Inhibition of autophagy in theca cells induces CYP17A1 and PAI-1 expression via ROS/p38 and JNK signalling during the development of polycystic ovary syndrome.

（多嚢胞性卵巣症候群における卵巣莢膜層でのオートファジー抑制は活性酸素-p38/JNKシグナル経路を介してCYP17A1およびPAI-1のmRNA発現を誘導する。）
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Chapter 0. Abstract

Polycystic ovary syndrome (PCOS) is a clinical syndrome characterized as hyperandrogenism, oligo/anovulation, and polycystic ovary. Insulin is involved in the pathogenesis of PCOS, but actual etiology of PCOS is still unknown. Autophagy is an intracellular system that degrades cytosolic proteins and organelles. Dysregulation of autophagy is involved in the pathogenesis of diabetes mellitus and nonalcoholic fatty liver disease which are associated with PCOS. But the relationship between autophagy and PCOS has not been clarified. In this study I found that the p62 and ubiquitin was significantly increased in theca cells of PCOS on immunohistochemistry. These findings suggest that autophagy is suppressed in the ovaries of PCOS. Using bovine theca cells as in vitro model, autophagy inhibition by palmitic acid and chloroquine increased p62 and ubiquitin protein levels and induce the expression of Cytochrome P450 17A1 (CYP17A1) and Plasminogen activator inhibitor type-1 (PAI-1) mRNA and Androstenedione (ASD) secretion. Furthermore, palmitic acid and chloroquine exposure significantly increased reactive oxygen species (ROS) and activated p38 and JNK signaling pathway. Inhibition of p38 and JNK signaling significantly reduced CYP17A1 and PAI-1 mRNA expression. In conclusion, I showed that the inhibition of autophagy in theca cells contributed the pathogenesis of PCOS, reading CYP17A1 and PAI-1 mRNA expression via ROS/p38 and JNK signaling pathway.
Chapter 1. Introduction

Polycystic ovary syndrome (PCOS) is the most prevalent reproductive disorder causing anovulatory infertility. The prevalence rate is 8 to 13% in women of reproductive age (1). The Rotterdam diagnostic criteria for PCOS are internationally endorsed and are based on two of three features: oligo- or anovulation, hyperandrogenism (clinical or biochemical), and polycystic ovary (2). A considerable proportion of women with PCOS display insulin resistance (3), but the actual aetiology of PCOS is unknown.

Recent studies showed that cytochrome P450 17A1 (CYP17A1) and plasminogen activator inhibitor (PAI-1) play important roles in the pathogenesis of PCOS. CYP17A1, localized in theca cells, converts progesterone to 17α-hydroxy pregnenolone and then androstenedione (ASD) by 17α-hydroxylase and C17-20 lyase activities (4, 5), and CYP17A1 expression in theca cells in PCOS women is up-regulated (6). Therefore, CYP17A1 in theca cells plays an important role in hyperandrogenism in the pathogenesis of PCOS (7).

PAI-1 is a molecule that prevents plasminogen activation driven by tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) (8, 9). In normal ovarian physiology, the plasminogen system allows for a window of t-PA excess, which causes the conversion of plasminogen to plasmin within the follicular fluid and ultimately results in proteolytic breakdown of the follicular wall and ovulation (10). Interestingly, Devin et al. reported that transgenic mice that constitutively express a stable form of human PAI-1 exhibit a hypertrophied theca layer with fibrosis, a characteristic of human PCOS ovaries (11). In other words, PAI-1 plays important roles in the fibrosis of PCOS ovaries. However, the cause of PAI-1 upregulation is unclear.
Autophagy is an intracellular system that degrades cytosolic proteins and organelles (12, 13), maintaining cell and tissue homeostasis by digesting unwanted or damaged components, recycling materials, and producing energy. Dysregulation of autophagy has been shown to be involved in the pathogenesis of human metabolic diseases, such as diabetes mellitus (DM) and non-alcoholic fatty liver disease (NAFLD) (14, 15). Although these metabolic diseases are strongly associated with PCOS (16, 17), the relationship between autophagy and PCOS is unknown. Hence, I investigated (1) the activity of autophagy in PCOS ovaries and (2) the role of autophagy in androgen production and fibrosis in vitro.

**Chapter 2. Materials and methods**

2.1 Human specimens

Immunohistochemical analysis was performed on the ovaries of 4 PCOS and 4 control patients. Normal ovaries were obtained from women with regular menstrual cycles without hormonal treatment, who underwent radical or extended hysterectomy for cervical carcinoma. PCOS ovaries were obtained from woman diagnosed with PCOS according to the Rotterdam diagnostic criteria, who underwent extended hysterectomy or hysterectomy for endometrial carcinoma. All experimental procedures were approved by the University of Toyama review board. All methods were performed in accordance with the guidelines and regulations of the review board at the University of Toyama, which are based on the ethical standards established in the 1964 declaration of Helsinki and its later amendments.

2.2 Histopathology
Paraffin-embedded tissues were cut to 4 µm-thick sections and mounted on slides. Slides were deparaffinized in xylene, rehydrated through a graded series of ethanol, and washed in water. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) by microwaving for 10 min and then cooling to room temperature. Slide staining with the first and second antibodies was performed according to the manufacturer’s instructions. The immunostaining was performed using specific antibodies to p62 (MBL, Nagoya, Japan, Cat# PM045, 1:200 dilution), ubiquitin (Cell Signaling Technology, MA, USA, Cat# 3936, 1:800 dilution) and PAI1 (Abcam, Cambridge, UK, Cat# ab66705, 1:200 dilution). The tissues were also stained with Azan Mallory. The percent of fibrotic tissue in the interstitial area was analyzed using ImageJ software. All micrographs were taken with a BZ-X810 microscope (Keyence, Osaka, Japan).

2.3 Theca cell culture

Bovine ovaries were collected immediately after slaughter at a local abattoir. The ovaries were placed in an ice-cold buffered salt solution and transferred to the laboratory less than 90 min after collection. Theca cells were isolated from the ovaries under sterile conditions, as described previously (18). Briefly, small antral follicles (2-5 mm diameter) with a clear surface were cut into halves, and the theca interna was removed in situ using fine forceps. The resultant thin thecal layer was minced and subsequently treated with high-glucose Dulbecco’s Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, MA, USA) containing 2.5 mg/ml collagenase (Fujifilm Wako Pure Chemical, Osaka, Japan), 0.4 IU/ml DNase (Invitrogen, CA, USA), 10% (v/v) foetal bovine serum (FBS) (Sigma-Aldrich, MO, USA), and 1% (v/v) penicillin-streptomycin-amphotericin B suspension (PSA) (Fujifilm Wako Pure Chemical). Isolated theca cells were grown in high-glucose
DMEM supplemented with 10% FBS and 1% PSA for 24—48 h. Twenty-four hours before performing the experiments, theca cells were placed in low-serum DMEM containing 1% FBS and 1% PSA.

2.4 Palmitic acid (PA) treatment

Bovine serum albumin (BSA)-conjugated PA was prepared as previously reported (19). Briefly, palmitic acid (P0500, Sigma-Aldrich) was dissolved in 95% (v/v) ethanol at 60°C to yield a stock concentration of 100 mM, conjugated with BSA (Cat# 01281-26, Nacalai Tesque, Kyoto, Japan) at a 3:1 molar ratio, and incubated for 1 h at 37°C. Theca cells were treated with or without PA for 24 h. In the experiments studying the role of p38 or JNK, theca cells were stimulated with PA in the absence or presence of the p38 inhibitor (10 µM, SB202190, Sigma-Aldrich) or JNK inhibitor (20 µM, SP600125, Sigma-Aldrich) for 30 min before adding PA.

2.5 Reverse transcription (RT) and quantitative real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted from the theca cells using an ISOGEN-II (Nippon Gene, Tokyo, Japan). RT was performed using Rever Tra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Tokyo, Japan). Approximately 0.5–1 µg of total RNA was reverse-transcribed in a 20-µL volume. For the quantification of various mRNA levels, real time PCR was performed using the Mx3000P Real-time PCR System (Agilent Technologies, CA, USA) according to the manufacturer’s instructions. The PCR primers used with the SYBR Green methods were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosomal DNA contaminants. The SYBR Green thermal cycling conditions were 1 cycle of
95°C for 30 s, and 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. The relative mRNA levels were calculated using the standard curve method and were normalized to the mRNA levels of beta-actin (forward, 5′-GCAGGAGTACGAGTCCG-3′ and reverse, 5′-TGTCACCTCACCCTCCAG-3′). The primer sequences used for RT-PCR were as follows: CYP17A1 (forward, 5′-GATCGGTATGCTGAGCG-3′ and reverse, 5′-CATGGGATCCGGGACGTTAG-3′) and PAI-1 (forward, 5′-CTGCGAAATTGAGGATGC-3′ and reverse, 5′-GGTGAGAAAACCACGTTGC-3′).

2.6 Western blot analysis

Treated cells were lysed with lysis RIPA buffer (Cat# 182-02451, Fujifilm Wako Pure Chemical) containing complete protein inhibitor (Cat# 11697498001, Roche, Basel, Switzerland) and phosphatase inhibitor cocktail 2 (Cat# P5726, Sigma-Aldrich). The protein concentration of the sample was measured by the Bradford assay using quick start Bradford (Cat# 500-0205, Bio-Rad, CA, USA). An equal amount of protein (20-30 µg) was resolved on 10% SDS-PAGE. Separated proteins were transferred onto a PVDF membrane (Cat# 162-0175, Bio-Rad), and then the membrane was blocked in 5% BSA (Cat# 01281-26, Nacalai Tesque) for 1 h and subsequently incubated with rabbit antibody to p62 (dilution; 1:1000, Cat# PMO45, MBL), rabbit antibody to Microtubule-associated proteins 1A/1B light chain 3B (LC3-B) (dilution; 1:1000, Cat# NB100-2220, Novus Biologicals), rabbit antibody to total p38 MAPK (dilution; 1:1000, Cat# 9212, Cell Signalling Technology, MA, USA), rabbit antibody to phosphor-specific (Thr180/Thr182) p38 MAPK (dilution; 1:500, Cat# 9211, Cell Signalling Technology), rabbit antibody to total JNK MAPK (dilution; 1:1000, Cat# 9252, Cell Signalling Technology), rabbit antibody to phosphor-
specific JNK MAPK (dilution; 1:500, Cat# 4668, Cell Signalling Technology), or mouse antibody to beta-actin (ACTB, dilution; 1:2000, Cat# 3700, Cell Signalling Technology) as the primary antibody for 2 h. Then, membranes were treated with the secondary anti-rabbit (dilution; 1:2000, Cat# 7074, Cell Signalling Technology) or anti-mouse (dilution; 1:2000, Cat# 7076, Cell Signalling Technology) IgG HRP-linked antibody for 1 h. Protein bands were detected by a chemiluminescence assay using SuperSignal (Cat# 34580, Thermo Fisher Scientific). The bands were finally quantified using NIH ImageJ software.

2.7 ASD assay

The bovine ASD concentration in cell culture supernatants was measured by the Androstenedione ELISA Kit (Abnova, Taipei, Taiwan). The absorbance was measured at 450 nm using an iMark Microplate Absorbance Reader (Bio-Rad).

2.8 Electron microscopy imaging

Bovine theca cells were immersion fixed in 2% (v/v) glutaraldehyde (0.1 M phosphate buffer, pH 7.4) for 60 min. Samples were post-fixed in 1% osmium tetroxide for 60 min at 4 °C, dehydrated in graded alcohol, embedded in 100% epoxy resin (Nisshin EM, Tokyo, Japan) in beam capsules, and allowed to harden in a 65°C oven for 48 h. After hardening, epoxy resin blocks were ultrathin-sectioned at a 100 nm thickness and placed on 150 mesh copper grids. Grids were next counterstained with saturated uranyl acetate and lead citrate and then viewed and imaged at X1500-6000 magnification using a transmission electron microscope (JEOL JEM-1400TC, Jeol .Ltd, Tokyo, Japan).
2.9 Measurement of reactive oxygen species (ROS) in bovine theca cells

Intracellular ROS generation was detected using a CellROX Green Reagent (Thermo Fisher Scientific) fluorescent probe. Pretreated cells in 24-well plates were incubated with CellROX (5 μmol/L) at 37°C for 60 min and then washed with PBS. The cell fluorescence was observed using a BZ-X810 (Keyence) and analysed by hybrid cell count module software/BZ-H4C (Keyence).

2.10 Statistical analysis

All experiments were repeated at least three times. Non-normally distributed data were analysed by nonparametric tests (Mann-Whitney U test) using JMP software (SAS Institute Inc., Cary, NC, USA). A p-value <0.05 was considered statistically significant.

Chapter 3. Results

3.1 Autophagic turnover was impaired in ovaries from women with PCOS

When autophagy is impaired, aggregated proteins are increased and tagged with p62 and ubiquitin (20). Therefore, to assess autophagy activity, I investigated the level of p62 and ubiquitin as markers of aggregated protein in human ovaries using immunohistochemistry. There was a significant increase in the frequency of cells that stained positive for p62 and the ubiquitin observed (p < 0.05) in theca cells and granulosa cells in the ovaries from women with PCOS compared with the control (Fig 1A and 1B). The intensity of immunostaining in theca cells was clearer than that in granulosa cells. These findings suggested that aggregated proteins accumulate in theca cells. Next, I examined the PAI-1 protein expression as a marker of fibrosis (21). The number of cells that stained positive
for PAI-1 was significantly increased (p < 0.05) in the theca cell layer in the PCOS cases compared with the control cases (Fig 1C). In addition, Azan staining revealed that the area of fibrotic tissue in the interstitium was significantly increased (p < 0.05) in PCOS cases compared to that in the control cases (Fig 1E). These findings suggested that elevated PAI-1 expression induced tissue fibrosis in the ovaries of women with PCOS.

3.2 Palmitic acid impaired autophagic turnover in bovine theca cells

I considered PA, one of the free fatty acids (FFAs), as a candidate substance to impair autophagic turnover, because PA is one of the main substances of lipotoxicity associated with metabolic syndrome and impairs autophagic turnover (15). It is ethically difficult to obtain human ovarian theca cells. Thus, I examined whether PA impaired autophagy in bovine theca cells in vitro. PA treatment significantly increased the p62 level (p < 0.05) and LC3-Ⅱ/LC3-Ⅰ ratio (p < 0.05) in a concentration-dependent manner (Fig 2A, 2B, and 2C). Elevation of the LC3-Ⅱ/LC3-Ⅰ ratio indicates not only autophagy activation but also inhibition of autophagosome degradation, which is a late-stage autophagy process (22). To clarify whether autophagy was activated or inhibited, I conducted the autophagy flux assay using chloroquine (CQ), which is an inhibitor of lysosome function and autophagy (23, 24). If the LC3-Ⅱ level is increased in the presence of lysosome inhibitors, this indicates that autophagy is activated. However, if the LC3-Ⅱ level is unchanged, this indicates that autophagosome accumulation occurred due to inhibition of autophagic degradation. Therefore, the LC3-Ⅱ ratio of no treatment with lysosomal inhibitor to treatment correctly shows autophagy activity (25). The autophagy flux assay revealed that the LC3-Ⅱ ratio was significantly decreased (p < 0.05) after PA treatment (Fig 2D and 2E), indicating inhibition of
autophagy turnover. These data clearly show that PA inhibited the late-stage autophagy
autophagosome degradation steps.

3.3 Palmitic acid increased CYP17A1 and PAI-1 mRNA expression and ASD secretion in bovine
theca cells

Next, I examined whether PA induced a PCOS-like hormonal or cytokine environment by
evaluating CYP17A1 and PAI-1 mRNA expression in bovine theca cells. CYP17A1 and PAI-1
mRNA were significantly elevated (p < 0.05, p < 0.05) after PA-treatment compared to the control
(Fig. 2F and 2G). Furthermore, secretion of ASD was significantly elevated (p < 0.05) in the culture
supernatant of PA-treated bovine theca cells (Fig. 2H). These findings suggest that PA induced
autophagy impairment and contributed to hyperandrogenism and fibrosis in ovaries.

3.4 Inhibition of the late stage of autophagy by chloroquine-induced CYP17A1 and PAI-1 mRNA
expression and ASD secretion

To clarify that autophagy impairment directly induces CYP17A1 and PAI-1 mRNA, and ASD
secretion, I investigated whether CQ, an inhibitor of the late stage of autophagy (23), induced
CYP17A1 and PAI-1 mRNA expression. CQ inhibited the late stage of autophagy because of the
significantly increased p62 levels (p < 0.05) and LC3- II / LC3- I ratios (p < 0.05) by 50 µM of CQ
treatment (Fig. 3A, 3B, and 3C). Furthermore, CQ treatment significantly elevated CYP17A1 (p <
0.05) and PAI-1 (p < 0.05) mRNA in bovine theca cells (Fig. 3D and 3E). ASD secretion was also
significantly elevated (p < 0.05) in the culture supernatant of CQ-treated theca cells (Fig. 3F). These
findings indicate that inhibition of the late stage of autophagy contributed to hyperandrogenism and fibrosis in ovaries.

3.5 Palmitic acid disrupts mitochondria and induces ROS production

There are numerous reports about how impairment of autophagy induces mitochondria dysfunction and reactive oxygen species (ROS) production (26-28). Therefore, I examined whether inhibition of autophagy by PA injured mitochondria and induced ROS. Transmission electron microscopy (TEM) revealed that PA and CQ treatment in theca cells resulted in a reduction of electron density in intracellular vesicles compared with starvation-treated theca cells (Fig 4Ab, 4Ac, and 4Ad). Aggregated proteins and cytoplasmic organelles in autophagosomes are regularly digested by lysosomal enzymes. This finding indicated that PA treatment disrupted lysosomal function (29). TEM showed that mitochondria in PA-treated theca cells were crucially swelled compared with those in BSA and CQ treatment conditions (Fig 4Ae, 4Ag, and 4Ah), suggesting mitochondria dysfunction (30). Next, I assessed ROS production by the CellROX assay. This experiment revealed that ROS production was significantly elevated by PA and CQ treatment in theca cells compared with BSA-treated controls (Fig. 4B).

3.6 p38 and JNK signalling pathways were involved in CYP17A1 and PAI-1 mRNA upregulation by PA and CQ treatment.

Stress-induced mitogen-activated protein kinases (SAPKs), JNK and p38, are activated by ROS in various cells (31, 32). Therefore, p38 and JNK might be key regulators of CYP17A1 and PAI-1 expression in PA-treated bovine theca cells. Firstly, I assessed the phosphorylation of p38 and JNK.
PA treatment significantly increased the phosphorylation of p38 and JNK in a dose-dependent manner (Fig 5A and 5B) and in CQ-treated theca cells (Fig 6A and 6B). Next, I studied the effect of p38 and JNK signal pathways on CYP17A1 and PAI-1 mRNA in PA- and CQ-treated bovine theca cells. Inhibitors of p38 and JNK significantly attenuated not only CYP17A1 and PAI-1, but also ASD in PA-treated theca cells (Fig 5C, 5D, and 5E) and in CQ-treated theca cells (Fig 6C, 6D, and 6E). These data show that p38 and JNK are key regulators of CYP17A1 and PAI-1 mRNA upregulation following PA and CQ treatment.

Chapter 4. Discussion

In this study, I obtained two new important findings. First, p62 and ubiquitin, aggregated proteins and autophagy impairment markers, were accumulated in the theca cell layer of the PCOS ovary. Second, dysregulation of late-stage autophagy in theca cells induced CYP17A1 and PAI-1 mRNA upregulation and androgen secretion via the ROS/p38 and JNK signalling pathway.

I reported in this study for the first time that p62 and ubiquitin are accumulated in the theca cell layer in the ovaries in women with PCOS. This suggests that autophagy is impaired in the theca cell layer in PCOS, and accumulation of p62 and ubiquitin were observed in autophagy deficient mice (20). A Gonzalez-Rodriguez et al. reported that the late stage of autophagy is impaired in the livers of individuals with non-alcoholic steatohepatitis, which is related to PCOS (17). They showed that saturated free fatty acids (FFAs), especially PA, were assumed to be the cause of autophagy impairment (15). FFA also plays an important role in insulin resistance (33). In addition, PA is elevated in the serum and follicular fluid of PCOS patients and induces androgen production(34, 35). Therefore, it is possible that PA plays a part in the pathogenesis of PCOS. Our in vitro experiment
revealed that PA treatment of theca cells increased the p62 and LC3-II/LC3-I ratio. To distinguish whether autophagy was activated or inhibited, I investigated the autophagy flux assay, which measures LC3-II accumulation in the presence of lysosomal function inhibitor (25). The autophagy flux assay showed that LC3-II accumulation only increased in the control treatment group in the presence of CQ but not in the PA treatment group. These findings showed that PA impaired autophagic degradation in bovine theca cells, resulting in autophagosome accumulation.

Secondly, I showed that impairment of late-stage autophagy in theca cells by PA and CQ treatment induced CYP17A1 and PAI-1 mRNA upregulation and androgen production via the ROS/p38 and JNK signalling pathway. Not only PA treatment but also CQ treatment increased CYP17A1 and PAI-1 mRNA expression in bovine theca cells, leading to androgen production. It is well-known that inhibition of autophagy induces ROS (36-38). ROS then activates p38 and JNK, which are known as stress-associated protein kinases in various cells (31, 32). The p38 and JNK signalling pathways play an important role in insulin resistance and fibrosis, typical findings in PCOS (39, 40). PA and CQ treatment increased ROS production and phospho-p38 and JNK. Both p38 and JNK inhibitor strongly inhibited CYP17A1 and PAI-1 mRNA expression. These findings strongly indicate that p38 and JNK are involved in the pathogenesis of PCOS. It is suggested that autophagy activation or ROS inhibition decrease CYP17A1 and PAI-1 expression via ROS/p38 and JNK signalling pathways. It is known that autophagy disorder and ROS induces endoplasmic reticulum (ER) stress (41, 42). Takahashi et al. reported that endoplasmic reticulum stress was elevated in the PCOS (43, 44). Thus, ER stress might be upregulated in our cellular model. Further studies are needed in order to explore these possibilities.
This study has three limitations. First, I could not assess autophagy activity in the ovaries of women with PCOS. In this study, I could only elucidate that aggregated proteins were increased in PCOS ovaries by evaluating p62 and ubiquitin. Second, I could explain the association between autophagy and PCOS only in vitro. To investigate the association between autophagy and PCOS in vivo, I should produce theca-cell specific autophagy deficient mice. Third, PA is also known to stimulate p38 and JNK through the TLR4 receptor (45, 46). Therefore, the activation of p38 and JNK were induced by not only autophagy inhibition but also the TLR4/p38 and JNK signalling pathway. Further studies are needed to address these limitations.

This study clearly showed that aggregated proteins were increased in the theca cell layer of the PCOS ovary, and dysregulation of late-stage autophagy in theca cells induced androgen production and fibrosis via the ROS/p38 and JNK signalling pathway. Our study suggests that the ROS/p38 and JNK signalling pathway might be an important therapeutic target for PCOS.

Chapter 5. Source of funding

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Chapter 6. Acknowledgments

I thank Ms. Matsushita and Ms. Ushijima for her excellent technical assistance.
Chapter 7. References


11. Devin JK, Johnson JE, Eren M, Gleaves LA, Bradham WS, Bloodworth JR, Jr., et al. Transgenic overexpression of plasminogen activator inhibitor-1 promotes the development of


Chapter 8. Figure legends

Figure 1: P62, ubiquitin, and PAI-1 protein expression levels in the ovaries of control and PCOS patients determined by immunohistochemical analysis. (A-C) Cross-sections of ovaries were stained with an anti-p62, anti-ubiquitin, and anti-PAI-1 antibody and counterstained with hematoxylin. (D) Controls for background level stained with isotype IgG and hematoxylin. (E) Cross-sections of ovaries were stained with Azan stain. Fibrotic tissue was stained blue. (A-C, and E) (a-d) show the representative sections. Lower panels (c, d) show highly magnified views corresponding to (a, b). (e) The quantitative analysis of (A-C) immunohistochemical staining and (E) Azan staining. (D) Right panel (b) shows a highly magnified view corresponding to the left panel (a). The scale bars in (A-C, and E) (a, b) and (E) (a) indicate 200 µm, while those in (A-C, and E), (c, d), and (E) (b) indicate 50 µm. *p < 0.05. GC, granulosa cell layer; TC, theca cell layer; NC, negative control.

Figure 2: Effects of PA on autophagy and Cyp17A1 and PAI-1 mRNA expression in theca cells. (A) Representative western blot analysis of p62 and LC3- II /LC3- I in theca cells treated with different doses of PA (100, 200, and 500 µM) for 24 h. (B and C) Densitometric analysis of p62 and LC3- II /LC3- I ratio. (D and E) The autophagy flux assay. Theca cells treated with 200 µM PA for 24 h in the absence or presence of 50 µM Chloroquine (CQ) for the last 2 h were subjected to immunoblotting. (D) Representative western blot analysis of p62 and LC3- II /LC3- I . (E) LC3 flux was assessed by subtracting the amount of LC3- II in the absence of CQ from the amount of LC3- II in the presence of CQ for each of the conditions. CYP17A1 (F) and PAI-1 (G) mRNA expression in theca cells treated with different doses of PA (100, 200, and 500 µM) for 24 h. (H) ASD in the
culture supernatant was measured by ELISA. *p < 0.05. Data are expressed as the mean ± SEM (n = 4 in each experiment).

Figure 3: Effect of CQ on autophagy and Cyp17A1 and PAI-1 mRNA expression in bovine theca cells. (A) Representative western blot analysis of p62 and LC3- II /LC3- I in theca cells treated with different doses of CQ (10, 20, and 50 µM) for 24 h. (B and C) Densitometric analysis of p62 and LC3- II /LC3- I ratio. (D and E) CYP17A1 and PAI-1 mRNA expression in theca cells treated with different doses of CQ (10, 20, and 50 µM) for 24 h. (F) ASD in the culture supernatant was measured by ELISA. *p < 0.05. Data are expressed as the mean ± SEM (n = 4 in each experiment).

Figure 4: Transmission electron microscopy (TEM) images and assessment of ROS production. (A) Representative TEM images from theca cells treated with control condition (a, e), starvation condition for 2 h (b, f), 200 µM PA for 24 h (c, g), or 20 µM CQ for 24 h (d, h). Lower panels (e, f, g, h) show highly magnified views corresponding to (a, b, c, and d). (B) Representative fluorescence images of intracellular ROS measured using CellROX Oxidative Stress Reagents in theca cells treated with control conditions (a), 200 µM PA for 24 h (b), or 20 µM CQ for 24 h (c). (d) Quantitative analysis was performed (n = 5). *p < 0.05. Data are expressed as the mean ± SEM.

Figure 5: The involvement of p38 and JNK pathway in the effects of PA. Representative western blot analysis and densitometric analysis of p38 (A) and JNK (B) in theca cells treated with different doses of PA (100, 200, and 500 µM) for 24 h. CYP17A1 (C) and PAI-1 (D) mRNA expression in theca cells incubated with 200 µM PA for 24 h in the absence or presence of 10 µM p38 inhibitor
(SB202190) and 20 μM JNK inhibitor (SP600125). (E) ASD in the culture supernatant was measured by ELISA. *p < 0.05. Data are expressed as the mean ± SEM (n = 4 in each experiment).

Figure 6: The involvement of the p38 and JNK pathway in the effects of CQ. Representative western blot analysis and densitometric analysis of p38 (A) and JNK (B) in theca cells treated with different doses of CQ (10, 20, and 50 μM) for 24 h. CYP17A1 (C) and PAI-1 (D) mRNA expression in theca cells incubated with 20 μM CQ for 24 h in the absence or presence of 10 μM p38 inhibitor (SB202190) and 20 μM JNK inhibitor (SP600125). (E) ASD in the culture supernatant was measured by ELISA. *p < 0.05. Data are expressed as the mean ± SEM (n = 4 in each experiment).
Figure 1.
Figure 2.

A)  

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p62  
LC3 I  
LC3 II  
actin

B)  

p62/Actin  

C)  

LC3- II/LC3- I  

D)  

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LC3 I  
LC3 II  
actin

E)  

LC3 Ratio (mg/mL)  

F)  

Cyp17A1/Actin mRNA  

G)  

PAI-1/Actin mRNA  

H)  

Androstenedione  

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Figure 3.

A) [Image of Western blot showing CQ (µM) 0, 10, 20, 50 with bands for p62, LC3 I, LC3 II, and actin.]

B) p62/Actin

C) LC3 II/LC3 I

D) Cyp17A1/Actin mRNA

E) PAI-1/Actin mRNA

F) Androstenedione

[Bar graphs showing CQ (µM) 0, 10, 20, 50 with p62, LC3 II/LC3 I, Cyp17A1, PAI-1, and Androstenedione levels.]
Figure 4.

A) TEM

BSA  Starvation  PA  CQ

B) ROS

a) BSA  b) PA  c) CQ

d) Fluorescent intensity (Ray)

![Image of TEM and ROS data](image-url)
Figure 5.

A) p-P38  
P38

B) p-JNK  
JNK

C) CYP17A1/Actin mRNA  
D) PAI-1/Actin mRNA  

E) Androstenedione

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Figure 6.

A) p-P38

B) p-JNK

C) CYP17A1/Actin mRNA

D) PAI-1/Actin mRNA

E) Androstenedione