Increase of essential amino acids in the bovine uterine lumen during preimplantation development

Anna E Groebner, Isabel Rubio-Aliaga, Katy Schulke, Horst D Reichenbach, Hannelore Daniel, Eckhard Wolf, Heinrich H D Meyer and Susanne E Ulbrich

Physiology Weihenstephan, Z I E L Research Center for Nutrition and Food Sciences, Technische Universitaet Muenchen, Weihenstephaner Berg 3, 85354 Freising, Germany, Molecular Nutrition Unit, Z I E L Research Center of Nutrition and Food Sciences, Technische Universitaet Muenchen, Gregor-Mendel-Straße 2, 85354 Freising, Germany, Institute of Animal Breeding, Bavarian State Institute for Agriculture, Prof.-Duerrwaechter-Platz 1, 85586 Grub, Germany and Chair for Molecular Animal Breeding and Biotechnology, and Laboratory for Functional Genome Analysis (LAFUGA), Gene Center, Ludwig-Maximilians-Universitaet Muenchen, Feodor-Lynen-Strasse 25, 81377 Muenchen, Germany

Correspondence should be addressed to S E Ulbrich; Email: ulbrich@wzw.tum.de

Abstract

Amino acids (AAs) are crucial for the developing conceptus prior to implantation. To provide insights into the requirements of the bovine embryo, we determined the AA composition of the uterine fluid. At days 12, 15, and 18 post-estrus, the uteri of synchronized pregnant and non-pregnant Simmental heifers were flushed for the analysis of 41 AAs and their derivatives by liquid chromatography–tandem mass spectrometry. The ipsilateral endometrium was sampled for quantitative PCR. In addition to a pregnancy-dependent increase of the essential AAs ($P<0.01$), we detected elevated concentrations for most non-essential proteinogenic AAs. Histidine (His) and the expression of the His/peptide transporter solute carrier 15A3 ($SLC15A3$) were significantly increased at day 18 of pregnancy in vivo. In addition, $SLC15A3$ was predominantly stimulated by trophoblast-derived interferon-$\gamma$ in stroma cells of an in vitro co-culture model of endometrial cells. Our results show an increased concentration of AAs most likely to optimally provide the elongating pre-attachment conceptus with nutrients.

Reproduction (2011) 141 685–695

Introduction

Amino acids (AAs) are components of enzymes, cytokines, and several hormones that further contribute to multiple cellular and metabolic functions (Supplementary Table 1, see section on supplementary data given at the end of this article). They further play important roles in catalytic functions, synthesis of purines, and pyrimidines as well as organic osmolytes. Mammals rely on essential AAs for proper growth and development, but even semi-essential AAs such as $\ell$-arginine (Arg), $\ell$-histidine (His), and $\ell$-tyrosine (Tyr) may become limited during a number of developmental stages such as during rapid growth and development or stressful conditions. Various transporter systems that carry cationic, anionic, aromatic, or aliphatic AAs with different affinities for their substrates and with overlapping substrate specificity have been identified as AA delivery systems (reviewed in Van Winkle (2001)) and allow selective accumulation of nutrients and further components in the uterine lumen as shown previously for cycling and early pregnant ewes (Gao et al. 2009a, 2009b).

Cattle exhibit a late implantation starting with the initial apposition at day 17 post-insemination (King et al. 1981). During the prolonged preimplantation phase, the growing conceptus is solely dependent on the nutrient supply from secretions of the uterine glands and selective transport of nutrients into the uterine lumen. A specific temporal supply with essential and non-essential AAs is vital to promote blastocyst formation and hatching both in vivo and in vitro (Liu & Foote 1995, Partridge & Leese 1996, Steeves & Gardner 1999). Depletion or an insufficient supply of essential as well as non-essential AAs resulted in altered development of bovine embryos in vitro (Steeves & Gardner 1999). For instance, a reduction of $\ell$-methionine (Met) decreased intracellular concentrations of glutathione in in vitro produced bovine embryos, although this did not affect apoptosis, methylation status, or cleavage rate (Bonilla et al. 2010). Whereas non-essential AAs seem to be crucial for cleavage of the zygote, blastocoeel formation, and blastocyst hatching, essential AAs are particularly important for the complex development of the inner cell mass of murine embryos (Lane & Gardner 1997).
In ruminants, the developing trophoblast undergoes enormous growth prior to tight attachment to the maternal endometrium with the transport of nutrients governed by the endometrium into the uterine lumen. The expression of AA transporter systems in the trophoectoderm and primitive endoderm of the conceptus permits the AA uptake from the uterine lumen and adequate proliferation and differentiation (Gao et al. 2009a, 2009b). In cattle, the composition of AAs in the uterine fluid during the secretory phase has recently been investigated (Hugentobler et al. 2007); however, physiological concentrations of AAs during the bovine preimplantation phase have not been determined yet. Thus, we analyzed whether the presence of the rapidly elongating bovine conceptus would lead to a modulation of the composition of intrauterine AAs prior to implantation. To inquire the participation of endometrial transport mechanisms, the expression levels of AA transporters and metabolic enzymes were additionally investigated.

Results

Total protein (TP) content (Fig. 1A) and total free AAs (Fig. 1B; calculated by the sum of all measured AAs) varied in uterine flushings of pregnant and non-pregnant heifers. The overall abundance of TP was affected by the status (P=0.04) as well as by the day of the cycle (P=0.04) and declined from days 15 to 18 in pregnant and non-pregnant animals. Overall total AA amounts were affected by the day of the cycle (P=0.03) and were greatest at days 12 and 15 and declined thereafter from days 15 to 18. The amounts of TP and total AAs significantly correlated (Fig. 1C; r = 0.82, P<0.0001).

Non-essential neutral AAs in the uterine lumen and expression of the respective transporters in the endometrium

The small neutral AA glycine (Gly; Fig. 2A) was most abundant in the uterine lumen (236 nmol/mg TP ±32), followed by taurine (Tau; Fig. 3A; 163 nmol/mg TP ±19), l-serine (Ser; Fig. 2B; 100 nmol/mg TP ±22), and l-glutamine (Gln; Fig. 2E; 85 nmol/mg TP ±11). Pregnant animals had greater levels of Gly at day 15 (P<0.05) than the respective controls (Fig. 2A). Ser (Fig. 2B) increased continuously from days 12 to 18 in uterine flushings of the pregnant animals (day P=0.01) and were 2.2- and 2.4-fold greater in pregnant than in non-pregnant animals at days 15 and 18 respectively (status P=0.0008). For l-proline (Pro; Fig. 2C), greater abundances were detected at days 15 and 18 in pregnant animals (status P=0.001). l-alanine (Ala; Fig. 2D) had overall greater abundances in uterine flushings from pregnant than non-pregnant animals (status P=0.004). In addition, Ala amounts were influenced by the day of the cycle (P=0.02). For both Gln and l-asparagine (Asn; Fig. 2F), the amounts were greater at day 18 in uterine flushings of pregnant animals (Gln: day P=0.0001, status P=0.02, and Asn: P=0.002, status P=0.02). The expression of the solute carrier 1A5 (SLC1A5), which mainly transports small neutral AAs such as Ala, Ser, and l-cysteine, increased from days 12 to 18 (day P=0.0001; Table 1).

Although l-glutamine (Glu; Fig. 2G) and l-aspartate (Asp; Fig. 2H) amounts in the uterine flushings were larger in pregnant animals at day 18 (status P=0.03 and P=0.04 respectively), similar levels for both the groups were detected at day 15. Moreover, amounts of Asp were affected by the day of the estrous cycle (day P=0.008). The acidic AA transporter SLC1A1 and the glutamic-pyruvate-transaminase (GPT; Table 1) mRNA levels were influenced neither by the day of the cycle nor by the pregnancy status in the endometrium. In conceptuses, the GPT mRNA levels were not different between days 15 and 18 (P>0.05) (mean ΔCq ± S.E.M.: 23.0 ±0.0 and 23.0 ±0.1 respectively; data not shown). However, GPT mRNA was expressed slightly greater in conceptuses (mean Cq 26.0) than in the corresponding endometrium samples (mean Cq 26.8) (data not shown). The cytosolic enzyme glutathione synthetase (GSS) catalyzes the formation of glutathione as a primary intracellular antioxidant. A pregnancy-dependent difference in mRNA expression levels was not observed (Table 1).
Non-essential neutral amino acids

![Graphs of non-essential neutral amino acids](image)

**Essential neutral AAs in the uterine lumen and expression of the respective transporters in the endometrium**

Although L-threonine (Thr) levels (Fig. 4A) declined in the uterine lumen of non-pregnant animals over time, increasing amounts were detected in pregnant animals, reaching 2.1- and 3.9-fold greater amounts than in non-pregnant animals at days 15 and 18 respectively (status P<0.0001, day×status P=0.001). In the uterine flushings of non-pregnant animals, the aromatic AAs L-phenylalanine (Phe; Fig. 4B), Tyr (Fig. 4C), and L-tryptophan (Trp; Fig. 4D) remained almost constant over time. The Phe levels increased 3.7-fold in pregnant animals from days 12 to 18 and were 2.1- and 3.8-fold higher in pregnant versus non-pregnant animals at days 15 and 18 respectively (day P<0.0001, status P=0.0001, day×status P<0.0001). The Tyr and Trp amounts also increased in pregnant animals during the preimplantation period (5.3- and 4.0-fold respectively; Tyr: day P<0.0001, status P<0.0001, day×status P<0.0001; and Trp: day P=0.001, status P=0.0007, day×status P=0.004). Whereas Tyr levels were 2.0- and 4.5-fold greater in pregnant versus non-pregnant animals at days 15 and 18, Trp abundances were 2.0- and 2.8-fold at days 15 and 18 in pregnant versus non-pregnant animals. All branched chain AAs (BCAAs) L-leucine (Leu; Fig. 4E), L-valine (Val; Fig. 4F), and L-isoleucine (Ile; Fig. 4G) increased consistently and were 1.8-, 1.9-, and 17.6-fold greater at day 15 and 2.3-, 3.0-, and 46-fold greater in uterine flushings of day 18 in pregnant versus non-pregnant animals. In contrast, the expression of mRNAs for both large neutral AA transporters, SLC7A5 (Table 1) and SLC7A8, were not affected by day, status, or their interaction.

**Basic AAs and respective AA derivatives related to the urea cycle**

The basic AAs L-lysine (Lys; Fig. 5A) and Arg (Fig. 5C) were more abundant in uterine flushings from pregnant heifers. At day 18, the Lys and Arg levels were increased 5.8- and 13.5-fold, respectively, compared with day 12 due to effects of day (Lys P<0.0001, Arg P<0.0001), status (Lys P=0.002, Arg P=0.01), and their interaction (Lys P=0.002, Arg P=0.02). His levels (Fig. 5B) were not affected by day in cyclic heifers, but increased 4.8-fold between days 12 and 18 of pregnancy and were 1.7- and 3.9-fold greater in uterine flushings of pregnant versus non-pregnant animals at days 15 and 18 (day P<0.0001, status P<0.0001, day×status, P=0.0001). Ornithine (Orn) resulting from the metabolism of Arg by arginase was not affected by day or status (data not shown). However, L-citrulline (Cit; Fig. 5D) formed from Orn...
within the urea cycle was more abundant in uterine flushings from pregnant heifers on day 15 (day \( P = 0.04 \)). The abundance of the cationic transporter SLC7A1 mRNA was not affected by day or status (Table 1). However, expression of the putative His/peptide transporter, SLC15A3, increased in pregnant animals from days 12 to 18 and was 5.5-fold greater in pregnant than in cyclic heifers by day 18 (day \( P = 0.0003 \), status \( P = 0.002 \), day \( \times \) status \( P = 0.008 \); Table 1). Conceptuses showed a similar expression of SLC15A3 (mean \( C_q \) 22.6) compared with the maternal endometrium (mean \( C_q \) 22.9), and the transcript abundance did not differ between days 15 and 18 (\( P > 0.05 \); mean \( \Delta C_q \) ± S.E.M.: 26.6 ± 0.3 and 26.1 ± 0.1 respectively; data not shown).

**Non-proteinogenic AAs in the uterine lumen**

In the uterine lumen of non-pregnant animals, Tau (Fig. 3A) increased between days 15 and 18 of the cycle. At day 18, the amount of Tau was 1.5-fold greater in the uterine flushings of non-pregnant versus pregnant animals (day \( P = 0.008 \), day \( \times \) status \( P = 0.02 \)). The expression of the \( \beta \)-AA transporter SLC6A6 (Table 1) in the endometrium increased in animals between days 12 and 18 (day \( P = 0.002 \)). Cystathionine (Cth; Fig. 3B), which is an intermediate product in Met metabolism, decreased in uterine flushings from days 12 to 18 post-estrus in both the treatment groups (day \( P = 0.002 \); Table 1). Ethanolamine (EtN; Fig. 3C) originates from Ser and is a precursor molecule for \( \delta \)-phosphoethanolamine (PEtN; Fig. 3D), which is a component of phospholipids in biological membranes. Intrauterine EtN varied with the day of the cycle (day \( P = 0.009 \)) with 1.4- and 1.7-fold increased amounts in the uterine flushings of pregnant heifers at days 15 and 18. The amount of PEtN was 2.2-fold greater in the uterine flushings of pregnant heifers at day 18. Hydroxy-\( L \)-proline (Hyp) amounts were different with the day of the cycle (day \( P = 0.01 \); Fig. 3E). The co-product of Gly synthesis sarcosine (Sar) was 2.0-fold increased in uterine flushings of pregnant heifers (day \( P < 0.05 \); Fig. 3F). AA derivatives that were not depicted in Fig. 3: \( L \)-methyl-\( L \)-histidine (M1His), \( L \)-3-methyl-\( L \)-histidine (M3His), \( L \)-\( \alpha \)-amino-n-butyric acid (Abu), \( L \)-\( \alpha \)-aminoacidic acid (Aad), argininosuccinic acid (Asa), \( \beta \)-alanine (bAla), \( L \)-carnosine (Car), \( O \)-phospho-L-serine (Pser), homocitrulline (Hcit), delta-hydroxylysine

**Table 1** mRNA expression of amino acid transporter and metabolic enzymes in days 12, 15, and 18 of pregnant and non-pregnant heifers. Data shown represent means \( \Delta C_q \) ± S.E.M. Different superscript letters indicate significant differences over time in control and pregnant animals respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day</th>
<th>SLC1A1</th>
<th>SLC1A5</th>
<th>SLC6A6</th>
<th>SLC7A1</th>
<th>SLC7A5</th>
<th>SLC7A8</th>
<th>SLC15A3</th>
<th>GPT</th>
<th>GSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>21.7 ± 0.7</td>
<td>17.6 ± 0.5*</td>
<td>17.7 ± 0.6*</td>
<td>21.5 ± 0.2</td>
<td>16.6 ± 0.9</td>
<td>19.3 ± 0.7</td>
<td>20.0 ± 0.5</td>
<td>16.2 ± 0.6</td>
<td>15.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>22.5 ± 0.4</td>
<td>18.4 ± 0.3*</td>
<td>19.2 ± 0.3</td>
<td>21.6 ± 0.2</td>
<td>16.9 ± 0.2</td>
<td>19.6 ± 0.3</td>
<td>20.2 ± 0.2</td>
<td>16.9 ± 0.4</td>
<td>16.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>22.6 ± 0.4</td>
<td>19.0 ± 0.4*</td>
<td>19.5 ± 0.4*</td>
<td>21.8 ± 0.3</td>
<td>17.3 ± 0.5</td>
<td>19.8 ± 0.3</td>
<td>20.5 ± 0.4</td>
<td>17.0 ± 0.4</td>
<td>16.2 ± 0.4</td>
</tr>
<tr>
<td>Pregnant</td>
<td>12</td>
<td>22.1 ± 0.5</td>
<td>17.8 ± 0.3*</td>
<td>18.2 ± 0.3</td>
<td>21.5 ± 0.2a</td>
<td>16.7 ± 0.7</td>
<td>19.4 ± 0.3</td>
<td>20.0 ± 0.4a</td>
<td>16.5 ± 0.5</td>
<td>16.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>22.0 ± 0.2</td>
<td>18.8 ± 0.3b</td>
<td>18.9 ± 0.2b</td>
<td>22.0 ± 0.1b</td>
<td>16.6 ± 0.3</td>
<td>19.2 ± 0.2</td>
<td>20.7 ± 0.3a</td>
<td>16.7 ± 0.2</td>
<td>17.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>22.1 ± 0.2</td>
<td>19.7 ± 0.4b</td>
<td>19.6 ± 0.2b</td>
<td>21.9 ± 0.1b</td>
<td>17.7 ± 0.5</td>
<td>19.8 ± 0.2</td>
<td>23.0 ± 0.3b</td>
<td>16.8 ± 0.2</td>
<td>17.4 ± 0.4</td>
</tr>
<tr>
<td>( P ) value</td>
<td>Day</td>
<td>0.7</td>
<td>0.001</td>
<td>0.001</td>
<td>0.3</td>
<td>0.2</td>
<td>0.5</td>
<td>0.0003</td>
<td>0.5</td>
<td>0.07</td>
</tr>
<tr>
<td>Status</td>
<td>0.7</td>
<td>0.2</td>
<td>0.7</td>
<td>0.3</td>
<td>0.9</td>
<td>0.8</td>
<td>0.002</td>
<td>1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Day ( \times ) status</td>
<td>0.6</td>
<td>0.8</td>
<td>0.7</td>
<td>0.7</td>
<td>0.8</td>
<td>0.9</td>
<td>0.008</td>
<td>0.8</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>
Intrauterine amino acids during early pregnancy

Gene expression following interferon-τ treatment in an in vitro co-culture model of endometrial cells

To determine whether the trophoblast-derived interferon-τ (IFNT) stimulates SLC15A3 expression as observed in vivo, the transcript abundance was determined following recombinant IFNT stimulation within an in vitro co-culture model of endometrial glandular and stromal cells. Interestingly, SLC15A3 mRNA expression was increased in co-cultivated glandular epithelium (36-fold, \( P=0.001 \)) and foremost in co-cultivated stroma cells (177-fold, \( P<0.0001 \)) compared with the respective untreated co-cultured cells (Table 2). The SLC15A3 mRNA was expressed 6.1-fold greater in stroma cells (mean ΔCq 17.9) than in glandular epithelial cells (mean ΔCq 15.3) following IFNT treatment in the in vitro co-culture model.

Discussion

Malnutrition during pregnancy can severely affect embryonic/fetal development and may have negative effects on metabolic imprinting, thereby affecting the susceptibility to chronic diseases during adult life (Waterland & Jirtle 2004). An optimal supply of nutrients is thus critical for the pre-attachment conceptus and successful pregnancy outcomes. This study investigated changes for the first time in profiles of essential and non-essential AAs in bovine uterine flushings during early gravidity prior to conceptus attachment.

In accordance with Schultz et al. (1971), TP content varied in the uterine flushings of pregnant and non-pregnant heifers and decreased over the analyzed time points. Interestingly, total free AAs varied concomitantly, as total AA variation correlated well with the TP content (\( r=0.82, P<0.001 \)). Thus, to circumvent possible inaccuracies resulting from the flushing procedure due to differential consistency of the bovine uterine fluid or due to differential solubility of particular components over the analyzed time points, we normalized the AA data with respect to the respective TP.

Significant variation in abundances of Gly, Ser, Thr, Met, His, Tau, and of the aromatic AAs Phe and Tyr in addition to BCAAs Val, Ile, and Leu has been demonstrated in uterine fluids of cycling cows, comparing days 6, 8, and 14 after estrous (Hugentobler et al. 2007). In comparison, our data show that the differences on days 12, 15, and 18 of the estrous cycle within the pregnancy status is shown by different superscript letters (x, y, and z in cyclic animals and a, b, and c in pregnant animals) and was regarded as significantly different if \( P<0.05 \). Treatment effects at the respective time points are indicated (*) if \( P<0.05 \).
Basic amino acids and components of the urea cycle respectively

<table>
<thead>
<tr>
<th></th>
<th>L-lysine (Lys)</th>
<th>L-histidine (His)</th>
<th>L-arginine (Arg)</th>
<th>L-citrulline (Cit)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>Day</td>
<td>Control</td>
<td>Pregnant</td>
<td>Control</td>
<td>Pregnant</td>
</tr>
<tr>
<td>P</td>
<td>Status</td>
<td>Status</td>
<td>P</td>
<td>Status</td>
</tr>
<tr>
<td>12</td>
<td>P&lt;0.0001</td>
<td>Status P =0.002</td>
<td>Control</td>
<td>Pregnant</td>
</tr>
<tr>
<td>15</td>
<td>P&lt;0.0001</td>
<td>Status P =0.002</td>
<td>Control</td>
<td>Pregnant</td>
</tr>
<tr>
<td>18</td>
<td>P&lt;0.0001</td>
<td>Status P =0.002</td>
<td>Control</td>
<td>Pregnant</td>
</tr>
</tbody>
</table>

Figure 5 Basic AAs and components of the urea cycle in bovine uterine flushings are shown. L-lysine (A), His (B), Arg (C), and Cit (D) in uterine flushings of cycling and pregnant heifers at days 12, 15, and 18 post-estrus are presented as nmol/mg TP ± S.E.M. The effect of the day of the estrous cycle within the pregnancy status is shown by different superscript letters (x, y, and z in cyclic animals and a, b, and c in pregnant animals) and was regarded as significantly different if P<0.05. Treatment effects at the respective time points are indicated (*) if P<0.05.

Gao et al. 2009e), Gly was the predominant AA in the uterine lumen. Gly is inter-convertible to Ser and Ala and is furthermore necessary for protein and DNA synthesis. It most prominently serves as an energy source within the citrate cycle (Hobbs & Kaye 1985). However, in contrast to the ovine uterus (Gao et al. 2009e), a pregnancy-dependent increase did not occur possibly due to species-specific exigencies of Gly during trophoblast elongation. As ruminants share a number of regulatory pathways but obviously differ in definite components during this important phase, it is of prime importance to understand individual species differences and specific requirements with respect to the AA composition (Spencer et al. 2008).

The mammalian target of rapamycin (MTOR) signaling pathway actively regulates cell proliferation and migrations, as well as translation of mRNA to protein. Concentrations of energy substrates and AAs and other components such as progesterone and IFNT affect MTOR signaling (Long et al. 2005, Gao et al. 2009b). Among the BCAAs, leucine is the major AA regulating protein synthesis (Buse & Reid 1975) by affecting phosphorylation of proteins 4E-BP1 and S6K1 that are substrates of MTOR (Anthony et al. 2000). We detected elevated concentrations of all BCAAs and aromatic AAs due to pregnancy. These AA might enter the uterine lumen via the sodium-independent L-System transporter (preference for Leu) expressed in the microvillous and basal membrane of the ovine endometrium in order to support the development of the conceptus as shown in sheep (Gao et al. 2009b). The MTOR signaling influences the activity of members of several transport systems (e.g. System L, A, and Taut), modulates translation, and influences in this manner AA uptake in the placenta (Roos et al. 2009). We suggest a regulatory effect of MTOR on AA transport in maternal and conceptus tissues during the preimplantation period. The L-system is regarded as the main route for the transport of BCAAs and aromatic neutral AAs across plasma membranes, each displaying a different affinity (Grillo et al. 2008). The L-system transporters are heterodimeric and covalently associated with the glycoprotein 4F2hc/CD98. They act as obligatory exchangers and induce an overall change in relative composition of distinct AAs. An accumulation of distinct AAs is achieved by unidirectional transport systems (Verrey 2003). For both transporters analyzed in this study (SLC7A5: LAT1, and SLC7A8: LAT2), neither day nor status significantly affected expression levels. SLC7A5, located in the epithelium and stroma cells in sheep endometrium, exceeded the expression of SLC7A8 of pregnant ewes (Gao et al. 2009b); however, in the endometrium of cattle, SLC7A8 revealed a more pronounced expression without exhibiting a pregnancy-dependent regulation. Generally, SLC7A8 is abundantly located at the basal membrane of epithelium and acts in concert with cationic transporters to mediate the reabsorption of cationic AAs, whereas SLC7A5 is mainly expressed in apical membranes of epithelia. Possibly, reabsorption of cationic AAs might play a more prominent role in cattle than in the ewe prior to implantation (Gao et al. 2009b).

During early pregnancy, we found an increased presence of almost all AAs, foremost the essential AAs in the uterine lumen (Fig. 6). These essential AAs within the uterine lumen originate from blood and are transported through the vascular wall and the endometrial tissue to nourish the fast growing conceptus. We assume that the developing conceptus induces the increase in AA transport processes via several signaling molecules, primarily and most likely through the effects of progesterone and IFNT (Farin et al. 1990, Godkin et al.

Table 2 mRNA expression of interferon-τ (IFNT)-treated and -untreated co-cultured bovine glandular and stroma cells analyzed separately are depicted. mRNA expression of SLC15A3 is shown as means ΔCq±s.e.m. The relative increase of transcript abundance following IFNT treatment is depicted.

<table>
<thead>
<tr>
<th>mRNA expression (log2)</th>
<th>ΔCq mean ± s.e.m.</th>
<th>Fold increase</th>
<th>IFNT treatment</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC15A3 expression in vitro</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glandular epithelial cells</td>
<td>15.3±0.8</td>
<td>20.0±0.8</td>
<td>36</td>
<td>0.001</td>
</tr>
<tr>
<td>Stroma cells</td>
<td>17.0±0.4</td>
<td>25.0±0.4</td>
<td>177</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Reproduction (2011) 141 685–695
1997). SLC15A3 (PHT2) is located in lysosomal membranes in which it mediates the electrogenic co-transport of short chain peptides in addition to free His out of lysosomes (Daniel & Kottra 2004). In cooperation with other cationic transporters located at membranes directed toward the uterine lumen, His might be transported into the uterine fluid. Increased SLC15A3 expression has already been demonstrated in the endometrium of day 18 pregnant cows (Klein et al. 2006). We affirmed that IFNT increased the expression mainly in stroma cells in vitro, which may relate to the elevated His concentrations found in vivo. As histidine decarboxylase was not expressed at detectable levels in endometria of any group (data not shown), we presume that His is not involved in histamine biosynthesis, but rather contributes to protein synthesis for the elongating conceptus. Ongoing studies will provide evidence as to whether other conceptus-derived signaling molecules affect enrichment of AAs in the lumen.

AA transport occurs via a variety of rheogenic co-transporters and exchangers with overlapping substrate specificity and differences in the affinity of their substrates. Although some transport systems are mainly expressed at basolateral membranes to perform the transport from the maternal blood (System L: SLC7A8) into the cell, others are rather located at the apical side (System ASC: SLC1A5; Castagna et al. 1997). The majority of transporters analyzed in this study revealed no pregnancy-dependent regulation. For SLC1A5 and SLC6A6 transcripts, we observed an increase over time, possibly induced by progesterone exposure as previously demonstrated in the ewe for SLC1A5 (Gao et al. 2009b). Blood flow increases from days 14 to 18 in the uterine artery supplying the gravid uterine horn with sufficient nutrients (Ford et al. 1979, Silva & Ginther 2010). This may enable an accumulation of nutrients even in the absence of increases in expression of genes for most solute carriers.

Cationic AAs utilize distinct carrier systems (y\(^+\), y\(^+\)L, b\(^{0+}\), N) for transport. Na\(^+\)-independent transport of cationic AAs (Arg, Lys, Orn, and protonated His) by the y\(^+\) transport system occurs with high affinity and low capacity in various tissues (MacLeod 1996). Endometrial SLC7A1 transcript analysis failed to detect an effect of pregnancy, but it may become a subject of regulation following implantation as demonstrated for the ewe (Gao et al. 2009a). Arg represents a semi-essential AA since it is formed via the urea cycle to a certain extent, although the requirements must be complemented through dietary supplements. The gaseous signaling molecule, nitric oxide (NO), arises from Arg and is the major component contributing to vasodilatation. It is further hypothesized to play a role in implantation events by regulating blood flow, tissue remodeling, and immune suppression in species exhibiting invasive placentation (Purcell et al. 2009). In addition, polyamines are synthesized from Arg via Orn and control DNA as well as protein synthesis. They are key regulators of angiogenesis that stimulate vascular functions, which has been shown during the first half of pregnancy in the ewe (Kwon et al. 2003). Inhibition of polyamine synthesis is involved in the development of intrauterine growth restriction in rat (Ishida et al. 2002). In this study, Arg increased in the uterine lumen of early gravid cows and exceeded Orn, but as demonstrated earlier (Gao et al. 2009c), NOS amounts declined. Thus, as shown in the ewe, our results suggest an intensified requirement for polyamines during the preimplantation period in cattle (Kwon et al. 2004).
Cth abundance declined significantly in both cyclic and pregnant heifers to undetectable levels on day 18. An increase in Tau in the uterine fluid was observed at day 18 only in cycling heifers. Tau is a metabolic product of sulfurous AAs. Although it is not a component of proteins, it fulfills many regulatory functions such as osmoregulation, membrane stabilization, antioxidation, and modulation of Ca$^{2+}$-flux. In the uterine fluid, concentration of Tau is fairly high in contrast to the bovine blood plasma or oviduct fluid (Hugentobler et al. 2007). Lowered Tau amounts in rat uterine epithelial cells were confirmed during the estrous phase as well as during pregnancy and epithelial cells contain large amounts of Tau at diestrus (Lobo et al. 2001). We demonstrated an increase in the expression of transcripts for AA transporter for $\beta$-AA (which include Tau and $b$Ala) SLC6A6 (TAUT) over time, which could account for elevated Tau in the uterine lumen. However, the transcript abundance did not differ from the endometrium of pregnant animals. Thus, mechanisms to inhibit Tau uptake into the uterine lumen must be implicit. The recession of Tau in the uterine luminal fluid during pregnancy might be a consequence from a loss of osmotic force to antagonize the effects of steroid hormones that encourage water retention (Phoenix & Wray 1994).

In animal husbandry, metabolic imbalances may not only negatively affect health and productivity of dairy cattle during early lactation but also alter the nutrient composition of the uterine fluid. In this study, we present a comprehensive and quantitative analysis of the physiological AA composition in the uterine lumen of early pregnant cattle. These results are critical to our understanding of specific requirements of the bovine pre-attachment conceptus during this critical period. Most notably, all essential AAs accumulated with advancing stages of pregnancy when the trophoblast undergoes continuous elongation. Apart from the His/peptide transporter SLC15A3, increasing amounts of AAs in the uterine fluid were not accompanied by increases in the expression of transcripts for specific AA transporters in endometria of pregnant heifers. This may indicate a relevance for an adjusted blood flow, allowing an adequate delivery of nutrients. The results of this study provide the basis for further studies to determine whether local deprivation or imbalances in nutrients occurs with possible negative consequences on the development of bovine conceptuses during the pre- and peri-implantation periods of pregnancy.

Materials and Methods

Pretreatment of animals

All experiments were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction and with the European Convention on Animal Experimentation. Cyclic Simmental heifers (Bos taurus, Deutsches Fleckvieh) were synchronized to estrus by injecting i.m. 500 $\mu$g of a single dose of the prostaglandin F$_{2\alpha}$-analog cloprostenol (Estrumate; Essex Tierarznei, Munich, Germany) at diestrus as described previously (Ulbrich et al. 2009b). Pregnant groups were inseminated after estrus detection, whereas the cyclic control groups received supernatant of centrifuged sperm from the same bull. Blood samples were taken to determine serum progesterone by RIA (Prakash et al. 1987). All animals had concentration of progesterone >6 ng/ml at the time of slaughter, thus assuring that luteolysis had not commenced. At day 12, 15, or 18 post-insemination, animals were slaughtered ($n=4–7$ per group); the uterus was removed and flushed with 100 ml PBS (pH 7.4) for the recovery of proteins, AAs, and other components in uterine secretions, as well as conceptuses in pregnant heifers. Animals from the pregnant group were included in the study only if a conceptus was present. The flushing fluid was centrifuged at 800 g for 10 min and the supernatant was stored at $-20^\circ$C until further usage (Ulbrich et al. 2009b). Intercarnuncular endometrium from the middle part of the ipsilateral uterine horn was sampled for gene expression analysis as described previously (Bauersachs et al. 2005).

Analysis of AAs and TP in the uterine lumen

In 40 $\mu$l of uterine flushing fluids, 41 AAs and their derivatives (Supplementary Table 1) were labeled by the isobaric tagging for relative and absolute quantification methodology using the AA45/32 Starter Kit according to the manufacturer’s instructions (Applied Biosystems, Carlsbad, CA, USA) and analyzed via liquid chromatography–tandem mass spectrometry (LC–MS/MS; 3200QTRAP LC/MS/MS, Applied Biosystems) as described previously (Kaspar et al. 2009). The data were analyzed using the Analyst 61666; 1.5 Software. TP content in uterine flushing fluids was determined by a conventional bicrocinohonic acid assay (Sigma–Aldrich). As TP content and total AA concentration (calculated by the sum of each individually measured AA) correlated well ($r=0.82$, $P<0.0001$), data are presented as mean nmol/mg TP±S.E.M.

Endometrial expression of genes for relevant transporters and metabolic enzymes

Total RNA from ipsilateral intercaruncular endometrial samples was isolated using TRizol reagent (Invitrogen Corporation) according to the manufacturer’s instructions. Quality of RNA was monitored by the Agilent 2100 Bioanalyzer (RNA 6000 Nano Assay Kit, Agilent Technologies, Böblingen, Germany). RNA integrity numbers ranged between 7 and 10 ($10=\text{intact RNA}$). The quantitative real-time PCR (qPCR) experiments were performed in accordance with the MIQE guidelines (Bustin et al. 2009). Quantitative PCR using the LightCycler DNA Master SYBR Green I protocol (Roche Diagnostics) was performed as described earlier (Ulbrich et al. 2009b). The sequences of commercially synthesized PCR primer pairs (Eurofins MWG Operon, Ebersberg, Germany), the sequence
Statistical analysis

For statistical analysis, the SAS program package release 9.1.3 (2002; SAS Institute, Inc., Cary, NC, 75 USA) was used. The data from uterine flushing fluids and endometria from cyclic and pregnant uteri were compared using the least-square ANOVA general linear models procedure to determine the effects of the day (day) of the estrous cycle and pregnancy, pregnancy status (cyclic or pregnant; status), and day by status interaction. The differences between the days (within cyclic and pregnant animals respectively) are shown by using different superscript letters (x, y, and z in cyclic animals and a, b, and c in pregnant animals, whereby same superscript letters indicate that there is no statistical difference between groups) and treatment effects at the respective significant time points are marked with asterisks (*). Values of $P<0.05$ were regarded as significantly different. Graphs were plotted using Sigma-Plot 8.0 (SPSS, Inc., Chicago, IL, USA).

In vitro co-culture of glandular and stroma cells

To test the effect of IFNT on glandular epithelial and stroma cells in vitro, co-culture experiments of these cell types were performed as described earlier (Ulbrich et al. 2009a). Approximately $10^6$ stromal cells were seeded at the bottom of a 6-well cell culture plate (Nunclon $\Delta$ Surface, Nunc, Wiesbaden, Germany) and $10^6$ glandular epithelial cells in culture inserts (Anopore membrane, 0.2 $\mu$m, Nunc) freshly coated with Growth Factor Reduced Matrigel (BD Biosciences, San Jose, CA, USA). Cells were cultured in 400 $\mu$l medium (DMEM/F12 with 10% FCS) at 37°C in a humidified atmosphere of 5% CO$_2$ in air. Immunocytochemistry using specific antibodies against cytokeratin (for glandular epithelial cells) and vimentin (for the stromal cells) showed that the purity of each cell population was >90% (data not shown). When the cells reached 90% confluence, they were cultured serum free for 24 h after which the IFNT stimulation of the cells was performed. A native phenotype of in vitro co-cultivated cells populations was previously confirmed by transmission electron microscopy (Ulbrich et al. 2010). Epithelial cells showed distinct characteristics, including microvilli on the apical surface and interdigitations in the lateral part between cell as well as desmosomes and tight junctions. Glandular epithelial and stroma cells in co-culture were stimulated with recombinant bovine IFNT (antiviral activity, $4.8 \times 10^4$ U/ml medium; PBL Biomedical Laboratories, Piscataway, NJ, USA) for 4 h. The medium with the respective diluent served as a control. Following stimulation, the medium supernatant in the wells and inserts was discarded and the glandular epithelial cells were washed with PBS. TRIzol (500 $\mu$l; Invitrogen) was added, shortly incubated, and mixed vigorously using a pipette. The cell lysates were kept at $-80^\circ$C until processing. RNA extraction and qPCR experiments were carried out as detailed for the endometrial samples.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-10-0533.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

We greatly acknowledge the support of the German Research Foundation (UL 350/1-2, FOR 478) and – in part – by the Federal Ministry of Education and Research (FUGATOplus-REMEDY) and the Gender Issue Incentive Fund (Technische Universität München).

Acknowledgements

The authors sincerely thank Gabriele Schmidt and Ronny Scheundel for conducting the LC–MS/MS, Stefan Kempf for gene expression analysis, and Eva Englberger for cell culture experiments.

References


Received 22 December 2010
First decision 21 February 2011
Accepted 7 March 2011