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Inhibitory effects of stress-activated nitric oxide on antioxidant enzymes and testicular steroidogenesis

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Abstract

The messenger role of nitric oxide (NO) in immobilization stress-induced inhibition of testicular steroidogenesis has been previously suggested. In accord with this, here, we show that the intratesticular injection of isosorbide dinitrate (ISDN; 2×2.5 mg/testis), an NO donor, mimicked the action of stress on serum testosterone concentrations and hCG-stimulated testosterone production in rat testicular tissue. When added in vitro, ISDN inhibited testicular 3β-hydroxysteroid dehydrogenase and 17α-hydroxylase/lyase. Immobilization stress and injections of ISDN also decreased the activity of catalase, glutathione peroxidase, glutathione transferase, and glutathione reductase in the interstitial compartment of testis. When stressed rats were treated concomitantly with bilateral intratesticular injections of N^{ω}-nitro-L-arginine methyl ester, a non-selective NOS inhibitor (2×600 μg/testis), the activities of antioxidative enzymes, as well as serum testosterone concentration, were partially normalized. These results indicate that stress-induced stimulation of the testicular NO signalling pathway leads to inhibition of both steroidogenic and antioxidant enzymes.

Keywords: Antioxidant enzymes; Nitric oxide; Steroidogenesis; Stress; Testosterone

1. Introduction

Nitric oxide (NO) is produced by testicular tissues and acts as an autocrine/paracrine messenger in local regulation of steroidogenesis [1]. Several lines of evidence have also suggested that NO mediates the stress-induced downregulation of testicular steroidogenesis. For example, in-vitro androgenesis by decapsulated testes from stressed rats exposed to human chorionic gonadotrophin (hCG) was significantly reduced. This inhibition was accompanied by an elevation in the testicular nitrite, a stable oxidation product of NO pathway [2,3]. The impaired testicular steroidogenesis in stressed rats coincided with a significant inhibition of 3β-hydroxysteroid dehydrogenase (3β-HSD) [4,5], 17α-hydroxylase/lyase (P450c17) [5–7], and NADPH-P450 reductase [8].

In addition to steroidogenic enzymes, the rise in intratesticular NO concentration may affect the activity of some antioxidant enzymes, leading to an imbalance of the prooxidant versus antioxidant status in the interstitial tissue and an increase in the production of reactive oxygen species. Namely, it is shown that NO donors, *S*-nitroso-*N*-acetylpenicillamine (SNAP) and sodium nitropruside (SNP), inhibited catalase activity in a concentration-dependent manner [9]. Furthermore, SNAP inhibited bovine glutathione peroxidase in a dose- and time-dependent manner [10]. It is also possible that inhibition of P450-dependent enzymes is accompanied by facilitation of the prooxidant status in the interstitial tissue and therefore by an additional inhibition of the antioxidant enzyme system in this compartment of the testis [11,12]. If the activity of the antioxidative enzymes is impaired in stress rats, this may result in facilitation of lipid peroxidation, which in turn could further inhibit the activity of the steroidogenic membrane-bound enzymes [11,12]. Thus, it is reasonable to speculate that the antigonadal effects of acute stress result from a dual inhibitory action of NO signalling pathway, on steroidogenic and antioxidant enzymes.

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To address these issues, we examined the activities of several antioxidant enzymes in the interstitial compartment of rats previously exposed to acute immobilization stress (2 h; hereafter described as stress). In order to establish the dependence of their activities on NO signalling pathway, the stressed rats were also treated concomitantly with N^{ω}-nitro-L-arginine methyl ester (L-NAME), a non-selective NO synthase (NOS) inhibitor [13]. In addition, we treated animals with isosorbide dinitrate (ISDN), an NO donor, and we examined the effects of these treatments on the testicular steroidogenesis and antioxidant enzyme activities. This was particularly important in experiments with ISDN, since the observation about the concomitant rise in NO production with downregulated testosterone production in stressed rats published earlier [8] did not establish any direct dependence of steroidogenesis on the level of intratesticular NO. The results of the present investigations indicate that the activities of both antioxidant and steroidogenic enzymes were downregulated by acute stress and that this inhibition is mediated by NO.

2. Materials and methods

2.1. Chemicals

Antitestosterone (No-250) and antiprogesterone (No-337) serum were kindly supplied by G.D. Niswender (Colorado State University, Fort Collins, CO). Medium 199 was purchased from GIBCO Laboratories (Gaithersburg, MD), human chorionic gonadotropin (hCG, 3000 IU/mg) from Organon (West Orange, NJ), and [(1,2,6,7^{3}H(N)] testosterone as well as [(1,2,6,7^{3}H(N)] progesterone from New England Nuclear (Brussels). All other chemicals were from Sigma (St. Louis, MO).

2.2. Animals and treatments

All experiments were performed in adult male Wistar rats bred in our laboratory and raised under controlled environmental conditions (temperature 22 ± 2°C; 14 h light/10 h dark) with food and water ad libitum. Rats were handled daily for a 1 week acclimation period prior to the experiments. Animals were immobilized on a wooden board for 2 h according to Kvetnansky et al. [14], starting at 8:00 a.m. The effects of NO donor on the activities of testicular antioxidant enzymes, as well as the testicular steroidogenic capacity, were investigated following an intratesticular injection of ISDN into each testis. ISDN (2.5 mg/testis) was injected at the beginning of experiment and 1 h later, while the control animals were injected with saline at the same time. To establish the possible involvement of NO in stress-induced change of antioxidant enzyme activities, the animals were subjected to stress, accompanied by two bilateral intratesticular injections of L-NAME (600 μg/testis) or saline, one at the beginning of the immobilization period and a second 1 h later. All experiments were approved by the Local Animal Ethical Committee of the University of Novi Sad and were conducted in accordance with the principles and procedures of the NIH Guide for Care and Use of Laboratory Animals.

2.3. In-vitro incubations and hormone assays

Testes from experimental and control rats were quickly removed, decapsulated, and incubated individually in vials containing the saturating concentration of hCG (20 ng/ml) in a total volume of 5 ml of medium 199 enriched with 0.1% bovine serum albumin (M199-BSA). Following incubation for 3 h at 34°C in a shaking water bath oscillating at 100 cycles/min under an atmosphere of 95% O_2–5% CO_2, incubation media were centrifuged for 10 min at 1500 g. Individual samples of supernatants were stored at -20°C prior to measurement of testosterone and nitrite. Testosterone and progesterone were measured by radioimmunoassay. Since the antitestosterone serum has a high cross-reactivity with dihydrotestosterone, assay values are referred to as testosterone + dihydrotestosterone (T + DHT) levels. All samples from the single experiment were run in one assay (sensitivity: 6 pg/tube; the intra- and inter-assay coefficients of variation were 5.8 and 7.5%, respectively). For progesterone measurement, each experiment was run also in one assay (sensitivity: 6 pg/tube; the intra- and inter-assay coefficients of variation were 6.8 and 10.7%, respectively). For nitrite measurements, sample aliquots of 1 ml were mixed with an equal volume of Greiss reagent containing 0.5% sulphanilamide and 0.05% naphthylethylenediamine in 2.5% phosphoric acid. The mixture was then incubated at room temperature for 10 min, and the absorbance was measured at 546 nm [15]. Nitrite concentrations were determined relative to a standard curve derived from increasing concentrations of sodium nitrite.

2.4. Characterization of antioxidant enzyme activities in denucleated fraction of interstitial cells

Testes from control and experimental groups were removed quickly, decapsulated and dissociated with collagenase (1.2 mg/ml) in M199-BSA, as previously described [16]. Interstitial cells were resuspended in a buffer containing 17 mM Tris and 140 mM NH_4Cl (pH 7.2) and incubated for 10 min at room temperature. This procedure eliminates red blood cells and interference of haemoglobin. The cell pellets were washed twice with M199-BSA and twice with 0.9% NaCl solution. Trypan Blue exclusion was used to assess the cell viability (over 90%). Denucleated fractions of intersti-

tial cells (separately for each animals) were obtained by homogenization of cell pellets in 50 mM phosphate buffer containing 0.25 M sucrose (pH 7.4) using a glass–glass homogenizer and centrifugation for 20 min at 1500 *g* (4°C). Protein contents in denucleated fractions of interstitial cell were estimated by the Bradford method [17], using BSA as a standard.

The activity of superoxide dismutase was measured by inhibition of superoxide radical production in a xanthine/xanthine-oxidase reaction, according to the method described by Misra and Fridovich [18] with some modification. The catalase activity was determined by the rate of H_2O_2 disappearance recorded at 240 nm, according to Clauborne et al. [19]. The GST activity was assayed using chlorodinitrobenzene as a substrate and measuring the absorbency change at 340 nm, according to Habig et al. [20]. Oxidation of NADPH at 340 nm, with tertz-butyle as a substrate, was employed in the modified method of Paglia and Valentine [21] for measuring the activity of glutathione peroxidase. The activity of glutathione reductase was measured by oxidation of NADPH at 340 nm. The reaction was initiated by the addition of denucleated fraction of interstitial cells, with the reaction media containing 1 mM NADPH, 1% BSA, 2% oxidated glutathione, all resuspended in 0.1 M phosphate buffer (pH 7.4)

2.5. Measurements of steroidogenic enzyme activities in post-mitochondrial testicular fractions

To prepare the post-mitochondrial fractions, testes from normal rats were decapsulated and homogenized in 50 mM phosphate buffer containing 0.25 M sucrose (pH 7.4), using a glass–glass homogenizer. After centrifugation (4°C for 20 min at 1500 *g*), the supernatants were mixed with dextran-coated charcoal in order to remove the endogenous steroids [22]. The samples were centrifuged at 1500 *g* for 10 min, and supernatants were further centrifuged at 12 000 *g* for 20 min. The protein content in the post-mitochondrial fractions was estimated by the Bradford method [17], using BSA as a standard.

The 17β-hydroxysteroid dehydrogenase (17βHSD) and P450c17 activities in post-mitochondrial fractions were measured as previously described and were estimated by conversion of Δ^4-androstendione to testosterone and progesterone to testosterone, respectively [5]. Briefly, in the final volume of 0.25 ml, the incubation solution contained 10 μM of steroid substrates, 1 mM NADPH, 0.1 M phosphate buffer (pH 7.4), and 0.1 ml post-mitochondrial fractions. Mixtures were incubated for 15 min at 37°C in a shaking water bath in a 95% O_2–5% CO_2 atmosphere. The 3βHSD activity was estimated through the conversion of pregnenolone to progesterone. In this assay, the incubation solution, with a final volume of 2 ml, contained 25 μM pregnenolone, 135 μM NAD^+, 100 mM phosphate buffer (pH 7.4), and 0.1 ml of the post-mitochondrial fraction. Mixtures were incubated for 10 min at 37°C in a shaking water bath in an atmosphere of 95% O_2–5% CO_2. The reaction conditions were defined in preliminary experiments, in which the enzyme activities were tested by varying the incubation times and concentrations of substrates, proteins, and coenzymes.

P450c17 and 17βHSD assays were done under initial velocity conditions and in the presence of saturated concentration of corresponding steroid substrate, whereas 3βHSD activity was measured under unsaturated concentration of pregnenolone, with a K_m value of 8.43 ± 1.76 μM, estimated in post-mitochondrial fractions from normal rats. Enzyme reactions were initiated by the addition of 0.1 ml of post-mitochondrial fractions and terminated by placing the tubes in an ice-cold bath. For the in-vitro effects of ISDN, the compound was added in incubation media (1 mM, 5 mM). After the end of incubation, the supernatants were stored at −20°C until assayed for progesterone and testosterone by radioimmunoassay.

2.6. Calculations

The non-parametric Mann–Whitney test was used for a statistical analysis of the results, and $P < 0.05$ or higher was considered as a significant difference.

3. Results

3.1. Parallelism between the effects of stress and injection of NO donor on the testicular steroidogenesis

It has been shown previously that the stress-induced downregulation of steroidogenesis coincides with a rise in NO in intertesticular tissue [2]. To address the dependence of testicular steroidogenesis on the NO signalling pathway more directly, in further studies we used ISDN, an NO donor. Serum testosterone levels were significantly decreased in stressed animals and this inhibition was mimicked by injection of ISDN (Table 1).

Table 1
Effect of immobilization stress (IMO) and intratesticular isosorbide dinitrate (ISDN) treatment on serum testosterone concentration[a]

	Control	IMO	ISDN
T + DHT (ng/ml)	5.13 ± 1.02	2.03 ± 0.37*	2.37 ± 0.47*
Number of animals	6	7	7

[a] Rats were immobilized for 2 h, or two bilateral intratesticular injections of ISDN (2.5 mg/testis) were applied at the beginning of experiment and 1 h later. Animals were sacrificed 2 h after the beginning of the experiment. Data shown are means ± S.E. Significance: *$P < 0.05$ vs. controls.

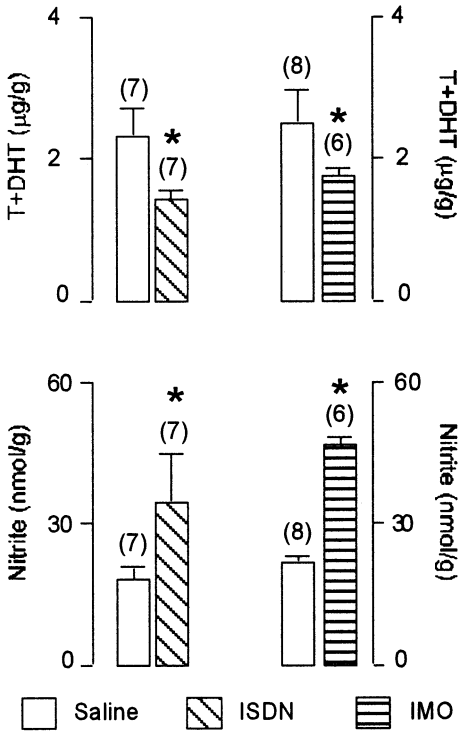

Fig. 1. Effect of immobilization stress (IMO) and isosorbide dinitrate (ISDN) injection on in-vitro steroidogenic capacity and nitrite production in testicular tissue. Saline or ISDN (2.5 mg/testis) was injected into each testis at the beginning of experiment and 1 h later. Animals were sacrificed 2 h after the beginning of experiment. Testes were incubated for 3 h in the presence of 20 ng/ml hCG. Bars represent means ± SE. The number in parentheses refers to the number of rats per group. $*P < 0.05$ vs. control group.

In further studies, the capacity of testicular tissue to produce testosterone and NO was examined. In vitro stimulating effect of hCG on testosterone production was significantly reduced in stressed animals and was accompanied with a significant increase in nitrite levels (Fig. 1, right panels). Injections of ISDN into testes also decreased in vitro hCG-stimulated testosterone production and increased nitrite levels (Fig. 1, left panels). These observations further indicate a parallelism in the actions of NO donor and immobilization stress on testicular steroidogenesis.

In vitro effects of NO donor on steroidogenic enzyme activities is shown in Fig. 2. The capacity of the post-mitochondrial fractions to metabolize pregnenolone to progesterone was significantly reduced in the presence of 5 mM ISDN in the incubation medium (Fig. 2A), suggesting that 3βHSD activity was inhibited by NO. Also, addition of 1 mM and 5 mM ISDN in the incubation media inhibited metabolism of progesterone to testosterone (Fig. 2B), whereas metabolism of Δ^4-androstendione to testosterone was not affected (Fig. 2C). This suggests that ISDN inhibits the P450c17 activity, but not the 17βHSD activity.

3.2. Parallelism between the effects of stress and injection of NO donor on the testicular antioxidant enzymes activities

Stress induced a significant fall in the activities of catalase (Fig. 3B), glutathione peroxidase (Fig. 3C), and glutathione transferase (Fig. 3D), whereas no significant differences were observed in testicular activity of superoxide dismutase comparing to the respective controls (Fig. 3A). In addition, stress effected the glutathione reductase activity [stressed rats: 3.94 ± 0.25 U/mg (6) vs. controls: 4.53 ± 0.15 U/mg (6), $P < 0.05$]. Injections of ISDN reduced catalase and glutathione transferase activities in a manner comparable to that observed in stressed rats. However, the effects of stress were more pronounced. As in stressed rats, no significant difference was observed in testicular activity of superoxide dismutase in comparison to controls (Fig. 3).

Parallelism between the effect of stress and injection of NO donor on the testicular antioxidant enzyme activities raised the possibility that there is a link between NO production and the decreased activities of antioxidant enzymes in stress conditions. To address this hypothesis, animals subjected to stress were treated with two intratesticular injection of L-NAME, a nonselective NOS inhibitor, or with saline. Control animals were treated with saline at the same time as stressed rats. As shown in Table 2, stress was associated with a reduction in serum testosterone level, which was prevented by L-NAME treatment. In addition, L-NAME significantly reduced the stress-induced inhibition of catalase (Fig. 4B), glutathione peroxidase (Fig. 4C), and glutathione transferase activities (Fig. 4D), compared to the stressed animals. However, L-NAME was unable to completely abolish the stress-induced inhibition of catalase and glutathione transferase activities

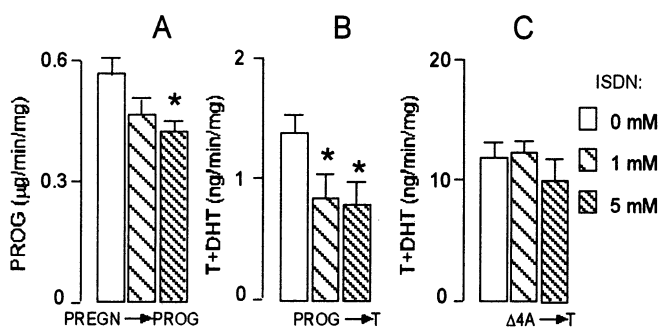

Fig. 2. In-vitro effects of isosorbide dinitrate (ISDN) on the 3βHSD, P450c17, and 17βHSD activities, which were estimated by conversion of pregnenolone to progesterone, progesterone to testosterone, and Δ^4-androstenedione to testosterone, respectively. Enzyme activities were estimated in post-mitochondrial testicular fractions obtained from six normal rats. Incubations were done without (controls) or with ISDN in incubation medium. Columns represent means ± SE of six to eight replicates per group. $*P < 0.05$ vs. controls.

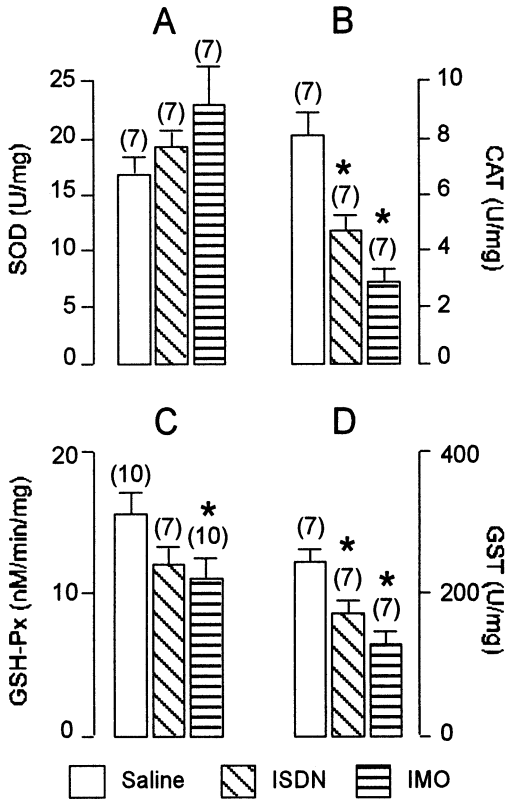

Fig. 3. Effects of immobilization stress and intratesticular ISDN injection on the activities of testicular antioxidant enzymes. Rats were immobilized for 2 h, or two bilateral intratesticular injections of ISDN (2.5 mg/testis) were applied at the beginning of experiment and 1 h later. Animals were sacrificed 2 h after the beginning the experiment. Denucleated fractions of interstitial tissue were used to determine the antioxidant enzyme activities. Columns represent means ± SE. The number in parentheses refers to the number of rats per group. $^{*}P < 0.05$ vs. control group. SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; GST, glutathione transferase.

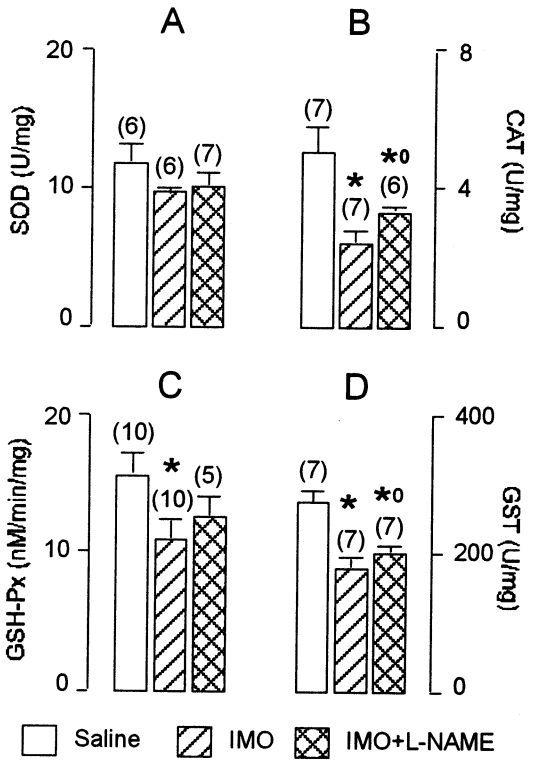

Fig. 4. Effects of immobilization stress and intratesticular L-NAME injection on the activities of testicular antioxidant enzymes. Saline or L-NAME (600 μg/testis) was injected into each testis at the beginning of, and 1 h into, the immobilization period. Animals were sacrificed at the end of a 2 h stress session. Denucleated fractions of interstitial tissue were used to determine the antioxidant enzyme activity. $^{*}P < 0.05$ vs. control group. $^{0}P < 0.05$ vs. immobilized group of rats. All other labelling as in Fig. 3.

4. Discussion

In normal physiological conditions, Leydig cells of testis are able to generate reactive oxygen species at a relatively high rate [23]. The cytochrome P450 enzymes of the steroidogenic pathway use molecular oxygen and electrons transferred from NADPH to hydroxylate the substrates [24]. In this process, superoxide anion and the other oxygen free radicals can be produced due to a leakage of electrons [11,24,25]. Testicular membranes are rich in unsaturated fatty acids and easily react with free radicals [23]. The lipid peroxidation process in turn causes a chain reaction that leads to deterioration of the membrane structure and integrity of cells [25].

In protection against peroxidative injury, testicular tissue relays on the actions of antioxidant vitamins. Their elevated levels in steroidogenic tissues indicate that the basal production of oxygen free radicals is high [11,26,27]. In addition to vitamins, the synergistic action of several testicular antioxidant enzymes is essential for controlling the balance of the prooxidant versus antioxidant status [28]. For example, superoxide dismutase catalyses the dismutation of superoxide anion radical to hydrogen peroxide, which is further metabolized by catalase [29]. Glutathione peroxidase and glu-

Table 2
Effect of immobilization stress (IMO) and intratesticular injection of L-NAME on serum testosterone concentration[a]

	Control	IMO	L-NAME+IMO
T+DHT (ng/ml)	5.96 ± 2.18	2.43 ± 0.28*	4.05 ± 0.74
Number of animals	6	6	6

[a] Saline or L-NAME (600 μg/testis) was injected into each testis at the beginning of experimental and 1 h into the immobilization period. Animals were sacrificed at the end of the 2 h immobilization session. Data shown are means ± S.E. Significance: $^{*}P < 0.05$ vs. controls.

tathione transferase function in the detoxification of reactive lipid peroxides [30].

Several lines of evidence indicate that the interactions between the testicular antioxidant and steroidogenic enzyme systems are complex and physiologically relevant. It was suggested that free radicals are involved in control of steroidogenesis in Leydig cells [31], corpus luteum [32], and adrenal cortex [12]. In cultured rat Leydig cells, free radicals mediate the cAMP-induced inactivation of the microsomal P450c17 [31]. Furthermore, it was shown that LH induces an acute increase in superoxide dismutase activity in mouse Leydig cells in vivo [33]. Lipid peroxides are also capable of inactivating P450 enzymes [11]. Therefore, the proper activity of the antioxidant enzyme system in steroidogenic tissue is very important for the maintenance of normal steroid hormone production.

At the present time, however, very little is known about the mechanism(s) and messenger(s) by which these two enzyme systems communicate. The discovery of NO and its messenger roles in steroidogenic tissue raised the possibility that this simple molecule may be involved in modulating the activity of both enzymatic systems. In our study, we used the stressed animals to address the possible role of NO in control of steroidogenic and antioxidant enzymatic systems. Previous results from our laboratory demonstrated that immobilization stress induces an acute inhibition of testicular steroidogenesis that is mediated by the NO pathway [2]. Here, we show that the stress-induced inhibition of steroidogenesis is mimicked by elevating NO levels in vivo. When added in vitro, ISDN-mediated elevation in NO led to inhibition of testicular 3β-HSD and P450c17 activities.

This is in line with results by Pomerantz and Petelka [34], showing that the inhibitory effect of NO on testosterone production in mouse Leydig cells occurs, at least in part, via inhibition of P450c17 activity. Also, NO donors inhibit hCG-stimulated steroidogenesis in MA-10 murine Leydig cells, as well as the conversion of cholesterol to pregnenolone that is catalyzed by the cholesterol side-chain cleavage enzyme P450scc [35]. Regarding the mechanism of the action of NO on steroidogenic enzymes, it was suggested that this messenger inhibits the activity of cytochrome P450 enzymes by its binding to heme iron [36], or to the sulphhydryl groups in these enzymes [37]. Addition of SNAP to mouse Leydig cells did not affect the expression of mRNA for key P450-associated steroidogenic enzymes. This finding by Pomerantz and Petelka [34] further indicates that NO interacts directly with the Fe haem of the P450 enzymes, leading to profound inhibition of P450c17.

Our results also indicate that stress induced a significant inhibition of several antioxidant enzymes in the interstitial compartment of the rat testis. The same effects were provoked by intratesticular injections of ISDN. Also, intratesticular injections of L-NAME, an NOS-inhibitor, partially abolished a decrease in the activities of these enzymes in stressed animals. These results indicate that NO mediates the stress-induced inhibition of the antioxidant defence system. In accord with this, it is reported that SNAP and SNP inhibited catalase activity in a concentration-dependent manner when applied directly to the enzymatic assay [9]. Brown [38] has also suggested that NO rapidly and reversibly inhibits catalase, with a K_i of 0.18 μM. It is likely that NO can bind reversibly to ferric ion and that such a reaction is responsible for the inhibition of this enzyme [39]. Furthermore, SNAP inhibit bovine glutathione peroxidase in a dose- and time-dependent manner, with an IC_{50} of about 2 μM estimated after 1 h incubation. A longer exposure to NO donor irreversibly inactivates this enzyme [10].

However, not all antioxidant enzymes that we have studied were affected by the NO signalling pathway. The unchanged activity of superoxide dismutase in stressed- and ISDN-treated rats could be explained by superoxide anion scavenger activity of NOS [40]. Specifically, it is shown that NO reacts rapidly with superoxide anion yielding peroxynitrite [41], a strong oxidative agent. Together with NO, peroxynitrite could inhibit enzymes that depend on metal cofactors or oxidizable amino acids for their activity [42]. As discussed, NO could inhibit catalase [38,39] and glutathione peroxidase directly [10], and our results concerning these two enzymes could be explained by the increased production of NO and possibly peroxynitrite.

In conclusion, these results demonstrate the complexity of acute stress on steroidogenic tissue and the role of NO in this process. The stress-induced activation of NO synthases and elevation of intratesticular NO lead to inhibition of both steroidogenic and antioxidant enzymes. This in turn changes the contributions of these two enzymatic systems in control of free radicals and their secondary actions. Any inhibition of antioxidant enzymes in interstitial tissue could favour the prooxidant status, leading to the inhibition of steroidogenesis by free radicals, in addition to that mediated by NO. Also, inhibition of P450-dependent enzymes could result in an increase in production of reactive oxygen species, leading to additional inhibition of the antioxidative enzymes. Such a complexity in the interactions of antioxidant and steroidogenic enzyme systems may provide a rationale for the occurrence of strong antigonadal effects during acute stress.

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