Dimethoate inhibits steroidogenesis by disrupting transcription of the steroidogenic acute regulatory (*StAR*) gene

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Abstract

Dimethoate is a widely used organophosphate insecticide that has been shown to disrupt reproductive function in animals. Although the pathogenesis of Dimethoate-induced reproductive toxicity remains to be determined, a reduction in serum testosterone levels is thought to play an important role in the development of Dimethoate-induced infertility. Since Leydig cells play a crucial role in male reproductive function by producing testosterone, the mouse MA-10 Leydig tumor cell line was used to determine if Dimethoate can directly block steroid hormone biosynthesis and to identify the site of steroidogenic inhibition. Dimethoate inhibited steroidogenesis in both a dose- and time-dependent manner without affecting total protein synthesis or protein kinase A activity. While it

decreased the activity of the P450 side chain cleavage (P450 scc) enzyme, a reduction in the activity of this enzyme alone could not account for the level of Bu₂cAMP-inhibited progesterone production. Instead, our results suggest that Dimethoate inhibited steroidogenesis primarily by blocking transcription of the steroidogenic acute regulatory (*StAR*) gene. This finding is significant since *StAR* protein mediates the rate-limiting and acutely-regulated step in steroidogenesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane. This study indicates that *StAR* may be an important target for environmental pollutants which disrupt steroidogenesis and impair reproductive function.

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Introduction

The recent increase in the agricultural use of organophosphorus pesticides may threaten the reproductive health of humans and wildlife. Since organophosphorus pesticides are rapidly degraded in the environment, they have largely replaced organochlorine insecticides, which can bioaccumulate and biomagnify. Unfortunately, studies have shown that organophosphate compounds have a much greater acute toxicity than organochlorine compounds and can cause male reproductive failure following repeated exposure (Pope 1999). Indeed, interest in the potential adverse reproductive effects of these compounds has heightened in recent years as a result of studies which show that several organophosphorus compounds including Dimethoate, methyl parathion, malathion, dichlorvos, chlorpyrifos and dimethyl-methylphosphate impair fertility, suppress libido, deteriorate semen quality and cause testicular degeneration in rodents following repeated exposure (Krause & Homola 1974, Krause 1977, Haas *et al.* 1983, Dunnick *et al.* 1984).

Not only is Dimethoate a commonly used agricultural insecticide and acaricide, but it is also listed (Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) priority list of hazardous

substances) as one of the contaminants at hazardous waste sites that pose the most significant potential threat to human health (United States Department of Health and Human Services 1997). In fact, investigators have shown that repeated exposure to Dimethoate decreases serum testosterone levels, testicular weight, and sperm motility and increases the percentage of dead and abnormal sperm in rats and rabbits (Salem *et al.* 1988, Afifi *et al.* 1991). Moreover, it accumulates in the testes where it persists for weeks even after its oral administration is stopped (Afifi *et al.* 1991). Since spermatogenesis and fertility are critically dependent upon the maintenance of adequate levels of testosterone, the ability of Dimethoate to reduce serum testosterone levels might contribute to the reduction in spermatogenesis and fertility observed in animals exposed to this pesticide.

Although organophosphates may reduce serum steroid hormone levels by increasing steroid catabolism and elimination, several studies have demonstrated that these compounds can directly inhibit steroid hormone production. Dichlorvos, dursban, diazinon, chlorpyrifos, furadan, and isopropyl bicyclic phosphate have all been shown to inhibit steroidogenesis in adrenal cells (Civen & Brown 1974, Civen *et al.* 1977). Importantly, these compounds block both adrenocorticotropin- and cAMP-stimulated

steroidogenesis but not pregnenolone-driven steroid production, indicating that they target the steroidogenic pathway between the formation of cAMP and the production of pregnenolone (Civen & Brown 1974, Civen *et al.* 1977). This finding is significant since this part of the pathway includes the true rate-limiting step in steroidogenesis, the transfer of cholesterol to the P450 side chain cleavage (P450 scc) enzyme, as well as the rate-limiting *enzymatic* step in steroidogenesis, the P450 scc enzyme itself.

The rate-limiting and acutely regulated step in hormone-stimulated steroidogenesis is the delivery of cholesterol from the outer to the inner mitochondrial membrane where the P450 scc enzyme initiates the synthesis of all steroid hormones (Stocco & Clark 1996). Numerous reports indicate that the steroidogenic acute regulatory (StAR) protein mediates this rate-limiting step in steroidogenesis. StAR protein is rapidly synthesized in response to cAMP, its active form has a very short half-life, and it rapidly increases the movement of cholesterol from the outer to the inner mitochondrial membrane (for review see Stocco & Clark 1996). Transfection of steroidogenic cells with *StAR* expression vectors or non-steroidogenic cells with *StAR* and the cholesterol side chain cleavage enzyme system increases steroidogenesis at least sixfold (Clark *et al.* 1994). Furthermore, mutations in the human *StAR* gene cause the disease lipoid congenital adrenal hyperplasia, a potentially lethal condition in which adrenal and gonadal steroidogenesis is greatly reduced (Lin *et al.* 1995).

As StAR protein mediates the transfer of cholesterol to the P450 scc complex, and the P450 scc enzyme catalyzes the conversion of cholesterol to pregnenolone, Dimethoate may block steroidogenesis by targeting these components of the steroidogenic pathway. Therefore, the present studies were undertaken to determine if Dimethoate could directly inhibit steroidogenesis in the mouse MA-10 Leydig tumor cell line and, if so, to determine the site of steroidogenic inhibition by measuring its effects on the activities and expression of the steroidogenic enzymes and StAR protein.

Materials and Methods

Chemicals

Waymouth's MB 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, USA). [1,2,6,7- ^3H (N)]-progesterone (specific activity (SA), 97 Ci/mmol) and [7- ^3H (N)]-pregnenolone (SA, 21 Ci/mmol) were obtained from New England Nuclear (Boston, MA, USA). Antibodies to progesterone were obtained from Holly Hills Biological (Hillsboro, OR, USA). SU 10603, cyanoketone, and antibodies to pregnenolone were generously provided by

Dr Focko Rommerts (Erasmus University, Rotterdam, The Netherlands). Percoll and dextran T70 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Nunc cell culture dishes, charcoal (Norit), trichloroacetic acid (TCA), scintiverse BD and sodium bicarbonate were obtained from Fisher Scientific (Houston, TX, USA). Acrylamide, bis acrylamide, and SDS were purchased from Bio-Rad (Hercules, CA, USA). Bovine serum albumin (BSA), Bu_2cAMP , 22(R)-hydroxycholesterol (22R-HC), pregnenolone, and progesterone were purchased from Sigma (St Louis, MO, USA). Dimethoate (480 g/l dimethoate) O,O-dimethyl S-methylcarbamoylmethyl phosphorodithioate was obtained from the BASF Corporation, Agricultural Products Group (Research Triangle Park, NC, USA). Rabbit antisera to amino acids 88–98 of mouse StAR protein was generated by Research Genetics (Huntsville, AL, USA). Rabbit antisera to amino acids 421–441 of rat P450 scc enzyme was purchased from Chemicon (Temecula, CA, USA). 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, USA). StAR cDNA was previously cloned in our laboratory (Clark *et al.* 1994). Bovine P450 scc cDNA was obtained from Dr Michael Waterman, Vanderbilt University (Nashville, TN, USA); mouse 3β -HSD I cDNA was provided by Dr Anita Payne, Stanford University (Stanford, CA, USA); mouse L-19 and 18S rRNA cDNAs were obtained from Dr Gail Cornwall, Texas Tech University Health Sciences Center (Lubbock, TX, USA).

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, USA). Cells were maintained in Waymouth's MB 752/1 medium + 15% horse serum at 37 °C and 5% CO_2 as described previously (Ascoli 1981). 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 nuclear run-on analysis, 50×10^6 cells were seeded onto 25×25 cm tissue culture dishes 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.

Treatment of cells

MA-10 cells were stimulated using maximal doses of Bu_2cAMP (1 mM). In some studies, optimal concentrations of 22R-HC (25 μM) or pregnenolone (10 μM)

were provided as steroidogenic substrate. All treatments were performed in serum-free media. Final concentrations of DMSO and ethanol used as chemical solvents were less than 0.4% and were included in controls.

Dose-response and time-course studies

In dose-response and time-course studies, the effects of Dimethoate on steroidogenesis and total cellular protein synthesis were determined. In dose-response studies, MA-10 cells grown in 96-well plates were stimulated with Bu₂cAMP for 2 h in the presence and absence of various concentrations of Dimethoate. The concentration of Dimethoate required to reduce progesterone production by 50% (IC₅₀) was calculated using linear regression analysis. Eadie/Hofstee plots ([progesterone] vs [progesterone]/[Dimethoate]) of the dose-response data were made (Nagy & Freeman 1990). The slope of the line equals the IC₅₀ value. In time-course studies, MA-10 cells grown in 96-well plates were stimulated with Bu₂cAMP in the presence or absence of various concentrations of Dimethoate for 2 or 4 h.

RIA

Quantitation of progesterone in the medium was performed by RIA as previously described (Resko *et al.* 1974). Quantitation of pregnenolone in the medium was performed essentially as described for progesterone except that pregnenolone-specific reagents were used. Analysis of RIA data was performed using a software program which was written by Mr Bennie Shaw (Texas Tech University Health Sciences Center). Data are expressed as ng/ml media. For progesterone, the intra-assay and interassay variations were 3% and 11% respectively. For pregnenolone, the intra-assay and interassay variations were 11% and 20% respectively. The progesterone antibody has 35% cross-reactivity with 5 α -androstane-3-17-dione. The pregnenolone antibody has 29%, 21% and 12% cross-reactivity with cortisol, 17 β -estradiol, and progesterone respectively.

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 μ Ci/ml Expre³⁵S³⁵S Protein Labeling Mix (SA, 1000 Ci/mmol; New England Nuclear). Determination of total protein by a modification of the Bradford method (Bradford 1976) was performed on identically plated cells that were not treated with Expre³⁵S³⁵S. Following treatment, media were removed and cells were solubilized for 2 h in 0.25 M NaOH at 37 °C. Next, an equal volume of cold 20% 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, USA) and rinsed with 5% TCA, dried and counted in a liquid scintillation counter. Results were expressed as counts per minute per sample, 2 or 4 h.

Protein kinase A activity determination

Protein kinase A (PKA) activity was measured with the SignaTECT cAMP-dependent protein kinase assay system (Promega, Madison, WI, USA) as described in the manufacturer's protocol. This assay measures the transfer of ³²P to the biotinylated PKA peptide substrate, Kemptide (LRRASLG). Cells grown in 100 mm plates were treated as described for 4 h. Following treatment, cells were collected in extraction buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β -mercaptoethanol, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin), homogenized at 1000 r.p.m. with 25 passes using a Potter Elvehjem homogenizer fitted with a Teflon pestle and centrifuged at 13 000 $\times$ g for 10 min. The supernatant was used directly for both the kinase assay and protein measurement by the Bradford method. Approximately 3 μ g protein were incubated for 10 min at 30 °C in 40 mM Tris-HCl, pH 7.4, 20 mM MgCl, 0.1 mg/ml BSA, 0.1 mM biotinylated Kemptide, 0.1 mM ATP, and 0.02 μ Ci/ μ l [γ -³²P]dATP (SA, 3000 Ci/mmol; New England Nuclear). In some cases, to ensure specificity for PKA activity, the PKA inhibitor PKI (Promega) was added. Reactions were stopped by the addition of 7.5 M guanidine-HCl. Ten microliters of the reaction mix were spotted onto a streptavidin-coated membrane that specifically binds biotinylated Kemptide. Unincorporated [γ -³²P]dATP and non-biotinylated protein were then removed by extensive washing. The incorporation of ³²P into biotinylated Kemptide bound to the membrane was determined by liquid scintillation counting. PKA activity was expressed as picomoles ³²P incorporated per minute per milligram protein.

Determination of P450 scc and 3 β -HSD activity and reversibility

The effects of xenobiotics on the combined activities of the P450 scc and 3 β -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, in some experiments cells were then rinsed with PBS⁺, allowed to recover for 24 h in serum-containing medium, and incubated again for 2 h with Bu₂cAMP and/or 22R-HC. Then, progesterone in the media was measured. To evaluate P450 scc enzyme activity, 22R-HC was provided as substrate to MA-10 cells in the presence and absence of appropriate xenobiotic as well as cyanoketone and SU 10603, inhibitors of 3 β -HSD and 17 α -hydroxylase/17,20-lyase (P450c17) respectively, for 2 h, and pregnenolone in the medium was measured. To evaluate 3 β -HSD enzyme activity, pregnenolone was provided as

substrate and MA-10 cells were treated in the presence and absence of the xenobiotic for 2 h, and progesterone in the media was measured.

Isolation of mitochondria and Western blot analysis

Cells were treated as described above for determination of PKA activity. Mitochondria were isolated by homogenization and differential centrifugation (Clark *et al.* 1994). Then, Western blot analysis of mitochondrial protein was performed as previously described (Wang *et al.* 1998). 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, USA) imaging system. Values obtained were expressed as integrated optical density units (IOD), as previously described (Stocco & Kilgore 1988).

Isolation of RNA and Northern blot analysis

Cells were treated as described above for determination of PKA activity. Then, total RNA was isolated using Trizol reagent (Gibco BRL, Grand Island, NY, USA), according to the manufacturer's 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 (Sutton *et al.* 1999). Twenty micrograms 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, USA) using [α -³²P]dCTP (SA, 3000 Ci/mmol; New England Nuclear), according to the manufacturer's 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.

Isolation of nuclei and nuclear run-on analysis

MA-10 cells grown in 25 × 25 cm tissue culture dishes were stimulated with Bu₂cAMP in the presence and absence of the appropriate xenobiotic for 4 h. Isolation of nuclei and nuclear run-on analysis were performed as described in Greenburg and Bender (1998). For isolation of nuclei following treatment, cells were harvested with a rubber policeman and centrifuged for 5 min at 500 × g,

4 °C. The cell pellet was resuspended in ice-cold Sucrose I buffer (0.32 M sucrose, 3 mM CaCl₂, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% (v/v) Nonidet P-40 (NP-40), 10 mM Tris-HCl, pH 8.0), and homogenized with 5 strokes of a Dounce homogenizer. To verify that nuclei were free of cytoplasmic tags, nuclei were inspected with an Olympus IMT-2 inverted microscope (Dexter Instrument Co., San Antonio, TX, USA). Then, the homogenate was layered onto a sucrose cushion consisting of Sucrose II buffer (2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT, 10 mM Tris-HCl, pH 8.0), and centrifuged for 45 min at 30 000 × g, 4 °C. The supernatant was discarded and the pellet containing nuclei was resuspended in ice-cold Glycerol Storage buffer (40% (v/v) glycerol, 5 mM MgCl₂, 0.1 mM EDTA, 50 mM Tris-HCl, pH 8.3), frozen on dry ice and stored in liquid nitrogen.

Equal numbers of nuclei were used in the *in vitro* transcription assay, and equal counts of RNA were hybridized to target StAR, L-19 and 18S cDNA inserts as well as linearized, empty pCMV-5 vector previously immobilized to nylon membranes (Hybond N+) using a Bio-Dot SF microfiltration apparatus (Bio-Rad) according to the manufacturer's protocol. Prehybridization and hybridization were performed under the same conditions described for Northern blots. Radioactivity was detected using a Phosphorimager 445 SI (Molecular Dynamics, Sunnyvale, CA, USA). Signals were quantitated using ImageQuant v4.1 software (Molecular Dynamics). Values were obtained from the phosphorimager as arbitrary units.

Statistical analysis

Statistically significant differences between treatments and controls were determined by one-way ANOVA and Fisher-protected least significant difference multiple comparison. Statistical analysis was performed using the software program Statview SE+Graphics (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Effects of Dimethoate on progesterone production, total cellular protein synthesis and protein kinase A activity

For dose-response and time-course studies, MA-10 cells were stimulated with Bu₂cAMP in the presence or absence of Dimethoate (Fig. 1). This pesticide decreased progesterone production in a dose-dependent manner (IC₅₀ = 21 ± 3.09 µg/ml) without producing a concomitant decrease in total protein synthesis, thus excluding acute toxicity and a general disruption in translation as mechanisms of steroidogenic inhibition. Since Dimethoate (50 µg/ml) significantly ($P < 0.05$) reduced steroidogenesis by 65% without affecting total protein synthesis, we chose

Figure 1 Effects of Dimethoate on progesterone production and total cellular protein synthesis in MA-10 cells. Cells were stimulated with Bu₂cAMP in the presence or absence of various concentrations of Dimethoate for 2 h and the medium was assayed for progesterone. In some cases, 5 μCi/ml Expre^{35S} 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 Materials and Methods. For progesterone production, each data point is the average ± S.E.M. from at least three separate experiments in which treatments were performed in quadruplicate. For protein synthesis, each data point is the mean ± S.E.M. of four replicates in a single experiment which was performed three times. Statistically significant differences are designated with an (a) $P < 0.05$ or (b) $P < 0.001$.

to use this dose for the remaining studies. This pesticide also significantly ($P < 0.001$) disrupted steroidogenesis over time without inducing a parallel decrease in total protein synthesis (Table 1).

A reduction in PKA activity might provide a simple explanation for the reduction in steroidogenesis. However, Dimethoate did not affect the ability of PKA in cell lysates

to phosphorylate the PKA-specific substrate, Kemptide (LRRASLG) (data not shown).

Effects of Dimethoate on P450 scc and 3β-HSD enzyme activity and expression, and steroidogenesis following a 24-h recovery

To determine if the inhibitory effect of Dimethoate on Bu₂cAMP-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 a substrate and cells were treated for 2 h with Dimethoate (50 μg/ml; Fig. 2A). 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.

Although Dimethoate significantly ($P < 0.01$) reduced Bu₂cAMP-stimulated steroidogenesis by 76%, Bu₂cAMP-stimulated progesterone production in these cells returned to control levels following a 24-h recovery, indicating that its effects on steroidogenesis were completely reversible (Fig. 2B). Dimethoate also significantly ($P < 0.05$) reduced 22R-HC-driven steroidogenesis by 63%, indicating that it inhibited P450 scc and/or 3β-HSD enzyme activity. However, as Bu₂cAMP+22R-HC-stimulated steroid production was only reduced by 43% after 2-h treatment, this shows that 22R-HC could partially reverse the inhibition of Bu₂cAMP-stimulated steroidogenesis.

To determine if Dimethoate specifically disrupted 3β-HSD, P450 scc or both steroidogenic enzyme activities, both pregnenolone-driven progesterone production (a measure of 3β-HSD activity) and 22R-HC-driven pregnenolone production (a measure of P450 scc activity) were measured following herbicide treatment for 2 h (Fig. 3).

Table 1 Time-course study of the effects of Dimethoate on progesterone production and total cellular protein synthesis in MA-10 cells. MA-10 cells grown in 96-well plates were stimulated with Bu₂cAMP in the presence and absence of 50 μg/ml Dimethoate for 2 or 4 h. Then, the medium was removed and assayed for progesterone, and total protein synthesis was also measured. For progesterone production, each data point is the average ± S.E.M. 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 ± S.E.M. of four replicates in a single experiment which was performed three times

	2 hours		4 hours	
	Progesterone (ng/ml)	Protein synthesis (c.p.m./mg × 10 ⁴)	Progesterone (ng/ml)	Protein synthesis (c.p.m./mg × 10 ⁴)
Control	1.92 ± 0.21	56 ± 5.9	2.8 ± 0.42	102 ± 6.5
Bu ₂ cAMP (1mM)	126 ± 20.3	65 ± 4.9	253 ± 13.1	94 ± 1.5
Dimethoate (50 μg/ml)	45 ± 14	47 ± 2.2	105 ± 18.5	79 ± 6.0

For progesterone production at 2 and 4 h, the difference between Bu₂cAMP and Dimethoate+Bu₂cAMP was statistically significant ($P < 0.001$).

Figure 2 Effects of Dimethoate on P450 scc and 3 β -HSD enzyme activity and steroidogenesis following a 24-h recovery in MA-10 cells. 22R-HC-stimulated cells grown in 96-well plates were treated for 2 h with Dimethoate (50 μ g/ml), and medium was assayed for progesterone. 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 Bu₂cAMP, and medium was assayed for progesterone. (A) Effects of 2-h treatment with Dimethoate on progesterone production. The difference between Bu₂cAMP and Dimethoate was statistically significant ($P < 0.01$). (B) Effects of 2-h treatment with Dimethoate on progesterone production following a 24-h recovery. Each data point represents the average $\pm$ S.E.M. from at least three separate experiments in which treatments were performed in quadruplicate.

Although Dimethoate did not alter 3 β -HSD enzyme activity, indicating that the herbicide was not acutely toxic to cells or mitochondria, it significantly ($P < 0.01$) reduced P450 scc activity by 49%.

To determine if the decrease in P450 scc enzyme activity might be due to a reduction in the levels of this enzyme, and to confirm that 3 β -HSD enzyme levels were not affected, the effects of Dimethoate on the expression of these enzymes were determined. As Fig. 4 shows, Dimethoate significantly blocked steroidogenesis by 81%. However, Western blot analysis of mitochondrial protein

Figure 3 Effects of Dimethoate on 3 β -HSD and P450 scc enzyme activity. To evaluate 3 β -HSD enzyme activity, cells grown in 96-well plates were treated with 50 μ g/ml Dimethoate in the presence of the substrate, pregnenolone (10 μ M), for 2 h. Then, medium was assayed for progesterone. To evaluate P450 scc enzyme activity, 22R-HC-stimulated cells were treated with 50 μ g/ml Dimethoate as well as with 5 μ M cyanoketone and 19 μ M SU 10603, inhibitors of 3 β -HSD and P450c17 respectively, for 2 h. The medium was assayed for pregnenolone. (A) Effects of Dimethoate on 3 β -HSD enzyme activity. (B) Effects of Dimethoate on P450 scc enzyme activity. Each data point represents the average $\pm$ S.E.M. from at least six separate experiments in which treatments were performed in quadruplicate. Statistically significant differences ($P < 0.01$) are designated with an asterisk.

revealed that it did not alter P450 scc or 3 β -HSD enzyme levels (Figs 5 and 6, upper panels). Moreover, Northern blot analysis revealed that Dimethoate did not affect P450 scc mRNA levels (Fig. 5, lower panel). Surprisingly, Dimethoate significantly ($P < 0.01$) reduced 3 β -HSD mRNA levels by 33% (Fig. 6, lower panel).

Effects of Dimethoate on StAR expression

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 Dimethoate on the levels of this protein were also

Figure 4 Effects of Dimethoate on progesterone production in MA-10 cells. Cells grown in 100-mm plates were treated with Dimethoate (50 µg/ml) for 4 h. Then, medium was assayed for progesterone. Each data point represents the average $\pm$ S.E.M. from six separate experiments in which treatments were performed in triplicate. Statistically significant differences ($P < 0.001$) are designated with an asterisk.

determined. Western blot analysis revealed that this pesticide dramatically reduced *StAR* protein levels by 83% (Fig. 7A).

To determine the effects of Dimethoate on *StAR* mRNA levels, Northern blot analysis was performed. *StAR* mRNA consists of the 1.6, 2.7, and 3.4 kb transcripts which comprise 18%, 10%, and 72% respectively of total *StAR* mRNA (Fig. 7B). Northern blot analysis revealed that Dimethoate reduced total *StAR* mRNA levels by 81%. Moreover, this pesticide significantly ($P < 0.01$) decreased levels of the most abundant 3.4 kb *StAR* transcript by 86% (Fig. 7B).

To determine if Dimethoate reduced *StAR* protein levels by blocking *StAR* transcription, nuclear run-on analysis was performed. As Fig. 8 shows, Bu₂cAMP increased the rate of *StAR* gene transcription fivefold. However, Dimethoate decreased *StAR* transcription by 55%, indicating that it primarily reduced *StAR* protein expression and steroidogenesis at the level of transcription.

Discussion

The present study showed that Dimethoate directly blocked Bu₂cAMP-stimulated steroid hormone production in MA-10 cells, an effect which most likely can be attributed to a reduction in *StAR* transcription. This observation is consistent with earlier reports which have shown that other organophosphate compounds can directly block the steroidogenic pathway between the formation of cAMP and the production of pregnenolone in rat adrenal cell primary cultures (Civen & Brown 1974, Civen *et al.* 1977). Since we have previously

A Western Analysis

B Northern Analysis

Figure 5 Effects of Dimethoate on P450 scc enzyme and mRNA levels. Cells were treated as described in Fig. 4. (A) Western blot analysis of mitochondrial protein was performed as described in Materials and Methods. In the upper panel, a representative Western blot is shown. (Lower panel) Immunospesific bands for the P450 scc enzyme were quantitated by computer assisted image analysis. (B) Northern blot analysis of total cellular RNA was performed as described in Materials and Methods. In the upper panel, representative Northern blots for P450 scc mRNA and 18S rRNA are shown. (Lower panel) Bands for P450 scc mRNA and 18S rRNA were quantitated and data expressed as P450 scc mRNA/18S rRNA. Each data point represents the average $\pm$ S.E.M. from three separate experiments in which treatments were performed in triplicate.

demonstrated with another organophosphorus compound, diethylumbelliferyl phosphate, that steroidogenesis in MA-10 cells can be inhibited by blocking *StAR* protein expression (Choi *et al.* 1995), these findings indicate the possibility that organophosphorus compounds as a class

A Western Analysis **B Northern Analysis**

Figure 6 Effects of Dimethoate on 3β-HSD enzyme and mRNA levels. Cells were treated as described in Fig. 4. (A) Western blot analysis of mitochondrial protein was performed as described in Materials and Methods. In the upper panel, a representative Western blot is shown. In the lower panel, immunospecific bands for the 3β-HSD enzyme were quantitated by computer assisted image analysis. (B) Northern blot analysis of total cellular RNA was performed as described in the Materials and Methods. In the upper panel, representative Northern blots for 3β-HSD mRNA and 18S rRNA are shown. In the lower panel, bands for 3β-HSD mRNA and 18S rRNA were quantitated and data expressed as 3β-HSD mRNA/18S rRNA. Each data point represents the average $\pm$ S.E.M. from three separate experiments in which treatments were performed in triplicate. Statistically significant differences ($P < 0.01$) are designated with an asterisk.

may block steroidogenesis through a common mechanism of action.

Previous studies concerning *StAR* gene expression in MA-10 cells have shown that cAMP increases *StAR* steady-state mRNA levels in parallel with *StAR* protein

A Western Analysis **B Northern Analysis**

Figure 7 Effects of Dimethoate on *StAR* protein and mRNA levels. Cells were treated as described in Fig. 4. (A) Western blot analysis of mitochondrial protein was performed as described in Materials and Methods. In the upper panel, a representative Western blot is shown. In the 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 Materials and Methods. In the 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' IOD/18S rRNA IOD or (lower panel) individual *StAR* transcript IOD/18S rRNA IOD. Each data point represents the average $\pm$ S.E.M. from three separate experiments in which treatments were performed in triplicate. Statistically significant differences are designated with (b) $P < 0.01$ or (c) $P < 0.001$.

Figure 8 Effects of Dimethoate on *StAR* gene transcription. Nuclear run-on analysis was performed on MA-10 cells grown in 25×25 cm tissue culture plates stimulated with Bu₂cAMP in the presence or absence of 50 μ g/ml Dimethoate for 4 h. In the upper panel, a representative experiment is shown. (Lower panel) Bands for *StAR*, *L-19* mRNA and *18S* rRNA were quantitated by computer assisted image analysis. *StAR* was normalized to *18S* for each treatment, and data are expressed as a percentage of *StAR*/*18S* of Bu₂cAMP-treated cells. Each data point represents the average $\pm$ S.E.M. of five separate experiments.

levels and steroid production (Clark *et al.* 1995), and that these events require new transcription (Clark *et al.* 1997). Therefore, a reduction in the rate of *StAR* gene transcription can account for the reduction in steroidogenesis. Although previous reports indicate that cAMP responsiveness is conveyed within the first 254 bp of the *StAR* promoter and that a number of transcription factors including steroidogenic factor-1 (SF-1) and CCAAT/enhancer binding protein- β are required for basal *StAR* transcription, the proteins involved in hormone-stimulated *StAR* protein expression are poorly defined (Reinhart *et al.* 1999). Therefore, further studies are needed to determine the mechanism by which Dimethoate reduces *StAR* transcription.

Tropic hormone-regulated steroidogenesis has an absolute requirement for the synthesis of new proteins. In fact, as *StAR* is a cycloheximide-sensitive protein, a general reduction in protein synthesis could have explained the decrease in *StAR* protein levels and thus steroid production (Garren *et al.* 1965). However, Dimethoate at concentrations as high as 100 μ g/ml failed to affect total protein synthesis in MA-10 cells (this study) or in the

human leukemia cell line, HL-60 (Marinovich *et al.* 1994), indicating that it did not inhibit steroidogenesis by causing acute cellular toxicity or by inducing a general disruption in translation. These studies indicate that Dimethoate may have disrupted steroidogenesis by targeting specific sites within the cell.

In Leydig cells, tropic hormone regulates steroid hormone production primarily through the cAMP second messenger pathway. Once synthesized, cAMP activates cAMP-dependent PKA which, in turn, phosphorylates several proteins involved in the regulation of steroidogenesis, including *StAR* protein, cholesterol ester hydrolyase, and perhaps other as yet unidentified proteins that must be phosphorylated for steroidogenesis to occur (Stocco & Clark 1993). While a reduction in PKA activity could have explained the inhibition of steroid production and reduction in *StAR* protein levels, the present results indicate that Dimethoate did not alter PKA activity. However, as the phosphorylation of *StAR* and other proteins were not directly measured and alternative mechanisms such as an increase in protein phosphatase activity might be involved, changes in protein phosphorylation status cannot be ruled out at this time.

Although the present study shows that Dimethoate reduced P450 scc activity following acute exposure, a reduction in *StAR* protein expression alone could account for Dimethoate's effects on steroidogenesis, suggesting that the inhibition of P450 scc activity has little physiological significance, its activity being distal to *StAR* function. The P450 scc enzyme is part of the cholesterol side chain cleavage enzyme system (CSCC) which also includes adrenodoxin reductase and adrenodoxin. The electrons required for the conversion of cholesterol to pregnenolone are transferred from NADPH to adrenodoxin reductase to adrenodoxin and finally to the P450 scc enzyme (Simpson 1979). Thus, Dimethoate could have directly targeted the enzyme's active site or affected the availability and/or activity of cofactors and reducing equivalents. However, further studies are necessary to determine how Dimethoate affects P450 scc enzyme activity.

Although Dimethoate slightly reduced 3 β -HSD mRNA levels, it failed to alter 3 β -HSD activity following acute exposure. Like Dimethoate, a reduction in 3 β -HSD mRNA levels has been observed following treatment with tumor necrosis factor- α and interleukin-1 β (Xiong & Hales 1997) although, again, the mechanism remains unclear. As the 3 β -HSD enzyme is chronically regulated and has a long half-life, a more protracted exposure to Dimethoate may reduce the levels of this enzyme and may ultimately contribute to the decline in the cell's steroidogenic capacity.

Three important differences in the regulation of *StAR* expression may render it susceptible to chemical modulation compared with the steroidogenic enzymes. First, in

Leydig cells StAR protein expression is mainly dependent upon luteinizing hormone (LH) stimulation, making its expression more vulnerable to chemicals which inhibit the LH signaling pathway. It has very low expression in the absence of LH and is rapidly synthesized to high levels following tropic hormone stimulation. In contrast, with the exception of P450c17, the steroidogenic enzymes are constitutively expressed at high levels, retain near normal steroidogenic capacity in the absence of LH for extended periods of time, and are only increased several-fold by LH after a lag of several hours (for review see Payne & O'Shaughnessy 1996). Secondly, as StAR protein mediates the rate-limiting step in steroidogenesis, steroid production is very sensitive to alterations in its expression. In contrast, with the exception of the P450 scc enzyme which can limit the cell's steroidogenic capacity (Nolan & Payne 1990), a reduction in steroidogenic enzyme levels does not cause a concomitant reduction in steroid production. In fact, inhibitors can reduce P450c17 levels by as much as 90% without decreasing the cell's steroidogenic capacity (Anakwe & Payne 1987). Finally, unlike the steroidogenic enzymes which have long half-lives and are chronically regulated, the active form of the StAR protein is highly labile and must be continuously synthesized for steroidogenesis to occur. Providing evidence that StAR protein may indeed be a target for endocrine disrupting chemicals, recent studies have shown that the herbicide Roundup, the pesticide lindane, and the antifungal drugs econazole and miconazole inhibit steroidogenesis by reducing StAR protein expression (Walsh & Stocco 1999, 2000a,b, Walsh *et al.* 2000).

In conclusion, Dimethoate inhibits steroid hormone production in MA-10 cells by reducing StAR transcription. As other compounds have been shown to reduce steroidogenesis by disrupting StAR protein expression, this may represent a common mechanism by which some endocrine disruptors inhibit steroidogenesis. Although Dimethoate has been shown to disrupt testicular function in laboratory animals, further studies are required to determine if Dimethoate and other contemporary use pesticides pose a real threat to humans and wildlife.

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