Effect of Immobilization Stress on Plasma Luteinizing Hormone, Testosterone, and Corticosterone Concentrations and on 3β-Hydroxysteroid Dehydrogenase Activity in the Testes of Adult Rats (43658)

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Abstract. **We have examined the effect of 3 hr of immobilization stress on plasma luteinizing hormone, testosterone, and corticosterone levels, and on the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) in microsomal and mitochondrial fractions of the testis from adult rats. Immobilization for 3 hr increased plasma corticosterone and reduced plasma testosterone concentrations by 57%. Plasma luteinizing hormone levels were lower, although not significantly ($P = 0.093$) so, in stressed animals. Immobilization (3 hr) reduced the V_{max} values of 3β-HSD in the mitochondria and in the microsomal fraction of the testis by 40% and 34%, respectively, but had no effect on the K_m values of 3β-HSD in the two cellular compartments. These results suggest that the inhibition of the activity of 3β-HSD may be partially responsible for the disruption of testicular steroidogenesis during immobilization stress.** [P.S.E.B.M. 1993, Vol 204]

The effects of stress on reproduction and the mechanism governing these effects depend on the type of stress, the magnitude and duration of the stress, and the frequency of its application (1). There have been conflicting reports about the effect of stress on the hypothalamic-pituitary-testicular axis. Immobilization or restraint stress in rats consistently lowers plasma testosterone concentrations (2, 3), whereas luteinizing hormone (LH) levels have been reported to be reduced (4, 5), increased (6), or unchanged (2, 3) in these animals. Thus, changes in plasma LH and testosterone concentrations may or may not be correlated during stress.

Immobilization stress does not affect the binding capacity or affinity of LH/human chorionic gonadotropin receptors on Leydig cells (3, 4). It appears that the effect of stress on testicular steroidogenesis occurs, in part, at a postreceptor site, since testicular interstitial cells from stressed rats produce subnormal amounts of testosterone in response to human chorionic gonadotropin, cAMP, or cholera toxin *in vitro* (3, 7).

Restraint stress inhibits the testicular activities of 17α-hydroxylase and 17,20-lyase in adult rats (2, 3). Whether immobilization stress alters the activity of other enzymes in the steroidogenic pathway is unknown. Although it may not be rate limiting, 3β-hydroxysteroid dehydrogenase (3β-HSD) is a key enzyme in the testicular steroidogenic pathway of the adult rat (8). In male rat fetuses, 3β-HSD activity in Leydig cells is closely correlated with steroidogenic activity and testosterone production (9, 10). Also, maternal stress (immobilization and high intensity illumination) is associated with reduced circulating levels of testosterone and 3β-HSD activity in Leydig cells of male fetuses (9). In adult normal and diabetic rats, 3β-HSD activity is closely correlated with plasma testosterone levels (11). The elevated levels of plasma testosterone in adult rats exposed to intermittent foot shock for 60 days was associated with a significant increase in testicular 3β-

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HSD activity (12). However, it is unknown whether the testicular activity of this enzyme is altered by acute immobilization stress.

In the present study, we determined the effect of 3 hr of immobilization stress on the testicular activity of 3β-HSD and correlated any changes in the activity of this enzyme with changes in plasma testosterone concentrations. We examined the effect of immobilization stress on the activity of this enzyme in both mitochondrial and microsomal fractions of the testis, because 3β-HSD activity is known to be present within these subcellular fractions of the ovary (13) and placenta (14).

Materials and Methods

Experimental Animals and Immobilization Protocol. All the experiments were conducted in accordance with the principles and procedures of the *NIH Guide for the Care and Use of Laboratory Animals.* Adult male rats (250–300 g) from Harlan Sprague-Dawley (Indianapolis, IN) were housed two per cage in a temperature (20–23°C)- and light (14:10-hr light:dark)-controlled room. Rats ($n = 7$) were subjected to immobilization stress or left undisturbed (unstressed controls, $n = 7$) for 3 hr beginning at 0600 hr. The immobilization protocol has been described previously (15). Animals were sacrificed by decapitation at the end of the immobilization stress period and trunk blood was collected in tubes containing 10% EDTA in phosphate-buffered saline (pH 7.4). Plasma samples were stored at −80°C until assayed for LH, testosterone, and corticosterone.

Preparation of Testicular Mitochondrial and Microsomal Fractions. Testes of the animals were excised on crushed ice, decapsulated and homogenized in buffer (0.01 *M* potassium phosphate buffer, pH 7.4, containing 0.25 *M* sucrose, 2.0 m*M* EDTA, and 20% glycerol). Homogenates were centrifuged at 1,200*g* for 20 min at 4°C and the pellets were discarded. Supernatants were then centrifuged at 12,000*g* for 20 min at 4°C and the resultant mitochondrial pellets were washed, resuspended, and stored in buffer at −80°C until assayed for 3β-HSD. The supernatants were further centrifuged at 105,000*g* for 60 min at 4°C and the resultant microsomal pellets were washed and stored in buffer at −80°C until assayed for 3β-HSD. Protein content in the microsomal and mitochondrial fractions was determined by the method of Bradford (16) using bovine serum albumin as standard.

Estimation of 3β-HSD Activity. 3β-HSD activity in mitochondrial and microsomal fractions was determined by the 3β-HSD digitonin radioassay procedure as described earlier (17). 3β-HSD activity was estimated in the fractions by the conversion of [7-^{3}H]pregnenolone to [7-^{3}H]progesterone. Digitonin effectively and selectively precipitates unconverted pregnenolone to leave [7-^{3}H]progesterone in the supernatant, as confirmed by thin layer chromatography (17). Incubations were performed for 5 min at 37°C in a shaking water bath. The reaction mixture consisted of 0.2 μCi [^{3}H] pregnenolone, with increasing concentrations of unlabeled pregnenolone (final concentration, 0.25–25 μ*M* pregnenolone) and 0.4 μmol of NAD^+. Reactions were initiated by the addition of 0.9 ml of the microsomal or mitochondrial suspension and terminated at 5 min by placing the tube in an ice bath and then transferring this mixture to a tube containing a solution of ice-cold pregnenolone (carrier, 1 mg). An aliquot of this mixture was then incubated with a 1% digitonin solution overnight at 4°C. Radioactivity in the supernatants was quantified in a liquid scintillation counter. In a preliminary experiment, incubation times of 5 or 10 min gave identical results.

Enzyme activity was plotted against substrate concentration using a curve-fitting computer software (ENZFITTER, Elsevier-Biosoft, Cambridge, UK). V_{max} and K_m values were calculated using Michaelis-Menten kinetics.

Radioimmunoassay. Plasma testosterone levels were measured by radioimmunoassay using a commercial kit obtained from Diagnostic Products Corp. (Los Angeles, CA) as reported earlier (3). All the samples were run in one assay. The minimum level of detection was 0.2 ng/ml. The assay had an intra-assay coefficient of variation of 6.6%.

Plasma corticosterone levels were measured using a commercial kit obtained from ICN Biomedicals, Inc. (Carson, CA) as reported previously (15). The minimum level of detection was 25 ng/ml. The intra-assay coefficient of variation was 3.7%.

Plasma LH levels were determined by the rat LH (rLH) radioimmunoassay kit supplied by National Institute Arthritis, Diabetes, and Digestive and Kidney Diseases (NIADDK) and National Hormone and Pituitary Program (NHPP). The sensitivity of the assay was 43 pg/ml. LH values are reported in terms of NIADDK-rLH-RP-3 standard. The intra-assay coefficient of variation was 8.7%.

Statistics. Plasma concentrations of hormones from control and stressed animals were compared by two-tailed *t* test for independent means. V_{max} and K_m data were analyzed by a 2 × 2 (control versus stressed × microsomal versus mitochondrial fractions) analysis of variance followed by Tukey's test for multiple comparisons between group means. Data are expressed as mean ± SE.

Results

Effect of 3 Hr of Immobilization Stress on Plasma Testosterone, Corticosterone, and LH Concentrations. Immobilization for 3 hr suppressed plasma concentrations of testosterone from a control level of 1.19 ± 0.23 ng/ml to 0.53 ± 0.06 ng/ml in stressed rats (*P*

< 0.05), but plasma LH concentrations were not altered significantly by immobilization stress (110 ± 31 ng/ml in controls vs 61 ± 11 ng/ml in stressed rats; $P = 0.093$).

Plasma corticosterone concentrations in stressed rats (322 ± 61 ng/ml) were greater ($P < 0.05$) than control levels (29 ± 0.3 ng/ml).

Effect of 3 Hr of Immobilization Stress on Testicular 3β-HSD Activity. The activity of 3β-HSD (V_{max} values) in testicular microsomal and mitochondrial fractions from stressed rats was significantly lower than in testicular fractions from control animals (Figs. 1 and 2; microsomal, controls: 1.49 ± 0.06 nmol/min/mg protein vs stressed: 0.99 ± 0.03 nmol/min/mg protein, $P < 0.01$; mitochondrial, controls: 0.65 ± 0.05 nmol/min/mg protein vs stressed: 0.39 ± 0.03 nmol/min/mg protein, $P < 0.01$). V_{max} values for mitochondrial preparations (whether they were from control or stressed animals) were more than 50% lower ($P < 0.01$) than those for microsomal preparations (Figs. 1 and 2). K_m values for 3β-HSD in testicular microsomal and mitochondrial fractions did not differ between control and stressed rats (Figs. 1 and 2; microsomal, controls: 3.17 ± 0.07 μM vs stressed: 3.19 ± 0.07 μM; mitochondrial 4.33 ± 0.21 μM vs stressed: 4.12 ± 0.17 μM). However, K_m values for 3β-HSD in microsomal fractions from control and stressed rats were lower ($P < 0.01$) than values from mitochondrial preparations.

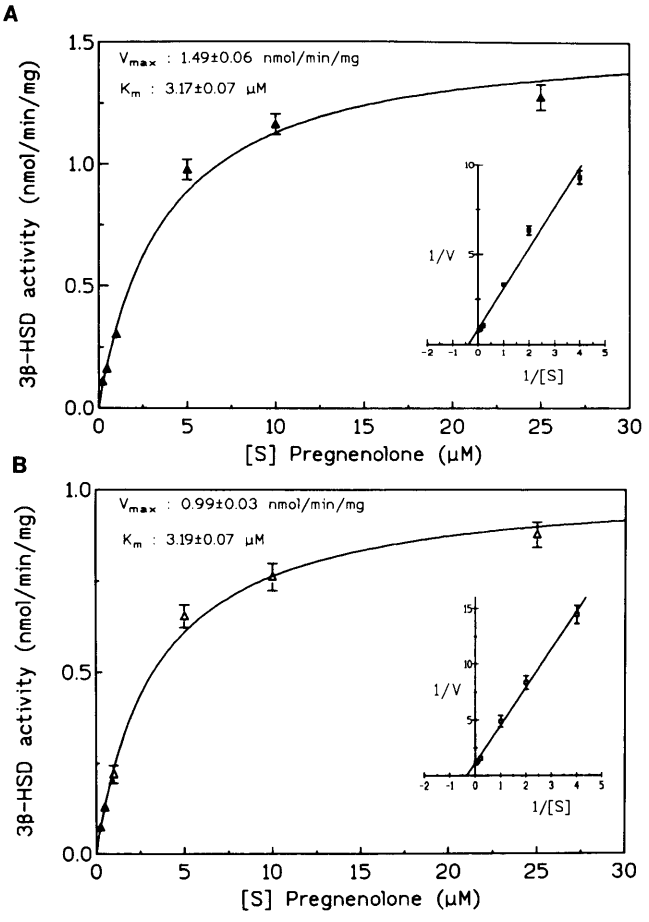

Figure 1. Effect of increasing concentrations of pregnenolone on the activity of 3β-HSD in microsomal preparations from the testes of control (A) and immobilization-stressed (B) rats. Inset figure is the double reciprocal Lineweaver-Burk transformation of the data.

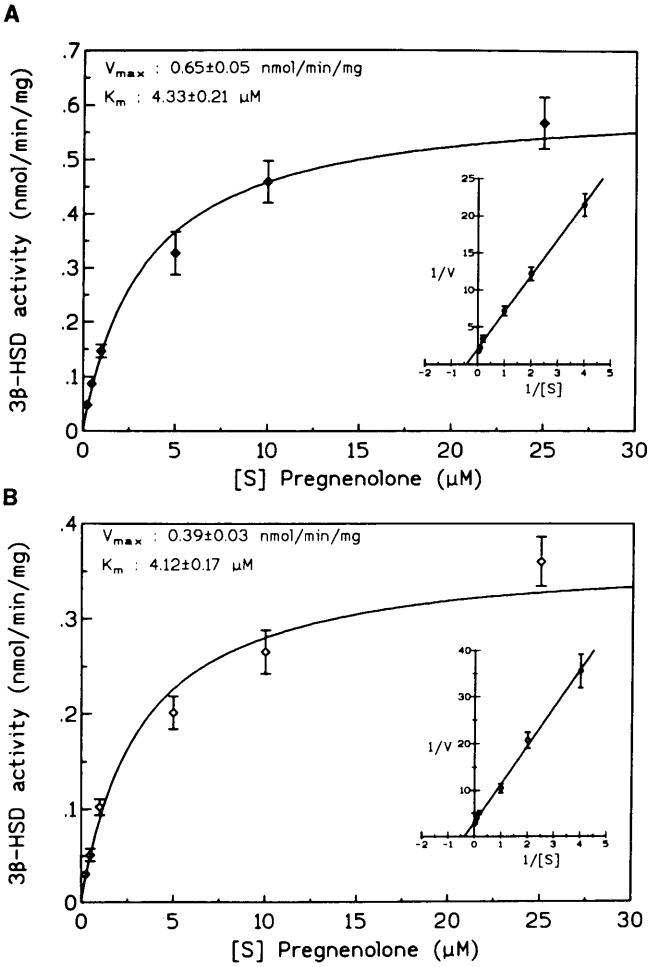

Figure 2. Effect of increasing concentrations of pregnenolone on the activity of 3β-HSD in mitochondrial preparations obtained from the testes of control (A) and immobilization-stressed (B) rats. Inset figure is the double reciprocal Lineweaver-Burk transformation of the data.

Discussion

In the present study, immobilization stress for 3 hr reduced plasma testosterone concentrations by 55%. The decline in circulating levels of testosterone was associated with a 34% and 40% reduction of 3β-HSD activity in testicular mitochondrial and microsomal preparations, respectively. These results suggest that one mechanism by which immobilization stress disrupts testicular steroidogenesis in the adult rat is by suppressing activity of this key steroidogenic enzyme (3β-HSD). These data, coupled with our earlier findings that the activities of both 17α-hydroxylase and 17,20-lyase are reduced by immobilization stress (18), suggest that three major enzymes in the steroidogenic pathway

between pregnenolone and testosterone are impaired during immobilization stress.

We have not yet defined the mechanism by which immobilization stress has such a potent inhibitory effect on the activities of 3β-HSD, 17α-hydroxylase, and 17,20-lyase. However, the reduced V_{max} levels of all three enzymes, coupled with the lack of an effect on K_m values, suggest that either a major portion of these enzymes is lost through unfolding or degradation, or inhibition occurs via a noncompetitive mechanism (e.g., loss of activator protein(s), presence of inhibitory protein(s), or post-translational modification(s)). Further study is needed to clarify this issue.

There is controversy concerning whether measured 3β-HSD activity in mitochondrial fractions of the adrenal cortex and ovary represents a separate distinct 3β-HSD or whether the mitochondrial activity results from microsomal contamination or is a redistributional artifact of the enzyme resulting from homogenization (13). This issue has not been resolved and must be noted relative to the present findings of significant 3β-HSD activity in testicular mitochondrial fractions. Activity of 3β-HSD in mitochondrial franctions was lower (by more than 50%) and K_m values higher than in microsomal fractions. The distribution of activity between the two compartments is similar to that reported for the placenta (14). The difference in K_m values between the microsomal and mitochondrial fractions may result from different 3β-HSD isozymes or different environmental milieu between these two compartments.

In the present study, mean plasma LH levels in animals after 3 hr of immobilization did not differ significantly ($P = 0.093$) from control values, but the trend was for levels to be lower in the stressed group. Thus, inadequate gonadotropin stimulation may have contributed to the reduced levels of 3β-HSD activity and plasma testosterone values. The marginally lower plasma LH concentrations in stressed rats may reflect changes in LH pulsatility. Frequent blood sampling over the entire stress period would be required in order to fully determine whether a 3-hr immobilization stress alters LH release. It should be noted, however, that if inadequate LH stimulation of the testis of stressed rats is responsible for reduced enzyme activity and lower testosterone production, this may not only result from lower LH secretion but also a greatly reduced testicular response to gonadotropin. Immobilization stress for as little as 30 min completely abolished the plasma testosterone response to human chorionic gonadotropin in adult male rats (2).

It is likely that hyperactivity of the hypothalamic-ACTH-adrenal axis is involved in mediating the effect of immobilization stress on the testes of rats. Acute immobilization causes an immediate and robust increase in ACTH and corticosterone secretion (15, 19–21). Glucocorticoid receptors are present in Leydig cells (22), and pretreatment of animals with RU 486 (a glucocorticoid receptor antagonist) partially blocked the inhibitory effects of immobilization stress on plasma testosterone concentrations (15). However, ACTH does not appear to have a direct action on testicular steroidogenesis in the rat (23).

In summary, we have shown that the suppression of testicular steroidogenesis in the rat during immobilization stress is associated with a significant reduction in testicular activity of 3β-HSD. Thus, the activities of three major enzymes (17α-hydroxylase, 17,20-lyase, and 3β-HSD) in the testicular steroidogenic pathway are impaired by exposure of the rat to acute immobilization stress. We are currently investigating whether other steroidogenic enzymes are similarly influenced by immobilization stress and the mechanism of their inhibition.

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