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Ibuprofen alters human testicular physiology to produce a state of compensated hypogonadism

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Concern has been raised over increased male reproductive disorders in the Western world, and the disruption of male endocrinology has been suggested to play a central role. Several studies have shown that mild analgesics exposure during fetal life is associated with antiandrogenic effects and congenital malformations, but the effects on the adult man remain largely unknown. Through a clinical trial with young men exposed to ibuprofen, we show that the analgesic resulted in the clinical condition named "compensated hypogonadism," a condition prevalent among elderly men and associated with reproductive and physical disorders. In the men, luteinizing hormone (LH) and ibuprofen plasma levels were positively correlated, and the testosterone/LH ratio decreased. Using adult testis explants exposed or not exposed to ibuprofen, we demonstrate that the endocrine capabilities from testicular Leydig and Sertoli cells, including testosterone production, were suppressed through transcriptional repression. This effect was also observed in a human steroidogenic cell line. Our data demonstrate that ibuprofen alters the endocrine system via selective transcriptional repression in the human testes, thereby inducing compensated hypogonadism.

ibuprofen | endocrine disruption | reproduction | hypogonadism | endocrinology

Much concern has been raised over declining male reproductive health, and the disruption of male endocrinology has been suggested to play a central role (1, 2). Male reproduction and general health rely on androgens, as well as on other hormones, which are mainly produced by testicular Leydig and Sertoli cells. In addition to the testis, the androgens act in many somatic organs, e.g., producing anabolic effects on muscle mass and influencing cognitive functions (3). Luteinizing hormone (LH) produced by the pituitary is the primary stimulator of testosterone production, and the testosterone/LH ratio is routinely used as a clinical marker of Leydig cell function. When Leydig cell function is compromised, normal or nearly normal testosterone levels can often be sustained by augmented LH levels, as observed in the clinical entity termed "compensated hypogonadism" (4). The essential importance of the pituitary–gonadal axis is emphasized by the recent association of hypogonadism with a wide range of risk factors and all-cause mortality in men (4, 5).

The so-called "over-the-counter" mild analgesics (hereafter simply called "analgesics"), such as acetaminophen/paracetamol, acetylsalicylic acid/aspirin, and ibuprofen, are among the most commonly used pharmaceutical compounds worldwide (6, 7). Increasing evidence from recent years shows that exposure to analgesics can generate negative endocrine and reproductive effects during fetal life (6). Nonetheless, no in-depth studies have analyzed the effect of mild analgesics on the human pituitary–gonadal axis. In this context, ibuprofen is especially interesting because of its increasing use in the general population and in particular by elite athletes (8–12).

Therefore in this study we focused on how ibuprofen, used in the general population for aches, pains, fever, and arthritis and heavily used by athletes (13), affects the pituitary–testis axis. Because of the intrinsic great challenge in identifying endocrine-disrupting effects of chemicals in the adult human, we performed a unique combination of three interconnected approaches: (*i*) a randomized, controlled clinical trial; (*ii*) an ex vivo organ model using adult human testis explants; and (*iii*) a standardized in vitro

Significance

Concern has been raised over declining male reproductive health in humans. Our study addresses this issue by extending data showing antiandrogen effects of analgesics and suggests that such compounds may be involved in adult male reproductive problems. Using a unique combination of a randomized, controlled clinical trial and ex vivo and in vitro approaches, we report a univocal depression of important aspects of testicular function, including testosterone production, after use of over-the-counter ibuprofen. The study shows that ibuprofen use results in selective transcriptional repression of endocrine cells in the human testis. This repression results in the elevation of the stimulatory pituitary hormones, resulting in a state of compensated hypogonadism, a disorder associated with adverse reproductive and physical health disorders.

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The authors declare no conflict of interest.

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model system based on a steroidogenic cell line of human origin to complement the ex vivo approach. The complementary results revealed that ibuprofen induces a state of compensated hypogonadism by modifying hormonal profiles through selective repression of gene expression.

Results

Ibuprofen Affects the Hormonal Balance in Adult Men. We conducted a randomized, controlled clinical trial of ibuprofen administration to identify its possible effects on pituitary–gonadal feedback in young men. During administration, ibuprofen levels in plasma ranged on average from 25 to 35 μg/mL (~$1.2–1.7 \times 10^{-4}$ M); the highest level measured was 100 μg/mL (4.85×10^{-4} M). The mean of this ibuprofen concentration was in the range measured after administration of 600 mg of ibuprofen to healthy volunteers (14). Samples drawn before administration showed that there were no initial differences between the placebo and ibuprofen groups for hormones or sex hormone-binding globulin (SHBG), a liver protein that specifically binds a substantial part of circulating testosterone (Fig. S1).

We investigated the levels of total testosterone and its direct downstream metabolic product, 17β-estradiol. Administration of ibuprofen did not result in any significant changes in the levels of these two steroid hormones after 14 d or at the last day of administration at 44 d (Fig. 1 *A* and *C*). The levels of free testosterone were subsequently analyzed by using the SHBG levels. Neither free testosterone nor SHBG levels were affected by ibuprofen during the administration (Fig. 1 *A* and *C*).

The pituitary gonadotropin hormones LH and FSH regulate the production of testosterone and peptide hormones by acting on Leydig and Sertoli cells, respectively. The levels of LH in the ibuprofen group had increased by 23% after 14 d of administration ($P = 0.05$) (Fig. 1*A*). This increase was even more pronounced at 44 d, at 33% ($P = 0.01$) (Fig. 1*C*). While a slight, nonsignificant increase in the average FSH concentration was observed at 14 d (+5%) (Fig. 1*B*), no difference in the average value of this hormone was seen at 44 d (Fig. 1*D*). These data suggested a link between pituitary LH levels and ibuprofen exposure. This assumption was confirmed when we investigated ibuprofen and LH levels in plasma and found they were significantly and positively correlated at 14 d ($r = 0.73$; $P = 0.003$) (Fig. 1*E*). We next calculated the free testosterone/LH ratio in the men. We found an 18% decrease ($P = 0.056$) in the ibuprofen group compared with the placebo group after 14 d (Fig. 1*A*) and a 23% decrease ($P = 0.02$) after 44 d (Fig. 1*C*). Taken together, these in vivo data suggest that ibuprofen induced a state of compensated hypogonadism during the trial, which occurred as early as 14 d and was maintained until the end of the trial at 44 d.

Inhibin B and anti-Müllerian hormone (AMH) are peptide hormones secreted to the blood from the Sertoli cells. The administration of ibuprofen did not change mean inhibin B levels (Fig. 1 *B* and *D*). To examine the stimulatory action of FSH on the Sertoli cells, we next examined the inhibin B/FSH ratio and found that it decreased by 4% after 14 d (Fig. 1*B*) and by 12% at the end administration at 44 d (Fig. 1*D*). Importantly, further investigation of Sertoli cell activity showed that AMH levels decreased significantly with ibuprofen administration, by 9% ($P = 0.02$) after 14 d (Fig. 1*B*) and by 7% ($P = 0.05$) after 44 d compared with the placebo group (Fig. 1*D*). The AMH data show that the hypogonadism affected not only Leydig cells but also Sertoli cells and also occurred as early as 14 d of administration.

Ibuprofen Inhibits Steroidogenesis ex Vivo and in Vitro. To determine the direct effect of ibuprofen on the testis, we next exposed adult testis explants from donors to doses of 10^{-9}–10^{-4} M, which corresponded to the oral doses producing mean plasma levels of $1.2–1.7 \times 10^{-4}$ M used among the men in the trial (see above). We first investigated testosterone production after 24 and 48 h of ibuprofen exposure to assess its effects on Leydig cell steroidogenesis. Inhibition of testosterone levels was significant and dose-dependent ($\beta = -0.405$, $P = 0.01$ at 24 h and $\beta = -0.664$, $P < 0.0001$ at 48 h) (Fig. 2*A*) and was augmented over time (10^{-4} M at 24 h and 10^{-5}–10^{-4} M at 48 h, −40%) (Fig. 2*A*). Examination of the effect of ibuprofen exposure on both the Δ4 and Δ5 steroid pathways (Fig. 2*B*) showed that it generally inhibited all steroids from pregnenolone down to testosterone and 17β-estradiol; the production of each steroid measured decreased at doses of 10^{-5}–10^{-4} M. Under control conditions, production of androstenediol and dehydroepiandrosterone (DHEA) was below the limit of detection except in one experiment with DHEA (Fig. 2*B*).

We next examined the gene expression involved in testicular steroidogenesis ex vivo and found that levels of expression of every gene that we studied except *CYP19A1* decreased after exposure for 48 h compared with controls (Fig. 2*C*). Suppression of gene expression concerned the initial conversion of cholesterol to the final testosterone synthesis. Hence, expression of genes involved in cholesterol transport to the Leydig cell mitochondria was impaired: Compared with controls, *TSPO*/*BZRP* fell significantly after exposure to 10^{-4} M; *StAR* expression was suppressed at both doses of 10^{-5} and 10^{-4} M (Fig. 2*C*); *CYP11A1*, *CYP17A1*, and *HSD17B3* were suppressed by 40–50% at doses of 10^{-5}–10^{-4} M; and *HSD3B2* was reduced by about 90%.

The data from the ex vivo testis model showed inhibition of both the Δ4 and Δ5 steroid pathways. A previous study reported androsterone levels decreased by 63% among men receiving 400 mg of ibuprofen every 6 h for 4 wk (15), suggesting a possible inhibitory effect on CYP17A1. We therefore exposed the NCI-H295R cells of human origin [which, although derived from the adrenal gland (instead of testis) and from a cancer originating in the cortex, are considered the best in vitro model of human steroidogenesis according to the Organization for Economic Cooperation and Development (OECD) (16)] to ibuprofen and, as a positive control, to the CYP17A1 antagonist abiraterone. The latter compound is clinically used as an androgen biosynthesis inhibitor (17). Before testing ibuprofen's action on steroidogenesis, we verified that none of the doses used had a toxic effect on the NCI-H295R cell line (Fig. S2 *A* and *B*). This in vitro work confirmed the global antagonistic effect of abiraterone on CYP17A1 (Fig. 3*A*). In contrast, ibuprofen significantly decreased androstenedione and other sex steroid levels downstream from the CYP17A1 lyase activity, including testosterone (Fig. 3 and Table S1), complementing the results in the ex vivo testis model. These data therefore reveal that ibuprofen, unlike abiraterone, is not a general CYP17A1 antagonist. It is noteworthy that the NCI-295R cell line was less sensitive to ibuprofen than were the ex vivo cells in the organ model, likely due to its different origin (6, 18).

Measuring the mRNA expression of genes involved in steroidogenesis in vitro showed that ibuprofen had a profound inhibitory effect on the expression of these genes (Fig. 3 *B*–*D*), consistent with that seen above in our ex vivo organ model. Taken together, these data examining effects on the endocrine cells confirm that ibuprofen-induced changes in the transcriptional machinery were the likely reason for the inhibition of steroidogenesis.

Ibuprofen Inhibits Leydig Cell Insulin-like Factor 3 in the Adult Human Testis ex Vivo. In addition to steroids, Leydig cells also produce insulin-like factor 3 (INSL3) (19). Its production increased at 24 h at the 10^{-9} M dose and subsequently decreased at the 10^{-7} M dose (Fig. 2*D*). These variations were transient, with no significant effect of ibuprofen observed after 48 h. *INSL3* expression decreased by 50% at a dose of 10^{-4} M, but expression of the LH receptor, luteinizing hormone/choriogonadotropin receptor (*LHCGR*), which is also Leydig cell specific, was not repressed (Fig. 2*E*). Nonetheless, overall the changes in gene expression indicate that the transcriptional machinery behind the endocrine action of Leydig cells was most likely impaired by ibuprofen exposure.

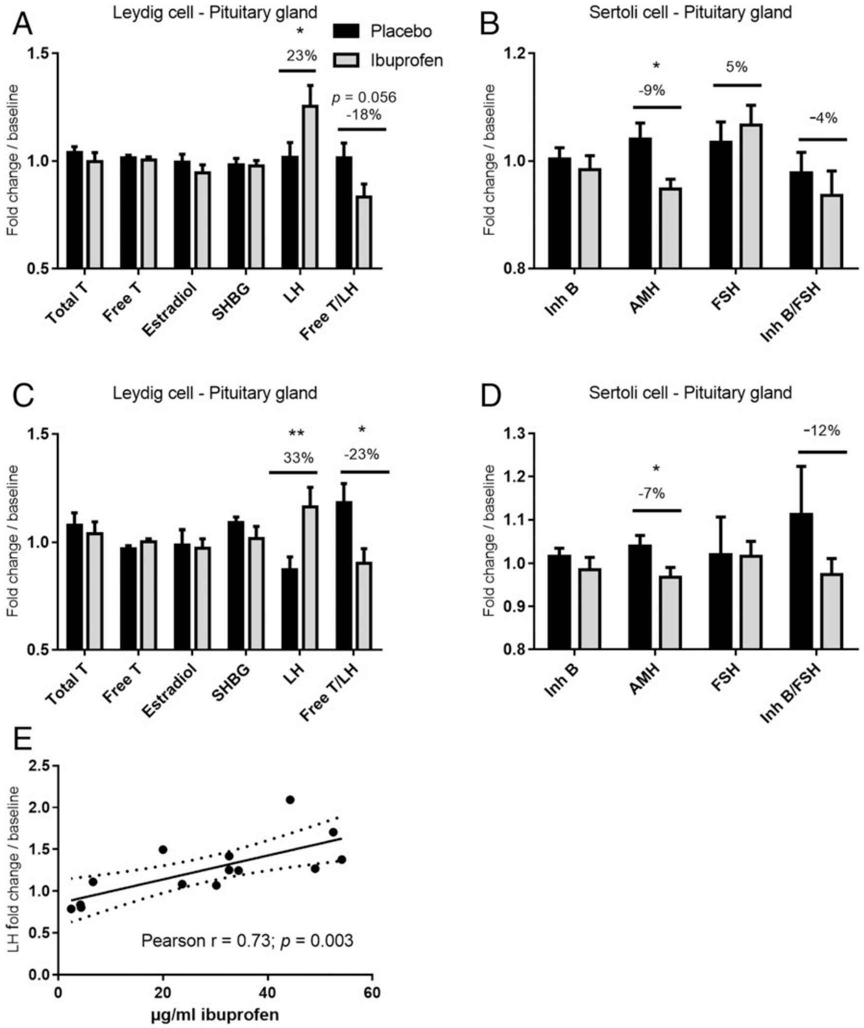

Fig. 1. Ibuprofen increases gonadotropins and decreases AMH in adult men. (*A*–*D*) Fold change in the Leydig cell–pituitary axis (*A* and *C*) and the Sertoli cell–pituitary axis (*B* and *D*) compared with baseline after 14 d (*A* and *B*) and at the end of the intervention after 44 d (*C* and *D*). Values are means ± SEM, and differences were analyzed with an unpaired Student's *t* test. (*E*) Correlation between absolute ibuprofen levels in plasma (*x* axis) and fold change in LH levels compared with baseline levels (*y* axis) 14 d after intervention. The slope and *P* value were calculated with a Pearson product-moment coefficient correlation. AMH, anti-Müllerian hormone; Free T, free testosterone; Inh B, inhibin B; SHBG, sex hormone-binding protein; Total T, total testosterone. $^*P \leq 0.05$, $^{**}P \leq 0.01$.

Ibuprofen Impairs Sertoli Cell Function in the Adult Human Testis ex Vivo. Data from the trial showed that ibuprofen affected Sertoli cells, inhibiting AMH and decreasing the inhibin B/FSH ratio. Ex vivo, increasing doses of ibuprofen resulted in an inverse correlation with inhibin B after 24 h ($\beta = -0.467$; $P = 0.01$), although none of the individual ibuprofen concentrations significantly inhibited this hormone (Fig. 4*A*). After 48 h of exposure, however, ibuprofen doses of 10^{-7}–10^{-4} M significantly decreased inhibin B production, resulting in a further significant negative association between ibuprofen concentrations and inhibin B ($\beta = -0.739$, $P < 0.0001$). AMH production was also negatively correlated with increasing ibuprofen concentration ($\beta = -0.451$; $P = 0.01$) (Fig. 4*B*). Accordingly, a dose of 10^{-4} M repressed gene expression of *AMH* and *INHBB* by ∼35% (Fig. 4*C*). Together, these data show that ibuprofen also directly impairs Sertoli cell function ex vivo by inhibiting transcription. Of note, no significant changes were found in the gene expression of the Sertoli cell-specific FSH receptor (*FSH-R*) or of *LAMA5* (Fig. 4*C*).

Ibuprofen Selectively Affects Peritubular Cells' Gene Expression in the Adult Human Testis ex Vivo. Peritubular cells lining the seminiferous wall play an important role in sustaining seminiferous tubule function (20). The peritubular cells are not broadly characterized in terms of specific markers. Nevertheless, we investigated the expression of a few genes that are assigned to these cells. We found that ibuprofen selectively repressed *ACTA2* and *MYH-11* by 50%, but two other peritubular cell markers, *THY1* and *KCNIP4*, did not change significantly (Fig. 5*A*).

Ibuprofen Spares the Spermatogenic Cells in the Adult Human Testis ex Vivo. Turning our attention to germ cells in the explants, we found no significant changes in the expression of genes involved specifically with spermatogenesis (Fig. 5*B*). The absence of a change in the germ cell complement by ibuprofen was confirmed by staining for caspase 3 after 48 h of exposure: Apoptosis did not increase significantly in the testis after exposure (Fig. 5 *C* and *D*), and the histopathology of the testis at the highest doses did not differ from that of controls (Fig. 5*D*).

Ibuprofen Suppresses Prostaglandin Production in the Adult Human Testis ex Vivo and in Vitro. As ibuprofen acts specifically on COX sites of prostaglandin H2 synthase (prostaglandin endoperoxide synthase or prostaglandin G/H synthase and cyclooxygenase, PTGS), and because prostaglandin receptors and synthesizing enzymes are widely distributed within the testis (21), we investigated prostaglandin D2 (PGD2) and E2 (PGE2) in our culture system. Ibuprofen produced a significant dose-dependent reduction of PGD2 ($\beta = -0.781$; $P < 0.0001$ at 24 h and $\beta = -0.797$; $P < 0.0001$ at 48 h) (Fig. 6*A*) and of PGE2 ($\beta = -0.707$; $P < 0.0001$ and $\beta = -0.627$; $P < 0.0001$, respectively) (Fig. 6*B*). *PTGS1* and *PTGS2* gene expression decreased similarly: *PTGS1* mRNA levels fell significantly by 24 and 48 h (Fig. 6*C*), and *PTGS2* mRNA was significantly repressed after 48 h of exposure. These ex vivo data show that ibuprofen suppressed both PTGS enzyme activity and *PTGS* gene expression in the adult testis.

To complement our ex vivo model system, we next screened the human NCI-H295R cell line for prostaglandin production. This screen showed that the NCI-H295R cells produced detectable levels of prostaglandins, which were decreased dose dependently with ibuprofen (Fig. 7*A*). As also shown in the experiments presented above, ibuprofen decreased the expression of PTGS1 and PTGS2 in the NCI-H295R cell line (Fig. 7*B*).

Discussion

The pituitary–gonadal axis plays key roles in growth, sex development, metabolism, musculoskeletal build-up, strength, mood, energy, immune system, libido, and reproduction (22–24). Fluctuations in or impaired fine-tuning of the axis can result in a wide range of endocrine disorders that may be local but severe, e.g., infertility (25), or affect the entire body, as seen with adverse outcomes involving this axis such as sexual symptoms (4), depression (26), coronary heart disease/heart attack (27), autoimmune diseases such as arthritis (28), and diabetes (29, 30). Testosterone forms a negative feedback loop that inhibits the production of both LH and gonadotropin-releasing hormone (GnRH) in the hypothalamus (31). While testosterone plays multiple roles outside the testes, the

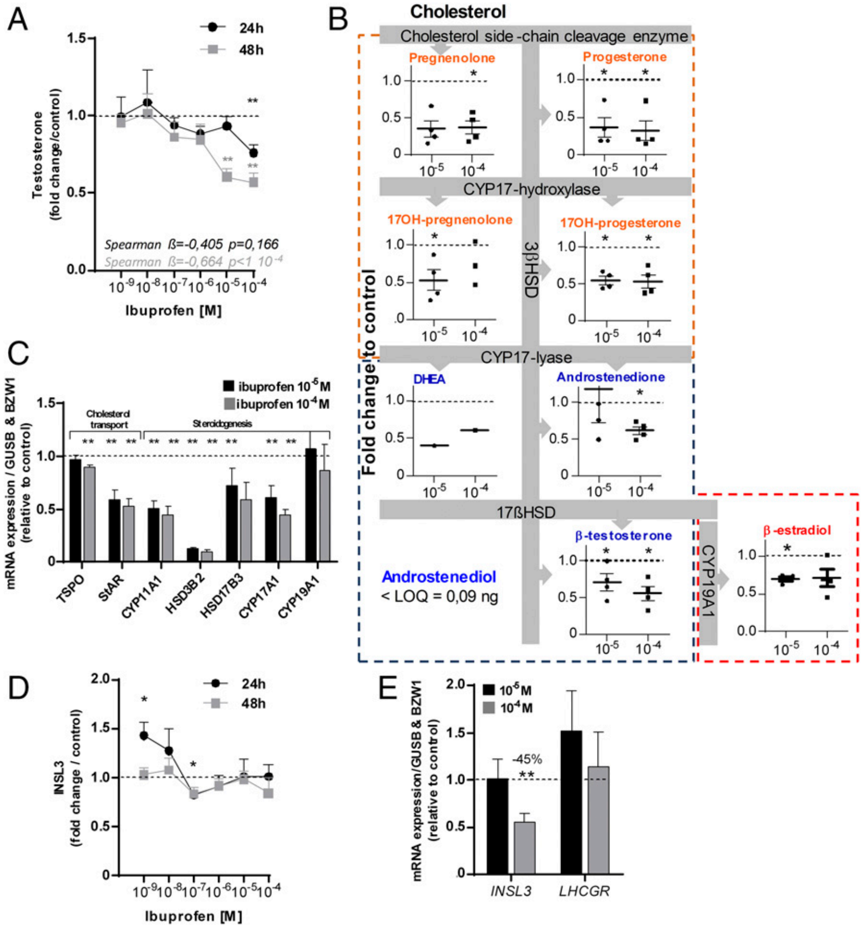

Fig. 2. Ibuprofen inhibits Leydig cell hormone production in human testicular explants. (*A*) Dose effect of ibuprofen for 24 and 48 h on testosterone production by the adult human testis. Values are means ± SEM of five independent experiments from different donors. Dose responses were analyzed for significance with the Mann–Whitney *U* test. The slopes and *P* values of these results were calculated with Spearman correlation. (*B*) Effect of 10^{-5} and 10^{-4} M ibuprofen on the Δ4 and Δ5 steroidogenic pathways. Steroid precursors were measured by GC-MS/MS. Values are means ± SEM of three independent experiments from different donors. Dose effects were analyzed for significance with the Mann–Whitney *U* test. DHEA, dehydroepiandrosterone. (*C*) Effect of 10^{-5} and 10^{-4} M ibuprofen on the gene expression of steroidogenic enzymes after 48-h exposure. Values are means ± SEM of five independent experiments from different donors. Each bar represents the mean ± SEM of the fold change in target gene expression relative to the reference genes *BZW1* and *GUSB*. Dose responses were analyzed for significance with the Mann–Whitney *U* test. (*D*) Dose effect of ibuprofen for 24 and 48 h on INSL3 production by the adult human testis. Values are means ± SEM of three independent experiments from different donors. Dose responses were analyzed for significance with the Mann–Whitney *U* test. (*E*) Specific Leydig cell hormone gene expression. Values are means ± SEM of five independent experiments from different donors. Each bar represents the mean ± SEM of the fold change in target gene expression relative to the reference genes *BZW1* and *GUSB*. Dose responses were analyzed for significance with the Mann–Whitney *U* test. BZW1, basic leucine zipper and W2 domains 1; CYP11A1, cytochrome P450 family 11 subfamily A member 1; CYP17A1, cytochrome P450 family 17 subfamily A member 1; CYP19A1, cytochrome P450 family 19 subfamily A member 1; GUSB, β-glucuronidase; INSL3, insulin-like factor 3; HSD17B3, hydroxysteroid 17-β dehydrogenase 3; HSD3B2, hydroxy-δ-5-steroid dehydrogenase 3 β- and steroid δ-isomerase 2; LHCGR, luteinizing hormone/choriogonadotropin receptor; StAR, steroidogenic acute regulatory protein; TSPO: translocation protein. $^*P \leq 0.05$, $^{**}P \leq 0.01$.

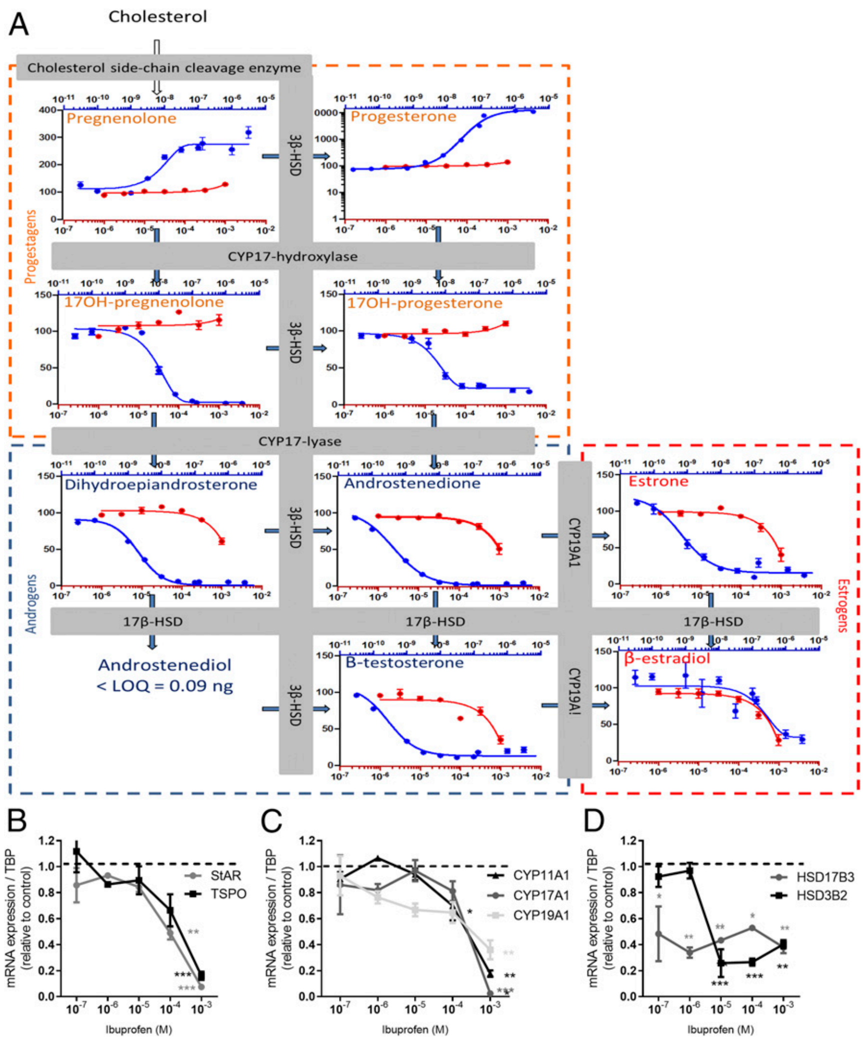

Fig. 3. The steroid screen identifies ibuprofen as an inhibitor of steroidogenesis in the human NCI-H295R cell line. (*A*) Effects of ibuprofen (red trace; $n = 9$–18) and abiraterone (blue trace; $n = 6$–15) exposure on the relative steroidogenic hormone production in the dose ranges of 10^{-6}–10^{-3} M and 3.15×10^{-11}–3.15×10^{-6} M (*x* axis), respectively, in the human NCI-H295R cell line, according to OECD standards. The position of each graph corresponds to the position of that particular steroid hormone in steroidogenesis. Steroid concentrations (*y* axis; % of control) are depicted as mean ± SEM with key enzymes shown in blue boxes. For statistics see Table S1. (*B*–*D*) Quantitative RT-PCR screen of steroidogenic gene expression in NCI-H295R cells after 48 h of culture with 10^{-7}–10^{-3} M ibuprofen. Values are means ± SEM of three independent experiments analyzed with one-way ANOVA followed by a post hoc Dunnett's test. CYP11A1, cytochrome P450 family 11 subfamily A member 1; CYP17A1, cytochrome P450 family 17 subfamily A member 1; CYP19A1, cytochrome P450 family 19 subfamily A member 1; HSD17B3, hydroxysteroid 17-β dehydrogenase 3; HSD3B2, hydroxy-δ-5-steroid dehydrogenase 3 β- and steroid δ-isomerase 2; StAR, steroidogenic acute regulatory protein; TSPO, translocation protein. $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$.

extratesticular actions of inhibin B are more subtle, working primarily to decrease FSH (32). Nonetheless, inhibin B is a key clinical marker of reproductive health (32). The function of AMH, also secreted by Sertoli cells, and its regulation through FSH remain unclear in men (33). It has, however, been shown that the AMH concentrations are lower in seminal plasma from patients with azoospermia than from men with normal sperm levels (32).

Our trial showed that ibuprofen use in men led to (*i*) elevation of LH; (*ii*) a decreased testosterone/LH ratio and, to a lesser degree, a decreased inhibin B/FSH ratio; and (*iii*) a reduction in the levels of the Sertoli cell hormone AMH. The decrease in the free testosterone/LH ratio resulted primarily from the increased LH levels, revealing that testicular responsiveness to gonadotropins likely declined during the ibuprofen exposure. Our data from the ex vivo experiments support this notion, indicating that the observed elevation in LH resulted from ibuprofen's direct antiandrogenic action. Accordingly, in the trial the average inhibin B levels did not differ significantly in the ibuprofen-treated men and the control group. This is consistent with a previous report that men who volunteered to take another nonsteroidal antiinflammatory drug (NSAID), acetylsalicylic acid, coadministered with human chorionic gonadotropin (hCG), which mimics LH, had lower levels of steroidal hormones than controls exposed to hCG but not to the analgesic (34).

AMH levels were consistently suppressed by ibuprofen both in vivo and ex vivo, indicating that this hormone is uncoupled from gonadotropins in adult men. The ibuprofen suppression of AMH further demonstrated that the analgesic targeted not only the Leydig cells but also the Sertoli cells, a feature encountered not only in the human adult testis but also in the fetal testis (35). It is noteworthy that ibuprofen repressed the expression of both *AMH* and *INHBB* as well as genes encoding essential proteins and enzymes involved in both cholesterol transport and steroidogenesis. Thus, ibuprofen displayed broad transcription-repression abilities involving steroidogenesis, peptide hormones,

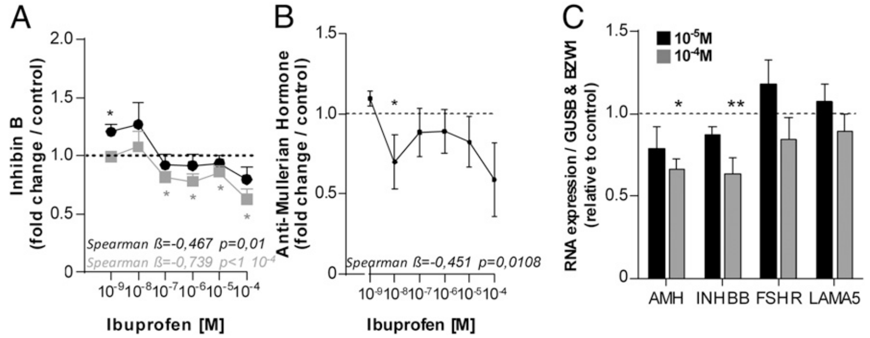

Fig. 4. Ibuprofen affects Sertoli cell activity in human testicular explants. (*A* and *B*) Dose effect of ibuprofen on the production of inhibin B after 24 and 48 h (*A*) and anti-Müllerian hormone (AMH) after 48 h (*B*) by the adult human testis. Values are means ± SEM of three independent experiments from different donors. Slopes and *P* values of Spearman correlation are indicated. (*C*) Quantitative RT-PCR performed after 48 h of culture treated with 10^{-5} and 10^{-4} M ibuprofen for specific Sertoli cell gene expression. Values are means ± SEM of five independent experiments from different donors. Each bar represents the mean ± SEM of the fold change in target gene expression relative to the reference genes *BZW1* and *GUSB*. Dose responses were analyzed for significance with the Mann–Whitney *U* test. AMH, anti-Müllerian hormone; BZW1, basic leucine zipper and W2 domains 1; FSHR, follicle-stimulating hormone receptor; GUSB, β-glucuronidase; INHBB, inhibin B subunit B; LAMA5, laminin subunit α5. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

and prostaglandin synthesis. However, these repressive abilities were selective, as a number of gene-expression patterns were spared by ibuprofen, namely prostaglandin inhibition in Leydig cells (*CYP19A1* and *LHCGR*), Sertoli cells (*LAMA5* and *FSHR*), peritubular cells (*KCNIP4* and *THY1*), and all those investigated in germ cells. Of note, the absence of an effect of ibuprofen on the expression levels of gonadotropin receptor genes (*LHCGR* and *FSHR*) indicates that the responsiveness of Leydig cells and Sertoli cells to the action of LH and FSH is likely not affected by ibuprofen. However, more investigation is required at this level.

Several compounds have been found to have unintentional antiandrogenic effects, and these are normally investigated in connection with fetal male development using rodent models (36, 37). Our approach, using ibuprofen as an example, demonstrates how a chemical compound, through its effects on the signaling compounds, can result in changes in the testis at gene level, resulting in perturbations at higher physiological levels in the adult human. The analgesics acetaminophen/paracetamol and ibuprofen have previously been shown to inhibit the post-exercise response in muscles by repressing transcription (38–40). However, the striking dual effect of ibuprofen observed here on both Leydig and Sertoli cells makes this NSAID the chemical compound, of all the chemical classes considered, with the broadest endocrine-disturbing properties identified so far in men. Previous ex vivo studies on adult testis have indeed pointed to an antiandrogenicity, only on Leydig cells, of phthalates (41), aspirin, indomethacin (42), and bisphenol A (BPA) and its analogs (43).

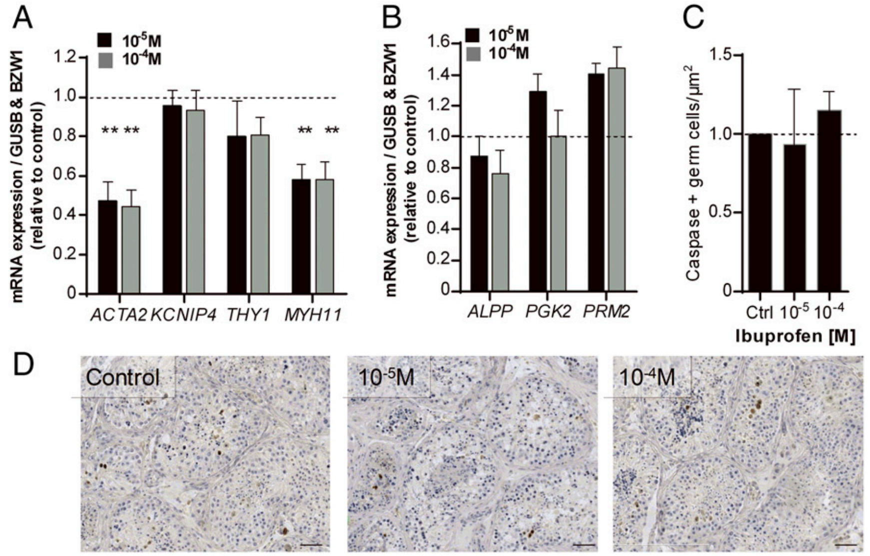

Fig. 5. Ibuprofen decreases gene expression in peritubular cells but does not affect germ cells or morphology in human testicular explants. (*A* and *B*) Quantitative RT-PCR performed after 48 h of culture treated with 10^{-5} and 10^{-4} M ibuprofen for gene expression in peritubular cells (*A*) and germ cells (*B*). Each bar represents the mean ± SEM of the fold change in target gene expression relative to the reference genes *BZW1* and *GUSB*. Values are means ± SEM of five independent experiments from different donors. A Mann–Whitney *U* test was performed. (*C*) Number of apoptotic germ cells. Values are means ± SEM of caspase^{+} cells in three independent experiments from different donors. (*D*) Immunostaining of apoptotic germ cells in testis explants cultured for 48 h in the presence of DMSO (control) or 10^{-5} or 10^{-4} M ibuprofen. Each micrograph shows representative areas of ibuprofen-induced morphology compared with corresponding area. (Scale bars: 50 μm.) ACTA2, actin α2 smooth muscle aorta; ALPP, alkaline phosphatase, placental; BZW1, basic leucine zipper and W2 domains 1; GUSB, β-glucuronidase; KCNIP4, potassium voltage-gated channel interacting protein 4; MYH11, myosin heavy polypeptide 11, smooth muscle; PGK2, phosphoglycerate kinase 2; PRM2, protamine 2; THY1, Thy-1 cell-surface antigen. $^{**}P \leq 0.01$.

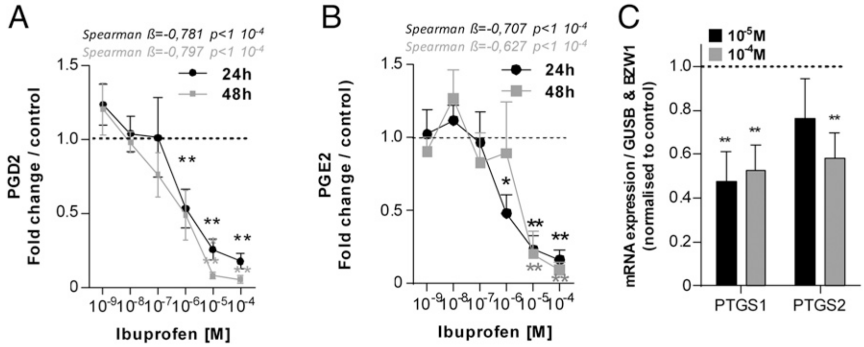

Fig. 6. Ibuprofen decreases PGE2 and PGD2 production and PTGS gene expression in human testicular explants. (*A* and *B*) Dose effect of ibuprofen exposure after 24 and 48 h on PGD2 (*A*) and PGE2 (*B*) production by adult human testicular explants. Values are means ± SEM of five independent experiments from different donors. Dose responses were analyzed for significance with the Mann–Whitney *U* test. Slopes and *P* values of Spearman correlation are indicated. (*C*) Quantitative RT-PCR was performed after 48 h of culture treated with 10^{-5} and 10^{-4} M ibuprofen. Each bar represents the mean ± SEM of the fold change in target gene expression relative to the reference genes *BZW1* and *GUSB*. Values are means ± SEM of five independent experiments from different donors. Differences in gene expression were analyzed with a Mann–Whitney *U* test. BZW1, basic leucine zipper and W2 domains 1; GUSB, β-glucuronidase; PTGS, prostaglandin-endoperoxide synthase. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

However, ibuprofen's effects were not restricted to Leydig and Sertoli cells, as data showed that the expression of genes in peritubular cells was also affected. Previous studies have shown that long-term fetal exposure to acetaminophen and acetylsalicylic acid in mice and rats targets primordial germ cell proliferation by blocking RNA synthesis and thus leads to reduced follicle reservoir and subsequent decreased fertility in adulthood (44–46). By contrast, in the present study using human testes, germ cells were the only cell category not altered by this analgesic in our ex vivo culture conditions. However, it must be noted that our ex vivo model systems can be used only for short-term exposure. Therefore, determining the effect on men that sustained exposure to ibuprofen would generate in terms of sperm production and fertility would require designing specific and challenging experiment(s). It is noteworthy that exposure to analgesics in men has been associated with increased time to pregnancy (47).

An important question is the exact relationship between the prostaglandin-inhibitory actions of ibuprofen and its effects on testosterone and gene expression. This has been investigated previously in studies on rodent and human testicular development, which showed no correlation between the endocrine-disruptive effects of analgesics and their prostaglandin-inhibitory actions (6, 48, 49). However, in the present study using testes from adult men, the suppression of androgens and prostaglandins occurred in parallel, and, because for several decades prostaglandins have been known to be involved in male reproduction (50), a link between the endocrine-disruptive properties of ibuprofen and the prostaglandin-inhibitory action of NSAIDs in the adult testis cannot be excluded.

In the clinical setting, compromised Leydig cell function resulting in increased insensitivity to LH is defined as compensated hypogonadism (4), an entity associated with all-cause mortality (5). Therefore, investigating ibuprofen-induced compensatory hypogonadism is crucial, as this clinical state is generally associated with smoking and aging (4, 51). Moreover, compensated hypogonadic men present with an increased likelihood of reproductive, cognitive, and physical symptoms (4, 52–54). Further characterizations of the state of compensated hypogonadism induced by ibuprofen, which was already established after 14 d of ibuprofen administration, are therefore important in determining the potential effects on healthy young men. Several reports have stressed the high level of long-term analgesic use among both amateur (55) and professional athletes; ibuprofen has been favored in this use and abuse (56–59). Of note, an inverse relationship was recently reported between

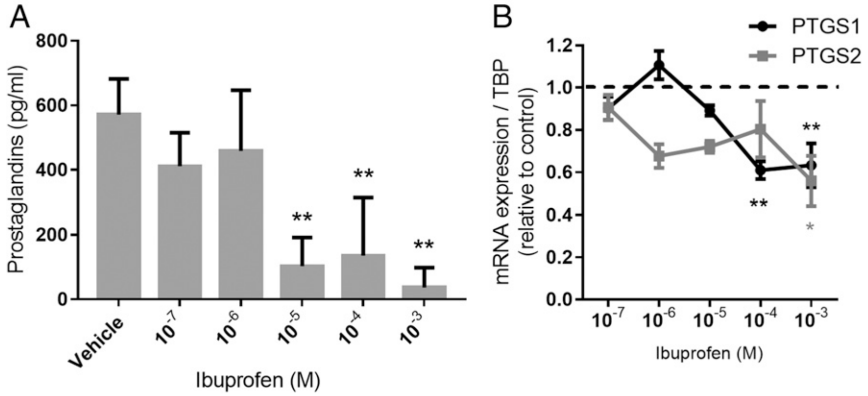

Fig. 7. Ibuprofen dose-dependently reduces prostaglandin levels and mRNA expression in human endocrine NCI-H295R cells. (*A*) Effects of ibuprofen on general prostaglandin production from NCI-H295R cells after 24 h. Values are means ± SEM of three independent experiments analyzed with one-way ANOVA followed by a post hoc Dunnett's test. (*B*) Quantitative RT-PCR screen of steroidogenic and *PTGS* gene expression in NCI-H295R cells after 48 h of culture with 10^{-7}–10^{-3} M ibuprofen. Values are means ± SEM of three independent experiments analyzed with one-way ANOVA followed by a post hoc Dunnett's test. PTGS, prostaglandin-endoperoxide synthase. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

endurance exercise training and male sexual libido, but the possibility that medication uptake might interfere in this observation could not be totally excluded (22). Moreover, ibuprofen appears to be the preferred pharmaceutical analgesic for long-term chronic pain and arthritis (60). Therefore it is also of concern that men with compensated hypogonadism may eventually progress to overt primary hypogonadism, which is characterized by low circulating testosterone and prevalent symptoms including reduced libido, reduced muscle mass and strength, and depressed mood and fatigue (4, 60, 61).

Materials and Methods

In Vivo Intervention Trial.

Design and participants. The in vivo study was designed as a double-blinded, placebo-controlled, randomized intervention trial in which ibuprofen or placebo was administered to subjects for 2 wk before and 30 d after a single exercise session. Staff not involved in the project prepared and distributed the medication in boxes weekly. Study personnel and participants were blinded to treatment, and all later analyses were performed blinded to the treatment type, participant, and time point. The study was part of a broader investigation also focusing on muscle biopsies, collected on days 0, 2, 7, and 30 postexercise, a subset of which is described elsewhere (62).

The study protocol was in compliance with the Helsinki Declaration, was approved by the Regional Scientific Ethical Committees of Copenhagen in Denmark (Ref: HD-2008-074), and was registered at ClinicalTrials.gov (no. NCT00832663). The study recruited 31 healthy white men, age 18–35 y. Subjects were included after an interview, a questionnaire assessing physical activity status, and the results of a screening blood sample. Exclusion criteria included body mass index above 30, knee injuries, peptic ulcers, signs of liver or kidney dysfunction, and participation in regular physical activity (especially strength training) apart from cycling as a means of transport. All individuals provided written informed consent to participate in the study. Subsequently, the subjects were assigned to either a placebo (17 subjects) or ibuprofen (14 subjects) group; the groups were matched for age, height, and weight.

Supplementation. One group of subjects received ibuprofen, 2 × 600 mg/d, (Ibumetin; Nycomed Denmark Aps) for a period of 6 wk, from 14 d before to 4 wk after the electrical stimulation exercise. Ibuprofen was detected only in participants to whom ibuprofen was distributed and only after administration began. The second group received placebo pills (which were visually indistinguishable from the ibuprofen pills) over the same period. Subjects received the medication in Medidos No. 1 boxes (KiBodan A/S), which were refilled every week. To verify compliance, ibuprofen levels in the blood were determined by HPLC at every blood-sampling time point (see below). Additionally, to monitor liver and kidney function, blood samples were analyzed for creatine, C-reactive protein, alkaline phosphates, and total cholesterol during the study. No subjects reported any adverse signs of taking the medication, nor did any blood parameters indicate or suggest adverse effects.

Blood samples and hormonal analysis. A 40-mL blood sample was collected from the antecubital vein of the nondominant arm when subjects arrived at the laboratory for screening. Samples were also taken at 3 and 2 wk before exercise (days −21 and −14), on the day of exercise before the exercise (day 0), after exercise (+2 h; day 0.1), and subsequently at 2, 4, 7, and 30 d after exercise. Hence, ibuprofen and placebo administration was ongoing for samples drawn on day 0, 0+2 h, 2, 4, 7, and 30. In the present study, we focused on samples drawn on day 0 after 14 d of supplementation. Plasma samples were stored at −80 °C until being assayed. The samples were analyzed for LH, FSH, testosterone, 17β-estradiol, AMH, inhibin B, and SHBG, as previously described in Aksglaede et al. (63), and ibuprofen as previously described in Farrar et al. (64). Of note, the LH measurements of one subject in the placebo group were aberrant and were discarded, thus decreasing the *n* value from 17 to 16.

Ex Vivo Organ Model: Testis Explant Assay. To determine the direct effect of ibuprofen on adult human testis physiology, we used a validated organ model assay (Testis Explant Assay) (65). Testes were obtained from prostate cancer patients who had not undergone any antihormonal treatment or from multiorgan donors (average age 46.9 ± 4.4 y). The protocol was approved by the local ethics committee (Agence de la Biomédine; authorization no. PFS09-015), and informed consent was obtained from all donors or their next of kin.

After collection of each testis, the organ was placed at 4 °C and rapidly processed for experimentation. Observation by transillumination allowed us to discard the rare testes not displaying spermatogenesis. Four 3-mm^3 testis explants were placed onto a polyethylene terephthalate insert (Falcon Labware) at the interface of air in 1 mL of DMEM (Sigma-Aldrich) in a well of a 12-well plate. The medium for exposure experiments contained 0.1% DMSO as a control and ibuprofen at different concentrations (purity >99%) (Sigma-Aldrich) in 0.1% DMSO. Four wells were analyzed for each condition. Exposures lasted 24 or 48 h, with a total medium change at 24 h, in a humidified atmosphere containing 5% CO_2 at 34 °C. The culture medium was then collected and stored at −80 °C. On the day of collection, three testis explants for each culture condition were randomly selected and fixed in neutral buffer, 4% formaldehyde, or Bouin's fixative for 2 h at 4 °C, embedded in paraffin, sliced into 5.0-μm-thick sections, and stored at 4 °C until immunostaining.

Hormone levels were assayed in duplicate in the culture medium. Testosterone levels were assayed with a specific RIA (Beckman Coulter). The intra- and interassay coefficients of variation were ≤8.6 and 11.9%, respectively. Control testis explants produced an average of 10.26 ± 2.50 ng testosterone per milliliter of explant after 24 h of culture and 8.47 ± 2.16 ng testosterone per milliliter of explant after 48 h of culture. INSL3 production was assayed with a commercial RIA (Phoenix France). Each sample was diluted twofold in RIA buffer before RIA. The intra- and interassay coefficients of variation were ≤15 and 7%, respectively, and the lower limit of detection was 20.17 pg/mL. Control testis explants produced an average of 0.29 ± 0.25 ng INSL3 per milliliter of explant after 24 h of culture and 0.20 ± 0.21 ng INSL3 per milliliter of explant after 48 h of culture. Inhibin B was assayed with a commercial ELISA kit (Beckman Coulter) according to the manufacturer's instructions. Each sample was diluted twofold in sample diluent solution before reactions. The intra- and interassay coefficients of variation for serum samples were ≤5.6 and 7.6%, respectively. Control testis explants produced an average of 570.43 ± 78.50 ng inhibin B per milliliter of explant after 24 h of culture and 529.44 ± 79.17 ng inhibin B per milliliter of explant after 48 h of culture. PGD2 and prostaglandin E2 (PGE2) were assayed by an ELISA method (Cayman Chemical Company), as was AMH (Immunotech).

RNA was extracted from testes with the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions and then was precipitated. Each total RNA sample (250 ng) was reverse transcribed with the Iscript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed according to the manufacturer's instructions with iTaq Universal SYBR Green Supermix (Bio-Rad) and a 2.5-μL cDNA template in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). The amplification program was as follows: an initial denaturation of 3 min at 95 °C; 40 cycles of 10-s denaturation at 95 °C; and 30 s at 62 °C for annealing and extension. Dissociation curves were produced with the thermal melting profile performed after the last PCR cycle. To avoid amplification of contaminating genomic DNA, primer pairs were selected on two different exons. Bzw1 and GusB mRNA were used as internal controls for normalization. Results were calculated by the ΔΔCT method as *n*-fold differences in target gene expression with respect to the reference gene and the calibration sample.

To analyze steroidogenesis in the explants, we performed solid-phase extraction (SPE) with C18 cartridges, reagents, and solvents from Solvent Documentation Synthesis. Standard reference steroids were from Sigma. Deuterated internal standards were from Steraloids. Quantification was performed by isotopic dilution. Samples were spiked with 400 pg of internal standards (etiocholanolone-d5, 17a-testosterone-d3, dihydrotestosterone-d3, 19-androstenedione-d3, progesterone-d9, 17a-methyltestosterone-d3, and 17b-estradiol-d3). Samples were applied to a C18 SPE column (2 g stationary phase) previously conditioned with 10 mL methanol and 10 mL water. The column was washed with water (5 mL) and then with cyclohexane (5 mL), and the steroids were eluted with methanol (10 mL). The extracts were dried (N2, 458C), and 400 pg of external standard (norgestrel) was added. Derivatization procedures and measurements in GC-MS/MS in electronization mode were performed as previously described (66, 67).

Cells were labeled with the primary rabbit antibody directed against cleaved caspase-3 (1/100; Cell Signaling) (41, 42) to enable detection of cells undergoing apoptosis in the explants. Slides were then scanned with a NanoZoomer slide scanner (Hamamatsu Photonics). Caspase-3$^+$ cells were counted by ImageJ software, and the results were expressed as percentages of the control values.

In Vitro: NCI-H295R Cell Line. The NCI-H295R human adrenocortical carcinoma cell line was obtained from ATCC (CRL-2128), and experiments aiming at completing the ex vivo experiments using human adult testis explants were conducted in accordance with OECD guidelines (16). Steroids were analyzed after exposure to ibuprofen, abiraterone, and PGD2 (Sigma-Aldrich). Toxicity was evaluated with the Alamar Blue assay (Sigma-Aldrich). Deuterated steroid analogs were obtained for analysis from CDN Isotopes and Toronto

Research Chemicals, and derivatization quality-control standards (7b-17β-estradiol-17-acetate), instrument control standards (estrone-3-methyl ether), and derivatization reagents (*N*-trimethylsilylimidazole, *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide, and 1,4-dithioerythritol) were obtained from Sigma-Aldrich. Steroid extraction and the subsequent quantification of steroid hormones were performed according to Holm et al. (68).

For prostaglandin secretion analysis, the NIC-H295R cells were plated in 12-well plates and were exposed overnight; the global prostaglandin synthesis was evaluated immediately afterward with a Prostaglandin Screening ELISA Kit (Cayman Chemical Company).

For transcriptional analysis with RT-qPCR, RNA was isolated from cells with the All Prep DNA/RNA kit (Qiagen). RNA integrity was measured on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific), and only 260/280 and 260/230 ratios >2.0 were accepted for further processing. Quantitative PCR was performed as previously described.

Statistical Analysis. For the trial, each individual's samples were normalized by division by the mean of the baseline samples drawn before the intervention. Hence, samples from each volunteer were normalized with the individual's own baseline values before the administration. Unpaired Student *t* tests were used to compare the placebo and ibuprofen groups after 14 and 44 d of administration. For the ex vivo experiments, data were compared using the Mann–Whitney *U* test and slopes with *P* values and Spearman correlation when indicated. For in vitro cell experiments, analysis was performed with one-way ANOVA followed by a post hoc Dunnett's multiple comparison test. All data are expressed as mean ± SEM, and differences were considered statistically significant when $P \le 0.05$.

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1. Bonde JP, et al. (2016) The epidemiologic evidence linking prenatal and postnatal exposure to endocrine disrupting chemicals with male reproductive disorders: A systematic review and meta-analysis. *Hum Reprod Update* 23:104–125.
2. Skakkebaek NE, et al. (2016) Male reproductive disorders and fertility trends: Influences of environment and genetic susceptibility. *Physiol Rev* 96:55–97.
3. Weinbauer GF, Luetjens CM, Simoni M, Nieschlag E (2010) Physiology of testicular function. *Andrology* (Springer, Berlin), pp 11–59.
4. Tajar A, et al.; EMAS Group (2010) Characteristics of secondary, primary, and compensated hypogonadism in aging men: Evidence from the European Male Ageing Study. *J Clin Endocrinol Metab* 95:1810–1818.
5. Holmboe SA, et al. (2015) The association of reproductive hormone levels and all-cause, cancer, and cardiovascular disease mortality in men. *J Clin Endocrinol Metab* 100:4472–4480.
6. Kristensen DM, et al. (2016) Analgesic use - prevalence, biomonitoring and endocrine and reproductive effects. *Nat Rev Endocrinol* 12:381–393.
7. Jégou B (2015) Paracetamol-induced endocrine disruption in human fetal testes. *Nat Rev Endocrinol* 11:453–454.
8. Van Wijck K, et al. (2012) Aggravation of exercise-induced intestinal injury by ibuprofen in athletes. *Med Sci Sports Exerc* 44:2257–2262.
9. Tscholl P, Feddermann N, Junge A, Dvorak J (2009) The use and abuse of painkillers in international soccer: Data from 6 FIFA tournaments for female and youth players. *Am J Sports Med* 37:260–265.
10. Tscholl P, Junge A, Dvorak J (2008) The use of medication and nutritional supplements during FIFA World Cups 2002 and 2006. *Br J Sports Med* 42:725–730.
11. Alaranta A, Alaranta H, Heliövaara M, Airaksinen M, Helenius I (2006) Ample use of physician-prescribed medications in Finnish elite athletes. *Int J Sports Med* 27:919–925.
12. Tscholl PM, Vaso M, Weber A, Dvorak J (2015) High prevalence of medication use in professional football tournaments including the World Cups between 2002 and 2014: A narrative review with a focus on NSAIDs. *Br J Sports Med* 49:580–582.
13. Mundie S, Jurejko J (2017) State of Sport: Fifa's former doctor says painkiller use risks footballers' health–BBC Sport. Available at www.bbc.com/sport/39333763. Accessed March 23, 2017.
14. Källström E, Heikinheimo M, Quiding H (1988) Bioavailability of three commercial preparations of ibuprofen 600 mg. *J Int Med Res* 16:44–49.
15. Ball KD, Levell MJ, Pickup ME (1982) The effect of ibuprofen on the excretion of steroid metabolites. *Clin Chim Acta* 124:23–29.
16. OECD (2011) *Test No. 456: H295R Steroidogenesis Assay* (OECD Publishing, Paris).
17. Ryan CJ, et al.; COU-AA-302 Investigators (2013) Abiraterone in metastatic prostate cancer without previous chemotherapy. *N Engl J Med* 368:138–148.
18. Hecker M, et al. (2006) Human adrenocarcinoma (H295R) cells for rapid in vitro determination of effects on steroidogenesis: Hormone production. *Toxicol Appl Pharmacol* 217:114–124.
19. Ivell R, Bathgate RAD (2002) Reproductive biology of the relaxin-like factor (RLF/INSL3). *Biol Reprod* 67:699–705.
20. Mayerhofer A (2013) Human testicular peritubular cells: More than meets the eye. *Reproduction* 145:R107–R116.
21. Frungieri MB, Calandra RS, Mayerhofer A, Matzkin ME (2015) Cyclooxygenase and prostaglandins in somatic cell populations of the testis. *Reproduction* 149:R169–R180.
22. Hackney AC (1996) The male reproductive system and endurance exercise. *Med Sci Sports Exerc* 28:180–189.
23. O'Shaughnessy PJ, Fowler PA (2011) Endocrinology of the mammalian fetal testis. *Reproduction* 141:37–46.
24. De Maddalena C, Vodo S, Petroni A, Aloisi AM (2012) Impact of testosterone on body fat composition. *J Cell Physiol* 227:3744–3748.
25. Jonas KC, Oduwole OO, Peltoketo H, Rulli SB, Huhtaniemi IT (2014) Mouse models of altered gonadotrophin action: Insight into male reproductive disorders. *Reproduction* 148:R63–R70.
26. Hintikka J, et al. (2009) Hypogonadism, decreased sexual desire, and long-term depression in middle-aged men. *J Sex Med* 6:2049–2057.
27. Kloner RA, Carson C, 3rd, Dobs A, Kopecky S, Mohler ER, 3rd (2016) Testosterone and cardiovascular disease. *J Am Coll Cardiol* 67:545–557.
28. Baillargeon J, et al. (2016) Hypogonadism and the risk of rheumatic autoimmune disease. *Clin Rheumatol* 35:2983–2987.
29. Dandona P, et al. (2008) Hypogonadotrophic hypogonadism in type 2 diabetes, obesity and the metabolic syndrome. *Curr Mol Med* 8:816–828.
30. Zitzmann M (2009) Testosterone deficiency, insulin resistance and the metabolic syndrome. *Nat Rev Endocrinol* 5:673–681.
31. Millar RP, et al. (2004) Gonadotropin-releasing hormone receptors. *Endocr Rev* 25:235–275.
32. Luisi S, Florio P, Reis FM, Petraglia F (2005) Inhibins in female and male reproductive physiology: Role in gametogenesis, conception, implantation and early pregnancy. *Hum Reprod Update* 11:123–135.
33. Young J, et al. (2005) Testicular anti-mullerian hormone secretion is stimulated by recombinant human FSH in patients with congenital hypogonadotropic hypogonadism. *J Clin Endocrinol Metab* 90:724–728.
34. Conte D, et al. (1999) Aspirin inhibits androgen response to chorionic gonadotropin in humans. *Am J Physiol* 277:E1032–E1037.
35. Ben Maamar M, et al. (2017) Ibuprofen results in alterations of human fetal testis development. *Sci Rep* 7:44184.
36. Christiansen S, et al. (2009) Synergistic disruption of external male sex organ development by a mixture of four antiandrogens. *Environ Health Perspect* 117:1839–1846.
37. Albert O, Jégou B (2014) A critical assessment of the endocrine susceptibility of the human testis to phthalates from fetal life to adulthood. *Hum Reprod Update* 20:231–249.
38. Burd NA, et al. (2010) Effect of a cyclooxygenase-2 inhibitor on postexercise muscle protein synthesis in humans. *Am J Physiol Endocrinol Metab* 298:E354–E361.
39. Trappe TA, et al. (2002) Effect of ibuprofen and acetaminophen on postexercise muscle protein synthesis. *Am J Physiol Endocrinol Metab* 282:E551–E556.
40. Markworth JF, Vella LD, Figueiredo VC, Cameron-Smith D (2014) Ibuprofen treatment blunts early translational signaling responses in human skeletal muscle following resistance exercise. *J Appl Physiol (1985)* 117:20–28.
41. Desdoits-Lethimonier C, et al. (2012) Human testis steroidogenesis is inhibited by phthalates. *Hum Reprod* 27:1451–1459.
42. Albert O, et al. (2013) Paracetamol, aspirin and indomethacin display endocrine disrupting properties in the adult human testis in vitro. *Hum Reprod* 28:1890–1898.
43. Desdoits-Lethimonier C, et al. (2017) Parallel assessment of the effects of bisphenol A and several of its analogs on the adult human testis. *Hum Reprod* 32:1465–1473.
44. Holm JB, et al. (2016) Intrauterine exposure to paracetamol and aniline impairs female reproductive development by reducing follicle reserves and fertility. *Toxicol Sci* 150:178–189.
45. Dean A, et al. (2016) Analgesic exposure in pregnant rats affects fetal germ cell development with inter-generational reproductive consequences. *Sci Rep* 6:19789.
46. Mukherjee AB, Chan M, Waite R, Metzger MI, Yaffee SJ (1975) Inhibition of RNA synthesis by acetyl salicylate and actinomycin D during early development in the mouse. *Pediatr Res* 9:652–657.
47. Smarr MM, et al. (2016) Urinary paracetamol and time-to-pregnancy. *Hum Reprod* 31:2119–2127.
48. Kristensen DM, et al. (2012) Paracetamol (acetaminophen), aspirin (acetylsalicylic acid) and indomethacin are anti-androgenic in the rat foetal testis. *Int J Androl* 35:377–384.
49. Mazaud-Guittot S, et al. (2013) Paracetamol, aspirin, and indomethacin induce endocrine disturbances in the human fetal testis capable of interfering with testicular descent. *J Clin Endocrinol Metab* 98:E1757–E1767.
50. Bygdeman M, Fredricsson B, Svanborg K, Samuelsson B (1970) The relation between fertility and prostaglandin content of seminal fluid in man. *Fertil Steril* 21:622–629.
51. Liu PY, et al. (2006) Aging attenuates both the regularity and joint synchrony of LH and testosterone secretion in normal men: Analyses via a model of graded GnRH receptor blockade. *Am J Physiol Endocrinol Metab* 290:E34–E41.
52. Ucak S, Basat O, Karatemiz G (2013) Functional and nutritional state in elderly men with compensated hypogonadism. *J Am Med Dir Assoc* 14:433–436.

53. Andersson A-M, Jørgensen N, Frydelund-Larsen L, Rajpert-De Meyts E, Skakkebaek NE (2004) Impaired Leydig cell function in infertile men: A study of 357 idiopathic infertile men and 318 proven fertile controls. *J Clin Endocrinol Metab* 89:3161–3167.
54. Ventimiglia E, et al. (2017) Primary, secondary and compensated hypogonadism: A novel risk stratification for infertile men. *Andrology* 5:505–510.
55. Locquet M, et al. (2016) Self-medication practice among amateur runners: Prevalence and associated factors. *J Sports Sci Med* 15:387–388.
56. Ziltener J-L, Leal S, Fournier P-E (2010) Non-steroidal anti-inflammatory drugs for athletes: An update. *Ann Phys Rehabil Med* 53:278–288.
57. Corrigan B, Kazlauskas R (2003) Medication use in athletes selected for doping control at the Sydney Olympics (2000). *Clin J Sport Med* 13:33–40.
58. Vaso M, Weber A, Tscholl PM, Junge A, Dvorak J (2015) Use and abuse of medication during 2014 FIFA World Cup Brazil: A retrospective survey. *BMJ Open* 5:e007608.
59. Wharam PC, et al. (2006) NSAID use increases the risk of developing hyponatremia during an Ironman triathlon. *Med Sci Sports Exerc* 38:618–622.
60. Moore RA, Derry S, Wiffen PJ, Straube S, Aldington DJ (2015) Overview review: Comparative efficacy of oral ibuprofen and paracetamol (acetaminophen) across acute and chronic pain conditions. *Eur J Pain* 19:1213–1223.
61. Dandona P, Rosenberg MT (2010) A practical guide to male hypogonadism in the primary care setting. *Int J Clin Pract* 64:682–696.
62. Mackey AL, et al. (2016) Activation of satellite cells and the regeneration of human skeletal muscle are expedited by ingestion of nonsteroidal anti-inflammatory medication. *FASEB J* 30:2266–2281.
63. Aksglaede L, Sørensen K, Petersen JH, Skakkebaek NE, Juul A (2009) Recent decline in age at breast development: The Copenhagen Puberty study. *Pediatrics* 123:e932–e939.
64. Farrar H, Letzig L, Gill M (2002) Validation of a liquid chromatographic method for the determination of ibuprofen in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* 780:341–348.
65. Roulet V, et al. (2006) Human testis in organotypic culture: Application for basic or clinical research. *Hum Reprod* 21:1564–1575.
66. Prevost S, Buisson C, Monteau S, André F, Le Bizec B (2004) Is GC-C-IRMS a possible analytical approach to clear up misuse situations for forbidden natural substances in edible tissues? *Proceedings of the Euroresidue V Conference* (Euroresidue V, Noordwijkerhout, The Netherlands).
67. Courant F, et al. (2008) Exposure assessment of prepubertal children to steroid endocrine disruptors. 2. Determination of steroid hormones in milk, egg, and meat samples. *J Agric Food Chem* 56:3176–3184.
68. Holm JB, et al. (2015) Aniline is rapidly converted into paracetamol impairing male reproductive development. *Toxicol Sci* 148:288–298.