Testosterone production in mice lacking inducible nitric oxide synthase expression is sensitive to restraint stress

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Weissman BA, Sottas CM, Zhou P, Iadecola C, Hardy MP. Testosterone production in mice lacking inducible nitric oxide synthase expression is sensitive to restraint stress. Am J Physiol Endocrinol Metab 292: E615–E620, 2007. First published October 10, 2006; doi:10.1152/ajpendo.00412.2006.—Immobilization stress (IMO) induces a rapid increase in glucocorticoid secretion [in rodents, corticosterone CORT] and this is associated with decreased circulating testosterone (T) levels. Nitric oxide (NO), a reactive free radical and neurotransmitter, has been reported to be produced at higher rates in tissues such as brain during stress. The biosynthesis of T is also known to be dramatically suppressed by NO. Specifically, the inducible isoform of nitric oxide synthase (iNOS) was directly implicated in this suppression. To assess the respective roles of CORT and NO in stress-mediated inhibition of T production, adult wild-type (WT) and inducible nitric oxide synthase knockout (iNOS−/−) male mice were evaluated. Animals of each genotype were assigned to either basal control or 3-h IMO groups. Basal plasma and testicular T levels were equivalent in both genotypes, whereas testicular weights of mutant mice were significantly higher compared with WT animals. Exposure to 3-h IMO increased plasma CORT and decreased T concentrations in mice of both genotypes. Testicular T levels were also affected by stress in WT and mutant males, being sharply reduced in both genotypes. However, the concentrations of nitrate and nitrite, the stable metabolites of NO measured in testicular extracts, did not differ between control and stressed WT and iNOS−/− mice. These results support the hypothesis that CORT, but not NO, is a plausible candidate to mediate rapid stress-induced suppression of Leydig cell steroidogenesis.

inducible nitric oxide synthase-null mice; glucocorticoid; androgen

STRESS IS DEFINED AS A DISRUPTION of homeostasis (47), and stimuli that challenge homeostasis are designated as stressors. Stressors can be divided into three general categories (45, 54, 56): 1) physical (e.g., 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 (IMO) is a mixture of physical and psychological stressors, restricting movement and isolating the individual from its group (44).

During stress, an adaptive response originating in the hypothalamic-pituitary-adrenal (HPA) axis is activated to sustain homeostasis (37, 41). The adaptive response alters the secretion of corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH), and luteinizing hormone (LH) as well as adrenal corticosteroids (41, 47). Stress from a variety of stimuli exerts a profound suppression of the reproductive axis (5, 10, 47, 54). In males, decreased plasma testosterone (T) is one of the first signs of stress (19), and a sharp rise in circulating glucocorticoid levels is viewed as a causative factor in the decline of steroidogenesis (1, 3, 20). LH, the main tropic stimulus of T production in Leydig cells, may be unchanged (8, 13, 52) or lower (4, 16), depending on the duration of the stress being investigated. In rats, acute IMO lowers T concentrations primarily at the testicular level with unchanged LH secretion, whereas chronic IMO has inhibitory effects on the hypothalamic-pituitary level and, by lowering plasma LH release, decreases plasma T concentrations (38).

Nitric oxide (NO) has been shown to potently reduce T production in vivo (2) and to directly suppress Leydig cell in vitro (15, 58). A short, 2-h session of IMO in rats produces a sharp decline of plasma T levels and blocks the LH-induced T production in hemitestic preparations incubated in vitro, and these actions have been attributed to endogenous NO activity (29). Further studies have led to the proposal that the involvement of NO in stress is due to activation of testicular inducible nitric oxide synthase (iNOS) (30, 31). In addition, multiple lines of evidence support the conclusion that IMO is associated with increased NOS mRNA and protein levels in the brain cortex (36) and paraventricular nucleus (7) and in the HPA axis (27).

NOS protein (14) and activity (6) are present in human and rat testis, respectively. On the basis of immunohistochemical techniques, endothelial (e)NOS has been detected in rat Sertoli, Leydig, and germ cells (60), and neuronal (n)NOS was identified in Leydig cells by immunocytochemistry (34). Whereas iNOS is induced by lipopolysaccharide (LPS) in testicular macrophages (TM) (59), constitutively expressed iNOS was proposed as a modulator of T production in Leydig cells (42). An NOS-related NADPH-diaphorase activity has been detected in mouse seminiferous tubules (49). In addition, a novel, testis-specific nNOS isoform (i.e., TnNOS) was described in human tissue (57). Therefore, it is evident that there is a database to suggest a physiological role of NO in testicular T production.

The aim of the present study was to assess the possible involvement of testicular iNOS and its product, namely NO, in the stress-induced reduction of Leydig cell steroidogenesis. To
this end, we subjected wild-type (WT) and iNOS-null (iNOS−/−) mice to 3 h of IMO. The rationale for evaluating the latter genotype was that if NO in general, and iNOS in particular is required for the decline in T production seen in stressed animals, iNOS−/− mice should have an attenuated or absent response. The data show that following 3 h of IMO, mice of both genotypes had similar patterns of increased plasma corticosterone (CORT) and reduced T concentrations. Moreover, although testicular T content sharply declined in stressed WT and iNOS−/− mice, the concentration of NO stable metabolites nitrate and nitrite (NOx) remained unaltered. These results indicate that testicular NO does not play an important role in the disruption of androgen synthesis following stress and are consistent with a direct CORT-mediated decline in T levels.

MATERIALS AND METHODS

Animals. Adult male mice C57BL/6 (25–30 g body wt, n = 39) were purchased from Charles River Laboratories (Wilmington, MA). The iNOS−/− mice (C57BL/6 congenic; n = 25) were obtained from an in-house colony (9, 26, 46) and housed five per cage under controlled environmental conditions (temperature 22 ± 2°C, 12:12-h light-dark cycle, with lights on from 0600 to 1800). All animals were handled to become adapted for ≥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 no. 04-059, renewed 8/25/05).

IMO. The animals were placed in wire mesh restrainers (4–9 cm in dimension), as described by McEwen et al. (40). The procedure effectively restricted movement. The start of IMO began at 10 AM, and the supernatants were stored at −20°C until assay. Testes were removed and stored at −70°C. The overall design was replicated three times.

Intratesticular T. Intratesticular T concentrations were measured using the method of Knorr et al. (28). In brief, testes were weighed and snap-frozen in liquid nitrogen. Frozen portions were then removed with a razor blade, weighed (range: 20–60 mg), and homogenized in 5 ml of 70% methanol using a glass-glass homogenizer (15 s × 2). The homogenates were transferred to 15-ml screw cap test tubes. Tracer steroid (1,000 cpm of tritiated T) was added to the homogenates to correct for recovery. The homogenates were left to incubate at room temperature, homogenates were centrifuged at 500 g, and the plasma supernatants were stored at −20°C until assay. Twenty microliters of a 30% solution of ZnSO4 were added to the homogenates. After 15 min of incubation at room temperature, homogenates were centrifuged at 4,000 rpm for 20 min. Supernatants were transferred to microcentrifuge tubes, 4–5 cadmium beads were added, and tubes were shaken overnight. Supernatants were transferred to microfuge tubes and centrifuged at room temperature for 10 min. Samples were kept at −20°C until assay. Duplicate aliquots (100 µl) were analyzed using the Griess method, with sodium nitrite (0.5–100 µM) to create a standard curve.

Plasma T and CORT concentrations. Plasma T concentrations were measured using a tritium-based RIA as previously described (12). Plasma CORT was measured by the RIA procedure of Spencer et al. (51), with an anti-CORT anti-B3-163 (Endocrine Sciences, Calabasas, CA). Values for interassay variation of the T and CORT RIAs were between 4 and 8%. The sensitivities of the assays for CORT and T were 10 ng/ml and 10 pg/ml, respectively.

Statistical analysis. Data are expressed as means ± SE. Statistical evaluation of plasma and testis parameters was performed by two-way analysis of variance (ANOVA) with genotype and treatment as the subclasses. If ANOVA indicated a significant difference of the means, multiple comparisons were applied to identify significant differences between groups by use of the least significant difference statistic. A two-tailed, nonpaired Student’s t-test analysis was used for comparing changes in testicular weights and T content. All calculations were performed using a computer program (STATISTICA for Windows; StatSoft, Tulsa, OK).

RESULTS

Testis weights. Testes of iNOS−/− mice weighed 21.5% more than those of WT animals 94.88 ± 1.35 (n = 25) vs. 78.07 ± 1.28 (n = 39) mg, P < 0.0001, respectively. These results agree with a previous report describing the male reproductive phenotype of iNOS−/− (35) and are consistent with the hypothesis that germ cell numbers are increased in the mutant due to deficient NO-mediated apoptosis.

Plasma T concentrations. Figure 1A depicts the effects of 3-h IMO on plasma T concentrations. No significant difference was found between the basal T concentration of WT (1.95 ±
0.48 ng/ml, n = 21) and congenic iNOS−/− mice (3.15 ± 1.27 ng/ml, n = 12). In contrast, the reduction of plasma T concentrations in both WT and mutant mice after 3-h IMO was significantly lower compared with basal control animals (P < 0.03). The post-IMO T levels in WT and iNOS−/− mice did not differ [0.305 ± 0.117 (n = 18) and 0.294 ± 0.057 (n = 13) ng/ml, respectively], indicating that genotype was unrelated to the stress response.

Testicular T levels. Testicular levels of T in all groups are presented in Fig. 1B, and the trends were similar to those shown for plasma T content (Fig. 1A). The concentrations of testicular T in WT and iNOS−/− mice, expressed as ng/mg tissue, did not differ statistically either in their basal (0.296 ± 0.066, n = 21 vs. 0.437 ± 0.235, n = 10) or in their stressed levels (0.068 ± 0.008, n = 18 vs. 0.053 ± 0.007, n = 13). However, decreased testicular T was observed in both genotypes after IMO (P < 0.05). The 71 and 40% reductions in testicular T content in WT and mutant mice, respectively, are in the same range reported for the rat model of IMO (43).

Testicular NOx levels. The values of testicular NOx were 22.1 ± 4.6 (n = 17, stressed) vs. 19.8 ± 3.1 (n = 21, basal control) and 24.1 ± 4.5 (n = 13, stressed) vs. 19.2 ± 3.5 (n = 12, basal control) nmol/g tissue in iNOS−/− and WT mice, respectively. No statistically significant difference was found between any of the groups (Fig. 2). This was consistent with published data on the low testicular NOS activity in the rat and mouse associated with very small rates of NO formation. These values range from <0.5 pmol-mg protein−1·min−1 (6), to 15 pmol-mg protein−1·min−1 (55), ≈20 pmol-mg protein−1·min−1 (24). Thus our results, showing the presence of NO metabolites at concentrations in the range of 10–20 nmol/g tissue, are in close agreement with the literature.

Plasma CORT concentrations. Evaluation of plasma CORT concentrations in control and stressed mice demonstrated that both WT and iNOS−/− animals responded as expected, namely, with a marked increase in the glucocorticoid level, as would be typical for stress (Fig. 3). Basal concentrations of CORT in WT mice (164.2 ± 23.3 ng/ml, n = 21) were not statistically different from those of iNOS−/− mice (99.0 ± 10.4 ng/ml, n = 12). The concentrations in the stressed animals were also not significantly different between the two genotypes (WT: 386.7 ± 32.6 ng/ml, n = 18 vs. iNOS−/−: 374.6 ± 19.1 ng/ml, n = 13). The robust elevations of plasma CORT levels over basal control were significant for both genotypes (P < 0.0001).

DISCUSSION

In the present investigation, WT and iNOS−/− mice were subjected to 3 h of IMO. On the basis of the hypothesis that testicular NO and iNOS are involved in the decline in T production that accompanies stress, iNOS-deficient animals should exhibit attenuated or absent response. Results presented herein show that, following 3 h of IMO, mice of both genotypes had identical patterns of reduced T associated with increased plasma CORT concentrations. Furthermore, although testicular T content sharply declined in stressed WT and mutant mice, the concentration of NOx, NO stable metabolites, remained unchanged. This suggests that, in contrast to CORT, signaling by testicular NO might not play a significant role in the stress-induced suppression of T production by Leydig cells.

Numerous reports have shown that acute stress decreases plasma T levels and that this is accompanied by an increase in the expression of nNOS and/or iNOS in the HPA axis (e.g., Refs. 7, 27, 36). Similarly, elevated concentrations of NOx have been observed in the brain under stress conditions (25, 39). In the tests, it has been proposed that NO is involved in the regulation of T biosynthesis by Leydig cells under normal (15, 42, 58) and stress conditions (29–31). Indeed, at micromolar concentrations, this reactive free radical abolishes steroidogenesis by Leydig cells in vitro (58). Because TMs reside in close proximity to Leydig cells in the interstitial tissue, they have been hypothesized to act as a source of NO, thereby modulating Leydig cell steroidogenesis under pathological conditions. Indeed, exposure of isolated TMs to LPS generates large amounts of NO (59), which could markedly reduce T production. Nevertheless, when C57BL/6 mice are subjected to IMO, the activity of their peritoneal macrophages is significantly decreased (11). Thus, following 3-h IMO, TMs of animals used herein are expected to release NO at quantities lower than those of unstressed mice. It follows, then, that NO generated by TMs could not be involved in stress-induced decline in T production.

An inverse relationship between NOx levels in plasma or testes and T content has been seen during the inflammatory response. As part of the inflammatory process, iNOS expres-
sion is induced and NO is formed (see Ref. 22 for example). It has been proposed, therefore, that testicular NO may be causally involved in the stress-induced decrease of T biosynthesis (29–31). Our aim was to evaluate the putative involvement of NO in general and iNOS in particular in IMO-elicited reduction of T secretion by examining the response of iNOS−/− mice to IMO.

To investigate the possible involvement of testicular NO-mediated pathways, the use of iNOS-deficient animals is preferable to the commonly used approach, namely, application of NOS inhibitors. In addition to the fact that there are no highly specific blockers for this family of enzymes, NOS inhibitors have been shown to affect the HPA system. For example, the NOS inhibitor N\(_{\text{G}}\)-nitro-L-arginine methyl ester (L-NAME) increases plasma ACTH levels induced by intracerebroventricular isoproterenol injection (48). This may increase CORT and thereby alter androgen production as a secondary effect. Similarly, several strains of mice (e.g., BALB/c, C3H/He, and DBA-2) respond to L-NAME with an increase in circulating ACTH and CORT concentrations (21). Therefore, NOS inhibition studies to date reveal confounding effects of NOS signaling on HPA function that may affect stress-mediated inhibition of testicular steroidogenesis. The possibility should be considered that in the iNOS knockout mice another member of the NOS family has taken over the function of iNOS. However, there are at present no reports demonstrating any compensatory upregulation of other NOS isoforms in all organs and tissues of iNOS-null mice that have been analyzed thus far. In fact, Sahrbacher et al. (50) reported on the failure to detect increased nNOS and eNOS mRNA in iNOS−/− mice. Therefore, in this special case of compensation, a noticeable elevation in testicular NOx concentrations following 3 h of IMO is expected. As reported above, no such increase was observed.

Mice lacking the gene for iNOS display a normal phenotype with standard growth, reproduction, and histology of all major organs and tissues (33) and equal levels of plasma LH, FSH, and T compared with WT animals (32, 35). Analysis of the male reproductive system, however, shows that iNOS deficiency results in a decreased frequency of germ cell apoptosis, increasing numbers of sperm, and overall testis size. Our efficiency results in a decreased frequency of germ cell apoptosis, male reproductive system, however, shows that iNOS defi-

In summary, our results show that IMO induced increased peripheral T concentrations when exposed to LPS injections (32). An IMO-induced activation of testicular iNOS, yielding increased output of NO, has been reported on the basis of an ex vivo paradigm (29–31). In this model, the IMO resulted in a diminished rate of T production that could be reversed by pretreatment with the selective iNOS inhibitor aminoguanidine (31). However, under these conditions, iNOS could have been expressed and activated in the tissue during the in vitro incubation period. The release of cytokines such as TNF-\(\alpha\) or IL-1β resulting from the killing of animals and preparing the tissues (18) could cause rapid NO production, thus leading to the suppression of T formation (53). Notably, those authors also reported on the in vivo administration of either L-NAME (29) or aminoguanidine (31). In both cases, the blockers used completely failed to reverse the decrease in T levels after 2 h of IMO and 3 h of incubation. Moreover, the latter paper describes a small IMO-induced increase in NOx concentration that was not affected by the iNOS inhibitor.

Recent research on neural regulation of testicular steroidogenesis has led to the proposal that mechanisms other than the NO pathway are involved in the stress-mediated decline in Leydig cell function (23). In this model, intracerebroventricular injections of agents such as isoproterenol or ethanol caused a rapid decrease in T concentration concomitant with elevated levels of NOx and the expression of the testicular NOS isoform TnNOS. However, pretreatment of rats with the nonspecific NOS inhibitor L-NAME, which blocked the isoproterenol-induced increase in TnNOS protein expression, did not restore the steroidogenic response to human chorionic gonadotropin. These results mirror the present observations in pointing away from NO as a mediator in the neural-testicular pathway regulating steroidogenesis.

Upregulation of nNOS activity was seen in the cortical and medullar regions of adrenal cortex of rats exposed to 6-h IMO, by 250–380% relative to control (27). Our findings show that after stress, mice lacking iNOS had NOx levels that were equivalent to WT. These observations gain added importance, considering the fact that under the same conditions the concentrations of circulating CORT levels are elevated and T production is dramatically reduced. The dynamics of stress-induced changes in T, CORT, and LH levels in mice have been investigated recently (17). It has been established that LH remains unchanged, whereas CORT levels increase, in stressed males starting at 15 min, reaching a higher plateau by 1 h. In the same time period, both plasma and testicular T concentrations decreased in stressed animals, starting from 30 min after IMO. Notably, in vitro CORT treatment of Leydig cells reduced intracellular cyclic adenosine monophosphate content by 15 min and T production by 30 min. It was concluded that the rapid changes in T suggests a suppression of its biosynthesis by glucocorticoid through a nongenomic mechanism involving a direct inhibitory action of CORT on Leydig cells.

In summary, our results show that IMO induced a marked reduction in plasma and testicular T concentrations, whereas testicular NOx levels remained unaltered. Given that there was no significant difference between the response of WT and iNOS knockout mice, the data indicate that the effect on T content is not mediated by NO pathways in general and the inducible isoform of NOS in particular. The likely candidate responsible for the observed decline in T levels is the glucocorticoid CORT, which undergoes a robust increase during stress.

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