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ORIGINAL ARTICLE

Regulation of leucine catabolism by metabolic fuels in mammary epithelial cells

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Abstract Lactation is associated with elevated catabolism of branched-chain amino acids (BCAA) in mammary glands to produce glutamate, glutamine, alanine, aspartate, and asparagine. This study determined effects of metabolic fuels 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 0.5 mM L-leucine and either L-[1-^{14}C]leucine or L-[U-^{14}C]leucine. The medium also contained 0–5 mM D-glucose, 0–2 mM L-glutamine, 0–4 mM DL-β-hydroxybutyrate, or 0–2 mM oleic acid. Rates of leucine decarboxylation were 60 % lower, but rates of α-ketoisocaproate production were 34 % higher, in the presence of 2 mM glucose than in its absence. All variables of leucine catabolism did not differ between 2 and 5 mM glucose or between 0 and 4 mM DL-β-hydroxybutyrate. Compared with 0–0.25 mM glutamine, 0.5 and 2 mM L-glutamine reduced leucine transport, transamination, and decarboxylation. In contrast, increasing the concentration of oleic acid from 0 to 2 mM dose-dependently stimulated leucine transamination, decarboxylation, and oxidation of carbons 2–6. Oleic acid also enhanced the abundance of cytosolic BCAA transaminase, while reducing the phosphorylated level (inactive state) of the E1α subunit of the mitochondrial branched-chain α-ketoacid dehydrogenase complex. Thus, hypoglycemia or ketosis in early lactation does not likely affect BCAA metabolism in mammary epithelial cells. Increasing circulating levels of BCAA and oleic acid may have great potential to increase the syntheses of glutamate, glutamine, aspartate, alanine, and asparagine by lactating mammary glands, thereby leading to enhanced production of milk for suckling neonates.

Keywords Leucine metabolism · Mammary epithelial cells · Energy substrates

Abbreviations

BCAA	Branched-chain amino acids
BCAT	Branched-chain amino acid aminotransferase
BCKA	Branched-chain α-ketoacids
BCKAD	Branched-chain α-ketoacid dehydrogenase
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
α-KIC	α-Ketoisocaproate

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Introduction

Lactation is associated with an increase in whole-body catabolism of branched-chain amino acids (BCAA) compared with nonlactating counterparts (DeSantiago et al. 1998; Viña and Williamson 1981). This metabolic change likely serves an important function in milk synthesis (Kim

and Wu 2009; Lei et al. 2012). Studies over the past 40 years have shown that BCAA catabolism by the mammary glands of sows (Spincer et al. 1969; Li et al. 2009), cows (Wholt et al. 1977), ewes (Davis et al. 1978), goats (Bequette and Douglass 2010), mice (DeSantiago et al. 1998), and rats (Viña and Williamson 1981) is markedly enhanced during early lactation. However, the underlying mechanisms for the up-regulation of BCAA catabolism are not known.

Branched-chain amino acids are not synthesized de novo in animal cells but are extensively degraded via inter-organ cooperation (Wu 2009). Catabolism of BCAA is initiated by BCAA aminotransferase (BCAT), which is present in both the cytoplasm and mitochondria (Chen et al. 2009; Conway and Huston 2000), to produce branched-chain α-ketoacids (BCKA) and glutamate. Subsequently, BCKA undergo oxidative decarboxylation by BCAA dehydrogenase (BCKAD) to form acyl-CoA for further oxidation (Harris et al. 2001; Li et al. 2009). Because the activity of BCKAD is relatively low in extrahepatic tissues (e.g., skeletal muscle, mammary tissue, and small intestine) due to the presence of the BCKAD protein primarily in the phosphorylated (inactive) state (Harper et al. 1984), a substantial portion of BCKA is released from cells (Chen et al. 2007; Wu and Thompson 1987, 1988b). The BCAA-derived glutamate is utilized for the synthesis of glutamine, alanine, aspartate, and asparagine (Li et al. 2009). These amino acids and their metabolites (e.g., arginine and proline) play critical roles in neonatal survival and growth (Haynes et al. 2009; Rhoads and Wu 2009), gene expression (Foster et al. 2012; Geng et al. 2011; Liu et al. 2012), intestinal function (Dai et al. 2011, 2012a, b; Ewaschuk et al. 2011), metabolic regulation (Gao et al. 2012; Satterfield et al. 2011, 2012; Wu et al. 2011a), immune response (Li et al 2007; Ren et al. 2011a, b), and whole-body homeostasis (Brosnan and Brosnan 2012; Bergen and Wu 2009; Xi et al. 2011a).

Mammals (including humans, cows, and sows) exhibit dynamic changes in the circulating levels of many energy substrates during lactation (de Boer et al. 1985; Doepel et al. 2009; Sartin et al. 1985). At present, little is known about the regulation of BCAA catabolism by metabolic fuels in mammary tissue. We hypothesize that increasing extracellular concentrations of energy substrates may inhibit leucine degradation in mammary epithelial cells. This hypothesis was tested using an established bovine mammary epithelial cell line (the Mac-T cell) and physiological concentrations of D-glucose, L-glutamine, β-hydroxybutyrate, and oleic acid. These substrates were used because their concentrations in plasma undergo marked changes during lactation (de Boer et al. 1985; Doepel et al. 2009; Plaizier et al. 2001; Robinson et al. 2008; Rukkwamsuk et al. 2000; Sartin et al. 1985).

Materials and methods

Materials

DL-β-Hydroxybutyric acid (sodium form), D-(+)-glucose, bovine insulin, and L-glutamine were purchased from Sigma Chemicals (St. Louis, MO, USA). Sodium oleate (powder) was obtained from Nu-Chek-Prep Inc. (Elysian, MN, USA). L-[1-^{14}C]leucine and L-[U-^{14}C]leucine, as well as DL-[1-^{14}C]hydroxybutyrate were purchased from American Radioactive Chemicals (St. Louis, MO, USA). Before use, L-[1-^{14}C]leucine and L-[U-^{14}C]leucine were purified using AG 1-×8 (acetate form, 200-400 mesh; Bio-Rad) as resin bed (0.6 × 6 cm) and deionized water (2 mL) as eluting solvent (Wu and Thompson 1988a, b). Soluene-350, a strong organic base formulated for compatibility with liquid scintillation cocktails, was obtained from Perkin Elmer (Branford, CT, USA). Gibco antibiotic–antimycotic solution (100×), sterile fetal bovine serum (FBS), SDS running buffer (20×), MOPS, and NuPage 10 % Bis–Tris gel (15 lane) were purchased from Invitrogen (Austin, TX, USA). The BCA Protein assay kit and SuperSignal West Dura Extended Duration Substrate were obtained from Pierce (Rockford, IL, USA). Rabbit anti-mitochondrial and cytosolic BCAT antibodies were prepared as described by Conway and Huston (2000). Rat anti-BCKAD antibody and rat anti-phosphorylated form of BCKAD E1α antibody were prepared as described by She et al. (2007). HPLC-grade water and methanol were obtained from Fisher Scientific (Houston, TX, USA).

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 DMEM medium supplemented with 1 % Gibco antibiotic–antimycotic liquid, 10 % FBS, and 0.1 mU/mL bovine insulin. Medium was changed every 2 days. Before 70–80 % confluence was reached, cells were harvested by trypsinization, washed twice with DMEM 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. 1994) for use in transport and metabolic studies. 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) containing 5 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), and physiological concentrations of other amino acids found in the plasma of lactating cows. Using enzymatic and chromatographic methods (Wu and Thompson 1987; Wu et al. 2007), we determined the concentrations (μM) of metabolites in the plasma of Holstein cows on day 30 of lactation (at 2 h after feeding): glucose 3,012; D-β-hydroxybutyrate 920; alanine 263; arginine 132; asparagine 43.0; aspartate 11.2; citrulline 82.6; cysteine 4.4; cystine 50.1; glutamate 47.2; glutamine 253; glycine 347; histidine 51.9; hydroxyproline 95.8; leucine 218; isoleucine 146; lysine 102; methionine 43.7; ornithine 45.4; phenylalanine 51.6; proline 168; serine 157; taurine 24.5; threonine 125; tryptophan 37.8; tyrosine 53.5; and valine 191.

After 1- to 5-min incubation at 37 °C, the solution was immediately transferred to a micro-centrifuge tube containing an oil mixture which was overlaid on 0.2 mL of 1.5 M $HClO_4$ solution (Wu and Flynn 1995). The tube was centrifuged, and the upper layer was thoroughly rinsed with saline to remove [^{3}H]inulin. The combined upper layer was analyzed for [1-^{14}C]-labeled α-ketoisocaproate (KIC) using chemical decarboxylation and collection of $^{14}CO_2$, as previously described (Wu and Thompson 1987). The bottom solution was analyzed for ^{14}C and ^{3}H radioactivities using a dual-channel counting program (Wu and Flynn 1995). The ratio of ^{14}C activity in the cell pellet to that in the incubation medium was 96:4. Thus, the amount of intracellular ^{14}C activity accurately represented leucine transport by bovine mammary epithelial cells.

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, the oxygenated (95 % O_2/5 % CO_2) Krebs bicarbonate buffer (1 mL) contained 2×10^6 viable cells, 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 0.1 mU/mL insulin, 5 mM D-glucose, 0.5 mM L-leucine, either L-[1-^{14}C]leucine or L-[U-^{14}C]leucine (approximately 2×10^5 DPM), and other amino acids at physiological concentrations present in the plasma of lactating cows (see above). L-[1-^{14}C]Leucine was used to determine net transamination, net release of KIC, and oxidative decarboxylation of leucine. Along with L-[1-^{14}C]leucine, L-[U-^{14}C]leucine was used to determine the oxidation of carbons 2–6 of leucine and the percentage of decarboxylated leucine oxidized to CO_2. To study effects of metabolic fuels on leucine oxidation, the incubation media also contained 0–5 mM D-glucose, 0–2 mM L-glutamine, 0–4 mM DL-β-hydroxybutyrate (equivalent to 0–2 mM D-β-hydroxybutyrate, which is the physiologic isomer), or 0–2 mM oleic acid. Four levels of each substance were added to the incubation medium based on their physiological concentrations found in the plasma of non-lactating and lactating cows.

After the cells were incubated for 2 h at 37 °C in a water bath (70 oscillations/min), $^{14}CO_2$ produced from the oxidation of [1-^{14}C]leucine or [U-^{14}C]leucine was collected as described previously (Wu and Thompson 1988a). Briefly, 0.2 mL Soluene-350 was injected through the rubber cap into a suspended center-well and 0.2 mL of 1.5 mM $HClO_4$ solution was injected also through the rubber cap into the incubation medium to liberate $^{14}CO_2$. This $^{14}CO_2$ was produced from enzymatic decarboxylation of [1-^{14}C]leucine-derived [1-^{14}C]KIC or all the carbons of L-[U-^{14}C]leucine. After the acidified medium was incubated at 37 °C for 1 h, suspended wells were transferred to scintillation vials containing 15 mL of cocktail for measurement of $^{14}CO_2$ (Wu and Thompson 1988b). To measure the net release of [1-^{14}C]leucine-derived [1-^{14}C]KIC by mammary epithelial cells, a new center-well received 0.2 mL Soluene-350. Thereafter, 0.35 mL of 30 % H_2O_2 was added through the rubber cap into the acidified incubation medium to chemically decarboxylate [1-^{14}C]KIC (Chen et al. 2007). Following 1-h incubation at 37 °C, the center-wells were collected for measurement of $^{14}CO_2$ (Wu and Thompson 1988b). Rates of net leucine transamination and oxidative decarboxylation were calculated on the basis of the specific activity of intracellular ^{14}C-labeled leucine (Wu and Thompson 1987), which was 78 % of the specific activity of ^{14}C-labeled leucine in incubation medium.

Determination of DL-β-hydroxybutyrate oxidation in cells

To determine whether β-hydroxybutyrate was oxidized by bovine mammary epithelial cells, the Krebs bicarbonate buffer (1 mL) contained 2×10^6 viable cells, 20 mM HEPES, 0.1 mU/mL insulin, 5 mM D-glucose, 0.5 mM L-leucine, 1–4 mM DL-β-hydroxybutyrate, and DL-[U-^{14}C]-β-hydroxybutyrate (approximately 2×10^5 DPM), and other amino acids at physiological concentrations present in the plasma of lactating cows (see above). After 2-h incubation, collection and analysis of $^{14}CO_2$ produced from the oxidation of [U-^{14}C]-β-hydroxybutyrate was performed as described above. Rates of CO_2 production were calculated on the basis of the specific activity of D-[U-^{14}C]-β-hydroxybutyrate in the incubation medium.

Determining effects of oleic acid on syntheses of amino acids in cells

Bovine mammary epithelial cells (2×10^6 viable cells) were incubated at 37 °C for 2 h in 1 mL Krebs bicarbonate buffer containing 20 mM HEPES, 5 mM D-glucose, 0.3 mM NH_4Cl, 0.1 mU/mL insulin, 0–2 mM oleic acid, 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 (see above). 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. The neutralized extracts were analyzed for amino acids, as described previously.

Western blot analysis

Western blotting analysis was used to determine the effect of oleic acid on the abundances of BCAT and BCKAD in bovine mammary epithelial cells (Li et al. 2009). Briefly, 2×10^6 viable cells were incubated at 37 °C for 2 h in the medium containing 0.5 mM leucine, 20 mM HEPES, 5 mM D-glucose, 0.1 mU/mL insulin, and 0 or 1 mM oleic acid, and other amino acids at physiological concentrations present in the plasma of lactating cows, as described previously. After 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 $10{,}000\times g$ 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. 2011). All samples were adjusted to an equal concentration of protein. Before electrophoresis, all samples were 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 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 water bath for 10 min (Hou et al. 2012). After cooling on ice, the sample solution was used for Western blot analysis (Kong et al. 2012). Each sample, which contained the same amount of protein (50 µg), was loaded onto NuPage 10 % Bis–Tris gel (Invitrogen) for SDS-PAGE. 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 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). The signals were detected on Fujifilm LAS-3000 (Tokyo, Japan). All data were normalized and expressed as the relative values to tubulin.

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

L-Leucine transport by cells

Leucine transport by bovine mammary epithelial cells was linear within 5 min [0.082 ± 0.006, 0.213 ± 0.019, and 0.406 ± 0.032 nmol/10^6 cells (means ± SEM, $n = 6$) at 1, 2.5 and 5 min, respectively]. Compared with the control group (no addition of any metabolic fuel to incubation medium), the addition of 2–5 mM D-glucose, 1–4 mM DL-β-hydroxybutyrate, or 0.5–2 mM oleic acid did not affect ($P > 0.05$) the rate of leucine transport by the cells (Table 1). The rate of leucine transport did not differ ($P > 0.05$) between 0 and 0.25 mM glutamine. However, compared with 0 mM glutamine, 0.5 and 2 mM glutamine dose-dependently reduced ($P < 0.05$) leucine uptake by bovine mammary epithelial cells. The rate of leucine transport was also lower ($P < 0.05$) in the presence of 2 mM glutamine than 0.5 mM glutamine.

Table 1 Rates of L-leucine transport by bovine mammary epithelial cells

Metabolic fuel	Concentration in incubation medium (mM)	L-Leucine transport
D-Glucose	0	0.40 ± 0.02
	2	0.42 ± 0.03
	3	0.41 ± 0.03
	5	0.40 ± 0.04
L-Glutamine	0.25	0.38 ± 0.03
	0.5	0.34 ± 0.02*
	2	0.29 ± 0.03*,†
DL-β-Hydroxybutyrate	1	0.40 ± 0.02
	2	0.42 ± 0.05
	4	0.39 ± 0.04
Oleic acid	0.5	0.40 ± 0.04
	1	0.43 ± 0.05
	2	0.41 ± 0.04

Values, expressed as nmol/10^6 cells per min, are means ± SEM, $n = 6$

* $P < 0.05$ versus the control (0 mM) group)

† $P < 0.05$ versus the 0.5 mM glutamine group

Effects of glucose on leucine catabolism in cells

Effects of glucose on leucine catabolism in mammary epithelial cells are shown in Table 2. Approximately 60 and 83 % of transaminated leucine were released as KIC by the cells incubated in the absence and presence of 2–5 mM glucose, respectively. Only 6–7 % of decarboxylated leucine was oxidized to CO_2. Compared with 0 mM glucose, the addition of 2 mM glucose to incubation medium reduced ($P < 0.05$) the rate of leucine oxidative decarboxylation by 60 %, while increasing ($P < 0.01$) the rate of KIC production by 34 % and the percentage of transaminated leucine released as KIC by 38 %. However, increasing extracellular concentrations of glucose from 2–5 mM did not affect ($P > 0.05$) any variable of leucine catabolism.

Effects of glutamine on leucine catabolism in cells

Effects of glutamine on leucine degradation in mammary epithelial cells are summarized in Table 3. In the presence of 5 mM glucose in incubation medium, the percentage of transaminated leucine released as KIC was approximately 80 %. Under this experimental condition, approximately 94 % KIC produced from leucine did not undergo further oxidation. Increasing extracellular concentrations of glutamine from 0 to 0.25 mM did not affect ($P > 0.05$) any variable of leucine catabolism. However, compared with 0 mM glutamine, 0.5 and 2 mM glutamine reduced ($P < 0.05$) leucine transamination and KIC release by 14 %, and 2 mM glutamine inhibited ($P < 0.05$) leucine oxidative decarboxylation by 11 %. The percentage of transaminated leucine released as KIC or the percentage of decarboxylated leucine oxidized to CO_2 did not differ ($P > 0.05$) among cells incubated with 0–5 mM glutamine.

Oxidation of β-hydroxybutyrate and its effects on leucine catabolism in cells

Bovine mammary epithelial cells extensively oxidized β-hydroxybutyrate, with the rates of CO_2 production being 0.41 ± 0.02, 0.73 ± 0.03, and 1.12 ± 0.06 nmol/10^6 cells per min (means ± SEM, $n = 6$), respectively, in the presence of 1, 2, and 4 mM DL-β-hydroxybutyrate. Increasing extracellular concentrations of DL-β-hydroxybutyrate from 0 to 4 mM did not affect ($P > 0.05$) any variable of leucine degradation in mammary epithelial cells (Table 4).

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

	Glucose concentration (mM)				Pooled SEM
	0	2	3	5	
CO_2 from all carbon (A)	0.93^a	0.37^b	0.38^b	0.35^b	0.058
CO_2 from carbon-1 (B)	0.71^a	0.28^b	0.29^b	0.26^b	0.043
CO_2 from carbon-2 to 6 (C)	0.22^a	0.09^b	0.09^b	0.09^b	0.019
Net release of α-KIC (D)	1.09^b	1.46^a	1.49^a	1.39^a	0.059
Net transamination (E = B + D)	1.80	1.74	1.79	1.66	0.053
Transaminated leucine released as α-KIC (D/E), (%)	61.1^b	83.8^a	83.0^a	83.9^a	2.17
Decarboxylated leucine oxidized to CO_2 (A–B)/(5 × B), (%)	6.43	6.97	6.28	6.72	0.709

Values, expressed as nmol/10^6 cells per 2 h, are means with pooled SEM, $n = 6$. The Krebs bicarbonate buffer (1 mL) contained 2 × 10^6 viable cells, 20 mM HEPES, 0.1 mU/mL insulin, 0–5 mM D-glucose, 0.5 mM L-leucine, L-[1-^{14}C]leucine or L-[U-^{14}C]leucine, and other amino acids at physiological concentrations present in the plasma of lactating cows

[a,b] Means sharing different superscript letters in a row differ ($P < 0.05$)

Table 3 Effect of glutamine on leucine catabolism in bovine mammary epithelial cells

	L-Glutamine concentration (mM)				Pooled SEM
	0	0.25	0.5	2	
CO_2 from all carbon (A)	0.47^{a}	$0.45^{a,b}$	$0.45^{a,b}$	0.43^{b}	0.014
CO_2 from carbon-1 (B)	0.36^{a}	$0.35^{a,b}$	$0.34^{a,b}$	0.32^{b}	0.011
CO_2 from carbon-2 to 6 (C)	0.12	0.11	0.13	0.11	0.010
Net release of α-KIC (D)	1.62^{a}	1.61^{a}	1.37^{b}	1.40^{b}	0.037
Net transamination (E = B + D)	1.99^{a}	1.95^{a}	1.70^{b}	1.72^{b}	0.045
Transaminated leucine released as α-KIC (D/E), (%)	81.5	81.9	79.9	81.2	0.865
Decarboxylated leucine oxidized to CO_2 (A–B)/(5 × B), (%)	6.43	6.40	7.56	7.12	0.731

Values, expressed as nmol/10^6 cells per 2 h, are means with pooled SEM, $n = 6$. The Krebs bicarbonate buffer (1 mL) contained 2×10^6 viable cells, 20 mM HEPES, 0.1 mU/mL insulin, 5 mM D-glucose, 0–2 mM L-glutamine, 0.5 mM L-leucine, L-[1-^{14}C]leucine or L-[U-^{14}C]leucine, and other amino acids at physiological concentrations present in the plasma of lactating cows. None of the measured variables ($P > 0.05$) differed among the cells treated with 0, 0.25, 0.5 and 2 mM L-glutamine

[a,b] Means sharing different superscript letters in a row differ ($P < 0.05$)

Table 4 Effect of DL-β-hydroxybutyrate on leucine catabolism in bovine mammary epithelial cells

	DL-β-Hydroxybutyrate concentration (mM)				Pooled SEM
	0	1	2	4	
CO_2 from all carbon (A)	0.50	0.47	0.52	0.50	0.017
CO_2 from carbon-1 (B)	0.38	0.34	0.37	0.36	0.012
CO_2 from carbon-2 to 6 (C)	0.12	0.13	0.15	0.14	0.015
Net release of α-KIC (D)	1.38	1.37	1.27	1.32	0.045
Net transamination (E = B + D)	1.76	1.70	1.64	1.68	0.049
Transaminated leucine released as α-KIC (D/E), (%)	78.2	80.0	77.1	78.3	0.847
Decarboxylated leucine oxidized to CO_2 (A–B)/(5 × B), (%)	6.06	9.13	8.43	8.00	1.011

Values, expressed as nmol/10^6 cells per 2 h, are means with pooled SEM, $n = 6$. The Krebs bicarbonate buffer (1 mL) contained 2×10^6 viable cells, 20 mM HEPES, 0.1 mU/mL insulin, 5 mM D-glucose, 0–4 mM DL-β-hydroxybutyrate, 0.5 mM L-leucine, L-[1-^{14}C]leucine or L-[U-^{14}C]leucine, and other amino acids at physiological concentrations present in the plasma of lactating cows. None of the measured variables differed ($P > 0.05$) among the cells treated with 0, 1, 2 and 4 mM DL-β-Hydroxybutyrate

Table 5 Effect of oleic acid on leucine catabolism in bovine mammary epithelial cells

	Oleic acid concentration (mM)				Pooled SEM
	0	0.5	1	2	
CO_2 from all carbon (A)	0.43^{d}	0.53^{c}	0.65^{b}	0.74^{a}	0.027
CO_2 from carbon-1 (B)	0.32^{d}	0.39^{c}	0.47^{b}	0.56^{a}	0.018
CO_2 from carbon-2 to 6 (C)	0.11^{d}	0.14^{c}	0.18^{b}	0.23^{a}	0.010
Net release of α-KIC (D)	1.39^{d}	1.59^{c}	1.80^{b}	2.04^{a}	0.059
Net transamination (E = B + D)	1.72^{d}	1.98^{c}	2.27^{b}	2.60^{a}	0.075
Transaminated leucine released as α-KIC (D/E) (%)	81.2	80.5	79.3	79.6	0.371
Decarboxylated leucine oxidized to CO_2 (A-B)/(5 × B) (%)	6.72	7.51	7.68	8.37	0.295

Values, expressed as nmol/10^6 cells per 2 h, are means with pooled SEM, $n = 6$. The Krebs bicarbonate buffer (1 mL) contained 2×10^6 viable cells, 5 mM glucose, 20 mM HEPES, 0.1 mU/mL insulin, 0–2 mM oleic acid, 0.5 mM L-leucine, L-[1-^{14}C]leucine or L-[U-^{14}C]leucine, and other amino acids at physiological concentrations present in the plasma of lactating cows

[a,b,c,d] Means sharing different superscript letters in a row differ ($P < 0.05$)

Effects of oleic acid on leucine catabolism in cells

Effects of oleic acid on leucine degradation in bovine mammary epithelial cells are summarized in Table 5. Compared with 0 mM oleic acid, increasing extracellular concentrations of oleic acid from 0 to 2 mM dose-dependently increased ($P < 0.05$) the rates of transamination, decarboxylation, and oxidation of carbons 2–6 of leucine in

Table 6 Effects of oleic acid on the syntheses of amino acids from BCAA in bovine mammary epithelial cells

Amino acid	Absence of BCAA and oleic acid from incubation medium	BCAA + Oleic acid at indicated concentration (mM)			
		0	0.5	1	2
Alanine	0.20 ± 0.01^{e}	0.53 ± 0.02^{d}	0.66 ± 0.03^{c}	0.84 ± 0.04^{b}	1.16 ± 0.06^{a}
Aspartate	0.21 ± 0.01^{e}	0.60 ± 0.03^{d}	0.73 ± 0.04^{c}	0.89 ± 0.05^{b}	1.24 ± 0.08^{a}
Asparagine	0.11 ± 0.01^{e}	0.38 ± 0.01^{d}	0.50 ± 0.01^{c}	0.62 ± 0.02^{b}	0.78 ± 0.03^{a}
Glutamate	0.44 ± 0.03^{e}	1.05 ± 0.06^{d}	1.31 ± 0.08^{c}	1.76 ± 0.10^{b}	2.12 ± 0.11^{a}
Glutamine	1.06 ± 0.05^{e}	1.94 ± 0.12^{d}	2.36 ± 0.14^{c}	2.82 ± 0.15^{b}	3.49 ± 0.18^{a}

Values, expressed as nmol/10^6 cells per 2 h, are means ± SEM, $n = 8$. Bovine mammary epithelial cells (2×10^6 viable cells) were incubated at 37 °C for 2 h in 1 mL Krebs bicarbonate buffer containing 20 mM HEPES, 5 mM D-glucose, 0.3 mM NH_4Cl, 0.1 mU/mL insulin, 0–2 mM oleic acid, 0 or 0.5 mM each of 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

[a,b,c,d,e] Means within a row sharing different superscript letters differ ($P < 0.05$)

the cells by 51, 75, and 109 %, respectively. The rate of net KIC production was 47 % higher ($P < 0.05$) in the presence of 0.5–2 mM oleic acid than in its absence. Neither the percentage of transaminated leucine released as α-KIC nor the percentage of decarboxylated leucine oxidized to CO_2 was affected by oleic acid ($P > 0.05$).

Effects of oleic acid on the syntheses of amino acids in cells

Bovine mammary epithelial cells readily synthesized glutamine, glutamate, aspartate, alanine, and asparagine from BCAA (Table 6). In the presence of 5 mM glucose, 0.5 mM each of three BCAA, and 0.3 mM NH_4Cl, increasing extracellular concentrations of oleic acid from 0 to 2 mM increased ($P < 0.05$) the syntheses of alanine, asparagine, aspartate, glutamate, and glutamine in a dose-dependent manner (Table 6). Among these amino acids, the rate of production of glutamine was the highest, followed by glutamate, aspartate, alanine, and asparagine in the descending order.

Effects of oleic acid on abundances of BCAT and BCKAD E1α in cells

Effects of oleic acid on BCAT and BCKAD E1α proteins in bovine mammary epithelial cells are illustrated in Figs. 1 and 2, respectively. These cells expressed both cytosolic and mitochondrial isoforms of the BCAT. Compared with its absence from incubation medium, oleic acid (1 mM) augmented ($P < 0.05$) the level of the cytosolic BCAT protein but had no effect ($P > 0.05$) on the mitochondrial BCAT isoform. In contrast, oleic acid reduced ($P < 0.05$) the abundance of the phosphorylated level of the mitochondrial BCKAD E1α subunit without affecting the total amount of the E1α polypeptide.

Discussion

Large amounts of BCAA are taken up by the lactating mammary glands of both ruminants (Davis et al. 1978) and non-ruminants (Spincer et al. 1969; Trottier et al. 1997), where these amino acids are extensively catabolized (DeSantiago et al. 1998; Li et al. 2009; Wholt et al. 1977). The initial reaction of BCAA degradation is catalyzed by BCAT to produce corresponding BCKA and glutamate. Glutamate is utilized for the synthesis of glutamine, alanine, aspartate, and asparagine by mammary tissue (Li et al. 2009), whereas BCKA is decarboxylated by BCKA dehydrogenase to generate acyl-CoA and CO_2 (Harper et al. 1984). There are two isoforms of BCAT (namely mitochondrial and cytosolic) in a variety of mammalian cells, whereas BCKAD is a multienzyme complex and localized exclusively in mitochondria (Harper et al. 1984; She et al. 2007). The activity of BCKAD is dependent on the phosphorylation (inactive) and de-phosphorylation (active) state of its E1α subunit, which are regulated by a tightly bound BCKAD kinase and a loosely bound phosphatase, respectively (Harper et al. 1984; Harris et al. 2001). Despite the reports that lactation is associated with increased catabolism of BCAA in the mammary gland (DeSantiago et al. 1998; Wholt et al. 1977), little is known about mechanisms regulating this physiological event in mammary epithelial cells.

Based on previous studies with skeletal muscle (Buse et al. 1973; Odessey and Goldberg 1972; Wu and Thompson 1987, 1988a, b) and mammary tissue (Abraham et al. 1964), glucose and ketone bodies are expected to modulate BCAA degradation in mammary epithelial cells. Compared with non-lactating counterparts, concentrations of β-hydroxybutyrate in the plasma of lactating dams are markedly elevated (de Boer et al. 1985; Rukkwamsuk et al. 2000), but concentrations of glucose are reduced (Sartin

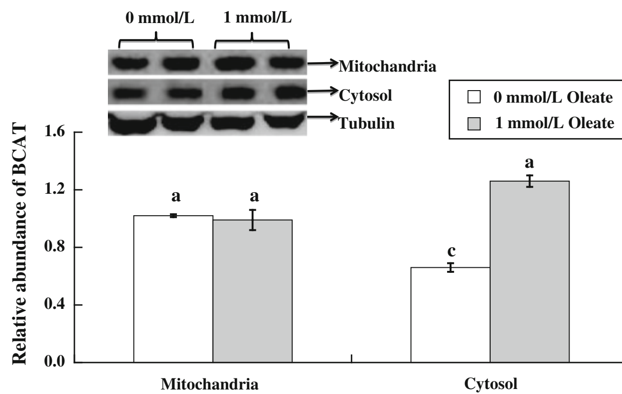

Fig. 1 Abundance of cytosolic and mitochondrial BCAT proteins in bovine mammary epithelial cells. Mammary epithelial cells were incubated for 2 h in the medium containing 0.5 mM L-leucine, 20 mM HEPES, 5 mM D-glucose, 0.1 mU/mL insulin, 0 or 1 mM oleic acid, and other amino acids at physiological concentrations present in the plasma of lactating cows. After 2-h incubation, cells were collected for analysis of cytosolic and mitochondrial BCAT. Values are means ± SEM, $n = 6$. Different letters indicate statistical significance ($P < 0.05$)

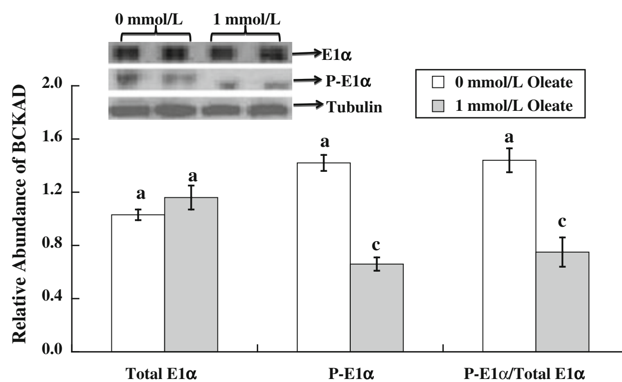

Fig. 2 Abundance of total and phosphorylated levels of mitochondrial phosphorylated BCKAD E1α subunit polypeptide in bovine mammary epithelial cells. Mammary epithelial cells were incubated for 2 h in the medium containing 0.5 mM L-leucine, 20 mM HEPES, 5 mM D-glucose, 0.1 mU/mL insulin, 0 or 1 mM oleic acid, and other amino acids at physiological concentrations present in the plasma of lactating cows. After 2-h incubation, cells were collected for analysis of cytosolic and mitochondrial BCAT. Values are means ± SEM, $n = 6$. Different letters indicate statistical significance ($P < 0.05$)

et al. 1985; Zhu et al. 2000). The results of this study indicate that physiological levels of D-glucose (3-5 mM) and D-β-hydroxybutyrate (1–2 mM) did not affect leucine catabolism in bovine mammary epithelial cells (Tables 2, 4). While the effect of glucose on these cells was similar to that reported for skeletal muscle, these two cell types responded differently to β-hydroxybutyrate. Specifically, ketone bodies markedly inhibit BCAA transamination and decarboxylation (Paul and Adibi 1978) by reducing glycolysis and pyruvate provision as well as the availability of coenzyme A (Wu and Thompson 1987, 1988a, b). Because β-hydroxybutyrate is readily oxidized in mammary epithelial cells, it is possible that the oxidation of the ketone body does not affect either glycolysis or the concentrations of pyruvate, α-ketoglutarate or coenzyme A in mammary epithelial cells due to multiple sources of these cofactors for BCAT and BCKAD. Indeed, despite the presence of ketoacidosis, rates of glucose utilization by the mammary glands of ruminants or nonruminants are very high during different phases of lactation (Annison and Linzell 1964; Annison et al. 1968; Linzell et al. 1967, 1969). For example, in goats (Annison and Linzell 1964) and sows (Linzell et al. 1969), glucose oxidation contributes to 40 and 54 % of the mammary CO_2 production, respectively. Nonetheless, the results of the present study suggest that hypoglycemia or elevated levels of ketone bodies in early lactation do not likely affect BCAA catabolism in mammary epithelial cells.

Little information is available regarding effects of glutamine on BCAA metabolism in mammary tissue. Lactation is associated with elevated uptake of glutamine by mammary glands and possibly kidneys, thereby reducing its concentration in arterial circulation (Viña and Williamson 1981; Zhu et al. 2000). In the mammary gland, the extracted glutamine is used primarily for milk synthesis because this tissue lacks glutaminase for glutamine hydrolysis (Li et al. 2009). The results of this study indicate that increasing extracellular concentrations of glutamine from 0 to 0.5 or 2 mM decreased leucine transamination by 14 % (Table 3), which is likely due to an inhibition of leucine transport by the cells (Table 1). Compared with 0 or 0.25 mM glutamine, 2 mM glutamine also reduced the rate of leucine oxidative decarboxylation by 11 % (Table 3) possibly due to an inhibitory effect on BCKAD activity. Glutamine is known to activate mTOR (Xi et al. 2011b) which may phosphorylate the BCKAD E1α subunit, thereby inhibiting its enzymatic activity. While the underlying mechanisms remain to be determined, it is clear that high levels of glutamine can inhibit BCAA transport and oxidative decarboxylation, as well as glutamine synthesis in mammary epithelial cells. However, elevated levels of glutamine (up to 2 mM) did not affect the oxidation of leucine carbons 2–6 or the activity of the Krebs cycle in bovine mammary epithelial cells (Table 3). Based on these results, it is unlikely that the increased oxidation of leucine to CO_2 in the mammary gland during lactation results from a decrease in plasma concentrations of glutamine from 0.5 to 0.25 mM. This view is further supported by our finding that neither oxidative decarboxylation nor the oxidations of carbons 2–6 of leucine differed between 0.25 and 0.5 mM glutamine (Table 3).

Lactation is associated with an increase in the circulating levels of long-chain fatty acids, including palmitate, stearate, oleate, and linoleate (Rukkwamsuk et al. 2000). Notably, the uptake of oleic acid by the mammary gland increases, while the uptake of palmitate and stearate

decreases, with advancing lactation (Annison et al. 1967). Available evidence shows that oleic acid is largely incorporated into milk fat in mammary tissue (Annison et al. 1967, 1968). However, some amounts of oleic acid are still available in mammary epithelial cells to exert its metabolic actions (Table 5), including the stimulation of leucine transamination, decarboxylation, and the oxidation of carbons 2–6 to CO_2 (Table 5). Similar results were also reported for leucine oxidation in rat diaphragm muscle (Buse et al. 1973). Elevated leucine transamination and decarboxylation could be explained by the increased abundance of the cytosolic BCAT (Fig. 1) and the reduced level of the phosphorylated BCKAD E1α subunit (Fig. 2). Consistent with this notion, there is evidence that oleic acid mediates PPARα (peroxisome proliferator-activated receptor α) activity, thereby inhibiting lipid accumulation in organs such as liver (Fan et al. 2009). Notably, activation of PPARα is linked to inactivation of BCKAD kinase (which phosphorylates the E1α subunit of BCKAD), thereby reducing the phosphorylation of the BCKAD E1α subunit (Harris et al. 2001).

The effect of oleic acid on leucine catabolism in mammary epithelial cells may have important nutritional and physiological significance. For example, activation of BCAA catabolism by oleic acid results in increased synthesis of glutamate, glutamine, alanine, aspartate, and asparagine in these cells (Table 6), the amino acids with important functions in the body. For example, glutamine, glutamate, and aspartate are preferential substrates for ATP production in the small intestine (Blachier et al. 2009; Brosnan and Brosnan 2012; Wu et al. 2011b). Glutamate and glutamine are also used for the intestinal syntheses of glutathione, arginine, and proline (Reeds and Burrin 2001; Wu et al. 2011a). Additionally, glutamine and aspartate are required for the synthesis of purines and pyrimidine, whereas glutamine is essential for the production of amino sugars (e.g., *N*-acetylglycosamine and *N*-acetylgalactosamine) (Wu et al. 2011b). Furthermore, physiological levels of glutamine activate the expression of a number of genes associated with proliferation, survival, and tight junction stabilization as well as anti-inflammatory and anti-apoptosis in the intestinal cells (Hou et al. 2011; Wang et al. 2008; Xi et al. 2011b). Although common ingredients for animal or human diets contain relatively high levels of BCAA, glutamate, and glutamine (Li et al. 2011a), large amounts of these AA are required for protein synthesis in the body (Wu 2009). Notably, these nutrients also have regulatory roles in cellular metabolism and signaling (Appuhamy et al. 2012; Li et al. 2011b; Suryawan and Davis 2011; Wilson et al. 2011; Yin et al. 2010). Available evidence shows that, under current feeding practices, milk-borne glutamine from mothers (e.g., sows and cows) is not sufficient to support their maximal lactation performance (Haynes et al. 2009; Kim and Wu 2009; Rezaei et al. 2011; Wu and Knabe 1994) or maximal protein accretion in neonates (Wu 2010). Therefore, enhancing the provision of glutamine and glutamate in milk through nutritional means (e.g., supplementation with oleic acid, BCAA, glutamine and glutamate) may be effective in promoting lactogenesis in the mammary gland. Future studies are warranted to test this hypothesis.

In summary, changes in circulating levels of glucose or ketone bodies within normal physiological ranges do not likely affect leucine catabolism in bovine mammary epithelial cells. Increasing extracellular concentrations of glutamine from 0.25 to 0.5 and 2 mM inhibit leucine transamination by 14 % and oxidative decarboxylation by 11 %, suggesting a modest effect of glutamine on BCAA degradation of glutamine synthesis in mammary tissue. In contrast, increasing extracellular concentrations of oleic acid from 0 to 2 mM substantially increases leucine transamination by 51 %, KIC release by 47 %, and leucine decarboxylation by 75 %, resulting in enhanced syntheses of glutamine, glutamate, alanine, aspartate, and asparagine by mammary epithelial cells. These findings may have important implications for designing new nutritional means to enhance milk production by lactating mammals.

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

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