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Annexin 1 (Lipocortin 1) Mimics Inhibitory Effects of Glucocorticoids on Testosterone Secretion and Enhances Effects of Interleukin-1β

Patricia O. Cover, Frederick Baanah-Jones, Christopher D. John, and Julia C. Buckingham

Faculty of Medicine, Department of Neuroendocrinology, Division of Neuroscience and Psychological Medicine, Imperial College of Science Technology and Medicine, Commonwealth Building, Hammersmith Hospital, London

Annexin 1 is an important mediator of glucocorticoid action in the hypothalamo-pituitary axis; however, little is known of its role in mediating glucocorticoid actions in the peripheral endocrine organs. Accordingly, we have carried out a preliminary study to investigate the effects of annexin 1 in vitro on the testicular secretion of testosterone, a process inhibited by both glucocorticoids and interleukin-1β (IL-1β). Luteinizing hormone (LH) and forskolin stimulated the release of testosterone from dispersed murine testicular cells in vitro. Their effects were reduced in cells from mice pretreated with dexamethasone (DEX). Similarly, preincubation of testicular cells from untreated mice with DEX, corticosterone, or 11-dehydrocorticosterone reduced LH-stimulated testosterone release, as did the 11β-hydroxysteroid dehydrogenase inhibitors, glycyrrhetinic acid and carbenoxolone. The inhibitory actions of the steroids were mimicked by annexin $1_{1–188}$ ($ANXA1_{1–188}$) (a stable annexin 1 analog). IL-1β produced a marked decrease in the response to LH, which was blocked by indomethacin, a nonselective cyclooxygenase inhibitor and an additive effect with DEX and $ANXA1_{1–188}$. These results confirm reports that glucocorticoids and IL-1β inhibit LH-stimulated testosterone release from mouse testicular cells. They also show, for the first time, that the effects of the steroids are mimicked by annexin 1 and that, in contrast to their mutually antagonistic effects in the neuroendocrine system, IL-1β and annexin 1 exert additive actions in the testis.

Key Words: Glucocorticoids; annexin 1; cyclooxygenase; testis; 11β-hydroxysteroid dehydrogenase; interleukin-1β.

Introduction

Glucocorticoids exert widespread effects in the body that are critical to the maintenance of homeostasis, particularly in conditions of physical (e.g., infection, inflammation) and emotional stress when they are released into the systemic circulation in substantial amounts *(1,2)*. However, when present in the circulation in excess amounts for long periods (e.g., in Cushing disease, in conditions of chronic stress, or as a result of long-term steroid therapy), glucocorticoids threaten the well-being of the individual by inducing immunosuppression and a number of other pathologies. These include disturbances in reproductive function characterized by oligospermia, amenorrhea, and consequently infertility *(3–5)*. The mechanisms by which glucocorticoids suppress fertility have been attributed largely to actions of the steroids on the hypothalamo-pituitary axis that disrupt secretion of the gonadotropins *(3,6)*. However, reports that glucocorticoid receptors are localized in the testis, particularly in the Leydig cells *(7)*, and that glucocorticoids depress testosterone release in a variety of preparations, suggest that glucocorticoid-induced infertility in the male may also reflect actions of the steroids on the gonads *(4,8)*.

In many tissues, the actions of glucocorticoids are modified at the cellular level by 11β-hydroxysteroid dehydrogenase (11βHSD) enzymes that catalyze the interconversion of cortisol and corticosterone and their biologically inert 11-keto forms, 11-dehydrocortisol and 11-dehydrocorticosterone (11DHC) *(9,10)*, and thereby regulate the local concentration of the steroid. Two enzymes have been described; type 1 (11βHSD1) may exhibit both 11β-dehydrogenase and 11β-reductase activities, and type 2 (11βHSD2) exhibits exclusively dehydrogenase activity and thus serves to inactivate the biologically active steroid *(11–14)*. The role of 11βHSD enzymes in the testis is not fully understood and appears to be subject to significant variance in species. Thus, Gao et al. *(15,16)* proposed that 11βHSD acts primarily as a dehydrogenase in rat Leydig cells and thereby protects the cells from the inhibitory effects of corticosterone on testosterone release. On the other hand, Leckie et al. *(17)* failed to detect 11βHSD2 mRNA in rat Leydig cells and provided evidence that 11βHSD1 acts predominantly as a reductase

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Author to whom all correspondence and reprint requests should be addressed: Dr. P. Cover, Faculty of Medicine, Department of Neuroendocrinology, Division of Neuroscience and Psychological Medicine, Imperial College of Science Technology and Medicine, Commonwealth Building, Hammersmith Hospital, Du Cane Road, London W12 0NN. E-mail: p.cover@ic.ac.uk

in these cells and thus augments glucocorticoid actions in the cells. Similarly, Condon et al. *(18)* failed to detect 11βHSD2 in the mouse testis.

The mechanisms downstream of the glucocorticoid receptor that lead to the suppression of testosterone secretion are also poorly understood, although there is evidence that the steroids modulate the expression of a number of the steroidogenic enzymes *(19–21)*. Studies in our laboratory have shown that annexin 1 (ANXA1, also known as lipocortin 1), a Ca^{2+} and phospholipid binding protein, is an important mediator of glucocorticoid action in the neuroendocrine system *(22)*, particularly in the context of the inhibitory influence of glucocorticoids on the actions of interleukin-1β (IL-1β) and other proinflammatory cytokines in the hypothalamopituitary axis that mediate the neuroendocrine responses to immune insults *(1,23–25)*. Annexin 1 is also a key mediator of the antiinflammatory *(26–28)*, antipyretic *(29)*, and antiproliferative *(30,31)* actions of the steroids and, thus, effectively suppresses the release and/or the activity of a variety of proinflammatory mediators, including the cytokines (e.g., IL-1β, IL-6), nitric oxide, and eicosanoids, in a variety of models of inflammation *(32,33)*. Little is known about the expression or function of annexin 1 as a mediator of glucocorticoid action in the peripheral endocrine organs, and its impact on testicular steroidogenesis has not yet been explored.

The relationship among glucocorticoids, annexin 1, and cytokines in the control of testicular function is a potentially interesting avenue for exploration. Functional studies have shown that IL-1 inhibits testosterone release from purified mouse *(34)* and rat *(35,36)* Leydig cells in vitro. Reports that resident macrophages in the rat testis are intimately associated with Leydig cells *(37–39)* and evidence that endotoxin augments the expression of IL-1β in the testis *(38)* raise the possibility that IL-1β fulfills a role as a paracrine mediator of macrophage–Leydig cell communication, particularly in conditions of infection or other challenges to the host defense system. Locally produced IL-1β may thus act in concert with glucocorticoids in the testis to augment the steroid-induced suppression of testosterone production, particularly in conditions of local or systemic inflammatory insults when glucocorticoid secretion is increased. Such an action contrasts markedly with the mutually antagonistic actions of the steroids and IL-1β in the neuroendocrine and host defense systems *(1,23–25,32)*.

The present study was designed to explore the complementary influences of glucocorticoids and IL-1β on testosterone secretion in an established in vitro system and to examine the potential role of annexin 1 in modulating steroidogenesis, using a stable analog, annexin $1_{1–188}$ ($ANXA1_{1–188}$), as a tool.

Results

Figure 1 shows the effects of luteinizing hormone (LH) on testosterone secretion in vitro. LH (50–400 mU/mL, 3 h)

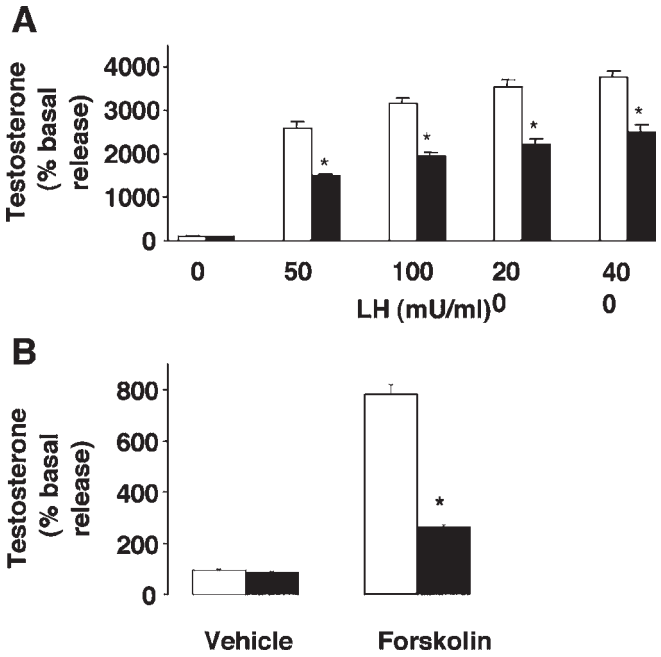

Fig. 1. Effects of LH and forskolin on testosterone release from dispersed testicular cells obtained from DEX-treated (20 mg/L of drinking water) (■) and control animals (received normal drinking water) (□). Cells were incubated for 3 h with or without LH and forskolin. (**A**) Effects of graded concentrations of LH (50–400 mU/mL); (**B**) effects of forskolin (50 μ*M*) or vehicle (veh). Each column represents the mean ± SEM ($n = 6$). *$p < 0.01$, vs vehicle pretreatment (analysis of variance [ANOVA] and Tukey-Kramer).

stimulated a dose-related release of testosterone from dispersed murine testicular Leydig cells (Fig. 1A). The adenylyl cyclase activator forskolin (50 μ*M*) (Fig. 1B) also stimulated the release of testosterone. These effects were reduced ($p < 0.01$) in cells obtained from mice pretreated with DEX (20 mg/mL in drinking water, 2.5 d) (Fig. 1). Initial studies showed that dispersed testicular cells obtained from untreated Tuck Ordinary mice also respond to LH (12.5–100 mU/mL, 2 h) with a dose-related release of testosterone (results not shown).

On the basis of these studies, a submaximal concentration of LH (25 mU/mL) was selected for further studies. Similarly, the concentrations of DEX (0.01 μ*M*), corticosterone (1 μ*M*), and glycyrrhetinic acid (50 n*M*) were selected from initial studies. DEX (0.01 μ*M*) (data not shown), 11DHC alone (1 μ*M*) (Fig. 2), and corticosterone (1 μ*M*) (Fig. 3) had no significant effect on basal testosterone production. However, when given in combination with a submaximal concentration of LH (25 mU/mL), these drugs significantly inhibited testosterone production ($p < 0.01$ vs LH alone) (Figs. 2–4). The inhibitory actions of the steroids on LH-stimulated testosterone release were mimicked by $ANXA1_{1–188}$, a stable annexin 1 analog that shows the full biologic activity of the full-length protein in the neuroendocrine system (8–1000

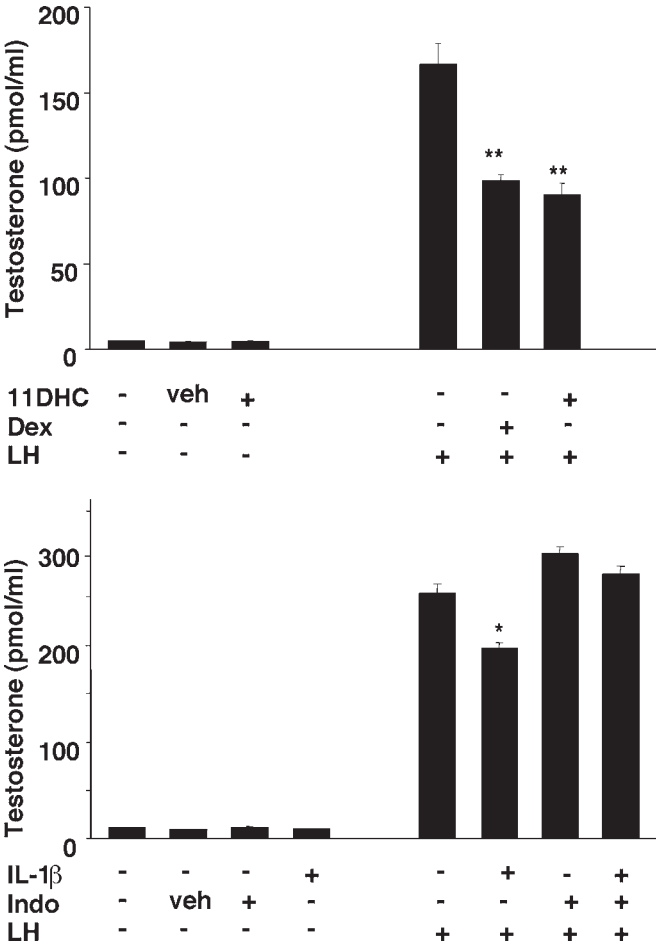

Fig. 2. Effects of 11DHC, DEX, IL-1β, and indomethacin (Indo) on basal and LH-stimulated testosterone release from dispersed testicular cells. Cells were preincubated for 2 h with or without drugs or vehicle (veh) followed by 2 h of incubation as for the preincubation with or without added LH (25 mU/mL). (**A**) Effects of 11DHC (1 μ*M*) and DEX (0.01 μ*M*); (**B**) effects of IL-1β (200 pg/mL) and Indo (10 μ*M*). Each column represents the mean ± SEM ($n = 6$). *$p < 0.01$; **$p < 0.001$ vs LH alone (ANOVA and Tukey-Kramer).

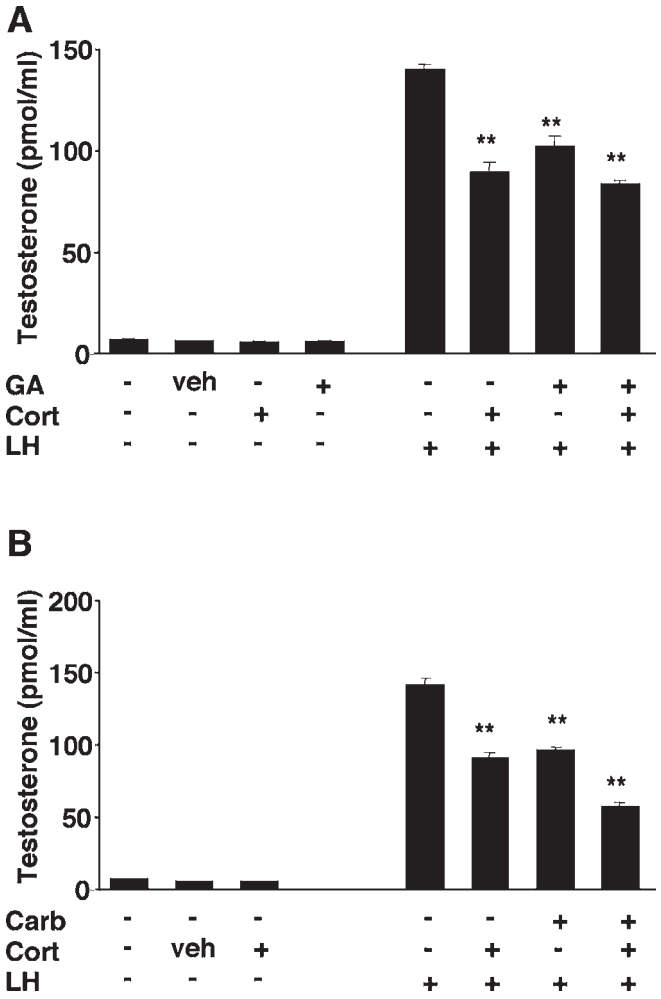

Fig. 3. Effects of 11β-hydroxysteroid inhibitors, glycyrrhetinic (GA) acid and carbenoxolone (Carb), and corticosterone (Cort) on basal and LH-stimulated testosterone release from dispersed testicular cells. Cells were preincubated for 2 h with or without drugs or vehicle (veh) followed by 2 h of incubation as for the preincubation with or without added LH (25 mU/mL). (**A**) Effects of GA (50 n*M*) and Cort (1 μ*M*); (**B**) effects of Carb (10 μ*M*) and Cort (1 μ*M*). Each column represents the mean ± SEM ($n = 6$). **$p < 0.001$ vs LH alone (ANOVA and Tukey-Kramer).

pg/mL). By contrast, $ANXA1_{1–188}$ (200 pg/mL) had no significant effects on basal testosterone production (Fig. 4A). Figure 5 illustrates the expression of ANXA1 protein in mice testes determined by Western blot analysis. In accordance with our earlier studies, two species of ANXA1 with molecular weights of 37 and 34 kDa were detected corresponding to the full-length protein and an N-terminally clipped metabolite *(40)*. Higher molecular weight fragments are probably multimers of ANXA1 *(41,42)*. LH-stimulated testosterone release ($p < 0.001$) was also inhibited by two 11-hydroxysteroid dehydrogenase inhibitors: glycyrrhetinic acid (50 n*M*) (Fig. 3A) and carbenoxolone (10 μ*M*) (Fig. 3B); the effects of the latter but not the former were additive with those of corticosterone (Fig. 3).

IL-1β (200 pg/mL) had no effect on basal testosterone secretion but produced a marked decrease in the response to LH ($p < 0.01$) (Fig. 2B), which was blocked by indomethacin (10 μ*M*), a nonselective cyclooxygenase (COX) inhibitor. The inhibitory effects of IL-1β on LH-stimulated testosterone release were additive with those of DEX (0.01 μ*M*) and $ANXA1_{1–188}$ (200 pg/mL) (Fig. 4B).

Discussion

These results confirm reports that glucocorticoids and IL-1β inhibit LH-stimulated testosterone release from mouse testicular cells and indicate that their effects are additive. They also show, for the first time, that the effects of the ste-

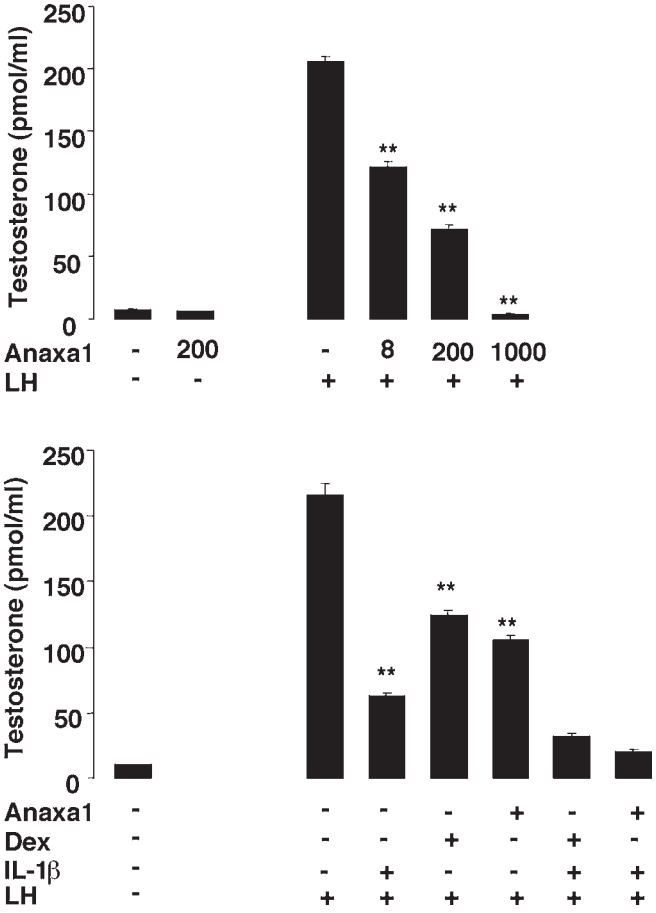

Fig. 4. Effects of IL-1β, DEX, and ANXA1$_{1–188}$ (ANXA1) on basal and LH-stimulated testosterone release from dispersed testicular cells. (**A**) Effects of graded concentration of ANAX1; (**B**) effects of IL-1β (200 pg/mL), DEX (0.01 μ*M*), and ANXA1 (200 pg/mL). Cells were preincubated for 2 h with or without drugs followed by 2 h of incubation as for the preincubation with or without added LH (25 mU/mL). Each column represents the mean ± SEM ($n = 6$). $^{**}p < 0.001$ vs LH alone (ANOVA and Tukey-Kramer).

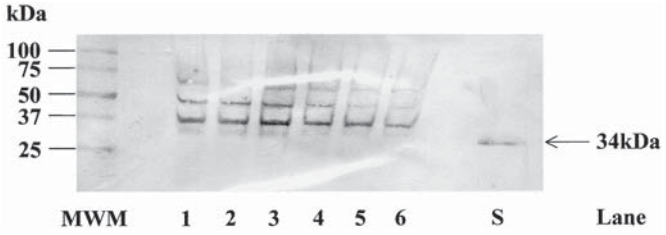

Fig. 5. Expression of ANXA1$_{1–188}$ (ANXA1) in testis by Western blot analysis. Lanes 1–6, expression in tissue extracts from six individual animals; lane MWM, molecular weight markers; lane S, human ANXA1 standard.

roids are mimicked by annexin 1 and that, in contrast to their mutually antagonistic effects in the neuroendocrine and host defense systems *(1,23–25)*, IL-1β and annexin 1 exert additive actions in the testis.

Our finding that treatment of mice prior to autopsy with DEX (20 mg/mL in drinking water, 2.5 d) reduced the ability of testicular cells to secrete testosterone in vitro in response to LH and forskolin confirms reports that elevations in circulating glucocorticoids impair testicular function *(4,8,43)*. The complementary observation that exposure of dispersed testicular cells to DEX or corticosterone in vitro reduces the evoked release of testosterone provides direct evidence for an action of the steroids within the testis; it thus accords with earlier reports based on studies on purified and cultured mouse Leydig cells *(20,44)* and on Leydig cells from other species *(8,43,45)*.

Reports that the glucocorticoid receptor is expressed in the Leydig cells *(7,8)* and that the glucocorticoid receptor antagonist mifepristone (RU 486) reverses the inhibitory effects of glucocorticoids on LH-induced testosterone production *(8)* suggests that the effects of the steroids are mediated via the glucocorticoid receptor. Several researchers have suggested that the events downstream of the glucocorticoid receptor involve suppression of the LH-induced transcription of steroidogenic enzymes, in particular cholesterol side-chain cleavage enzyme (P450scc) and 3βHSD *(20,21)*, which are key regulators of testosterone synthesis, and also of 17α-hydroxylase/C17-20 lyase (P450c17) *(19)*. Others have proposed that glucocorticoids downregulate the LH receptor *(43)*. It seems unlikely that the latter mechanism would be effective within the time frame of our in vitro study. Moreover, our finding that in vivo glucocorticoid treatment impaired the steroidogenic responses to both LH and forskolin suggests the principal target for the steroid is downstream of the LH receptor. The demonstration that excessive exposure to glucocorticoids can induce apoptosis of rat Leydig cells *(46)* and thus potentially contribute to the suppression of testosterone levels when glucocorticoid concentrations are raised suggests an alternative mechanism of glucocorticoid action, which clearly requires investigation using mouse tissue.

In the present study we have demonstrated, for the first time, in the mouse testis, expression of annexin 1, a protein that contributes to the regulatory actions of glucocorticoids in the host defense and neuroendocrine systems. We also have shown, for the first time, that annexin 1 effectively blocks LH-induced testosterone secretion and consequently mimics the actions of glucocorticoids. The protein was very highly potent in this regard and thus effective at low concentrations, comparable with those that suppress the release of corticotropic-releasing hormone and adrenocorticotropic hormone from the hypothalamus and pituitary gland, respectively *(40,47)*. Taken together, these data raise the possibility that locally produced annexin 1 plays a significant role in the regulation of testosterone secretion, possibly serving as a mediator of glucocorticoid action, as it does in other tissues *(22)*.

Interestingly, the inhibitory actions of corticosterone in our in vitro system were mimicked by 11DHC, the biologically inert metabolite of corticosterone. This response may have been consequent on the generation of corticosterone from the inert steroid and thus concurs with reports in the rat that the 11βHSD enzyme(s) in Leydig cells and liver show predominant reductase activity *(17,48,49)* and that the type 2 enzyme, which exhibits only dehydrogenase activity, is not expressed in the rat testis *(17)*. However, other data suggest that the directional activity of 11βHSD1, which is expressed relative weakly in the murine testis *(50,51)*, is sensitive to the steroid *milieu*. Thus, high levels of testosterone promote the reductase activity and augment the inhibitory actions of glucocorticoids on steroidogenesis; by contrast, high levels of corticosterone promote the dehydrogenase activity and thereby reduce the bioavailability of the steroid within the cell *(16)*. Such a mechanism could account for the ability of carbenoxolone or glycyrrhetinic acid to augment the inhibitory actions of corticosterone on testosterone secretion since the exogenous steroid would favor dehydrogenase activity. However, it would not explain our finding that both enzyme inhibitors suppressed steroidogenesis in the absence of exogenous glucocorticoids. Similar actions of carbenoxolone have been reported previously *(17,52)*, but the underlying mechanisms have not been explained. A direct action on the glucocorticoid receptor seems unlikely because, although carbenoxolone binds to the receptor, its affinity is very low, some 20,000–30,000 times less than that of DEX *(52)*. Moreover, our demonstration that the effects of corticosterone and carbenoxolone are additive suggests that the two drugs work via different mechanisms. Other possible explanations included blockade of steroidogenic enzymes and inhibition of prostaglandin breakdown, which, as discussed subsequently, would be expected to impair testosterone production *(53)*.

IL-1β is produced locally in the testis, both by the resident macrophage population in the interstitial compartment *(39)* and by blood leukocytes that appear in the microvasculature after an immune challenge *(54)*, and these cells may make a significant contribution to the rise in testicular IL-1β induced by systemic administration of endotoxin. Our data confirm reports *(35,37)* that IL-1β suppresses LH-induced testosterone secretion. They therefore support the premise that locally produced IL-1β may contribute to the fall in testosterone production associated with stressors that threaten the host defense system, such as infections. Synergistic actions of glucocorticoids and IL-1β have been described in other systems *(55,56)* and attributed to upregulation of cytokine receptors by the steroids *(57)* and/or cytokine regulation of 11βHSD *(58)*. However, such actions contrast markedly with the effects observed in the host defense system in which glucocorticoids and annexin 1 oppose the actions of proinflammatory cytokines released in response to an immune insult *(2,59)*. How glucocorticoids and annexin 1 act in concert with IL-1β in the testis remains to be explained. There are data to suggest that, like glucocorticoids, IL-1β inhibits LH/human chorionic gonadotropin–stimulated cyclic adenosine monophosphate formation *(35,60)* and the downstream expression of P450scc and P450c17 expression in adult murine Leydig cells *(34,61)*. However, the present data, which show that the effects of IL-1β are blocked by the nonselective COX inhibitor indomethacin advocate a role for prostaglandins, which have been shown by others to be powerful inhibitors of testosterone production *(35,62)*. That such actions should be enhanced by glucocorticoids and annexin 1 is surprising on two counts. First, glucocorticoids have been shown to inhibit the activity of phospholipase A_2 via an annexin 1–dependent mechanism *(22)* and, thus, to prevent the liberation of the eicosanoid precursor, arachidonic acid, from membrane phospholipids. Second, glucocorticoids suppress the cytokine-driven expression of the inducible form of COX (COX-2), the enzyme that plays a key role in the generation of prostaglandins from arachidonic acid in conditions of immune insults *(63)*.

In conclusion, our study shows, for the first time, that the inhibitory effects of glucocorticoids on testosterone secretion are mimicked by annexin 1. It also provides novel evidence that, in contrast to their actions in the neuroendocrine and host defense system, glucocorticoids and annexin 1 appear to act in concert with IL-1β in the testis. While the mechanisms underlying this complementary activity are obscure, it seems likely that the phenomenon is indicative of a mechanism through which testicular secretory activity is reduced until the termination of an immune insult. It may therefore contribute to the etiology of the infertility induced by stressors that threaten the host defense system.

Materials and Methods

Drugs

DEX sodium phosphate (David Bull Laboratories, Warwick, UK), forskolin, indomethacin, carbenoxolone, corticosterone, 18β-glycyrrhetinic acid, and 11DHC (Sigma), LH (LH-S1 standard for bioassay; Sigma), rat recombinant (IL-1β) (R & D Systems Europe), and recombinant human $ANXA1_{1–188}$ (a gift from Dr. Frank Carey, AstraZeneca) were used. Indomethacin, 11-DHC, corticosterone, and forskolin were initially dissolved in small amounts of ethanol and subsequently diluted in EBSS. The final concentration of ethanol never exceeded 0.3%, and appropriate vehicle controls were included in all experiments.

Animals and Drug Treatments

Adult male Tuck Ordinary (in vitro studies) or Balb/c (in vivo treatment) mice (8–11 wk old) were housed eight per cage in a quiet room with controlled lighting (lights on 8:00 AM to 8:00 PM), humidity (50%), and temperature (21–23ºC) for at least 7 d prior to the experiment. Food and water were available ad libitum. Experiments were started between 8.00 AM to 10:00 AM to avoid changes associated with the

circadian rhythm. Where appropriate, Balb/c mice were pretreated with DEX (20 mg/L in drinking water) for 2.5 d prior to autopsy; control animals received normal tap water. The study was carried out under license in accordance with the UK Scientific Procedures (Animals) Act (1986).

In Vitro Experiments

Static Incubation of Dispersed Testicular Cells

The preparation used was a modification of the method developed by Janszen et al. *(64)*. Briefly, the mice were killed by cervical dislocation. The testes were decapsulated and dispersed mechanically in Earle's balanced salt solution (EBSS) (pH 7.4, phenol red free; Sigma, Poole, Dorset, UK), after which the seminiferous tubules were allowed to settle. The medium containing mainly Leydig cells was harvested and the dispersion procedure repeated with the remaining tubules. The harvested cells were filtered using 100-µm gauze to remove any remaining seminiferous tubules. The resulting cell suspension was centrifuged (100*g*, 10 min), resuspended in 10 mL of EBSS, and the centrifugation repeated. The pellet was resuspended in 4 mL of EBSS and an aliquot taken for a cell count using a hemocytometer. Cell viability was assessed by trypan blue exclusion and was always >90%. The cells were then plated out in 96-well cell culture plates (Costar, Cambridge, MA) and preincubated for 2 h at a concentration of 10^6 cells/mL of EBSS, in the presence or absence of drugs. Cells were maintained at 34ºC in a humid atmosphere of 95% O_2/5% CO_2. The plates were centrifuged (2000*g*, 10 s), the supernatants discarded, and the cells incu-bated for a further 2 h, as before, in the presence of a sub-maximal dose of LH (25 mU/mL) (LH-S1 standard for bioassay; Sigma) or vehicle. At the end of the incubation period, cells were centrifuged (2000*g*, 10 s) and the supernatant fluid was collected and either assayed immmediately for immonoreactive testosterone or stored at –20ºC for subsequent testosterone measurement.

Radioimmunoassay of Testosterone

Testosterone released in vitro was measured by a modification of the radioimmunoassay method of Collins et al. *(65)*, using a well-characterized antiserum raised in rabbits against testosterone-2-oxime-bovine serum albumin (Guildhay, Surrey) and ^{3}H-labeled testosterone (Amersham International PLC, UK). Separation of bound and free steroid was achieved by the addition of dextran-coated charcoal. The inter- and intraassay coefficients of variation were 10.2 and 5.8%, respectively. Dilution curves of the samples were parallel to those of the standard curve, and samples were run in the same assay to avoid interassay variation.

Detection of Annexin 1 by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Western Blot Analysis

The method employed is described in detail elsewhere *(40)*. The protein content of the testicular extracts was estimated according to Bradford *(66)*. The proteins contained within the tissue extracts were separated by electrophoresis following application to sodium dodecyl sulfate (SDS)-polyacrylamide gels consisting of 10% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 0.001% (w/v) SDS, 0.1 *M* Tris/bicine, 2% (w/v) ammonium persulfate, and 0.1% (w/v) TEMED (Sigma). The separated proteins were transferred electrophoretically to nitrocellulose paper (Amersham Pharmacia, UK) and incubated overnight at 4ºC with a well-characterized anti-ANXA1 polyclonal antibody (anti-ANXA1 pAb, coded S0, diluted 1:5000 in 50 mL of phosphate-buffered saline [PBS]-Tween-20), which was raised in-house in sheep against full-length human recombinant ANXA1 and does not crossreact with other annexins *(27,31)*. Deposition of the antibody was visualized by a second antibody method involving sequential incubation at 4ºC with a peroxidase-conjugated donkey antisheep antibody (diluted 1:5000 in PBS-Tween-20, 2 h) (Sigma) and 50 mL of diaminobenzidine (0.05% [w/v] in PBS-Tween-20) to which 20 µL of 30% (v/v) hydrogen peroxide was added just before use. The molecular weights of the bands of immunoreactive ANXA1 were determined by comparison with the migration of molecular weight markers (Rainbow molecular weight markers, Amersham) and ANXA1 standard (human recombinant, *Escherichia coli* derived) *(67)*. The blots were scanned using a Fujix-Bax 1500 imaging system with a low-level light-sensitive camera (Raytek, Germany).

Statistical Analyses

The results, which were normally distributed, were analyzed using ANOVA followed by Tukey-Kramer multiple comparison tests. Replicate experiments gave a similar profile of data. However, as the result of variation between experiments, statistical comparisons were made only within experiments and differences were considered to be significant when $p < 0.05$.

Acknowledgment

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