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Oleic acid induces specific alterations in the morphology, gene expression and steroid hormone production of cultured bovine granulosa cells

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ABSTRACT

After parturition, one of the major problems related to nutritional management that is faced by the majority of dairy cows is negative energy balance (NEB). During NEB, excessive lipid mobilization takes place and hence the levels of free fatty acids, among them oleic acid, increase in the blood, but also in the follicular fluid. This accumulation can be associated with serious metabolic and reproductive disorders. In the present study, we analyzed the effects of physiological concentrations of oleic acid on cell morphology, apoptosis, necrosis, proliferation and steroid production, and on the abundance of selected transcripts in cultured bovine granulosa cells. Increasing oleic acid concentrations induced intracellular lipid droplet accumulation, thus resulting in a foam cell-like morphology, but had no effects on apoptosis, necrosis or proliferation. Oleic acid also significantly reduced the transcript abundance of the gonadotropin hormone receptors, *FSHR* and *LHCGR*, steroidogenic genes *STAR*, *CYP11A1*, *HSD3B1* and *CYP19A1*, the cell cycle regulator *CCND2*, but not of the proliferation marker *PCNA*. In addition, treatment increased the transcript levels of the fatty acid transporters *CD36* and *SLC27A1*, and decreased the production of 17-beta-estradiol and progesterone. From these data it can be concluded that oleic acid specifically affects morphological and physiological features and gene expression levels thus altering the functionality of granulosa cells. Suggestively, these effects might be partly due to the reduced expression of *FSHR* and thus the reduced responsiveness to FSH stimulation.

1. Introduction

After parturition, many high-yield dairy cows suffer from ovarian dysfunction like delayed cycle resumption and prolonged calving intervals due to different factors (Opsomer et al., 1998; Shrestha et al., 2004). One of the major risk factors is negative energy balance (NEB) (Beam and Butler, 1999; Opsomer et al., 2000). Due to NEB, animals can show suboptimal milk yield and are vulnerable to infections, metabolic diseases and subfertility. During NEB, fat from adipose tissue gets mobilized due to low blood glucose levels to meet the animal's energy requirements. This leads to increased plasma levels of free fatty acids like, palmitic acids, steric acid, oleic acid, but also of Beta hydroxyl butyrate (BHBA) (Rukkwamsuk et al., 2000). As a consequence, corresponding levels are also increased in the follicular fluid (Leroy et al., 2005). Excessive lipid mobilization is associated with metabolic and reproductive disorders (Roche, 2006), which may affect the cumulus-oocyte complex (COC) morphology and embryo quality (Jungheim et al., 2011; Leroy et al., 2005; Metwally et al., 2007). In bovine, also short-term fasting periods lead to increased free fatty acid plasma levels due to decreased blood glucose concentrations (Aardema et al., 2013). The levels of glucose and BHBA in follicular fluid are similar to those in the blood. However, while the levels of palmitic and steric acid did not reach the plasma

Abbreviations: BHBH, beta-hydroxy butyric acid; *CD36*, cluster of differentiation 36; CL, corpus luteum; CREB-CBP, cAMP regulatory element-binding protein complex; *CCND2*, cyclin-D2; *CYP11A1*, cytochrome P450, family 11, subfamily A, polypeptide 1; *CYP19A1*, cytochrome P450, family 19, subfamily A, polypeptide 1; FSH, follicle-stimulating hormone; *FSHR*, follicle-stimulating hormone receptor; *HSD3B1*, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1; IGF1, insulin-like growth factor 1; LH, luteinizing hormone; *LH/CGR*, luteinizing hormone/choriogonadotropin receptor; mTOR, mammalian target of rapamycin; NEB, negative energy balance; P4, progesterone; PBS, phosphate-buffered saline; *PCNA*, proliferating cell nuclear antigen; PI3K, phosphoinositid-3-kinase; PKA, protein kinase A; SGK, serum and glucocorticoid-induced kinase; *SLC27A1*, solute carrier family 27 (fatty acid transporter), member 1; *STAR*, steroidogenic acute regulatory protein.

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levels, the levels of oleic acid were nearly the same (200–300 μM, Aardema et al., 2013). Oleic acid promotes apoptosis and necrosis of human lymphocytes through the activation of caspase-3 (Cury-Boaventura et al., 2006). At high concentration oleic acid inhibits the proliferation of scar and normal fibroblasts and causes an excessive and continued inflammatory reaction by promoting the secretion of pro-inflammatory mediators (Jiang et al., 2012). In lymph-node carcinoma cells of the prostate (LNCaP), oleic acid significantly inhibits proliferation (Liu et al., 2009).

In a previous report it was shown that oleic acids reduced proliferation, but stimulated 17-beta-estradiol secretion in bovine granulosa cells (Vanholder et al., 2005). However, there are no data available on the effects of oleic acid on gene expression in bovine granulosa cells. The aim of the present study was to analyzed effects of elevated oleic acid concentrations as observed in the follicular fluid during negative energy balance or starvation on granulosa cell function on the molecular level. Therefore, during this work, we comprehensively studied the impact of oleic acid on cell morphology, cell cycle, proliferation, steroid hormone secretion, and on the expression of key genes involved in the function of granulosa cells in a serum-free, estrogen-producing granulosa cell long term culture model.

2. Material and methods

2.1. Materials

Unless otherwise indicated, all chemicals used were purchased from Sigma Aldrich (Steinheim, Germany).

2.2. Granulosa cells cultures

Granulosa cells were isolated and cultured as previously described, (Baufeld and Vanselow, 2013; Gutierrez et al., 1997; Hamel et al., 2005). Briefly, granulosa cell preparations (n = 3) were cell pools derived from 30 to 40 ovaries and 10–15 small to medium sized follicles (2–6 mm diameter), each. Only follicles containing clear, turbid free antral fluid were aspirated with the help of a syringe and an 18-G needle in 1× PBS that was supplemented with antibiotics. Viability of cells was measured in a hemocytometer as per the trypan blue exclusion method. The proportion of living cells was 60–80% in freshly isolated granulosa cell samples (data not shown). Only living cells were included in cell counts. Cells were seeded on 24-well collagen coated plates at $1.25x10^5$ cells per 0.5 ml of basal α-MEM supplemented with 2 mM L-Glutamin, 0.1% BSA, 0.084% sodium bicarbonate, 20 mM HEPES, transferrin (5 μg/ml), sodium selenite (4 ng/ml), 1 mM non-essential amino acids, penicillin (100 IU), streptomycin (0.1 mg/ml), 10 ng/ml insulin (above chemicals from Biochrom, Berlin, Germany), 20 ng/ml FSH, 50 ng/ml IGF-I and 2 μM androstenedione. For initial 48 h, cells were grown in α-MEM at 37 °C in the presences of 5% CO_2. After initial 48 h, the media were replaced with media (supplemented α-MEM) containing different concentrations of oleic acid ($C_{18}H_{34}O_2$). Oleic acid was dissolved in 100% ethanol as described in Vanholder et al. (2005) and diluted to stocks of 0.35 M and final concentrations of 100 μM, 200 μM and 400 μM in culture medium. All controls were treated with 0.11% of the solvent ethanol as vehicle corresponding to the ethanol percentage of the highest oleic acid treatment group (400 μM). Similar oleic acid concentrations were found also in follicular fluid after starvation in vivo (Aardema et al., 2013). Media were replaced every other day according to (Baufeld and Vanselow, 2013). After 8 days in culture the cells were lysed for RNA isolation and conditioned media were stored at-20 °C for steroid determination.

2.3. Staining of intracellular lipid droplets

Intracellular lipid droplets were stained with the Lipid Staining Kit Oil Red O according to the manufacturer's instructions. Briefly, cells were cultured in collagen-coated 96-well plates and treated with different concentrations of oleic acid as described. After treatment, the medium was removed and the cells were gently washed twice with 100 μl of PBS. After the addition of 100 μl of 10% formalin, the cells were incubated for 30 min at room temperature. Subsequently, the formalin was discarded and the cells were washed twice with 100 μl of water and incubated with 100 μl of 60% isopropanol for 5 min. Then, isopropanol was removed, Oil Red O working solution was added, and the cells were incubated at room temperature for 15 min. Oil Red O solution was removed and the cells were washed five times with water to remove excess Oil Red O solution. For nuclear staining, 100 μl of hematoxylin was added for 1 min, and the cells were washed five times with water to remove the excess hematoxylin. Then, 100 μl of water was added and the staining was evaluated in a Nikon TMS-F inverted microscope.

2.4. Flow cytometry analysis

Different phases of the cell cycle and the percentage of apoptotic and necrotic cells were determined by flow cytometry analysis. Briefly, after culturing and treatment with different oleic acid concentrations the medium was removed completely, 250 μl of accutase enzyme (Biochrom, Berlin, Germany) was added to each well of the 24-well plates and the cells were incubated at 37 °C in 5% CO_2 for 20 min. After microscopic control, cells from three wells were pooled, and transferred to a 1.5 ml micro centrifuge tube. To collect all remaining cells, the wells were washed with 300 μl of PBS and transferred to the same tube. After centrifugation at 300×g for 5 min at 4 °C, the supernant was removed and the sedimented cells were re-suspended in 300 μl PBS. The cells were then added to 10 ml of ethanol (70% v/v, ice cold) dropwise and stored at −20 °C for 1–2 h. Subsequently, the cells were subjected to centrifugation at 300×g at 4 °C for 5 min. The pellets were re-suspended in 1 ml of RNase solution (1 mg/ml) and incubated at 37 °C in 5% CO_2 for 30 min. For nuclear staining 100 μl of propidium iodide solution (70 μM final concentration) was added and the cells were incubated at 37 °C for 30 min. Then, propidium iodide fluorescence was quantified from single cells (10,000 counts) at 488 nm (argon laser band) and an emission at 600 nm ± 10 nm using a flow cytometer (EPICS-XL, Beckman-Coulter, Krefeld, Germany). The data were recorded and analyzed using the Multicycle software (Phoenix, USA) as described in previous studies (Darzynkiewicz et al., 1992; Lohrke et al., 1998).

2.5. RNA isolation and cDNA synthesis

Total RNA was isolated using the Nucleo Spin RNA II Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions and quantified using a NanoDrop1000 Spectrophotometer (Thermo Scientific, Bonn, Germany). cDNA was prepared with M-MLV reverse transcriptase and RNasin ribonuclease inhibitor (both Promega, Mannheim, Germany) using oligo-(dT) primers (2 ng/μl) mixed with random hexamer primers (4 ng/μl; both Roche, Mannheim, Germany) from 200 ng RNA as previously described (Baufeld and Vanselow, 2013).

2.6. Real-time RT-PCR (qPCR)

Quantification of *FSHR*, *LHGCR*, *CCND2*, *CYP19A1*, *STAR*, *CYP11A1*, *HSD3B1*, *CD36*, *PCNA*, *SLC27A1*, relative to *TBP* reference transcripts by real time PCR was performed using SensiFast SYBR No-ROX