NUTRITIONAL AND REGULATORY ROLES FOR BRANCHED-CHAIN AMINO ACIDS IN MILK PRODUCTION BY LACTATING SOWS

A Dissertation

by

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ABSTRACT

Both feeding and cell culture studies were conducted to test the hypothesis that branched-chain amino acids (BCAA) can enhance milk synthesis by porcine mammary epithelial cells (PMEC). In the first in vivo feeding trial, ten multiparous lactating sows were fed corn-soybean meal-based diets supplemented with 0%, 1.535%, or 3.07% BCAA for 29 d. The number of piglets was standardized to 9 per sow. On d 3,15 and 29 of lactation, body weights of sows and their piglets, as well as milk consumption of piglets were determined. Blood samples were obtained from sows and piglets at 2 and 1 h after feeding, respectively. The second feeding experiment was conducted with dietary supplements containing 0%, 1% or 2% monosodium glutamate (MSG). Data were analyzed by two-way ANOVA. In both feeding trials, plasma concentrations of aspartate, glutamine, citrulline, arginine, and BCAA were higher, compared to the control. Dietary supplementation with 1.535% and 3.07% BCAA increased: 1) concentrations of free and protein-bound glutamate plus glutamine in milk; 2) milk intake of piglets by 14% and 21%; and 3) daily weight gains of piglets by 19% and 28%. Dietary supplementation with 1% and 2% MSG increased: 1) concentrations of free and protein-bound glutamate plus glutamine in milk; 2) milk intake of piglets by 14% and 25%; and 3) daily weight gains of piglets by 23% and 44%. These results indicated that dietary supplementation with up to 3.07% BCAA or 2% MSG was safe and enhanced lactation in sows. To elucidate the mechanisms responsible for the effects of BCAA on milk production, PMEC were cultured in customized medium containing 0.1, 0.25, 0.5, 1 or 2 mM L-

leucine, L-isoleucine, or L-valine, or a mixture of those three BCAA. Elevating extracellular concentrations of BCAA from 0.1 to 2 mM increased the phosphorylation of proteins in the mechanistic target of rapamycin pathway, the rate of protein synthesis and cell proliferation, while decreasing the abundance of proteasome and ubiquitinated protein, and the rate of protein degradation in PMEC. The findings not only advance understanding of nutritional regulation of lactation but also provide a new strategy to improve milk production by livestock, women, and other mammals.

DEDICATION

I dedicate this dissertation to my wonderful family, especially, to my father and mother for instilling the importance of hard work and higher education.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

BACKGROUND

An increase in production unit size results from increasing economic pressure on the swine industry (Foxcroft et al., 2006). The economic profitability of a swine production unit is primarily determined by both the number and size of piglets produced per year per sow (Quiniou et al., 2002). However, increased litter size results in lower birth weights for individual piglets (Fix et al., 2010). The greatest proportion of mortality in commercial swine production occurs prior to weaning. Reviews of piglet survival during lactation indicate that pre-weaning mortality ranges between 10 and 20% of live-born pigs (Alonso-Spilsbury et al., 2007; Wu et al., 2006). Similarly, pre-weaning mortality was estimated at about 12.8% in U.S. commercial swine herds during 2008 (PIGCHAMP, 2008).

A variety of causes lead to pre-weaning mortality and poor performance, such as low birth weight of individual piglets (Alonso-Spilsbury et al., 2007), as well as inadequate provision of milk (Wolter et al., 2002). Milk is the sole source of nutrients for suckling piglets, and their maximal growth performance and survival largely depend on sufficient production of milk by sows (Wu et al., 2006). In turn, the weight achieved by the time of weaning determines adaptability of piglets to the nursery and how rapidly they reach market weight (Harrell et al., 1993). Indeed, secretion of milk by the mammary gland is the main determinant of neonatal growth rate; therefore, it is

important that milk output by high-producing sows is adequate to allow maximum neonatal growth (Boyd et al., 1995). For instance, arginine (Arg, a nutritionally essential AA for neonatal pigs) is deficient in sow's milk which limits growth performance of piglets (Wu et al., 2010). In addition, the amount of milk produced does not match energy and protein requirements of neonatal piglets in a litter. Previous results showed that milk from high-producing, primiparous females does not satisfy the energy requirements of piglets (9 to10 pigs/litter) after d 8 of lactation. This gap gradually increases throughout lactation so that maternal milk output is approximately 50% of neonatal needs at 21 d of age (Boyd et al., 1995).

It has been estimated that a sow must produce 18-20 kg milk/d to supply a litter of 9 piglets with enough energy to grow at a rate comparable to artificially-reared piglets of the same age (Harrell et al., 1993). In pigs, average growth rates of at least 450 g/d to 21 d of age (vs 230 g/d) are achievable with an adequate supply of nutrients (Harrell et al., 1993). The biological basis for this apparent limitation in milk secretion is unclear. It may result from an inadequate supply of nutrients to mammary tissue, a deficit in endocrine stimulation of milk synthesis, and (or) coordination of metabolism in extra-mammary tissues (e.g., adipose, muscle) to spare nutrients for use in milk synthesis.

Sows have a high capacity to utilize nutrients from the arterial circulation for milk production (Kim et al., 2000). However, due to inadequate feed intake by lactating sows, they do not meet physiological requirements for maximum milk production, thereby leading to a catabolic state in which their body reserves are mobilized to provide nutrients and energy for milk production during lactation (Boyd et al., 1995). Traditional

feeding strategies for sows currently practiced in swine enterprises are unable to support sufficient milk production for optimal growth and survival of neonatal pigs (Wu et al., 2010). Many studies have shown that high neonatal mortality and poor growth of pre-weaning pigs are highly correlated with insufficient milk production by sows. Most of the studies have focused on hormonal regulation of lactation; however, little attention has been paid to the development of nutritional strategies that enhance lactogenesis in sows or growth of suckling piglets.

Amino acids are building blocks of proteins that regulate metabolic pathways for whole body homeostasis (Wu, 2009). Sows require large amounts of amino acids to support tissue growth and milk synthesis by the mammary glands during lactation (Guan et al., 2002). Of the dietary essential amino acids whose carbon skeletons cannot be synthesized endogenously, branched chain amino acids (BCAA), including leucine, isoleucine and valine, have received much attention in recent years (Lei et al., 2012ab). BCAA are major components of milk proteins, and their uptake by the sow's mammary glands is much higher than their secretion into milk (Li et al., 2009; Trottier et al., 1997). This means that BCAA are catabolized extensively in lactating mammary tissue to provide amino groups for biosynthesis of other amino acids, such as glutamate and glutamine, which are necessary for piglet growth and digestive tract maturation (Lei et al., 2012ab). High levels of BCAA can also reduce degradation of arginine by arginase in mammary tissue, thereby increasing the bioavailability of arginine for synthesis of milk proteins (Kim et al., 2008). Recent studies have shown the important roles of BCAA in the regulation of mammary gland metabolism. For example, leucine increases

protein synthesis in mammary epithelial cells through activation of the mechanistic target of rapamycin (mTOR) cell signaling pathway (Wu, 2009). There is great potential for BCAA to increase milk synthesis in mammary tissue, thereby improving neonatal growth, development and survival.

The requirement of the lactating sow for BCAA involves more than just synthesis of milk proteins. Recommendations for appropriate amino acid requirements for optimal milk production by lactating sows relies on a better understanding of the utilization and metabolic fates of BCAA during lactation. Therefore, the objectives of this study are to: 1) evaluate survival and growth performance of piglets from sows receiving BCAA as a dietary -supplement; 2) determine regulatory roles of BCAA in protein synthesis by porcine mammary epithelial cells; and 3) elucidate biochemical and molecular mechanisms whereby BCAA increases milk synthesis.

INTRODUCTION

Milk is the secretory product of the mammary gland, compound, tubulo-alveolar structure with a merocrine/apocrine mode of secretion. Fats, proteins, and some amino acids are synthesized and secreted by epithelial cells of alveoli. The alveoli are connected to a duct system through which the secreted milk flows into the teat canal from which it can be removed by suckling or milking. Thus, the mammary gland is a highly organized organ which serves as a medium to supply nutrients from the mother to the neonate through suckling (Akers, 2002).

THE STRUCTURE OF THE MAMMARY GLAND

The classification of the mammary gland

One of the main distinctive adaptations in mammals is highly evolved secreting glands developed in the skin (e.g., hair follicles) and mammary glands. A gland is a secretory organ whose products can be secreted into a cavity or directly into the blood in order to be distributed to tissues. The structure of glands can be simple (e.g., coiled tubular or branched alveolar), or compound (e.g., branched tubulo-alveolar). The mammary gland is an example of compound, branched tubuloalveolar gland (Blackburn et al., 1989). Glands can be categorized based on their mode of action: 1) apocrine, products are synthesized by the cells of the gland without causing disintegration of the cell; 2) exocrine, a fluid is secreted and cells are not lost as part of the secretory process; 3) holocrine, secretion results from disintegrated of cells of the gland; and 4) merocrine, the gland is repeatedly functional and cells are not destroyed during the secretory process. The secretions from the mammary gland occur via both merocrine and apocrine modes (Cardiff and Allison, 2012).

Tissues of the mammary gland

In the lactating mammary gland, the parenchymal tissue is composed of epithelial structures (e.g., alveoli and ducts) and the associated stromal connective tissue. The stroma of a lactating gland is composed of connective tissue surrounding the epithelial structures (Cardiff and Allison, 2012). The cellular components of the connective tissue include fibroblasts, endothelial cells associated with blood vessels, and leukocytes, while non-cellular components include collagen and other connective-tissue

proteins. In addition, an extensive white adipose tissue exists as part of the stroma of the developing gland. This fat pad is considered as extra-parenchymal tissue and is noticeably larger during the early phases of fetal development and the decreases in mass through the remainder of pregnancy. Thus, the fat pad of a lactating mammary gland is smaller than during pregnancy (Cardiff and Allison, 2012).

Mammary glands are also considered accessory reproductive organs. As noted previously, they are compound, tubulo-alveolar skin glands which are located on the ventral surface of the mammal. The alveolus is the basic milk secreting structure which consists of a single layer of secretory epithelial cells surrounding a lumen. The epithelial layer is surrounded by a layer of contractile myoepithelial cells which play an important role in milk ejection. The alveolus is then surrounded by a basement membrane (Frandson et al., 2013).

Upon stimulation of mechanoreceptors in the teat skin, these receptors induce cholinergic nerve impulses which travel via segmental pathways in the central nervous system (CNS) to the paraventricular (PVN), and supraoptic nuclei (SON) in the hypothalamus resulting in release of oxytocin from the posterior pituitary gland. Oxytocin is a nonapeptide consisting of nine amino acids and is produced in the SON and PVN of the hypothalamus. Oxytocin is transported by carrier proteins (neurophysin I) from the cell bodies of PVN and SON through the pituitary stalk. From the pituitary, oxytocin is released from neurophysin and enters the blood for transport to the mammary gland where it binds to its receptors on the myoepithelial cells surrounding the alveoli.

This causes myoepithelial cells to contract and force milk from the alveoli into the teat canal from which it is expelled via nursing or milking (Crowley and Armstrong, 1992).

In pigs, the number of mammary glands varies from 6 to 20 (3-10 pairs). The arrangement of glands is in two parallel rows on either side of the ventral median line and extending from the pectoral to the inguinal region. Each gland is separate and independent of secretory tissue from adjacent glands. The heritability of teat number is rather low (0.10 to 0.20). Thus, it is difficult to select for high teat number in sows (Hartmann and Holmes, 1989).

Innervation in the mammary gland

As the mammary gland is a skin-derived structure that contains external innervation in the skin covering the gland. There are few internal innervations which control the blood vessels in the mammary gland. Innervation inside the gland is sparse compared with other tissues. Sympathetic nerves are present in the mammary gland and are associated with the arteries, but not the alveoli. Sensory nerves found in the teat and skin are critical for initiating the afferent pathway (neural pathway) of the milk ejection reflex. There is no parasympathetic innervation of the mammary gland. This is similar to other skin glands. There is no innervation of the secretory system. Myoepithelial cells are not innervated and do not contract in response to direct innervation, but rather contract in response to the blood-borne hormone, oxytocin (Frandson et al., 2013).

Blood and lymphatic vessels in the mammary gland

The blood supply to the mammary gland must leave and return to the body cavity through points where blood vessels exit the body cavity to reach the ventral portions of

the skin, such as the thoracic and inguinal canal (Cardiff and Allison, 2012). In pigs, blood supply to the mammary glands arises from two branches of the arterial system, the common carotid artery supplies the anterior glands and a branch of the abdominal aorta supplies the posterior glands. There is an anastomosis of the anterior and posterior mammary arteries and veins between the second and fourth inguinal glands so that the blood supplying the inguinal glands may pass forward through the anastomosis, whereas the blood supplying anterior glands may pass posteriorly through the anastomosis (Frandson et al, 2013).

The extracellular fluids are drained from the mammary tissue and conducted back to the circulatory system via the lymphatic network. Also, the lymphatics contain concentrated areas of leukocytes (particularly lymphocytes and macrophages) in lymph nodes; these leukocytes can mount an immune response to bacteria and foreign material. The lymphatic network serves to transport some nutrients such as vitamin K and lipids absorbed in the intestine. The lymphatic network originates in tissue spaces as very thin, closed endothelial tubes (lymphatic capillaries). These are analogous to blood capillaries, but are much more permeable, with little resistance to passage. They have no basement membrane. Lymph capillaries converge to form larger vessels. Lymph flow is unidirectional from the tissues through lymphatic vessels, eventually draining into the vena cava (Frandson et al., 2013).

DEVELOPMENT OF THE MAMMARY GLAND

Mammogenesis

The mammary gland is one of a few tissues in mammals which can undergo repeated cycles of growth, functional differentiation, and regression. The development of mammary gland structures is referred as mammogenesis. Mammogenesis begins during early fetal development and proceeds beyond initiation of lactation. Most mammogenesis occurs during pregnancy, and some pregnancy hormones such as estrogen and progesterone are responsible for mammary growth. In fact, high concentrations of such hormones mark the primary physiological mediators of mammogenesis during pregnancy (Knight et al., 1986; 1998).

Differences among species in how lactation fits into the overall reproductive cycle is one of many interesting aspects of lactation biology. For example, a sow typically is bred to farrow for the first time at about 12 months of age (gestation is about 114 days). The majority of mammary development occurs during this 114 day period. Lactation begins at farrowing. Usually, the piglets are weaned at 21 days, at which time the mammary gland of the sow undergoes involution and milk secretion ceases (King, 1993; 2000).

Many mammalian species experience lactational anestrus, that is, they do not experience estrous cycles during early lactation. The length of lactational anestrus varies among species. For example, in the sow, gestation is 114 days and the piglets suckle the sow for several weeks (generally 2 – 4 weeks) before weaning. Lactation in the sow inhibits normal reproductive cycling and the next estrus occurs several days after the

piglets are weaned. In fact, this management approach is used to synchronize estrus and, therefore, the time of the next farrowing (parturition). In this case the swine producer uses the normal relationships between reproductive function and lactation to optimize sow productivity. Lactation in swine, under modern management procedures, accounts for only one part of the total reproductive process, but is nevertheless a critical period for survival and growth of the newborn piglet (Brooks and Burke, 1998).

Lactation and reproductive processes are closely intertwined in mammals. Mammary development occurs only slightly during estrous cycles, while substantial development occurs while the female is pregnant and preparing to provide extra-uterine maternal nutritional support after birth of the piglet. Some factors involved in control of pregnancy are the same factors involved in regulating mammary development during pregnancy. For example, key pregnancy hormones, estrogen and progesterone, are the primary stimulators of growth of the mammary tissue. In addition, growth hormone, prolactin and glucocorticoids (mainly cortisol) also contribute to mammary gland development. Similarly, important hormone changes that occur in association with parturition are critical to the initiation of milk synthesis and secretion by the mammary tissue at birth (Akers, 2002; King, 1989).

During pregnancy, the interrelationships between mother and offspring occur in utero. After birth, the metabolic interrelationships occur outside of the uterus via production of milk by the mammary gland. During lactation, the mother undergoes substantial changes in metabolism to account for the increased demands of milk synthesis. From this perspective, the mammary gland replaces functions of the placental-

uterine unit in providing nutrients and protective factors for development of the offspring (Hughes and Varley, 1980). With the subsequent evolution of the placenta resulting in a much more extensive intrauterine development of the conceptus, the ability to lactate after expulsion of the offspring from the uterus becomes a critical part of the reproductive strategy of mammalian species that give birth to live young (Hughes and Varley, 1980).

Most of our understanding of the physiology of lactation (including structural development of mammary and control of nutrient supply to the mammary gland) has been acquired from studies of the dairy cow. That is because of the combined importance of efficient milk production and the consumption of milk as a major human nutritional component. Another reason is the relative ease of using the bovine model compared with the pig with regard to handling and milking as well as the availability of equipment that allows for precise and easy measurement of total milk output in the cow to assess mammary gland metabolism (Trottier et al., 1997).

Structural development of the mammary gland generally occurs in five phases. These phases are development during the fetal period, the prepubertal period, the postpubertal period, pregnancy, and lactation (Akers, 2000). These morphological changes are distinct and have been well characterized in cows and mice. Here, we will next review hormonal regulation of the development of the mammary gland.

Hormonal regulation of mammary gland development

During the fetal period

Testosterone injection into the pregnant mouse on d 8, 9, or 10 of pregnancy leads to masculinization of the mammary gland of female fetuses. The female fetuses are born without nipples. The mammary bud becomes exteriorized and is separated from the epidermis, as observed by d 14 or 15. Mammary development in male fetuses is not further affected by the testosterone treatment of the mother. Gonadectomy of male fetuses results in the female pattern of mammary development in male fetuses. Gonadectomy resulted from localized irradiation on d 13 of pregnancy of the region where the gonads form in the mouse fetus. This functionally castrates the fetus. By d 18 of pregnancy, mammary development in male mice includes formation of normal primary mammary cords. Gonadectomy of the female fetus by irradiation does not alter normal mammary development (Bocchinfuso and Korach, 1997). Administration of high doses of estrogen to female fetuses or to the mother results in a number of abnormalities of mammary development. These abnormalities include total suppression of development of the mammary bud, leaving only a nipple with no internal structures; partial inhibition of the mammary bud; formation of a cavity at the site of the mammary bud; abnormally shaped mammary buds; formation of multiple primary mammary cords; and excessive formation of the mesenchyme around the primary mammary duct (Cheng et al., 2004). Likewise, administration of growth hormone into the mouse fetus increases the size of the developing mammary gland in both male and female fetuses, suggesting

that this hormone is also involved in development of the mammary gland of the fetus (Etherton and Bauman, 1998).

During the pre-pubertal period

Daily injections of somatotropin (growth hormone) to heifers from 8 to 15.6 months of age resulted in increased mammary parenchyma and decreased extraparenchymal tissue compared to controls (Sejrsen et al., 1999). Growth hormone stimulates mammary growth through increasing the hepatic synthesis of insulin-like growth factor-1 (IGF-1), which is a potent mitogen for mammary cells. Therefore, growth hormone may be a major factor controlling mammary development during the prepartum period. However, administration of growth hormone during the prepubertal period does not increase milk yield during the first lactation (Blackburn et al., 1989).

Leptin, which is produced by white adipocytes, is another protein that influences mammary development indirectly and this may explain why excessive fattening impairs mammary development. For example, results from both in vitro and in vivo studies indicate that leptin decreases IGF-1-induced proliferation of bovine mammary epithelial cells. However, leptin could not mediate the effects of a high-plane of nutrition on mammary epithelial cells in prepubertal heifers due to the paucity of leptin receptors on those cells. It appears that leptin exerts its effects on mammogenesis through inhibition of IGF-1 mediated cell signaling in mammary epithelial cells that is linked to activation of macrophages in the mammary gland. Upon stimulation with leptin, macrophages increase their phagocytic activity and production of cytokines such as TNFβ and IL-6,

which are associated with inhibition of IGF-1-induced proliferation of many cell types (Shennan and Peaker, 2000).

During the post-pubertal period

The growth of mammary tissue during estrus is related to ovarian steroid hormones. Estrogen receptors and progesterone receptors both appear in cells of the gland around the time of puberty. However, the exact roles of estrogen and progesterone are not completely understood. Mammogenic hormones establish the conditions for specific growth patterns in mammary tissue. For example, concurrently elevated concentrations of estrogen and progesterone in blood in late gestation result in an exponential increase in parenchymal growth and in formation of alveoli. In contrast, the cyclic changes in those hormones associated with estrous cycles result primarily in duct elongation and formation of some lobular tissue, but not in formation of alveoli.

Mammary development is usually driven by a complex of hormones acting in concert. Effects of many mammogenic hormones are thought to be mediated through stromal cell-derived growth factors which act in a paracrine manner by eliciting mitogenic responses in the adjacent epithelial cells. Much of our current understanding of how mammogenic hormones and growth factors function arises from research in rodents. However, similarities are being noted in other animals and knowledge of hormone action in rodents can help in understanding similar processes in other animals (Akers, 2002). Estrogen is an important mammogenic factor, particularly in the postpubertal female. Estrogen receptors (particularly estrogen receptor α) appear in the gland around the time of puberty, coinciding with the period when the gland becomes

exposed to cyclic increases in concentrations of estrogens in blood. In rodents, estrogen acts via its receptors in the stromal tissue to stimulate production of growth factors, which in turn stimulate ductal development. The evidence available from studies of cattle also indicates that estrogen's action on mammary development may be mediated through the stroma (Ellis et al., 2000). Progesterone is another ovarian steroid hormone which plays a key role in mammary development. While progesterone receptors have been difficult to identify in the mammary fat pad, administration of progesterone stimulates proliferation of stromal cells under some physiological conditions. The stimulatory effect of progesterone on DNA synthesis in ductal epithelium is probably mediated indirectly through its effects on stromal cells. The major mammogenic effect of progesterone is mediated through binding to its receptors in epithelial cells and stimulating ductal side branching or alveolar bud formation, which are the hallmarks of postpubertal mammary development. Estrogen stimulation of progesterone receptor expression in epithelial cells is required for progesterone to exert its effects.

Progesterone, therefore, has a major role in alveolar morphogenesis and a lesser role in ductal morphogenesis. During estrous cycles, duct elongation and expansion of the parenchymal tissue into the fat pad occur in limited bursts associated with the periods when circulating concentrations of estrogen are high. During the luteal phase when concentrations of progesterone are greatest, relatively little growth of the mammary gland occurs in ruminants, but formation and maintenance of lobular structures in the mammary gland may be stimulated by progesterone, with little

regression of the mammary ductal system occurring between estrous cycles (Akers, 1990).

Synergy between estrogen and progesterone is observed during pregnancy when both hormones are present in high concentrations. Elevated concentrations of estrogen and progesterone in blood establish the conditions required for exponential growth of the mammary gland during pregnancy (Haslam and Shyamala, 1979). Lobuloalveolar development represents the greatest increase in mammary gland tissue mass during pregnancy. In the cow, circulating concentrations of progesterone are elevated throughout gestation, while circulating concentrations of unconjugated estrogens are greatest during the later stages of gestation when there is the greatest increase in mammary tissue mass. Estrogen and progesterone have direct effects on the mammary gland, probably mediated via autocrine and paracrine factors produced locally in the tissue. In addition, steroid hormones may have indirect effects via their impact on the secretion of prolactin by the anterior pituitary gland lactotrophs (Mepham, 1987).

Prolactin often is associated with initiation of lactation and galactopoeisis, but it also has mammogenic effects. Prolactin receptors are present in the mammary fat pad and the mammary epithelium. Prolactin may act on both epithelial and stromal components of the growing mammary tissue. Inhibition of prolactin secretion inhibits mammary gland development in pregnant goats, pigs and other species (Farmer and Palin, 2005).

Concentrations of prolactin are normally low during pregnancy. Mammary development during pregnancy may not be limited by low concentrations of prolactin in

blood (Farmer and Petitclerc, 2003), especially when the action of prolactin may be regulated by estrogen-induced changes in expression of receptors for prolactin in mammary tissue (Young et al., 1989).

Administration of growth hormone (somatotropin) to cattle is known to stimulate milk production during lactation. This effect is indirect in that growth hormone stimulates secretion of insulin-like growth factor-I (IGF-I) from the liver, which in turn mediates many of the galactopoeitic effects of growth hormone during lactation (Bauman and Vernon, 1993). Growth hormone also acts as a mammogenic hormone and can stimulate mammary growth at all stages of development. Direct effects of growth hormone on mammary tissue require the presence of growth hormone receptors on mammary cells. While this remains a point of controversy, there is evidence for growth hormone receptors in mammary epithelial or stromal cells in various species. Several lines of evidence indicate that growth hormone may act on mammary tissue in ruminants by stimulating stromal production of IGF-I which is mitogenic for mammary epithelial cells. The highest level of IGF-I expression in mammary tissue occurs in the fat pad and is greatest during the prepubertal allometric growth phase and during late pregnancy.

Mammary expression of IGF-I is regulated by growth hormone, estrogen and positive feedback stimulation from proliferating epithelial cells. The function of IGF-II in mammary growth is less clear than for IGF-I. Stromal cells probably produce IGF-II, which is regulated by stage of mammary development and hormonal stimulation (Akers, 2002).

Placental lactogens are secreted by the placenta and may have prolactin- or growth hormone-like activities, depending upon the species. In pregnant goats, concentrations of placental lactogen in maternal blood is closely correlated with the number of fetuses present and, therefore, placental mass. This graded concentrations of placental lactogen, in combination with other mammogenic hormones, may regulate the extent of mammary development during late pregnancy. In the dairy cow, there is a relationship between placental mass and subsequent milk production. However, the concentration of placental lactogen in maternal blood of the dairy cow is low and the effect of placental mass may result from other placental hormones, including estrogen (Schams et al., 1984). Other hormones also are required for mammary growth, including glucocorticoids, thyroid hormones and insulin (Tucker, 2000). Severely diabetic mice given estrogen and progesterone will develop extensive lobuloalveolar structures. Of note, insulin synergizes with estrogen and progesterone to increase mammary development. Normal concentrations of insulin in blood is not limiting for normal mammary development (Tucker, 2000).

During pregnancy

Optimal mammary growth requires both estrogen and progesterone. During pregnancy, both sromal and epithelial cells of the mammary gland express estrogen receptor alpha and progesterone receptors. During lactation, the epithelial cells of the mammary gland express estrogen receptor alpha, but there is no expression of progesterone receptors. In fact, progesterone receptor levels are inversely proportional to the secretory activity of the gland. Concurrently, changes in circulating concentrations of

estrogen and progesterone during pregnancy establish conditions for geometric cell multiplication of epithelial cells and lobuloalveolar growth, which is characteristic of mammary tissue development during pregnancy. In pigs, on d 30 of pregnancy, elevated estrogen levels signal to increase DNA concentrations in mammary glands. The mammary continues to grow throughout pregnancy but the peak in growth is between d 75 and 90 (Kensinger et al., 1982). In the cow, progesterone is elevated throughout gestation (required for maintenance of pregnancy), while estrogen is particularly elevated during the second half of gestation. Consequently, most mammary growth during the first half of gestation is ductal growth and lobular formation. In the second half of gestation, ductal growth continues, but most growth is lobuloalveolar (Tyler and Ensminger, 2006).

In hypophysectomized-ovariectomized goats, administration of estradiol (E2), progesterone (P4), prolactin (PRL), growth hormone (GH1), adrenocorticotrophic hormone (ACTH) and glucocorticoids, particularly cortisol, are required for lobuloalveolar development comparable to that in mid-pregnancy. This suggests that all of these hormones are involved in mammary development during pregnancy (Brisken, 2002). The roles of some of these and other hormones are discussed below.

Prolactin (PRL) and Growth Hormone (GH1) In virgin rats, transplantation of the pituitary to the kidney capsule releases the anterior pituitary lactotrophs from inhibition of secretion of PRL by dopamine and this results in marked stimulation of growth of the mammary gland. However, circulating levels of both PRL and GH are normally suppressed during gestation in most species (Barber et al., 1992). Although both PRL

and GH are required for mammary development, their concentrations in blood are not normally limiting. GH is implicated in mammary growth during the fetal stages and prepubertal stage of mammary development (Buskirk et al., 1997).

Placental lactogens (CSH1) are synthesized and secreted from the chorion of the placenta. Generally they have both PRL- and GH-like activities. However, there is great variation among species. Pigs and rabbits do not have placental lactogen (Neville, 2002). In the rat, during the first half of pregnancy, PRL and GH are the primary hormones involved in mammary development. Significant amounts of CSH1 are not secreted until d 12 of gestation in rats.

Hypophysectomy of the rat at d 12 of pregnancy or later, does not affect mammary cell numbers or maintenance of pregnancy (Barber et al., 1992). In goats, the concentration of placental lactogen in maternal blood is closely correlated with the number of fetuses (Hayden et al., 1980). One interpretation of this observation is that the level of total lactogenic hormone activity in maternal blood (PRL plus CSH1 or other hormones) regulates the extent of mammary development during late pregnancy. However, in the cow, concentrations of placental lactogen in maternal blood are very low (Capuco et al., 2001). Placental lactogen probably synergizes with E2, P4, PRL and GH1 in mammary development (Byatt et al., 1992). It must be borne in mind that the placenta also secretes estrogens and progesterone in a highly species-dependent manner (Tucker, 1994).

Relaxin is a hormone secreted during pregnancy in some species. Its role is usually associated with preparing the late pregnant reproductive tract for parturition. For

example, it is involved in softening of the cervix prior to parturition. Most effects of relaxin require the estrogen stimulation (Hurley et al., 1991). Relaxin also has a major mammogenic role in the sow. Removal of the ovary from late pregnant gilts (at d 80 or d 100 of pregnancy in association with administration of exogenous progesterone to maintain pregnancy) removes the source of relaxin in the pregnant pig. This led to substantially reduced mammary development during the last weeks of pregnancy (Hurley et al., 1991). Relaxin also stimulates mammary development in virgin gilts (Winn et al., 1994). It is interesting that, while the pig does not express a placental lactogen, the corpora lutea do secrete relaxin as a major mammogenic factor during pregnancy. The concentration of relaxin in the mother's blood is correlated with the number of corpora lutea on the ovaries in pigs. Additionally, the number of corpora lutea is directly related to the number of ovulated oocytes and the number of fetuses. Thus, even in the pig, there is a relationship between the number of fetuses and the level of mammogenic stimulus on development of the mammary gland (Hurley, 2001; Zaleski et al., 1996)).

Insulin is required to maintain normal mammary tissue function. Mammary cells are resistant to insulin before conception, and thereafter they become sensitive to insulin during gestation and lactation, and become insensitive again during involution of the mammary gland. Insulin stimulates mitosis of mammary cells in vitro, but is not absolutely essential in vivo (Spinka et al., 1999). Severely diabetic mice given estrogen and progesterone develop extensive lobuloalveolar structures. Still, insulin synergizes with estrogen and progesterone to increase mammary development. Concentrations of

insulin in blood decrease during gestation as do concentrations of prolactin and growth hormone; therefore; it can be surmised that insulin is not a major factor directly affecting mammary development (Richert et al., 2000).

Thyroid hormones (T3 and T4) are involved in overall metabolic rate and oxygen consumption by cells of the body. Their effect on mammary development is probably indirect or via the normal requirements of cell maintenance. Hypothyroidism retards ductal and lobuloalveolar growth in rat mammary tissue and administration of thyroid hormones restores the normal developmental pattern (Tucker, 1994).

During lactation

The number of cells in the lactating mammary gland is critical for milk production and they continue to increase after parturition. Mammary tissue weights and total DNA content continue to increase in early lactation. The impact of this increased mass of mammary tissue on milk production can be substantial in some species. For example, the total amount of DNA in mammary glands of rats during lactation is highly correlated with litter weight gain (Tucker, 2000). In rats, total mammary DNA can increase by over 100% during lactation, depending upon litter size (Tucker, 2000). In the sow, mammary gland weight and total mammary DNA increase by 55% and 100%, respectively, between d 5 and 21 of lactation when the sow nurses 9 or 10 piglets (Kim et al., 1999). In cows, mammary DNA increases by 65% from d 10 prepartum to d 10 postpartum, although how much of this increase occurred pre- and postpartum was not determined (Akers, 2002). So far there are no reports of cell numbers in the cow mammary gland throughout the lactating period (Tyler and Ensminger, 2006).

Litter size in some species (e.g., the rat or pig) is directly related to nursing intensity (a combination of number of nursing young and the intensity with which they nurse). Nursing intensity and parity have major effects on the extent of mammary growth during lactation (Hurley et al., 2001). There is considerable evidence that the mammary growth is enhanced by nursing (Farmer et al., 2012) and that mammary tissue of the lactating sows has substantial growth potential (Kim et al., 2000).

AUTOCRINE AND PARACRINE REGULATION OF MAMMARY GROWTH

Autocrine and paracrine factors (local growth factors) play a major role in growth of the mammary glands. Many effects of steroid hormones on growth of the mammary gland are mediated by local growth factors and those effects include an interaction between the developing mammary gland epithelial structures and the mammary fat pad. Mammary epithelial cells will only grow and organize when transplanted into the fat pad. The interaction may involve specific fatty acids from the fat pad which induce changes in proliferation and differentiation of epithelial cells (Hovey et al., 1999).

Stromal cells in the region of the terminal end buds (TEB) or terminal ductule lobular unit (TDLU) of mammary glands may be involved in dissolving collagen so that structures can expand. The enlarging ducts may promote mesenchymal growth and angiogenesis. Epithelial cells in the TDLU and developing ducts are probably interacting with each other to induce synthesis and assembly of the basement membrane (Talhouk et al., 1993; Akers, 2002).

Besides IGF-1, as noted previously, a number of other growth factors have positive or negative effects on mammary gland development. Local production of transforming growth factor-ß (TGFB) inhibits mammary growth, such as during the prepubertal period and between estrous cycles. Epidermal growth factor (EGF) and TGF-alpha (TGFA) produced in the mammary tissue stimulate mammary cell proliferation. Both EGF and TGFA bind to EGF receptors. The mammogenic action of estrogen and progesterone occurs in part by decreasing local production of the inhibitory TGFB, while increasing local production of TGFA and increasing EGF receptors in the epithelial cells. EGF receptors in stromal cells also are necessary for normal ductal growth (Plath-Gabler et al., 2001).

Other growth factors produced by stromal cells are known epithelial cell mitogens and may be involved in mediating effects of mammogenic hormones. These include hepatocyte growth factor (HGF), and members of the fibroblast growth factor (FGF) family such as acidic FGF and FGF7. Basic-FGF is also an epithelial cell mitogen, although the origin of this growth factor in mammary tissue is uncertain (Akers, 2002).

Fatty acids, particularly unsaturated fatty acids, stimulate growth of mammary epithelial cells and enhance in vitro effects of other growth factors such as IGF-I and EGF. Mammary stromal cells also are involved in dissolving collagen so that the epithelial structures can continue to grow. Several proteases are involved in tissue remodeling and growth of parenchymal tissue derived from stromal tissue. Extracellular matrix components (e.g. proteoglycans, hyaluronan, fibronectin, laminin and so forth),

which are important for mammary tissue growth and function, are produced by both epithelial cells and stromal cells (Neville et al., 1998).

LACTATION IN MAMMALS

Lactation is a critical component of the reproductive strategy of mammals and the physiology of lactation is intimately intertwined with the physiology of reproductive processes. Lactation is defined as the combined processes of milk secretion and milk removal. Milk secretion is the synthesis of milk by epithelial cells and passage of that milk from the epithelial cell into the lumen of the alveoli of the mammary gland. Milk removal is the passive removal from the cisterns and the ejection of milk from the lumen of the alveoli lumen (Akers, 2002).

Cycles of lactation

Mammals reproduce more than once, and therefore, lactate more than once. The mammary gland is one of the relatively few structures of the body which undergoes repeated cycles of structural development, functional differentiation, and regression (Mepham, 1983). The cycle includes the following events:

(1) the mammary gland undergoes mammogenesis, the most dramatic change in the structural development of the mammary gland that occurs during pregnancy; (2) milk synthesis and secretion begin after parturition (lactogenesis) and begins with synthesis and secretion of colostrum and then lactation lasting for weeks or months; (3) maintenance of milk secretion (galactopoiesis) until the young no longer need milk or milk is no longer removed from the gland; and (4) involution of the mammary gland

following cessation of milk secretion and the cycle can start again with stimulatory effects of hormones of a new pregnancy and peri-parturient events.

These phases of mammary development are at the heart of lactation biology (Akers, 2002). In essence, they include onset, maintenance and termination of lactation. These physiological processes are described in the following sections.

Onset of lactation

The onset of lactation can be divided into two stages: Stage 1 and Stage 2 lactogenesis. In pigs, Stage 1 lactogenesis occurs between d 90 and d 105 of pregnancy, and Stage 2 lactogenesis is between d 112 of pregnancy and early lactation which is concomitant with copious secretion of milk. By d 4 of lactation, mammary epithelial cells are fully differentiated (Kensinger et al., 1986).

Lactogenesis consists of a series of events in the differentiation process whereby cells are converted from a nonsecretory to a secretory state. Stage 1 consists of cytologic and enzymatic differentiation of alveolar cells and coincides with limited milk secretion before parturition. The prelactating alveolar epithelial cells have an irregular shaped nucleus, minimal RER, a small inconspicuous Golgi, few microvilli at the apical surface, few mitochondria, and perhaps 1 or 2 fat droplets. Immediately before and during parturition, there is dramatic hypertrophy of the RER, hypertrophy of the Golgi, appearance of large vesicles containing casein micelles, release of granular material (casein micelles) into the lumen, an increase in the number of cytoplasmic fat droplets and their release into the lumen, an increase in the number of microvilli at the apical cell membrane, and an increase in the number of mitochondria per cell. Cell polarity

becomes evident: the RER is primarily on the basal half of the cell, the nucleus is shifted to the basal portion of the cell, and the Golgi is apical to the nucleus (Kensinger et al., 1986).

Enzymatic changes include increased synthesis of acetyl CoA carboxylase, fatty acid synthetase, and other enzymes associated with lactation, as well as increases in the activities of transport systems for amino acids, glucose, and other substrates for milk synthesis. Synthesis of Alpha-lactalbumin, and therefore, lactose, does not begin until Stage 2 of lactogenesis. The mammary glands enter Stage 1 lactogenesis days or weeks (e.g., five weeks in pigs) prior to farrowing. This stage may be detected by measureable amounts of colostral proteins in plasma of blood. Colostrum production takes place during Stage 1 lactogenesis, whereas milk is not produced at that time because of high levels of progesterone in the plasma which inhibits lactation (Akers, 2002).

At farrowing, concentrations of progesterone in plasma decrease rapidly which relieves inhibition of milk synthesis, while increasing concentrations of prolactin in the plasma stimulate synthesis of milk components (e.g. Alpha-lactalbumin and lactose) and growth of the mammary gland (Hurley et al., 1991; Hartmann et al., 1984a). These changes are probably the main driving forces for the mammary gland entering Stage 2 lactogenesis characterized by the initiation of copious milk production, which begins 0 to 4 days before parturition and extends through a few days postpartum in swine. Other hormones such as estrogen, oxytocin and relaxin are also involved (Ford et al., 2003). Suckling of the mammary gland by piglets is an additional factor in initiating the onset of Stage 2 lactogenesis. This is different than other species such as cows and humans

where milk production is initiated irrespective to whether the glands are being suckled or not (Hartmann et al., 1984b).

Maintenance of lactation

In Stage 2 lactogenesis, milk removal is necessary to maintain milk production by individual mammary glands. If milk removal ceases, such as when a piglet dies, that mammary gland undergoes rapid involution. Involution is induced if milk stasis occurs for an extended period of time, often less than one day. Milk production cannot be restored in individual glands after the involution process reaches a “point of no return” after 40-60 h of milk stasis (Theil et al., 2005).

However, during the next reproductive cycle, all glands start to re-develop and they will again produce milk if suckled after parturition. Development of mammary glands and their synthesis of milk are regulated at the gland level by interactions between systemic factors (e.g., circulating hormones and nutrients) and local factors (e.g., hormone receptors in the membranes of mammary epithelial cells). Immediately after milk removal, systemic factors, of which prolactin is the most important, are responsible for stimulating milk production and the rate of milk synthesis is high during the first 30-35 min after a suckling bout. As filling of the gland progresses, the rate of synthesis of milk is inhibited by local factors (e.g. hormone receptors and transcription factors) controlled by milk stasis (Kim et al., 2001).

When the mammary gland is emptied once again at the next suckling, the inhibition of milk synthesis due to milk stasis is relieved and milk synthesis resumes at the maximum rate (Hurley, 2001). A lactogenic complex consisting of insulin,

glucocorticoids and prolactin is responsible for maintenance of lactation in many species. In pigs, in contrast to the onset of lactation, maintenance of lactation seems to be dependent on piglet suckling and prolactin (Lucy, 2008). Suckling by piglets induces release of prolactin from lactotrophs in the anterior pituitary gland into the blood, and upon binding to receptors in the mammary glands, prolactin elicits signals to stimulate milk production and mammary tissue growth (Akers, 2002).

The importance of prolactin for milk production by sows was elucidated by Farmer et al (2009). When sows were given bromocriptine to inhibit prolactin release, there was a marked reduction in milk yield, whereas milk production increased again when the bromocriptine treatment ceased. However, prolactin is normally not the limiting factor for milk production, and improvements in milk yield of sows using exogenous prolactin treatment has been attempted without success. No other hormones are known to be involved directly in maintaining lactation (Hurley, 2001). The hormone oxytocin is clearly necessary for milk letdown, but there is no knowledge of its direct role in maintaining milk synthesis (Akers, 2002). In contrast, growth hormone has a well described lactogenic effect in dairy cows, but administration of porcine growth hormone to lactating sows did not influence milk yield or maintenance of lactation (Lucy, 2008).

Termination of lactation

Mammary glands begin to involute rapidly if not suckled. This occurs in early lactation for mammary glands not suckled after piglets develop a teat preference or anytime throughout lactation if a piglet dies and the associated mammary gland is not suckled (Accorsi et al., 2002). Involution of the mammary gland occurs immediately

follows weaning and is characterized by apoptosis or loss of the alveolar epithelial cells and proteolytic degradation (Accorsi et al., 2002).

Rates of epithelial cell apoptosis vary with species (Akers, 2002). Apoptosis, usually caused by DNA fragmentation, is thought to be correlated with decreases in circulating concentrations of prolactin, GH1, and IGF1 (Accorsi et al., 2002). There is no fundamental difference between involution of individual mammary glands during lactation or after weaning (Kim et al., 2001). If milk stasis remains in one or more glands for one-half day, expression of prolactin receptors in the mammary gland decrease, thereby uncoupling the stimulatory effects of prolactin on milk synthesis (Theil et al., 2006). Furthermore, milk stasis induces a mechanism that leads to programmed cell death or apoptosis. Once started, apoptosis has severe consequences on the mammary gland. If milk stasis is relieved after 24 h, milk yield is reduced by approximately 25% throughout the remaining period of lactation. If milk stasis is not relieved within 50-60 h, the gland will become totally involuted and unfunctional (Theil et al., 2006).

SIGNIFICANCE OF MOTHER'S MILK TO NEONATES

Mammals have a highly developed brain and complex body systems. It usually takes a prolonged period of time for mammalian neonates to fully develop physiologically and anatomically. This developmental process occurs in utero during gestation and after birth. Prior to birth, the fetus is nurtured in a sterile, protected, moist, and warm environment of the uterus. The fetus is provided with all necessary nutrients and oxygen required for growth and development. At birth, the neonate is suddenly

exposed to an environment with many potentially adverse situations, and it also experiences a dramatic switch from primarily parenteral nutrition to exclusively enteral nutrition. This is associated with rapid changes in nutrient metabolism (Mepham, 1983). Because a newborn mammal has an underdeveloped ability to digest solid foods, it is greatly dependent on the mother's liquid milk for nutritional support (Akers, 2002).

Milk (colostrum or mature milk) contains water, lactose, fat, proteins, minerals, and vitamins (Noblet and Ettiene, 1989). Milk composition varies among species, strains or breeds within a species, stage of lactation, and different milking intervals (Table A-1). Colostrum is the first secretion from the mammary gland immediately and within a few days after parturition. Colostrum is characterized by high concentrations of immunoglobulins necessary for conferring passive immunity in support of the underdeveloped immune system of the newborn (Klobasa et al., 1987). In addition, milk is the only source of water, organic nutrients, and minerals for the suckling newborn and it has a high energy value to meet needs of the neonate. Besides immunoglobulins, colostrum and mature milk also contain nonnutrient substances (e.g., osteopontin, insulin-like growth factor I, and other bioactive factors) that are crucial for growth, development, health, and survival of the neonate (Klaver et al., 1981). Thus, milk is of supreme importance to survival, proper development, and dynamic growth of the neonate (Fox and McSweeney, 1998).

Lactation is an integral component of the highly successful reproductive strategy of mammalian species. Mammals reproduce multiple times in their lives. This means that the requirement to provide the newborn offspring with nutrients occurs repetitively.

A mammary gland that lactates continually is not energetically favorable, whereas a mammary gland that develops and functions in concert with the reproductive cycle of the animal is much more efficient. In fact, development of the mammary gland is closely linked to the reproductive cycle of all mammalian species (Mepham, 1983; Manjarin et al., 2014).

PROTECTIVE FACTORS IN MOTHER'S MILK

Analyses of the composition of milk revealed a variety of protective factors including antimicrobial lipids and proteins in milk. Most of the protective factors are not specifically produced by the mammary gland itself, but instead they are directly taken up from the mother's blood and transferred into milk. These proteins include lactoferrin which binds iron to prevent growth of microorganisms, lysozyme that hydrolyzes bacterial cell walls, lactoperoxidase which oxidizes bacterial components, and secretory immunoglobulins, IgA and IgM, in colostrum. These secretory factors are effectively involved in protecting the gastrointestinal tract from damage induced by environmental insults. The levels and activities of these factors are species specific. For instance, bovine milk contains low levels of taurine, lactoferrin, lysozyme, and IgA, but high levels of lactoperoxidase. In contrast, human milk has higher levels of taurine, lactoferrin, lysozymes, IgA, and antimicrobial activity, but lower amounts of lactoperoxidase than bovine milk (Akers, 2002).

MACRONUTRIENTS IN MILK AND THEIR CHARACTERISTICS

As noted previously, milk is a complex mixture of a wide range of compounds important to the neonate. This nutritious food is an emulsion of fat globules and a

suspension of casein micelles (casein, calcium, and phosphorous) in an aqueous phase that contains solubilized lactose, whey proteins, and minerals. The mother's blood is the sources of all nutrients needed for milk synthesis by epithelial cells of the mammary gland. Specifically, the precursors of milk components leave the blood and enter the extracellular fluid among capillaries and epithelial cells. These substances are taken up from the extracellular fluid through the basolateral membrane of the epithelial cell. Once inside the cell, they enter the appropriate synthetic pathways. In addition, some pre-formed proteins (e.g., immunoglobulins) and amino acids (e.g., BCAA and taurine), are transported intact across the mammary epithelial cells and incorporated into milk (Mepham, 1983).

Synthesis of proteins

Amino acids are absorbed through the basal membrane of mammary epithelial cells by specific amino acid transport systems. Once inside the cell, amino acids are covalently bound together to form proteins in the poly-ribosomes on the rough endoplasmic reticulum (RER). Proteins that are synthesized at the RER are either secreted proteins (e.g., casein, Beta-lactoglobulin, and Alpha-lactalbumin), membrane-bound proteins (e.g., extra-cellular matrix proteins involved in cell-cell contacts and membrane-bound enzymes), or intracellular proteins. Newly synthesized proteins are transferred from the RER to the Golgi apparatus where they are processed for transport out of the cell. Casein is secreted as a micelle, which is formed in the Golgi from casein, calcium and phosphorous. Caseins and other proteins undergo post-translational

processing in the Golgi. Proteins that remain in the cell include cellular enzymes, structural proteins (e.g., keratin), and fatty acid-binding proteins (Akers, 2002).

Milk proteins and lactose are transported to the apical area of the cell via secretory vesicles that bud off of the Golgi. These secretory vesicles are bounded by a lipid bilayer membrane and make their way to the apical membrane via a mechanism involving microtubules made of polymerized tubulin. Tubulin is one of several cytoskeletal proteins which form the cellular scaffolding, providing the cell with structure. Keratin is another cytoskeletal protein. The secretory vesicles do not transfer to the basolateral membrane if the epithelial cell is polarized. The apical membrane of the epithelial cell and the membrane of the secretory vesicle fuse resulting in an opening through which the vesicle contents are discharged into the lumen of the alveoli (Mepham, 1983).

Synthesis of lactose

Glucose enters the cell through the basolateral membrane via a specific transport mechanism (predominantly via GLUT1). Some glucose is converted to galactose. Both glucose and galactose enter the Golgi to form lactose via a series of enzyme-catalyzed reactions. The formation of lactose in the Golgi results in drawing water into the cytoplasm and then into the Golgi where it ultimately becomes a component of milk. The Golgi apparatus is involved in processing of milk proteins, synthesis of lactose, and osmotic gradients for transport of water and, therefore, very important in the synthesis of milk components. Lactose and much of the associated water in milk is released from

secretory vesicles along with the milk proteins into the lumen of the alveoli (Mepham, 1983).

Synthesis of fat

Precursors for synthesis of milk fats, including acetate, ß-hydroxybutyrate, preformed fatty acids, glycerol, and monoacylglycerides, are taken up by mammary epithelial cells at the basolateral membrane. The ketone bodies are important precursors of fatty acid synthesis in epithelial cells of the mammary gland in ruminants. All of those components enter the pathway for synthesis of triglycerides in the smooth endoplasmic reticulum (SER), leading to the formation of small lipid droplets. Numerous small lipid droplets fuse to yield larger droplets, which move toward the apical membrane. At the apical membrane, the large lipid droplet is exported out of the mammary epithelial cell through its apical membrane into the lumen of alveoli and duct system of the mammary gland (Mather and Keenan, 1998).

TRANSPORT OF MILK COMPONENTS NOT SYNTHESIZED IN MAMMARY EPITHELIAL CELLS

A number of components in maternal plasma are transported across the mammary epithelial cell into the duct system essentially unchanged. These substances include immunoglobulins which bind to specific receptors on the basolateral surface of the cells and are taken "into" the cell through the apical side of the cell via endocytic vesicles (or transport vesicles). In this process, the membrane of the transport vesicles fuses with the inner surface of the apical membrane of the cell and releases the immunoglobulin into the lumen of the alveolus. As the transport vesicles traverse the

cell, they do not seem to interact with the Golgi, secretory vesicles, or lipid droplets. Serum albumin may be transported across the mammary epithelial cells by this mechanism. Because there is no serum albumin receptor, serum albumin molecules are probably internalized into the cell along with the immunoglobulins in transport vesicles (Mepham, 1987).

Paracellular pathway for nutrient transport

Because of the tight junctions between epithelial cells, there is little or no flow of macromolecules between the cells, except perhaps water and ions. Passing of substances between the cells through the tight junction is known as paracellular transport. When the gland is inflamed, such as during mastitis or involution, or when oxytocin is causing milk ejection, the tight junctions open to allow lactose and potassium to move from the lumen into the extracellular space and for sodium and chlorine to move into the lumen from the extracellular space. This results in a change in electrical conductivity by the mammary epithelial cell, as well as increases concentrations of lactose and other milk-specific components in blood. For instance, lactose can be measured in the urine of a cow during the periparturient period. Milk proteins can be detected in the cow's blood during lactation and early involution (Akers, 2002).

Other components that can enter the lumen without passing through the epithelial cells include leukocytes. The leukocytes comprise the vast majority of somatic cells in milk. These cells pass between the epithelial cells via the paracellular pathway to enter the lumen of alveoli of the mammary gland (Akers, 2002). Sodium, potassium and chloride are major monovalent ions in milk which transported actively via Na^{+}-K^{+} pump

located on the basal and lateral membranes, but not the apical membranes, of mammary secretory cells. Ca^{++} is also actively transported but the mechanism is still poorly understood. Other macronutrients such as Iron, Zink, Cupper, Selenium, Iodine, Fluoride, Manganese and so forth are also actively transported to cells via their respective transporters on mammary epithelial cell membranes (Shennan and Peaker, 2000).

MAJOR MILK PROTEINS

All of the major milk proteins (except serum albumin and immunoglobulins) are synthesized and secreted by epithelial cells in the mammary gland from amino acids. The amino acids required for protein synthesis are either taken up from the blood (Klobasa et al., 1987) or synthesized from numerous precursors in mammary epithelial cells (Kim and Wu, 2009).

Caseins

Caseins are present in milk in the form of a micelle. The phosphate groups covalently bound to the casein molecules bind calcium via ionic bonds. After caseins are phosphorylated, Ca^{2+} binds the phosphate to initiate polymerization of the micelle particles. This structure is essential for micelle formation. The casein micelle (140 nanometers in diameter) functions as a source of nutrients for the neonate, supplying amino acids, calcium and phosphate. They are composed of alpha, beta, gamma, and kappa caseins. Alpha-caseins are in multi phosphorylated forms (s2, s3, s4, s5, and s6). Beta-casein is the major casein in the sow's milk. Kappa-casein (a glycoprotein) is distributed throughout the casein micelle and acts to stabilize the micelle. Gamma-

caseins are C-terminal fragments of ß-casein which are released after proteolytic degradation by plasmin when this milk is within the alveoli of the mammary gland. Destabilization of the casein micelle by proteases is part of the mechanism involved in digestion of milk proteins in the stomach and intestine of neonates (Mather, 2000).

Major whey proteins

Beta-lactoglobulin

This protein comprises of approximately 50 % of the total whey proteins in milk and it is the major whey protein in milk from ruminants and pigs. Beta-lactoglobulin is not found in the milk of many species. The function of Beta-Lactoglobulin is unknown, but it may be a fatty acid or lipid binding protein. Beta-lactoglobulin does have sequence similarities to retinol-binding proteins. Generally, Beta-Lactoglobulin is found in the milk of species which transport high levels of immunoglobulins during colostrum formation; however, the specific relationship between the presence of Beta-Lactoglobulin and immunoglobulin transport remains unclear (Mather, 2000).

Alpha-lactoglobulin

This protein accounts for about 25 % of the total whey proteins in milk. It is the B protein of the lactose synthase enzyme complex. Therefore, Alpha-lactoglobulin plays a role in the control of milk production and secretion. Alpha-lactoglobulin may have other nonspecific effects (e.g., binding Ca^{++} and Zn^{+}) to affect the integrity of milk fat membranes (Mather, 2000).

Minor whey proteins

Serum albumin

Serum albumin in milk is derived from maternal serum and is not synthesized in the mammary gland. There are suggestions that this protein enters the milk via the paracellular pathway, or taken up along with other components such as immunoglobulins. There does not seem to be a specific mechanism for transport of serum albumin. Increases in the concentration of serum albumin in milk occur especially during mastitis and during involution of the mammary gland. The function of serum albumin in milk is unknown, but this protein can bind to fatty acids and some small molecules (Mepham, 1987).

Immunoglobulins (Ig)

These proteins include IgG1, IgG2, IgA, and IgM. Immunoglobulins are present in very high concentrations in colostrum, but in much lower concentrations in milk. Immunoglobulins provide passive immunity to offspring by being transported from the mother's colostrum to the general circulation of the neonate. They also serve as part of the immune system of the mammary gland. Secretory component (SC) is the part of the IgA receptor which is hydrolyzed by a protease, and remains attached to IgA during the process of secretion from the cell (Mepham, 1987).

Other milk proteins

Lactoferrin (LF)

Lactoferin is an iron-binding protein with antibacterial properties. LF is present

at relatively low concentrations in milk from cows during lactation, but at relatively high concentrations when cows experience mastitis or involution of the mammary gland. Lactoferrin may also be an immunomodulatory protein, as it is the major nonspecific disease resistance factor found in the mammary gland (Meisel, 1997).

Lactoperoxidase

Lactoperoxidase is a member of the heme peroxidase family of enzymes that break down hydrogen peroxide into water. This protein also has antibacterial and anti-oxidant properties (Meisel, 1997).

Lysozyme

Lysozyme is an enzyme that cleaves the carbohydrate polymers of the bacterial cell wall to act as a bactericidal protein. This enzyme has a high activity in the human milk and possibly horse milk, but its activity is low in cow's milk (Mepham, 1987).

Beta2-microglobulin

Beta2-Microglobulin was initially discovered as a crystalline precipitate in resuspended casein and was first called lactolin. It is part of major histocompatability complex II (MHC II). While the function of Beta2-microglobulin in milk is not known, this protein is present in the epithelial cell in association with a protein that binds immunoglobulin G. Beta 2-microglobulin may be involved in function of the IgG receptor or in transport of IgG during colostrum formation (Meisel, 1997).

Milk has numerous other enzymes, including proteases, protease activators, nucleases, glycosidases, and D-amino acid oxidases (Klobasa et al., 1987). They can degrade proteins, amino acids, carbohydrates, and nucleotides present in milk.

MILK PROTEIN SYNTHESIS AND THE ROLE OF mTOR

The major role of mechanistic target of rapamycin (mTOR), particularly mTORC1, in the regulation of protein synthesis in animal cells has been well defined (Wang and Proud, 2006). Protein synthesis is inhibited by the association of the nonphosphorylated eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) with the eukaryotic translation initiation factor 4E (eIF4E), preventing the formation of the translation initiation complex. Insulin increases phosphorylation of mTOR, which in turn phosphorylates 4EBP1 at multiple sites and subsequently causes the release of eIF4E from the 4EBP1-eIF4E complex. Once released from this complex, eIF4E forms the active translation initiation complex with other translation initiation factors. The complete translation initiation complex then binds the 40S ribosomal subunit, generating the 43S pre-initiation complex which initiates translation of mRNA into protein (Kimball and Jefferson, 2006).

The activated mTOR complex enhances mRNA translation by additional mechanisms: (1) increased activation of the 40S ribosomal subunit through phosphorylation of the ribosomal protein S6 kinase (S6K1); (2) phosphorylation of the eukaryotic elongation factor-2 kinase (EEF2K); and (3) enhanced ATP production (Kimball and Jefferson, 2006). Specifically, activated mTOR phosphorylates ribosomal protein S6 (RPS6, a component of the 40S ribosomal subunit), resulting in the initiation of polypeptide synthesis. Because active, non-phosphorylated EEF2K inhibits translation by hindering the activity of the elongation factor 2 (eEF2), inhibition of EEF2K by mTOR frees eEF2, thereby increasing mRNA translation. Moreover, activation of

mTOR stimulates expression of glucose transporter 1 and amino acid transporters in epithelial cells of the mammary gland (Schieke et al., 2006). These nutrients can be used for mitochondrial ATP synthesis, which drives the translation processes (Proud, 2007). The mTOR complex is activated by certain amino acids, particularly leucine (a branched-chain AA) (Wu, 2010). Additionally, leucine enhances overall protein synthesis by serving as a precursor for the synthesis of glutamate, glutamine, aspartate, asparagine, and protein (Lei et al. 2012).

The activation of transcription for milk protein synthesis by Jak2-Stat5 cell signaling has also been reported where binding of prolactin and insulin to their receptors induces the phosphorylation of STAT5. Upon phosphorylation, STAT5 dimers translocate to the nucleus and induce transcription of several genes, including ELF5 and genes coding for milk proteins. ELF5 protein enhances the activity of STAT5, thereby increasing transcription of genes encoding milk proteins (Rosen, 1999).

AMINO ACID METABOLISM IN THE MAMMARY GLAND AND THEIR ROLES IN LACTATION

Amino acid utilization by mammary tissue is greatly affected by: a) concentrations of amino acids in plasma; b) mechanisms of amino acid uptake into mammary cells;, and c) intracellular metabolism of the amino acid. Each of these control systems is further influenced by a variety of factors. Concentrations of amino acids in plasma are affected by diet and physiological state of the animal. There are multiple mechanisms responsible for amino acid transport by mammary epithelial cells. In addition, intracellular metabolism of amino acids involves many competing biochemical

pathways. Thus, uptake and intracellular metabolism of amino acids by the mammary gland are more complex than simply being incorporated into milk proteins (Oddy et al., 1988). For example, uptake of "nutritionally nonessential amino acids" varies considerably with stage of lactation, generally exhibiting a deficit in uptake relative to their amounts in milk proteins. Some "nutritionally essential amino acids" are taken up stoichiometrically (e.g., Lys, Met, Phe, Tyr, and Trp) in relation to milk protein output, while others, including BCAA and arginine, are taken up by the mammary gland in amounts greater than rates of milk protein production (Trottier et al., 1997). Some of these amino acids are extensively used by the mammary tissue either as a potential energy source or as a source of carbon and nitrogen for synthesis of other nonessential amino acids, such as glutamine and glutamate (Wu, 2010).

Amino acids are not only building blocks for protein, but they are also key regulators of metabolic pathways that are vital for whole body homeostasis (Jobgen et al. 2006; Wu and Meininger 2000) as well as lactogenesis (Bequette et al., 1998). In primiparous sows, limited food intake and decreased blood flow to lactating mammary glands lead to reduced provision of nutrients (including amino acids) to mammary tissues for their own growth and milk synthesis (Kim et al., 1999). Of note, arginine is the common substrate for the generation of nitric oxide (NO; a major vasodilator and angiogenic factor) and polyamines (key regulators of protein synthesis and lactogenesis) (Wu and Morris, 1998). Compelling evidence shows that endogenous synthesis of arginine alone cannot meet the need for maximal milk production by lactating sows and

arginine is also deficient in sow's milk with respect to supporting maximum growth of suckling piglets (Mateo et al., 2008; Wu et al., 2004).

The BCAA are actively involved in regulation of cell-cycle progression, cell differentiation (Kimura and Ogihara, 2005), cell apoptosis (D'Anona et al., 2010), and milk protein synthesis (Toerien et al., 2010). Lactating sows have high requirements for BCAA to support milk production (Wu, 2009). The uptake of BCAA by porcine mammary glands (76 g/d on d 13–20 of lactation) is much greater than their output in milk protein (46 g/d) (Wu, 2010). Thus, the lactating porcine mammary gland catabolizes about 30 g BCAA/d (40% of the BCAA taken up from arterial plasma) (Lei et al., 2012). In contrast, sow's milk contains high concentrations of free and peptide-bound glutamine plus glutamate (Mateo et al., 2008), which are crucial for growth, development, and normal function of the neonatal small intestine (Kim and Wu, 2009). On d 10 of lactation, the lactating porcine mammary gland takes up 16 g/d glutamine from the arterial circulation (Trottier et al., 1997), but produces more than twice (36 g/d) that amount of glutamine in milk (Haynes et al., 2009). Therefore, extraction of glutamine by the lactating mammary gland is much less than its output in milk and a large amount of glutamine (20 g/d) must be synthesized by mammary tissue to support the production of milk proteins (Li et al., 2009). Mammary tissue does not contain pyrroline-5-carboxylate dehydrogenase or proline oxidase activity; therefore, it cannot convert arginine, ornithine, or proline into glutamate or glutamine (O'Quinn et al., 2002). Instead, BCAA are utilized for glutamine synthesis in the lactating mammary

gland through BCAA transaminase and glutamine synthetase, asreported for skeletal muscle (Wu et al., 1989) and placentae (Self et al., 2004).

Catabolism of BCAA in mammary tissue

The overall pathway of BCAA catabolism in the lactating mammary gland of sows is depicted in Figure B-1. The catabolism of BCAA by the mammary gland and mammary epithelial cells is initiated by BCAA transaminase in the presence of α-ketoglutarate to form branched-chain Alpha-ketoacids (BCKA) and glutamate (Li et al., 2009). Thus, concentrations of BCKA in milk may be relatively high, and milk-born BCKA may serve as energy substrates for the small intestine of suckling neonates. In support of this view, there is evidence that substantial amounts of BCKA are utilized by enterocytes of the small intestine (Wu, 2009) and the splanchnic bed of animals (Wu, 1998). The BCAA transaminase exists as mitochondrial and cytosolic isoforms. Both isoforms are expressed in mammary epithelial cells (Desantiago et al., 1998). BCAA transaminase is activated by octanoate, a medium-chain fatty acid.

The BCAA-derived BCKA undergoes oxidative decarboxylation by the mitochondrial BCKA dehydrogenase complex (BCKAD). This enzyme complex consists of BCKA decarboxylase (E1 which requires thiamine pyrophosphate as a cofactor), dihydrolipoamide acyltransferase (E2 which requires lipoate and coenzyme A as cofactors), and dihydrolipoamide dehydrogenase [E3 which requires flavine adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD) as cofactors] (Brosnan and Brosnan, 2006). The BCKAD E1 has two subunits: E1a and E1b. BCKAD is regulated by phosphorylation and dephosphorylation in cells. Protein levels for BCAT

and BCKAD E1α, as well as dephosphorylation of BCKAD E1α are enhanced by leucine. Increasing concentrations of leucine from 0.5 to 2 mM increased protein levels for mitochondrial BCAA transaminase and total BCKAD E1α by 39% and 42%, respectively, while decreasing the abundance of phosphorylated BCKAD E1α by 33% (Lei et al., 2012). As a result, the ratio of phosphorylated BCKAD E1α to total BCKAD E1α (PE1α/ total E1α value) is reduced by 51% (Lei et al., 2012). Lactation increases mammary tissue BCAT activity by a factor of ten and sustains the BCKAD complex in the fully activated state (Desantiago et al., 1998). For comparison, only 20% of BCKAD exists in an active state in mammary tissue of non-lactating cows (Brosnan and Brosnan, 2006). Thus, the mammary gland is a quantitatively significant site for BCAA catabolism during lactation (Wu, 2009).

The BCAA transaminase catalyzes the transamination of BCAA with α-ketoglutarate to form glutamate and BCKA in mammary epithelial cells. The BCAA-derived glutamate is either amidated to form glutamine or transaminated with pyruvate (or oxaloacetate) to produce alanine (or aspartate) (Li et al., 2009). Rates of synthesis of alanine, aspartate, asparagine, glutamate, and glutamine from BCAA increase as the concentrations of leucine increase from 0 to 5 mM, with the values being the highest for glutamine, followed by glutamate, aspartate, alanine, and asparagine in descending order (Lei et al., 2012). Glutamine synthetase and phosphate-dependent glutaminase are the two key enzymes involved in glutamine synthesis and degradation, respectively in most animal cells (Wu et al., 2011). Interestingly, glutaminase activity is absent from tissues of the lactating mammary gland; therefore, the epithelial cells maximize production and

release of glutamine (Li et al., 2009). The de novo synthesis of glutamate and glutamine helps to explain the high abundance of these two amino acids in both free and peptide-bound forms in milk (Wu and Knabe, 1994). Accordingly, BCAA likely play an important role in milk synthesis by mammary epithelial cells of lactating mammals (Lei et al., 2012). Approximately 30 g/d BCAA must be degraded to form 20 g/d glutamine in the mammary glands of lactating sows (Lei et al., 2012).

DIETARY REQUIREMENTS OF AMINO ACIDS BY NEONATES

Protein deposition in the body of neonates is affected by both the quality and the quantity of dietary protein. Relatively high intakes of protein and energy are required by young animals for supporting their rapid growth rates. The energy density of the diet could influence the voluntary feed intake of animals. To meet the requirement for energy, feed intake generally increases when dietary energy is low. Similarly, when diets contain suboptimal levels of protein, food intake also increases to minimize deficiencies in amino acids. These studies have been performed extensively with young pigs.

The capacity of the gut of neonatal pigs limits their feed intake. Thus, piglets may not be able to consume sufficient amounts of a diet with low energy density to maintain their optimal growth rate. Many amino acids cannot be synthesized by piglets and must be provided in the diet. Therefore, an adequate supply of all amino acids must be taken into consideration when recommending requirements of dietary protein (Wu, 2010). The composition of amino acids in milk and rates of milk consumption by suckling piglets can be used to estimate dietary requirements piglets weighing 1 to 5 kg.

Their maximum growth rates may be achieved by increasing dietary provisions of glutamine, glycine, arginine, and proline beyond that provided in milk (Wang et al. 2013, 2014; Wu et al. 2014). Current growth models cannot be used to accurately estimate energy or amino acid requirements for neonatal pigs (< 20 kg body weight) because there is not sufficient information on their energy or amino acid metabolism. Experimental data on optimal dietary requirements of other amino acids by neonatal pigs between birth and weaning are not available. Thus, NRC-recommended intakes of dietary amino acids for swine (NRC, 2012) may not be ideal for piglets. This is exemplified by the dietary requirements for arginine by young pigs (see discussion below). Rather, reductions in dietary lysine requirements for piglets between 5 and 20 kg body weight have been proposed based on results from biochemical and nutritional studies (Wu, 2014). Specifically, optimal amounts of dietary lysine (based on true ileal digestible amounts) are 1.19% and 1.01% for 5-10 and 10-20 kg pigs, respectively, which is consistent with a progressive decrease in the fractional rate of skeletal-muscle protein synthesis (Wu, 2013).

Sow's milk is thought to provide adequate amino acids for growth of neonatal pigs. However, the amount of milk produced by sows during lactation does not provide adequate amounts of all amino acids for maximum growth of piglets (Rezaei et al., 2011). Hodge (1974) and Boyd et al. (1995) demonstrated that artificially reared neonatal pigs grow at a rate that is at least 50% greater than that of sow-reared piglets. Beginning at eight days of age, piglets exhibit sub-maximal growth, which may have resulted from inadequate intake of protein or energy from sow's milk (Boyd et al.,

1995). Furthermore, arginine is a nutritionally essential amino acid for maximum rates of growth of young mammals (Wu et al., 2004). There are low levels of arginine in sow's milk (Wu and Knabe 1994); therefore, neonatal pigs must synthesize substantial amounts of arginine to achieve maximum growth rates. Compelling evidence shows that endogenous synthesis of arginine in young pigs is inadequate for their maximum growth. Recently, Wu (2014) proposed that an ideal, highly digestible diet for 5-10 and 10-20 kg piglets contain 1.19% and 1.01% arginine based on true digestible amounts in the ilium.

Importance of glutamine, glutamate and aspartate in neonates

Glutamine is used extensively in the rat small intestine (Windmueller and Spaeth, 1974). Additionally, in vivo studies have shown that glutamate and aspartate are actively oxidized to CO2 via the Krebs cycle in the intestinal mucosa (Stoll et al., 1999; Wu et al., 1995). Based on these findings, dietary glutamine, glutamate and aspartate are now regarded as essential for the maintenance and function of the small intestine (Xi et al., 2011).

Dietary glutamate is a preferential substrate for glutathione (a major antioxidant) synthesis in the gut mucosa (Reeds et al., 1997), and glutamine serves as the nitrogen moiety for the syntheses of purines, pyrimidines, and amino sugars (e.g., N-acetylglycosamine and N-acetylglactosamine) (Wu et al., 2011). Purines and pyrimidines are necessary for the synthesis of DNA, whereas amino sugars are required for the production of mucosal mucin and, therefore, the maintenance of intestinal-mucosal integrity (Reeds and Burrin, 2001).

As noted previously, the catabolism of glutamine and glutamate in enterocytes is of supreme importance for the synthesis of ornithine, citrulline, and arginine in most of mammalian neonates, including human infants and young pigs (Wu, 2009). The endogenous synthesis of arginine from its precursors compensates for a marked deficiency of arginine in the milk of most species, including humans, cows, sheep, and pigs (Kim and Wu, 2004; Geng et al., 2011). Arginine stimulates protein synthesis in muscle, maintains an active hepatic urea cycle for ammonia detoxification, and regulates the uptake and utilization of nutrients by tissues (Wu et al., 2009).

Physiological levels of glutamine enhance intestinal expression of genes that are related to: i) antioxidative responses (Wang et al., 2008); ii) proliferation and survival of mucosal cells (Weiss et al., 1999); c) expression of tight-junction proteins (Wang et al. 2014) and mucosal integrity (Roads and Wu, 2009); and iii) prevention of inflammation and apoptosis (Roads and Wu, 2009). Glutamine can also modulate the transport of many nutrients (including amino acids, small peptides, fatty acids, glucose, vitamins and minerals) by enterocytes at transcriptional and translational levels (Wu, 2010). Also, through activation of the mechanistic target of rapamycin (mTOR) signaling pathway, glutamine stimulates protein synthesis and inhibits protein degradation in enterocytes, thereby promoting cell growth (Xi et al., 2011) and migration (Roads and Wu, 2009). Studies using the swine model demonstrate that milk-borne glutamine is insufficient for maximal growth of neonates (Haynes et al., 2009). Sow's milk contains 4.3-10.8 g/L glutamine which increases as lactation advances. Based on milk consumption, piglets with a normal birth-weight obtain 2.9- 3.5 g/day glutamine from milk during the

suckling period (Lei et al., 2012). Due to extensive catabolism of glutamine in the small intestine, less than 36% of milk-derived glutamine (i.e., 1.14 g) is accreted in the body of the piglet. As a consequence, the rate of endogenous synthesis of glutamine in the suckling piglet is relatively high ($\geq$ 0.88 g/kg body weight per day) to sustain maximum growth (Wu, 2009). Another milk-derived amino acid closely related to glutamine is glutamate, which can spare glutamine oxidation by the intestine (Wu et al., 1995) and maintain gut function (Rezaei et al., 2013).

A suckling piglet can obtain 3.3-3.8 g/L glutamate from milk, but more than 95% of that amount is removed by the portal-drained viscera (Stoll et al., 1999). Thus, glutamate in milk is virtually not available for protein synthesis in extra-intestinal tissues (Lei et al., 2012). Although a young pig can obtain 6-7 g of glutamine plus glutamate from milk daily, oral administration of glutamine (1 g/kg body weight per day) can enhance daily weight gains and anti-inflammatory responses in the animal (Wu et al., 2011). The beneficial effects of glutamate have also been reported for older pigs, as supplementation of up to 4% mono-sodium glutamate significantly increased growth in postweaning pigs (Rezaei et al., 2013). Therefore, it seems logical to provide lactating dams with an abundant supply of BCAA for the synthesis of glutamine and glutamate in mammary tissue which can be secreted in milk (Lei et al., 2012). These amino acids promote the growth, development and health of neonates (Wu, 2009).

BCAA AND LACTATION

Effects of BCAA on growth and proliferation of mammary epithelial cells

BCAA modulates cell metabolism through the synthesis of macromolecules

(DNA and protein), that regulate growth and proliferation of a variety of cell types, including pancreatic cells (Xu et al., 1998), hepatocytes (Kimura and Ogihara, 2005), fibroblasts (Gonçalves and Marcondes, 2010), embryonic kidney cells (Kim et al., 2002), and mammary epithelial cells (Kim et al., 2009). This indicates that BCAA are not only building blocks for synthesis of proteins, but also nutrient signals that regulate mechanism affecting cell proliferation including the cell cycle, DNA synthesis, and protein synthesis (Lei et al., 2012).

The cell cycle consists of interphase and mitosis phases with a series of sequential rapid and highly complex events leading to cell growth and division. During the interphase step of the cell cycle, the synthesis of cellular macromolecules, particularly proteins, is the primary event that initiates the cell cycle (Fingar et al., 2002, 2004). Based on the stage of cell division, the interphase is divided into three sequential phases [Gap 1 (G1), DNA synthesis (S), and Gap 2 (G2)] and is the longest period of the cell cycle compared with the mitotic (M) phase. During the three phases of interphase, the cell grows by synthesizing proteins and cytoplasmic organelles and duplication of its chromosomes during S and G2 phases, followed by cell mitosis (Blow and Tanaka, 2005). At the M stage of the cell cycle, nuclear and cytoplasmic division occurs. Thus, an increase in cell size through synthesis of macromolecules (particularly protein) is necessary for cell division. At the end of each cell cycle, two daughter cells are produced by mitosis. The process of cell differentiation, which is critically influenced by intracellular levels of polyamines, requires activation of the necessary biochemical machinery (Wu et al., 2011; Wu et al., 2010).

During lactation, extensive constitutive proteins are synthesized by cells of the mammary gland that account for 40 to 70% of total mammary protein synthesis that includes synthesis of constitutive tissue proteins and milk proteins) (Bequette et al., 1998). In lactating sows, the synthetic rate for mammary constitutive proteins increases to a peak level on Day 14 of lactation (7.8 g/kg wet tissue/day), and then decreases to 2.7 g/kg wet tissue/day on Day 21 and to 0.5 g/kg wet tissue/day on Day 28 of lactation (Bequette et al., 1998). Consistent with protein synthesis, the net rate of DNA synthesis changes in a similar manner during lactation (Kim et al., 1999). Interestingly, the highest rate of milk production by sows occurs around Day 14 of lactation, as estimated by the weigh-suckle-weigh (Rezaei et al., 2011) and deuterium oxide dilution (Thodberg and Sørensen, 2006) methods. It is likely that the synthesis of constitutive proteins in mammary tissue is proportionally correlated with the number of functional mammary epithelial cells, which is a consequence of cell proliferation. As mentioned earlier, the physiological process of increased proliferation of epithelia cells of the mammary gland can be up-regulated by BCAA (Kim et al., 2009) although the underlying mechanisms remain unknown.

Effects of BCAA on the functional differentiation of mammary epithelial cells

Mature secretory cells must possess the proper biochemical pathways for synthesis and secretion of milk (Kim and Wu, 2009; Kim et al., 2009). These metabolic pathways develop during the process of cell differentiation and have important implications for epigenetic regulation of lactogenesis (Wang et al., 2012). The mTOR cell signaling pathway plays an important role in regulation of cellular differentiation in

response to certain nutrients, including BCAA (Jankiewicz et al., 2006). Branched-chain amino acids, especially leucine and its metabolites, stimulate secretion of insulin by pancreatic β- cells (Li et al., 2007) partly through phosphorylation of molecules downstream of mTOR, particularly p70 S6K and 4E-BP1. Results from both in vitro and in vivo studies show that unlike differentiating mammary epithelial cells, undifferentiated and predifferentiated cells have low levels of phosphorylated mTOR. Also, treatment with rapamycin abrogates development of mammary alveoli, β-casein secretion and olume of the mammary gland (Jankiewicz et al., 2006). Further, mTOR-mediated expression of the Id2 protein (inhibitor of DNA-binding protein) is a prerequisite for cell differentiation (Jankiewicz et al., 2006). These results indicate that mTOR is necessary for functional differentiation of mammary secretory cells and normal development of the mammary gland. Such a mechanism explains the stimulatory effects of BCAA on protein synthesis and secretion by mammary epithelial cells (Lei et al., 2012).

Effects of BCAA on the longevity of mammary epithelial cells

Mammary involution is a dramatic cellular remodeling process associated with cell aging and apoptosis (Appuhamy et al., 2012). An increase in mitochondrial biogenesis occurs in response to the onset of lactation to meet the need for ATP in biosynthetic pathways. For example, in lactating mice, the density of mitochondrial inner membranes in mammary epithelial cells increases 7-fold and the activity of mammary cytochrome C oxidase increases 4- to 5-fold (Rosano et al., 1976). These results demonstrate increases in the number and activity of mitochondria in the

mammary gland during lactation. However, increased rates of mitochondrial respiration are required for the mammary gland to support ATP provision, but high metabolic needs can be deleterious because endogenous free radicals are also produced concurrently to adversely affect normal cellular functions (Dai et al. 2013). More specifically, accumulation of free-radical results in oxidative damage of cellular lipids, proteins and DNA, which further leads to cell aging and impaired synthetic capacity of the mammary epithelial cells (Appuhamy et al., 2012). Thus, controlling mitochondrial respiration and optimizing the defense system against generation of reactive oxygen species are crucial for prolonging mammary cell survival and milk production. Through activating the mTOR signaling pathway to promote the synthesis of anti-oxidative proteins, BCAA may beneficially increase the life span of mammary epithelial cells. The anti-aging role of BCAA is associated with the enhanced growth of new mitochondria (Alvers et al., 2009). In support of this view, leucine, isoleucine and valine can extend the chronological longevity of Saccharomyces cerevisiae, implicating that these nutrients are capable of promoting eukaryotic cell survival (Alvers et al., 2009). Similarly, provision of BCAA to cardiac and skeletal myocytes increases mitochondrial DNA concentration and expression of major genes related to activation of mitochondrial biogenesis and reductions in production of ROS (D'Anona et al., 2010). These genes include peroxisome proliferator-activated receptor γ coactivator-1α and sirtuin 1. The result is prevention of oxidative damage, and possibly apoptosis, in metabolically active cells which extends their survival in the face of increased mitochondrial respiration for generation of ATP (Lei et al., 2012).

Effects of BCAA on milk synthesis process in mammary epithelial cells

Intracellular protein synthesis

Production of milk proteins depends on the balance between rates of intracellular protein synthesis and proteolysis (Kim and Wu, 2009). The mammary gland has a high capacity for protein synthesis during lactation. Large amounts of milk proteins and a variety of constitutive proteins (e.g., structural proteins and enzymes) are synthesized endogenously by the lactating mammary gland (Grinari et al., 1997). The mammary glands of sows and cows produce 420 and 950 g milk protein, respectively, each day at the peak of lactation (Lei et al., 2012). However, the efficiency of converting dietary protein into milk protein is less than 30% and 10% in sows and cows, respectively (Bell and Bauman, 2006; Wu et al. 2014). The factors that regulate this process in the mammary gland include: a) entry of dietary amino acids into the portal circulation; b) uptake of amino acids and peptides; c) concentrations of lactogenic hormones and nutrients that regulate metabolism of amino acids and peptides; and d) activation of signaling pathways that regulate syntheses of proteins, lipids and lactose (Boyd et al., 1995). Because the metabolic capacity of the mammary gland for milk protein synthesis is much higher than now achieved under current feeding systems and management (Kim and Wu, 2009), there is great potential for enhancing milk production in animals and humans. The regulatory roles of hormones in controlling the synthesis of milk proteins are well studied (Rhoads and Grudzien-Nogalska, 2007). However, the functions of amino acids in this regard are largely unknown. Previous studies showed that BCAA promotes milk protein production (Richert et al., 1997), but inconsistent results have

been reported by the same laboratory (see Wu et al. 2014 for discussion). It is unclear whether those nutrients simply serve as building blocks of peptides and proteins or function as signaling molecules in the control of metabolic pathways for lactogenesis (Lei et al., 2012). Dairy cows that received intragastric infusion of casein (a BCAA-rich protein) as a source of amino acids had 10% higher production of milk protein (Bell and Bauman, 2006). Interestingly, this effect of casein was amplified (28% increase in the production of milk protein compared with the control group) by intragastric administration of casein together and intravenous infusion of insulin, which indicates a synergistic action of amino acids and insulin on milk protein synthesis (Bell and Bauman, 2006). Intravenous infusion of either total essential amino acids or leucine activates the mTOR signaling pathway, but infusion of methionine plus lysine or histidine had no effect (Toerien et al., 2010). In contrast, either a lack of all amino acids or selective deprivation of leucine inhibited phosphorylation of the mTOR substrates, 4E-BP1 and p70 S6K1, thereby decreasing the synthesis of total and cell-specific proteins (e.g., Beta-lactoglobulins) in both bovine and murine mammary epithelial cell lines (Moshel et al., 2006). Addition of either complete amino acids or leucine to the amino acid-free basal medium restored protein synthesis in the cells, but such effects were blocked by rapamycin or LY294002 (a phosphatidylinositol 3-kinase inhibitor) in a dose dependent manner (Moshel et al., 2006). Collectively, these results indicate that leucine (and/or BCAA) is signaling molecules in the translational machinery that controls milk protein synthesis, in addition to serving as a substrate for synthesis of polypeptides. This is consistent with findings from both in vitro and in vivo studies

which indicate that BCAA (particularly leucine) have anabolic effects on protein synthesis in skeletal muscle and other tissues (Gringari et al., 1997; Bell and Bauman, 2006; Richert et al., 1997; Tischler et al., 1982; Garlick and Grant, 1988; Mitch and Clark, 1984).

Intracellular protein degradation

The control of intracellular protein degradation in the lactating mammary gland is very important to maximize the production of milk proteins. However, the rates or regulation of protein degradation in mammary epithelial cells are poorly understood. Nonetheless, there is an inhibitory effect of BCAA on proteolysis in skeletal muscle and whole body under catabolic conditions (Wang et al., 2012; Tischler et al., 1982; Tayek et al., 1986). α-Ketoisocaproate (a product of leucine transamination) and 4-methyl-2-oxopentanoate (a metabolite of α-ketoisocaproate) also suppress proteolysis in muscle tissue (Tischler et al., 1982; Mitch and Clark, 1984); however, the underlying mechanisms are unknown. High concentrations of α-ketoisocaproate and 4-methyl-2-oxopentanoate (> 0.25 mmol/L) are required for inhibiting muscle protein degradation (Tischler et al., 1982; Mitch and Clark, 1984), but it is not known whether they mediate this effect in the presence of physiological levels of leucine in muscle cells.

Multiple proteolytic mechanisms are known to be responsible for the catabolism of proteins, including: a) the calcium-activated pathway; b) the ATP- and ubiquitin-dependent proteasome pathway; and c) the lysosomal pathway (Ciechanover, 2012). The calcium-activated pathway plays a major role in degrading myofibrillar protein. In contrast, most of the intracellular proteins are hydrolyzed by the proteasomal pathway,

which is enhanced by a variety of catabolic conditions, including trauma, sepsis, cancer, and starvation. The lysosomal system is mostly responsible for the degradation of engulfed particles that enter lysosomes via a process known as autophagy (Meijer et al. 2014). The short-term effect of BCAA on proteolysis has been associated with decreased activities of lysosomal proteases whereas the long-term effect of these amino acids might be mediated through the proteasome pathway (Busquets et al., 2000). In addition, activation of the mTOR signaling pathway results in inhibition of intracellular proteolysis in enterocytes (Yao et al., 2011), but this effect has not been reported for mammary epithelial cells. Based on the aforementioned evidence, BCAA may inhibit protein degradation in mammary epithelial cells via activation of the mTOR signaling pathway (Lei et al., 2012). However, there is a need for experiments to test this hypothesis.

Effect of dietary supplementation of BCAA on lactation and the growth performance of neonates

Limitation of glutamine and glutamate supply in lactating animals

Glutamate and glutamine are preferred sources of energy for cells of the small intestineand large amounts are required for maturation of the neonatal gut (Wu, 2009). Although regular diets for animals contain relatively high levels of glutamine and glutamate (Li et al., 2011; Hou et al., 2010; Wu et al., 2010), dietary supplementation with 1% glutamine to lactating sows significantly increased milk production (Wu et al., 2011). The underlying mechanisms may involve the cooperation of several organs, including the small intestine, skeletal muscle and lactating mammary gland (Lei et al.,

2012). At present, little is known about efficacy of dietary supplementation with glutamine versus BCAA to sustain high levels of glutamate and glutamine in milk. Studies with rats have shown that glutamine catabolism in the small intestine increases during lactation compared with virgin and nonlactating animals (Ardawi et al., 1988). Likewise, Okine et al. (1995) reported that enterocytes from cows in early stages of lactation oxidize more glutamine than cells from cows at either the mid- or late-stage of lactation. Consequently, the small intestine of lactating dams degrades approximately 70% of dietary glutamine (Wu et al., 2011) and only a small percentage is available for the lactating mammary gland (Doepel et al., 2007). This explains the need to increase glutamine and glutamate availability in milk. Supplementing BCAA to the diet of lactating dams may provide an efficient and effective strategy to overcome the problem of suboptimal milk production in lactating mammals (Li et al., 2009).

Effects of dietary BCAA supplementation on production of milk in lactating animals

The amount and quality of nutrients secreted by the mammary gland is the major determinant of growth and survival of piglets (Boyd et al., 1995). Lei et al. (2012) suggested that BCAA stimulates secretion of milk and modifies its nutrient composition.

The mammary glands of lactating sows produce 125% more glutamine in milk than is taken up from arterial plasma (Li et al., 2009), while the uptake of glutamate by the mammary gland is almost equal by the amount of glutamate present in milk (Shennan and Peaker, 2000). The extra glutamine could not be derived from proline because porcine mammary tissue lacks proline oxidase (O'Quinn et al., 2002). The synthesis of aspartate and asparagine has important implications since the uptake of

these two amino acids by the mammary gland of lactating sows accounts for only 50% of their output in milk (Trottier et al., 1997). Aspartate and asparagine are the third most abundant nonessential amino acids in porcine milk proteins (Wu and Knabe, 1994; Davis et al., 1994). As a result, BCAA substantially supports protein synthesis in lactating mammary tissue through stimulating the synthesis of nutritionally nonessential amino acids. As summarized in Table A-2, many studies have used BCAA supplementation as a strategy to improve lactation and litter performance in pigs, but the results are inconsistent. Several studies showed that supplementing BCAA to the diets of lactating sows enhances milk yield and neonatal growth. First, Richert et al. (1997a) found that weaning weights and weight gains of litters of high-producing lactating sows increased as dietary valine increased from 0.85 to 1.15%. Subsequently, they confirmed that increasing dietary valine (0.64-1.44%) to lactating sows nursing 10 or more piglets increased weaning weights and weight gains of litters (Richert et al., 1997b). This effect of dietary valine on lactation was also confirmed by Moser et al. (2000). Furthermore, Paulicks et al. (2003) observed that dietary supplementation of valine (0.85- 1.45%) to lactating sows increased milk production and milk protein content, in comparison with the control group (0.55% valine). Similarly, increasing dietary intake of valine, isoleucine, or total branched-chain amino acids by lactating sows increased milk synthesis, composition and growth performance of piglets (Richert et al., 1997).

Consistent with the findings from swine, duodenal infusion of leucine to lactating cows increased concentrations of casein, whey proteins, and total proteins in milk (Rulquin and Pisulewski, 2006). These results from animal studies have important

implications for enhancing milk production in women who have impaired lactogenesis under various stressful conditions (e.g., premature birth, maternal complications, and high or low ambient temperatures) and in response to low intake of dietary protein (Dewey, 2001).

CONCLUDING REMARKS

The synthetic capacity of the mammary gland depends largely on the number and efficiency of functional mammary epithelial cells and it directly correlates with subsequent growth of suckling neonates. In the lactating mammary gland, BCAA are extensively transaminated with α-ketoglutarate to produce glutamate and BCKA, with glutamate being an essential substrate for synthesis of other NEAA such as glutamine, alanine, aspartate and asparagine. Moreover, some of the BCAA carbons are either oxidized to provide ATP in mitochondria or utilized for lipid synthesis in cytoplasm.

BCAA enhance secretion of insulin by β-cells of the pancreas (Li et al., 2007) and also stimulate the mTOR cell signaling pathway to initiate synthesis of polypeptidesand inhibit protein degradation (Suryawan, and Davis, 2011). At present, little is known about hormonal or nutritional regulation of BCAA catabolism in mammary tissue. Emerging evidence from animal studies indicate that dietary supplementation with valine, isoleucine or a mixture of BCAA stimulates milk synthesis and lactation in sows (Richert et al., 1997a and 1997b; Moser et al., 2002; Paulicks et al., 2003). Interestingly, in contrast to enteral provision (Rulquin and Pisulewski, 2006), intravenous administration of BCAA was not found to affect milk protein synthesis or milk production by lactating cows. Thus, the route of supplementation with BCAA or

dietary composition of amino acids may be a critical factor influencing physiological responses. It is possible that BCAA inhibits catabolism of amino acids by both mucosal cells and luminal microorganisms of the small intestine (Dai et al., 2010; Dai et al., 2011; Hou et al., 2011), thereby increasing the entry of dietary amino acids to the portal circulation for utilization by extra-intestinal cells and tissues. One of the beneficial outcomes would be to increase the availability of arginine for synthesis of nitric oxide (Meininger and Wu, 2002), a major vasodilator (Wu and Meininger, 2009), to increase blood flow and associated increases in the uptake of nutrients by the lactating mammary gland.

Collectively, the findings on BCAA metabolism and nutrition in mammary tissue have not only advanced our knowledge of the biology of lactation, but they also have practical implications for improving milk production by female animals and women in both developed and developing countries. Furthermore, it is important to define optimal requirements for BCAA by lactating livestock and other mammals. Basic research on the biology of lactation is essential to achieve the biological potential for neonatal growth and survival, improve the efficiency of livestock production, and sustain animal agriculture worldwide.

CHAPTER II

DIETARY SUPPLEMENTATION WITH BRANCHED-CHAIN AMINO ACIDS ENHANCES MILK PRODUCTION BY LACTATING SOWS AND GROWTH OF SUCKLING PIGLETS

SYNOPSIS

The growth rate of suckling pig is critically dependent on milk yield and the concentration of nutrients in milk. In lactating sows, large amounts of amino acids are mobilized from the body for milk synthesis. Under current dietary regimens, milk secretion is insufficient to sustain the maximal growth of neonatal pigs. This study tested the hypothesis that dietary supplementation of branched-chain amino acids (BCAA; L-leucine, L-isoleucine and L-valine) to lactating sows enhanced their production of milk and the growth performance of their suckling piglets. Thirty multiparous sows (Landrace x Large White) were assigned randomly into one of three groups: 1) control (a corn- and soybean meal-based diet), 2) basal diet + 1.535% BCAA; and 3) basal diet + 3.07% BCAA. Diets were made isonitrogenous by addition of appropriate amounts of L-alanine. Lactating sows had free access to drinking water and their respective diets. The number of live-born piglets was normalized to 9 per sow at d 0 of lactation (the day of parturition). On d 3, 15 and 29 of lactation, body weights and milk consumption of piglets were measured and blood samples obtained from sows and piglets at 2h after feeding. Data were analyzed by 2-way ANOVA. Feed intake did not differ among the three groups of sows. Concentrations of aspartate, glutamine, citrulline, arginine, and

BCAA were higher ($P < 0.05$) in the plasma of BCAA-supplemented sows and their piglets than those in the control group. Compared with the control, dietary supplementation with 1.535% and 3.07% BCAA increased ($P < 0.05$): 1) concentrations of free and protein-bound BCAA, glutamate and glutamine in milk ($P < 0.01$); 2) milk production by 14% and 21%, 3) daily weight gains of piglets by 19% and 28%, respectively; and 4) reduced the rate of preweaning mortality by 50% and 70%, respectively. These results indicate that dietary supplementation with up to 3.07% BCAA enhanced milk production in lactating sows which improved growth and survival of their piglets.

INTRODUCTION

The major goal of the swine industry is to minimize neonatal mortality and improve weaning weights of neonatal pigs as their adaptability to weaning and rates of growth to market weight are positively affected by weaning weight (Boyd et al., 1995). The total net profit of a swine production unit is primarily determined by both the number and size of piglets produced per year per sow (Quiniou et al., 2002). Recent attempts to increase litter size have led to increased prevalence of low birth weight piglets (Fix et al., 2010), which results in a higher rate of mortality and a longer period of time to reach an optimum market weight, when compared with normal-birth-weight piglets (Rezaei et al., 2011; Wu et al. 2006). Pork quality is also affected by birth weight to a large extent (Fix et al., 2010). The greatest proportion of mortality in commercial swine production occurs prior to weaning and ranges between 10 and 20% of live-born pigs (Wu et al., 2006). For example, pre-weaning mortality was estimated at 13% in U.S.

commercial swine herds during 2008 (PIGCHAMP, 2008) due to poor performance of piglets, including low birth weight and inadequate provision of milk (Boyd et al., 1995; Wolter et al., 2002).

Insufficient intake of dietary amino acids limits milk production by lactating sows. For example, increasing the content of crude protein (23% vs 16.2%) and all amino acids, including leucine plus isoleucine plus valine (e.g., 4.61% vs 2.28%), in the diet of lactating sows enhances milk yield and piglet growth (Dunshea et al, 2005). It is unknown whether this effect is due to specific amino acids (e.g., BCAA) or simply total amino-acid nitrogen. Results from several studies indicate that dietary supplementation with valine (Richert et al. 1996, 1997a; Moser et al. 2000; Paulicks et al. 2003) or isoleucine (Richert et al. 1997b), but not leucine (Moser et al. 2000), increase litter weight of suckling piglets. BCAA are major AA components of milk protein (Davis et al., 1994; Wu et al., 1994), and also provide the amino group for synthesis of glutamate, glutamine, aspartate and asparagine all of which are abundant amino acids in milk (Li et al., 2009). To date, dietary supplementation with a single BCAA to sows has yielded inconsistent results on milk production (Richert et al., 1997ab; Moser et al., 2000) possibly due to a dietary imbalance of the three BCAA (Wu et al., 2014). For example, dietary supplementation of lactating sows with 0.4% isoleucine enhanced growth of suckling piglets when the ratio of isoleucine to leucine in the complete diet was 0.88 (Richert et al., 1997a), but isoleucine failed to exert a beneficial effect when the ratio of isoleucine to leucine in the complete diet was 0.55 (Moser et al., 2000). Little is known

about effects of dietary supplementation with leucine plus isoleucine plus valine on lactation performance in any mammal, including sows.

L-Leucine activates the mechanistic target of rapamycin (mTOR) cell signaling pathway to stimulate protein synthesis in skeletal muscle (Dennis et al., 2011; Suryawan and Davis, 2011; Suryawan et al., 2012) and mammary epithelial cells (Lei et al., 2012b). We hypothesized that BCAA would increase milk synthesis in mammary tissue of lactating sows, thereby improving neonatal growth, development and survival. The present study tested this hypothesis by determining effects of BCAA supplementation to lactating sows on: 1) growth performance of neonates; 2) yield, composition and nutrient composition of milk; 3) biochemical metabolites in milk as well as plasma from sows and piglets. To prevent an imbalance among BCAA, we supplemented the diet of lactating sows with a mixture of L-leucine, L-isoleucine and L-valine in proportion to their physiological ratios in the lumen of their small intestine.

MATERIALS AND METHODS

Animals housing and management for feeding trials

The experimental protocol for this study was approved by the Texas A&M University Institutional Animal Care and Use Committee. Thirty multiparous sows (Yorkshire × Landrace; parity = 2 and 3) were housed individually in 1.8 × 2.5 m pens with plastic coated perforated flooring. During the entire gestation, they were fed 2 kg/d a corn- and soybean meal-based diet containing 12.0% crude protein and 12.9 MJ/kg metabolizable energy (Li et al., 2010). Immediately after farrowing, the sows were assigned randomly to one of three groups, and fed a corn- and soybean meal-based diet

(Table A-3) supplemented with 0% (control), 1.535% (0.32% L-isoleucine plus 0.82% L-leucine plus 0.395% L-valine), or 3.07% BCAA (0.64% L-isoleucine plus 1.64% L-leucine plus 0.79% L-valine). Diets were provided to sows twice a day (3 kg/meal) and they had free access to their respective diets. These supplemental doses of BCAA were based on the following rationale. First, the ratios of isoleucine:leucine:valine in the lumen of the small intestine of lactating sows were determined to be 1.00:2.56:1.23 (R. Rezaei and G. Wu, unpublished results). Second, the content of leucine in the basal diet was 1.64%. Thus, the supplemental doses of 0.82% and 1.64% leucine amounted to 50% and 100% of the leucine content in the basal diet. There were 10 sows per group. Diets were made isonitrogenous and isoenergetic by addition of appropriate amounts of L-alanine at the expense of corn starch (Table A-4). Supplemental AA were obtained from Ajinomoto Co., Inc. (Tokyo, Japan). Before feeding sows, complete diets were mixed thoroughly using a Hobart feed mixer. On the day of farrowing (d = 0), cross fostering of newborn pigs was performed to minimize within-litter variation, and the number of piglets per sow was standardized to nine, yielding a total of 270 piglets for this experiment. The number of male and female piglets per treatment group was the same. The ambient temperature in the farrowing facility was maintained at 26°C throughout the trial. Heat lamps were used to provide heat to neonatal pigs in each pen. Lactating sows had free access to drinking water and their respective diets. Feed intake of the sows was recorded daily. Teeth of piglets were clipped and their tails docked at d 3 of age. At this age, each piglet was also ear notched for identification and given an intramuscular iron dextran injection (200 mg per piglet). On d 3, 15 and 29 of lactation, all sows and

piglets were weighed. During the entire experiment, piglets had no access to the feed provided to sows and were not provided with any creep feed. The growth performance and survival rates of piglets were determined.

Collection of blood samples from sows and piglets

Heparinized tubes were used to draw blood from sows and piglets on d 3, 15, and 29 of lactation. Specifically, blood samples (10 ml) were obtained from all sows via the caudal auricular vein in the ear 2h post-feeding, and blood samples (2 ml) were obtained via the jugular vein 1 h after nursing from two randomly selected piglets in each litter. Blood samples were immediately centrifuged at 10,000 x g for 1 min, and the supernatant fluid (plasma) stored at -80°C for subsequent analyses.

Collection of milk from sows

To obtain milk samples, 20 IU oxytocin was administered via the ear vein of sows to induce milk letdown into the teat canal and milk was expressed manually (Wu and Knabe, 1994). Milk samples (50 ml/gland) were collected from three different mammary glands (front, middle, and rear). Equal volumes of milk (10 ml) from each gland of a sow were mixed, and the samples stored at -80° C for subsequent analyses.

Milk consumption of piglets

Milk consumption of piglets was determined using the weigh-suckle-weigh method as previously described (Kim and Wu, 2004). Briefly, on d 3, 15, and 29 of lactation, after blood sampling, piglets were separated from their mothers for 1.5 h and then returned to the sows for nursing. Piglets were weighed before and after nursing to calculate milk intake. This procedure was repeated four times.

Analysis of milk composition

The frozen whole-milk samples were thawed at 4°C, and then used for determination of total milk fat, lactose, crude protein, DM and ash according to AOAC (Association of Official Analytical Chemists, Washington DC 1990) procedures. Additionally, protein was determined by the modified Lowry procedure using purified porcine IgG as the standard (Wu and Knabe, 1994). Milk lactose was determined using the colorimetric method of Marier and Boulet (1959) that involved the reaction with phenol and sulfuric acid and then reading against the blank at 490 nm. Total milk lipids were extracted using a mixture of isopropylalcohol:heptane:1N H_2SO_4 (Dole, 1956).

Analysis of free amino acids in plasma

Plasma samples (0.5 mL) were deproteinized with an equal volume of 1.5 M $HClO_4$ and neutralized with 0.25 mL of 2 M K_2CO_3. The neutralized samples were analyzed for free AA by HPLC methods involving precolumn derivatization with o-phthal-dialdehyde (Wu et al., 1997).

Analysis of free and protein-bound amino acids in milk

For analysis of free AA, milk samples (0.5 mL) were deproteinized with an equal volume of 1.5 M $HClO_4$ and neutralized with 0.25 mL of 2 M K_2CO_3. The neutralized samples were analyzed for free AA, as described above. Composition of protein-bound AA in milk was determined as described by Dai et al. (2014).

Analysis of glucose, ammonia, urea, lactate, pyruvate and ketone bodies in plasma and milk

Deproteinized and neutralized plasma or milk samples were analyzed for

glucose, ammonia, urea, lactate, pyruvate, and ketone bodies by enzymatic methods (Tekwe et al., 2013). The enzymes were hexokinase,glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, urease, lactate dehydrogenase, and β-hydroxybutyrate dehydrogenase for the assays of glucose, ammonia plus urea, lactate plus pyruvate, acetoacetate plus β-hydroxybutyrate, respectively. We also measured cholesterol, triglycerides, free fatty acids and glycerol in sows and piglet's plasma and milk colorimetrically according to manufacturer's manual.

Determination of plasma thiobarbituric acid reactive substances (TBARS)

To evaluate the oxidative status of sows and piglets, their plasma was analyzed for TBARS. Thiobarbituric acid forms a reaction with malondialdehyde (a naturally occurring product of lipid peroxidation) under high temperature and acidic conditions and can be measured colorimetrically. The technical procedure was performed as recommended by the manufacturer (Cayman Chemical, Ann Arbor MI). Briefly, 100 µL of thawed plasma was placed into a labeled glass tube and mixed with the reagents of the commercial kit (Cayman Chemical, Cat # 10009055). Each tube was capped and incubated at 95°C for 60 min. The tubes were then cooled in an ice bath for 10 min. Thereafter, the tubes were centrifuged at 1,100 × g for 15 min and the supernatant fluid was obtained for the measurement absorbance at 530-540 nm (Draper et al., 1993).

STATISTICAL ANALYSIS

Results are expressed as means ± pooled SEM. Data were analyzed statistically using two-way ANOVA software available online at https://housseinassaad.shinyapps.io/TwoWayANOVA/. Data on rates of piglet mortality

were analyzed by X^2-analysis. Values of $P \leq 0.05$ indicated statistical significance. All statistical analyses were performed by using the 64-bit version of the R 3.1.0 software on Linux.

RESULTS

Feed intake and body weights of lactating sows

There were no differences in feed intake by sows among groups which was approximately 32 g/kg BW per day throughout the 29-d period of lactation (Table A-6). With advanced lactation, sows mobilized body reserves to provide nutrients for milk production. On the day of farrowing, body weights of sows did not differ among the three treatment groups (Table A-7). However, on d 29 of lactation, sows in the 3.07% BCAA group were heavier ($P < 0.05$) than those in the control and 1.535% BCAA groups (Table A-7). Overall, sows in the control and 1.535% BCAA groups lost ($P < 0.05$) 26 and 16 kg body weight between d 0 and 29 of lactation, respectively, whereas there was no difference in the body weights of sows in the 3.07% BCAA group during the same period (Table A-8). Most (approximately 60%) of the weight losses in sows occurred during the fourth week of lactation.

Milk intake, growth and mortality of sow-reared piglets

Milk intake of piglets was 16.5% and 20.5% higher in the 1.535% and 3.07% BCAA groups, respectively, compared with control piglets (Table A-6). Piglet BW at d 0 of lactation did not differ among treatment groups (Table A-7). However, the BW of piglets nursed by sows fed the BCAA-supplemented diets during lactation were greater ($P < 0.01$) at d 15 and 29 of lactation, compared with piglets from control-fed sows.

Average daily weight gains of piglets from sows fed the BCAA-supplemented diets during lactation were consistently greater ($P < 0.01$) throughout the 29-d lactation compared with piglets from control-fed sows (Table A-8). Piglets in the 3.07% BCAA group had a greater weight gain ($P < 0.01$) than piglets in the 1.535% BCAA group. Of note, rates of preweaning mortality of piglets in the 1.535% and 3.07% BCAA groups were 50% and 33% ($P < 0.05$) of the value for the control group, respectively (Table A-9).

Milk composition

Concentrations of DM, CP, fat and carbohydrate were higher ($P < 0.01$) in milk from BCAA-supplemented sows as compared to control sows (Table A-10). Lactose accounted for 89.3% to 89.8% of total carbohydrate in each group. Composition of milk did not differ between the 1.535% and 3.07% groups. Concentrations of DM, fat and CP in milk were higher on d 3 than on d 15 or d 29 of lactation ($P < 0.05$), but did not differ between d 15 and 29 of lactation. There was no treatment × day interaction effect on milk composition (Table A-10).

Concentrations of AA in the plasma of piglets and sows

Concentrations of AA in plasma of sows and piglets at d 3, 15 and 29 of lactation are summarized in Tables A-11 and A-12, respectively. BCAA supplementation to sows resulted in greater ($P < 0.001$) concentrations of Pro, BCAA, citrulline, Arg, Asn, Glu and Gln in plasma, compared with values for sows in the control group. Except for Asn, b-Ala and Lys, concentrations of all other AA in the plasma from sows were greater ($P < 0.05$) for the 3.07% BCAA group than the 1.535% BCAA group (Table A-11). The

effect of day of lactation was significant for all AA in plasma from sows except for Cys, Glu, Gln, Gly, Lys, Met, Pro and Tau. However, no treatment × day effect was detected for any AA except Asn in plasma of sows. Similar results were obtained for concentrations of AA in plasma from piglets (Table A-12).

Concentrations of protein-bound and free AA in milk

Concentrations of most free AA, including Ala, Arg, Asp, Asn, Cit, Glu, Gln, BCAA and Pro, were greater ($P < 0.05$) in milk from BCAA-supplemented, as compared with the control sows (Table A-13). Except for Ala and Trp, the abundance of all free AA was greatest in milk from sows in the 3.07% BCAA group (Table A-13). There was a significant effect of day on concentrations of all free AA in milk, but a treatment × day interaction effect was detected only for Ser (Table A-13). Concentrations of all protein-bound AA were higher ($P < 0.01$) in milk of BCAA-supplemented sows, compared with control sows. Concentrations of all protein-bound AA were affected by day of lactation. There was no treatment × Day effect for any AA in milk protein (Table A-14).

Metabolites in the plasma of sows and piglets and in milk

Effects of BCAA supplementation on concentrations of ammonia, urea, glucose, lactate and pyruvate in the plasma of sows and piglets and in sow's milk are summarized in Table A-15. Concentrations of glucose were higher in plasma from BCAA-supplemented sows ($P=0.018$) and piglets nursed by those sows ($P < 0.001$) than sows and piglets in the control group. No differences in concentrations of glucose in milk were detected among the three groups of sows. BCAA supplementation did not affect

concentrations of ammonia, urea, lactate or pyruvate in the plasma of sows or piglets or in sow's milk. Concentrations of lactate in plasma and milk from sows increased as lactation advanced, but there were no changes in other measured metabolites. No treatment × day of lactation interaction had an effect on concentrations of ammonia, urea, lactate or pyruvate in plasma from sows and piglets or in milk from d 3, 15 or 29 of lactation. Acetoacetate and β-hydroxybutyrate were not detected in plasma of sows and piglets or in milk on the same days of lactation (detection limit = 25 μM). Concentrations of lipids in plasma and milk are summarized in Table A-16.

Plasma TBARS concentrations in sows and piglets

Concentrations TBARS in the plasma of sows increased ($P < 0.001$) as lactation progressed, but the opposite changes were detected in plasma from piglets (Table A-17). BCAA supplementation reduced ($P= 0.004$) the concentrations of TBARS in plasma from sows compared with control sows (Table A-17). Concentrations of TBARS in plasma from piglets did not differ among the three treatment groups.

DISCUSSION

The mammary gland undergoes metabolic and histological changes during lactation (Kensinger et al., 1982; Shennan and Peaker, 2000). During lactation, sows are capable of utilizing nutrients from the arterial circulation for milk production (Guan et al., 2004; Kim et al., 2000). However, due to reduced feed intake of lactating sows, they cannot meet the nutritional requirements for milk production, thereby leading to a catabolic state in which their body reserves are mobilized to provide precursors and energy for milk production (Boyd et al., 1995). This results in substantial weight loss for

lactating sows, which can negatively affect their subsequent reproductive performance (Hughes and Varley, 1980). Results of the present study demonstrate that supplementing 1.535% and 3.07% BCAA to the typical corn- and soybean meal-based diet of multiparous sows during the entire 29d lactation period reduced their weight loss, while increasing milk yield as well as concentrations of total AA, lipids and lactose in milk, and improved piglet growth performance. Therefore, findings of the present study provide a new and effective strategy for nutritional management of lactating sows. To our knowledge, this is the first study demonstrating effects of dietary supplementation with a mixture of BCAA (without increasing dietary protein levels) on lactational performance of lactating sows and associated effects on growth and survival of piglets.

Lactating mammals extensively degrade BCAA (Wholt et al., 1977; Viña and Williamson, 1981); therefore, the requirements for dietary BCAA by lactating sows to support milk synthesis are great (Wu, 2014). There are reports that supplementing L-valine to the diets of lactating sows enhances milk yield and piglet growth. For example, Richert et al. (1997a) found that litter weaning weight and litter weight gain in high-producing lactating sows increased as total content of valine and isoleucine in the diet increased from 0.72% to 1.42% and from 0.50% to 1.2%, respectively. Also, they showed that increasing total valine content from 0.61% to 1.15% in the diet for lactating sows nursing 10 or more piglets increased litter preweaning weights (Richert et al., 1997b). This beneficial effect of dietary valine (an increase from 0.75% to 1.15% valine) on lactation was confirmed by Moser et al. (2000). Furthermore, Paulicks et al. (2003) observed that supplementing up to 1% valine to a basal diet containing 0.45% valine for

lactating sows increased milk production and total milk protein content, in comparison with the control group. Of note, supplementing either 0.4% leucine to a basal diet containing 1.57% leucine or 0.4% isoleucine to a basal diet containing 0.68% isoleucine for lactating sows did not affect milk production or piglet growth (Moser et al., 2000). In contrast, duodenal infusion of leucine to lactating cows increased concentrations of casein, whey proteins, and total proteins in milk (Rulquin and Pisulewski, 2006). Ratios of leucine, isoleucine and valine in the complete diet may affect lactational performance. To prevent a BCAA imbalance resulting from the addition of one single BCAA to the ration, dietary supplementation with all three BCAA provides a more effective strategy to improve milk production in sows and perhaps other mammals.

Voluntary feed intake of sows was not affected by dietary supplementation with up to 3.07% BCAA (Table A-6). As reported previously (Kim et al., 2009), the body weight of sows is markedly reduced with advanced lactation (Table A-7). Interestingly, dietary supplementation with 1.535% BCAA and 3.07% BCAA reduced weight loss of sows in a dose-dependent manner (Table A-8) which may be attributed to effects of BCAA (mainly leucine) to stimulate muscle protein synthesis and inhibit muscle proteolysis during catabolic conditions (Donato et al., 2007; Biolo et al., 1997) and those effects may enhance subsequent reproductive performance. In the present study, we did not measure the component of weight loss in lactating sows, but the loss likely occurred primarily in white adipose tissue and secondarily in skeletal muscle (Kim et al., 2009). It is likely that by activating the MTOR cell signaling pathway, BCAA supplementation improves the efficiency of utilization of dietary nutrients for milk production.

Approximately 40% to 45% of dietary BCAA are utilized by the small intestine of sows in the first pass (Wu et al., 2014). Because degradation of BCAA in the liver is limited due to its low BCAA transaminase activity, most of the BCAA that enters the portal circulation bypasses this organ to become available for utilization by other tissues, including skeletal muscle and mammary glands (Tover et al., 2001). Skeletal muscle of lactating mammals actively transports extracellular BCAA and converts them into BCKA and glutamate (Harper et al., 1984). Glutamate is subsequently amidated to form glutamine and undergoes transamination with pyruvate and oxaloacetate to yield alanine and aspartate, respectively (Li et al., 2009; Yin et al., 2010). This explains why dietary BCAA supplementation increased concentrations of glutamate, glutamine, alanine and aspartate in the plasma of lactating sows. Utilization of arterial glutamine by the small intestine of lactating sows is expected to promote intestinal synthesis of citrulline (the precursor of arginine), thereby contributing to an increase in the concentrations of both citrulline and arginine in maternal blood (Table A-11).

Besides skeletal muscle and small intestine, the mammary gland of sows takes up and catabolizes large amounts of extracellular BCAA (Li et al., 2009; Manjarin et al., 2012). For example, during lactation, 76-80 g/d BCAA are utilized by the mammary gland, with 46-53 g/d BCAA being secreted in milk (Trottier et al., 1997; Nielsen et al., 2002). Thus, approximately 30 g/d BCAA is metabolized in the mammary gland. We reported that BCAA are nitrogenous precursors for synthesis of glutamate, glutamine, aspartate, and alanine in the lactating gland, which contains BCAA transaminase, BCKA dehydrogenase, glutamine synthetase, glutamate-oxaloacetate aminotransferase,

glutamate-pyruvate aminotransferase, and asparagine synthetase (Li et al., 2009; Conway and Hutson, 2000). Similarly, Matsumoto et al. (2013) found that dietary supplementation with leucine increased concentrations of glutamate and glutamine in milk of rats. The BCKA produced can be either oxidized to provide ATP or utilized for fat synthesis. Of note, dietary supplementation with 2 g/d β-hydroxy-β-methyl butyrate (a leucine metabolite) enhances fat content in colostrum by 41% and body weight of suckling pigs by 7% on d 21 of lactation (Nissen et al., 1994). Furthermore, BCAA stimulate protein synthesis in mammary epithelial cells through the mTOR pathway (Lei et al., 2012b). The results of the present study advance understanding of these new and important findings that dietary BCAA supplementation increases concentrations of free and peptide-bound glutamate, glutamine, aspartate, and alanine, as well as concentrations of protein and fat in sow's milk.

As noted previously, inadequate provision of dietary protein and some amino acids (e.g., arginine, glutamine, glutamate, glycine, leucine, and proline) limits maximal growth of young pigs (Manjarin et al., 2014; Wang et al. 2014ab; Wu et al., 2014). An increase in the provision of these amino acids from sow's milk enhances their concentrations in plasma of suckling piglets, thereby improving growth of suckling piglets. The underlying mechanisms may involve the supply of substrates for protein synthesis as well as activation of the mTOR cell signaling pathway to increase protein synthesis and inhibit protein degradation (Columbus et al., 2014; Suryawan and Davis, 2014). In support of this view, arginine and glycine are known to be severely deficient in sow’s milk relative to their requirements for piglet growth (Davis et al., 1994; Wang et

al., 2013; Wu and Knabe, 1994). Additionally, approximately 70% and 95% of glutamine and glutamate in sow's milk are utilized via catabolic and synthetic pathways in the small intestine of piglets and, therefore, do not enter the portal circulation (Reeds and Burrin, 2001; Stoll et al., 1999; Wu et al., 2011). Thus, endogenous synthesis of these two amino acids, which are highly abundant in tissue protein, is necessary for piglet growth (Rezaei et al., 2013; Wu et al., 2014). Furthermore, arginine, glutamine, glycine, and leucine stimulate the phosphorylation of mTOR, S6K1, and 4EBP1 proteins in porcine tissues, including mammary tissue (Appuhamy et al., 2012), skeletal muscle (Escobar et al., 2006; Suryawan and Davis, 2011; Yao et al., 2008), and the small intestine (Wang et al., 2014b; Xi et al., 2011). Taken together, these functional amino acids are expected to play an important role in improving the growth of neonatal pigs and the efficiency of milk utilization (Wu, 2013).

In summary, supplementing a mixture of BCAA to the diet for lactating sows increases concentrations of amino acids in plasma and milk of sows, and plasma of piglets, while reducing oxidative stress in both sows and piglets. BCAA supplementation does not affect feed intake by sows or milk consumption by piglets (per kilogram body weight), but enhances the growth of suckling piglets likely through stimulating tissue protein synthesis. Dietary supplementation with appropriate proportions of leucine, isoleucine and valine can prevent an imbalance among BCAA in the diet and provide an effective means to improve milk synthesis by lactating sows and, in turn, growth and survival of piglets.

CHAPTER III

DIETARY SUPPLEMENTATION WITH MONOSODIUM GLUTAMATE ENHANCES MILK PRODUCTION BY LACTATING SOWS AND GROWTH OF SUCKLING PIGLETS

SYNOPSIS

L-Glutamate is a predominant product of extensive catabolism of branched-chain amino acids (BCAA) in the lactating mammary gland and a major nutrient in the milk of all mammals. Results provided in Chapter II indicate that dietary supplementation with BCAA enhances glutamate provision and lactational performance in sows. Although glutamate has been traditionally regarded to be a nutritionally nonessential amino acid for mammals, emerging evidence shows that a typical corn- and soybean meal-based diet may not provide sufficient glutamate to support maximum protein synthesis in growing swine. Therefore, the present study was conducted to test the hypothesis that increasing dietary content of glutamate through addition of monosodium glutamate (MSG) enhances milk production by lactating sows and the growth of their offspring. Thirty multiparous sows (Landrace x Large White) were assigned randomly into one of three dietary groups: 1) control (a corn- and soybean meal-based diet), 2) basal diet + 1 % MSG; and 3) basal diet + 2 % MSG. Diets were made isonitrogenous by addition of appropriate amounts of L-alanine. Lactating sows had free access to drinking water and their respective diets. The number of live-born piglets was normalized to 9 per sow at d 0 of lactation (the day of farrowing). On d 3, 15 and 29 of lactation, body weight and

milk consumption of piglets were measured, and blood samples obtained from sows and piglets at 2h and 1h after feeding, respectively. Data were analyzed by 2-way ANOVA. Feed intake of sows did not differ among the three groups of sows (P > 0.05). Concentrations of aspartate, glutamine, citrulline, arginine, and glutamate were higher (P < 0.05) in plasma of MSG-supplemented sows and their piglets than for controls. Compared with the control, dietary supplementation with 1% and 2 % MSG increased (P < 0.05): 1) concentrations of free and protein bound glutamate plus glutamine in milk (P < 0.01); 2) milk intake of piglets by 14-25% (P < 0.001); and 3) daily weight gains of piglets by 23% and 44%, respectively. These results indicate that dietary supplementation with up to 2% MSG enhances milk production to support growth of piglets nursing lactating sows.

INTRODUCTION

Amino acids are not only building blocks for protein, but are also key regulators of metabolic pathways that are vital for whole body homeostasis (Jobgen et al., 2006; Wu and Meininger, 2000) and lactogenesis (Bequette et al., 1998). In primiparous sows, limited food intake and inadequate blood flow to lactating mammary glands result in reduced provision of nutrients (including AA) to the mammary gland for synthesis of components of milk (Kim et al., 1999; Kim and Wu, 2009). Sow's milk contains high concentrations of free and peptide-bound glutamine plus glutamate (Wu et al., 2011) which are crucial for growth, development, and normal function of the small intestine of neonates (Kim and Wu, 2009). On d 10 of lactation, the lactating porcine mammary gland takes up 16 g/d glutamine from the arterial circulation (Trottier et al., 1997), but

produces 36 g/d glutamine in milk (Haynes et al., 2009). Therefore, extraction of glutamine by the lactating mammary gland is much less than its output in milk and a large amount of glutamine (20 g/d) must be synthesized by mammary tissue to support the production of milk proteins (Li et al., 2009). Mammary tissue does not contain pyrroline-5-carboxylate dehydrogenase or proline oxidase activity; therefore, it cannot convert arginine, ornithine, or proline into glutamate or glutamine (O'Quinn et al., 2002). Instead, BCAA are utilized for glutamate and glutamine synthesis in the mammary gland through BCAA transaminase and glutamine synthetase (Li et al., 2009). The de novo synthesis of glutamate and glutamine helps to explain the high abundance of these two amino acids in both free and peptide-bound forms in milk (Wu and Knabe, 1994).

Sow's milk is thought to provide adequate amino acids needed for the growth of neonatal pigs. However, it has been shown that the amount of milk produced by sows during lactation does not provide adequate amounts of all amino acids for maximal growth of piglets (Rezaei et al., 2011). For example, Hodge (1974) and Boyd et al. (1995) demonstrated that artificially reared neonatal pigs grow at a rate that is at least 50% greater than that of sow-reared piglets. Beginning at 8 d of age, piglets exhibit sub-maximal growth, which may have resulted from inadequate intake of protein or energy from sow's milk (Boyd et al., 1995). Whether the suboptimal growth of piglets results from inadequate provision of all or some individual amino acids in diet remain unknown. However, increasing the provision of L-arginine (Wu et al., 2004), glycine (Wang et al., 2014), L-glutamine (Haynes et al., 2009) and branched-chain amino acids

(BCAA; see Chapter II) in sow's milk enhances growth of the piglets. L-Glutamate is a major metabolic fuel, a substrate for synthesis of citrulline and arginine, and an inhibitor of glutamine degradation in the small intestine (Boutry et al., 2011; Reeds and Burrin, 2001; Wu, 2009). At present, little is known about effects of glutamate on milk production by any lactating mammal. Although glutamate has been traditionally regarded as a nutritionally nonessential amino acid for mammals, emerging evidence shows that a typical corn- and soybean meal-based diet may not provide sufficient glutamate to support maximum protein synthesis in growing swine (Rezaei et al., 2013). The current study was conducted to test the hypothesis that increasing dietary provision of glutamate through addition of monosodium glutamate (MSG) enhances milk production by lactating sows and the growth of their suckling piglets.

MATERIALS AND METHODS

Animals housing and management for feeding trials

The experimental protocol for this study was approved by the Texas A&M University Institutional Animal Care and Use Committee. Thirty multiparous sows (Yorkshire × Landrace; parity = 2 and 3) were housed individually in 1.8 × 2.5 m pens with plastic coated perforated flooring. During the entire gestation, they were fed 2 kg/d a corn- and soybean meal-based diet containing 12.0% crude protein and 12.9 MJ/kg metabolizable energy (Li et al., 2010). Immediately after farrowing, the sows were assigned randomly to be fed a corn- and soybean meal-based diet (Table A-3) supplemented with either 0% (control), 1%, or 2% MSG. Diets were provided to sows twice a day (3 kg/meal) and they had free access to their respective diets. There were 10

sows per group. Diets were made isonitrogenous and isocaloric by addition of appropriate amounts of L-alanine at the expense of cornstarch (Table A-18). The content of sodium in the three diets was made the same through the addition of appropriate amounts of NaCl (Table A-18). Supplemental AA were obtained from Ajinomoto Co., Inc. (Tokyo, Japan). Before being fed to sows, complete diets were mixed thoroughly using a Hobart mixer. On the day of farrowing (d = 0), cross fostering of newborn pigs was performed to minimize within-litter variation, and the number of piglets per sow was standardized to nine, yielding a total of 270 piglets for this experiment. The number of male and female piglets per treatment group was the same. The ambient temperature in the farrowing facility was maintained at 26°C throughout the trial. Heat lamps were used to provide warmth for neonatal pigs in each pen. Lactating sows had free access to drinking water and their respective diets. Feed intake of the sows was recorded daily. Teeth of piglets were clipped and their tails docked at d 3 of age. At this age, each piglet was also ear notched for identification and received an intramuscular iron dextran injection (200 mg per piglet). On d 3, 15 and 29 of lactation, all sows and piglets were weighed. During the entire experiment, piglets had no access to the feed provided to sows and piglets were not provided with any creep feed. The growth performance and survival rates of piglets were determined.

Collection of blood samples from sows and piglets

Heparinized tubes were used to draw blood from sows and piglets on d 3, 15, and 29 of lactation. Specifically, blood samples (10 ml) were obtained from all sows via the

caudal auricular vein in the ear 2h post-feeding, and blood samples (2 ml) were obtained from the jugular vein of two randomly selected piglets in each litter 1 h after nursing. Blood samples were immediately centrifuged at 10,000 x g for 1 min, and the supernatant fluid (plasma) was stored at -80°C for subsequent analyses.

Collection of milk from sows

To obtain milk samples, 20 IU oxytocin was administered via the ear vein of sows to induce letdown of milk into the teat canal from which it was expressed manually (Wu and Knabe, 1994). Milk samples (50 ml/gland) were collected from three different mammary glands (front, middle, and rear). Equal volumes of milk (10 ml) from each gland of a sow were mixed, and thereafter the samples were stored at -80°C for subsequent analyses.

Milk consumption of piglets

Milk consumption of piglets was determined using the weigh-suckle-weigh method as previously described (Kim and Wu, 2004). Briefly, on d 3, 15, and 29 of lactation, after blood sampling, piglets were separated from their mothers for 1.5 h and then returned to the sows for nursing. Piglets were weighed before and after nursing to calculate their milk intake and this procedure was repeated four times.

Analysis of milk composition

The frozen whole-milk samples were thawed at 4°C, and then used for determination of total milk fat, lactose, crude protein, DM and ash according to AOAC (1990) procedures. Additionally, protein was determined by the modified Lowry procedure using purified porcine IgG as the standard (Wu and Knabe, 1994). Milk

lactose was determined by the colorimetric method of Marier and Boulet (1959) that involved the reaction with phenol and sulfuric acid and then reading against the blank at 490 nm. Total milk lipids were extracted by using a mixture of isopropylalcohol:heptane:1N H_2SO_4 (Dole, 1956).

Analysis of free amino acids in plasma

Plasma samples (0.5 mL) were deproteinized with an equal volume of 1.5 M $HClO_4$ and neutralized with 0.25 mL of 2 M K_2CO_3. The neutralized samples were analyzed for free AA by HPLC methods involving precolumn derivatization with o-phthal-dialdehyde (Wu et al., 1997).

Analysis of free and protein-bound amino acids in milk

For analysis of free AA, milk samples (0.5 mL) were deproteinized with an equal volume of 1.5 M $HClO_4$ and neutralized with 0.25 mL of 2 M K_2CO_3. The neutralized samples were analyzed for free AA, as described above. Composition of protein-bound AA in milk was determined as described by Dai et al. (2014).

Analysis of glucose, ammonia, urea, lactate, pyruvate and ketone bodies in plasma and milk

Deproteinized and neutralized plasma or milk samples were analyzed for glucose, ammonia, urea, lactate, pyruvate, and ketone bodies by enzymatic methods (Tekwe et al., 2013). The enzymes hexokinase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, urease, lactate dehydrogenase, and β-hydroxybutyrate dehydrogenase were used for assays of glucose, ammonia plus urea, lactate plus pyruvate, and acetoacetate plus β-hydroxybutyrate, respectively. We also measured

cholesterol, triglycerides, free fatty acids and glycerol in sows and piglet's plasma and milk colorimetrically according to manufacturer's manual.

Determination of plasma thiobarbituric acid reactive substances (TBARS)

To evaluate the oxidative status of sows and piglets, their plasma was analyzed for TBARS. Thiobarbituric acid forms a reaction with malondialdehyde (a naturally occurring product of lipid peroxidation) under high temperature and acidic conditions and can be measured colorimetrically. The technical procedure was performed as recommended by the manufacturer (Cayman Chemical, Ann Arbor MI). Briefly, 100 µL of thawed plasma was placed into a labeled glass tube and mixed with the reagents of the commercial kit (Cayman Chemical, Cat # 10009055). Each tube was capped and incubated at 95°C for 60 min. The tubes were then cooled in an ice bath for 10 min. Thereafter, the tubes were centrifuged at 1,100 × g for 15 min and the supernatant fluid was obtained for the measurement of absorbance at 530-540 nm (Draper et al., 1993).

STATISTICAL ANALYSIS

Results are expressed as means ± pooled SEM. Data were statistically analyzed using two-way ANOVA software available online at https://housseinassaad.shinyapps.io/TwoWayANOVA/. Data on rates of piglet mortality were analyzed by X^2-analysis. Values of $P \leq 0.05$ are taken to indicate statistical significance. All statistical analyses were performed using the 64-bit version of the R 3.1.0 software on Linux.

RESULTS

Feed intake and body weights of lactating sows

There was no difference in feed intake by sows among groups which was approximately 32 g/kg BW per day throughout the 29 d period of lactation (Table A-20). With advanced lactation, sows mobilized body reserves to provide nutrients for milk production. On the day of farrowing, body weights of sows did not differ among the three treatment groups (Table A-21). However, on d 29 of lactation, sows in the 1% and 2% MSG groups were heavier ($P < 0.05$) than those in the control group (Table A-21). Overall, sows in the control, 1% MSG, and 2% MSG groups lost ($P < 0.05$) 21.3, 17.3 and 13.4 kg between d 0 and d 29 of lactation, respectively (Table A-22). Most (approximately 60%) of the weight losses in sows occurred in the fourth week of lactation. The sows used in this study did not exhibit any adverse response to MSG supplementation.

Milk intake, growth and mortality of sow-reared piglets

Milk intake of piglets was 14% and 25% higher in the 1% and 2% MSG groups, respectively, compared with the control sows (Table A-21). Piglet BW at d 0 of lactation did not differ among treatment groups (Table A-22). However, the BW of piglets nursed by sows fed the MSG-supplemented diets were greater ($P < 0.01$) at d 15 and 29 of lactation, compared with piglets from control-fed sows. Average daily weight gains of piglets from sows fed the MSG-supplemented diet were greater ($P < 0.01$) throughout the 29 d lactation, compared with piglets from control-fed sows (Table A-22). Piglets in the 1% and 2% MSG groups had 27% and 42% greater weight gains ($P < 0.01$) than

piglets in the control group. Of note, rates of preweaning mortality for piglets in 1% and 2% MSG groups were 4.4% and 3.3%, respectively, which were lower ($P < 0.05$) than the 16.7% preweaning mortality for piglets nursed by control sows (Table A-23).

Milk composition

Concentrations of DM, CP, fat and carbohydrate were higher ($P < 0.01$) in milk from MSG-supplemented sows, as compared to control sows (Table A-24). Lactose accounted for 89.3% to 89.8% of total carbohydrate in each treatment group. Composition of milk did not differ among treatment groups. Concentrations of DM, fat and CP in milk were higher on d 3 than on d 15 or d 29 of lactation ($P < 0.05$), but did not differ between d 15 and 29 of lactation. There was no treatment x day interaction effect for milk composition (Table A-24).

Concentrations of free AA in the plasma of sows and piglets

Concentrations of AA in plasma of sows and piglets at d 3, 15 and 29 of lactation are summarized in Tables 8 and 9, respectively. MSG supplementation to sows during lactation increased ($P < 0.001$) concentrations of BCAA, Pro, Arg, Asn, Glu and Gln, in plasma compared with values for control sows. Concentrations of Cys, Gly, His, and Lys in maternal plasma was not affected by treatment (Table A-25). The effect of day of lactation was significant ($P < 0.05$) for all AA in plasma of sows except for Cys, Glu, Gln, Gly, Lys, Met, Pro and Tau. Similar results were observed for concentrations of free AA in plasma from control and MSG-supplemented piglets, except that significant treatment x day effects were detected for beta-Ala, Tau, and Trp (Table A-26).

Concentrations of protein-bound and free AA in milk

Concentrations of many free AA in sow's milk, including Ala, Arg, Asp, Asn, Cit, Glu, Gln, BCAA and Pro, were higher ($P < 0.05$) in MSG-supplemented sows, compared with the control group, with values being highest for the 2% MSG group (Table A-27). There was a significant effect of day for all free AA in milk. However, treatment × day interaction effects ($P < 0.05$) were detected only for Gln and Ser (Table A-27). All protein-bound AA, including Arg, Cit, Asp plus Asn, and Glu plus Gln, were higher ($P < 0.05$) in milk of MSG-supplemented as compared to control sows. Concentrations of all protein-bound AA were affected by day of lactation, but there was no treatment × Day effect for any AA (Table A-28).

Metabolites in the plasma of sows and piglets and in milk

Effects of MSG supplementation on concentrations of ammonia, urea, glucose, lactate and pyruvate in the plasma of sows and piglets and in sow's milk are summarized in Table A-29. Concentrations of glucose in plasma of MSG-supplemented sows were higher ($P < 0.05$) than those for control sows. No effect of treatment on concentrations of glucose in sow's milk or plasma of piglets was detected. MSG supplementation did not affect concentrations of ammonia, urea, lactate or pyruvate in the plasma of sows or piglets or in sow's milk. Only concentrations of lactate in plasma and milk of sows increased as lactation advanced. No treatment × day of lactation interactions were detected for concentrations of ammonia, urea, lactate and pyruvate plasma and milk of sows and plasma of piglets on d 3, 15 or 29 of lactation. Acetoacetate and β-hydroxybutyrate were not detected in plasma or milk of sows and plasma of piglets

during lactation (detection limit = 25 μM). Concentrations of lipids in plasma and milk are summarized in Table A-30.

Plasma TBARS concentrations in sows and piglets

Concentrations of TBARS in plasma of sows increased ($P < 0.001$) as lactation progressed, but the opposite changes occurred in plasma from piglets (Table A-31). MSG supplementation reduced ($P < 0.01$) the concentrations of TBARS in plasma from sows compared with control sows (Table A-31). Concentrations of TBARS in plasma from piglets were not affected by treatment or day of lactation.

DISCUSSION

Glutamate is a relatively abundant amino acid in typical corn- and soybean meal-based diets for animals (Li et al., 2011). However, 95% to 97% of glutamate in enteral diets is extensively catabolized in the small intestine to provide energy and synthesize nitrogenous substances with enormous physiological importanceincluding protein, glutathione, arginine and proline. In addition, there is growing evidence that glutamate inhibits the catabolism of glutamine and BCAA in enterocytes (Boutry et al., 2011; Reeds and Burrin, 2001) and possibly some amino acids (including nutritionally essential amino acids) by intestinal bacteria (Dai et al., 2013; Rezaei et al., 2013). Thus, glutamate has multiple functions in regulating intestinal nitrogen metabolism to support integrity of the gut mucosa and spare dietary AA for utilization by extra-intestinal tissues. In support of this view, dietary supplementation with MSG increased concentrations of some AA (including BCAA, glutamine, arginine, proline, and tryptophan) in plasma of lactating sows. Although it had been a long-standing view that

animals have no requirements for dietary glutamate, compelling evidence from this study indicates that a sufficient supply of glutamate in diets is required to maximize lactational performance of sows. Thus, the present work represents a new paradigm shift in understanding of protein nutrition in animals.

Results of this study indicated that supplementing the diet of multiparous sows with up to 2% MSG during the entire 29 d period of lactation increased milk yield and concentrations of total AA and other nutrients in milk which improved growth performance of piglets. It is well known that insufficient milk production by sows limits maximal growth performance of neonatal pigs; however, it was not known whether a lack of all AA or certain AA was responsible for suboptimal milk production by sows fed conventional corn- and soybean meal-based diets. Besides arginine (Wu et al., 2004), glutamine (Haynes et al., 2009), and glycine (Wang et al., 2014), glutamate is the latest addition to the family of synthesizable AA that enhances milk production by sows.

The mammary gland of lactating sows produce glutamate from BCAA and α-ketoglutarate in mitochondria and cytoplasm and subsequently converts glutamate into glutamine by glutamine synthetase in the cytoplasm (Li et al., 2009). In contrast, the lactating gland does not hydrolyze glutamine into glutamate or form glutamate from proline due to the absence of glutaminase or proline oxidase, respectively (O'Quinn et al., 2002). Additionally, formation of glutamate from arginine in mammary tissue is very limited because of low activity of pyrroline-5-carboxylate dehydrogenase (O'Quinn et al., 2002). As a result, nutritionally significant quantities of glutamate in mammary epithelial cells are derived only from maternal blood and de novo synthesis from BCAA.

Because dietary BCAA in typical corn- and soybean meal-based diets are inadequate for maximizing milk production by lactating sows (Chapter II), increasing glutamate in maternal blood is likely an effective strategy to increase the availability of glutamate for protein synthesis in the lactating mammary gland.

Whole colostrum and mature milk from sows contain 13.8 and 5.3 g/L glutamate, respectively (Wu et al., 2011). Given daily milk yields of 4.7 and 7.7 L/sow on d 3 and 15 of lactation, respectively, the lactating sow produces 65 and 41 g glutamate on d 3 and 15 of gestation, respectively. Because accurate data on the extraction of arterial glutamate by the mammary gland of lactating sows are not available, it is unknown whether maternal blood supplies sufficient glutamate for synthesis of milk proteins. Uptake of AA by the lactating gland is positively correlated with their concentrations in the arterial blood (Manjarin et al., 2014). Thus, dietary supplementation with MSG, which increases concentrations of glutamate in maternal plasma increases the availability of glutamate for the synthesis of glutamine, alanine, aspartate, asparagine, and protein in mammary epithelial cells. This helps to explain how dietary supplementation with MSG enhanced concentrations of protein in sow's milk in this study (Tables A-24 and A-28).

Besides protein, concentrations of lactose and fat in sow's milk were increased by dietary supplementation with MSG (Table A-24). Glutamate, glutamine, alanine, aspartate, proline and arginine are substrates for gluconeogenesis in pigs. Increases in the concentrations of those AA in the maternal plasma contributed to the increase in circulating concentrations of glucose in lactating sows (Table A-29), which, in turn,

increased substrate for lactose synthesis by mammary tissue. These AA may also be utilized to synthesize fatty acids by white adipose tissue (Jobgen et al., 2006), and uptake of fatty acids by the lactating gland promotes the formation of triacylglycerides in mammary epithelial cells. Furthermore, an increase in circulating levels of arginine in MSG-supplemented sows is expected to enhance blood flow to the mammary gland via increases in the production of nitric oxide (a major vasodilator), thereby increasing the provision of all nutrients for milk synthesis in mammary epithelial cells (Kim and Wu, 2009).

The weight gain of piglets is positively correlated with increased volume and nutrient composition of milk (Noblet and Etienne, 1989). Results of the present study indicate that piglets reared by MSG-supplemented sows have faster rates of growthand, therefore, heavier final BW at weaning (Tables A-21 and A-22). The increases in milk consumption and growth of piglets indicate greater production of milk by sows. Improved nutritional status of piglets improves their antioxidative capacity (Table A-31) as well as their digestive and immune functions (Wu et al., 2014), thereby reducing the rate of preweaning mortality (Table A-23). Because the voluntary feed intake of sows was not affected by treatment (Table A-20), increased concentrations of free and protein-bound AA in milk likely result from increased efficiency of use of dietary nutrients for protein synthesis by MSG-supplemented sows. Indeed, arginine and glutamine activate the mechanistic target of rapamycin cell signaling pathway to stimulate protein synthesis and inhibit proteolysis in skeletal muscle and other tissues (Wu, 2013). This mechanism

can explain our finding that dietary supplementation with MSG reduced the loss of body weight by lactating sows.

Mateo et al. (2008) reported that dietary supplementation of lactating sows with arginine enhances its concentration in maternal plasma, milk production, and piglet growth. At present, the use of exogenous arginine in diets for lactating sows is limited by its relatively high costs. MSG is a very cheap ingredient relative to arginine and, therefore, is expected to be cost-effective in promoting the growth of sucking piglets. An advantage of the use of MSG over arginine is that MSG increases concentrations of glutamine in maternal plasma and ameliorates loss of body weight, whereas arginine does not (Mateo et al., 2008). It is possible that glutamine and arginine, whose concentrations were increased in plasma of MSG-supplemented sows, have a synergetic effect to improve protein balance in skeletal muscle and reduce fat mobilization in white adipose tissue. The underlying mechanisms remain to be elucidated in future investigations.

In summary, supplementing a typical corn- and soybean meal-based diet with MSG for lactating sows enhances milk synthesis and growth of their suckling piglets by increasing efficiency of milk protein synthesis and reducing oxidative stress. The increased litter weight gain results from increased provision of AA, including glutamine, arginine and poline, fat and lactose from sow's milk. Given a plethora of beneficial effects of glutamate on lactation, MSG can be safely and effectively used to improve lactational performance of sows and, therefore, growth of suckling piglets. Thus, glutamate is truly a functional AA in lactating sows.

CHAPTER IV

BRANCHED-CHAIN AMINO ACIDS REGULATE INTRACELLULAR PROTEIN TURNOVER IN PORCINE MAMMARY EPITHELIAL CELLS

SYNOPSIS

Dietary supplementation with branched-chain amino acids (BCAA; leucine, isoleucine, and valine) enhances lactational performance in sows (Chapter II). This study tested the hypothesis that BCAA stimulate protein synthesis and inhibits protein degradation in mammary epithelial cells. Porcine mammary epithelial cells (PMEC) were cultured at 37°C in Dulbecco's modified Eagle medium containing 5 mM D-glucose, 1 mM phenylalanine, L-[ring-2,4-^{3}H]phenylalanine, 0.1 (control), 0.25, 0.5, 1, or 2 mM L-leucine, L-isoleucine or L-valine or a mixture of the three BCAA. The culture medium also contained physiological concentrations of other amino acids found in plasma of lactating sows. Proliferation, protein synthesis, proteolysis, β-casein production, mechanic target of rapamycin (mTOR) signaling, and the ubiquitin-proteasome pathway were determined for PMEC. Proliferation of cells and abundances of phoshporylated mTOR, 4E-BP1, and S6K1 proteins increased ($P < 0.05$), but the abundances of ubiquitinated protein and proteasome decreased ($P < 0.05$) when extracellular concentrations of leucine, isoleucine, valine, or a mixture of BCAA were increased from 0.1 to 2 mM. Compared with the control, 0.25, 0.5, 1 or 2 mM BCAA enhanced ($P < 0.01$) protein (including β-casein) synthesis, while decreasing ($P < 0.05$)

protein degradation in PMEC in a dose-dependent manner. Collectively, our results indicate that physiological concentrations of BCAA regulate protein turnover in mammary epithelial cells to favor net protein synthesis through stimulating the mTOR signaling pathway and inhibiting the ubiquitin-proteasome pathway. These findings advance basic understanding of molecular mechanisms in epithelial cells of the mammary gland whereby dietary BCAA supplementation enhances milk production by lactating sows.

INTRODUCTION

Lactation is essential for survival, growth and development of mammalian neonates. Milk protein synthesis occurs in mammary epithelial cells and depends on the availability of amino acids (Manjarin et al., 2014; Reynolds et al., 1994). In recent years, branched-chain amino acids (BCAA; L-leucine, L-isoleucine and L-valine) have received much attention as they can regulate cell cycle progression (Xu et al., 1998), cell differentiation (Kimura and Ogihara, 2005; Gonçalves and Gomes-Marcondes, 2010), and protein synthesis in muscle (Kimball et al., 1999; Escobar et al., 2006, 2010). BCAA are actively taken up by mammary epithelial cells (Lei et al., 2013), and activate the mechanic target of rapamycin (mTOR) cell signaling pathway (Suryawan and Davis, 2011). Appuhamy et al. (2012) reported that, compared with Dulbecco's modified Eagle's F12 Ham medium (DMEM-F12) containing 17.5 mM glucose, 0.7 mM arginine, 0.45 mM leucine, 0.42 mM isoleucine, and 0.45 mM valine (~ 3 to 5 times physiological concentrations of glucose and amino acids in the plasma of lactating cows and sows (Lei et al., 2012; Mateo et al., 2008), the omission of leucine or isoleucine reduced mTOR

phosphorylation and protein synthesis in incubated bovine mammary tissue. Similar results were obtained for mouse CID and bovine L-1 cells (mammary epithelial cell lines) cultured in DMEM-F12 containing the same high concentrations of glucose and amino acids vs the DMEM-F12 without leucine (Moshel et al., 2006). In these studies (Appuhamy et al., 2012; Moshel et al., 2006), the investigators did not determine protein degradation in mammary tissue or cells. Comparison of results obtained from cell cultures in the presence of very high concentrations of amino acids (e.g., BCAA) and in their complete absence may not be nutritionally or physiologically relevant.

Net synthesis of milk proteins by mammary epithelial cells depends on the balance between intracellular protein synthesis and degradation (Xi et al., 2012; Kong et al., 2014; Tan et al., 2011). The mTOR pathway is a master regulator of protein synthesis and may also play a role in inhibiting protein degradation (Meijer et al., 2014). Among intracellular proteolytic systems, the ATP-ubiquitin-proteasome-dependent pathway plays a major role in degrading proteins in animal cells (Ciechanover, 2012). At present, no information is available about effects of individual or a mixture of BCAA within physiological concentrations on protein turnover (synthesis and degradation) in mammary epithelial cells cultured in the presence of the physiological concentration of glucose. Based on our recent findings that dietary supplementation with BCAA enhances milk production by lactating sows (Chapter II), the present study was conducted with porcine mammary epithelial cells (PMEC) to test the hypothesis that BCAA stimulate protein synthesis and inhibit proteolysis to favor net synthesis of protein by these cells.

MATERIALS AND METHODS

Reagents, antibodies, cell line

L-[ring-2,4-^{3}H]phenylalanine was purchased from American Radiolabel Chemicals. Dulbecco's modified Eagle's F12 Ham medium (DMEM-F12), sterile fetal bovine serum (FBS), and Gibco antibiotic-antimycotic liquid were purchased from Invitrogen. Insulin and amino acids were obtained from Sigma. Customized DMEM containing 5 mM D-glucose, 0.1 mM L-leucine, 0.1 mM L-isoleucine, 0.1 mM L-valine, and physiological concentrations of other amino acids found in plasma of lactating sows (Chapter II) was procured from Gibco. The BCA protein assay kit and SuperSignal West Dura Extended Duration Substrates were obtained from Pierce. Rabbit-derived primary antibodies against mTOR and phoshporylated mTOR (Ser2481), p70 S6K, 4E-BP1, 20S proteasome, and mouse primary antibodies against phoshporylated 4E-BP1 (Thr70), phoshporylated p70 S6K (Thr389), and ubiquitin were purchased from Cell Signaling. Mouse primary antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Chemicon. Secondary antibodies of peroxidase-labeled donkey anti-rabbit or anti-mouse IgG were obtained from Jackson Immuno Research. The ß-casein antibody was obtained from Santa Cruz (sc-30042) and prolactin from Sigma-Aldrich (Cat # 6520). PMEC were obtained as described in the following section.

Preparation and culture of PMEC

Aseptic techniques were used to isolate PMEC from three different mammary glands of a 9-month-old nonpregnant and nonlactating female pig. The gilt was sacrificed in a local slaughterhouse to obtain mammary glands. Mammary tissue was

disinfected by three cycles of quick dipping (2 seconds) in 75% ethanol and rinsing with 1x phosphate-buffered saline (PBS). After visible fat tissue was removed from the disinfected mammary tissue, fat-free mammary tissue (10 g) was minced into small pieces (0.1 cm in diameter) using surgical scissors. The tissue pieces were placed in a 50ml falcon tube followed by addition of 40 ml DMEM-F12 medium with 2 mM L-glutamine (Sigma Cat. # D8437). The medium also contained 1 mg/ml collagenase A (Sigma Cat. # C9407, St. Louis, MO, USA), 0.05% hyaluronidase (Sigma Cat. # H3506), and 1X antifungal/anibiotics mixture (Gibco Cat. # 15240-062). The tissue pieces were dissociated at 37°C by gentle agitation (250 rpm on a rocker) for 14 h. The tissue digestion solution was transferred into 80-micron cell strainer meshes (80-mesh; BD Biosciences Cat. # 352360) to remove undissociated tissue, fat and debris. Cells that passed through the strainer meshes were collected into a 50ml conical tube. The tube was centrifuged at 800 x *g* for 3 min at 4°C, and the cells were washed three times with Dulbecco's PBS (PBS without Ca^{2+} or Mg^{2+}; Sigma Cat. # D1408) containing 1X antifungal/antibiotics mixture. The cell pellet (PMEC) was washed three times with 10 ml DMEM-F12 (pH 7.4) containing 5% FBS and 1x antifungal/antibiotics mixture. The isolated PMEC were cultured in growth medium in four Petri dishes (Olympus Cat. # 25-202). Each dish contained 10 ml DMEM-F12, 10% FBS, 5 μg/mL insulin (Gibco 12585-014), 1 μg/mL hydrocortisone (Sigma Cat# H0888), 5 ng/mL epidermal growth factor (BD Cat # 354001), 50 μg/ml Gentamycin (Gibco Cat. # 15710-064), and 1x antifungal/antibiotics mixture. Every 24 h, medium was removed and the cells were washed three times with PBS containing 1x antifungal/antibiotics mixture. Thereafter,

fresh growth medium (10 ml) was added to the dish for continuous culturing. At 80% confluence, the cells were collected using 0.25% trypsin and 1 mM EDTA (Life Technology Cat. # 25300-054; diluted with PBS supplemented with 1x antifungal/antibiotics mixture). The trypsinization step took ~ 5 min. The cells were then stored in liquid nitrogen as passage 1. Some passage-1 cells continued to be cultured (1×10^6 cells) in 10 ml fresh growth medium until 80% confluence as described above. This resulted in passage-2 cells. Cells at passage 5 were used for the current study. All cell cultures were carried out at 37°C in a 5% CO_2/95% air incubator.

To freeze PMEC in liquid nitrogen, culture medium was removed from the Petri dish and the cells were washed three times with 1x PBS through centrifugation. Then, 1 mL of 0.25% trypsin and 1 mM EDTA (prepared in PBS supplemented with 1x antifungal/antibiotics mixture) was added to the cell pellet. The Petri dish was cultured for 5 min, and cell adhesion was monitored under an inverted phase-contrast microscope. Once cells were loosened, 5 mL complete growth DMEM-F12 medium was added to the Petri dish. The whole solution was transferred to a 50-ml conical tube, and the tube was centrifuged for 2 min at 600 x g. The supernatant fluid was removed, and the cells were gently re-suspended in 45 ml pre-warmed PBS. The cells were washed one more time with PBS, and the cell pellet was re-suspended in 1 mL solution consisting of 95% FCS and 5% DMSO (Sigma Cat. # D8418). The concentration of PMEC in this solution was 1×10^6/ml. An aliquot of the solution (1 mL) was transferred to a screw-top Cryovial (Corning Cat. # 25704). The Cryovials were quickly placed in a freezing chamber (Bel-Art-Product Cat. F18844) filled with isoproponol (Sigma Cat. #

270490) and then transferred to a -80°C freezer. After 16 h, the Cryovials were removed from the freezer and immediately placed in a liquid nitrogen chamber for long-term storage.

Determination of cell proliferation

PMEC (1×10^{6} cells/well) were cultured for 3 days in 6-well plates containing 2 mL DMEM-F12, 5% FBS, 0.2 μM prolactin, 5 μg/mL insulin, 1 μg/mL hydrocortisone, 50 μg/mL Gentamycine, and 1x antifungal/antibiotics mixture (pH, 7.4), with the medium being changed every 24h. Cells were then used to determine effects of BCAA on their proliferation, as described by Wang et al. (2014). Briefly, PMEC (1×10^{4}/cm^{2}) were seeded in 24-well culture plates containing 0.5 mL DMEM-F12/well. After overnight culture, the cells were maintained for 6 h in 0.5 ml customized DMEM containing 5 mM D-glucose, 0.1 mM L-leucine, 0.1 mM L-isoleucine, 0.1 mM L-valine, and physiological concentrations of other amino acids found in the plasma of lactating sows (Chapter II) . Thereafter, the cells were cultured in 0.5 ml of fresh customized DMEM containing 5% FBS, 0.2 μM prolactin, 5 μg/mL insulin, 1x antifungal/antibiotics mixture, 5 mM D-glucose, 0.1 (control), 0.25, 0.5, 1 or 2 mM Leu, Ile, or Val or a mixture of those three BCAA. We chose 0.1 mM for each of the three BCAA as the control, based on the concentrations of leucine (0.17 mM), isoleucine (0.09 mM) and valine (0.22 mM) in the plasma of sows at d 15 of lactation (Chapter II). Dietary supplementation with 1.64% leucine plus 0.64% isoleucine plus 0.79% valine increased the concentrations of leucine, isoleucine, and valine in the plasma of sows on Day 15 of lactation (2 h after feeding) to 0.40, 0.20, and 0.42 mM, respectively (Chapter II).

Culture medium was changed every 2 days. Cell numbers were determined on Days 2, 4 and 6, as described by Wang et al. (2014). Briefly, at the end of the pre-specified time period, culture medium was aspirated and cells were washed three times with PBS. The cells were then fixed in 50% ethanol for 30 min, followed by staining with 0.2% solution of Janus Green B in PBS (pH 7.1-7.2) for 30 min at room temperature. Dye was aspirated off and plates were washed using cold PBS. After 0.5 ml of 0.5 N HCl was added to each well, the plates were gently shaken by hand for 5 min. Thereafter, 300 µl of each colored supernatant fluid was collected and transferred to a 96-well plate and read against blanks (0.5 N HCl) at 595 nm in a microplate reader (Molecular Device, Sunnyvale, CA).

Determination of protein synthesis in PMEC

The PMEC (1×10^5 cells/well) were cultured for 3 days in 6-well plates containing 2 ml DMEM-F12, 5% FBS, 0.2 µM prolactin, 5 µg/mL insulin, 1 µg/mL hydrocortisone, 50 µg/ml Gentamycin, and 1x antifungal/antibiotics mixture (pH, 7.4), with the medium being changed every 24h. Cells were then used to determine effects of BCAA on protein synthesis, as described by Kong et al. (2014). Briefly, the cells were maintained for 6 h in 2 ml customized DMEM, and then cultured for 4 days in the customized DMEM containing 5% FBS, 0.2 µM prolactin, 5 µg/mL insulin, 1x antifungal/antibiotics mixture, and 0.1, 0.25, 0.5, 1 or 2 mM Leu, Ile, Val or a mixture of the three BCAA. There were eight replicate sets of wells per a BCAA or a mixture of BCAA at each dose. After the 4-day culture, the medium was removed and the cells were washed once with 2 ml medium. Then, cells were cultured for 3 h in 2 ml

customized DMEM containing 5% FBS, 0.2 μM prolactin, 5 μg/mL insulin, and 1 mM L-phenylalanine, 0.8 μCi L-[ring-2,4-^{3}H]phenylalanine (American Radiolabeled Chemicals). The medium also contained 0.1 (control), 0.25, 0.5, 1 or 2 mM Leu, Ile or Val or a mixture of these three BCAA. At the end of a 3h culture period, the medium was collected and cells were washed rapidly three times with 2 ml ice-cold PBS. The medium and the cell pellet were separately mixed with 2 ml of 10% trichloroacetic acid (TCA) (Wu and Thompson, 1990). Cells in each well were scraped and the whole solution was collected into a 15-ml tube and centrifuged at 3,000g for 5 min. The TCA-treated culture medium and the TCA-treated cell extracts were combined and centrifuged at 600 x g for 10 min. The supernatant fluid was removed and the protein pellet washed three times with 5 ml of 10% TCA, and then dried in air at 37°C. After the pellet was dissolved in 0.5 ml of 1 M NaOH, 0.4 ml of the solution was transferred to a 20-ml scintillation vial containing 19 ml of Hionic Fluor Scintillation cocktail (PerkinElmer, MA, USA). The ^{3}H- phenylalanine generated radioactivity was determined by a liquid scintillation counting after overnight standing at room temperature. The specific radioactivity of ^{3}H-phenylalanine in the medium was used to calculate the rate of protein synthesis in cells.

Determination of protein degradation in PMEC

PMEC were cultured as described for the measurement of protein synthesis, except that beginning on Day 4 of culture in the customized DMEM, cells were cultured for 24 h in 2 ml of DMEM containing 0.1 mM L-phenylalanine, L-[ring-2,4-^{3}H]phenylalanine (0.8 μCi/well) and 0.1, 0.25, 0.5, 1 or 2 mM Leu, Ile, or Val or a

mixture of these three BCAA. After the 24 h culture to label cellular proteins, cells were washed three times with 2 ml DMEM medium containing 1 mM L-phenylalanine to deplete intracellular free [^{3}H]phenylalanine (Wu and Thompson, 1990). The cells were then cultured for 3 h in 2 ml customized DMEM containing 1 mM L-phenylalanine. The customized medium also contained 0.1 (control), 0.25, 0.5, 1 or 2 mM Leu, Ile, or Val or a mixture of these three BCAA. At the end of a 3h culture period, the culture medium was collected into a new tube. Then, 2 ml of 10% TCA was added separately to each culture medium and each well in the plates. Cells were then scraped from the well thoroughly. The whole TCA extract of the cells was collected into a 15-ml tube. TCA-treated culture medium and TCA-treated cell extract were centrifuged separately at 3,000 x g for 5 min. The supernatant fluid from the TCA-treated culture medium was used for the measurement of ^{3}H-phenylalanine. The TCA-treated cell extract (protein pellet) was washed three times with 5 ml of 10% TCA and dried in air at 37°C. The protein pellet was then dissolved in 0.5 ml of 1 M NaOH and 0.4 ml of the solution transferred to a 20-ml scintillation vial containing 19 ml Hionic Fluor Scintillation cocktail for measurement of ^{3}H-phenylalanine. For determining [^{3}H]phenylalanine released from prelabeled proteins into culture medium, the collected medium was centrifuged at 3,000 x g for 2 min to remove any dead cells. An aliquot (1 ml) of the supernatant fluid was transferred to a 15 ml tube containing 2 ml 10% TCA. After the tubes were centrifuged at 3,000 x g for 5 min, 1 ml of the supernatant fluid was transferred to a 20ml scintillation vial containing 18 ml Hionic Fluor Scintillation cocktail for ^{3}H measurement. The percentage of protein-bound [^{3}H]phenylalanine

released into culture medium (namely [^{3}H]phenylalanine in medium ÷ [^{3}H]phenylalanine in cell protein ×100) was calculated to indicate protein degradation in PMEC.

Western blot analysis for the mTOR pathway, the ubiquitin-proteasome pathway, and ß-casein expression

PMEC were cultured as described for the measurement of protein synthesis, except that the medium contained no [^{3}H]phenylalanine. At the end of the 4-day culture in the presence of 0.1 (control), 0.25, 0.5, 1 or 2 mM Leu, Ile, Val or a mixture of the three BCAA, cells were lysed using 500 µl of lysis buffer to extract protein. Protein concentrations of cell homogenates were measured using the BCA method, with bovine serum albumin as the standard. All samples were adjusted to an equal concentration of protein. The supernatant fluid (containing cell proteins) was diluted with 2× sodium dodecyl sulfate (SDS) sample buffer (0.63 ml of 0.5 M Tris–HCl pH 6.8, 0.42 ml 75% glycerol, 0.125 g SDS, 0.25 ml ß-mercaptoethanol, 0.2 ml 0.05% solution of bromphenol blue, and 1 ml water to a final volume of 2.5 ml) and heated in boiling water for 5 min. The solution was cooled on ice and then used for western blot analysis. Aliquots of samples were loaded onto SDS polyacrylamide gels. After separation on 4% to 12% gels, proteins were transferred to a nitrocellulose membrane (BioRad, Hercules, CA, USA) under 12 V current overnight, using the Bio-Rad Transblot apparatus (Hercules, CA, USA). Membranes were blocked in 5% fat-free milk in Tris-Tween buffered saline (TTBS; 20 mM Tris/150 mM NaCl, pH 7.5, and 0.1% Tween-20) for 3 h and then incubated with one of the following primary antibodies overnight at 4°C with gentle rocking: mTOR (cell signaling, 1:1000), phosphorylated mTOR (Ser2448; cell signaling,

1:1000), 4EBP1 (cell signaling, 1:1000), phosphorylated 4EBP1 (Ser65; cell signaling, 1:1000), S6K1 (cell signaling, 1:1000), phosphorylated S6K1 (Thr389; cell signaling, 1:1000), ubiquitin (1 : 1000), proteasome (1 : 10000), ß-casein (1:200) or GAPDH (cell signaling, 1:1000). After washing three times with TTBS, the membranes were incubated at room temperature for 3 h with secondary antibody at a dilution of 1:50,000 (horseradish peroxidase-conjugated goat anti-rabbit IgG, cell signaling or peroxidase-labeled rabbit anti-goat IgG, Kirkegaard & Perry Lab). Finally, the membranes were washed with TTBS, followed by development using Super signal West Dura Extended Duration Substrate according to the manufacturer's instructions (Pierce, Rockford, IL, USA). The blot was scanned for detection of fluorescence using the BioRad Gel Detection system. All data were normalized as relative values of targeted protein abundance to GAPDH abundance.

STATISTICAL ANALYSIS

Results were expressed as means and pooled SEM. Data were statistically analyzed by one-way ANOVA (Assaad et al., 2014) and all tables were generated using the one-way ANOVA software available online at https://housseinassaad.shinyapps.io/TableReport/. The Duncan multiple comparisons test was used to identify effects of treatment. Values of $P < 0.05$ were taken to indicate statistical significance. All statistical analyses were run using the R 3.1.0 software for Linux (64-bit).

RESULTS

Effects of Leu, Ile, or Val or a mixture of BCAA on cell proliferation and protein concentrations of PMEC

The number of PMEC increased ($P < 0.05$) as the time of culture increased from 2 to 6 days (Table A-32). Cell numbers also increased ($P < 0.05$) on Days 2, 4 and 6 of culture as extra-cellular concentrations of Leu, Ile, or Val or a mixture of the three BCAA increased from 0.1 to 2 mM. Concentrations of proteins in PMEC treated with 0.25, 0.5, 1 and 2 mM of Leu, Ile, or Val or a mixture of the three BCAA were greater ($P < 0.05$) than for the control, with values being greatest at 1 and 2 mM BCAA (Table A-33).

Effects of Leu, Ile, or Val or a mixture of BCAA on protein synthesis and protein degradation in PMEC

Increasing the extracellular concentrations of Leu (Table A-34), Ile (Table A-35), or Val (Table A-36) or a mixture of BCAA (Table A-37) from 0.1 to 2 mM increased ($P < 0.01$) protein synthesis and reduced ($P < 0.05$) protein degradation in PMEC. The rates of protein synthesis were 26%, 40%, 65%, and 77% higher ($P < 0.05$), while the rates of protein degradation were 18%, 30%, 53%, and 53% lower ($P < 0.01$) in cells treated with 0.25, 0.5, 1, 2 mM Leu, respectively, compared with the control. Treatment with 1 and 2 mM Ile increased ($P < 0.05$) protein synthesis by 23% and 43%, whereas Ile at 0.25 to 2 mM decreased ($P < 0.05$) protein degradation, as compared with the control. Val increased ($P < 0.05$) protein synthesis and decreased ($P < 0.05$) protein degradation at all doses. Of note, compared with 0.1 mM BCAA, 0.25 to 2 mM BCAA increased (P

< 0.05) protein synthesis and decreased ($P < 0.05$) proteolysis in PMEC in a dose-dependent manner.

Effects of Leu, Ile, or Val or a mixture of BCAA on the mTOR signaling pathway and β-casein expression in PMEC

Figures B2-B5 illustrate effects of Leu, Ile, or Val or a mixture of BCAA on the abundance of total and phoshporylated mTOR, S6K1, and 4E-BP1 proteins, as well as the abundance of β-casein. Increasing extracellular concentrations of Leu, Ile, or Val from 0.1 to 2 mM did not affect ($P > 0.05$) total lmTOR, S6K1, or 4E-BP1 proteins in PMEC. However, in comparison the control, 0.25, 0.5, 1 or 2 mM Leu increased ($P < 0.05$) the abundances of: 1) phosphorylated mTOR by 20%, 38%, 61%, and 63%; 2) phosphorylated 4E-BP1 by 20%, 36%, 51%, and 54%; 3) phosphorylated S6K1 by 29%, 43%, 48%, and 57%; and 4) β-casein by 35%, 60%, 68%, and 89%, respectively (Fig B-2). Addition of 1 and 2 mM Ile increased ($P < 0.05$) the abundance of phosphorylated mTOR, 4E-BP1 and S6K1, while 0.25 to 2 mM Ile increased ($P < 0.05$) β-casein, in comparison with the control (Fig B-3). Compared with the control, addition of 0.25 to 2 mM Val to culture medium also increased ($P < 0.05$) the abundance of phosphorylated mTOR, 4E-BP1, S6K1, and β-casein (Fig B-4). Furthermore, compared with 0.1 mM BCAA, 0.25 to 2 mM BCAA increased ($P < 0.05$) the abundance of: 1) phosphorylated mTOR by 33%, 45%, 69%, and 73%; 2) phosphorylated 4E-BP1 by 23%, 28%, 51%, and 56%; 3) phosphorylated S6K1 by 43%, 46%, 58%, and 63%; and 4) β-casein by 34%, 64%, 73%, and 93%, respectively (Fig B-5). A mixture of the three BCAA had a

greater ($P < 0.05$) effect on increasing the abundance of phosphorylated mTOR, S6K1, or 4E-BP1 proteins in PMEC than any individual BCAA.

Effects of Leu, Ile, or Val or a mixture of BCAA on the ubiquitin-proteasome pathway in PMEC

The effects of Leu, Ile, or Val or a mixture of BCAA on the abundance of ubiquitin, ubiquitinated proteins, and proteasome in PMEC are shown in Figures B6-B9 respectively. As indicated by its manufacturer, the ubiquitin antibody from Cell Signaling recognized both free and protein-conjugated ubiquitin. When compared with the control, addition of 0.25, 0.5, 1 or 2 mM Leu to culture medium reduced ($P < 0.01$) the abundance of: 1) ubiquitinated proteins by 19%, 34%, 44%, and 48%; and 2) proteasome by 21%, 34%, 42%, and 46%, respectively (Fig B-6). The abundance of free ubiquitin in PMEC was not affected by individual or the mixture of BCAA. Compared with the control, the three BCAA had a greater effect to reduce the abundance of both ubiquitinated protein and proteasome in PMEC than any individual BCAA.

DISCUSSION

The capacity of the lactating mammary gland to synthesize milk is greatly influenced by many factors, including the number and activity of functional mammary epithelial cells, mammary cell differentiation, degree of premature involution of the mammary gland, availability of adequate precursors (e.g. amino acids and energy), concentrations of lactogenic hormones, and inducers of key cell signaling pathways (Boyd et al., 1995; Kim and Wu, 2009). Lactogenic hormones (prolactin, growth hormone, insulin, and glucocorticoids) affect proliferation, functional differentiation, and

involution of mammary epithelial cells, as well as transcriptional and translational regulation of gene expression in these cells (Boyd et al., 1995; Rhoads and Grudzien-Nogalska, 2007; Xu et al., 1998). In recent years, there has been growing interest in the regulation, by amino acids, of milk synthesis in mammary epithelial cells as BCAA are capable of stimulating cell cycle progression, cell differentiation (Xu et al., 1998; Kimura and Ogihara, 2005; Gonçalves and Gomes-Marcondes, 2010), and cell apoptosis (D'Anona et al., 2010; Toerien et al., 2010; Moshel et al., 2006). The present study focused on effects of BCAA on the mTOR cell signaling pathway, the ubiquitin-proteasome pathway, and intracellular protein turnover in PMEC.

L-Leucine stimulates protein synthesis and inhibits proteolytic degradation in skeletal muscle (Tischler et al.. 1982; Mitch and Clark, 1985), as well as protein synthesis in brain and heart (Swian et al., 1990). Additionally, results of in vitro studies have shown that compared with 0.45 mM leucine (~ 3 to 4 times leucine concentration in the plasma of lactating cows and sows), the complete absence of leucine from culture medium containing 17.5 mM glucose reduced protein synthesis in bovine mammary tissue (Appuhamy et al., 2012), as well as bovine and mouse mammary epithelial cell lines (Moshel et al., 2006). The nutritional relevance of those findings is not known, because leucine is always present in the blood of animals. To date, no information is available about effects of physiological concentrations of BCAA on protein synthesis in mammary epithelial cells in the presence of the physiological concentration of glucose. Additionally, results of studies regarding effects of BCAA on rates of protein degradation or proteolytic systems in these cells are not available. Because accumulation

of protein in the cell is regulated by the balance between the rates of protein synthesis and degradation and it is necessary for cell growth and mitogenesis (Fingar et al., 2004 and 2006), the present study determined whether BCAA regulate protein synthesis and protein degradation in mammary epithelial cells. The findings were to increase understanding of cellular mechanisms whereby dietary BCAA supplementation enhances milk production by lactating sows and perhaps other mammals. As the ATP-ubiquitin-proteasome-dependent pathway is responsible for bulk degradation of protein in cells (Ciechanover, 2012) and mTOR is the master regulator of protein synthesis (Suryawan and Davis, 2011), the present study was directed toward those pathways in PMEC treated with various physiological concentrations of BCAA.

A novel and important observation from the current study is that individual or a mixture of BCAA reduced the abundance of ubiquitinated protein and proteasome, as well as the rate of protein degradation in PMEC. The ATP- and ubiquitin-dependent proteasome system is ubiquitous in animal cells (Tomko and Hochstrasser, 2013). The most common form of the proteasome is the 26S proteasome with a molecular mass of 2,000 kDa, which contains one 20S proteasome and two 19S regulatory caps (Voges et al., 1999). Ubiquitin-dependent protein degradation involves: (a) activation of ubiquitin by a multienzyme system; (b) binding of multiple ubiquitin moieties to Lys residues of a protein substrate; and (c) hydrolysis of the protein substrate by 26S proteasome (Finley, 2009). The ubiquitin antibody used in the present study detected both free and protein-conjugated ubiquitin. Our findings that the abundance of ubiquitinated protein, but not free ubiquitin, was reduced by individual or a mixture of BCAA in PMEC suggests

inhibition of either ubiquitin activation or binding of multiple ubiquitin moieties to Lys residues of the target protein. Nonetheless, the decrease in the abundances of ubiquitinated proteins and proteasome indicated that BCAA reduced degrade of intracellular proteins in PMEC to. This view is further supported by our actual measurement of hydrolysis of [^{3}H]Phe-labeled proteins into TCA-soluble [^{3}H]Phe fractions. Like any other proteins, the proteasome is subject to degradation by specific proteases whose expression may be enhanced by BCAA through an mTOR-dependent mechanism or whose enzymatic activity may be activated allosterically by BCAA as reported for glutamate dehydrogenase (Tomita et al., 2011). Further studies are necessary to test those new hypotheses.

As a serine/threonine (Ser/Thr) protein kinase, mTOR is a highly conserved signaling protein in eukaryotes that has a critical role in regulating protein synthesis, cell growth, metabolism, and proliferation by sensing and responding to a variety of nutritional and hormonal stimuli (Xu et al., 1998; Kim et al., 2002; Jankiewicz et al., 2006; Debnath et al., 2003). mTOR is a member of the phosphatidylinositol-3-kinase (PI3K)-related kinase family and phosphorylates its downstream effectors S6K1 and 4E-BP1, which consequently control translation efficiency in cells (Suryawan and Davis, 2011). Eukaryotic initiation factors (eIF) control mRNA translation by stimulating initiation of the translation process (Suryawan and Davis, 2011). Both L-isoleucine and L-leucine can increase mTOR, S6K1, and 4EBP1 phosphorylation ($P < 0.05$) in bovine mammary tissue, as compared with the complete absence of those amino acids from culture medium (Appuhamy et al., 2012). The present study revealed that physiological

levels of BCAA activate the mTOR cell signaling pathway and stimulate protein (including casein) synthesis in PMEC, and demonstrated, for the first time to our knowledge, that L-valine had similar effects to those of L-leucine and L-isoleucine to enhance protein synthesis in PMEC. Furthermore, a mixture of BCAA exerted a greater effect on activating the mTOR signaling pathway and protein synthesis in PMEC than any single BCAA. Whether BCAA exerts a direct or an indirect effect on mTOR phosphorylation is not known and warrants further investigation.

In summary, individual or a mixture of BCAA at physiological concentrations activate the mTOR pathway and suppress the ubiquitin-proteasome pathway in PMEC, resulting in increased protein (including β-casein) synthesis, decreased protein degradation, and enhanced cell proliferation. These findings provide a molecular mechanism whereby dietary BCAA supplementation enhances milk production by lactating sows. These results advance understanding of nutritional regulation of lactation and have important implications for improving milk protein synthesis in livestock, women, and other mammals.

CHAPTER V

CONCLUSION AND SUMMARY

Increasing efficiency and reducing costs of pork production are continually required to enhance the profitability of the global swine industry. Among livestock species, the pig exhibits the highest rate of neonatal mortality, which is currently 12% in the U.S. This problem results from the lack of brown adipose tissue for thermogenesis in swine, very low to no fat depots at birth (only 1% of the body weight versus 10% in human newborns), and inadequate milk production by sows.

Milk is the sole source of nutrients for neonatal mammals during the suckling period. Hodge (1974) and Boyd et al. (1995) demonstrated that artificially reared neonatal pigs grow at least 50% faster than sow-reared piglets. Additionally, Harrell et al. (1993) have shown that a sow needs to produce at least 18 kg/d milk to supply piglets with enough energy to grow at a rate comparable to artificial-reared piglets of the same age. However, inadequate protein in sow's milk is another major factor limiting maximum growth of piglets (Wu et al., 2014). A heavier piglet in the first week after birth is more likely to survive to weaning. Thus, a major goal of rearing young pigs is to enhance their growth during the suckling period (Rezaei et al., 2013).

The composition of sow's colostrum and milk limits the potential for lean tissue growth of piglets (Wu et al., 1994). Based on the amino acid composition of sow's milk, arginine requirements of piglets for growth, and metabolic studies, it is clear that a deficiency of arginine in milk is a major factor limiting maximum growth of young pigs

(Wu and Knabe, 1994; Wu and Knabe, 1995). Thus, arginine is a nutritionally essential amino acid for young mammals (Mateo et al., 2008). Emerging evidence also shows that sufficient provision of glutamine, glutamate, and glycine in diets is required for maximum growth of milk-fed piglets (Wang et al., 2014; Wu et al., 2014). Additionally, increasing the availability of amino acids (e.g., branched-chain amino acids; BCAA) that are not synthesized by animals is beneficial for promoting milk synthesis by lactating mammals and the growth of their offspring (Li et al., 2009; Lei et al., 2012a).

The pre-weaning body weight of pigs is closely related to their post-weaning health and growth, and, therefore, the time from weaning to marketing. While piglets are exposed to many stressors immediately after birth, gastrointestinal problems are among the most severe. Milk-born amino acids are major sources of energy for the small intestine and are necessary for maintenance of anti-oxidative reactions in the intestine. The neonate instantly transits from primarily parenteral to enteral nutrition, which requires that the small intestine be the major organ for digestion and absorption of nutrients in the diet. However, the gut of the neonatal pig is underdeveloped and highly susceptible to injury and oxidative stress. Thus, the main losses in pig production occur between birth and weaning. Most pre-weaning mortality in piglets takes place during the first week after birth. In a study by Glastonbury (1976), mean total piglet mortality prior to weaning was 20% (n=10,000 piglets), and most losses occurred within the first 4 d after birth. There is evidence that milk production by sows is inversely correlated with prewaning piglet mortality (Rezaei et al., 2011). This further underscores the importance

of developing strategies that insure sufficient milk production by sows for sustaining the swine industry in the U.S. and worldwide.

As noted previously, the survival and growth of piglets are critically dependent on the availability of sufficient provision of nutrients in maternal milk. The synthetic capacity of the mammary gland for milk for all mammals is determined by coordinate actions of hormonal and nutritional factors, as well as functionality of mammary epithelial cells during gestation and lactation. In the current swine industry, dietary regimens for lactating sows are not adequate for efficient produce sufficient milk to support maximum survival or growth of preweaning piglets. As a result, a high rate of neonatal mortality and a suboptimal rate of growth remain two major problems in the swine industry worldwide (Wu et al., 2006). These problems are particularly severe for low-birth-weight piglets in a litter.

To date, few mechanism-based methods have been developed to improve lactation in mammals. Sows require tremendously high amounts of amino acids during lactation to support milk synthesis. Among the amino acids, BCAA are taken up in amounts in excess than their output in milk (Manjarin et al., 2014). The carbon skeleton and nitrogen of BCAA can be used to synthesize other amino acids (e.g., glutamate and glutamine) whose requirements by neonates to meet their physiological needs are particularly high. More importantly, BCAA take part in the regulation of metabolic pathways including DNA transcription, mRNA translation, and proteolysis which increase net protein synthesis and cell growth. Thus, dietary supplementation with valine to lactating sows enhances milk yield and piglet growth (Richert et al., 1997; Paulicks et

al., 2003). Similar results have been reported for supplementation of diets of lactating cows with leucine (Rulquin and Pisulewski, 2006). However, dietary supplements for sows with a single BCAA have yielded inconsistent results on milk production (Richert et al. 1997a,b; Moser et al., 2000) possibly due to dietary imbalances for the three BCAA (Wu et al., 2014). For example, dietary supplementation of diets for lactating sows with 0.4% isoleucine could enhance growth of suckling piglets when the ratio of isoleucine to leucine in the complete diet was 0.88 (Richert et al. 1997a), but not at a ratio of 0.55 (Moser et al., 2000). Little is known about effects of dietary supplementation with leucine plus isoleucine plus valine on lactation performance in any mammal, including sows.

In view of the foregoing published results, research for this dissertation was conducted to test the hypothesis that BCAA enhance milk protein synthesis and lactation performance in sows. To achieve this goal, in vivo feeding (Chapters II and III) and in vitro cell culture (Chapter IV) experiments were performed. In the first feeding study, lactating sows fed a corn- and soybean meal-based diets supplemented with 0%, 1.535%, or 3.07% BCAA throughout a 29-d period of lactation had piglets that grew faster and had less neonatal mortality. The sows fed the BCAA supplemented diet also lost less body weight which enhances subsequent reproductive performance. Concentrations of ree Arg, Gln, Glu, Asn, Asp and BCAA in plasma were also greater in BCAA sows and their piglets, as compared with control sows. Similarly, concentrations of most free and protein-bound amino acids were greater in milk from BCAA-supplemented sows. BCAA supplementation also increased concentrations of fats and lactose in sow's milk, while

reducing the plasma levels of TBARS which are indicative of oxidative stress. This finding may have important implications for lactation performance, as oxidative stress leads to lactation failure in mammals.

Because glutamate is a predominant product of the extensive catabolism of BCAA in the lactating mammary gland (Li et al., 2009) and a major nutrient in milk of all mammals (Wu et al., 2014), we hypothesized that glutamate mediates, at least in part, the beneficial effect of BCAA supplementation to improve milk production in sows. Although glutamate has been traditionally regarded as a nutritionally nonessential amino acid for mammals, there is emerging evidence that a typical corn- and soybean meal-based diet does not provide sufficient glutamate to support maximum protein synthesis in growing swine (Wu et al., 2014). This view may also apply to diets for lactating sows. Thus, the second feeding trial was conducted with lactating sows fed corn- and soybean meal-based diets supplemented with 0, 1% or 2% MSG between d 0 and 29 of lactation, as described for the first feeding experiment. Overall, the milk yield, body condition and antioxidative capacity, as well as the preweaning growth and survival of their piglets were improved for sows in response to dietary MSG supplementation in a dose-dependent manner. Collectively, these findings indicate important roles for BCAA and their metabolite glutamate in improving lactational performance in sows as well as growth and survival of their piglets.

The cell culture experiments investigated biochemical pathways responsible for stimulatory effects of BCAA on milk synthesis in sows (Chapter IV). Mammary epithelial cells are secretory units of mammary glands. These cells synthesize milk and

secrete it upon stimulation by prolactin. We isolated these cells from a sexually mature gilt for biochemical studies and focused on regulatory effects of BCAA on mTOR cell signaling, the ubiquitin-proteasome pathway for degradation cellular proteins, and intracellular protein turnover in PMEC. mTOR has a critical role in regulating protein synthesis, rate of cell growth, metabolism, and proliferation by sensing and responding to a variety of nutritional and hormonal stimuli. (Xu et al., 1998; Kim et al., 2002; Jankiewicz et al., 2006; Debnath et al., 2003). As a member of the phosphatidylinositol-3-kinase (PI3K)-related kinase family, mTOR phosphorylates its downstream effectors S6K1 and 4E-BP1, which consequently control translation efficiency in cells (Suryawan and Davis, 2011). Eukaryotic initiation factors (eIF) controls mRNA translation by stimulating initiation of the translation process through the formation of the eIF4F complex (Suryawan and Davis, 2011). Compared with the control (0.1 mM BCAA), treatment of cells with 0.25 to 2 mM of individual or mixture of BCAA decreased the abundance of ubiqitinated proteins and proteasome, as well as the rate of protein degradation, while increasing phosphorylation of mTOR, 4EBP1 and S6K1 proteins, as well as the rate of protein (including β-casein) synthesis in PMEC. Thus, increasing extracellular concentrations of BCAA from 0.1 to 2 mM increased the rate of proliferation of PMEC in a dose-dependent manner.

Taken together, our results indicate that physiological levels of BCAA regulate protein synthesis and decrease protein degradation in PMEC through activating mTOR cell signaling and inhibiting the ubiquitin-proteasome pathway. This proposed mechanism is illustrated in Figure B-10. Collectively, results of this dissertation research

provide compelling evidence that BCAA play important roles in enhancing milk production by lactating sows. The findings significantly advance understanding of nutritional regulation of lactation and provide a new strategy to improve milk protein synthesis in livestock, women, and other mammals.

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APPENDIX A

Table A-1. Composition of mature milk of domesticated and wild mammals

Species	Fat	Casein	Whey protein	Total protein	NPN subs	Lactose	Total CH_2O	Ca	Ash	DM[a]	References
Antelope[b]	72	48	14	62	18	42	47	2.6	13	212	47,48,60
Baboon	46	4.7	7.3	12	3.0	60	77	0.44	3.0	141	5,6,20-24
Bat	133	x	x	80	5.0	34	40	x	6.8	265	26,27,29
Bear (black)	220	88	57	145	7.5	3.0	27	3.6	19	419	11,24,43,58
Bear (grizzly)	185	68	67	135	7.0	4.0	32	3.4	13	372	11,24,48,57
Bear (polar)	331	71	38	109	5.6	4.0	30	3.0	12	488	18,42,21-24
Beaver	182	85	23	108	6.3	17	22	2.4	20	338	3,21,40,62
Bison	35	37	8.0	45	3.0	51	57	1.2	9.6	150	12,35,40
Buffalo	77	38	7.0	45	5.2	40	47	1.9	8.0	192	12,35,40,48
Blue whale	423	73	36	109	6.6	10	13	3.4	16	568	16,24,42,44
Camel	45	29	10	39	5.6	49	56	1.4	7.0	153	3,12,22,40
Cat (domestic)	108	31	60	91	10	42	49	1.8	6.2	264	3,21,21,48
Chimpanzee	37	4.8	7.2	12	2.0	70	82	0.36	11	144	12,22,58,60
Cow (domestic)[c]	37	28	6.0	34	2.2	49	56	1.2	7.1	136	19-24,48,58
Coyote	107	x	x	99	x	30	32	x	9.0	247	3,22,48
Deer	197	94	10	104	14	26	30	2.6	14	359	3,12,40,48
Dog (domestic)	95	51	23	74	23	33	38	2.0	12	242	3,20-24,40
Dolphin	330	39	29	68	3.0	10	11	1.5	7.5	420	12,40,44,49
Donkey (Ass)	14	11	9.0	20	3.2	61	68	0.91	4.5	110	3,12,20-24
Elephant	116	19	30	49	4.1	51	60	0.80	7.6	237	12,20-24,45
Ferret	80	32	28	58	6.7	38	44	x	8.0	197	6,21,12,40
Fin whale	286	82	38	120	6.2	2.0	26	3.0	16	454	24,31,44
Fox	63	x	x	63	4.0	47	50	3.4	10	190	1,2,40,48
Giant panda	104	50	21	71	10	12	15	1.3	9.4	209	38,58
Giraffe	125	48	8.0	56	2.2	34	40	1.5	8.7	232	2,12,20,40
Goat (domestic)	45	27	5.0	32	5.8	43	47	1.4	7.9	137	2,24,48,52
Goat (mountain)	57	24	7.0	31	5.3	28	32	1.3	12	136	3,12,33,40
Gorilla	19	13	9.0	22	1.9	62	73	3.2	6.0	122	12,46,61
Guinea pig	39	66	15	81	12	30	36	1.6	8.2	176	12,36,39,40
Hamster	126	58	32	90	11	32	38	2.1	14	279	3,12,24,60
Horse (domestic)	19	13	12	25	3.6	62	69	0.95	5.1	122	20-24,48,60
Human[d]	42	4.4	6.6	11	2.8	70	80	0.32	2.2	138	3,12,48,60
Kangaroo	21	23	23	46	4	<0.01	47	1.6	12	130	3,6,40,48
Lion	189	57	36	93	6.6	27	34	0.82	14	337	3,9,12,40
Llama	42	62	11	73	9.6	60	66	1.7	7.5	198	12,35,37,40
Mink	80	x	x	74	12	69	76	1.3	10	252	12,29,48,59
Moose	105	x	x	135	18	33	38	3.6	16	312	8,13,23,48
Mouse (Lab)	121	70	20	90	11	30	36	2.5	15	273	3,12,25,48
Mule[e]	18	x	x	20	3.0	55	62	0.76	4.8	108	12,20-24,48
Musk ox	110	35	18	53	7.0	27	33	3.0	18	221	40,48,55
Opossum	61	48	44	92	4.6	16	20	4.2	16	194	15,17,30
Peccary	36	40	15	55	5.7	66	71	1.2	6.4	174	3,48,53,60
Pig (domestic)	80	28	20	48	5.4	52	58	3.1	9.2	201	20-24,48,60
Pronghorn	130	x	x	69	7.2	40	43	2.5	13	262	3,12,48
Rabbit	183	104	32	136	11	18	21	6.3	20	371	3,12,40,48
Rat (Lab)	126	64	20	84	6.3	30	38	3.2	15	269	3,12,48,58
Reindeer	203	86	15	101	14	28	35	3.1	14	367	12,20-24,48
Rhesus monkey	40	11	5.0	16	1.6	70	82	0.40	26	166	14,34,48
Rhinoceros	4.0	11	3.0	14	2.3	66	72	0.56	3.7	96	20-24,35,48
Sea lion	349	x	x	136	4.1	0.0	6.0	0.76	6.4	502	44,48,50,56
Seal (fur)	251	46	43	89	6.9	1.0	24	0.70	5.0	376	10,42,44,56
Seal (gray)	532	50	52	102	10	1.0	26	2.0	7.0	677	42,44,48
Seal (harp)	502	38	21	59	2.4	8.9	23	1.2	3.9	590	7,12,40,54
Seal (hooded)	404	x	x	67	5.0	0.0	10	1.2	8.6	496	12,42,44,48
Sheep (domestic)	74	46	9.0	55	2.7	48	55	1.9	9.2	194	20-24,40,48
Sperm whale[f]	153	32	50	82	6.3	20	22	1.5	8.0	270	19,44,48
Squirrel (gray)	121	50	24	74	16	30	34	3.6	12	257	3,12,41
Tree shrew	170	x	x	85	19	15	20	x	8.0	302	3,40,60
Water buffalo	74	32	6.0	38	5.8	48	55	1.9	7.8	181	4,6,12,35
Water shrew	200	x	x	100	x	1.0	30	x	20	350	3,12,20-24
White whale	220	82	38	120	3.7	2.0	18	3.6	16	378	12,44,48
Wolf	96	x	x	92	4.8	32	35	4.0	25	253	3,12,32,60
Yak	68	36	7.0	43	2.5	50	54	1.3	8.0	176	6,12,35,48
Zebra	21	12	11	23	2.6	74	82	0.8	3.5	132	35,40,48,51

Values are g/kg whole milk. Nonprotein nitrogen = total nitrogen – protein nitrogen. Nitrogen content in milk protein is 15.67% (19-24). The amount of non-protein nitrogenous substances (g/kg whole milk) is calculated as the amount of nonprotein nitrogen (g/kg whole milk) x 6.25. Caseins include α_{S1}-casein, α_{S2}-casein, β-casein, γ-casein, and *k*-casein. Whey proteins include α-lactoalbumin, β-lactoglobulin, serum albumin, immunoglobulins, lactoferrins, lysozymes, amino acid oxidases, xanthine oxidase, and other enzymes.

AA = amino acids; Ca = calcium; DM = dry matter; NPN subs = non-protein nitrogenous substances (including free amino acids, small peptides, urea, ammonia, uric acid, creatine, creatinine, and other low-molecular weight nitrogenous substances). The symbol "x" denotes the lack of data in the literature.

[a] Including fat, protein, NPN, lactose plus other carbohydrates, and minerals (total ash). When data on total carbohydrates have not been reported, ratios of lactose to other carbohydrates in milk are estimated to be 15:1 (g/g) (14, 38, 57, 58).

[b] Gemsbok antelope;

[c] Concentrations of urea, creatinine, and amino sugars are 317, 127, and 392 mg/L whole mike, respectively.

[d] Concentrations of urea, creatinine, and amino sugars are 274, 209, and 1111 mg/L whole mike, respectively.

[e] Mule is a domesticated, hybrid animal produced by crossing a female horse with a male donkey.

[f] Pygmy sperm whale.

Cited references: 1. Ahlstrøm and Wamberg (2000); 2. Aschaffenburg et al. (1962); 3. Ben Shaul (1962); 4. Bonfatti et al. (2013); 5. Buss (1978); 6. Claeys et al. (2014); 7. Cook and Baker (1969); 8. Cook et al. (1970); 9. De Waal et al. (2004); 10. Dosako et al. (1983); 11. Farley and Robbins (1995); 12. Fox and McSweeney (1998); 13. Franzmann et al. (1976); 14. Goto et al. (2010); 15. Green et al. (1996); 16. Gregory et al. (1955); 17. Gross and Bolliger (1959); 18. Hedberg et al. (2011); 19. Jenness and Odell (1978); 20. Jenness (1979); 21. Jenness and Holt (1987); 22. Jenness and Sloan (1970); 23. Jenness (1974); 24. Jenness (1986); 25. Knight et al. (1986); 26. Korine and Arad (1999); 27. Kunz et al. (1983); 28. Kunz and Lönnerdal (1993); 29. Kunz et al. (1995); 30. Kuy et al. (2007); 31. Lauer and Baker (1969); 32. Lauer et al. (1969a); 33. Lauer et al. (1969b); 34. Lönnerdal et al. (1984); 35. Medhammar et al. (2012); 36. Mepham and Beck (1973); 37. Morin et al (1995); 38. Nakamura et al. (2003); 39. Nelson et al (1951); 40. Neville and Watters (1983); 41. Nixon and Harper (1972); 42. Oftedal (1993); 43. Oftedal et al. (1993); 44. Oftedal et al. (2014); 45. Osthoff et al. (2007); 46. Osthoff et al. (2009); 47. Osthoff et al. (2012); 48. Park and Haenlein (2006); 49. Peddemors et al. (1989); 50. Riet-Sapriza et al. (2012); 51. Schryver et al. (1986); 52. Selvaggi et al. (2014); 53. Sowls et al. (1961); 54. Stewart et al. (1983); 55. Tenner (1956); 56. Trillmich and Lechner (1986); 57. Urashima et al. (1997); 58. Urashima et al. (2001); 59. Wamberg and Tauson (1998); 60. Widdowson (1984); 61. Whittier et al. (2011); 62. Zurowski et al. (1974).

Table A-2. Effects of dietary BCAA supplementation to lactating sows on growth of suckling piglets

BCAA, Lys and CP content in basal diet (%)					BCAA supplementation (%)			Total BCAA in supplemental diet (%)			Milk DM yield	Litter weigh gain of piglets	Ref.
Leu	Ile	Val	Lys	CP	Leu	Ile	Val	Leu	Ile	Val			
1.36	0.5	0.72	0.9	14.5	0	0.35-0.7	0	1.36	0.85-1.2	0.72	↑	↑	1
1.36	0.5	0.72	0.9	14.5	0	0	0.35-0.7	1.36	0.5	1.07-1.42	↑	↑	1
0.95	0.58	0.61	0.8	14.2	0	0	0.26-0.37	0.95	0.58	0.77-0.98	x	NC[a]	2
0.95	0.58	0.61	0.8	14.2	0	0	0.26-0.37	0.95	0.58	1.15	x	NC[b]	2
0.95	0.58	0.61	0.8	14.2	0	0	0.26-0.37	0.95	0.58	1.15	x	↑[c]	2
0.95	0.58	0.94	1.2	20.5	0	0	0.23-43	0.95	0.58	1.17-1.37	x	NC[d]	2
0.95	0.58	0.94	1.2	20.5	0	0	0.23-43	0.95	0.58	1.17-1.37	x	NC[e]	2
0.95	0.58	0.94	1.2	20.5	0	0	0.23-43	0.95	0.58	1.17-1.37	x	↑[f]	2
1.31	0.64	0.75	0.90	14.3	0	0	0.1-0.4	1.31	0.64	0.85-1.15	x	↑	3
1.57	0.68	0.8	0.9	15.5	0.4	0	0	1.97	0.68	0.8	NC	NC	4
1.57	0.68	0.8	0.9	15.5	0	0.4	0	1.97	1.08	0.8	NC	NC	4
1.57	0.68	0.8	0.9	15.5	0	0	0.4	1.97	0.68	1.2	NC	↑	4
1.18	0.65	0.45	1.01	15.5	0	0	0.1-1.0	1.18	0.65	0.55-1.45	↑	↑	5

[a]All sows with the average total number of pigs weaned/litter being 9.8 to 10.0.
[b]Sows with the average total number of pigs weaned/litter being 8.61 to 8.91.
[c]Sows with the average total number of pigs weaned/litter being 10.4 to 10.6.
[d]All sows with the average total number of pigs weaned/litter being 9.95 to 10.0.
[e]All sows with the average total number of pigs weaned/litter being 8.7-8.91.
[f]All sows with the average total number of pigs weaned/litter being 10.5.

NC = no effect
The sign “x” denotes the lack of data.

[1] Richert et al. (1997a); [2] Richert et al. (1997b); [3] Richert et al. (1996); [4] SA Moser et al. (2000); [5] Paulicks et al. (2003)

Table A-3. Composition of the basal diet for lactating sows on an as-fed basis.

Item	Basal diet[1], %
Ingredient	
Corn grain	57.50
Soybean meal, 44.5% CP	27.00
Cornstarch	3.07
Sugarcane molasses	2.78
Potassium chloride	0.10
Salt	0.35
Vitamin-mineral premix[2]	3.00
Vegetable oil	3.00
Dicalcium phosphate	2.50
Limestone	0.70
Chemical composition	
DM, %	90.0
ME, Mcal/kg	3.32
CP[3], %	17.5
Ca, %	1.04
Available P, %	0.54
Total P, %	0.79

[1] This basal lactation diet was provided ad libitum from farrowing to d 29 of lactation.
[2]The vitamin premix provided the following per kilogram of complete diet (as-fed basis): 46.7 mg of Mn as manganous oxide; 75 mg of Fe as iron sulfate; 103.8 mg of Zn as zinc oxide; 9.5 mg of Cu as copper sulfate; 0.72 mg of I as ethylenediamine dihydroiodide; 0.23 mg of Se as sodium selenite; 7,556 IU of vitamin A as vitamin A acetate; 825 IU of vitamin D3; 61.9 IU of vitamin E; 4.4 IU of vitamin K as menadione sodium bisulfate; 54.9 µg of vitamin B12; 13.7 mg of riboflavin; 43.9 mg of D-pantothenic acid as calcium pantothenate; 54.9 mg of niacin; and 1,650 mg of choline as choline chloride.
[3]The basal diet provided the following amino acids (%, as-fed basis): alanine, 0.94; arginine, 1.09; asparagine, 0.79; aspartate, 1.12; cysteine, 0.30; glutamine, 1.68; glutamate, 1.56; glycine, 0.86; histidine, 0.44; isoleucine, 0.74; leucine, 1.64; lysine, 0.90; methionine, 0.28; phenylalanine, 0.87; proline, 1.45; serine, 0.84; threonine, 0.65; tryptophan, 0.21; tyrosine, 0.71; and valine, 0.82.

Table A-4. Supplemental BCAA and L-alanine to the basal corn-soybean diet[1]

Treatment	Supplemental amino acid (g/100 g diet)				Total nitrogen
	L-Isoleucine	L-Leucine	L-Valine	L-Alanine	(mmol N/100 g diet)
Control	0.00	0.00	0.00	2.149	24.12
1.535% BCAA	0.32	0.82	0.395	1.07	24.12
3.07% BCAA	0.64	1.64	0.79	0.00	24.12

[1]The diets were made isonitrogenous with the addition of L-Ala at the expense of corn starch.

Table A-5. Number of sows in each parity

Treatment	Parity 2	Parity 3
Control	4	6
1.535 % BCAA	4	6
3.07 % BCAA	4	6

Values are number of sows in parities 2 or 3 for each group.

Table A-6. Milk consumption of piglets and feed intake by sows during lactation

Variable	Control			1.535% BCAA			3.07% BCAA			Pooled SEM	*P*-value		
	d 3	**d 15**	**d 29**	**d 3**	**d 15**	**d 29**	**d 3**	**d 15**	**d 29**		**Treatment**	**Day**	**T×D[1]**
Milk consumption by piglets, ml/kg BW/d	284	231	153	315	252	178	342	277	193	6.4	<0.01	<0.01	0.291
ADFI[2] by sows, g/kg BW/d	**D 3 - 15**	**d 15 - 29**	**d 0 - 29**	**d 3 - 15**	**d 15 - 29**	**d 0 - 29**	**d 3 - 15**	**d 15 - 29**	**d 0 - 29**				
	29.7	34.2	32.0	29.9	33.7	31.8	29.7	34.0	31.9	3.1	0.997	0.069	0.996

Values are means with pooled SEM, n = 10 sows/treatment group. See Table 6 for the number of piglets on d 3, 15 and 29 of lactation.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.
[2]ADFI = Average daily feed intake

Table A-7. Body weights of sows and piglets during lactation

Variable	Control				1.535% BCAA				3.07% BCAA				Pooled SEM	*P*-value		
	d 0	d 3	d 15	d 29	d 0	d 3	d 15	d 29	d 0	d 3	d 15	d 29		Treatment	Day	T×D[1]
Sow BW, kg	187	184	173	161	185	182	175	169	187	185	183	181	9.1	0.198	0.010	0.791
Piglet BW, kg	1.40	1.84	4.34	9.54	1.41	1.94	5.05	11.5	1.42	2.03	5.34	12.9	0.10	<0.001	<0.010	<0.010

Values are means with pooled SEM, n = 10 sows/treatment group. See Table 6 for the number of piglets on d 3, 15 and 29 of lactation.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.

Table A-8. Body-weight changes of sows and piglets during lactation

Variable	Control				1.535% BCAA				3.07% BCAA				Pooled SEM	*P*-value		
	d 0-3	d 3-15	d 15-29	d 0-29	d 0-3	d 3-15	d 15-29	d 0-29	d 0-3	d 3-15	d 15-29	d 0-29		Treatment	Day	T×D[1]
ADG of piglets, g/d	147	208	371	281	177	259	460	348	203	276	540	396	6.7	<0.001	<0.001	<0.010
BW loss of sows, kg	3.4	11.1	11.7	26.2	3.3	7.4	6.0	16.7	1.3	2.5	2.2	6.0	0.98	<0.001	<0.001	<0.010

Values are means with pooled SEM, n = 10 sows/treatment group. See Table 6 for the number of piglets on d 3, 15 and 29 of lactation.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.

Table A-9. Rates of survival and mortality of sow-reared piglets during lactation

Group	Number of piglets				Rate of mortality (%)			
	d 0	d 3	d 15	d 29	d 0[1]	d 0-3	d 0-15	d 0-29
Control	90	78	77	76	0	13.3	14.4	15.6
1.535% BCAA	90	84	84	84	0	6.7	6.7	6.7
3.07% BCAA	90	86	86	86	0	4.4	4.4	4.4

Values are the numbers of live piglets.
Data were analyzed by X^2-analysis, which revealed that BCAA supplementation reduced the rate of preweaning mortality (P = 0.021) as compared to control.
[1]Time of assignment to treatment groups.

Table A-10. Composition of milk in control and BCAA-supplemented sows during lactation

Variable	Control			1.535% BCAA			3.07% BCAA			Pooled SEM	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29		Treatment	Day	T×D[1]
DM, %	20.8	19.6	19.4	22.4	20.7	20.5	22.8	21.1	21.0	0.478	<0.01	0.023	0.983
CP, %	5.72	4.93	4.78	6.10	5.16	5.06	6.24	5.19	5.10	0.193	<0.01	0.011	0.985
Fat, %	8.63	8.04	8.12	9.12	8.44	8.48	9.26	8.66	8.74	0.245	<0.01	0.002	0.952
Lactose, %	4.99	5.16	5.10	5.61	5.59	5.39	5.71	5.66	5.57	0.206	<0.01	0.329	0.996
Carb, %	5.58	5.76	5.62	6.24	6.16	6.02	6.37	6.32	6.22	0.206	<0.01	0.329	0.981
Ash, %	0.87	0.87	0.88	0.94	0.94	0.94	0.93	0.93	0.94	0.0251	<0.01	0.884	0.993

Values are means with pooled SEM, n = 10 sows/treatment group.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.
Carb = carbohydrate (including lactose); CP = N% x 6.25.

Table A-11. Concentrations of free amino acids in the plasma of sows during lactation

Variable	Control			1.535% BCAA			3.07% BCAA			Pooled SEM	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29		Treatment	Day	T×D[1]
Ala	369	492	517	482	600	607	596	640	649	42	<0.001	<0.001	0.485
Arg	103	121	150	147	173	177	161	181	182	15	<0.001	0.001	0.632
Asn	58	63	80	83	85	85	96	92	95	5.9	<0.001	0.052	0.037
Asp	32	34	36	34	33	36	35	34	38	2.0	0.353	0.005	0.789
b-Ala	17	18	19	18	18	19	18	19	19	0.59	0.021	0.038	0.056
Cit	60	64	74	70	78	90	86	85	91	6.7	<0.001	0.004	0.597
Cys	229	235	236	231	235	239	243	252	255	14	0.100	0.555	0.986
Gln	564	567	572	620	628	632	654	664	670	26	<0.001	0.717	0.990
Glu	84	72	80	113	121	125	132	140	143	18	<0.001	0.494	0.461
Gly	947	964	1003	949	981	1018	1029	981	1023	54	0.429	0.343	0.822
His	76	69	80	80	75	78	91	79	80	4.2	0.005	0.008	0.161
Ile	70	91	114	130	139	146	169	195	219	14	<0.001	<0.001	0.545
Leu	148	167	210	260	286	295	341	397	403	30	<0.001	0.011	0.831
Lys	124	126	130	138	144	147	156	164	163	8.1	<0.001	0.265	0.984
Met	37	35	34	41	41	41	41	42	50	3.0	<0.001	0.354	0.083
Orn	69	76	102	76	77	101	78	77	105	6.1	0.465	<0.001	0.882
Phe	72	77	96	73	73	95	79	73	97	4.7	0.684	<0.001	0.66
Pro	237	246	247	300	306	320	358	376	385	17	<0.001	0.162	0.945
Ser	136	137	147	138	141	151	139	142	154	9.1	0.675	0.030	0.997
Tau	55	61	58	58	62	61	58	66	67	5.1	0.149	0.119	0.921
Thr	101	102	127	108	101	132	109	119	132	9.4	0.151	<0.001	0.692
Trp	49	49	52	53	51	57	54	60	64	3.8	<0.001	0.038	0.482
Tyr	58	74	91	79	90	92	82	91	99	6.3	<0.001	<0.001	0.194
Val	182	216	234	276	326	357	374	416	473	32	<0.001	<0.001	0.852

Values, expressed as µmol/L, are means with pooled SEM, n = 10 sows/treatment group.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction

Table A-12. Concentrations of free amino acids in plasma of piglets during lactation

Variable	Control			1.535% BCAA			3.07% BCAA			Pooled SEM	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29		Treatment	Day	T×D[1]
Ala	636	649	649	760	687	685	764	692	702	24	<0.001	0.002	0.036
Arg	216	136[b]	127[b]	238	171	173	272	179	183	12	<0.001	<0.001	0.881
Asn	78	89	101	106	110	117	119	120	124	4.8	<0.001	<0.001	0.132
Asp	14	19	20	18	21	22	19	23	25	1.2	<0.001	<0.001	0.754
b-Ala	26	26	27	28	28	26	28	26	27	1.0	0.504	0.336	0.011
Cit	132	76	81	164	82	90	178	107	109	5.4	<0.001	<0.001	0.149
Cys	166	165	158	166	169	162	172	171	162	7.1	0.510	0.130	0.973
Gln	709	542	538	748	605	602	809	646	688	32	<0.001	< 0.001	0.190
Glu	83	83	85	101	107	103	112	116	118	10	<0.001	0.240	0.906
Gly	1080	1091	1093	1152	1047	1060	1060	1160	1188	32	<0.001	0.647	0.968
His	98	95	108	97	95	112	97	95	114	4.6	0.854	<0.001	0.885
Ile	126	128	127	169	179	188	185	198	201	11	<0.001	0.132	0.732
Leu	198	178	186	223	227	233	240	242	247	18	<0.001	0.674	0.596
Lys	202	234	232	215	251	243	220	251	242	15	0.206	0.001	0.990
Met	78	80	77	83	85	79	86	89	91	3.4	<0.001	0.413	0.485
Orn	136	95	109	141	103	110	148	98	113	6.1	0.193	<0.001	0.613
Phe	93	93	96	95	96	98	96	96	97	3.1	0.014	0.794	0.022
Pro	500	509	510	526	534	540	541	568	573	14	<0.001	0.060	0.783
Ser	251	294	301	269	294	299	269	291	302	12	0.703	<0.001	0.617
Tau	113	128	123	141	137	157	143	170	167	8.0	<0.001	0.001	0.039
Thr	261	241	243	273	249	253	296	260	269	14	0.004	<0.001	0.962
Trp	42	46	48	43	48	59	44	51	59	2.2	0.029	<0.001	<0.001
Tyr	128	136	135	141	139	136	146	143	140	6.8	<0.001	0.532	0.002
Val	226	232	231	321	329	330	351	349	352	16	<0.001	0.787	0.547

Values, expressed as µmol/L, are means with pooled SEM, n = 20 piglets/treatment group.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction

Table A-13. Composition of free amino acids in sow's milk during lactation

Variable	Control			1.535% BCAA			3.07% BCAA			Pooled SEM	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29		Treatment	Day	T×D[1]
Ala	235	511	635	279	597	730	301	609	663	50	0.025	<0.01	0.682
Arg	36	64	72	52	73	91	54	87	109	6.7	<0.01	<0.01	0.220
Asn	29	108	227	46	132	259	51	141	267	11	<0.01	<0.01	0.829
Asp	158	462	480	192	526	593	313	546	639	30	<0.01	<0.01	0.075
b-Ala	15	30	41	15	30	45	15	28	41	2.6	0.534	<0.01	0.634
Cit	6.8	38	46	18	49	68	31	59	78	4.7	<0.01	<0.01	0.302
Cys	116	192	362	120	199	358	123	204	370	19	0.706	<0.01	0.994
Gln	326	1060	3517	439	1170	4025	472	1210	4196	112	<0.01	<0.01	0.024
Glu	366	1010	991	419	1140	1070	447	1200	1190	43	<0.01	<0.01	0.261
Gly	294	812	1180	307	830	1240	358	843	1300	57	0.096	<0.01	0.844
His	787	682	436	769	689	465	824	688	486	35	0.269	<0.01	0.737
Ile	5.2	17	21	14	31	38	29	43	52	3.7	<0.01	<0.01	0.546
Leu	23	43	51	52	77	93	64	85	103	8.1	<0.01	<0.01	0.772
Lys	32	52	68	35	66	86	50	66	85	4.5	<0.01	<0.01	0.063
Met	2.9	20	23	4.2	21	28	4.2	22	28	2.2	0.059	<0.01	0.448
Orn	29	45	64	30	48	67	30	52	58	3.9	0.548	<0.01	0.178
Phe	20	35	38	25	36	39	27	38	40	2.8	0.040	<0.01	0.776
Pro	34	82	114	46	115	147	49	123	168	12	<0.01	<0.01	0.195
Ser	45	221	403	51	231	474	63	238	381	18	0.012	<0.01	<0.001
Tau	1020	1280	1410	1110	1290	1430	1230	1290	1490	99	0.212	<0.01	0.722
Thr	86	133	426	89	140	437	85	145	420	23	0.862	<0.01	0.962
Trp	3.6	11	17	5.3	12	21	5.7	12	19	1.1	0.012	<0.01	0.378
Tyr	35	63	75	42	63	76	49	66	79	5.7	0.114	<0.01	0.689
Val	54	108	134	96	132	160	149	166	229	13	<0.01	<0.01	0.150

Values, expressed as µmol/L of whole milk, are means with pooled SEM, n = 10 sows/treatment group.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction

Table A-14. Composition of amino acids in proteins and peptides of sow's milk during lactation

Variable	Control			1.535% BCAA			3.07% BCAA			Pooled SEM	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29		Treatment	Day	T×D[1]
Ala	27.8	15.2	14.6	29.0	20.1	18.4	29.7	19.6	18.8	1.9	0.015	<0.001	0.690
Arg	15.0	7.48	7.30	17.0	13.7	13.1	19.6	16.4	15.5	1.5	<0.001	<0.001	0.961
Asp	16.2	10.1	9.72	17.5	13.0	11.8	18.2	14.8	13.9	1.1	<0.001	<0.001	0.157
Asn	17.6	9.90	9.48	18.6	13.5	10.6	19.1	16.0	13.3	1.0	<0.001	<0.001	0.142
Cys	6.82	3.22	3.08	7.02	3.48	3.35	7.56	3.89	3.73	0.50	0.030	<0.001	0.975
Glu	41.1	23.5	21.8	44.3	26.2	24.7	46.8	29.7	28.5	2.4	<0.001	<0.001	0.341
Gln	35.2	20.2	19.3	37.4	23.0	22.4	40.1	26.1	26.2	2.2	<0.001	<0.001	0.336
Gly	19.3	9.48	9.21	20.7	9.85	9.64	21.5	10.2	9.73	1.2	<0.001	<0.001	0.892
His	9.09	5.33	5.26	10.5	5.67	5.48	11.2	5.84	5.66	0.78	<0.001	<0.001	0.937
Ile	19.6	11.8	11.0	21.1	13.0	12.6	23.6	14.8	13.9	1.8	<0.001	<0.001	0.975
Leu	42.7	26.5	25.3	44.8	26.0	25.2	46.3	27.3	26.8	3.1	<0.001	<0.001	0.876
Lys	36.3	24.1	23.2	39.5	26.6	24.8	40.7	28.1	25.1	2.3	<0.001	<0.001	0.997
Met	9.90	6.18	5.97	10.6	6.77	6.34	11.3	6.98	6.56	0.70	<0.001	<0.001	0.413
Phe	15.8	9.83	9.45	16.7	10.4	10.1	17.2	11.5	10.8	1.1	<0.001	<0.001	0.827
Pro	42.1	32.8	31.2	44.6	35.6	34.5	46.8	37.2	36.0	3.7	<0.001	0.001	0.998
Ser	31.6	16.8	15.3	33.0	18.2	16.1	33.8	19.7	18.3	2.5	<0.001	<0.001	0.947
Thr	24.6	14.1	12.7	26.1	14.9	13.7	27.3	15.6	14.1	1.9	<0.001	<0.001	0.945
Trp	6.01	3.32	3.18	6.57	3.58	3.39	6.65	3.79	3.54	0.26	<0.01	<0.001	0.702
Tyr	18.7	11.9	10.6	19.2	12.4	11.4	20.7	13.5	12.0	1.5	<0.001	<0.001	0.946
Val	27.8	15.2	14.4	30.3	16.8	16.0	32.5	17.4	16.1	2.1	<0.001	<0.001	0.296

Values, expressed as mmol/L of whole milk, are means with pooled SEM, n = 10 sows/treatment group.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.

Table A-15. Concentrations of metabolites in the plasma of sows and piglets and in milk during lactation

Variable	Control			1.535% BCAA			3.07% BCAA			Pooled SEM	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29		Treatment	Day	T×D[1]
Sow's plasma													
Ammonia, µM	60	62	62	61	64	65	64	67	68	6.4	0.372	0.665	0.984
Urea, mM	2.88	2.97	3.07	2.74	2.83	2.94	2.59	2.68	2.80	0.28	0.224	0.487	0.992
Glucose, mM	5.38	5.41	5.53	5.47	5.51	5.55	5.59	5.64	5.69	0.12	0.018	0.261	0.978
Lactate, mM	1.27	1.49	1.69	1.28	1.52	1.76	1.25	147	1.66	0.21	0.979	<0.001	0.965
Pyruvate, µM	135	148	161	136	146	166	134	148	170	14	0.963	<0.001	0.987
Piglet's plasma													
Ammonia, µM	64	65	65	66	65	67	66	68	69	2.6	0.173	0.607	0.992
Urea, mM	2.37	2.57	2.79	2.36	2.45	2.55	2.33	2.42	2.51	0.25	0.533	0.20	0.964
Glucose, mM	5.32	5.34	5.44	5.41	5.64	5.73	5.65	5.84	5.84	0.13	<0.001	0.015	0.723
Lactate, mM	2.37	2.38	2.40	2.44	2.44	2.45	2.52	2.49	2.51	0.15	0.367	0.987	0.981
Pyruvate, µM	154	155	156	157	157	157	158	159	160	10	0.807	0.976	0.976
Milk													
Ammonia, mM	1.59	1.64	1.66	1.65	1.71	1.72	1.70	1.78	1.81	0.17	0.383	0.677	0.986
Urea, mM	7.23	4.79	5.26	7.04	4.65	5.09	6.92	4.67	5.14	0.58	0.832	<0.001	0.997
Glucose, mM	0.54	0.589	0.592	0.577	0.62	0.629	0.574	0.633	0.64	0.06	0.384	0.163	0.972
Lactate, mM	0.142	0.17	0.195	0.136	0.167	0.192	0.147	0.175	0.199	0.03	0.909	0.026	0.966
Pyruvate, µM	65	67	73	67	65	74	62	69	79	9.4	0.937	0.111	0.932

Values are means with pooled SEM, n = 10 sows/treatment group for plasma and milk, n = 20 piglets/treatment group
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction

Table A-16. Concentrations of lipids in the plasma of BCAA-supplemented sows and their piglets and in milk during lactation

Variable	Control			1.535% BCAA			3.07% BCAA			Pooled	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29	SEM	Treatment	Day	T×D[1]
Sow's plasma													
Cholesterol mM	2.28	2.24	2.27	2.21	2.23	2.25	2.20	2.24	2.20	0.29	0.742	0.894	0.997
TG, mM	0.449	0.451	0.463	0.434	0.444	0.450	0.436	0.445	0.449	0.06	0.499	0.273	0.979
FFA, mM	0.574	0.581	0.589	0.566	0.569	0.573	0.561	0.566	0.566	0.09	0.676	0.421	0.998
Glycerol mM	0.489	0.505	0.484	0.477	0.462	0.481	0.471	0.467	0.476	0.07	0.555	0.336	0.989
Piglet's plasma													
Cholesterol mM	1.86	1.96	1.99	1.86	1.92	1.94	1.88	1.95	1.96	0.19	0.689	0.708	0.995
TG, mM	0.421	0.429	0.433	0.427	0.434	0.439	0.429	0.437	0.443	0.07	0.234	0.279	0.667
FFA, mM	0.323	0.329	0.332	0.328	0.330	0.335	0.326	0.334	0.337	0.05	0.341	0.456	0.976
Glycerol mM	0.428	0.435	0.442	0.436	0.440	0.444	0.431	0.439	0.445	0.07	0.807	0.976	0.998
Milk													
Cholesterol mM	1.11	1.09	1.07	1.16	1.13	1.10	1.20	1.16	1.12	0.14	0.297	0.521	0.995
TG, mM	45.4	43.1	42.9	52.3	51.6	50.7	57.2	54.1	53.3	4.36	0.034	0.389	0.987
FFA, mM	0.621	0.607	0.596	0.737	0.726	0.717	0.745	0.735	0.729	0.08	0.029	0.132	0.778
Glycerol mM	0.379	0.358	0.349	0.384	0.377	0.368	0.390	0.384	0.377	0.09	0.454	0.141	0.966

Values are means with pooled SEM, n = 10/treatment group for sows, n = 20 piglets/treatment group, and n = 10 sows/treatment group for milk.
Data were analyzed by two-way ANOVA.
[1]T × D = Treatment × Day interaction effect.

Table A-17. Concentrations of TBARS in plasma of sows and piglets during lactation

Animals	Control			1.535% BCAA			3.07% BCAA			Pooled SEM	*P*-value		
	d 3	**d 15**	**d 29**	**d 3**	**d 15**	**d 29**	**d 3**	**d 15**	**d 29**		**Treatment**	**Day**	**T×D[1]**
Sows	1.99	2.37	2.84	1.76	2.17	2.59	1.70	2.02	2.41	0.18	0.004	<0.001	0.982
Piglets	4.90	4.06	3.61	5.03	3.96	3.04	5.11	4.15	3.14	0.48	0.804	<0.001	0.829

Values expressed as μmol/L are means with pooled SEM, n = 20 piglets/treatment group and n = 10 sows/treatment group.
Data are analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction

Table A-18. Supplemental monododium glutamate (MSG) or alanine to the basal diet for lactating sows[1]

Treatment	MSG	NaCl	L-Alanine	Cornstarch
		kg/100 kg diet		
Control	0.00	0.271	1.054	0.675
1 % MSG[1]	1.00	0.136	0.527	0.337
2 % MSG[2]	2.00	0.00	0.00	0.00

[1]Equivalent to 0.87% of supplemental glutamate in the final diet or (55% of glutamate in the basal diet)
[2]Equivalent to 1.74% of supplemental glutamate in the final diet or (110% of glutamate in the basal diet)
The molecular weight (MW) of glutamic acid = 147.1; The MW of MSG = 169.1; and the MW of Na = 23.0

Table A-19. Number of sows in each parity

Treatment	Parity 2	Parity 3
Control	5	5
1% MSG	5	5
2% MSG	5	5

Values are numbers of sows in parities 2 or 3 for each group

Table A-20. Milk consumption by piglets and feed intake by sows during lactation

Variable	Control			1% MSG			2% MSG			Pooled SEM	*P*-value		
	d 3	**d 15**	**d 29**	**d 3**	**d 15**	**d 29**	**d 3**	**d 15**	**d 29**		**Treatment**	**Day**	**T×D[1]**
Milk consumption by piglets, ml/kg BW/d	287	236	156	327	262	184	352	290	205	6.6	<0.001	<0.001	0.309
ADFI by sows[2], g/kg BW/d	**d 3-15**	**d 15-29**	**d 0-29**	**d 3-15**	**d 1-29**	**d 0-29**	**d 3-15**	**d 15-29**	**d 0-29**				
	30.3	33.9	32.8	30.9	34.5	32.1	30.5	34.6	32.8	4.1	0.998	0.144	0.995

Values are means with pooled SEM, n = 10 sows/treatment group. See Table 6 for the number of piglets on d 3, 15 and 29 of lactation.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.
[2]ADFI = Average daily feed intake

Table A-21. Body weights of sows and piglets during lactation

Variable	Control				1% MSG				2% MSG				Pooled SEM	*P*-value		
	d 0	d 3	d 15	d 29	d 0	d 3	d 15	d 29	d 0	d 3	d 15	d 29		Treatment	Day	T×D[1]
Sow BW, kg	189	185	179	168	187[a]	184	179	170	185	182	176	172	11	0.226	0.010	0.865
Piglet BW, kg	1.37	1.79	4.23	9.64	1.37	1.88	5.37	11.8	1.38	2.01	5.91	13.3	0.21	<0.001	<0.001	<0.001

Values are means with pooled SEM, n = 10 sows/treatment group. See Table 6 for the number of piglets on d 3, 15 and 29 of lactation.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.

Table A-22. Changes in body weights of sows and piglets during lactation

Variable	Control				1% MSG				2% MSG				Pooled SEM	*P*-value		
	d 0-3	d 3-15	d 15-29	d 0-29	d 0-3	d 3-15	d 15-29	d 0-29	d 0-3	d 3-15	d 15-29	d 0-29		Treatment	Day	T×D[1]
Piglet ADG, g/d	140	203	386	285	170	291	461	361	210	325	515	405	9.7	<0.001	<0.001	<0.001
Sow BW loss, kg	3.9	6.8	11.1	21.3	3.4	5.5	8.4	17.3	3.1	5.4	4.3	13.4	1.3	<0.001	<0.001	<0.001

Values are means with pooled SEM, n = 10 sows/treatment group. See Table 6 for the number of piglets on d 3, 15 and 29 of lactation.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.

Table A-23. Rates of mortality of sow-reared piglets during lactation

Group	Number of piglets				Rate of mortality (%)			
	d 0	d 3	d 15	d 29	d 0[1]	d 0-3	d 0-15	d 0-29
Control	90	77	75	75	0	14.4	16.7	16.7
1 % MSG	90	86	86	86	0	4.4	4.4	4.4
2 % MSG	90	87	87	87	0	3.3	3.3	3.3

Values are the numbers of live piglets
Data were analyzed by X^2-analysis, which revealed that MSG supplementation reduced the rate of preweaning mortality (P = 0.001)
[1]Time of assignment to treatment groups

Table A-24. Composition of milk from control and MSG-supplemented sows during a 29 d period of lactation

Variable	Control			1% MSG			2% MSG			Pooled SEM	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29		Treatment	Day	T×D[1]
DM, %	20.7	19.5	19.3	22.6	20.4	20.2	23.0	20.7	20.4	0.54	<0.001	0.111	0.989
CP, %	5.65	5.01	4.72	6.19	5.12	5.01	6.30	5.33	5.12	0.16	<0.001	0.027	0.990
Fat, %	8.52	8.11	8.06	9.24	8.36	8.23	9.66	8.60	8.47	0.44	<0.001	0.001	0.960
Lactose, %	5.06	4.94	5.02	5.60	5.39	5.37	5.48	5.29	5.26	0.31	<0.001	0.355	0.997
Carb, %	5.67	5.51	5.64	6.28	6.02	6.05	6.14	5.87	5.89	0.27	<0.001	0.284	0.985
Ash, %	0.86	0.87	0.88	0.89	0.90	0.91	0.90	0.90	0.92	0.02	<0.01	0.903	0.992

Values are means and pooled SEM. n = 10 per treatment group.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.
Carb = carbohydrate (including lactose); CP = N% x 6.25.

Table A-25. Concentrations of free amino acids in the plasma of sows during lactation

Variable	Control			1% MSG			2% MSG			Pooled SEM	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29		Treatment	Day	T×D[1]
Ala	411	549	576	538	669	676	664	713	724	51	<0.001	<0.001	0.559
Arg	115	135	168	164	193	198	180	202	209	19	<0.001	0.001	0.719
Asn	65	71	91	93	96	96	109	104	107	6.7	<0.001	0.046	0.039
Asp	37	39	42	39	39	42	40	39	45	3.4	0.418	0.004	0.822
b-Ala	17	18	18	17	18	19	18	18	19	0.60	0.233	0.031	0.159
Cit	67	72	83	78	88	101	96	95	101	8.2	<0.001	0.001	0.668
Cys	230	227	231	236	239	248	239	246	248	18	0.299	0.681	0.991
Gln	602	606	611	667	677	683	708	720	727	31	<0.001	0.669	0.984
Glu	89	90	92	133	142	147	156	164	168	14	<0.001	0.519	0.722
Gly	1078	1088	1110	1082	1102	1204	1134	1162	1196	52	0.523	0.394	0.909
His	74	78	79	78	80	86	78	83	86	6.7	0.511	0.664	0.789
Ile	74	81	89	78	87	93	85	90	97	6.4	<0.001	0.218	0.792
Leu	140	145	153	154	158	171	160	168	176	8.1	<0.001	0.198	0.849
Lys	116	121	123	122	130	138	127	136	145	12	0.169	0.362	0.989
Met	36	38	39	37	40	41	36	42	43	5.2	0.266	0.421	0.844
Orn	72	84	113	84	85	112	87	85	116	7.1	0.384	<0.001	0.831
Phe	75	80	86	76	83	94	78	86	95	5.3	0.626	<0.001	0.692
Pro	253	263	270	333	340	355	397	417	427	22	<0.001	0.209	0.956
Ser	125	131	135	130	131	138	126	130	141	9.9	0.699	0.159	0.989
Tau	54	60	56	57	60	60	57	66	65	5.0	0.122	0.136	0.949
Thr	106	109	111	108	114	116	89.5	92.6	96.9	9.7	0.211	0.187	0.779
Trp	47	48	52	53	55	61	55	61	68	4.2	<0.001	0.046	0.471
Tyr	60	56	62	61	64	62	63	65	66	6.2	0.184	0.157	0.628
Val	175	201	217	192	226	223	207	236	245	12	<0.001	<0.001	0.848

Values, expressed as µmol/L, are means with pooled SEM, n = 10 sows/treatment group.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.

Table A-26. Concentrations of free amino acids in plasma of piglets during lactation

Variable	Control			1 % MSG			2 % MSG			Pooled SEM	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29		Treatment	Day	T×D[1]
Ala	629	631	653	775	693	692	780	701	712	30	<0.001	0.002	0.031
Arg	207	141	131	224	189	191	249	197	201	13	<0.001	<0.001	0.826
Asn	86	98	111	117	121	129	131	132	136	5.3	<0.001	<0.001	0.187
Asp	15	21	22	20	23	24	21	25	28	1.3	<0.001	<0.001	0.668
b-Ala	27	27	30	32	31	28	31	28	30	1.1	0.504	0.323	0.035
Cit	128	73	77	146	91	99	163	118	121	6.3	<0.001	<0.001	0.217
Cys	173	168	164	166	170	162	172	171	162	8.1	0.449	0.165	0.988
Gln	688	514	520	724	612	619	755	637	659	27	<0.001	<0.001	0.223
Glu	87	85	90	110	117	119	124	128	130	5.8	<0.001	0.331	0.922
Gly	1103	1024	1008	1066	1050	1058	1060	1073	1094	37	<0.001	0.725	0.979
His	95	92	99	94	93	103	94	92	105	4.2	0.826	<0.001	0.898
Ile	120	122	126	140	144	154	155	162	165	9.7	<0.001	0.187	0.789
Leu	192	185	183	225	218	224	231	235	240	20	<0.001	0.566	0.652
Lys	199	212	226	193	205	218	190	212	224	13	0.307	0.001	0.978
Met	79	80	78	83	85	82	86	89	91	3.6	<0.001	0.368	0.549
Orn	140	93	91	156	96	94	153	98	95	8.9	0.669	0.598	0.788
Phe	98	104	106	96	107	103	96	102	103	3.4	0.157	0.614	0.293
Pro	512	532	533	551	559	566	567	596	602	16	<0.001	0.079	0.799
Ser	235	246	242	247	251	259	245	254	251	9.9	0.798	0.398	0.689
Tau	98	113	109	125	121	139	137	163	160	6.2	<0.001	0.001	0.028
Thr	243	266	270	253	275	283	278	287	296	16.6	<0.001	<0.001	0.898
Trp	43	46	48	46	51	59	47	53	60	3.4	0.038	<0.001	<0.001
Tyr	120	122	129	125	132	133	124	125	135	9.8	0.568	0.559	0.798
Val	220	223	226	239	236	241	247	255	259	11	<0.001	0.801	0.679

Values, expressed as µmol/L, are means with pooled SEM, n = 20 piglets/treatment group.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.

Table A-27. Composition of free amino acids in sow's milk during lactation

Variable	Control			1 % MSG			2 % MSG			Pooled SEM	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29		Treatment	Day	T×D[1]
Ala	261	567	705	310	662	810	335	676	736	56	0.016	<0.001	0.723
Arg	40	71	79	58	82	101	60	97	121	9.7	<0.001	<0.001	0.598
Asn	32	120	252	51	146	287	57	156	297	15	<0.001	<0.001	0.778
Asp	176	512	533	213	584	658	348	607	709	40	<0.001	<0.001	0.066
b-Ala	16	34	45	16	33	49	17	35	46	2.8	0.625	<0.001	0.701
Cit	7.5	42	51	20	54	76	35	65	87	7.2	<0.001	<0.001	0.556
Cys	129	213	401	133	221	397	136	226	410	25.4	0.729	<0.001	0.986
Gln	335	1180	3580	488	1300	3890	524	1350	4050	146	<0.001	<0.001	0.039
Glu	406	1120	1100	465	1260	1180	496	1340	1320	59	<0.001	<0.001	0.338
Gly	326	901	1310	340	921	1380	397	936	1440	71	0.125	<0.001	0.908
His	802	707	484	844	765	516	856	764	539	44	0.321	<0.001	0.819
Ile	5.8	19	23	6.1	21	27	6.4	27	27	4.1	0.279	<0.001	0.668
Leu	25	49	58	37	55	67	41	54	69	9.2	0.044	<0.001	0.848
Lys	35	57	75	39	73	95	55	74	95	5.1	<0.001	<0.001	0.133
Met	3.2	22	25	4.6	23	32	4.6	24	31	3.3	0.069	<0.001	0.556
Orn	31	50	71	33	54	74	34	58	65	5.6	0.487	<0.001	0.661
Phe	22	38	42	27	40	44	30	42	45	3.1	0.036	<0.001	0.809
Pro	37	91	126	51	128	164	54	136	187	15	<0.001	<0.001	0.195
Ser	47	225	427	66	256	526	71	265	423	19	0.023	<0.001	<0.001
Tau	1142	1420	1568	1229	1431	1590	1372	1435	1664	136	0.326	<0.001	0.819
Thr	90	142	467	93	149	479	88	156	460	30	0.778	<0.001	0.996
Trp	3.8	12	19	4.9	14	23	5.3	15	22	1.3	0.021	<0.001	0.456
Tyr	37	66	80	46	70	82	54	73	81	7.8	0.235	<0.001	0.789
Val	57	120	149	69	129	157	74	134	160	9.4	<0.001	<0.001	0.148

Values, expressed as µmol/L of whole milk, are means with pooled SEM, n = 10 sows/treatment group.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.

Table A-28. Composition of amino acids of proteins and peptides in sow's milk during lactation

Variable	Control			1 % MSG			2 % MSG			Pooled SEM	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29		Treatment	Day	T×D[1]
Ala	27.9	15.4	14.8	30.5	17.7	16.9	31.3	18.9	18.0	1.5	0.006	0.005	0.934
Arg	14.7	7.53	7.26	16.3	8.03	7.81	18.0	9.24	8.86	0.86	<0.001	<0.001	0.924
Asp	16.3	10.2	9.84	17.0	10.8	10.4	18.4	11.8	11.4	1.2	<0.001	<0.006	0.861
Asn	17.2	10.4	9.66	18.1	11.0	10.8	19.2	11.2	11.0	1.0	<0.001	<0.005	0.848
Cys	6.77	3.30	3.21	7.14	3.77	3.63	7.28	4.01	4.05	0.54	0.030	<0.001	0.975
Glu	41.7	23.5	23.0	43.4	25.4	24.3	45.8	26.4	25.7	1.5	<0.001	0.026	0.964
Gln	35.3	20.7	20.5	36.9	21.9	21.7	39.5	23.1	22.7	1.3	<0.001	0.024	0.952
Gly	19.1	9.39	9.24	20.4	10.8	10.1	21.0	11.6	11.2	0.95	0.042	0.002	0.853
His	9.13	5.40	5.22	9.66	5.89	5.63	10.5	6.34	6.20	0.62	0.006	<0.001	0.876
Ile	19.3	12.1	11.7	20.4	14.0	13.6	21.5	16.2	14.8	1.3	<0.001	<0.001	0.702
Leu	42.3	26.1	25.5	43.7	27.7	26.2	45.9	29.0	28.2	1.5	<0.001	<0.001	0.883
Lys	36.8	24.0	23.6	37.4	27.9	26.1	39.2	28.6	27.2	1.7	0.005	0.001	0.991
Met	10.1	6.23	6.09	10.8	6.65	6.27	12.6	7.18	6.84	0.51	0.038	<0.001	0.439
Phe	16.1	10.3	9.28	17.3	10.8	9.61	18.7	11.3	9.93	0.65	0.030	<0.001	0.879
Pro	42.8	33.2	32.0	45.2	37.3	36.5	47.3	38.5	37.4	2.9	<0.001	0.002	0.989
Ser	32.1	17.0	16.4	33.8	18.2	17.5	35.2	19.3	18.7	1.6	0.024	0.004	0.995
Thr	25.0	14.3	13.5	26.9	15.4	14.6	28.3	16.6	16.1	1.3	0.038	0.005	0.971
Trp	6.07	3.40	3.26	6.48	3.73	3.55	6.91	3.87	3.72	0.24	<0.001	0.003	0.702
Tyr	18.9	12.1	11.0	20.3	13.6	13.2	21.8	14.0	13.4	1.0	0.013	0.006	0.876
Val	27.5	15.0	14.6	28.9	16.4	15.3	30.2	17.8	17.1	1.2	0.010	0.034	0.949

Values, expressed as mmol/L of whole milk, are means with pooled SEM, n = 10 sows/treatment group.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.

Table A-29. Concentrations of metabolites in the plasma of sows and piglets and in milk during lactation

Variable	Control			1 % MSG			2 % MSG			Pooled SEM	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29		Treatment	Day	T×D[1]
Sow's plasma													
Ammonia, μM	61	63	63	60	61	64	65	65	66	7.2	0.399	0.689	0.982
Urea, mM	2.73	2.81	2.94	2.79	2.90	2.98	2.82	2.89	2.90	0.31	0.287	0.501	0.975
Glucose, mM	5.15	5.23	5.36	5.39	5.44	5.45	5.49	5.50	5.52	0.14	0.033	0.308	0.990
Lactate, mM	1.16	1.47	1.56	1.20	1.59	1.71	1.22	1.69	1.78	0.24	0.988	<0.001	0.988
Pyruvate, μM	127	133	154	128	135	158	130	141	163	12	0.970	0.001	0.975
Piglet's plasma													
Ammonia, μM	61	62	63	62	64	64	63	65	67	3.1	0.221	0.715	0.991
Urea, mM	2.19	2.26	2.44	2.25	2.35	2.51	2.29	2.48	2.59	0.29	0.566	0.280	0.977
Glucose, mM	5.36	5.34	5.39	5.31	5.41	5.45	5.40	5.45	5.50	0.188	0.514	0.161	0.997
Lactate, mM	1.08	1.09	1.15	1.12	1.14	1.18	1.20	1.27	1.30	0.18	0.415	0.995	0.983
Pyruvate, μM	146	149	150	151	150	155	150	153	156	14	0.926	0.998	0.975
Milk													
Ammonia, mM	1.49	1.55	1.61	1.55	1.62	1.69	1.59	1.70	1.76	0.21	0.569	0.753	0.980
Urea, mM	7.09	4.96	4.99	6.91	5.06	5.14	7.15	5.17	5.19	0.59	0.811	<0.001	0.992
Glucose, mM	0.52	0.57	0.58	0.58	0.60	0.61	0.59	0.61	0.62	0.06	0.426	0.196	0.984
Lactate, mM	0.155	0.159	0.167	0.149	0.171	0.180	0.154	0.178	0.188	0.043	0.841	0.244	0.972
Pyruvate, μM	62	64	69	63	67	70	66	69	75	7.8	0.966	0.239	0.979

Values are means with pooled SEM, n = 10/treatment group for sows, n = 20 piglets/treatment group, and n = 10 sows/treatment group for milk.
Data were analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.

Table A-30. Concentrations of lipids in the plasma of MSG-supplemented sows and their piglets and in milk during lactation

Variable	Control			1% MSG			2% MSG			Pooled	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29	SEM	Treatment	Day	T×D[1]
Sow's plasma													
Cholesterol mM	2.33	2.29	2.24	2.27	2.20	2.20	2.26	2.19	2.23	0.33	0.599	0.871	0.996
TG, mM	0.458	0.447	0.449	0.445	0.452	0.439	0.440	0.436	0.433	0.08	0.379	0.456	0.998
FFA, mM	0.559	0.573	0.577	0.554	0.560	0.566	0.568	0.560	0.559	0.07	0.586	0.327	0.979
Glycerol mM	0.458	0.469	0.474	0.466	0.461	0.467	0.461	0.476	0.473	0.08	0.431	0.556	0.990
Piglet's plasma													
Cholesterol mM	1.91	1.94	1.96	1.83	1.89	1.90	1.82	1.85	1.86	0.23	0.558	0.553	0.876
TG, mM	0.417	0.422	0.427	0.423	0.429	0.428	0.425	0.430	0.436	0.09	0.421	0.385	0.998
FFA, mM	0.314	0.321	0.322	0.321	0.326	0.329	0.321	0.324	0.327	0.08	0.546	0.477	0.981
Glycerol mM	0.412	0.423	0.433	0.421	0.431	0.435	0.418	0.429	0.433	0.09	0.777	0.658	0.997
Milk													
Cholesterol mM	1.18	1.12	1.11	1.19	1.17	1.13	1.21	1.17	1.14	0.18	0.388	0.229	0.988
TG, mM	44.8	43.7	41.8	53.7	52.5	51.4	59.6	57.5	54.1	4.79	0.032	0.287	0.995
FFA, mM	0.618	0.612	0.602	0.746	0.739	0.730	0.761	0.744	0.741	0.09	0.021	0.176	0.991
Glycerol mM	0.367	0.360	0.351	0.377	0.365	0.361	0.381	0.376	0.368	0.07	0.268	0.321	0.999

Values are means with pooled SEM, n = 10/treatment group for sows, n = 20 piglets/treatment group, and n = 10 sows/treatment group for milk.
Data were analyzed by two-way ANOVA.
[1]T × D = Treatment × Day interaction effect.

Table A-31. Concentrations of TBARS in plasma of sows and piglets during lactation

Animals	Control			1 % MSG			2 % MSG			Pooled SEM	*P*-value		
	d 3	d 15	d 29	d 3	d 15	d 29	d 3	d 15	d 29		Treatment	Day	T×D[1]
Sows	1.83	2.44	2.97	1.65	2.09	2.31	1.59	1.98	2.25	0.19	0.018	<0.001	0.979
Piglets	5.15	4.15	3.54	5.09	3.89	2.95	5.13	3.84	2.94	0.61	0.544	<0.001	0.911

Values, expressed as µmol/L, are means with pooled SEM, n = 20 piglets/treatment group and n = 10 sows/treatment group.
Data are analyzed by two-way ANOVA.
[1]Treatment (T), Day (D) and T × D = Treatment × Day interaction.

Table A-32. Effects of L-leucine, L-isoleucine, or L-valine or a mixture of BCAA on proliferation of porcine mammary epithelial cells (PMEC)

Day of culture	L-Leucine concentrations in culture medium (mmol/L)					Pooled SEM
	0.1	0.25	0.5	1	2	
Day 2	1.71^{d}	2.76^{c}	3.26^{b}	4.17^{a}	4.26^{a}	0.23
Day 4	0.76^{d}	1.22^{c}	1.68^{b}	2.19^{a}	2.24^{a}	0.16
Day 6	0.33^{c}	0.66^{b}	0.75^{ab}	0.79^{a}	0.81^{a}	0.05
Day of culture	L-Isoleucine concentrations in culture medium (mmol/L)					Pooled SEM
	0.1	0.25	0.5	1	2	
Day 2	1.71^{c}	2.19^{c}	2.88^{b}	3.33^{ab}	3.61^{a}	0.24
Day 4	0.76^{d}	1.17^{c}	1.48^{b}	1.92^{a}	2.01^{a}	0.15
Day 6	0.33^{d}	0.65^{c}	0.7^{bc}	0.74^{ab}	0.77^{a}	0.03
Day of culture	L-Valine concentrations in culture medium (mmol/L)					Pooled SEM
	0.1	0.25	0.5	1	2	
Day 2	1.71^{d}	2.59^{c}	3.16^{b}	3.67^{a}	3.89^{a}	0.23
Day 4	0.76^{d}	1.21^{c}	1.64^{b}	2.15^{a}	2.20^{a}	0.15
Day 6	0.33^{c}	0.66^{b}	0.73^{ab}	0.77^{ab}	0.81^{a}	0.06
Day of culture	Concentrations of a mixture of each of the three BCAA in culture medium (mmol/L)					Pooled SEM
	0.1	0.25	0.5	1	2	
Day 2	1.71^{d}	2.89^{c}	3.44^{b}	4.29^{a}	4.67^{a}	0.25
Day 4	0.76^{d}	1.37^{c}	1.74^{b}	2.33^{a}	2.45^{a}	0.17
Day 6	0.33^{d}	0.71^{c}	0.79^{bc}	0.86^{ab}	0.91^{a}	0.06

Values, expressed as %/day are means with pooled SEM, n = 8 per treatment group.
Means in a row without a common superscript letter differ (P<0.05).

Table A-33. Effects of L-leucine, L-isoleucine, or L-valine or a mixture of BCAA on concentrations of protein in porcine mammary epithelial cells (PMEC)

Leu in medium (mmol/L)	0.1	0.25	0.5	1	2	Pooled SEM
Protein content in cells (μg/ml)	584[d]	876[c]	1120[b]	1230[ab]	1270[a]	64
Ile in medium (mmol/L)	0.1	0.25	0.5	1	2	Pooled SEM
Protein content in cells (μg/ml)	584[d]	772[c]	998[b]	1110[ab]	1170[a]	61
Val in medium (mmol/L)	0.1	0.25	0.5	1	2	Pooled SEM
Protein content in cells (μg/ml)	584[d]	809[c]	1030[b]	1190[a]	1210[a]	64
Mixture of three BCAA in medium (mmol/L)	0.1	0.25	0.5	1	2	Pooled SEM
Protein content in cells (μg/ml)	584[d]	918[c]	1160[b]	1300[ab]	1330[a]	74

Values are means with pooled SEM, n = 8 per treatment group.
Means in a row without a common superscript letter differ (P<0.05).

Table A-34. Effects of L-leucine on synthesis and degradation of proteins in primary mammary epithelial cells (PMEC)

Variable	Concentrations of L-leucine in culture medium (mmol/L)					Pooled SEM
	0.1	0.25	0.5	1	2	
Protein synthesis (nmol Phe/mg protein/3 h)	39.3[c]	49.5[b]	55.0[b]	65.0[a]	69.5[a]	4.7
Protein degradation (%/3 h)	7.13[a]	5.85[b]	5.02[b]	4.07[c]	3.35[c]	0.48

Values are means and pooled SEM, n = 8 per treatment group.
Means in a row without a common superscript letter differ (P<0.05)

Table A-35. Effects of L-isoleucine on synthesis and degradation of proteins in primary mammary epithelial cells (PMEC)

Variable	Concentrations of L-isoleucine in culture medium (mmol/L)					Pooled SEM
	0.1	0.25	0.5	1	2	
Protein synthesis (nmol Phe/mg protein/3 h)	39.0[c]	41.6[bc]	45.9[bc]	48.2[b]	56.0[a]	3.7
Protein degradation (%/3 h)	7.10[a]	6.05[b]	5.20[bc]	4.27[cd]	4.03[d]	0.46

Values are means and pooled SEM, n = 8 per treatment group.
Means in a row without a common superscript letter differ ($P<0.05$).

Table A-36. Effects of L-valine on synthesis and degradation of proteins in primary mammary epithelial cells (PMEC)

Variable	Concentrations of L-valine in culture medium (mmol/L)					Pooled SEM
	0.1	0.25	0.5	1	2	
Protein synthesis (nmol Phe/mg protein/3 h)	39.1^{d}	48.4^{c}	54.0^{bc}	60.1^{ab}	63.0^{a}	3.4
Protein degradation (%/3 h)	7.12^{a}	5.90^{b}	4.78^{c}	3.58^{d}	3.32^{d}	0.49

Values are means and pooled SEM, n = 8 per treatment group.
Means in a row without a common superscript letter differ (P<0.05).

Table A-37. Effects of a mixture of three BCAA on synthesis and degradation of proteins in primary mammary epithelial cells (PMEC)

Variable	Concentrations of a mixture of each of the three BCAA in culture medium (mmol/L)					Pooled SEM
	0.1	0.25	0.5	1	2	
Protein synthesis (nmol Phe/mg protein/3 h)	39.0^{d}	50.5^{c}	58.0^{b}	65.6^{a}	72.5^{a}	3.5
Protein degradation (%/3 h)	7.15^{a}	5.78^{b}	4.62^{c}	3.40^{d}	3.05^{d}	0.52

Values are means and pooled SEM, n = 8 per treatment group.
Means in a row without a common superscript letter differ (P<0.05).

APPENDIX B

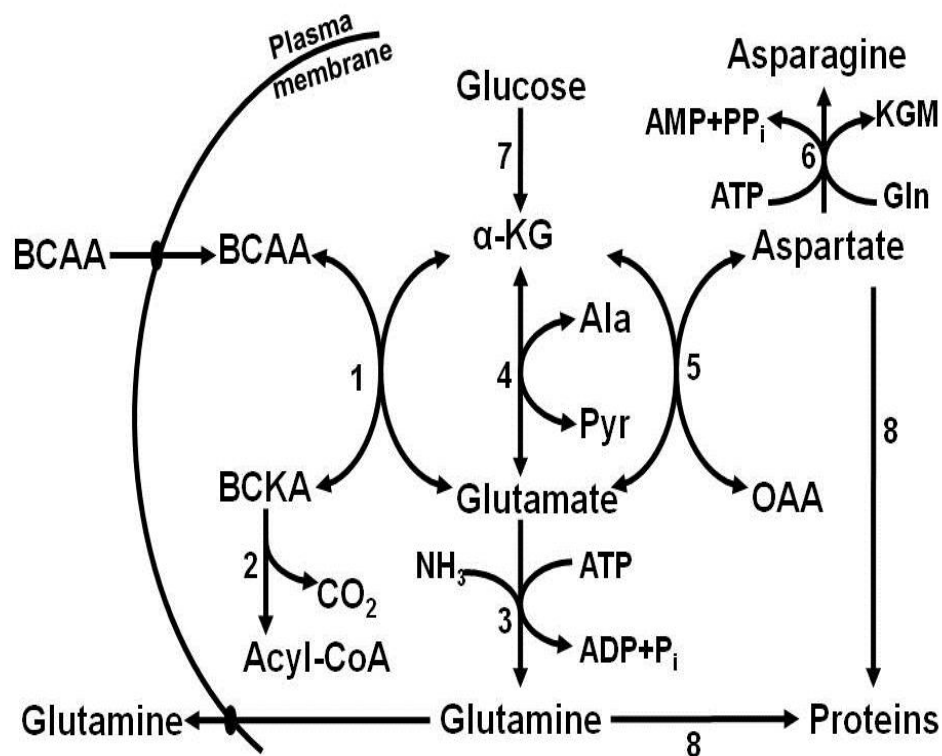

Figure B-1. Pathways for BCAA catabolism and amino acid synthesis in lactating porcine mammary tissue. Enzymes that catalyze the indicated reactions are: 1) BCAT= branched-chain aminotransferase; 2) BCKAD = branched-chain alpha-keto acid dehydrogenase; 3) GS = glutamine synthetase; 4) GOT = glutamate-oxaloacetate transaminase; 5) GPT = glutamate-pyruvate transaminase; 6) AS = asparagine synthetase; 7) glucose metabolism via glycolysis and the Krebs cycle; 8) protein synthesis. The corresponding a-ketoacids of leucine, isoleucine and valine are a-ketoisocaproate, a-keto-b-methylvalerate, and a-ketoisovalerate, respectively. Mammary tissue takes up BCAA and releases glutamine through specific transporters on the plasma membrane.

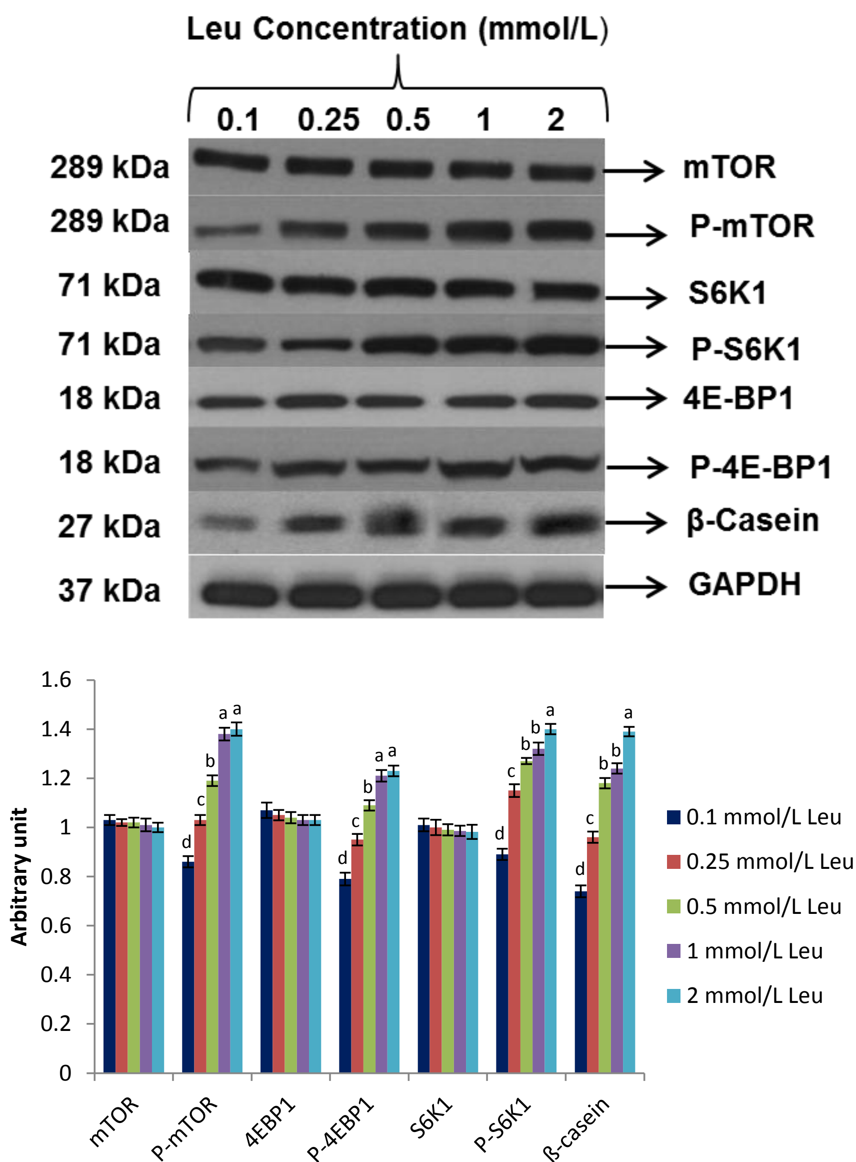

Figure B-2. Relative abundance of total and phosphorylated mTOR, S6K1, 4E-BP1, as well as β-casein in PMEC cultured in the presence of 0.1 to 2 mmol/L L-leucine. Values are mean ± SEM, n = 8. Different letters within each variable indicate statistical difference ($P < 0.05$).

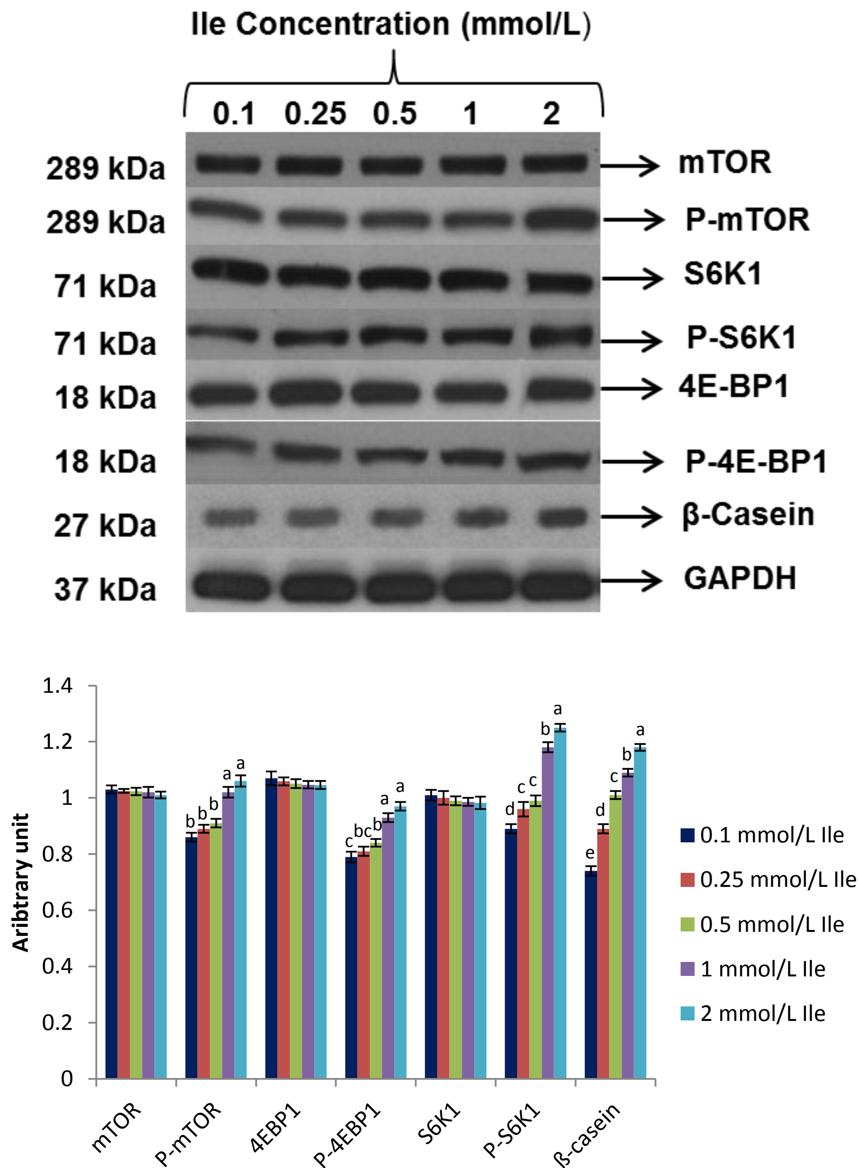

Figure B-3. Relative abundance of total and phosphorylated mTOR, S6K1, 4E-BP1, as well as β-casein in PMEC cultured in the presence of 0.1 to 2 mmol/L L-isoleucine. Values are mean ± SEM, n = 8. Different letters within each variable indicate statistical difference ($P < 0.05$).

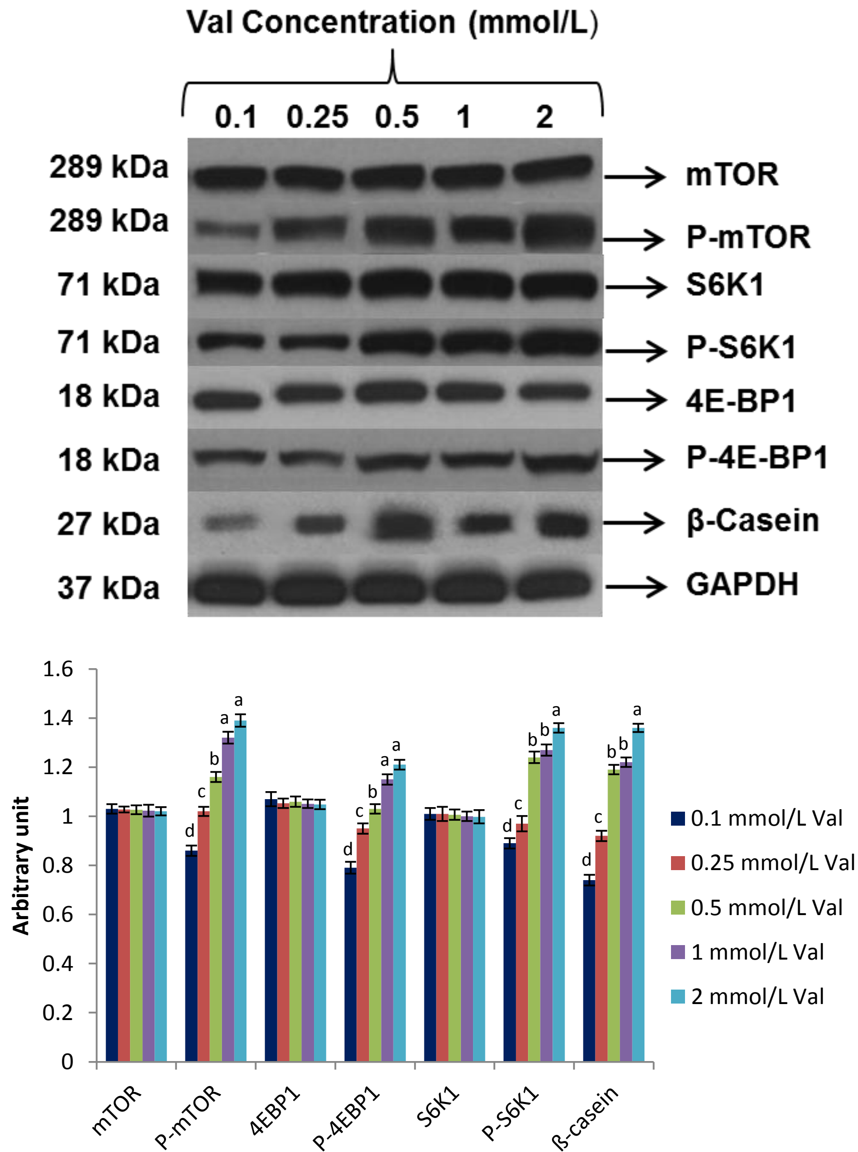

Figure B-4. Relative abundance of total and phosphorylated mTOR, S6K1, 4E-BP1, as well as β-casein in PMEC cultured in the presence of 0.1 to 2 mmol/L L-valine. Values are mean ± SEM, n = 8. Different letters within each variable indicate statistical difference ($P < 0.05$).

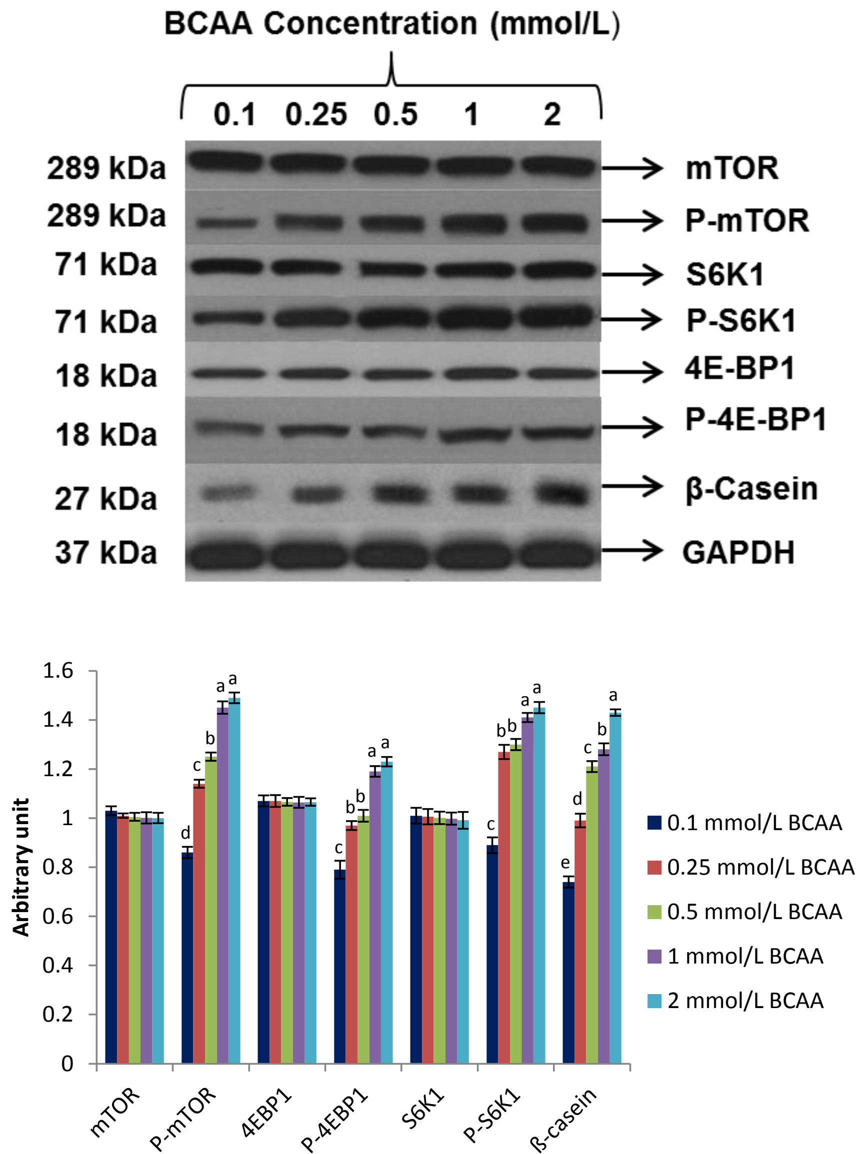

Figure B-5. Relative abundance of total and phosphorylated mTOR, S6K1, 4E-BP1, as well as β-casein in PMEC cultured in the presence of a mixture of the three BCAA (leucine, isoleucine and valine) at 0.1 to 2 mmol/L. Values are mean ± SEM, n = 8. Different letters within each variable indicate statistical difference ($P < 0.05$).

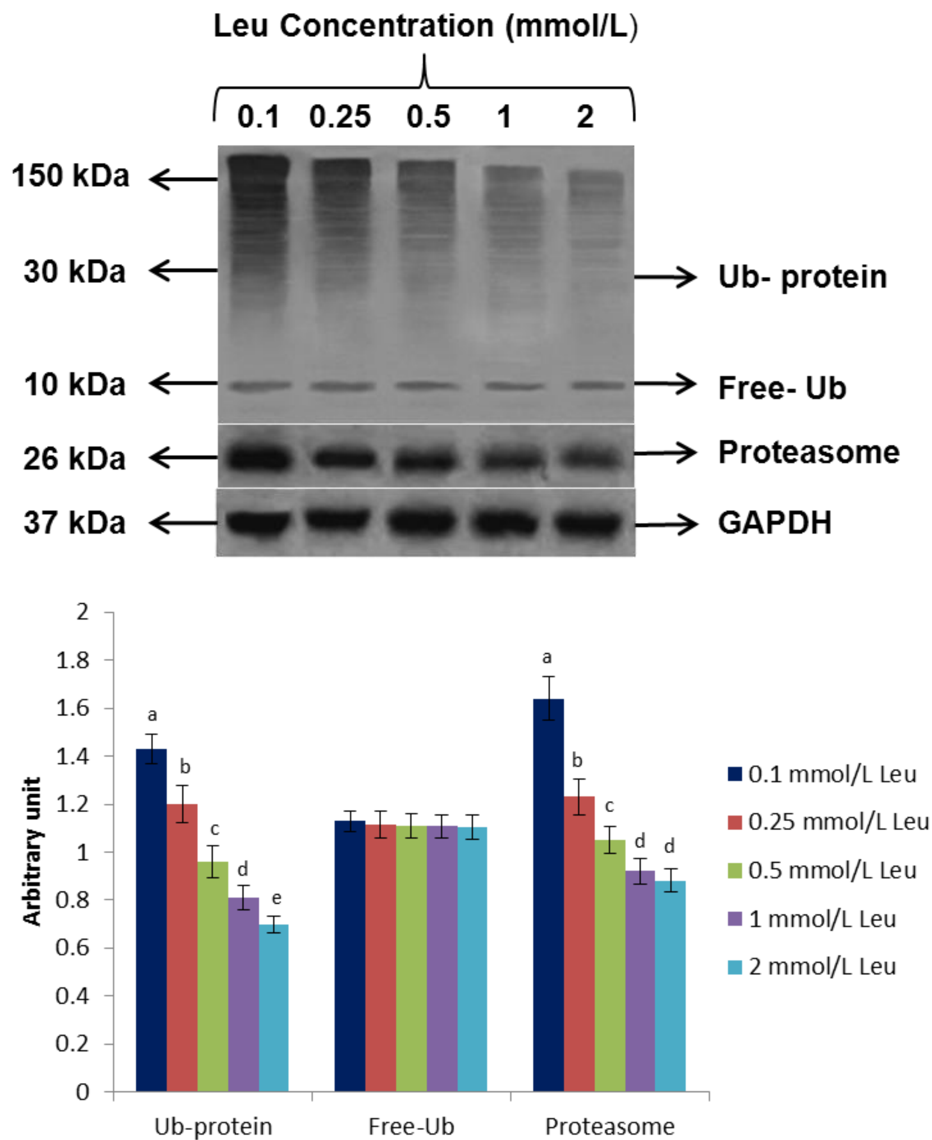

Figure B-6. Relative abundance of ubiquitinated proteins (Ub-protein), free ubiquitin (Free-Ub) and proteasome in mammary epithelial cells treated with 0.1 to 2 mmol/L L-leucine. Values are mean ± SEM, n = 8. Different letters within each variable indicate statistical difference ($P < 0.05$).

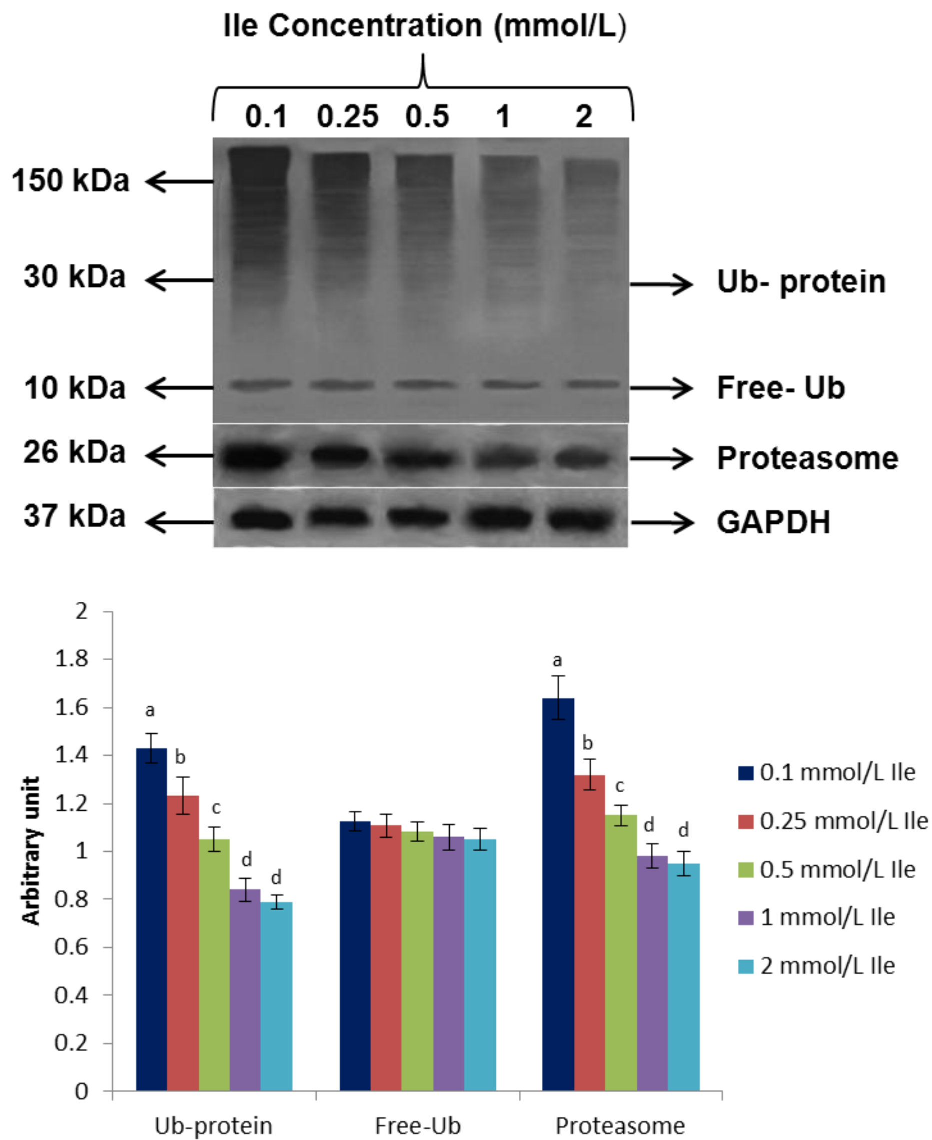

Figure B-7. Relative abundance of ubiquitinated proteins (Ub-protein), free ubiquitin (Free-Ub) and proteasome in mammary epithelial cells treated with 0.1 to 2 mmol/L L-isoleucine. Values are mean ± SEM, n = 8. Different letters within each variable indicate statistical difference ($P < 0.05$).

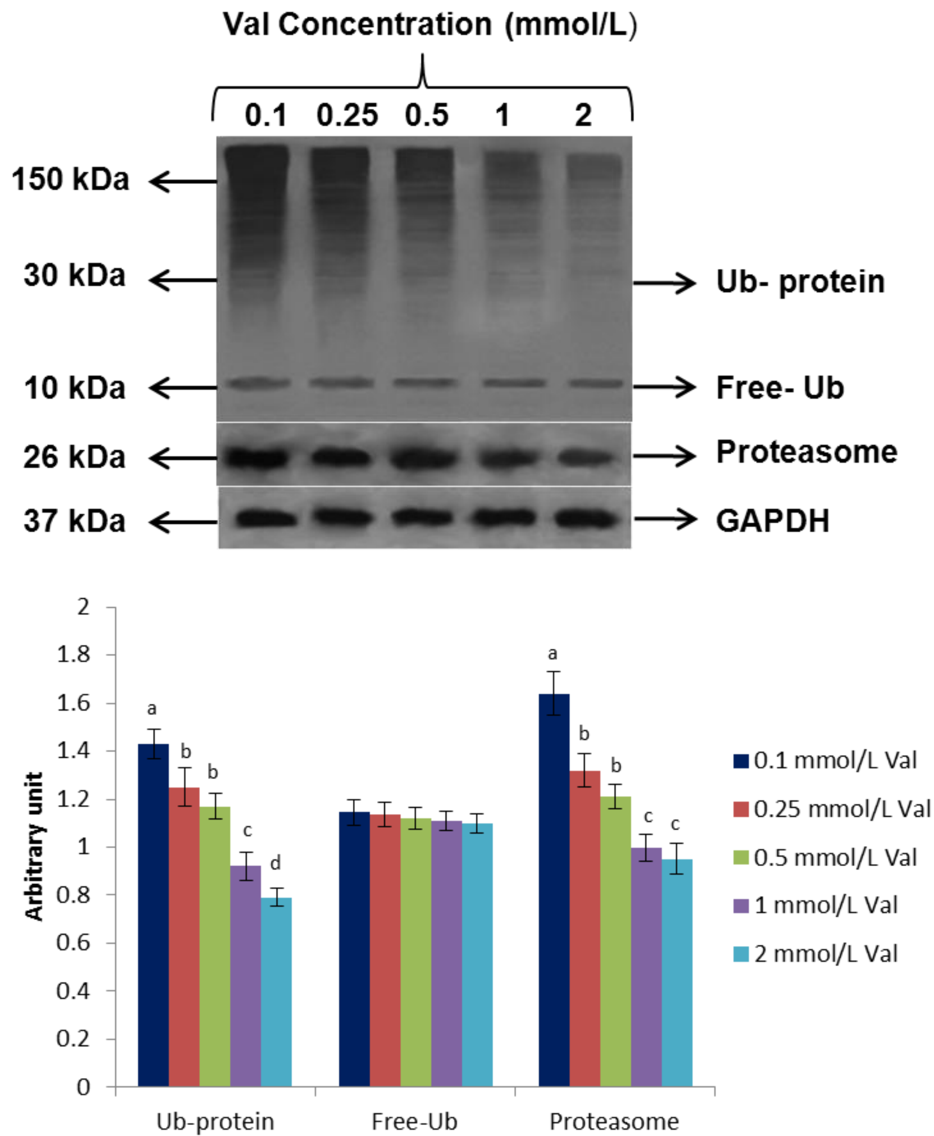

Figure B-8. Relative abundance of ubiquitinated proteins (Ub-protein), free ubiquitin (Free-Ub) and proteasome in mammary epithelial cells treated with 0.1 to 2 mmol/L L-valine. Values are mean ± SEM, n = 8. Different letters within each variable indicate statistical difference ($P < 0.05$).

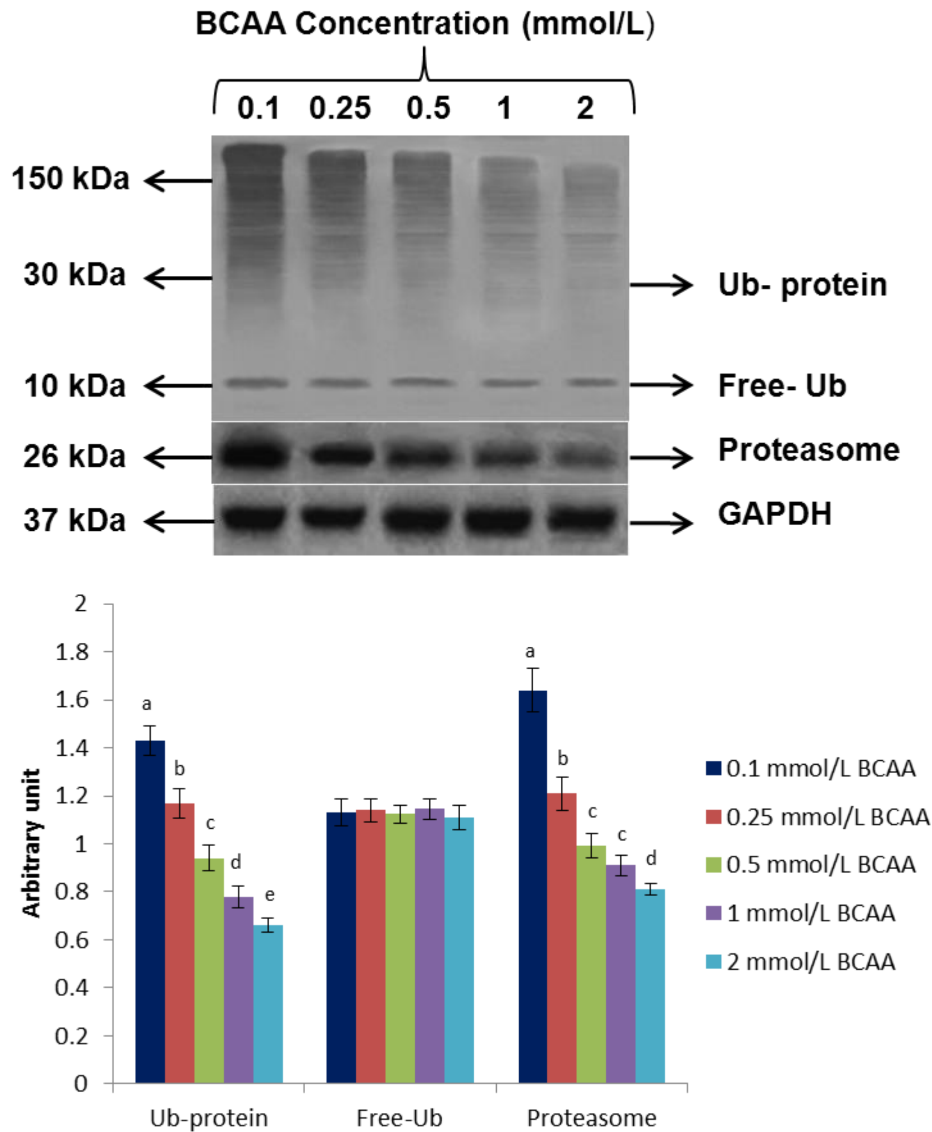

Figure B-9. Relative abundance of ubiquitinated proteins (Ub-protein), free ubiquitin (Free-Ub) and proteasome in PMEC cultured in the presence of a mixture of the three BCAA (leucine, isoleucine, valine) at 0.1 to 2 mmol/L. Values are mean ± SEM, n = 8. Different letters within each variable indicate statistical difference ($P < 0.05$).

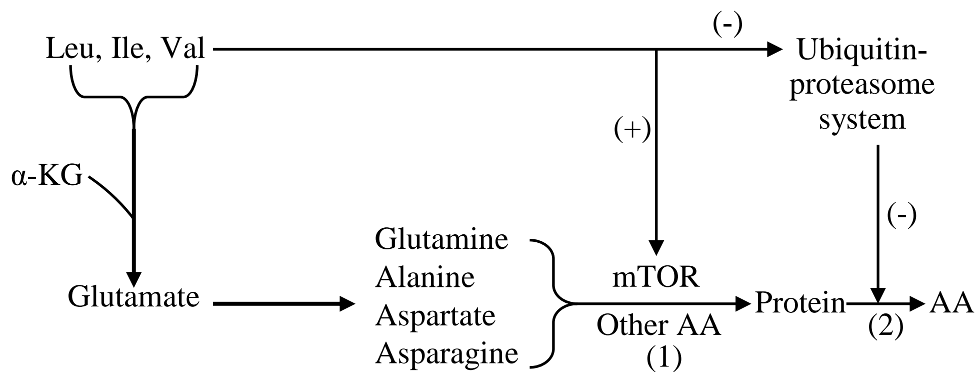

Figure B-10. A proposed mechanism whereby BCAA increases milk synthesis in mammary epithelial cells. BCAA increased phosphorylation of mTOR, 4EBP1 and S6K1 proteins which stimulated protein synthesis, while decreasing the abundance of ubiqitinated proteins and proteasome to inhibit protein degradation in PMEC. Formation of glutamate, glutamine, alanine, aspartate and asparagine also contributes to enhanced protein synthesis in the cells. α-KG = α-ketoglutarate. mTOR = mechanistic target of rapamycin. The signs (+) and (-) denote activation and inhibition, respectively. (1), protein synthesis; (2) protein degradation.