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Ibuprofen inhibits key genes involved in androgen production in theca–interstitial cells

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Abstract

Objective: To study the effects of ibuprofen on androgen production, gene expression, and cell viability in rat theca–interstitial cells exposed to the proinflammatory stimuli interleukin-1β (IL-1β) and lipopolysaccharide (LPS).

Design: Animal study.

Setting: University-based research laboratory.

Patient(s)/Animal(s): Theca-interstitial cells were isolated from 30 day old female Sprague Dawley rats.

Intervention(s): Theca cells were cultured with pro-inflammatory media containing IL-1β and LPS and compared with cells cultured in control media.

Main Outcome Measure(s): Androstenedione quantification was performed on conditioned cell culture medium using liquid chromatography-mass spectrometry. Theca cell viability was assessed using PrestoBlue cell viability assay. The gene expression of *Cyp17a1*, *Cyp11a1*, and *Hsd3b* was analyzed using quantitative polymerase chain reaction.

Result(s): Both proinflammatory stimuli IL-1β and LPS increased androstenedione concentration in cell culture medium, and these effects were mitigated with ibuprofen. Both inflammatory agents in addition increased the expression of key genes involved in androgen

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synthesis: *Cyp17a1*, *Cyp11a1*, and *Hsd3b*; the addition of ibuprofen to the culture medium inhibited these effects. Theca cell viability increased with IL-1β and LPS. Ibuprofen inhibited the IL-1β-mediated increase in cell viability but did not reverse the effects of LPS.

Conclusion(s): In conclusion, our findings support the hypothesis that many of the alterations induced by inflammatory stimuli in theca–interstitial cells are abrogated by the addition of ibuprofen.

Keywords

Androgen excess; inflammation; polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is a common chronic reproductive endocrine disorder characterized by oligoanovulation and androgen excess (1–5). A leading concept explaining PCOS is theca cell dysfunction, the source of androgens in the human ovary (4). The PCOS ovaries exhibit increased androgen production via overexpression of messenger RNA (mRNA) for key genes involved in androgen biosynthesis (6) and theca cell hyperplasia (5).

The underlying cause of theca cell dysfunction and androgen excess has yet to be determined although a growing body of evidence suggests that chronic systemic inflammation plays a contributory role (7–11). For example, several key inflammatory mediators such as C-reactive protein (12, 13), tumor necrosis factor-α (14), and interleukin 6 (15) are elevated in women with PCOS independent of known confounders including obesity. In addition, treatment of women with PCOS using agents with anti-inflammatory properties such as statins (16–19) and resveratrol (20) has been found to decrease androgen levels and improve several PCOS symptoms.

Prior in vitro work supports the hypothesis that proinflammatory mediators including interleukin-1β (IL-1β) and lipopolysaccharide (LPS) may increase ovarian androgen production via theca cell, the androgen-producing cell of the human ovary (7). Incubation of rat theca–interstitial cells (TICs) with either IL-1β or LPS elicited a dose-dependent increase in theca viability and androgen production similar to the theca cell dysfunction seen in PCOS (7). Transcriptional analyses suggest that these changes likely occur because of the increased expression of key androgenic genes *Cyp17a1*, *Cyp11a1*, and *Hsd3b* (7). In the present study, we investigated if the widely available over-the-counter anti-inflammatory drug ibuprofen inhibits the enhanced ovarian androgen production and theca cell hyperplasia induced by proinflammatory agents LPS and IL-1β. The primary mechanism of action of ibuprofen is through the inhibition of cyclooxygenase (COX)-induced prostaglandin synthesis. A paucity of data exists regarding the effects of ibuprofen on androgen production in the theca cell although ibuprofen appears to have suppressive effects on androgen production in the Leydig cell, the androgen-producing cell of the testis (21). A randomized controlled clinical trial of healthy men revealed that exposure to ibuprofen (2×600 mg/day for 6 weeks) resulted in an elevated luteinizing hormone (LH) level and decreased testosterone/LH ratio (21). This investigation revealed that ibuprofen decreased androgen production in a dose-dependent fashion in the testis via transcriptional repression of key androgenic genes (21). Considering that the ovarian theca cell is analogous to the testicular Leydig cell, we hypothesized that the cotreatment of TICs with LPS or IL-1β and

ibuprofen would inhibit the increased androgen production and theca cell number previously demonstrated with LPS or IL-1β treatment alone (7).

MATERIALS AND METHODS

Isolation of Rat TICs

Female Sprague Dawley rats were obtained at the age of 24 days from Envigo (Placentia, CA). Starting at the age of 27 days, the rats were injected subcutaneously with 17β-estradiol (1 mg in 0.3-mL sesame oil) (Millipore Sigma, St. Louis, MO) for 3 days to stimulate ovarian development and growth of antral follicles as described previously (22). For each experiment, 12 rats were used for the isolation of theca cells, and each experiment was repeated at least three times. Sample size was determined from a prior study showing a dose-dependent increase in TIC viability and androgen production when incubated with LPS or IL-1β (7). All rats survived to the final day of injections, and no behavioral or physical abnormalities were noted before euthanasia; therefore, all rats were included in the experiments (n = 36). All rat ovaries were pooled before TIC isolation; therefore, no randomization of rats or blinding of researchers was deemed necessary for the conduct of the experiments. Twenty-four hours after the last injection, the animals were anesthetized using ketamine (Ketaset, Zoetis, Kalamazoo, MI) and xylazine (intraperitoneal) (Akorn, Lake Forest, IL) and euthanized by intracardiac perfusion using sterile 0.9% sodium chloride solution. The ovaries were collected in ice-cold M199 isolation medium (Millipore Sigma, St. Louis, MO) containing 2-mM L-glutamine, 25-mM HEPES, 1% antibiotic and antimycotic, and 0.1% bovine serum albumin (BSA, Millipore Sigma, St. Louis, MO) at pH 7.25. All experimental procedures involving animals were performed in accordance with the accepted standards of animal care as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and a protocol approved by the Institutional Animal Care and Use Committee at the University of California, San Diego (Protocol ID S14262).

The collection and purification of ovarian TICs were performed as described previously (22). Briefly, the ovaries were removed from the animals and dissected free of oviducts and fat under a dissecting microscope. The ovaries were gently punctured with a 26-gauge needle in ice-cold M199 isolation medium. The remaining ovaries were washed twice with ice-cold M199 isolation medium (Millipore Sigma, St. Louis, MO) and then were minced and digested in 5 mL of collagenase–DNase solution containing 21.1 mg of collagenase (Worthington, Lakewood, NJ), 1.5 mg of DNase (Worthington, Lakewood, NJ), and 50 mg of BSA in M199 isolation medium at 37 °C for 60 minutes. After a 60-minute collagenase–DNase digestion, TICs were purified using discontinuous Percoll (Millipore Sigma, St. Louis, MO) gradient centrifugation. The purified TICs were then washed twice with ice-cold M199 isolation medium, and the pellet was resuspended in a known volume of ice-cold serum-free McCoy's 5A culture medium (Gibco, Life Technologies, Carlsbad, CA) supplemented with 1% antibiotic/antimycotic mix, 0.1% BSA, and 2-mM glutamine (Gibco, Life Technologies, Carlsbad, CA). Theca–interstitial cells were counted with a hemocytometer, and cell viability determined by trypan blue exclusion was routinely in the 90%–95% range.

Inflammatory Stimuli Protocol

Theca–interstitial cells were cultured on human fibronectin-coated plates (Corning Life Sciences, Tewksbury, MA) at 37 °C in an atmosphere of 5% CO_2 in humidified air in McCoy's 5A culture medium (Gibco, Life Technologies, Carlsbad, CA). To study the effects of inflammatory stimuli on the growth of TICs, TICs were incubated for up to 96 hours in human fibronectin-coated 96-well plate at a density of 1.0×10^4 cells/well. To study the effects of inflammatory stimuli on gene expression, TICs were incubated for 48 hours in human fibronectin-coated 24-well plate at a density of $1.0–2.0 \times 10^5$ cells/well. Theca–interstitial cells were incubated in the presence of a vehicle (control), recombinant rat IL-1β (1 ng/mL) (Gibco, Life Technologies, Carlsbad, CA), or LPS (100 ng/mL) (Millipore Sigma, St. Louis, MO) with and without 0.1 mM of ibuprofen (Millipore Sigma, St. Louis, MO).

Cell Viability Assay

Cell viability was estimated using PrestoBlue cell viability reagent 10X solution (Thermo Fisher Scientific, Carlsbad, CA) according to the manufacturer's instructions. Briefly, TICs in cell culture medium were seeded at 1×10^4 cells/well in a 96-well fibronectin-coated plates treated with vehicle (control) or different doses of LPS and IL-1β for 96 hours and culture medium only for background control. One hour before the end of the culture period, 10X PrestoBlue was added into 96 wells including medium-only wells (blanks) and incubated at 37 °C for 60 minutes. Cell viability was evaluated by fluorescence, which was determined with the use of a microplate reader (FLUOstar Omega, BMG, Durham, NC) with excitation and emission wavelengths of 570 and 610 nm, respectively. Relative cell viability was expressed as a percentage relative to the untreated control cells. This experiment was repeated three times using six to eight replicates for each experiment.

Ribonucleic acid Isolation and quantitative polymerase chain reaction

Ribonucleic acid (RNA) was isolated using the MagMAX-96 Total RNA Isolation Kit (Applied Biosystems, Foster City, CA) and KingFisher robot (Thermo Scientific, Vantaa, Finland). Reverse transcription of total RNA to complementary DNA (cDNA) was performed using the High Capacity cDNA Reverse Transcription Kit for real-time polymerase chain reaction quantitative polymerase chain reaction (qPCR) (Applied Biosystems, CA). Quantitative polymerase chain reaction were set up in 20-μL volumes, consisting of 5-μL cDNA (5–25 ng), 2.5-μL forward and 2.5-μL reverse 200-nM primers, and 10 μL of PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The gene specific primers used in this study are listed in Table 1. Quantitative polymerase chain reaction was run on an ABI 7300 (Applied Biosystems, Foster City, CA). The transcript levels of *Cyp11a1*, *Hsd3b*, and *Cyp17a1* were quantified using the Ct method with *Hprt* as the housekeeping reference gene. Each experiment was performed three times with three to four replicates.

Androstenedione Measurement

After 48 hours of treatment, the conditioned culture medium was collected for androstenedione quantification and kept frozen at −80 °C before testing. Androstenedione concentration was quantified using liquid chromatography-mass spectrometry. Cell culture

medium was thawed, and 100 μL was aliquoted into a 16 × 125-mm glass tube. After the addition of medium, 100 μL of water (Burdick & Jackson, Muskegon, MI) containing the internal standard, testosterone-d3 at 20 pg/μL (Cerilliant, Round Rock, TX), was added; samples were mixed by vortexing; and 3 mL of methyl tert-butyl ether (Fisher Scientific, Fairlawn, NJ) was added. The samples were rotated for 5 minutes in and centrifuged for 2 minutes at 800 × g. After centrifugation, the supernatant was removed and evaporated using nitrogen (45 °C), and the extract was redissolved with 60 μL of 95% water/5% acetonitrile (Burdick & Jackson, Muskegon, MI) with 0.2% formic acid (Alfa Aesar, Ward Hill, MA) and transferred to glass autosampler vials. Androstenedione concentrations were determined using an Agilent 1260 high performance liquid chromatography (Santa Clara, CA) coupled to a Thermo Q Exactive HF (Bremen, Germany) mass spectrometer. Briefly, 40 μL of the extract was injected, and compounds were separated over a 12.5-minute method via a reverse phase gradient with a flow rate of 0.4 mL/minute on an ACE C18 (2.1 × 100 mm, 3 μm) column held at 35 °C. Mobile phase A was comprised of water with 0.2% formic acid, and mobile phase B was comprised of acetonitrile with 0.2% formic acid. At the start of the run, the mobile phase was held at 97% A for 0.4 minutes and decreased to 1% A over 7.1 minutes, and the composition was held for 0.7 minutes before returning to starting conditions. The compounds were introduced to the mass spectrometer via positive-mode electrospray ionization and mass spectrometry spectra collected using both full scan (resolution, 45,000; AGC target, 1E6; scan range, 100–620 m/z) and parallel reaction monitoring (resolution, 15,000; isolation window, 1.5; AGC target, 100 ms) of 287.20056 (androstenedione) and 292.23480 (testosterone-D3) m/z. All data were acquired using Thermo XCalibur software and processed using Thermo Quan browser software. Concentrations were determined with linear regression analysis using the analyte to internal standard peak area ratios with $1/x^2$ weighting.

Statistical Analysis and Bioinformatic Analysis

All determinations were performed in duplicate, triplicate, or quadruplicate, and each experiment was repeated at least three times. Values were expressed as mean ± standard error of mean. Statistical analysis was performed using JMP 13.0 software (SAS, Cary, NC). Significant differences ($P<.05$) between groups were determined using one-way analysis of variance followed by post hoc pairwise comparisons of individual means (Dunnett's test). Normality of distribution was assessed by the Shapiro–Wilk W test. In the absence of normality and/or unequal variance, data were appropriately transformed, and/or nonparametric testing (Kruskal–Wallis, Dunn) was performed.

RESULTS

Ibuprofen Inhibits IL-1β- and LPS-Mediated Increase in Androgen Production in TICs

We examined the effects of IL-1β and LPS on TIC androgen production by measuring androstenedione concentrations in culture medium before and after 48 hours with and without pharmacologic concentrations of ibuprofen (21). Consistent with findings from our prior study (7), IL-1β and LPS significantly increased androstenedione concentration in TIC culture medium compared with control condition ($P<.05$) (Fig. 1A and B). In contrast, this incremental change in androstenedione was eliminated by ibuprofen treatment and

not different from that observed under control conditions (Fig. 1A and B). These findings suggest that ibuprofen inhibits the androgenic effects of these proinflammatory stimuli.

Ibuprofen Inhibits IL-1β- and LPS-Mediated Increased Expression of Steroid Hormone Biosynthesis Genes in TICs

To investigate the underlying mechanism of increased androgen production in TICs cultured in the presence of proinflammatory stimuli and the mitigation of these effects with ibuprofen, we examined the expression of key genes involved in androgen production using qPCR. Similar to our prior study, LPS- and IL-1β-treated TICs exhibited an increased expression of genes encoding for key androgenic enzymes including 17α-hydroxylase and 17,20-lyase (*Cyp17a1*) (Fig. 2A and B), cholesterol side-chain cleavage enzyme P450scc (*Cyp11A1*) (Fig. 2C and D), and 3-β-hydroxysteroid dehydrogenase (*Hsd3b1*) (Fig. 2E and F). In contrast, the addition of ibuprofen to IL-1β- and LPS-treated TICs abrogated the effects on androgenic gene expression (Fig. 2A and F) and interestingly decreased *Cyp17a1* expression to below basal levels (Fig. 2 A and B). Ibuprofen alone decreased the basal expression of these key androgenic genes compared with controls (Fig. 2A and F). Taken together, these data suggest that ibuprofen-mediated changes in the transcriptional machinery of key androgenic genes were the reason for the differences in androgen production in TICs exposed to proinflammatory stimuli with and without ibuprofen.

Ibuprofen Inhibits IL-1β-Mediated Increase in TIC Number but Does not Reverse the Effects of LPS

Compared with controls, IL-1β treatment demonstrated an increase in TIC number after 48 hours, and the addition of ibuprofen inhibited these effects (Fig. 3A). In contrast, LPS treatment increased the TIC number compared with controls although the addition of ibuprofen did not reverse these effects (Fig. 3B).

DISCUSSION

In the present study, we demonstrated that proinflammatory stimuli IL-1β and LPS can trigger alterations in androgen production, androgenic gene expression, and cell viability in TICs, consistent with our prior findings (7). In addition, we showed that nonsteroidal anti-inflammatory treatment, using ibuprofen, mitigates many of the alterations in TICs induced by these proinflammatory stimuli. We chose a nonspecific inhibitor of prostaglandin synthesis, ibuprofen, for these experiments because of the known role of prostaglandins as key mediators in physiologic inflammatory processes including the tightly regulated response of the ovarian follicle to the LH surge (23–25). Prostaglandins (including a key paracrine mediator in the ovary, prostaglandin E$_2$) are synthesized via the release of arachidonic acid from membrane phospholipid stores where they are converted to bioactive prostaglandins by a COX (COX-1 and COX-3 encoded, respectively, by PTGS1 and PTGS2 genes) (24). Ibuprofen is a COX inhibitor that inhibits two isoforms of COX, COX-1 and COX-2. In the ovarian follicle, PTGS1 is expressed constitutively, and PTGS2 expression is inducible by hormones, cytokines, and growth factors (24). In a prior study (7), we demonstrated an increase in *PTGS2* mRNA expression in theca cells exposed to IL-1β and LPS compared with controls, which additionally paralleled an increase in androgens

and androgenic gene expression (dataset available at https://data.mendeley.com/datasets/hyzpkbv376/draft#file-0d7286f6-adde-400a-a8f1-aab2d5bd29a1).

While both inflammatory treatments, IL-1β and LPS, increased androstenedione production and androgenic gene expression in TICs, IL-1β treatment showed a more pronounced effect (Figs. 1 and 2). Interestingly, we found a relatively wide variability in androstenedione concentration in culture medium treated with LPS and IL-1β (Fig. 1A and B). The possible explanations for this variability include variations in cell concentration among experiments/replicates and differences in the potency of inflammatory stimuli across experiments. In addition, IL-1β increased theca cell viability to a significant extent compared with control conditions, whereas LPS did not demonstrate a statistically significant increase in theca cell viability (Fig. 3). We suggest that the differences in the magnitude of the effects of IL-1β and LPS on theca cells and, thus, the altered response to ibuprofen are because of the differences in mechanisms through which these substances affect the inflammasome pathway (26). The inflammasomes are multiprotein complexes of the innate immune system, which, when activated, lead to a multitude of inflammatory responses including effects on cellular proliferation (27), apoptosis (28), and proteolytic processing of proinflammatory cytokines (including IL-1β) (26, 29–31). Both LPS and IL-1β are known mediators of the inflammasome pathway. Lipopolysaccharide is known to act more proximally as an inducer of the pathway via binding to toll-like receptor 4, whereas activated IL-1β is a potent proinflammatory end product (28, 30–32). Thus, it is expected that these two proinflammatory stimuli would demonstrate overlapping as well as divergent effects on TICs. Comparing the unique effects of LPS and IL-1β may highlight potential mechanisms through which these mediators potentiate their effects. Indeed, the transcriptional analysis of TICs exposed to LPS vs. IL-1β revealed that 2,222 genes were significantly regulated by LPS or IL-1β but not by both (7).

Notably, the gene expression analysis of ibuprofen alone compared with control conditions demonstrated a reduction in *Cyp17a1*, *Cyp11a1*, and *Hsd3b* to subbasal levels (Fig. 2). Kristensen et al. (21) demonstrated similar findings in the Leydig cells of ex vivo adult human testis exposed to ibuprofen, suggesting parallel mechanisms through which ibuprofen acts on the theca and Leydig cells. In their study, the addition of ibuprofen in addition coincided with a reduction in *PTGS1* and *PTGS2* mRNA as well as prostaglandin D2 and E2 levels in the culture system (21), highlighting a likely mechanism through which testosterone and gene expressed were repressed. A significant next question to address will be the precise relationship between the prostaglandin-inhibitory actions of ibuprofen and its effects on androgens and androgenic gene expression in the theca cell.

One of the strengths of our study is the use of pharmacologic concentrations of ibuprofen (0.1 mM) in theca cell culture medium. This concentration was chosen to correspond to the mean serum levels of ibuprofen on the basis of prior human studies of healthy male patients who ingested 600 mg of ibuprofen twice daily for 6 weeks (21, 33). While the men included in the aforementioned study did not endorse any adverse effects of the drug during the study (21), it is significant to note the well-known adverse effects of chronic nonselective nonsteroidal anti-inflammatory drug use. These include gastrointestinal effects, renal toxicity, antiplatelet effects, and risks in pregnancy including premature

closure of the ductus arteriosus and impairment of fetal kidney function. The potential sources of bias in our study include the lack of blinding during outcomes assessment (i.e., qPCR, androstenedione measurement, and cell viability assay) and data analysis. The outcomes reported are objective measurements; thus, the lack of blinding is unlikely to significantly influence results. However, future validation studies should be conducted by outside research groups to confirm our findings and ensure that the lack of blinding did not lead to overestimation of the treatment effects. In addition, future in vivo animal studies are needed to assess the impact of inflammatory stimuli and ibuprofen therapy on ovarian androgen production.

In conclusion, our findings support the hypothesis that the alterations induced by inflammatory stimuli in TICs are abrogated by the addition of ibuprofen. These findings not only help to clarify the relationship between inflammation and androgen production in TICs but in addition highlight future areas for research.

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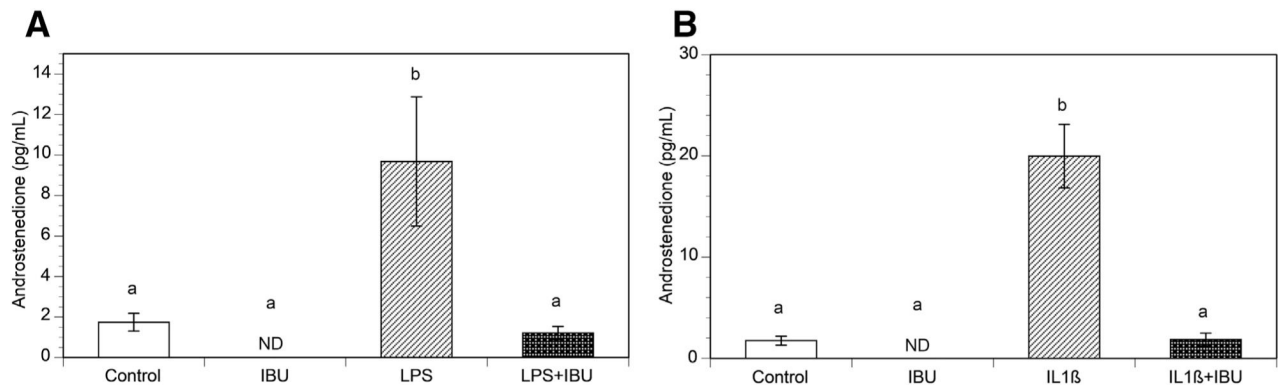
FIGURE 1.

Lipopolysaccharide (LPS) and interleukin 1β (IL-1β) increase the androstenedione concentration in theca–interstitial cells (TICs), and these findings are reversed with the addition of ibuprofen (IBU). Rat TICs are cultured in the presence of IL-1β or LPS +/− IBU for 48 hours in three experiments using at least three replicates in each treatment. The conditioned culture medium is then harvested, and the androstenedione concentrations (pg/mL) are determined by tandem mass spectrometry (*P<.05). ND = not detectable.
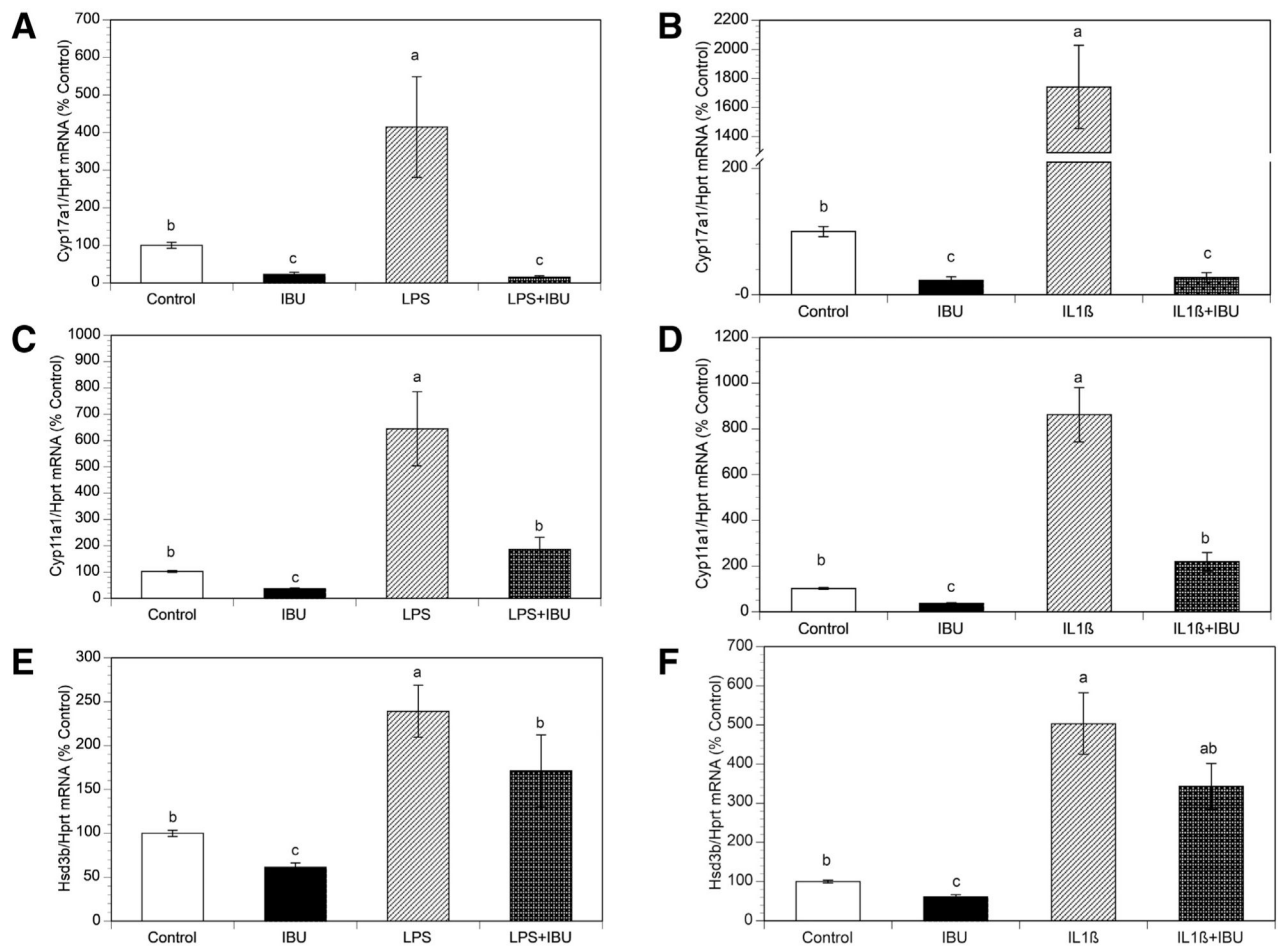
FIGURE 2.

Lipopolysaccharide (LPS) and interleukin 1β (IL-1β) increase the expression of key androgen biosynthesis genes, and ibuprofen (IBU) inhibits these effects. Rat theca–interstitial cells (TICs) are cultured for 48 hours in the presence of LPS (**A**, **C**, and **E**) or IL-1β (**B**, **D**, and **F**) +/− IBU. Ribonucleic acid is then extracted and reverse transcribed to complementary DNA, and quantitative polymerase chain reaction is performed for *Cyp17a1* (**A** and **B**), *Cyp11a1* (**C** and **D**), *Hsd3b* (**E** and **F**), and *Hprt1* (reference gene). The experiment is conducted three times using at least three replicates. The gene transcript levels are displayed as % control, Hprt1 (P<.05).
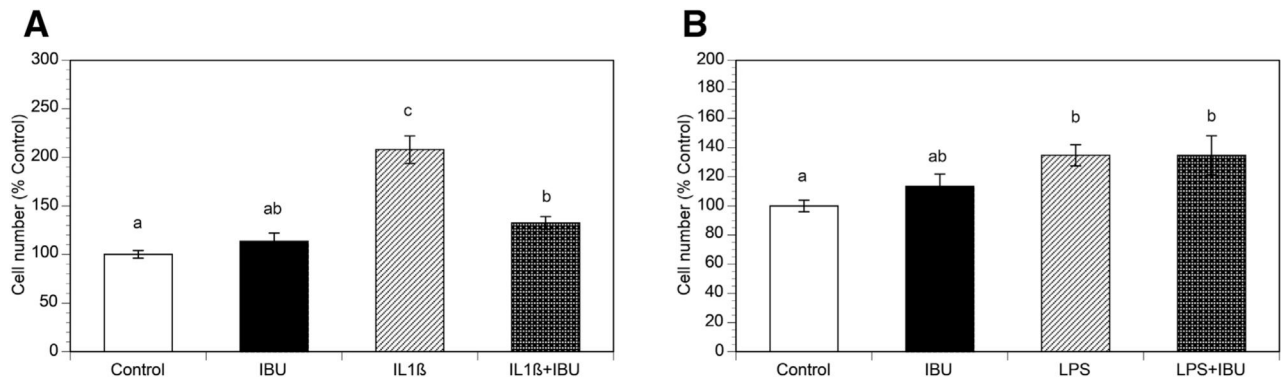
FIGURE 3.

Lipopolysaccharide (LPS) and interleukin 1β (IL-1β) increase the number of viable theca–interstitial cells (TICs). Ibuprofen (IBU) inhibits the effects of IL-1β but not the effects of LPS. Rat TICs are cultured IL-1β and LPS for 96 hours. Cell viability is assessed by fluorescence. The experiment is conducted three times using at least six replicates. The viable theca cell number (% of control) is displayed after exposure to LPS +/− IBU (A) and IL-1β +/− IBU (B) (P<.05).

TABLE 1

Primer sequences used in this study.

Gene symbol	RefSeq	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)	Product size (bp)
Hprt1	NM_012583	TTGTTGGATATGCCCTTGACT	CCGCTGTCTTTTAGGCTTTG	105
Hsd3b1	NM_001007719	ATATTGGAGGCCTGCGTCG	CGGCCATCCTTTTGCTGTA	166
Cyp11a1	NM_017286.3	GCTGGAAGGTGTAGCTCAGG	CACTGGTGTGGAACATCTGG	224
Cyp17a1	NM_012753.2	ACTGAGGGTATCGTG GATGC	CCGTCAGGCTGGAGATAGAC	187