

Müllerian Inhibiting Substance Inhibits Testosterone Synthesis in Adult Rats

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ABSTRACT: Müllerian inhibiting substance (MIS) is a gonadal hormone that causes regression of the Müllerian ducts during male sexual differentiation. Postnatally, MIS inhibits the proliferation and differentiation of immature Leydig cells, and transgenic mice that overexpress MIS have decreased serum testosterone concentrations. To elucidate the effects of MIS on androgen regulation in the postnatal testis, we examined testosterone synthesis in adult Sprague-Dawley rats following intratesticular and intraperitoneal injections of MIS. Intratesticular MIS injection achieved high local concentrations of MIS (574.0 ± 60.0 ng/mL) at 4 hours, with a corresponding decline in serum testosterone concentrations to 0.7 ± 0.1 ng/mL, compared to 1.1 ± 0.2 ng/mL with intraperitoneal MIS and 1.6 ± 0.1 ng/mL with intratesticular vehicle (IT-Veh) ($P < .001$). Intratesticular administration of MIS (IT-MIS) resulted in much higher serum and testicular interstitial fluid MIS concentrations than the intraperitoneal route. To directly examine the testosterone production rate in MIS-treated animals, we isolated Leydig cells from MIS and vehicle-injected testes.

Primary Leydig cells exposed to MIS had a lower testosterone production rate and decreased expression of p450c17 (hydroxylase/lyase) and luteinizing hormone (LH) receptor mRNAs than that of vehicle-injected controls or the noninjected contralateral testis. In conclusion, intratesticular administration of MIS caused a decline in serum testosterone concentrations by decreasing the rate of testosterone biosynthesis, confirming that MIS can regulate adult Leydig cell androgen production. The ability of MIS to down-regulate mRNA expression of the p450c17 and LH receptor genes suggests that this effect is mediated transcriptionally. These data indicate that, in addition to its role in embryonic differentiation of the male reproductive tract, MIS has a regulatory function in the postnatal testis. We conclude that one such function is for MIS to directly inhibit adult Leydig cell steroidogenesis.

Key words: Anti-Müllerian hormone, primary Leydig cells, p450c17, testis, androgen biosynthesis, steroidogenesis.

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Müllerian inhibiting substance (MIS), also termed anti-Müllerian hormone (AMH), is a 140-kd glycoprotein belonging to the transforming growth factor (TGF)- β family of growth and differentiation factors (Cate et al, 1986). In the rat, MIS is first expressed in testicular Sertoli cells at gestational day 13 and in ovarian granulosa cells after birth, but it continues to be expressed in both males and females throughout life (Josso et al, 1993; Lee and Donahoe, 1993). During male sexual differentiation, MIS has the critical role of inducing regression of the Müllerian ducts, the anlagen of the female internal reproductive tracts. More recently, this gonadal

hormone has been recognized to have additional paracrine roles in the postnatal testis. Binding sites have been demonstrated on human sperm (Fallat et al, 1998), and MIS has been reported to improve the motility and viability of fresh and cryopreserved sperm (Siow et al, 1998). Moreover, both MIS and its Type II receptor are expressed in the adult testis; the ligand is localized to Sertoli cells, while the receptor is expressed in both Sertoli and Leydig cells (Munsterberg and Lovell-Badge, 1991; Lee, 2000). Transgenic mice overexpressing MIS have impaired Leydig cell differentiation, reduced adult Leydig cell number and volume, and decreased testosterone levels (Racine et al, 1998). Conversely, mice with targeted deletions of either MIS or its receptor develop Leydig cell hyperplasia and neoplastic foci (Behringer et al, 1994; Mishina et al, 1996). In vitro studies have demonstrated direct effects of MIS on thymidine uptake in primary progenitor Leydig cells (Lee et al, 1999), suggesting that MIS inhibits the proliferation of immature Leydig cells. MIS has also been shown to decrease steroidogenesis in primary Leydig cells (Rouiller-Fabre et al, 1998; Lee, 2000) and Leydig cell tumor lines (Teixeira et al, 1999), consistent with a regulatory role for MIS in androgen production.

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The testicular phenotype in the MIS transgenic and knockout mice and the cell-type-specific expression of MIS and its receptor in the adult testis led us to hypothesize that MIS could exert direct paracrine control of mature Leydig cell-differentiated function. To elucidate the effects of MIS on testosterone steroidogenesis in the adult testis, we used an *in vivo* model in which we administered a single dose of MIS to sexually mature male rats. We examined the consequences of a local transient elevation of MIS on serum testosterone concentrations, Leydig cell testosterone production rates, and the expression of steroidogenic enzymes. Our studies demonstrate that MIS inhibits the testosterone production rate and down-regulates mRNA expression of p450c17 and the luteinizing hormone (LH) receptor in an *in vivo* model, confirming a potential role for MIS in the control of androgen biosynthesis.

METHODS

Animals and Experimental Protocol

These studies were conducted according to a protocol approved by the Rockefeller University Animal Care and Use Committee (91200-R2). Sixty- to 65-day-old male Sprague-Dawley rats (Charles River Laboratories) were anesthetized with metofane, then given a single intratesticular (IT-MIS) or intraperitoneal (IP-MIS) injection of recombinant human MIS, 75 μg in 50 μL of Dulbecco phosphate buffered saline ($n = 6$ per experimental group). Control rats received intratesticular injections of phosphate buffered saline (IT-Veh). All intratesticular injections were administered in the right testis with careful attention to avoiding injury of the testicular vessels. The contralateral noninjected left testis was also examined for several of the endpoints as an untreated comparison to the MIS-injected testis.

The MIS was a gift from Drs David MacLaughlin and Patricia Donahoe. MIS is produced recombinantly in Chinese hamster ovary cells transfected with the human MIS gene and purified by immunoaffinity chromatography in the Pediatric Surgical Research Laboratory at Massachusetts General Hospital (MacLaughlin et al, 1991). The recombinant human MIS is quantified in a urogenital ridge bioassay and an ELISA specific for human and nonhuman primate MIS (Donahoe et al, 1977; Hudson et al, 1990). Recombinant human MIS has been shown in 2 different MIS bioassays in the rat and mouse (di Clemente et al, 1992; MacLaughlin et al, 1992) and in other *in vitro* systems to be bioactive across species (Vigier et al, 1989; Racine et al, 1998; Lee et al, 1999).

Trunk or tail blood was collected at 2, 4, 6, 8, 12, 24, and 48 hours for serum testosterone and MIS determinations by radioimmunoassay (RIA) and ELISA, respectively (Cochran et al, 1981; Hudson et al, 1990). At designated time points (4, 8, 12, 24, and 48 hours), the rats were euthanized by CO_2 asphyxiation for the collection of testes for RNA extraction, extraction of testicular interstitial fluid for MIS and testosterone measurements, or isolation of primary Leydig cells. Testicular interstitial fluid was obtained from freshly collected testes by the method

of Turner et al (1984). In brief, the caudal end of the testis was punctured twice with a 23-gauge needle, taking care to avoid damage to blood vessels and seminiferous tubules. The punctured testes were then centrifuged at $50 \times g$ for 15 minutes to drain and collect the interstitial fluid that was stored frozen at -20°C until assay.

MIS and Testosterone Assays

MIS values were measured in a primate-specific ELISA that detects only the exogenously administered human MIS and not endogenous rodent MIS (Hudson et al, 1990; Lee et al, 1996). The ELISA is performed on 96-well plates using a mouse monoclonal (6E11) as the first antibody and a rabbit polyclonal (MGH-4) as the second sandwich antibody. Samples were measured in duplicate at 4 serial dilutions as described previously (Hudson et al, 1990; Lee et al, 1996). The limit of sensitivity of the assay is 0.5 ng/mL, and the intra- and interassay coefficients of variation are 9% and 15%, respectively. The MIS ELISA does not recognize related members of the gene family such as TGF- β , activin, or inhibin.

Testosterone concentrations in conditioned media and serum were measured by a previously validated RIA (Cochran et al, 1981). The limit of sensitivity of the assay is 34 pg/mL, and the intra- and interassay coefficients of variation are 14% and 13%, respectively.

Cell Isolation and Culture

Adult Leydig cells were isolated according to the method of Klinefelter et al (1993). Briefly, testes were removed and perfused with 1 mg/mL collagenase in Medium-199 via the testicular artery before decapsulation. Decapsulated testes were dispersed with collagenase in Medium-199 for 10 minutes at 34°C with shaking. The dispersed cells were further purified in a Beckman JE-6B elutriation chamber (Palo Alto, Calif) at a flow rate of 16 mL/min at 2000 rpm. The partially purified adult Leydig cells were then subjected to density gradient centrifugation in Percoll and collected between densities of 1.07 and 1.09 g/mL. The purity of the Leydig cell preparations determined by 3β -hydroxysteroid dehydrogenase histochemistry (Payne et al, 1980) was estimated to be greater than 95% in the cell preparations isolated for these studies. The freshly purified Leydig cells were cultured for 3 hours in serum-free, phenol red-free media (DMEM/Ham F12) at 34°C to assess steroidogenic capacity. The Leydig cells were incubated with a maximally stimulating concentration of ovine LH (100 ng/mL) or a substrate-saturating concentration of 22(R)-hydroxycholesterol (22(R)OH-CHOL [20 μM]), a water-soluble cholesterol. The testosterone production rate by the primary Leydig cells was calculated by assaying the testosterone concentrations in spent media by RIA.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated using a single-step extraction method with guanidinium/phenol (Tri Reagent, Sigma, St Louis, Mo) and quantified by spectrophotometric analysis. RNA samples with $A_{260/280}$ ratios greater than 1.6 were subjected to semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for p450c17, steroid acute regulatory protein (StAR), and the LH receptor, as described previously (Shan et al, 1995; Ak-

ingbemi et al, 2000). Forward and reverse primer pairs for each amplified gene were designed using PRIMER software (Whitehead Institute of Biomedical Research, Cambridge, Mass) to span at least 1 intron-exon junction and to generate a product distinguishable in size from that of the internal control ribosomal protein S16. Oligonucleotides were synthesized by the Rockefeller University DNA and Protein Sequencing Facility using the Gene Assembler Special (LKB, Rockville, Md). For each PCR, a tube without reverse transcriptase was included as a separate control for contaminating genomic DNA. For each pair of primers, preliminary experiments were conducted to establish the amplification cycle range for logarithmic product formation and to determine the optimal amount of template RNA for product formation in the linear range of the assay. The PCR products were separated on 2% agarose gels in Tris-borate EDTA buffer. The signal intensities in each band were evaluated by image analysis using a gel documentation system (Kodak EDAS120 gel documentation system, Eastman Kodak Co, New Haven, Conn) and normalized to that of ribosomal protein S16 as the internal control.

Type	Expected Product Size
p450c17	
5'-TCATCAAGAAGGGAAAAGAA-3' (forward)	294 bp
5'-TGAAGCAGATAGCACAGATG-3' (reverse)	
StAR	
5'-TTGGGCATACTCAACAACCA-3' (forward)	389 bp
5'-ATGACACCGCTTTGCTCAG-3' (reverse)	
LH receptor	
5'-AGAGTGATTCCCTGGAAAGGA-3' (forward)	273 bp
5'-TCATCCCTTGGAAAGCATTC-3' (reverse)	
S16	
5'-AAGTCTTCGGACGCAAGAAA-3' (forward)	148 bp
5'-TTGCCAGAAAGCAGAACAG-3' (reverse)	

Statistical Analysis

All experiments were repeated at least 3 times. The data were analyzed by a nonparametric Kruskal-Wallis analysis of variance because of the small sample size (Sokal and Rohlf, 1995), followed by multiple comparisons testing to identify significant differences among the groups. The data shown in the tables and graphs are the means and standard deviations of the results from all the experiments conducted for these studies unless otherwise stated in the figure legends.

RESULTS

MIS Levels in Serum and Interstitial Fluid

Intratesticular administration of 75 μ g MIS increased serum MIS concentrations to 66.0 ± 21.3 ng/mL by 2 hours, the first time point examined (Table 1). MIS con-

Table 1. Serum MIS concentrations*

Time Point	MIS (ng/mL)		
	Vehicle	Intraperitoneal	Intratesticular
2 h	0.3 ± 0.2	27.0 ± 8.4	66.0 ± 21.3 †
4 h	0.6 ± 0.2	22.0 ± 12.8	65.0 ± 6.2 ‡
6 h	0.8 ± 0.6	36.0 ± 15.0	43.0 ± 5.0
8 h	1.2 ± 0.9	70.0 ± 24.5	85.0 ± 16.7
12 h	0.7 ± 0.2	49.0 ± 8.8	31.0 ± 10.5 †
24 h	0	12.5 ± 3.4	15.6 ± 5.4

* MIS indicates Müllerian inhibiting substance. Serum MIS concentrations measured by ELISA (Hudson et al, 1990) from 2 to 24 hours after a single injection of intratesticular vehicle, intraperitoneal MIS (75 μ g), or intratesticular MIS (75 μ g). The data shown are the mean MIS values (\pm SD) in serum collected from 6 animals in each group for each time point. Within each MIS-injected group, serum MIS at the 8-hour time point was significantly higher than all other time points ($P = .01$ to $.001$).

† $P < .01$ intratesticular compared to intraperitoneal.

‡ $P < .001$ intratesticular compared to intraperitoneal.

centrations remained elevated for 12 hours, with a peak of 85 ng/mL at 8 hours, then fell thereafter to undetectable levels by 48 hours. After intraperitoneal administration, peak concentrations of MIS in serum were observed at 8 hours (70.0 ± 24.5 ng/mL), followed by a progressive decline to undetectable levels by 48 hours. Within each group, serum MIS was significantly higher at 8 hours than at any other time point. Delivery of MIS by the intratesticular route resulted in higher serum concentrations of MIS than intraperitoneal injection at 2 hours ($P < .001$), 4 hours ($P < .001$), and 12 hours ($P < .01$). The time course of these studies revealed a rapid clearance of the circulating recombinant human MIS, with a half-life between 4 and 12 hours. Rat MIS does not cross-react with the antibodies used in this primate-specific MIS ELISA; thus, endogenous rodent MIS is not detected, and MIS values in the vehicle-injected group were undetectable or at the lower limits of the assay. The serum values of MIS attained in this study are within the physiologic range in mice measured in a rodent-specific assay with a lower sensitivity than the human assay (Al-Attar et al, 1997). In this assay, serum MIS concentrations in neonatal mice average 160 ng/mL and can be as high as 220 ng/mL, and adult values are approximately 15 ng/mL at 2 months and decline to the lower limits of the assay (1.6 ng/mL). These values are comparable to physiologic serum concentrations in humans that are as high as 400 ng/mL in young boys and up to 20 ng/mL in adult men (Lee et al, 1996).

Four hours after the injections, testicular interstitial fluid MIS concentrations in the right IT-injected testis peaked and were much higher than those in the contralateral noninjected testis (574.0 ± 60.0 ng/mL vs 41.8 ± 9.6 ng/mL, $P < .001$) (Table 2). Interstitial fluid MIS concentrations fell rapidly in the injected testis between 4 and 8 hours. Thereafter, interstitial fluid MIS concentrations de-

Table 2. Testicular interstitial fluid MIS concentrations*

Time Point	MIS (ng/mL)					
	Vehicle		Intraperitoneal		Intratesticular	
	Left	Right	Left	Right	Left	Right
4 h	0	0	17.9 ± 13.4	8.2 ± 6.0	41.8 ± 9.6	574.0 ± 60†
8 h	0	0	25.0 ± 8.7	25.4 ± 8.7	119.0 ± 4.0	73.0 ± 33
24 h	0	0	27.6 ± 5.5	28.0 ± 9.1	12.3 ± 3.7	32.0 ± 13

* MIS indicates Müllerian inhibiting substance. Mean (\pm SD) MIS concentrations in testicular interstitial fluid collected 4, 8, and 24 hours after administration of vehicle or MIS (75 μ g). All vehicle injections were administered in the right testis. MIS was delivered intraperitoneally or by intratesticular injection to the right testis. The data represent the mean testosterone values in interstitial fluid collected from 6 animals in each group at each time point.

† $P < .001$; IT-MIS, right injected testis vs left noninjected testis.

clined progressively in both testes and were nearly undetectable by 48 hours. As expected, much lower interstitial fluid MIS concentrations were achieved with intraperitoneal administration of the same dose of MIS for the first 8 hours (Table 2). By 24 hours, however, interstitial fluid MIS concentrations from either testis of both MIS-treated groups were not significantly different. Endogenous concentrations of MIS within the testis have not been determined by RIA but have been quantified in the less sensitive urogenital ridge bioassay, which requires at least 0.5 μ g of MIS for detectable bioactivity (Donahoe et al, 1977; MacLaughlin et al, 1991). In this bioassay, MIS activity is measurable in testicular fragments up to 21 days of age, while pubertal and adult testes are inactive (Donahoe et al, 1976). These studies showed that prepubertal testicular fragments have the microgram amounts of MIS that are needed for full bioactivity in the assay. Thus, the interstitial fluid concentrations of MIS achieved by the intratesticular injections are within the physiological concentrations in immature testes.

Serum and Interstitial Fluid Testosterone Levels

Serum testosterone concentrations were assayed at 2, 4, 6, 8, 12, 24, and 48 hours after the injection of MIS (Table 3). All of the mean serum testosterone values were within the normal range of serum testosterone concentrations in normal young adult rats (Corpechot et al, 1981; Turner et al, 1985), except for the 4-hour IT-injected group. Thus, although several values in the treatment groups are significantly different from those in the vehicle group, these results may be without biological consequence. By contrast, serum testosterone concentrations in the IT-MIS-injected animals were below the normal physiologic range and were significantly lower than those of the vehicle-treated group (IT-MIS = 0.7 ± 0.1 ng/mL, IT-Veh = 1.6 ± 0.1 ng/mL, $P < .001$) at the 4-hour time point (Table 3). The intratesticular concentration of MIS was highest at this time point (Table 2). The group that received intraperitoneal injections of MIS had intermediate serum values of testosterone (1.1 ± 0.2 ng/mL).

Testosterone concentrations were measured in interstitial fluid collected from testes of animals in the MIS- and vehicle-injected group at 4, 8, and 24 hours after injection. In the group that received IT-MIS, interstitial fluid was collected from both the injected testis (IT-MIS) and the noninjected contralateral testis (IT-Contra). Testosterone concentrations in the interstitial fluid collected from the MIS-injected ipsilateral testis declined 4 hours after MIS administration (IT-MIS = 27.3 ± 5.9 ng/mL, IT-Veh = 87.9 ± 23.0 ng/mL, $P < .01$) (Figure 1) and were restored to concentrations comparable to the vehicle-treated group by 24 hours (data not shown). By contrast, testosterone levels in interstitial fluid collected from the IP-injected animals or from the noninjected contralateral testis (IT-Contra) of IT-injected animals were no different from those of vehicle-treated controls at all time points studied. These results indicate that serum and interstitial fluid concentrations of testosterone declined in concert with the increased intratesticular concentrations of MIS. In this in vivo model, the effects of a single injection of MIS are reversible, with recovery of the serum and intra-

Table 3. Serum testosterone concentrations*

Time Point	Testosterone (ng/mL)		
	Vehicle	Intraperitoneal	Intratesticular
2 h	1.3 ± 0.1	4.2 ± 1.1‡	2.9 ± 0.8†
4 h	1.6 ± 0.1	1.1 ± 0.2	0.7 ± 0.1§
6 h	2.3 ± 0.4	3.5 ± 1.5	2.3 ± 0.7
8 h	3.7 ± 1.4	1.7 ± 0.3†	2.8 ± 0.1
12 h	1.1 ± 0.2	3.0 ± 0.7‡	3.6 ± 1.6‡
24 h	3.1 ± 1.0	4.4 ± 0.4	3.3 ± 0.4

* Mean (\pm SD) serum testosterone concentrations from 2 to 24 hours after intraperitoneal injection of Müllerian inhibiting substance (MIS) (75 μ g) or intratesticular administration of vehicle or MIS (75 μ g) to the right testis. Serum testosterone was measured in 6 animals for each treatment group at each time point. Because serum testosterone concentrations fluctuate in the rat, only the 4-hour intratesticular concentration is outside the physiologic range of 1 to 4 ng/mL.

† $P < .05$; ‡ $P < .01$; § $P < .001$; P values between treated group and vehicle group.

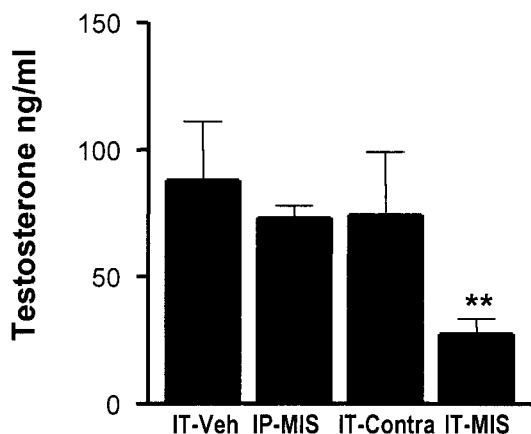


Figure 1. Testicular interstitial fluid testosterone concentrations. Testicular interstitial fluid was collected from the following groups: vehicle-treated animals (IT-Veh), animals administered Müllerian inhibiting substance (MIS) by intraperitoneal injections (IP-MIS), and both the contralateral noninjected (IT-Contra) and ipsilateral MIS-injected (IT-MIS) testes of animals administered unilateral intratesticular injections of MIS. The testosterone concentration in the interstitial fluid was measured by radioimmunoassay (RIA) (Cochran et al, 1981). Each bar represents the mean value (\pm SD) for 4 animals treated in each group. (** $P < .01$ IT-MIS vs IT-Veh).

testicular testosterone concentrations as the levels of intratesticular MIS declined.

Testosterone Production by Purified Leydig Cells

To determine if the transiently lowered concentrations of testosterone were caused by a change in the rate of testosterone biosynthesis, Leydig cell steroidogenic capacity was assessed after *in vivo* administration of MIS. Primary Leydig cells were isolated 4 hours after administering intratesticular MIS or vehicle at the time point with the highest interstitial fluid concentrations of MIS and lowest concentrations of testosterone. The Leydig cells were incubated for 3 hours, and testosterone was assayed in the conditioned media to assess the *in vitro* androgen production rate.

Under basal conditions, testosterone production was unchanged after *in vivo* MIS treatment (Figure 2). The addition of stimulatory doses of LH during the incubation increased the testosterone production rate in the vehicle-treated group 15-fold above the basal rate (302.6 ± 60.2 ng/ 10^6 cells/3 h). In the presence of LH, Leydig cells from MIS-injected testes had a 42% lower testosterone production rate (176.0 ± 20.6 ng/ 10^6 cells/3 h) than cells from vehicle-injected animals. Leydig cells from the contralateral noninjected testis of the rats receiving unilateral MIS injections were unaffected (276.9 ± 113.3 ng/ 10^6 cells/3 h). The addition of 22(R)OH-CHOL as a nonlimiting water-soluble substrate increased testosterone production rates by 25-fold in the vehicle-treated group (476.5 ± 53.4 ng/ 10^6 cells/3 h), while testosterone production was decreased by 24% (361.0 ± 41.5 ng/ 10^6 cells/

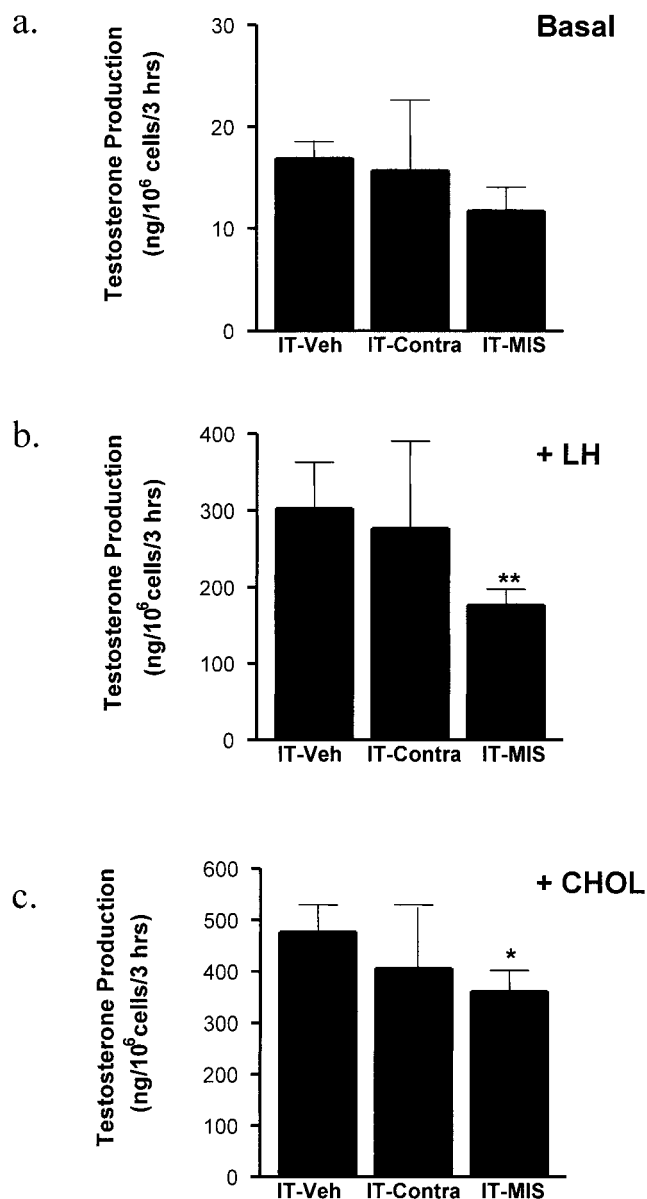


Figure 2. Primary Leydig cell testosterone production rate after *in vivo* Müllerian inhibiting substance (MIS). Four hours after injection, primary Leydig cells were purified from the testes of animals treated with vehicle (IT-Veh) or intratesticular MIS (contralateral noninjected [IT-Contra] or ipsilateral-injected testis [IT-MIS]). The freshly isolated Leydig cells were incubated in triplicate for 3 hours at 34°C with medium alone (Basal); with 100 ng/mL of ovine luteinizing hormone (LH); or with 20 μ M 22(R)-hydroxycholesterol (CHOL). Each experimental treatment was repeated 4 times. The graphs represent the mean (\pm SD) testosterone production rate of all the replicate experiments for each condition (IT-MIS compared to IT-Veh, ** $P < .01$; * $P < .05$).

3 h) in the MIS group. Leydig cells from the contralateral testis had an intermediate rate of testosterone production (406.0 ± 123.4 ng/ 10^6 cells/3 h). These results demonstrate a rapid and direct inhibition of *in vitro* androgen production in primary Leydig cells freshly isolated from testes exposed to high concentrations of MIS *in vivo*.

A. Densitometric analysis

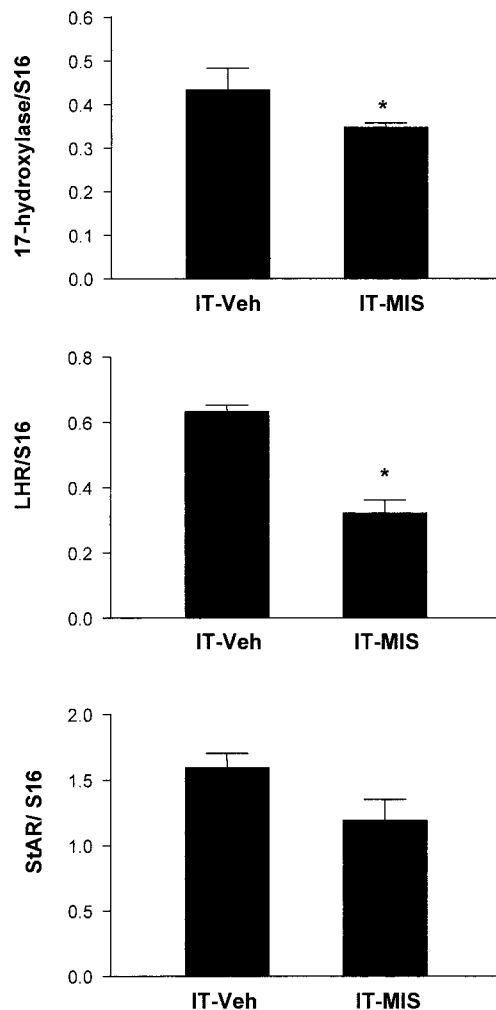


Figure 3. mRNA expression of p450c17, luteinizing hormone (LH) receptor, and steroid acute regulatory protein in purified Leydig cells after in vivo Müllerian inhibiting substance (MIS) administration. Total RNA was extracted from freshly isolated Leydig cells 4 hours after intratesticular injection of MIS or vehicle. Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using primers specific for p450c17 (17-hydroxylase); LH receptor (LHR); and steroid acute regulatory protein (StAR). (A) The intensity of the signals was quantified by densitometry and normalized to ribosomal protein S16. The graphs represent the mean values (\pm SD) for 3 replicate experiments ($n = 3$). (* $P < .05$ IT-Veh vs IT-MIS). (B) An ethidium bromide stained gel showing a representative PCR analysis for each gene.

mRNA Expression of P450c17, LH Receptor, and StAR

The capacity of Leydig cells to synthesize testosterone is primarily regulated by LH. Binding of LH to its receptor stimulates various intracellular events, including StAR-mediated mitochondrial transport of cholesterol and the 17α -hydroxylase activity of p450c17. We investigated the mechanism underlying the MIS-mediated inhibition of testosterone synthesis by examining the mRNA expression of several genes important for this process. Semi-

B. A representative RT-PCR analysis

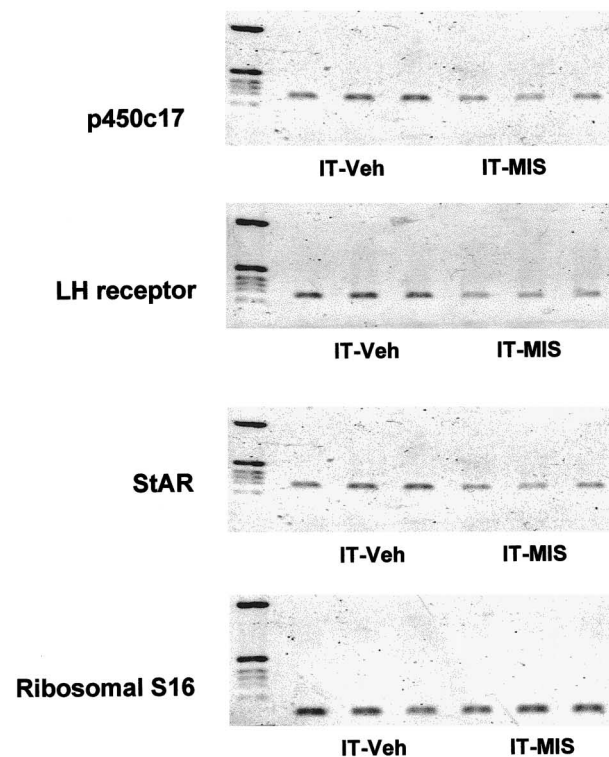


Figure 3. Continued.

quantitative RT-PCR was performed to compare the expression of p450c17, LH receptor, and StAR in Leydig cells purified from animals administered MIS or vehicle by intratesticular injection. The Leydig cells were isolated at the 4-hour time point. The mRNA expression of p450c17 and LH receptor were lower in the MIS-treated cells compared to vehicle ($P < .05$) (Figure 3A). No significant change was observed in StAR mRNA levels. Figure 3B shows a representative RT-PCR analysis. Northern analysis of total testicular RNA confirmed down-regulation of the mRNA transcripts for p450c17 and LH receptor but not StAR in MIS-treated animals compared to vehicle (data not shown). These results are in accord with in vitro data in Leydig cell tumor lines (Teixeira et al, 1999), which show that the inhibition of testosterone production by MIS is, in part, transcriptionally mediated.

DISCUSSION

Our data demonstrate a rapid but reversible inhibition of adult rat Leydig cell steroidogenesis following a single injection of MIS that achieved concentrations of MIS within the physiological range for young animals. In this

in vivo model, intratesticular administration of MIS resulted in much higher local concentrations of MIS in the injected testis compared to the contralateral testis with rapid equilibration in serum. The high local concentrations of MIS caused a decline in the testosterone production rate that was also reflected in lower serum testosterone concentrations. The contralateral testis, which was exposed to the same serum concentration of MIS but a lower local concentration, had a minimal decrement in testosterone production. The interstitial fluid MIS concentrations decreased eightfold from 4 to 8 hours, with a corresponding rise in testosterone concentrations to values comparable to those of vehicle-treated animals. These findings suggest that the effects of MIS on androgen steroidogenesis are reversible and may be dose dependent. Previous studies that characterized the effects of MIS in the MA-10 Leydig cell tumor line reported sustained down-regulation of p450c17 mRNA levels and progesterone production for 48 hours in culture (Teixeira et al, 1999). These studies, however, employed 105 nM MIS (14.7 $\mu\text{g/mL}$), a supraphysiologic concentration that is 25-fold higher than the peak interstitial fluid concentrations achieved by direct intratesticular injection of MIS in this study. Moreover, MIS in cell cultures is quite stable, with high concentrations maintained for greater than 24 hours, whereas testicular interstitial fluid and serum MIS concentrations decline rapidly in the MIS-injected animals and are undetectable after 48 hours. The short half-life of exogenous MIS in the rats was unexpected because previous studies using less purified bovine MIS had estimated a half-life of 36 hours in serum (Donahoe and MacLaughlin, unpublished data). Our work demonstrates that MIS has paracrine effects on Leydig cell steroidogenesis that require adequate local concentrations of MIS. The inhibition of testosterone production observed in our in vivo injection studies using concentrations of MIS that fall within the physiological range confirms the relevance of these findings to the regulation of androgen biosynthesis in the postnatal testis.

In vitro studies have shown that MIS inhibits testosterone production and p450c17 mRNA expression in primary Leydig cells (Rouiller-Fabre et al, 1998; Lee, 2000) and down-regulates p450c17 transcriptional activity in Leydig tumor cell lines (Teixeira et al, 1999). Our study demonstrates that direct intratesticular injection of MIS decreases the expression of p450c17 and the LH receptor and regulates the androgen production rate. In this model, the effects of exogenously administered MIS can be seen despite previous exposure of the Leydig cells to endogenous rodent MIS. Moreover, with pooled data, the expected variability in experimental conditions and biological responsiveness in these in vivo studies masks the greater impact of MIS on androgen production that is seen in individual experiments. We speculate that sustained ex-

posure of the Leydig cells to MIS as found physiologically within the testis might exert a more dramatic and prolonged inhibition of androgen production. This conclusion is supported by the finding of decreased testosterone concentrations in transgenic mice that overexpress MIS (Behringer et al, 1990; Racine et al, 1998).

The disparate effects of MIS on androgen production by Leydig cells harvested from the directly injected vs the noninjected testis indicate that the local concentration of MIS is critical for this action. These findings also suggest that MIS primarily functions as a paracrine hormone in the testis rather than centrally via regulation of the hypothalamic-pituitary-gonadal axis. Although mice transgenic for MIS have elevated concentrations of LH and follicle-stimulating hormone (FSH) (Racine et al, 1998), this may be secondary to the reduced concentrations of testosterone and estradiol rather than a direct effect of MIS. In the testis, MIS appears to inhibit p450c17 directly as well as indirectly by down-regulating LH receptor expression. LH stimulates testosterone production via its G-protein-coupled receptor that interacts with adenylate cyclase to increase cyclic adenosine monophosphate (AMP). Cyclic AMP then acts as a second messenger, inducing de novo synthesis of StAR and up-regulating the steroidogenic enzymes. Although not the rate-limiting enzyme for testosterone production, p450c17 is one of the key enzymes in the pathway. Sufficient inhibition of its activity will result in decreased testosterone production, as seen in clinical disorders caused by mutations in the p450c17 gene (Auchus and Miller, 1999). The expression of this enzyme is induced by LH (Payne, 1990) and IGF-1 (Le Roy et al, 1999) and is down-regulated by testosterone and estradiol (Payne and Youngblood, 1995; Majdic et al, 1996). Estradiol, however, regulates Leydig cell steroidogenesis by several different pathways (Bartke et al, 1977; Saez et al, 1978; Onoda and Hall, 1981; Majdic et al, 1996; Abney, 1999), including an estrogen receptor independent one involving direct competitive inhibition of 17α -hydroxylase activity (Onoda and Hall, 1981). Future studies will further elucidate the various mechanisms by which MIS modulates Leydig cell steroidogenesis.

MIS and its Type II receptor are both developmentally regulated and exhibit stage-specific expression in mature testes (Hirobe et al, 1992; Baarends et al, 1995). MIS is expressed at higher levels in the immature than in the mature testis, although there appears to be a transient rise at puberty (Lee, 2000), while expression of the MIS Type II receptor mRNA is down-regulated after birth, then induced again peripubertally (Baarends et al, 1995; Teixeira et al, 1996). In the adult testis, MIS mRNA is generally expressed at low levels in Sertoli cells but is maximal during Stage VII of the spermatogenic cycle (Baarends et al, 1995; Teixeira et al, 1996). By contrast, the MIS Type

II receptor mRNA increases to its highest levels at Stages VI and VII, then rapidly decreases at Stage VIII (Baarends et al, 1995). We speculate that in the mature testis, the varying levels of expression of MIS and its receptor help modulate the local production of androgens. In immature rats, the actions of MIS may prevent the premature onset of steroidogenesis by suppressing androgen production until the seminiferous tubules are sufficiently differentiated to support spermatogenesis.

The differentiation of Leydig cells in the postnatal testis can be divided into 3 discrete stages. Initially, the Leydig cells differentiate as mesenchymal-like progenitors, then they acquire more distinctive Leydig cell characteristics to become immature pubertal Leydig cells (Ewing and Keeney, 1993; Benton et al, 1995). Finally, as mature adult Leydig cells, they acquire maximal steroidogenic capacity and are fully differentiated. Establishment of the adult Leydig cell number depends on proliferation of the progenitor and immature Leydig cells and their subsequent differentiation, primarily under the stimulus of LH and FSH (Kerr and Sharpe, 1985; Saez, 1994). MIS has been shown to be inhibitory for this process. MIS-deficient mice develop Leydig cell hyperplasia (Behringer et al, 1994), while transgenic mice overexpressing MIS exhibit a block in differentiation with decreased mature Leydig cell numbers (Racine et al, 1998), indicating that MIS has a major role in Leydig cell development in the postnatal testis. In vitro studies have demonstrated that MIS decreases DNA synthesis of progenitor Leydig cells (Lee et al, 1999), suggesting that absence of the antiproliferative actions of MIS is responsible for the Leydig cell hyperplasia found in MIS-deficient mice. The current studies demonstrate that MIS also has a role in the regulation of Leydig cell-differentiated function by its ability to directly inhibit androgen production.

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