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Saturated fatty acid exposure induces androgen overproduction in bovine adrenal cells

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Abstract

Background—Polycystic ovary syndrome (PCOS) is mainly defined by hyperandrogenemia, from ovarian and adrenal origin, and is characterized by insulin resistance (IR). Studies found that raising in vivo non-esterified fatty acid (NEFA) levels, which induces lipotoxicity, increases androgen levels and IR. The aim of this study was therefore to determine the effects of in vitro over-exposure to NEFA on androgen synthesis in a bovine adrenocortical cell model.

Methods—Bovine fasciculata/reticularis cells were cultured for 2 days in the absence or presence of ACTH (10 nmol/L) or Forskolin (fsk, 10 µmol/L), alone or in combination with the saturated fatty acid (FA) palmitate (100 µmol/L). Steroid production was measured in medium and corrected for initial cell seeding count. CYP17 protein expression and ERK1/2 phosphorylation were assessed by Western blotting.

Results—Under unstimulated conditions, dehydroepiandrosterone (DHEA) levels were barely detected and no difference was observed after palmitate exposure, which was also the case for CYP17 expression and ERK1/2 phosphorylation. Under stimulation, palmitate exposure increased DHEA production by 38% and 69%, for ACTH and fsk, respectively, as compared to untreated conditions ($P < 0.05$). In palmitate-treated vs untreated cells, fsk-stimulated ERK1/2 phosphorylation was reduced by 46% ($P = 0.0047$), but stimulated CYP17 expression was not significantly affected.

Conclusion—In a model of androgen-producing cells, under stimulated conditions, overexposure to saturated FAs significantly increases androgen production and reduces MEK/ERK activation. Therefore, this study is the first to demonstrate that lipotoxicity can directly trigger androgen overproduction in vitro, in addition to its well-described impact on IR, which strongly supports a central role of lipotoxicity in PCOS pathophysiology.

Keywords

Polycystic ovary syndrome; Androgens; Adrenal glands; Fatty acids; Mitogen-activated protein kinase; Steroid 17 α -hydroxylase

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1. Introduction

The polycystic ovary syndrome (PCOS) is a common endocrinopathy affecting 6–10% of childbearing aged women [1], and is defined by hyperandrogenism and ovarian dysfunction [1]. Of note, PCOS hyperandrogenemia is both of ovarian (60%) and adrenal (40%) origin [2], and both organs display an exaggerated androgenic response to LH or ACTH [3,4], which suggests that the cause of PCOS is not limited to the gonadal axis but affects all androgen-producing cells. On the other hand, most PCOS women develop insulin resistance with compensatory hyperinsulinemia [5] and other metabolic disorders such as dyslipidemia, metabolic syndrome, impaired glucose tolerance and type 2 diabetes [6–8]. Furthermore, many studies found that insulin sensitization through lifestyle or use of insulin sensitizing drugs improves PCOS manifestations and androgen levels, as well as the androgenic hyper-responsiveness to LH or ACTH that characterizes PCOS [3,4].

However, the existence of lean normo-insulinemic PCOS women [9,10] and the finding that hyperandrogenism is driven by circulating insulin levels even in these women [11] suggests that the compensatory hyperinsulinemia observed in most PCOS women aggravates hyperandrogenism, but does not cause their androgenic hyper-responsiveness to physiological stimuli (insulin, LH and ACTH) [12]. Furthermore, restoration of ovulatory cycles and decrease in androgen levels following treatment with a PPAR γ agonist in non-obese normo-insulinemic PCOS women, which occurred without change in insulin levels [13], suggest that PCOS hyperandrogenism may be caused by a defect in systemic or intracellular non-esterified fatty acid (NEFA) metabolism. The main mechanism of action of the thiazolidinedione PPAR γ agonists is indeed to improve NEFA storage in peripheral adipose tissue and increase intracellular NEFA oxidation [14], which directly improves lipotoxicity, i.e. the deleterious impacts of NEFA on exposed tissues [15]. In line with this hypothesis, it has been recently shown that PCOS women display both functional and morphological adipose tissue anomalies together with their insulin resistance status [16]. Moreover, Mai et al. [17,18] observed increased circulating androgens and associated precursors following heparin/intralipid infusion, which increases circulating NEFA levels and lipotoxicity, both in normal men and women.

Several studies suggest that the MAPK pathway of insulin signaling, and more specifically the MEK/ERK signaling molecules, can play a critical role in steroidogenesis. Most of these studies concluded that the MEK/ERK pathway can act as an inhibitory factor of the different steps leading to androgen biosynthesis, including an inhibition of CYP17 expression, both in ovaries [19–21] and in a human adrenocortical cell line [22]. CYP17 is the main enzyme driving steroidogenesis toward androgen production in ovarian and adrenal glands because it has a strong 17,20 lyase activity. Furthermore, the existence of a constitutive downregulation of the MEK/ERK pathway was shown in ovarian thecal cells from PCOS women when compared to healthy controls [21]. This downregulation was correlated with increased androgen production along with increased CYP17 expression. To this regard, it is worth noting that MAPK activity was found to be reduced in rat fibroblasts exposed in vitro to palmitate, a saturated NEFA [23].

However, little is known regarding the impact on androgen biosynthesis of intracellular metabolic defects, and more specifically lipotoxic phenomena, either in adrenal or ovarian androgen-secreting cells. Furthermore, fundamental studies characterizing the cellular mechanisms of lipotoxicity in steroidogenic tissues are scarce. As mentioned, the cause of PCOS is likely to affect both adrenal and ovarian tissue, which makes adrenocortical tissue a suitable model for the study of PCOS pathogenesis. Accordingly, the aim of the present study was to determine the effects of in vitro over-exposure to NEFA on androgen synthesis and CYP17 protein expression in a bovine adrenocortical cell model. In addition, this study aimed at assessing the impact of such lipotoxic treatment on oxidative stress and MEK/ERK phosphorylation.

2. Material and methods

2.1. Reagents, assay kits and antibodies

Chemicals and peptides used in the present study were obtained from the following sources: 6-wells polystyrene tissue culture plate dishes from Becton Dickinson (Franklin Lakes, NJ); MEM Eagle's medium, fetal bovine serum albumin (BSA) and collagenase from Invitrogen Life Technologies (Burlington, ON, Canada); DMEM/Ham's F12 from Wisent (St. Bruno, QC, Canada), deoxyribonuclease, Percoll and Forskolin from Sigma-Aldrich (St. Louis, MO); and ACTH 1–24 peptide (Cortrosyn) from Organon (Toronto, Ontario, Canada). Horseradish peroxidase-conjugated antirabbit or anti-mouse antibodies and enhanced chemiluminescence system were purchased from Amersham Pharmacia Biotech (Oakville, Canada). Polyvinylidene difluoride (PVDF) membranes were purchased from Roche (Montreal, QC, Canada).

Dehydroepiandrosterone (DHEA) and cortisol ELISA kits were obtained from Diagnostics Biochem Canada, Inc. (London, ON, Canada). 8-isoprostane kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Anti-CYP17, reacting with bovine enzyme, was a gift from Dr. Alain Bélanger (CHUL, Québec, Canada). Fat free BSA and sodium palmitate were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of A-grade purity.

2.2. Primary cell culture

The protocol for isolation of fasciculata/reticularis cells from bovine adult adrenal glands was adapted from the methods previously described by Gallo-Payet and Payet [24] and Bidey [25]. Bovine adrenal glands were collected from non-pregnant adult cows at a local slaughterhouse. After removal of adrenal glands (3/experiment) and throughout isolation and dissociation processes, tissues were submerged in sterile ice-cold MEM medium containing HEPES (8 mmol/L), L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 g/mL) and NaHCO_3 (1.39 mmol/L). During entire dissection, glands were processed on ice. Briefly, after removal of fat, a tissue slicer was used to remove the capsule, the zona glomerulosa and the medulla. The remaining zona fasciculata and reticularis tissues were cut into 1 mm³ pieces and cells were dispersed from tissue with three successive enzymatic digestions using type IA collagenase (500 U/mL) and type I DNase (160 U/mL) and incubations at 37 °C. Cells were disrupted by gentle aspiration and centrifuged. Cell pellet

was resuspended in iso-osmotic Percoll solution (1.07 g/mL) and centrifuged to eliminate contaminating red blood cells. Fasciculata/reticularis cells were washed in MEM medium and resuspended in DMEM-Ham's F12 medium containing HEPES (8 mmol/L), penicillin, streptomycin, L-glutamine, NaHCO₃ (1.39 mmol/L) and 10% fetal bovine serum. After initial cell count, cells were seeded in duplicate into 6 wells polystyrene plates at a density of 6×10^5 cells per well. Cells were cultured at 37 °C in a humidified atmosphere (95% air–5% CO₂).

2.3. Cell treatment

Culture medium was changed 12 h after initial seeding. Cells were examined under a phase-contrast microscope (Leica Corp., Deerfield, IL) equipped with a 20× objective. Cells were then incubated for 48 h either in the absence or presence of 10 nmol/L ACTH [24], twice daily for 2 days, or in the absence or presence of 10 µmol/L Forskolin (fsk), once on the first day of experiment. Cells were also exposed twice daily for 2 days to palmitate (100 µmol/L) complexed with BSA, at a ratio of 6:1, or to the vehicle containing BSA. At this concentration, we determined in previous experiments that palmitate exerts its optimal effect while having no visible consequence on cell viability (based on cell counts and microscopic aspects). On the morning of the third day, medium was removed by aspiration and stored at –80 °C until assayed for steroids and 8-isoprostane content. To minimize artifactual formation of 8-isoprostane resulting from lipid auto-oxidation, samples were stored at –80 °C and assays were performed within the following week. Cells were immediately processed for protein expression or phosphorylation assessments.

2.4. Assays

DHEA, cortisol and androstenedione were measured using commercial ELISA kits and pregnenolone, 17α-hydroxypregnenolone (17OH-pregnenolone), 17OH-progesterone and 11-deoxycortisol were purified and assessed using liquid chromatography plus tandem mass spectrometry (LC-MS/MS) methods by the clinical laboratory of the Centre Hospitalier Universitaire de Sherbrooke (CHUS), as described by Fink and Cyr [26]. In order to evaluate NEFA-induced oxidative stress following palmitate entrance in bovine fasciculata cells [27], lipid peroxidation was assessed by measuring 8-isoprostane formation using a commercial EIA kit. 8-isoprostanes are produced by the random oxidation of cell membrane phospholipids, in particular arachidonic acid, by oxygen radicals.

For conditions not stimulated with ACTH or fsk, DHEA levels were under the lower limit of detection established by the manufacturer and were thus arbitrary set to the lower detection limit of this kit, i.e. 0.1 ng/mL. Other steroid results were all within the limits of detection, which were set by the manufacturer at 0.4 µg/dL, 0.04 ng/mL and 2.7 pg/mL for cortisol, androstenedione and 8-isoprostane, respectively. According to the CHUS clinical laboratory protocols, all LC-MS/MS results were within the limit of detection, and the lower limit of quantification was 0.2 ng/mL for all steroids. Results are corrected for 6×10^5 cells and palmitate exposure effects are expressed as percentage of palmitate-treated over untreated conditions. Steroid secretion results were corrected for initial cell seeding count in order to minimize for artificial over estimation of the data due to losses of functional cells that can occur following final washing procedure.

2.5. Western blotting

Briefly, total proteins were solubilized in cell lysis buffer containing Tris-EDTA (50 mmol/L Tris HCl, 1 mmol/L EDTA), 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate, 0.5% deoxycholic acid, 1% Igepal and phosphatase/protease inhibitor cocktail. Protein samples were stored at -80°C until assessment of Western blot analysis. Samples from an equivalent amount of protein (20 μg for CYP17 and ERK1/2) were separated on 8% or 10% SDS-polyacrylamide gels, for ERK1/2 and CYP17 respectively, and proteins transferred electrophoretically onto PVDF membranes. Membranes were blocked with 5% fat-free powdered milk and 0.05% Tween 20 in Tris-buffered saline (pH 7.5). After three washes with 0.05% TBS-Tween 20, membranes were incubated with anti-CYP17 (1:5000) or Thr202/Tyr204 phosphorylated sites ERK1/2 antibody (1:2000) for 1 h at room temperature. Detection was performed by reaction with horseradish peroxidase conjugated antirabbit (CYP17) or anti-mouse (ERK1/2 and actin) secondary antibody (1:15000; 1 h at room temperature) and visualized by enhanced chemiluminescence plus Western blotting detection reagent kit according to the manufacturer's instructions. Immunoreactive bands were scanned by laser densitometry and expressed in arbitrary units. Quantification of blots was relatively expressed as a ratio of densitometric values obtained for equal protein loading as determined by actin (1:5000) or total ERK1/2 (1:2000) antibodies.

2.6. Statistical analysis

Results of duplicate conditions for each experiment were averaged for all analyses. All data were normally distributed as determined by Kolmogorov-Smirnov normality tests and are presented as mean $\pm$ SEM. Differences between ACTH- or forsk-stimulated steroid levels and unstimulated levels, without palmitate treatment, were tested using two-tailed paired *t*-tests (paired by adrenal gland experiments), using the natural log of level ratios. Log-transformation was necessary to render the ratios normally distributed. Differences between palmitate-treated conditions and their corresponding untreated control conditions were also tested using two-tailed paired *t*-tests, using untransformed ratios of levels. No correction for multiple comparisons was applied because it was pre-specified that each palmitate condition will be compared only with its control condition. All statistical analyses were performed using JMP 7.0 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Steroid production

Table 1 presents net steroid concentrations in the medium, corrected for initial cell seeding counts, and the change in steroid levels following palmitate treatment for each condition, which is expressed as a percentage of levels of the corresponding untreated condition. Of note, pregnenolone, 17OH-pregnenolone, 17OH-progesterone and 11-deoxycortisol were assessed using LC-MS/MS, providing more accurate and sensitive results. However, with this technique a small increase of the low steady state steroid concentration, even if not physiologically relevant, results in dramatic but aberrant changes when reported as percentages. For this reason, results of these steroids expressed as percentage of palmitate-treated under untreated levels, without stimulation, were not reported in Table 1 (N/A, not applicable).

As expected, Table 1 shows that all steroid concentrations in the medium were significantly and markedly increased under ACTH stimulation, by 5–78-folds ($P \leq 0.001$), as well as under fsk stimulation, by 6–63-folds ($P \leq 0.005$). Under conditions not stimulated with ACTH or fsk, palmitate exposure did not significantly change production of DHEA, androstenedione and cortisol. However, following stimulation by ACTH or fsk, palmitate increased pregnenolone production by about 20%, as compared to the absence of palmitate, but this was not statistically significant ($P = 0.12$ and 0.27 , respectively). In presence of palmitate, cells significantly increased their production of the two other Δ^5 -steroids, 17OH-pregnenolone and DHEA, by 46% and 38% under ACTH stimulation ($P = 0.02$ and 0.05 , respectively) and by about 70% under fsk stimulation ($P = 0.01$ and 0.007 , respectively). Production of the Δ^4 -steroids was not affected by palmitate exposure, except for a significant reduction in androstenedione production by 32% under ACTH stimulation, as compared to ACTH-stimulated androstenedione production without palmitate ($P = 0.02$). This decrease was not observed however when androstenedione production was stimulated with fsk.

Regarding glucocorticoids, 11-deoxycortisol production was significantly decreased by 25% when cells were exposed to palmitate, as compared to no exposure, both under ACTH ($P = 0.05$) and fsk ($P = 0.02$) stimulation. Similarly, cortisol levels in cell medium were significantly reduced by 25% following treatment with palmitate and stimulation with fsk, as compared to fsk alone ($P = 0.003$); but this decrease was less pronounced under ACTH stimulation (by 15%) and not statistically significant ($P = 0.15$).

3.2. CYP17 protein expression

In order to further substantiate the observations on palmitate-induced androgen overproduction under ACTH and fsk stimulation, protein expression of the main enzyme driving androgen biosynthesis, CYP17, was specifically measured (Fig. 1). ACTH and fsk stimulations greatly enhanced CYP17 expression in comparison to unstimulated conditions, for which expression was barely detectable (Fig. 1A). Accordingly, untreated conditions were not quantified and results are not represented in Fig. 1C. Results expressed as percentage of palmitate-treated over untreated condition show that under ACTH stimulation, palmitate did not modify CYP17 expression, although a tendency was observed under fsk stimulation ($148.3 \pm 22\%$; $P = 0.08$) (Fig. 1C).

3.3. ERK1/2 phosphorylation

Since steroidogenesis appears to be also modulated by the MEK/ERK pathway, which was reported to be defective in PCOS thecal cells, ERK1/2 phosphorylation was evaluated. In comparison to untreated condition, ERK1/2 phosphorylation was increased both by ACTH and fsk stimulations (Fig. 1B). Under fsk stimulation, this phosphorylation was significantly decreased following palmitate treatment, by $45.9 \pm 10.5\%$ as compared to phosphorylation level without palmitate exposure ($P = 0.0047$). However, no significant modification was observed with palmitate treatment in the presence of ACTH (Fig. 1D).

3.4. Isoprostane production

In order to assess palmitate-induced oxidative stress in bovine fasciculata/reticularis cells, 8-isoprostane levels in the medium were measured. Fig. 2 shows that palmitate increased the release of 8-isoprostane significantly and similarly for all conditions, i.e. by $31.0 \pm 8.0\%$ in absence of stimulation ($P = 0.01$), $34.1 \pm 6.8\%$ in the presence of ACTH ($P = 0.004$) and $27.6 \pm 3.5\%$ under fsk stimulation ($P < 0.001$). Notably, under basal conditions (without palmitate), 8-isoprostane levels were not significantly affected by ACTH or fsk stimulation ($+5\%$ and $+11\%$, respectively).

4. Discussion

To our knowledge, this is the first study to observe a direct effect of NEFA on androgen production in an androgen-producing cellular model. The present study clearly shows that under ACTH or fsk treatment, overexposure of fasciculata/reticularis cells to NEFA induces preferential production of androgens and their precursors at the expense of glucocorticoid formation. This effect does not seem to be fully explained by an increase in CYP17 protein expression, but the shift toward production of the Δ^5 -steroids resulting from CYP17 enzymatic activities (17OH-pregnenolone and DHEA), suggests that CYP17 activity was stimulated by palmitate exposure. Importantly, these findings were concomitant with a decrease in ACTH- and fsk-activation of the MAPK pathway, a defect previously described in PCOS thecal cells. Another interesting finding of this study is that palmitate triggers lipid-induced oxidative stress as determined by elevation of 8-isoprostane formation.

The concept of lipotoxicity as a possible direct cause of hyper-androgenism, and not only through induction of PCOS insulin resistance, is a relatively recent concept in the literature [17,28,29]. Initial human studies have already shown that fatty acid infusion significantly increases adrenal and ovarian androgen production, both in men and women [17,18]. It is important to determine whether NEFA can directly trigger exaggerated androgen production because it would mean that lipotoxicity could directly cause both the hyperandrogenism and insulin resistance that characterize women with PCOS, and therefore strongly support a central role of lipotoxicity in PCOS pathophysiology [29]. Accordingly, it is our hypothesis that women are predisposed to PCOS because of a primary defect in NEFA intracellular metabolism in androgen-producing cells, which promotes NEFA overflow. This defect could be associated or not with defects in NEFA storage in adipose tissue, resulting in NEFA systemic spillover. It follows that while some lean PCOS women do not develop systemic insulin resistance, they do display a severe enough NEFA-induced androgenic hyper-responsiveness in order to develop hyperandrogenemia. However, following weight gain most PCOS women will exceed their adipose tissue's ability to efficiently store NEFA. Hence, they will develop NEFA spillover, insulin resistance and compensatory hyperinsulinemia [15], which will further stimulate androgen biosynthesis and uncover PCOS predisposition.

4.1. Lipotoxicity and androgenic enzyme activity

The main result of this study is the increase in 17OH-pregnenolone and DHEA production when androgen-secreting cells are over-exposed to the saturated FA palmitate. We did not

observe an increase in DHEA with palmitate in the absence of ACTH or Forskolin stimulation, but this might only be due to the fact that baseline levels in our model were too low to be accurately detected with our techniques. On the other hand, we observed a decrease in 11-deoxycortisol and cortisol production after palmitate treatment. Thus, in this in vitro model, it seems that palmitate diverts steroidogenesis from glucocorticoids toward androgen production, as illustrated in Fig. 3. Importantly, if this were to occur in vivo, the retro-feedback of cortisol on the adrenal axis would keep cortisol levels normal, but at the expense of further increase in adrenal androgen levels.

CYP17 is the main enzyme of androgenesis and possesses two activities: a 17 α -hydroxylase activity responsible for 17OH-pregesterone and 17OH-pregnenolone synthesis, and a 17,20 lyase activity that converts these two precursors respectively into androstenedione and DHEA [30]. However, the main androgen produced by bovine and primate adrenal glands is DHEA [31]. Thus, since the Δ^5 -androgen pathway is the one that is primarily active, it would explain why, in our cell model, we did not observe major effects of palmitate treatment on Δ^4 -androgen production.

Both transcriptional and post-translational mechanisms can affect CYP17 activity. Among the existing transcriptional mechanisms, it has been shown that CYP17 gene expression [32] and mRNA stability [33] are increased in ovarian thecal cells from PCOS women when compared to healthy women. However, we were unable, in the present study, to observe a significant increase in CYP17 protein expression following palmitate treatment, although there was a non-significant 50% increase when cells stimulated with forskolin were exposed to palmitate. In order to comprehensively assess CYP17 gene expression, it will be important to perform real time PCR analyses in future studies. Moreover, since post-translational mechanisms affecting CYP17 enzymatic activity has been described, it will be important to investigate more closely these mechanisms as well. Indeed, CYP17 post-translational modifications have been suggested to be involved in PCOS hyperandrogenism [34]. In NCI-H295 human adrenal cell line and in COS-1 cell line transfected with a CYP17 expression vector, it has been shown that CYP17 serine/threonine phosphorylation can increase the 17,20 lyase to 17 α -hydroxylase activity ratio by increasing its affinity toward the electron donor partners P450 oxidoreductase (POR) and cytochrome b5 [30]. Interestingly, it has also been shown that intracellular overflow of NEFAs generates certain FA metabolites (e.g. diacylglycerols and ceramides) that activate serine/threonine kinases [35,36], which could be one possible mechanism for NEFA-induced hyperandrogenism.

In addition, 8-isoprostane assessments clearly suggest that the treatment of fasciculata/reticularis cells with palmitate induces an increase in their level of oxidative stress. These cell types are not biologically programmed to store or metabolize high quantities of NEFA as would do adipocytes, for instance. Our results confirm that the excess of NEFAs that is added to the medium is taken up by the cells and exceed their ability to appropriately oxidize them, thus inducing lipotoxic events such as oxidative stress. Other lipotoxic consequences are therefore likely to take place, such as the formation of fatty acids metabolites like diacylglycerol and ceramides, which would be important to assess in future studies.

4.2. Mechanisms of lipotoxic effects on androgenesis

The main purpose of this study was a proof of concept for the direct impact of excessive saturated NEFA exposure on androgen biosynthesis in androgen-producing cells, and not to tackle underlying mechanisms. Nevertheless, we analyzed the effects of NEFA exposure on the MAPK (MEK/ERK) pathway because Nelson-Degrave et al. [21] previously described that the activation of this pathway was reduced in PCOS thecal cells, which was correlated with increased CYP17 mRNA expression and DHEA production. Similarly, we found that NEFA overexposure significantly reduce ERK1/2 activation under Forskolin stimulation in fasciculata/reticularis cells, which was also associated with increased DHEA production.

Several studies have shown that the MAPK pathway plays an important role in steroidogenesis regulation. The study performed in human thecal cells by Nelson-Degrave et al. showed that MEK/ERK pathway exerts an inhibition of CYP17 gene expression [21], as opposed to the cAMP dependant stimulatory effects. Crosstalk between PKA/cAMP and MEK/ERK pathways has been previously described in human adrenal NCI-H295R cell line [37]. In this study, Sewer et al. have shown the existence of a dual specificity phosphatase protein, the mitogen-activated protein kinase phosphatase-1 (MKP-1), which expression could be induced by PKA/cAMP dependant signals. This phosphatase can in turn decrease MEK/ERK pathway activation, thus releasing MEK/ERK inhibitory effect on CYP17 gene expression. The antagonistic effects of these two pathways on CYP17 expression probably contribute to the establishment of a physiological balance, which could be destabilized in favor of stimulating pathways in pathologies such as PCOS. Since our results suggest that NEFA overexposure induces a defect in the MAPK pathway in androgen-producing cells, future studies should assess NEFA-induced changes in stimulating and inhibitory signaling molecules of this pathway.

Some of our experiments resulted in non-significant effects under ACTH stimulation while Forskolin stimulation in the same conditions produced significant results. These apparent discrepancies could usually be explained by higher variability in the results following ACTH stimulation than after fsk stimulation, but trends were in the same directions. Under palmitate exposure, the higher CYP17 protein expression following fsk stimulation as compared to ACTH stimulation is however puzzling. This difference was not significant and might only be due to random effects.

In conclusion, this study found that in a model of androgen-producing cells, and under stimulated conditions, overexposure to saturated fatty acids shifts steroidogenesis toward androgen production. Study results also suggest that underlying mechanisms could involve changes in MEK/ERK activation state. Therefore, this study is the first to demonstrate that lipotoxicity can directly trigger androgen overproduction in vitro, in addition to its well-described impact on insulin resistance, which strongly supports a central role of lipotoxicity in PCOS pathophysiology.

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Fig. 1.

Impact of palmitate treatment on CYP17 expression and on ERK1/2 phosphorylation in bovine fasciculata/reticularis cells cultured under untreated, ACTH or fsk stimulation. Cells were seeded in duplicate at a density of 6×10^5 cells and were cultured for 2 days in the absence (control) or in the presence of ACTH (10 nmol/L) or fsk (10 μ mol/L), alone or in combination with palmitate (100 μ mol/L), as described in Section 2. Duplicates of each condition were pooled for total protein extraction and cell lysates were subjected to Western blotting using specific antibody against CYP17 (1:5000) and actin (1/5000). Representative gels of (A) CYP17 and actin and (B) phospho-ERK1/2 and total ERK1/2 protein expression. Graphical representation of (C) CYP17/actin and of (D) phospho-ERK1/2 corrected by total ERK1/2 protein levels. Results are expressed as densitometric values obtained for 20 μ g loading of protein. Results are shown as mean $\pm$ SEM and as percentage of palmitate-treated over untreated condition. Untreated condition was set at 100%; $n = 7$ different experiments. (Black: in absence of palmitate – white: in presence of palmitate). ** $P < 0.01$ in comparison with their respective controls.

Fig. 2.

Impact of palmitate treatment on 8-isoprostane formation in bovine fasciculata/reticularis cells cultured under unstimulated, ACTH- or fsk-stimulated conditions. (Black: in absence of palmitate – white: in presence of palmitate). Cells were seeded in duplicate at a density of 6×10^5 cells in Petri dishes and treated for 2 days in the absence (control) or in the presence of ACTH (10 nmol/L) or fsk (10 μ mol/L), alone or in combination with palmitate (100 μ mol/L), as described in Section 2. 8-Isoprostane formation was measured in culture medium using commercial EIA kits. Results are expressed as means $\pm$ SEM and as percentage of palmitate-treated over untreated condition. Untreated condition was set at 100%. $n = 6$ different experiments. ** $P < 0.01$ in comparison with their respective untreated condition.

Fig. 3. Overall effect of palmitate over-exposure on steroidogenesis. (1) CYP17A1's 17-hydroxylase activity; (2) CYP17A1's 17,20 lyase activity; (3) 3- β hydroxysteroid dehydrogenase activity; (4) CYP21A1's 21-hydroxylase activity and (5) CYP11B2's 11-hydroxylase activity.

Table 1

Effect of palmitate treatment on steroid production in bovine fasciculata/reticularis cells.

	N	Net concentration (steroid value) untreated conditions			Palmitate effect (% of corresponding untreated condition)		
		Control	ACTH	Forskolin	Control	ACTH	Forskolin
<i>5-Steroids</i>							
Pregnenolone (ng/mL/6 × 10 ⁵ cells)	6	0.4 ± 0.08	1.5 ± 0.2	2.1 ± 0.3	N/A	120.9 ± 11.0%	117.1 ± 13.5%
17OH-pregnenolone (ng/mL/6 × 10 ⁵ cells)	6	0.2 ± 0.03	13.9 ± 2.8	10.4 ± 2.8	N/A	146.0 ± 13.5% *	172.1 ± 19.5% *
DHEA (ng/mL/6 × 10 ⁵ cells)	7	0.1 ± 0.0	1.2 ± 0.4	0.8 ± 0.2	99.1 ± 0.5%	137.7 ± 15.5% *	168.7 ± 17.1% **
<i>4-Steroids</i>							
17OH-progesterone (ng/mL/6 × 10 ⁵ cells)	6	0.4 ± 0.1	10.5 ± 3.8	13.8 ± 4.4	N/A	103.3 ± 16.8%	92.8 ± 13.5%
Androstenedione (ng/mL/6 × 10 ⁵ cells)	6	0.7 ± 0.05	20.8 ± 7.3	11.2 ± 3.4	100.7 ± 1.7%	77.7 ± 6.5% *	96.3 ± 6.8%
<i>Glucocorticoids</i>							
11-Deoxycortisol (ng/mL/6 × 10 ⁵ cells)	6	1.7 ± 0.4	135.2 ± 33.7	98.7 ± 23.3	N/A	75.3 ± 9.3% *	74.8 ± 7.1% *
Cortisol (µg/dL/6 × 10 ⁵ cells)	6	17.9 ± 3	387.5 ± 173.7	439.9 ± 212.1	88.3 ± 7.1%	85.3 ± 8.5%	74 ± 4.6% **

Cells were cultured for 2 days in the absence (control) or in the presence of ACTH (10 nmol/L) or forsk (10 µmol/L), alone or in combination with palmitate (100 µmol/L), as described in Section 2. Results are expressed as means ± SEM or as percentages of palmitate-treated over untreated condition.

N/A, not applicable: results of these steroids expressed as percentage of palmitate-treated under untreated levels, without stimulation, are not reported. Since LC-MS/MS technique provide a more sensitive result, in these conditions, a small increase of the low steady state steroid concentration results in dramatic but aberrant changes when reported as percentages.

* P 0.05 compared to the corresponding condition untreated with palmitate.

** P < 0.01 compared to the corresponding condition untreated with palmitate.