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Influence of arginine on enzymes related to arginine metabolism in bovine mammary epithelial cells in vitro

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Abstract: Bovine mammary epithelial cells (BMEC) were used to evaluate the effects of different levels of Arginine (Arg) on enzymes related to Arg metabolism. A series of 7 Arg concentrations in the medium as treatments were: T0 (0.00 mg L⁻¹) as control group, and T0.25 (69.50 mg L⁻¹), T0.5 (139.00 mg L⁻¹), T1 (278.00 mg L⁻¹), T2 (556.00 mg L⁻¹), T4 (1112.00 mg L⁻¹) and T8 (2224.00 mg L⁻¹) as experiment groups respectively. The qPCR and ELISA analysis showed that the nitric oxide (NO) concentration, the expressions of endothelial nitric oxide synthase (eNOS) in mRNA and enzyme level were all increased in response to enhanced Arg doses such that the T8 was the greatest group ($P < 0.05$). Four folds of Arg concentration improved gene expression and synthesis of arginase which then decreased when excessive Arg was supplied ($P < 0.05$). The expressions of ornithine aminotransferase (OAT) mRNA and enzyme in T1 and T2 groups were significantly greater than that in the

other groups ($P < 0.05$). Two folds of Arg was the optimum level for ornithine decarboxylase (ODC) gene expression and enzyme synthesis among all seven treatments ($P < 0.05$). These somewhat various effects of Arg concentrations on four kinds enzymes in different Arg metabolic pathways suggest that Arg might participate in regulating bovine mammary physiological function with an optimum concentration by influencing the enzymes in related metabolic pathways.

Key words: arginine, metabolic enzyme, bovine mammary epithelial cell, metabolic pathway, enzyme interaction.

INTRODUCTION

In mammals, functional amino acids are involved in key metabolic pathways promoting growth and development, improving milk yield as well as reproductive performance (Kim et al. 2007; Wang et al. 2013). Each of them plays a unique and irreplaceable role in regulating a wide array of physiological activities. Arginine (Arg), one of functional amino acids with many biological functions like protein synthesis, physiological metabolism and immune response (Wu et al. 2007; Mateo et al. 2008), has two main metabolic pathways in mammary tissue (O'Quinn et al. 2002, Fig. 1). One is the L-Arg/NO pathway where Arg is catalyzed by nitric oxide synthase (NOS) to nitric oxide (NO), the other is the L-Arg/Orn pathway wherein Arg is converted to ornithine (Orn) by arginase which further metabolizes to proline or glutamine through transamination of ornithine aminotransferase (OAT) or to polyamines (putrescine, spermidine, and spermine) mainly through decarboxylation of ornithine decarboxylase (ODC).

Interestingly, ruminant mammary amino acid flow assays established that the uptake of Arg was greatly in excess of the requirement for Arg residues in milk protein (Mephram and Linzell. 1966; Bequette et al. 1998). The Arg uptake to output ratio (U: O) averaged 2.5 and did not alter with protein supply (Lapierre et al. 2012). Mammary redundant Arg seems to get involved in other metabolic pathways and exert unknown biological functions apart from milk protein synthesis. To date, our laboratory has revealed the effect of Arg on the cellular proliferation (Xu et al. 2012), on casein synthesis (Chen et al. 2013) and on related transcriptional regulators (Wang et al. 2014) of bovine mammary epithelial cells (BMEC) in vitro. Arg appears to mediate the casein synthesis through regulating cellular proliferation and transcriptional regulators. Further study on arginase inhibition found that the casein synthesis was regulated by Arg/Orn pathway, which implies the involvement of enzymes related to Arg metabolism in the casein synthesis of BMEC (Wang et al. 2017). Previous studies have identified the interaction of related enzymes in Arg metabolic pathways. The NOS activity was significantly boosted with the addition of arginase inhibitor in bovine pulmonary artery endothelial cells (Chicoine et al. 2004) while was sufficiently decreased after taking ODC inhibitor in mice (Inoh et al. 2009). In addition, in vivo and in vitro studies have confirmed that NO would directly inhibit ODC activity (Bauer et al. 1999). Nevertheless, direct demonstration for Arg on the mammary gland epithelium and enzymes involved in Arg metabolism is still lacking, and specific regulation of optimum Arg concentration on enzymes both in Arg/Orn and Arg/NO pathways remains unclear. To this end, we examined the role of a series of Arg concentrations on metabolism-related enzymes among metabolic pathways in BMEC in order to provide some novel knowledge for understanding the regulatory

mechanisms on Arg metabolism related to casein synthesis.

MATERIALS AND METHODS

Experimental design and treatments

This study was approved by the Inner Mongolia Agricultural University Animal Care and Use Committee. A single factor complete randomized experiment design was carried out by varying Arg doses in the present work. Approximately 5×10^4 mL⁻¹ BMEC cells were inoculated in 6-well plate with 2 ml Arg-free DMEM / F12 medium. When cells reached 70 % confluence, BMEC cells were incubated with treatment medium in different concentrations of Arg for 24h. Based on the results of optimum Arg concentration demanded for milk protein synthesis in BMEC (Xu *et al.* 2012), 278 mg L⁻¹ of Arg was set as the absolute requirement for milk protein synthesis. The design included a series of 7 Arg concentrations in the culture medium: T0 (0 mg L⁻¹, control), T0.25 (69.50 mg L⁻¹), T0.5 group (139.00 mg L⁻¹), T1 (278.00 mg L⁻¹), T2 (556.00 mg L⁻¹), T4 (1112.00 mg L⁻¹) and T8 (2224.00 mg L⁻¹), respectively equivalent to 0 (control), 0.25, 0.5, 1, 2, 4, and 8 folds of the optimum Arg concentration for milk protein synthesis (278 mg L⁻¹). Each treatment was run in triplicate.

Preparation of the cell culture medium

As Arg exists in commercial cell culture medium, the composition of the medium without Arg used in this work was prepared according to that of the DMEM/F12 medium (11320082, Gibco, Life company, US). Briefly, amino acids (Table 1), vitamins and minerals were dissolved in ultra-pure water in turn and the pH was set to 7.2 with NaHCO₃. For the experimental treatments, the Arg was added into the medium under a series of concentrations

in accordance with the experimental design described above.

Mammary tissue sampling and culture of BMEC

A multiparous Chinese Holstein dairy cow in mid-lactation (Inner Mongolia Agricultural University Farm) was chosen for mammary biopsy and epithelial cell isolation (Bionaz et al., 2012). Obvious pieces of fat and connective tissue were completely removed. Deep layer mammary tissue was collected and immediately rinsed in PBS buffer solution supplemented with penicillin/streptomycin (P0389/S6501, Sigma-Aldrich Corp., Saint Louis, MO). The 0.5% of collagenase I (17100017, Gibco, New York, USA) was introduced for 3 h digestion at 37°C with 5 % of CO₂ followed by filtering through a 74 µm sieve and centrifuging. The mixed cells were inoculated at a density of 5×10^5 mL⁻¹ in 25 cm² culture flasks with 5 mL cell growth medium. Then the adherent cells were digested with 0.25% of trypsin-EDTA (25200072, Gibco, New York, USA) and fibroblasts were removed to purify BMEC. The BMEC cells identification was carried out according to the methods described by O' Quinn et al. (2002) and Xu et al. (2012).

Examination of mRNA expression of Arg metabolism-related enzymes

The treatment medium was discarded after 24 h incubation, and the cell total RNA was extracted using RNA prep pure Cell/Bacteria Kit (DP430 spin column, TIANGEN BIOTECH Co. Ltd., Beijing, China). RNA concentration and OD260/280 value were confirmed with NanoDrop1000 spectrophotometer (NanoDrop, Technologies, US). RNA was reverse-transcribed into cDNA with the PrimeScripTM RT reagent Kit (RR037A, TaKaRa, Japan). cDNA was used as the template for amplification using iQTM SYBR® Green Supermix kit (170-8882, Bio-Rad, USA) on a MyiQTM System (Bio-Rad). PCR primers

were reported in table 2 and all Ct values were normalized to the internal reference gene *GAPDH*. The relative expression of gene mRNA was calculated using the $2^{-\Delta\Delta C_t}$ method (Pfaffl *et al.* 2001).

Measurement of Arg metabolism-related enzymes synthesis

After incubating with each treatment medium in 6-well plate for 24 h, total cellular protein content was extracted with the RIPA protein lysate (TIANGEN Biotech, Beijing, China) and concentration detected via the BCA protein kit (P0010, Enhanced, Beyotime Biotechnology, Shanghai, China). Then the total protein concentration was normalized, the concentrations of NO (BV2183, BIOTANG, USA), eNOS (BV2402, BIOTANG, USA), arginase (BV2361, BIOTANG, USA), ODC (MBS028141, MyBioSource, USA) and OAT (MBS9351120, MyBioSource, USA) were assessed via bovine ELISA kits according to the manufacturer's instructions. Specifically, after preparing the required dilute washing buffer and standards as well as enzyme-labelled plate, 50 μ L of standards, 40 μ L enzyme sample with 10 μ L sample diluent and zero standard controls were added to the plate sealed with the adhesive cover incubating for 30 min at 37 °C. Before and after adding 50 μ L of enzyme-labelled dilution to plate except control well, each well was aspirated and washed 4 times with dilute washing buffer for 1 min. To remove residual washing buffer, the plate was inverted and tapped on paper towels between each wash. Then, 50 μ L chromogenic reagent A and B were added in turn and incubated in dark for 30 min at 37 °C. The reaction was stopped with 50 μ L of stop solution with the wells turning from blue to yellow in color. Lastly, the optical density (OD) was determined using a micro-plate reader at 450nm within 15 min. Both intra-assay and inter-assay CV are less than 15% for OAT and ODC ELISA kits,

intra-assay CV is less than 8% and inter-assay CV is less than 12% for NO, eNOS and arginase ELISA kits ($CV = SD/mean \times 100\%$). The R^2 values of each ELISA standard curve are all greater than 0.99 which indicated the linear equations could be used to calculate the concentration of NO and the enzymes.

Statistical analysis

Data were analysed using one-way ANOVA with post-hoc Duncan multiple comparison test and curve estimation procedure in SPSS_{13.0}. We used a completely randomized design with Arg dose as the only factor. Statistically significant difference was declared at $P < 0.05$.

RESULTS

The mRNA expression of enzymes related to Arg metabolism

The results of the relative expression quantities of *eNOS*, *arginase*, *OAT* and *ODC* gene were displayed in Fig. 2. It was obvious that, compared with the control, the relative mRNA expression of the *eNOS* quadratically increased with the enrichment of Arg doses in the medium ($P < 0.01$, $R^2 = 0.979$), with a marked greater expressions of T4 and T8 group among all treatments ($P < 0.05$, Fig. 2a). The mRNA expressions of *arginase*, *OAT* and *ODC* shared a similar change manner which was characterized by upgrading from the low concentration of Arg to different optimum Arg levels (T4, T1&T2 and T2 group, respectively) but downgrading from maximum expression along with further increase of Arg levels in the medium. Briefly, the responses of *arginase* ($P < 0.01$, $R^2 = 0.976$) and *OAT* ($P < 0.05$, $R^2 = 0.976$) mRNA expression to Arg dose were cubic. For *arginase* gene expression (Fig. 2b), T4 group (1112.00 mg L⁻¹) was greatest among all groups, followed with T8 group greater than other groups ($P < 0.05$). However, there was no difference among the remaining treatments including the control ($P > 0.05$). And for *OAT* gene expression (Fig. 2c), both T1 and T2

group were greater than all other groups ($P < 0.05$), between which no difference was detected ($P > 0.05$). The T4 and T8 group were greater than the other three groups, which were all greater than the control except the T0.25 group ($P < 0.05$, Fig. 2c). In addition, there was a cubic trend ($P = 0.088$, $R^2 = 0.856$) between *ODC* gene expression and increasing levels of Arg (Fig. 2d). The T1 and T2 group, among all treatments, were significantly greater than the control where the T2 group was the greatest ($P < 0.05$). However, the remaining four levels of Arg (T0.25, T0.5, T4 and T8 group) were not different compared with the control ($P > 0.05$).

The synthesis of enzymes related to Arg metabolism

The results of NO, eNOS, arginase, OAT and ODC synthesis were displayed in Fig. 3. The concentrations of NO and eNOS in all treatments ranged from 69.50 mg L⁻¹ (T0.25 group) to 2224.00 mg L⁻¹ (T8 group) were dramatically greater than the control ($P < 0.05$) except for T0.25 group in eNOS concentration ($P > 0.05$). The NO synthesis had a cubic relationship ($P < 0.01$, $R^2 = 0.992$) with Arg dose where the T8 group was greatest among all treatments ($P < 0.05$) and greater than that of T2 and T4 group, between which no difference was noticed ($P > 0.05$, Fig. 3a). It was notable that the synthesis of eNOS had a quadratic response to Arg dose ($P < 0.01$, $R^2 = 0.908$) exactly as *eNOS* gene expression, and likewise, was greatest in the T8 group among all treatments (Fig. 3b). As shown in Fig. 3c, a cubic response of arginase concentration to Arg level ($P < 0.05$, $R^2 = 0.921$) was characterized by a greatest amount of arginase in the T4 group. And the arginase concentration in T8 group was greater than that in the control ($P < 0.05$) and the remaining groups ($P < 0.05$), among which no difference was found ($P > 0.05$). In addition, there was a cubic response trend both for concentration of OAT ($P > 0.05$, $R^2 = 0.701$) and ODC ($P > 0.05$, $R^2 = 0.683$) to Arg dose. Specifically, no difference among T0.5, T1 and T2 group for OAT synthesis was detected ($P > 0.05$), but all three groups were greater than the other groups including the control ($P < 0.05$, Fig. 3d). And

from Fig. 3e, two folds of Arg addition (T2 group, 556.00 mg L⁻¹) was the optimum concentration for ODC synthesis among all treatments ($P < 0.05$), followed with T4 group which was greater than the other groups ($P < 0.05$) where no difference was noticed among them ($P > 0.05$).

DISCUSSION

The increase in the quantity of proteins will lead to phenotypic enhancement. Cao et al. (2004) indicated that the significant inhibition of eNOS activity attributing to the addition of AGE-BSA (AGE-modified bovine serum albumin) might partially because of the reduced eNOS expression, which implies that the synthesis of enzyme protein could represent enzyme activity to some extent that enough enzyme protein expression provides the basis for the biological activities required for enzyme activity. Multiple studies determined a series of Arg concentrations in blood plasma of lactating dairy cows respectively were 17.68 mg L⁻¹ (Whitelaw et al. 1986), 11.71 mg L⁻¹ (Plaizier et al. 2001), 12.69 mg L⁻¹ (Jing et al. 2013) and 10.80 mg L⁻¹ (Nichols et al. 2016), which ranged from 10.80 to 17.68 mg L⁻¹. Those apparent low Arg concentrations in lactating bovine plasma compared with that of Arg supplied in present work confirmed that Arg participated among these pathways under a normal physiological conditions. As for the Arg concentrations in bovine mammary gland tissue, Nichols et al. (2016) detected a 3083.34 mg h⁻¹ of mammary gland uptake of Arg in lactating dairy cows, the arteriovenous difference multiplied by the 24 h estimated plasma flow rate, suggesting all Arg levels added in present study could be uptaken by bovine mammary gland within unit hour.

There are two major metabolic pathways in relation to NO and enzyme eNOS, arginase, OAT and ODC during Arg catabolism. The ability of Arg metabolism is dependent partially on the presence of enzymes related to Arg metabolic pathways in various tissues (Davenport

et al. 1990), and Arg is also capable of selectively regulating enzymes in Arg/Orn or Arg/NO pathway (Morris, 2004). In addition, Quinn et al (2002) quantified Arg catabolism via arginase and NOS pathways in the mammary tissue of sows, and the results showed that proline, Orn, urea, glutamate, glutamine, CO₂ and polyamines were formed from Arg, accounting for 46, 31, 17, 2.3, 1.5, 0.22, and 0.30%, respectively, of the metabolized Arg carbons. Relatively small amounts of Arg were utilized for NO and citrulline synthesis, with citrulline accounting for 2% of the metabolized Arg carbons. The ratio of Arg metabolic distribution in sow could lead to prediction that the amounts of Arg participated between Arg/NO and Arg/Orn pathways in bovine are about 98% and 2%, respectively, and the amounts of Arg participated between Orn/Citrulline and Orn/Polyamines pathways are about 49.8% and 0.52%, respectively. Our results indicating changes in enzyme expressions at various Arg levels could reflect the distribution of Arg pathways to some degree and the important role of Arg in physiologically metabolic regulation. However, accurate distribution of Arg metabolism pathways required specific experiments such as isotope labeling research.

The present results indicated that the gene expression and protein synthesis of enzymes involved in Arg metabolic processes showed a highly consistency, which were promoted in a dose-dependent manner both in mRNA and protein level. eNOS, mainly existing in epithelial cells, is a rate-limiting enzyme in the L-Arg/NO pathway catalyzing the sole substrate Arg to NO (Zate et al. 1991; Caso et al. 2004). The synthesis of catalyzed enzyme and end product are expected to be improved when the supplement of substrate is increased. In the present study, the synthesis of NO and eNOS was observed to have a cubic and quadratic relationship with Arg dose, which confirmed that expectation. In other aspect, the L-Arg/NO pathway may also serve for dumping overdosed Arg to some extent. The marked increase of NO and eNOS expression in response to Arg dose was consistent with multiple studies that NO production in patients with septic shock (Luiking et al. 2015) and the NOS activity of rat vascular

endothelial cells (Yu, 2006) were increased after L-Arg infusion whereas the activity of NOS was sufficiently inhibited when limiting L-Arg absorption in mouse astrocytes (Manner et al. 2003) that indirectly illustrated the interaction between Arg and NOS.

From low concentration range up to various optimum Arg levels in medium, the mRNA and protein expressions of arginase, OAT and ODC in BMEC were elevated as the increasing of Arg concentration, but were decreased when Arg concentration in medium continuously improved. Those results indicated that there was an optimum concentration for the peak yield of each enzyme. To be specific, in the present study, the four folds of Arg concentration ($1112.00 \text{ mg L}^{-1}$) was the optimum level for promoting the L-Arg/Orn metabolic pathway through improving arginase synthesis arginase expression. The decline occurred in T8 group, however, might attribute to the activation of other metabolic pathways like the L-Arg/Agmatine pathway that Arg is catalyzed by arginine decarboxylation to agmatine which regulates intracellular polyamines by inducing the translation of ODC antizyme proteins to inhibit ODC activity, contributing to a reduction of arginase activity (Demougeot et al. 2005). Several findings proved the mutual interaction between Arg and arginase, which showed consistency with our present work. Topal et al. (2006) indicated that the activity of arginase increased to a certain extent when adding Arg into the human endothelial cells while a lack of arginase could lead to hyperargininemia (Cederbaum et al. 1979). The arginase and Arg concentration in different organs of seedling also had a similar directly proportional relationship (Gao et al. 2008).

Orn serves as the common substrate for both OAT and ODC in two different branches of L-Arg/Orn pathway (Cantón et al. 2005; Todd et al. 2001). OAT regulates the interconversion of Orn into glutamine in OAT-containing tissues (Civen et al. 1967) and, hence, is involved in the metabolism of Arg related to nitrogen homeostasis. And ODC, widely existing in growing tissues, responds quickly to a range of nutritional irritations (Heby et al. 1990). Gonzalez et al.

(1991) found that the ODC viability of mammary tumor tissue was significantly restored when rats with Arg-restricted diet was fed 2% Orn. We believed that the inhibition of ODC was mainly due to restriction of Arg that elicited suppression on Orn production which acts as the substrate for ODC. And the supply of Orn could substitute for Arg naturally recovering the deficiency of substrate for ODC. A specific concentration of Arg in the medium might promote the enzyme synthesis of BMEC as its role in protein deposition, it was however that, much more high concentration of Arg might limit the enzyme synthesis partially because the efficiency of transfer of absorbed amino acids into protein decreased as protein production accumulated (Raggio et al. 2004), which could provide possible reason for the decreasing of OAT and ODC synthesis when Arg reached the optimum concentration of highest yield in the present study. Furthermore, Wu et al (2004) demonstrated that both proline and Orn, not Arg, were converted into polyamines in placental tissue throughout pregnancy, suggesting that proline was the major amino acid for polyamine synthesis in the porcine placenta. Excessive production of proline owing to excessive Arg addition in the medium could result in over-synthesis of polyamines, as the end product, that might exert suppression on OAT and ODC. Thus, the decline of OAT and ODC beyond optimum Arg addition level indicate that the cells have tight systems to control the productions of pyrroline-5-carboxylate and putrescine, probably preventing an over-production of polyamines and proline.

There are a variety of interactions among enzymes related to Arg metabolism (Fig. 1). Arginase could affect the synthesis of NO by competing NOS at certain physiological concentrations (Müllner, et al. 2002). This idea was in accordance with the fact that the enrichment of arginase activity in porcine coronary restrained the formation of NO while the addition of arginase inhibitor could enhance the NO concentration to recover the normal systolic and diastolic function (Hein et al. 2003). Competitive inhibition between pathways might also be the main reason for negative relationship between NOS and ODC (Bauer et al.

1999), which is characterized by suppression of metabolites where a large production of NO in L-Arg/NO pathway was able to competitively inhibit the activity of ODC in L-Arg/Orn pathway. In addition, along the L-Arg/Orn pathway, citrulline is derived partially from Orn, one of Arg metabolic products via arginase, through the breakdown of proline or glutamine by OAT to recycle Arg. So the branch pathway linking Arg, glutamine, and proline is bidirectional, which noticed a possible interaction existing between arginase and OAT. However, an inverse relationship between OAT activity and Orn levels in mice plasma and liver was found that the overexpression of OAT decreased Orn concentration rather than exerting effects on Arg homeostasis, which implies that OAT could only restrict Arg metabolism without participating in the regulation of Arg balance (Ventura et al. 2009). In contrast, OAT-null mice presented increased Orn concentration and would even die 24-48 h after birth if they do not receive Arg supplementation, suggesting the reaction favors Orn consumption at the whole-body level (Cadoret et al. 2001). Mutually reinforcement does exist between arginase and ODC, which might be due to an intermediary role of Orn in the L-Arg/Orn pathway. It is thought to be part of the mechanism promoting Arg positive metabolism and is further underscored by studies where the application of arginase appeared to enhance the activity and expression of ODC enzyme (Gobert et al. 2004) whereas intravenous infusion of ODC inhibitor in plasma contributed to a reduction of arginase activity in rats spontaneously hypertensive model (Demougeot et al. 2005). In general, polyamines induced by Arg positive metabolism with the interaction of enzyme arginase and ODC are required for DNA synthesis and protein metabolism in mammals, which might have been one factor associated with the improvement in the proliferation and development of mammary acinar tissue providing the physiological basis for the synthesis of mammary casein. This partially explains the study of Oka et al. (1974) who found that the improvement of arginase activity in mammary tissue of lactating Holstein dairy cow significantly boosted the

synthesis of milk protein.

CONCLUSION

Different concentrations of Arg promoted the synthesis of NO and expression of four kinds of enzymes related to Arg metabolism both in mRNA and protein level in BMEC to different extents. This suggests that Arg may affect the bovine mammary physiological metabolism by influencing the enzymes related to Arg metabolic pathways, which plays a crucial role in promoting mammary gland development and protein synthesis.

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Tables

Table 1. Concentration of each amino acid in arginine free DMEM/F12 medium (mg L⁻¹)

Amino acid	Concentration	Amino acid	Concentration
L-Glycine	18.75	L-Lysine	91.25
L-Alanine	4.45	L-Methionine	17.24
L-Arginine	-- ^a	L-Phenylalanine	35.48
L-Aspartic acid	6.65	L-Proline	17.25
L-Cysteine	17.56	L-Serine	26.25
L-Glutamic acid	7.35	L-Threonine	53.45
L-Tryptophan	9.02	L-Valine	52.85
L-Histidine	31.48	L-glutamine	365.00
L-Isoleucine	54.47	L-Tyrosine	55.79
L-Leucine	59.05		

^aArginine in DMEM/F12 medium used in this work was 0.00 mg L⁻¹. Different levels of arginine were added into the medium before the culture started.

Table 2. Primers for genes of arginine metabolism-related enzymes qPCR analysis

Items	Genebank ID or sources	Sequences of primers (5' -3')	Products (bp)
GAPDH ^a	<u>NM_001034034.1</u>	F: GGG TCA TCA TCT CTG CAC CT R: GGT CAT AAG TCC CTC CAC GA	176
eNOS ^b	<u>NM_181037</u>	F: TAC CAG CCG GGG GAC CAC ATA GGC R: CTC CAG CTG CTC CAC AGC CAC AGA C	126
Arginase	<u>NM_007482.3</u>	F: GCT TGC GAG ACG TAG ACC R: CAT CAC CTT GCC AAT CCC	104
ODC ^c	(Mei <i>et al.</i> 2006)	F: GCA GGA TCC ACC ATG AAC AAC TTT GGT AA A R: GCC GAG ATC TCA GAA GAA GAA ACT TC	120
OAT ^d	<u>NM_177487.2</u>	F: TGA TGA GTG CGG CTA AAT R: AAC CTG GTA ACC TTG GAA A	245

^aGlyceraldehyde-3-phosphate dehydrogenase.^bEndothelial nitric oxide synthase.^cOrnithine decarboxylase.^dOrnithine aminotransferase.

Figure captions**Fig. 1** Interaction of arginine metabolic enzymes in bovine mammary epithelial cells

Arginine (Arg) metabolism has two main pathways in mammary tissue. One is the L-Arg/NO pathway where Arg is catalyzed by nitric oxide synthase (NOS) to nitric oxide (NO) and citrulline, the other is the L-Arg/Orn pathway wherein Arg is converted to polyamines (putrescine, spermidine, and spermine) mainly through decarboxylation of ornithine decarboxylase (ODC) or to ornithine (Orn) by arginase which further metabolizes to proline or glutamine through transamination of ornithine aminotransferase (OAT) breaking down into citrulline to reform Arg. Note: the blue minuses denote competitive inhibition between NO and ODC and arginase or inverse relationship between OAT and Orn; the red plus signs denote mutually reinforcement relationship between ODC and arginase.

Fig. 2 (a, b, c, d) Effect of different arginine levels on gene expression of eNOS, arginase, OAT and ODC in bovine mammary epithelial cells

(a) the relative eNOS gene expression quantities; (b) the relative arginase gene expression quantities; (c) the relative OAT gene expression quantities; (d) the relative ODC gene expression quantities.

eNOS, endothelial nitric oxide synthase; ODC, ornithine decarboxylase; OAT, ornithine aminotransferase. T_x denotes different concentrations of arginine in the medium: T0 (0.00 mg L⁻¹), T0.25 group (69.50 mg L⁻¹), T0.5 group (139.00 mg L⁻¹), T1 group (278.00 mg L⁻¹), T2 group (556.00 mg L⁻¹), T4 group (1112.00 mg L⁻¹) and T8 group (2224.00 mg L⁻¹). The small bars represent standard deviations (SD)

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Fig. 3 (a, b, c, d, e) Effect of different arginine levels on synthesis of NO and enzyme eNOS, arginase, OAT and ODC in bovine mammary epithelial cells

(a) the amount of NO synthesis; (b) the synthesis of enzyme eNOS; (c) the synthesis of enzyme arginase; (d) the synthesis of enzyme OAT; (e) the synthesis of enzyme ODC.

NO, nitric oxide; eNOS, endothelial nitric oxide synthase; ODC, ornithine decarboxylase; OAT, ornithine aminotransferase. Tx denotes different concentrations of arginine in the medium: T0 (0.00 mg L⁻¹), T0.25 group (69.50 mg L⁻¹), T0.5 group (139.00 mg L⁻¹), T1 group (278.00 mg L⁻¹), T2 group (556.00 mg L⁻¹), T4 group (1112.00 mg L⁻¹) and T8 group (2224.00 mg L⁻¹). The small bars represent standard deviations (SD).

Labeled Figures

Figure 1

Figure 2

Figure 3