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

Vengala Rao Yenuganti, Torsten Viergutz, Jens Vanselow *

Institute of Reproductive Biology, Leibniz Institute for Farm Animal Biology (FBN), 18196 Dummerstorf, Germany

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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.
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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.

* Corresponding author.
E-mail address: vanselow@fbn-dummerstorf.de (J. Vanselow).

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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

(Bioline, Luckenwalde, Germany) and gene-specific primers (Table 1) in a LightCycler 96 instrument (Roche) as described previously (Baufeld and Vanselow, 2013). Briefly, we amplified 0.25 and 0.5 µl of cDNA of each sample in a 12-µl reaction mixture as follows: pre-incubation at 95 °C for 5 min, 40 cycles of denaturation for 20 s at 95 °C, annealing for 15 s at 60 °C, extension at 72 °C for 15 s and single-point fluorescence acquisition for 10 s. To ensure amplification of correct products melting points of products were routinely analyzed. Initially, all amplicons were cloned and sequenced for authenticity. These cloned plasmids were used as external standards. For preparation of external standard curve we prepared five fresh dilutions with concentrations of 5×10^{-12} to 5×10^{-16} g DNA/reaction. These were co-amplified with each run of samples. After amplification, values of 0.25 and 0.5 µl of cDNA were averaged considering different dilutions. The size of product was routinely controlled by agarose gel electrophoresis. The abundance of transcripts was calculated relatively to TBP as an appropriate housekeeping control (Baddela et al., 2014).

2.7. Quantification of 17-beta-estradiol and progesterone concentrations in culture media

After, 8 days of culture, media conditioned for 2 days from individual samples were collected and the concentrations of 17-beta-estradiol and progesterone were determined using radioimmunoassay (RIA) with rabbit-raised antibodies purified by chromatography as described previously (Schneider and Brüssow, 2006). The levels of 17-beta-estradiol were determined with a modified competitive 3H-RIA. The tracer, [2,4,6,7-3H]estradiol-17β, was purchased from Hartmann Analytic (Braunschweig, Germany). The lower limit for detection of 17-beta-estradiol was 3 pg/ml and intra- and interassay coefficients of variation were 6.9% and 9.9%, respectively. To prepare the standards, 17-beta-estradiol was dissolved in 100% ethanol and diluted in RIA buffer. The analysis of media was done with 10 µl of undiluted samples in duplicates. The levels of progesterone were quantified by a competitive 3H-radioimmunoassay. The tracer, [1,2,6,7-3H(N)] progesterone, was purchased from PerkinElmer (Boston, MA, USA). The minimum detection limit was 7 pg/ml and intra- and interassay coefficients of variation were 7.6% and 9.8%, respectively. Progesterone standard dilutions were prepared in RIA buffer after dissolving the steroid in 100% ethanol. For analysis, media were diluted 1:40 in RIA-buffer and 10 µl of the dilution was measured in duplicates. The levels of radioactivity were measured in a liquid scintillation counter (LSC) that also contains an integrated RIA-calculation programme (TriCarb 2900 TR; PerkinElmer).

2.8. Cell viability assay

The cell viability assay indicating the numbers of viable cells was performed as previously described (Baufeld and Vanselow, 2013). Briefly, cells were grown in 96-well plates and treated with oleic acid as described. After 8 d in culture, the medium was replaced with 100 µl of fresh medium and 20 µl of CellTiter 96 AQueous One Solution Reagent (Promega, Mannheim, Germany) in each well. Subsequently, the cells were incubated for 1 h at 37 °C in 5% CO_2 and the OD was measured at 450 nm in a MR5000 plate reader (Dynatech, Denkendorf, Germany).

2.9. Statistical analyses

Data of treatment groups were analyzed by one-way ANOVA (ANOVA, all pairwise multiple comparison procedures, Holm-Sidak method) or unpaired *t*-test using the Sigma Plot 11.0 Statistical Analysis System (Jandel Scientific, San Rafael, CA, USA). All experimental data are presented as means ± SD (standard deviation) of three independent experiments. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Cell morphology, lipid droplet formation and expression of fatty acid transporter transcripts

In vitro treatment of granulosa cells with increasing concentrations of oleic acid induced dramatic morphological and structural changes. At 400 µM, nearly all cells showed a typical morphology (Fig. 1) that has been described as "foam cell-like" in other cell types [2,32,35]. Oil Red O staining of the cells revealed numerous lipid droplets inside the cells, in contrast to untreated controls. The numbers of lipid droplets however, varied between different concentration groups. In the samples that received 400 µM oleic acid Oil Red O

Table 1
Primers used for transcript quantification by real-time PCR.

Name	Sequence	Size (bp)	Accession No.
CCND2	For: CGCAGGGCCGTGCCGGACGCCAAC Rev: CACGGCCCCCAGCAGCTGCAGATGG	279	NM_001076372
CD36	For: GCTCCTTAAGCCATTCTTGGAT Rev: CACCAGTGTCAACGCACTTT	151	NM_001278621.1
CYP11A1	For: AGAGAATCCACTTTCGCCACATC Rev: GGTCTTTCTTCCAGGTTCCTGAC	237	NM_176644
CYP19A1	For: GCTTTTGGAAGTGCTGAACCCAAGG Rev: GGGCCCAATTCCCAGAAAGTAGCTG	172	NM_174305
FSHR	For: TCACCAAGCTTCGAGTCATCCCAAA Rev: TCTGGAAGGCATCAGGGTCGATGTA	189	NM_174061
HSD3B1	For: TGTTGGTGGAGGAGAAGGATCTG Rev: GCATTCCTGACGTCAATGACAGAG	208	NM_174343
LHCGR	For: GCATCCACAAGCTTCCAGATGTTACGA Rev: GGGAAATCAGCGTTGTCCCATTGA	205	NM_174381
PCNA	For: GTGAACCTGCAGAGCATGGACTCGT Rev: CGTGTCCGCGTTATCTTCAGCTCTT	192	NM_001034494
STAR	For: TTGTGAGCGTACGCTGTACCAAG Rev: CTGCGAGAGGACCTGGTTGATG	236	NM_174189.2
SLC27A1	For: GGTGGTGAGCGGTGAGGACAGCA Rev: CGGAAAGGCCGAAGAGGTCCCGC	244	NM_001033625.2
TBP	For: GCCTTGTGCTTACCCACCAACAGTTC Rev: TGTCTTCCTGAAACCCTTCAGAATAGGG	200	NM_001075742.1

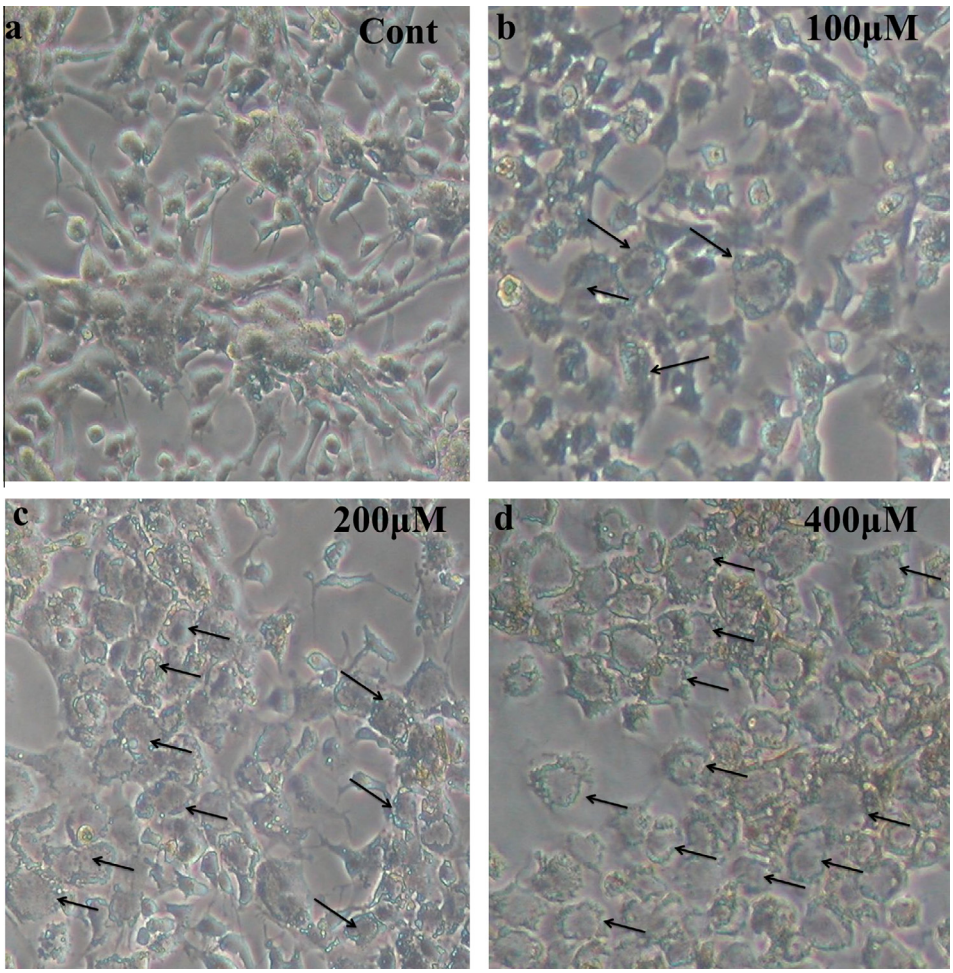

Fig. 1. Effects of oleic acid on the morphology of cultured granulosa cells. The different panels represent cultured bovine granulosa cells with oleic acid supplementation: (a) control, (b) 100 µM, (c) 200 µM, and (d) 400 µM oleic acid. Photomicrographs were captured using a Nikon TMS-F inverted microscope. The arrows indicate examples of cells with foam cell like morphology.

staining was more intense as compared with the 100 µM and 200 µM treatment groups (Fig. 2). As a first mechanistic approach, the transcript abundance of possibly involved fatty acid transporters *CD36* and *SLC27A1* was determined in treated (400 µM oleic acid) and untreated granulosa cells. The data showed that oleic acid treatment induced a significant increase of *CD36* and *SLC27A1* transcripts (Fig. 2e, f).

3.2. Effects on apoptosis, necrosis and proliferation, and on the expression of associated transcripts

Flow cytometry analysis of untreated granulosa cells and cells treated with different concentrations of oleic acid showed no significant differences of the proportions of cells at different phases of the cell cycle (Fig. 3a–h). The percentage of cells in the sub G0/G1 phase of the cell cycle indicated that only a small number of cells underwent necrosis with 4.67% ± 1.04%, 3.3% ± 0.12%, 4.83% ± 0.7% and 6.12% ± 0.67% in the controls and in the 100 µM, 200 µM and 400 µM treatment groups, respectively, however without significant differences. There were virtually no signs of apoptosis in controls and oleic acid treated cells. The proliferation activity of the cells was also low under all experimental conditions as indicated by the low percentage of cells in the S-phase or G2/M-phase, but high percentage in the G0/G1-phase. CellTiter 96 AQueous One Solution Cell Proliferation Assay results showed no change in the numbers of viable cells treated with oleic acid compared with untreated cells. Real-time PCR results showed that *PCNA* was not significantly altered, whereas *CCND2* transcript levels were significantly reduced by oleic acid treatment at all concentrations (Fig. 4).

3.3. Expression of gonadotropin receptors and transcripts involved in steroidogenesis

The data showed that oleic acid reduced the levels of *FSHR* and *LHCGR* transcripts at all concentrations, but maximum down regulation was observed in case of 400 µM (Fig. 5). Quantitative PCR analysis of transcripts involved in steroidogenesis, such as *STAR*, *CYP11A1*, *HSD3B1* and *CYP19A1*, also revealed significant down-regulation with maximum inhibition at 400 µM (Fig. 6).

3.4. 17-beta-estradiol and progesterone production of oleic acid treated granulosa cells

17-beta-estradiol and progesterone levels in granulosa cell conditioned media were determined after treatment with different oleic acid concentrations. The results showed that all concentrations of oleic acid gradually reduced the production of 17-beta-estradiol, whereas that of progesterone was significantly decreased only in case of 400 µM (Fig. 7).

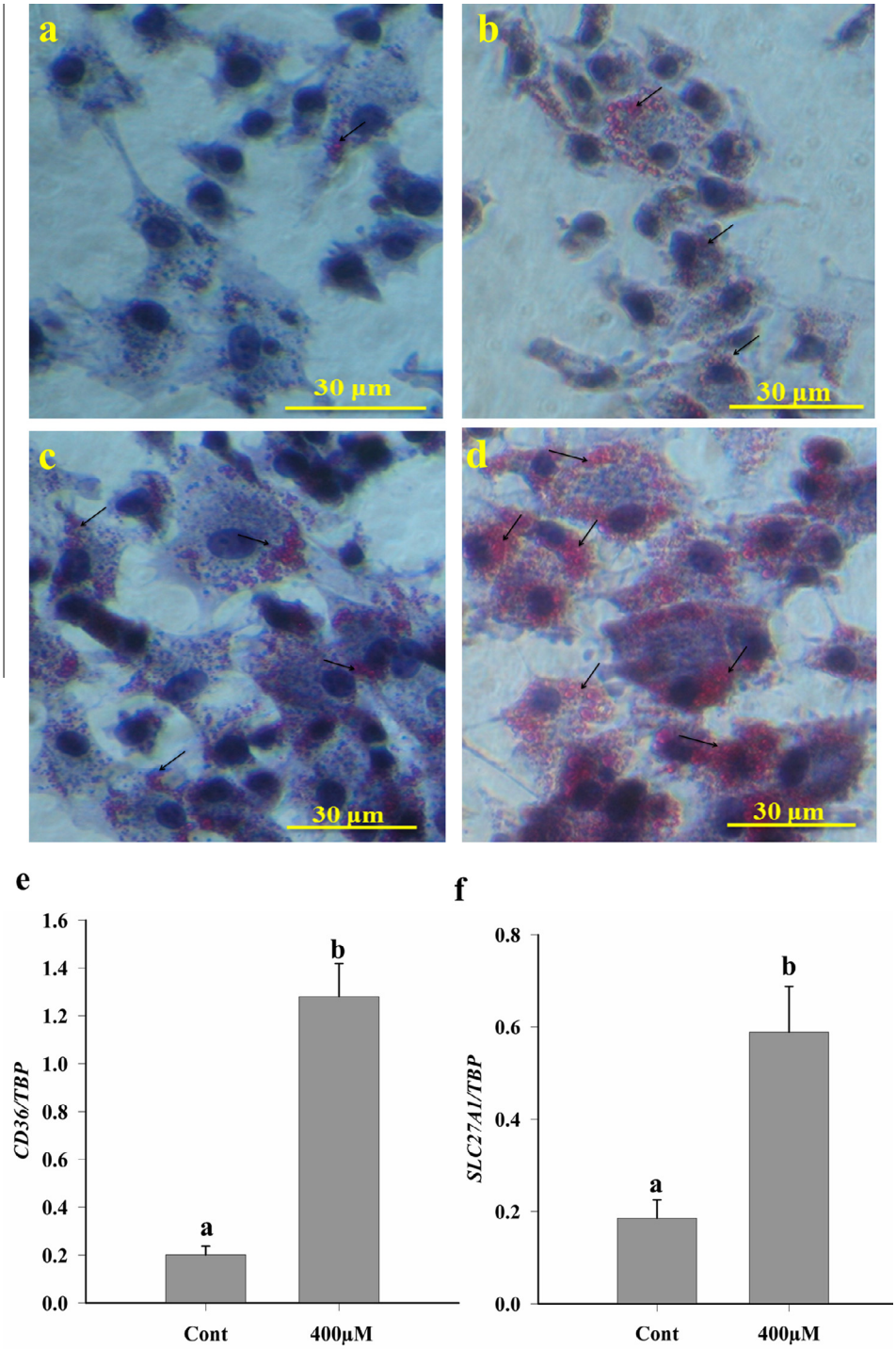

Fig. 2. Lipid droplet formation and expression of *CD-36* and *SLC27A1* transcripts in oleic acid treated granulosa cells. The different panels represent cultured granulosa cells under control conditions (a) and with 100 µM (b), 200 µM (c), and 400 µM (d) oleic acid supplementation. Photomicrographs were captured using a Nikon TMS-F inverted microscope. The arrows indicate intracellular lipid droplets. Diagrams (e) and (f) indicate relative mRNA levels of *CD36* and *SLC27A1* in vehicle controls (Cont) and in cells treated with 400 µM oleic acid. Scale bars in (a) to (d) indicate 30 µm. The data in (e) and (f) are shown as means ± SD (n = 3). Different letters indicate significant differences between groups (unpaired *t*-test; $P < 0.05$).

4. Discussion

4.1. Oleic acid induced intracellular lipid droplet accumulation and the transformation into foam cells without changing the proportion of apoptotic, necrotic and proliferative cells

In the present study, we describe for the first time that oleic acid induced specific and profound morphological, physiological and molecular transformations of bovine granulosa cells. The marked morphological changes closely resemble foam cells, which were previously observed in smooth muscle cells and macrophages (Aqel et al., 1984; Doran et al., 2008; Ma et al., 2011; Oh et al., 2012). Foam cells are characterized by the accumulation of lipid droplets, which can be identified by the Oil Red O staining (Ii et al., 2008; Lee et al., 2009; Xu et al., 2010). In smooth muscle cells, it was observed that oleic acid can induce foam cell formation

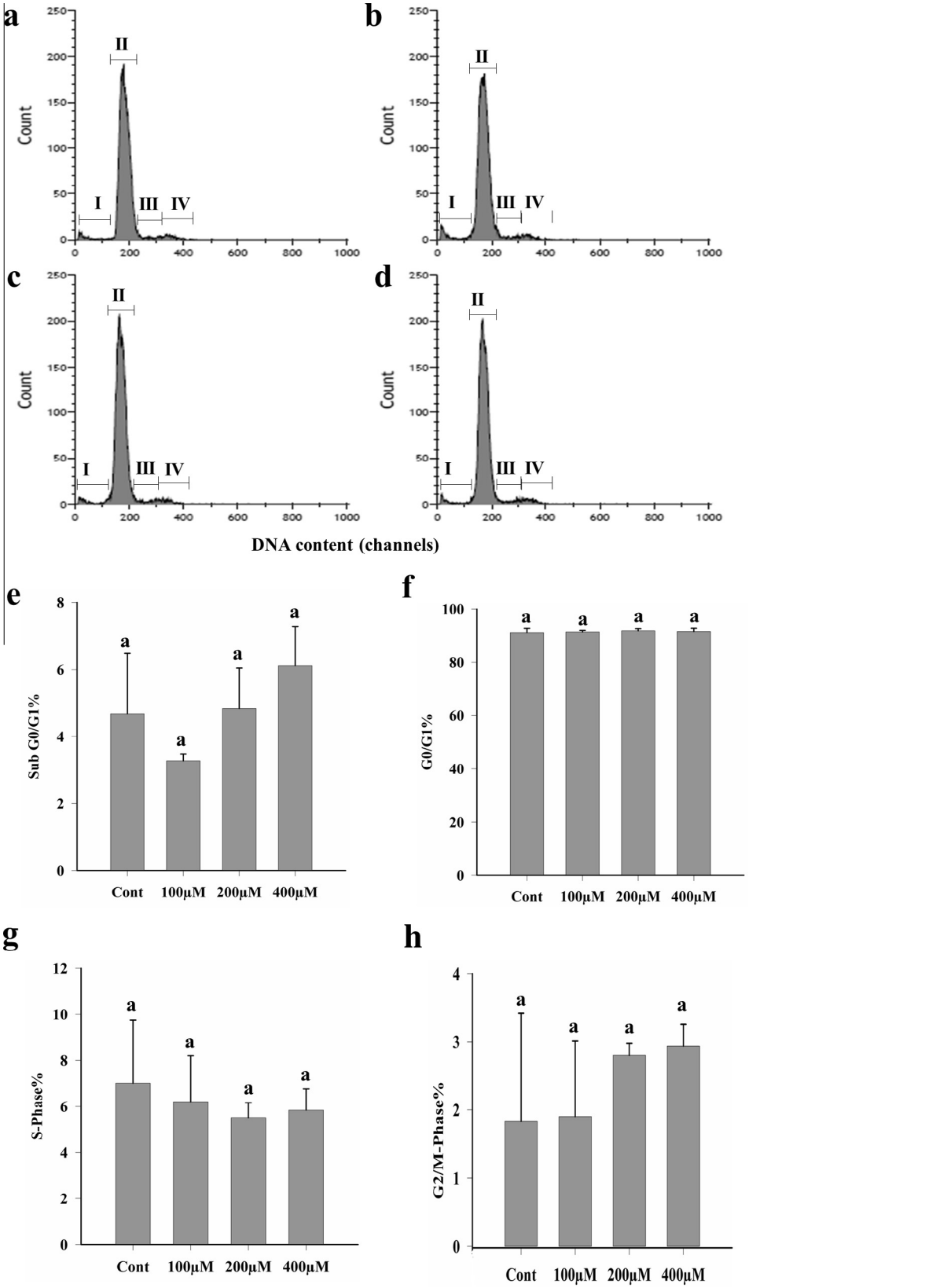

Fig. 3. Effects of oleic acid on cell cycle phases of cultured granulosa cells. The cell cycle distribution was analyzed by propidium iodide staining and flow cytometric analysis using the Multicycle program. The DNA histograms (a–d) demonstrate granulosa cells after culture without (a, control) and with oleic acid (b, 100 μM; c, 200 μM and d, 400 μM). Region (I), peak (II), region (III) and peak (IV) indicate the portion (%) of cells from the sub-G0/G1-phase, from the G0/G1-phase, from the S-phase and from G2/M-phase of the cell cycle, respectively. The diagrams (e–h) represent the evaluation of three experiments. Cont, 100 μM, 200 μM and 400 μM indicate the vehicle control group and the groups treated with different concentrations of oleic acid. The data are shown as means ± SD (n = 3). Significant differences between groups (Holm Sidak test; $P < 0.05$) were not found.

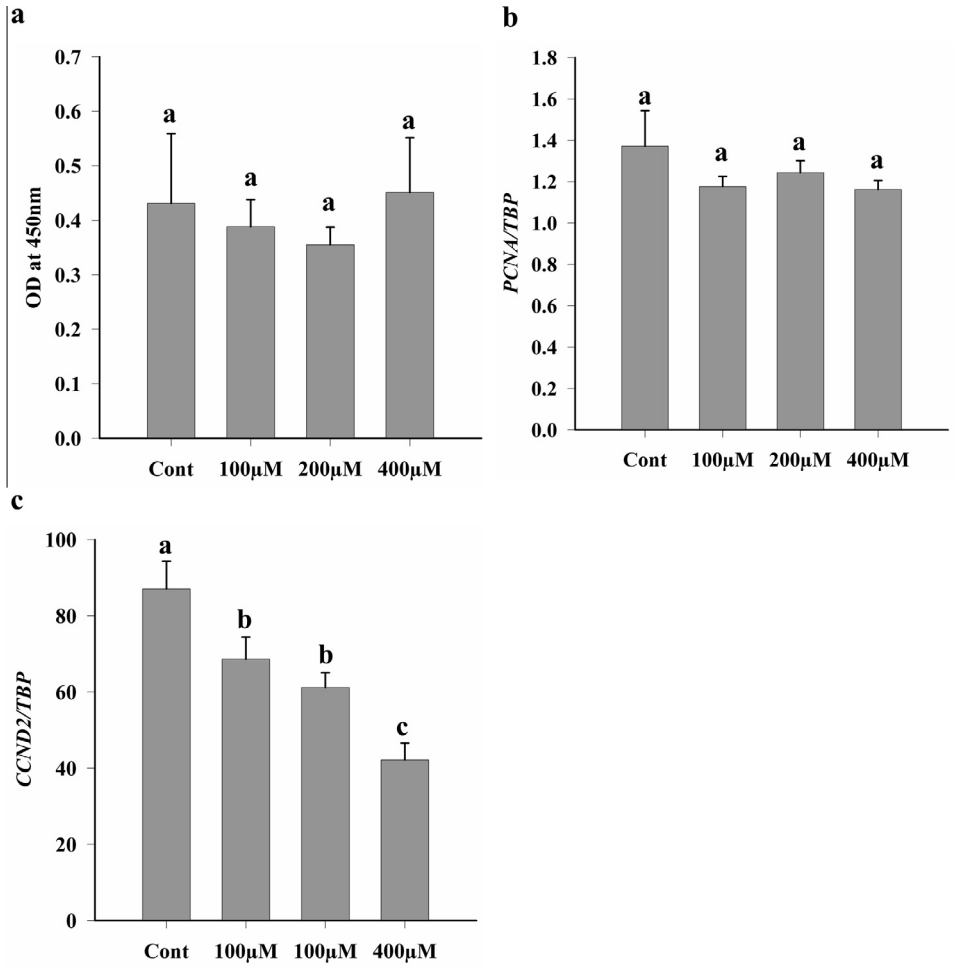

Fig. 4. Effects of oleic acid on viability/proliferation and on transcript abundance of genes involved in proliferation and cell cycle regulation. The results of the cell viability/proliferation assay (MTT assay) after oleic acid treatment are shown in (a). The transcript abundance of *PCNA* and *CCND2* in oleic acid treated granulosa cells are shown in (b) and (c), respectively. Cont, 100 µM, 200 µM and 400 µM indicate the vehicle control and the groups treated with different concentrations of oleic acid. The data are shown as means ± SD (Holm Sidak test; n = 3). Different letters indicate significant differences ($P < 0.05$).

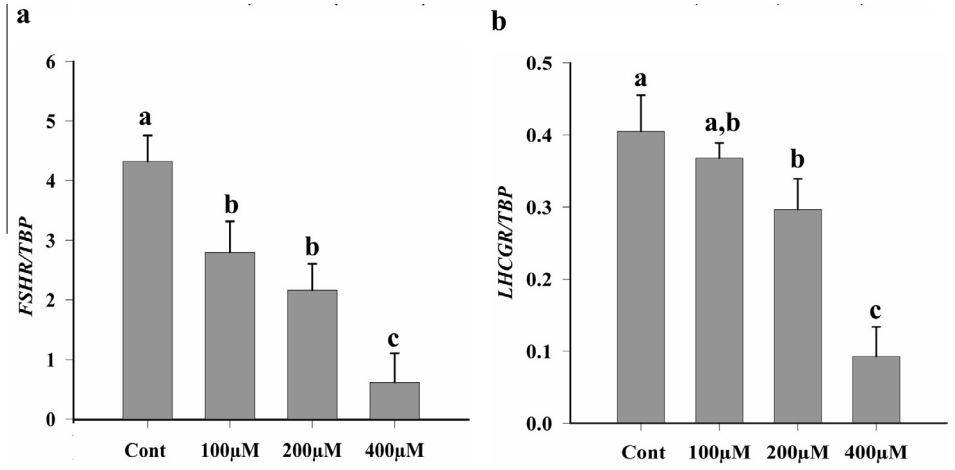

Fig. 5. Effects of oleic acid on gonadotropin receptor transcript abundance. Diagrams in (a) and (b) indicate the relative abundance of *FSHR* and *LHCGR* transcripts in cultured granulosa cells after treatment with different amounts of oleic acid. Cont, 100 µM, 200 µM and 400 µM indicate the vehicle control and the groups treated with different oleic acid concentrations. The data are shown as means ± SD (n = 3). Different letters indicate significant differences (Holm Sidak test; $P < 0.05$).

by increasing lipid droplet accumulation in a concentration-dependent manner (Ma et al., 2011). In addition to this, oleic acid also increased lipid droplet formation in Huh7 cells (Rohwedder et al., 2014). In a recent paper it has been described that lipid droplet accumulation in the cumulus cell layer may have a protective function (Lolicato et al., 2015). Here, we describe that oleic acid induced the expression of *CD36* and *SLC27A1*, which are involved in the transport of fatty acids in different cell types

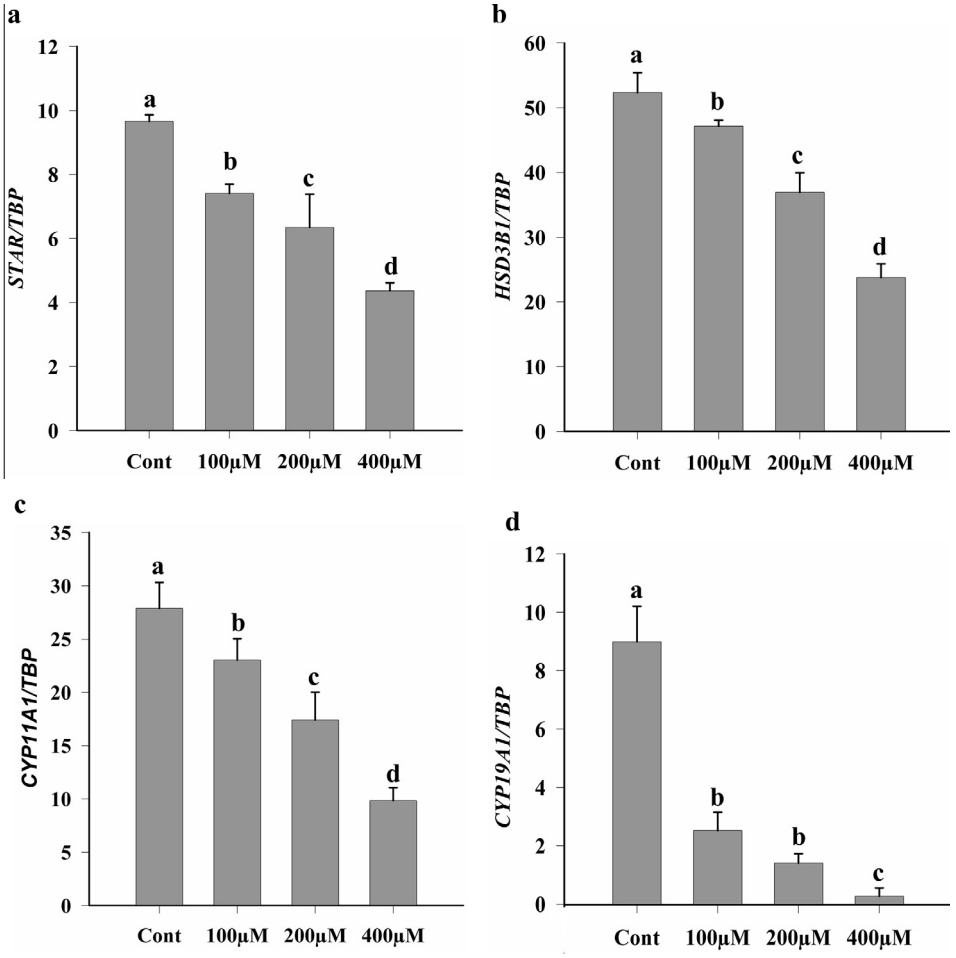

Fig. 6. Effects of oleic acid on the expression of key genes of estrogen and progesterone biosynthesis. Relative abundance of *STAR* (a), *CYP11A1* (b), *3β HSD1* (c) and *of CYP19A1* (d) transcripts was considerably lower in oleic acid treated granulosa cells as compared with the untreated controls. Cont, 100 μM, 200 μM and 400 μM indicate the vehicle control and the groups treated with different oleic acid concentrations. The data are shown as means ± SD (n = 3). Different letters indicate significant differences (Holm Sidak test; $P < 0.05$).

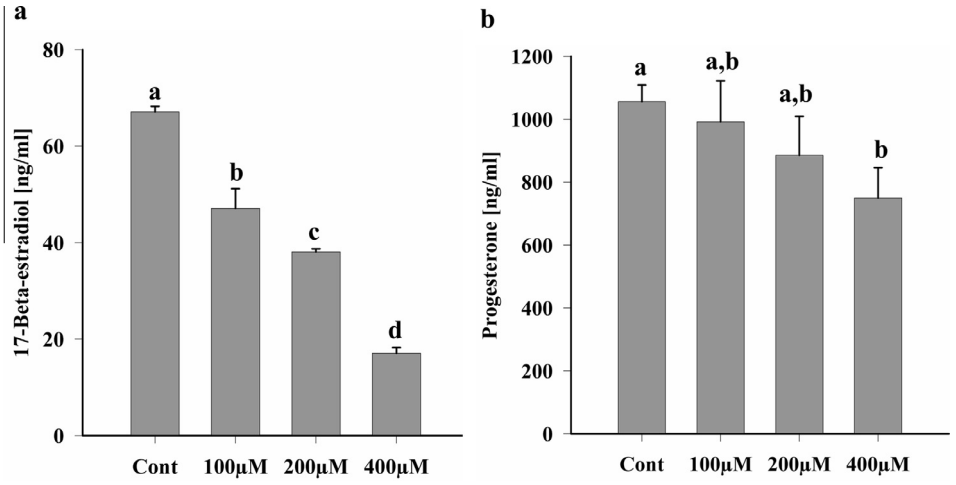

Fig. 7. Effects of oleic acid on the 17-beta-estradiol and progesterone production of cultured bovine granulosa cells. 17-beta-estradiol (a) and progesterone (b) concentrations were determined in media conditioned by granulosa cells after treatment with different oleic acid concentrations. The levels of estradiol and progesterone were analyzed by RIA after 8 days in culture. Cont, 100 μM, 200 μM and 400 μM indicate the vehicle control and the groups treated with different oleic acid concentrations. The data are shown as means ± SD (n = 3). Different letters indicate significant differences (Holm Sidak test; $P < 0.05$).

(Bonen et al., 2007). Our results are consistent with other studies in bovine mammary epithelial cells, where oleic acid induced *CD36* mRNA expression (Yonezawa et al., 2004) and where it was shown that inhibition of CD36 lead to inhibition of lipid droplet formation (Ma et al., 2011). In the light of these studies, our findings suggest that oleic acid may also induce the accumulation of lipid droplets

via elevated *CD36* and *SLC27A1* synthesis in bovine granulosa cells. However, it is not yet clear, whether or how lipid droplet accumulation is causally involved in other effects induced by oleic acid treatment as altered gene expression and reduced steroid production.

In spite of these dramatic morphological changes, our results also showed that treatment with oleic acid did not induce cell death (apoptosis or necrosis). Also the number of viable granulosa cell was not affected even at highest oleic acid concentrations. This result is consistent with some previous studies, where oleic acid was reported to have no effects even at high concentrations in CHO cells and β-islet cells on cell viability, but can even prevent palmitate-induced apoptosis (Listenberger et al., 2001, 2003; Maedler et al., 2001). In contrast, however, it has also been reported that oleic acid promotes apoptosis and necrosis in human lymphocytes, where it mediates cell death by the activation of caspase-3 (Cury-Boaventura et al., 2006). From our results, we conclude that oleic acid at physiological concentrations does not significantly affect the viability of cultured granulosa cells or the proportion of apoptotic and necrotic cells. With regards to gene expression, however, cell cycle regulation might be slightly affected, because the abundance of *CCND2* transcripts showed significant down regulation in cultured granulosa cells.

4.2. The decreased steroid hormone production might be due to the down-regulation of associated key transcripts by oleic acid

To determine effects of oleic acid on granulosa cell function, we analyzed key genes involved in gonadotropin signaling such as *FSHR* and *LHCGR*, and in steroidogenesis such as *STAR*, *CYP11A1*, *HSD3B*, and *CYP19A1*. Our data showed that oleic acid decreased the expression of these genes. FSHR has been frequently shown to be present on granulosa cells (Camp et al., 1991; Sprengel et al., 1990; Zeleznik et al., 1974). The binding of FSH leads to the activation of FSHR, which in turn can induce nearly 500 target genes (Escamilla-Hernandez et al., 2008; Hsueh and Rauch, 2012) that are involved in processes of cytodifferentiation and proliferation thus leading to the development of preovulatory follicles (Erickson, 1983; Hirshfield, 1991).

FSH also activates the transcription of genes involved in the cascade of steroidogenesis, as *STAR*, encoding steroidogenic acute regulatory protein, which is involved in the initial step of steroidogenesis, the transport of cholesterol into the mitochondria. Subsequent steps are catalyzed by cholesterol side chain cleavage enzyme encoded by *CYP11A1*, and by the 3 beta hydroxyl steroid dehydrogenase transcribed from *HSD3B1*, and *CYP19A1*, encoding aromatase, the key enzyme of estrogen synthesis (Clarke et al., 1993; Eimerl and Orly, 2002; Ke et al., 2004, 2005). According to our data, the treatment of granulosa cells with oleic acid significantly reduced the expression of *FSHR* and *LHCGR* along with *STAR*, *CYP11A1*, *HSD3B1* and *CYP19A1*. Accordingly, the levels of progesterone and 17-beta-estradiol found in granulosa cell conditioned media were also lower compared with the control. Very likely, the reduced production of progesterone and particularly of 17-beta-estradiol might be due to the reduced transcription of *STAR*, *CYP11A1*, *HSD3B1* and *CYP19A1*.

These results appear contradictory to previously published data (Vanholder et al., 2005), from which it was concluded that oleic acid inhibited proliferation, but stimulated the production of 17-beta-estradiol in cultured bovine granulosa cells. However, our study and the study by Vanholder et al. (2005) are based on very different experimental approaches.

In the study of Vanholder et al. (2005) granulosa cells were harvested from large follicles and cultured for two days. In the present study, which was strongly focused on effects of oleic acid on gene expression granulosa cells were obtained from small to medium sized follicles and were cultured for 8 days, because it has been demonstrated that the abundance of *CYP19A1* transcripts

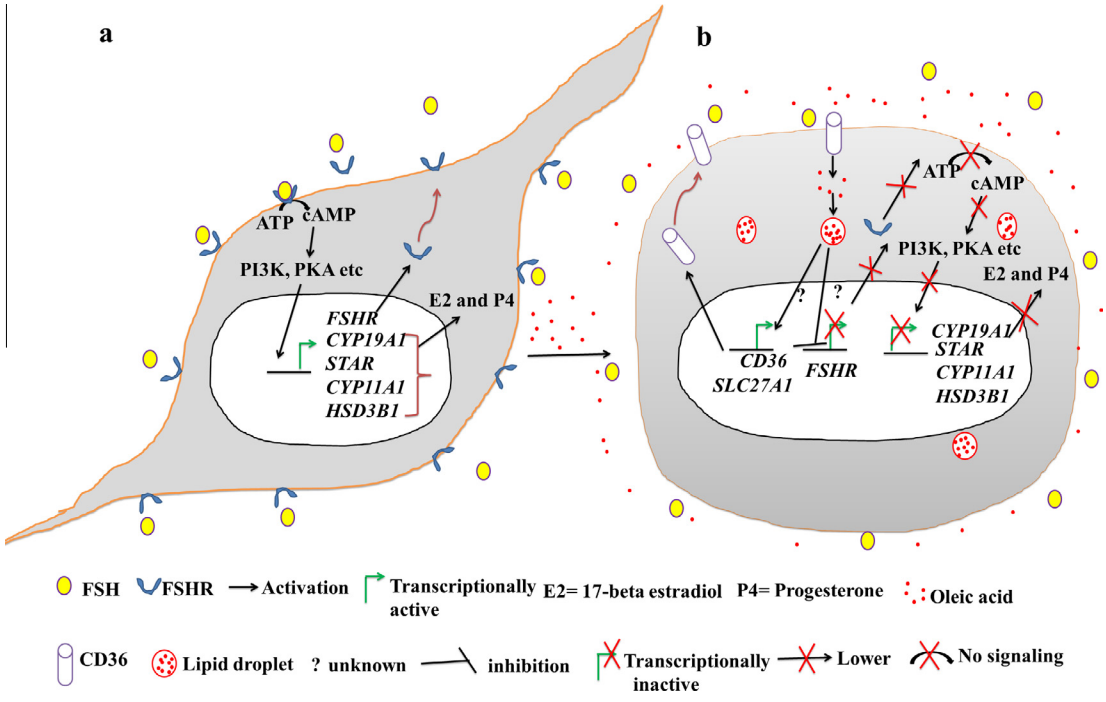

Fig. 8. Hypothetic involvement of FSH signaling in oleic acid effects. The schematic graphics show putative FSH signaling pathways in cultured granulosa cells under standard conditions and with oleic acid supplementation. (A) Under standard culture conditions (i.e. with FSH, IGF1 and A4 supplementation) granulosa cells display a fibroblast like structure and show considerable expression of *FSHR* receptor thus inducing different genes involved in steroidogenesis and other granulosa cell functions like *LHCGR*, *CCND2*, *CYP19A1*, *STAR*, *CYP11A1* and *HSD3B1* via PKA (Protein kinase A) and/or PI3K (Phosphoinositide 3-kinase) activation. (B) Treatment with oleic acid induces morphological changes, increased expression of *CD36* and *SLC27A1*, and decreased expression of FSH regulated genes. Also the expression of FSHR was largely down-regulated, thus suggesting that the cells become less sensitive to FSH and as a consequence downstream processes like estradiol and progesterone production were also compromised.

or of 17-beta-estradiol production in granulosa cells isolated from large follicles are rapidly (i.e. within the first 48 h) down-regulated under culture conditions (Baufeld and Vanselow, 2013; Gutierrez et al., 1997). In contrast, *CYP19A1* expression preferentially directed from the granulosa cell-specific promoter region 2 (Hamel et al. 2005), and as a consequence, 17-beta-estradiol production is considerably restored after 6 days in culture and further increases to day 8 of culture (data not shown), if granulosa cells descend from small- to medium-sized follicles (Gutierrez et al., 1997; Silva and Price, 2000). Accordingly, the observed effects of oleic acid on in vitro differentiated granulosa cells in the present work reflect long-term effects, thus including profound effects on cell type-specific gene expression profiles. In contrast, the effects observed by Vanholder et al. (2005) might be explained by rapid mechanisms including translational and/or enzyme activity. Possible changes of transcript abundance have not been determined in previous studies.

4.3. Oleic acid-induced alterations might be due to reduced sensitivity to gonadotropic hormones

An important clue to understand the multiple and specific effects of oleic acid on granulosa cells is provided by the observation that oleic acid clearly reduced the expression levels of *FSHR*. In rat granulosa cells, it was shown that FSH induces the expression of *LHCGR* via protein kinase A (PKA) and phosphoinositide3-kinase (PI3K) pathways (Law et al., 2013). *LHCGR* is highly expressed in granulosa cells during the preovulatory stage to enable responsiveness to the LH surge thus leading to ovulation, oocyte maturation and corpus luteum formation (Zhang et al., 2001). Thus, reduced *FSHR* expression might be responsible for the observed decrease in *LHCGR* transcripts abundance. FSH in cooperation with activin also mediates *CCND2* expression, which is required for granulosa cell proliferation (Hsueh et al., 1984; Park et al., 2005; Sicinski et al., 1996). Accordingly, follicular maturation is seriously compromised in *CCND2* deficient mice (Sicinski et al., 1996). The down regulation of FSHR may therefore affect the above signaling pathways thus leading to compromised granulosa cell function.

In the present study, we showed that oleic acid induced specific morphological and physiological changes and alterations in the gene expression levels of cultured bovine granulosa cells. These data support the hypothesis that during NEB increased oleic acid levels in the follicular fluid can directly affect the functionality of granulosa cells and thus the follicular development and differentiation, which might be responsible for the compromised fertility of dairy cows under these conditions. Suggestively, these alterations might be primarily attributed to the reduced expression of FSHR, which is absolutely essential for the development and maturation of ovarian follicles (Fig. 8).

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References

Aardema, H., Lolicato, F., van de Lest, C.H., Brouwers, J.F., Vaandrager, A.B., van Tol, H.T., Roelen, B.A., Vos, P.L., Helms, J.B., Gadella, B.M., 2013. Bovine cumulus cells protect maturing oocytes from increased fatty acid levels by massive intracellular lipid storage. Biol. Reprod. 88, 164.

Aqel, N.M., Ball, R.Y., Waldmann, H., Mitchinson, M.J., 1984. Monocytic origin of foam cells in human atherosclerotic plaques. Atherosclerosis 53, 265–271.

Baddela, V.S., Baufeld, A., Yenuganti, V.R., Vanselow, J., Singh, D., 2014. Suitable housekeeping genes for normalization of transcript abundance analysis by real-time RT-PCR in cultured bovine granulosa cells during hypoxia and differential cell plating density. Reprod. Biol. Endocrinol. 12, 118.

Baufeld, A., Vanselow, J., 2013. Increasing cell plating density mimics an early post-LH stage in cultured bovine granulosa cells. Cell Tissue Res. 354, 869–880.

Beam, S.W., Butler, W.R., 1999. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. J. Reprod. Fertil. Suppl. 54 (411–24), 411–424.

Bonen, A., Chabowski, A., Luiken, J.J., Glatz, J.F., 2007. Is membrane transport of FFA mediated by lipid, protein, or both? Mechanisms and regulation of protein-mediated cellular fatty acid uptake: molecular, biochemical, and physiological evidence. Physiology (Bethesda) 22 (15–29), 15–29.

Camp, T.A., Rahal, J.O., Mayo, K.E., 1991. Cellular localization and hormonal regulation of follicle-stimulating hormone and luteinizing hormone receptor messenger RNAs in the rat ovary. Mol. Endocrinol. 5, 1405–1417.

Clarke, T.R., Bain, P.A., Greco, T.L., Payne, A.H., 1993. A novel mouse kidney 3 beta-hydroxysteroid dehydrogenase complementary DNA encodes a 3-ketosteroid reductase instead of a 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4-isomerase. Mol. Endocrinol. 7, 1569–1578.

Cury-Boaventura, M.F., Gorjao, R., de Lima, T.M., Newsholme, P., Curi, R., 2006. Comparative toxicity of oleic and linoleic acid on human lymphocytes. Life Sci. 78, 1448–1456.

Darzynkiewicz, Z., Bruno, S., Del, B.G., Gorczyca, W., Hotz, M.A., Lassota, P., Traganos, F., 1992. Features of apoptotic cells measured by flow cytometry. Cytometry 13, 795–808.

Doran, A.C., Meller, N., McNamara, C.A., 2008. Role of smooth muscle cells in the initiation and early progression of atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 28, 812–819.

Eimerl, S., Orly, 2002. Regulation of steroidogenic genes by insulin-like growth factor-1 and follicle-stimulating hormone: differential responses of cytochrome P450scc side-chain cleavage, steroidogenic acute regulatory protein, and 3b-hydroxysteroid dehydrogenase/isomerase in rat granulosa cells. Biol. Reprod. 67, 900–910.

Erickson, G.F., 1983. Primary cultures of ovarian cells in serum-free medium as models of hormone-dependent differentiation. Mol. Cell Endocrinol. 29, 21–49.

Escamilla-Hernandez, R., Little-Ihrig, L., Orwig, K.E., Yue, J., Chandran, U., Zeleznik, A. J., 2008. Constitutively active protein kinase A qualitatively mimics the effects of follicle-stimulating hormone on granulosa cell differentiation. Mol. Endocrinol. 22, 1842–1852.

Gutierrez, C.G., Campbell, B.K., Webb, R., 1997. Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle- stimulating hormone, and morphological characteristics. Biol. Reprod. 56, 608–616.

Hamel, M., Vanselow, J., Nicola, E.S., Price, C.A., 2005. Androstenedione increases cytochrome P450 aromatase messenger ribonucleic acid transcripts in non-luteinizing bovine granulosa cells. Mol. Reprod. Dev. 70, 175–183.

Hirshfield, A.N., 1991. Development of follicles in the mammalian ovary. Int. Rev. Cytol. 124 (43–101), 43–101.

Hsueh, A.J., Adashi, E.Y., Jones, P.B., Welsh Jr., T.H., 1984. Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocr. Rev. 5, 76–127.

Hsueh, A.J., Rauch, R., 2012. Ovarian kaleidoscope database: ten years and beyond. Biol. Reprod. 86, 192.

Ii, H., Oka, M., Yamashita, A., Waku, K., Uozumi, N., Shimizu, T., Sato, T., Akiba, S., 2008. Inhibition of cytosolic phospholipase A(2) suppresses production of cholesteryl ester through the reesterification of free cholesterol but not formation of foam cells in oxidized LDL-stimulated macrophages. Biol. Pharm. Bull. 31, 6–12.

Jiang, Q., Wu, B.Y., Chen, X.D., 2012. Effect of oleic acid on the proliferation and secretion of pro-inflammatory mediators of human normal fibroblasts and scar fibroblasts. Zhonghua Shao Shang Za Zhi 28, 444–450.

Jungheim, E.S., Macones, G.A., Odem, R.R., Patterson, B.W., Lanzendorf, S.E., Ratts, V. S., Moley, K.H., 2011. Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during in vitro fertilization. Fertil. Steril. 95, 1970–1974.

Ke, F.C., Chuang, L.C., Lee, M.T., Chen, Y.J., Lin, S.W., Wang, P.S., Stocco, D.M., Hwang, J.J., 2004. The modulatory role of transforming growth factor beta1 and androstenedione on follicle-stimulating hormone-induced gelatinase secretion and steroidogenesis in rat granulosa cells. Biol. Reprod. 70, 1292–1298.

Ke, F.C., Fang, S.H., Lee, M.T., Sheu, S.Y., Lai, S.Y., Chen, Y.J., Huang, F.L., Wang, P.S., Stocco, D.M., Hwang, J.J., 2005. Lindane, a gap junction blocker, suppresses FSH and transforming growth factor beta1-induced connexin43 gap junction formation and steroidogenesis in rat granulosa cells. J. Endocrinol. 184, 555–566.

Law, N.C., Weck, J., Kyriss, B., Nilson, J.H., Hunzicker-Dunn, M., 2013. Lhcgr expression in granulosa cells: roles for PKA-phosphorylated beta-catenin, TCF3, and FOXO1. Mol. Endocrinol. 27, 1295–1310.

Lee, T.S., Lin, C.Y., Tsai, J.Y., Wu, Y.L., Su, K.H., Lu, K.Y., Hsiao, S.H., Pan, C.C., Kou, Y.R., Hsu, Y.P., Ho, L.T., 2009. Resistin increases lipid accumulation by affecting class A scavenger receptor, CD36 and ATP-binding cassette transporter-A1 in macrophages. Life Sci. 84, 97–104.

Leroy, J.L., Vanholder, T., Mateusen, B., Christophe, A., Opsomer, G., de, K.A., Genicot, G., Van, S.A., 2005. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. Reproduction 130, 485–495.

Listenberger, L.L., Han, X., Lewis, S.E., Cases, S., Farese Jr., R.V., Ory, D.S., Schaffer, J.E., 2003. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc. Natl. Acad. Sci. U. S. A. 100, 3077–3082.

Listenberger, L.L., Ory, D.S., Schaffer, J.E., 2001. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. J. Biol. Chem. 276, 14890–14895.
Liu, J., Shimizu, K., Kondo, R., 2009. Anti-androgenic activity of fatty acids. Chem. Biodivers. 6, 503–512.
Lohrke, B., Viergutz, T., Shahi, S.K., Pohland, R., Wollenhaupt, K., Goldammer, T., Walzel, H., Kanitz, W., 1998. Detection and functional characterisation of the transcription factor peroxisome proliferator-activated receptor gamma in lutein cells. J. Endocrinol. 159, 429–439.
Lolicato, F., Brouwers, J.F., de Lest, C.H., Wubbolts, R., Aardema, H., Priore, P., Roelen, B.A., Helms, J.B., Gadella, B.M., 2015. The cumulus cell layer protects the bovine maturing oocyte against fatty acid-induced lipotoxicity. Biol. Reprod. 92, 16.
Ma, S., Yang, D., Li, D., Tang, B., Yang, Y., 2011. Oleic acid induces smooth muscle foam cell formation and enhances atherosclerotic lesion development via CD36. Lipids Health Dis. 10, 53.
Maedler, K., Spinas, G.A., Dyntar, D., Moritz, W., Kaiser, N., Donath, M.Y., 2001. Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. Diabetes 50, 69–76.
Metwally, M., Cutting, R., Tipton, A., Skull, J., Ledger, W.L., Li, T.C., 2007. Effect of increased body mass index on oocyte and embryo quality in IVF patients. Reprod. Biomed. Online 15, 532–538.
Oh, J., Riek, A.E., Weng, S., Petty, M., Kim, D., Colonna, M., Cella, M., Bernal-Mizrachi, C., 2012. Endoplasmic reticulum stress controls M2 macrophage differentiation and foam cell formation. J. Biol. Chem. 287, 11629–11641.
Opsomer, G., Coryn, M., Deluyker, H., de Kruif, A., 1998. An analysis of ovarian dysfunction in high-yielding dairy cows after calving based on progesterone profiles. Reprod. Domest. Anim. 33, 193–204.
Opsomer, G., Grohn, Y.T., Hertl, J., Coryn, M., Deluyker, H., de, K.A., 2000. Risk factors for post partum ovarian dysfunction in high producing dairy cows in Belgium: a field study. Theriogenology 53, 841–857.
Park, Y., Maizels, E.T., Feiger, Z.J., Alam, H., Peters, C.A., Woodruff, T.K., Unterman, T. G., Lee, E.J., Jameson, J.L., Hunzicker-Dunn, M., 2005. Induction of cyclin D2 in rat granulosa cells requires FSH-dependent relief from FOXO1 repression coupled with positive signals from Smad. J. Biol. Chem. 280, 9135–9148.
Roche, J.F., 2006. The effect of nutritional management of the dairy cow on reproductive efficiency. Anim. Reprod. Sci. 96, 282–296.
Rohwedder, A., Zhang, Q., Rudge, S.A., Wakelam, M.J., 2014. Lipid droplet formation in response to oleic acid in Huh-7 cells is mediated by the fatty acid receptor FFAR4. J. Cell Sci. 127, 3104–3115.
Rukkwamsuk, T., Geelen, M.J., Kruip, T.A., Wensing, T., 2000. Interrelation of fatty acid composition in adipose tissue, serum, and liver of dairy cows during the development of fatty liver postpartum. J. Dairy Sci. 83, 52–59.
Schneider, F., Brüssow, K.P., 2006. Effects of a preovulatory administered depot gonadotrophin-releasing hormone agonist on reproductive hormone levels and pregnancy outcome in gilts. Reprod. Fertil. Dev. 18, 857–866.
Shrestha, H.K., Nakao, T., Higaki, T., Suzuki, T., Akita, M., 2004. Resumption of postpartum ovarian cyclicity in high-producing Holstein cows. Theriogenology 61, 637–649.
Sicinski, P., Donaher, J.L., Geng, Y., Parker, S.B., Gardner, H., Park, M.Y., Robker, R.L., Richards, J.S., McGinnis, L.K., Biggers, J.D., Eppig, J.J., Bronson, R.T., Elledge, S.J., Weinberg, R.A., 1996. Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. Nature 384, 470–474.
Silva, J.M., Price, C.A., 2000. Effect of follicle-stimulating hormone on steroid secretion and messenger ribonucleic acids encoding cytochromes P450 aromatase and cholesterol side-chain cleavage in bovine granulosa cells in vitro. Biol. Reprod. 62, 186–191.
Sprengel, R., Braun, T., Nikolics, K., Segaloff, D.L., Seeburg, P.H., 1990. The testicular receptor for follicle stimulating hormone: structure and functional expression of cloned cDNA. Mol. Endocrinol. 4, 525–530.
Vanholder, T., Leroy, J.L., Soom, A.V., Opsomer, G., Maes, D., Coryn, M., de Kruif, A., 2005. Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation in vitro. Anim. Reprod. Sci. 87, 33–44.
Xu, S., Huang, Y., Xie, Y., Lan, T., Le, K., Chen, J., Chen, S., Gao, S., Xu, X., Shen, X., Huang, H., Liu, P., 2010. Evaluation of foam cell formation in cultured macrophages: an improved method with Oil Red O staining and DiI-oxLDL uptake. Cytotechnology 62, 473–481.
Yonezawa, T., Yonekura, S., Kobayashi, Y., Hagino, A., Katoh, K., Obara, Y., 2004. Effects of long-chain fatty acids on cytosolic triacylglycerol accumulation and lipid droplet formation in primary cultured bovine mammary epithelial cells. J. Dairy Sci. 87, 2527–2534.
Zeleznik, A.J., Midgley Jr., A.R., Reichert Jr., L.E., 1974. Granulosa cell maturation in the rat: increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone in vivo. Endocrinology 95, 818–825.
Zhang, F.P., Poutanen, M., Wilbertz, J., Huhtaniemi, I., 2001. Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. Mol. Endocrinol. 15, 172–183.