Inhibition of arginase via jugular infusion of *N*^w-hydroxy-nor-L-arginine inhibits casein synthesis in lactating dairy cows

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ABSTRACT

A previous in vitro study revealed that Arg elicits positive effects on casein synthesis through alterations of the Arg-ornithine pathway in bovine mammary epithelial cells. The main purpose of this work was to determine the effects of arginase inhibition using *N*^w-hydroxy-nor-L-arginine (nor-NOHA) on milk protein synthesis in vivo. Six healthy Chinese Holstein cows with similar body weight (550.0 ± 20 kg; means $\pm$ standard deviation), parity (4), body condition score (3.0), milk yield (21.0 ± 1.0 kg), and days in milk (80 ± 2) were selected and randomly assigned to 3 treatments in a replicated 3×3 Latin square design with 22 d for each period (7 d for infusion and 15 d for wash-out). The treatments were (1) control: saline infusion; (2) nor-NOHA: infusion of 125 mg/L of nor-NOHA; (3) nor-NOHA + Arg: infusion of 125 mg/L of nor-NOHA with 9.42 g/L of Arg. The activity of enzymes related to Arg metabolism, milk protein synthesis, and expression of AA transporters was determined. The infusion of nor-NOHA decreased the activity of arginase but had no effect on the activity of ornithine decarboxylase and nitric oxide synthase in serum, and these responses were the same at the gene expression level in mammary gland. In addition, the infusion of nor-NOHA also reduced protein and fat synthesis in milk but had no effect on milk yield. When Arg was infused with nor-NOHA, the activity of total arginase, ornithine decarboxylase, and nitric oxide synthase, and the concentration of casein, protein, and fat in milk did not change compared with the nor-NOHA group, but the milk protein yield, the expression of some Arg transporters (*SLC7A5* and *SLC7A8*), and milk yield increased. Overall, results verified previous in vitro findings indicating that synthesis of casein protein is

closely regulated by the Arg-ornithine pathway in bovine mammary gland.

Key words: arginine, *N*^w-hydroxy-nor-L-arginine, milk casein synthesis, jugular vein infusion

INTRODUCTION

The synthesis and secretion of milk protein in mammary gland is affected by several factors including nutrition, genetics, environment, and management. As the raw materials of milk protein synthesis, some EAA (lysine, methionine, and histidine) elicit regulation on the synthesis of milk protein (Prizant and Barash, 2008; Kim and Wu, 2009). Recently, some studies revealed that Arg, a conditionally essential AA, also has some positive effects on the synthesis of milk protein (Mabjeesh et al., 2002; Doepel and Lapierre, 2011). Some recent studies (Chen et al., 2013; Wu et al., 2016) have demonstrated that the addition of Arg in culture medium increased casein protein synthesis in bovine mammary epithelial cells (BMEC). Moreover, Wang et al. infused Arg through jugular vein in dairy cows to measure the effects of Arg infusion on milk protein synthesis in vivo. They found the infusion of Arg (37.66 g/d) increased milk protein yield significantly [M. Z. Wang, Q. Y. Xu, G. Zhou, J. Zhang, and H. R. Wang (College of Animal Science and Technology, Yangzhou University, Yangzhou, China), and J. J. Looor (Department of Animal Science and Division of Nutritional Science, University of Illinois, Urbana), unpublished data].

Arginine can be degraded through 3 different ways (Wu et al., 2009) in mammals: (1) Arg-Orn, which is regulated by arginase; (2) Arg-nitric oxide, which is regulated by nitric oxide synthase (NOS); and (3) Arg-aggmatine, which involves arginine decarboxylase. The conversion of Arg to Orn is reported to be the main pathway in mammary tissue (O'Quinn et al., 2002). The final products of the Arg-Orn pathway are polyamines (putrescine, spermidine, and spermine). Chen et al. (2015) reported that putrescine had some positive effects on the proliferation of cells, and the addition of

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spermidine and spermine elicited a positive effect on the synthesis of milk protein (Kong et al., 2014).

As the first rate-limiting enzyme in the Arg-Orn-Polyamine pathway (Wu et al., 2000), we hypothesized that arginase is the main regulator of milk casein synthesis. Decreasing activity of the Arg-Orn pathway with an arginase-specific inhibitor led to a decrease in the synthesis of casein and mammary cell proliferation. However, addition of Orn to the BMEC cultured with arginase inhibitor resulted in a recovery of casein synthesis and rate of cellular proliferation (Wang et al., 2017). These in vitro results provided strong indication that the Arg-Orn pathway is a key regulator of the synthesis of casein. To verify the in vitro results, the present study explored the effects of arginase inhibition on milk production, casein concentration in milk, and mammary gene expression of AA transporters through infusing arginase inhibitor in the jugular vein of lactating dairy cows.

MATERIALS AND METHODS

Animals and Treatments

All animal procedures were approved by the Yangzhou University Animal Care and Use Committee of Jiangsu Province, China. The experiment was carried out at the Experimental Farm of Yangzhou University, Yangzhou city, Jiangsu province, China. Six Chinese Holstein lactating cows with similar BW (550.0 ± 20 kg), parity (4), BCS (3.0), milk yield (21.0 ± 1.0 kg), and DIM (80 ± 2 d) were selected for this experiment. The indwelling catheters (L13712, Jiangxi Huali Medical Instrument Company, Ganzhou, China) were placed in a jugular vein and flushed with heparin and physiological saline (750 IU/mL) twice daily during the week before infusion. The animals were fed a common diet (provided by the Experimental Farm of Yangzhou University) as a TMR according to NRC (2001). The composition and nutrient levels of basal diets are reported in Table 1. The experimental cows were fed twice daily at 0600 and 2000 h, milked thrice daily at 0700, 1500, and 2300 h. Cows were housed in a freestall barn, and had ad libitum access to the TMR and fresh water.

Cows were randomly assigned to 3 treatments in a replicated 3×3 Latin square design with 22 d for each period (d 1 to 7 for infusion and d 8 to 22 for washout). The treatments were as follows: (1) saline (control); (2) saline + 125 mg/L of *N*^ω-hydroxy-nor-L-arginine (**nor-NOHA**); (3) saline + 125 mg/L of nor-NOHA + 9.42 g/L of L-Arg (**nor-NOHA + Arg**). The dosage of infused nor-NOHA in the nor-NOHA group and nor-NOHA + Arg group per day was designed according

to some previous studies in different animals (Reid et al., 2007; Kövamees et al., 2014). The dosage of Arg infused to cows per day was designed on the basis of the study of Wang et al. (2014). Wang et al. (2014) found that the mRNA expression of *CSN1S1*, *CSN1S2*, *CSN2*, and *CSN3* in BMEC cultured with different concentrations of Arg were different. Moreover, the cubic regression analysis shown that the expression of casein genes was highest when the concentration of Arg in medium was 434.44 mg/L, which was almost 1.56 times that (278 mg/L) in the control group. In a later experiment in lactating dairy cows [M. Z. Wang, Q. Y. Xu, G. Zhou, J. Zhang, and H. R. Wang (College of Animal Science and Technology, Yangzhou University, Yangzhou, China), and J. J. Looor (Department of Animal Science and Division of Nutritional Science, University of Illinois, Urbana)], the authors infused Arg through the jugular vein to evaluate the results in vitro. In the pre-experiment, they infused 37.66 g/d of Arg to make 104.91 g/d of supplementation of Arg to the mammary gland, which was 1.56 times that in the control group, and they found the infusion of 37.66 g/L of Arg increased the concentration of protein in milk. We infused this dosage of Arg in this study.

Table 1. Composition and nutrient levels of basal diets (DM basis)

Item	Concentration
Ingredient (g/kg)	
Alfalfa	209.00
Chinese wildrye	38.00
Corn silage	246.00
Cracked corn	277.00
Cottonseed meal	37.00
Soybean meal	135.00
Corn DDGS ¹	38.00
CaHPO ₄	3.00
NaCl	5.00
Premix ²	12.00
Total	1,000.00
Nutrient ³	
NE _L (Mcal/kg)	1.51
CP (g/kg)	166.80
NFC (g/kg)	445.40
NDF (g/kg)	283.90
ADF (g/kg)	177.90
Ether extract (g/kg)	32.60
Ca (g/kg)	11.60
Total P (g/kg)	6.00

¹Corn DDGS = corn distillers dried grains with solubles that contain 2.31 Mcal/kg of NE_L, 280 g/kg of CP, 390 g/kg of NDF, 150 g/kg of ADF, 110 g/kg of ether extract, 2 g/kg of Ca, and 8 g/kg of total P.

²The premix provided the following per kilogram of diet: 25 mg of CuSO₄, 75 mg of FeSO₄·H₂O, 105 mg of ZnSO₄·H₂O, 0.0024 mg of Co, 0.016 mg of Na₂SeO₃, 12,000 IU of vitamin A, 10,000 IU of vitamin D₃, 25 mg of vitamin E, 36 mg of nicotinic acid, and 1,000 mg of choline.

³The NE_L in the diet was calculated according to the NE_L of ingredients and their percentages; concentrations of the other nutrients were measured values.

The perfusate was manufactured by Cambridge Biological Company (Nanjing, China). The solutions were infused continuously through a peristaltic pump (Longer, Hebei, China) for 8 h/d (from 0600 to 1400 h), and the total perfusion for each cow was 4 L in 8 h.

Detection of the Activity of Enzymes Associated with Arg Metabolism in Blood Serum

In the last day of the infusion period, blood from the coccygeal artery (Cant et al., 1993) was collected into serum tubes simultaneously every 3 h from 0600 to 1500 h. Samples were centrifuged (10 min, $2,810 \times g$, at 4°C) 2 to 3 h after collection, and the serum aliquoted and stored at 20°C for the analysis of enzyme activities. The serum sample was mixed thoroughly before analyses. The activity of total arginase and NOS was determined with an arginase (DARG-200, BioAssay Systems, Hayward, CA) and NOS kit (A014-2, Jianchen, Nanjing, China) following the manufacturer's protocols [arginase kit (<https://www.bioassaysys.com/Datasheet/DARG.pdf>) and NOS kit (<http://www.njjcbio.com/uploadfile/product/big/20140217125016525.pdf>)] In addition, the activity of ornithine decarboxylase (ODC) was measured with the method reported by Ngo et al. (1987).

Data Collection and Milk Sample Analyses

Milk production was recorded at each milking and sampled on the last 2 d of each infusion period (d 6 and 7). Milk samples were collected 3 times a day and a pool was made from each sample in proportion to milk yield at each milking. A subsample of milk was used for measuring protein, fat, and nonfat milk solids (Bentley FTS/FCM 400 Combi, Bentley Instruments Inc., Chaska, MN). Another subsample was centrifuged at $2,810 \times g$ at 4°C for 10 min to separate milk fat for the determination of casein concentrations in milk. The concentrations of α -CN, β -CN, and κ -CN were detected using the bovine ELISA kits from Cloud-Clone Corp. (SEJ333Bo, SEJ332Bo, and SEJ331Bo, respectively, Cloud-Clone Corp., Houston, TX).

RNA Extraction and PCR for Gene Expression

Mammary Gland Biopsy. At the end of each infusion period, cows were given a small dose of general anesthetic (0.025 mg of Xylazine/kg of BW, 20 mg/mL) before biopsy. Mammary gland tissue samples were harvested using a published biopsy method (Bionaz and Loor, 2007). Tissue was collected in 1.5-mL cryovials and frozen immediately in liquid N before storage at -80°C until RNA extraction.

RNA Extraction and Reverse Transcription.

Frozen mammary tissue was quickly minced and weighed (0.5–1.0 g) and immediately subjected to RNA extraction with ice-cold TRIzol (15596018, Thermo Fisher Scientific, Waltham, MA) as described previously including a DNase digestion step (Loor et al., 2006). Total RNA was precipitated with isopropanol and washed with 70% ethanol and resuspended in UltraPure DNase/RNase-Free Distilled Water (10977015, Thermo Fisher Scientific). The RNA integrity was assessed by agarose gel electrophoresis analysis of 28S and 18S rRNA subunits. The RNA concentration was measured with a Nanodrop spectrophotometer (Thermo Fisher Scientific). The purity of RNA (A260/A280) was higher than 1.9. A portion of the RNA was then diluted to 100 ng/ μL before reverse transcription and cDNA synthesis with the High-Capacity cDNA Reverse Transcription Kit (4368813, Applied Biosystems, Waltham, MA). Each cDNA was synthesized in a 20- μL reaction (including 2 μL of $10 \times \text{RT buffer}$, 0.8 μL of 100 mM deoxynucleotide triphosphate mix, 2.0 μL of $10 \times \text{RT Random Primers}$, 1.0 μL of MultiScribe reverse transcriptase, 1 μL of RNase inhibitor, 3.2 μL of nuclease-free H_2O , and 10 μL of RNA sample). The mixture was incubated at 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and kept at 4°C for reverse transcription (RT)-PCR analysis.

RT-PCR Analysis. Sufficient cDNA was prepared for the detection of all the selected genes. The RT-PCR analysis was performed using the Power SYBR Green PCR Master Mix (4367659, Applied Biosystems). A total of 10 μL of $2 \times \text{Fast SYBR Green Master Mix}$, 0.8 μL of 10 μM forward and reverse primers, 1 μL of cDNA template, and 7.4 μL of RNase-free water was used for each reaction. Each sample was run in triplicate and the reactions were performed in the ABI Prism 7500 Detection Instrument (Applied Biosystems) using the protocol below: 30 s at 95°C , 10 s at 95°C , 20 s annealing temperature, and 30 s at 72°C for 40 cycles. The same conditions were performed on an equal amount of RNase-free water as a negative control. Gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ (delta cycle threshold) method (Livak and Schmittgen, 2001).

Primer Design of Selected Genes. Primer sequences of genes are listed in Table 2. Commonly used reference genes such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β -actin (*ACTB*) were selected as internal control genes. Arginase 1 (*ARG1*), arginase 2 (*ARG2*), endothelial nitric oxide synthase (*NOS3*), inducible nitric oxide synthase (*NOS2*), ornithine decarboxylase 1 (*ODC1*), α_{S1} -casein (*CSN1S1*), α_{S2} -casein (*CSN1S2*), β -casein (*CSN2*), κ -casein (*CSN3*), and insulin-like growth factor 2 (*IGF2*) were

chosen to allow for comparison with previous in vitro results (Wang et al., 2017). In addition, 6 additional genes associated with transport mechanisms also were selected: solute carrier family 7 member 1 (*SLC7A1*), solute carrier family 7 member 2 (*SLC7A2*), solute carrier family 7 member 5 (*SLC7A5*), solute carrier family 7 member 6 (*SLC7A6*), solute carrier family 7 member 7 (*SLC7A7*), and solute carrier family 7 member 8 (*SLC7A8*). All primers were designed with Primer Express software 1.5 (Applied Biosystems) fixing the amplicon length to 100 to 150 bp with low specific binding at the 3' end. All the sequences were present in both databases. Primer products were tested before RT-PCR. The cDNA samples of different bovine tissues were mixed and used for RT-PCR analysis as described

above. The thermal-cycling conditions were same as in the RT-PCR analysis. The PCR products were then run in a 2% agarose gel stained with GoldView (G8142, Solarbio, Beijing, China).

Statistical Analysis

Normalized RNA expression data were analyzed by ANOVA using general linear model procedures of SPSS 16.0 with treatment, period, and square as fixed effects. Cow was the random effect. A 1-way ANOVA was used to test for differences among treatments, whereas the Tukey multiple comparison test was done to determine differences between treatments; differences were considered significant if $P \leq 0.05$.

Table 2. Primers of genes for real-time PCR analysis¹

Gene ²	ID number	Sequence ³ (5' to 3')	Product size (bp)	Annealing temperature (°C)
<i>ARG1</i>	NM_001046154.1	F: TAATGGAAGTGAATCCGTCTC R: ACTTTGGTGGGCTAAGGTAAT	145	57
<i>ARG2</i>	NM_001017942.1	F: TACGACCAACTTCCAACCTCC R: TCTGTCACCACAAACGCCTCA	139	61
<i>NOS3</i>	NM_181037.3	F: GCATGGATGAGTATGACGTGGTGT R: GAAGCGGATCTTGTAACCTCTGTGC	185	59
<i>NOS2</i>	NM_001076799.1	F: GGAAGTGGCTACGGAAGTGG R: GGTGAAGCGTGTCTTGGAAA	146	63
<i>ODC1</i>	NM_174130.2	F: GATATTGGTGGTGGCTTTCTCT R: GTCCAGTGCTGGGTTGATTAC	162	58
<i>CSN1S1</i>	BC109618	F: TCACAGTATGAAAGAGGGAA R: AGCCAATAGGATTAGGGA	189	56
<i>CSN1S2</i>	NM_174528.2	F: AGGAACGCAAATGAAGAG R: GGAGTAATGGGAACAGCA	150	55
<i>CSN2</i>	NM_181008	F: TGAGGAACAGCAGCAAAC R: CAGAGGCAGAGGAAGGTG	148	56
<i>CSN3</i>	NM_174294	F: CGCTGTGAGAAAGATGAA R: AGACCGCAGTTGAAGTAA	115	54
<i>IGF2</i>	NM_174087.3	F: TTCGCCCTCGTGCCTGCTATG R: CGGCTGCGTCCGTTTATG	100	61
<i>ACTB</i>	NM_173979.3	F: ACTGTTAGCTGCGTTACACCCTT R: TGCTGTCACCTTCACCGTTCC	190	62
<i>GAPDH</i>	NM_001034034.1	F: GGTCACCAGGGCTGCTTT R: CTGTGCCGTTGAAGTTGC	176	59
<i>SLC7A1</i>	NM_001135792	F: TCAACCAGCCTCCTAGCACT R: GGCACCTTGAATGAGAGCTT	147	60
<i>SLC7A2</i>	XM_010820288	F: CTCGCTCCTCCGTGATAAAT R: ATTCAGCGATCACCTCCATT	168	63
<i>SLC7A6</i>	NM_001075937	F: GCCTTTGGTTTCAGGGTATGC R: CTGGATAGGTGCCAGCTT	128	60
<i>SLC7A7</i>	NM_001075151	F: GGAGCCTCGACTCACTTTGA R: GTCCCTCATGTTGAGCACAG	133	60
<i>SLC7A5</i>	NM_173892	F: CTGATGGAGTATGCGAAGCA R: GGGTCCTGGGTCTTTGTGTA	164	55
<i>SLC7A8</i>	NM_001034428	F: CTGCGAAGTCAATCAGAACCA R: CTCCAGCAGCCTTTCAGAGT	134	63

¹All of the primers used in this experiment were synthesized in Invitrogen (Nanjing, China).

²*ARG1* = arginase 1; *ARG2* = arginase 2; *NOS3* = endothelial nitric oxide synthase; *NOS2* = inducible nitric oxide synthase; *ODC1* = ornithine decarboxylase 1; *CSN1S1* = α_{S1} -casein; *CSN1S2* = α_{S2} -casein; *CSN2* = β -casein; *CSN3* = κ -casein; *IGF2* = insulin like growth factor 2; *ACTB* = β -actin; *SLC7A1* = solute carrier family 7 member 1; *SLC7A2* = solute carrier family 7 member 2; *SLC7A5* = solute carrier family 7 member 5; *SLC7A6* = solute carrier family 7 member 6; *SLC7A7* = solute carrier family 7 member 7; *SLC7A8* = solute carrier family 7 member 8.

³F = forward; R = reverse.

Table 3. Effects of *N*^ω-hydroxy-nor-L-arginine (nor-NOHA) and nor-NOHA + Arg infusion on activity of enzymes related to Arg metabolism in blood

Enzyme ¹	Treatment ²			SEM	P-value
	Control	Nor-NOHA	Nor-NOHA + Arg		
Total arginase (U/L)	7.43 ^a	5.64 ^b	5.70 ^b	0.23	<0.01
ODC (nmol/h)	28.28	27.04	27.96	1.38	0.66
NOS (U/mL)	21.99	23.62	24.43	1.00	0.08

^{a,b}Different superscripts within a row represent significant differences ($P < 0.05$).

¹ODC = ornithine decarboxylase; NOS = nitric oxide synthase.

²Control = saline; nor-NOHA = saline + 125 mg/L of nor-NOHA; nor-NOHA + Arg = saline + 125 mg/L of nor-NOHA + 9.42 g/L of Arg.

RESULTS

Effects of nor-NOHA Infusion on the Activity of Enzymes Associated with Arg Metabolism in the Coccyeal Artery Blood

As shown in Table 3, the activity of total arginase in the group infused with nor-NOHA was lower than in the control ($P < 0.01$), and the total arginase activity did not change when Arg was supplied in the perfusate compared with the nor-NOHA group ($P = 0.97$). However, the activity of ODC ($P = 0.66$) and total NOS ($P = 0.08$) was not affected by different treatments.

Effect of nor-NOHA Infusion on Gene Expression of Enzymes Related to Arg Metabolism

Compared with the control, the mRNA expression of *ARG1* ($P = 0.01$) and *ARG2* ($P = 0.02$) was lower in the nor-NOHA group, the mRNA expression of *ARG1* ($P = 0.04$) and *ARG2* ($P = 0.01$) was also lower in the nor-NOHA + Arg group (Figure 1). Expression of *ARG1* ($P = 0.53$) and *ARG2* ($P = 0.78$) between nor-NOHA and nor-NOHA + Arg was not different. No difference was observed in *ODC1* ($P = 0.11$), *NOS3* ($P = 0.06$), and *NOS2* ($P = 0.91$) expression among groups.

Effects of nor-NOHA Infusion on Milk Production and Milk Quality

Compared with the control, the concentration of milk protein was lower in nor-NOHA ($P = 0.02$), and no difference was observed in milk protein between nor-NOHA and nor-NOHA + Arg ($P = 0.09$; Table 4). However, the milk protein yield in nor-NOHA was lower than that in nor-NOHA + Arg ($P = 0.02$), and no difference in protein yield was found between the control and nor-NOHA ($P = 0.06$). Moreover, the milk fat concentration was not affected by different treatments ($P = 0.04$), but the yield of milk fat was lower in nor-NOHA compared with the control ($P = 0.04$), and

no difference was observed in milk fat yield between nor-NOHA and nor-NOHA + Arg ($P = 0.11$). Furthermore, the infusion of nor-NOHA + Arg had no effects on milk yield compared with control ($P = 0.11$) but led to greater milk yield compared with nor-NOHA ($P < 0.01$). No difference in milk yield between the control and nor-NOHA was detected ($P = 0.35$). Solids-not-fat was not affected by treatments ($P = 0.82$).

Effects of nor-NOHA Infusion on Casein Concentration in Milk

With the infusion of nor-NOHA, the concentration of both α -casein ($P < 0.01$) and β -casein ($P < 0.01$) was lower compared with control (Table 5). In the infused Arg + nor-NOHA group, the concentration of α -casein

Figure 1. Effects of *N*^ω-hydroxy-nor-L-arginine (nor-NOHA) and nor-NOHA + Arg infusion on gene expression (by delta cycle threshold method, 2^{-ΔΔCt}) of enzymes associated with Arg metabolism in the bovine mammary gland. Control = saline; nor-NOHA = saline + 125 mg/L of nor-NOHA; nor-NOHA + Arg = saline + 125 mg/L of nor-NOHA + 9.42 g/L of Arg. Enzymes: ARG1 = arginase 1; ARG2 = arginase 2; ODC = ornithine decarboxylase; NOS3 = endothelial nitric oxide synthase; NOS2 = inducible nitric oxide synthase. Bars with different letters (a,b) for a given enzyme represent significant differences ($P < 0.05$). Error bars = SD.

Table 4. Effects of *N*^ω-hydroxy-nor-L-arginine (nor-NOHA) and nor-NOHA + Arg infusion on milk production and quantity in lactating dairy cows

Item	Treatment ¹			SEM	<i>P</i> -value
	Control	Nor-NOHA	Nor-NOHA + Arg		
Milk yield (kg/d)	20.84 ^{ab}	20.13 ^b	21.91 ^a	0.51	<0.01
Milk fat (%)	3.24	3.05	3.06	0.08	0.04
Milk protein ² (%)	3.67 ^a	3.49 ^b	3.37 ^b	0.18	0.02
Milk fat yield (kg/d)	0.68 ^a	0.62 ^b	0.67 ^{ab}	0.02	0.04
Milk protein yield (kg/d)	0.79 ^{ab}	0.69 ^b	0.81 ^a	0.04	0.01
Solids nonfat (%)	10.13	9.94	9.69	0.69	0.82

^{a,b}Different superscripts within a row represent significant differences ($P < 0.05$).

¹Control = saline; nor-NOHA = saline + 125 mg/L of nor-NOHA; nor-NOHA + Arg = saline + 125 mg/L of nor-NOHA + 9.42 g/L of Arg.

²Concentration of milk protein was calculated with the Nx = 6.38, a conversion ratio transforming concentration of N to protein.

($P = 0.38$) and β -casein ($P = 0.48$) did not change compared with nor-NOHA. In addition, no difference was observed in κ -casein concentrations among treatments ($P = 0.95$).

on the expression of *SLC7A1* ($P = 0.09$), *SLC7A2* ($P = 0.47$), *SLC7A6* ($P = 0.19$), and *SLC7A7* ($P = 0.43$).

DISCUSSION

Effects of nor-NOHA Infusion on Gene Expression of Casein in Mammary Tissue

As shown in Figure 2, the mRNA expression of *CSN1S1*, *CSN1S2*, and *CSN2* in response to nor-NOHA and nor-NOHA + Arg was lower compared with the control group ($P < 0.01$). No difference was observed in expression of *CSN1S1* ($P = 0.60$), *CSN1S2* ($P = 0.76$), and *CSN2* ($P = 0.05$) between nor-NOHA and nor-NOHA + Arg. Furthermore, the expression of *CSN3* was not affected by treatments ($P = 0.86$).

Effects of nor-NOHA Infusion on mRNA Expression of AA Transporters

Compared with the control ($P < 0.01$) or nor-NOHA group ($P < 0.01$), infusion of nor-NOHA + Arg led to greater expression of both *SLC7A5* and *SLC7A8* (Table 6). However, no difference was observed in expression of *SLC7A5* ($P = 0.88$) and *SLC7A8* ($P = 0.50$) between the control and nor-NOHA. Treatment had no effect

Effects of nor-NOHA Infusion on Enzymes Related to Arg Metabolism

N^ω-Hydroxy-nor-L-arginine is a natural and powerful inhibitor of arginase and has no effects on the activity of NOS (Custot et al., 1997). Similar to a previous in vitro study (Wang et al., 2017), the activity of arginase in coccygeal artery blood and the expression of *ARG1* and *ARG2* in mammary tissue decreased after infusing nor-NOHA for 7 d. Reid et al. (2007) infused nor-NOHA in mice and reported lower activity of total arginase in blood. In addition to the effects on the activity of arginase in blood, nor-NOHA was also reported to had efficient negative effects on the activity of arginase in different tissues like mammary artery, heart, kidney, and aorta (Kropf et al., 2005; Shemyakin et al., 2012; Rodríguez-Gómez et al., 2015). The inhibition of the Arg-Orn pathway through inhibition of arginase also can affect other enzymes related to Arg metabolism. Benson et al. (2011) reported that the production of Orn synthesized from Arg decreased when the activity

Table 5. Effects of *N*^ω-hydroxy-nor-L-arginine (nor-NOHA) and nor-NOHA + Arg infusion on concentration of caseins in milk (mg/mL)

Item	Treatment ¹			SEM	<i>P</i> -value
	Control	Nor-NOHA	Nor-NOHA + Arg		
α -Casein	13.93 ^a	13.16 ^b	13.37 ^{ab}	0.23	0.01
β -Casein	11.56 ^a	9.89 ^b	10.24 ^b	0.48	0.01
κ -Casein	3.19	3.15	3.02	0.56	0.95

^{a,b}Different superscripts within a column represent significant differences ($P < 0.05$).

¹Control = saline; nor-NOHA = saline + 125 mg/L of nor-NOHA; nor-NOHA + Arg = saline + 125 mg/L of nor-NOHA + 9.42 g/L of Arg.

Table 6. Effects of *N*^ω-hydroxy-nor-L-arginine (nor-NOHA) and nor-NOHA + Arg infusion on gene expression of AA carriers in mammary gland (fold-change relative to control $2^{-\Delta\Delta C_t}$)

Item	Treatment ¹			SEM	P-value
	Control	Nor-NOHA	Nor-NOHA + Arg		
<i>SLC7A1</i>	1.04	0.51	3.83	1.29	0.09
<i>SLC7A2</i>	1.01	0.83	1.04	0.17	0.47
<i>SLC7A6</i>	1.11	0.72	1.25	0.26	0.19
<i>SLC7A7</i>	1.03	1.18	1.22	0.14	0.43
<i>SLC7A5</i>	1.01 ^b	1.09 ^b	3.54 ^a	0.17	<0.01
<i>SLC7A8</i>	1.02 ^b	0.63 ^b	2.78 ^a	0.33	<0.01

^{a,b}Different superscripts within a column represent significant differences ($P < 0.05$).

¹Control = saline; nor-NOHA = saline + 125 mg/L of nor-NOHA; nor-NOHA + Arg = saline + 125 mg/L of nor-NOHA + 9.42 g/L of Arg.

of total arginase was inhibited. Along with lower activity of ODC, the decrease of Orn and ODC caused a decrease of putrescine production. In the present study, the supplementation of nor-NOHA via the jugular vein had no effects on the activity and gene expression of ODC, which was different from a previous study in T cells (Munder et al., 2006). Furthermore, the infusion of nor-NOHA had no effects on the activity of NOS and the mRNA expression of *NOS2* and *NOS3*, which was similar to a study in mice (Reid et al., 2007).

As substrate of arginase and NOS, Arg has been reported to elicit some regulation on enzymes related to Arg metabolism; for example, the limitation of extracellular L-arginine inhibited the expression of *iNOS* and *ARG* (Morris, 2004). In inflammatory macrophages, El-Gayar et al. (2003) found that activity of NOS was

related to the concentration of Arg in growth medium. In the present study, the addition of Arg to the infusate with nor-NOHA had no effect on the activity of NOS or, the expression of *NOS3* and *NOS2* compared with the control group. Those results were opposite to Topal et al. (2006) who reported that the supplementation of Arg to human epithelial cells treated with nor-NOHA increased NOS activity. In bovine pulmonary artery endothelial cells treated with nor-NOHA, Arg also increased NOS activity (Chicoine et al., 2004). These differences may be due to the complex in vivo environment. In addition, Sugano et al. (1982) reported that an increase in dietary Arg concentration led to greater activity of arginase in mouse liver. Gonzalez and Byus (1991) reported that the supplementation of Arg to a mouse diet enhanced the activity of ODC. The fact that infusion of Arg plus nor-NOHA in the present study did not have effects on both the activity and gene expression of arginase and NOS might be related to the high potency of nor-NOHA for inhibition of arginase activity.

Effects of nor-NOHA Infusion on Casein Synthesis, Milk Production, and Quality

Previous research revealed that milk yield is closely related to mammary blood flow and concentration of nutrients in blood (Renaudeau et al., 2002). Arginine elicits positive effects on milk synthesis in addition to protein synthesis (Boyd et al., 1995; Jobgen et al., 2006). For instance, Mateo et al. (2008) reported that pig litter weight and sow milk yield were significantly improved by adding 1% Arg to diets. Despite the lack of effect of nor-NOHA on milk yield compared with the control, milk yield in response to infusion of nor-NOHA + Arg increased and was higher than that in the nor-NOHA groups.

The lower activity of arginase in response to nor-NOHA strongly indicated that the Arg-Orn pathway

Figure 2. Effects of *N*^ω-hydroxy-nor-L-arginine (nor-NOHA) and nor-NOHA + Arg infusion on gene expression of casein in bovine mammary tissue (delta cycle threshold method, $2^{-\Delta\Delta C_t}$) of enzymes associated with Arg metabolism in the bovine mammary gland. Control = saline; nor-NOHA = saline + 125 mg/L of nor-NOHA; nor-NOHA + Arg = saline + 125 mg/L of nor-NOHA + 9.42 g/L of Arg. Genes: *CSN1S1* = α_{S1} -casein; *CSN1S2* = α_{S2} -casein; *CSN2* = β -casein; *CSN3* = κ -casein. Bars with different letters (a,b) within a casein gene represent significant differences ($P < 0.05$). Error bars = SD.

was inhibited. Furthermore, the infusion of Arg plus nor-NOHA resulted in the activation of the Arg-NO pathway and an increase of NO production. As reported previously (Meininger and Wu, 2002), NO, which is the product of the Arg-NO pathway, had positive effects on growth and development of blood vessels in the mammary gland. Nitric oxide is a potent vasorelaxant of the vasculature of the mammary gland, and exogenous NO had positive effects on blood flow to the mammary gland (Lacasse et al., 1996). Kim and Wu (2009) added 50 mg/d of diethylenetriamine-NO (a NO donor) to diets of lactating sows and detected an increase in milk production. Thus, these kinds of effects could partly explain the milk yield increase in cows infused with nor-NOHA + Arg.

Polyamines are the final products of the Arg-Orn pathway, and were reported to elicit a positive regulation of milk protein synthesis and secretion (Wu and Morris, 1998; Kong et al., 2014). As a rate-limiting enzyme of the Arg-Orn pathway, arginase activity also was reported to elicit positive effects on the synthesis of milk protein in mammary gland (Yip and Knox, 1972). In the present study, the decrease in activity of arginase with the infusion of nor-NOHA led to a decrease of milk protein percentage, a response that also was detected in sow mammary gland (Mateo et al., 2008). As a main constituent of milk protein, casein is regarded as an important nutrient in milk. In the present study, the concentration of α -casein and β -casein in milk, and gene expression of *CSN1S1*, *CSN1S2*, and *CSN2* in mammary gland also decreased when arginase activity was reduced by nor-NOHA. These results were similar to previous in vitro findings (Wang et al., 2017).

Some studies indicate that Arg plays an important role in increasing the casein synthesis in BMEC (Wang et al., 2014; Wu et al., 2016). However, in this study the addition of Arg into the perfusate with nor-NOHA had no effect on concentration of α -casein and β -casein in milk compared with nor-NOHA. The mRNA expression of *CSN1S1*, *CSN1S2*, and *CSN2* also was not affected. As indicated in the previous section, the lack of response to supplemental Arg when nor-NOHA was infused could be related to a high efficiency of the compound for inhibition of arginase.

Effects of Arginase Inhibition on Gene Expression of AA Transporters

It is well established that both the ratio of EAA and the amounts supplied are vital determinants of the synthesis and secretion of milk protein (Bequette et al., 1998; Burgos et al., 2010). In addition, the amount

and activation of AA carrier proteins on the cell plasma membrane are essential for the positive response in synthesis of protein when AA supply increases (Hundal and Taylor, 2009; Wempe et al., 2012a).

In addition to being the substrate for protein synthesis, some AA can regulate physiological function of cells by modulating the expression of protein carriers (Wempe et al., 2012b). In the present study, no difference in *SLC7A1*, *SLC7A2*, *SLC7A6*, and *SLC7A7* expression was observed among treatments. The infusion of nor-NOHA also had no effect on gene expression of *SLC7A5* and *SLC7A8* compared with the control, but when Arg was added to the infusate, expression of *SLC7A5* and *SLC7A8* increased. Furthermore, Zeng et al. (2012) recently reported that Arg could increase expression of *SLC7A8* to promote growth and proliferation of porcine intestinal epithelial cells through activation of the mammalian target of rapamycin (**mTOR**) pathway.

The mTOR protein is a conserved serine/threonine kinase that has a central role in the regulation of cell growth and metabolism (Chi, 2012). The mTOR senses and integrates diverse environmental signals, including nutrients (protein, AA, and glucose) and growth factors, and it is activated when the supply of AA is adequate (Han et al., 2012). Across different animal species, Wall et al. (2013) and Columbus et al. (2015) found that some branched-chain AA play an important role in protein synthesis by activating mTOR. Wang et al. (2014) also detected that increasing availability of Arg can enhance casein synthesis in BMEC. In another study, Arg was reported to reduce the production of IL-1, IL-6, and TNF- α (Wu et al., 2016) by increasing mTOR expression.

The AA carriers sodium-coupled neutral AA transporter 2, proton-assisted AA transporter, *SLC7A5*, and *SLC7A8* play a role in the positive regulation of mTOR in response to exogenous AA availability (Nicklin et al., 2009; Heublein et al., 2010; Levy et al., 2011). In the present study, the infusion of nor-NOHA + Arg increased the expression of both *SLC7A5* and *SLC7A8* in bovine mammary gland tissue; hence, it was expected that production of milk protein would have also increased due to the activation of mTOR and the ensuing increase in gene expression of AA transporters. However, the lack of production responses in response to nor-NOHA + Arg compared with nor-NOHA indicate that the regulation of protein synthesis via Arg might not depend entirely on the mTOR pathway. Furthermore, AA transporters such as *SLC7A5* and *SLC7A8* may have an additional AA-sensing role related to mTOR activation such as control of cell proliferation and cellular protein synthesis.

CONCLUSIONS

The jugular infusion of nor-NOHA reduced the activity of arginase in blood of caudal artery and both the content of protein and casein in milk. The supplementation of Arg to mammary gland, which should promote the synthesis of milk protein, played no roles in the production of both milk protein and casein in cows infused with nor-NOHA, but increased the expression of some AA transporters (*SLC7A1*, *SLC7A5*, and *SLC7A8*), which could elicit some positive effects on the synthesis of protein in mammary gland. Keeping consideration of the results above, although the changes of activity of enzymes related in Arg metabolism in mammary gland were unclear after infusing with nor-NOHA in the present study, we could propose that, the Arg-Orn pathway in mammary gland should be reduced significantly by inhibition of arginase activity through nor-NOHA, which resulted in the invalidity of Arg addition. Combined with the results of gene expression of enzymes related in Arg metabolism in mammary tissue, there is support for previous in vitro data that the synthesis of casein protein should be closely regulated by the Arg-Orn pathway in the bovine mammary gland.

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