Effect of Myxothiazol on Leydig Cell Steroidogenesis: Inhibition of Luteinizing Hormone-Mediated Testosterone Synthesis but Stimulation of Basal Steroidogenesis

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Studies of MA-10 Leydig cells have shown that intact mitochondria with active respiration are essential for LH-induced Leydig cell steroidogenesis. To further elucidate the role played by mitochondria in steroidogenesis, we examined the effects of the perturbation of the mitochondrial electron transport chain with myxothiazol (MYX) on testosterone production by primary cultures of Brown Norway rat Leydig cells. Analysis of the steroidogenic pathway revealed that cAMP production and the activities of each of 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase/C17–20 lyase, and 17β-hydroxysteroid dehydrogenase were inhibited by MYX and that LH-stimulated testosterone production was suppressed. In contrast to the inhibition of LH-stimulated testosterone production by MYX, the incubation of Leydig cells with MYX in the absence of LH stimulated testosterone production. Although testosterone production was increased, steroidogenic acute regulatory protein was decreased in response to MYX, not increased as could be expected. Additional electron transport chain inhibitors had stimulatory effects on testosterone production that were similar to those of MYX, strongly suggesting that the effect of MYX on basal testosterone production is related to its effect on the mitochondrial electron transport chain. Finally, incubation of the cells with a combination of MYX and the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N′,N′,N′′-tetraacetic acid tetrakis(acetoxy)methyl ester suppressed MYX-mediated increased basal steroidogenesis but had no effect on hydroxycholesterol-mediated steroidogenesis. Taken together, these results indicate that inhibition of the mitochondrial electron transport chain can block LH-stimulated testosterone production through suppression of a number of steps of the steroidogenic pathway but also stimulates basal testosterone production through a calcium-mediated mechanism. (Endocrinology 148: 2583–2590, 2007)

LEYDIG CELL STEROIDOGENESIS is regulated primarily by LH secreted from the pituitary gland. Acting chronically, LH maintains normal Leydig cell morphology and expression of steroidogenic enzymes (1–4). Acting acutely, LH stimulates cholesterol transfer to the inner mitochondrial membrane in which it is metabolized to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) (2). Both the chronic and acute effects of LH are dependent primarily on the cAMP-protein kinase A signaling pathway (5). A key regulatory role is played by mitochondria; the rate-limiting step in the biosynthesis of steroid hormones is the transport of cholesterol to the inner mitochondrial membrane, a process that is dependent on the actions of steroidogenic acute regulatory protein (STAR) (6) and peripheral-type benzodiazepine receptor (PBR) (7). The conversion of cholesterol to pregnenolone at the inner mitochondrial membrane is catalyzed by the P450scc enzyme. After its synthesis in the mitochondria, pregnenolone moves out of the mitochondria to the smooth endoplasmic reticulum in which it is further converted into testosterone through reactions catalyzed by a series of three enzymes, 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase/C17–20 lyase (P450c17), and 17β-hydroxysteroid dehydrogenase (17β-HSD) (3, 4).

In previous studies of MA-10 Leydig tumor cells, perturbation of electron transport in mitochondria was found to inhibit steroidogenesis, suggesting that mitochondria must actively respire for successful steroidogenesis and, as a corollary, that alterations in the state of mitochondria may be involved in regulating steroid biosynthesis (8). Building on the findings with MA-10 cells, we wished to relate steroidogenesis to mitochondrial function in primary cultures of freshly isolated Leydig cells. To this end, the mitochondrial toxin myxothiazol (MYX) was used to disrupt mitochondrial function in primary cultures of freshly isolated Brown Norway rat Leydig cells. MYX was originally isolated as an antibiotic from the myxobacterium Myxococcus fulus (9, 10) and found to be an effective growth inhibitor of yeast and fungi at very low concentrations (0.01–3 μg/ml). MYX suppresses oxygen consumption of the cell (11) by blocking electron transport through cytochrome b-c1 of mitochondrial complex III (12) and has been widely used as a specific

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Abbreviations: AA, Antimycin A; AOS, azoxystrobin; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxy)methyl ester; dbCAMP, dibutyryl cAMP; DPI, diphenyleneiodonium; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; KOM, kroxoxin-methyl; MYX, myxothiazol; PBR, peripheral-type benzodiazepine receptor; P450c17, 17α-hydroxylase/C17–20 lyase; P450scc, P450 cholesterol side-chain cleavage enzyme; ROT, rotenone; SDS, sodium dodecyl sulfate; STAR, steroidogenic acute regulatory protein; TTFA, 2-thenoyltrifluoroacetone.

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inhibitor of mitochondrial complex III in various cellular systems (13, 14).

We report herein that the disruption of mitochondrial function by MYX has a significant suppressive effect on LH-mediated testosterone production by Brown Norway rat Leydig cells, consistent with the finding that suppression of the mitochondrial electron transport chain suppresses progesterone production by MA-10 cells (8). Additionally, we observed that MYX significantly stimulates basal steroidogenesis (i.e., steroidogenesis in the absence of LH stimulation) by rat Leydig cells. These results suggest that in addition to their well-established central role in hormone-mediated steroidogenesis, mitochondria may play a role in steroidogenesis that does not completely rely on the classical hormone-stimulated mechanism.

Materials and Methods

Reagents

MYX, 2-thienyltrifluoroacetone (TTFA), antimycin A (AA), azoxyspro- tobin (AOS), kreosin-methyl (KOM), diphenylhexamethione (DPI), sodium azide (NaN3), dibutyryl cAMP (dbcAMP), isobutyl-methylxan- theline (IBMX), 25-hydroxycholesterol, cycloheximide, and 1,2-bis(2-aminophenoxo)ethane-N,N,N′,N′-tetracetic acid tetrakis acetoxymethyl ester (BAPTA-AM) were obtained from Sigma-Aldrich (St. Louis, MO). M-199 medium was from Gibco (Grand Island, NY). Type III collagenase was from Worthington (Freehold, NJ). All the ste- roids, including pregnenolone, progesterone, 17-hydroxyprogesterone, androstenedione, and testosterone, were purchased from Steraloids (Newport, RI). [1,2,6,7,16,17-3H(N)]testosterone (115.3 Ci/mmol) was from PerkinElmer Life Sciences, Inc. (Boston, MA). Testosterone anti- body was obtained from MP Biomedicals (Solon, OH). STAR antibody was from ABR Inc. (Golden, CO). P450scc antibody was purchased from Chemicon International (Temecula, CA). MAPKs (ERK1/2) and phos- pho-ERK1/2 antibodies were from Cell Signaling Technology, Inc. (Bever- ley, MA). The Western blot detection kit and [3H]cAMP assay kit were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Bovine LH (USDA-bLH-B-6) was provided by the U.S. Department of Agricultur- al Animal Hormone Program (Beltsville, MD).

Animals

Brown Norway rats 4 months of age were obtained from Harlan Sprague Dawley (Indianapolis, IN) through the National Institute on Aging (Bethesda, MD) and housed in the animal facilities of the Johns Hopkins School of Public Health (22 C, 14-h light, 10-h dark cycle), with access to feed and water ad libitum. Animal handling and care were in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University.

Leydig cell isolation and treatment with electron transport chain inhibitors

Leydig cells were isolated from 4-month-old Brown Norway rats as previously described (15). In brief, the testicular artery was cannulated and perfused with type III collagenase (1 mg/ml) in dissociation buffer [M-199 medium with 2.2 g/liter HEPES, 0.1% BSA, 25 mg/liter trypsin inhibitor, 0.7 g/liter sodium bicarbonate (pH 7.4)]. Testes were then decapsulated and dissociated at room temperature with a 1:1000 dilution of collagenase C to a lower concentration (0.25 mg/ml) of the collagenase, with slow speed shaking (90 cycles/min). Seminiferous tubules were removed by filtration through 100 μm pore nylon mesh. The dissociated cells in the filtrate were subjected to centrifugal elutriation (16 ml/min, 2000 rpm). The fraction enriched with Leydig cells was collected and centrifuged (27,000 × g, 1 h) on a 90% Percoll gradient formed in situ. Leydig cells with a density of 1.07 and heavier were harvested for subsequent assay of testosterone production. Leydig cells, enumerated after their staining for 3ß-HSD activity, were about 95% pure in all experiments. The viability of the cells, assessed by trypan blue exclusion, was greater than 90%.

To assess the time and dose effect of MYX on testosterone production, freshly isolated Leydig cells (106) were incubated with MYX (1 μM to 10 μM) with or without LH (100 ng/ml) in 200 μl M-199 medium [containing 2.2 g/liter HEPES, 0.1% BSA, 2.2 g/liter sodium bicarbonate (pH 7.4)] at 34 C for up to 2 h. In all but the dose-response study, MYX was used at a concentration of 1 μM. To study the specificity of the MYX effect, the electron transport chain inhibitors rotenone (ROT, 1 μM), TTF1 (1 μM), AA (1 μM), KOM (100 μM), ACS (10 μM), DPI (1 μM), and NaN3 (1 mM) were used. The inhibitors were first dissolved in dimethyl sulfoxide and then diluted into culture medium. The final concentration of dimethyl sulfoxide in the medium was less than 0.2%, a concentration that was shown to have no effect on Leydig cell steroidogenesis in preliminary experiments. To examine the effect of MYX on the ste- roidogenic pathway, cells were incubated with MYX (1 μM) plus db-cAMP (2 μM), 22-hydroxycholesterol (20 μM), 25(R)-hydroxycholesterol (25 μM), or pregnenolone (12.5 μM) for 2 h. To assess the effect of protein synthesis on MYX action, cells were preincubated with the protein synthesis inhibitor cycloheximide (0.5 μM) for 30 min before adding MYX and LH. To assess the effect of calcium on MYX action, cells were preincubated with the calcium-erelating agent BAPTA-AM (20 μM) for 30 min before adding MYX, LH, or hydroxycholesterol. Cultures were maintained in 20 μM BAPTA-AM for the duration of the 2-h incubation. After the incubations, the cells and media were frozen at −80 C for subsequent testosterone assay by RIA. The sensitivity and intra- and interassay coefficients of variation of the RIA were 13 pg/tube and 8.9 and 13.6%, respectively.

cAMP production

To assay the effect of MYX on LH-stimulated cAMP production, Leydig cells were preincubated with or without MYX (1 μM) for 2 h. The medium was then removed and fresh phenol-red-free M-199 medium (50 μl) containing LH (100 ng/ml) was added to the plates. The medium also contained 100 μM isobutyl-methylxanthine to inhibit phosphodieste- rase activity. After a 20-min incubation, 50 μl Tris buffer [0.05 M (pH 7.5) containing 4 mM EDTA, 2 mg/ml theophylline] was added to the culture medium and the plates were frozen on dry ice and stored at −80 C until cAMP assay. The cAMP concentration was assayed by a cAMP [3H] assay kit system (Amersham, Piscataway, NJ) according to the manufacturer’s instructions. The sensitivity of the assay was 0.05 pmol per assay tube.

Steroidogenic enzyme activities and testosterone metabolism

Steroidogenic enzyme activities were assayed using HPLC to mea- sure steroids according to a previously published method (17). In brief, the cells were incubated with pregnenolone (25 μM), progesterone (12.5 μM), or androstenedione (12.5 μM), substrates for 3ß-HSD, P450sc17, and 17ß-HSD, respectively, in the presence or absence of MYX for 2 h. The steroid products of each enzyme were assayed by HPLC after extraction of the culture medium with ether. 11ß-Hydroxy-androstenedione (200 ng/tube) was mixed with the samples before the extraction as an internal standard to correct for recovery and HPLC loading. After ether was evaporated, the contents in the tubes were dissolved in 50 μl methanol and injected into a reverse-phase HPLC C18 column (Waters-Millipore Associates Inc., Milford, MA) with methanol/tetrahydrofuran/water (28:16:56) as the mobile phase. Peaks of the five 3α- ketosteroids were detected by an online UV absorbance detecting system at 240 nm. In- dividual steroids were determined by integrated peak areas and cor- rected by recoveries monitored by the internal standard.

The effect of MYX on the ability of Leydig cells to metabolize tes- tosterone was assayed by incubation of Leydig cells (about 106 cells) with
Myxothiazol (MYX) inhibits LH-stimulated testosterone production, the effects of MYX on critical steps of the steroidogenic pathway were examined. Preincubation of Leydig cells with MYX (1 μM) for 2 h inhibited the ability of Leydig cells to produce cAMP in response to subsequent LH stimulation (Fig. 2A). Likewise, when cells were incubated with MYX and dbcAMP, 22-hydroxycholesterol (HC), pregnenolone (Preg; 12.5 μM), or 17β-HSD (12.5 μM androstenedione) in the presence or absence of MYX (1 μM) for 2 h. Testosterone was assayed in the medium. Panel C, Leydig cells were incubated with substrate for 3β-HSD (25 μM pregnenolone), P450c17 (12.5 μM progesterone), or 17β-HSD (12.5 μM androstenedione) in the presence or absence of MYX for 2 h. The steroid products (progesterone, 17α-hydroxyprogesterone, androstenedione, and testosterone) were quantified by HPLC and summed. The enzyme activities were expressed as nanomoles of substrate converted during the 2-h incubation. The bars represent the mean ± SEM of four experiments. *, Significantly different from the values obtained from cells incubated without MYX at P < 0.05.

Western blot analysis

To examine the effect of MYX on the levels of StAR, P450scc, and the phosphorylation of MAPK (ERK1/2), 106 cells were incubated with MYX (1 μM) and/or LH (100 ng/ml) for 2 h. The cells were lysed with Tris-sodium dodecyl sulfate (SDS) buffer (75 mM Tris, 2% SDS, 50 mM dithiothreitol [pH 6.8]), sonicated on ice, and then mixed with 3× SDS loading buffer (New England BioLab Inc., Ipswich, MA). Protein from equal numbers of cells (2×10^6) was separated on a 10% polyacrylamide SDS gel and then transferred onto a nitrocellulose membrane. After incubation with primary antibody (1:400) and horseradish peroxidase-conjugated secondary antibody (1:5000), the signals were detected by the enhanced chemiluminescence Western blot kit from Amersham and quantified by densitometry scanning of the film. For some membranes, the bound antibodies were stripped by Restore Western blot stripping buffer (Pierce, Rockford, IL) and reblotted with new antibodies.

Statistical analyses

Data are expressed as the mean ± SEM of three to four different pools of cells that came from different animals. Statistical differences were determined by one-way ANOVA. If group differences were revealed by ANOVA (P < 0.05), differences between individual groups were determined with the Student-Newman-Keuls test by using SigmaStat software. Values were considered significant at P < 0.05.

Results

Effect of MYX on LH-stimulated Leydig cell testosterone production and the steroidogenic pathway

To examine the effect of MYX on LH-stimulated Leydig cell steroidogenesis, isolated cells were incubated for 2 h with increasing concentrations of MYX (0–10 μM) in the presence of maximally stimulating LH (100 ng/ml). As seen in Fig. 1, testosterone production was inhibited significantly as a function of MYX concentration.

To begin to elucidate the mechanism by which MYX inhibits LH-stimulated testosterone production, the effects of MYX on the production and the steroidogenic pathway were examined. Preincubation of Leydig cells with MYX (1 μM) for 2 h inhibited the ability of Leydig cells to produce cAMP in response to subsequent LH stimulation (Fig. 2A). Likewise, when cells were incubated with MYX and dbcAMP, 22-hydroxycholesterol (HC), pregnenolone (Preg; 12.5 μM), or 17β-HSD (12.5 μM androstenedione) in the presence or absence of MYX (1 μM) for 2 h. Testosterone was assayed in the medium. Panel C, Leydig cells were incubated with substrate for 3β-HSD (25 μM pregnenolone), P450c17 (12.5 μM progesterone), or 17β-HSD (12.5 μM androstenedione) in the presence or absence of MYX for 2 h. The steroid products (progesterone, 17α-hydroxyprogesterone, androstenedione, and testosterone) were quantified by HPLC and summed. The enzyme activities were expressed as nanomoles of substrate converted during the 2-h incubation. The bars represent the mean ± SEM of four experiments. *, Significantly different from the values obtained from cells incubated without MYX at P < 0.05.

FIG. 1. Effects of MYX on LH-stimulated testosterone production. Leydig cells were incubated with increasing doses of MYX (0–10 μM) in the presence of maximally stimulating LH (100 ng/ml) for 2 h. Testosterone was assayed in the medium after incubation. Each bar represents the mean ± SEM of four experiments. *, Significantly different from the values obtained from cells incubated without MYX (0) at P < 0.05.

FIG. 2. Effects of MYX on cAMP (panel A), testosterone production (panel B), and steroidogenic enzyme activities (panel C). Panel A, After preincubation with or without MYX (1 μM) for 2 h, Leydig cells were incubated with maximally stimulating LH (100 ng/ml) for 20 min. Total cAMP was assayed in the medium plus cells. Control (C) cells were incubated without LH or MYX. Panel B, Leydig cells were incubated with dbcAMP (2 mM), 25-hydroxycholesterol (HC; 25 μM), or pregnenolone (Preg; 12.5 μM) in the presence or absence of MYX (1 μM) for 2 h. Testosterone was assayed in the medium. Panel C, Leydig cells were incubated with substrate for 3β-HSD (25 μM pregnenolone), P450c17 (12.5 μM progesterone), or 17β-HSD (12.5 μM androstenedione) in the presence or absence of MYX for 2 h. The steroid products (progesterone, 17α-hydroxyprogesterone, androstenedione, and testosterone) were quantified by HPLC and summed. The enzyme activities were expressed as nanomoles of substrate converted during the 2-h incubation. The bars represent the mean ± SEM of four experiments. *, Significantly different from the values obtained from cells incubated without MYX at P < 0.05.
droxycholesterol, 25-hydroxycholesterol, or pregnenolone, testosterone production was significantly reduced in each case (Fig. 2B and data not shown), as were the activities of each of 3β-HSD, P450c17, and 17β-HSD (Fig. 2C). These results suggest that MYX inhibits LH-stimulated testosterone production at multiple sites along the steroidogenic pathway.

Because the mitochondria provide the free energy for the synthesis of ATP, we reasoned that decreased levels of ATP could contribute to the inhibitory effect of MYX on the multiple steps of hormone-mediated steroidogenesis. Indeed, we observed that in response to a 2-h incubation of Leydig cells with MYX plus LH, intracellular ATP content was reduced significantly, by more than 70% (Fig. 3).

**Effect of MYX on basal (unstimulated) Leydig cell testosterone production**

In light of the inhibitory effects of MYX on LH-stimulated testosterone production by Leydig cells, the observed effects of MYX on basal (unstimulated) testosterone production by these cells, shown in Fig. 4, were unexpected. In this study, the cells were incubated for 2 h with increasing concentrations of MYX without LH in the medium. Basal testosterone production increased significantly from control in response to MYX concentrations of 0.01–10 μM (Fig. 4A). In response to MYX at 1 μM, progressive increases in testosterone production were seen from 15 to 120 min of incubation (Fig. 4B).

We hypothesized that increased STAR protein levels might correlate with the increased basal testosterone production in response to MYX. To test this hypothesis, StAR protein levels were analyzed by Western blot (Fig. 5A). Whereas StAR protein were elevated at 2 h of LH stimulation, StAR protein levels decreased in response to MYX, indicating that MYX effects on basal testosterone production are unlikely to involve enhanced StAR protein synthesis. Neither LH nor MYX exposure resulted in changes in P450scc protein levels from control values within the 2 h incubation (Fig. 5A), arguing against enhanced or diminished P450scc expression as playing a significant role in either of the observed effects of MYX. Consistent with these findings, treatment with the protein translation inhibitor cycloheximide suppressed LH-stimulated steroidogenesis and had no effect on MYX-stimulated testosterone production (data not shown), suggesting that MYX increases testosterone production by a mechanism other than elevation of StAR protein expression.

We next tested the possibility that the increased basal testosterone seen with MYX exposure might result from reduced testosterone metabolism/degradation. To this end, Leydig cells were incubated with 3H-testosterone in the presence or absence of 1 μM MYX or 100 ng/ml LH for 2 h. The 3H-testosterone left in the medium after the incubation was quantified after its separation from testosterone metabolites by HPLC. Both MYX and LH treatments were found to increase, not decrease, the rate of 3H-testosterone metabolism/degradation (Fig. 5B), suggesting that increased basal testosterone induced by MYX is unlikely to result from reduced testosterone metabolism.

In addition to the classic cAMP-protein kinase A pathway, LH and growth factors have been shown to activate the MAPK signaling pathway in Leydig cells (18, 19). MAPK signaling has been shown to stimulate the transfer of cholesterol across the mitochondrial membrane by a StAR-independent mechanism (19). To examine whether MYX causes increased basal testosterone production through MAPK signaling, the phosphorylation of p42/p44 MAPK was analyzed by Western blot (Fig. 5A). Whereas MAPK phosphorylation increased in response to LH stimulation, MAPK phosphorylation decreased in response to MYX, indicating that MYX inhibits LH-stimulated MAPK signaling.

**Fig. 4. Effects of MYX on basal testosterone production. A, Leydig cells were incubated with increasing doses of MYX (0–10 μM) in the absence of LH for 2 h. B, Leydig cells were incubated with MYX at 1 μM for 0 min to 2 h. Testosterone was assayed in the medium. Each bar represents the mean ± SEM of four experiments. *, Significantly different from the values obtained from cells incubated without MYX (0) at P < 0.05.**

**Fig. 3. Effect of MYX on intracellular ATP content. Leydig cells were incubated with LH or LH plus MYX for 2 h. Intracellular ATP content in cells was analyzed by an ATP bioluminescence assay kit. Each bar represents the mean ± SEM of four experiments. *, Significantly different from the values obtained from cells incubated without MYX at P < 0.05.**
(ERK1/2) was analyzed by Western blots (Fig. 6A). In either the presence or absence of LH, MYX (1 µM) suppressed the phosphorylation of ERK1/2 to below detectable levels within 2 h, without affecting total ERK1/2 protein levels. This result suggests that the increased basal testosterone production by MYX-treated cells occurs by a mechanism(s) that does not involve increasing ERK1/2 phosphorylation. Figure 6B shows that a 2-h incubation of Leydig cells with MYX alone resulted in significantly reduced intracellular ATP content. This supports the contention that MYX stimulation of testosterone production is unlikely to occur via a phosphorylation-dependent mechanism, which is consistent with the results observed with ERK1/2 or StAR.

We hypothesized that the mechanism by which MYX acts to stimulate basal testosterone production (as well to inhibit LH-stimulated testosterone production) is through its inhibition of the mitochondrial electron transport chain and reasoned that if this is the case, other electron transport chain inhibitors also should affect testosterone production. To test this, we examined the effects on basal testosterone production of ROT, which inhibits complex I; TTFA, which inhibits complex II; AA, AOS, and KOM, all of which inhibit complex III; NaN3, which acts on complex IV; and DPI, which serves as a general inhibitor of flavin protein. Of these seven mitochondrial inhibitors, five (ROT, TTFA, AA, AOS, KOM) were as effective in stimulating testosterone production as MYX (Fig. 7). NaN3 and DPI failed to stimulate testosterone production. These results strongly support the contention that MYX effects on testosterone result from its effects on the mitochondrial electron transport chain.

To examine the possibility that inhibition of the mitochondrial electron transport chain stimulates basal steroidogenesis through a calcium-dependent mechanism, we incubated Leydig cells with the intracellular calcium chelator BAPTA-AM in addition to MYX, LH, or 22-hydroxycholes-
terol. As shown in Fig. 8, incubation of the cells with BAPTA-AM significantly inhibited MYX/LH-mediated steroidogenesis but had no effect on basal or 22-hydroxycholesterol-mediated steroidogenesis. These findings implicate a role for calcium in MYX-stimulated basal testosterone production.

Discussion

Based on previous studies showing that perturbation of the mitochondrial electron transport chain resulted in suppression of progesterone production by MA-10 Leydig cells (8), we hypothesized that inhibition of the electron transport chain would suppress LH-stimulated testosterone production by freshly isolated Leydig cells. We show that the short-term (2 h) incubation of Leydig cells with MYX, which binds to the cytochrome bc1 segment of complex III and thereby blocks the transfer of the electrons from ubiquinol to cytochrome c (12), resulted in inhibition of LH-stimulated testosterone production as a function of MYX concentration. This inhibition was associated with the reduced ability of the cells to produce cAMP and with reduced activities of each of 3β-HSD, P450c17, and 17β-HSD. These results suggest that MYX inhibits LH-stimulated testosterone production at multiple sites along the steroidogenic pathway, presumably downstream of its inhibition of the mitochondrial electron transport chain. The observation that intracellular ATP content was reduced significantly by MYX is consistent with MYX’s presumed effect on electron transport and concomitantly on mitochondrial membrane potential.

Because ATP is required for several steps in steroidogenesis, MYX, by disrupting mitochondria, would be expected to have effects at a number of steps of the steroidogenic pathway. For example, the decreases seen in cAMP production and ERK1/2 phosphorylation when cells were incubated with MYX both might be a consequence of reduced ATP production. In turn, reduced ATP may directly affect 17β-HSD activity (20). An additional consequence of ATP deficiency could be reduced StAR-dependent cholesterol transport because actively respiring mitochondria appear to be necessary for this step in the acute stimulation of Leydig cell steroidogenesis (8).

The finding that MYX inhibited steroidogenesis (testosterone production) in freshly isolated Leydig cells is consistent with the earlier observation that inhibition of the mitochondrial electron transport chain in MA-10 cells suppressed steroidogenesis (progesterone production) by these cells (8). However, our observation that MYX inhibited steroidogenic enzymes downstream of the mitochondria was not observed in the studies of MA-10 cells (8), suggesting that the mechanism by which inhibition occurred might differ in the two cell types. In the current study, we found that using 25-hydroxycholesterol or pregnenolone as substrates resulted in decrease in testosterone production by MYX-incubated cells. This is in contrast to the finding in MA-10 Leydig cells in which the use of 22(R)-hydroxycholesterol, a different hydrophilic cholesterol substrate, resulted in normal progesterone production (8). It seems unlikely that the cholesterol substrate accounts for the difference, however, because in unpublished studies, we obtained the same results with 22(R)-hydroxycholesterol as with 25-hydroxycholesterol. In our study, MYX inhibited the activities of 3β-HSD, P450c17, and 17β-HSD. MA-10 cells do not contain the final two enzymes of the testosterone synthesis pathway, P450c17 and 17β-HSD, so no direct comparison at this level is possible between the systems. However, 3β-HSD activity was not inhibited in the MA-10 cells (8). It may be our use of the novel agent, myxothiazol, that accounts for the difference. Moreover, system-specific differences between the two cell types cannot be discounted. Nonetheless, the results of our studies, in combination with those of Allen et al. (8), indicate clearly the importance of mitochondrial electron transport chain fidelity and ATP for steroidogenesis.

The inhibitory effects of MYX on steps of the steroidogenic pathway may explain the inhibition of LH-stimulated steroidogenesis but not the paradoxical enhancement of basal testosterone production. In light of the known effects of MYX on the mitochondrial electron transport chain, we hypothesized that the stimulatory effect of MYX on basal testosterone

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**Fig. 8.** Effects of the intracellular calcium chelator BAPTA-AM on Leydig cell testosterone production. Cells were preincubated for 30 min in the presence or absence of BAPTA-AM (20 μM). The cultures were then incubated with MYX (1 μM), LH (100 ng/ml), or 22-hydroxycholesterol (HC; 20 μM) in the presence or absence of BAPTA-AM (20 μM) for 2 h. Cells incubated in the absence of MYX, LH, or HC served as controls (C). Testosterone was assayed in the medium. The figure shows testosterone concentrations; the inset shows the same data as percentage of vehicle. **Bars** represent the mean ± SEM from three experiments. *Significantly different from the values obtained from cells incubated without BAPTA-AM at P < 0.05.
production was likely to be related to its ability to inhibit the mitochondrial electron transport chain. In fact, we found that a number of electron transport chain inhibitors, including ROT, TTFA, AA, AOS, and KOM also stimulated Leydig cell basal testosterone production. Thus, the inhibition of the mitochondrial electron transport chain serves as a common theme in this increase of testosterone production.

We sought to identify a more specific mechanism by which inhibition of the electron transport chain results in increased testosterone. Our observation that testosterone metabolism/degradation was increased in response to MYX exposure is inconsistent with the possibility that the MYX-mediated increased basal testosterone production results from reduced testosterone metabolism/degradation. The evidence that we present also argues against the possibility that MYX increases basal testosterone production through stimulation of Leydig cell MAPK signaling or ATP content. If MYX increases basal testosterone production via a STAR-mediated pathway, it should be sensitive to cycloheximide-mediated suppression. In fact, we failed to observe any significant effect of cycloheximide on MYX-stimulated testosterone production (our unpublished results), suggesting that MYX increases basal testosterone production by a mechanism other than increasing STAR protein synthesis. This was confirmed by Western blot, which showed that MYX results in decreased, not increased, STAR protein content in the Leydig cells. Moreover, because ATP production is a good marker of mitochondrial membrane potential, which is critical for STAR-mediated cholesterol transport to the mitochondria, diminished ATP levels in the presence of MYX also argues that MYX stimulates testosterone production via a STAR-independent mechanism.

How then might perturbation of the mitochondrial electron transport chain lead to increased testosterone production? The rate-limiting step in the acute regulation of Leydig cell testosterone production is cholesterol transport into the mitochondria. This may be the target site of stimulatory MYX activity because, as with LH and dbcAMP, MYX results in increased testosterone production within minutes and thus is not dependent on gene expression. In addition to STAR, PBR also has been shown to play a major role in cholesterol transport into mitochondria in steroidogenic cells (7). There is evidence that PBR and STAR may function coordinately in this process, with STAR functioning as an initiator of cholesterol transport and PBR as a gate for cholesterol entry into mitochondria (21, 22). Thus, a possible explanation for MYX-mediated steroidogenesis is through PBR stimulation at the mitochondrial membrane. Several lines of evidence support this possibility. First, as with MYX activity, the effect of PBR on cholesterol transport is cycloheximide insensitive (23). Second, PBR is involved in the regulation of the mitochondrial membrane potential and in cellular oxygen sensing (24), two processes strongly dependent on the electron transport chain. Third, change in the redox status of the Leydig cell has been shown to modify PBR and its ability to transport cholesterol (25). The inhibition of the electron transport chain modifies the mitochondrial redox state (26). It is possible, therefore, that MYX and the other electron transport chain inhibitors may increase cholesterol transport by stimulating PBR activity in a redox-dependent manner.

Finally, the data presented herein suggest the involvement of calcium in MYX-mediated testosterone synthesis. It has been recognized for some time that mitochondria are able to accumulate calcium and more recently that mitochondria are able to regulate intracellular calcium levels (27, 28). Inhibition of the electron transport chain has been shown to result in increased intracellular calcium levels in a number of cell types (29, 30). This has implications for Leydig cell steroidogenesis. Several groups have shown that intracellular Ca2+ concentrations increase in parallel with hormone-stimulated Leydig cell testosterone production and that suppression of this Ca2+ increase by chelation suppresses steroidogenesis (31, 32). Additionally, Ca2+-dependent stimulation of testosterone synthesis has been observed to be cAMP independent (31, 33). We reasoned that if elevated intracellular calcium levels resulting from MYX inhibition of mitochondrial electron transport is involved in stimulating testosterone production, MYX-mediated steroidogenesis should be sensitive to calcium chelation. Indeed, as shown in Fig. 8, incubation of Leydig cells with the intracellular calcium chelator BAPTA-AM significantly inhibited MYX-stimulated steroidogenesis but did not affect basal or hydroxycholesterol-mediated steroidogenesis. These observations provide support for a model wherein inhibition of mitochondrial metabolism stimulates mitochondrial calcium release, which in turn stimulates testosterone production.

In summary, we have shown that MYX is able to suppress LH-stimulated Leydig cell testosterone production by inhibiting multiple steps of the steroidogenic pathway. Unexpectedly, MYX also was found able to stimulate basal testosterone production. Thus, in addition to its well-established central role in hormone-mediated steroidogenesis, mitochondria may play a role in steroidogenesis that does not rely on the classical hormone-stimulated cellular cholesterol-transfer machinery. The effect of MYX on basal testosterone production was shared by multiple electron transport chain inhibitors, which is consistent with the contention that MYX exerts its effects by inhibition of the mitochondrial electron transport chain. MYX was found to stimulate basal testosterone production by Leydig cells after only brief exposure of the cells, suggesting that MYX’s effects are nongenomic. Such nongenomic activity would likely involve early cell-signaling events. The use of electron transport inhibitors may be a useful tool in investigating the two models proposed above, the stimulation of PBR-mediated steroidogenesis and/or mitochondrial calcium release-mediated steroidogenesis.

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