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Full Length Article

Contribution of PPARγ in Modulation of LPS-Induced Reduction of Milk Lipid Synthesis in Bovine Mammary Epithelial Cells

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Abstract

Lipid content of milk is recognized as an indicator of milk quality. Lipopolysaccharides (LPS)-induced milk fat depression has also been observed in lactating dairy cows. However, it is not completely clear how the LPS regulate the milk lipid synthesis? Our objective was to examine the molecular mechanism in bovine mammary epithelial cells (bMEC). The bMEC were treated with oleic acid to induce milk lipid synthesis. The intracellular triglyceride in bMEC layer and the secreted triglyceride in culture medium were detected by enzymatic colorimetric assay. The expression and activity of the peroxisome proliferator-activated receptor gamma (PPARγ), the expression of the *FASN* (fatty acid synthetase), *ACACA* (acetyl-CoA carboxylase-1), *ACBP* (acyl-CoA-binding protein), and LPL (lipoprotein lipase) were investigated. The results showed that LPS decreased the level of lipid synthesis via down-regulating the transcription, translation, and nuclear translocation level of the PPARγ. The results also indicated that the transcription level of the PPARγ target genes, *FASN*, *ACACA*, *ACBP*, and *LPL* were significantly down-regulated in bMEC after LPS treatment. These results indicate that PPAR*γ* participate in LPS-induced reduction of milk lipid synthesis in bMEC. Our data may provide new insight into the mechanisms of milk fat depression caused by LPS. © 2019 Friends Science Publishers

Keywords: Dairy cow; Milk lipid synthesis; Lipopolysaccharides; Peroxisome proliferator-activated receptor gamma; Mammary epithelial cells

Introduction

Peroxisome proliferator-activated receptor gamma (PPARγ) plays an important role in lipid homeostasis, insulin sensitivity, and inflammation (Kawaiand and Rosen, 2010). Expression of PPARγ and the PPARγ coactivator 1, increases 2.5- and 3-fold in bovine mammary gland of lactating animals (Bionaz and Loor, 2008). PPARγ is putative transcription factor controlling the network of lipogenic genes. Kadegowda *et al.* (2009) reported that PPARγ in bovine mammary cells up regulate the mRNA expression of PPARγ lipogenic target genes, such as FASN (fatty acid synthetase), ACACA (acetyl-CoA carboxylase-1), and SCD (stearoylcoenzyme A desaturase). Kadegowda *et al*. (2009) also reported that PPAR is activated by palmitate and stearate in bovine MacT cells. Palmitic acid and acetic acid could induce milk lipid synthesis and markedly upregulate the transcription of PPARγ, FASN, ACACA, and ACD in bovine mammary epithelial cells (bMEC) (Liu *et al.*, 2016). These evidence support PPAR*γ* likely being the central regulatory point for milk fat synthesis.

Milk fat depression (MDF) is indicated by a decrease in milk fat by 0.2% or more (Mccarthy *et al.*, 2018). Lipopolysaccharides (LPS) are the major component of the outer membrane of Gram-negative bacteria. The concentrations of rumen lipopolysaccharides showed a strong negative relationship with milk fat content (Zebeli and Ametaj, 2009). LPS could induce milk fat depression by decreasing the plasma non esterified fatty acid (NEFA) and lipoprotein lipase (LPL) expression and activity (Yoon, 2014). Elevated interleukin 1 beta (IL-1β) level in mammary gland was observed in LPS-induced mastitis (Miao *et al.*, 2012). Matsunaga *et al.* (2018) found that IL-1β could inhibit milk lipid synthesis in bMEC *via* activation of signal transducer and activator of transcription (STAT) 3 and inactivation of STAT5. Besides above pathways, it is not completely clear how the LPS regulate the milk lipid synthesis in bMEC? We hypothesize that PPAR*γ* may be a key factor in LPS-induced the decrease of milk lipid synthesis in bMEC. In the current study, we analyzed the change of PPAR*γ* pathway after LPS challenge to elucidate the mechanisms of LPS regulates the milk lipid synthesis in bMEC.

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Materials and Methods

Cell Culture

Several immortalized bovine mammary epithelial cell lines have been established, such as hormone-adapted bovine mammary epithelial cell line (BMEC+H), immortalized bovine mammary cell line with both epithelial and myoepithelial characteristics (ET-C), and immortalized bovine mammary alveolar cells (Mac-T) (Hosseini *et al.*, 2013). However, these cell lines cannot synthesize milk lipids efficiently. In the present study, fibroblasts-free bMEC culture was obtained by differential trypsinization according to previous study (Xu *et al.*, 2017). The percentage of cytokeratin 18 (CK 18)-positive cells was calculated using flow cytometry according to previous study (Smalley, 2010). Primary antibody used in this study was mouse anti-CK 18 monoclonal antibody (1:1000; Abcam, Cambridge, C.A., U.S.A.). Secondary antibody used in this study was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:500; Abcam, Cambridge, C.A., U.S.A.). The bMEC were treated with oleic acid to induce milk lipid synthesis. BODIPY (493/503, Invitrogen, Carlsbad, C.A., U.S.A.) was used to visualize milk fat in bMEC according to previous study (Spangenburg *et al.*, 2011).

Cell Treatment

LPS (Sigma-Aldrich, St. Louis, M.O., U.S.A.) was added to the medium in 6-well culture plate at the 10 μg/ml concentration during 0-48 h. The culture medium and cell layer were collected to analyze the level of milk lipid and the activity of PPARγ pathway after LPS challenge.

Measurement of Triglyceride (TG)

The intracellular TG in bMEC layer and the secreted TG in culture medium were detected by enzymatic colorimetric assay using the TG assay kit (Nanjing Jiancheng Bioengeneering Institute, Nanjing, China) according to the manufacturer's protocol.

RT-PCR

FASN, *ACACA*, *ACBP* (acyl-CoA-binding protein), and *LPL* (lipoprotein lipase) are PPARγ target genes. These genes play vital role in regulating milk lipid synthesis. Therefore, the mRNA level of *FASN*, *ACACA*, *ACBP*, *LPL* and *PPARG* genes were evaluated by quantitative RT-PCR. Total RNA from bMEC layer was extracted with TRIzol reagent (Sigma-Aldrich). High-quality RNA samples were reverse-transcribed to cDNA using a reverse-transcription kit (TaKaRa, Kyoto, Japan). The mRNA expression level was evaluated using a SYBR Green PCR Kit (TaKaRa). Quantitative RT-PCR was conducted on a real-time PCR analyzer (Bio-Rad, Hercules, CA, USA). The relative expression level of genes was calculated by normalizing to *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) using the $2^{-\triangle\triangle CT}$ method (Taylor *et al.*, 2010). Primer sequences used in the study were listed in Table 1.

Western Blotting

The translation level of PPARγ in bMEC was performed as Western blotting protocol. Cells were lysed with a lysis buffer. Then, the protein concentration was determined by the method of Bradford (Biyotime, Shanghai, China). The protein samples were mixed in 1:1 ratio with the SDS-polyacrylamide gel standard loading buffer. Samples were run on 12% Tris-Glycine precast gel (BeyoGel™ SDS-PAGE Precast Gel) in SDS-PAGE running buffer. After protein transfer, PVDF membranes were blocked by incubation with blocking buffer. The rabbit anti-PPARγ polyclonal antibody (1:1000; Abcam) and rabbit anti-β-actin polyclonal antibody (1:2000, Bioss, Beijing, China) were used as primary antibodies. HRP-labeled goat anti-rabbit IgG (1:500; Abcam) were used as secondary antibody. The immunoreactive bands were detected using ECL Western Blotting Substrate (Solarbio, Beijing, China). The images of the protein bands were obtained with a Bio-Rad ChemiDoc™ XRS+ (Bio-Rad).

Immunostaining

Nuclear translocation for PPARγ in bMEC was performed as immunofluorescence staining protocol. Briefly, coverslips were incubated for 1 h with rabbit anti-PPARγ polyclonal antibody (1:1000; Abcam), followed by 1h with alexa fuor 488-labeled goat anti-rabbit IgG (1:500; Abcam). Nuclei were stained with DAPI (Sigma-Aldrich) for 1 min after removal of secondary antibodies. Finally, the coverslips were observed and photographed using a confocal laser-scanning microscope (Leica, Mannheim, Germany).

Statistical Analysis

The results were expressed as the mean ± standard deviation and were analyzed using S.P.S.S. 19.0 software (S.P.S.S., IBM, Armonk, NY, USA). The differences were considered significant at *P*-values of < 0.05 and highly significant at *P*-values of < 0.01.

Results

Cell Culture Results

As shown in Fig. 1, purified bMEC with epithelial-like shape were typically observed (Fig. 1A). The flow cytometry data showed that the ratio of CK18-positive cells is 99.04% (Fig. 1B). In addition, intracellular lipid droplets fluorescence staining result (yellow; Fig. 1C) showed that oleic acid could induce high lipid production ability of

Table1: Primer sequences used in the study

Gene name	Sequence	GenBank Accession No.	Length (bp)
FASN	F: GTGGGCTTGGTGAACTGTCT R: AGGACTTCGGGTCTGTCTCA	NM_001012669.1	112
ACACA	F: AACGCAGGCATCAGAAGATT R: CGCACTCACATAACCAACCA	NM_174224.2	119
ACBP	F: AAGACCAAGCCAGCAGATGA R: GTCCCTTTCAGCTCATTCCA	NM_001113321.1	146
LPL	F: ACACTTGCCACCTCATTCCT R: TACATTCCTGTCACCGTCCA	NM_001075120.1	109
PPARG	F: GCATTTCCACTCCGCACTAT R: ACAGGCTCCACTTTGATTGC	NM_181024.2	118
GAPDH	F: GGCATCGTGGAGGGACTTATG R:GCCAGTGAGCTTCCCGTTGAG	NM_001034034.2	171

bMEC. These results suggest that the bMEC culture model is suitable for investigating the milk lipid synthesis function.

TG Synthesis and Secretion

As shown in Fig. 2, LPS could decrease milk lipid synthesis and secretion in bMEC. Total TG content at 1, 3, 6, 12, 24, and 48 h after LPS treatment was 0.90-, 0.72-, 0.57-, 0.38-, 0.49-, and 0.52-fold lower than the control, respectively.

PPARγ Expression and Activity

The transcription, translation, and translocation of PPARγ were detected in this study. As shown in Fig. 3, the transcription level (Fig. 3A) and translation level (Fig. 3B and 3C) of PPARγ were down-regulated in bMEC treated with LPS. The transcription level of PPARγ at 6 h after LPS treatment was 6.3-fold lower than the control ($P < 0.01$). The translation level of PPARγ at 48 h after LPS treatment was 4.6-fold lower than the control ($P < 0.01$). Consistent with the above results, LPS could inhibit the translocation of PPARγ from the cytoplasm to the nucleus (Fig. 3D). These results suggest that LPS could inhibit the expression level and activity of PPARγ in bMEC.

PPARγ Target Genes Transcription Level

ACBP, *LPL*, *FASN*, and *ACACA* gene are PPARγ target genes. These genes play vital role in regulating milk fat synthesis in bovine mammary gland. Therefore, the mRNA level of *ACBP*, *LPL*, *FASN*, and *ACACA* genes were evaluated by quantitative RT-PCR. As shown in Fig. 4, LPS could inhibit the transcription of *ACBP*, *LPL*, *FASN*, and *ACACA* genes in a time-dependent manner. The transcription level of *ACBP* at 24 h after LPS treatment was 3.0-fold lower than the control ($P < 0.01$). The transcription level of *LPL* at 48 h after LPS treatment was 5.3-fold lower than the control ($P < 0.01$). The transcription level of *FASN* at 24 h after LPS treatment was 4.1-fold lower than the control ($P < 0.01$). The transcription level of *ACACA* at 12 h after LPS treatment was 2.9-fold lower than the control ($P < 0.01$). These results

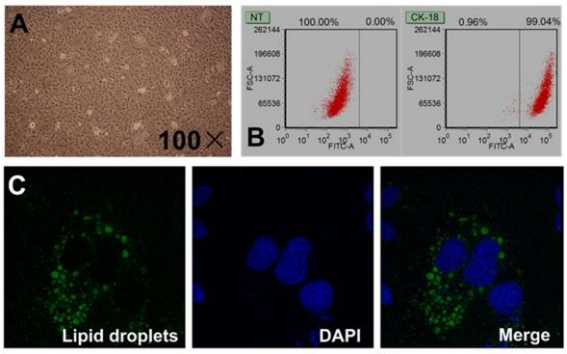

Fig. 1: Characterization of the bMECs. **A**: Morphology of the purified bMECs (100×). **B**: Flow cytometry analysis of CK-18 positive cells. **C**: The result of BODIPY staining of lipid droplets (yellow) in the bMECs (400×)

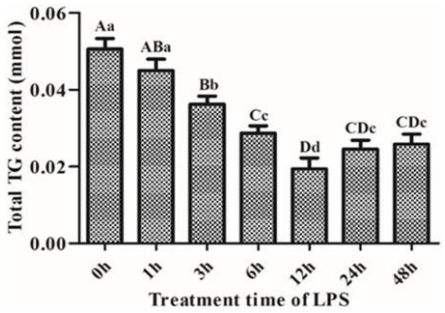

Fig. 2: LPS decreases TG content in the bMECs in a time-dependent manner. The data with different capital letters between two groups showed very significant differences ($P < 0.01$); data with different lower-case letters between two groups showed significant differences ($P < 0.05$); data with the same letters between two groups showed no significant differences ($P > 0.05$)

suggest that LPS could inhibit the uptake of long-chain (predominantly C_{18}) fatty acids and *de novo* synthesis (from C_4 to C_{14}) of fatty acids in bMEC.

Discussion

As a major component in milk, lipids in milk supply calories required for calves and infants (Rudolph *et al.*,

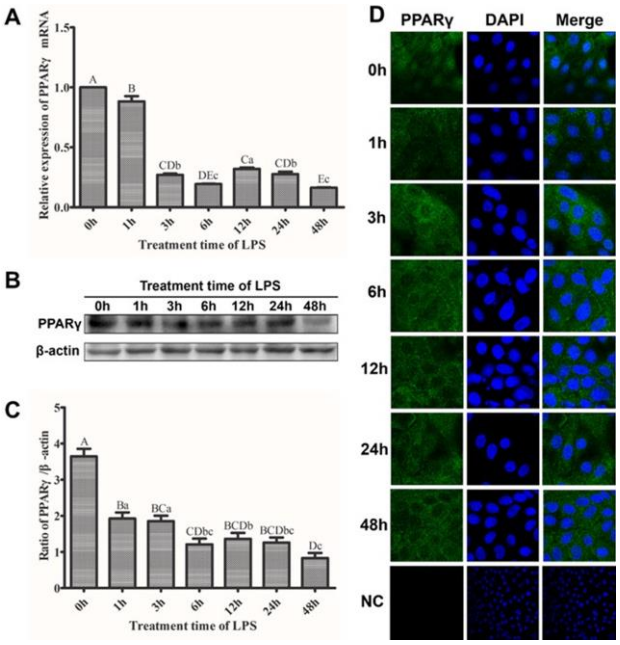

Fig. 3: LPS inhibits the transcription, translation and translocation of PPARγ in the bMECs. **A**: The relative transcription level of *PPARG* gene. **B**: The western blotting result of PPARγ. **C**: The relative expression level of PPARγ. **D**: The nuclear translocation of PPARγ (green) in bMECs (400×). 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 ($P > 0.05$)

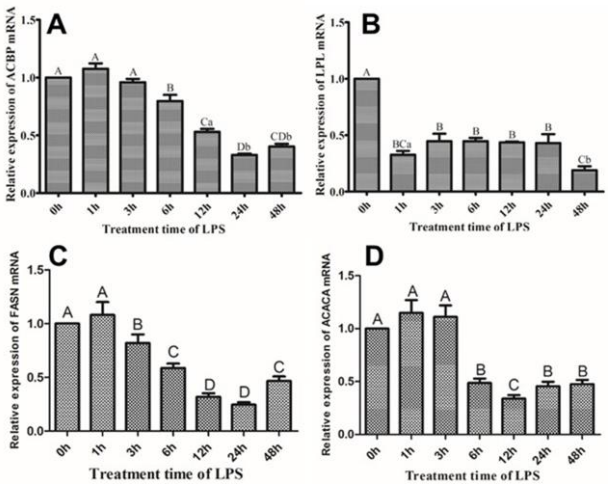

Fig. 4: LPS regulates the transcription of PPARγ target genes in the bMECs. **A**: The transcription level of *ACBP* gene. **B**: The transcription level of *LPL* gene. **C**: The transcription level of *FASN* gene. **D**: The transcription level of *ACACA* gene. The data with different capital letters 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 ($P > 0.05$)

2006). Milk fat depression have major economic impact in the dairy industry. Milk fat arise from two sources: the *de novo* fatty acids synthesis in mammary gland and the uptake of long-chain fatty acids from circulation (Rudolph *et al.*, 2007). Numerous lipogenic enzymes (*i.e.*, FASN, ACACA, LPL, ACBP, and CD36) are involved in the biosynthesis of milk lipids (Bionaz and Loor, 2008). Evidents suggests that PPARγ is associated with milk fat synthesis (Bionaz and Loor, 2008; Kawaiand and Rosen, 2010; Ma, 2012). LPS could induce milk fat depression via regulating the body and blood fat metabolism. However, the specific roles and the mechanisms of PPARγ in regulating LPS-induced reduction of milk lipid biosynthesis are still unknown.

PPARs are ligand-activated transcription factors, including α, δ, and γ. The three subtypes of PPAR alter lipid metabolism in different ways. PPARγ play vital role in adipocyte differentiation, and fatty acids synthesis (Kawaiand and Roser, 2010). Peroxisome proliferators (including herbicides, leukotriene antagonists, plasticizers, and hypolipidemic drugs) can activate PPAR (Ma, 2012). Furthermore, Bionaz *et al.* (2012) found that long-chain fatty acids activate PPAR and its target genes participate in regulating lipid metabolism in bovine kidney cells. Activated PPARγ can coordinate up-regulation of lipogenic gene networks in bMEC (Bionaz and Loor, 2008). That indicate the role of PPARγ in regulating milk fat synthesis. PPARγ in bMEC upregulates the expression of its target genes, such as *FASN*, *ACACA*, and *SCD* (Kadegowda *et al.*, 2009). Therefore, we hypothesize that PPAR*γ* may be a key factor in LPS-induced the decrease of milk lipid synthesis in bMEC.

Cell culture was a convenient tool for dissecting milk fat synthesis process. In the present study, near-fibroblast-free bMEC cultures were obtained by differential trypsinization. The flow cytometry data showed that the ratio of CK18-positive cells is 99.04%. The bMEC were treated with oleic acid to induce milk lipid synthesis. The results of BODIPY staining and TG detection showed that oleic acid could induce high lipid production ability of bMEC. Therefore, the lactating bMEC culture model is suitable for investigating the milk fat synthesis function. The TG content of the bMEC and culture medium were detected by enzymatic colorimetric assay. We found that LPS could decrease milk lipid synthesis and secretion. PPAR*γ* is a nuclear receptor and transcription factor that plays important role in milk fat synthesis and mastitis therapy in dairy cows (Kadegowda *et al.*, 2009). Therefore, the expression and activity of PPAR*γ* were detected in this study. We found that LPS could inhibit the expression and nuclear translocation of PPAR*γ*. The results suggest that PPAR*γ* participate in LPS-induced reduction of TG synthesis and secretion in bMEC.

ACBP, *LPL*, *FASN*, and *ACACA* gene are PPAR*γ* downstream target genes. As an intracellular carrier of acyl-CoA esters, ACBP can bind C_{12}-C_{22} acyl-CoA

esters with very high affinity (Burton *et al*., 2005). LPL is an enzyme playing an important role in milk lipid synthesis. It catalyzes the complete hydrolysis of TG yielding glycerol and fatty acids (Mead *et al*., 2002). ACACA supplies malonyl-CoA to FAS and is committed to de novo lipogenesis in mammary gland tissue (Mao *et al*., 2009; Ma, 2012). Therefore, the mRNA level of *ACBP*, *LPL*, *FASN*, and *ACACA* gene were evaluated by quantitative RT-PCR. Consistent with the PPARγ, we found that LPS could inhibit the transcription of *ACBP*, *LPL*, *FASN*, and *ACACA* gene in a time-dependent manner. The results suggest that LPS could inhibit the long-chain fatty acids uptake and *de novo* synthesis of short- and medium-chain fatty acids in bMEC via inhibiting the expression and activity of the PPARγ.

Conclusion

LPS can decrease the level of triglyceride synthesis in bMEC via down-regulating the expression and activity of the PPARγ. Our data may provide new insight into the mechanisms of milk fat depression caused by LPS.

Acknowledgements

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