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Review Article

Arginine Regulatory Mechanisms on Casein Synthesis in Dairy Cows

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Arginine, Milk protein, Molecular mechanism, Signaling pathway, Bovine

ABSTRACT

As one functional amino acid in various tissues, arginine (Arg) has received increased interest due to its key roles in metabolism and physiology. Arg not only regulates lactation performance (milk yield and milk composition) by serving as a building block to synthesize milk protein, but also acts as a signaling molecule coordinating casein protein gene transcription and translation. A number of studies have characterized various possible pathways by which mammary total milk protein synthesis can be affected. Changes in the abundance of milk protein genes, efficiency of mRNA translation altered by phosphorylation of translation factors in individual cells, the abundance of the translational apparatus in secretory cells, or the number of secretory cells are responsible for nutritional modification of total milk protein synthesis. This paper reviews advances in the molecular regulatory mechanism whereby Arg regulates casein synthesis and mammary development, focusing on novel mechanisms, including transcription factors, microRNA and metabolic enzymes. Our aim is to highlight fundamental information that can aid in the improvement of approaches to enhance milk protein quality.

INTRODUCTION

Milk protein is a key indicator of milk quality and safety of dairy products; hence, its regulation is at the core of the competitiveness of the dairy industry. Milk protein is mainly composed of casein, whey protein, and a small amount of milk fat globule membrane protein. Casein, consisting of αs1-, αs2-, β-, and κ-caseins, accounts for 76%-86% of total milk protein (Swaisgood, 2003) (Table I), derived from casein phosphorylation, glycosidation and hydrolysis. Thus, research on casein synthesis regulation is essential and necessary to the dairy industry in terms of improving total milk protein content.

Table I. Composition of milk protein in dairy cattle.

Types	Composition	Content (%)
Casein	αs1-Casein	34-40
	αs2-Casein	11-15
	β-Casein	25-35
	κ-Casein	8-15
Whey protein	α-Lactalbumin	2-4
	β-Lactalbumin	7-12
	Serum albumin	0.5-2
Lactoferrin		Nil
Immunoglobulin (Ig)	IgA, IgG1, IgG2, IgM	Nil

This table is cited from advanced dairy chemistry by Swaisgood (2003).

The mammary gland is a site of extensive synthesis and degradation of amino acids (AA) (Manjarin *et al*., 2014). Milk protein yield accounts for approximately 90% of net mammary AA up-take by mammary acinar epithelial cells (Cant *et al*., 2018). Thus, as raw materials for milk protein synthesis, the availability and balance of AA is critical for improving milk protein synthetic efficiency and milk protein content during lactation. Previous work on

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AA metabolism in dairy cattle has focused on achieving an optimal balance in the diet based on an ideal protein amino acid (IPAA) concept that, conceptually, is needed to maintain and enhance lactation performance (Bequette *et al*., 1998; Hanigan *et al*., 2002).

Besides its well-established role in protein synthesis and the urea cycle, Arginine (Arg) (a conditionally-essential AA) is also a functional AA involved in numerous physiological activities including maintenance of optimal growth and nitrogen retention in a number of animal species (Wu, 2009). Indeed, Arg is unique in terms of its mammary gland metabolism in that, in dairy cows, its uptake is 2 to 3 times the amount secreted in milk protein, and this relationship is not altered by changes in dietary protein supply (Lapierre *et al*., 2009). In contrast, the mammary uptake to output ratio (U:O) for the other 9 essential amino acids (EAA) varies only between 1 and 1.4 (Mepham, 1982). Thus, the large uptake of Arg coupled with its relatively lower content in milk protein (3.4% of CP) (crude proteion) (Swaisgood, 1995) points to substantial Arg catabolism by mammary tissue. This paper reviews the effects of Arg on casein synthesis in mammary gland, and explores molecular mechanisms among milk protein synthesis processes in order to aid in the improvement of approaches to enhance milk protein quality.

BIOLOGICAL ACTIVITY AND CATABOLIC PATHWAYS OF ARGININE

Free Arg can be generated from dietary protein (Castillo *et al*., 1993), turnover of body proteins (approximately 85% of Arg in the circulation), and *de novo* synthesis (Castillo *et al*., 1995). Despite the fact that the dairy cow can synthesize Arg, it is normally considered as a conditionally-EAA especially for gestating and lactating cows allowing for optimal reproductive and productive performance. This, in part, is due to the fact that *de novo* synthesis is not sufficient to meet the requirements of the high-producing cow (NRC, 2001). As depicted in Figure 1, Arg is well-known as a direct precursor for the synthesis of various signaling molecules such as ornithine (Orn), nitric oxide (NO), urea and polyamines. All of these are important compounds coordinating peripheral blood flow, cell division, tissue growth and protein synthesis and, hence, play an irreplaceable role in regulating reproduction and lactation (Bequette *et al*., 1998).

There are three main catabolic pathways for Arg in mammary tissue (O'Quinn *et al*., 2002) (Fig. 1). One is the Arg/NO pathway where Arg is metabolized by nitric oxide synthase (NOS) to NO, a signal transduction molecule involved in maintenance of vascular permeability and plasma flow. Another is the Arg/Orn pathway wherein Arg is converted to Orn by arginase prior to being further metabolized to proline or glutamine through transamination via ornithine aminotransferase (OAT) or to polyamines (putrescine, spermidine, and spermine) mainly through decarboxylation via ornithine decarboxylase (ODC). The third pathway of Arg metabolism involves arginine: Glycine amidino transferase (AGAT), which catalyzes the reaction of Arg to Orn and creatinine (Mateo *et al*., 2008).

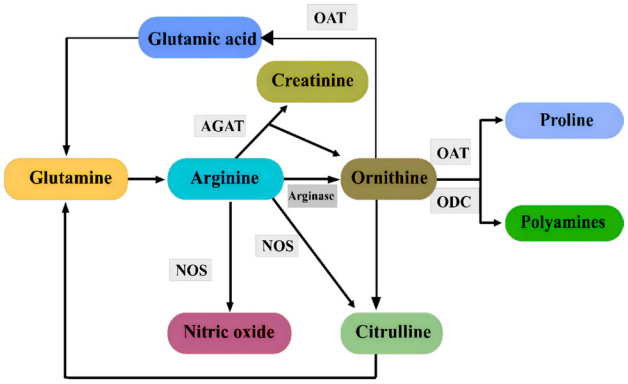

Fig. 1. Arginine (Arg) metabolic pathways in mammary tissue. In the Arg/NO pathway, nitric oxide synthase (NOS) uses Arg to synthesize nitric oxide (NO) and citrulline. In the Arg/Orn pathway, Arg is converted to Ornithine (Orn) via arginase, and is further metabolized to proline or glutamine through transamination via ornithine aminotransferase (OAT) or to polyamines mainly through decarboxylation via ornithine decarboxylase (ODC). The third pathway of Arg metabolism is the arginine: Glycine amidino transferase (AGAT) in which Arg is used to synthesize Orn and creatinine.

ARGININE REGULATION OF CASEIN SYNTHESIS *IN VITRO* AND *IN VIVO*

In vitro *studies of arginine regulation of casein synthesis*

Total cell proliferation and percentage of secretory cells are positively correlated with milk production, hence, are important determinants of milk protein yield. Some studies support the role of Arg in promoting casein synthesis via its effect on cell proliferation. For instance, Greene *et al*. (2013) reported that Arg at both physiological (0.2 mmol/L) and supra-physiological (0.8 mmol/L) concentrations increased proliferation of human endometrial RL95-2 cells. Supplying Arg at 0.4-6.4 mmol/L had a positive effect on the proliferation of bovine mammary epithelial cells (BMEC), which peaked at an Arg concentration of 3.2 mmol/L in the medium (Wang *et al*., 2014).

Milk protein synthesis in mammary cells is directly

determined by the efficiency of mRNA and protein expression of four kinds of caseins. Indeed, Arg regulating milk protein synthesis is partly achieved by manipulation of transcriptional, translational and post-translational processes of casein proteins via several well-known signaling pathways. Our *in vitro* studies were the first to demonstrate that casein synthesis in BMEC was affected by Arg supplementation in a dose-dependent manner with an optimal concentration. The mRNA abundance of caseins and mechanistic target of rapamycin (mTOR) targets as well as the abundance of α- and β-casein protein was optimal at 3.2 mmol/L of Arg in the medium (two times the profile of Arg in casein) (Wang *et al*., 2014; Chen *et al*., 2013). The fact that total casein abundance and the content of β-casein were enhanced after supplementing Arg during an lipopolysaccharide (LPS) challenge in BMEC (Wu *et al*., 2016) indicates that even during inflammatory or stress conditions, increasing the supply of Arg can elicit a positive effect on protein synthesis.

The dose-dependent increase in casein synthesis observed *in vitro* is partly due to its role as one of the backbones for protein synthesis. However, repression of casein synthetic ability beyond a certain level of Arg supplementation can be partly explained by the fact that the transfer efficiency of absorbed AA into protein is decreased as protein synthesis reaches an upper limit within mammary cells (Raggio *et al*., 2004).

In vivo *studies of arginine regulation of casein synthesis*

Nucleic acid content has been used as indicator of mammary development status and the capacity for protein synthesis (Hackett and Tucker, 1968). The onset of lactation in many species is always associated with an increase in ratio of RNA to DNA (Akers *et al*., 1981), while the decline in milk production after peak lactation is accompanied by a decrease in total RNA amount in the mammary epithelium (Capuco *et al*., 2001). Arg is capable of increasing functional properties of mammary cellular activity and DNA-RNA concentrations, both of which are positively correlated with promoting mammary gland alveolar duct enlargement and elongation that fundamentally determine milk secretion capacity (Iizuka *et al*., 1997).

Alkareem *et al*. (2013) reported that glandular volume density, number of terminal end buds and length of mammary ducts in mice were all increased significantly in response to Arg loading dose at 0.57-1.44 mmol/kg body weight (BW) compared with unsupplemented controls. Zhou *et al*. (2021) reported that Arg could promote rat mammary tissue development as indicated by enhanced mammary acinar area. A beneficial effect of Arg on mammary development is further confirmed by a study in which an Arg-deficient diet decreased mammary total content of DNA and RNA in gestating rats. The negative effect extended to an inhibition on both mammary gland development and mammary protein synthesis during lactation (Pau and Milner, 1982). These data demonstrated that Arg is uniquely required for optimal mammary gland growth and a greater capacity for protein synthesis.

The stimulatory effects of Arg on casein synthesis *in vivo* are substantiated by the fact that Arg-supplemented sows had a greater milk protein content on day 7 of lactation (Mateo *et al*., 2008). Deletion of Arg from an abomasal AA infusion did not impair milk protein yield, but decreased the mammary uptake of Arg (by 3.2 mmol/h) (Doepel and Lapierre, 2011). Authors concluded that examination of net balance of nutrients across the mammary gland would be needed to explain how cows coped with this deficiency of Arg to maintain milk protein response. Jugular vein infusions of Arg performed by our laboratory observed that Arg induced a greater mRNA abundance of casein genes (*CSN1S1* and *CSN1S2*) as well as abundance of α- and κ-casein proteins (Zhou *et al*., 2016). Similarly, a significant increase of β-casein content in rat mammary gland with supplemental dietary Arg was detected (Zhou *et al*., 2021). Unlike work with Arg, infusions of EAA into cows that increased milk protein yield by 29% over controls had no effect on mRNA abundance of β-casein (Nichols *et al*., 2017). Authors concluded that such lack of effect might have been partly due to the variation in the composition of casein between species.

MECHANISM OF ARGININE REGULATION OF PROTEIN AND CASEIN SYNTHESIS

Arginine regulates mammary gland development and amino acid availability

Mammary glands exhibit a series of developmental stages that encompass proliferation, differentiation and involution, and mammary epithelial cell turnover is typified by proliferation and apoptosis (Stefanon *et al*., 2002). The potential for milk production of mammary glands is shaped by both mammary epithelial cell number and their secretory activity (Forsyth, 1986), thus, establishing a direct link between the growth status of mammary gland and milk yield and milk protein content. The decline in daily milk yield with advancing lactation is due primarily to a decrease in secretory cell number rather than a change in cellular milk synthesizing activity (Capuco *et al*., 2001), which underscores the important role of mammary cell proliferation in milk production.

Arg is a constituent AA for protein synthesis in cells and is considered to be an EAA for young developing mammals, suggesting its essentiality for cellular growth

(Flynn *et al*., 2002). Arg supplementation enhances angiogenesis (Holanda *et al*., 2019) and the amount of nutrients provided to mammary glands (Kim and Wu, 2009) that are required for optimal mammogenesis. In turn, these effects can determine the secretion of nutrients via colostrum and milk (Krogh *et al*., 2016). A deficiency of dietary Arg impairs the growth and development of mammary glands in rats (Pau and Milner, 1982). In addition, the increase in cell proliferation and decreased incidence of cell apoptosis with the greater supply of Arg are likely associated with its metabolites including polyamines and NO (Oka and Perry, 1974). These are important during lactogenesis because they stimulate vasodilation, blood flow, and nutrient uptake by the mammary glands (Meininger and Wu, 2002; Lenaerts *et al*., 2007).

Studies in epithelial colorectal adenocarcinoma cells indicated that Arg deprivation decreased cell proliferation and enhanced the susceptibility to apoptosis, and those changes were restored by subsequent supply of Arg or citrulline (Lenaerts *et al*., 2007). Polyamines exert their cellular effect via the ability of binding nucleic acids and proteins, and have been implicated in the promotion of an anti-apoptotic state in various cell lines (Igarashi and Kashiwagi, 2000; Seiler and Raul, 2005). NO can stimulate phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling as well as the expression of the enzyme ODC, which is responsible for converting Orn to the first polyamine putrescine, hence, promoting cell survival (Kawasaki *et al*., 2003). Taken together, the Arg-NO/polyamine pathway can enhance lactation performance partly via promoting mammary cellular growth and survival, both of which are important for mammary gland development.

There are three factors involved in nutrient provision to the lactating mammary gland: blood nutrient concentration, blood flow, and cellular uptake (Baumrucker, 1984). In response to supply of Arg, mammary glands are able to modify their blood flow rate and net clearance of AA out of plasma, hence, potentially affecting milk yield and milk protein content (Cant *et al*., 2018). Arg supplementation to multiparous sows during gestation and lactation increased mammary plasma flow by 32% (Krogh *et al*., 2016). Increased portal vein blood flow of growing pigs was determined after portal infusion of 0.35 mmol/kg BW of Arg (Tan *et al*., 2012). Subtraction of Arg from a mixture of EAA infused into the abomasum tended to decrease mammary blood flow (Doepel and Lapierre, 2011). Infusion of NO also increased the rate of blood flow to mammary glands in lactating goats (Lacasse and Prosser, 2003), while infusion of an NOS inhibitor into the external iliac artery of lactating cows decreased mammary plasma flow (Cieslar *et al*., 2014). Together, these researches indicate that the Arg-induced elevation in blood flow to the mammary glands is partly via enhancing the synthesis of the vasodilator NO, hence, increasing supply of nutrients for milk production.

The net uptake of AA, a consequence of bidirectional transport across the plasma membranes of mammary epithelial cells, can be used to estimate rates of AA catabolism and associations with cellular mammary pathways of milk synthesis and nutrient metabolism (Cant *et al*., 2018). Although the Arg content of milk protein is relatively low (3.4% of CP) (Swaisgood, 1995), mammary uptake of Arg is relatively high. Doepel and Lapierre (2011) reported that mammary uptake of Arg was greater with a mixture of EAA including Arg infused than that of the Arg-free group. It is noteworthy that this kind of bidirectional AA transport across the membrane relies on transport systems. A maximal increase of milk yield of 10% to 15% might be limited by competition of substrates for specific transport systems (Clark *et al*., 1978), and the mammary excess uptake of Arg may be a result of a shared transport system (Baumrucker, 1984).

Different intracellular pools of Arg exist in different cell types, suggesting diversity of functions of Arg transporters (Closs, 2002). About 70% of the intracellular Arg transport occurs via the y^+ system (Grillo and Colombatto, 2004), a group of five cationic transporters designated as CATs (SLC7 family), among which CAT1 transports Arg in mammary epithelial and endothelial cells. Studies with bovine, murine and porcine mammary glands have reported that *CAT1* coding gene expression was increased with the onset of lactation (Alemán *et al*., 2009) and was associated with changes in β-casein mRNA abundance (Manjarin *et al*., 2011). Arg deprivation enhanced transcription and translation of the *CAT1* coding gene and CAT1 protein, respectively, which might have resulted from the preservation of its availability and utilization under this condition (Fernandez *et al*., 2003). Thus, it seems that increasing the supply of Arg regulates the synthesis of casein protein in part via its influence on blood flow and intracellular AA concentrations taken up by mammary gland via the various AA transport systems.

Arginine regulates signal transduction pathways of casein synthesis

Transcriptional and translational alterations of various signaling pathways such as JAK/STAT, mTOR, and ISR are involved in casein synthesis. Thus, Arg can regulate milk protein synthesis partly through alterations in abundance of components in those signaling pathways. An intact Janus-activated kinase (JAK)- signal transducer and activator of transcription (STAT) signaling is required for the induction of NOS and the production of NO

(Stempelj *et al*., 2007), and has been implicated in various physiological activities including growth, inflammatory reactions and transcriptional regulation of casein synthesis.

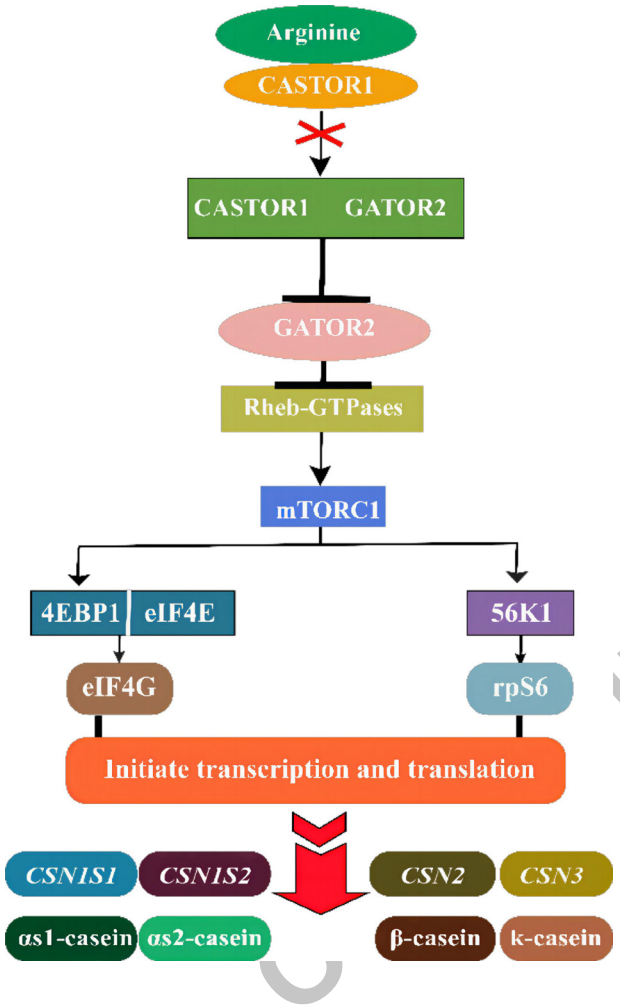

Fig. 2. Arginine (Arg) -induced mechanistic target of rapamycin complex1 (mTORC1) signaling pathway in the context of casein synthesis. Arg disrupts the interaction of the cellular arginine sensor for mTORC1 (CASTOR) dimers with GATOR2 complex, allowing free GATOR2 to inhibit GATOR1 complex, and further activates mTORC1 through the active Rag GTPases to localize mTORC1 to lysosomes. Active mTORC1 activates transcription and translation of caseins through its interactions with eukaryotic initiation factor 4E-binding protein 1 (4EBP1), the eukaryotic initiation factor 4E (eIF4E), and ribosomal S6 kinase 1 (S6K1). Briefly, mTORC1 phosphorylates translation inhibitor 4EBP1, releasing it from eIF4E to free eIF4E to join the eukaryotic translation initiation factor 4G (eIF4G). mTORC1 phosphorylates and activates S6K1, which in turn stimulates the initiation of protein synthesis through activation of ribosomal protein S6 (rpS6).

Different growth factors, prolactin and AA are reported as activators of STAT via JAK-mediated phosphorylation, and activated STAT5 binds to gamma interferon activation sequence sites to modulate the transcriptional activity of target genes such as casein protein genes (Gouilleux *et al*., 1994). Another two AA-sensing mechanisms have been identified in mammalian cells and may be responsible for the milk protein stimulation during AA supplementation. One is through uncharged tRNA that activates phosphorylation of eIF2α as part of the integrated stress response (ISR), from a substrate for GDP/GTP exchange to an inhibitor of the exchange, which slows initiation of mRNA translation in response to AA deficiencies and other cellular stresses (Proud, 2005). The other involves the mTOR signaling pathway that mediates cellular anabolic responses to cytokines, AA, and intracellular energy state that stimulates mRNA translation by phosphorylation of the S6K1 and 4EBP1 (Tan *et al*., 2010) (Fig. 2).

Among all upstream components of the mTOR pathway in the nutrient-sensing branch, branched-chain AA and particularly Arg exert the most effective stimulatory effect on protein synthesis by regulating translation initiation. The cellular arginine sensor for mTORC1 (CASTOR proteins) was recently identified as potential Arg sensor (Chantranupong *et al*., 2016). Arg disrupts the interaction of CASTOR dimers with GATOR2 complex by binding at the interface of two aspartate kinase, chorismate mutase, TyrA domains of the CASTOR1 homodimer and, hence, allowing free GATOR2 to inhibit the GATOR1 complex. Less inhibition on GATOR1 further facilitates the activated Rag heterodimers to localize mTOR complex 1 (mTORC1) to the surface of lysosomes where the Rheb-GTPase is located, which promotes the activation of mTORC1. Despite available data, the molecular mechanism of activation of the AA-induced upstream mTOR signaling pathway requires further research.

The positive effect of Arg and its metabolites on cell proliferation, survival and protein synthesis through JAK/STAT and mTOR-related signaling pathways have been extensively studied in various species. Both, *in vitro* with BMEC and *in vivo* through Arg jugular infusion studies in our laboratory demonstrated that the mRNA abundance of JAK2, STAT5, mTOR and S6K1 was the highest at 3.2 mmol/L of Arg in culture medium (two times the profile of Arg in casein protein), while *4EBP1* gene expression was significantly down-regulated, consistent with its negative regulation of protein translation (Wang *et al*., 2014). Similarly, Bauchart *et al*. (2010) reported that Arg enhances the activation of mTOR, S6K1, and 4EBP1 in a time- and dose-dependent manner in porcine neonatal intestinal epithelial cells (IEC), underscoring an increase in cell survival and protein synthesis with a maximal response at a physiological concentration of 0.1-0.5

mmol/L of Arg.

Studies in rat IEC (Ban *et al*., 2004) and in chicken enterocytes (Yuan *et al*., 2015) revealed a similar stimulation on mTORC1 downstream targets in response to greater supply of Arg. Consistent with the results *in vitro*, a study in piglets reported that dietary Arg treatment increased intestinal protein synthesis associated with the activation of mTOR downstream signaling (Corl *et al*., 2008). Wang *et al*. (2018) speculated that Arg supplementation increased the rate of protein synthesis in mouse C2C12 myoblasts in an NO-dependent manner, while Kong *et al*. (2012) reported that greater supply of Arg increased protein synthesis and decreased protein degradation of porcine conceptus trophectoderm cells independent of NO. The different responses to NO in the context of Arg regulation of protein synthesis via mTOR signaling might be associated with species-specific mechanisms.

Arginine regulates miRNA metabolism related to casein synthesis

A microRNA (miRNA) is a small non-coding RNA molecule containing about 21-25 nucleotides that functions in RNA silencing and post-transcriptional regulation of gene expression. miRNAs are abundant in many cell types and appear to target about 60% of the genes in mammals (Friedman, 2009). Numerous miRNAs are evolutionarily conserved, which implies that they perform important biological functions (Bartel and David, 2018). Recent studies have suggested a vital role for miRNA in lactation physiology across various species. Zidi *et al*. (2010) examined the 3 untranslated regions (UTR) of caprine *CSN1S1*, *CSN1S2*, *CSN2*, and *CSN3* via resequencing and identified five single nucleotide polymorphisms that might create or disrupt miRNA target sites. These data suggested that polymorphisms at miRNA target sites might have some effect on the control casein expression. Wang *et al*. (2012) demonstrated that the expression pattern of miRNA associated with cellular proliferation, lipid metabolism, and innate immunity in bovine mammary tissue was altered by stage of lactation. Izumi *et al*. (2014) observed different expression profiles of some immune- and development-related miRNA in rat milk. Thus, together the available data indicate that miRNA might constitute a critical regulatory step in the physiological control of mammary gland function across stages of lactation.

Our laboratory screened 8 miRNAs related to Arg-mediated casein synthesis (Fig. 3) whose expression was greater in mammary cells incubated with 2-fold more Arg than controls (3.2 mmol/L *in vitro* and 216.2 mmol/d *in vivo*) (Zhang *et al*., 2020). Among the miRNA studied, miR-743a negatively regulates *mdh2*, encoding malate dehydrogenase (MDH), at a post-transcriptional level by directly targeting its 3'UTR (Shi and Gibson, 2011). Greater expression of miR-743a in response to increased supply of Arg might inhibit *mdh2* mRNA abundance and MDH activity, and, hence, alleviate the oxidative stress status of mammary epithelial cells. This idea is supported by the positive effect of Arg on antioxidant capacity in mammary gland tissue of lactating sows (Holanda *et al*., 2019).

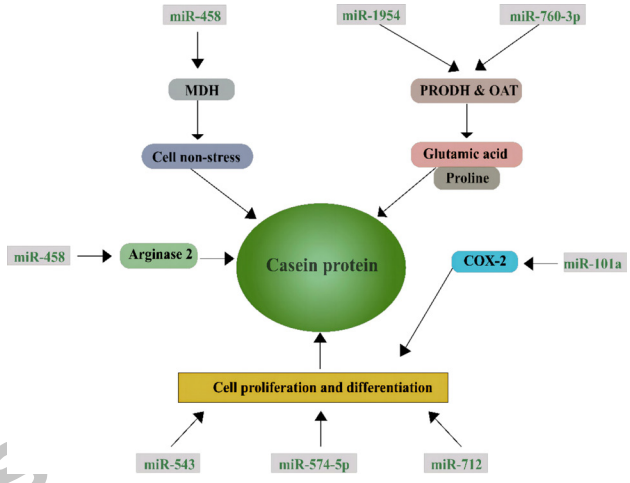

Fig. 3. Mechanism of the regulation of 8 screened-miRNA by arginine (Arg) supplementation and effects on casein synthesis. Greater expression of miR-743a negatively regulates dehydrogenase (MDH) in response to supply of Arg leading to inhibition of MDH activity and, hence, alleviation of the oxidative stress status of mammary epithelial cells. miR-1954 and miR-760-3p are both predicted to target proline dehydrogenase (PRODH) and ornithine aminotransferase (OAT). Their induced expression with Arg supplementation suggested that Arg promotes cell proliferation and casein synthesis partly via inhibition on formation of glutamine and proline in the Arg/Orn pathway. miR-101a has been shown to regulate cell proliferation partly via alteration of the expression of cyclooxygenase-2 (Cox-2) that is critical for mammary gland development. Furthermore, miR-543, miR-574-5p and miR-712 target genes that are related to cell proliferation and differentiation, which suggests that Arg promotes cellular proliferation associated with those four miRNA candidates. The opposite response of miR-468 when the supply of Arg increases both *in vitro* and *in vivo* might be related to the regulation of arginase 2.

The expression of miR-1954 and miR-760-3p, both predicted to target proline dehydrogenase (PRODH) and OAT, was induced by Arg supplementation suggesting that Arg promotes cell proliferation and casein synthesis partly via inhibition of the formation of glutamine and proline in the Arg/Orn pathway. Furthermore, miR-543, miR-

574-5p and miR-712 target genes that are related to cell proliferation and differentiation. A significantly greater expression of the *Ki67* gene (a marker of cell proliferation) after overexpression of miR-543 was detected in mouse myoblast cells. An inhibition of colorectal cancer development and arterial occlusion was reported after down-regulation of miR-574-5p and miR-712 (Ji *et al*., 2013; Son *et al*., 2013). Together, these data suggest that Arg promotes cellular proliferation associated with those miRNAs.

miR-101a has been shown to regulate cell proliferation partly via alteration in the expression of cyclooxygenase-2 (Cox-2), which is critical for mammary gland development (Tanaka *et al*., 2009). However, overexpression of miR-101a suppressed β-casein expression in murine mammary epithelial cells, which was inconsistent with our results where Arg supplementation led to greater miR-101a and casein expression. And an opposite performance for miR-468 in response to the supply of Arg both *in vitro* and *in vivo* might be related to the regulation of arginase 2 on Arg metabolism, but detailed mechanism is not fully understood.

Arginine regulates arginine metabolism-related enzymes and metabolites

Arg participates in various physiological activities as a substrate for metabolic enzymes including arginase, NOS, AGAT, and arginine decarboxylase (ADC). In terms of quantity, however, Arg catabolism via AGAT and ADC in mammals only accounts for 1% and 2% of total amount of Arg that is metabolized, respectively, which suggests that a relatively large amount of Arg is utilized in the Arg/NO and Arg/Orn pathway through the activity of arginase and NOS. There are two distinct isoforms of arginase, of which type I, cytosolic arginase, is expressed primarily in the liver and plays an important role in the detoxification of ammonia (Morris, 1992). Type II, mitochondrial arginase, is expressed mainly in mammary gland tissue and is involved in biosynthesis of molecules such as Orn, proline and urea (Gotoh *et al*., 1996). The enzyme NOS consists of three different subtypes, namely, inducible nitric oxide synthase, endothelial nitric oxide synthase and neuronal nitric oxide synthase (Bauchart-Thevret *et al*., 2010), metabolizing Arg to NO and citrulline.

In vitro work from our laboratory demonstrated that Arg at an optimum concentration regulates certain mammary gland functions by influencing enzymes and metabolites in the Arg/NO and Arg/Orn pathway (Hu *et al*., 2018). Specifically, the marked elevation in NO and NOS expression in response to increasing Arg dose suggests that both the synthesis of enzyme and production of the metabolite increase as the supply of substrate is increased. A four-fold greater supplementation of Arg (6.38 mmol/L) was the optimum concentration promoting the Arg/Orn metabolic pathway through improving arginase synthesis. A decrease in pathway activity occurred when Arg reached an eight-fold concentration (12.77 mmol/L), which might have been attributed to the activation of other metabolic routes such as the Arg/agmatine pathway in which Arg is metabolized to agmatine via ADC. This compound regulates intracellular polyamine synthesis by inducing the translation of ODC antizyme proteins to inhibit its activity, contributing to a reduction of arginase activity (Demougeot *et al*., 2005). Competitive inhibition between Arg/Orn and Arg/NO pathway may also be a reason for the decrease of arginase activity that induced NOS expression in response to excess Arg supplementation. At high levels, Arg is able to exert an inhibition of arginase activity through producing nor-NOHA which serves as an arginase inhibitor.

Arg plays an essential role in maintaining its own homeostasis by selectively controlling several key enzymes involved in its metabolism (Morris, 2004), which suggests that it can influence mammary casein synthesis partly by exerting regulation of enzymes and metabolites in various tissues. Yip and Knox (1972) reported that the synthesis of milk protein was correlated with the activity of arginase in mammary gland tissue, which was in accordance with a study of Oka and Perry (1974) in which the increase in arginase activity in mammary tissue of lactating dairy cows enhanced the synthesis of milk protein via its influence on the biosynthesis of spermidine. Subsequent research revealed that suppression of arginase activity markedly decreased the abundance of αs- and β- casein in BMEC, but such response was not detected when NOS activity was inhibited (Wang *et al*., 2017). This indicated that, from a perspective of enzyme activity, the Arg/Orn pathway was more important in terms of regulation of casein synthesis. In agreement with an *in vitro* study, jugular infusion of the arginase inhibitor nor-NOHA reduced mRNA and protein expression of αs- and β- casein (Ding *et al*., 2018), demonstrating that Arg regulates mammary casein production through effects on enzyme activity and production of metabolites in the Arg/Orn pathway.

CONCLUSION

The quality of milk is closely related to human health. The content of milk protein is a core index related milk quality. Number of studies have been done to clarify the regulatory mechanism on milk protein synthesis. As a functional amino acid, Arg exerts a regulatory effect on casein synthesis through several mechanisms. Arg promotes

mammary gland development via coordinating cellular growth and apoptosis, hence, allowing optimal conditions for casein synthesis during lactation. The regulatory role of Arg on casein production occurs through the activation of transcriptional and translational signaling mechanisms, miRNA, and effects on metabolism-related enzymes and metabolites in the Arg/Orn pathway. Additional research should be performed to better understand whether and how these miRNA candidates and enzymes play roles in the regulation of casein synthesis via Arg to enhance efficiency of production and quality of dairy products.

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IRB approval

IRB of Yangzhou University, Jiangsu Province, China approved the study (approval number: 202103202).

Ethical statement

The use of animals and experimental procedures were approved by the Animal Care and Use Committee of Yangzhou University, Jiangsu Province, China (No. 201406018).

Statement of conflict of interest

The authors have declared no conflict of interest.

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