Evidence to Suggest Nitric Oxide Is an Interstitial Regulator of Leydig Cell Steroidogenesis

Catherine Welch, Mary E. Watson, Merrily Poth, Timothy Hong, and Gary L. Francis

Recent studies have suggested that nitric oxide (NO) may function as both an intracellular and intercellular signal that affects neural and immunological activity, vascular tone, platelet adhesion, and production of some hormones. Arginine analogs such as N^G-monomethyl-L-arginine (L-NMMA) and Nω-nitro-L-arginine methyl ester (L-NAME) act to inhibit the intracellular formation of NO and have been used to study the effects of decreased NO formation on physiological systems. A single in vivo study has suggested that a similar analog, N^G-nitro-L-arginine, increases serum testosterone (T), but the organ site and mechanism of action were not investigated. The present study was performed to investigate the effects of NO synthase inhibitors on Leydig cell function. L-NMMA and L-NAME, but not the inactive enantiomer (D-NMMA), increased both basal and human chorionic gonadotropin (hCG)-stimulated T production while decreasing guanosine 3′:5′-cyclic monophosphate (cGMP). There was no effect on either adenosine 3′:5′-cyclic monophosphate (cAMP) accumulation or specific hCG binding. These results suggest that NO formation, which is inhibited by L-NMMA and L-NAME, is important in the regulation of Leydig cell T production by interstitial cells of the testis, and that changes in cGMP levels might be involved in this process.

RECENT STUDIES have suggested that nitric oxide ([NO] synthesized from the precursor arginine) may function as both an intercellular and intracellular signal in many physiological systems. Increased NO directly affects neural and immunological activity, vascular tone, platelet adhesion, and the production of at least one hormone, insulin.[1-7]

The formation of NO is decreased by arginine analogs such as N^G-monomethyl-L-arginine (L-NMMA) and Nω-nitro-L-arginine methyl ester (L-NAME), which are competitive inhibitors of the enzyme NO synthase. These compounds have been used to study the effects of decreased NO formation on many cellular systems.[4-7]

A single in vivo study has suggested that a similar arginine analog, N^G-nitro-L-arginine, increases serum testosterone (T) in the rat, but the organ site and mechanism of action were not investigated.[7] Based on these observations, we hypothesized that NO might be produced by the interstitial cells of the testis, either by Leydig cells themselves, macrophages, or perhaps other cells as well. If so, this basal NO formation could be important in regulating the T response to gonadotropin stimulation, and blocking NO synthesis with arginine analogs would be expected to stimulate T production. The present study was performed to investigate the effect and mechanism of action of the arginine analogs, L-NMMA and L-NAME on Leydig cell steroidogenesis.

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MATERIALS AND METHODS

Animal Care and Procedures

This study was performed in accordance with Army regulation no. 70-18 and the guide for the use and care of laboratory animals from the National Institutes of Health, and was approved by the animal research committee and institutional review board of Walter Reed Army Medical Center, Washington, DC.

Leydig Cell–Enriched Cultures

Based on the method of Dufau and Catt,[8] testes were aseptically removed from Sprague–Dawley male rats (either 20 to 40 days of age [immature] or 60 to 75 days of age [mature]) and placed into iced medium 199 containing bovine serum albumin (1 mg/mL), penicillin (50 U/mL), and streptomycin (0.05 g/mL). This medium was used for all incubations and is simply referred to as medium. Testes were decapsulated and digested in collagenase (0.25 mg/mL, 37°C, 10 minutes). The interstitial cell preparation was separated from tubular elements by filtration through a 60-mesh cell sieve. We chose not to use percoll gradient separation because we wished to retain other interstitial cells, particularly macrophages, in our preparation. We believed this might be important because macrophages are a potential source of NO formation in the testis.[9] This Leydig cell–enriched preparation was centrifuged, washed, and diluted to a concentration of 1×10^7 cells/mL before characterization. Leydig cells were quantified by staining for the presence of 3-β-hydroxysteroid dehydrogenase,[10] and macrophages were quantified by staining for the presence of nonspecific esterase activity.[11] In the immature preparation, Leydig cells comprised 33% to 45% of the total cell number and macrophages comprised 10% to 15%. In mature preparations, Leydig cells comprised 10% to 25% and macrophages 10% to 15% of the total cell number.

T Production

Leydig cell–enriched preparations (1×10^6 cells) were incubated 4 hours (2 mL, 37°C, 5% CO_2) in control media or media containing L-NMMA (0.1 to 1.0 mmol/L), D-NMMA (1.0 mmol/L), or L-NAME (1.0 mmol/L). After 4 hours, human chorionic gonadotropin ([hCG] 0.1 U/mL unless otherwise specified) was added to stimulate T production. After 16 hours, media were removed and analyzed for T content by direct radioimmunoassay.

Cell viability, as determined by Trypan Blue exclusion performed in normal saline, was greater than 95% in all experiments.

Cyclic Nucleotide Formation

To determine the effect on adenosine 3′:5′-cyclic monophosphate (cAMP) formation, cells (1×10^6) were incubated 4 hours in control media containing isobutyl-methylxanthine (0.1 mg/mL) or media containing isobutyl-methylxanthine and L-NMMA. After 4 hours, hCG (0.1 U/mL, where indicated) was added for 15 minutes. Following incubation, cells were centrifuged ($1,000 \times g$, 10 minutes, 4°C) and extracted with ethyl alcohol (95%, 2 mL, 0°C, 15 minutes). The extracts were evaporated and reconstituted for determination of cAMP levels by direct radioimmunoassay.

The production of guanosine 3′:5′-cyclic monophosphate (cGMP) was similarly studied except that 3×10^6 cells were incubated for 1 to 24 hours in control media or media containing L-NMMA, D-NMMA, or L-NAME. Where indicated, hCG (0.1 U/mL) was then added for 15 minutes. Cells were similarly extracted, and cGMP levels were determined by radioimmunoassay.

Specific hCG Binding

Specific hCG binding was determined using intact Leydig cell cultures.[12] Cells (1×10^6) were incubated 4 hours in control media (n = 20) or media containing L-NMMA (1.0 mmol/L, n = 20). After 4 hours, cultures in each treatment group were divided into two sets of 10 each. Total binding was determined using the first set. Cells were incubated with ^{125}I-hCG (2 ng/mL, 87.1 Ci/g) at 37°C for 1 hour. Nonspecific binding was determined using the second set. Cells were incubated with ^{125}I-hCG plus unlabeled hCG (0.1 μmol/L). After incubation, cells were extensively washed with Hanks balanced salt solution (0°C) and the radioactivity in each cell pellet was determined. Specific binding was calculated as the difference between total and nonspecific binding.

Nitrite Determinations

For the determination of nitrite production, either Leydig cell–enriched preparations (0.1 to 1×10^7 cells) or whole decapsulated testes were incubated 4 hours in control media or media containing 1.0 mmol/L L-NMMA. At the end of incubation, media were removed and analyzed for nitrite content using the Greiss reagent.[13]

Radioactive Materials

The ^{125}I-hCG (87.1 Ci/g) used for binding experiments was obtained from New England Nuclear (Boston, MA) and used within 15 days of receipt. Radioimmunoassay materials for the determination of T levels were obtained from ICN Biomedicals (Costa Mesa, CA), and those for cAMP and cGMP assays were obtained from Amersham (Arlington Heights, IL).

Chemicals and Reagents

The hCG used for cell stimulation and L-NAME were obtained from Sigma Chemical (St Louis, MO). L-NMMA and D-NMMA were obtained from Calbiochem (La Jolla, CA). Unless otherwise stated, all chemicals and reagents were of reagent quality and obtained from standard vendors.

Statistical Analyses

Statistical significance for the difference between group means was determined using one-way ANOVA. The *P* values are as indicated.

RESULTS

Studies using immature rat Leydig cell cultures were performed first. Figure 1 shows that L-NMMA at all three concentrations tested significantly increased basal T production by approximately 50% ($P < .005$ *v* control) and hCG-stimulated T production by approximately 35% ($P < .05$ *v* hCG alone).

Figure 2 shows that L-NMMA significantly increased T production by Leydig cells, which were stimulated with lower concentrations of hCG as well (0.00025 to 0.1 U/mL).

Figure 3 shows that D-NMMA (the inactive enantiomer of NMMA) had no effect on either basal or hCG-stimulated T production. In contrast, L-NAME (another active NO synthase inhibitor) also increased basal ($P < .05$) and hCG-stimulated ($P = .06$) T production.

Figure 4 shows the effects of L-NMMA, D-NMMA, and L-NAME on cGMP formation during 4-hour incubations. As shown in Fig 4A, basal cGMP production was significantly decreased when either L-NMMA or L-NAME (1.0 mmol/L) but not D-NMMA was added ($P < .005$ *v* control). As shown in Fig 4B, cGMP levels were reduced with the addition of hCG, and when either L-NMMA or L-NAME (1.0 mmol/L), but not D-NMMA was added together with hCG, the levels of cGMP were further decreased ($P < .005$ *v* hCG alone). To better understand the temporal significance of this effect, cells were incubated with L-NMMA (1.0 mmol/L) for up to 24 hours. cGMP levels were reduced by L-NMMA at all time points between 2 and 24 hours (control 8.5 ± 1.0 *v* L-NMMA 0.7 ± 0.05 pmol/10^6 cells at 24 hours, $P < .005$).

Figure 5 shows the effect of L-NMMA on cAMP formation. L-NMMA (0.1 to 1.0 mmol/L) had no effect on the formation of either basal or hCG-stimulated cAMP.

The effect of L-NMMA on hCG binding was also studied. L-NMMA (1.0 mmol/L) had no effect on specific hCG binding (control 7.8 ± 0.2 *v* L-NMMA 7.0 ± 0.5 pg/10^6 cells).

The amount of nitrite produced by cultures of immature Leydig cells was directly measured, but it was less than 1

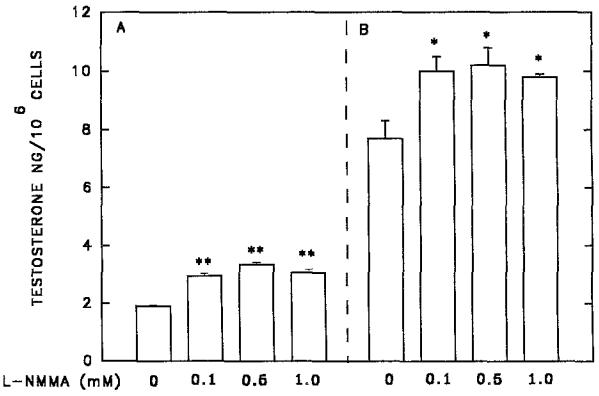

Fig 1. Effect of L-NMMA on (A) basal and (B) hCG-stimulated T production by immature rat Leydig cells. Cells were incubated 4 hours with L-NMMA (0.1 to 1.0 mmol/L), after which hCG, 0.1 U/mL was added (B) for 16 hours. Results are the mean ± SEM of five replicate cultures. *$P < .05$; **$P < .005$.

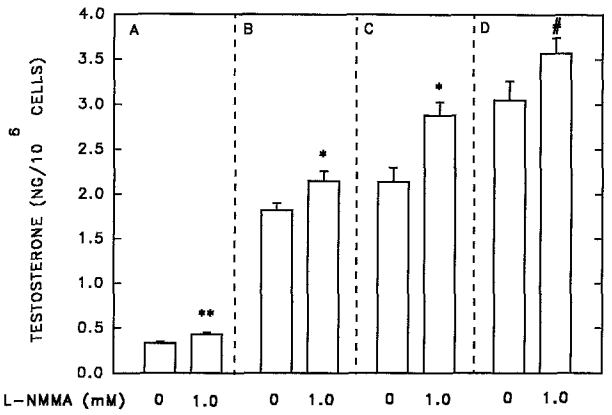

Fig 2. Effect of L-NMMA on T production by immature rat Leydig cells stimulated with different concentrations of hCG. Cells were incubated 4 hours with L-NMMA (1.0 mmol/L), after which hCG at specified concentrations was added for 16 hours. (A) Basal T production; (B) hCG, 0.00025 U/mL; (C) hCG, 0.005 U/mL; (D) hCG, 0.1 U/mL. Results are the mean ± SEM of five replicate cultures. #$P = .07$; *$P < .05$; **$P < .005$.

nmol (lower level of sensitivity) for both control and L-NMMA (1.0 mmol/L) cultures even though up to 1×10^7 cells were used in each incubation. Whole decapsulated testes were then studied in which nitrite levels were measurable (control 2.9 ± 0.1 *v* L-NMMA 2.7 ± 0.4 nmol/testis) but similar in control and L-NMMA cultures.

To determine if the effects of L-NMMA and L-NAME were specific to immature Leydig cells, similar studies were repeated using Leydig cells obtained from mature rats. Figure 6 shows the effects of L-NMMA and L-NAME on T production by cultures of mature Leydig cells. As expected, the amount of T produced by mature Leydig cells was greater than that previously shown using immature cells. L-NMMA significantly increased both basal ($P < .005$ *v* control) and hCG-stimulated T production ($P < .005$ *v* hCG alone). L-NAME increased basal T (but this only

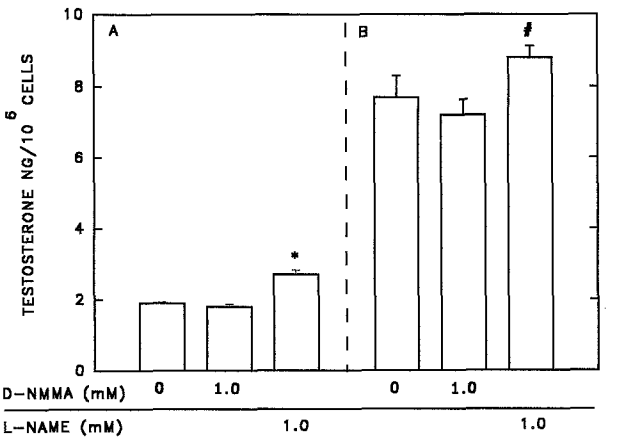

Fig 3. Effect of D-NMMA and L-NAME on (A) basal and (B) hCG-stimulated T production by immature rat Leydig cells. Cells were incubated 4 hours in media containing either D-NMMA (1.0 mmol/L) or L-NAME (1.0 mmol/L), after which hCG (0.1 U/mL) was added (B) for 16 hours. Results are the mean ± SEM of five replicate cultures. #$P = .06$; *$P < .05$.

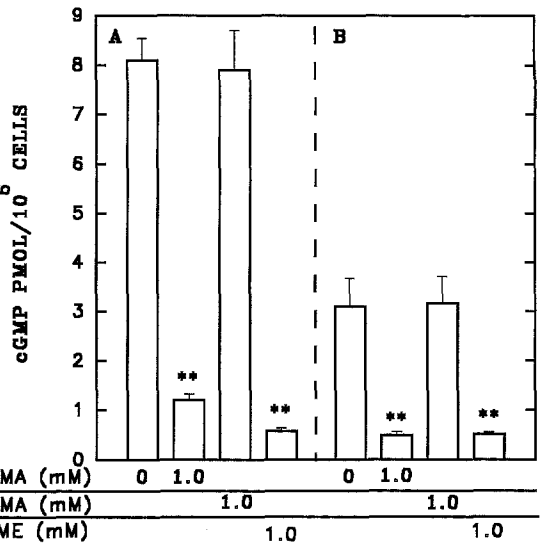

Fig 4. Effect of L-NMMA, D-NMMA, and L-NAME on cGMP formation by immature rat Leydig cells. (A) Cells were incubated 4 hours with either L-NMMA (1.0 mmol/L), D-NMMA (1.0 mmol/L), or L-NAME (1.0 mmol/L), after which hCG 0.1 U/mL was added (B) for 15 minutes. Results are the mean ± SEM of five replicate cultures. **$P < .005$.

approached statistical significance: $P < .07$ *v* control) and had no effect on hCG-stimulated T production.

Figure 7 shows the effects of L-NMMA and L-NAME on cGMP formation in cultures of mature Leydig cells. There was a significant decrease in both basal ($P < .005$ *v* control) and hCG-stimulated cGMP ($P < .005$ *v* hCG alone) when either L-NMMA or L-NAME (1.0 mmol/L) was added.

DISCUSSION

Previous studies have shown that increased formation of NO may decrease hormone production by some endocrine

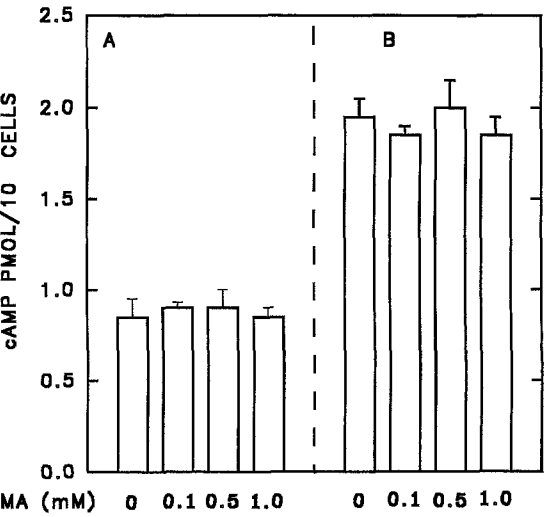

Fig 5. Effect of L-NMMA on (A) basal and (B) hCG-stimulated cAMP formation by immature rat Leydig cells. Cells were incubated 4 hours with L-NMMA (0.1 to 1.0 mmol/L), after which hCG 0.1 U/mL was added (B) for 15 minutes. Results are the mean ± SEM of five replicate cultures.

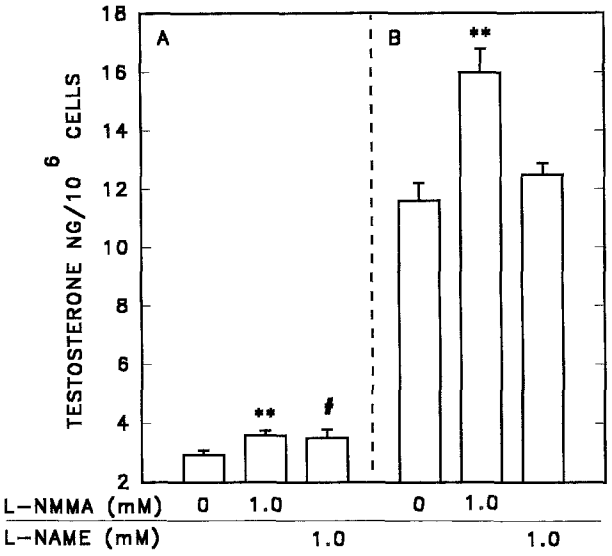

Fig 6. Effect of L-NMMA and L-NAME on (A) basal and (B) hCG-stimulated T production by mature Leydig cells. Cells were incubated 4 hours with either L-NMMA (1.0 mmol/L) or L-NAME (1.0 mmol/L), after which hCG 0.1 U/mL was added (B) for 16 hours. Results are the mean ± SEM of five replicate cultures. #P = .07; **P < .005.

tissues. In the pancreas, NO decreases islet cell insulin production[5,6] perhaps by binding to and inhibiting the activity of Krebs cycle enzymes.

Other studies have shown that NO, acting as either an intracellular or intercellular signal, affects many cell types and can change neural and immunological activity, vascular tone, and platelet adhesion.[1-4] These studies have suggested a different mechanism for NO action, which is to increase the activity of soluble guanyl cyclase and the generation of intracellular cGMP.[9]

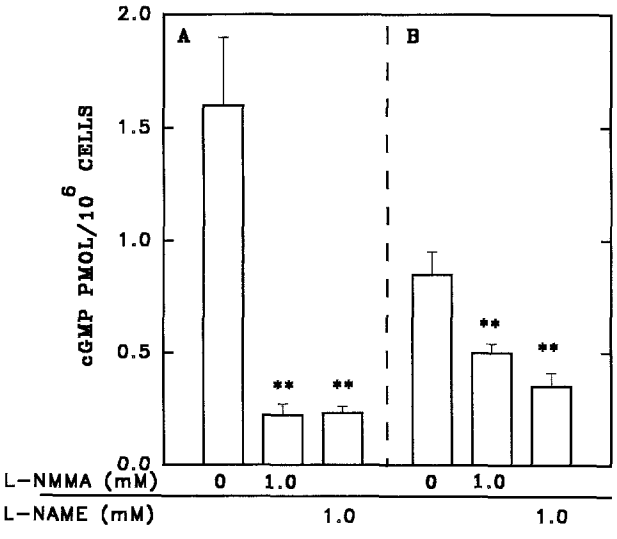

Fig 7. Effect of L-NMMA and L-NAME on basal (A) and (B) hCG-stimulated cGMP accumulation by mature rat Leydig cell cultures. Cells were incubated 4 hours with either L-NMMA (1.0 mmol/L) or L-NAME (1.0 mmol/L), after which hCG 0.1 U/mL was added (B) for 15 minutes. Results are the mean ± SEM of five replicate cultures. *P < .05; **P < .005.

To our knowledge, only one previous study has suggested that NO may affect testicular function. In that study, in vivo administration of an inhibitor of NO formation increased serum T levels in the rat.[7] However, the organ site and mechanism of action were not defined. These observations suggest that NO might be involved in the regulation of T production, but there could be several mechanisms by which this might occur. NO could control testicular vascular tone, neural activity, or Leydig cell steroidogenesis itself in addition to having effects at the level of the hypothalamus or pituitary. The present study was performed to evaluate whether, and if so, how, these inhibitors of NO formation affect Leydig cell T production.

The results of this study show that at least two different inhibitors of NO formation, L-NMMA and L-NAME, but not D-NMMA, the inactive enantiomer, increase rat Leydig cell T synthesis and decrease cGMP accumulation in Leydig cell–enriched cultures. This is not associated with a change in specific hCG binding or cAMP generation.

L-NMMA and L-NAME affect both immature and mature Leydig cell cultures. Cells from animals of both ages were studied because the activity of 5-α-reductase is reduced in adult as compared with immature Leydig cells.[14] Since L-NMMA and L-NAME affect both cell preparations, it is unlikely that these compounds act by changing the activity of 5-α-reductase.

In addition to increasing T, L-NMMA and L-NAME decreased cGMP production. This is consistent with a mechanism of action that involves the inhibition of NO formation, decreased guanyl cyclase activity, and decreased production of cGMP. However, to our knowledge, there is no direct evidence that cGMP is a regulator of Leydig cell T production. In addition, we were unable to show any effect of up to 0.5 mg/mL dibutryl-cGMP on either basal (0.24 ± 0.02 v 0.21 ± 0.01 ng/10^6 cells) or hCG-stimulated (0.96 ± 0.11 v 1.09 ± 0.09 ng/10^6 cells) T production. L-NMMA and L-NAME could have decreased cGMP production in Leydig cells themselves or perhaps in macrophages, an important cellular component of the interstitium. Macrophages comprised approximately 10% to 15% of our cell preparation and are a potential source of NO.[2,9,15-22]

However, there is evidence that concomitant changes in T and cGMP do occur in Leydig cells and that these changes may be functionally related. Atrial natriuretic peptide has been shown to stimulate mouse Leydig cell T production and cGMP accumulation.[23] It was postulated that these changes might be linked through the calcium/calmodulin system. We did not investigate a role for calcium or compounds such as atrial natriuretic peptide in our system, and it is possible such factors could be important in understanding a possible link between cGMP and the regulation of T production.

One of the end products of NO metabolism, nitrite, was analyzed to determine whether and if so in which cells NO was being produced. Nitrite levels were undetectable, even using 1 × 10^7 cells. Whole testes were used in similar incubations with which nitrite production was measureable (suggesting that NO formation can occur in the interstitial

cells of the testis), but levels were similar in control and L-NMMA cultures. Although we are unable to provide direct evidence that NO formation in our cultures is affected by L-NMMA or L-NAME, our data do not exclude this possibility. It is well accepted that NO can act as an intracellular messenger and change cellular metabolism at concentrations (picomolar) below the sensitivity of our assay system.[24,25] In addition, it is possible that NO could have been further metabolized to compounds other than nitrite and thereby escaped detection.[13]

In conclusion, the present study has shown that L-NMMA and L-NAME, two inhibitors of NO formation, increase T and decrease cGMP production by Leydig cell–enriched cultures. These observations suggest that NO is an interstitial regulator of Leydig cell T production. Further investigation is necessary to define the individual roles and cell of origin of NO and cGMP, and to determine whether calcium or other factors are also involved.

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REFERENCES

1. Moncada S, Palmer RMJ, Higgs EA: Nitric oxide: Physiology, pathophysiology, and pharmacology. Pharmacol Rev 43:109-142, 1991

2. Ding AH, Nathan CF, Stuehr DJ: Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. J Immunol 141:2407-2412, 1988

3. Stuehr DJ, Nathan CF: Nitric oxide. J Exp Med 169:1543-1555, 1989

4. Kilbourn RG, Gross SS, Jubran A, et al: N^G-methyl-L-arginine inhibits tumor necrosis factor–induced hypotension: Implications for the involvement of nitric oxide. Proc Natl Acad Sci USA 87:3629-3632, 1990

5. Welsh N, Eizirik DL, Bendtzen K, et al: Interleukin-1β–induced nitric oxide production in isolated rat pancreatic islets requires gene transcription and may lead to inhibition of the Krebs cycle enzyme aconitase. Endocrinology 129:3167-3173, 1991

6. Southern C, Schulster D, Grenn IC: Inhibition of insulin secretion by interleukin-1β and tumor necrosis factor-α via an L-arginine–dependent nitric oxide generating mechanism. FEBS Lett 276:42-44, 1990

7. Adams ML, Nock B, Truong R, et al: Nitric oxide control of steroidogenesis: Endocrine effects of N^G-nitro-L-arginine and comparisons to alcohol. Life Sci 50:35-40, 1991

8. Dufau ML, Catt KJ: Gonadotropic stimulation of interstitial cell functions of the rat testis in vitro. Methods Enzymol 39:252-271, 1975

9. Bromberg Y, Pick E: Cyclic GMP metabolism in macrophages. Regulation of cyclic GMP levels by calcium and stimulation of cyclic GMP synthesis by NO-generating agents. Cell Immunol 52:73-83, 1980

10. Payne AH, Downing JR, Wong K: Luteinizing hormone receptors and testosterone synthesis in two distinct populations of Leydig cells. Endocrinology 106:1424-1429, 1980

11. Miller GA, Morahan PS: Use of nonspecific esterase stain, in Adams DO, Edelson PJ, Koren HS (eds): Methods for Studying Mononuclear Phagocytes. New York, NY, Academic, 1981, pp 367-373

12. Mather JP, Saez JM, Haour F: Regulation of gonadotropin receptors and steroidogenesis in cultured porcine Leydig cells. Endocrinology 110:933-940, 1982

13. Green LC, Wagner DA, Glogowski J, et al: Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. Anal Biochem 126:131-138, 1982

14. Moger WH: Serum 5α-androstane-3α, 17β-diol, androsterone, and testosterone concentrations in the male rat, influence of age and gonadotropin stimulation. Endocrinology 100:1027-1032, 1977

15. Hutson JC: Changes in the concentration and size of testicular macrophages during development. Biol Reprod 43:885-890, 1990

16. Hutson JC, Stocco DM: Comparison of cellular and secreted proteins of macrophages from the testis and peritoneum on two-dimensional polyacrylamide gels: Evidence of tissue specific function. Regul Immunol 2:249-253, 1989

17. Lombard-Vignon N, Grizard G, Boucher D: Influence of rat testicular macrophages on Leydig cell function in vitro. Int J Androl 15:144-159, 1991

18. Molenaar R, Rommerts FFG, Van Der Molen HJ: Characteristics of Leydig cells and macrophages from developing testicular cells. Ann NY Acad Sci 438:618-621, 1984

19. Niemi M, Sharpe RM, Brown WRA: Macrophages in the interstitial tissue of the rat testis. Cell Tissue Res 243:337-344, 1986

20. Sharpe RM: Intratesticular control of steroidogenesis. Clin Endocrinol 33:787-807, 1990

21. Skinner MK: Cell-cell interactions in the testis. Endocr Rev 12:45-77, 1991

22. Yee JB, Hutson JC: Effects of testicular macrophage conditioned medium on Leydig cells in culture. Endocrinology 116:2682-2684, 1985

23. Mukhopadhyay A, Helbing J, Leidenberger F: The role of Ca^{2+} and calmodulin in the regulation of atrial natriuretic peptide–stimulated guanosine 3′,5′-cyclic monophosphate accumulation by isolated mouse Leydig cells. Endocrinology 125:686-692, 1989

24. Malinski T, Taha Z: Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor. Nature 358:676-678, 1992

25. Malinski T, Radomski M, Taha Z, et al: Direct electrochemical measurement of nitric oxide released from human platelets. Biochem Biophys Res Commun 194:960-965, 1993