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Rapid Glucocorticoid Mediation of Suppressed Testosterone Biosynthesis in Male Mice Subjected to Immobilization Stress

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ABSTRACT: Physical and psychosocial stress challenge homeostasis, increasing glucocorticoid secretion (in rodents, corticosterone [CORT]) while decreasing testosterone (T) levels. The dynamics of stress-induced changes in T, CORT, and luteinizing hormone (LH) concentrations in mice have not been investigated previously. In particular, it remains to be established whether there is a rapid effect of CORT that is directly mediated by glucocorticoid receptors (GRs) in the testis. Therefore, serum and intratesticular T, serum CORT, and LH levels were measured during acute immobilization (IMO) stress, using the C57BL/6 strain of mice. The effects of testicular GR blockade were investigated by administration of the GR antagonist, RU486, via intratesticular (IT) or intraperitoneal (IP) injection. CORT levels increased in stressed males starting at 15 minutes, reaching a fivefold higher plateau by 1 hour compared with controls ($P < .01$). Conversely, starting from 30 minutes on, both serum and intratesticular T levels decreased in stressed males to 30% and 8% of control values, respectively, by 6 hours ($P < .01$). In contrast, LH was unchanged by IMO stress for up to 6 hours. Intratesticular treatment with RU486 partially prevented the IMO-induced decline in T levels. CORT treatment reduced intracellular cyclic adenosine monophosphate (cAMP) content in Leydig cells by 15 minutes and T production by 30 minutes in vitro. We conclude that 1) the rapid changes in T suggest a suppression of T biosynthesis by glucocorticoid through a nongenomic mechanism, lowering the production of cytoplasmic cAMP; 2) changes in gonadotropic stimulation of Leydig cells are unlikely to explain the suppression of T levels during acute stress; and 3) the results are consistent with a direct inhibitory action of CORT on Leydig cells.

Key words: Leydig cell, cyclic AMP, glucocorticoid receptor.
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Stress is defined as a disruption of homeostasis (Rivier and Rivest, 1991), and stimuli that challenge homeostasis are designated as stressors. Stressors can be divided into 3 general categories (Pacak et al, 1998; Van de Kar and Blair, 1999; Tilbrook et al, 2000): 1) physical (for example, restraint, foot shock, and exercise); 2) psychosocial, including isolation, anxiety, fear, or mental frustration; and 3) metabolic, including upright tilt, heat exposure, hypoglycemia, and hemorrhage. Stress has been further subdivided based on duration: acute (single, intermittent, and time-limited exposures) and chronic (intermittent-and-prolonged or continuous exposures). Stressors used in research are often of a mixed type. For example, immobilization stress is a mixture of physical and psychological stressors, restricting movement and isolating the individual from its group (Pacak and Palkovits, 2001).

During stress, an adaptive response originating in the hypothalamus-pituitary-adrenal (HPA) axis is activated to sustain homeostasis (Moberg, 1987; Xiao et al, 1999). The adaptive response alters the secretion of corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH), and luteinizing hormone (LH), as well as adrenal corticosteroids (Moberg, 1987; Rivier and Rivest, 1991). Stress from a variety of stimuli exerts a profound suppression of the reproductive axis (Brann and Mahesh, 1991; Rivier and Rivest, 1991; Chrousos and Gold, 1992; Tilbrook et al, 2000). In males, decreased serum testosterone (T) is one of the first signs of stress (Fenster et al, 1997), and a sharp rise in serum glucocorticoid levels is viewed as a causative factor in the decline of steroidogenesis (Orr and Mann, 1990; Monder et al, 1994b; Gao et al, 1996). Luteinizing hormone (LH), the main tropic stimulus of T production in Leydig cells, may be unchanged (Collu et al, 1979; Charpenet et al, 1981; Srivastava et al, 1993) or lower (Demura et al, 1989; Lopez-Calderon et al, 1991), depending on the duration of the stress being investigated. In rats, acute immobilization (IMO) stress lowers T concentrations primarily at the testicular level with unchanged LH secretion, while chronic IMO stress has inhibitory effects on the hypothalamic-pituitary level and, by lowering serum LH release, decreases serum T concentrations (Maric et al, 1996).

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Table 1. *Design of the glucocorticoid receptor blockade experiment*

	Group						
	1	2	3	4	5	6	7
Stress	None	No	No	IMO*	IMO	IMO	IMO
Injection	None	IT†	IP‡	IT	IP	IT	IP
Agent	None	V§	V	V	V	RU‖	RU
RU486, μg	...	...	...	...	...	16¶	16
Volume, μL	...	10#	100	20	100	20	100

* Immobilization for 3 hours.
† IT, intratesticular.
‡ IP, intraperitoneal.
§ V, vehicle.
‖ RU, RU486.
¶ Sixteen micrograms in 20 μL per mouse via IT injection.
# Eight micrograms in 10 μL per testis via IT injection.

The aims of this study were to define hormonal profiles under acute IMO stress in mice and to elucidate the mechanisms for stress-induced declines in T levels. Since glucocorticoid is thought to inhibit Leydig cell function through a GR-mediated pathway (Orr and Mann, 1992; Monder et al, 1994b; Gao et al, 1996; McEwen, 2000), we employed RU486 (mifepristone), a GR antagonist (Cadepond et al, 1997), to evaluate the effects of GR blockade at the testicular level. We asked whether rapid declines in androgen production are caused by reduced gonadotropic stimulation of Leydig cells by LH, as opposed to increased glucocorticoid activity. The results support the hypothesis that stress-induced increases in serum glucocorticoid levels directly inhibit Leydig cell function and implicate a rapid nongenomic pathway of glucocorticoid action.

Materials and Methods

Animals

Adult male mice C57BL/6 (25–30 g body weight) were purchased from Charles River Laboratories (Wilmington, Mass) and housed 5 per cage under controlled environmental conditions (temperature 22°C ± 2°C; 12:12 hour light:dark, with lights on from 0600 to 1800 hours). All animals were handled to become adapted for at least 3 days prior to the beginning of the experiment. The animal protocol was approved by the Animal Care and Use Committee of Rockefeller University (protocol number 03-048, renewed September 25, 2003).

Immobilization Stress

The animals were placed in wire mesh restrainers (4 × 9 cm in dimension) as described by McEwen and colleagues (McEwen and Sapolsky, 1995). The procedure effectively restricted movement. The start of IMO stress began at 10 AM and the treatment durations were 15 and 30 minutes and 1, 3, and 6 hours (n = 5 per time point). Control animals were left undisturbed in their cages for the duration of the experiment and sampled at the same time points (n = 5 per group). At the end of each stress period, trunk blood was collected after decapitation in tubes containing heparin and centrifuged at 500 × *g* and the sera were stored at −20°C until assay. Testes were removed and stored at −70°C. The overall design was replicated 4 times.

GR Blockade

To investigate the involvement of GRs in the glucocorticoid-induced T decrease during IMO, RU486 [17β-hydroxy-11β-(4-dimethy-aminophenyl-1)-17α-(1-prop-1-ynyl)-oestra-4, 9-diene-3-one, Roussel, UCLAF, France] was administered in vivo either by IT or IP injection using a 29½ gauge syringe needle (Baulieu, 1994) prior to the stress session. Details of the design for this experiment are presented in Table 1. The dose of RU486, 16 μg, was selected on the basis of a previous study conducted in rats (Orr and Mann, 1992). RU486 was first dissolved in absolute ethanol and subsequently diluted with the vehicle, 45% aqueous 2-hydroxypropyl-β-cyclodextrin (catalog number 0926, Sigma Chemical Co, St Louis, Mo), to attain the needed concentrations (the final concentration of ethanol was 0.8%) (Roozendaal et al, 2002). Animals (n = 5 per group) were subjected to IMO stress for 3 hours as described above. At the end of the stress period, animals were sacrificed by decapitation and trunk blood was collected for hormonal assays. Leydig cells were harvested for direct measurement of steroidogenesis in vitro after IT treatment with RU486 in vivo. The cells were isolated, respectively, from untreated control, IT vehicle, IMO plus IT RU486, and IMO plus IT vehicle groups, and then (1×10^4/mL) incubated in a microcentrifuge tube at 34°C with 100 ng LH in Dulbecco modified Eagle medium (DMEM):F12 culture medium for 3 hours. Spent media were collected for assay of testosterone concentrations. The overall design was replicated 4 times.

cAMP Measurement

The ability of Leydig cells to produce cyclic adenosine monophosphate (cAMP) in response to elevated glucocorticoid during IMO stress was measured. Briefly, purified Leydig cells (1×10^5/200 μL) were preincubated in 96-well Falcon culture plates (Becton Dickinson and Co, Franklin Lakes, NJ) under 5% CO_2: 5% O_2: 90% N_2 34°C air for 2 hours. The medium was then carefully removed, and 200 μL fresh phenol-red-free DME/F12 medium was added, buffered with 15 mM HEPES and 26 mM sodium bicarbonate (Leydig cell culture medium [LCM]) and

containing a 1.44 μM final concentration of corticosterone (CORT) (500 ng/mL) that is achieved during stressful conditions in vivo. The cells were incubated with CORT for 0–60 minutes. After incubation with CORT, media were moved and cells were then incubated in 200 μL fresh phenol-red-free LCM with LH 100 ng/mL for 20 minutes. At the end of the incubation, 50 μL TET buffer (0.05 M Tris, 4 mM EDTA) was added immediately to the plates. The preparations were frozen in liquid N_2 and kept at −80°C until cAMP and T assay. cAMP assay was assayed with a kit (catalog TRK432, Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's directions. The sensitivity of the assay was 0.05 pmol per assay tube.

Leydig Cell Purification

Leydig cells were purified by the procedure of Klinefelter et al (Klinefelter et al, 1987) with modifications. In brief, testes were removed, decapsulated, and dispersed in 10 mL of medium 199 (M-199) with 0.25 mg/mL collagenase in a shaking water bath at 34°C for 10 minutes. To terminate collagenase dispersion, 1% bovine serum albumin (BSA) M-199 buffered with 15 mM HEPES and 4 mM sodium bicarbonate plus 25 μg/mL soybean trypsin inhibitor (SBTI) was added to dilute the original suspension 1:5. Tubes were then capped and inverted several times. Seminiferous tubules were allowed to settle, and the supernatant containing the interstitial cells was collected by aspiration. The tubes containing the settled seminiferous tubules were refilled with 1% BSA, and the procedure was repeated several times to further harvest the interstitial cells. The cells were pelleted in 250-mL tubes by centrifugation at 800 × *g* for 20 minutes at 4°C and then fractionated using a continuous Percoll gradient (55% Percoll in Hanks balanced salt solution [HBSS] buffered with 15 mM HEPES and SBTI) in a total volume of 35 mL. The gradients were formed in situ by centrifugation in a Beckman JA-20 fixed angle rotor at 22 045 × *g* for 30 minutes at 10°C. A tube containing density marker beads and 35 mL of 55% Percoll solution (Sigma) was used as a reference. Leydig cells were recovered starting at a density of 1.07 gm/mL to the top of the red blood cell layer. HBSS was added to dilute the Percoll in the resulting cell suspension, and the cells were pelleted at 200 × *g* for 10 minutes at 4°C. The cell pellets were then resuspended in 2–4 mL of 1% BSA buffered HBSS and gently layered on top of a discontinuous BSA gradient, containing 15 mL each of 10%, 5%, and 2.5% BSA buffered HBSS. The gradient was centrifuged at 60 × *g* for 10 minutes at 4°C. Supernatants were aspirated until 15 mL of cell suspension remained at the bottom of the tube. The bottom BSA layer was transferred to another tube and diluted with HBSS by 1:3. After centrifugation at 200 × *g* for 10 minutes at 4°C, the final pellet was resuspended in LCM. Cells were counted in a hemacytometer (Hausser Scientific, Horsam, Pa). The Leydig cell purity was approximately 97%, as determined by histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) using 0.4 mM etiocholan-3β-ol-17-one as the enzyme substrate (Payne et al, 1980). As expressed in Table 2, fractions of Leydig cells isolated by this method were comparable to cells obtained by the earlier unmodified procedure that incorporates an elutriation step (Klinefelter et al, 1987).

Table 2. *Properties of mouse Leydig cells isolated by 2 separate methods*

	BSA*	ELUT†
Purity, %‡	97.2 ± 0.3	95.4 ± 1.7
Yield§	0.08 ± 0.02	0.06 ± 0.02
T production‖	$3.9 \times 10^3 \pm 91$	$3.5 \times 10^3 \pm 71$

* BSA, Percoll bovine serum albumin method.
† ELUT, elutriator purified (Klinefelter et al, 1987).
‡ Purity is expressed as a percentage of 3β-hydroxysteroid dehydrogenase positive cells.
§ Yield is expressed as $\times 10^6$ Leydig cells per testis.
‖ Testosterone production is expressed as ng per 10^6 cells with 3 hours of luteinizing hormone stimulation.

Intratesticular Testosterone

Intratesticular testosterone concentration was measured using the method of Knorr et al (Knorr et al, 1970). In brief, testes were homogenized in 5 mL of 70% methanol using a glass-glass homogenizer. The homogenates were transferred to 15-mL screw cap test tubes. Tracer steroid (1000 counts per minute [cpm] of tritiated testosterone) was added to the homogenate to correct for recovery. The homogenates were left standing overnight at room temperature. The tubes were centrifuged at 1800 × *g*, and the supernatant was aspirated and dried under nitrogen to remove the methanol. The water layer was extracted twice with high-performance liquid chromatography–grade diethylether. The ether extracts were then resuspended in 400 μL of radioimmunoassay (RIA) buffer, and 100 μL was removed for measurement of recovery (the cpm value in 100 μL × 4 ÷ 1000). The remaining 300 μL was used for RIA.

Serum T, CORT, and LH Concentrations, and Measurement of T Production

Serum T concentrations were measured using a tritium-based RIA as previously described (Cochran et al, 1981). Serum CORT was measured by the RIA procedure of Spencer et al (Spencer et al, 1996), with an anti-CORT antiserum B3-163 (Endocrine Sciences, Calabasas, Calif). Serum LH concentrations were assayed by the method of Chandrashekar and Bartke (Chandrashekar and Bartke, 1988) using rat LH standards, NIDDK-r-LH-19; LH antibody and National Institute of Diabetes & Digestive & Kidney Disease-anti-rLH-S-11 from the National Hormone and Pituitary Program; and IgG antiserum (ICN Pharmaceuticals, Costal Mesa, Calif). Radioactive ^{125}I-rat LH was produced using the Iodogen method (catalog 28601, Pierce Chemical Co, Rockford, Ill). Values for interassay variation of the T, LH, and CORT RIAs were between 4% and 8%. The sensitivities of the assays for LH, CORT, and T were 0.12 ng/mL, 10 ng/mL, and 10 pg/mL, respectively.

Statistics

Data were expressed as the mean plus or minus standard error of mean. Statistical evaluation of serum and testis parameters was performed by 2-way analysis of variance (ANOVA) with time and treatment as the subclasses. Student-Newman-Keuls multiple comparisons testing was applied to identify significant differences between groups. The Dunn-Sidak method was used

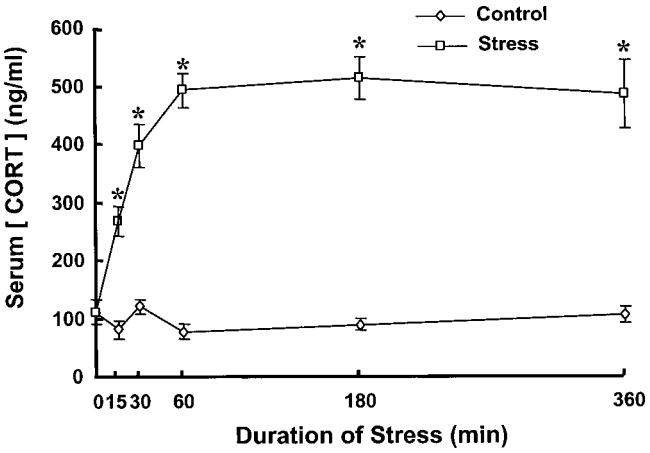

Figure 1. Corticosterone response to immobilization stress in mice. Data are expressed as means plus or minus standard error of means of 4 experiments (n = 15–20). *Significant difference between the control and stress groups ($P < .01$).

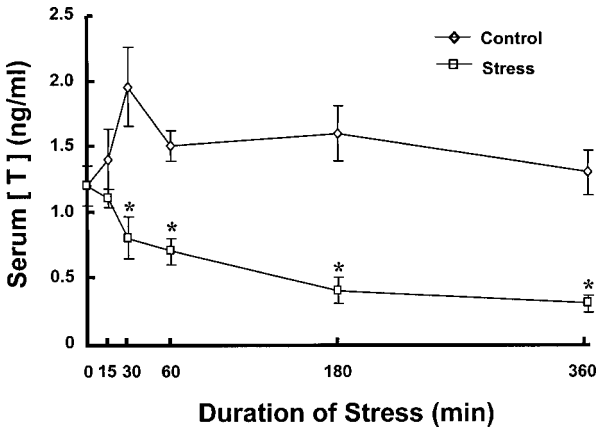

Figure 2. Effects of immobilization on serum testosterone concentrations. Data are expressed as the mean plus or minus standard error of means of 4 experiments (n = 15–20). *Significant difference between the control and stress groups ($P < .01$).

to calculate an experiment-wise error rate for the Leydig cell T production in vitro after treatment of RU486 in vivo (Rohlf, 1995). All calculations were performed using a software package (InStat, San Diego, Calif). Differences were regarded as significant at $P < .05$.

Results

Based on the knowledge of probable physiological fluctuation in serum and testis hormone levels, 2 variables, both treatment of stress and time, were included in the statistical analysis using 2-way ANOVA.

CORT

The increase in CORT levels in stressed males was significant by 15 minutes, and then a plateau was reached by 1 hour (492 ± 30 ng/mL) that was fivefold higher compared with its correspondence control (n = 20 animals/time point, $P < .01$, Figure 1). Significant effects of time and interaction between time and treatment subclasses were detected by the 2-way ANOVA. This indicated that CORT levels in control and stress males varied distinctly as a function of time.

Testosterone

Both serum (Figure 2) and testicular (Figure 3) T concentrations were lower starting at 30 minutes in IMO-stressed males and decreased further to 30% and 8% of control values, respectively, by 6 hours (n = 20 animals/time point, $P < .01$). Significant effects of time and interaction were again recorded by the 2-way ANOVA, suggestive of suppressed baseline variability of testosterone parameters over time in the stressed group.

LH

In contrast to the changes in T and CORT concentrations, serum LH levels were unchanged by IMO stress (n = 20 animals/time point, $P > .05$) (Figure 4). This implied that decreased gonadotropic stimulation of Leydig cells was unlikely to explain the suppression of T levels during IMO stress.

Testicular GR Blockade by RU486

To test the direct inhibitory action of glucocorticoid on T production, RU486 was administered to the stressed animals with a dose of 16 μg. Serum CORT levels in all stressed groups went up to 564 ± 23 ng/mL, compared with 110 ± 14 ng/mL in unstressed controls (n = 20 animals per group, $P < .01$), irrespective of the agent delivered (RU486 or vehicle) or route of injection (systemic or intratesticular, data not shown). This indicated that administration of RU486 did not block the systemic increases in serum CORT induced by IMO stress. The

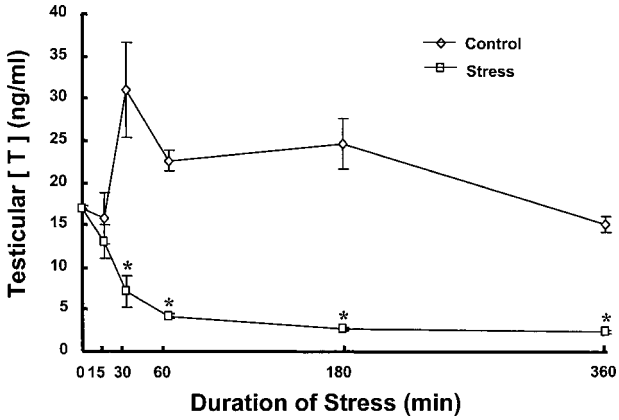

Figure 3. Effect of immobilization on testicular testosterone concentrations. Data are expressed as means plus or minus standard error of means of 4 experiments (n = 15–20). *Significant difference between the control and stress groups ($P < .01$).

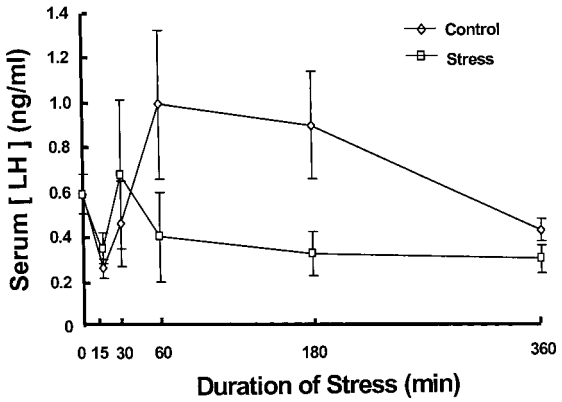

Figure 4. Effects of immobilization on serum luteinizing hormone levels. Data are expressed as means plus or minus standard error of means of 4 experiments (n = 15–20). There were no significant differences among the groups.

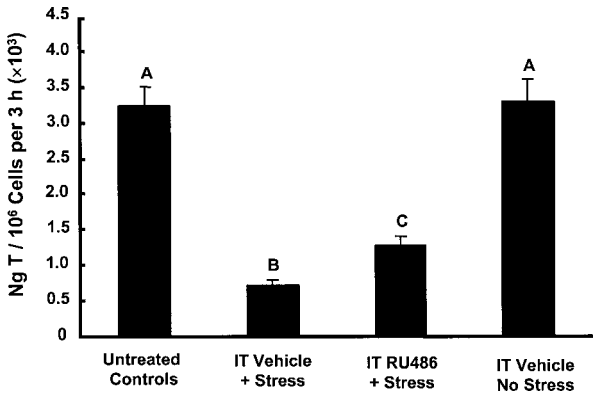

Figure 6. Leydig cell testosterone production in vitro after administration of RU486 in vivo. Shared letters designate groups that were not significantly different at $P \leq .05$. Although testosterone levels in the IT RU486 (16 μg dose) group were lower compared with unstressed controls ($P \leq .05$), they were significantly higher than the intratesticular vehicle plus immobilization group ($P \leq .05$). This indicated that the suppression was partly blocked by RU486.

average serum T level in all unstressed animals (including the untreated and vehicle injection groups) was 0.50 ± 0.3 ng/mL. T levels among these 3 groups were equivalent, implying that the injection itself did not disturb hormone levels. IMO stress decreased serum T concentrations in groups receiving either IT or IP injection of vehicle to 0.09 ± 0.01 ng/mL, about 20% of the value in unstressed animals (n = 20 animals per group, $P < .01$, Figure 5). Testicular administration of RU486 increased serum T levels in stressed animals to 0.33 ± 0.02 ng/mL compared with vehicle-injected controls (n = 20 animals per group, $P < .01$). In contrast, T levels in animals that received systemic IP injections were unchanged relative to vehicle-treated controls. LH-stimulated T production (× 10^3 ng/mL) by Leydig cells in vitro, after IT administration of RU486 and IMO stress in vivo, was lower compared with unstressed controls (3.20 ± 0.50 ng/mL) but higher compared with the IT vehicle plus IMO group (1.40 ± 0.20 ng/mL vs 0.70 ± 0.10 ng/mL, Figure 6). Taken together, the results indicate that RU486 partially blocked the suppressive effects of CORT on T production and that the blockade was only effective when testicular, rather than systemic.

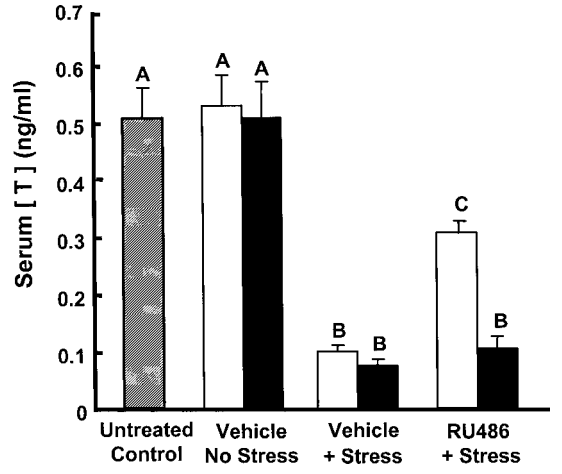

Figure 5. Serum testosterone (T) concentrations after glucocorticoid receptor blockade. The hatched bar represents the untreated control, and open and closed bars refer to intratesticular and intraperitoneal injection, respectively. Shared letters designate groups that were not significantly different at $P \leq .05$. The T levels in unstressed groups (untreated and vehicle-injected groups) were equivalent ($P > .05$). The overall T levels in stressed animals were significantly lower compared with unstressed males, and T values in vehicle-injected controls were decreased by 80% to 0.09 ± 0.008 ng/mL ($P \leq .05$). Administration of RU486 by IT elevated T levels in stressed animals ($P \leq .05$).

cAMP and T Production

cAMP (Figure 7) and T (Figure 8) production were measured using isolated Leydig cells that were cultured with

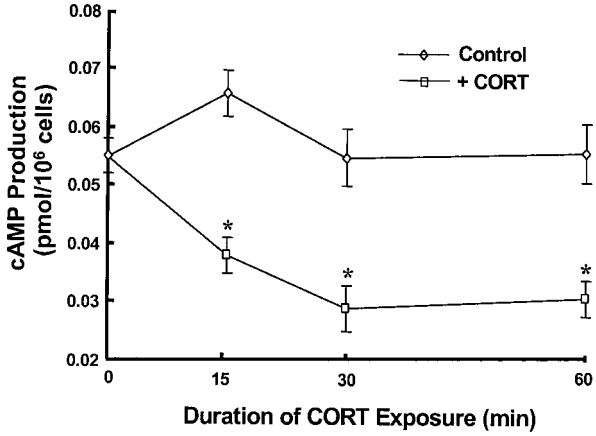

Figure 7. Total cyclic adenosine monophosphate content of Leydig cells from corticosterone (CORT)-treated and non-CORT-treated groups. Cells were incubated with or without CORT (1.44 μM) for 0, 15, 30, and 60 minutes first, and cells were then stimulated with luteinizing hormone (100 ng/mL) for 20 minutes. Data are expressed as means plus or minus standard error of means of 3 experiments. *Significant difference between the 2 groups ($P < .05$).

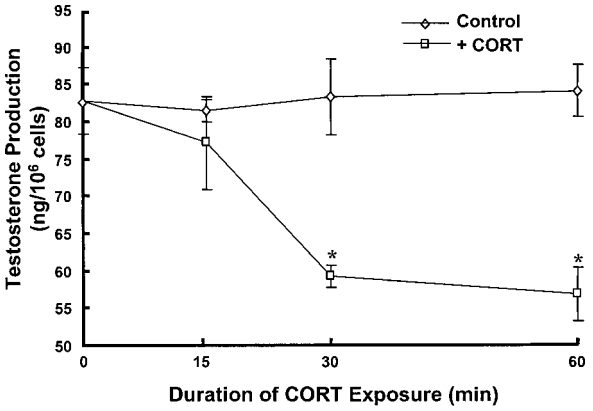

Figure 8. Testosterone production by Leydig cells after treatment with corticosterone in vitro. Cells were incubated with or without corticosterone (1.44 μM) for 0, 15, 30, and 60 minutes first, and cells were then stimulated with luteinizing hormone (100 ng/mL) for 20 minutes. Data are expressed as means plus or minus standard error of means of 3 experiments. *Significant difference between the 2 groups ($P < .05$).

CORT (1.44 μM) for 0, 15, 30, and 60 minutes and then with LH (100 ng/mL) for 20 minutes. cAMP levels in CORT-treated groups were significantly lower relative to control, and rates of T production were also lower, at 30 and 60 minutes. This indicated that rates of cAMP and T production in Leydig cell are rapidly suppressed by a direct action of CORT.

Discussion

In adult mice, IMO stress induced a sharp fall in both serum and testicular T levels relative to unstressed controls. Leydig cells are the main site of testosterone biosynthesis in males and are regulated by the pituitary gonadotropin LH (Dufau, 1988). Under IMO stress, diminished stimulation of the Leydig cell by LH level may ultimately lower T production. However, as observed in the present set of experiments, the declines in serum and testicular T levels associated with stress are not always accompanied by decreases in serum LH concentrations. Orr and colleagues have shown that IMO stress does not affect the binding kinetics of the LH receptor in rat Leydig cells during the period when T production is inhibited (Orr et al, 1994). It is likely, therefore, that IMO stress, as an acute stressor, does not decrease T production by inhibiting LH stimulation of Leydig cells, which agrees with data from other laboratories (Charpenet et al, 1981; Mann and Orr, 1990; Srivastava et al, 1993; Orr et al, 1994). This contrasts with chronic stress where there is evidence for suppression of the HPA axis over a long term (Veldhuis, 1997).

Stress and other conditions that elevate circulating adrenocorticotropin hormone (ACTH) (Gomez et al, 1996) and CORT levels lead to depressed T levels in all known animal species and in men (Armario and Castellanos, 1984; Bernier et al, 1984; Orr and Mann, 1992; Monder et al, 1994b; Gao et al, 1996). The levels of T in circulation are set by the steroidogenic capacity of individual Leydig cells and the total numbers of Leydig cells per testis. Excessive exposure to CORT initiates apoptosis in rat Leydig cells, potentially contributing to suppression of T levels caused by the decline in steroidogenic capacity (Gao et al, 2002). This process first becomes detectable 12 hours after termination of stressor and is thus not considered a complicating factor in the present 6-hour study design. Leydig cells express GRs and are the primary targets of glucocorticoid action in the testis (Ortlip et al, 1981). The GR is a 94-kd ligand-activated intracellular transcriptional regulator belonging to the nuclear receptor superfamily (Mangelsdorf et al, 1995). The GR mediates glucocorticoid action and thereby modulates metabolism of carbohydrates, proteins, and fats; suppression of the immune/inflammatory response; activation of the central nervous system; and regulation of cardiovascular function (Landers and Spelsberg, 1992; Ge et al, 1997). According to a genomic model of action, glucocorticoid enters the cell and binds to the GR, forming a steroid-receptor complex. Following its formation, the complex enters the nucleus and acts as a transcriptional regulator. Transcriptional and posttranscriptional regulation induced by GRs may cause either positive or negative changes in the steady-state levels of specific mRNAs and proteins modulating cell function (Landers and Spelsberg, 1991; Schmid et al, 1995).

In the Leydig cell, following the above genomic model, glucocorticoid is thought to directly inhibit processes critical to the biosynthesis of T by suppressing expression levels of the cholesterol side-chain cleavage enzyme (P450scc), 3β-HSD, and 17α-hydroxylase/17-20-lyase ($P450_{17\alpha}$) (Welsh et al, 1982; Hales and Payne, 1989; Payne and Sha, 1991; Srivastava et al, 1993). In our study, an almost fivefold increase in serum CORT levels was associated with a fall in T levels during IMO stress, whereas LH concentrations were unchanged. The inverse association between glucocorticoid and T levels suggests that CORT causes reductions in T levels during IMO stress.

RU486 (mifepristone), a GR antagonist, is postulated to block the suppressive action of glucocorticoid on T production (Baulieu, 1994). The antiglucocorticoid function of RU486 has been attributed to its high affinity for GR, masking the DNA binding domain of the receptor (Groyer et al, 1987; Lefebvre et al, 1988), although the antagonist can also act at steps subsequent to DNA binding on transcription and/or translation (Guiochon-Mantel et al, 1988; Beck et al, 1993; Edwards et al, 1995). Although the effects of GR blockade on T biosynthesis were analyzed previously in rats (Orr and Mann, 1992; Monder

et al, 1994a), the present study was the first to investigate the effects of a testicular blockade. RU486 was administered to IMO-stressed mice and partially prevented the stress-induced decreases in T levels, indicating that blockade of CORT binding to GRs in Leydig cells suppresses the inhibitory effects of glucocorticoid action. The higher efficiency seen with local, IT administration of RU486 compared with systemic is consistent with a direct action of glucocorticoid on the testis. This was further confirmed by the fact that LH-stimulated T production in Leydig cells purified after IMO stress was higher in groups receiving RU486 by IT injection.

To our knowledge there have been no previous studies addressing the time course of stress-mediated inhibition of T levels. The durations of experimentally imposed stress reported in the literature have ranged from 2 hours to over 10 hours (Bernier et al, 1984; Monder et al, 1994a; Orr et al, 1994; Maric et al, 1996), indicating that a period of hours is required for the operation of genomic events initiated by glucocorticoid action. However, the data reported herein showed that CORT levels were sharply increased as early as 15 minutes, and T levels decreased starting at 30 minutes after imposition of IMO stress. The rapidity of the changes in T levels raises the possibility that suppression of T biosynthesis by glucocorticoid occurs through a nongenomic mechanism, not involving decreased expression of steroidogenic enzymes initially. The nongenomic effects of steroids are characterized by a rapid intracellular response to hormone (often within minutes) that does not require protein synthesis. Such rapid nongenomic effects include the activation of protein kinases, opening of ion channels, induction of phospholipid turnover, and increases in intracellular calcium and cAMP levels (Wehling, 1997). Glucocorticoid is reported to have nongenomic effects (Wehling, 1997; Revelli et al, 1998), which might explain the rapid reduction of T induced by CORT. Increased intracellular production of cAMP is part of the LH signaling transduction pathway leading to a rapid increase in Leydig cell steroidogenetic activity. Therefore, a fast acting CORT-mediated decline in intracellular cAMP may account for decreased T levels during acute IMO stress. In our study, CORT reduced the production of the cAMP in Leydig cells from 15 minutes onward and decreased T production from 30 minutes on after incubation with CORT in vitro. This time course is consistent with the rapid changes in serum T observed during IMO stress in vivo. There is a delay in the decline of T levels with respect to the decrease in cAMP formation after CORT exposure. The temporal ordering of the declines may be significant since cyclic AMP is the second messenger involved in the acute regulation of steroidogenesis. Therefore, these results pointed strongly in the direction of a nongenomic action of glucocorticoid. It is reasonable to infer from the present data that both the genomic and nongenomic modes of glucocorticoid action are applicable to the suppression of T levels during stress. However, our results do not exclude the possibility that other mechanisms contribute to the stress-induced declines in T concentrations. For example, catecholamine, opiates, and/or neuroendocrine pathways may also be involved in T suppression (Rivier and Rivest, 1991; Rivier, 2002).

In summary, IMO stress was found to suppress androgen secretion in mice. LH levels were unchanged, but T levels declined in the presence of elevated serum CORT concentrations. Local intratesticular administration of RU486 partially reversed the IMO stress-induced decrease in T levels, confirming that glucocorticoid and its receptor are involved in steroidogenic suppression. This indicates that glucocorticoid-mediated inhibition of Leydig cell steroidogenesis is a direct action at the testicular level. Suppression of intracellular cAMP levels in Leydig cells is implicated in the rapid response pathway induced by IMO stress.

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