filtration to purify SAL1 protein. By dot blot analysis of each fraction, fractions 9 to 18 containing

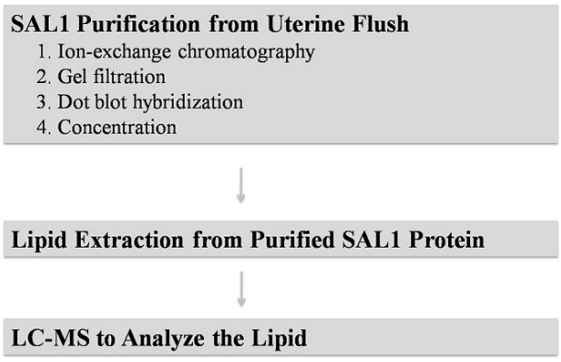

Fig. 1. Schematic overview illustrating identification of ligand for SAL1 in the uterus in pigs.

SAL1 protein were identified (Fig. 2A), pooled, and concentrated. Immunoblot analysis of concentrated SAL1 solution detected a band of SAL1 with a relative molecular mass of 20,000 (Fig. 2B), indicating that SAL1 protein was successfully purified from the uterine secretions.

2. Characterization of Natural Ligand of Uterine Luminal SAL1 Protein

To characterize the lipid extracted from purified SAL1 protein, we performed LC-MS. HPLC chromatogram of SAL1 ligand extraction showed one major peak eluting at retention time (RT) = 13.885 min (Fig. 3A). Lipid sample eluting at RT = 13.885 appeared to be constituted by one molecule with m/z 349 (Fig. 3B), which was predicted to be a type of steroid hormones or prostaglandins.

DISCUSSION

The uterine endometrial epithelial cells including luminal epithelia and glandular epithelia secrete various molecules, such as protease inhibitors, growth factors, cytokines, and adhesion molecules to constitute uterine secretions or histotrophs, which is important for conceptus development particularly in the species having long pre-implantation period (Geisert and Yelich, 1997). During the peri-implantation period in pigs, conceptus signals for maternal recognition of pregnancy lead to compositional change in uterine secretions to support development of the conceptus. Our previous studies (Ka *et al*., 2009; Seo and Ka, unpublished data) showed that SAL1 is a component of uterine secretions and increased in the uterine luminal fluid at the time of maternal recognition of pregnancy, which play an important role for normal conceptus development. However, the function of SAL1 in the uterus in pigs remains unknown.

Members of the lipocalin protein family show low homology in amino acid sequences between them but share structural β-barrel feature (Flower, 1996). Due to their β-barrel structure, lipocalins can bind to small hydrophobic molecules. Physiological function of lipocalins varies depending on both their tissue distribution and their endogenous ligand i.e. retinol transport, cryptic coloration, olfaction, pheromone transport, and the enzymatic synthesis of prostaglandins. Retinol-binding protein (RBP), a well-studied lipocalin known to act as a transporter of retinol to developing conceptuses, was purified from uterine luminal

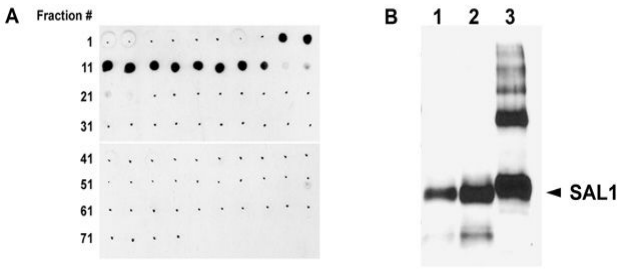

Fig. 2. Purification of SAL1 from uterine flushing using ion exchange chromatography and gel filtration. A. Dot blot hybridization for SAL1. Four milliliters of uterine flush obtained from pig on D12 of pregnancy was separated by ion-exchange chromatography and gel filtration to purify SAL1 protein. Two microliters of each fraction was dotted on nitrocellulose membrane. Fractions containing SAL1 was detected using anti-SAL1 serum. B. Immunoblot analysis for SAL1 from purified protein solution. 1, 2 μl purified protein solution; 2, 4 μl purified protein solution; 3, recombinant SAL1 protein.

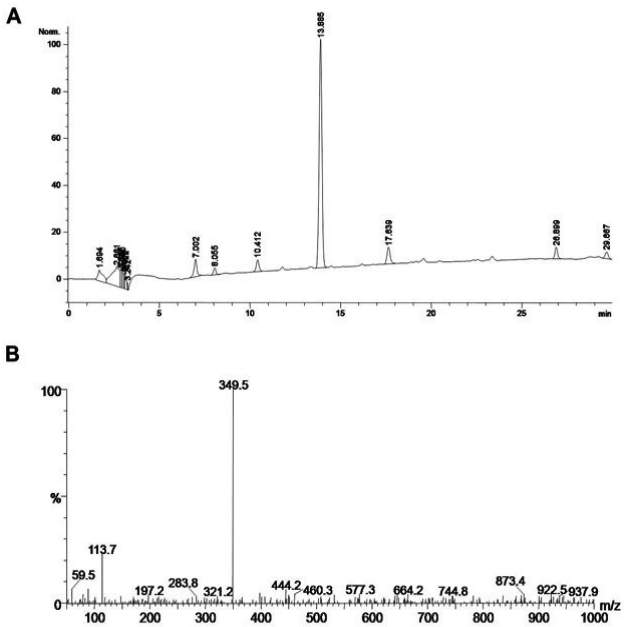

Fig. 3. Identification of ligand for purified SAL1 in the porcine uterus by liquid chromatography - mass spectrometry. A. Total ion chromatogram of ligand extraction from purified SAL1. Ligand extract was separated on a Capcell pak C18 column. The flow rate of the mobile phases was 1 ml/min. HPLC chromatogram of SAL1 ligand extraction showed one major peak eluting at retention time (RT) = 13.885 min. B. Mass spectrum of peak with RT = 13.885 min. Mass spectrometer coupled with electrospray ionization source was operated in the negative ion mode. Lipid sample eluting at RT = 13.885 appeared to be constituted by one molecule with m/z 349.

fluid in pigs using chromatography (Clawitter *et al*., 1990). Mass spectrometry of ligand extracted from natural protein showed that boar SAL1 protein purified from submaxillary gland has pheromones, 5 α-androst-16-en-3-one and 5 α-androst-16-en-3 α-ol, as endogenous ligand (Marcheses *et al*., 1998; Loebel *et al*., 2000). Similarly to these studies, we carried out chromatography to purify SAL1 protein present in uterine lumen in pigs and mass spectrometry to characterize endogenous ligand extracted from purified SAL1. In the present study, we used uterine flushes obtained from pigs on D12 of pregnancy to purify SAL1 protein because SAL1 protein was abundantly detected during this period (Seo and Ka, unpublished data). Immunoblot analysis with purified protein solution detected a band of SAL1 with a relative molecular mass of 20,000, which is similar in size to that in boar SAL1 (Marcheses *et al*., 1998), indicating that SAL1 protein was successfully purified from the uterine secretions. It has been known that many other lipocalins as well as boar SAL1 have lipophilic molecule as endogenous ligand. Thus, we hypothesized that ligand of SAL1 in uterine lumen in pigs may also be small lipophilic molecule. For this reason, we extracted lipid from purified SAL1 to characterize the ligand. LC chromatogram with lipid extracted from purified SAL1 showed one prominent peak, indicating successful extraction of lipid from purified SAL1.

Information on the ligand of SAL1 is essential to understand the function of SAL1 in the uterus during pregnancy in pigs. Although boar SAL1 protein is known to act as a pheromone-binding protein in submaxillary gland, it may not the case for the function of SAL1 in the uterus because male sex pheromones, 5 α-androst-16-en-3-one and 5 α-androst-16-en-3 α-ol, have not been shown to be present in the uterine lumen during early pregnancy in pigs. Instead, steroid hormones, including progesterone, unconjugated estrogen (estrone, estradiol, estriol), conjugated estrogen (estrone sulfate and estradiol sulfate), unconjugated androgen (androstenedione, dihydrotestosterone, and testosterone), which are present in uterine lumen and show a similar structure to male sex pheromones, might be the ligand of uterine SAL1 in pigs (Bazer *et al*, 1979; Heap *et al*., 1979). In agreement with this, results of this study showed that a small lipophilic molecule with m/z 349 was determined as the ligand of SAL1, which is similar in size to that of steroid hormones. Other lipid molecules such as prostaglandins (Spencer *et al*., 2004) and lysophosphatidic acids (Seo *et al*., 2008) present in the uterine lumen at the time of SAL1 production might also be possible candidate for SAL1 ligands. Further study to characterize the ligand of uterine SAL1 in pigs is needed. Currently, we are working on the generation of recombinant SAL1 to determine the ligand(s) for SAL1 *in vitro*.

In summary, we characterized, in part, the endogenous ligand of SAL1 released from uterine endometrium in pigs using chromatography and mass spectrometry. Although further characterization of the ligand is still needed, information on the lipophilic ligand that SAL1 binds to in the uterine luminal fluid is useful to understand the function of SAL1 in the uterus during the implantation period in pigs.

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