Roundup Inhibits Steroidogenesis by Disrupting Steroidogenic Acute Regulatory (StAR) Protein Expression

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Recent reports demonstrate that many currently used pesticides have the capacity to disrupt reproductive function in animals. Although this reproductive dysfunction is typically characterized by alterations in serum steroid hormone levels, disruptions in spermatogenesis, and loss of fertility, the mechanisms involved in pesticide-induced infertility remain unclear. Because testicular Leydig cells play a crucial role in male reproductive function by producing testosterone, we used the mouse MA-10 Leydig tumor cell line to study the molecular events involved in pesticide-induced alterations in steroid hormone biosynthesis. We previously showed that the organochlorine insecticide lindane and the organophosphate insecticide Dimethoate directly inhibit steroidogenesis in Leydig cells by disrupting expression of the steroidogenic acute regulatory (StAR) protein. 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 cytochrome P450 side chain cleavage (P450scc) enzyme initiates the synthesis of all steroid hormones. In the present study, we screened eight currently used pesticide formulations for their ability to inhibit steroidogenesis, concentrating on their effects on StAR expression in MA-10 cells. In addition, we determined the effects of these compounds on the levels and activities of the P450scc enzyme (which converts cholesterol to pregnenolone) and the 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme (which converts pregnenolone to progesterone). Of the pesticides screened, only the pesticide Roundup inhibited dibutyryl [$(Bu)_2$]cAMP-stimulated progesterone production in MA-10 cells without causing cellular toxicity. Roundup inhibited steroidogenesis by disrupting StAR protein expression, further demonstrating the susceptibility of StAR to environmental pollutants. *Key words*: chemical mixtures, cytochrome P450 side chain cleavage, environmental endocrine disruptor, 3β-hydroxysteroid dehydrogenase, Leydig cells, Roundup, steroid hormones, steroidogenesis, steroidogenic acute regulatory protein. *Environ Health Perspect* 108:769–776 (2000). [Online 12 July 2000]
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The biosynthesis of all steroid hormones begins with the cleavage of the side chain of cholesterol to form pregnenolone. This reaction is catalyzed by the P450scc component of the cholesterol side chain cleavage enzyme system (CSCC) located on the matrix side of the inner mitochondrial membrane (*1*). Although this constitutes the rate-limiting enzymatic step in steroidogenesis, the true rate-limiting step is the delivery of cholesterol to the inner mitochondrial membrane and the P450scc enzyme (*2*). Because the aqueous diffusion of cholesterol is extremely slow, cholesterol cannot readily diffuse to the inner mitochondrial membrane at rates capable of sustaining physiologically relevant levels of steroid production (*3*). To illustrate this point, maximal steroid production can be achieved in the absence of stimulation by providing steroidogenic cells with water-soluble cholesterol analogs, which can freely diffuse to the inner mitochondrial membrane (*4*). Thus there are mechanisms that mobilize cholesterol from cellular stores to the mitochondria and which transfer cholesterol from the outer to the inner mitochondrial membrane.

Although the delivery of cholesterol from cellular stores to the mitochondria is essential to maintain maximal rates of steroid production, the intramitochondrial transfer of cholesterol is the key hormonally regulated step. Using protein synthesis inhibitors such as cycloheximide and puromycin, investigators have shown that hormone regulated steroid production requires rapid *de novo* protein synthesis (*5*). Furthermore, cycloheximide treatment, while permitting cholesterol accumulation in the outer mitochondrial membrane of steroidogenic cells, almost completely blocks cholesterol movement to the inner mitochondrial membrane (*6*). Thus, a hormone-stimulated, rapidly synthesized, cycloheximide-sensitive protein is required to mediate the rate-limiting step in steroidogenesis, the intramitochondrial transfer of cholesterol.

Numerous studies have been performed to identify and characterize this acute regulatory factor. Although several proteins have been proposed as the acute regulator [reviewed by Stocco and Clark (*7*)], one of these candidate proteins was first described and characterized by Orme-Johnson et al. (*8*) as a mitochondrial phosphoprotein that is rapidly synthesized in response to hormone stimulation in rat adrenal cells. Our laboratory has described a similar protein in mouse MA-10 Leydig tumor cells and has since purified, cloned, sequenced, and expressed this protein and named it the steroidogenic acute regulatory (StAR) protein. The StAR protein fulfills all of the criteria of the putative acute regulatory factor (*7,9,10*). Perhaps the most compelling argument for the role of StAR in steroidogenesis comes from the finding that, in humans, mutations in the StAR gene cause the disease lipoid congenital adrenal hyperplasia (lipoid CAH), a condition in which cholesterol and cholesterol esters accumulate and the newborn is unable to synthesize adequate levels of steroid hormones. Furthermore, StAR knockout mice have been generated, and their phenotype mirrors that of human lipoid CAH (*11*). These observations indicate that StAR plays an indispensable role in the transfer of cholesterol to the P450scc.

Because StAR protein mediates the rate-limiting step in steroidogenesis, we hypothesized that, when compared to the steroidogenic enzymes, StAR protein may be particularly susceptible to modulation by environmental pollutants for a number of reasons. First, unlike the steroidogenic enzymes that are chronically regulated and have long half-lives (*12*), StAR protein is not an enzyme, is acutely regulated, and its active precursor form is highly labile. Second, StAR protein expression is critically dependent on trophic hormone stimulation, making it susceptible to xenobiotics that disrupt components of the trophic hormone signaling pathway. In contrast, with the exception of cytochrome P450 17α-hydroxylase/17,20-lyase (P450c17), the steroidogenic enzymes retain near-normal steroidogenic enzyme capacity even in the absence of trophic hormone stimulation (*12*). Third, StAR mediates the rate-limiting step in steroidogenesis,

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rendering steroidogenesis extremely sensitive to disruptions in its expression. Conversely, with the exception of P450scc, which can be limiting, the steroidogenic enzymes are present in excess amounts (*13*). Fourth, because StAR functions upstream of steroidogenic enzyme activity, the effects of the xenobiotic on steroidogenic enzyme activity may be of little importance if the xenobiotic also blocks StAR protein expression. Finally, we recently showed that two pesticides, the organochlorine insecticide lindane (Sigma, St. Louis, MO), and the organophosphate insecticide Dimethoate (BASF Corp., Agricultural Products Group, Research Triangle Park, NC), both of which lower serum testosterone levels in animals, block steroid hormone biosynthesis in Leydig cells by reducing StAR protein expression (*14,15*). These findings raise the possibility that other pesticides may also inhibit steroidogenesis by targeting StAR expression.

Several currently used pesticides disrupt steroid hormone levels and/or reproductive system function in animals (*16–19*). One billion pounds of active ingredients and several times this amount of inert ingredients are used annually in the United States alone; therefore, the possibility that these compounds can affect the reproductive health of humans and wildlife in their natural habitats is of great concern (*20*). Little information is available regarding the effects of pesticides, including Ammo (Zeneca Agricultural Products, Wilmington, DE) and Ambush (Zeneca Agricultural Products) and the herbicides Banvel (Sanex, Inc., Burlington, Ontario, Canada), Cotoran (Ciba-Geigy Corporation, Greensboro, NC), Cyclone (Zeneca Agricultural Products), Fusilade (Zeneca Agricultural Products), Dual (Ciba-Geigy), and Roundup (Monsanto Co., St. Louis, MO) on endocrine system function, despite their widespread use. Therefore, the present study was performed to determine if these pesticides can disrupt steroid hormone biosynthesis in the mouse MA-10 Leydig tumor cell line and to determine the site of steroidogenic inhibition.

Materials and Methods

Chemicals. We purchased 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^-) from Gibco Life Technologies (Gaithersburg, MD). We obtained [1,2,6,7-N^3-H(N)]-progesterone [specific activity (SA) 97 Ci/mmol] and [7-3H(N)]-pregnenolone (SA 21 Ci/mmol) from New England Nuclear (Boston, MA). Antibodies to progesterone were obtained from Holly Hills Biological (Hillsboro, OR). SU 10603, cyanoketone, and antibodies to pregnenolone were generously provided by F. Rommerts, Erasmus University (Rotterdam, The Netherlands). We obtained Percoll and Dextran T70 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), dibutyryl [$(Bu)_2$]cAMP, 22(*R*)-hydroxycholesterol (22*R*-HC), pregnenolone, and progesterone were purchased from Sigma. We purchased rabbit antisera to amino acids 88–98 of mouse StAR protein from Research Genetics (Huntsville, AL) and rabbit antisera to amino acids 421–441 of rat P450scc enzyme from Chemicon (Temecula, CA). Antisera to purified mouse 3β-hydroxysteroid dehydrogenase I (3β-HSD) was a generous gift from A. Capponi, University of Geneva (Geneva, Switzerland). We purchased horseradish peroxidase conjugated goat antimouse IgG from Amersham (Arlington Heights, IL). StAR cDNA was previously cloned in our laboratory (*21*). Bovine P450scc cDNA was a generous gift from M. Waterman, Vanderbilt University (Nashville, TN); mouse 3β-HSD I cDNA was a generous gift from A. Payne, Stanford University (Stanford, CA); and mouse 18S rRNA cDNA was a generous gift from G. Cornwall, Texas Tech University Health Sciences Center (Lubbock, TX).

Pesticide formulations selected for study included:

- Ammo (300 g/L cypermethrin): (*R,S*)-α-cyano-3-phenoxybenzyl(1*R,S*)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
- Ambush (240 g/L permethrin): 3-phenoxybenzyl(1*R,S*)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
- Fusilade (120 g/L fluazifop-*p*-butyl): (*R*)-2-[4-(5-trifluoromethyl-2-pyridyloxy)phenoxy]propionic acid
- Cyclone (240 g/L paraquat): 1,1′-dimethyl-4,4′-bipyridinium
- Roundup (180 g/L glyphosate): *N*-(phosphonomethyl) glycine
- Banvel (480 g/L dicamba): 3,6-dichloro-*o*-anisic acid
- Cotoran (480 g/L fluometuron): 1,1-dimethyl-3-(α,α,α-trifluoro-*m*-tolyl) urea
- Dual (958 g/L metolachlor) 2-chloro-6′ethyl-*N*-(2-methoxy-1-methylethyl)acet-*o*-toluidine.

Glyphosate and α- and γ-HCH were obtained from Sigma.

MA-10 cell culture. The mouse MA-10 Leydig tumor cell line was a generous gift from M. Ascoli, University of Iowa College of Medicine (Iowa City, IA). We maintained cells in Waymouth's MB 752/1 medium + 15% horse serum at 37°C, 5% CO_2, as described previously (*22*). For dose–response, time–course, steroidogenic enzyme activity, reversibility, and mixture 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. We stimulated MA-10 cells using a maximal stimulatory dose of $(Bu)_2$cAMP (1 mM). In some studies, optimal concentrations of 22*R*-HC (25 μM) or pregnenolone (10 μM) were provided as a steroidogenic substrate. All treatments were performed in serum-free media. Final concentrations of DMSO and ethanol used as chemical solvents were < 0.4 %.

Dose–response and time-course studies. We stimulated MA-10 cells with $(Bu)_2$cAMP in the presence or absence of the appropriate xenobiotic for 2 hr (in dose–response studies), or 4 hr (in time-course studies), and we measured steroid levels and total protein synthesis. We calculated the concentration that inhibits 50% (IC_{50}) values as the slope of the linear regression line obtained from Eadie/Hofstee plots of steroidogenesis dose–response data. For steroid determination in Roundup-treated cells, each data point is the average ± SE of the means from at least three separate experiments in which treatments were performed in quadruplicate. For progesterone production in cells treated with other pesticides, each data point is the mean ± SE of four replicates in a single experiment that was repeated once.

Radioimmunoassay (RIA). We quantified progesterone by RIA as previously described (*23*). Standard curves were prepared in serum-free Waymouth's medium. Analysis of RIA data was performed using a computer program specifically designed for this purpose. Data are expressed as nanograms per milliliter media.

Determination of total cellular protein synthesis. To determine the effects of compounds on total protein synthesis, cells were treated as described previously with the inclusion of 5 μCi/mL Expre^{35}S^{35}S protein labeling mix (SA 1,000 Ci/mmol; New England Nuclear). We determined total protein content using a modification of the Bradford method (*24*) on identically plated cells that were not treated with Expre^{35}S^{35}S. After treatment, medium was removed and

cells were solubilized for 2 hr in 0.25 M NaOH at 37°C. Next, we added an equal volume of cold 20% trichloroacetic acid (TCA) 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), rinsed with 5% TCA, dried, and counted in a liquid scintillation counter. Results were reported as counts per minute per milligram protein (2 or 4 hr). Each data point is the mean ± SE of four replicates in a single experiment, which was performed three times.

Determination of P450scc and 3β-HSD activity and reversibility. We determined the effects of xenobiotics on the combined activities of the P450scc and 3β-HSD enzymes by adding 22*R*-HC to MA-10 cells in the presence or absence of the xenobiotic for 2 hr and measuring progesterone production. To determine reversibility, cells were then rinsed with PBS^+, allowed to recover for 24 hr in serum-containing medium, and incubated again for 2 hr with $(Bu)_2$cAMP and/or 22*R*-HC. Progesterone in the medium was then measured. To evaluate P450scc enzyme activity, 22*R*-HC was provided as substrate to MA-10 cells in the presence and absence of the appropriate xenobiotic as well as cyanoketone and SU 10603, inhibitors of 3β-HSD and P450c17, respectively, for 2 hr, and pregnenolone in the medium was measured. To evaluate 3β-HSD enzyme activity, pregnenolone was provided as substrate, and MA-10 cells were treated in the presence and absence of the xenobiotic for 2 hr, and progesterone in the media was measured. Each data point represents the average ± SE of the means from at least three separate experiments in which treatments were performed in quadruplicate.

Isolation of mitochondria and Western blot analysis. MA-10 cells were stimulated with $(Bu)_2$cAMP in the presence or absence of Roundup for 4 hr, and progesterone in the media was measured by RIA. We isolated mitochondria by homogenization of the cells followed by differential centrifugation (*21*). Western blot analysis of mitochondrial protein was performed as previously described (*25*). After detection of StAR, membranes were 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 P450scc 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, as previously described (*26*). Each data point represents the average ± SE of the means from three separate experiments in which treatments were performed in triplicate.

Isolation of RNA and Northern blot analysis. Cells were treated as described for Western blot analysis. The media were retained and progesterone was measured by RIA. We isolated total RNA using Trizol reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol. RNA was quantitated and resuspended in RNA sample buffer (0.1 × borate buffer, 48% formamide, 6.4% formaldehye, 5.3% glycerol, and 0.27% bromophenol blue). We performed Northern blot analysis as previously described (*27*). We loaded 20 µg total RNA into each well. Labeling of cDNA probes for mouse StAR, P450scc, 3β-HSD, and 18S rRNA was achieved by random priming (Prime-It II; Stratagene, La Jolla, CA) using [α-^{32}P] dCTP (SA 3,000 Ci/mmol; New England Nuclear) according to the manufacturer's protocol. After hybridization, the blots were washed twice in 2 × standard saline citrate (SSC), 1% SDS at room temperature for 30 min and once in 0.1 × SSC, 0.1% SDS at 65°C for 30 min. After 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 P450scc, 3β-HSD, and 18S rRNA cDNA. We quantitated the bands of interest, and values obtained were expressed as described previously. Each data point represents the average ± SE of the means from three separate experiments in which treatments were performed in triplicate.

Isolation of nuclei. MA-10 cells were stimulated with Bu_2cAMP in the presence or absence of Roundup for 4 hr. After 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 $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT, 0.5% v/v Nonidet P-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, we inspected them using an Olympus IMT-2 inverted microscope (Dexter Instrument Co., San Antonio, TX). Then the homogenate was layered onto a sucrose cushion consisting of sucrose buffer II (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 $MgCl_2$, 0.1 mM EDTA, 50 mM Tris-HCl, pH 8.3), frozen on dry ice, and stored in liquid nitrogen.

Nuclear run-on analysis. Nuclei were thawed at room temperature. An equal volume of 2 × reaction buffer (5 mM $MgCl_2$, 0.3 M KCl, 5 mM DTT, 10 mM Tris-HCl, pH 8.0) containing 50 µCi/mL [α-^{32}P] uridine triphosphate (SA 3,000 Ci/mmol; New England Nuclear), and 0.5 mM cold ATP, guanosine triphosphate, and cytidine triphosphate (Clontech, Palo Alto, CA) was added. Transcription complexes were elongated by incubating samples in a shaking water bath for 30 min at 30°C. After incubation, the reaction mixture was digested successively with 40 µg/mL DNAse I for 5 min at 30°C and 160 µg/mL proteinase K for 30 min at 42°C. DNAse I and proteinase K were obtained from Sigma. RNA was extracted with 5:1 phenol-chloroform, pH 4.3 (Fisher Scientific), precipitated by the addition of 10% TCA, collected onto 0.45 µm Millipore HA nitrocellulose filters using a vacuum manifold, and rinsed free of unincorporated nucleotides with 5% TCA. RNA captured onto filters was treated with 25 µg/mL DNAse I for 30 min at 37°C, eluted from filters with elution buffer (1% SDS, 5 mM EDTA, 10 mM Tris-HCl) for 10 min at 65°C, and treated with 30 µg/mL proteinase K for 30 min at 37°C. The resultant mixture was extracted with 5:1 phenol-chloroform, pH 4.3, and subjected to a 10-min digestion on ice with 0.200 M NaOH before quenching the reaction with 0.290 M Hepes. We precipitated RNA by adding 1/10 (v/v) 3 M sodium acetate and 2.5 vol 100% ethanol then centrifuged it for 30 min at 10,000 × *g*, 4°C. The resultant RNA pellet was resuspended in water and an aliqout was counted using a liquid scintillation counter. We used equal numbers of nuclei in the *in vitro* transcription assay. Equal counts of RNA were hybridized to target StAR, P450scc, and 18S cDNA inserts and linearized, empty pCMV-5 vector previously immobilized to nylon membranes (Hybond N+) using a Bio-Dot SF microfiltration apparatus (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Prehybridization and hybridization were performed under the same conditions described for Northern blots. We detected radioactivity using a Phosphorimager 445 SI (Molecular Dynamics, Sunnyvale, CA). Signals were quantitated using ImageQuant version 4.1 software (Molecular Dynamics) in volume mode, which integrates the intensity of each pixel within the defined area. Values were obtained as arbitrary units. Each data point represents the average ± SE of five separate experiments.

Protein kinase A (PKA) activity determination. Cells were treated and homogenized as described in "Isolation of Mitochondria and Western Blot Analysis." We measured

PKA activity with the SignaTECT cAMP-dependent protein kinase assay system (Promega, Madison, WI), as described in the manufacturer's protocol. Three separate experiments were performed in which treatments were performed in triplicate.

Mixture studies. We stimulated cells with $(Bu)_2$cAMP in the presence or absence of the indicated pesticides for 2 hr and we measured progesterone. Each data point represents the average ± SE of the means from three separate experiments in which treatments were performed in triplicate.

Statistical analysis. Statistically significant differences were determined by one-way analysis of variance and Fisher–protected least-square difference multiple comparison using the software program Statview SE + Graphics (Abacus Concepts, Inc., Berkeley, CA).

Results

Progesterone production and total cellular protein synthesis. Initial studies were performed to determine the effects of several pesticide formulations on steroidogenesis and total protein synthesis (Figure 1). Unlike the other pesticides, Roundup decreased progesterone production in a dosage-dependent manner (IC_{50} = 24.4 ± 0.67 µg/mL) without inducing a parallel decrease in total protein synthesis, indicating that this herbicide did not cause acute cellular toxicity or a general disruption in translation. Because 25 µg/mL Roundup significantly ($p < 0.01$) reduced steroidogenesis by 65% without affecting total protein synthesis, we chose to use this dose for the remaining studies. As Table 1 shows, Roundup also significantly ($p < 0.001$) disrupted steroidogenesis over time without inducing a parallel decrease in total protein synthesis. Interestingly, the active ingredient in Roundup, glyphosate, did not alter steroidogenesis or total protein synthesis at any dose tested (0–100 µg/mL; data not shown). These studies indicate that Roundup may have targeted specific components of the steroidogenic pathway to inhibit steroid production.

P450scc and 3β-HSD enzyme activity, expression, and steroidogenesis. To determine if the inhibitory effect of Roundup on $(Bu)_2$cAMP-stimulated progesterone production might be due to an inhibition of the activities of the steroidogenic enzymes, P450scc and/or 3β-HSD, 22*R*-HC was provided as a substrate and cells were treated for 2 hr with Roundup. We used 22*R*-HC, which can readily diffuse to the P450scc enzyme, bypassing the need for StAR-mediated cholesterol transfer, to determine steroidogenic capacity. Although Roundup significantly ($p < 0.01$) reduced $(Bu)_2$cAMP-stimulated steroidogenesis by 84%, $(Bu)_2$cAMP-stimulated progesterone production in these cells returned to control levels after a 24-hr recovery, demonstrating that Roundup's effects were completely reversible (Figure 2). The herbicide also significantly ($p < 0.05$) reduced 22*R*-HC-driven steroidogenesis by 71%, indicating that it inhibited P450scc and/or 3β-HSD enzyme activity. However, when cells were stimulated with $(Bu)_2$cAMP in the presence of 22*R*-HC, steroid production was reduced by only 58%. Therefore, because 22*R*-HC could partially rescue $(Bu)_2$cAMP-stimulated steroid production, a reduction in steroidogenic enzyme activity alone could not account for the observed level of steroidogenic inhibition.

To determine if Roundup specifically disrupted 3β-HSD, P450scc, or both steroidogenic enzyme activities, both pregnenolone-driven progesterone production (a measure of 3β-HSD activity) and 22*R*-HC-driven pregnenolone production (a measure of P450scc activity) were measured after herbicide treatment for 2 hr (Figure 3). Although Roundup did not alter 3β-HSD enzyme activity, indicating that the herbicide was not acutely toxic to cells or mitochondria, it

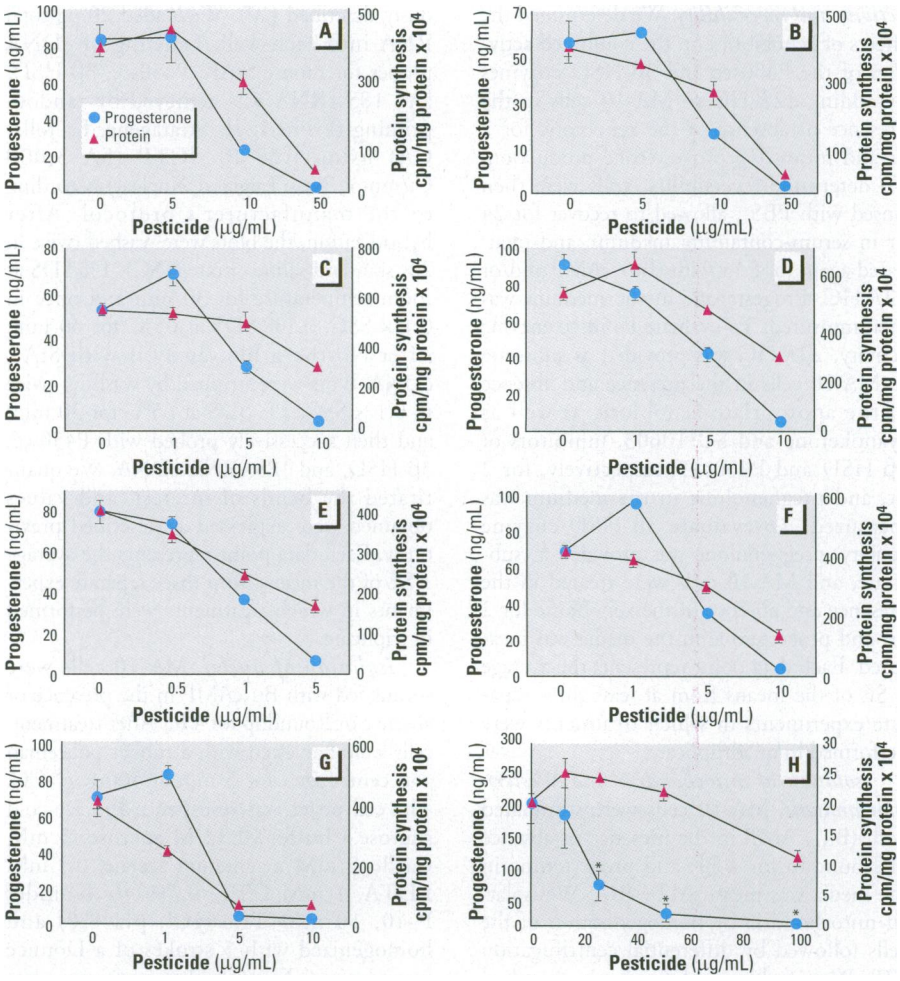

Figure 1. Effects of currently used pesticides on progesterone production and total cellular protein synthesis in MA-10 cells. (*A*) Ambush. (*B*) Ammo. (*C*) Banvel. (*D*) Cotoran. (*E*) Cyclone. (*F*) Dual. (*G*) Fusilade. (*H*) Roundup.
*Statistically significant differences ($p < 0.01$).

Table 1. Time-course study of the effects of Roundup on progesterone production and total cellular protein synthesis in MA-10 cells.

	2 hr		4 hr	
	Progesterone (ng/mL)*	Protein synthesis (cpm/mg × 10^4)	Progesterone (ng/mL)*	Protein synthesis (cpm/mg × 10^4)
Control	1.92 ± 0.21	56 ± 5.9	2.8 ± 0.42	102 ± 6.5
Bu_2cAMP (1 mM)	126 ± 20.3	65 ± 4.9	253 ± 13.1	94 ± 1.5
Roundup (25 µg/mL)	21 ± 5.0	40 ± 2.3	47 ± 7.8	75 ± 2.2

*For progesterone production at 2 and 4 hr, the difference between $(Bu)_2$cAMP and Roundup ± $(Bu)_2$cAMP was statistically significant ($p < 0.001$).

significantly ($p < 0.001$) reduced P450scc activity by 61%.

To determine if the decrease in P450scc enzyme activity might have been due to a reduction in the levels of this enzyme, and to confirm that 3β-HSD enzyme levels were not affected, we determined the effects of Roundup on the expression of these enzymes. Although this herbicide significantly ($p < 0.001$) blocked steroidogenesis by 94% (Figure 4), Western blot analysis of mitochondrial protein revealed that it did not alter P450scc or 3β-HSD enzyme protein levels (Figures 5 and 6). Moreover, Northern blot analysis also revealed that it did not affect P450scc mRNA levels (Figure 5). Interestingly, Roundup significantly ($p < 0.05$) reduced 3β-HSD mRNA levels by 33% (Figure 6). Because a reduction in P450scc activity alone cannot account for the observed level of steroidogenic inhibition, the data suggest that this herbicide also blocked steroidogenesis before the P450scc enzyme, potentially by reducing cholesterol availability.

StAR protein and mRNA levels. Because StAR protein mediates the transfer of cholesterol to the inner mitochondrial membrane, we determined the effects of Roundup on the expression of this protein. Western blot analysis revealed that Roundup significantly ($p < 0.01$) reduced StAR protein levels by 90% (Figure 7). Because StAR levels were reduced in proportion to steroid levels, and StAR functions upstream of the steroidogenic enzymes, a reduction in StAR protein levels alone could account for the observed level of steroidogenic inhibition.

To determine if Roundup reduced StAR protein levels by decreasing StAR mRNA levels, we performed Northern blot analysis (Figure 7). StAR mRNA consists of the 1.6, 2.7, and 3.4 kb transcripts, which make up 18, 10, and 72%, respectively, of total StAR mRNA. Northern blot analysis revealed that Roundup did not alter StAR mRNA levels, indicating that Roundup disrupted StAR protein expression post-transcriptionally.

Although the importance of the three StAR transcripts is unknown at this time, Roundup preferentially increased levels of the 1.6 and 2.7 StAR transcripts (Figure 7). In fact, this herbicide significantly ($p < 0.05$) increased levels of the 1.6 and 2.7 kb transcripts by 2- and 2.3-fold, respectively.

Roundup may have increased StAR transcript levels by increasing the rate of StAR gene transcription, altering the post-transcriptional expression of StAR (e.g., increasing StAR transcript stability), or by causing a combination of the two processes. As Figure 8 shows, Bu_2cAMP increased the rate of StAR gene transcription 5-fold. However, when cells were stimulated with Bu_2cAMP in the presence of Roundup, StAR transcription increased 8-fold, indicating that Roundup may have increased StAR transcript levels by increasing their synthesis.

PKA activity. A reduction in PKA activity might partly explain the observed reduction in StAR expression and steroidogenesis. To determine if Roundup disrupts PKA activity, cells were treated for 4 hr. Roundup did not affect the ability of PKA present in cell lysates to phosphorylate the PKA-specific substrate (data not shown), demonstrating that Roundup inhibited StAR protein expression distal to PKA activation.

Effects of a mixture of pesticides on steroidogenesis. We previously showed that Dimethoate and α-, δ-, and γ-HCH (lindane) inhibit steroidogenesis primarily by disrupting StAR protein expression (*14,15*). However, unlike Roundup, these compounds reduced StAR expression primarily by reducing StAR mRNA levels. As shown in Figure 9, when tested individually at concentrations that were not maximally inhibitory, each pesticide inhibited steroidogenesis by 25%. However, when pesticides were tested together in a mixture at the same concentrations, they inhibited steroidogenesis by only 50%, indicating that components in the mixture may have interacted antagonistically.

Discussion

We previously showed that lindane and Dimethoate inhibited steroidogenesis by disrupting StAR protein expression (*14,15*). Although these pesticides likely impacted different cellular pathways that regulate the expression of StAR, they impinged on the steroidogenic pathway at the level of StAR protein. Therefore, we hypothesized that a disruption in StAR expression might also account for the reduction in steroidogenesis following treatment with other currently used pesticides. In support of this hypothesis, the present study showed that Roundup decreased steroidogenesis by disrupting StAR expression post-transcriptionally. Although StAR has largely been overlooked as a target for environmental pollutants in the past, the

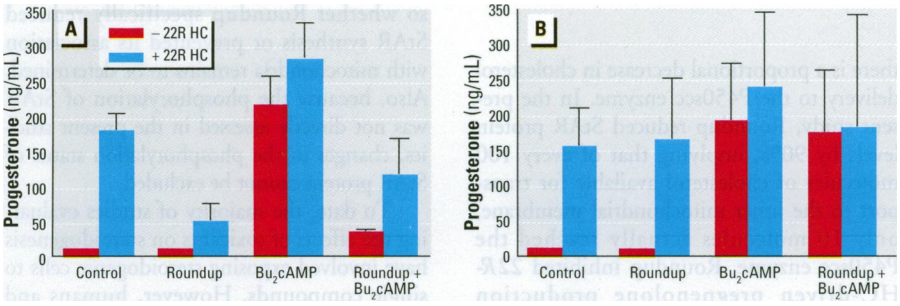

Figure 2. Effects of Roundup on P450scc and 3β-HSD enzyme activity and steroidogenesis in MA-10 cells. (*A*) Effects of 2-hr treatment with Roundup on progesterone production. The difference between $(Bu)_2$cAMP and Roundup + $(Bu)_2$cAMP was statistically significant ($p < 0.01$). (*B*) Effects of 2-hr treament with Roundup on progesterone production after a 24-hr recovery.

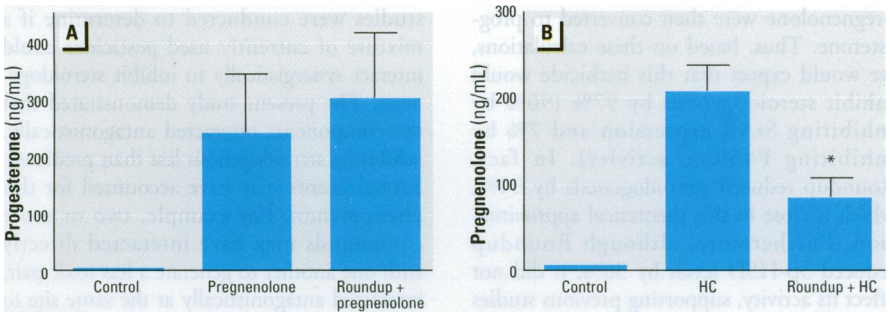

Figure 3. Effects of Roundup on P450scc and 3β-HSD enzyme activity. (*A*) Effects of Roundup on 3β-HSD enzyme activity. (*B*) Effects of Roundup on P450scc enzyme activity.
*Statistically significant differences ($p < 0.001$).

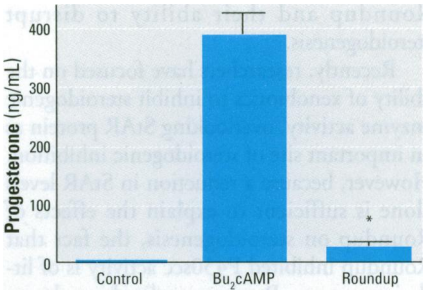

Figure 4. Effects of Roundup on progesterone production in MA-10 cells. Cells grown in 100-mm plates were treated as described for Western and Northern blot analysis in "Materials and Methods."
*Statistically significant differences ($p < 0.001$).

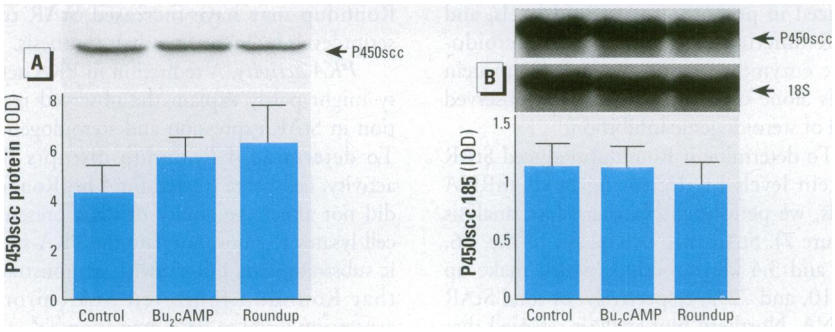

Figure 5. Effects of Roundup on P450scc enzyme and mRNA levels. (*A*) Western blot analysis. (Upper panel) A representative Western blot; (lower panel) quantitation of immunospecific bands for the P450scc enzyme. (*B*) Northern blot analysis. (Upper panel) Representative Northern blots for P450scc mRNA and 18S rRNA; (lower panel) bands for P450scc mRNA and 18S rRNA were quantitated and data expressed as P450scc mRNA/18S rRNA.

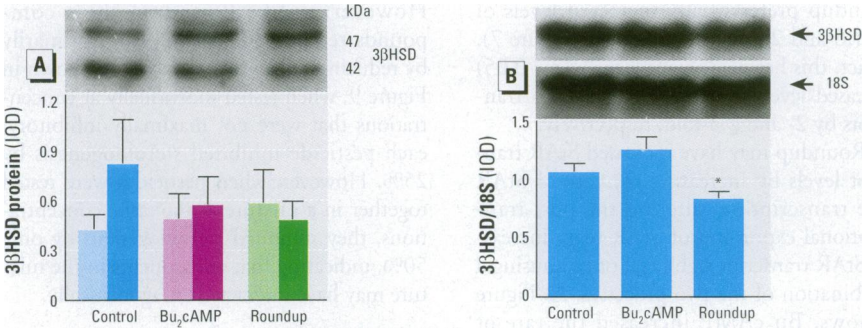

Figure 6. Effects of Roundup on 3β-HSD enzyme and mRNA levels. (*A*) Western blot analysis. (Upper panel) A representative Western blot; (lower panel) quantitation of immunospecific bands for the 3β-HSD enzyme. Lighter shades indicate 47-kDa exposure; darker shades indicate 42 kDa exposure. (*B*) Northern blot analysis. (Upper panel) Representative Northern blots for 3β-HSD mRNA and 18S rRNA; (lower panel) bands for 3β-HSD mRNA and 18S rRNA were quantitated and data expressed as 3β-HSD mRNA/18S rRNA.
*Statistically significant differences ($p < 0.05$).

present findings suggest that StAR may be susceptible to at least some environmental pollutants that inhibit steroidogenesis and impair fertility.

Although Roundup decreased steroidogenesis, the active ingredient of this herbicide, glyphosate, did not alter steroid production, indicating that at least one other component of the formulation is required to disrupt steroidogenesis. Because the formulation of Roundup is proprietary, further studies are needed to identify the components in Roundup and their ability to disrupt steroidogenesis.

Recently, researchers have focused on the ability of xenobiotics to inhibit steroidogenic enzyme activity, overlooking StAR protein as an important site of steroidogenic inhibition. However, because a reduction in StAR levels alone is sufficient to explain the effects of Roundup on steroidogenesis, the fact that Roundup inhibited P450scc activity is of little importance. Previous studies have shown that the accumulation of the 30-kDa form of StAR protein in mitochondria reflects the amount of cholesterol delivered to the inner mitochondrial membrane over time. Thus, for a given decrease in StAR protein levels there is a proportional decrease in cholesterol delivery to the P450scc enzyme. In the present study, Roundup reduced StAR protein levels by 90%, implying that of every 100 molecules of cholesterol available for transport to the inner mitochondrial membrane, only 10 molecules actually reached the P450scc enzyme. Roundup inhibited 22*R*-HC-driven pregnenolone production (P450scc activity) by 71%, indicating that of the 10 molecules of cholesterol that reached the enzyme, only 3 were converted to pregnenolone. Because Roundup did not alter 3β-HSD activity, these 3 molecules of pregnenolone were then converted to progesterone. Thus, based on these calculations, we would expect that this herbicide would inhibit steroidogenesis by 97% (90% by inhibiting StAR expression and 7% by inhibiting P450scc activity). In fact, Roundup reduced steroidogenesis by 94%, which is close to this theoretical approximation. Furthermore, although Roundup reduced 3β-HSD levels by 30%, it did not affect its activity, supporting previous studies which have shown that the steroidogenic enzymes, with the exception of P450scc, are present in excess amounts. For example, in one study, testosterone production was unaffected, even though levels of P450c17 were reduced by 90% (*28*). In contrast, because StAR mediates the rate-limiting step in steroid biosynthesis, steroidogenesis is sensitive to small changes in StAR protein expression. The present study illustrates the importance of StAR-mediated cholesterol transfer in environmental pollutant-inhibited steroidogenesis.

Although the transcriptional regulation of StAR has been the subject of intense research since StAR was cloned in 1994 [reviewed by Reinhart et al. (*29*)], the post-transcriptional regulation of StAR has received considerably less attention and is poorly understood. StAR protein is regulated post-transcriptionally by oxysterols, prostaglandin F2α, and endotoxin, indicating that this may be an important level at which StAR expression is controlled physiologically (*30–32*). At least three post-transcriptional events are required for StAR to have full steroidogenic activity and may have been targeted by Roundup, including translation of the StAR mRNA into protein, association of StAR with the outer mitochondrial membrane (*33*), and phosphorylation of the StAR protein. Although the mechanism by which Roundup disrupted StAR protein expression post-transcriptionally remains unclear, inhibition of *de novo* protein synthesis and PKA activity were ruled out. The precursor 37-kDa form of StAR cannot easily be measured, so whether Roundup specifically reduced StAR synthesis or prevented its association with mitochondria remains to be determined. Also, because the phosphorylation of StAR was not directly assessed in the present studies, changes in the phosphorylation status of StAR protein cannot be excluded.

To date, the majority of studies evaluating the effects of toxicants on steroidogenesis have involved exposing steroidogenic cells to single compounds. However, humans and wildlife are typically exposed to large numbers of chemicals simultaneously. Because enhanced steroidogenic inhibition may result from interactions between different chemicals present in chemical mixtures, studies were conducted to determine if a mixture of currently used pesticides could interact synergistically to inhibit steroidogenesis. The present study demonstrated that the components interacted antagonistically, inhibiting steroidogenesis less than predicted. Several events may have accounted for this phenomenon. For example, two or more compounds may have interacted directly with one another to generate a less toxic pair, interacted antagonistically at the same site to cancel each other's effects, and/or acted additively at one site, which contributes only 50% to overall steroid production. Finally,

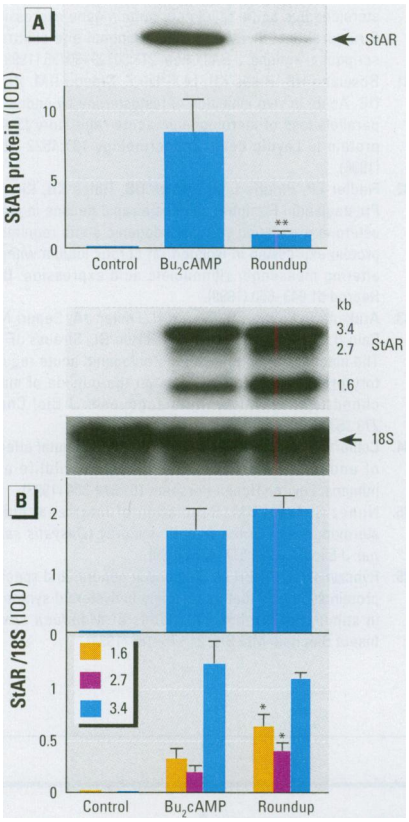

Figure 7. Effects of Roundup on StAR protein and mRNA levels. IOD, integrated optical density units. (*A*) Western blot analysis. (Upper panel) A representative Western blot; (lower panel) quantitation of immunospecific bands for the StAR protein. (*B*) Northern blot analysis. (Upper panel) Representative Northern blots for StAR mRNA and 18S rRNA; (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.

*Statistically significant differences ($p < 0.05$). **Statistically significant differences ($p < 0.01$).

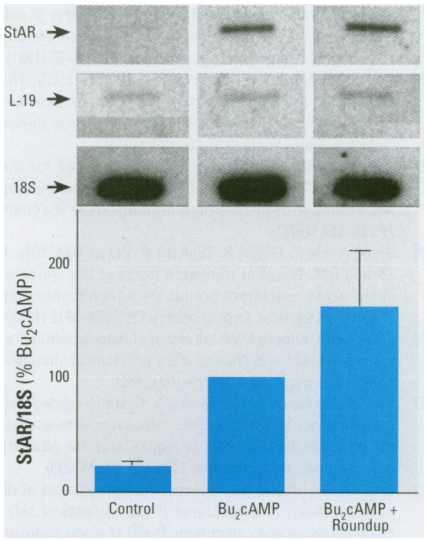

Figure 8. Effects of Roundup on StAR gene transcription. Upper panel, a representative experiment is shown. Lower panel, bands for StAR, L19 mRNA and 18S rRNA were quantitated by computer assisted image analysis.

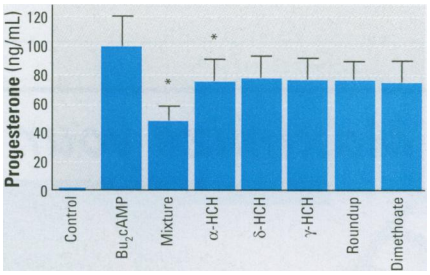

Figure 9. Effects of a mixture of Roundup, α-, δ- and γ-HCH, and Dimethoate on steroid production.

*Statistically significant differences ($p < 0.05$).

one compound could have altered the metabolism of another compound to generate a less toxic metabolite.

As a result of the important role that StAR plays in the steroidogenic pathway, it may prove to be a useful biomarker to evaluate endocrine system function in sentinel wildlife species. In fact, a disruption in StAR expression may represent the first event in the sequence of time-related changes that underlie pesticide-induced toxicity and lead to disturbances at the cellular and whole-organism level. Environmental pollutants have purportedly caused a wide range of adverse effects including decreased fertility in shellfish, fish, birds, and mammals and decreased hatching success in fish, birds, and reptiles (*34*). In many of these cases, abnormal steroid hormone levels have been measured, indicating the possibility that these toxicants may block steroid hormone synthesis. Although StAR has not yet been identified in submammalian species, several reports indicate the probability that a StAR-like protein may exist in these species. For example, hormone-stimulated steroidogenesis in fish and insects requires the *de novo* synthesis of a hormone-regulated, rapidly synthesized, cycloheximide-sensitive, and highly labile protein that may mediate cholesterol transfer for steroid hormone biosynthesis (*35,36*). A disruption in this StAR-like protein might contribute to the development of reproductive dysfunction in these species. If this putative StAR-like protein is identified and characterized, it may enhance our understanding of the mechanism of pollutant-induced steroidogenic inhibition in these species.

Not only does StAR play an important role in steroid production in the gonads, but it is also indispensable for steroidogenesis in the adrenal glands. As a result, a disruption in StAR protein expression may impair more than just fertility. The adrenal glands synthesize glucocorticoids and mineralocorticoids, and a reduction in StAR expression in the adrenal gland may affect carbohydrate metabolism, immune system function, and water balance. Because many toxicants that reduce StAR expression and steroidogenesis in the ovary and testis also reduce StAR expression and steroidogenesis in the adrenal gland, a disruption in StAR protein expression may underlie many of the toxic effects of environmental pollutants (*29*).

In conclusion, Roundup disrupted steroidogenesis in Leydig cells through a post-transcriptional reduction in StAR protein expression. The use of StAR as an end point in studies concerning endocrine disruption merits further consideration.

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