Research Article

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High levels of fatty acids inhibit β-casein synthesis through suppression of the JAK2/STAT5 and mTOR signaling pathways in mammary epithelial cells of cows with clinical ketosis

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Abstract

Ketosis is a metabolic disease of dairy cows often characterized by high concentrations of ketone bodies and fatty acids, but low milk protein and milk production. The Janus kinase 2 (JAK2)-signal transducer and activator of transcription 5 (STAT5) and the mechanistic target of rapamycin (mTOR) signaling pathways are central for the regulation of milk protein synthesis. The effect of high levels of fatty acids on these pathways and β-casein synthesis are unknown in dairy cows with clinical ketosis. Mammary gland tissue and blood samples were collected from healthy ($n = 15$) and clinically-ketotic ($n = 15$) cows. In addition, bovine mammary epithelial cells (BMEC) were treated with fatty acids, methionine (Met) or prolactin (PRL), respectively. In vivo, the serum concentration of fatty acids was greater ($P > 0.05$) and the percentage of milk protein ($P > 0.05$) was lower in cows with clinical ketosis. The JAK2-STAT5 and mTOR signaling pathways were inhibited and the abundance of β-casein was lower in mammary tissue of cows with clinical ketosis ($P > 0.05$). In vitro, high levels of fatty acids inhibited the JAK2-STAT5 and mTOR signaling pathways ($P > 0.05$) and further decreased the β-casein synthesis ($P > 0.05$) in BMEC. Methionine or PRL treatment, as positive regulators, activated the JAK2-STAT5 and mTOR signaling pathways to increase the β-casein synthesis. Importantly, the high concentration of fatty acids attenuated the positive effect of Met or PRL on mTOR, JAK2-STAT5 pathways and the abundance of β-casein ($P > 0.05$). Overall, these data indicate that the high concentrations of fatty acids that reach the mammary cells during clinical ketosis inhibit mTOR and JAK2-STAT5 signaling pathways, and further suppress β-casein synthesis.

The ability of bovine mammary epithelial cells (BMEC) to synthesize milk proteins is one of the most important determinants of milk production in dairy cows (Boutinaud *et al.*, 2004). Ketosis is one of the most common metabolic diseases of dairy cows in early lactation (Littledike *et al.*, 1981). The rapid increase in milk production coupled with insufficient energy intake causes a state of negative energy balance (NEB) (Vanholder *et al.*, 2015) resulting in elevated concentrations of plasma fatty acids and ketone bodies [acetoacetate and β-hydroxybutyrate (BHB)] leading to ketosis (Ingvartsen, 2006; Ospina *et al.*, 2010). Besides the liver, the mammary gland can take up fatty acids (Glascock and Welch, 1974) and directly influence milk quality (Palmquist *et al.*, 1993). As a result, ketosis is not only associated with decreased milk production but also greater milk fat and lower milk protein percentage (Duffield *et al.*, 2009; Kayano and Kataoka, 2015; Zhang *et al.*, 2019a; 2019b).

Caseins are the most important proteins in milk (Rezaei *et al.*, 2016). Synthesis of casein is a complex process that is closely regulated by the Janus kinase 2 (JAK2)-signal transducer and activator of transcription 5 (STAT5) and the mechanistic target of rapamycin (mTOR) signaling pathways (Yang *et al.*, 2000; Jiang *et al.*, 2015). The JAK2-STAT5 signaling pathway controls casein gene transcription through activation of STAT5 (Imada and Leonard, 2000; Yang *et al.*, 2015), which upon transfer into the nucleus induces transcription of casein genes (Yamashita *et al.*, 2001). The hormone prolactin (PRL) and amino acids (AA) can activate the mTOR signaling pathway to mediate milk protein synthesis in part by regulating
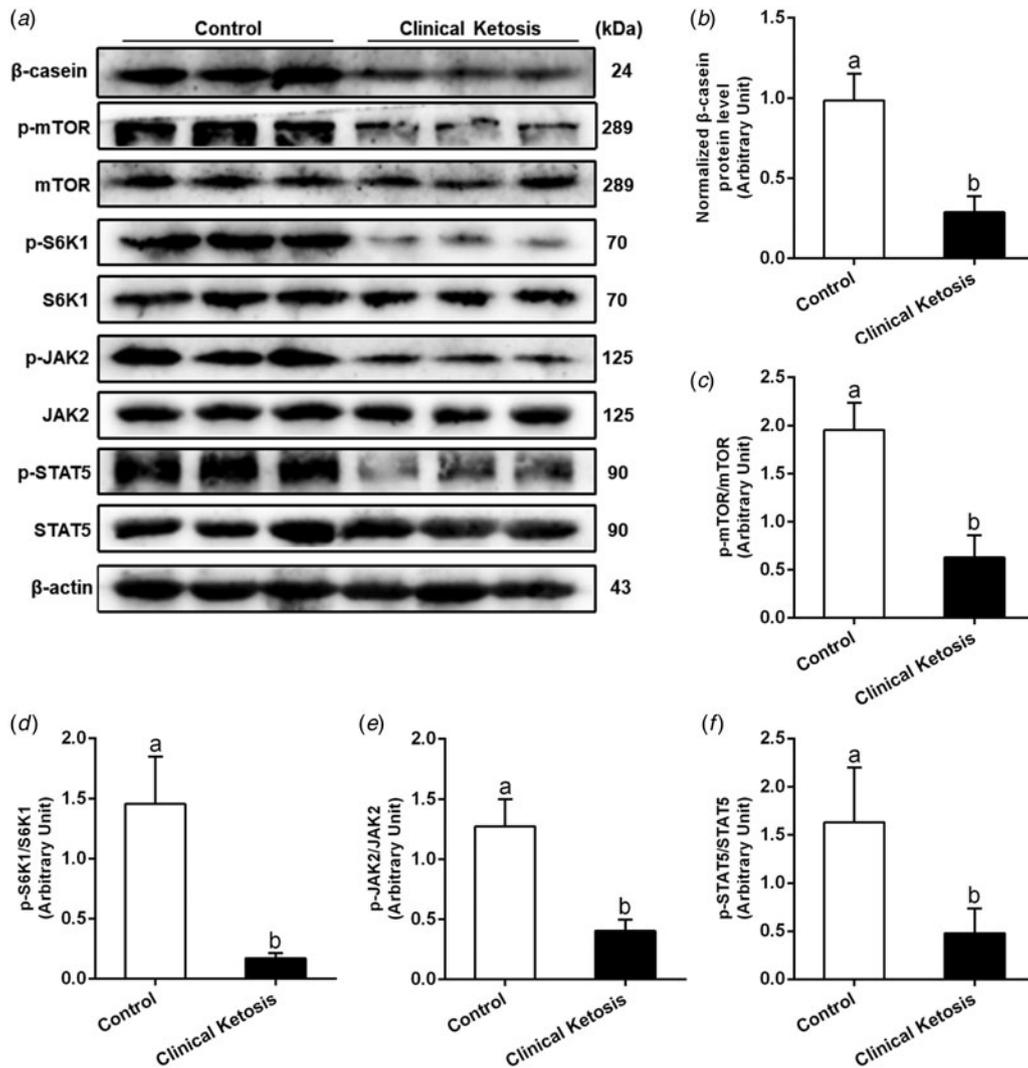
Fig. 1. The abundance of mTOR, JAK2-STAT5 signaling and β-casein are lower in the mammary gland of clinically ketotic cows. (a) Western blot analysis of β-casein, p-mTOR, mTOR, p-S6K1, S6K1 p-JAK2, JAK2, p-STAT5 and STAT5 in mammary gland of clinically-ketotic cows. (b) Relative protein abundance of β-casein. (c–f) The ratio of p-mTOR/mTOR, p-S6K1/S6K1, p-JAK2/JAK2 and p-STAT5/STAT5 in BMEC. The data were analyzed with paired t tests and expressed as the mean ± SEM ($n = 15$ per group). In the bar charts, different superscript lowercase letters indicate significant differences ($P < 0.05$).

casein transcription (Pauloin and Chanat, 2012; Zhang et al., 2018). Phosphorylated mTOR is indicative of mTOR activation in BMEC (Chiang and Abraham, 2005). Active mTOR can trigger phosphorylation of downstream molecules including ribosomal protein S6 kinase 1 (S6K1) and initiation factor eukaryotic factor 4E-binding protein 1 (4EBP1), which promote translation culminating in milk protein synthesis (Wang and Proud, 2006).

Besides the potential for high concentrations of fatty acids for creating a lipotoxic environment within cells, the inverse association between high concentrations of circulating plasma fatty acids and low milk protein synthesis suggest that fatty acids might play a role in the control of milk protein synthesis under ketotic conditions. The general hypothesis we sought to evaluate was that high concentrations of fatty acids inhibit JAK2-STAT5 and mTOR signaling pathways and further suppress the abundance of β-casein. This study provides a theoretical basis for elucidating the molecular mechanism of low milk protein percentage in ketosis cows and provides a scientific basis for the regulation of casein synthesis in the dairy industry.

Materials and methods

The experiment was approved by the Ethics Committee for the Use and Care of Animals, Jilin University [2017 clinical trial (201711036)] (Changchun, China).

Animals

Lactating Holstein dairy cows in this study were chosen from a 7000-cow dairy farm located in Changchun City, China. All cows received a routine physical examination to ensure the absence of other co-morbidities, and the chosen cows had a similar number of lactations (median = 3, range = 2 to 4) and days in milk (median = 6 d, range = 3 to 10 d). Cows were classified as suspected of being clinically ketotic based on signs of depression, low feed intake and low milk yield, and positive results for ketone bodies in milk using a nitroprusside test (Duffield et al., 2009; Piechotta et al., 2012). Subsequently, the blood concentration of BHB in these cows was measured to confirm the initial

diagnosis. According to clinical symptoms and serum BHB concentrations (Oetzel, 2004), 15 cows with serum BHB greater than 3 mM (clinical ketosis) and 15 cows with serum BHB concentration lower than 0.6 mM (healthy controls) were randomly selected. The basic description of the cows is reported in online Supplemental Table S1. Cows had ad libitum access to the same diet (online Supplemental Table S2) offered twice a day at 0830 and 1600 h.

Milking and milk samples

Milk samples were collected as previous description (Sun *et al.*, 2019). Cows had access to a constant supply of fresh water and were milked twice daily at 0800 and 1530 h. Milk yield was electronically recorded and samples collected at each milking for 5 d for storage at 4°C with a preservative (1 mg/ml potassium dichromate). Milk fat, protein, and lactose were analyzed by infrared analysis with a 4-channel spectrophotometer (Multispec, Foss Food Technology Corp., Eden Prairie, MN).

Blood sample and mammary tissue collection

Blood samples were collected by jugular venipuncture (no anti-coagulant) between 0700 and 0800 h before feeding and the serum was separated after centrifugation at $3,500 \times g$ for 15 min at 4°C and stored at −80°C until analysis. Concentrations of glucose, BHB and fatty acids in serum were determined using a Hitachi 7170 autoanalyzer (Hitachi, Tokyo, Japan) with commercially-available kits (Randox Laboratories, Crumlin, UK).

Percutaneous biopsies of 15 cows with clinical ketosis and 15 healthy cows were obtained from the right or left rear quarter of the mammary gland according to published procedures (Bionaz and Loor, 2008). Briefly, the skin of the biopsy site was washed and sterilized with 75% alcohol and iodine. The area at the biopsy site was anesthetized by subcutaneous injection of 3 mL of lidocaine hydrochloride (20 mg/ml). After making a skin incision, we performed blunt dissection of the mammary capsule to ensure tissue obtained during the biopsy was mammary parenchyma. Immediately after removal of the biopsy instrument from the capsule, pressure was applied to the wound until visual signs of bleeding were absent. The skin incision was closed with 4 or 5 Michel clips (11 mm; Henry Schein, http://www.henryschein.com). The incision site was sprayed with topical antiseptic (Povidone Iodine Ointment, 10%; Taro Pharmaceuticals). Rectal temperature, milk yield, and feed intake were recorded daily for 7 d to monitor post-operative health condition. Tissue samples for quantitation of protein were frozen immediately in liquid nitrogen and stored at −80°C until use.

Primary cell culture

Primary mammary epithelial cell isolation from lactating mammary tissue of 3 dairy cows was as described previously (Wang *et al.*, 2014). Immediately after exsanguination, parenchyma samples were aseptically removed from the core of the mammary gland. The tissue was minced into small pieces and digested with 0.5% collagenase III (C8490, Solarbio, Beijing, China) for 3 h at 38°C. The resulting solution was filtered sequentially with a 74-μm strainer and centrifuged at $150 \times g$ for 10 min.

The cells were cultured with complete Dulbecco's modified Eagle's medium containing: Nutrient Mixture F-12 medium (DMEM/F-12, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (Grand Island, New York, USA),
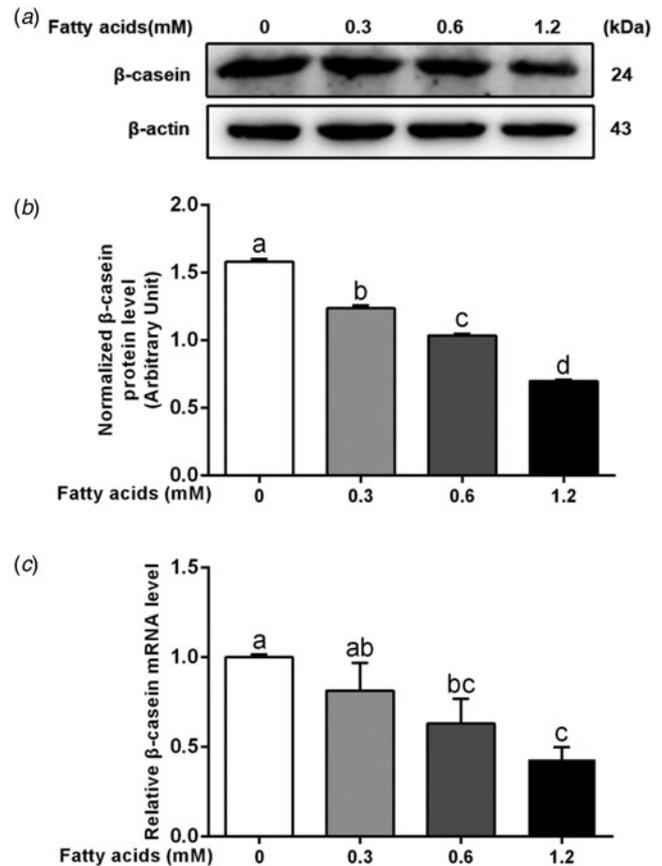

Fig. 2. Fatty acids negative regulates β-casein content in BMEC. The cells were incubated in DMEM/F-12 and cultured with 0, 0.3, 0.6, or 1.2 mM of a fatty acids-2% fatty acid-free bovine serum albumin (BSA) mixture for 24 h. (a) Western blotting analysis of β-casein in BMEC. (b) Relative protein abundance of β-casein. (c) Relative mRNA abundance of β-casein in BMEC. Results were representative of at least three independent experiments. The data of control group was used to normalize the data of each treatment group. Comparisons among groups were calculated using one-way ANOVA with subsequent Bonferroni correction. The data presented are the mean ± SEM. In the bar charts, different superscript lowercase letters indicate significant differences ($P < 0.05$), while the same letters represent no significant difference ($P > 0.05$).

500 ng/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA), 1 μg/ml PRL (100-07, Peprotech, Rocky Hill, NJ), 10 ng/ml epidermal growth factor (Peprotech, Rocky Hill, NJ), and 100 IU/ml penicillin/streptomycin (Baoman Biotechnology, Shanghai, China) in an incubator at 37°C under 5% CO_2. Prior to experimental treatments, cells were detached with trypsin for purification. After passaging three times, cells were seeded into a six-well tissue culture plate (2 ml per well) with DMEM/F-12 supplemented with 10% fetal bovine serum and 100 IU/ml penicillin/streptomycin. The medium was changed once every 24 h.

Fatty acids and methionine (Met)/PRL treatments

The fatty acids components (oleic, linoleic, palmitic, stearic, and palmitoleic acids; Sigma Aldrich) used in this study were chosen based on profiles reported in the blood of ketotic cows (Yamdagni and Schultz, 1970). The fatty acids were diluted in 0.1 M KOH at 60°C and pH of the solution adjusted to 7.4 with hydrochloric acid (1 M). The NEFA stock (52.7 mM) solution contained oleic (22.9 mM), linoleic (2.6 mM), palmitic (16.8 mM), stearic (7.6 mM) and palmitoleic acids (2.8 mM) (Li *et al.*, 2012). Before treatments, cells were serum-starved for 12 h. Then, the BMEC were cultured with 0, 0.3, 0.6, or 1.2 mM fatty acids and 2% fatty
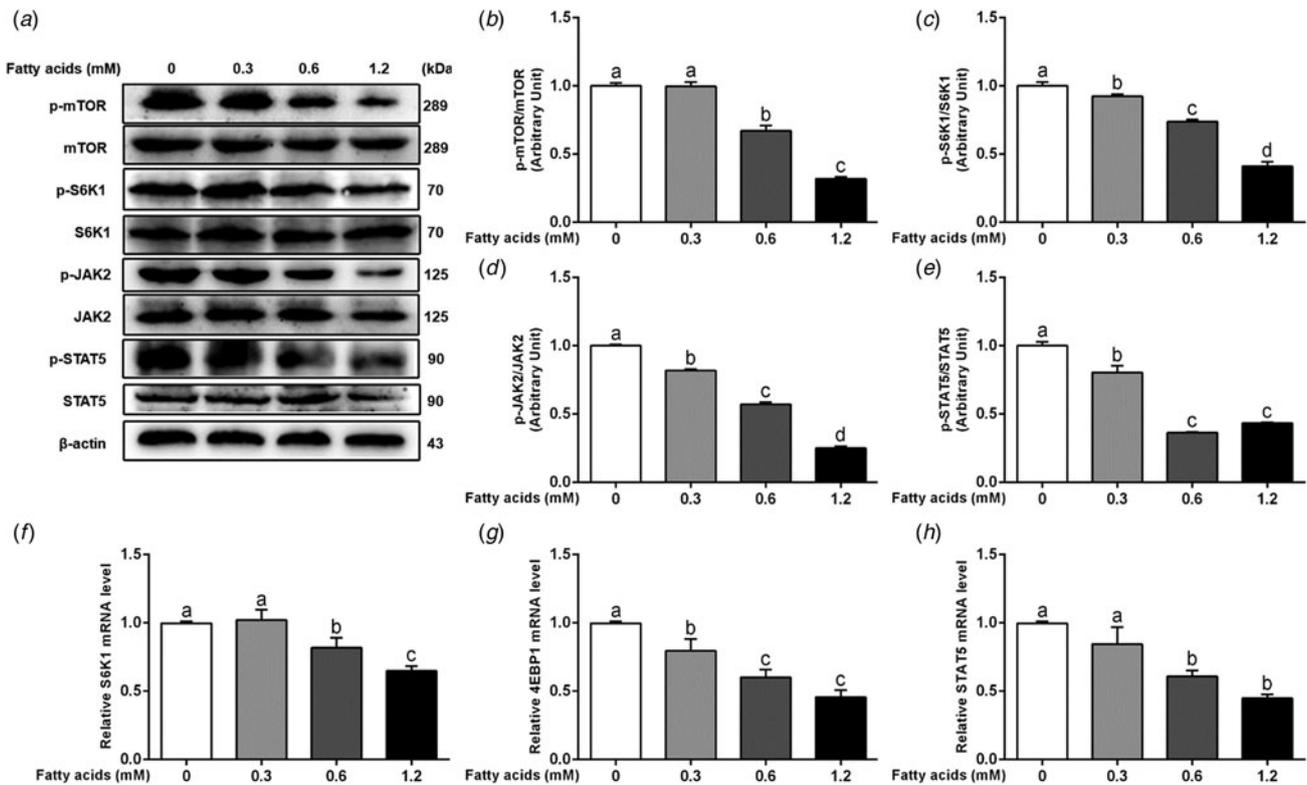
Fig. 3. The negative effect of fatty acids on mTOR and JAK2-STAT5 signaling pathways in bovine mammary epithelial cells (BMEC). The cells were incubated in DMEM/F-12 and cultured with 0, 0.3, 0.6, or 1.2 mM of a fatty acids-2% fatty acid-free BSA mixture for 24 h. (a) Western blotting analysis of p-mTOR, mTOR, p-S6K1, S6K1 p-JAK2, JAK2, p-STAT5 and STAT5 in BMEC. (b–e) The ratios of p-mTOR/mTOR, p-S6K1/S6K1, p-JAK2/JAK2 and p-STAT5/STAT5 in BMEC. (f–h) Relative mRNA abundance of S6K1, 4EBP1 and STAT5 in BMEC. Results were representative of at least three independent experiments. The data of control group was used to normalize the data of each treatment group. Comparisons among groups were calculated using one-way ANOVA with subsequent Bonferroni correction. The data presented are the mean ± SEM. In the bar charts, different superscript lowercase letters indicate significant differences ($P < 0.05$), while the same letters represent no significant difference ($P > 0.05$).

acid-free bovine serum albumin (BSA) for 24 h at 37°C under 5% CO_2. In addition, cells were cultured with Met (0.6 mM; Lu et al. 2012) and PRL (5 µg/ml; Zhang et al., 2019a, 2019b) for 24 h or co-cultured with 1.2 mM fatty acids and 0.6 mM Met or 5 µg/ml PRL for 24 h before collection for analyses. Cell culture was performed in triplicate.

RNA extraction and quantitative reverse-transcription PCR assay

Total RNA from cultured BMEC was extracted using Trizol. Purity was assessed: the OD_{260}/OD_{280} ratio of the total RNA was determined to be 1.9. One µg total RNA was reverse-transcribed to cDNA using a commercial kit (TaKaRa Biotechnology Co., Ltd, Tokyo, Japan). The abundance of mRNA was measured via SYBR green plus a reagent kit (Roche, Norwalk, CT, USA) with the 7500 Real-Time PCR System (Applied Biosystems). Primer sets are reported in online Supplemental Table S3. The relative abundance of target genes was normalized to β-actin, and analyzed using the $2^{-\Delta\Delta CT}$ method. The PCR reaction was performed in triplicate from each of the 3 individual cell preps.

Protein extraction and western blotting

Total protein in BMEC was extracted using a protein extraction kit (Sangon Biotech Co., Ltd, Shanghai, China) and the content quantified using a BCA Protein Assay Kit (P1511; Applygen Technologies). Total protein (30 µg per lane) from each sample was separated by 12% SDS-PAGE and electro-transferred to a polyvinylidenedifluoride membranes (PVDF; 0.45 µm). Then, the PVDF membranes were washed, blocked, and incubated overnight at 4°C with primary antibodies (online Supplemental Table S4), respectively. Subsequently, membranes were washed and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (1 : 5,000, Boster, Wuhan, China) at room temperature for 45 min. Immunoreactive bands were visualized by enhanced chemiluminescence solution (Pierce Biotechnology Inc., Chicago, USA). Lastly, the bands were imaged using a protein imager (ProteinSimple, San Jose, CA, USA).

Statistical analysis

All data were tested for normality and homogeneity of variance. In vivo data from Western Blotting were normally distributed and analyzed with paired t-tests. Other in vivo data did not have a normal distribution, thus, were analyzed with the Wilcoxon-signed-rank-test. In the in vitro studies, comparisons of groups were done by one-way ANOVA, and the Bonferroni's multiple comparison post-test was applied to examine all possible pairwise comparisons. Statistical analysis was performed using GraphPad Prism 6.01 (Graph Pad Software, San Diego, CA) or SPSS (Statistical Package for the Social Sciences) 19.0 software (IBM, Armonk, NY). All data are expressed as the mean ± SEM; $P < 0.05$ was considered statistically significant.
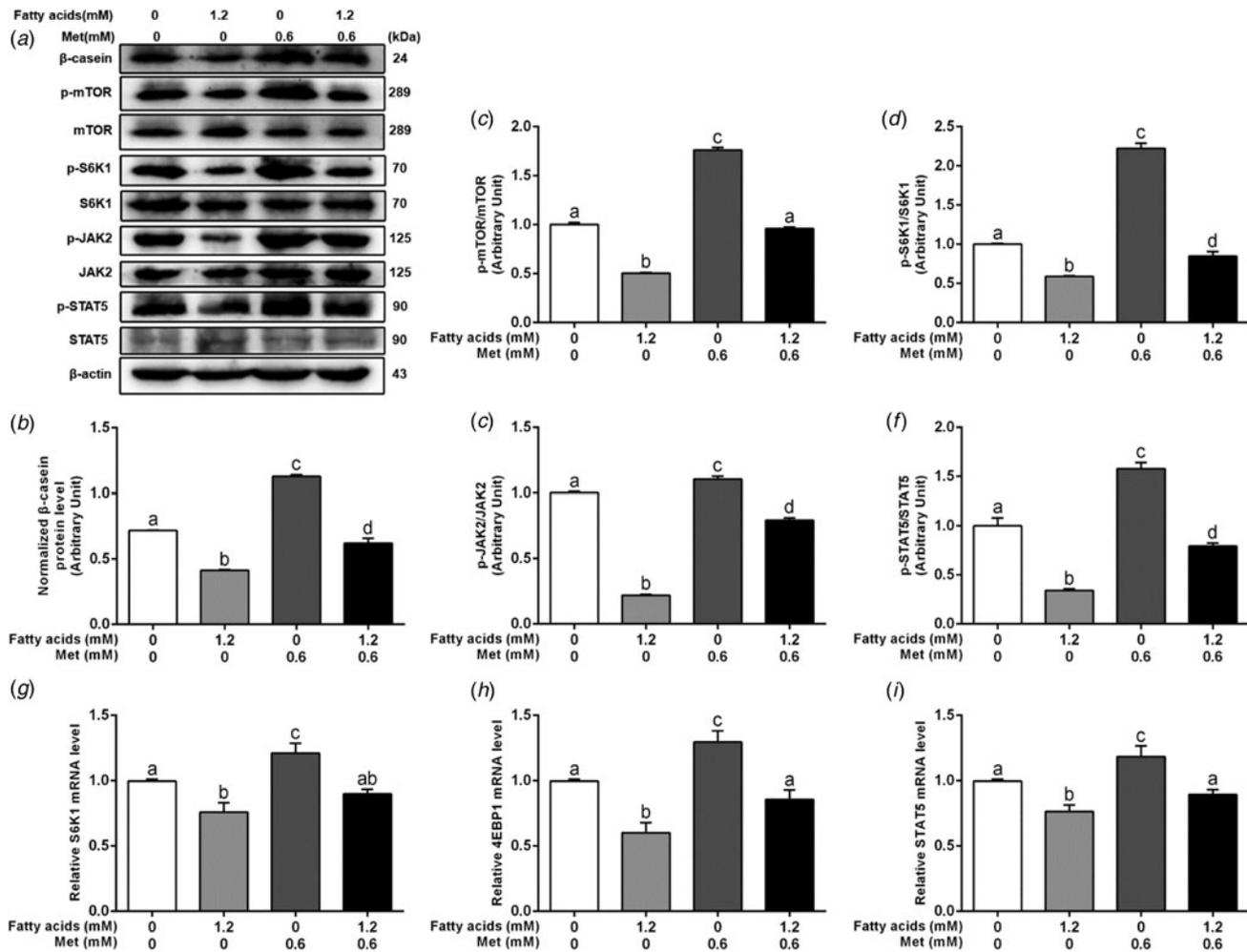
Fig. 4. Exogenous methionine (Met) alleviated the inhibition of fatty acids on milk protein synthesis in bovine mammary epithelial cells (BMEC). The cells were incubated in DMEM/F-12 with 2% fatty acid-free BSA, and cultured with 0, 1.2 mM fatty acids, 0.6 mM Met or co-cultured with 1.2 mM fatty acids and 0.6 mM Met for 24 h. (a) Western blot analysis of β-casein, p-mTOR, mTOR, p-S6K1, S6K1, p-JAK2, JAK2, p-STAT5 and STAT5. (b) Relative protein abundance of β-casein. (c–f) The ratio of p-mTOR/mTOR, p-S6K1/S6K1, p-JAK2/JAK2 and p-STAT5/STAT5. (g–i) Relative mRNA abundance of S6K1, 4EBP1 and STAT5. Results were representative of at least three independent experiments. The data of control group was used to normalize the data of each treatment group. Comparisons among groups were calculated using one-way ANOVA with subsequent Bonferroni correction. The data presented are the mean ± SEM. In the bar charts, different superscript lowercase letters indicate significant differences ($P < 0.05$), while the same letters represent no significant difference ($P > 0.05$).

Results

Baseline characteristics and blood parameters

As shown in online Supplemental Table S1, the milk production and milk protein concentration were lower in cows with clinical ketosis ($P < 0.05$). The milk fat was greater in cows with clinical ketosis ($P < 0.05$). The plasma concentration of fatty acids and BHB was greater ($P < 0.05$) while glucose concentration was lower ($P < 0.05$) in cows with clinical ketosis.

Decreased β-casein content in the mammary gland of clinically-ketotic cows

Compared with the control cows, the ratios of p-mTOR/mTOR, p-S6K1/S6K1, p-JAK2/JAK2 and p-STAT5/STAT5 were significantly lower in the cows with clinical ketosis ($P < 0.05$; Fig. 1a and c–f). Furthermore, the protein abundance of β-casein in the mammary tissue of cows with clinical ketosis was also markedly lower ($P < 0.05$; Fig. 1a and b). These results suggest that the suppression of mTOR and JAK2-STAT5 pathways decreases the synthesis of β-casein, and further leads to lower milk protein percentage.

Effect of fatty acids on the content of β-casein, mTOR and JAK2-STAT5 signaling in BMEC

The protein and mRNA abundance of β-casein were markedly decreased in the 0.6 and 1.2 mM fatty acids treatment groups ($P < 0.05$; Fig. 2a–c). Additionally, compared with the control, the ratios of p-mTOR/mTOR, p-S6K1/S6K1, p-JAK2/JAK2 and p-STAT5/STAT5 decreased gradually in response to incremental fatty acids (0.6 and 1.2 mM) ($P < 0.05$; Fig. 3a–e). Exogenous fatty acid treatment markedly downregulated the mRNA abundance of S6K1, 4EBP1, and STAT5 ($P < 0.05$; Fig. 3f–h). These results suggested that fatty acids are a negative regulator of mTOR and JAK2-STAT5 signaling pathways and β-casein content in BMEC.

Excessive fatty acids significantly alleviated the activation of exogenous Met/PRL on mTOR and JAK2-STAT5 signaling and β-casein content

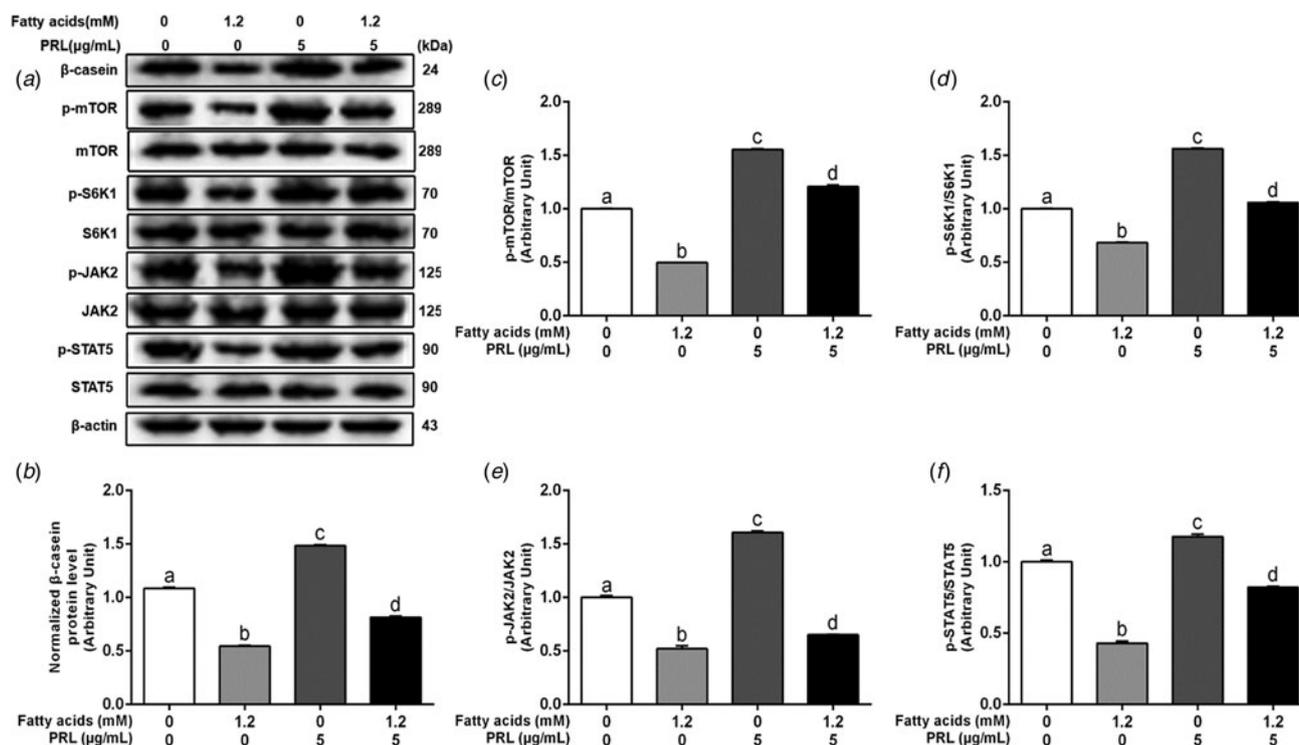Although exogenous Met and PRL significantly increased β-casein protein abundance, exogenous fatty acids significantly

Fig. 5. Exogenous prolactin (PRL) alleviated the inhibition of fatty acids on milk protein synthesis in bovine mammary epithelial cells (BMEC). The cells were incubated in DMEM/F-12 with 2% fatty acid-free BSA, and cultured with 0, 1.2 mM fatty acids, 5 µg/mL PRL or co-cultured with 1.2 mM fatty acids and 5 µg/ml PRL for 24 h. (a) Western blot analysis of β-casein, p-mTOR, mTOR, p-S6K1, S6K1, p-JAK2, JAK2, p-STAT5 and STAT5. (b) Relative protein abundance of β-casein. (c–f) The ratio of p-mTOR/mTOR, p-S6K1/S6K1, p-JAK2/JAK2 and p-STAT5/STAT5. Results are representative of at least three independent experiments. The data of control group was used to normalize the data of each treatment group. Comparisons among groups were calculated using one-way ANOVA with subsequent Bonferroni correction. The data presented are the mean ± SEM. In the bar charts, different superscript lowercase letters indicate significant differences ($P < 0.05$), while the same letters represent no significant difference ($P > 0.05$).

diminished the positive effect of Met and PRL on β-casein protein abundance ($P < 0.05$; Figs. 4a and b, 5a and b). The phosphorylation of mTOR, S6K1, JAK2 and STAT5 were enhanced by Met and PRL, and these effects were markedly diminished by co-treatment with fatty acids ($P < 0.05$; Figs. 4a and c–f, 5a and c–f). Additionally, exogenous fatty acids also significantly inhibited the positive effect of Met on mRNA abundance of S6K1, 4EBP1 and STAT5 ($P < 0.05$; Fig. 4g–i). These data reveal that high concentration of fatty acids inhibits mTOR and JAK2-STAT5 signaling, and further suppresses the content of β-casein in BMEC.

Discussion

The inhibition of mTOR and JAK2-STAT5 signaling pathways and lower abundance of β-casein in mammary tissue of cows with clinical ketosis, along with the inhibitory effect of fatty acids on mTOR and JAK2-STAT5 signaling pathways and β-casein synthesis in BMEC partly explain the lower milk protein percentage detected during clinical ketosis. Because previous studies have demonstrated that mTOR and JAK2-STAT5 signaling pathways play important roles in milk protein synthesis in the bovine mammary gland (Yang et al., 2000; Bionaz and Loor, 2011), results from the present study suggest a mechanistic link between excessive fatty acid influx into mammary gland during ketosis and the reduction in milk protein percentage.

Casein is not merely an important component of milk protein but also a significant index of milk quality (Gao et al., 2017). β-casein accounts for a high percentage of milk protein, thus,

the factors that affect β-casein abundance have a large effect on milk protein synthesis in BMEC (Davies and Law, 1983). In this study, the lower protein abundance of β-casein in mammary tissue and the lower milk protein percentage in cows with clinical ketosis are consistent with a previous study (Duffield et al., 2009). The high concentrations of fatty acids along with the increase in lipotoxicity they induced within BMEC can inhibit the activity of enzymes involved in milk protein synthesis (Ingvartsen, 2006; Ospina et al., 2010). Thus, we speculate that fatty acids might be one of the factors which regulate the abundance of β-casein. The very potent negative effect of fatty acids on milk protein synthesis was underscored in the present study by data from co-incubations of fatty acids and Met or PRL. Exogenous Met (0.6 mM) treatment increases the abundance of β-casein in BMEC (Lu et al., 2012). Furthermore, PRL is one of the major hormones that regulate milk protein synthesis (Hennighausen et al., 1997). Thus, both in vivo and in vitro data clearly reinforce negative effects of high availability of fatty acids on casein content, and partly explain the reduction in milk protein percentage during clinical ketosis.

The JAK2-STAT5 and mTOR signaling pathways are central to the regulation of milk protein synthesis (Bionaz and Loor, 2011; Jiang et al., 2015). Phosphorylated JAK2-STAT5 stimulates milk protein synthesis at the transcriptional level (Yamashita et al., 2001). The mTOR signaling pathway is also an essential signaling pathway involved in the long-term regulation of milk protein synthesis (Bionaz and Loor, 2011). In this study, the phosphorylation levels of JAK2, STAT5 and mTOR were significantly decreased in the mammary gland of dairy cows with clinical ketosis.

Furthermore, high concentrations of fatty acids markedly downregulated the phosphorylation levels of JAK2 and STAT5 (along with lower mRNA abundance), mTOR, and S6K1, and mRNA abundance of 4EBP1 and S6K1 in BMEC. These results indicate that high levels of fatty acids inhibit JAK2-STAT5 and mTOR pathway in the mammary gland of dairy cows with clinical ketosis, which further underscores the sensitivity of the β-casein synthesis machinery to intracellular fatty acids.

The role of exogenous AA availability and lactogenic hormones on the regulation of cellular protein synthesis through mTOR and JAK2-STAT5 signaling pathways are well-known (Kim, 2009; Yang *et al.*, 2015). For example, exogenous Met (0.6 mM) treatment was used to activate mTOR and JAK2-STAT5 signaling pathways and further increased the abundance of β-casein in BMEC (Burgos *et al.*, 2013; Yang *et al.*, 2015). The hormone PRL can trigger the dimerization of PRL receptor and activate JAK2 and STAT5 (Imada and Leonard, 2000), thus, the results we observed with Met and PRL on JAK2-STAT5 and mTOR pathways and β-casein content support a role for these pathways in the control of protein synthesis in BMEC. Furthermore, the mTOR inhibitor rapamycin induced a strong attenuation of the PRL-induced β-casein synthesis in mouse mammary epithelial cells (Pauloin and Chanat, 2012). The fact that fatty acids treatment diminished the activation by Met and PRL of p-TOR, p-S6K1, JAK2 and STAT5 along with the upregulation of S6K1, 4EBP1, STAT5, and β-casein to some extent offers additional support for the involvement of fatty acids in the inhibition of mTOR and JAK2-STAT5 pathways and β-casein synthesis in BMEC.

Nutrients such as AA not only function as signaling molecules regulating the abundance of casein *via* JAK2-STAT5 and mTOR signaling pathways in BMEC but also serve as building blocks and anabolic factors for milk protein synthesis (Arriola Apelo *et al.*, 2014). AA are small polar compounds that need the help of transporters to pass through the cell membrane, and some AA transporters act as sensors as well as carriers (Wang *et al.*, 2017). In the context of mechanisms linking fatty acids and AA signaling, one intriguing possibility is that above a certain concentration, the fatty acids may interfere with AA transport into cells or interfere in the sensing of extracellular AA concentrations. For instance, a recent study revealed that the bovine mammary tissue G protein-coupled receptor TAS1R1/TAS1R3 can sense and transport AA into mammary cells to elicit activation of mTOR signaling in part *via* increases in intracellular Ca^{2+} (Wang *et al.*, 2017; Zhou *et al.*, 2018). Clearly, a growing body of data is revealing complex mechanisms in the control of milk protein synthesis. Further research should help to clarify the biological relevance of fatty acids in the control of milk protein synthesis.

In summary, the milk protein percentage is negatively associated with the concentration of fatty acids in clinically ketotic cows. High concentration of fatty acids inhibits mTOR and JAK2-STAT5 signaling pathways in mammary epithelial cells of cows with clinical ketosis, which further decreases the β-casein content, thereby leading to a decrease of milk protein percentage. This can partly explain the lower milk protein percentage detected during clinical ketosis.

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