PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 75 (2000) 229–236

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Econazole and miconazole inhibit steroidogenesis and disrupt steroidogenic acute regulatory (StAR) protein expression post-transcriptionally

Lance P. Walsh [a], Connye N. Kuratko [b], Douglas M. Stocco [a,*]

[a] *Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA*
[b] *Department of Pathology, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA*

Accepted 28 September 2000

Abstract

The imidazole antifungal drugs econazole and miconazole have previously been shown to disrupt steroidogenesis in Leydig and adrenal cells by inhibiting 17α-hydroxylase/17,20-lyase (P450c17) enzyme activity, thus reducing the conversion of progesterone to androstenedione. However, a recent study in Y-1 adrenal cells indicated that these compounds may also reduce the availability of cholesterol to the cytochrome P450 side chain cleavage ($P450_{scc}$) enzyme, the first enzyme in the steroidogenic pathway. Since the steroidogenic acute regulatory protein (StAR) mediates the transfer of cholesterol from the outer to the inner mitochondrial membrane where the $P450_{scc}$ enzyme resides, an action which constitutes the rate-limiting and acutely-regulated step in steroidogenesis, we hypothesized that these drugs may also reduce StAR expression and/or activity. Our studies demonstrate that these drugs reversibly inhibited $(Bu)_2$cAMP-stimulated progesterone production in a dose- and time-dependent manner in MA-10 cells without affecting total protein synthesis or $P450_{scc}$ and 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme expression or activity. In contrast, they dramatically decreased $(Bu)_2$cAMP-stimulated StAR protein expression post-transcriptionally. This study indicates that StAR protein is susceptible to inhibition by at least some imidazole compounds that inhibit steroidogenesis.

Keywords: StAR protein; Imidazole; Steroidogenesis

1. Introduction

Econazole and miconazole are commonly used imidazole compounds effective in the treatment of cutaneous mycoses. Miconazole is also effective against severe pulmonary fungal disease which occurs frequently in immunosuppressed patients and in patients with terminal cancer. Imidazole antifungal drugs disrupt ergosterol biosynthesis in yeast and cholesterol biosynthesis in humans by inhibiting sterol 14α-demethylase, a microsomal cytochrome P450-dependent enzyme system [1]. In yeast, this causes an accumulation of 14α-methylsterols in fungal membranes, resulting in the close packing of phospholipid acyl chains, thus limiting fungal growth [2]. Imidazole compounds (at nanomolar to micromolar concentrations) have been shown to reduce the activities of a variety of cytochrome P450-dependent steroid metabolizing enzymes, including P450-dependent 17α-hydroxylase/17,20-lyase (P450cI7) steroidogenic enzyme activity in Leydig and adrenal cells [3,4]. However, recent studies in Y-1 adrenal cells have shown that these compounds also inhibit acute pregnenolone production without affecting cytochrome P450 cholesterol side chain cleavage (P450scc) enzyme activity [5], suggesting that they may block the availability of cholesterol to the $P450_{scc}$ enzyme. Although these compounds reduce de novo cholesterol biosynthesis, this cannot account for the reduction in steroidogenesis observed during acute stimulation since newly synthesized cholesterol only contributes a small percentage to the amount of steroids made during this period [6]. Since the intrami-

* Corresponding author. Tel.: +1-806-7432505; fax: +1-806-7432990.
E-mail address: doug.stocco@ttmc.ttuhsc.edu (D.M. Stocco).

0960-0760/01/$ - see front matter
PII: S0960-0760(00)00170-9

tochondrial transfer of cholesterol constitutes the rate-limiting and acutely-regulated step in steroidogenesis, it may be an important site of steroidogenic inhibition.

The steroidogenic acute regulatory (StAR) protein mediates the rate-limiting and acutely regulated step in steroidogenesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane where the $P450_{scc}$ enzyme initiates the synthesis of all steroid hormones (for review see [7]). StAR protein is a family of 37 and 30-kDa mitochondrial phosphoproteins expressed in adrenal and gonadal steroidogenic cells that is rapidly synthesized in response to trophic hormone stimulation and whose active form is highly labile. Transfection of steroidogenic cells with StAR and non-steroidogenic cells with StAR and the cholesterol side-chain cleavage enzyme system results in a six-fold increase in steroidogenesis [8]. Further, mutations in the StAR gene cause the disease Lipoid Congenital Adrenal Hyperplasia (lipoid CAH), a condition that results from the inability of the new-born to synthesize adequate amounts of steroids [9].

The present study was performed to determine if econazole and miconazole reduce StAR activity and/or expression. Using MA-10 Leydig tumor cells, we evaluated the effects of these drugs on progesterone production, and on StAR, $P450_{scc}$ and 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme activity, protein and mRNA levels.

2. Materials and methods

2.1. Chemicals

Waymouth's NIB 752/1 medium, horse serum, gentamicin sulfate, lyophilized trypsin-EDTA, phosphate-buffered saline with Ca^{2+} and Mg^{2+}, (PBS^{+}), and phosphate buffered saline without Ca^{2+} and Mg^{2+}, (PBS^{-}) were purchased from Gibco Life Technologies (Gaithersburg, MD). [1,2,6,7-N-^{3}H(N)]-progesterone (Specific Activity (SA), 97 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Antibodies to progesterone were obtained from Holly Hills Biological (Hillsboro, OR). Percoll and Dextran T70 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Nunc cell culture dishes, charcoal (Norit), trichloroacetic acid, scintiverse BD and sodium bicarbonate were obtained from Fisher Scientific (Houston, TX). Acrylamide, bis acrylamide, and SDS were purchased from Bio-Rad (Hercules, CA). Bovine serum albumin (BSA), $(BU)_2$cAMP, 22(R)-hydroxycholesterol (22R-HC), econazole 1-(2-[(4-chlorophenyl)methoxy]-2-[2,4-dichlorophenyl] ethyl)-1H-imidazole and miconazole 1-(2,4dichloro-p-[(2,4-dichlorobenzyl)-oxylphenethyl)imidazole were purchased from Sigma (St Louis, MO). Rabbit antisera to amino acids 88–98 of mouse StAR protein was generated by Research Genetics (Huntsville, AL). Rabbit antisera to amino acids 421–441 of rat $P450_{scc}$ enzyme was purchased from Chemicon (Temecula, CA). Antisera to purified mouse 3β-hydroxysteroid dehydrogenase I (3β-HSD) was a gift from Dr Allesandro Capponi, University of Geneva (Geneva, Switzerland). Horseradish peroxidase conjugated donkey anti-rabbit IgG was purchased from Amersham (Arlington Heights, IL). StAR cDNA was previously cloned in our laboratory [8]. Bovine $P450_{scc}$ cDNA was obtained from Dr Michael Waterman, Vanderbilt University (Nashville, TN); Mouse 3β-HSD I cDNA was provided by Dr Anita Payne, Stanford University (Stanford, California); Mouse L-19 and 18S rRNA cDNAs were obtained from Dr Gail Cornwall, Texas Tech University Health Sciences Center (Lubbock, TX).

2.2. MA-10 cell culture

The mouse MA-10 Leydig tumor cell line was a gift from Dr Mario Ascoli, University of Iowa College of Medicine (Iowa City, IA). Cells were maintained in Waymouths MB 752/1 medium + 15% horse serum at 37°C, 5% CO_2 as described previously [10]. For dose-response, time-course, steroidogenic enzyme activity and reversibility studies, 75 000 cells were seeded into each well of a 96-well plate and grown overnight. For the remaining studies, 1.5×10^6 cells were plated into 100 mm culture dishes and grown until 80% confluent. For all experiments, medium was removed, cells were washed twice with PBS^{+}, and serum-free Waymouth's medium containing the appropriate treatment was placed on the cells.

2.3. Treatment of cells

Stimulation of MA-10 cells was performed using a maximal stimulatory dose of $(BU)_2$cAMP (1 mM). In some studies, optimal concentrations of 22R-hydroxycholesterol (25 μM) were provided as steroidogenic substrate. All treatments were performed in serum-free media. Final concentrations of the DMSO and ethanol used as chemical solvents were $< 0.4\%$.

2.4. Dose-response and time-course studies

In dose-response and time-course studies, the effects of xenobiotics on steroidogenesis and total cellular protein synthesis were determined. In dose-response studies, $(BU)_2$cAMP-stimulated MA-10 cells grown in 96-well plates were treated for 2 h with various amounts of xenobiotics. The concentrations of econa-

zole and miconazole required to reduce progesterone production by 50% (IC50) were calculated using linear regression analysis. Eadie/Hofstee plots ([Progesterone] versus [Progesterone]/[econazole or miconazole]) of the dose-response data were made [11]. The slope of the line equals the IC_{50} value. In time-course studies, $(Bu)_2$cAMP-stimulated MA-10 cells grown in 96 well plates were treated in the presence or absence of each xenobiotic for 2 or 4 h.

2.5. RIA

Quantitation of progesterone in the medium was performed by RIA as previously described [12]. Analysis of RIA data was performed using a software program which was written by Bennie Shaw (Texas Tech University Health Sciences Center, Lubbock, TX). Data are expressed as ng/ml media.

2.6. Determination of total cellular protein synthesis

To determine the effects of compounds on total protein synthesis, cells grown in 96-well plates were treated as described above with the inclusion of 5 μCi/ml Expre^{35}S ^{35}S Protein Labeling Mix (SA, 1000 Ci/mmol; New England Nuclear; Boston, MA). A zero time point control was taken in which the Expre^{35}S^{35}S Protein Labeling Mix was added and immediately removed. Also, determination of total protein content by a modification of the Bradford method [13] was performed on identically plated cells that were not treated with Expre^{35}S^{35}S. Following treatment, media was removed and cells were solubilized for 2 h in 0.25 M NaOH at 37°C. Next, an equal volume of cold 20% trichloroacetic acid (TCA) was added and protein was precipitated overnight at 4°C. TCA precipitable material was transferred onto glass fiber filters using a 1225 Sampling Manifold (Millipore; Bedford, MA) and rinsed with 5% TCA, dried and counted in a liquid scintillation counter. Results were reported as: counts per minute/mg protein/2 or 4 h minus counts per minute/mg protein/0 h.

2.7. Determination of $P450_{scc}$ and 3β-HSD activity and reversibility

The effects of xenobiotics on the combined activities of the $P450_{scc}$ and 3β-HSD enzymes were determined by adding 22R-HC to MA-10 cells in the presence or absence of the xenobiotic for 2 h and measuring progesterone production. To determine reversibility, cells were then rinsed with PBS^+, allowed to recover for 24 h in serum containing medium, and incubated again for 2 h with $(Bu)_2$cAMP and/or 22R-HC. Progesterone in the media was measured by RIA.

2.8. Isolation of mitochondria and Western blot analysis

MA-10 cells grown in 100 min plates were stimulated with $(Bu)_2$cAMP in the presence or absence of econazole or miconazole (25 μM) for 4 h. Mitochondria were isolated by homogenization and differential centrifugation [8]. Then, Western blot analysis of mitochondrial protein was performed as previously described [14]. Following detection of StAR, the membrane was stripped in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol at 70°C for 30 min, washed in 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl twice for 10 min, and then successively probed with $P450_{scc}$ or 3β-HSD antisera. The bands of interest were quantitated using a BioImage Visage 2000 (BioImage Corp., Ann Arbor, MI) imaging system. Values obtained were expressed as integrated optical density units (IOD) as previously described [15].

2.9. Isolation of RNA and Northern blot analysis

Cells were treated as described above for Western blot analysis. Total RNA was isolated using Trizol reagent (Gibco BRL, Grand Island, NY), according to the manufacturers protocol. RNA was quantitated and resuspended in RNA sample buffer (0.1 × borate buffer, 48% formamide, 6.4% formaldehyde, 5.3% glycerol and 0.27% bromophenol blue). Northern blot analysis was performed as previously described [16]. A total of 20 μg of total RNA were loaded into each well. Labeling of cDNA probes for mouse StAR, $P450_{scc}$, 3β-HSD, and 18S rRNA was achieved by random priming (Prime-It II; Stratagene, La Jolla, CA) using [α-^{32}P] dCTP (SA, 3000 Ci/mmol; New England Nuclear, Boston, MA), according to the manufacturers protocol. After hybridization, the blots were washed twice in 2 × SSC, 1%SDS at room temperature for 30 min, and once in 0.1 × SSC, 0.1% SDS at 65°C for 30 min. Following Northern blot analysis with StAR cDNA, blots were stripped by washing twice in 0.1 × SSC, 1% SDS at 65°C for 30 min, and then successively probed with $P450_{scc}$, 3β-HSD, and 18S rRNA cDNA. The bands of interest were quantitated and the values obtained were expressed as described above.

2.10. Statistical analysis

Statistically significant differences between treatments and controls were determined by one-way ANOVA and Fisher-PLSD multiple comparison using the software program Statview SE + Graphics™ (Abacus Concepts, Berkeley, CA).

3. Results

3.1. Effects of econazole and miconazole on progesterone production and total cellular protein synthesis

For dose response studies, $(BU)_2$cAMP-stimulated MA-10 cells were treated with econazole or miconazole (0–100 μM) for 2 h (Fig. 1). These drugs decreased progesterone production in a dosage-dependent manner (IC_{50} in μM econazole = 12.6 ± 1.16, miconazole = 12.5 ± 1.57). To exclude acute toxicity and a general disruption in translation as mechanisms of steroidogenic inhibition, total protein synthesis was also measured. Econazole and miconazole decreased total protein synthesis only at high concentrations (100 μM). In fact, at 25 μM, both compounds significantly ($P < 0.05$) reduced steroidogenesis by 73% without affecting total protein synthesis.

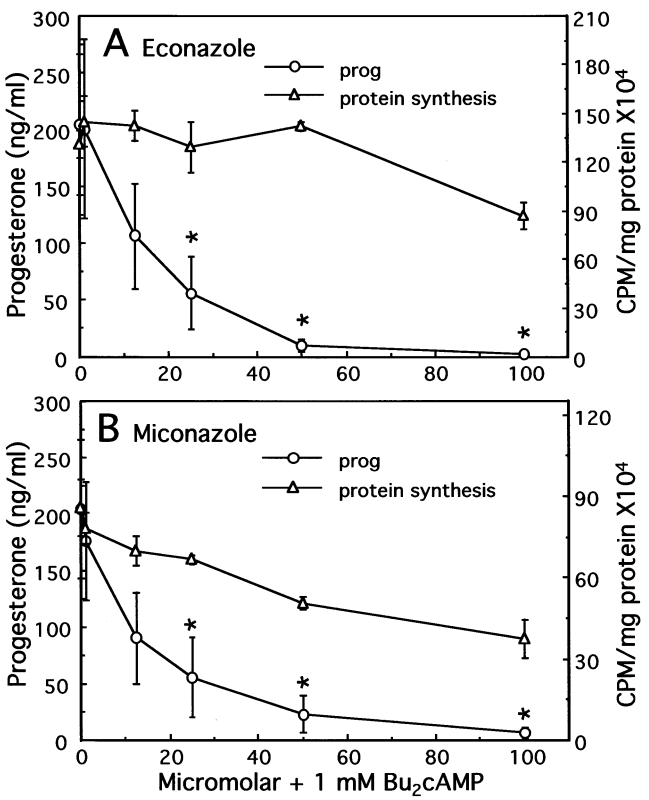

Fig. 1. Effects of econazole and miconazole on progesterone production and total cellular protein synthesis in MA-10 cells. MA-10 cells grown in 96 well plates were stimulated with $(Bu)_2$cAMP in the presence or absence of various concentrations of econazole or miconazole for 2 h. At the end of the incubation period, the medium was removed and assayed for progesterone. In some cases, 5 μCi/ml Expre^{35}S^{35}S Protein Labeling Mix was added to the cells. In these cases, following treatment, the medium was removed, cells were rinsed, and total cellular protein synthesis was assayed as described in Section 2. Panel A, effects of econazole on progesterone production and total protein synthesis. Panel B, effects of miconazole on progesterone production and total protein synthesis. For progesterone production, each data point is the average ± SEM of the means from at least three separate experiments in which treatments were performed in quadruplicate. For protein synthesis, each data point is the mean ± SEM of four replicates in a single experiment which was performed three times. Statistically significant differences were designated with an: (a) $P < 0.05$; or (b) $P < 0.01$.

For time-course studies, $(BU)_2$cAMP-stimulated cells were treated with econazole or miconazole (25 μM) for 2 or 4 h (Table 1). Econzole inhibited steroidogenesis by 80% ($P < 0.001$) at both 2 and 4 h while miconazole inhibited steroidogenesis by 64% ($P < 0.001$) at both these times inducing a parallel decrease in total protein synthesis. At 2 h, the morphology of MA-10 cells treated with these drugs was indistinguishable from untreated cells. However, by 4 h, cells treated with econazole and miconazole exhibited numerous membrane blebs (data not shown).

3.2. Effects of econazole and miconazole on $P450_{scc}$ and 3β-HSD enzyme activity, expression and steroidogenesis following a 24 h recovery

To determine if the inhibitory effect of econazole and miconazole on $(Bu)_2$cAMP-stimulated progesterone production might be due to an inhibition of the activities of the steroidogenic enzymes, $P450_{scc}$ and/or 3β-HSD, 22R-HC was provided as substrate and cells were treated for 2 h with econazole or miconazole (50 μM; Fig. 2, Panels A and B). The water soluble cholesterol analog 22R-HC was used since it can readily diffuse to the $P450_{scc}$ enzyme located on the inner mitochondrial membrane, bypassing the need for StAR-mediated cholesterol transfer. Then, to determine if the effects of the drugs on steroidogenesis were reversible, cells were rinsed, allowed to recover for 24 h in serum containing medium, and treated again for 2 h with $(BU)_2$cAMP and/or 22R-HC (Fig. 2, Panels C and D). Although both compounds significantly ($P < 0.001$) reduced $(Bu)_2$cAMP-stimulated steroidogenesis in this set of experiments by 95% at 2 h, $(Bu)_2$cAMP-stimulated progesterone production in these cells returned to control levels following a 24 h recovery. Moreover, these compounds did not alter $P450_{scc}$ or 3β-HSD enzyme activity after a 2 h treatment or following a 24 h recovery.

To confirm that econazole and miconazole do not affect the $P450_{scc}$ and 3β-HSD steroidogenic enzymes, further studies were performed to determine the effects of these pesticides on $P450_{scc}$ and 3β-HSD enzyme and mRNA levels. $(BU)_2$CAMP-stimulated cells were treated with econazole or miconazole (25 μM) for 4 h. Both drugs significantly ($P < 0.001$) blocked steroidogenesis by 95% (Fig. 3). Western blot analysis of mitochondrial protein confirmed that these drugs did not alter $P450_{scc}$ and 3β-HSD enzyme levels (data not shown). Moreover, Northern blot analysis of total cellular RNA revealed that these drugs did not affect $P450_{scc}$ and 3β-HSD mRNA levels (data not shown).

Table 1
Time-course study on the effects of econazole and miconazole on progesterone production and total cellular protein synthesis in MA-10 cells[a]

	2 h		4 h	
	Progesterone (ng/ml)	Protein synthesis (CPM/mg × 10^4)	Progesterone (ng/ml)	Protein synthesis (CPM/mg × 10^4)
Control	1.80 ± 0.04	29 ± 1.5	2.15 ± 0.05	42.5 ± 3.1
BU_2cAMP (1 mM)	143 ± 13	21 ± 4.8	278 ± 19	84.1 ± 6.7
Econazole (25 μM)	24.6 ± 1.1	18 ± 1.6	54.2 ± 10	61.5 ± 3.9
Miconazole (25 μM)	52.4 ± 8.4	16 ± 7.5	99.6 ± 13	58.2 ± 6.1

[a] $(Bu)_2$cAMP-stimulated MA-10 cells grown in 96 well plates were treated with 25 μM econazole or miconazole for 2 or 4 h. At the end of the incubation period, the medium was removed and assayed for progesterone. The effects of econazole or miconazole on total protein synthesis was also determined as described in Fig. 1. For progesterone production and protein synthesis, each data point is the mean ± SEM of four replicates in a single experiment which was performed three times. For progesterone production at 2 and 4 h, the difference between $(Bu)_2$cAMP and econazole or miconazole + $(Bu)_2$cAMP was statistically significant ($P < 0.001$).

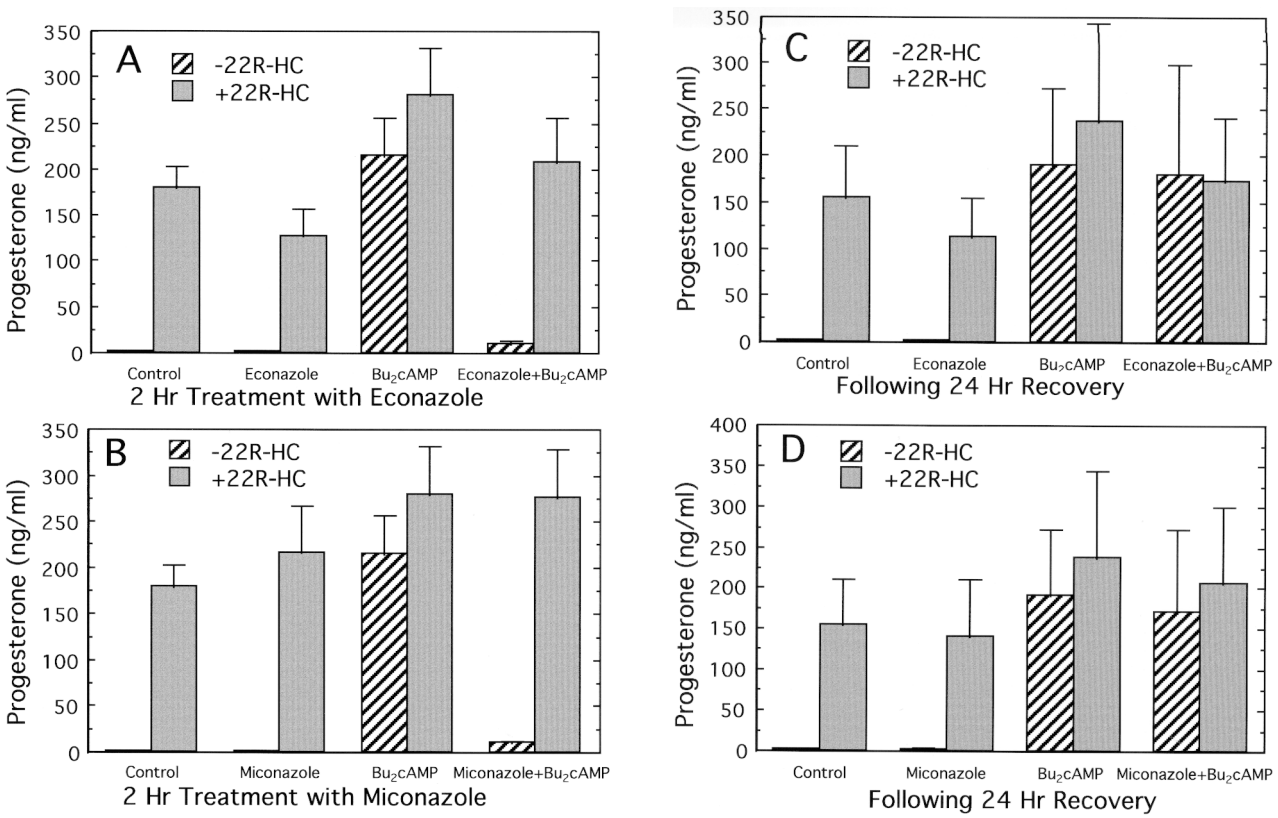

Fig. 2. Effects of econazole and miconazole on $P450_{scc}$ and 3β-HSD enzyme activity and steroidogenesis following a 24 h recovery in MA-10 Cells. MA-10 cells grown in 96 well plates were treated for 2 h with 22R-HC in the presence or absence of econazole or miconazole (50 μM). Then, the medium was assayed for progesterone by RIA. To determine reversibility, cells were rinsed with PBS', incubated in serum-containing medium for 24 h, and stimulated again for 2 h with 22R-HC and/or Bu_2cAMP. Then, medium was removed and assayed for progesterone. Panels A and B, effects of 2 h treatment with econazole or miconazole. The difference between $(Bu)_2$cAMP and econazole or miconazole + $(Bu)_2$cAMP was statistically significant ($P < 0.001$). Panels C and D, effects of 2 h treatment with econazole or miconazole on progesterone production following a 24 h recovery. Each data point represents the average ± SEM of the means from at least three separate experiments in which treatments were performed in quadruplicate.

3.3. Effects of econazole and miconazole on StAR expression in MA-10 cells

Since StAR protein mediates the transfer of cholesterol to the inner mitochondrial membrane, an action which constitutes the rate-limiting step in steroidogenesis, the effects of econazole and miconazole on the levels of StAR protein were also determined. $(Bu)_2$cAMP-stimulated cells were treated with econazole and miconazole (25 μM) for 4 h as described above. Western blot analysis revealed that econazole and miconazole significantly ($P < 0.01$) reduced StAR protein levels by 79 and 94%, respectively (Fig. 4, Part A).

To determine the effects of econazole and miconazole on StAR mRNA levels, cells were treated for 4 h with 25 μM of each compound as described above. StAR mRNA consists of the 1.6, 2.7, and 3.4 kb transcripts which comprise 22, 6 and 72%, respectively of total StAR mRNA (Fig. 4(B)). Northern blot analysis revealed that econazole significantly ($P < 0.05$) increased levels of StAR mRNA 2.4-fold. In contrast, miconazole did not alter StAR mRNA levels. Although the importance of the three different StAR transcripts is unknown at this time, econazole significantly ($P < 0.01$) increased levels of the 1.6 and 2.7 StAR transcripts by three-and 4.8-fold, respectively (Fig. 4, Part B, Lower panel). In contrast, miconazole did not affect the levels of any StAR transcript. Econazole was significantly ($P < 0.05$) different from miconazole with respect to its ability to increase the 1.6 and 2.7 kb transcripts.

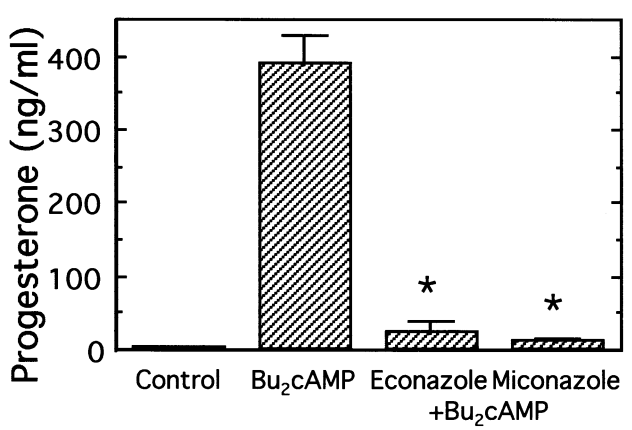

Fig. 3. Effects of econazole and miconazole on progesterone production in MA-10 cells. MA-10 cells grown in 100 mm plates were stimulated with $(Bu)_2$cAMP in the presence or absence of econazole or miconazole (25 μM) for 4 h. Then, medium was removed and assayed for progesterone. Each data point represents the average ± SEM of the means from six separate experiments in which treatments were performed in triplicate. Statistically significant differences ($P < 0.001$) were designated with an asterisk (*).

4. Discussion

The present study demonstrates that econazole and miconazole reduced Bu_2cAMP stimulated progesterone production in MA-10 cells by blocking StAR protein expression post-transcriptionally. This finding agrees with previous observations that these compounds inhibit steroidogenesis by blocking the availability of

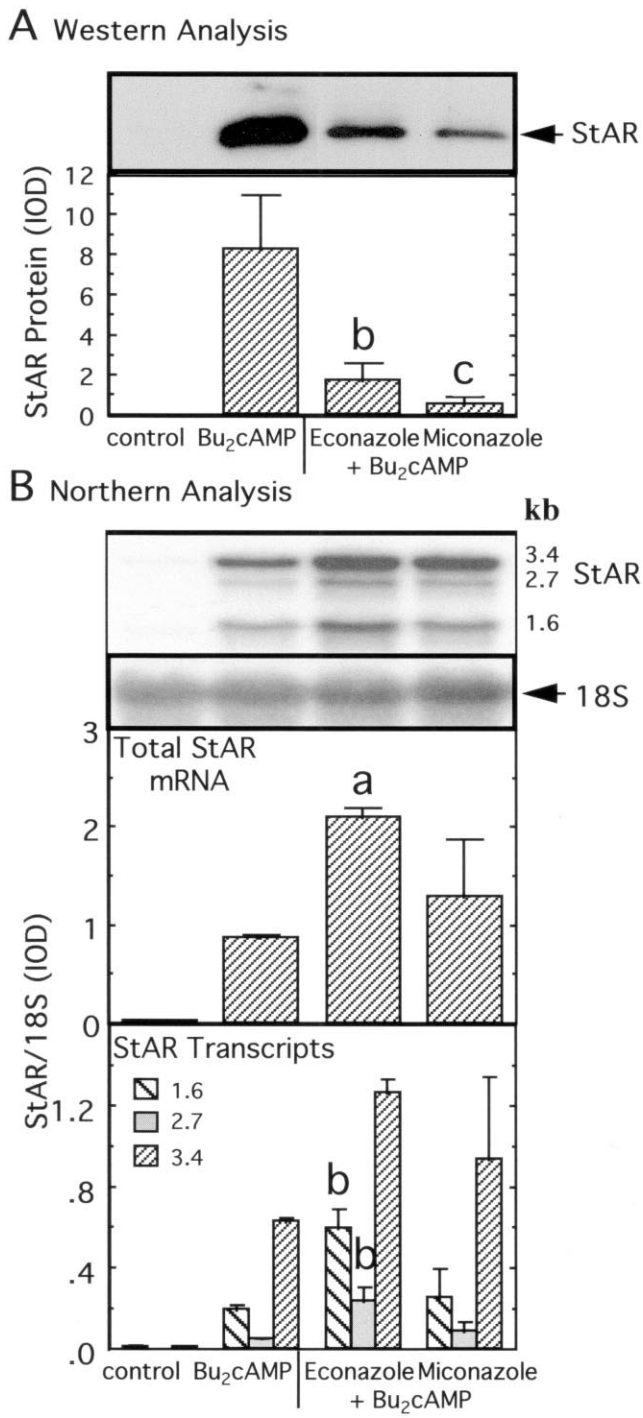

Fig. 4.

cholesterol to the $P450_{scc}$ enzyme, not by affecting $P450_{scc}$ or 3β-HSD steroidogenic enzyme activity [3,5].

Although the mechanism by which these drugs disrupted the post-transcriptional expression of StAR remains to be elucidated, they have been shown to block a number of cytochrome P450-dependent enzymes and non-cytochrome P450 dependent enzymes that are known to regulate StAR protein expression and/or steroidogenesis. Numerous studies have shown that Ca^{2+}, K^+, calmodulin, and arachidonic acid and its metabolites can modulate StAR expression and/or steroidogenesis [17–19]. Given that several studies have shown that econazole and miconazole inhibit leukotriene biosynthesis, two distinct Ca^{2+} entry pathways, a Ca^{2+}, pump, a K^+ channel, and calmodulin, it is not possible to specify which, if any, of these diverse activities accounts for the inhibition of StAR protein expression [20–22]. In fact, inhibition of arachidonic acid release and conversion to its lipoxygenase metabolites has been shown to reduce StAR protein levels by reducing StAR gene expression [18,19]. Since econazole significantly increased StAR mRNA levels, these drugs probably did not reduce StAR protein expression by affecting arachidonic acid release and metabolism alone. While econazole may have increased StAR mRNA levels by increasing StAR transcript stability or by upregulating the synthesis of StAR mRNA, clearly, more work would be required to determine how econazole increased StAR mRNA levels and the mechanism by which both compounds reduced StAR protein production.

Although numerous membrane blebs were observed in MA-10 cells treated for 4 h with econazole and miconazole (25 μM), $P450_{scc}$/3β-HSD enzyme activity and total protein synthesis were not affected, indicating that these drugs were not acutely toxic to cells or to mitochondria. Further, steroidogenesis was completely restored following a 24 h recovery in cells treated initially for 2 h with either drug. Preliminary evidence obtained in our laboratory suggests that both drugs are toxic to MA-10 cells following chronic treatment, and that miconazole (>8 h, 25 μM) can induce apoptosis in these cells [23], an observation which is consistent with these findings.

Unlike econazole and miconazole, which have no effect on $P450_{scc}$ enzyme activity in MA-10 cells (this study) or in Y-1 cells, ketoconazole, a related imidazole compound, has been shown to inhibit $P450_{scc}$ enzyme activity in MA-10 cells [24]. Previous studies have shown that many forms of P450 display broad substrate specificities, but individual isoforms exhibit strict regio- and stereospecificity to a particular compound, which could account for the differences in the abilities of these compounds to inhibit $P450_{scc}$ activity [25].

Not only do imidazole compounds block the conversion of lanosterol to ergosterol in yeast by inhibiting sterol 14α-demethylase, but they also reduce the conversion of lanosterol to cholesterol in mammalian cells by inhibiting this same enzyme [26]. Since cholesterol is the precursor of all steroid hormones, econazole and miconazole might block steroidogenesis upstream of StAR action by reducing cholesterol biosynthesis. However, this probably does not account for the decrease in steroidogenesis observed in the present study since de novo cholesterol biosynthesis only contributes to the production of a small percentage of steroids during acute (0–4 h) steroidogenesis [6]. In fact, during the first 4 h of stimulation in MA-10 cells, the majority (60–70%) of steroids produced are obtained from intracellular stores of free and esterified cholesterol with the remaining steroid derived from either de novo cholesterol biosynthesis or from lipoproteins [6]. However, this might contribute to the steroidogenic inhibition during more prolonged treatments (>4 h) when cells preferentially use both newly synthesized and lipoprotein derived cholesterol [6].

The rate-limiting step in steroidogenesis has often been overlooked as a site of steroidogenic inhibition by xenobiotics. This work demonstrates that StAR protein is susceptible to inhibition by at least some imidazole compounds that inhibit steroidogenesis.

Fig. 4. Effects of econazole and miconazole on StAR protein and mRNA levels. Cells were treated as described in Fig. 3. (A) Western blot analysis of mitochondrial protein was performed as described in Section 2. Upper panel, a representative Western blot is shown. Lower panel, immunospecific bands for the StAR protein were quantitated by computer assisted image analysis. (B) Northern blot analysis of total cellular RNA was performed as described in Section 2. Upper panel, representative Northern blots for StAR mRNA and 18S rRNA are shown. Middle and Lower panels, bands corresponding to the 3.4, 2.7, and 1.6 kb transcripts of StAR mRNA and 18S rRNA were quantitated and data expressed as (Middle panel) the sum of StAR transcripts' IODs/18S rRNA IOD or (Lower panel) individual StAR transcript IOD/18S rRNA IOD. Each data point represents the average ± SEM of the means from three separate experiments in which treatments were performed in triplicate. Statistically significant differences were designated with: (a) $P<0.05$; (b) $P<0.01$; or (c) $P<0.001$.

Acknowledgements

The authors would like to acknowledge the technical assistance of Deborah Alberts, Dr James Hutson, Dr Yevgeniya Lukyanenko, and Dr Jua-Jin Chen.

References

[1] H. van den Bossche, G. Willemsens, W. Cools, W.F. Lauwers, L. Le Jeune, Biochemical effects of miconazole on fungi. II. Inhibition of ergosterol biosynthesis in *Candida albicans*, Chem. Biol. Interact. 21 (1978) 59–78.

[2] H. Van den Bossche, J.M. Ruysschaert, F. Defrise-Quertain, G. Willemsens, F. Cornelissen, P. Marichal, W. Cools, J. Van Cutsem, The interaction of miconazole and ketoconazole with lipids, Biochem. Pharmacol. 31 (1982) 2609–2617.
[3] M. Ayub, M.J. Levell, Inhibition of testicular 17 alpha-hydroxylase and 17,20-lyase but not 3 beta-hydroxysteroid dehydrogenase-isomerase or 17 beta hydroxysteroid oxidoreductase by ketoconazole and other imidazole drugs, J. Steroid Biochem. 28 (1987) 521–531.
[4] M. Ayub, M.J. Levell, Inhibition of human adrenal steroidogenic enzymes in vitro by imidazole drugs including ketoconazole, J. Steroid Biochem. 32 (1989) 515–524.
[5] D.M. Zisterer, D.C. Williams, Calmidazoliurn and other imidazole compounds affect steroidogenesis in Y1 cells: lack of involvement of the peripheral-type benzodiazepine receptor, J. Steroid Biochem. Mol. Biol. 60 (1997) 189–195.
[6] D.A. Freeman, M. Ascoli, Studies on the source of cholesterol used for steroid biosynthesis in cultured Leydig tumor cells, J. Biol. Chem. 257 (1982) 14231–14238.
[7] D.M. Stocco, B.J. Clark, Regulation of the acute production of steroids in steroidogenic cells, Endocr. Rev. 17 (1996) 221–244.
[8] B.J. Clark, J. Wells, S.R. King, D.M. Stocco, The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR), J. Biol. Chem. 269 (1994) 28314–28322.
[9] D. Lin, T. Sugawara, J.F. Strauss, III, B.J. Clark, D.M. Stocco, P. Saenger, A. Rogol, W.L. Miller, Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis, Science 267 (1995) 1828–1831.
[10] M. Ascoli, Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses, Endocrinology 108 (1981) 8895.
[11] L. Nagy, D.A. Freeman, Effect of cholesterol transport inhibitors on steroidogenesis and plasma membrane cholesterol transport in cultured MA-10 Leydig tumor cells, Endocrinology 126 (1990) 2267–2276.
[12] J.A. Resko, R.L. Norman, G.D. Niswender, H.G. Spies, The relationship between progestins and gonadotropins during the late luteal phase of the menstrual cycle in rhesus monkeys, Endocrinology 94 (1974) 128–135.
[13] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[14] X. Wang, Z. Liu, S. Eimerl, R. Timberg, A.M. Weiss, J. Orly, D.M. Stocco, Effect of truncated forms of the steroidogenic acute regulatory protein on intramitochondrial cholesterol transfer, Endocrinology 139 (1998) 3903–3912.
[15] D.M. Stocco, M.W. Kilgore, Induction of mitochondrial proteins in MA-10 Leydig tumour cells with human choriogonadotropin, Biochem. J. 249 (1988) 95103.
[16] H.G. Sutton, A. Fusco, G.A. Cornwall, Cystatin-related epididymal spermatogenic protein colocalizes with luteinizing hormone-beta protein in mouse anterior pituitary gonadotropes, Endocrinology 140 (1999) 2721–2732.
[17] B.A. Cooke, Signal transduction involving cyclic AMP-dependent and cyclic AMP-independent mechanisms in the control of steroidogenesis, Mol. Cell. Endocrinol. 151 (1999) 25–35.
[18] X. Wang, L.P. Walsh, D.M. Stocco, The role of arachidonic acid on LH-stimulated steroidogenesis and steroidogenic acute regulatory protein accumulation in MA-10 mouse Leydig tumor cells, Endocrine 10 (1999) 7–12.
[19] X. Wang, L.P. Walsh, A.J. Reinhart, D.M. Stocco, The role of arachidonic acid in steroidogenesis and steroidogenic acute regulatory (StAR) gene and protein expression, J. Biol. Chem. 275 (2000) 20204–20209.
[20] M. Montero, J. Alvarez, J. Garcia-Sancho, Agonist-induced Ca^{2+} influx in human neutrophils is secondary to the emptying of intracellular calcium stores, Biochem. J. 277 (1991) 73–79.
[21] C. Villalobos, R. Fonteriz, M.G. Lopez, A.G. Garcia, J. Garcia-Sancho, Inhibition of voltage-gated Ca^{2+} entry into GH3 and chromaffin cells by imidazole antimycotics and other cytochrome P450 blockers, Faseb. J. 6 (1992) 2742–2747.
[22] J. Alvarez, M. Montero, J. Garcia-Sancho, High affinity inhibition of Ca(2 +)dependent K^+ channels by cytochrome P-450 inhibitors, J. Biol. Chem. 267 (1992) 11789–11793.
[23] L.P. Walsh, D.M. Stocco, Econazole and miconazole inhibit steroidogenesis through a post-transcriptional reduction in steroidogenic acute regulatory (StAR) protein expression and induction of Leydig cell apoptosis, Biol. Reprod. 60 (1999) 156.
[24] L.R. Chaudhary, D.M. Stocco, Inhibition of hCG- and cAMP-stimulated progesterone production in MA-10 mouse Leydig tumor cells by ketoconazole, Biochem. Int. 18 (1989) 251–262.
[25] G.D. Szklarz, J.R. Halpert, Molecular basis of P450 inhibition and activation: implications for drug development and drug therapy, Drug Metab. Dispos. 26 (1998) 1179–1184.
[26] J.L. Raucy, S.T. Carpenter, J.M. Trzaskos, Identification of lanosterol 14 alpha-methyl demethylase in human tissues, Biochem. Biophys. Res. Commun. 177 (1991) 497–503.