

LPS-induced reduction of triglyceride synthesis and secretion in dairy cow mammary epithelial cells *via* decreased SREBP1 expression and activity

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Sterol regulatory element binding protein 1 (SREBP1) has a central regulatory effect on milk fat synthesis. Lipopolysaccharides (LPS) can induce mastitis and cause milk fat depression in cows. SREBP1 is also known to be associated with inflammatory regulation. Thus, in the current study, we hypothesized that LPS-induced milk fat depression in dairy cow mammary epithelial cells (DCMECs) operates *via* decreased SREBP1 expression and activity. To examine the hypothesis, DCMECs were isolated and purified from dairy cow mammary tissue and treated with LPS (10 µg/ml). LPS treatment of DCMECs suppressed lipid-metabolism-related transcription factor SREBP1 mRNA expression, nuclear translocation and protein expression, leading to reduced triglyceride content. The transcription levels of acetyl-CoA carboxylase-1 and fatty acid synthetase were significantly down-regulated in DCMECs after LPS treatment, suggesting that acetyl-CoA carboxylase-1 and fatty acid synthetase involved in *de novo* milk fat synthesis was regulated by SREBP1. In summary, these results suggest that LPS induces milk fat depression in dairy cow mammary epithelial cells *via* decreased expression of SREBP1 in a time-dependent manner.

Keywords: Dairy cow mammary epithelial cells, lipopolysaccharides, sterol regulatory element binding protein 1, milk fat synthesis.

Many researchers have investigated the synthesis and regulation mechanism of milk fat, because milk fat is important for the nutritional quality and flavor of milk. Milk fat consists predominantly of triglyceride (TG; >95%), diglyceride (2%), phospholipids (1%), cholesterol (0.5%) and small amount of free fatty acids (FFA) (~0.1%) in all mammals (Staniewski et al. 2012). Milk fat is the major material basis for the nutritional quality in milk, which can provide nutrition and energy to human beings. Mammary epithelial cells can synthesize and secrete FA. There are two main sources of milk FA: short chain (4–8 C) and medium chain (10–14 C), and a portion of 16-C FA that arise *de novo* almost exclusively from DCMECs using acetic acid, β-hydroxybutyric acid and glycerol as precursor substances that are catalyzed by acetyl-CoA carboxylase-1 (ACC1) and fatty acid synthase (FAS). Remaining 16-C FA and long chain FA (>16 C) are

obtained from the blood of cows and are produced by DCMECs using lipoprotein lipase (LPL), acetyl-CoA binding protein (ACBP), CD36 and other FA transport enzymes (Bionaz and Looor, 2008). Lipid-metabolism-related transcription factor sterol regulatory element binding protein 1 (SREBP1) regulates the synthesis of dairy cow milk fat by regulating expression of the above-mentioned FA-synthesizing genes, SREBP1 plays an important role in milk fat uptake, transport, and *de novo* synthesis (Ma, 2012; Rincon et al. 2012).

Lipopolysaccharides (LPS) from the cell wall of Gram-negative bacteria are also referred to as a bacterial endotoxin. Bacteria will release LPS during clinical disease such as ruminal acidosis, mammary and uterine infection as well as during heat stress (Gott, 2011). LPS is harmful to dairy cows, causing systemic and local inflammatory reactions, and leading to a serious decrease in milk fat percentage and yield, and consequent major economic losses in the dairy farming and processing industries. It is known that LPS can reduce the content of milk fat synthetic precursors in the

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blood. LPS also induces acute phase reactions of the liver, resulting in damage to the liver that then does not provide sufficient amounts of apolipoproteins required for VLDL assembly. Since VLDL particles are an important source of fatty acids for milk fat production, LPS-induced acute phase reactions of the liver can lead to a reduction in milk fat synthesis.

In addition, meta-analysis of ruminant mastitis differential gene expression spectra has indicated that SREBP1 may be involved in the adaptive response to mammary infection (Genini et al. 2011). SREBP1 also has a role in inflammatory regulation, and has been attracting increased research attention (Spann et al. 2012; Wei and Espenshade, 2012; Oishi et al. 2017). It is unclear whether LPS can regulate the expression and activity of SREBP1, and hence influence the synthesis of milk fat in DCMECs. Thus, in the current study, we hypothesized that LPS-induced milk fat depression in DCMECs operates *via* decreased SREBP1 expression and activity. These results will provide a scientific basis to study further the regulatory mechanism of milk fat synthesis and improve the nutritional quality of cow's milk.

Materials and methods

Isolation of DCMECs and LPS treatment

Primary DCMECs were cultured and purified as described previously (Xu et al. 2017). Experiments were performed using the 5th passage DCMECs. Forty-eight hours before treatment, insulin and hydrocortisone concentrations were reduced to 0%, and the concentration of other components was unchanged. LPS (*Escherichia coli* 055:B5; 10 µg/ml; Sigma) was added to the new medium. DCMECs were treated with LPS and collected for subsequent analysis. Experiments were repeated three times.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA from DCMECs was extracted with TRIzol reagent (Sigma). The concentration and mass of RNA were measured at 260/280 nm using an ultraviolet spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA with OD_{260/280} 1.8–2.0 was reverse-transcribed to cDNA using PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa Biotechnology, Tokyo, Japan). qRT-PCR primers were designed using Primer 5.0 software (Applied Biosystems, Foster City, CA, USA; sequences are given in online Supplementary Table S1). qRT-PCR was performed using the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), and SYBR Premix Ex Taq (TaKaRa Biotechnology). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene (Wu et al. 2015). The relative expression level of genes was calculated by normalizing to GAPDH using the $2^{-\Delta\Delta Ct}$ method (Huang et al. 2012; Li et al. 2014).

Western blotting

Western blotting for SREBP1 in DCMECs was performed as described previously (Liu et al. 2016; Song et al. 2016). Primary antibodies: mouse anti-SREBP1 monoclonal antibody (ab3259; 1:1000 dilution; Abcam); rabbit anti-β-actin antibody (bs-0061R; 1:2000 dilution; Bioss, Woburn, MA, USA). Secondary antibodies: horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (H + L) (ab6789; 1:5000 dilution; Abcam); HRP-labeled goat anti-rabbit IgG (H + L) (ab205718; 1:5000 dilution; Abcam). The ECL signal was detected using a gel imaging system, and OD values of western blotting images were analyzed using Image-Pro Plus 6.0 software (IPP; Media Cybernetics, Bethesda, MD, USA).

Nuclear translocation assay of SREBP1

Nuclear translocation for SREBP1 in DCMECs was performed as described previously (Huang et al. 2013). Primary antibodies: mouse anti-SREBP1 monoclonal antibody (ab3259; 1:1000 dilution; Abcam). Secondary antibodies: Alexa Fluor 488-labeled goat anti-mouse IgG (ab150117; 1:500 dilution; Abcam). The coverslips were photographed using laser confocal microscopy (Leica).

BODIPY493/503 staining of lipid droplets

Staining for lipid droplets in DCMECs was performed as described previously (Lee et al. 2012).

Determination of TG content

The total protein concentration of cells was determined by the Enhanced BCA Protein Assay Kit (Pierce, Rockford, IL, USA). TG concentration of the DCMECs was quantification using the Tissue Triglyceride Assay Kit (Applygen Technologies, Beijing, China) at 550 nm. The TG concentration of DCMECs was corrected to concentration per mg protein (He et al. 2012; Shi et al. 2016).

Statistical analysis

Each sample was assessed in triplicate and the experimental data were expressed as the mean ± SD. The data were analyzed by one-way analysis of variance using SPSS version 19.0 software (SPSS, IBM, Armonk, NY, USA). The differences were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

Results

LPS decreases transcription, translation and nuclear translocation of SREBP1

The mRNA expression level of SREBP1 was reduced in DCMECs treated with LPS for 3 h, and gradually decreased further with LPS treatment time ($P < 0.01$ or $P < 0.05$). Transcription of SREBP1 was lowest at 12 h after LPS

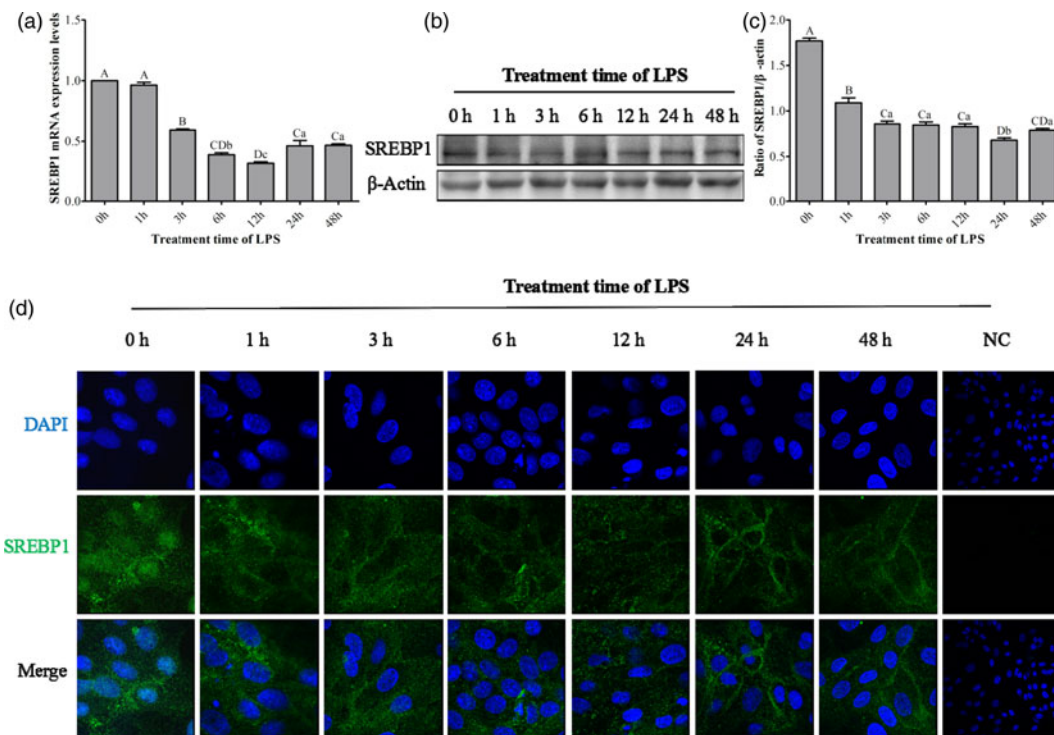


Fig. 1. Transcription, translation and nuclear translocation of SREBP1 in DCMECs. (a) Transcription levels of SREBP1. (b) Western blotting of SREBP1. (c) The grayscale analysis of SREBP1. (d) Nuclear translocation assay of SREBP1. Note: The data with different capital letters between two groups showed very significant differences ($P < 0.01$); data with different lower-case letters between two groups showed significant differences ($P < 0.05$); data with the same letters between two groups showed no significant differences ($P > 0.05$).

treatment, and then began to increase gradually ($P < 0.01$). However, the trend for increased SREBP1 transcription after LPS treatment for 24 and 48 h was not obvious, and the difference was not significant ($P > 0.05$) (Fig. 1a).

Western blotting revealed that protein expression of SREBP1 was down-regulated in DCMECs treated with LPS for 1 h, and gradually decreased further with LPS treatment time ($P < 0.01$). Expression of SREBP1 protein was lowest at 24 h after LPS treatment, and then began to increase gradually ($P < 0.05$) (Fig. 1b, c).

Immunofluorescence revealed that after treatment with LPS for 1 h, the intracellular trafficking of mature SREBP1 to the nucleus was inhibited. The levels of intracellular trafficking of mature SREBP1 to the nucleus were inhibited least at 24 h after LPS treatment, and then began to increase gradually (Fig. 1d). These results suggest that treatment with LPS decreases expression and activity of SREBP1 in DCMECs in a time-dependent manner.

LPS decreases transcription levels of FAS and ACC1

Transcription of FAS was down-regulated in DCMECs treated with LPS for 3 h, and gradually decreased further with LPS treatment time ($P < 0.01$). Transcription of FAS was lowest at 24 h after LPS treatment, and then began to increase gradually ($P < 0.01$) (Fig. 2a). The transcriptional changes of ACC1 are consistent with those of FAS (Fig. 2b).

LPS decreases milk fat synthesis

The content of TG was decreased in DCMECs treated with LPS for 3 h, and gradually decreased further with LPS treatment time ($P < 0.01$ or $P < 0.05$). The content of lipid droplets was lowest at 12 h after LPS treatment, and then began to increase gradually ($P < 0.05$) (Fig. 3). However, the trend for increased milk fat synthesis after LPS treatment for 24 and 48 h was not obvious and the difference was not significant ($P > 0.05$). The results of the lipid droplets BODIPY493/503 staining was consistent with the results of the determination of TG content (data are given in online Supplementary Fig. S1). These results suggest treatment with LPS can decrease milk fat synthesis in DCMECs in a time-dependent manner.

Discussion

SREBP1 belongs to the basic helix-loop-helix-leucine zipper family of transcription factors, and is essential for the regulation of FA and cholesterol biosynthetic gene expression (Jeon and Osborne, 2012). Genome-wide association scans and functional genomics analyses have established that SREBP1 is a key regulator of milk fat synthesis and secretion (Ogorevc et al. 2009). It can regulate the synthesis of fat in the body by controlling the expression of the relevant lipid-synthesizing-related enzymes involved in the

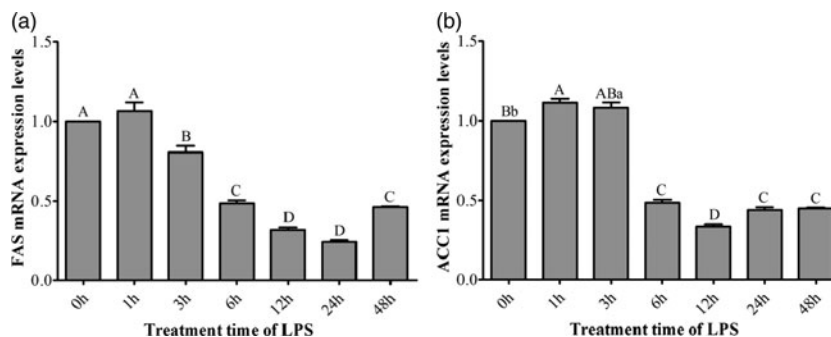


Fig. 2. Transcription levels of FAS and ACC1 in DCMECs. (a) Transcription levels of FAS. (b) Transcription levels of ACC1. Note: The data with different capital letters between two groups showed very significant differences ($P < 0.01$); data with different lower-case letters between two groups showed significant differences ($P < 0.05$); data with the same letters between two groups showed no significant differences ($P > 0.05$).

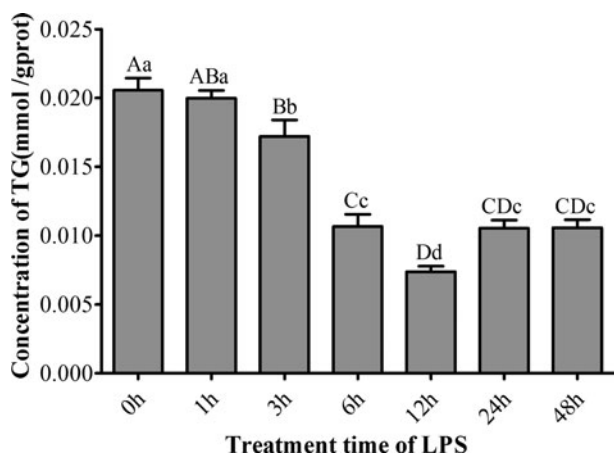


Fig. 3. Concentration of TG in DCMECs. Note: The data with different capital letters between two groups showed very significant differences ($P < 0.01$); data with different lower-case letters between two groups showed significant differences ($P < 0.05$); data with the same letters between two groups showed no significant differences ($P > 0.05$).

synthesis and uptake of cholesterol, FA, TG and phospholipids. Normally, SREBP1 in the endoplasmic reticulum is an inactive precursor. When cells are stimulated by liver X receptor, specificity protein 1 and oxysterols, SREBP1 is activated. Following activation, the amino terminal fragments of SREBP1 translocate to the nucleus and initiate transcription of target genes in combination with the SREs within the promoters of target genes (Zhang et al. 2015). Numerous cell culture experiments and genetically modified mouse models have shown that the major target genes of SREBP1 are some of the rate-limiting enzymes in the FA and cholesterol biosynthesis pathways. After treatment of DCMECs with SREBP1 siRNA, ACC1 and FAS content decreased by 40–65% (Ma & Corl, 2012). It has also been found that the mRNA expression levels of SREBP1 and milk fat synthesis enzymes in dairy cows are up-regulated in lactation, indicating that SREBP1 has a central regulatory effect on milk fat synthesis (Farke et al. 2008).

Milk fat synthesis involves the de novo synthesis of FA as well as the incorporation of de novo and preformed FA into TG. The TG accumulates to form lipid droplets, primarily in the mammary gland tissues of mammals. The activation of these metabolic pathways requires the coordinated regulation of a network of genes encoding lipogenic enzymes, such as the lipid-metabolism-related transcription factor SREBP1, as well as the de novo FA synthesis genes FAS and ACC1. FAS and ACC1 are the key enzymes for de novo synthesis of milk fat in DCMECs, and the gene promoter region of both has the binding site of SREBP1. Acetyl-CoA synthetase converts acetate into acetyl-CoA and begins de novo synthesis of milk fat. ACC1 catalyzes the conversion of acetyl-CoA to malonyl-CoA, a step that is the rate-limiting step in milk fat synthesis. FAS utilizes acetyl-CoA as a substrate to catalyze a series of carbon chain extension reactions to add the two carbon units from malonyl-CoA to the gradually elongated fatty acyl chain. SREBP1 translocates to the nucleus where it activates lipogenic genes by binding to the SREBP1 response element of the ACC1 and FAS genes (Kim et al. 2010). The mRNA transcription levels of ACC1 and FAS, as well as TG secretion and lipid droplets formation represent the DCMECs' capacity for milk fat synthesis. In this study, we found that SREBP1 affected the mRNA expression levels of the FAS and ACC1 genes in accordance with changes in TG content and lipid droplets accumulation, further confirming that SREBP1 acts on its target genes to regulate milk fat synthesis in DCMECs.

LPS is an important pathogen-associated molecular pattern that reduces the concentration of milk fat synthetic precursors in the blood of dairy cows, and can also be identified by pattern recognition receptors (e.g. Toll-like receptor 4) on the surface of mammary epithelial cells, resulting in up-regulated DNA binding activity of nuclear factor (NF)- κ B and increased expression of inflammatory cytokines including TNF α , IL-1 β , IL-6 and IL-8. This creates a highly inflammatory cytokine state in the mammary tissue and even in the body as a whole (Miao et al. 2012). The binding site of NF- κ B has been found in the promoter region of SREBP1 (Zhang et al. 2005). These results

suggest that the inflammatory signaling pathway has the potential to regulate lipid metabolism.

In this study, LPS decreased the concentration of TG and the formation of lipid droplets in DCMECs, and decreased the transcriptional and nuclear translocation of lipogenic transcription factor SREBP1 in DCMECs. At the same time, LPS decreased the expression of milk fat de novo synthesis-related enzyme genes in DCMECs. Moreover, these results suggest that LPS affects the synthesis of dairy cow milk fat by down-regulating expression of SREBP1 and milk fat de novo synthesis of related enzyme genes. Nevertheless, expression of milk-fat-synthesis-related genes and proteins and TG is not always decreased after DCMECs are treated with LPS, but with the prolongation of treatment, there is a tendency for increased expression of milk-fat-synthesis-related genes and proteins and TG. This may be due to the fact that LPS is identified by the pattern recognition receptor on the surface of mammary epithelial cells to up-regulate the DNA binding activity of NF- κ B, increase the transcription of SREBP1, and enable SREBP1 to participate in inflammatory regulation. However, this still needs validation. This may explain why cow mastitis can further aggravate the depression of milk fat.

In conclusion, our study suggests that LPS induces milk fat depression in DCMECs via decreased SREBP1 expression and activity in a time-dependent manner.

Conflict of interests

All the authors in this article claim no conflicts of interest.

Supplementary material

The supplementary material for this article can be found at <https://doi.org/10.1017/S0022029918000547>.

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References

- Bionaz M & Looor JJ 2008 Gene networks driving bovine milk fat synthesis during the lactation cycle. *BMC Genomics* **9** 366
- Farke C, Meyer HH, Bruckmaier RM & Albrecht C 2008 Differential expression of ABC transporters and their regulatory genes during lactation and dry period in bovine mammary tissue. *Journal of Dairy Research* **75** 406–414
- Genini S, Badaoui B, Sclep G, Bishop SC, Waddington D, Laan MH, Klopp C, Cabau C, Seyfert HM, Petzl W, Jensen K, Glass EJ, Greeff AD, Smith HE, Smits MA, Olsaker I, Boman GM, Pisoni G, Moroni P, Castiglioni B, Cremonesi P, Corvo MD, Foulon E, Focuras G, Rupp R & Giuffra E 2011 Strengthening insights into host responses to mastitis infection in ruminants by combining heterogeneous microarray data sources. *BMC Genomics* **12** 315–316
- Gott PN 2011 *Endotoxin Tolerance in Lactating Dairy Cows*. Columbus, USA: The Ohio State University
- He YH, He Y, Liao XL, Niu YC, Wang G, Zhao C, Wang L, Tian MJ, Li Y & Sun CH 2012 The calcium-sensing receptor promotes adipocyte differentiation and adipogenesis through PPAR γ pathway. *Molecular and Cellular Biochemistry* **361** 321–328
- Huang JG, Gao XJ, Li QZ, Lu LM, Liu R, Luo CC, Wang JL, Qiao B & Jin X 2012 Proteomic analysis of the nuclear phosphorylated proteins in dairy cow mammary epithelial cells treated with estrogen. *In Vitro Cellular and Developmental Biology-Animal* **48** 449–457
- Huang YL, Zhao F, Luo CC, Zhang X, Si Y, Sun Z, Zhang L, Li QZ & Gao XJ 2013 SOCS3-mediated blockade reveals major contribution of JAK2/STAT5 signaling pathway to lactation and proliferation of dairy cow mammary epithelial cells in vitro. *Molecules* **18** 12987–13002
- Jeon TI & Osborne TF 2012 SREBPs: metabolic integrators in physiology and metabolism. *Trends in Endocrinology and Metabolism* **23** 65–72
- Kim YM, Shin HT, Seo YH, Byun HO, Yoon SH, Lee IK, Hyun DH, Chung HY & Yoon G 2010 Sterol regulatory element-binding protein (SREBP)-1-mediated lipogenesis is involved in cell senescence. *Journal of Biological Chemistry* **285** 29069–29077
- Lee JS, Mendez R, Heng HH, Yang ZQ & Zhang K 2012 Pharmacological ER stress promotes hepatic lipogenesis and lipid droplet formation. *American Journal of Translational Research* **4** 102–113
- Li N, Zhao F, Wei C, Liang M, Zhang N, Wang C, Li QZ & Gao XJ 2014 Function of SREBP1 in the milk fat synthesis of dairy cow mammary epithelial cells. *International Journal of Molecular Sciences* **15** 16998–17013
- Liu L, Zhang LI, Lin YE, Bian Y, Gao X, Qu B & Li Q 2016 14-3-3 γ regulates cell viability and milk fat synthesis in lipopolysaccharide-induced dairy cow mammary epithelial cells. *Experimental and Therapeutic Medicine* **11** 1279–1287
- Ma L & Corl BA 2012 Transcriptional regulation of lipid synthesis in bovine mammary epithelial cells by sterol regulatory element binding protein-1. *Journal of Dairy Science* **95** 3743–3755
- Miao J, Fa Y, Gu B, Zhu W & Zou S 2012 Taurine attenuates lipopolysaccharide-induced dysfunction in mouse mammary epithelial cells. *Cytokine* **59** 35–40
- Ogorevc J, Kunej T, Razpet A & Dovc P 2009 Database of cattle candidate genes and genetic markers for milk production and mastitis. *Animal Genetics* **40** 832–851
- Oishi Y, Spann NJ, Link VM, Muse ED, Strid T, Edillor C, Kolar MJ, Matsuzaka T, Hayakawa S, Tao J, Kaikkonen MU, Carlin AF, Lam MT, Manabe I, Shimano H, Saghatelian A & Glass CK 2017 SREBP1 contributes to resolution of pro-inflammatory TLR4 signaling by reprogramming fatty acid metabolism. *Cell Metabolism* **25** 412–427
- Rincon G, Islastrejo A, Castillo AR, Bauman DE, German BJ & Medrano JF 2012 Polymorphisms in genes in the SREBP1 signalling pathway and SCD are associated with milk fatty acid composition in Holstein cattle. *Journal of Dairy Research* **79** 66–75
- Shi X, Li D, Deng Q, Peng Z, Zhao C, Li X, Wang Z, Li X & Liu G 2016 Acetoacetic acid induces oxidative stress to inhibit the assembly of very low density lipoprotein in bovine hepatocytes. *Journal of Dairy Research* **83** 442–446
- Song Y, Li N, Gu J, Fu S, Peng Z, Zhao C, Zhang Y, Li X, Wang Z, Li X & Liu G 2016 β -Hydroxybutyrate induces bovine hepatocyte apoptosis via an ROS-p38 signaling pathway. *Journal of Dairy Science* **99** 9184–9198
- Spann NJ, Garmire LX, McDonald JG, Myers DS, Milne SB, Shibata N, Reichart D, Fox JN, Shaked I, Heudobler D, Raetz CR, Wang EW, Kelly SL, Sullards MC, Murphy RC, Merrill AH, Jr, Brown HA, Dennis EA, Li AC, Ley K, Tsimikas S, Fahy E, Subramaniam S, Quehenberger O, Russell DW & Glass CK 2012 Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. *Cell* **151** 138–152

- Staniewski B, Kielczewska K, Smoczyński M, Baranowska M, Czerniewicz M & Brandt W** 2012 Effect of high pressures on the composition of milk fat triacylglycerols. *Milchwissenschaft-milk Science International* **67** 18–21
- Wei S & Espenshade PJ** 2012 Expanding roles for SREBP in metabolism. *Cell Metabolism* **16** 414–419
- Wu Q, Liu MC, Yang J, Wang JF & Zhu YH** 2015 Lactobacillus rhamnosus GR-1 ameliorates Escherichia coli-induced inflammation and cell damage via attenuation of ASC-independent NLRP3 inflammasome activation. *Applied and Environmental Microbiology* **82** 1173–1182
- Xu DD, Wang G, He XJ, Wang JF, Yang B, Sun ZP, Sun DB, He QY, Zhang X & Wu R** 2017 17 β -Estradiol and progesterone decrease MDP induced NOD2 expression in bovine mammary epithelial cells. *Veterinary Immunology Immunopathology* **188** 59–64
- Zhang C, Shin DJ & Osborne TF** 2005 A simple promoter containing two Sp1 sites controls the expression of sterol-regulatory-element-binding protein 1a (SREBP-1a). *Biochemical Journal* **386** 161–168
- Zhang M, Zhang S, Hui Q, Lei L, Du X, Gao W, Zhang R, Liu G, Li X & Li X** 2015 β -Hydroxybutyrate facilitates fatty acids synthesis mediated by sterol regulatory element-binding protein1 in bovine mammary epithelial cells. *Cellular Physiology and Biochemistry* **37** 2115–2124