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ORIGINAL ARTICLE

Hormonal regulation of leucine catabolism in mammary epithelial cells

Jian Lei · Dingyuan Feng · Yongliang Zhang · Sudath Dahanayaka · Xilong Li · Kang Yao · Junjun Wang · Zhenlong Wu · Zhaolai Dai · Guoyao Wu

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Abstract Branched-chain amino acids (BCAA) are actively taken up and catabolized by the mammary gland during lactation for syntheses of glutamate, glutamine and aspartate. Available evidence shows that the onset of lactation is associated with increases in circulating levels of cortisol, prolactin and glucagon, but decreases in insulin and growth hormone. This study determined the effects of physiological concentrations of these hormones on the catabolism of leucine (a representative BCAA) in bovine mammary epithelial cells. Cells were incubated at 37 °C for 2 h in Krebs buffer containing 3 mM D-glucose, 0.5 mM L-leucine, L-[1-^{14}C]leucine or L-[U-^{14}C]leucine, and 0–50 μU/mL insulin, 0–20 ng/mL growth hormone 0–200 ng/mL prolactin, 0–150 nM cortisol or 0–300 pg/mL glucagon. Increasing extracellular concentrations of insulin did not affect leucine transamination or oxidative decarboxylation, but decreased the rate of oxidation of leucine carbons 2–6. Elevated levels of growth hormone dose dependently inhibited leucine catabolism, α-ketoisocaproate (KIC) production and the syntheses of glutamate plus glutamine. In contrast, cortisol and glucagon increased leucine transamination, leucine oxidative decarboxylation, KIC production, the oxidation of leucine 2–6 carbons and the syntheses of glutamate plus glutamine. Prolactin did not affect leucine catabolism in the cells. The changes in leucine degradation were consistent with alterations in abundances of BCAA transaminase and phosphorylated levels of branched-chain α-ketoacid dehydrogenase. Reductions in insulin and growth hormone but increases in cortisol and glucagon with lactation act in concert to stimulate BCAA catabolism for glutamate and glutamine syntheses. These coordinated changes in hormones may facilitate milk production in lactating mammals.

Keywords Insulin · Growth hormone · Cortisol · Prolactin · Glucagon · Lactation · Leucine

Abbreviations

BCAA	Branched-chain amino acids
BCAT	Branched-chain amino acid transferase
BCKA	Branched-chain α-ketoacids
BCKAD	Branched-chain α-ketoacid dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-Piperazineethanesulfonic acid
KIC	α-Ketoisocaproate

Introduction

The mammary gland extensively degrades branched-chain amino acids (BCAA) for the syntheses of glutamate, glutamine, alanine and aspartate during lactation (Li et al. 2009). Available evidence shows that BCAA catabolism is initiated by BCAA aminotransferase (BCAT), which exists in both mitochondria and cytoplasm (DeSantiago et al.

J. Lei (✉) · D. Feng · Y. Zhang · G. Wu
College of Animal Science, South China Agricultural University, Guangzhou 510642, People's Republic of China
e-mail: sgleijian@163.com

J. Lei · S. Dahanayaka · X. Li · K. Yao · J. Wang · G. Wu (✉)
Department of Animal Science and Faculty of Nutrition, Texas A&M University, College Station, TX 77843, USA
e-mail: g-wu@tamu.edu

J. Wang · Z. Wu · Z. Dai · G. Wu
State Key Laboratory of Animal Nutrition, China Agricultural University, Beijing 100193, China

1998; Harper et al. 1984). Branched-chain α-ketoacids (BCKA), products of BCAA transamination, undergo irreversible decarboxylation, which is catalyzed by the mitochondrial BCKA dehydrogenase (BCKAD) (Ichihara et al. 1973). BCKAD is a multienzyme complex, including E1 subunit (branched-chain α-ketoacid decarboxylase, an $\alpha 2\beta 2$ structure with thiamine pyrophosphate cofactor), E2 subunit (dihydrolipoyl transacylase with lipoate cofactor) and E3 subunit (dihydrolipoyl dehydrogenase with flavin adenine dinucleotide cofactor) (Harris et al. 2001). The E1α subunit is exclusively regulated by a tightly bound kinase and loosely bound phosphatase, leading to phosphorylation (inactivation) and de-phosphorylation (activation) of BCKAD, respectively (She et al. 2007). The abundance of BCAT is greater in the lactating mammary gland than in other tissues, including skeletal muscle, liver and small intestine (Li et al. 2009). In contrast, the activity of BCKAD is lower in mammary tissue compared with the liver and small intestine, indicating interorgan cooperation for BCAA degradation in lactating mammals (Lei et al. 2012a).

During lactation, BCAA catabolism in mammary tissue is increased markedly (DeSantiago et al. 1998; Viña and Williamson 1981) due to enhanced expression of BCAT (Harper et al. 1984; Ichihara et al. 1973). In rats, lactation increases both mRNA and protein levels for BCAT and BCKAD in mammary tissue, resulting in a tenfold increase in BCAT activity and the presence of the BCKAD complex in a fully activated state (DeSantiago et al. 1998). Elevated activities of BCAT and BCKAD enable the mammary gland to become a major site for BCAA removal during lactation. Thus, large amounts of BCAA are taken up by the mammary glands of livestock species, including sows, ewes and cows (Lei et al. 2012a).

There are marked changes in circulating levels of hormones during lactation. For example, in cows, lactation is associated with an increase in plasma concentrations of cortisol (Marinelli et al. 2007), prolactin (Chew et al. 1984a) and glucagon (Sartin et al. 1985), but a reduction in insulin (Sartin et al. 1985; de Boer et al. 1985) and growth hormone (Chew et al. 1984b). Results of studies with non-lactating animals indicate that BCAA catabolism in multiple peripheral tissues (e.g., liver, skeletal muscle and kidney) can be regulated by glucocorticosteroids (Beaufrere et al. 1989), insulin (Buse and Buse 1967; Hutson et al. 1980), growth hormone (Gibney et al. 2007) and glucagon (Buse et al. 1972). At present, little is known about hormonal regulation of BCAA degradation in mammary tissue. Therefore, this study was conducted with an established bovine mammary epithelial cell line (Lei et al. 2012b) to determine the effects of physiological concentrations of these hormones on leucine (a representative BCAA) catabolism.

Materials and methods

Materials

Hydrocortisone, glucagon and insulin were purchased from Sigma Chemicals (St. Louis, MO, USA). Recombinant bovine growth hormone and human prolactin were obtained from Genway Inc. (San Diego, CA, USA). The sources and preparations of other materials (including amino acids, reagents, culture medium and Western blot membranes) were the same as previously provided (Lei et al. 2012b).

Culture of cells

The bovine mammary epithelial cell line (the Mac-T cell) was obtained from American Type Culture Collection (Manassa, VA, USA). Cells were seeded in a 75-cm^2 (T-75) polystyrene flask containing 10 mL of Dulbecco's modified Eagle's medium supplemented with 1 % Gibco antibiotic–antimycotic liquid, 10 % fetal calf serum and 0.1 mU/mL bovine insulin. The medium was changed every 2 days. Before 70–80 % confluence was reached, cells were harvested by trypsinization, washed twice with Dulbecco's modified Eagle's medium, and then suspended in 10 mL of the Krebs bicarbonate buffer (pH 7.4, gassed with 95 % O_2/5 % CO_2; Wu et al. 1996) for use in transport and metabolic studies, as previously described (Lei et al. 2012b). An aliquot of this medium (100 μL) was removed for viable cell counts using a hemocytometer and trypan blue (Wu et al. 1996).

Determination of leucine transport by cells

Bovine mammary epithelial cells (2×10^6 viable cells) were added to 0.2 mL of oxygenated Krebs bicarbonate buffer (pH 7.4; Wu et al. 1994) containing 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 3 mM D-glucose, 0.5 mM L-leucine, 0.05 μCi L-[U-^{14}C]leucine, 0.05 μCi [^{3}H]inulin (an extracellular marker that does not enter cells), physiological concentrations of other amino acids found in the plasma of lactating cows (Lei et al. 2012b) and one of the following hormones: 0–50 μU/mL insulin, 0–15 nM cortisol, 0–200 ng/mL prolactin, 0–20 ng/mL growth hormone or 0–300 pg/mL glucagon. The concentrations of the hormones were chosen on the basis of published data on: (1) concentrations of insulin in the serum of late-pregnant cows (38 μU/mL) or early-lactating cows (13 μU/mL; Sartin et al. 1985); (2) concentrations of growth hormone in the plasma of late-pregnant cows (8–9 ng/mL) or lactating cows (4–5 ng/mL) (Chew et al. 1984b); (3) concentrations of prolactin in the plasma of late-pregnant cows (20–60 ng/mL) or calving cows

(100–150 ng/mL) (Chew et al. 1984a); (4) concentrations of cortisol in the plasma of pregnant cows (10 nM) or calving cows (38 nM; Marinelli et al. 2007); and (5) concentrations of glucagon in the plasma of non-pregnant non-lactating cows (147 pg/mL) (Sartin et al. 1985) and lactating cows (187 pg/mL) (de Boer et al. 1985). At the end of a 5-min incubation period, the amount of L-[U-^{14}C]leucine in cells was determined to indicate leucine transport (Lei et al. 2012b).

Determination of leucine degradation in cells

Bovine mammary epithelial cells suspended in the Krebs bicarbonate buffer (pH 7.4; gassed with 95 % O_2/5 % CO_2) were centrifuged at 600 g for 5 min. The cells were washed again with 10 ml of the Krebs bicarbonate buffer by centrifugation and then suspended in 1 mL of this buffer at a concentration of 20 × 10^6/mL. To study leucine catabolism, 2 × 10^6 viable cells were incubated at 37 °C in 1 mL of oxygenated (95 % O_2/5 % CO_2) Krebs bicarbonate buffer containing 20 mM HEPES, 3 mM D-α-glucose, 0.5 mM L-leucine, either L-[1-^{14}C]leucine or L-[U-^{14}C] leucine (approximately 2 × 10^5 DPM), other amino acids at physiological concentrations present in the plasma of lactating cows (Lei et al. 2012b) and one of the following hormones: 0–50 μU/mL insulin, 0–20 ng/mL growth hormone, 0–200 ng/mL prolactin, 0–150 nM cortisol or 0–300 pg/mL glucagon. After a 2-h incubation, collection of $^{14}CO_2$ produced from ^{14}C-labeled leucine and calculation of rates of leucine catabolism were performed as previously described (Lei et al. 2012b; Wu and Thompson 1988). Based on cell variation (Lei et al. 2012b) and statistical power calculation (Fu et al. 2010), there were at least six independent experiments for treatment with each hormone.

Determining the effects of hormones on syntheses of amino acids

Bovine mammary epithelial cells (2 × 10^6 viable cells) were incubated at 37 °C for 2 h in 1 mL of Krebs bicarbonate buffer containing 20 mM HEPES, 3 mM D-glucose, 0.3 mM NH_4Cl, 0 or 0.5 mM each of three BCAA (leucine, isoleucine and valine) and other amino acids (except for the absence of alanine, aspartate, asparagine, glutamate and glutamine) at physiological concentrations found in the plasma of lactating cows (Lei et al. 2012b). The incubation medium contained one of the following hormones: 0–50 (μU/mL) insulin, 0–20 ng/mL growth hormone, 0–200 ng/mL prolactin, 0–150 nM cortisol or 0–300 pg/mL glucagon. After a 2-h incubation, the reaction was terminated by addition of 0.2 mL of 1.5 M $HClO_4$, followed by addition of 0.1 mL of 2 M K_2CO_3 (Dai et al. 2012b). The neutralized extracts were analyzed for amino acids (Dai et al. 2012a; Li et al. 2011b).

Western blot analysis

Western blot analyses of the abundances of BCAT and BCKAD in mammary epithelial cells were performed as previously described (Lei et al. 2012b). Briefly, 2 × 10^6 viable cells were incubated at 37 °C for 2 h in oxygenated (95 % O_2/5 % CO_2) Krebs bicarbonate buffer (1 mL) containing 20 mM HEPES, 3 mM D-α-glucose, 0.5 mM L-leucine, other amino acids at physiological concentrations present in the plasma of lactating cows (Lei et al. 2012b) and one of the following hormones: 0 or 10 ng/mL growth hormone, 0 or 50 nM cortisol or 0 or 150 pg/mL glucagon. After a 2-h incubation, cells were collected and lysed in 0.1 mL buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 0.5 mM sodium vanadate, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, 50 mM β-glycerophosphate, 1 mM benzamidine and 0.1 mM phenylmethylsulfonyl fluoride). The cell lysates were centrifuged at 10,000×*g* for 10 min at 4 °C, and the supernatant fluid was used for determination of protein concentration using the BCA method and bovine serum albumin as standard (Yao et al. 2012). All samples were adjusted to an equal concentration of protein. Before electrophoresis, all samples were diluted with 2× sodium dodecyl sulfate (SDS) 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 β-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 a 75 °C waterbath for 10 min. After cooling on ice, the sample solution was used for Western blot analysis (Kong et al. 2011). Each sample, which contained the same amount of protein (50 μg), was loaded onto NuPage 10 % Bis–Tris gel (Invitrogen) for SDS-PAGE (Xi et al. 2011). After electrophoresis, proteins in the gel were transferred to a nitrocellulose membrane under 12 V overnight, using the Bio-Rad Transblot apparatus. Membranes were blotted in 5 % fat-free dry 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 the following primary antibodies (Conway and Hutson 2000; She et al. 2007): overnight at 4 °C with gentle rocking: antibodies for tubulin (1:10,000), mitochondrial BCAT (1:10,000), cytosolic BCAT (1:10,000), total BCKAD E1α (1:10,000) or phosphorylated BCKAD E1α (1:50,000). After washing three times with TTBS, the membranes were incubated at room temperature for 3 h with a secondary antibody (peroxidase-labeled donkey anti-rat, anti-rabbit or anti-mouse IgG, Jackson Immuno Research) at 1:50,000. Finally, the membranes were washed with TTBS, followed by development using SuperSignal West Dura Extended Duration

Substrate according to the manufacturer's instructions (Pierce, Rockford, IL, USA). The signals were detected on Fujifilm LAS-3000 (Tokyo, Japan). All data were normalized to the values for tubulin in the same samples (Yao et al. 2012).

Statistical analysis

Values are expressed as mean ± SEM, with the number of independent experiments (n) given in table and figure legends. Results were analyzed statistically using SPSS (Statistical Package for Social Scientists, vision 18.0, SPSS Inc., Chicago, USA). Specifically, data on leucine metabolism and transport were analyzed by one-way and two-way ANOVA, respectively. The Duncan's multiple-range test was used to compare means of treatment groups when significant main effects were detected in one-way ANOVA (Wei et al. 2012). The Western blotting data were analyzed by the paired t test. P values ≤ 0.05 were considered to be significant.

Results

Effects of hormones on leucine transport

Insulin, growth hormone and prolactin did not affect ($P > 0.05$) leucine transport by mammary epithelial cells (Table 1). In contrast, cortisol and glucagon increased ($P < 0.05$) leucine transport by the cells. The rate of leucine transport was higher ($P < 0.05$) in the presence of 150 nM cortisol, compared with 300 pg/mL glucagon.

Table 1 Effects of hormones on L-leucine transport by bovine mammary epithelial cells

Hormone	Concentration in incubation medium	L-Leucine transport
Insulin, μU/mL	0	0.38 ± 0.03^{d}
	10	0.40 ± 0.03^{d}
	20	0.37 ± 0.04^{d}
	50	0.39 ± 0.03^{d}
Growth hormone, ng/mL	5	0.40 ± 0.03^{d}
	10	0.39 ± 0.04^{d}
	20	0.38 ± 0.03^{d}
Prolactin, ng/mL	50	0.39 ± 0.03^{d}
	100	0.37 ± 0.04^{d}
	200	0.40 ± 0.04^{d}
Cortisol, nM	20	0.51 ± 0.03^{c}
	50	0.65 ± 0.04^{b}
	150	0.79 ± 0.04^{a}
Glucagon, pg/mL	50	0.41 ± 0.03^{d}
	150	0.48 ± 0.04^{c}
	300	0.50 ± 0.03^{c}

Values expressed in nmol/10^6 cells per min are mean ± SEM, $n = 8$

Means sharing different superscript letters in a column differ ($P < 0.05$)

Effects of insulin on leucine catabolism

Effects of insulin on leucine catabolism by mammary epithelial cells are shown in Table 2. Addition of insulin (up to 50 μU/mL) to the incubation medium did not affect ($P > 0.05$) the rates of leucine transamination, KIC production and CO_2 production from leucine oxidative decarboxylation (i.e., leucine carbon-1). However, compared with the control, insulin at 20 and 50 μU/mL decreased ($P < 0.05$) the rates of CO_2 production from the oxidation of leucine carbons 2–6. As a result, the percentage of decarboxylated leucine oxidized to CO_2 was decreased ($P < 0.05$) by 31 %. The percentage of transaminated leucine released as KIC was not affected ($P > 0.05$) by insulin. Compared with the absence of insulin, insulin at 50 μU/mL did not affect ($P > 0.05$) the syntheses of glutamate, glutamine, alanine and aspartate from BCAA in mammary epithelial cells (Table 3).

Effect of growth hormone on leucine catabolism

The effects of growth hormone on leucine catabolism in mammary epithelial cells are summarized in Table 4. Increasing extracellular concentrations of growth hormone reduced ($P < 0.05$) leucine transamination, leucine

Table 2 Effects of insulin on leucine catabolism in bovine mammary epithelial cells

	Insulin concentration (μU/mL)				Pooled SEM
	0	10	20	50	
CO_2 from all carbons (A)	0.46^{a}	0.45^{a}	0.40^{b}	0.38^{b}	0.013
CO_2 from carbon-1 (B)	0.36	0.34	0.32	0.32	0.011
CO_2 from carbons 2 to 6 (C)	0.11^{a}	0.11^{a}	0.08^{b}	0.06^{b}	0.005
Net release of KIC (D)	1.39	1.42	1.24	1.39	0.040
Net transamination (E = B + D)	1.75	1.77	1.57	1.71	0.038
Transaminated leucine released as KIC (D/E), %	79.0	80.5	78.8	81.4	0.86
Decarboxylated leucine oxidized to CO_2 (A − B)/(5 × B), %	5.78^{a}	6.02^{a}	5.06^{ab}	4.00^{b}	0.36

Values expressed in (nmol/10^6 cells per 2 h) unless indicated otherwise are mean ± SEM, $n = 6$

Means sharing different superscript letters in a row differ ($P < 0.05$)

Table 3 Effects of hormones on the syntheses of amino acids from BCAA in bovine mammary epithelial cells

Amino acid	Absence of BCAA from incubation medium	Presence of BCAA in incubation medium (0.5 mM leucine, 0.5 mM isoleucine and 0.5 mM valine)					
		No addition	50 μU/ml Insulin	20 ng/mL Growth hormone	200 ng/mL Prolactin	150 nM Cortisol	300 pg/mL Glucagon
Alanine	0.18 ± 0.01^{e}	0.48 ± 0.02^{c}	0.50 ± 0.04^{c}	0.40 ± 0.01^{d}	0.52 ± 0.02^{c}	0.77 ± 0.04^{a}	0.62 ± 0.03^{b}
Aspartate	0.20 ± 0.01^{e}	0.56 ± 0.04^{c}	0.58 ± 0.05^{c}	0.47 ± 0.02^{d}	0.55 ± 0.03^{c}	0.93 ± 0.06^{a}	0.75 ± 0.04^{b}
Asparagine	0.10 ± 0.01^{e}	0.34 ± 0.02^{c}	0.35 ± 0.02^{c}	0.28 ± 0.01^{d}	0.37 ± 0.02^{c}	0.56 ± 0.02^{a}	0.42 ± 0.02^{b}
Glutamate	0.41 ± 0.02^{e}	0.94 ± 0.05^{c}	0.98 ± 0.06^{c}	0.78 ± 0.04^{d}	1.01 ± 0.07^{c}	1.64 ± 0.09^{a}	1.31 ± 0.05^{b}
Glutamine	0.97 ± 0.04^{e}	1.82 ± 0.10^{c}	1.90 ± 0.12^{c}	1.44 ± 0.08^{d}	1.94 ± 0.13^{c}	2.90 ± 0.16^{a}	2.36 ± 0.14^{b}

Values expressed in nmol/10^6 cells per 2 h are mean ± SEM, $n = 8$

Means within a row sharing different superscript letters differ ($P < 0.05$)

Table 4 Effects of growth hormone on leucine catabolism in bovine mammary epithelial cells

	Growth hormone concentration (ng/mL)				Pooled SEM
	0	5	10	20	
CO_2 from all carbons (A)	0.46^{a}	0.39^{b}	0.31^{c}	0.25^{d}	0.014
CO_2 from carbon-1 (B)	0.35^{a}	0.30^{b}	0.26^{c}	0.22^{d}	0.010
CO_2 from carbons 2 to 6 (C)	0.12^{a}	0.08^{b}	0.06^{c}	0.03^{d}	0.001
Net release of KIC (D)	1.57^{a}	1.32^{b}	1.13^{c}	0.87^{d}	0.056
Net transamination (E = B + D)	1.92^{a}	1.62^{b}	1.38^{c}	1.09^{d}	0.070
Transaminated leucine released as KIC (D/E), %	81.8	81.1	81.2	79.4	0.53
Decarboxylated leucine oxidized to CO_2 (A − B)/(5 × B), %	6.67^{a}	5.48^{b}	4.28^{c}	2.71^{d}	0.37

Values expressed in (nmol/10^6 cells per 2 h) unless indicated otherwise are mean ± SEM, $n = 6$

Means sharing different superscript letters in a row differ ($P < 0.05$)

oxidative decarboxylation, KIC production and oxidation of leucine carbons 2–6 in a dose-dependent manner. The percentages of transaminated leucine released as KIC was not affected ($P > 0.05$) by growth hormone. However, the percentage of decarboxylated leucine oxidized to CO_2 was suppressed ($P < 0.05$) by growth hormone in a dose-dependent manner. Compared with the absence of growth hormone (the no addition group), growth hormone at 20 ng/mL reduced ($P < 0.05$) the syntheses of glutamate, glutamine, alanine and aspartate from BCAA in mammary epithelial cells (Table 3).

The effects of growth hormone on the abundances of mitochondrial and cytosolic BCAT as well as total and phosphorylated BCKAD E1α in mammary epithelial cells are shown in Fig. 1. Growth hormone at 10 ng/mL decreased ($P < 0.01$) the abundance of the cytosolic BCAT protein by 44 %, but increased ($P < 0.01$) the level of phosphorylated BCKAD E1α by 51 %. Consequently, the P-E1α/total E1α ratio was elevated ($P < 0.01$) by 40 %. There was no effect ($P > 0.05$) of growth hormone on the protein abundances of mitochondrial BCAT and total BCKAD E1α in the cells.

Effects of prolactin on leucine catabolism

Increasing extracellular concentrations of prolactin from 0 to 200 ng/mL did not affect ($P > 0.05$) any parameters of leucine catabolism, including leucine transamination, KIC production, leucine oxidative decarboxylation, the percentage of transaminated leucine released as KIC or the percentage of decarboxylated leucine oxidized to CO_2 in mammary epithelial cells (Table 5). Likewise, rates of the syntheses of glutamate, glutamine, alanine and aspartate from BCAA did not differ ($P > 0.05$) between 0 and 200 ng/ml prolactin in these cells (Table 3).

Effect of cortisol on leucine catabolism

The effects of cortisol on leucine catabolism in mammary epithelial cells are summarized in Table 6. Increasing extracellular concentrations of cortisol from 0 to 150 nM increased ($P < 0.05$) leucine transamination, leucine oxidative decarboxylation and KIC production in a dose-dependent manner. The rate of production of CO_2 from carbons 2–6 was higher ($P < 0.05$) in the presence of 50

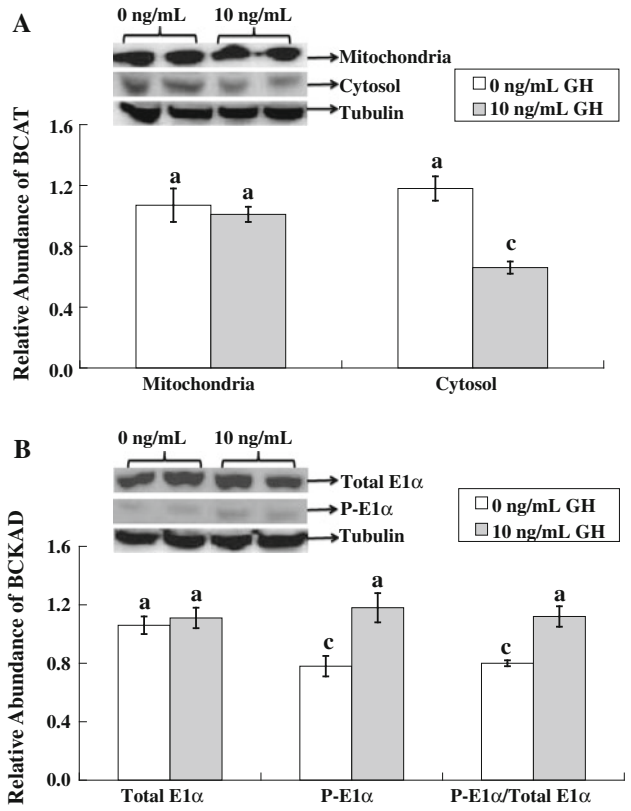

Fig. 1 Effects of growth hormone on the abundances of the mitochondrial and cytosolic BCAT proteins (**a**), as well as total BCKAD E1α and phosphorylated BCKAD E1α (**b**). Values are mean ± SEM, $n = 6$. Means with different letters within a parameter differ ($P < 0.05$)

Table 5 Effects of prolactin on leucine catabolism in bovine mammary epithelial cells

	Prolactin concentration (ng/mL)				Pooled SEM
	0	50	100	200	
CO_2 from all carbons (A)	0.40	0.39	0.41	0.37	0.021
CO_2 from carbon-1 (B)	0.32	0.29	0.30	0.28	0.016
CO_2 from carbons 2 to 6 (C)	0.08	0.10	0.10	0.09	0.009
Net release of KIC (D)	1.49	1.43	1.29	1.40	0.047
Net transamination (E = B + D)	1.82	1.72	1.60	1.67	0.049
Transaminated leucine released as KIC (D/E), %	82.3	83.5	80.4	83.3	0.94
Decarboxylated leucine oxidized to CO_2 (A − B)/(5 × B), %	5.64	6.49	6.78	6.46	0.61

Values expressed in (nmol/10^6 cells per 2 h) unless indicated otherwise are mean ± SEM, $n = 6$

For each variable, means within a row did not differ ($P > 0.05$)

Table 6 Effects of cortisol on leucine catabolism in bovine mammary epithelial cells

	Cortisol concentration (nM)				Pooled SEM
	0	20	50	150	
CO_2 from all carbons (A)	0.45^{d}	0.56^{c}	0.69^{b}	0.79^{a}	0.028
CO_2 from carbon-1 (B)	0.36^{d}	0.45^{c}	0.56^{b}	0.66^{a}	0.027
CO_2 from carbons 2 to 6 (C)	0.10^{b}	0.11^{b}	0.13^{a}	0.13^{a}	0.006
Net release of KIC (D)	1.30^{d}	1.49^{c}	1.80^{b}	2.22^{a}	0.075
Net transamination (E = B + D)	1.66^{g}	1.94^{e}	2.36^{c}	2.87^{a}	0.098
Transaminated leucine released as KIC (D/E), %	78.9	77.0	76.5	77.1	0.40
Decarboxylated leucine oxidized to CO_2 (A − B)/(5 × B), %	5.18^{a}	5.19^{a}	4.99^{a}	4.06^{b}	0.35

Values expressed in (nmol/10^6 cells per 2 h) unless indicated otherwise are mean ± SEM, $n = 6$

Means sharing different superscript letters in a row differ ($P < 0.05$)

and 150 nM cortisol than 0 and 20 nM cortisol. The percentages of transaminated leucine released as KIC was not affected ($P > 0.05$) by cortisol. However, the addition of 150 nM cortisol to the incubation medium reduced ($P < 0.05$) the percentage of decarboxylated leucine oxidized to CO_2, compared with 0–50 nM cortisol. In comparison with the absence of cortisol, cortisol at 150 nM enhanced ($P < 0.05$) the syntheses of glutamate, glutamine, alanine and aspartate from BCAA in mammary epithelial cells (Table 3).

Compared with the absence of cortisol, cortisol at 50 nM increased ($P < 0.01$) the abundance of the cytosolic BCAT protein by 56 %, but decreased ($P < 0.01$) the level of phosphorylated BCKAD E1α by 41 % in mammary epithelial cells (Fig. 2). As a result, the ratio of P-E1α to total E1α was reduced ($P < 0.01$) by 40 %. Cortisol did not affect ($P > 0.05$) the abundance of the mitochondrial BCAT protein or the level of total BCKAD E1α in these cells (Fig. 2).

Effects of glucagon on leucine catabolism

The effects of glucagon on leucine catabolism in mammary epithelial cells are summarized in Table 7. The rates of leucine degradation did not differ ($P > 0.05$) between 0 and 50 pg/mL glucagon. Increasing the concentration of glucagon from 50 to 150 pg/mL increased ($P < 0.05$) leucine transamination, KIC production and leucine oxidative decarboxylation, without affecting the percentage of transaminated leucine released as KIC or the percentage of

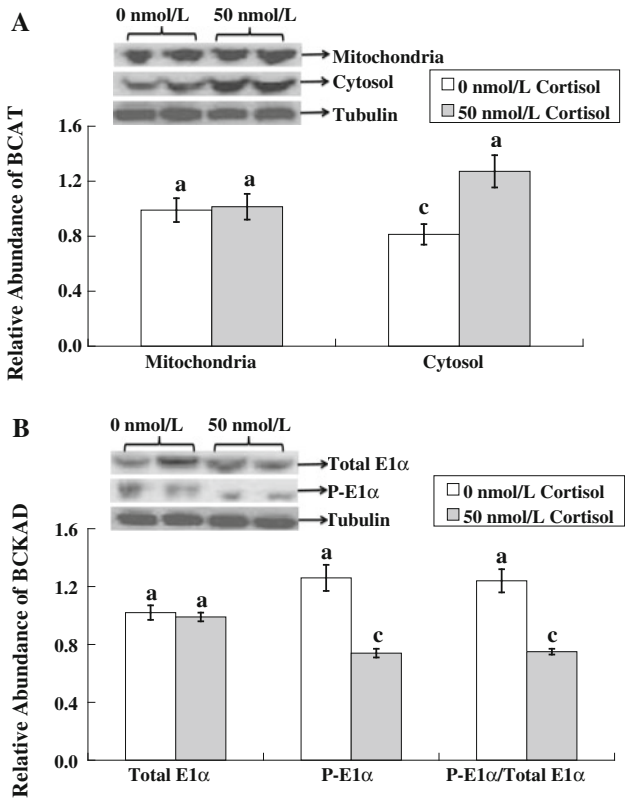

Fig. 2 Effects of cortisol on the abundances of the mitochondrial and cytosolic BCAT proteins (**a**), as well as total BCKAD E1α and phosphorylated BCKAD E1α (**b**). Values are mean ± SEM, $n = 6$. Means with *different letters* within a parameter differ ($P < 0.05$)

Table 7 Effects of glucagon on leucine catabolism in bovine mammary epithelial cells

	Glucagon concentration (pg/mL)				Pooled SEM
	0	50	150	300	
CO_2 from all carbons (A)	0.41^{c}	0.44^{c}	0.61^{a}	0.69^{a}	0.028
CO_2 from carbon-1 (B)	0.33^{b}	0.35^{b}	0.46^{a}	0.53^{a}	0.021
CO_2 from carbons 2 to 6 (C)	0.09^{b}	0.09^{b}	0.15^{a}	0.16^{a}	0.010
Net release of KIC (D)	1.14^{b}	1.26^{b}	1.51^{a}	1.53^{a}	0.052
Net transamination (E = B + D)	1.47^{b}	1.61^{b}	1.97^{a}	2.06^{a}	0.065
Transaminated leucine released as KIC (D/E), %	76.5	78.1	76.8	74.4	0.95
Decarboxylated leucine oxidized to CO_2 (A − B)/(5 × B), %	5.47	5.33	6.75	6.24	0.52

Values expressed in (nmol/10^6 cells per 2 h) unless indicated otherwise are mean ± SEM, $n = 6$

Means sharing different superscript letters in a row differ ($P < 0.05$)

decarboxylated leucine oxidized to CO_2. The rates of leucine degradation did not differ ($P > 0.05$) between 150 and 300 pg/mL glucagon. Compared with the absence of glucagon, glucagon at 300 pg/mL enhanced ($P < 0.05$) the syntheses of glutamate, glutamine, alanine and aspartate from BCAA in mammary epithelial cells (Table 3).

Glucagon at 150 pg/mL increased ($P < 0.01$) the abundances of the mitochondrial BCAT protein and total BCKAD E1α by 95 and 25 %, respectively, while decreasing ($P < 0.01$) the level of phosphorylated BCKAD E1α by 33 % in mammary epithelial cells (Fig. 3). As a result, the ratio of phosphorylated BCKAD E1α to total BCKAD E1α was reduced ($P < 0.05$) by 56 % in response to the glucagon treatment. Glucagon did not affect ($P > 0.05$) the abundance of the cytosolic BCAT protein in the cells.

Discussion

BCAA are not only substrates for protein synthesis, but are also signaling molecules to regulate metabolic pathways (Li et al. 2011a; Wilson et al. 2011; Wu 2009; Yin et al. 2010). Both metabolic and enzymological studies have

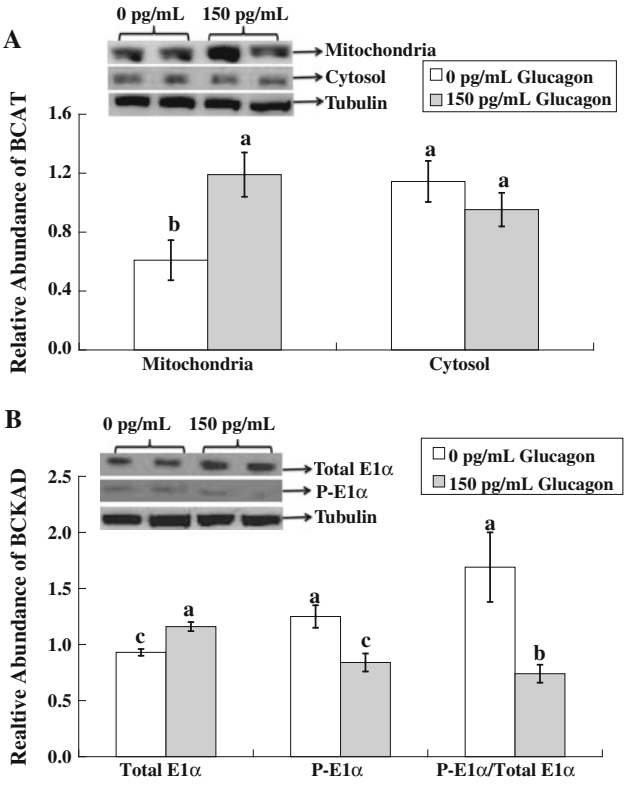

Fig. 3 Effects of glucagon on the abundances of the mitochondrial and cytosolic BCAT proteins (**a**), as well as total BCKAD E1α and phosphorylated BCKAD E1α (**b**). Values are mean ± SEM, $n = 6$. Means with *different letters* within a parameter differ ($P < 0.05$)

shown that BCAA provide an amino group for the syntheses of glutamate, glutamine, aspartate and alanine in the lactating mammary gland (Brosnan and Brosnan 2012; Li et al. 2009; Ren et al. 2011a, b). These amino acids participate in a wide variety of metabolic reactions and play critical roles in neonatal health, growth and development (Haynes et al. 2009; Suryawan and Davis 2011; Wang et al. 2008; Wu et al. 2011a, b). Available evidence from studies with lactating sows shows that catabolism of BCAA is necessary for the synthesis of glutamate and glutamine by the lactating mammary gland to support lactogenesis (Li et al. 2009; Wu 2010). These biochemical reactions are enhanced in response to lactation, but the underlying mechanisms are unknown. Understanding hormonal regulation of BCAA degradation in mammary epithelial cells is expected to design new means to enrich glutamine plus glutamate in milk and promote lactogenesis (Kim and Wu 2009; Wu and Knabe 1994). Thus, this work extends the concept of functional amino acids in nutrition and health of humans and other animals (Fernstrom 2012; Geng et al. 2011; Gao et al. 2012; Hou et al. 2011, 2012; Liu et al. 2012; Satterfield et al. 2011, 2012; Tan et al. 2010, 2011, 2012; Wang et al. 2012).

The results of the present study indicate that physiological levels of insulin did not affect leucine transport (Table 1), leucine transamination or leucine decarboxylation, but reduced the oxidation of leucine carbons 2–6 via the Krebs cycle in mammary epithelial cells (Table 2). Likewise, Hutson et al. (1980) reported an inhibitory effect of insulin on leucine oxidation in perfused skeletal muscle from fed rats. Similarly, insulin had no effect on the uptake of leucine by mammary gland explants obtained from pregnant mice (Anderson and Rillema 1976) or the oxidative decarboxylation of leucine in the mammary gland of lactating rats (Viña and Williamson 1981) or sheep (Sinclair et al. 2009). Also, in lactating sheep treated with hyperinsulinemic euglycemic clamp, the proportion of whole-body leucine flux to the mammary gland was not altered (Sinclair et al. 2009). However, insulin inhibited the oxidation of [2-^{14}C]leucine and [U-^{14}C]leucine to CO_2 in the rat mammary tissue (Viña and Williamson 1981), which may help increase conversion of the carbon skeleton of leucine into lipids in the lactating gland. In support of this view, leucine is metabolized to produce acetyl-CoA or acetoacetate, which is efficiently utilized for lipogenesis in the lactating mammary tissue (Abraham et al. 1964). Thus, elevated levels of insulin can enhance lipogenesis in mammary glands (Jones et al. 1984) by suppressing the irreversible oxidation of leucine carbons. Conversely, a decrease in this hormone during lactation may promote the oxidation of BCAA as metabolic fuels, thereby sparing glucose while reducing the efficiency of utilization of dietary amino acid for milk protein synthesis.

Growth hormone affects mammalian BCAT activity in a tissue-specific manner (Shirai and Ichihara 1971). Gibney et al. (2007) also reported that administration of a supraphysiological dose of growth hormone to humans reduced whole-body leucine oxidation. Similarly, we found that growth hormone inhibited leucine transamination and oxidative decarboxylation in mammary epithelial cells (Table 4). These results may be explained by the following putative mechanisms. First, growth hormone has an anabolic effect on protein synthesis in cells (Bush et al. 2002), thereby decreasing the availability of leucine for catabolism. Second, the lipolytic action of growth hormone can result in elevated availability of free fatty acids for oxidation to generate acetyl-CoA, which then has an inhibitory effect on leucine oxidation in cells (Gibney et al. 2007; Wu and Thompson 1987). Third, the abundance of the cytosolic BCAT protein and the degree of BCKAD activation were dramatically reduced in mammary epithelial cells treated with growth hormone (Fig. 1). Similar effects of oleic acid have recently been reported for this cell type (Lei et al. 2012b). Intracellular concentrations of cAMP are elevated in response to growth hormone (Gibney et al. 2007), thereby stimulating cAMP-dependent kinase to phosphorylate BCKAD. At present, it is not known how an acute treatment of the cells with growth hormone for 2 h could rapidly lead to a reduction in the abundance of the cytosolic BCAT protein. We suggest that a covalent modification of the cytosolic BCAT protein in response to growth hormone may reduce the reactivity of the enzyme with the antibody used, resulting in lowered levels of the native BCAT protein. Nonetheless, a decrease in circulating levels of growth hormone during lactation may play a permissive role for elevated catabolism of BCAA in mammary epithelial cells. Because growth hormone reduces the synthesis of glutamate and glutamine from BCAA, increasing the concentrations of glutamate, glutamine and BCAA in the plasma of growth hormone-treated lactating cows may help further enhance protein synthesis and milk production by the lactating gland.

The catabolic effect of elevated levels of glucocorticoids on protein metabolism has been reported in mammals (Flynn et al. 2009). In rat skeletal muscle, glucocorticoids activate BCKAD to enhance BCAA degradation (Block et al. 1987). Similarly, glucocorticoids stimulate whole-body leucine oxidation in adult humans (Beaufrere et al. 1989) as well as BCAT activities in rat kidney and skeletal muscle (Shirai and Ichihara 1971). Conversely, a reduction in cortisol brought about by adrenalectomy results in a decrease of BCAT activity by 50 % (Shirai and Ichihara 1971). In the present study, increasing extracellular concentrations of cortisol increased the rates of leucine transamination and oxidative decarboxylation in mammary epithelial cells in a dose-dependent manner (Table 6). This

stimulatory effect of cortisol on leucine catabolism results, in part, from increases in abundance of the cytosolic BCAT protein and de-phosphorylation of the E1α subunit of the BCKAD (Fig. 2), as reported for skeletal muscle (Harris et al. 2001). These results indicate that cortisol is capable of upregulating BCAA transport [an initial step in the utilization of AA by cells (Suryawan et al. 2012)] and metabolism in mammary tissue. Thus, an increase in circulating levels of glucocorticoids with advanced lactation is responsible, in part, for enhanced catabolism of leucine in mammary epithelial cells.

Little information is available concerning the effect of prolactin on BCAA catabolism in mammary tissue. Anderson and Rillema (1976) reported that prolactin did not affect leucine uptake by mouse mammary tissue slice, but did stimulate leucine incorporation into protein during a 4-h incubation period. Presumably, the prolactin-induced increase in the utilization of leucine for protein synthesis would reduce the availability of this amino acid for catabolism. Prolactin signal transduction mechanisms in the mammary gland involve the Jak/Stat pathway (Watson and Burdon 1996). Interestingly, we did not observe an effect of physiological levels of prolactin on leucine degradation in mammary epithelial cells (Table 5). Thus, the activities of key enzymes (e.g., BCAT and BCKAD) in the pathway of BCAA degradation may not be regulated via the Jak/Stat pathway. In addition, our results suggest that prolactin is unlikely to be a major factor influencing BCAA catabolism in the lactating mammary gland.

Glucagon is another hormone that can affect BCAA metabolism in animal cells (Buse et al. 1972). Specifically, glucagon stimulates the uptake of leucine, isoleucine and valine by liver, while increasing intracellular proteolysis, leading to increased availability of these amino acids for catabolism (Mallette et al. 1969). Similarly, the results of the present study indicate a stimulatory effect of glucagon on leucine transamination and oxidative decarboxylation in mammary epithelial cells (Table 4). In support of these metabolic data, the Western blot analysis revealed that glucagon enhanced the abundances of the mitochondrial BCAT protein and E1α subunit of BCKAD, while reducing the level of the phosphorylated E1α (Fig. 3). This is in sharp contrast to the report of Robson et al. (1984) that the mammary glands of lactating rats lack a glucagon receptor. Likewise, lipogenesis in rat mammary tissue is not sensitive to glucagon (Jones et al. 1984). Interestingly, administration of glucagon can retard the growth of mammary aplastic carcinoma and prolong the survival of mice (Pavelić and Pavelić 1980), suggesting the presence of glucagon receptors on their mammary epithelial cells. It is possible that species differences exist in glucagon receptors or their translocation from the cytoplasm to the plasma membrane in response to extracellular glucagon. Elevated levels of glucagon in the plasma of lactating mammals may play an important role in stimulating BCAA catabolism and the synthesis of glutamate ad glutamine by mammary epithelial cells.

In conclusion, at increased concentrations in incubation medium, insulin and growth hormone inhibit, but cortisol and glucagon stimulate, leucine catabolism in mammary epithelial cells. In contrast, prolactin has no effect on leucine degradation in these cells. Reductions in circulating levels of insulin and growth hormone but increases in cortisol and glucagon with lactation may play an important role in enhancing BCAA catabolism for glutamate and glutamine syntheses in the lactating mammary gland. These coordinated changes in the endocrine status contribute to the regulation of milk production in mammals, including cows, sows and humans.

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Conflict of interest The authors declare that they have no conflict of interest.

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