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The involvement of nitric oxide in stress-impaired testicular steroidogenesis

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Abstract

The participation of the nitric oxide (NO) pathway in downregulation of testicular steroidogenesis in normal and stressed rats was investigated both in vivo and in vitro. In Leydig cells from normal animals, isosorbide dinitrate, an NO donor, decreased the human chorionic gonadotropin (CG)-stimulated and progesterone-derived androgen production. Also, the intratesticular injection of a precursor of NO, arginine (10 mg/testis), transiently decreased serum androgen levels and inhibited human CG-stimulated androgen production in acute testicular cultures. These effects were eliminated in rats cotreated with N^{ω}-nitro-L-arginine methyl ester (L-NAME) (2×600 μg/each testis). Acute immobilization stress (2 h) decreased serum androgen levels and inhibited human CG-stimulated androgen production in vitro. These effects were accompanied by a significant increase in nitrite, a stable oxidation product of NO, in testicular cultures. Bilateral intratesticular injection of L-NAME prevented the stress-induced decrease of human CG-stimulated androgen production, and significantly reduced the nitrite levels. These results implicate NO in normal and stress-impaired testicular steroidogenesis. © 1998 Elsevier Science B.V.

Keywords: Nitric oxide (NO); Stress, acute; Steroidogenesis, testicular

1. Introduction

Nitric oxide (NO), a free radical generated from L-arginine by nitric oxide (NO) synthase (EC 1.14.13.39), acts as an intracellular and/or intercellular messenger in a number of tissues (Moncada et al., 1991; Collier and Vallance, 1991). Within the endocrine tissues, NO was found to influence the secretion of hypothalamic and pituitary hormones (Kishimoto et al., 1996; Schmidt and Walter, 1994), insulin (Schmidt et al., 1992) and adrenal steroid hormones (Adams et al., 1992; Giordano et al., 1996). Estradiol secretion by human granulosa luteal cells in culture is also inhibited by NO (Van Voorhis et al., 1994), as well as aromatase activity, the latter due to acute direct inhibition of the aromatase and to sustained indirect inhibition in the expression of the messenger RNA for this enzyme (Snyder et al., 1996). Similar effects were observed in rat luteinized ovarian cells in vitro, suggesting a messenger role of this radical in luteal regression (Olson et al., 1996).

Recent studies have also indicated a potential role of this messenger in the control of testicular functions. Several lines of evidence are consistent with the paracrine mode of NO actions in testicles. NO synthase is localized in the vascular endothelium of the rat testis (Burnett et al., 1995). The constitutive type of NO synthase is present in the Leydig cells of human testis (Davidoff et al., 1995), which also express a soluble guanylyl cyclase/cyclic guanosine monophosphate pathway, including an active soluble guanylyl cyclase (Davidoff et al., 1997). Furthermore, the messenger RNA for an inducible NO synthase is expressed in these cells (Tatsumi et al., 1997) and in Sertoli cells as well (Stephan et al., 1995). Finally, cultured rat Leydig cells produce a low level of NO and interleukine-1β enhances its production (Tatsumi et al., 1997). The inhibitory effect of the NO pathway on testosterone production was observed in vivo (Adams et al., 1992, 1994). Also, the NO donors inhibit steroidogenesis in Leydig cells in vitro, presumably by inhibiting the conversion of cholesterol to pregnenolone by the choles-

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terol side-chain cleavage enzyme, P450scc (EC 1.14.15.6) (Punta et al., 1996). On the other hand, the NO synthase inhibitors, N^G-monomethyl-L-arginine and N^ω-nitro-L-arginine methyl ester (L-NAME), increase testosterone synthesis and decrease cyclic GMP accumulation in enriched Leydig cells (Welch et al., 1995).

Although this and other evidence implies that NO is a messenger involved in the control of hormonal secretion, the mechanism for activation of the NO signalling pathway and the conditions under which this pathway is activated have not yet been well characterized. The finding that immobilization stress increases the messenger RNA for neuronal NO synthase in the paraventricular nucleus of the hypothalamus (Calza et al., 1993), indicates a possible role of this radical in stress-mediated signals. A marked increase in neuronal NOS activity of the hypothalamo–pituitary–adrenal axis and adrenal medulla after immobilization stress has also been reported (Kishimoto et al., 1996). An earlier study indicates that subcutaneous injection of L-NAME significantly increases plasma corticosterone in rats, suggesting an inhibitory action of NO on corticosterone secretion (Adams et al., 1992). Thus, it is likely that NO represents an inhibitory rather than a facilitatory factor in the control of steroidogenesis, and that this effect of NO is found throughout steroidogenic tissue. We extended these studies by addressing the involvement of the NO signalling system in the downregulation of testicular steroidogenesis in normal rats and in rats exposed to immobilization stress. Results of our in vivo and in vitro studies indicate that NO plays a regulatory role in human chorionic gonadotropin (CG)-stimulated testosterone secretion in unstressed and in stress-impaired testicular steroidogenesis.

2. Material and methods

2.1. Chemicals

Antitestosterone serum (No. 250) was kindly supplied by G.D. Niswender (Colorado State University, Fort Collins, CO). Medium 199 was purchased from GIBCO Laboratories (Gaithersburg, MD), human CG (Pregnyl, 3000 IU/mg) from Organon and [(1,2,6,7^{3}H(N)]testosterone was obtained from New England Nuclear. All other chemicals were from Sigma Chemical (St. Louis, MO).

2.2. Animals

Experiments were performed in adult male Wistar rats (about 250 g) 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. The animals were handled daily for one week prior to experiments. The immobilization stress was done according to Kvetnansky et al. (1970). All experiments were approved by the Local Animal Ethical Committee of the University of Novi Sad, and were conducted in accordance with principles and procedures of the NIH Guide for Care and Use of Laboratory Animals.

2.3. In vitro steroidogenesis

Testes from control and experimental rats were removed quickly, decapsulated and incubated individually in plastic vials containing 5 ml of 0.1% bovine serum albumin in medium 199 with or without 20 ng/ml human CG. Incubations were carried out at 34°C for 3 h in a 95% O_2–5% CO_2 atmosphere. The medium was decanted and centrifuged at $1500 \times g$ for 10 min and the supernatant was stored at −20°C until assayed for testosterone content. Androgen production was also studied in a crude suspension of Leydig cells. Testes removed from normal rats immediately after killing, were decapsulated and dissociated with collagenase (1.2 mg/ml) as previously described (Kovačević and Sarač, 1993). Trypan blue exclusion was used to asses cell viability (over 90%). Aliquots (10^6 cells) of crude suspensions of Leydig cells were added to plastic tubes containing a maximal concentration of human CG (10 ng/tube) and isosorbide dinitrate (0, 0.1, 0.5, 5 mM/tube) in medium 199 containing 0.1% bovine serum albumin, so that the final incubation volume was 0.5 ml. Parallel incubations were performed with 2 μM progesterone added as a steroid substrate. Following incubation for 2 h in a shaking water bath (34°C at 100 cycles/min, under an atmosphere of 95% O_2–5% CO_2) all tubes were centrifuged for 10 min at $1500 \times g$ and samples of the supernatants were stored at −20°C prior to measurement of androgen content.

2.4. Assays for testosterone and nitrite

Testosterone in the incubation medium and serum levels were measured by radioimmunoassay. Since the antitestosterone serum has a high cross-reactivity with dihydrotestosterone, assay values are referred to as testosterone + dihydrotestosterone levels. All samples from a single experiment were run in one assay. The assay sensitivity is 6 pg/tube and the intra-assay and inter-assay coefficients of variation are 5–8% and 7.5% respectively. For nitrite measurements, aliquots of 1 ml were mixed with an equal volume of Greiss reagent (1% sulphanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid). The mixture was incubated at room temperature for 10 min and absorbance was measured at 546 nm (Green et al., 1982). Nitrite concentrations were determined from a standard curve derived from increasing concentrations of sodium nitrite.

Table 1
Effects of intratesticular injection of arginine and L-NAME individually and in combination on serum testosterone levels

Treatment	Testosterone + dihydrotestosterone (ng/ml)	Number of animals per group
Control	4.44 ± 0.62	12
Arginine	2.65 ± 0.38[a]	11
L-NAME	3.17 ± 0.33	11
Arginine + L-NAME	5.14 ± 0.9	11

For experimental design see caption of Fig. 1. Data shown are means ± S.E.M.
[a]Significant at $P < 0.05$ vs. controls.

2.5. Calculations

The nonparametric Mann–Whitney test was used for statistical analysis of results with $P < 0.05$ or higher as significant difference.

3. Results

3.1. Effects of intratesticular injection of arginine and L-NAME on testosterone production

To examine the in vivo effects of arginine, a substrate for NO synthase, and L-NAME, a NO synthase inhibitor, both drugs were injected bilaterally in the testes, and the animals were divided into four groups. The first group was injected with arginine (10 mg of arginine in 50 μl of saline/testis once), the second group was injected with L-NAME (600 μg of L-NAME in 50 μl of saline/testis), at the beginning of experiment and 60 min later. The third group was initially injected with both arginine (10 mg/testis) and L-NAME (600 μg/testis), followed by an injection of L-NAME (600 μg/testis) 60 min later. The control animals were injected twice with saline (0.1 ml). Each group was killed by decapitation 2 h after beginning of the experiment, trunk blood was collected, testes from control and experimental rats were removed quickly, and human CG-stimulated testosterone production was analyzed in decapsulated testes during a 3-h incubation.

As shown in Table 1, the bilateral injection of arginine induced a significant fall in serum testosterone levels compared to those in the control rats. No decrease in serum testosterone concentrations was observed after combined injections of L-NAME + arginine, suggesting that L-NAME blocks the arginine-induced fall in testosterone production. In parallel to this, human CG-stimulated testosterone production was reduced in cultures from arginine-treated animals (Fig. 1). The intratesticular injection of L-NAME normalized the arginine-evoked suppression of human CG-stimulated androgen production (Fig. 1). To examine the time course of the arginine-induced downregulation of testosterone production, animals were injected into each testis with arginine (10 mg/testis), L-NAME (600 μg/testis), or saline and killed 30, 60, or 120 min after the injection. Thereafter human CG-stimulated testosterone production in vitro was measured, and the results are presented in Fig. 2. Arginine-treated animals had a significant decrease in human CG-stimulated testosterone production 120 min after injection. In contrast, a transient increase in human CG-stimulated testosterone production was observed in cultures from L-NAME-treated animals killed 60 min after injection.

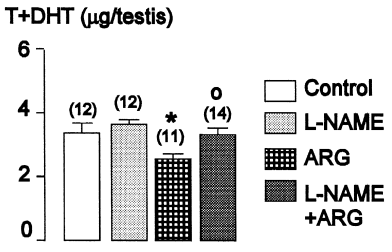

Fig. 1. Effects of intratesticular injections of L-NAME and arginine, individually or in combination, on in vitro human CG-stimulated testosterone production. L-NAME was injected into each testis (600 μg/testis) at the beginning and after 1 h of the experimental period. Arginine (ARG) was injected into each testis (10 mg/testis) only at the beginning of the experiment. In combined injections, L-NAME, 2×600 μg/testis and arginine, 1×10 mg/testis were injected. Animals were killed 2 h after the beginning of treatment. Testes from control and treated rats were incubated for 3 h in the presence of human CG (hCG; 20 ng/ml). T+DHT is testosterone+dihydrotestosterone. Columns represent means ±S.E.M. Number in parenthesis refers to the number of testes. Significance: * $P < 0.05$ vs. controls; $^{o}P < 0.02$ vs. arginine-treated group.

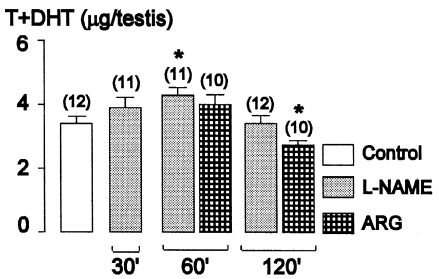

Fig. 2. Time course of in vitro human CG-stimulated testosterone production after in vivo intratesticular injection of L-NAME or arginine. Saline, L-NAME (600 μg/testis) or arginine (ARG; 10 mg/testis) was injected into each testis. All groups of animals were killed at the same time. Injections of L-NAME and arginine were given 30, 60 and 120 min before killing, while controls received saline 120 min before killing. Testes from controls, L-NAME-, or arginine-treated rats were incubated for 3 h in the presence of human CG (20 ng/ml). Columns represent means ± S.E.M. Number in parenthesis refers to the number of testes. Significance: * $P < 0.05$ vs. controls. Other abbreviations as in Fig. 1.

Table 2
Effects of immobilization stress and L-NAME treatment on serum testosterone levels

Treatment	Testosterone + dihydrotestosterone (ng/ml)	Number of animals per group
Control	4.85 ± 0.70	7
Immobilization stress	2.19 ± 0.28[a]	8
L-NAME	6.41 ± 1.51	9
Immobilization stress + L-NAME	2.48 ± 0.48[a]	7

For experimental design see Section 3. Data shown are means ± S.E.M.
[a]Significant at $P < 0.05$ vs. controls.

Thus, both in vivo and in vitro testosterone production are suppressed by arginine and this downregulation of steroidogenesis is prevented by L-NAME, which itself has a transient stimulatory effect. Since arginine is a substrate for NO synthase and L-NAME, a specific NO synthase inhibitor, these results strongly support a role for the NO signalling system in testicular steroidogenesis.

3.2. Effects of immobilization stress on steroidogenesis and nitrite levels

We have reported previously (Marić et al., 1996) that immobilization stress is accompanied by a decrease in androgen production. In order to investigate the possible involvement of NO in stress-induced decreases of testicular steroidogenesis, the animals were subjected to acute (2 h) immobilization, accompanied by two intratesticular injections of L-NAME (600 μg/testis) or saline, one at the beginning of the immobilization period and one 60 min later. Control animals were treated with L-NAME and saline at the same time as stressed rats. At the end of the immobilization period, all animals were killed, serum samples were collected, and testes were quickly removed for analysis of in vitro basal and human CG-stimulated androgen production and nitrite levels.

Immobilization induced a significant decrease in serum testosterone concentrations compared to the control concentrations (Table 2). In parallel to this, immobilization stress was associated with a significant decrease in basal and human CG-stimulated testosterone production in vitro (Fig. 3 bottom panels). Bilateral intratesticular injection of L-NAME prevented the stress-induced decrease of human CG-stimulated, but not basal, androgen production. Also, bilateral injection of L-NAME did not alter serum testosterone levels in stressed rats. These results indicate a parallelism between the actions of arginine and immobilization stress on androgen production in vivo and in vitro. Also consistent with the hypothesis that an NO pathway is involved in the stress-induced inhibition of steroidogenesis, is the fact that downregulation of human CG-stimulated testosterone secretion in vitro was accompanied by a significant increase in nitrite levels, and this action was partially inhibited by L-NAME (Fig. 3, upper panels).

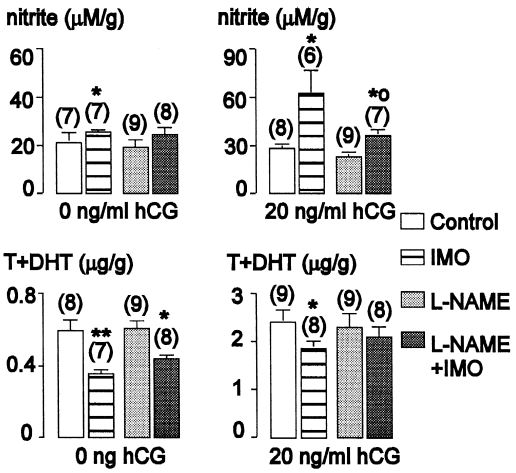

Fig. 3. Effect of immobilization stress and intratesticular L-NAME treatment on nitrite levels and basal and human CG-stimulated testosterone production. Saline or L-NAME (600 μg/testis) was injected into each testis at the beginning and 1 h into the immobilization period (IMO), and animals were killed at the end of the 2-h immobilization session. Testes were incubated for 3 h in the absence, or in the presence, of human CG (20 ng/ml). Columns represent means ± S.E.M. Number in parenthesis refers to the number of testes. Significance: * $P < 0.05$ vs. controls, $^{o}P < 0.05$ vs. immobilized group of rats. Other abbreviations as in Fig. 1.

In the experiments described above, the steroidogenic potency of the decapsulated testes was evaluated immediately following the treatments. Although the role of NO in

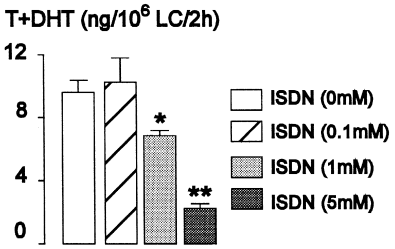

Fig. 4. Effect of isosorbide dinitrate on in vitro human CG-stimulated testosterone production of Leydig cells in suspension. Freshly dispersed Leydig cells (10^6/tube) were incubated for 2 h in the presence of 10 ng human CG and increasing concentrations (0–5 mM) of isosorbide dinitrate (ISDN). Data shown (mean ± S.E.M.) are for seven assay replicates. Significance: * $P < 0.02$; ** $P < 0.009$ vs. controls. Other abbreviations as in Fig. 1.

3.3. Effects of isosorbide dinitrate on in vitro steroidogenesis

In the experiments described above, the steroidogenic potency of the decapsulated testes was evaluated immediately following the treatments. Although the role of NO in

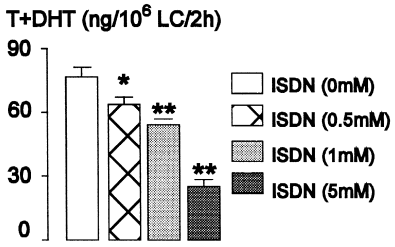

Fig. 5. Effect of isosorbide dinitrate on in vitro testosterone production by rat Leydig cells in the presence of progesterone as a substrate. Leydig cells were incubated for 2 h in the presence of 2 μM progesterone and increasing concentration (0–5 mM) of isosorbide dinitrate. Data shown (mean ± S.E.M.) are for seven assay replicates. Significance: $* P < 0.05$; $** P < 0.001$ vs. controls. Other abbreviations as in Fig. 1Fig. 4.

such a system was clearly shown, this procedure does not distinguish the direct from the indirect effects of NO on steroidogenesis. To confirm the hypothesis that NO is a de facto messenger for Leydig cell function, we further analyzed the effects of increasing concentrations of isosorbide dinitrate (an organic nitrate that produces NO) on in vitro human CG-stimulated testosterone production by a suspension of Leydig cells (Fig. 4). In concentrations of 0.1 to 5 mM, isosorbide dinitrate significantly inhibited human CG-stimulated testosterone production compared to that in the controls, with an IC_{50} of about 3 mM. Fig. 4 shows that at 1 and 5 mM concentrations, isosorbide dinitrate induced 28% and 76% inhibition of agonist-induced testosterone production, respectively, whereas it was ineffective in a concentration of 100 μM. The inhibitory effect of isosorbide dinitrate on testosterone production by Leydig cells in suspension was also observed in the presence of progesterone as a substrate (Fig. 5). In this experiment, testosterone production was significantly lower (16%, 29% and 67%, respectively) at all three concentrations as compared to control values. The inhibitory effect of isosorbide dinitrate on Leydig cell steroidogenesis was not associated with cytotoxicity, since cell viability was not altered in the presence of isosorbide dinitrate after 2 h of incubation.

4. Discussion

Steroidogenesis in Leydig cells is controlled by hypothalamo–pituitary hormones, as well as by a variety of other factors produced in the testes that acts in an autocrine and/or paracrine fashion (Saez, 1994). Recent investigations have focused on central and peripheral factors mediating the inhibitory actions on androgen production and the androgen-controlled cellular functions. For example, endogenous opioid peptides are important messenger molecules involved in local paracrine control of testicular function, especially in mediating stress-induced changes in testicular steroidogenesis (Akinbami et al., 1994; Kostić et al., 1997). A number of observations indicate a possible role of the NO pathway in the downregulation of androgen production (see Section 1). However, it was not clear what represents the signal for activation of the testicular NO pathway. Does this messenger act directly on Leydig cells, or indirectly, through another locally produced signalling molecule(s)? Our results indicate that stress is an efficient mechanism of activation of the testicular NO pathway, and that NO itself is a sufficient inhibitor of human CG-stimulated androgen production. The results further suggest that this system is also operative in control animals, and thus that NO may be a negative-feedback signalling molecule in the homeostatic control of testicular steroidogenesis.

Stress is a complex physiological situation that leads to activation of the hypothalamo–pituitary–adrenal system as well as activation of several other central and peripheral systems (Rivier and Rivest, 1991). It is well documented that plasma levels of glucocorticoids increase during stress (Srivastava et al., 1993; Orr and Mann, 1992) and that adrenal glucocorticoids may mediate some of the effects of stress on androgen production (Orr and Mann, 1992). The inhibition of testosterone production after exposure to acute (2–3 h) immobilization stress is not associated with a concomitant decrease in serum gonadotropin concentrations (Orr et al., 1994; Marić et al., 1996; Kostić et al., 1997), nor with the reduction in binding capacity and affinity of gonadotropin receptors on Leydig cells (Orr et al., 1994). Thus, the effect of acute stress is probably mediated at a postreceptor site(s), i.e., by inhibition of the steroidogenic pathway of the Leydig cells. The stress-induced reduction in testosterone production appears to be related to inhibition of the activities of steroidogenic enzymes (Srivastava et al., 1993; Orr et al., 1994; Marić et al., 1996), raising the possibility that NO could be such a factor acting directly on Leydig cell steroidogenesis.

In our study, the possible involvement of NO in stress-mediated inhibition of testicular steroidogenesis was investigated by measuring the levels of nitrite, a stable oxidation product of NO, in the incubation media obtained from the testes of rats previously exposed to acute immobilization stress and of stressed rats treated concomitantly with L-NAME. Consistent with the suggestion that increased NO production is involved in the impaired testicular steroidogenesis of stressed rats, a significant elevation in nitrite levels was accompanied by inhibition of human CG-stimulated testosterone production and this elevation was reduced by L-NAME. The present results, however, show that L-NAME treatment of stressed rats did not completely reverse the accompanying increase in nitrite levels. Since L-NAME shows selectivity towards the constitutive isoforms of NO synthase rather than to the inducible NO synthase (Lambert et al., 1992), the latter form of the enzyme may be involved in the stress-mediated inhibition of steroidogenesis. Accordingly, exposure of rat Leydig cells to interleukin-1β markedly increased the NO production and expression of inducible NO synthase messenger

RNA in these cells as early as 3 h after the addition of the cytokine (Tatsumi et al., 1997).

The possibility of involvement of NO in downregulation of testicular steroidogenesis is indirectly supported by results of our in vivo experiments. In these experiments, the bilateral intratesticular injection of NO synthase substrate arginine decreased serum testosterone levels, as well as the human CG-stimulated androgen production in cultures obtained immediately after the experiment. This is consistent with a report that intraperitoneal injection of the arginine methyl ester suppresses testosterone secretion (Adams et al., 1994). As in the stressed rats, the concomitant intratesticular administration of L-NAME counteracted the arginine-induced inhibition of serum testosterone levels and in vitro human CG-stimulated testosterone production, while L-NAME itself induced a significant but transient increase of human CG-stimulated testosterone production. Furthermore, subcutaneous injection of L-NAME increased serum and testicular interstitial testosterone levels without altering serum luteinizing hormone values (Adams et al., 1992). These results suggest a role of NO pathway in the control of androgen secretion in both normal animals and those stressed by immobilization.

The results also showed that addition of isosorbide dinitrate, an organic nitrate that produces NO, to a suspension of Leydig cells inhibits human CG-stimulated and progesterone-supported testosterone production. The inhibitory effect of isosorbide dinitrate is not a consequence of the cytotoxicity of this compound in the concentrations used in our experiments, since the viability of cells was not altered after a 2-h incubation with isosorbide dinitrate. These observations are in accordance with data presented by other authors, showing an inhibitory effect of NO donors on in vitro steroidogenesis (Punta et al., 1996) and increased basal and human CG-stimulated testosterone production by rat Leydig cell cultures incubated with NO synthase inhibitors (Welch et al., 1995). NO exerts many of its functions by reacting with methyl- and thiol-containing proteins, which can result in both activation and inhibition of the target protein (Stamler, 1994). It has been suggested that cytochrome P450 enzymes are inhibited by NO binding to its heme iron (Quaroni et al., 1996), or by reacting with the sulfahydryl group of cysteins present in these enzymes (Snyder et al., 1996). It is probable, therefore, that NO reduces the activity of steroidogenic enzymes containing cytochrome P450. According to Punta et al. (1996), the conversion of cholesterol to pregnenolone, which is catalyzed by the enzyme P450scc, is at least one step that is inhibited by NO. These results were obtained by incubation of MA-10 Leydig cells in the presence of pregnenolone or 22R-OH cholesterol. However, in these cells the final product of steroidogenesis is progesterone, since 17α-hydroxylase, 17,20 lyase (P450c17; EC 1.14.99.9) is not present. In our experiments, it was most probably the inhibition of P450c17 activity in Leydig cells that was demonstrated, since progesterone-supported testosterone production was inhibited by the addition of isosorbide dinitrate. However, inhibition of P450c17 does not exclude a possible inhibition of P450scc during human CG-stimulated testosterone production by a suspension of Leydig cells. Further experiments with isolated Leydig cells are necessary to find possible steps in the steroidogenic pathway which could be inhibited by NO.

In summary, these results provide what is, to our knowledge, the first demonstration of the involvement of NO in impaired steroidogenesis after exposure of rats to acute immobilization stress. The data also suggest that NO is involved in the local regulation of testicular steroidogenesis of normal rats. Further experiments should clarify which extracellular signalling pathway(s) is involved in activating the testicular NO system under normal conditions and during acute stress. Also, additional experiments are needed to identify the steroidogenic enzymes inhibited by NO.

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References

Adams, M.L., Nock, B., Truong, R., Cicero, T.J., 1992. Nitric oxide control of steroidogenesis: endocrine effects of N^G-nitro-L-arginine and comparison to alcohol. Life Sci. 50, PL35–PL40.

Adams, M.L., Meyer, E.R., Sewing, B.N., Cocero, T.J., 1994. Effects of nitric oxide-related agents on rat testicular function. J. Pharmacol. Exp. Ther. 269, 230–237.

Akinbami, M.A., Taylor, M.F., Collins, D.C., Mann, D.R., 1994. Effect of a peripheral and central acting opioid antagonist on the testicular response to stress in rats. Neuroendocrinology 59, 343–348.

Burnett, A.L., Ricker, D.D., Chamness, S.L., Maguire, M.P., Crone, J.K., Bredt, D.S., Snyder, S.H., Chang, T.S.K., 1995. Localization of nitric oxide synthase in the reproductive organs of the male rat. Biol. Reprod. 52, 1–7.

Calza, L., Giardino, L., Ceccatelli, S., 1993. NOS mRNA in the paraventricular nucleus of young and old rats after immobilization stress. NeuroReport 4, 627–630.

Collier, J., Vallance, P., 1991. Physiological importance of nitric oxide. Br. Med. J. 302, 1289–1290.

Davidoff, M.S., Middendorff, R., Mayer, B., Holstein, A.F., 1995. Nitric oxide (NOS-I) in Leydig cells of the human testis. Arch. Histol. Cytol. 58, 17–30.

Davidoff, M.S., Middendorff, R., Mayer, B., de Vente, J., Koesling, D., Holstein, A.F., 1997. Nitric oxide/cGMP pathway components in the Leydig cells of the human testis. Cell. Tissue Res. 287, 161–170.

Giordano, M., Vermeulen, M., Trevani, A.S., Dran, G., Andonegui, G., Geffner, J.R., 1996. Nitric oxide synthase inhibitors enhance plasma levels of corticostrone and ACTH. Acta Physiol. Scand. 157, 259–264.

Green, L.C., Wagner, A.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. Anal. Biochem. 126, 131–138.

Kishimoto, J., Tsuchiya, T., Emson, P.C., Nakayama, Y., 1996. Immobilization-induced stress activates neuronal nitric oxide synthase (nNOS)

mRNA and protein in hypothalamic–pituitary–adrenal axis in rats. Brain Res. 720, 159–171.

Kostić, T., Andrić, S., Kovačević, R., Marić, D., 1997. The effect of opioid antagonists in local regulation of testicular response to acute stress in adult rats. Steroids 62, 703–708.

Kovačević, R., Sarać, M., 1993. Bromocriptine-induced inhibition of hydroxylase/lyase activity of adult rat Leydig cells. J. Steroid Biochem. Mol. Biol. 46, 841–845.

Kvetnansky, R., Weise, V.K., Kopin, I.J., 1970. Elevation of adrenal tyrosine hydroxylase and phenylethanolamine-*N*-methyl transferase by repeated immobilization of rats. Endocrinology 87, 744–749.

Lambert, L.E., French, J.F., Whitten, J.P., Baron, B.M., McDonald, I.A., 1992. Characterization of cell selectivity of two novel inhibitors of nitric oxide synthesis. Eur. J. Pharmacol. 216, 131–134.

Marić, D., Kostić, T., Kovačević, R., 1996. Effects of acute and chronic immobilization stress on rat Leydig cell steroidogenesis. J. Steroid Biochem. Mol. Biol. 58, 351–355.

Moncada, S., Palmer, R.M.J., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109–142.

Olson, L.M., Jones-Burton, C.M., Jablonka-Shariff, A., 1996. Nitric oxide decreases estradiol synthesis of rat luteinized ovarian cells: possible role for nitric oxide in functional luteal regression. Endocrinology 137, 3531–3539.

Orr, T.E., Mann, D.R., 1992. Role of glucocorticoids in the stress-induced suppression of testicular steroidogenesis in adult male rat. Horm. Behav. 26, 350–363.

Orr, T.E., Taylor, M.F., Bhattacharyya, A.K., Colins, D.C., Mann, D.R., 1994. Acute immobilization stress disrupts testicular steroidogenesis in adult male rats by inhibiting the activities of 17α-hydroxylase and 17,20-lyase without affecting the binding of LH/hCG receptors. J. Androl. 15, 302–308.

Punta, K.D., Charreau, E.H., Pignataro, O.P., 1996. Nitric oxide inhibits Leydig cell steroidogenesis. Endocrinology 137, 5337–5343.

Quaroni, L., Reglinski, J., Wolf, R., Smith, W.E., 1996. Interaction of nitrogen monoxide with cytochrome P-450 monitored by surface-enhanced resonance Raman scattering. Biochim. Biophys. Acta 1296, 5–8.

Rivier, C., Rivest, S., 1991. Effect of stress on the activity of the hypothalamic–pituitary–gonadal axis: peripheral and central mechanisms. Biol. Reprod. 45, 523–532.

Saez, J.M., 1994. Leydig cells: endocrine, paracrine and autocrine regulation. Endocr. Rev. 15, 574–626.

Schmidt, H.H.W., Walter, U., 1994. NO at work. Cell 78, 919–925.

Schmidt, H.H.W., Warner, T.D., Ishii, K., Sheng, H., Murad, F., 1992. Insulin secretion from pancreatic β cells caused by L-arginine-delivered nitrogen oxides. Science 255, 721–723.

Snyder, G.D., Holmes, R.W., Bates, J.N., Van Voorhis, B.J., 1996. Nitric oxide inhibits aromatase activity: mechanisms of action. J Steroid Biochem. Mol. Biol. 58, 63–69.

Srivastava, R.K., Taylor, M.F., Mann, D.R., 1993. Effect of immobilization stress on plasma luteinizing hormone, testosterone, and corticosterone concentrations and 3β-hydroxysteroid dehydrogenase activity in the testis of adult rats. Proc. Soc. Exp. Biol. Med. 204, 231–235.

Stamler, J.S., 1994. Redox signalling nitrosylation and related target interactions of nitric oxide. Cell 78, 931–936.

Stephan, J.P., Guillemois, C., Jegou, B., Bauche, F., 1995. Nitric oxide production by Sertoly cells in response to cytokines and lipopolysaccharide. Biochem. Biophys. Res. Commun. 213, 218–224.

Tatsumi, N., Fujisawa, M., Kanzaki, M., Okuda, Y., Okada, H., Arakawa, S., Kamodona, S., 1997. Nitric oxide production by cultured rat Leydig cells. Endocrinology 138, 994–998.

Van Voorhis, B.J., Dunn, M.S., Snyder, G.D., Weiner, C.P., 1994. Nitric oxide: an autocrine regulator of human granulosa–luteal cell steroidogenesis. Endocrinology 135, 1799–1806.

Welch, C., Watson, M.E., Poth, M., Hong, T., Francis, G.L., 1995. Evidence to suggest nitric oxide is an interstitial regulator of Leydig cell steroidogenesis. Metabolism 44, 234–238.