Role of the testis in the response of the pituitary-testicular axis to nitric oxide-related agents

F Gaytán1, C Bellido2, R Aguilar2, C Morales3, N van Rooijen4 and E Aguilar2

Departments of 1Cell Biology, 2Physiology and 3Pathology, School of Medicine, University of Córdoba, Córdoba, Spain and 4Department of Cell Biology, Free University, Amsterdam, The Netherlands

(Correspondence should be addressed to F Gaytán, Department of Cell Biology, School of Medicine, 14071 Córdoba, Spain)

Abstract
Nitric oxide (NO) is generated from the guanidine group of L-arginine by NO synthases (NOS) in a wide variety of tissues, including endocrine organs. In order to discriminate between central and local effects of NO-related agents on the pituitary-testicular axis, adult rats were injected intraperitoneally with 1 g/kg body weight (bw) L-arginine methyl ester (L-AME, an exogenous substrate of NOS), 0.5 mg/kg bw sodium nitroprusside (SNP, an NO donor) or intratesticularly with 2 mg/testis L-AME, 2 μg/testis SNP or 25 μl vehicle, and killed at 60 or 120 min after treatment. Both intraperitoneal and intratesticular administration of L-AME had the same effects: a decrease in the serum concentrations of LH and testosterone and in those of testosterone in the testicular interstitial fluid. However, treatment with SNP was more effective when given intratesticularly, inducing a decrease in serum and interstitial fluid testosterone concentrations, without significant changes in LH concentrations. Furthermore, when rats were injected intraperitoneally with 4 mg L-AME (the same dose as that given intratesticularly), serum LH concentrations were not changed. In addition, L-AME administration was not effective in modifying serum LH concentrations in castrated rats. To test the possible role of Leydig cells, the effects of systemic administration of L-AME were studied in rats depleted of Leydig cells by treatment with ethylene dimethane sulphonate. In these animals L-AME significantly decreased serum LH concentrations. To study the role of macrophages in this system, rats depleted of testicular macrophages by the liposome-suicide approach were injected intraperitoneally (1 g/kg bw) or intratesticularly (2 mg/testis) with L-AME or vehicle, 10 days after macrophage depletion, and killed at 120 min after treatment. The effects of L-AME on serum LH concentrations were blocked when the drug was administered intratesticularly.

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Introduction
Nitric oxide (NO) is a widely distributed intra- and intercellular messenger involved in many physiological processes. NO is generated from L-arginine by NO synthases (NOS) through the L-arginine–NO pathway (1). A growing body of evidence indicates that NO is involved in the regulation of endocrine processes, such as hypothalamic-pituitary-adrenal and -gonadal axis functions (2). Treatment with the NOS competitive inhibitors nitro-L-arginine, N⁶-monomethyl-L-arginine or N⁷-nitro-L-arginine methyl ester (L-NAME) increases testosterone concentrations in both serum and interstitial fluid (3–5). Furthermore, treatment with NO donors such as isosorbide dinitrate (ISDN) or sodium nitroprusside (SNP) or with the NOS substrates L-arginine or L-arginine methyl ester (L-AME) decreases both luteinizing hormone (LH) and testosterone concentrations (4). The mechanisms proposed to explain the role of NO on the hypothalamic-pituitary-testicular axis involve effects at different levels (6). At the hypothalamic level, most studies have reported stimulatory effects (7–9), although inhibitory effects have been also reported (10). At the pituitary level, NO inhibits LH secretion (11). Finally, at the testicular level, NO has been reported to inhibit Leydig cell steroidogenesis (4, 5, 12).

Testicular androgens are the main regulators of pituitary LH secretion. In the last few years, several studies have reported that testicular non-androgenic factors are also involved in the regulation of LH secretion (13–15). In the testis, NO can be produced by several cell types closely related to androgen-producing cells such as endothelial cells, autonomic nerve endings, macrophages, mast cells or Leydig cells themselves (5, 16, 17). Previous studies have found that NO-related agents modify serum LH concentrations, although the mechanisms involved and the role of the testis are not clear.

Due to the wide effects of NO-related agents when administered systemically (for instance, on the vascular
system and at the different levels of the hypothalamic-pituitary-testicular axis), the effects observed in vivo could be the consequence of antagonistic actions at different levels. It is therefore of interest to study the effects of local administration of low doses of NO-related agents, thus circumventing or minimizing the systemic actions of these drugs. In this context, we have compared the effects of the systemic and local administration of NO-generating agents, in order to analyse the possible role of the l-arginine–NO pathway on the regulation of LH secretion.

Materials and methods

Animals and chemicals

Adult Wistar male rats (body weight (bw) 280–300 g) were used. The animals were maintained under controlled conditions of light (14 h lights/10 h darkness) and temperature (22 ± 1 °C). An exogenous NOS substrate L-AME and an NO donor SNP were used (Sigma Chemical Co., St Louis, MO, USA). Ethylene dimethane sulphonate (EDS) was prepared following previously described methods (18). Liposomes containing dichloromethylene diphosphonate (Cl2MDP) were prepared as previously reported (19). Cl2MDP was a kind gift from Boehringer Mannheim (Germany) and phosphatidylcholine (Lipoid EPC) from Lipoid KG (Germany). Anti-macrophage ED1 and ED2 antibodies were a kind gift from Dr C D Dijkstra (Amsterdam, The Netherlands).

Experimental designs

Experiment 1 This experiment was designed to compare the systemic and local effects of an exogenous NOS substrate (L-AME) and an NO donor (SNP) on the pituitary-testicular axis. For this, animals were injected intraperitoneally with 1 g/kg bw L-AME, 0.5 mg/kg bw SNP or vehicle and killed at 60 or 120 min after treatment (ten animals per group). These doses have previously been found to be effective (4, 20). Additional animals were injected intratesticularly under light ether anaesthesia, with a 30 gauge needle in both testes with 2 mg/testis L-AME, 2 μg/testis SNP or vehicle (25 μl) and killed at 60 or 120 min after treatment (ten animals per group). Trunk blood and interstitial fluid were collected (21) and stored until assayed. The serum follicle-stimulating hormone (FSH), LH and testosterone concentrations, as well as the testosterone concentration in the testicular interstitial fluid (TIF) were measured.

Experiment 2 This experiment was designed to analyse the role of the testis in the effects of L-AME on LH secretion. In order to eliminate the possibility that the effects of intratesticularly administered L-AME were due to diffusion of the drug to the systemic circulation, rats were injected intraperitoneally with 4 mg/rat L-AME (the same dose as that given intratesticularly) or vehicle. In addition, the effects of L-AME were studied in castrated or sham-operated rats. Operations were performed through a scrotal incision under light ether anaesthesia. After 10 days rats were injected intraperitoneally with 1 g/kg bw L-AME or vehicle and killed at 120 min (ten animals per group). Trunk blood was collected and stored at −20 °C until assayed for LH and testosterone concentrations.

Experiment 3 In order to analyse the possible role of Leydig cells in the effects of L-AME on LH secretion, and to eliminate the possibility that the results found in castrated animals were due to the high LH concentrations present in these animals rather than to the lack of the testis, the effects of L-AME on serum LH concentrations were studied in rats depleted of Leydig cells by administration of EDS. Animals were injected intraperitoneally with 80 mg/kg bw EDS or vehicle (DMSO : water, 1 : 3). Ten days later blood was obtained by jugular puncture under light ether anaesthesia and serum was stored to determine basal LH and testosterone concentrations. Thereafter, the animals were injected with 1 g/kg bw L-AME or vehicle and killed at 120 min. Trunk blood was collected and serum stored at −20 °C until assayed for LH and testosterone concentrations. Some testes were fixed for 24 h in Bouin–Hollande’s fluid, embedded in paraffin, cut at 5 μm, stained with haematoxylin and eosin and viewed under light microscopy to confirm the absence of Leydig cells in EDS-treated rats.

Experiment 4 To analyse the possible role of testicular macrophages in this system, the effects of L-AME on LH and testosterone secretion were studied in rats depleted of testicular macrophages by administration of liposome-entrapped Cl2MDP. Animals were injected intratesticularly with 100 μl Cl2MDP-liposomes or the same volume of 0.9% NaCl in both testes. Ten days later animals were injected intraperitoneally with 1 g/kg bw or intratesticularly with 2 mg/testis L-AME or vehicle and killed at 120 min. Trunk blood was collected and serum stored at −20 °C until assay for LH and testosterone concentrations. Some testes were carefully dissected and processed for immunohistochemistry. to assess the effectiveness of macrophage depletion.

Immunohistochemistry

The testes from rats injected intratesticularly with NaCl or Cl2MDP-liposomes 10 days before were decapsulated, covered with OCT embedding medium, frozen in liquid nitrogen and stored at −70 °C until sectioned. Cryostat sections (5 μm thick) were cut, air-dried for 10 min and fixed in cold acetone (4 °C) for 10 min and processed for immunohistochemistry. Endogenous peroxidase was inhibited by incubation in 0.2% hydrogen peroxide in...
methanol for 30 min. After rehydration in graded ethanol, the sections were rinsed in PBS, blocked with 10% normal rabbit serum for 2 h and incubated overnight with the primary antibodies (ED1 or ED2 or both) diluted 1:600 in PBS. The sections were then processed according to the avidin–biotin–peroxidase (ABC) method (22). Briefly, sections were treated sequentially with rabbit anti-mouse IgG–biotin conjugate (Sigma, Poole, Dorset, UK; 1:1000 for 1 h at room temperature) and ABC complex (Vector Labs, Burlingame, CA, USA; 1 h at room temperature). Tissue-bound peroxidase was visualized by incubation in 0.03% diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, USA), 0.01% hydrogen peroxide in 0.1 M Tris buffer (pH 7.6 for 1 min). Sections were counterstained with 1% neutral red.

RIAs

Serum concentrations of LH and FSH were measured in duplicate in 25 μl samples using double-antibody RIA kits supplied by the NIH (Bethesda, MD, USA) according to previously described methods (23). Rat LH-I-9 and FSH-I-8 were labelled with 125I by the chloramine T method (24). LH and FSH concentrations were expressed as μg/l serum of the reference preparation LH-rat-RP-3 and FSH-rat-RP-2. All samples were analyzed in the same assay, and the intra-assay coefficients of variation were 7% and 6% for LH and FSH respectively. The sensitivity of the assays were 7.5 and 20 pg/tube for LH and FSH respectively.

Statistical analyses were carried out by ANOVA and Tukey’s test for multiple comparisons among means or Student’s t-test when two means had to be compared. Significance was considered at the 0.05 level.

Results

Effects of intraperitoneal versus intratesticular treatment with SNP or L-AME

The animals treated intraperitoneally with L-AME showed decreased serum concentrations of LH and testosterone at both 60 and 120 min, whereas the concentrations of testosterone in the TIF were decreased only at 60 min after treatment (Fig. 1). Treatment with SNP had slight effects and affected only serum testosterone concentrations that were decreased at 120 min after treatment.

Intratesticular treatment with L-AME induced a decrease in the serum concentration of LH at 120 min after treatment. Testosterone concentrations in both serum and TIF were decreased at 60 and 120 min after treatment (Fig. 1). Treatment with SNP induced a decrease in serum testosterone concentrations at both 60 and 120 min after injection, whereas testosterone concentrations in the TIF were decreased only at 120 min after treatment. Serum concentrations of FSH were not changed in any group (data not shown). Otherwise, the administration of 4 mg/rat L-AME intraperitoneally did not modify the serum concentrations of LH or testosterone or the concentration of testosterone in TIF (Fig. 2).

Effects of L-AME in castrated rats

As expected, castrated animals showed highly increased serum LH concentrations. The administration of L-AME did not decrease serum LH concentrations in castrated rats, whereas it was decreased in sham-operated animals (Fig. 3).

Effects of L-AME in Leydig cell-depleted rats

The testes of rats treated with EDS showed an absence of Leydig cells at 10 days after treatment. This was confirmed by histological examination of testis sections and by the extremely low testosterone concentrations found in these animals (Fig. 4). Basal serum LH concentrations were highly increased in EDS-treated rats. Intraperitoneal treatment with L-AME significantly decreased serum LH concentrations in both DMSO- and EDS-treated rats (Fig. 4).

Effects of L-AME in macrophage-depleted rats

The testes of control (NaCl-injected) rats showed abundant ED1- and ED2-positive cells in the interstitial areas (Fig. 5A and B). Labelling with ED2 antibodies was more intense than with ED1 antibodies. On the contrary, ED1- and ED2-positive cells were nearly absent in CI2MDP-liposome-injected rats (Fig. 5C).

The basal serum concentrations of LH and testosterone 10 days after macrophage depletion are shown in Fig. 6A and B. Rats depleted of testicular macrophages showed increased LH and testosterone concentrations when compared with NaCl-injected animals. The effects of L-AME treatment were dependent on the route of administration. When the drug was injected intraperitoneally, serum LH concentrations were decreased (Fig. 6C). However, when the drug was administered intratesticularly, serum LH concentrations were not changed (Fig. 6D).

Discussion

Intraperitoneal treatment with the NOS substrate L-AME
decreased both LH and testosterone concentrations. The effects of L-AME are due to the generation of NO, since it is blocked by concomitant administration of the NOS inhibitor L-NAME (4). It is likely that in certain situations and/or locations, NO synthesis could be limited by extracellular L-arginine concentrations (6, 26). Furthermore, the effects of the NO donor SNP were similar to those of L-AME, although variable. It was more effective when given intratesticularly, inducing a decrease in testosterone concentrations, without a change in those of LH. Differences between both NO-generating drugs administered in this study (SNP and L-AME) could be due to differences in their kinetics or in the amount of NO generated. Previous studies have shown that the response to another NO donor ISDN was delayed with respect to the response to L-AME (4).

The co-existence of decreased concentrations of both LH and testosterone in L-AME-treated rats could be due to central effects, decreasing LH secretion which in turn led to a decrease in testosterone synthesis, or to a dual mechanism: central effects on LH secretion and a direct gonadal effect, inhibiting Leydig cell steroidogenic activity. Previous experiments have also reported a decrease in serum LH concentrations after systemic administration of L-AME (4). A recent study in humans has reported that the administration of L-AME was ineffective in modifying serum LH concentrations (27). However, in this study, the dose was about 0.4 g/kg bw, which is lower than the minimal effective dose in the rat. Although interspecies differences probably do exist, these authors did not find any effects on blood pressure which also suggests that the quantity of NO generated was too small to affect the vascular system, and consequently its failure to affect pituitary function was not surprising. On the other hand, the existence of direct gonadal effects of NO-related agents is also supported by several studies. Experiments analyzing the time-course of the effects of L-AME on hormone concentrations suggest that the decreased testosterone secretion does not seem to be due to a prior decrease in LH concentrations (4). Furthermore, treatment with the NOS inhibitor L-NAME (3) increased testosterone con-

![Figure 1](http://example.com/figure1.png)

Figure 1 Serum concentrations of (A) LH and (B) testosterone (T), and (C) testosterone concentrations in the TIF in rats injected intraperitoneally or intratesticularly with L-AME, SNP or vehicle at 60 and 120 min after treatment. Data represent the mean ± S.E.M. for n = 9–10. a and b, significant differences at 0.01 and 0.05 levels respectively versus vehicle-treated rats (ANOVA and Tukey’s test).
centrations without affecting those of LH, and in vitro studies have shown that L-NAME increased testosterone secretion by isolated Leydig cells (5), whereas NO donors inhibit Leydig cell steroidogenesis (12). It is therefore unclear to what extent the effects of L-AME are due to direct inhibition of steroidogenesis in the testis or to gonadotrophin suppression.

The final effects observed after systemic administration of NO-related agents are difficult to analyse because of the involvement of actions at different levels, due to the ubiquity of the L-arginine–NO pathway. In this sense, changes in serum hormone concentrations following systemic administration of high doses of L-AME are the consequence of the integration of stimulatory and inhibitory actions at hypothalamic, pituitary and testicular levels, in addition to the effects on the vascular system.

To our knowledge, this is the first study comparing the effects of systemic and local administration of NO-related agents on the pituitary-testicular axis. In general, the effects of L-AME were equivalent when given either intraperitoneally or intratesticularly. The data from this study indicated that the effects of intratesticular injection of L-AME were not due to diffusion of the drug to
the systemic circulation. This was supported by the lack of effects of a dose of 4 mg/rat L-AME when given intraperitoneally, whereas it was effective when given intratesticularly. This agrees with previous studies (4) which have shown that the minimal effective intraperitoneal dose of L-AME was 1 g/kg bw, corresponding to about 300 mg/rat, and no response was found with a dosage of 0.1 g/kg bw (about 30 mg/rat), which is 7.5-fold greater than the intratesticular dose given in this study (4 mg/rat). The existence of a role of the testis in the effects of L-AME on LH secretion was also suggested by the lack of effects of L-AME in castrated rats. The absence of response to L-AME was not due to the hyperstimulated status of pituitary LH-secreting cells in castrated rats, because it was effective in EDS-treated rats which showed similar

Figure 5 Micrographs from cryostat sections from the testes of rats injected with (A and B) NaCl or (C) Cl2MDP-liposomes and immunostained with (A) ED1, (B) ED2 or (C) ED1/ED2 antibodies. Macrophages are indicated by arrows. T, seminiferous tubules. Bar in (A) = 10 μm.

Figure 6 Basal serum concentrations of (A) LH and (B) testosterone in rats treated 10 days before with Cl2MDP-liposomes or NaCl, to deplete testicular macrophages, and serum LH concentrations in Cl2MDP-liposomes and NaCl-treated rats at 120 min after (C) intraperitoneal treatment with 1 g/kg bw L-AME or vehicle and (D) intratesticular treatment with 2 mg/testis L-AME or vehicle. Data represent the mean±S.E.M. for n = 10. a, significant differences at 0.01 level versus vehicle-treated rats (Student’s t-test or ANOVA and Tukey’s test).
serum LH concentrations. Since locally generated NO is most unlikely to reach the systemic circulation in adequate concentrations, the central effects of intratesticularly injected L-AME could be mediated by testis-derived factors.

It is clearly established that the main inhibitory regulators of LH secretion in the male are Leydig cells through testosterone secretion. However, other testicular cell types have also been implicated in the modulation of serum LH concentrations (13–15). Testicular macrophages or cytokines potentially released by testicular macrophages have been reported to modulate LH secretion and testicular steroidogenesis (28, 29). This is in accordance with the data from this study indicating that macrophage-depleted rats showed increased serum LH concentrations. Since testosterone concentrations were also increased, changes in LH concentrations cannot be attributed to primary changes in testicular steroidogenesis. The mechanisms for the increase in serum LH concentrations in macrophage-depleted rats are unknown. In these animals, testosterone concentrations were slightly increased and the functioning of the seminiferous tubules and the secretion of FSH was not modified, which suggests that changes are specific for LH. It is tempting to speculate that testicular macrophages release factors that, directly or indirectly, modulate LH secretion.

Surprisingly, the effects of L-AME on serum LH concentrations in macrophage-depleted rats were different depending on the route of administration. In macrophage-depleted rats, the effects of L-AME on serum LH concentrations were blocked when the drug was administered intratesticularly, whereas L-AME decreased serum LH concentrations when the drug was given systemically. Although the reasons for these differences are unknown, this suggests that, in spite of the equivalence of the final effects observed after systemic and local administration of L-AME, different mechanisms are involved, and that macrophages play some role in the response to intratesticularly administered L-AME. Since intratesticular administration of L-AME seemed to obviate the central effects of the drug, this suggests a role for the testicular L-arginine–NO pathway and macrophages in the modulation of serum LH concentrations. Although the presence of NOS activity in testicular macrophages has not been demonstrated, macrophages from other territories express immunoreactive NOS (30). In addition, macrophages could mediate the effects of NO generated in other testicular cell types. Several interstitial cell types have been reported to show NOS activity in the testis, such as endothelial cells and Leydig cells (16, 29).

NO is a potent vasodilator and the effects of NO-related agents seem to be due, at least in part, to its effects on the microcirculation. In the ram, intratesticular administration of L-NAME has variable effects on local blood flow and vasomotion, whereas the administration of SNP increases local blood flow and abolishes vasomotion (31). Otherwise, in the rat, the effects of L-NAME on testosterone secretion are blocked by the administration of vasodilators that seem to act by NO-independent mechanisms (4). Similarly, changes in the volume of TIF have been reported after administration of NO-related agents (31). Although TIF volume was not measured in this study, testosterone concentrations in TIF were, in general, well correlated with serum concentrations of testosterone, which suggests that the observed changes in testosterone concentrations in TIF were not due in a significant amount to changes in TIF volume.

Summarizing, the existence of measurable effects of the intratesticular administration of low doses of NO-related agents allows us to study the possible role of the L-arginine–NO pathway in the pituitary-testicular axis, circumventing the systemic effects of these drugs. Furthermore, the data reinforced the idea that testicular factors, other than testosterone, modulate serum LH concentrations and suggest that testicular macrophages are involved in this regulatory loop.

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