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REVIEW ARTICLE

Proline metabolism in the conceptus: implications for fetal growth and development

G. Wu · F. W. Bazer · S. Datta · G. A. Johnson · P. Li · M. C. Satterfield · T. E. Spencer

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Abstract Although there are published studies of proline biochemistry and nutrition in cultured cells and postnatal animals, little is known about proline metabolism and function in the conceptus (embryo/fetus, associated placental membranes, and fetal fluids). Because of the invasive nature of biochemical research on placental and fetal growth, animal models are often used to test hypotheses of biological importance. Recent evidence from studies with pigs and sheep shows that proline is a major substrate for polyamine synthesis via proline oxidase, ornithine aminotransferase, and ornithine decarboxylase in placentae. Both porcine and ovine placentae have a high capacity for proline catabolism and polyamine production. In addition, allantoic and amniotic fluids contain enzymes to convert proline into ornithine, which is delivered through the circulation to placental tissues. There is exquisite metabolic coordination among integrated pathways that support highest rates of polyamine synthesis and concentrations in placentae during early gestation when placental growth is most rapid. Interestingly, reduced placental and fetal growth are associated with reductions in placental proline transport, proline oxidase activity, and concentrations of polyamines in gestating dams with either naturally occurring or malnutrition-induced growth retardation. Conversely, increasing proline availability in maternal plasma through nutritional or pharmacological modulation in pigs and sheep enhances concentrations of proline and polyamines in placentae and fetal fluids, as well as fetal growth. These novel findings suggest an important role for proline in conceptus metabolism, growth and development, as well as a potential treatment for intrauterine growth restriction, which is a significant problem in both human medicine and animal agriculture.

Keywords Proline · Placenta · Fetus · Nutrition · Fetal growth

Abbreviations

IUGR	Intrauterine growth retardation
NO	Nitric oxide
OAT	Ornithine aminotransferase
ODC	Ornithine decarboxylase
POX	Proline oxidase

Introduction

Despite advanced prenatal care for mothers and fetuses, intrauterine growth retardation (IUGR; impaired growth and development of the mammalian embryo/fetus or its organs during pregnancy) remains a significant problem in both human medicine and animal agriculture (Murphy et al. 2006; Wu et al. 2006). Because birth weight is one of the most sensitive and important measures of fetal growth, IUGR is often diagnosed as birth weight below the tenth

G. Wu · F. W. Bazer · P. Li · M. C. Satterfield · T. E. Spencer
Department of Animal Science, Texas A&M University, College Station, TX 77843, USA

S. Datta
Department of Statistics, Texas A&M University, College Station, TX 77843, USA

G. A. Johnson
Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, TX 77843, USA

G. Wu (✉)
Kleberg Center, Texas A&M University, 2471 TAMU, Room 212, College Station, TX 77843-2471, USA
e-mail: g-wu@tamu.edu

percentile of the birth-weight-for-gestational-age reference curve in clinics (de Onis et al. 1998). In animal studies, IUGR can be defined as fetal or birth weight less than two standard deviation of the mean body weight for gestational age. IUGR infants represent 11% of all newborns in developing countries (de Onis et al. 1998) and also a large number of all newborns in developed nations, e.g., 2–5% in the U.S. (Scholl and Johnson 2000). In addition, although considerable effort has been directed toward defining nutrient requirements of livestock species over the past 30 years, IUGR frequently occurs in many species (e.g., cattle, pigs, and sheep) worldwide as a result of suboptimal nutrition during gestation, placental insufficiency, and poor management (Wu et al. 2006). Notably, among domestic animals, pigs exhibit the most severe naturally occurring IUGR, with the birth weights of 15–20% of newborns being less than 1.1 kg (Wu et al. 2006). Compelling evidence from both epidemiological and animal studies have linked IUGR with metabolic disorders and the etiology of many adult-onset diseases in humans and animals (Murphy et al. 2006; Wu et al. 2004a). These findings have prompted us to focus on elucidating the mechanisms responsible for the nutritional regulation of placental and fetal growth.

Although there are published studies of proline biochemistry and nutrition in cultured cells and postnatal animals (Hu et al. 2007; Liu et al. 2006; Phang 1985; Wu 1996, 1997), as well as proline transport by placentae (Boyd and Lund 1981), little is known about proline metabolism in the conceptus (see Fig. 1). In view of evidence from published studies that suggests crucial roles for proline in cellular signaling transduction and metabolic control, we chose to quantify developmental changes in

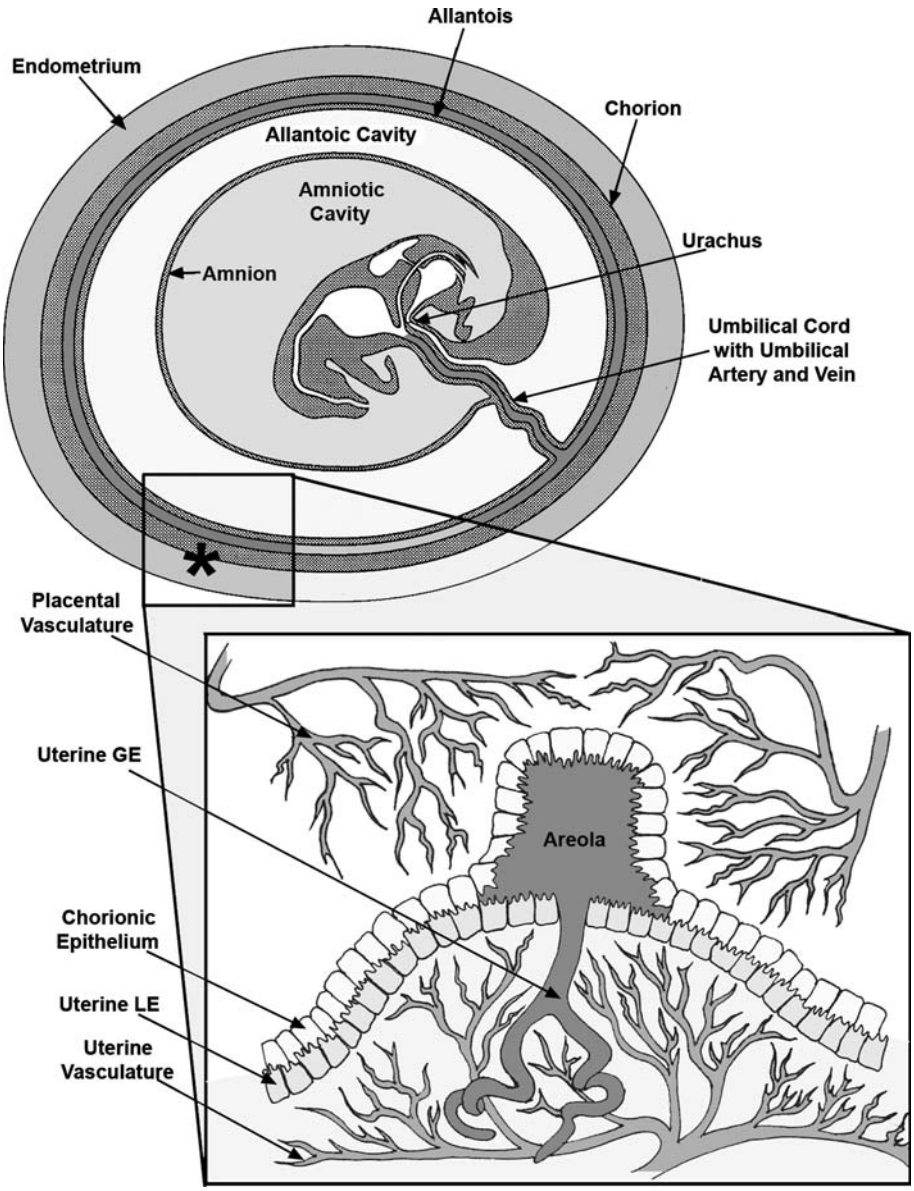

Fig. 1 The porcine and ovine conceptus. The fetus is suspended within amniotic fluid in the amniotic sac and can derive nutrients by drinking amniotic fluid. The interface between the maternal uterus and placenta in pigs allows for close apposition of vasculature for transplacental transport of nutrients (e.g., amino acids) and gases, as well as for transport, via specialized structures called areolae, of macromolecules across the placenta and into the fetal-placental circulation. Nutrients and gases transported from the maternal capillaries are transferred into the placental capillaries and transported to the heart via the umbilical vein for distribution to all tissue of the fetal-placental unit. Macromolecules transported across the areolae also go to the heart via the umbilical circulation for utilization by various tissues and cells. All nutrients transferred across the placenta may be cleared via the kidney and into the bladder from which they can enter the allantoic sac via the urachus for metabolism, degradation or re-uptake into the placental circulation and redistribution to affect development and function of fetal-placental tissues

concentrations, synthesis, and degradation of proline in the conceptus. Because of the invasive nature of biochemical research on placental and fetal growth, animal models (e.g., pigs, sheep, rats, and mice) are often used to test hypotheses of biological importance (Kwon et al. 2004b; Wu et al. 2004a, b). The major objective of this article is to both review recent findings and present new experimental results regarding proline metabolism in the conceptus, an emerging area of interdisciplinary research involving reproduction and nutrition.

Dynamic exchanges of nutrients in the conceptus

In mammalian species, the placenta develops in response to interactions between the embryonic chorion and maternal endometrium and is the essential organ for metabolic exchange between mother and fetus (Georgiades et al. 2002). Placentae undergo marked growth and rapid formation of new blood vessels (angiogenesis) during the first half of pregnancy (Reynolds and Redmer 2001). In the pig, which possesses a noninvasive, diffuse type of epitheliochorial placentation (Friess et al. 1980), the placenta grows most rapidly between Days 20 and 60 of gestation and placental development is maximal by Day 70 of gestation (Knight et al. 1977). The ovine placenta, classified as syndesmochorial (Dunlap et al. 2006), is comprised of both interplacentomal chorioallantois and placentomes, with each placentome consisting of a maternal uterine caruncle and a fetal cytoledon (Fig. 2). Placental angiogenesis is necessary to increase placental-fetal blood flow and, therefore, the supply of nutrients from mother to fetus (Ford 1995; Vonnahme et al. 2001). Thus, placental growth is a critical factor for controlling fetal survival, growth, and development (Bell and Ehrhardt 2002).

The developing fetus is surrounded by the amniotic sac containing amniotic fluid that the fetus may actively drink and into which fecal material and small amounts of urine are discharged from the rectum and urethra, respectively (Schmidt 1992). There is also the very important allantoic sac (in some animal species, such as pigs, sheep, and cattle) that is connected to the fetus via the urachus (Bazer 1989). The allantoic fluid then becomes a reservoir for water and other nutrients (e.g., amino acids), as well as many proteins

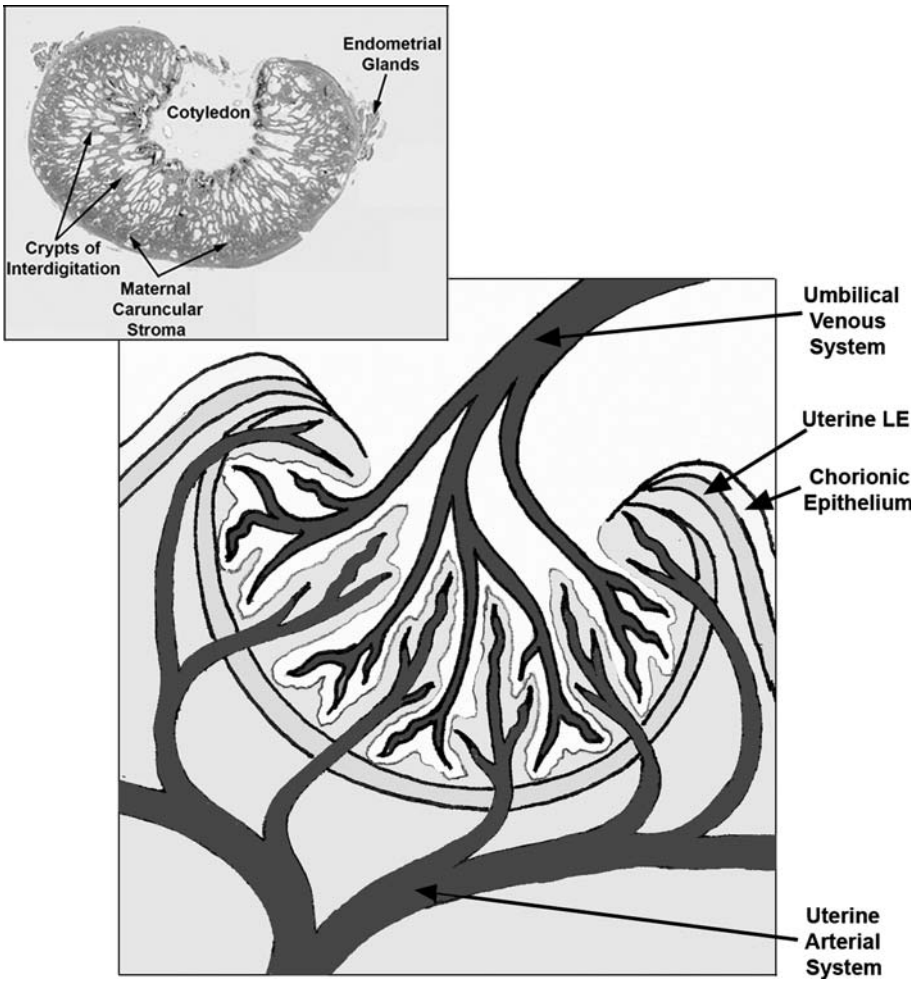

Fig. 2 The ovine placentome. In ewes, there are 70–90 placentomes representing the maternal uterine caruncle and the placental cotyledon. The maternal caruncles and fetal cotyledons are perfused with approximately 90% of the uterine arterial blood and placental umbilical vein blood, respectively. Within the placentomes, there is countercurrent exchange of nutrients and oxygen from the maternal uterine arterial system to the placental umbilical venous system which delivers these nutrients and gases to the fetal heart for distribution throughout the fetal-placental tissues

that can influence fetal-placental development and functions (Bazer 1989). There is a progressive increase in arteriovenous O_2 difference in the uterine circulation during pregnancy (Caton and Bazer 1978). Approximately 90% of blood flow to the uterus and placenta is directed to the placentomes from which the fetal-placental vasculature receives nutrients into the umbilical vein (Caton et al. 1983). Amniotic and allantoic fluids derive, in part, from (1) the transport of water and solutes across the placenta from the endometrium and (2) uterine secretions (Bazer 1989; Schmidt 1992). The amniotic fluid provides a unique aqueous environment in which the fetus develops symmetrically. In addition, amniotic fluid is a significant source of nutrients for the gut and other fetal tissues (Ross and Nijland 1998). With an increase in the transport capacity of fetal enterocytes with gestation (Sagawa et al. 1979; Wu et al. 2004a), amniotic fluid swallowed by the fetus provides a significant source of amino acids and other substances for supporting the proliferation and differentiation of intestinal epithelial cells. The nutritional significance of amniotic fluid is illustrated by the finding that esophageal ligation, which prevents the entry of this fluid into the small intestine, results in IUGR in both fetal pigs and lambs (Sangild et al. 2002; Trahair et al. 1995).

Although the allantoic sac was traditionally considered as a reservoir for fetal wastes, it is now clear that allantoic fluid nutrients can be absorbed by the allantois into the fetal-placental circulation and utilized by fetal tissues (Bazer 1989). Recent studies with pigs have shown that the allantois plays an important role in the accumulation of nutrients and metabolism of both uteroferrin (a progesterone-induced iron-binding protein) and iron (Buhi et al. 1983), suggesting a hitherto unrecognized function of the allantois in fetal nutrition. In support of this notion, both porcine and ovine allantoic fluids are unusually rich in the arginine-family of amino acids during early pregnancy that are crucial for placental and fetal growth and development (Kwon et al. 2003a; Wu et al. 1996). Particularly, concentrations of arginine are 4–6 mM in porcine allantoic fluid at Day 40 (term, 114 days; Wu et al. 1996, 1998a), whereas concentrations of citrulline and glutamine are 10 and 24 mM, respectively, in ovine allantoic fluid on Day 60 of gestation (term, 147 days; Kwon et al. 2003a). Importantly, these amino acids are interconvertable with proline in a cell- and tissue-specific manner (Wu et al. 2007b), and proline metabolism bridges the tricarboxylic acid cycle and the urea cycle in mammals (Phang 1985).

Changes in proline concentrations in the conceptus

There are marked changes in concentrations of proline in porcine and ovine conceptuses during the first half of pregnancy (Kwon et al. 2003a; Wu et al. 1995). In porcine placentae, concentrations of proline increase from 0.41 to 0.58 mM between Days 20 and 40 of gestation, remain at these high concentrations through Day 60 of gestation, and decline thereafter (Wu et al. 2005). Interestingly, concentrations of proline in ovine placentomes increase, to a much greater extent, from 0.43 to 3.38 mM (calculated on the basis of water content of 81%; Kwon et al. 2003a) between Days 30 and 60 of gestation (Table 1), compared with porcine placentae. Notably, concentrations of proline in the placentae are three to tenfold greater than those in maternal plasma (Wu et al. 2005), therefore necessitating placental transport of proline by sodium-dependent protein transporters (Regnault et al. 2005). Placental transport systems for proline include the transport system IMINO (primarily for proline), as well as systems A, ASC, and B^o for neutral amino acids (Regnault et al. 2005). In both porcine placentae (Wu et al. 2005) and ovine placentomes (Table 1), the increases in proline concentrations are associated with increases in proline transport.

Concentrations of proline in porcine amniotic fluid increase from 88 to 212 μM between Days 30 and 60 of gestation, as the fluid volume increases from 2 to 105 ml (Wu et al. 1995). Similarly, concentrations of proline in ovine amniotic fluid increase from 89 to 172 μM between Days 30 and 60 of gestation, as the fluid volume increases from 2 to 190 ml (Kwon et al. 2003a). Thus, the total amounts of proline in porcine and ovine amniotic fluids increase from ~0.18 μmol to 22.3 and 32.7 μmol, respectively, between Days 30 and 60 of pregnancy. In both porcine and ovine amniotic fluids, concentrations of proline decline after Day 60 of gestation (Kwon et al. 2003a; Wu et al. 1995). In contrast, during this period, concentrations of proline in porcine allantoic fluid decrease progressively from 261 to 46 μM as the fluid volume increases from 209 to 322 ml (Wu et al. 1995), whereas concentrations of proline in ovine allantoic fluid increase from 211 to 573 μM at the same fluid volume (31 ml; Kwon et al. 2003a). Thus, although there are species differences in placental transport and concentrations of proline between pigs and sheep, highest values are associated with the period of rapid placental growth during early gestation.

Metabolic pathways for proline utilization in the conceptus

In animals, proline is synthesized from pyrroline-5-carboxylate (P5C) by cytosolic NAD(P)H-dependent P5C reductase, which is widely spread in tissues (Phang 1985). There are two pathways for P5C synthesis from ornithine and glutamate (Wu and Morris 1998). P5C is formed from

Table 1 Proline metabolism in ovine placentome during gestation

	Day of gestation							
	30	40	60	80	100	120	140	SEM
Placentome								
Proline transport (nmol/g tissue per min)	3.76e	21.3a	11.6b	13.0b	8.82c	7.09d	6.82d	0.71
Proline (nmol/g tissue)	351e	2,734a	1,542b	1,619b	1,146c	738d	705d	82
Pyrroline-5-carboxylate (nmol/g tissue)	18.4e	156a	97.2b	102b	58.5c	39.3d	37.9d	4.4
Methionine (nmol/g tissue)	196e	752a	562b	554b	432c	330d	326d	29
S-Adenosylmethionine (nmol/g tissue)	22.4e	73.8a	60.4b	57.2b	44.6c	35.7d	33.9d	2.6
POX activity (nmol/g tissue per min)	157e	416a	313b	272c	220d	168e	163e	7.6
OAT activity (nmol/g tissue per min)	283e	769a	598b	486c	409d	305e	292e	19
P5CS activity (pmol/g tissue per min)	131d	246b	383a	257b	202c	154d	142d	8.5
Ornithine synthesis from proline (nmol/g tissue per h)	237e	719a	584b	439c	325d	266e	251e	22
Polyamine synthesis from proline (pmol/g tissue per h)	693e	2,583a	1,837b	1,402c	1,018d	742e	720e	81
Ornithine synthesis from arginine (nmol/g tissue per h)	47.5e	120a	95.1b	78.4c	62.6d	50.3e	51.8e	4.6
Polyamine synthesis from arginine (pmol/g tissue per h)	173e	429a	251b	196c	127d	93.6e	87.6e	10
Allantoic fluid								
POX activity (nmol/ml per min)	10.9e	30.5a	24.4b	19.7c	15.0d	11.6e	10.4e	1.2
OAT activity (nmol/ml per min)	13.4e	33.8a	28.2b	24.0c	19.7d	14.2e	12.8e	1.0
Pyrroline-5-carboxylate (nmol/ml)	3.88e	10.6a	8.34b	8.19b	6.62c	5.29d	5.17d	0.47
Amniotic fluid								
POX activity (nmol/ml per min)	11.3e	32.8a	26.7b	21.9c	16.4d	12.0e	11.7e	1.1
OAT activity (nmol/ml per min)	3.42e	11.9a	8.21b	6.44c	5.19d	3.66e	3.78e	0.34
Pyrroline-5-carboxylate (nmol/ml)	2.64e	8.35a	6.92b	6.77b	5.28c	4.04d	3.91d	0.28

Data are means with pooled SEM, $n = 4$ ewes on each Day of gestation. Columbia crossbred ewes were mated to Suffolk rams when detected as being in estrus (Day 0) and at 12 and 24 h later. Proline transport and metabolism (measured at 2 mM), concentrations of amino acids and pyrroline-5-carboxylate, and activities of proline oxidase (*POX*), ornithine aminotransferase (*OAT*), and pyrroline-5-carboxylate synthase (*P5CS*) were measured, as we described (Wu et al. 1997). Arginine metabolism and arginase activity were measured at 2 and 10 mM arginine, respectively (Kwon et al. 2003b). Data were analyzed by one-way ANOVA using the PROC GLM procedures of SAS (SAS Institute, Cary, NC, USA). Differences among means were determined by the Student–Newman–Keuls multiple comparison test (Steel et al. 1997). Means with different letters within the same row are different ($P < 0.05$)

ornithine by mitochondrial vitamin B6-dependent ornithine aminotransferase (OAT), which is expressed in all cells (usually at high levels) except for red blood cells. P5C is also generated from glutamate via mitochondrial NADPH-dependent P5C synthase (a bifunctional enzyme), which is present predominantly in enterocytes of the mammalian small intestine (except for cats and ferrets; Wu et al. 1998) and, to a much lesser extent, in mammalian placentae (Hu et al. 1999) and endothelial cells (Wu et al. 2000b). Consistent with this view, both porcine placentae (Table 2) and ovine placentomes (Table 1) contain P5C synthase activity for ornithine and proline synthesis from extracellular glutamate. However, the subsequent conversion of ornithine into citrulline is absent from both porcine placentae and ovine placentomes during pregnancy, because they do not have ornithine carbamoyltransferase activity (the present study) as measured using established methodology (Wu 1997). The lack of this enzyme in placentae maximizes the use of ornithine for the synthesis of polyamines in the conceptus.

Ornithine can be produced from arginine by cytosolic type-I and mitochondrial type II arginase (Wu and Morris 1998). Both isoforms are present in ovine placentomes (Kwon et al. 2003b), but are absent from the porcine placenta (Wu et al. 2005) throughout gestation. Thus, large amounts of arginine are catabolized in ovine placentomes for proline synthesis, and active placental transport of citrulline (an effective precursor of arginine) helps conserve arginine for supporting fetal growth and development. This explains why citrulline (10 mM) is more abundant than arginine (0.82 mM) in ovine allantoic fluid on Day 60 of pregnancy (Kwon et al. 2003a). Likewise, the absence of arginase from porcine placentae maximizes the net transfer of arginine from mother to fetus, which provides a metabolic basis for the high abundance of arginine (4–6 mM) in porcine allantoic fluid on Day 40 of gestation (Wu et al.

Table 2 Proline metabolism in porcine conceptus with normal fetal growth or IUGR at Day 60 of gestation

	Normal fetal growth	IUGR	SEM
Placenta			
Weight (g)	171	134*	6.9
Proline transport (nmol/g tissue per min)	30.8	24.1*	2.2
Proline (nmol/g tissue)	453	388*	17
Pyrroline-5-carboxylate (nmol/g tissue)	12.6	10.3*	0.85
Ornithine (nmol/g tissue)	215	176*	10
Arginine (nmol/g tissue)	618	502*	42
Polyamine (nmol/g tissue)	283	213*	17
POX activity (nmol/g tissue per min)	705	591*	46
OAT activity (μmol/g tissue per min)	1.47	1.08*	0.09
ODC activity (pmol/g tissue per min)	225	163*	14
Ornithine synthesis from proline (μmol/g tissue per h)	1.95	1.40*	0.12
Polyamine synthesis from proline (nmol/g tissue per h)	2.42	1.97*	0.16
Allantoic fluid			
Volume (ml)	368	345	29
Proline (nmol/ml)	65.9	52.7*	4.8
Pyrroline-5-carboxylate (nmol/ml)	10.6	8.24*	0.63
Ornithine (nmol/ml)	642	507*	44
Arginine (nmol/ml)	670	523*	42
POX activity (nmol/ml per min)	82.5	67.4*	5.3
OAT activity (nmol/ml per min)	88.4	73.1*	6.0
Amniotic fluid			
Volume (ml)	123	109	8.6
Proline (nmol/ml)	225	170*	15
Pyrroline-5-carboxylate (nmol/ml)	11.8	9.46*	0.67
Ornithine (nmol/ml)	61.2	47.9*	3.5
Arginine (nmol/ml)	126.3	90.2*	8.8
POX activity (nmol/ml per min)	80.7	64.3*	6.1
OAT activity (nmol/ml per min)	22.4	16.1*	1.4

Data are means with pooled SEM, $n = 6$ gilts (two fetuses from middle positions of uterine horns per gilt). Sexually mature crossbred gilts (Yorkshire × Landrace dams and Duroc × Hampshire sires) were mated to intact boars when detected as being in estrus (Day 0) and at 12 and 24 h later. All measurements were made at Day 60 of gestation. Fetal weights were 126 and 94 g (SEM = 4.5 g; $P < 0.05$), respectively, in the normal fetal growth and IUGR groups. Proline transport and metabolism (measured at 2 mM), concentrations of amino acids and P5C, and enzyme activities were measured, as we described (Wu et al. 2005). Data were analyzed by ANOVA with fetuses nested within gilts using SAS (SAS Institute, Cary, NC, USA). * $P < 0.05$ versus the normal fetal growth group

1996; Wu et al. 1998a). In contrast, the presence of arginase in ovine placentomes and fetal fluids catalyzes the formation of ornithine from arginine in the conceptus (Kwon et al. 2003b).

The above findings raised an intriguing question about the intracellular source(s) of ornithine for polyamine synthesis in placentae. Our subsequent research to answer this question led to the discovery of the presence of mitochondrial proline oxidase (POX) in porcine placentae for proline degradation (Wu et al. 2005). This pathway for proline utilization is also highly active in ovine placentomes (Table 1). The equilibrium of the placental OAT reaction favors the formation of ornithine from P5C (Wu et al. 2005). The proline-derived ornithine exits mitochondria and is converted into putrescine, spermidine and spermine in the cytosol via ODC, spermidine synthase, and spermine synthase, respectively (Fig. 3; Wu et al. 2005). These results suggest that POX is coupled with OAT in placental mitochondria and that the proline-derived ornithine readily enters the cytoplasm to serve as the substrate for ODC. Notably, compared with arginine, proline plays a quantitatively more important role in ornithine and polyamine synthesis in ovine placentomes (Table 1).

POX is the only known enzyme for initiating proline degradation in animal cells (Adams and Frank 1980; Phang 1985). Evidence from our studies indicates that this protein is a rate-controlling enzyme in placental synthesis of polyamines (Wu et al. 2005). In addition, results from the present work demonstrate that POX activity is greater in porcine placentae (Table 2) than in ovine placentomes (Table 1). This further indicates a quantitatively more important role for proline in polyamine synthesis in the porcine placenta, when compared with ovine placentomes, as noted above. Interestingly, the small intestine of neonatal pigs, which grows rapidly like the placenta, also lacks arginase activity (Dillon et al. 1999; Wu 1997) but

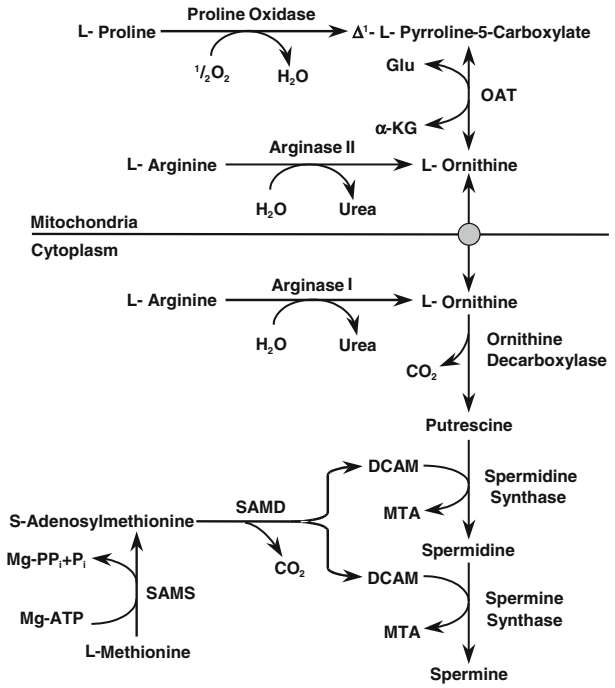

Fig. 3 Polyamine synthesis in placentae. Arginase I and II are present in ovine placentomes but absent from the porcine placenta. Proline is the major amino acid for polyamine synthesis in both the porcine placenta and ovine placentomes throughout pregnancy. *DCMA* decarboxylated 5-adenosylmethionine, *Glu* glutamate, *α-KG* α-ketoglutarate, *MTA* methylthioadenosine, *OAT* ornithine aminotransferase, *SAMD* S-adenosylmethionine decarboxylase, *PPi* inorganic pyrophosphate. This figure was originally published in Wu et al. (2005), and was reproduced with permission from the Society for the Study of Reproduction

expresses high levels of POX for polyamine synthesis (Wu et al. 2000a). Thus, consistent with its important role in DNA synthesis and cellular signalling (Phang 1985; Hu et al. 2007), there is active metabolism of proline for P5C production in rapidly proliferating cells. Although human placenta has been reported to have arginase activity (Ishikawa et al. 2007), whether this organ expresses POX is currently unknown.

Almost all cell types, perhaps except for red blood cells, express POX (Phang 1985; Wu et al. 1997, 2005). In pigs, POX activity is highest in the small intestine, followed by liver, kidney, and placenta (Dillon et al. 1999; Wu et al. 1997). Interestingly, P5C is not converted into glutamate in porcine placentae (Wu et al. 2005) or ovine placentomes (the present study) due to the absence of P5C dehydrogenase. This metabolic coordination maximizes the availability of P5C for the synthesis of ornithine and therefore polyamines (Fig. 3). The oxidation of proline by POX to yield P5C and the conversion of P5C into proline by P5C reductase constitutes an intracellular proline-P5C cycle that spans two compartments: mitochondrion and cytoplasm. This cycle plays an important role in the regulation of cellular redox state, DNA synthesis, gene expression, apoptosis, and cell proliferation (Hu et al. 2007; Liu et al. 2006; Phang 1985; Wu 1996).

A novel and interesting finding from our studies is that allantoic and amniotic fluids of pigs and sheep contain both POX and OAT (Tables 1, 2, 3, 4). These two proteins may be secreted from allantoic and amniotic membranes of the placenta as well as the uterus. As in the placenta, the equilibrium of the OAT reaction favors the generation of ornithine from proline (Wu et al. 2005). The production of P5C and ornithine in fetal fluids represents a hitherto unrecognized pathway for extracellular metabolism of proline in animals. The high activity of POX extensively catabolizes proline to generate P5C in allantoic and amniotic fluids, therefore explaining relatively low concentrations of proline but relatively high concentrations of P5C in these fluids. Indeed, proline is the second least abundant among nutritionally nonessential amino acids in allantoic and amniotic fluids of both pigs and sheep throughout pregnancy (Kwon et al. 2003a; Wu et al. 1995), whereas concentrations of P5C in porcine and ovine fetal fluids are 10- to 20-fold higher than those in maternal plasma of pregnant gilts (Wu et al. 2005) and ewes (0.50 μM). Whether P5C is a signaling molecule that regulates placental metabolism and function remains to be determined. Nonetheless, the discovery of POX in the conceptus opens a new avenue for future studies of the role of proline in fetal growth and development.

Coordination of metabolic pathways for polyamine synthesis from proline in the conceptus

Transport of proline across the plasma membrane represents the first step for its utilization by the placenta. Results of our studies show that the rates of placental transport of proline are highest during early gestation in pigs (Wu et al. 2005) and sheep (Table 1). The active transport of proline ensures an adequate supply of intracellular proline for metabolism by the placenta. In addition, the activities of POX oxidase, OAT and ODC are maximal at Day 40 of gestation, which would maximize production of ornithine and subsequently all polyamines from proline, in both the porcine placenta (Wu et al. 2005) and ovine placentomes (Table 1). Furthermore, concentrations of ornithine in allantoic fluid are highest at Day 40 of gestation in both pigs (Wu et al. 1996) and sheep (Kwon et al. 2003a), and the dynamic exchange of nutrients between this fluid and the placenta provides an additional source of ornithine for enhancing the placental synthesis of polyamines. Moreover, the production of glutamate (a major substrate for OAT) and glutamine (a stimulator of ODC activity) from branched-chain amino

Table 3 Proline metabolism in the conceptus of gilts fed protein-adequate (PA) or protein-deficient (PD) diets between Days 0 and 60 of gestation

	PA gilts	PD gilts	SEM
Placenta			
Weight (g)	179	140*	6.9
Proline transport (nmol/g tissue per min)	28.3	21.6*	1.8
Proline (nmol/g tissue)	437	364*	15
Pyrroline-5-carboxylate (nmol/g tissue)	12.6	8.81*	0.79
Ornithine (nmol/g tissue)	229	173*	12
Arginine (nmol/g tissue)	630	524*	36
Polyamine (nmol/g tissue)	295	216*	17
POX activity (nmol/g tissue per min)	686	523*	42
OAT activity (μmol/g tissue per min)	1.31	0.82*	0.07
ODC activity (pmol/g tissue per min)	204	152*	11
Ornithine synthesis from proline (μmol/g tissue per h)	1.81	1.33*	0.10
Polyamine synthesis from proline (nmol/g tissue per h)	2.39	1.64*	0.13
Allantoic fluid			
Volume (ml)	358	331	24
Proline (nmol/ml)	62.4	50.8*	3.2
Pyrroline-5-carboxylate (nmol/ml)	11.5	8.02*	0.61
Ornithine (nmol/ml)	629	534*	38
Arginine (nmol/ml)	659	547*	46
POX activity (nmol/ml per min)	79.3	15.8*	2.8
OAT activity (nmol/ml per min)	84.4	40.7*	4.7
Amniotic fluid			
Volume, ml	118	102	7.3
Proline (nmol/ml)	206	165*	12
Pyrroline-5-carboxylate (nmol/ml)	9.91	7.35*	0.50
Ornithine (nmol/ml)	63.2	45.0*	3.1
Arginine (nmol/ml)	113.5	87.2*	6.4
POX activity (nmol/ml per min)	72.4	50.6*	4.8
OAT activity (nmol/ml per min)	20.6	14.8*	1.0

Data are means with pooled SEM, $n = 6$ gilts (11–14 fetuses/gilt for placental and fetal weights; two fetuses/gilts for other measurements). Gilts (Yorkshire × Landrace dams and Duroc × Hampshire sires) were mated to intact boars when detected as being in estrus (Day 0) and at 12 and 24 h later, and were fed 13 or 0.5% protein diets between Days 0 and 60 of gestation as we described (Wu et al. 1998). All measurements were made at Day 60 of gestation. Fetal weights were 122 and 101 g (SEM = 6.7 g; $P < 0.05$), respectively, in the normal fetal growth and IUGR groups. Proline transport and metabolism (measured at 2 mM), concentrations of amino acids and P5C, as well as enzyme activities were measured, as we described (Wu et al. 2005). Data were analyzed by ANOVA with fetuses nested within gilts using SAS (SAS Institute, Cary, NC, USA).
* $P < 0.05$ versus the normal fetal growth group

acid catabolism, as well as their concentrations in placentae and allantoic fluid, are highest during early pregnancy (Self et al. 2004; Wu et al. 1996). Finally, placental concentrations of methionine and its metabolite S-adenosylmethionine, which provides a methyl group for the synthesis of spermidine and spermine from putrescine (Fig. 3), are highest at Day 40 of gestation in both pigs (Wu et al. 2005) and sheep (Table 1). Collectively, our results indicate metabolic coordination among several integrated pathways that support maximal polyamine synthesis in the placenta during early pregnancy, when placental growth and morphological changes are most rapid (Kwon et al. 2003b; Knight et al. 1977). This common phenomenon from two divergent mammalian species supports the notion that polyamines play a crucial role in promoting conceptus development (Wu et al. 2004a). Thus, modulation of the polyamine-synthetic pathways may provide a potentially novel and useful means to regulate fetal growth and survival.

Proline catabolism in the small intestine of the pregnant dam and its fetus

The small intestine is not only the terminal site for the digestion and absorption of nutrients, but is also a major organ responsible for whole-body proline homeostasis in animals. There is little uptake of arterial proline by the small intestine (Wu 1998). However, this organ plays a quantitatively important role in the degradation of dietary proline (Stoll et al. 1998; Wu et al. 2000a). Results from our studies with pigs indicate that the rate of proline catabolism in enterocytes is greater in gilts at Day 60 of gestation than in nonpregnant gilts, with rates of ornithine synthesis from 2 mM proline being 1.86 ± 0.15 and 1.34 ± 0.09 μmol/g tissue per h (mean ± SEM, $n = 6$; $P < 0.05$), respectively. Using established methods (Wu et al. 1994; Dekaney et al. 2003), we determined that, on Day 60 of gestation, OAT activities were 8.19 ± 0.44 (Dekaney et al. 2001) and 6.23 ± 0.57 μmol/g tissue per min (mean ± SEM, $n = 6$), whereas POX

Table 4 Reduced activities of proline-metabolic enzymes in placentome and fetal fluid of ewes underfed between Days 28 and 112 of gestation

	Control	50% NRC	SEM
Placentome			
Proline transport (nmol/g tissue per min)	7.48	6.25*	0.51
Proline (nmol/g tissue)	833	607*	58
Pyrroline-5-carboxylate (nmol/g tissue)	42.6	31.2*	2.9
Ornithine (nmol/g tissue)	340	269*	22
Arginine (nmol/g tissue)	1,752	1,204*	97
Polyamine (nmol/g tissue)	873	705*	62
Arginase activity (nmol/g tissue per min)	8.26	6.14*	0.45
POX activity (nmol/g tissue per min)	197	118*	13
OAT activity (nmol/g tissue per min)	358	273*	26
ODC activity (pmol/g tissue per min)	8.9	6.05*	0.64
P5CS activity (pmol/g tissue per min)	174	128*	9.3
Ornithine synthesis from proline (nmol/g tissue per h)	296	191*	24
Polyamine synthesis from proline (pmol/g tissue per h)	826	530*	67
Allantoic fluid			
Arginase activity (nmol/ml per min)	0.75	0.41*	0.05
POX activity (nmol/ml per min)	15.4	3.76*	0.42
OAT activity (nmol/ml per min)	16.2	10.4*	1.2
Pyrroline-5-carboxylate (nmol/ml)	5.46	3.08*	0.34
Amniotic fluid			
Arginase activity (nmol/ml per min)	1.39	0.70*	0.09
POX activity (nmol/ml per min)	18.2	7.76*	0.93
OAT activity (nmol/ml per min)	4.83	3.06*	0.26
Pyrroline-5-carboxylate (nmol/ml)	4.17	2.65*	0.31

Data are means with pooled SEM, $n = 6$ ewes at Day 112 of gestation. On Day 28, Suffolk ewes with singleton pregnancies (identified using ultrasonography) were randomly assigned to receive 100% (Control) or 50% (underfed) of NRC nutrient requirements between Days 28 and 112 of gestation, as we described (Kwon et al. 2004a). Proline transport and metabolism (measured at 2 mM), concentrations of amino acids and pyrroline-5-carboxylate, as well as enzyme activities in placenta and fetal fluids were measured at Day 112 of gestation, as previously described (Wu et al. 2005). Data were analyzed by unpaired *t* test (SAS Institute, Cary, NC, USA). * $P < 0.05$ versus the normal fetal growth group

activities were 0.62 ± 0.05 and 0.48 ± 0.03 μmol/g tissue per min (mean ± SEM, $n = 6$), respectively, in porcine and ovine small intestines. On the basis of the analysis of proline pharmacokinetics as reported for arginine studies (Wu et al. 2007a), we found that 60% of orally administered proline entered the portal circulation in gilts ($n = 6$) on Day 60 of gestation, with the remaining proline being largely degraded by the small intestine in first pass. Such an extensive intestinal catabolism of proline may necessitate adequate provision of proline to the diet for gestating gilts.

Although proline is synthesized in mammals via the interorgan metabolism of amino acids (Wu et al. 2007b), it may become a conditionally essential nutrient during pregnancy to support optimal growth and development of the conceptus. In this regard, it is noteworthy that an increase in concentrations of proline in maternal plasma through dietary supplementation with arginine is associated with improved fetal survival and the litter size of viable piglets in gilts (Mateo et al. 2007). However, traditionally, little attention has been paid to this important aspect of proline nutrition and physiology owing to a lack of the fundamental knowledge. Indeed, the National Research Council (NRC 1998) currently does not recommend a value for dietary proline requirement by gestating pigs. This guideline should be revised on the basis of recent evidence for proline as a functional amino acid in improving animal nutrition and production (Li et al. 2007; Wu et al. 2007b; Wu and Meininger 2002).

Reduced proline catabolism in placentae with IUGR

In pigs, natural IUGR is associated with reduced placental growth and impaired cellular signaling (Wang et al. 2008; Wu et al. 2006). However, the underlying mechanisms remain poorly understood. Thus, we conducted studies to determine concentrations of proline and related metabolites (ornithine, arginine, and polyamines), as well as POX and OAT activities in placentae, allantoic fluid, and amniotic fluid of fetuses experiencing normal growth or IUGR. Interestingly, proline transport, concentrations of proline and related metabolites (P5C, ornithine, arginine, and polyamines), as well as POX and OAT activities, were lower in placentae, allantoic fluid, and amniotic fluid of IUGR fetuses, when compared with littermates with normal growth (Table 2). A decrease in leucine transport has also been reported for porcine placentae with natural IUGR (Finch et al. 2004). In addition, placental ODC activity and placental synthesis of polyamines from proline were lower

in littermates with natural IUGR (Table 2). Both synthesis and secretion of the enzymes by the conceptus are likely impaired in IUGR fetuses, resulting in decreased availabilities in placentae and fetal fluids. Similar findings were obtained in porcine IUGR induced by dietary protein deficiency between Days 0 and 60 of gestation (Table 3). Particularly, proline concentrations in fetal plasma and allantoic fluid, as well as ODC activities in endometrium and placenta, were reduced in protein-deficient gilts, in comparison with control gilts fed 13% protein (Wu et al. 1998a, b). Also, POX activity in fetal fluids of gilts fed a 0.5%-protein diet were decreased by 80%, compared with gilts fed a protein-adequate diet (Table 3). Reduced availability of proline and polyamines in the conceptus may contribute to reduced placental and fetal growth both in natural IUGR (Wu et al. 2006) and in response to dietary protein deficiency (Schoknecht et al. 1994). However, the underlying mechanisms remain to be elucidated.

Results of our recent studies demonstrate that maternal undernutrition (50% of global nutrient restriction; NRC 1985) in pregnant ewes for both the first half of gestation and throughout gestation resulted in reduced concentrations of proline and polyamines in maternal plasma and the conceptus, as well as IUGR (Kwon et al. 2004a). This adverse pregnancy outcome could be ameliorated by realimentation during the second half of gestation in association with increased concentrations of proline and polyamines in maternal plasma and fetal fluids (Kwon et al. 2004a). As in pigs, maternal undernutrition in pregnant ewes decreased concentrations of proline, ornithine, arginine, and polyamines in placentomes (Kwon et al. 2004a), as well as the activities of POX, OAT and OCT in placentomes and fetal fluids (Table 4). Interestingly, intramuscular administration of Viagra (Sildenafil citrate) to control-fed or underfed ewes between Days 28 and 112 of gestation enhanced concentrations of proline and polyamines in maternal plasma and fetal fluids, as well as fetal growth (Satterfield et al. 2007). It is likely that the Viagra treatment, which acts to enhance intracellular cGMP availability by inhibiting phosphodiesterase-5 (an enzyme that hydrolyzes cGMP; Jobgen et al. 2006), increases utero-placental blood flows via the protein kinase G signalling (Wareing et al. 2005) and therefore the placental transfer of proline from mother to fetus. The action of Viagra on relaxing the vascular smooth muscle is similar to that of physiological levels of endothelium-derived NO, which stimulates cGMP generation from GTP by activating guanylyl cyclase (Wu and Meininger 2000).

In conclusion, proline homeostasis in the fetus is regulated by many factors, including intracellular protein turnover, proline synthesis and degradation, and placental transport of proline. Evidence from both porcine and ovine models suggests that there is exquisite metabolic coordination among integrated pathways that support highest rates of polyamine synthesis from proline in the placenta during early pregnancy. Knowledge about proline metabolism in the conceptus provides a new framework for future studies to define the roles of proline in fetal-placental growth and development. As a functional amino acid, enteral or parenteral supplementation with proline may have important implications for preventing both IUGR and fetal origins of adult-onset diseases in humans and livestock species.

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