TRIAZINE-MEDIATED DISRUPTION OF BLTK1 LEYDIG CELL STEROIDOGENESIS

By

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ABSTRACT

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Triazines are broad spectrum herbicides implicated in the etiology of testicular dysgenesis eliciting perturbations of gonad development and reproductive function. In rodents triazines alter the onset of puberty, cause reproductive senescence, and disrupt reproductive tract development including dysregulation of androgen-dependent tissue development. Disruption of testosterone biosynthesis is thought to underlie the effects of triazines in males across vertebrate species. However, the current approaches used to identify developmental and reproductive toxicants involve *in vivo* studies evaluating apical endpoints that are generally descriptive and do not contribute to the elucidation of mechanism of action. As such, the mechanism by which triazines disrupt steroidogenesis remains unknown. The objectives of this study were to establish a model for the evaluation of triazines effects on steroidogenesis that is amenable to mechanism determination and to identify a possible mode of action for triazine-elicited disruption of steroidogenesis.

BLTK1 Leydig cells were characterized and demonstrated to be a viable *in vitro* model for the evaluation of endocrine disruptor-mediated effects on steroidogenesis. BLTK1 cells express all necessary steroidogenic enzymes essential for hormone biosynthesis and maintain low basal levels of testosterone (T) production, inducible by recombinant human chorionic

gonadotropin (rhCG) and forskolin (FSK). The time- and concentration-dependent effects of triazine herbicides, atrazine (ATR), propazine (PRO), simazine (SIM) and terbuthylazine (TBA), and their chlorometabolites, desethylatrazine (DEA), desisopropylatrazine (DIA) and diamino-*s*-chlorotriazine (DACT), were evaluated in BLTK1 Leydig cells. Triazines and their chlorometabolites induced concentration-dependent increases in basal progesterone (P) and T levels. Triazines also elicited the differential gene expression of several steroidogenic enzymes required for steroidogenesis. These results were consistent with the cumulative risk assessment "Common Mechanism Group" designation that PRO, SIM, DEA, DIA and DACT have ATR-like effects. Using ATR as the representative triazine, whole-genome microarrays identified differential gene expression at later time points (> 12 hr) with affected genes associated with steroidogenesis and cholesterol metabolism. Finally, the effects of ATR on rhCG-mediated induction of steroidogenesis revealed antagonism of P and T levels, despite potentiation of intracellular cAMP levels. The inhibition of cAMP-specific phosphodiesterases was identified as underlying increases in cAMP levels. However, the ATR-mediated super-induction of cAMP levels was not causative of T antagonism in the presence of rhCG. Disruption of phosphorylation cascades likely contribute to ATR-mediated effects on steroidogenesis, with observed effects on protein kinase A (PKA) target proteins. This study has established BLTK1 cells as a novel *in vitro* steroidogenic model for the evaluation of endocrine disrupting chemicals and the evaluation of triazines in BLTK1 cells has expanded our knowledge of triazine-mediated disruption of steroidogenesis.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AR	androgen receptor
ATR	atrazine
cAMP	3,5’-cyclic adenosine monophosphate
cGMP	3,5’-cyclic guanosine monophosphate
CMG	common mechanism group
CO_2	carbon dioxide
CREB	cAMP response element binding protein
DACT	diamino-*s*-chlorotriazine
DAVID	database for annotation, visualization and integrated discovery
DEA	desethylatrazine
DEHP	diethylhexyl phthalate
DIA	desisopropylatrazine
DHT	dihydrotestosterone
DMEM	Dulbecco’s modified eagle medium
DMSO	dimethyl sulfoxide
E2	17β-estradiol
EC_{50}	effective concentration causing 50% of the maximal response
EDC	endocrine disrupting compound
EIA	enzyme immunoassay
EPA	Environmental Protection Agency (United States)

ER	estrogen receptor
FBS	fetal bovine serum
FSK	forskolin
GPCR	G-protein-coupled receptor
HPG	hypothalamic-pituitary-gonadal
IBMX	3-isobutyl-1-methylxanthine
IPA	Ingenuity® pathway analysis
LH	luteinizing hormone
LHCGR	luteinizing hormone/chorionic gonadotropin receptor
MEHP	monoethylhexyl phthalate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
P	progesterone
PCR	polymerase chain reaction
PDE	phosphodiesterase
cPDE	cAMP-specific phosphodiesterase
PKA	protein kinase A
PRO	propazine
QRTPCR	quantitative real-time PCR
rhCG	recombinant human chorionic gonadotropin
RNA	ribonucleic acid
SIM	simazine
StAR	steroidogenic acute regulatory protein
T	testosterone
TBA	terbuthylazine

CHAPTER 1

CHAPTER 1

REVIEW OF LITERATURE: TRIAZINE-MEDIATED TOXICITY AND STEROIDOGENESIS

INTRODUCTION

Chloro-*s*-triazines are broad-spectrum herbicides gaining public concern due to their endocrine disruptive effects in aquatic and mammalian species. While the mode of action by which triazines elicit their effects remains unknown, it is accepted that they target the hypothalamic-pituitary-gonadal (HPG) axis eliciting altered steroid hormone levels, disruption of gonadal morphometry and compromised reproduction. Establishing a physiologically relevant model to evaluate *de novo* steroid hormone biosynthesis (steroidogenesis) that is amenable to elucidating a mode of action is necessary to characterize how triazines disrupt hormone levels.

TRIAZINE HERBICIDE APPLICATION AND EXPOSURE

Chloro-*s*-triazines, referred to herein as triazines, are broad-spectrum herbicides characterized by a conserved chlorinated triazine ring backbone (Figure 1). Triazines are used to control broadleaf and grassy weeds as well as algae through inhibition of photosynthesis (*1, 2*). More specifically, high affinity, irreversible binding to the plastoquinone binding site on photosystem II in the thylakoid membrane of chloroplasts inhibits electron transfer, effectively blocking photosynthesis thereby eliminating target plants and algae (*1, 2*).

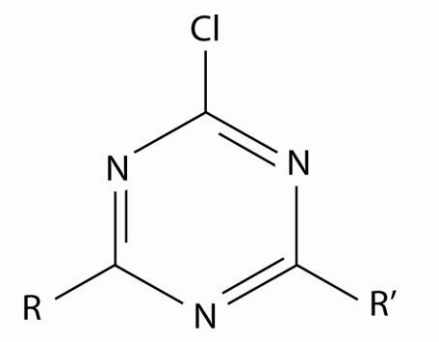

FIGURE 1. STRUCTURE OF THE CHLORO-*S*-TRIAZINE BACKBONE.

Several triazines are registered for use in the United States including atrazine (ATR) and simazine (SIM) for use on agricultural crops, propazine (PRO) for indoor grown ornamental plants, and terbuthylazine (TBA) for use as an algicide (Figure 2) (*3*). ATR was introduced by Ciba-Geigy (then Novartis and currently Syngenta) in 1958, and is currently the most widely used triazine with nearly 80 million pounds applied annually, predominantly on corn crops in the Midwestern United States (*4, 5*). ATR is highly mobile, readily transported from groundwater to streams and lakes, and is persistent in both soil and water with a half-life ranging from 146 to 742 days in aerobic soil and water, respectively (*6*). It was the most common pesticide contaminant detected in water in the United States between 1992 and 2001, often detected above the U.S. Envionmental Protection Agency's (EPA) suggested maximum contamination level (MCL) of 3 ppb (*6*). In the Midwestern United States where use of ATR is the greatest, storm runoff can result in peaks of ≥20 µg/L ATR in watersheds (*7*). Similarly, SIM

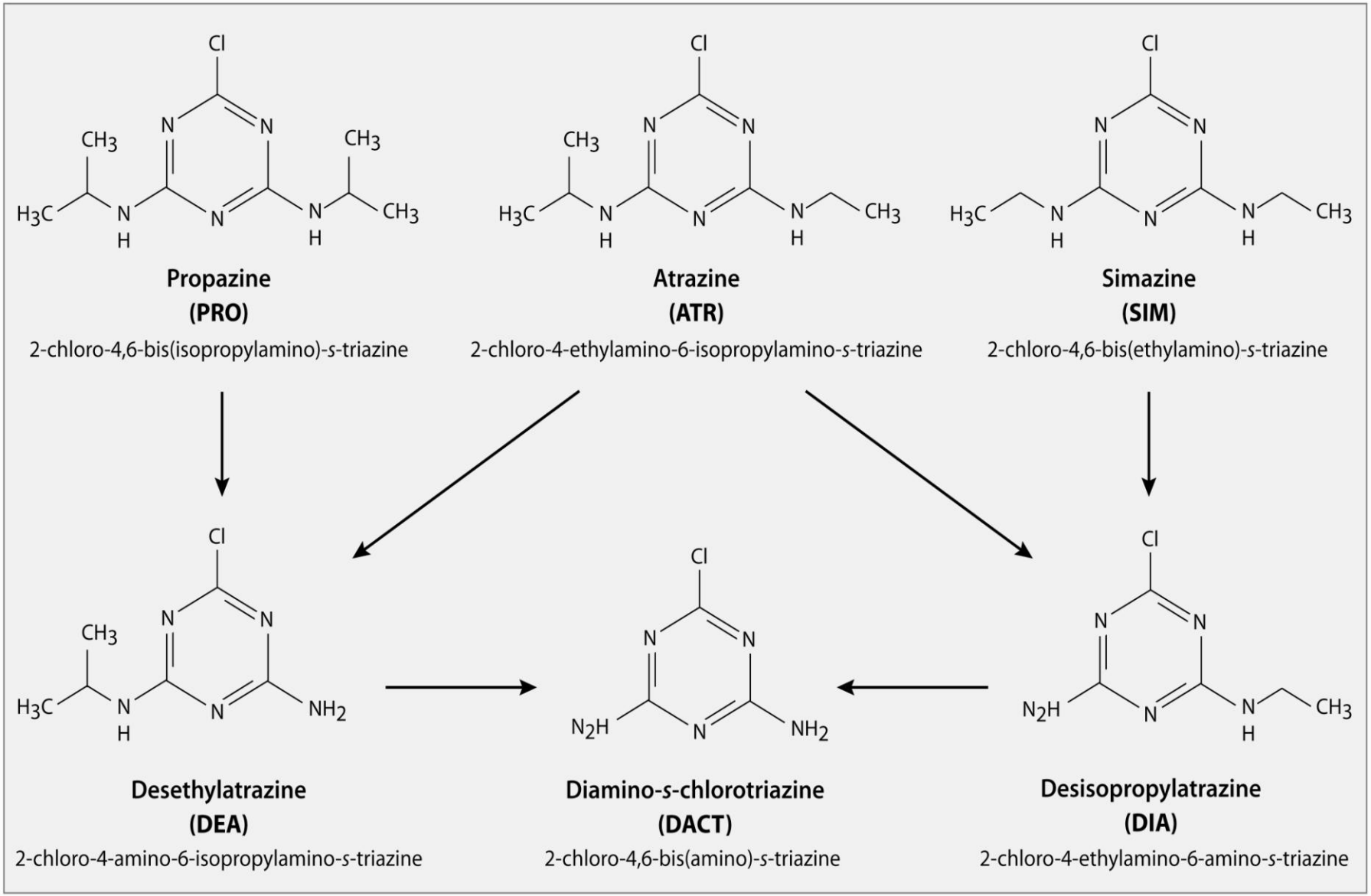

FIGURE 2. STRUCTURES OF THE CMG TRIAZINES AND CHLOROMETABOLITES.

FIGURE 2 (cont'd)
Simazine (SIM), Atrazine (ATR) and Propazine (PRO) are dealkylated in the environment by photodegradation, microbial, or fungal degradation, and in mammalian systems by cytochrome P450 enzymes (shown as black arrows) predominantly into the chlorometabolites desisopropylatrazine (DIA), desethylatrazine (DEA) and diamino-*s*-chlorotriazine (DACT). These six chemicals comprise the triazine common mechanism grouip (CMG).

is also a groundwater contaminant found above its MCL of 4 ppb with a soil half-life of 91 days and water half-life of 664 days (*6, 8*). Ubiquitous groundwater contamination caused the European Union to cancel ATR registration in 2003 ceasing its use in most of Europe with TBA (Figure 3) having been introduced in its place (*9*). However, based on a risk assessment carried out by the EPA in 2006 the re-registration of ATR was approved, and 60 other countries also continue to use ATR (*10, 11*). Given the new body of scientific information, the EPA initiated a new comprehensive re-evaluation of ATR, including more accurate methods to monitor human exposure (*12, 13*).

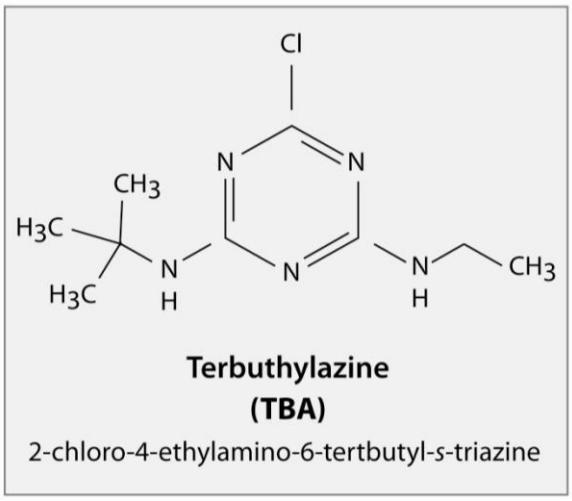

FIGURE 3. STRUCTURE OF TERBUTHYLAZINE.
Structure of terbuthylazine (TBA) is used as an ATR alternative in the European Union, and as an algicide in the United States.

Dealkylation of ATR and SIM in the environment by microbial and fungal degradation, as well as photodegradation, form three major chlorinated metabolites (chlorometabolites): desethylatrazine (DEA), desisopropylatrazine (DIA) and diaminotriazine (DACT; Figure 2), which are commonly found with ATR and SIM in groundwater (*6, 14, 15*). As such, triazine exposure estimations require measurements of not only the parent triazine, but also the chlorometabolites (*16*).

In mammals, triazines are widely distributed throughout the body and rapidly metabolized primarily by cytochrome P450s, namely CYP1A1/2, into DEA, DIA and DACT (*16-19*). Evaluation of human urine by mass spectrometry also identified hydroxylated, dealkylated, and glutathione-derived mercapturic acid metabolites of ATR, but the dealkylated chlorometabolites DEA, DIA and DACT were the most abundant, consistent with rodent studies (*16*). More specifically, urinary ATR metabolite composition was up to ~70% DACT, ~20% DEA, and ~5% DIA at environmental exposure levels below the MCL (*16*). Maximum plasma levels in mice are achieved within 1 hr, with maximum DACT plasma concentrations within 2 hrs post-dose (*18*). Chlorometabolite concentrations of ~2 µM are reported in serum with less than 1 µM of parent compound 3 hrs following administration of 200 mg/kg ATR or an equimolar dose of SIM or PRO in male Wistar rats (*20*). Most ATR and chlorometabolite dose is excreted within 24 hrs in mice and rats, with the exception of substantial DACT detected in urine up to 72 hrs post-dose (*18, 19*). Estimations of human daily doses from occupational exposure to ATR during mixing, loading and application ranged from 1.8 to 6.1 µg/kg/day, suggesting exposures can be as high as 6.1 ppb (*21*).

Exposure to triazines is of concern due to their endocrine disruptive effects (*20, 22, 23*). The EPA has grouped ATR, SIM and PRO as well as their major chlorometabolites (DEA, DIA and DACT) into a "Triazine Common Mechanism Group" for cumulative risk assessment. Common mechanism groups (CMGs) are defined for cumulative risk assessment as two or more chemicals that cause a common toxic effect(s) using the same sequence of major biochemical events, interpreted as mode of action (*24*). The triazine CMB was established based on the conserved disruption of the HPG axis by all chemicals. More specifically, luteinizing hormone (LH) surge was suppressed by all compounds in female rodents (*24-26*). The inclusion of the chlorometabolites assumes toxic equipotency to the parent triazines (*25*), highlighting the need to study the effects of chlorometabolites independent of the parent triazines.

TRIAZINE-MEDIATED TOXICITY

Many studies on triazine-mediated toxicity have focused on ATR as the representative triazine since it is most widely used. Despite few studies evaluating PRO, SIM, TBA or chlorometabolites, effects elicited by ATR are considered significant for all chemicals in the triazine CMG. Due to the accumulation of ATR in water systems, the effects of ATR on a variety of vertebrate classes, including aquatic species such as fish and frogs, have been widely evaluated and compared to effects in mammalian species, namely rodents (*27, 28*).

Due to the structural similarities between the specific site of ATR action at plastoquinone binding sites on photosystem II in plants and mammalian electron transport chain (ETC) complex I and III quinone binding sites, the potential for ATR to interfere with mitochondrial respiration has been investigated (*29, 30*). Mitochondrial dysfunction

characterized by decreased ETC complex III activity has been documented in rats, although it was only observed in one study with chronic administration of ATR in drinking water for 5 months, and only in skeletal muscle (*30*). It is generally accepted that ATR does not target mitochondrial ETC complexes in mammalian systems (*13*).

Species-specific effects of ATR exposure including immunomodulation (*31-33*), oxidative stress (*34*) and tumor promotion (*35*) have been documented. For example, ATR-mediated immunomodulation includes reduced ability of silver catfish fingerlings to withstand pathogenic microorganism challenge (*32*), but increased T cell cytolytic activity in Balb/c mice (*31*). Meanwhile, ATR increases hepatic lipid peroxidation and oxidative stress with concomitant elevation of antioxidant enzyme expression only in fish (*34, 36, 37*). Mammary tumors resulting from perturbations of LH surge have only been identified in female Sprague-Dawley rats (*35, 38, 39*). However, tumor promotion is not considered significant to human risk assessment, and ATR is not categorized as a human carcinogen, due to differences in hormonal control of estrus in Sprague-Dawley rats mediated by LH surge compared to mediation by estrogen in humans (*40, 41*). Despite the lack of relevance of mammary tumor promotion to other species, the most consistent and widely reported effects of ATR are on hormone-mediated reproductive development and function.

Reproductive toxicity elicited by ATR includes changes in hormone levels and altered reproductive function in many vertebrate species with generally 'estrogenic effects' despite negligible interaction on estrogen receptor activity (*17, 37, 38, 42-45*). As such, the mode of action by which ATR elicits endocrine disruptive effects remains unknown. In female rodents ATR suppresses LH surge (*46*), alters estrous cycling (*47*), delays vaginal opening, uterine growth

and puberty (*48-50*), and disrupts steroidogenesis (*51*). Meanwhile, in male rodents ATR delays puberty, alters testicular morphology and impairs androgen-dependent organ development (*52-57*). Most of these ATR-mediated effects are attributed to changes in steroid hormone levels associated with disruption of the HPG axis (Figure 4) (*40, 58*).

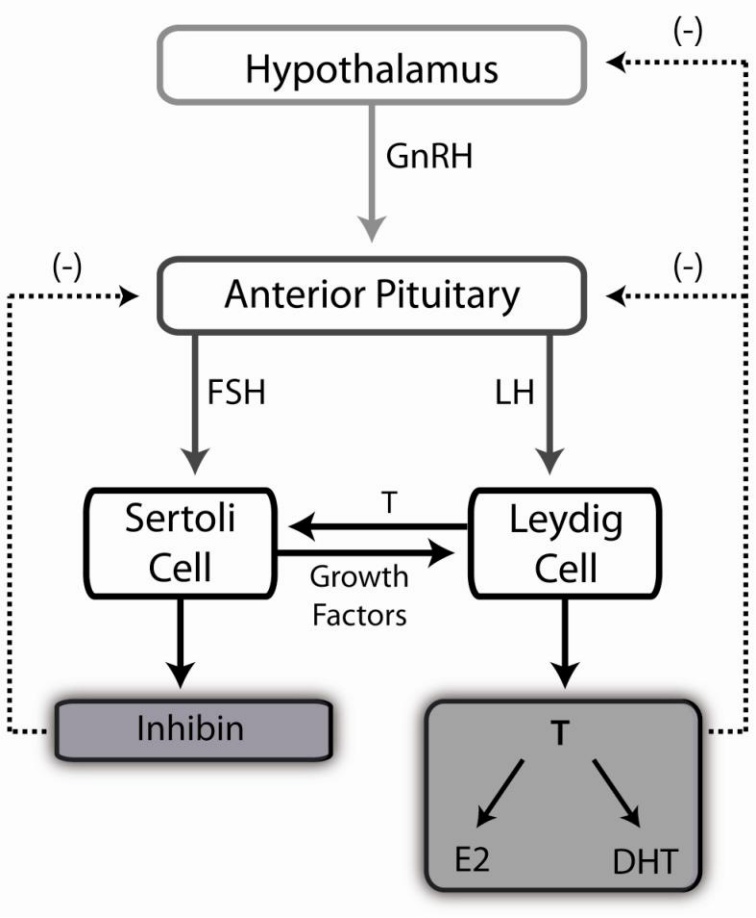

FIGURE 4: THE MALE HYPOTHALAMIC-PITUITARY-GONADAL (HPG) AXIS.
The HPG axis is the endocrine regulatory framework consisting of stimulation and feedback inhibitory hormone activity between the hypothalamus, pituitary and gonads. Briefly, the hypothalamus produces gonadotropin-releasing hormone (GnRH), which stimulates the anterior pituitary to secrete follicle-stimulating hormone (FSH) and LH. LH stimulation of Leydig cells promotes T production, which both stimulates Sertoli cells and feedback inhibits (-) GnRH and LH production. T can also be metabolized in Leydig cells by aromatization into 17β-estradiol (E2) or by 5α-reduction into 5α-dihydrotestosterone (DHT).

ATR-elicited reproductive and developmental effects in aquatic wildlife species are consistent with rodent studies including disruption of steroidogenesis and gonadal development, reduced fertility, and reproductive senescence (*40, 44, 59-62*). For example, in male goldfish ATR exposure decreased plasma androgen concentrations and altered the morphology of the testes, while elevated ovary atresia was evident in females (*63*). Gonad abnormalities in both sexes of ATR-treated fathead minnow resulted in reduced egg production and ultimately decreased reproduction (*61*). Meanwhile, ATR-elicited reproductive toxicity is characterized by feminization comprising elevated estrogen levels, demasculinization characterized by decreased androgen concentrations, and hermaphroditism in male *Xenopus laevis* (*27, 40, 44, 60, 64, 65*). All these effects noted in frogs can be attributed to disruption of steroidogenesis. For example, hermaphroditism in frogs is linked to a lack of 5α-reduced androgens such as 5α-dihydrotestosterone (DHT), which is produced from testosterone (T) by 5α-reductase enzymes in the testes (*60*). Furthermore, both T and 5α-reduced androgens are essential during male gonad development and for progression of spermatogenesis in Sertoli cells (*66*). In fact, ATR elicits disruption of androgen levels and reproductive development (*52, 53, 55*) as well as decreased sperm number and motility in rodents and humans (*56, 67, 68*). Consequently, the leading hypothesis proposed in the literature is that ATR's endocrine disruptive effects in males are due to alterations in steroidogenesis, namely affecting progesterone (P) and T levels (*45, 52, 53, 57*).

BIOLOGY OF TESTICULAR STEROIDOGENESIS

Despite the consensus that ATR is an endocrine disruptor that dysregulates steroidogenesis and alters reproductive function, the underlying mechanism and intracellular target(s) of ATR remain unknown. Several fungicides, pesticides and phthalates are known to elicit reproductive tract abnormalities including compromised reproductive fitness and increase cancer incidence due to altered steroid hormone levels (*69-72*). As such, it is recognized that steroidogenesis is a potential target for drugs, chemicals, natural products and environmental contaminants that adversely impact reproductive development and fertility (*73, 74*). The mechanisms involved in endocrine disrupting chemical (EDC)-mediated changes in steroid levels remain largely unknown but could involve disrupting steroidogenic enzyme expression, activation and/or activity (*69*). Developmental exposure to high doses of EDCs has been associated with testicular dysgenesis syndrome (TDS) in humans, comprised of adverse effects including cryptorchidism, hypospadias, low sperm count and testicular cancer, similar effects have been characterized in rodents and wildlife species with similar etiology (*75-78*).

While both the adrenal glands and the testes/ovaries have the capacity to carry out steroidogenesis, in males the testes are the primary organ producing T and may be a target for ATR. In mammals, the male gonads contain terminally differentiated adult Leydig cells that are the primary source of circulating T in the body. Leydig cells have an intracellular organelle structure adapted to serve in steroidogenesis, including abundant smooth endoplasmic reticulum and many mitochondria and lipid droplets, which provide the precursor to steroid synthesis: cholesterol (*79*). The primary function of Leydig cells is the production of androgens

including DHT and T, which are essential for the descent of the testes during development and for progression of spermatogenesis (*66*).

Leydig cell steroidogenesis is stimulated by the chief gonadotropin tropic stimulator, LH secreted from the anterior pituitary activating the G-protein-coupled LH/chorionic gonadotropin receptor (LHCGR; Figure 5) (*66*). Alternatively, human chorionic gonadotropin (hCG), produced by the placenta during pregnancy, with 85% sequence similarity to LH (*80*), can also stimulate the LHCGR and is often used for research due to a longer half-life (~24 hrs compared to ~4 hrs for LH (*81*)). High affinity binding of either gonadotropin to LHCGR expressed on the surface of Leydig cells, results in dissociation of the G-protein alpha stimulatory subunit, which in turn activates adenylyl cyclase (*66*). The stimulation of adenylyl cyclase activity, converting ATP into cAMP, rapidly increases intracellular cAMP levels (*82, 83*). The cAMP levels are a function of both synthesis and degradation, with cyclic nucleotide phosphodiesterase enzymes responsible for the turnover of cAMP (*66*). cAMP binding activates protein kinase A (PKA), resulting in the phosphorylation and activation of steroidogenic enzymes promoting the synthesis of steroid hormones (*83*). Leydig cell steroidogenesis can be divided in to two phases: (1) an acute phase comprising LHCGR activation and induction of intracellular cAMP and PKA signaling events, and (2) a longer term effect involving differential gene expression and increased steroidogenic enzyme expression that sustains steroidogenesis (*66*). While the acute phase induces immediate T production, sustained hormone biosynthesis requires both phases. Therefore, any studies examining disruption of steroidogenesis should examine both phases.

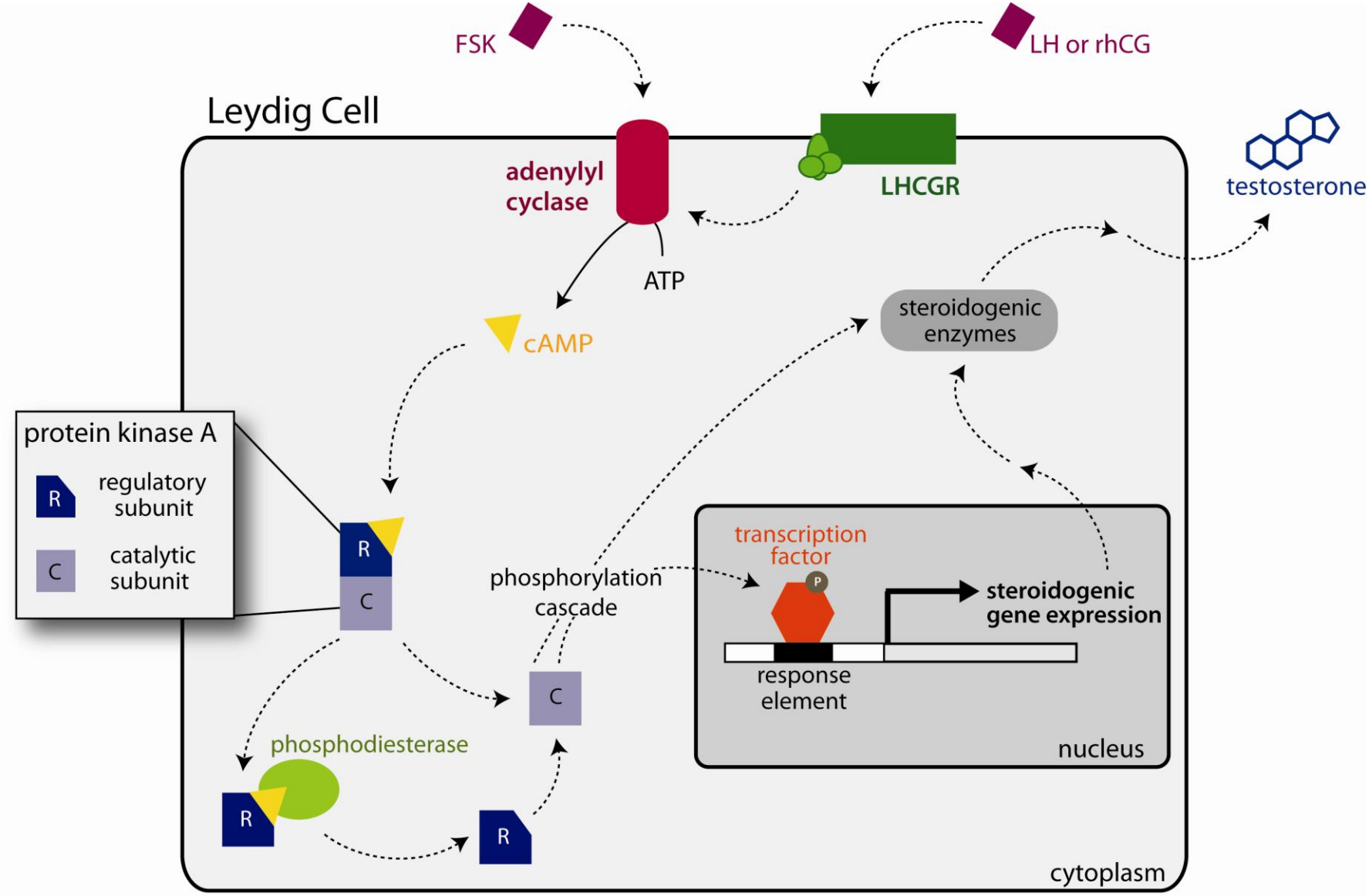

FIGURE 5: STIMULATION OF STEROIDOGENESIS IN LEYDIG CELLS.

FIGURE 5 (cont'd)
Leydig cell steroidogenesis can be stimulated upon activation of the G-protein-coupled LHCGR (green) by LH or rhCG or stimulation of adenylyl cyclase by forskolin (FSK; purple). Subsequent increases in intracellular cAMP (yellow) activate protein kinase A (PKA; blue) resulting in the dissociation of the PKA regulatory subunit (dark blue) from the catalytic subunit (light blue). The released catalytic subunit phosphorylates steroidogenic enzymes (grey) and other factors (ie. transcription factors; orange) eliciting increased expression and activation of steroidogenic enzymes ultimately resulting in the production of testosterone. *For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.*

IN VITRO STEROIDOGENIC MODELS

Current approaches to identify developmental and reproductive toxicants involve examining offspring after administering pregnant animals with maternally toxic doses (*84*). Apical endpoints such as nipple retention, ano-genital distance, vaginal opening and balano-preputial separation are assessed in addition to evaluating reproductive fitness (*85, 86*). Such animal-based testing is expensive, time consuming, generally descriptive, and does not significantly contribute to the elucidation of mechanisms (*87-92*). *In vitro* evaluation of steroidogenesis is important for elucidating mechanisms of potential reproductive and developmental toxicants (*73, 74*).

Steroidogenesis model development has focused on Leydig cells as they are the only cell type to synthesize T and E2 directly from cholesterol (*72*). Ovarian steroidogenesis is a two-cell system with both theca and granulosa cells producing P, but only theca cells express enzymes to metabolize P to T, and subsequent aromatization of T to estrogens or 5α reduction to DHT occurs only in granulosa cells (*93*). Steroidogenic theca cell line development has not been

successful, but there are several rodent models of Leydig cell origin including mouse MA-10 and mLTC-1 cells as well as rat R2C cells. MA-10 and mLTC1 cells, established from transplantable M548OP Leydig tumors carried in C57Bl/6 mice exhibit P induction by rhCG but lack the enzymes required for subsequent metabolism to T (*94-96*). Recent studies report mLTC-1 cells can produce low levels of androgens when stimulated with very high concentrations of rhCG (*97*), however, the high concentration of rhCG required to elicit even minimal T levels limits the physiological relevance of this system. The rat R2C Leydig cell line exhibits high basal steroidogenic activity but lacks functional LHCGR expression (*98*), making R2C cells not a suitable model system to study EDC-mediated effects on gonadotropin-stimulated steroidogenesis since they are not inducible.

In addition to Leydig cells or ovarian theca and granulosa cells, the adrenal gland is the only other organ expressing the cytochrome side chain cleavage enzyme (P450scc) capable of *de novo* steroidogenesis in the mammalian system. The adrenal glands produce mainly glucocorticoids, and subsequently mineralocorticoids, from progestagens (Figure 6). The adrenal cortex, comprised of three different cell types (called zones), normally only produces dehydroepiandrosterone (DHEA) sulfate and androstenedione (precursors of T) and does not express sufficient levels of the 17-beta-hydroxysteroid dehydrogenase necessary for T biosynthesis (*99*). Human H295R cells (ATCC CLR-2128), isolated from a pluripotent adenocortical carcinoma, produce detectable basal cortisol (glucocorticoid), aldosterone (mineralocorticoid), androgens and E2 (*100*). H295R cells are used as the steroidogenesis assay in the U.S. EPA's Endocrine Disruptor Screening Program to screen for effects of chemicals on T and E2 biosynthesis (*101*). However, H295R cells do not express functional ACTH receptors (a

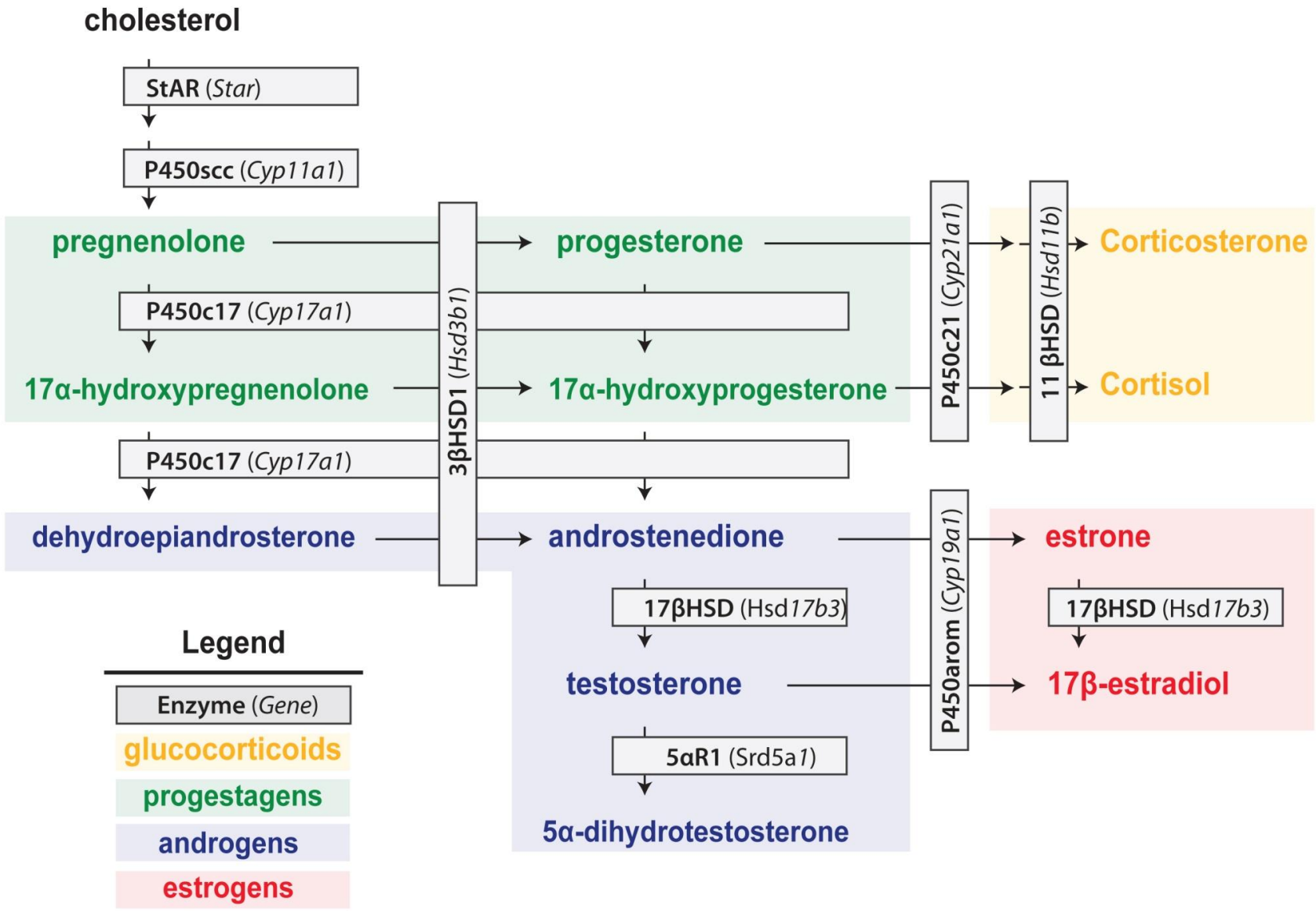

FIGURE 6: OVERVIEW OF *DE NOVO* STEROIDOGENESIS PATHWAYS.

FIGURE 6 (cont'd)
Only steroidogenic cells of the testes (Leydig cells), ovaries (theca and granulose), and adrenal gland (adrenal cortex) express the P450 side chain cleavage (P450scc) enzyme required to cleave cholesterol forming pregnenolone, the commitment step for steroid hormone biosynthesis. Metabolism to various classes of steroid hormone including progestagens (green), glucocorticoids (yellow), androgens (blue), and estrogens (red) is carried out by specific cell types in various tissues.

member of the cell surface 7-transmembrane domain superfamily of G-protein coupled receptors), and therefore steroidogenesis must be stimulated using FSK. Furthermore, the relatively high T and E2 production, in addition to the glucocorticoid and mineralocorticoid production suggest that H295R cells have the characteristics of zonally undifferentiated fetal adrenal cells (*100*).

CONCLUSIONS

Although ATR-mediated endocrine disruption has been widely evaluated demonstrating targeted effects on the HPG axis, most studies are descriptive in nature and the mechanism by which the CMG triazines elicit these effects remains unknown. Furthermore, data suggest that steroidogenesis may specifically be a target of triazines but sufficient models for mechanistically evaluating steroidogenesis are lacking. Collectively, these data highlight the need for mechanistic evaluation of CMG triazines and chlorometabolites in a physiologically relevant steroidogenesis model that is amenable to mechanism determination. Establishing a

Leydig cell model for the evaluation of ATR-mediated disruption of steroidogenesis will provide important insight to help understand the mechanism of triazine-elicited endocrine disruption.

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CHAPTER 2

CHAPTER 2

RATIONALE, HYPOTHESIS AND SPECIFIC AIMS

RATIONALE

Triazines are broad spectrum herbicides implicated in eliciting endocrine disruptive effects including perturbation of male reproductive development function. Current approaches used to evaluate reproductive toxicants involve evaluating apical endpoints that are generally descriptive and do not contribute to the elucidation of mechanism of action. Incidentally, the mechanism of action for triazines remains unknown. However, it has been suggested that disruption of the HPG, specifically steroidogenesis in the gonads, may underlie the effects of triazines across vertebrate species. The objectives of this study are to establish a model for the evaluation of triazines effects on steroidogenesis that is amenable to mechanism determination and to identify a possible mode of action for triazine-elicited disruption of steroidogenesis.

HYPOTHESIS

Triazines alter testosterone biosynthesis in BLTK1 Leydig cells by disrupting cAMP-mediated steroidogenic gene expression.

SPECIFIC AIMS

In order to test the hypothesis, a Leydig cell model is required that will allow the comprehensive examination of triazine-elicited steroidogenic disruption. This model will be amenable to testing the hypothesis with the use of gene expression microarrays and enzyme-linked immunoassays and will include the following aims:

1. Establish and characterize BLTK1 murine Leydig cells as a novel model for the evaluation of steroidogenesis.

2. Characterize the time- and concentration-dependent effects of triazine herbicides and their major chlorometabolites on steroidogenesis in BLTK1 Leydig cells.

3. Determine a mode of action by which Atrazine disrupts steroidogenesis.

CHAPTER 3

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CHAPTER 3

BLTK1 MURINE LEYDIG CELLS: A NOVEL STEROIDOGENIC MODEL FOR EVALUATING THE EFFECTS OF REPRODUCTIVE AND DEVELOPMENTAL TOXICANTS

ABSTRACT

Leydig cells are the primary site of androgen biosynthesis in males. Several environmental toxicants target steroidogenesis resulting in both developmental and reproductive effects including testicular dysgenesis syndrome. The aim of this study was to evaluate the effect of several structurally diverse endocrine disrupting compounds (EDC) on steroidogenesis in a novel BLTK1 murine Leydig cell model. We demonstrate that BLTK1 cells possess a fully functional steroidogenic pathway that produces low basal levels of testosterone (T), and express all the necessary steroidogenic enzymes including *Star, Cyp11a1, Cyp17a1, Hsd3b1, Hsd17b3 and Srd5a1*. Recombinant human chorionic gonadotropin (rhCG) and forskolin (FSK) elicited concentration- and time- dependent induction of cAMP, progesterone (P) and T, as well as the differential expression of *Star, Hsd3b6, Hsd17b3 and Srd5a1* mRNA levels. The evaluation of several structurally diverse male reproductive toxicants including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), atrazine, prochloraz, triclosan, monoethylhexyl phthalate (MEHP), glyphosate and RDX in BLTK1 cells suggest different modes of action perturb steroidogenesis. For example, prochloraz and triclosan anti-fungals reduced rhCG-induction of

T, consistent with published *in vivo* data, but did not alter basal T levels. In contrast, atrazine and MEHP elicited modest induction of basal T but antagonized rhCG-mediated induction of T levels, whereas TCDD, glyphosate and RDX had no effect on basal or rhCG-induction of T in BLTK1 cells. These results suggest that BLTK1 cells maintain rhCG-inducible steroidogenesis and are a viable *in vitro* Leydig cell model to evaluate the effects of EDCs on steroidogenesis. This model can also be used to elucidate the different mechanisms underlying toxicant-mediated disruption of steroidogeneis.

INTRODUCTION

Steroidogenesis is a potential target for drugs, chemicals, natural products and environmental contaminants that adversely impact reproductive development and fertility (*1, 2*). The mechanisms involved in altering steroid levels remain largely unknown but could involve disrupting enzyme expression and/or activity that affects testosterone (T) biosynthesis (*3*). For example, fungicides, pesticides and phthalates elicit reproductive tract abnormalities, compromise reproductive fitness, and cancer as a result of altering steroid levels (*3-6*). Developmental exposure to high doses of endocrine disrupting chemicals (EDCs) has been associated with testicular dysgenesis syndrome (TDS) in humans, comprised of adverse effects including cryptorchidism, hypospadias, low sperm count and testicular cancer, such effects have been characterized in rodents and wildlife species with similar etiology (*7-10*).

Current approaches to identify developmental and reproductive toxicants involve examining offspring after dosing pregnant animals to maternally toxic levels (*11*). Apical endpoints such as nipple retention, ano-genital distance, vaginal opening and balano-preputial

separation are assessed in addition to evaluating reproductive fitness (*12, 13*). To comply with the European Commission's Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) initiative, approximately $2.9B and 41 million animals are required for reproductive toxicity testing alone using current approaches (*14-17*). Such animal-based testing is expensive, time consuming, generally descriptive, poses ethical concerns, and does not significantly contribute to the elucidation of mechanisms (*18-23*). Furthermore, they are contrary to the replacement, reduction and refinement of animal testing. Alternative short-term assays such as the sliced testes assay and the human H295R adenal corticocarcinoma cell-based assay used in Tier 1 of the Endocrine Disruptor Screening Program, still require animals or is based on adrenal steroidogenesis, which is not the primary site of sex hormone production (*24-27*). Therefore, *in vitro* gonadal-based steroidogenesis assays that support ranking and prioritization for further investigation are needed.

The "Toxicity Testing in the 21st Century: a Vision and a Strategy" report proposed testing should be less reliant on whole animal studies with more effort devoted to systems-oriented, computational models developed using *in vitro* and *in vivo* data that could screen chemicals, metabolites and mixtures for potential toxicity (*19*). In response, the U.S. Environmental Protection Agency (USEPA), the National Institute of Environmental Health Sciences National Toxicology Program, and the U.S. Food and Drug Administration have initiated ToxCast, Tox21 and EDSP21 programs in collaboration with the National Institute of Health Chemical Genomics Center, as well as other high-throughput service providers, to use *in silico* and *in vitro* assays to screen chemicals for potential toxicity (*28-30*). These programs use informatic platforms to integrate data from thousands of chemicals that are robotically

screened in diverse assays to develop predictive computational models of toxicity. Similarly, the European Union has established ChemScreen and ReProTect to develop simple, rapid *in vitro/in silico* screening systems for reproductive toxicants (www.chemscreen.eu; www.reprotect.eu) (*31*). Pathways and over-represented gene functions identified in dose-response genomic and high-throughput screening studies may also be used to determine points of departure in provisional peer reviewed toxicity values (PPRTVs; http://hhpprtv.ornl.gov) for chemicals not comprehensively evaluated by the Integrated Risk Information System (IRIS; http://www.epa.gov/iris/index.html) or where there is minimal safety data. Despite this broad spectrum of programs, which includes assays for cell viability, apoptosis, mitochondrial function, receptor-mediated activity and DNA damage in human, mouse and rat cell lines, as well as cell-free systems, none assess steroidogenesis in a high-throughput format (*32*).

In mammals, the induction of steroidogenesis has been thoroughly studied. Luteinizing hormone (LH) secreted from the anterior pituitary induces gonadal steroidogenesis. LH binds G-protein-coupled LH-hCG receptors (LHCGR) on the surface of Leydig cells. Subsequent dissociation of the $G\alpha_s$ subunit stimulates adenylyl cyclase activity producing cAMP that induces gene expression and enzyme activity increasing steroid biosynthesis. BLTK1 cells, isolated from a testicular tumor that developed in a transgenic mouse expressing the mouse inhibin α promoter/simian virus 40 T-antigen fusion gene (*33, 34*), retain functional LHCGR-mediated steroidogenesis. They are stable over multiple passages, easily transfected and exhibit excellent growth characteristics. In this study, recombinant human chorionic gonadotropin (rhCG) and forskolin (FSK) are used as positive controls for the induction of steroidogenesis, as measured by increases in progesterone (P), T and 17β-estraidiol (E2) levels in media. We investigate

murine BLTK1 Leydig cells as a novel model for evaluating the effects of chemicals on steroidogenesis. Our results demonstrate that BLTK1 cells can be used to screen substances that alter intracellular cAMP, steroidogenic gene expression and sex steroid levels.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENT

Mouse Leydig BLTK1 (BLT-1 cells, clone K1) cells were isolated from a testicular tumor that developed in a transgenic mouse expressing the mouse inhibin α promoter/simian virus 40 T-antigen fusion gene (*34*). Cells were maintained in phenol-red free Dulbecco's modified eagle medium nutrient mixture F-12 (DMEM/F-12 media; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Thermo Scientific HyClone, Logan, UT), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen) and incubated under standard conditions (5% CO_2, 37^oC). For the evaluation of steroidogenic enzyme and receptor expression, cells were grown to 80% confluency and harvested without any treatment. For cAMP, progesterone (P), testosterone (T) and estradiol (E2) determination, cells were grown to 80% confluency, transferred into 24-well tissue culture plates (Sarstedt, Newton, NC) and incubated overnight. Cells were treated with DMSO (Sigma, St. Louis, MO) vehicle, or 0.1, 0.3, 1, 3, 10, 30 or 100 ng/ml rhCG (obtained from A.F. Parlow, NIDDK's National Hormone & Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) or 0.1, 0.3, 1, 3, 10, 30 or 100 µM FSK (Sigma) and media was collected at indicated times. Time course studies were conducted with DMSO vehicle, 3 ng/ml rhCG, or 10

μM FSK and media was collected at 1, 2, 4, 8, 12, 24 or 48 hrs. Gene expression studies used the same study design, concentrations and time points with cells seeded into T-25 flasks (Sarstedt).

MTT CYTOTOXICITY ASSAY

BLTK1 cells in 96 well plates (Starstedt) were treated with 1, 3, 10, 30, 100, 300 or 600 ng/ml rhCG (positive control), 1, 3, 10, 30, 100, 300 or 600 μM FSK (positive control) or 1, 3, 10, 30, 100, 300 or 600 μM of test compound in triplicate, respectively. Media was aspirated after 24 hrs and replaced with 50 μL of fresh MTT reagent (5 mg/mL thiazolyl blue tetrazolium bromide (Sigma) in phosphate buffered saline). Following a 3 hr incubation, MTT reagent was aspirated and replaced with 150 μL DMSO. Cells were incubated for 2 hrs followed by absorbance measurements at 595 and 650 nm (A595-A650) using an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA). Results are reported as percentage of control calculated from the relative absorbance of treated versus DMSO controls where 100% indicates no cytotoxicity.

WESTERN BLOTTING

Lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton and protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN)). Protein concentration was determined spectrophotometrically by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Extracts (50 μg) were resolved on a 10% sodium dodecyl sulfate/polyacrylamide gel and transferred to Immobilon membrane (Millipore, Billerica, MA). Membranes were incubated with 1 μg/ml primary antibody [rabbit anti-StAR (Santa Cruz Biotechnology, Santa Cruz, CA; Catalog No. 25806), rabbit anti-Cyp11a1 (Millipore; Catalog No. AB1244), goat anti-

Cyp17a1 (Santa Cruz; Catalog No. SC-46081), goat anti-3β-HSD (Santa Cruz; Catalog No. SC-30820), goat anti-Srd5a1 (Santa Cruz; Catalog No. SC-20399) and rabbit anti-Cyp19a1 (Abcam, Cambridge, MA; Catalog No. ab51924)] at 4^{o}C overnight. Membranes were washed and incubated with donkey anti-rabbit or donkey anti-goat IgG conjugated with horseradish peroxidase (HRP; Santa Cruz; Catalog No. SC-2077 and SC-2056, respectively) for 1 hr. Enhanced Chemiluminescence (Thermo Scientific Pierce, Rockford, IL) was used to visualize detected protein.

ENZYME IMMUNOASSAYS

Enzyme immunoassay (EIA) kits for T (Cayman Chemical Company, Ann Arbor, MI; limit of detection 6 pg/ml, cross-reactivity 27% for 5α-dihydrotestosterone and 19% for 5β-dihydrotestosterone), E2 (Cayman Chemical Company; limit of detection 20 pg/ml, cross-reactivity 14% for estradiol-3-glucuronide, 12% for estrone and 10% for estradiol 17-glucuronide), and P (ALPCO Diagnostics, Salem, NH) were used according to the manufacturer's instructions. Intracellular cAMP was measured by EIA (Cayman Chemical Company; limit of detection 3 pmol/ml cAMP, cross-reactivity 1.5% for cGMP) following hydrochloric acid extraction. All kits consisted of 96-well pre-coated antibody plates, for which samples compete for binding with conjugated hormone. Following incubation, plates were washed and measured at 420 nm for cAMP, T and E2, or 405 nm for P, using an Emax precision microplate reader (Molecular Devices). Standards (0.3 – 750 pmol/ml cAMP, 0 - 60 ng/ml P, 3.9 - 500 pg/ml T or 6.6 – 4,000 pg/ml E2) were used to generate a standard curve for quantification.

RNA ISOLATION AND GENE EXPRESSION

Total RNA was extracted from cell pellets using RNeasy Mini Kits (Qiagen, Valencia, CA) according to the manufacturer's protocol with an additional RNase-free DNase (Qiagen) digestion. RNA was quantified at 260 nm (A260) and purity assessed using the A260/A280 ratio, as well as by denaturing gel electrophoresis. First-strand cDNA was synthesized from RNA (1 μg) using SuperScript II reverse transcriptase (Invitrogen) and anchored oligo-dT primer (Invitrogen) as described by the manufacturer. For RT-PCR evaluation of steroidogenic enzyme and receptor expression, the cDNA was used as template for PCR amplification with gene specific primers (Table 1). Quantitative real-time PCR (QRTPCR) was used to quantify concentration- and time-dependent expression of specific genes. Reactions in 96-well plates consisted of 30 μl, including 1 μl of cDNA template, 0.1 μM forward and reverse gene-specific primers, 3 mM $MgCl_2$, 1.0 mM dNTPs, 0.025 IU AmpliTaq Gold, and 1 X SYBR Green PCR Buffer (Applied Biosystems, Foster City, CA) using an Applied Biosystems PRISM 7500 Sequence Detection System. Dissociation curve analysis assured single product amplification. To control for differences in RNA loading, quality and cDNA synthesis, samples were standardized to the geometric mean of three housekeeping genes: *Actb*, *Gapdh* and *Hprt* (*35*). Results were quantified using a standard curve generated on the same 96-well plate and amplified by using purified cDNA product as template specific for each gene (serial 10X dilutions from 108 to 101 copies). The slope of the standard curve was used to assess amplification efficiency as described by the manufacturer with all amplification efficiencies >90%. Fold changes were calculated relative to time-matched vehicle. Relative expression was scaled such that time-matched vehicle control expression equaled one for graphing purposes.

TABLE 1. PRIMER SEQUENCES FOR QRTPCR

	Gene Symbol	RefSeq	Forward Primer	Reverse Primer	Product Size
Receptors	*Ahr*	NM_013464	ACCAGAACTGTGAGGGTTGG	TCTGAGGTGCCTGAACTCCT	155
	Ar	NM_013476	AAGCAGGTAGCTCTGGGACA	GAGCCAGCGGAAAGTTGTAG	117
	Era	NM_007956	AGGGAAATCTTGAGCCCCTA	AGCAAAATTAGCTGCCCTGA	1733
	Lhcgr	NM_013582	GACCAAAAGCTGAGGCTGAGA	CAATGTGGCCATCAGGGTAGA	78
	Pgr	NM_008829	ACGTCTTAGCCCCAGGTTTT	CTGGAAGTCGCCGTAAAGAG	1604
	Ppara	NM_011144	GTCCTCAGTGCTTCCAGAGG	GGTCACCTACGAGTGGCATT	107
	Pparg	NM_011146	TTTTCAAGGGTGCCAGTTTC	AATCCTTGGCCCTCTGAGAT	198
	Nr3c1 (Gr)	NM_008173	ACCTGCTGGGTAAACTGTGG	TTGCTATCGCACCACTGAAG	112
	Nr5a1 (sf-1)	NM_139051	TACCCAGGGAGGAGGAGTCT	TGTCTCCTGCCCACTTCTCT	166
	Nr5a2(Lrh-1)	NM_030676	GCATGCCAAAAGAGCCTAAG	AAGAGGGTTACTGCCCGTTT	730
Steroidogenic Enzymes	*Cyp11a1*	NM_019779	CCCATCCTCTTCAACTTCCA	ACCTGGGCAGGTAATCACAG	172
	Cyp17a1	NM_007809	GATCGGTTTATGCCTGAGCG	CTCCGAAGGGCAAATAACTG	82
	Cyp19a1	NM_007810	GACAGGCACCTTGTGGAAAT	CGGATAAGTAATGCCCCAGA	102
	Hsd3b1	NM_008293	GGTGCAGGAGAAAGAACTGC	ACAGCAGCAGTGTGGATGAC	186
	Hsd3b6	NM_013821	TTTTTTTGAGGTATTGACAAGTATTTATTG	TCCCCATTCAGAGCATGTATAGC	89
	Hsd17b3	NM_008291	GAGTTGGCCAGACATGGACT	AGCTTCCAGTGGTCCTCTCA	97
	Star	NM_011485	CTCGGTGCTTTAAGGTGAGC	GATTCCCGGGATGGTTCTAT	64
	Srd5a1	NM_175283	ACCTGGTGTGTCCTGAAAGG	CCTGGGTTCCAACTTTAGCA	115
Housekeeping Genes	*Hprt*	NM_013556	AAGCCTAAGATGAGCGCAAG	TTACTAGGCAGATGGCCACA	104
	Gapdh	NM_008084	GTGGACCTCATGGCCTACAT	TGTGAGGGAGATGCTCAGTG	125
	Actb	NM_007393	GCTACAGCTTCACCACCACA	TCTCCAGGGAGGAAGAGGAT	123

DOSE-RESPONSE MODELING AND STATISTICAL ANALYSES.

ToxResponse uses particle swarm optimization to identify the best-fit across five model classes: sigmoidal, exponential, linear, quadratic and Gaussian (*36*). The best fitting model was then used to calculate EC50 values. All statistical analyses were carried out using SAS v9.1 (SAS Institute, Cary, NC) by analysis of variance (ANOVA), with Dunnett's or Tukey's post hoc tests for concentration-response and time course data, respectively. Differences between treatment groups were considered significant when $p < 0.05$ relative to time-matched DMSO control.

RESULTS

STEROIDOGENIC ENYZME EXPRESSION IN BLTK1 CELLS

Steroidogenesis involves the conversion of cholesterol to progestagens (pregnenolone, progesterone (P), 17α-hydroxypregnenolone and 17α-hydroxyprogesterone), followed by further metabolism to androgens (dehydroepiandrosterone, androstenedione, testosterone (T) and 5α-dihydrotestoeterone) and estrogens including estrone and 17β-estradiol (Figure 7). Steroidogenic enzyme mRNA and protein were detected in BLTK1 cells by RT-PCR and/or western blotting with all required steroidogenic enzymes expressed (Figure 8). In addition, mRNA for several potential regulatory factors including luteinizing hormone G-coupled receptor (LHGCR, data not shown), estrogen receptor (ER), androgen receptor (AR) and steroidogenic factor 1 (SF-1), peroxisome proliferator-activated receptors (PPARα and PPARγ), the pregnane X

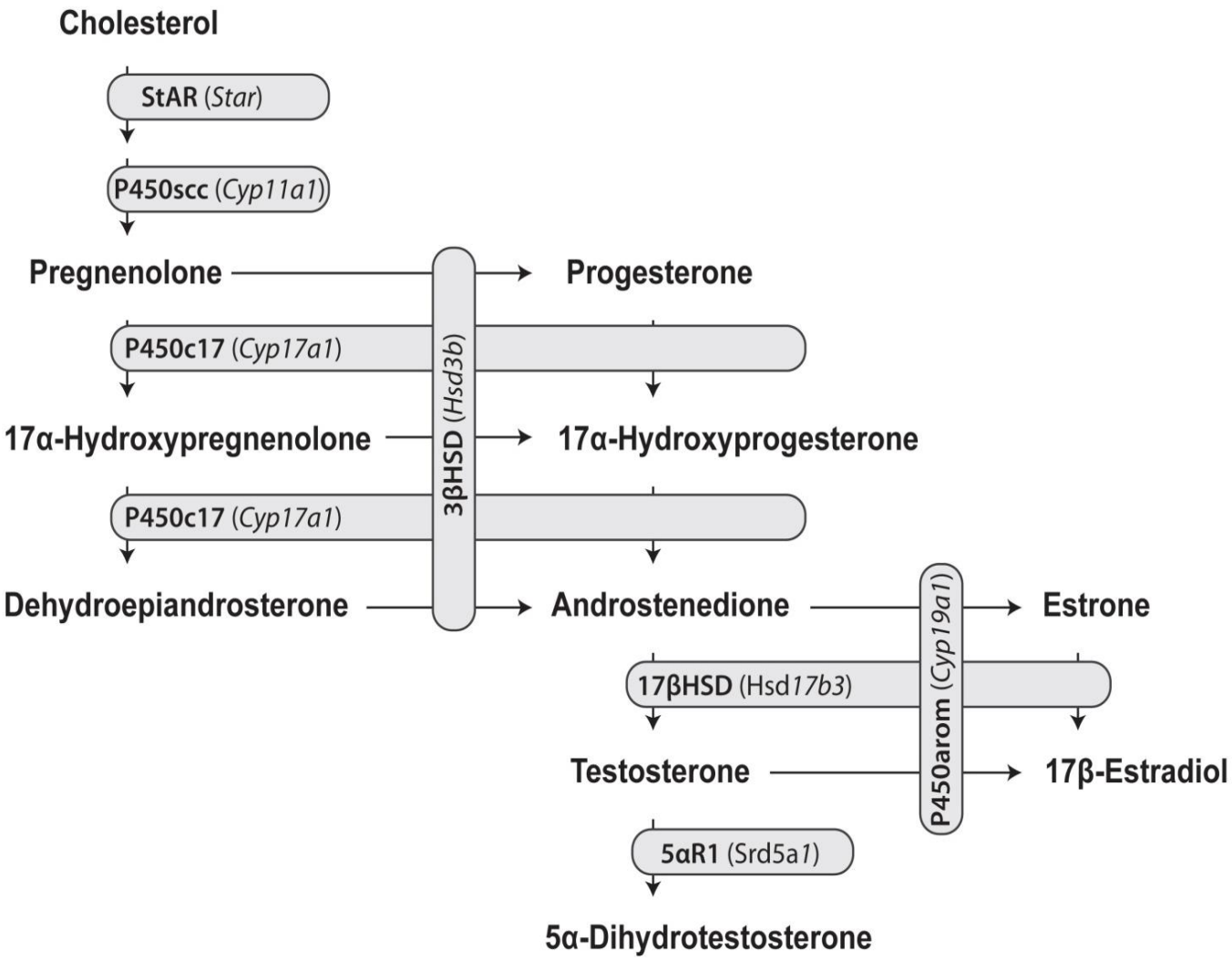

FIGURE 7. THE STEROIDOGENIC PATHWAY

Steroid hormones are biosynthesized from cholesterol. Cholesterol is mobilized and delivered to the inner mitochondrial membrane by StAR, where it is cleaved by P450scc to produce pregnenolone. Subsequent enzymatic events in the smooth endoplasmic reticulum include metabolism by P450c17 and 3βHSD to produce progestagens (i.e., progesterone (P), 17α-hydroxypregnenolone and 17α-dhydroxyprogesterone). Androgens (i.e., dehydroepiandrosterone and androstenedione) are produced by P450c17, then converted to testosterone (T) by 17βHSD and finally reduced to 5α-dihydrotestosterone (DHT) by 5αR1. Androgen metabolism by P450arom also yields estrogens (i.e., estrone and 17β-estradiol (E2)).

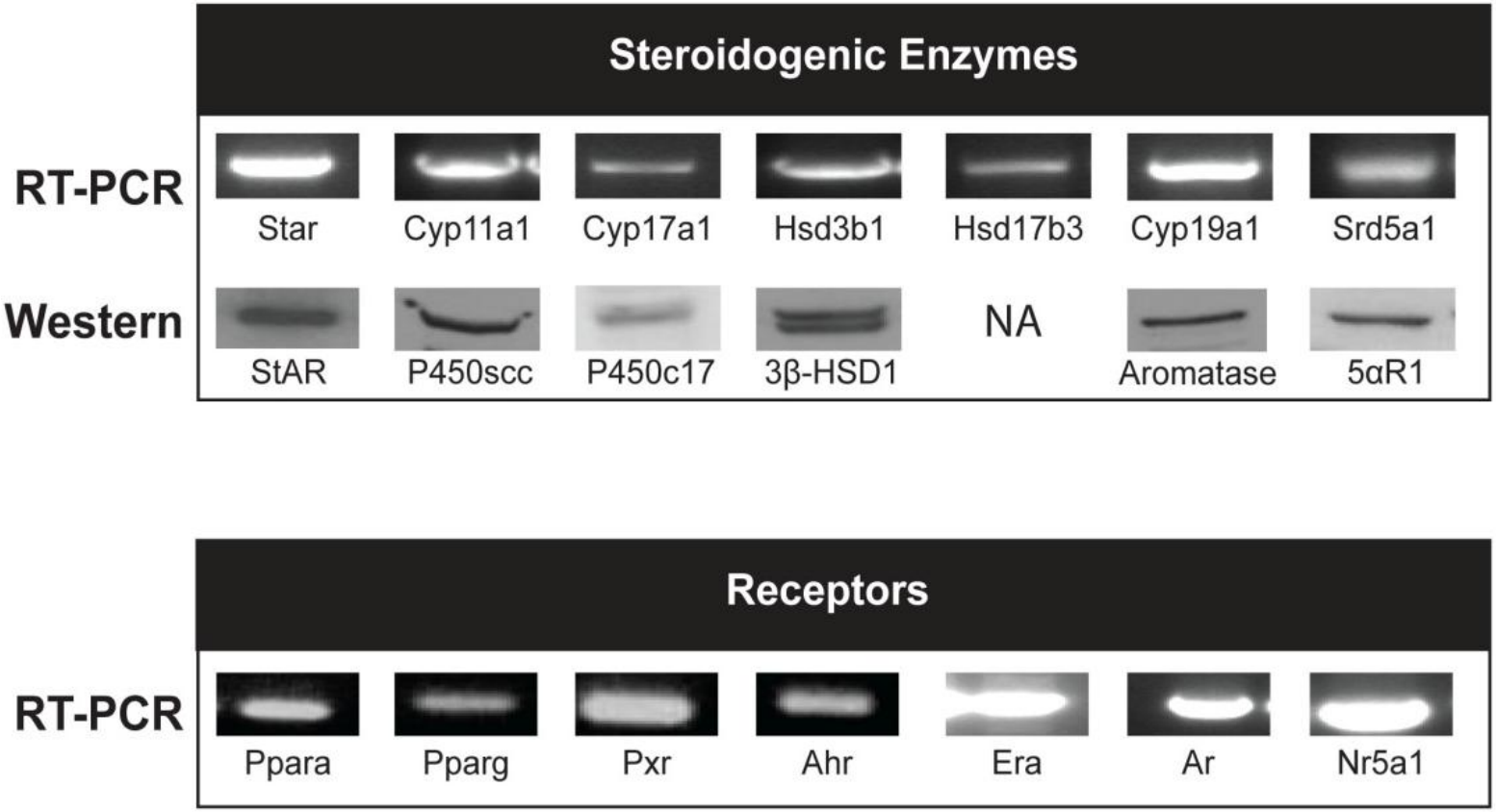

FIGURE 8. STEROIDOGENIC ENZYME AND RECEPTOR EXPRESSION IN BLTK1 CELLS

Expression of steroidogenic enzymes and receptors in untreated BLTK1 cells was evaluated by RT-PCR and western blotting. mRNA and/or protein expression was detected for all steroidogenic enzymes. PPARα, PPARγ, PXR, AhR, ERα, AR and SF-1 expression was also confirmed. NA: antibody not available

receptor (PXR), and the aryl hydrocarbon receptor (AhR) were also detected. However, mRNA for progesterone receptor (PR), glucocorticoid receptor (GR) or the liver receptor homologue 1 (LRH-1) was not detected in BLTK1 cells despite verification of RT-PCR primer specificity and functionality in mouse Hepa1c1c7 cells (data not shown).

INDUCTION OF STEROIDOGENESIS BY FSK AND rhCG

Temporal profiles of intracellular cAMP as well as P and T levels in media were evaluated in response to 3 ng/ml rhCG or 10 μM FSK by EIA (Figure 9). Intracellular cAMP was induced by FSK at 30 min (~120 pmol/ml, ~10-fold) and by 1 hr in response to rhCG (635 pmol/ml, 60-fold). However, levels quickly diminished such that no intracellular cAMP was detected by 8 hrs. Maximum P levels (200 ng/ml, 8-fold) were observed at 2 hrs in response to rhCG and FSK, followed by a steady decline due to metabolism to androgens and estrogens. In contrast, T levels gradually increased reaching a maximum of ~200 pg/ml (7-fold) at 48 hrs, with significant increases detected as early as 1 hr post-treatment.

Concentration-dependent induction of intracellular cAMP and secreted P and T was evaluated at 4 hrs when cAMP can still be detected (Figure 10). 17β-Estradiol (E2) was evaluated at 48 hr (Figure 10), as it was not consistently detected at 4 hrs (data not shown). cAMP, P and T were induced 25-, 10- and 4-fold, respectively, at 4 hrs, while E2 was induced ~4-fold by 48 hr. The EC_{50} for cAMP induction was >24 ng/ml for rhCG and >29 μM for FSK. Meanwhile, EC_{50} values of 1 ng/ml rhCG and 9 μM FSK were conserved for both P and T induction, while E2 EC_{50}s were 10 ng/ml for rhCG and 9 μM for FSK.

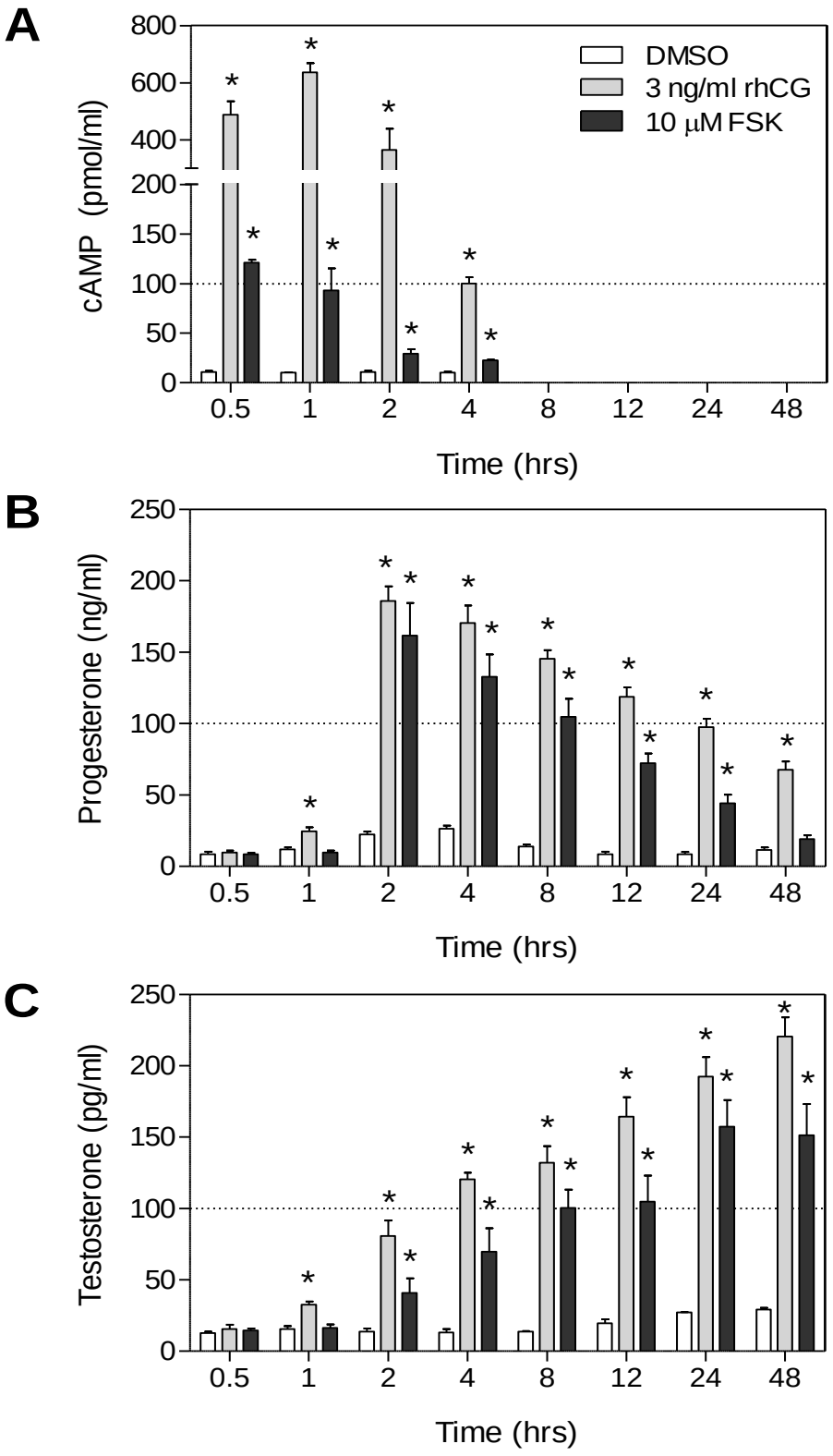

FIGURE 9. TEMPORAL rhCG- AND FSK-INDUCED cAMP, P AND T LEVELS.

FIGURE 9 (cont'd)
Intracellular cAMP (A) as well as P (B) and T (C) levels in media were evaluated in response to 3 ng/ml rhCG (light bars) or 10 μM FSK (dark bars) relative to DMSO vehicle controls (white bars). Both treatments elicited similar temporal profiles consisting of maximal cAMP levels reached early at 0.5-1 hr followed by decrease and was undetectable by 8 hr, while P reached maximum induction at 2 hrs followed by a gradual decrease over time. In contrast, T levels steadily increased throughout the time course. Data represent mean ± standard error of 3 replicates, **p* < 0.05 vs. DMSO controls.

Intracellular cAMP levels are not only regulated by synthesis but also degradation, which is regulated by cyclic nucleotide phosphodiesterase enzymes (PDEs) (*37*). The PDE inhibitor IBMX maximizes cAMP levels in order to further induce steroidogenesis. However, IBMX co-treatment with rhCG or FSK did not increase T levels further, albeit rhCG and FSK potencies were greater (Figure 11; T EC_{50} with and without IBMX by FSK: 0.1 μM vs. 9.4 μM and rhCG: 0.1 ng/ml vs. 0.9 ng/ml, respectively).

rhCG- AND FSK-ELICITED STEROIDOGENIC GENE EXPRESSION

The effect of rhCG and FSK on the gene expression of steroidogenic enzymes including steroidogenic acute regulatory protein (*Star*) were evaluated by QRTPCR at 24 hrs. rhCG elicited concentration-dependent induction of *Star*, *Hsd3b6*, *Hsd17b3* and *Srd5a1* mRNA levels (Figures 12-14). In contrast, only *Star* and *Cyp17a1* mRNA exhibited concentration-dependent induction by FSK at 24 hrs. *Star*, carrying out the rate-limiting step in steroidogenesis, was induced ~20-fold by rhCG and ~10-fold by FSK. Temporal gene expression (Figures 12-14) also revealed differences in the induction kinetics of *Star*, *Hsd3b6* and *Cyp19a1* by rhCG and FSK. Most notably were differences in the induced levels of *Hsd17b3* and *Srd5a1*. These differences likely

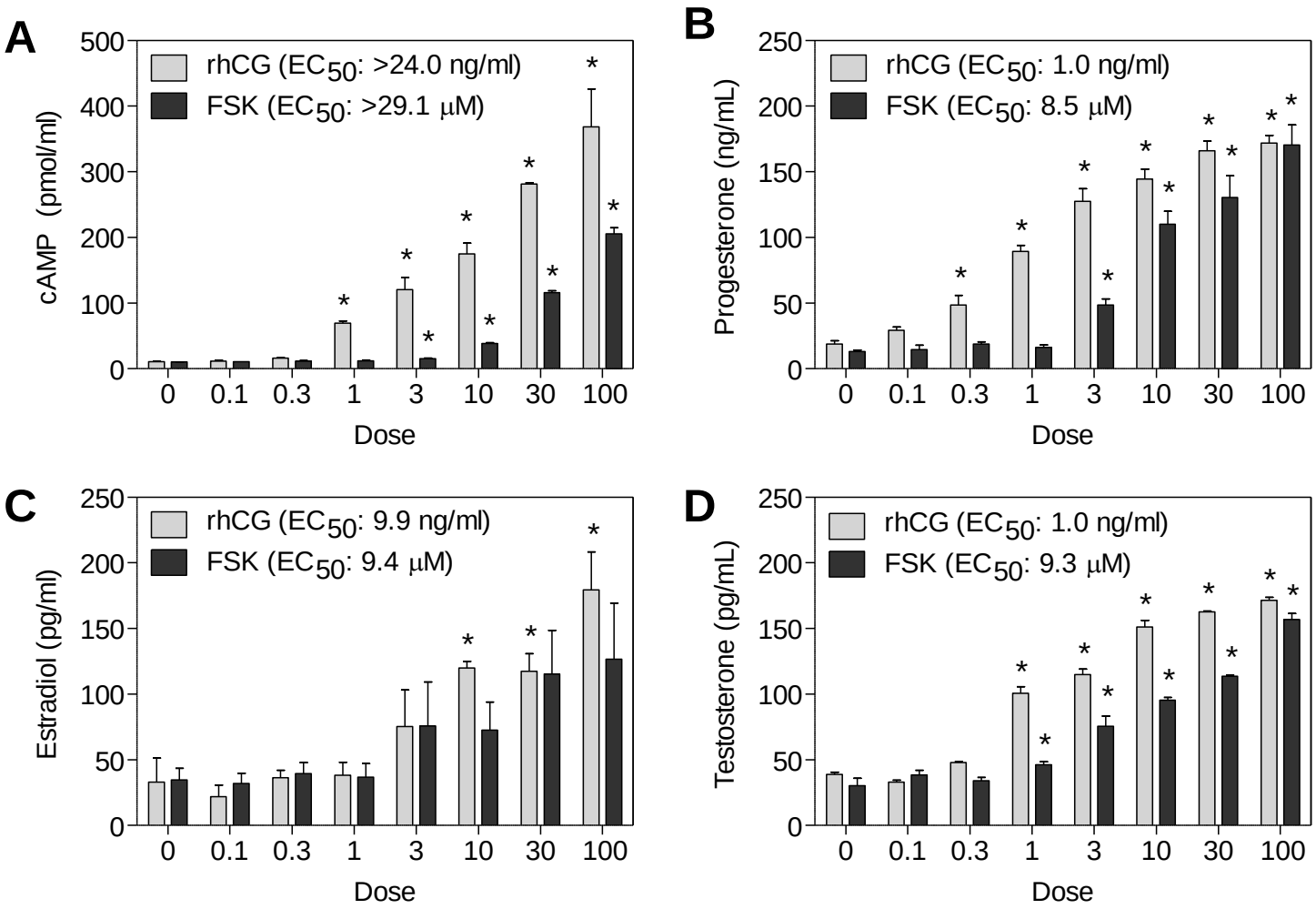

FIGURE 10. CONCENTRATION-DEPENDENT INDUCTION OF cAMP, P, T AND E2 BY rhCG AND FSK

Intracellular cAMP concentrations (A) and secreted P (B) and T (C) levels in media induced by rhCG (ng/ml; light bars) and FSK (μM; dark bars) at 4 hrs. E2 (D) levels in media were measured 48 hrs after treatment. EC_{50} values were calculated by the ToxResponse modeler. Data represent mean ± standard error of 3 replicates, *$p < 0.05$ vs. DMSO controls.

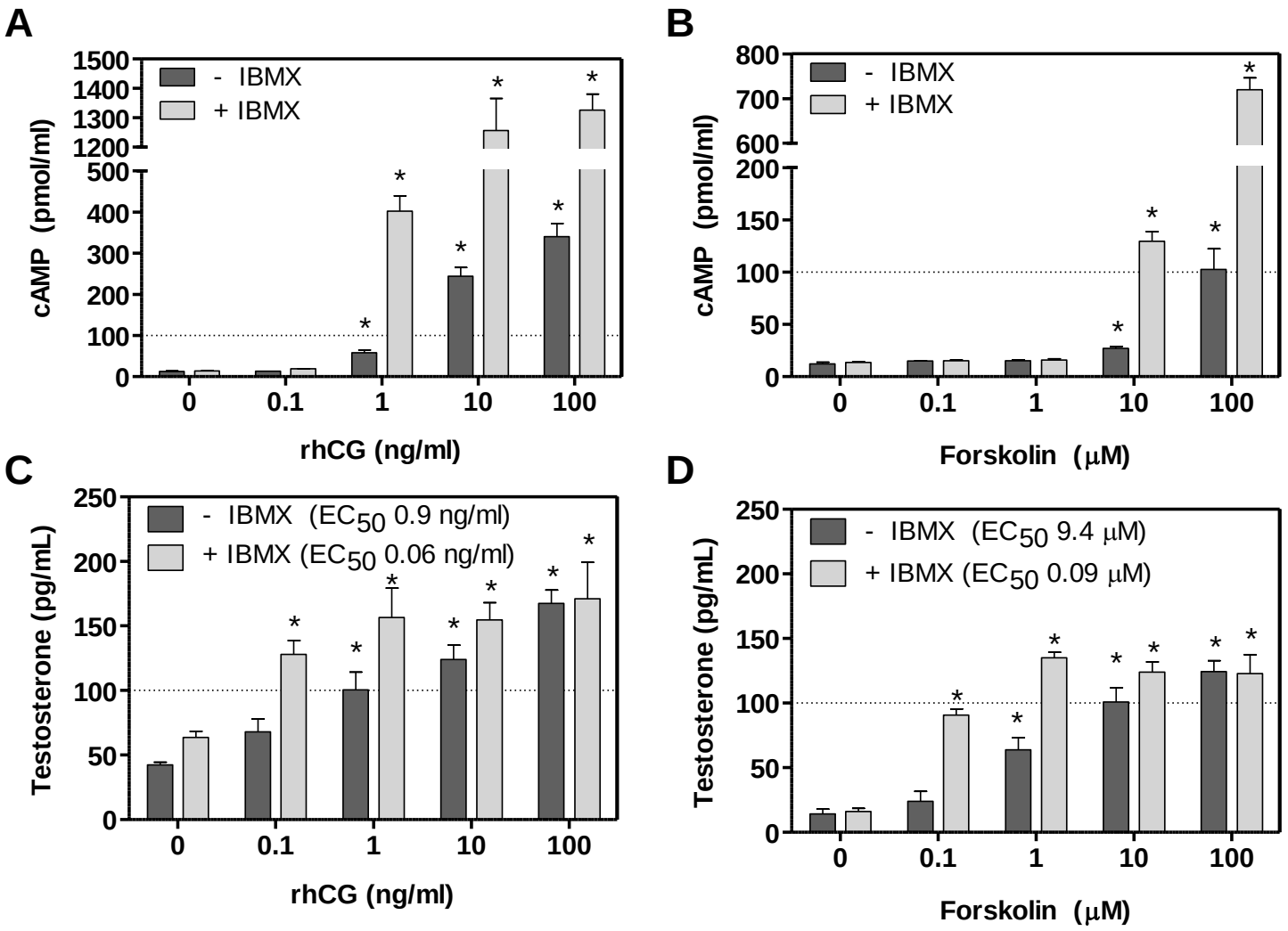

FIGURE 11. EFFECT OF THE PDE INHIBITOR IBMX ON rhCG- AND FSK-ELICITED INDUCTION OF T

Concentration-dependent induction of cAMP (**A**, **B**) and T (**C**, **D**) by rhCG (0.1 – 100 ng/ml) or FSK (0.1 – 100 μM) alone (dark bars) or in the presence of 0.2 mM IBMX (light bars) for 4 hrs. IBMX did not affect T induction, cAMP levels were increased, and the potency of rhCG and FSK were enhanced. Data represent mean ± standard error of 3 independent replicates, *$p < 0.05$ vs. DMSO controls.

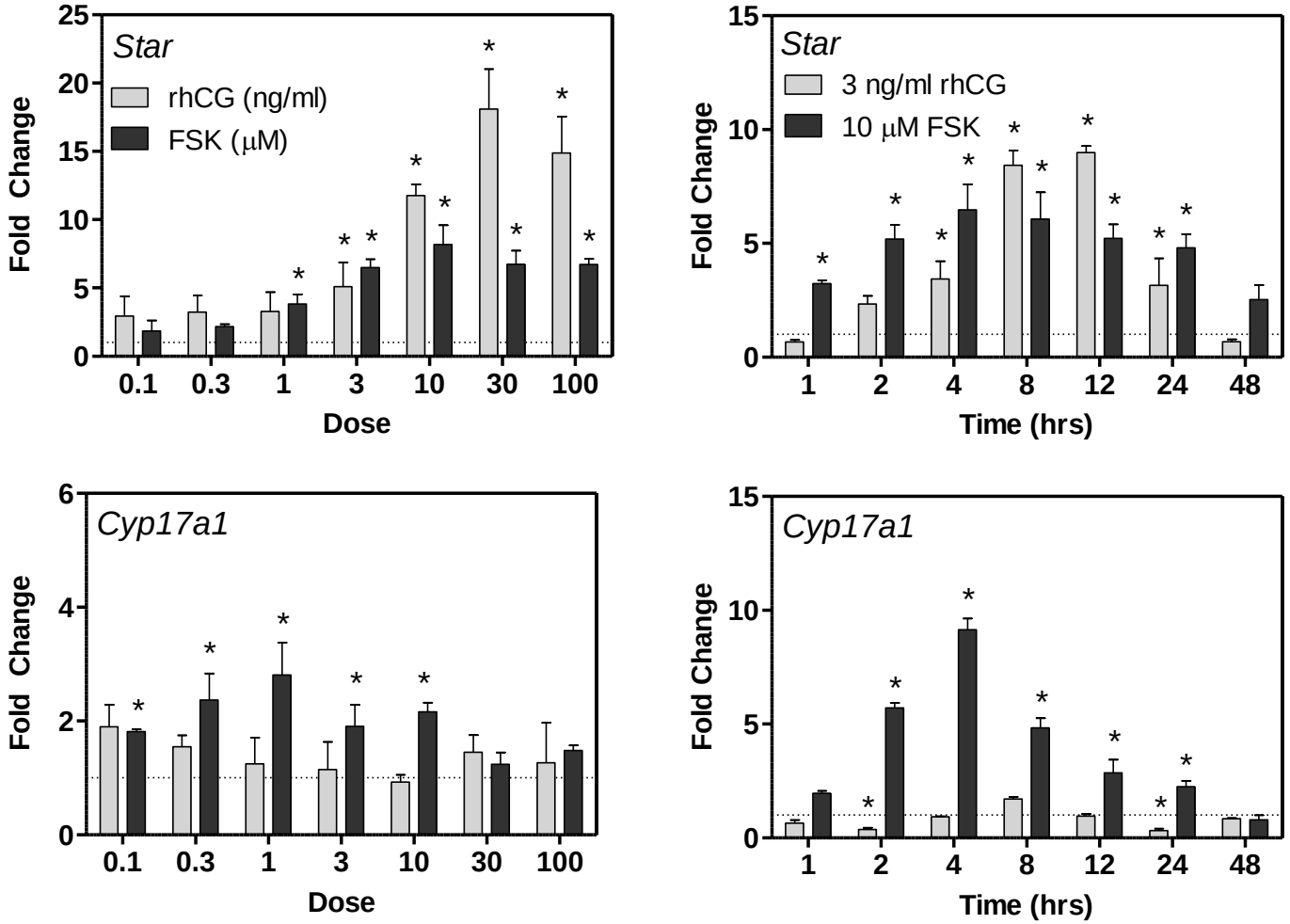

FIGURE 12. DIFFERENTIAL GENE EXPRESSION OF PROGESTAGEN BIOSYNTHETIC ENZYMES

The concentration-dependent (24 hrs, left column) and temporal (right column) effects of rhCG (ng/ml, light bars) and FSK (μM, dark bars) on *Star* and *Cyp17a1* mRNA levels examined using QRTPCR. Cyp11a1 mRNA expression was not affected by treatment (data not shown). Data represent mean ± standard error of 3 replicates, *$p < 0.05$ vs. DMSO controls.

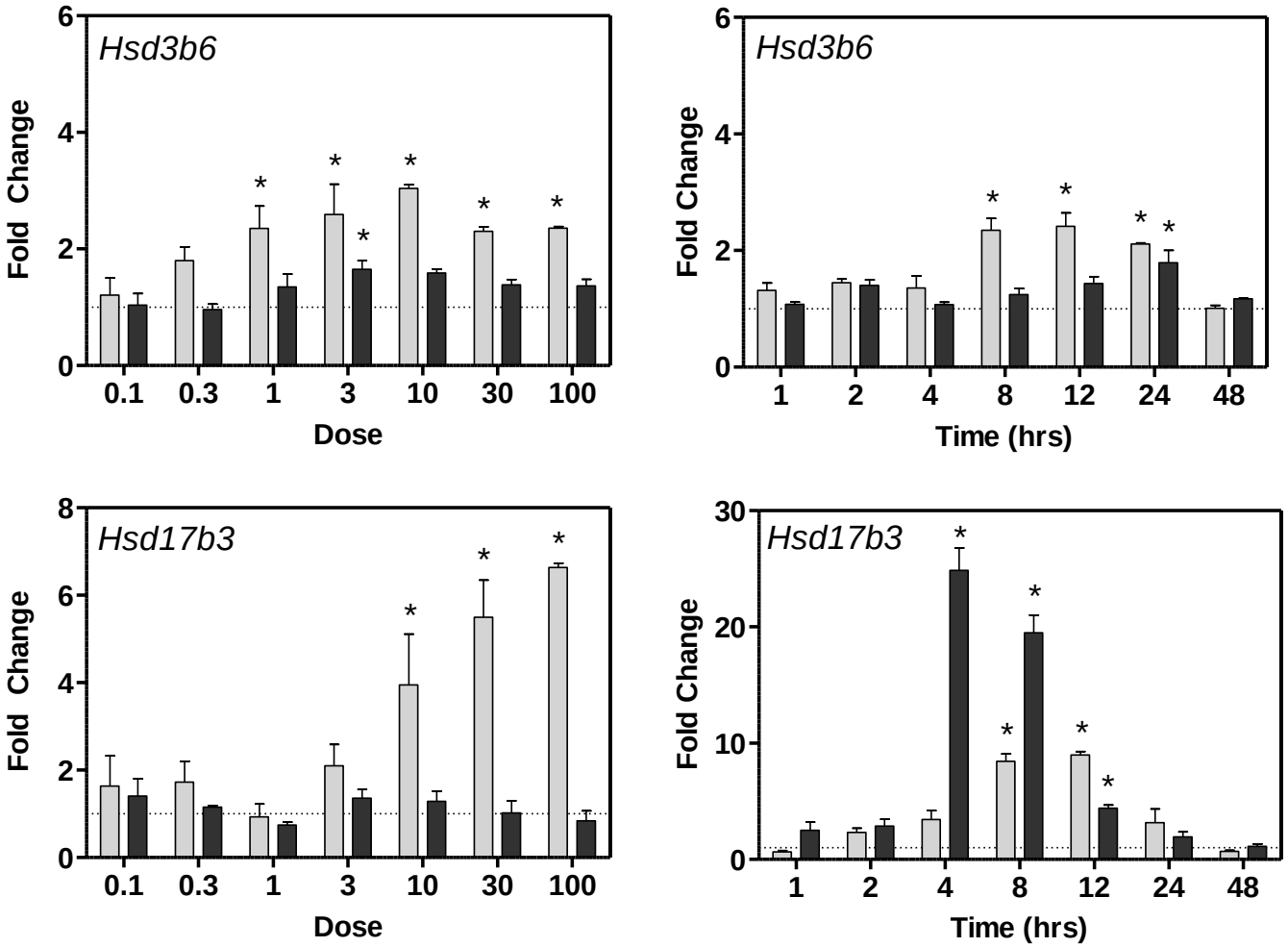

FIGURE 13. DIFFERENTIAL GENE EXPRESSION OF ANDROGEN PRODUCING ENZYMES

The concentration-dependent (24 hrs, left column) and temporal (right column) effects of rhCG (ng/ml, light bars) and FSK (µM, dark bars) on *Hsd3b6* and *Hsd17b3* mRNA levels examined using QRTPCR. Data represent mean ± standard error of 3 replicates, *$p < 0.05$ vs. DMSO controls.

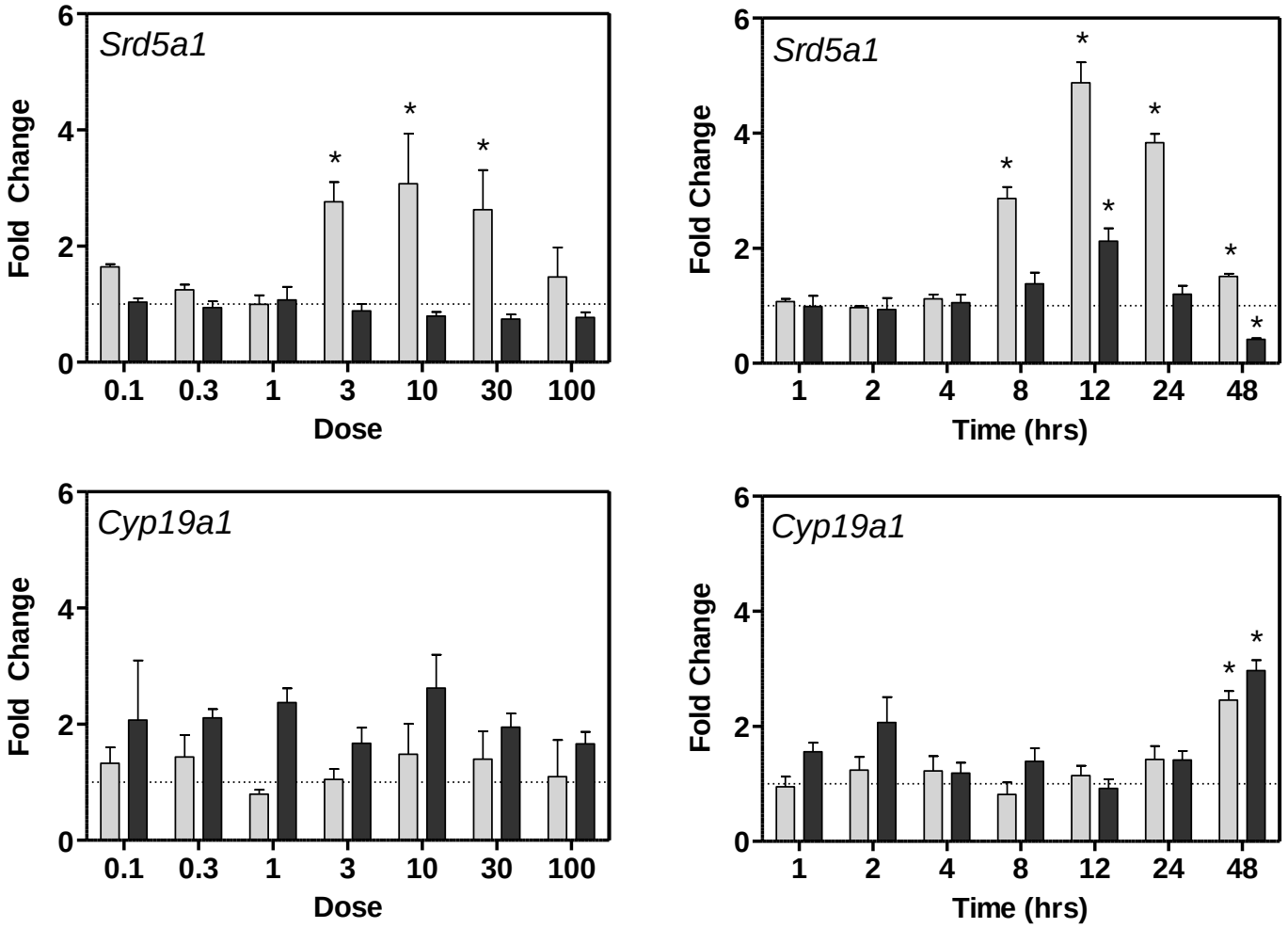

FIGURE 14. DIFFERENTIAL GENE EXPRESSION OF T METABOLIZING ENZYMES

The concentration-dependent (24 hrs, left column) and temporal (right column) effects of rhCG (ng/ml, light bars) and FSK (µM, dark bars) on *Srd5a1* and *Cyp19a1* mRNA levels examined using QRTPCR. Data represent mean ± standard error of 3 replicates, *$p < 0.05$ vs. DMSO controls.

result from different pathway activation. FSK directly induces cAMP levels by activating adenylyl cyclase whereas rhCG induction via the LHCGR activates adenylyl cyclase and can also activate alternate signal transduction pathways such as the release of arachidonic acid and its metabolites (*38, 39*). Furthermore, *Cyp17a1* induction was only seen with FSK, while rhCG inhibited expression at 2 and 24 hrs. There were negligible effects on *Cyp11a1* and *Hsd3b1* in both concentration-response and temporal evaluation (data not shown). More detailed studies are warranted to elucidate the mechanisms responsible for the differential effects of rhCG and FSK on steroidogenic gene expression.

EFFECTS OF EDCs ON TESTOSTERONE LEVELS

The effect of selected EDCs reported to elicit reproductive toxicity (Table 2) was assessed in BLKT1 cells by evaluating T levels. They include the triazine herbicide Atrazine (*40, 41*), the broad spectrum herbicidal active ingredient glyphosate used in commercially available Round-up (*42, 43*), the conazole anti-fungal Prochloraz (*44, 45*), the anti-fungal/anti-bacterial Triclosan (*46, 47*), and the diethylhexyl phthalate (DEHP) metabolite monoethylhexyl phthalate (MEHP) (*48-50*). None of these chemicals elicited significant cytotoxicity in the concentration range examined (Figure 15). Compounds were evaluated for concentration-dependent effect on basal T levels and at a single co-treatment concentration to investigate potential interactions with rhCG-mediated stimulation of T levels (Figure 16).

T levels were induced 2-fold (60 pg/ml) and 10-fold (250 pg/ml) by Atrazine and MEHP, respectively. No other examined chemical altered basal T levels. However, steroidogenesis in Leydig cells is stimulated by LH via the LHCGR during late gestation first measurable at gestational day (GD) 15 and peaking at GD 18 in the mouse (*51*), and throughout gestation in

TABLE 2. REPORTED EVALUATIONS OF EDCs THAT ALTER T LEVELS

Common Name	Chemical Name	CAS #	Molecular Weight (g/mol)	Reproductive Toxicity (References)
Atrazine	2-chloro-4-ethylamino-6-isopropylamino-s-triazine	1912-24-9	215.68	decreased serum T, dysregulation of steroidogenesis (*40, 41*)
Triclosan	2,4,4'-trichloro-2'-hydroxydiphenyl ether	3380-34-5	289.54	anti-androgenic, decreased T production by Leydig cells, decreased serum T levels (*46, 47*)
Prochloraz	N-propyl-N-(2,4,6-trichlorophenoxy)ethyl-imidazole-1-carboxamide	67747-09-5	376.67	decreased serum T levels, AR antagonism, altered androgen-dependent gene expression (*44, 45*)
Dioxin (TCDD)	2,3,7,8-tetrachlorodibenzo-*p*-dioxin	1746-01-6	321.97	decreased intratesticular T, inhibition of steroidogenic gene expression (*52-54*)
Glyphosate	N-(phosphonomethyl) glycine	1071-83-6	169.07	inhibition of steroidogenic enzymes expression, decreased T production (*42, 43*)
Monoethylhexyl Phthalate (MEHP)	2-(2-ethylhexoxycarbonyl) benzoic acid	4376-20-9	278.35	decreased serum T levels, Leydig cell hyperplasia (*49, 50*)
RDX	1,3,5-trinitroperhydro-1,3,5-triazine	121-82-4	222.12	altered reproductive capacity (*55-57*)

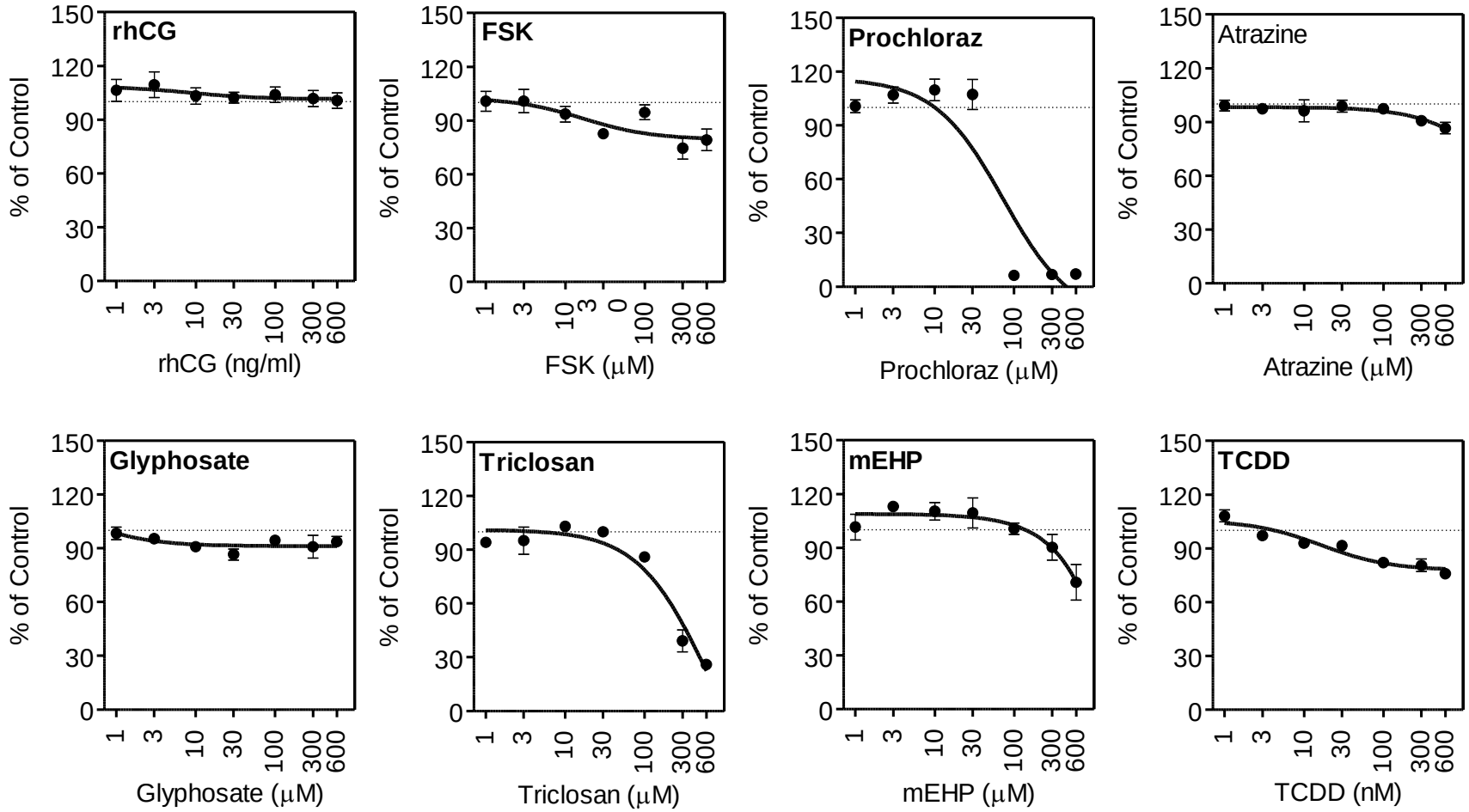

FIGURE 15. MTT EVALUATION OF EDC CYTOTOXICITY

FIGURE 15 (cont'd)
BLTK1 cell viability following 24 hr treatment with rhCG (A), FSK (B), prochloraz (C), ATR (D), glyphosate (E), triclosan (F), MEHP (G) and TCDD (H) using MTT assay. Cells exhibited >90% viability at concentrations used for subsequent effects on steroidogenesis. Data represent mean ± standard error of 3 independent replicates.

humans, with a similar programming window for androgen-dependent masculinization extrapolated between humans and rodents (*58*)). LH stimulation is also crucial during puberty and adulthood to stimulate T biosynthesis to support Sertoli cell spermatogenesis. Consequently, co-treatment studies with 3 ng/ml rhCG were conducted to mimic Leydig cell stimulation. Atrazine (300 µM) inhibited rhCG-induced T levels ~30% (rhCG alone ~100 pg/ml T vs. Atrazine + rhCG ~70 pg/ml T). Similarly, 100 µM MEHP inhibited rhCG-elicited T induction ~20% (rhCG alone ~100 pg/ml vs. MEHP co-treatment ~80 pg/ml). In contrast, Prochloraz did not significantly alter basal T levels, but co-treatment decreased rhCG induced T to control levels (rhCG ~100 pg/ml vs. prochloraz + rhCG ~50 pg/ml vs. DMSO ~40 pg/ml). Similarly, 30 µM Triclosan had no effect, yet co-treatment decreased rhCG-elicited T induction ~25% (rhCG alone ~100 pg/ml vs. triclosan + rhCG co-treatment ~75 pg/ml). Glyphosate, TCDD and RDX did not induce, or alter rhCG induction of T.

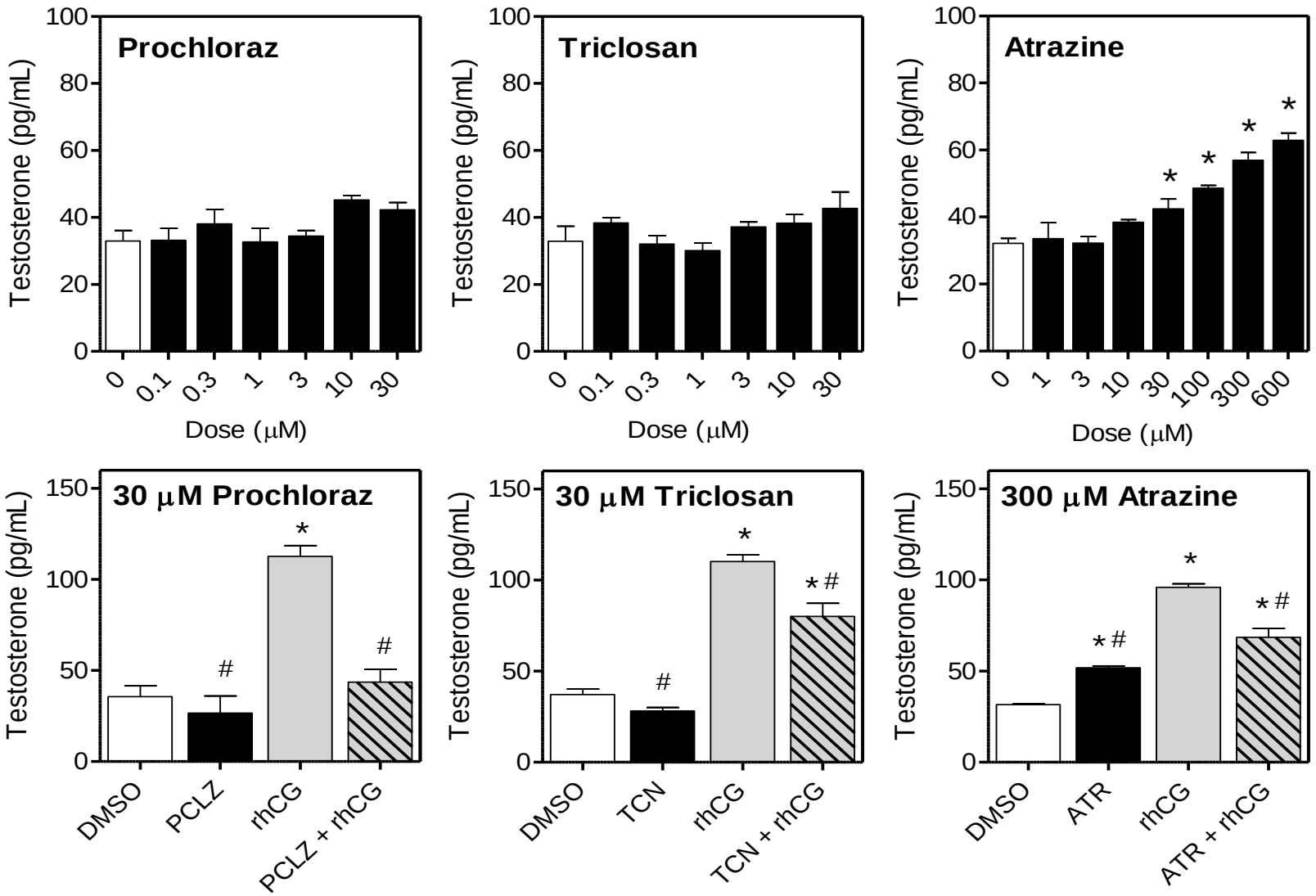

FIGURE 16. EFFECTS OF EDCS ON T LEVELS

FIGURE 16 (cont'd)

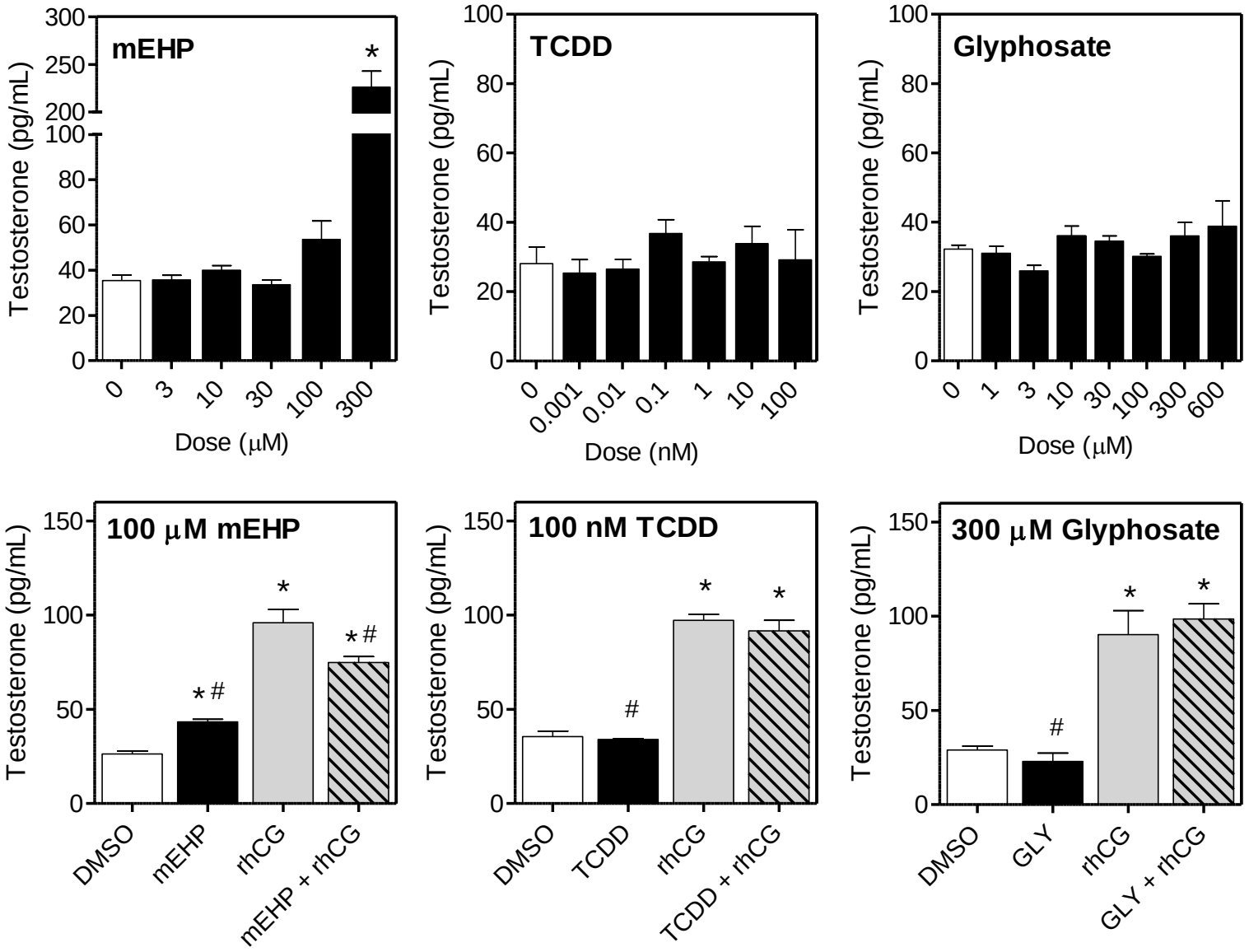

FIGURE 16 (cont'd)
The concentration-dependent effects of Prochloraz, Atrazine, glyphosate, Triclosan, MEHP and TCDD on T levels were evaluated after 4 hrs (top row, black bars) relative to DMSO controls (white bars). The effect of 30 µM Prochloraz, 300 µM Atrazine, 300 µM glyphosate, 30 µM Triclosan, 100 µM MEHP and 100 nM TCDD on rhCG-induced (3 ng/ml rhCG) T levels 4 hrs after treatment (bottom row). RDX had no effect on basal or rhCG induced T levels (data not shown). Data represent mean ± standard error of 3 replicates, $*p < 0.05$ vs. DMSO controls, $^{\#}p < 0.05$ vs. rhCG.

DISCUSSION

The *in vitro* evaluation of steroidogenesis is important for screening potential reproductive and developmental toxicants (*1, 2*). Current models include human H295R adrenacarcinoma cells and several rodent Leydig cell lines including MA-10s, mLTCs and R2Cs. Human H295R cells (ATCC CLR-2128), isolated from a pluripotent adenocortical carcinoma in a 48 year old black female, produce detectable basal aldosterone, cortisol, androgens and E2 (*59*), and are part of the Endocrine Disruptor Screening Program to screen for effects on T and E2 biosynthesis (*60*). However, H295R cells do not express functional ACTH receptors (a member of the cell surface 7-transmembrane domain superfamily of G-protein coupled receptors), and therefore steroidogenesis must be stimulated using FSK. Moreover, besides glucocorticoids and mineralocorticoids, the adrenal cortex normally only produces dehydroepiandrosterone (DHEA) sulfate and androstenedione (precursors of testosterone) and does not express the 17-beta-hydroxysteroid dehydrogenase necessary for T biosynthesis (*61*). As such it has been suggested that H295R cells have the characteristics of zonally undifferentiated human fetal adrenal cells (*59*). Only Leydig cells synthesize T and E2 directly

from cholesterol (*6*). In ovarian steroidogenesis, theca and granulosa cells produce P, while only theca cells metabolize P to T, and subsequent aromatization to estrogens or 5α reduction to DHT occurs in granulosa cells (*62*). Steroidogenic theca cell line development has not been successful and as such rodent models are of Leydig cell origin such as mouse MA-10 and mLTC-1 models as well as rat R2C cells. MA-10 and mLTC1 cells were established from transplantable M548OP Leydig tumors carried in C57Bl/6 mice that exhibit P induction by rhCG, but lacked the enzymes required for subsequent metabolism to T (*63-65*). However, recent studies report mLTC-1 cells can produce low levels of androgens, with 100 IU/ml hCG eliciting a maximum of 600 pmol/well P and 10 pmol/well T in a similar 24-well plating format as used herein (*66*). These levels are lower than those observed in resting, unstimulated BLTK1 cells. Rat R2C cells lack functional LHCGR expression and are not rhCG inducible, but exhibit high basal steroidogenesis activity (*67*).

In contrast, BLTK1 cells produce progestagens, androgens and E2 in response to trophic hormone stimulus via LHCGR, the 7-transmembrane G-protein coupled cell surface receptor, similar to T biosynthesis *in vivo* following LH secretion from the anterior pituitary (*37*). Although human steroidogenesis prefers the Δ5 pathway over the Δ4 pathway in mice, the enzymatic steps are highly conserved facilitating extrapolation of murine BLTK1 results to humans (*6*). Gestation is the critical period of exposure to EDCs that targets fetal Leydig cells causing reproductive and development toxicity. Fetal Leydig cells are observed as early as GD12.5 in mice or week 6 in humans (*34, 51*). Murine fetal Leydig cells do not express measurable LHCGR until GD 15.5, and murine fetal T production is LH-independent (*68*). However, human fetal Leydig cells do express LHCGR and are dependent on LH- and hCG-stimulation of

steroidogenesis for masculinization during development (*51*). Though BLTK1 cells are not murine fetal Leydig cells, they retain rhCG-inducible steroidogenesis characteristic of adult murine Leydig cells and both fetal and adult human Leydig cells. The ability to evaluate rhCG-inducible steroidogenesis provides a unique functional feature that is important for screening EDC interactions that may occur during human fetal and adult exposure.

During gestation, LH stimulates Leydig cell proliferation and T production, while during puberty and adulthood LH stimulates T biosynthesis to indirectly support spermatogenesis by contributing to Sertoli cell stimulation. rhCG also promotes cell proliferation or apoptosis, but no such effects were observed on BLTK1 cells (data not shown). rhCG is used clinically as an LH substitute to treat LH deficiency and in research to stimulate steroidogenesis (*69*), due to its longer half-life (~24 hrs compared to ~4 hrs for LH) (*70*). Like LH, rhCG-induces steroidogenesis via the LHCGR, and initiates the same signaling pathways resulting in a robust *in vivo* and *in vitro* steroidogenic response (*69, 71, 72*). Interestingly, LH/rhCG levels required for maximum cAMP synthesis are 15-fold higher than the level needed for maximal T production (*37*), suggesting that minimal changes in cAMP can affect steroidogenesis. Our results demonstrate that IBMX co-treatment increased rhCG potency consistent with the potentiation of cAMP induction with no effect on maximum T induction, suggesting maximum steroidogenic capacity has been achieved. It also highlights the important role of phosphodiesterases in the regulation of steroidogenesis. For example, Leydig cells isolated from phosphodiesterase 8A (PDE8A) null mice are more sensitive to LH induction but induced T levels were comparable in wild type and PDE8A null mice (*73*). These data are consistent with IBMX-treated BLTK1 cells and the enhanced potency of rhCG and FSK but comparable induced T levels.

The acute effect of LH/rhCG is activation of cAMP signaling that induces rapid mobilization of cholesterol stores and induction of steroidogenic enzyme activity. However, long term effects include the induction of steroidogenic gene expression (*37*). Differential gene expression is necessary as increased steroidogenesis in response to trophic hormone requires *de novo* protein synthesis, such as StAR induction (*67*). Consequently, in addition to effects on T biosynthesis, comprehensive screening should also include effects on cAMP as well as changes in steroidogenic gene expression.

BLTK1 cells express all the necessary enzymes required for T biosynthesis and metabolism, as well as LHCGR and several other regulators of steroidogenesis. rhCG and FSK, a direct activator of adenylyl cyclase, induce steroidogenesis in BLTK1 murine Leydig cells as indicated by time-dependent increases in cAMP, P, T, and E2 levels, and steroidogenic gene expression. Robust induction of intracellular cAMP occurred rapidly after rhCG and FSK treatment. Overall, rhCG and FSK elicited comparable induction of intracellular cAMP, steroidogenic enzyme mRNA expression, and altered P, T and E2 levels (with and without IBMX). Furthermore, BLTK1 cells exhibited robust and consistent induction of steroidogenesis for up to 30 passages (data not shown) while H295R cell assays are limited to passages 5-10 (*26, 60*).

Intracellular cAMP, as well as P, T and E2 levels in media, exhibited concentration-dependent induction following rhCG and FSK treatment in the absence and presence of IBMX. Selected EDCs also altered steroidogenesis in BLTK1 cells consistent with reports in other models. For example, prochloraz, a residential and commercial imidazole fungicide that inhibits C17-20 lyase (Cyp17a1) and aromatase (Cyp19a1), as well as antagonizes AR and ER activity (*3,*

44, 74), completely blocked T induction by rhCG. However, prochloraz alone did not alter basal T levels most likely due to the low basal level of production in BLTK1 cells. In contrast, prochloraz reduced basal T levels in H295R adrenal cells, which have relatively high basal T levels (*26*). Similarly, ATR, which decreases circulating T levels and antagonizes rhCG-induced T production in primary Leydig cells (*40, 75-78*), inhibited rhCG-induced T levels ~30% in BLTK1 cells. However, atrazine alone induced modest increases in T levels, consistent with results in rat primary Leydig cell cultures (*40*). This modest induction of basal T is consistent with atrazine acting as a phosphodiesterase inhibitor (*79*). Triclosan did not alter basal levels when administered alone but decreased rhCG-induced T levels in co-treatment studies in agreement with its reported anti-androgenic activity (*46, 47, 80*). Despite the confirmed expression of the aryl hydrocarbon receptor (AhR) in BLTK1 cells, and previous studies *in vivo* suggesting AhR-mediated inhibition of steroidogenic expression and reduction of fetal intratesticular T levels (*52-54*), TCDD had no effect on T levels in BLTK1 cells. A recent study in mouse primary Leydig cells has also demonstrated that TCDD does not alter Leydig cell steroidogenesis (*81*). Collectively, these differences in agonist and antagonist profiles suggest EDC-elicited effects on steroidogenesis use various modes of action that warrant further investigation. Moreover, these results highlight the need for more comprehensive screens that not only monitor effects on T and E2 levels, but also examine effects on steroidogenic enzyme expression and activity, or even alternative signaling cascades in order to elucidate EDC-specific adverse outcome pathways that are relevant to humans and wildlife.

BLTK1 cells can also be used to evaluate the concentration-dependent effects of specific metabolites. The mono-ester metabolite, MEHP, is thought to be responsible for DEHP-elicited

in vivo reproductive and developmental toxicity in rodents at high doses. MEHP increased T levels in BLTK1 cells, consistent with results obtained in mouse mLTC-1 Leydig cells (*49*). Furthermore, the inhibition of rhCG-induced T levels by MEHP is also reported in mouse MA-10 Leydig cell line, consistent with the proposed antagonistic effect of phthalates on T levels (*48, 82*). Although, RDX elicits reproductive and developmental effects in zebra fish, earthworms and crickets, no effects on T levels were observed in BLTK1 cells suggesting RDX may not elicit steroidogenesic effects in mammalian systems or that other indirect interactions may be involved (*55, 56*). Glyphosate also had no effect on T levels in BLTK1 cells, despite previous studies showing effects on steroidogenesis in rat and mouse models (*42, 43*). However, these studies evaluated Roundup formulations, which are complex mixtures with glyphosate as the active pesticide ingredient. Glyphosate alone only has marginal effects compared to the Roundup formulation (*43*), suggesting formulations as well as active and inert components should be examined. Nevertheless, more comprehensive temporal and concentration-response follow-up studies are required that include assessing cAMP, P, T, E2 levels and gene expression prior to any conclusions regarding the steroidogenic effects of these chemicals.

In summary, current protocols and models are inadequate to screen the universe of chemicals, metabolites and mixtures that may alter steroidogenesis (*1, 2, 4*). BLTK1 cells are a novel complementary rhCG-inducible, Leydig-based model that can be used to assess effects on steroidogenic gene expression, intracellular cAMP, and P, T and E2 levels in media. Their consistent response characteristics and inducibility over 30 passages also make this cell line attractive for high-throughput screening. Comprehensive characterization of effects on intermediate steroid biosynthesis, including pregnenolone, 17-hydroxyprogesterone, DHEA,

androstenedioine, estrone and DHT, as well as the differential expression of steroidogenic enzymes will also facilitate the elucidation of modes of action relevant to adverse outcome pathways in humans and other relevant species.

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CHAPTER 4

Forgacs AL, D'Souza ML, Huhtaniemi IT, Rahman NA and Zacharewski TR: **Triazine herbicides and their chlorometabolites alter steroidogenesis in BLTK1 murine Leydig cells**. *Toxicol Sci* 2013, 134(1): 155-167.

CHAPTER 4

TRIAZINE HERBICIDES AND THEIR CHLOROMETABOLITES ALTER STEROIDOGENESIS IN BLTK1 MURINE LEYDIG CELLS

ABSTRACT

The triazine herbicides, Atrazine (ATR), Simazine (SIM), Propazine (PRO) and Terbuthylazine (TBA) as well as their chlorinated metabolites have been implicated in the etiology of testicular dysgenesis by altering steroidogenesis. To further investigate their effects on testosterone biosynthesis, BLTK1 cells were used to evaluate steroid hormone levels as well as genome-wide gene expression. BLTK1 cells are a novel murine Leydig cell line possessing an intact steroidogenic pathway with constitutive low basal testosterone (T) levels that can be induced by recombinant human chorionic gonadotropin (rhCG). Triazines (ATR, SIM, PRO and TBA) and their chlorometabolites (DEA, DIA and DACT) induced concentration-dependent (1, 3, 10, 30, 100, 300 and 600 μM) increases in progesterone (P) and T levels relative to DMSO at 24 hrs. Temporal analysis (300 μM at 1, 2, 4, 8, 12, 24 or 48 hrs) elicited comparable P and T profiles by all compounds with varying efficacies (ATR > TBA > PRO > DEA > DIA > DACT >> SIM) that were similar to rhCG. ATR and TBA elicited time- and concentration-dependent induction of *Star*, *Hsd3b6* and *Hsd17b3* mRNA levels while *Hsd3b1*, *Cyp17a1* and *Srd5a1* mRNA expression was repressed. PRO elicited similar, albeit weaker effects while SIM had negligible effects consistent with their induction of P and T levels. Whole-genome microarrays identified 797 differentially regulated genes elicited by 300 μM ATR, primarily at later time points (> 12 hr)

with over-represented functions associated with steroidogenesis and cholesterol metabolism. These results indicate that changes in P and T levels can be partially attributed to triazine-elicited alterations in steroidogenic gene expression.

INTRODUCTION

Chloro-*s*-triazines are widely used broad-spectrum herbicides that block photosynthesis by inhibiting electron transfer at the reducing site of photosystem II in the chloroplasts of broadleaf and grassy weeds (*1, 2*). Atrazine (ATR), simazine (SIM), propazine (PRO) and terbuthylazine (TBA) are triazine herbicides registered for use in the United States: ATR for agricultural crops and grasses, SIM for grasses, fruit and agricultural crops, PRO for indoor greenhouse use, and TBA as a slimicide for ornamental fountains (*3-6*). PRO and TBA use is restricted in the U.S., and therefore human exposure is negligible (*3, 7*). In contrast, ATR and SIM are widely distributed agricultural herbicides with potential exposure to both the parent triazine and its major metabolites (*7*). ATR and SIM are biotransformed in the environment by microbial and fungal degradation, as well as photodegradation, to form three major chlorinated metabolites (chlorometabolites): des-ethyl-*s*-Atrazine (DEA), des-isopropyl-*s*-Atrazine (DIA) and diaminochlorotriazine (DACT; Figure 2) (*8, 9*). Furthermore, cytochrome P450 metabolism produces the same chlorometabolites detectable in urine, serum and various tissues of exposed animals (*10-12*).

ATR, SIM, DEA, DIA and DACT persist in the environment and are mobile in soil and ground water (*13*). ATR and SIM have been reported above the U.S. maximum contaminant levels in groundwater (*7, 14*). For example, ATR and its metabolites were the most common

pesticide contaminant detected in water, found in 75% of the streams and 40% of the groundwater samples tested in agricultural and urban areas in the U.S. between 1992 and 2001 (*15*). Groundwater contamination resulted in the cancelation of ATR and SIM registrations and cessation of their use in most of Europe (*16-18*) with the European Union approving the use of TBA as an agricultural alternative (*19*). In contrast, the Environmental Protection Agency (EPA) has approved the continued use of triazines in the U.S. (*6*).

ATR, SIM and PRO as well as their major chlorometabolites (DEA, DIA and DACT) are categorized as a Common Mechanism Group (CMG), based on their disruption of the hypothalamic-pituitary-gonadal (HPG) axis (*7, 20*). CMGs are defined for cumulative risk assessment as two or more chemicals that cause a common toxic effect(s) using the same sequence of major biochemical events, interpreted as mode of action (*21*). Moreover, the major chlorometabolites are classified as toxicologically equipotent to the parent triazines by the CMG (*7*). Therefore, all triazines and their major metabolites require further examination regarding potential endocrine disrupting activities.

The endocrine disrupting effects of triazines and their metabolites have been extensively studied in mammalian models and aquatic species. In amphibians, reptiles, and fish, ATR reduced reproductive capacity, disrupted steroidogenesis and altered gonadal development resulting in hermaphroditism and male feminization (*22-26*). Effects on steroidogenesis in amphibians and fish suggest disruption of testosterone (T) biosynthesis may underlie the effects of ATR in males (*23, 24, 27*). Rodent studies also report that ATR disrupts male and female reproductive tract development and function by altering steroid levels that affect estrus, androgen-dependent tissue development, puberty onset, reproductive

senescence and overall fertility (*28-31*). PRO, SIM, DEA, DIA and DACT elicit similar effects in rodents that are generally characterized as ATR-like with a limited number of studies examining TBA (*32-35*). Collectively, these results suggest that triazines and their chlorometabolites target the HPG axis (*30, 36*).

This study examines the effects of ATR, SIM, PRO and TBA as well as their primary chlorometabolites (DEA, DIA and DACT) on steroidogenesis in BLTK1 murine Leydig cells. Leydig cells are the primary site for T biosynthesis required for proper male development and reproduction. Briefly, luteinizing hormone (LH) stimulation induces the expression and activation of steroidogenic enzymes that increases progesterone (P) levels and metabolism to T (*37, 38*). BLTK1 cells, a subclone of a murine Leydig cell line isolated from a testicular tumor in a inhibin α/SV40Tag transgenic mouse, produce low basal P and T levels which are inducible by recombinant hCG (rhCG) and forskolin (*39, 40*). Unlike other murine Leydig cell lines such as BLTC-1 (*41*), MA-10 (*42*) and mLTC-1 (*43*) that do not proceed beyond P biosynthesis, BLTK1 cells produce appreciable basal levels of T which can be induced by rhCG, an LH analog (*40*). In this study, we have evaluated the time- and concentration-dependent effects of ATR, SIM, PRO and TBA and their major chlorometabolites on P and T production and gene expression in BLTK1 cells. Our results confirm that changes in P and T levels can be partially attributed to triazine-elicited alterations in steroidogenic gene expression.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENT

BLTK1 cells were maintained in phenol-red free Dulbecco's modified eagle medium nutrient mixture F-12 (DMEM/F-12 media; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Thermo Scientific HyClone, Logan, UT), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen) and incubated under standard conditions (5% CO_2, 37^oC). Cells were seeded at 80% confluency in 24 well tissue culture plates (Sarstedt, Newton, NC) for measurement of P and T levels in media. Concentration-response studies at 24 hrs were conducted with DMSO (vehicle; Sigma, St. Louis, MO), 0.1, 0.3, 1, 3, 10, 30 or 100 ng/ml rhCG (obtained from A.F. Parlow, NIDDK's National Hormone & Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) or 1, 3, 10, 30, 100, 300 or 600 µM of ATR (Sigma, St. Louis, MO), DIA, DEA, DACT, PRO, SIM or TBA (Accustandard, New Haven, CT). Time course studies were conducted with DMSO (vehicle), 300 µM ATR, DIA, DEA, DACT, PRO, SIM, or TBA and rhCG (3 ng/ml) as a positive control. The entire volume of media from wells was collected at 1, 2, 4, 8, 12, 24 or 48 hrs, with different wells used for each time point, such that cumulative steroid production was evaluated for the time points indicated. Cells were grown to 80% confluency in T-25 flasks (Sarstedt) for RNA isolation.

MTT CYTOTOXICITY ASSAY

BLTK1 cells, plated into 96 well tissue culture plates (Starstedt), were treated with 1, 3, 10, 30, 100, 300 or 600 ng/ml rhCG or 1, 3, 10, 30, 100, 300 or 600 µM of ATR, PRO, SIM, TBA, DEA, DIA or DACT. After 24 hrs, media was aspirated and 50 µL of freshly prepared MTT reagent

(5 mg/mL thiazolyl blue tetrazolium bromide (Sigma) in phosphate buffered saline) was added to each well. Cells were incubated for 3 hrs, then MTT reagent was aspirated and 150 µL DMSO was added to each well for 2 hrs before spectrophotometric measurement of product at 595 and 650 nm (A595-A650) using an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA). Results are reported as percentage of control calculated from relative absorbance of treated versus controls where 100% indicates no cytotoxicity.

ENZYME IMMUNOASSAYS

T (Cayman Chemical Company, Ann Arbor, MI) and P (ALPCO Diagnostics, Salem, NH) EIA kits were used following manufacturer's instructions, as previously described (*40*). Briefly, conjugated hormone and sample compete for antibody on 96-well pre-coated plates. Following incubation, the plates were washed and a reaction was initiated to yield a spectrophotometrically detectable product (412 nm for T, 450 nm for P) using an Emax precision microplate reader. A standard curve (3.9 - 500 pg/ml T and 0 - 60 ng/ml P) was generated for P and T quantification. All chemicals were confirmed to have no cross-reactivity with the EIA kits (data not shown).

RNA ISOLATION AND QRTPCR

Treated cells (T-25 flasks) were trypsinized (Invitrogen) and stored as pellets at -80˚C. RNA was extracted using RNeasy Mini Kits (QIAGEN, Valencia, CA) according to the manufacturer's protocol with an additional RNase-free DNase (QIAGEN) digestion. RNA was spectrophotometrically quantified at 260 nm (A260) and purity was assessed using the A260/A280 ratio as well as denaturing gel electrophoresis.

RNA (1 µg) was reverse transcribed by SuperScript II (Invitrogen) using an anchored oligo-dT primer (Invitrogen) as described by the manufacturer. QRTPCR reactions consisted of 30 µl reaction volumes that included 1 µl of cDNA template, 0.1 µM forward and reverse gene-specific primers, 3 mM $MgCl_2$, 1 mM dNTPs, 0.025 IU AmpliTaq Gold, and 1 X SYBR Green PCR Buffer (Applied Biosystems, Foster City, CA) carried out in 96 well plates and amplified using an Applied Biosystems PRISM 7500 Sequence Detection System. Dissociation curve analysis assessed single product amplification. To control for differences in RNA loading, quality and cDNA synthesis, samples were standardized to the geometric mean of three housekeeping genes (*Actb*, *Gapdh* and *Hprt*) (*44*). Primer sequences for all genes evaluated are available in Table 1. Results were quantified using a standard curve generated on the same 96 well plate used for samples by amplifying a purified cDNA template specific for each gene (serial 10X dilutions from 10^8 to 10^1 copies). Standard curve slope was used to assess amplification efficiency, as described by the manufacturer, with all amplification efficiencies between 80-100%. Fold changes were calculated relative to time-matched vehicle. Relative expression levels were scaled such that the expression level of the time-matched vehicle control group equaled one for graphing purposes.

MICROARRAY ANALYSIS

The temporal changes in gene expression elicited by 300 µM ATR were evaluated using Agilent mouse 4 X 44K oligonucleotide microarrays according to the manufacturer's protocol (Agilent Manual: G4140-90050 v5.0.1; array product number: G4122F; Agilent Technologies, Santa Clara, CA). Briefly, 1 µg total RNA from ATR-treated BLTK1 cells and time-matched DMSO controls was labeled independently with Cy3 and Cy5 dye and co-hybridized in an independent

reference design. This design involves comparing treated samples to controls with two independent labelings per sample (dye swap) for a total of 14 arrays per replicate (7 time points with dye swap). Three replicates of this design were performed for a total of 42 arrays. Microarrays were scanned at 532 nm (Cy3) and 635 nm (Cy5) using a GenePix 4000B scanner (Molecular Devices, Union City, CA), using GenePix Pro 6.0 to extract features and background intensities. All images and data were loaded into the TIMS dbZach data management system (*45*). All microarray data passed our quality assurance protocols (*46*) and were normalized in SAS v9.1 (SAS Institute) using a semi-parametric approach (*47*). Posterior probabilities were determined on a per gene and per time point basis (*48*) to determine model-based *t*-values (SAS v9.1) which were then used to determine P1(*t*) values by an empirical Bayes method using R (R, Institute for Statistics and Mathematics, WU Wien). Data were filtered and prioritized in a MySQL v5.1.40 database using a filtering criteria of |fold change| > 1.5 and P1(*t*) > 0.999 to identify differentially expressed genes.

Functional annotation was carried out using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA; www.ingenuity.com) and the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/). Using IPA, genes were evaluated on a per time point basis to identify significantly enriched (*p*-value < 0.05) functions. The IPA Transcription Factor tool was used to identify predicted activation or inhibition of transcription factors. K-means clustering was conducted using the TM4 MultiExperiment Viewer v.4.6.0 (*49*). DAVID was used to identify significantly enriched (*p*-value < 0.05) Gene Ontology (GO) biological process functional annotation terms (GOTERM_BP_FAT).

STATISTICAL ANALYSES AND DOSE-RESPONSE MODELING

EIA and QRTPCR concentration-response data were analyzed using SAS v9.1 (SAS Institute, Cary, NC) by analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. Differences between groups were considered significant when $p < 0.05$. EIA and QRTPCR data from time course studies were also analyzed by SAS v9.1 by ANOVA, but followed by Tukey's *post hoc* test, using a significance cutoff of $p < 0.05$. Dose-response modeling was carried out using a particle swarm optimization algorithm (ToxResponse modeler (*50*)) to identify the best-fit across five model classes (sigmoidal, exponential, linear, quadratic and Gaussian). All EC_{50} values included in this study were determined from sigmoidal concentration-response curves.

RESULTS

EFFECT OF TRIAZINES AND THEIR CHLOROMETABOLITES ON P AND T LEVELS

The effect of triazine herbicides and their primary chlorometabolites on P and T levels in media were assessed after 24 hrs of treatment with non-cytotoxic concentrations of triazines and chlorometabolites (Figure 17). ATR, PRO, SIM or TBA as well as DEA, DIA, and DACT, elicited concentration-dependent increases in P levels at 24 hrs, albeit with lower efficacy and potency relative to rhCG (Figure 18). Each triazine and chlorometabolite induced P at 100-600 µM, with a maximum induction of ~3.5-fold (90 ng/nl) in response to 600 µM ATR relative to vehicle controls. In comparison, P levels were induced up to 170 ng/ml (~6-fold) following treatment with 100 ng/ml rhCG. The EC_{50} for P induction by rhCG was 0.8 ng/ml while triazine and chlorometabolite EC50s were ~33 µM. Difference in efficacy was more evident on T (Figure 19),

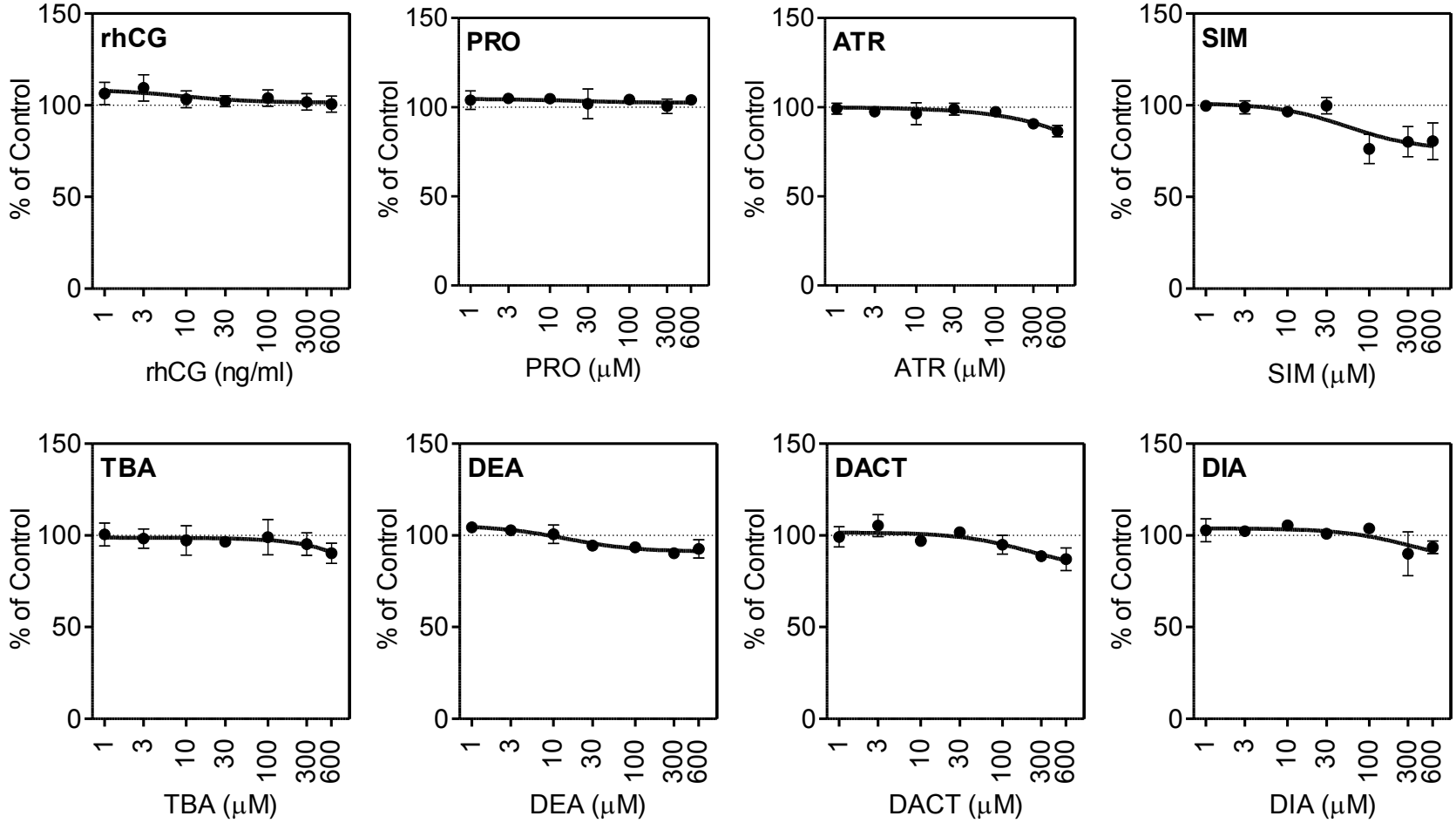


FIGURE 17. EVALUATION OF TRIAZINE AND CHLOROMETABOLITE CYTOTOXICITY.

BLTK1 cells were treated for 24 hrs with 1-600 ng/ml rhCG or 1-600 μM of PRO, ATR, SIM, TBA, DEA, DACT or DIA followed by MTT reagent. No significant cytotoxicity was detected for any treatment group, cells exhibited >90% viability even at the highest doses. Data are plotted as % control of means ± standard error, n=3.

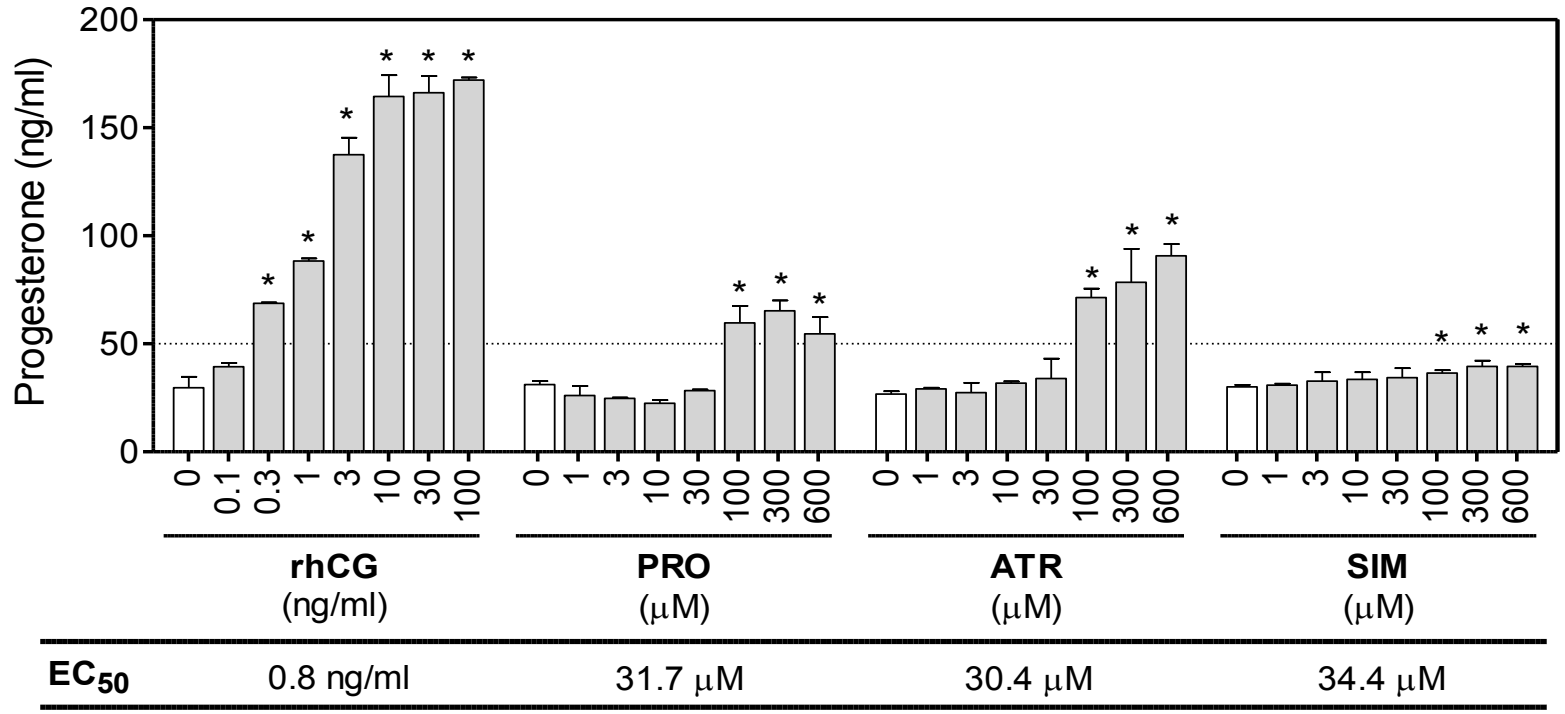
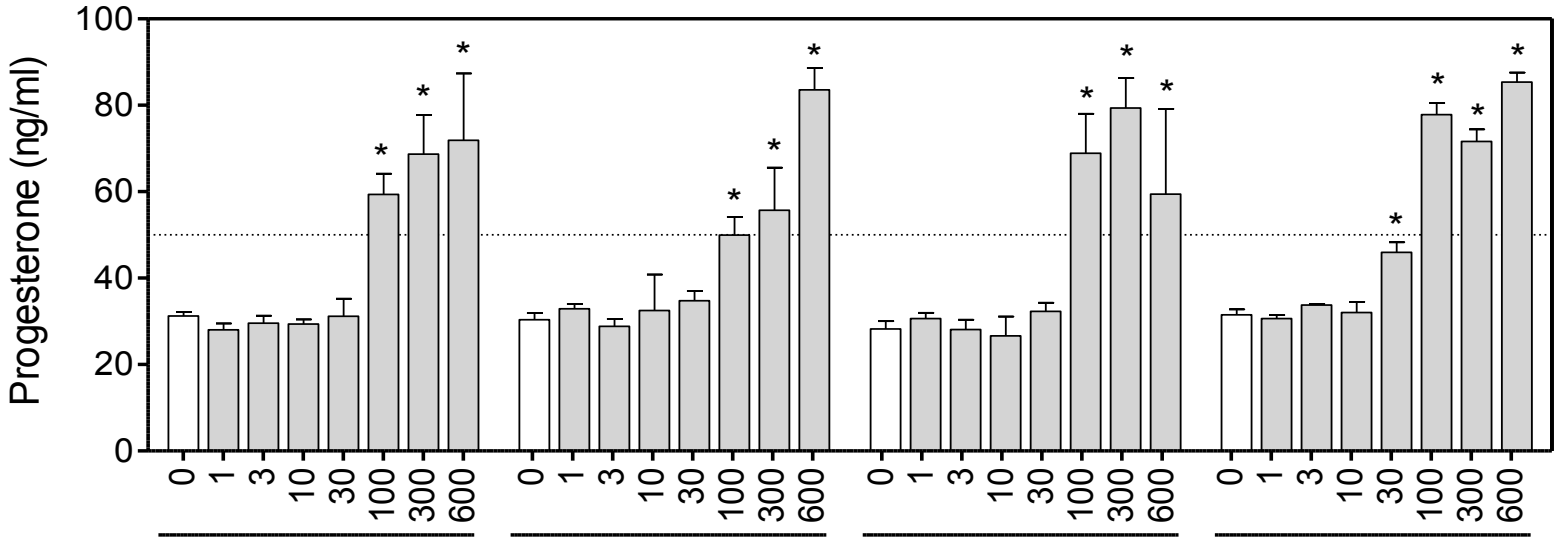


FIGURE 18. CONCENTRATION-RESPONSE EFFECTS OF TRIAZINES AND CHLOROMETABOLITES ON P LEVELS.

Concentration-dependent induction of P levels measured in the media by EIA 24 hrs post-treatment using DMSO as the vehicle control (open bars), 0.1-100 ng/ml rhCG or 1-600 μM PRO, ATR, SIM, TBA, DEA, DACT or DIA (solid bars). All triazines and chlorometabolites elicited dose-dependent induction of P levels, albeit with lower efficacy compared to rhCG. Data are plotted as means ± standard error and were analyzed using ANOVA followed by Dunnett's *post hoc* test, n=3, *p<0.05.

FIGURE 18 (cont'd)



	DEA	DACT	DIA	TBA
EC_{50}	35.0 μM	36.9 μM	34.7 μM	33.3 μM

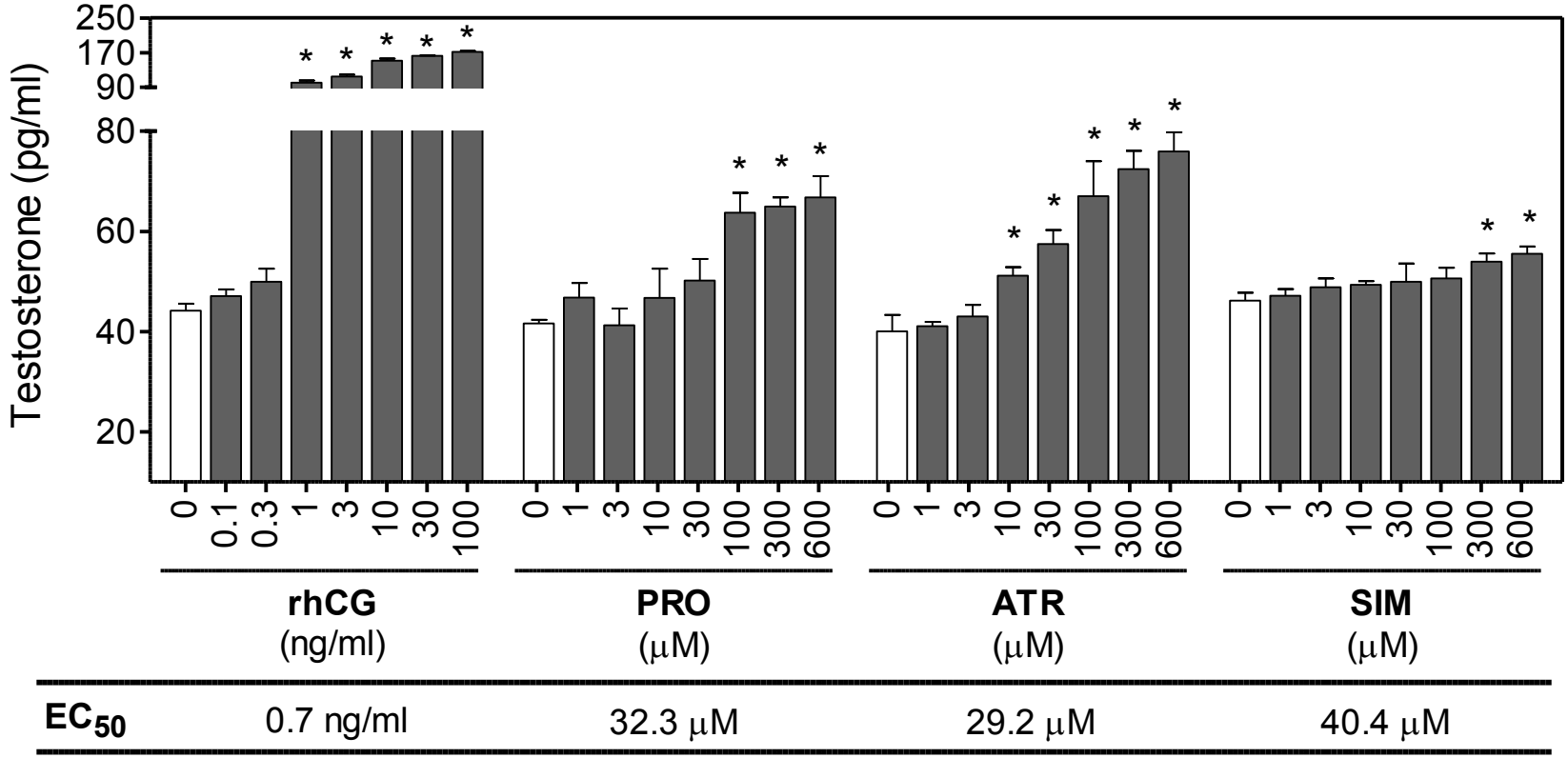
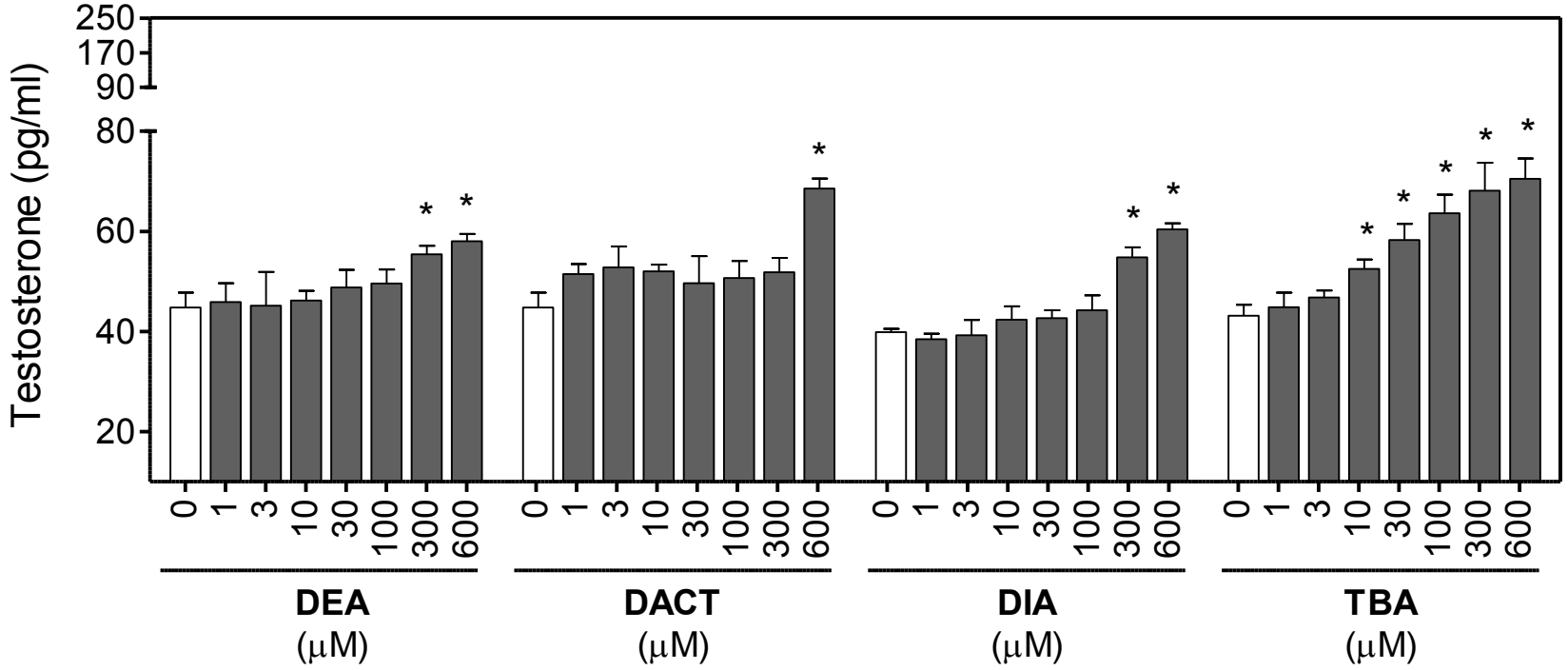


FIGURE 19. CONCENTRATION-RESPONSE EFFECTS OF TRIAZINES AND CHLOROMETABOLITES ON T LEVELS.

Concentration-dependent induction of T levels measured in the media by EIA 24 hrs post-treatment using DMSO as the vehicle control (open bars), 0.1-100 ng/ml rhCG or 1-600 μM PRO, ATR, SIM, TBA, DEA, DACT or DIA (solid bars). All triazines and chlorometabolites elicited dose-dependent induction of T levels, albeit with lower efficacy compared to rhCG. Data are plotted as means ± standard error and were analyzed using ANOVA followed by Dunnett's *post hoc* test, n=3, *p<0.05.

FIGURE 19 (cont'd)



	DEA (μM)	DACT (μM)	DIA (μM)	TBA (μM)
EC_{50}	44.8 μM	-	41.9 μM	33.1 μM

where 100 ng/ml rhCG induced levels ~4-fold (170 pg/ml) while ATR only elicited a ~2-fold (75 pg/ml) increase. SIM was the least efficacious with a modest 1.3-fold induction of T. EC_{50} values for T induction were 0.7 ng/ml for rhCG, and 30-45 μM for the triazines and chlorometabolies. DACT only had T induction at 600 μM and therefore an EC_{50} value could not be determined using the ToxResponse modeler. Overall, each compound elicited concentration-dependent induction of P and T levels, with ATR exhibiting the greatest efficacy.

Based on the 24 hr concentration-response analysis, 300 μM was used to examine the time-dependent effects of the parent triazines and their primary chlorometabolites on the induction of P and T levels. ATR, TBA and PRO elicited comparable temporal P and T profiles, albeit with lower potency compared to rhCG (Figure 20) with efficacy similar to levels in the concentration-response studies. Like rhCG, triazines and their chlorometabolites induced maximum P levels by 2 hrs, which subsequently decreased, while rhCG maintained P levels for 2-4 hrs before decreasing. Concomitantly, T increases were inversely proportional to the time-dependent decreases in P levels. However, rhCG-mediated efficacy was significantly greater compared to any triazine or chlorometabolite. Although SIM and DACT elicited comparable temporal profiles, the effects on P and T levels were not statistically significant. Overall, ATR, SIM, PRO, TBA, DEA, DIA and DACT elicited comparable temporal effects on P and T levels with differences in efficacy.

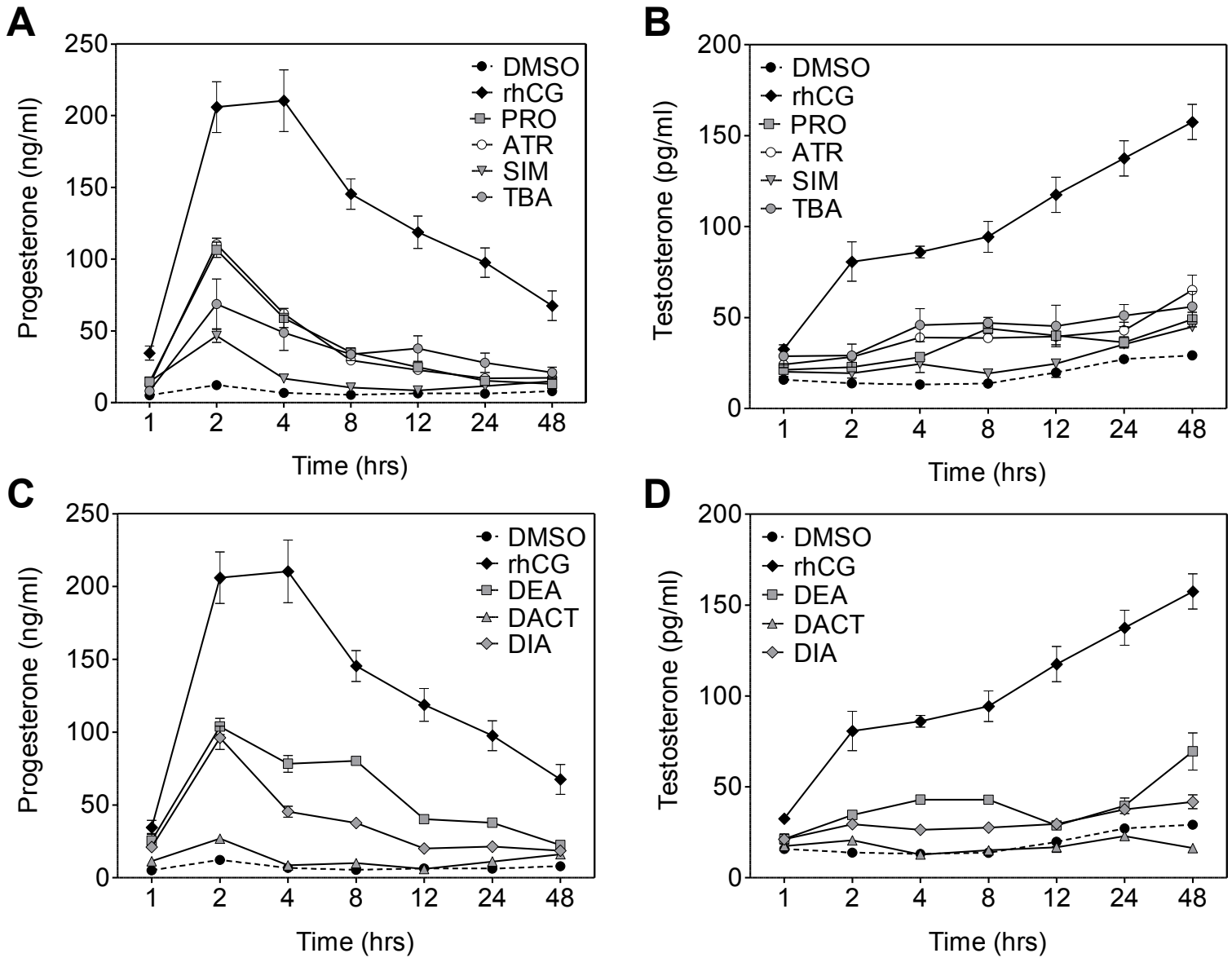


FIGURE 20. TIME-DEPENDENT EFFECTS OF TRIAZINES AND CHLOROMETABOLITES ON P AND T LEVELS.

FIGURE 20 (cont'd)
BLTK1 cells were treated with 3ng/ml rhCG or 300 µM of ATR, PRO, SIM or TBA **(A and B)**, or chlorometabolite (i.e., DEA, DACT, or DIA) **(C and D)** and evaluated for effects on P and T levels relative to time-matched DMSO (vehicle control). P **(A and C)** and T **(B and D)** levels were measured at each time point by EIA. P levels in media increased within 2 hr of treatment followed by a gradual decrease over time. In contrast, T levels exhibited a concomitant time-dependent increase following treatment. Data were plotted as mean ± standard error. Statistical significance was determined using ANOVA followed by Tukey's *post hoc* test, n=3.

TRIAZINE EFFECTS ON STEROIDOGENIC ENZYME GENE EXPRESSION

The expression of steroidogenic enzymes involved in the metabolism of cholesterol to progestagens, androgens and estrogens was evaluated by QRTPCR for parent triazines and rhCG. ATR, PRO and TBA elicited concentration-dependent differential expression of several steroidogenic genes at 24 hrs (Figure 21). Compared to the ~20-fold rhCG-mediated induction of *Star*, only ATR, PRO and TBA induced *Star* mRNA levels (4-, 3-, and 3.5-fold, respectively), while SIM had negligible effects. Meanwhile, *Hsd3b6* mRNA induction was comparable (~3-fold) for ATR, PRO and TBA, similar to rhCG. Interestingly, *Hsd3b1* was repressed 10-, 10- and 4-fold in response to 600 µM ATR, TBA and PRO, respectively, while rhCG had no significant effect on the expression of this gene. Although *Cyp17a1* was also repressed (8-, 7- and 4-fold decrease by ATR, PRO and TBA, respectively), rhCG and SIM elicited negligible effects. *Srd5a1* was induced ~3-fold by rhCG, but repressed ~2-fold by ATR, PRO and TBA. *Hsd17b3* mRNA levels were induced ~6-fold by both rhCG and ATR treatment, while PRO, SIM and TBA had negligible effects. Only TBA repressed *Cyp19a1* (5-fold). None of the treatments altered *Cyp11a1* expression.

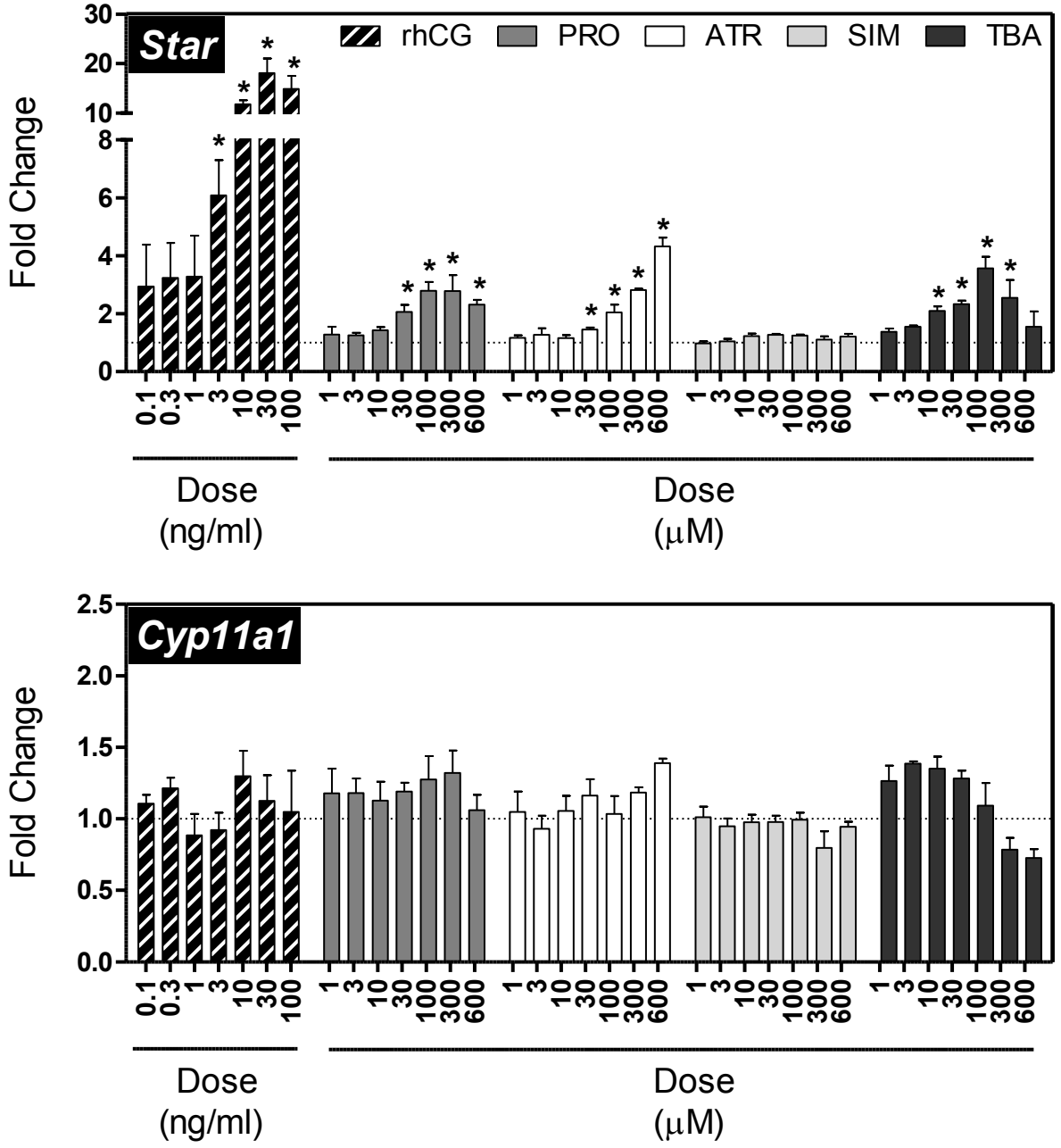


FIGURE 21. CONCENTRATION-RESPONSE EVALUATION OF TRIAZINE- AND RHCG-MEDIATED GENE EXPRESSION.

The concentration-dependent effects of rhCG (black striped bars), PRO (medium grey bars), ATR (white bars), SIM (light grey bars), or TBA (black solid bars) on steroidogenic enzyme gene expression examined 24 hr post-treatment using QRTPCR. Data are plotted as mean ± standard error of three independent replicates, *$p < 0.05$ vs DMSO controls.

FIGURE 21 (cont'd)

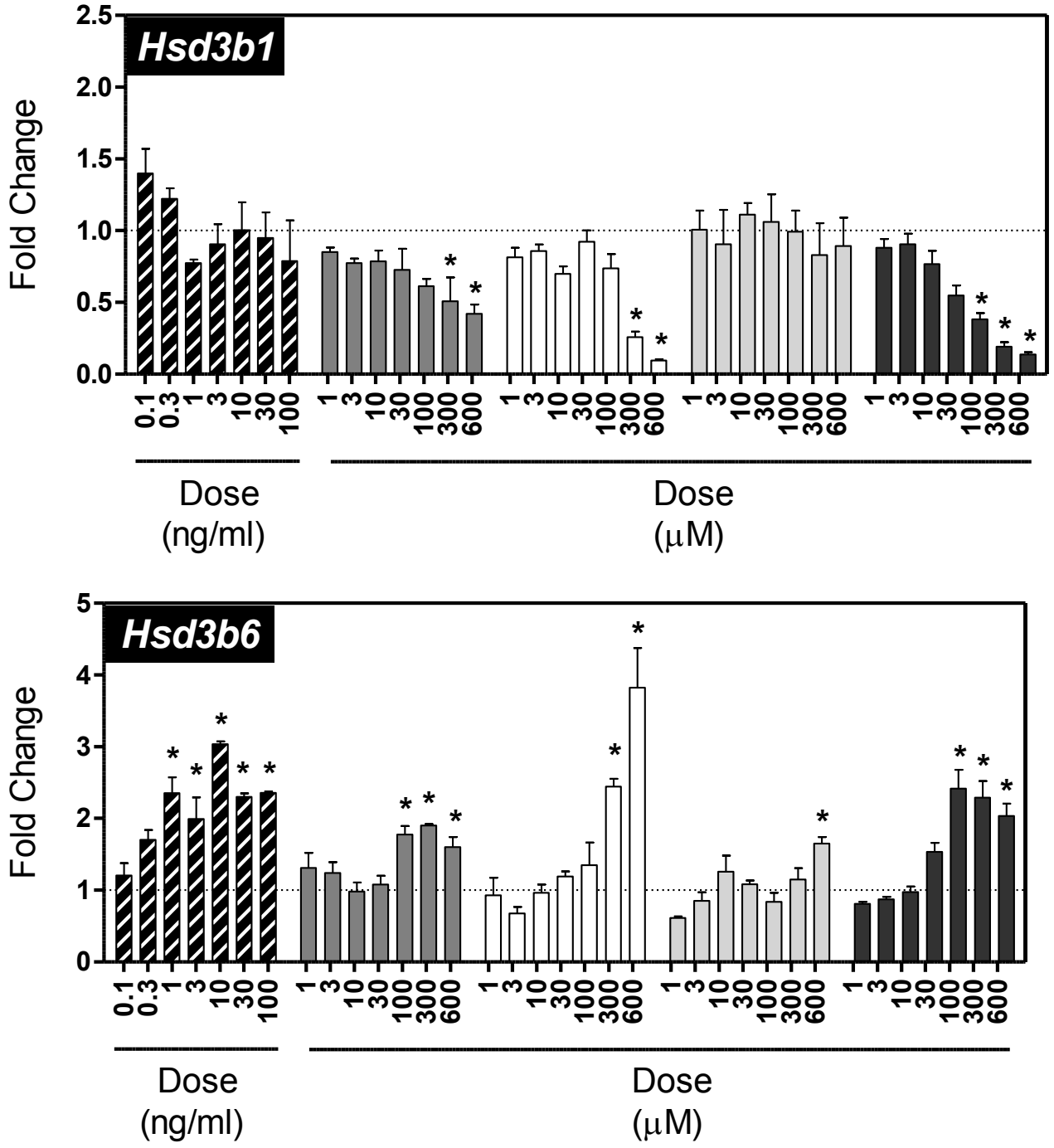


FIGURE 21 (cont'd)

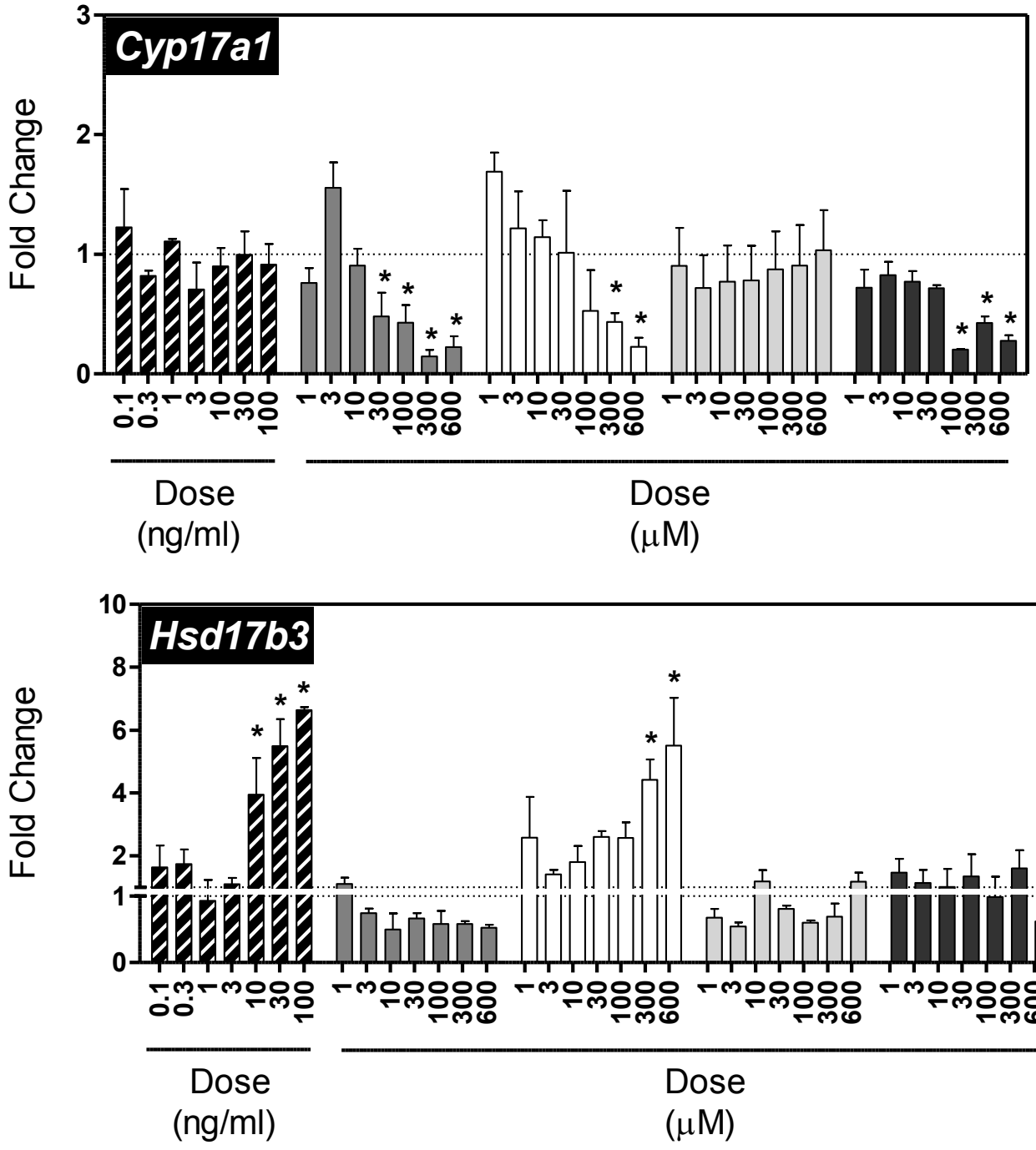
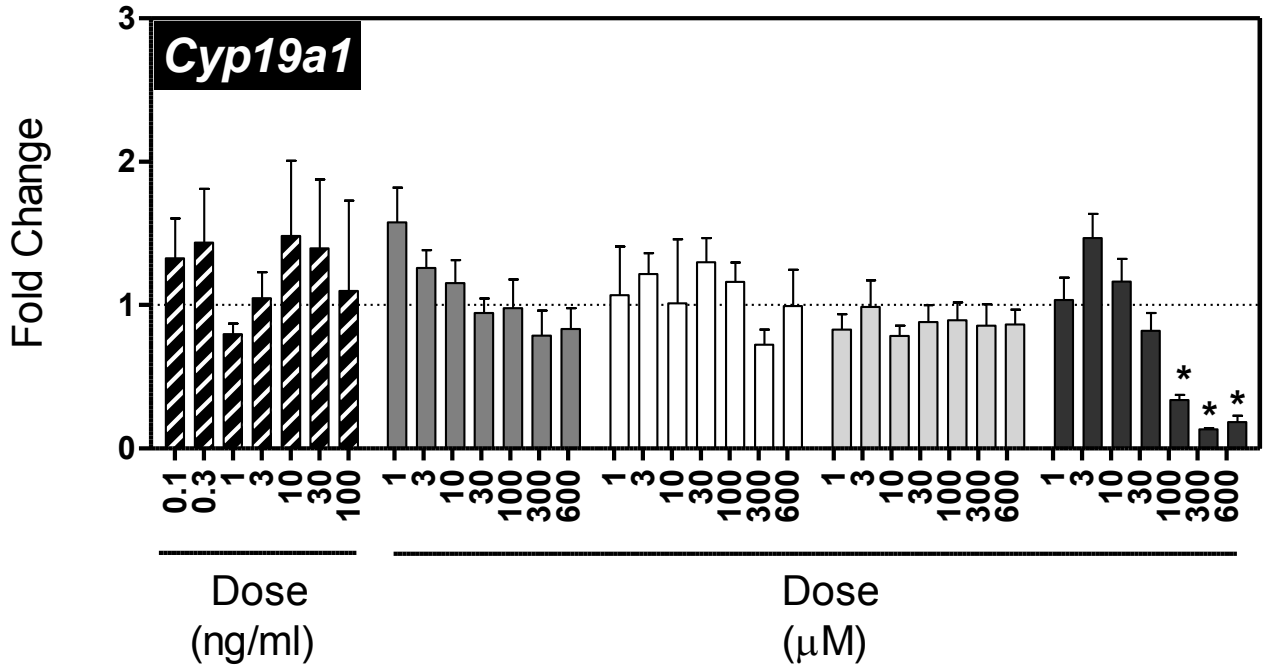
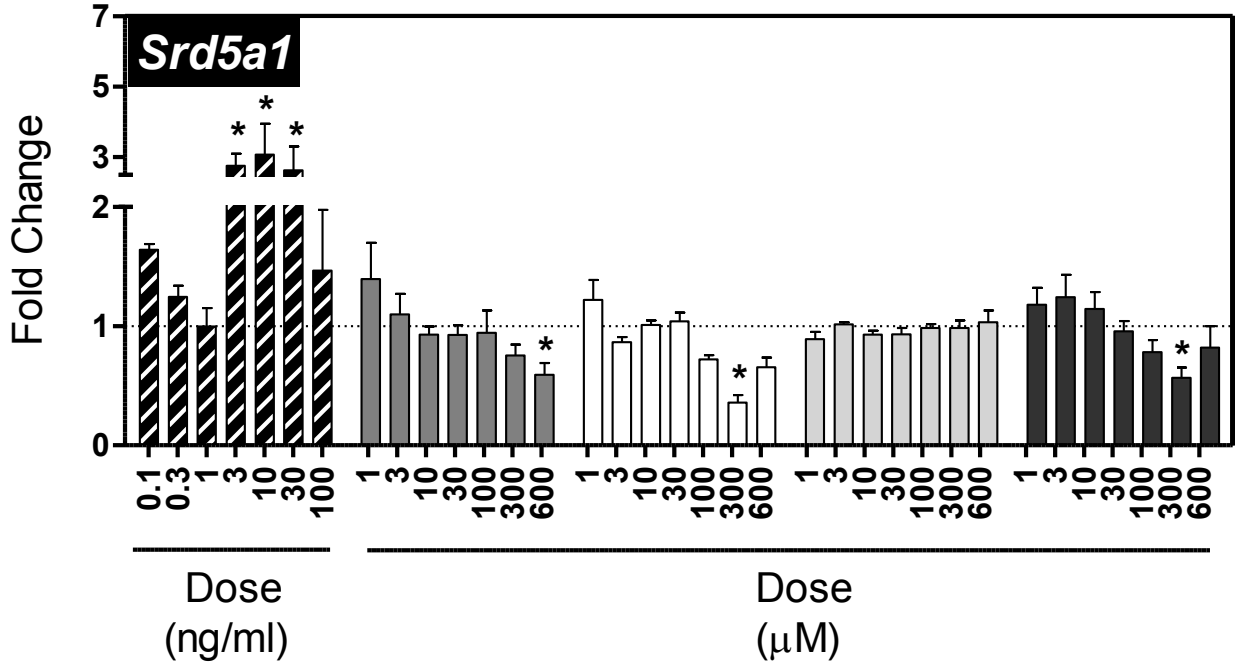


FIGURE 21 (cont'd)





The temporal effects of ATR, PRO, SIM and TBA on steroidogenic mRNA levels were also examined (Figure 22). Despite some differences in triazines- vs. rhCG-elicited concentration-response profiles at 24 hrs, time-dependent ATR-, PRO- and TBA-elicited steroidogenic gene expression profiles were comparable to rhCG, albeit with differing efficacies. In general, *Cyp11a1* and *Cyp19a1* were unaffected by treatment while *Star*, *Hsd3b6*, *Hsd17b3* and *Cyp17a1* were induced by rhCG and triazines, in agreement with the 24 hr dose-response profiles. For example, *Star* was induced 2-4-fold by ATR, PRO, SIM and TBA and 10-fold by rhCG with maximum expression achieved at 8-12 hrs. Similarly *Cyp17a1* also showed peak induction at ~8 hrs with repression evident by 24-48 hrs for both rhCG and ATR. Furthermore, *Srd5a1* exhibited divergent regulation with rhCG inducing expression 5-fold from 8-24 hrs while ATR, PRO and TBA elicited >80% repression from 24-48 hrs. Overall, SIM elicited negligible effects on the expression of the steroidogenic genes examined.

WHOLE-GENOME EVALUATION OF ATR-MEDIATED DIFFERENTIAL GENE EXPRESSION

Due to the similarities among the triazines and chlorometabolites only ATR was investigated for genome-wide differential gene expression as it had greater efficacy. Agilent microarrays contain ~41,000 features, representing 21,308 unique genes, with 797 differentially expressed (|fold change| > 1.5 and P1(*t*) > 0.999) by 300 µM ATR (Figure 23a). Differential gene expression increased over the course of the study with 23 genes at 1 hr, 18 at 2 hr, 117 at 4hr, 134 at 8 hr, 284 at 12 hr, 377 at 24 hr, and 493 at 48 hrs (Figure 23B). *Star* induction measured by microarray was validated by QRTPCR, demonstrating comparable time-dependent profiles and levels of induction from 4-48 hrs (Figure 23C). K-means analysis of the 797 differentially expressed genes identified three distinct clusters with 317 induced, 362 repressed and

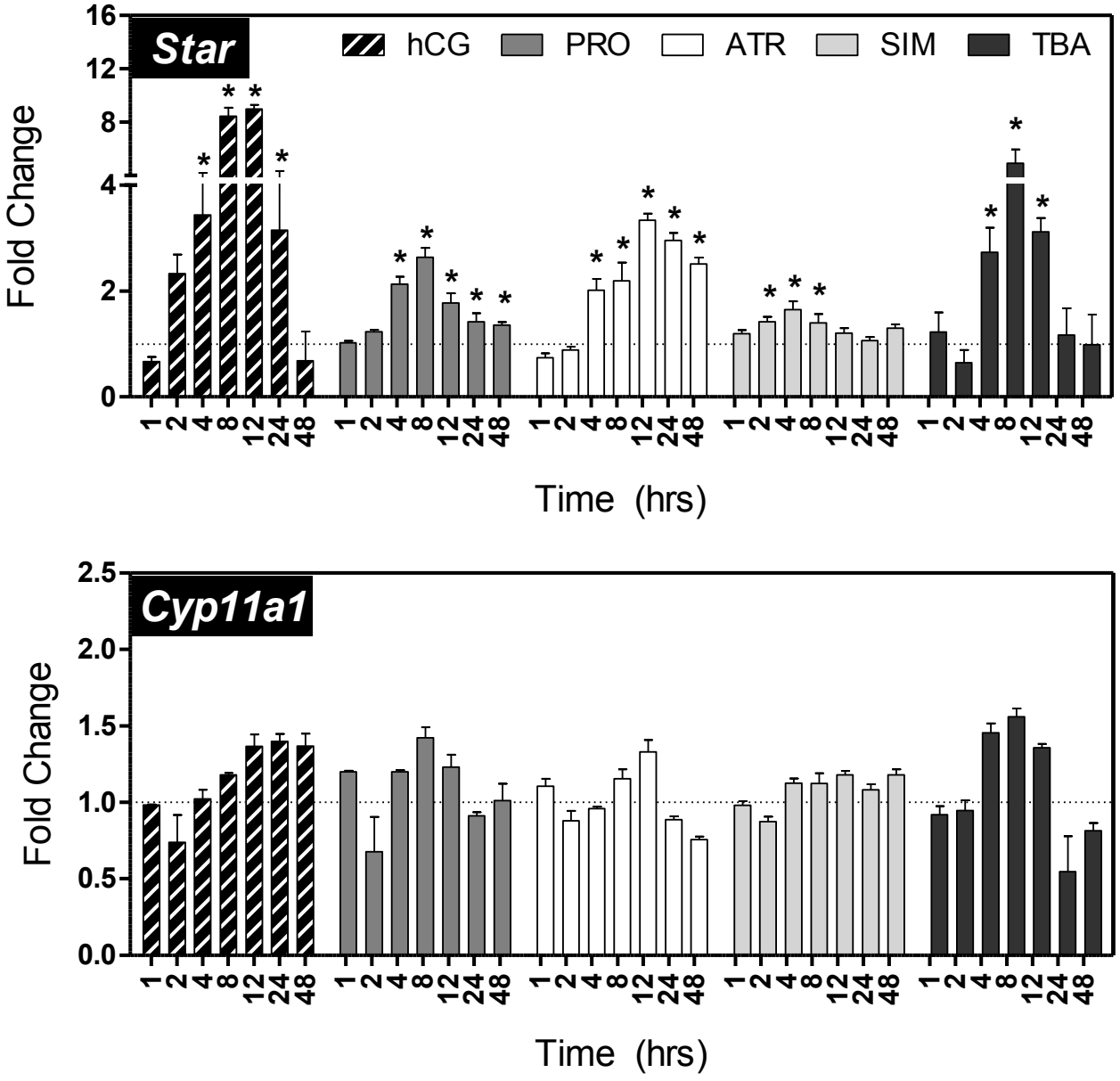


FIGURE 22. TIME-DEPENDENT EFFECTS OF TRIAZINES AND rhCG ON STEROIDOGENIC GENE EXPRESSION.

Temporal gene expression profiling of steroidogenic enzymes in response to 3 ng/ml rhCG (black striped bars) or 300 µM of PRO (medium grey bars), ATR (white bars), SIM (light grey bars) or TBA (black solid bars), respectively, was examined by QRTPCR. Data are plotted as mean ± standard error of three independent replicates, *p < 0.05 vs. time-matched DMSO controls.

FIGURE 22 (cont'd)

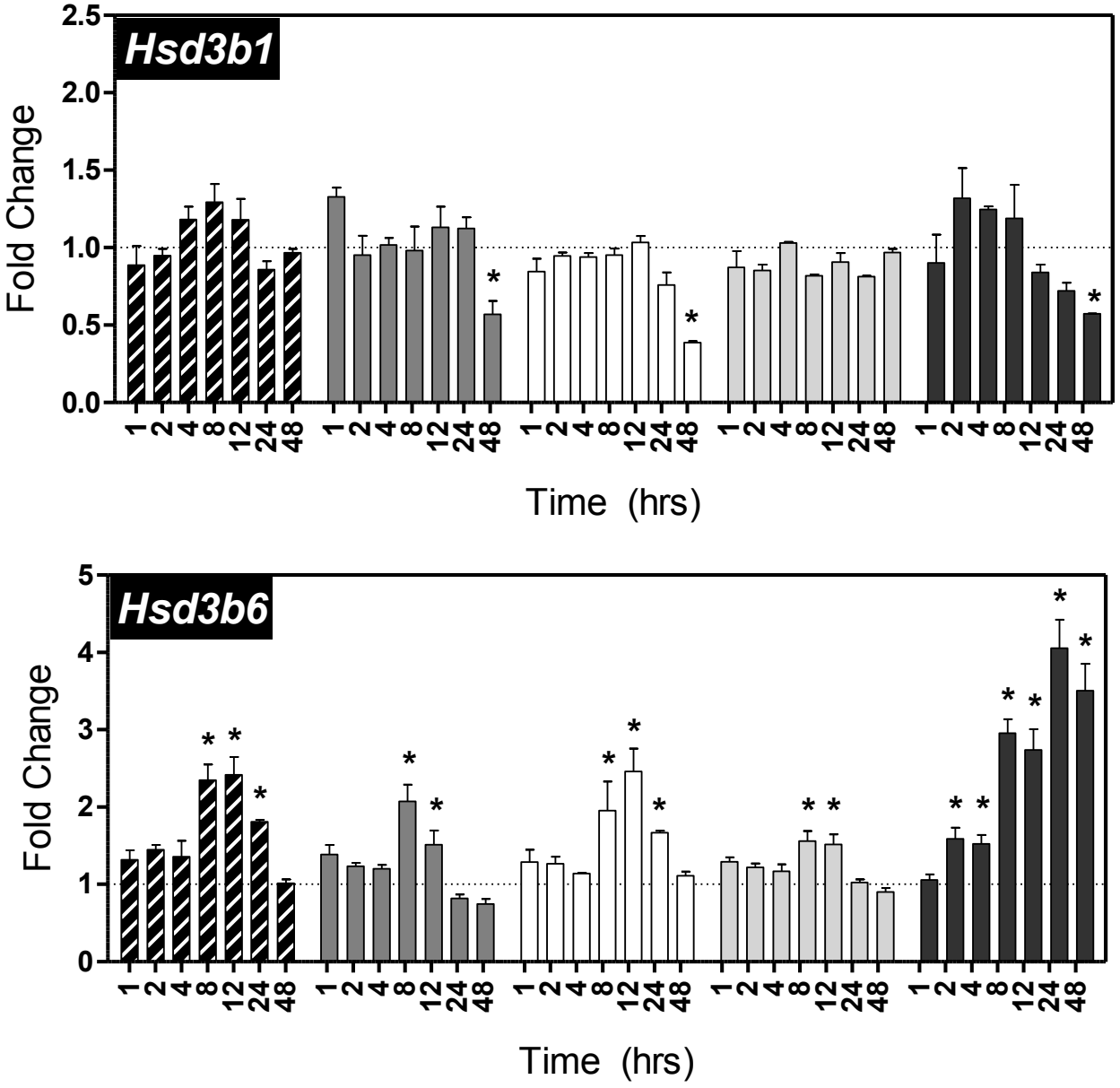


FIGURE 22 (cont'd)

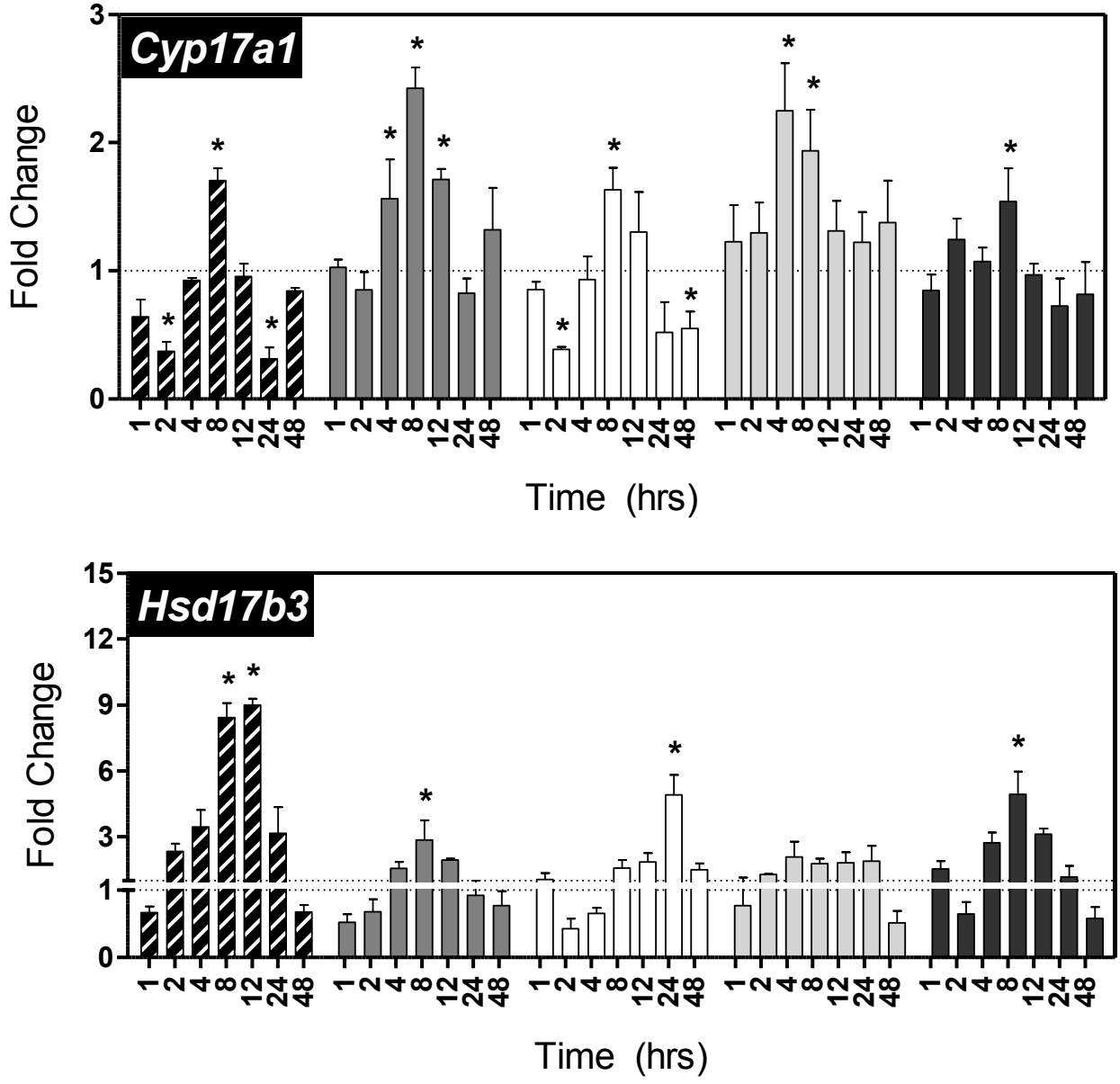
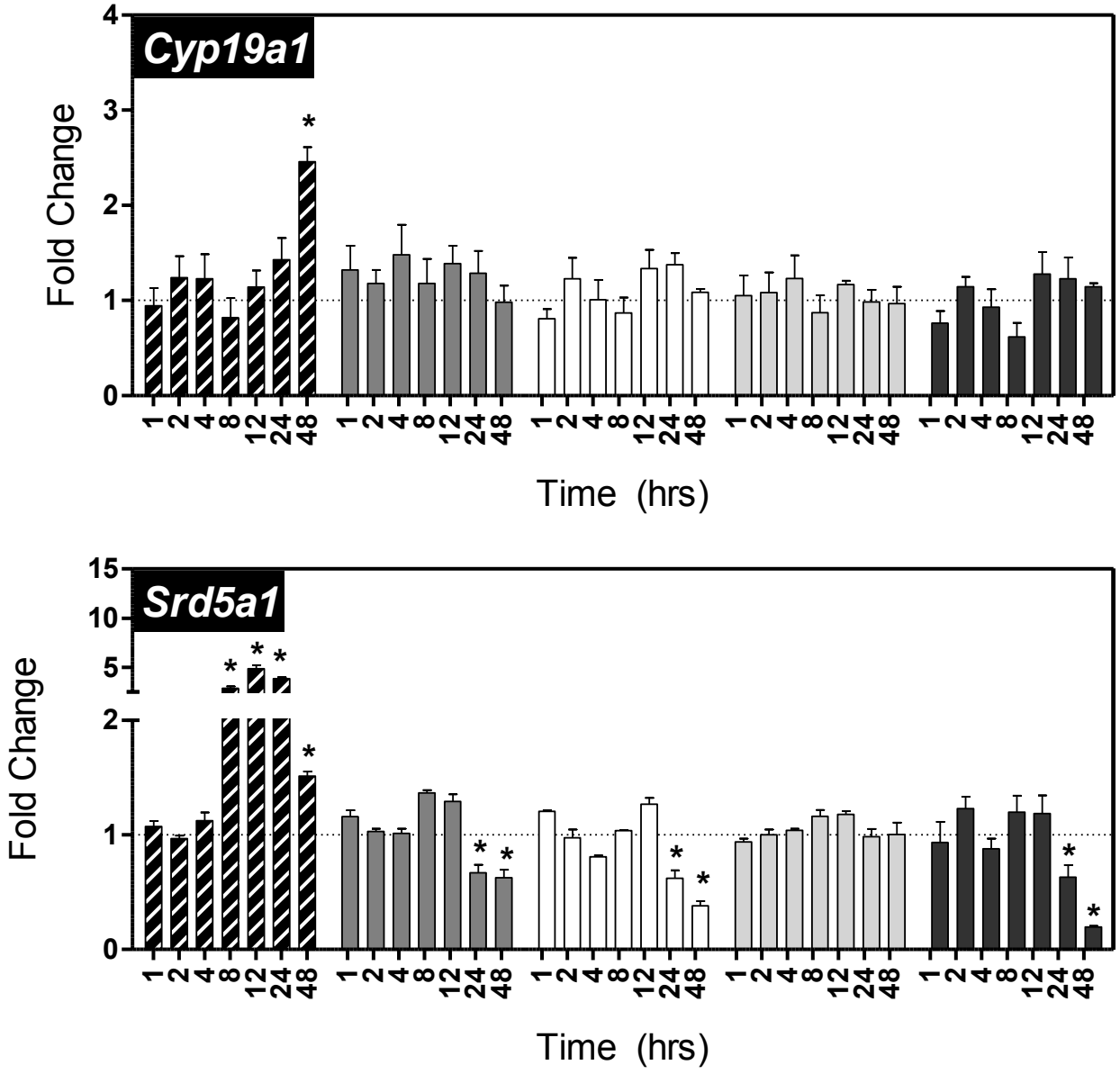


FIGURE 22 (cont'd)



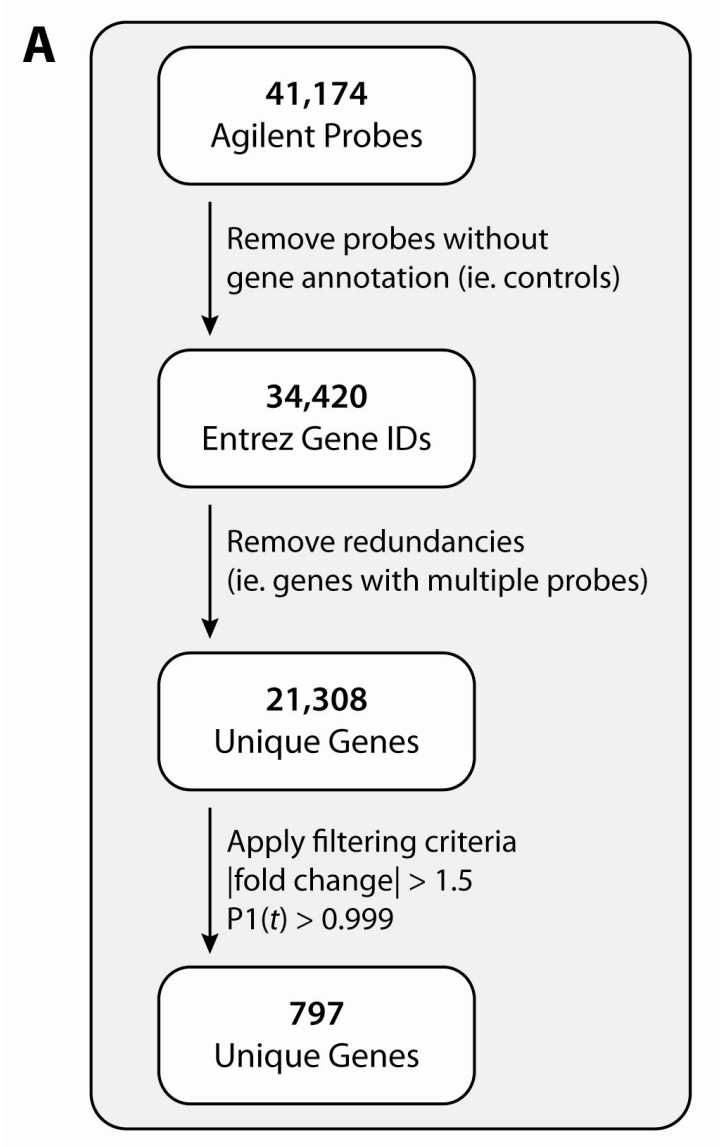
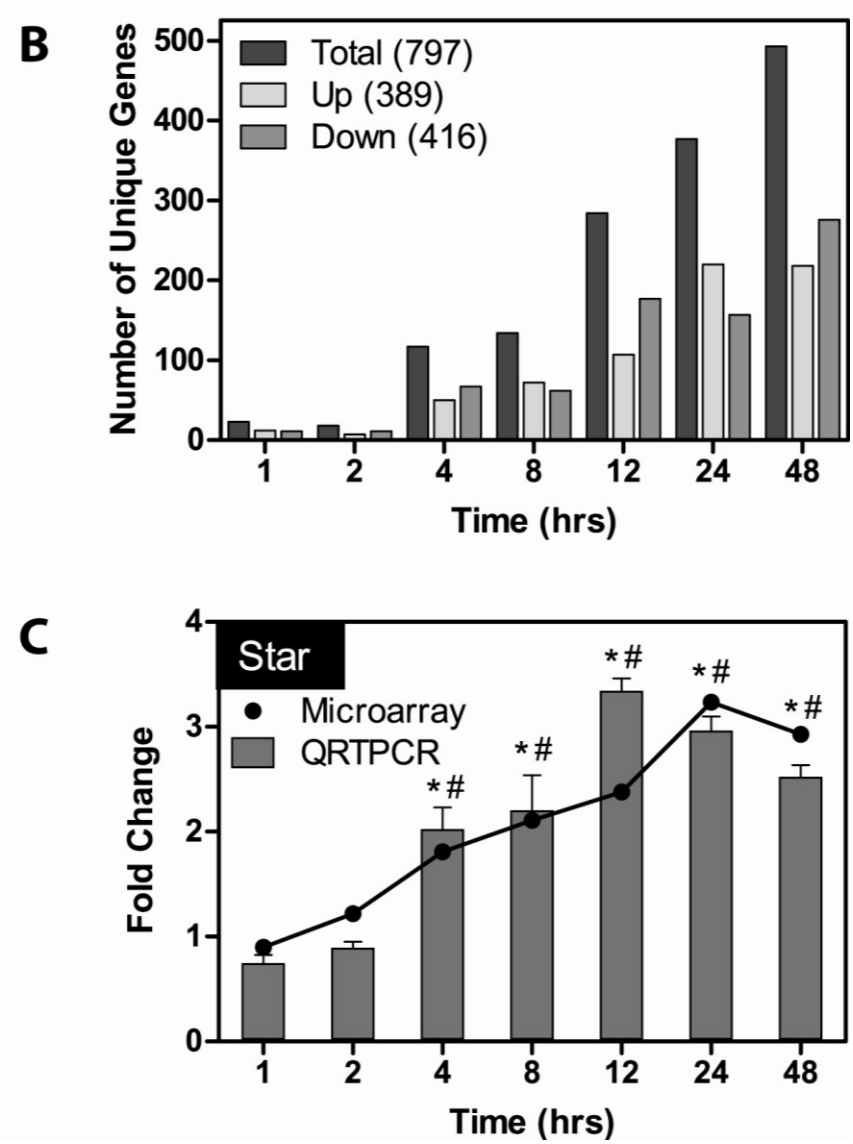


FIGURE 23. TEMPORAL WHOLE-GENOME EVALUATION OF ATR-MEDIATED DIFFERENTIAL GENE EXPRESSION.

Gene expression elicited by 300 µM ATR was evaluated using Agilent whole-genome microarrays. Filtering of microarray data (**A**), and summary of the number of genes exhibiting differential expression per time point (**B**; |fold change| > 1.5 and P1(t) > 0.999). Evaluation of *Star* expression by microarray (line) and QRTPCR (bars) (**C**). QRTPCR validation of *Star* plotted as mean ± standard error of three independent replicates, $*p < 0.05$ vs. time-matched DMSO controls. Microarray data are plotted as mean fold change of three independent replicates, $^{\#}P1(t) > 0.999$.

FIGURE 23 (cont'd)



118 exhibiting induction from ~4-24 hrs that tapered by 48 hrs (Figure 24). The Ingenuity® Transcription Factor Tool predicted enrichment of androgen receptor (AR) activity (24 and 48 hr, p-value 7.59e-03) based on 19 differentially expressed AR target genes at 24 and 48 hrs, of which 10 were repressed and 9 exhibited induction. The AR target genes contributing to the

prediction of AR signaling activation were all differentially expressed at later time points, coinciding with increasing T levels detected at 24 and 48 hrs (Figure 20). However, AR mRNA expression levels were not altered by ATR, suggesting that the activation of AR signaling was a result of androgen binding rather than increased expression.

Functional annotation was conducted using DAVID and Ingenuity® Pathway Analysis (IPA) to identify enriched gene ontology (GO) terms and over-represented functions, respectively (Table 3). Enriched GO terms were associated with hormone metabolism and regulation (including steroidogenic gene expression evaluated by QRTPCR) as well as cholesterol biosynthesis and metabolism. Examples included cholesterol *de novo* biosynthesis related genes such as *Hmcgr*, *Hmgcs1*, *Lss*, *Sqle* and *Cyp51* (all showing 1.5 to 2-fold induction at 8-12 hrs), cholesterol transport and uptake (*Star* up to 3-fold from 4-48 hrs and *Ldlr* 2-fold at 8 and 12 hrs), and metabolism into steroids (as demonstrated by QRTPCR of steroidogenic enzyme expression, Figure 22). IPA transcription factor analysis also predicted the activation of Srebf1 (Table 4) and Srebf2 (p-value 1.70e-05), which regulate gene expression associated with sterol homeostasis. The predicted activation of Srebf1 and Srebf2 was consistent with the intermediate up-regulation of their target genes as identified in K-means clustering. In addition, *Lhcgr*, the cell surface receptor for LH/rhCG, was up-regulated 1.6-fold at 24 hrs.

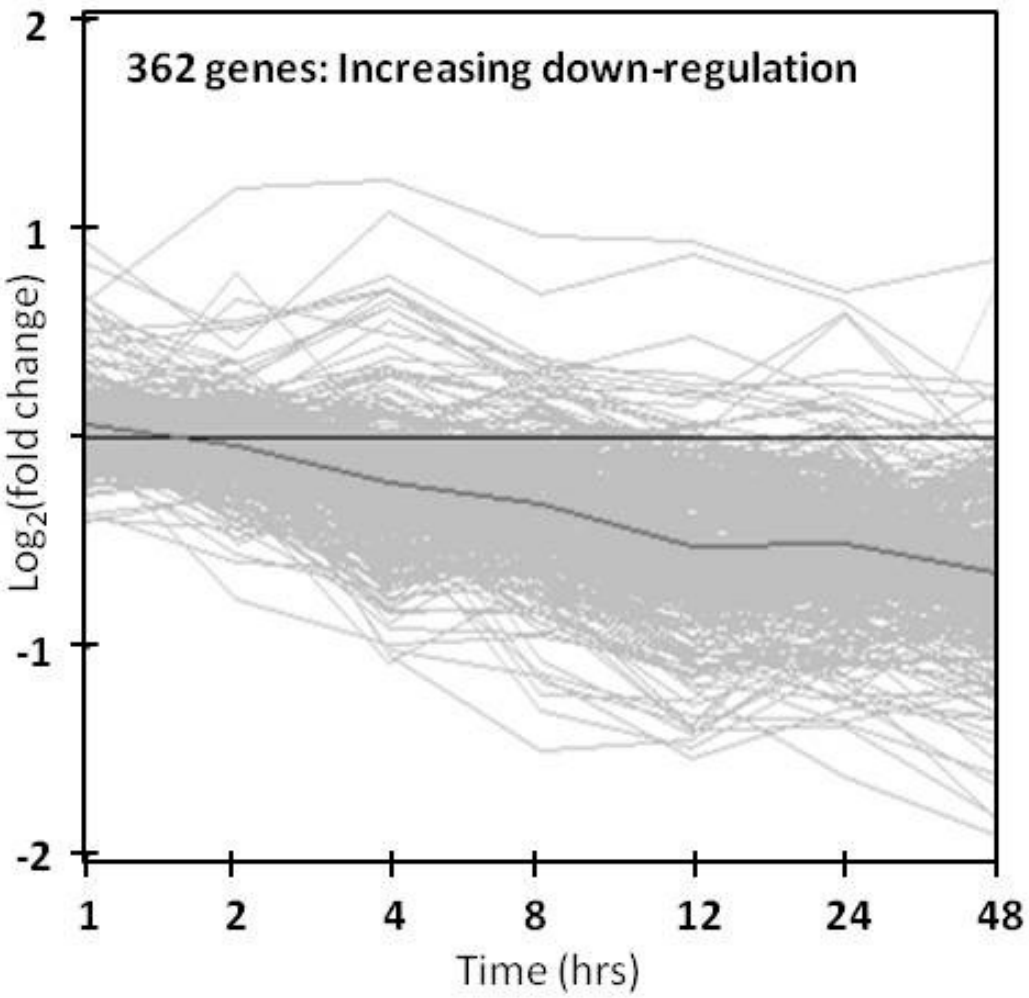


FIGURE 24. K-MEANS CLUSTERING OF ATR-ELICITED TEMPORAL WHOLE-GENOME GENE EXPRESSION.

ATR (300 uM) elicited the differential expression (|fold change| > 1.5 and P1(t) > 0.999) of 797 genes. K-means clustering using a Pearson correlation distance metric, identified three distinctive profiles: increasing down-regulation, increasing up-regulation and intermediated up-regulation. Light grey lines illustrate the profile of each gene within the cluster, while the dark grey line illustrates the average trend for the group.

FIGURE 24 (cont'd)

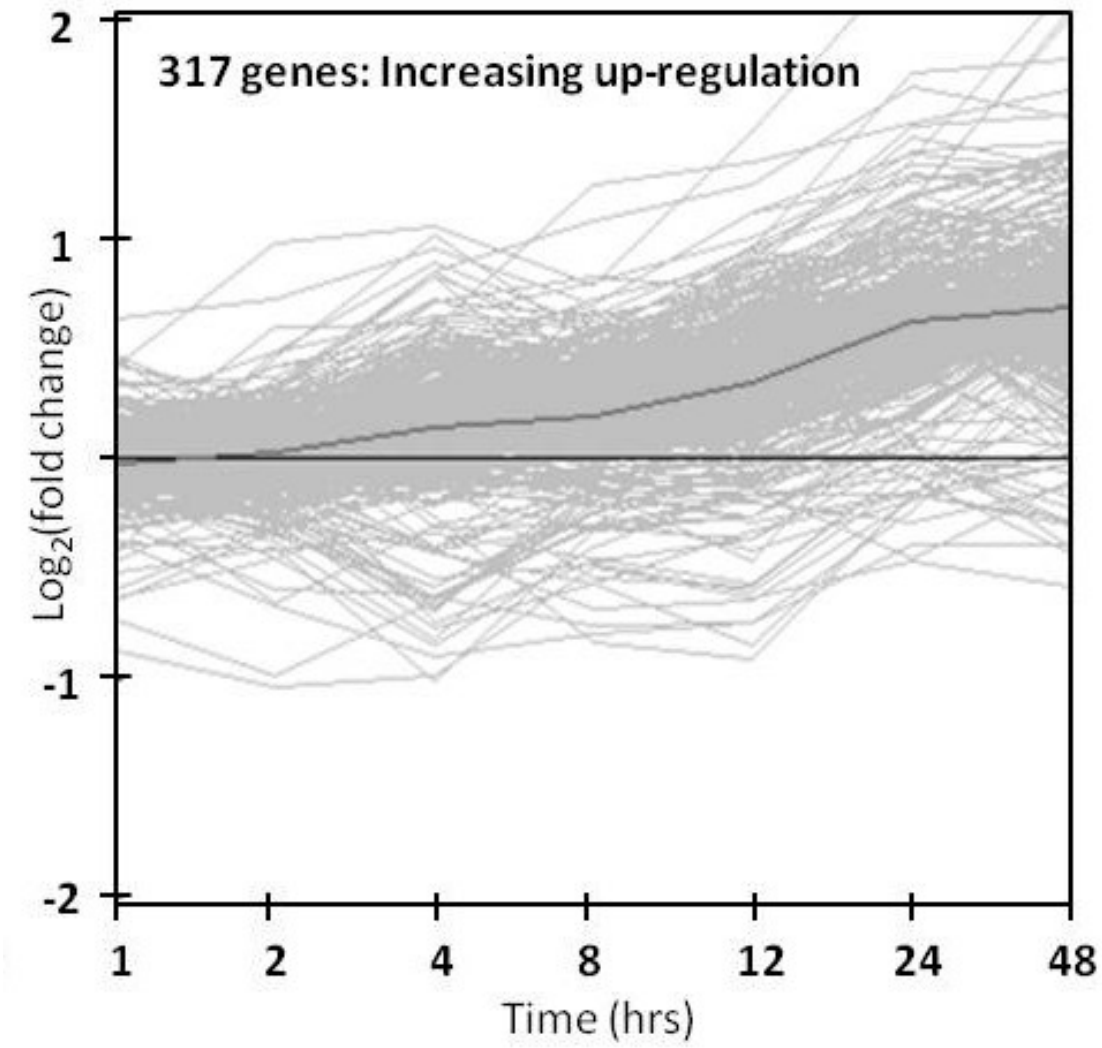


FIGURE 24 (cont'd)

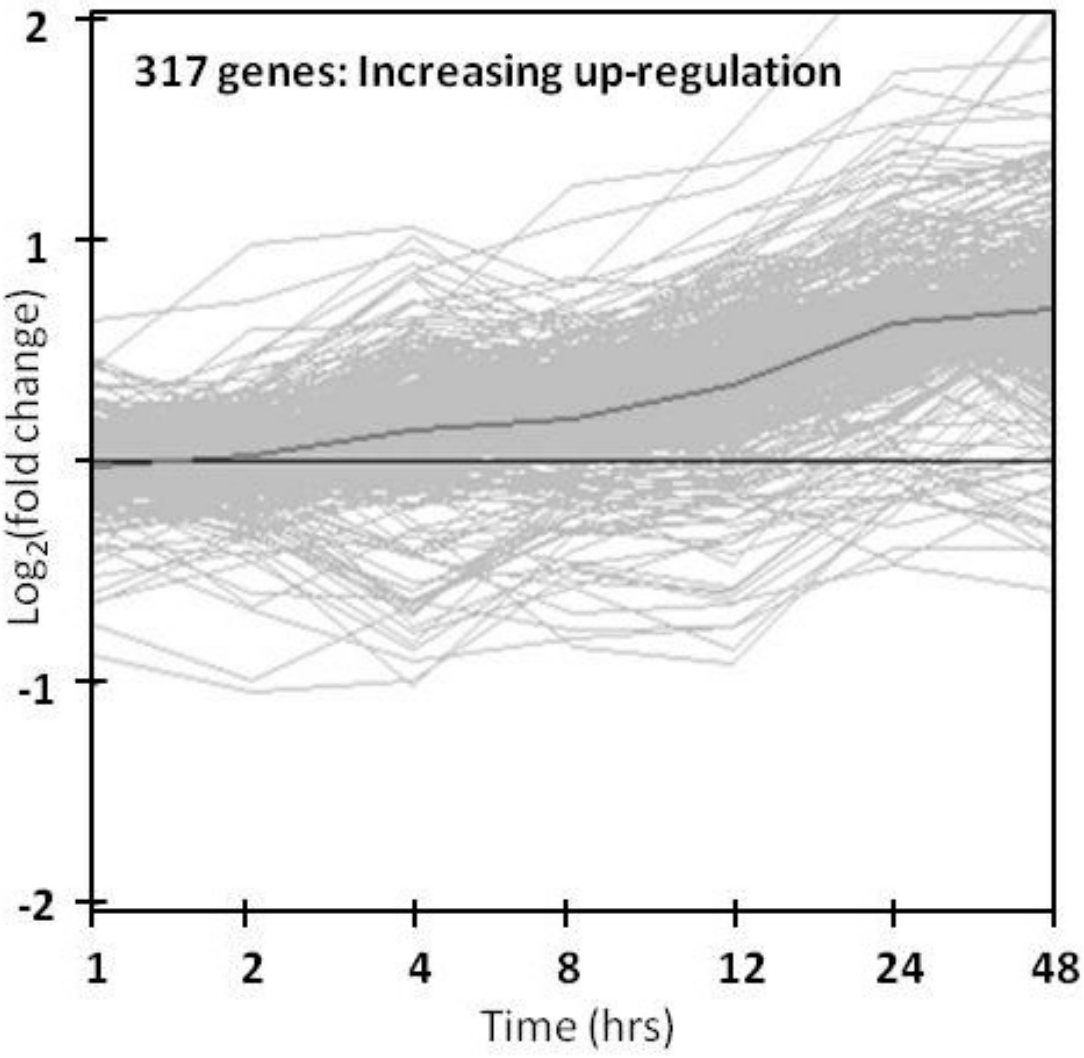


TABLE 3. ENRICHED FUNCTIONAL ANNOTATION TERMS FOR ATR-ELICITED DIFFERENTIALLY REGULATED GENES

Functional Annotation Terms	*p*-value	Number of Genes
Top 5 GO Terms (DAVID)		
Regulation of Hormone Levels (GO:0010817)	5.73e-05	17
Hormone Metabolic Process (GO:0042445)	6.28e-05	14
Cholesterol Biosynthetic Process (GO:0006695)	2.37e-04	7
Cellular Hormone Metabolic Process (GO:0034754)	2.09e-04	10
Cholesterol Metabolic Process (GO:0008203)	1.05e-04	12
Top 5 Functional Terms (IPA)		
Cancer	1.6e-17 – 1.4e-03*	276
Reproductive System Disease	1.3e-11 – 1.3e-03*	167
Reproductive System Development & Function	1.3e-11 – 1.5e-03*	102
Tissue Development	1.5e-09 – 1.4e-03*	178
Tissue Morphology	2.5e-08 – 1.3e-03*	192

*p-value range provided by IPA for each time point the function is enriched.

IPA also identified functions associated with reproduction as over-represented. This included the induction of *Rad18*, *Insl6* and *Prnp* (1.5-fold at 48 hrs), required for spermatogenesis, as well as the repression of *Srd5a1* and *Srd5a3* (2-fold at 48 hr) required for male reproductive development. Tissue development and morphology was also over-represented and included several actin regulatory proteins genes such as *Avil* (induced 2-fold at 48 hr), *Fmn1* (induced ~2-fold 8-12 hr), and *Cald1* (down-regulated ~2-fold 8-48 hrs). The predicted activation of Creb1 and Atf4 (Table 4) suggest cAMP-mediated gene expression, and included the induction of *Vegfa*, *Gadd45a*, and *Bcat1* consistent with changes in cell morphology and growth. Induction of the phosphodiesterase (Pde) genes *Pde4b* (up 1.8-fold 12-48 hr), *Pde4dip* (up 1.6-fold at 24 hr), and *Pde10a* (up to 3-fold 4-48hr) are also indicative of

cAMP signaling. The predicted activation of CCAAT/enhancer-binding proteins (Cebp), Cebpa (p-value 4.38e-05) and Cebpb (Table 4), as well as ATR-mediated differential expression of *Cebpd* (repressed 1.5-fold at 12 hr) and *Cebpg* (up-regulated 1.5-fold at 24 hrs) further suggest ATR-mediated changes in cellular growth and differentiation. Interestingly, BLTK1 cells exhibited morphological changes (e.g., contracted cell bodies, increased pseudopod length) in response to rhCG at 24 hrs (Figure 25). MTT (Figure 17) and trypan blue staining (data not shown) indicated no significant rhCG- or ATR-elicited cytotoxicity with no effects on cell growth or doubling time over the 48 hr time course based on cell counting (data not shown).

TABLE 4. IPA PREDICTED TRANSCRIPTION FACTOR ACTIVATION/DEACTIVATION

Transcription Factor	*p*-value	Time Points Enriched	Predicted Activation* (time point in hrs)	Number of Genes*
Atf4	1.68e-12	4, 8, 12, 24, 48	Activated (4, 12, 24, 48)	25
Foxo4	1.54e-07	4, 8,12, 24, 48	-	-
Cebpb	5.31e-07	4, 8,12, 24, 48	-	-
Creb1	5.29e-06	4, 8, 12, 24, 48	Activated (4, 48)	27
Srebf1	4.87e-06	4, 8, 12	Activated (8, 12)	14

* Transcription factors are predicted to be activated or deactivated based on the differential expression of known target genes. Time point of activation/deactivation (in parentheses) and the number of genes contributing to prediction are provided.

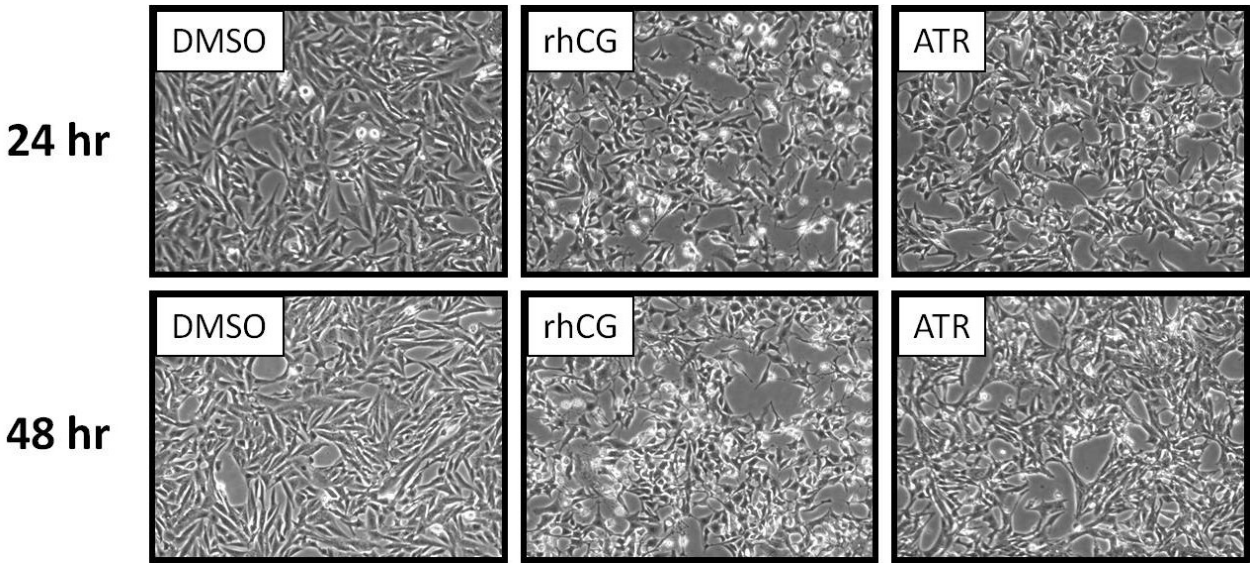


FIGURE 25. BLTK1 CELL MORPHOLOGY IN RESPONSE TO rhCG AND ATR TREATMENT. Cells were photographed using a phase/contrast microscope at 100x magnification 24 and 48 hrs after treatment with DMSO, 3 ng/ml rhCG or 300 µM ATR.

DISCUSSION

The endocrine disruptive effects of triazines have been extensively studied *in vivo* (*24, 28, 30, 32-35, 51*), however their mechanism remains unresolved. ATR, SIM, PRO, TBA, and DACT all induce mammary gland tumors in female Sprague-Dawley rats, which was the common mode of action used to define them as a common mechanism group (CMG) (*20, 30*). The CMG also includes the chlorometabolites DEA, DIA and DACT, which are found in drinking water and food and elicit comparable HPG axis disruption (*20, 33, 36*). At the time, only ATR, SIM and PRO were deemed to have uses that result in exposure of the general public thus excluding TBA from the CMG. ATR and SIM exposure was of greatest risk due to widespread use

and persistence in ground and surface water (*15*). Estimations of human ATR exposure based on urine levels of turf applicators is ~2 µM ATR equivalents (including metabolites) while acute exposure is approximately half this amount (*12*). ATR metabolite profiles in human urine consist of 50% DACT, 30% DEA and 5% DIA (*12*). Rodent studies suggest similar ATR metabolism, primarily by CYP1A1/2 (*10, 11*). Maximum plasma levels in mice are achieved within 1 hr, with maximum DACT plasma concentrations within 2 hrs post-dose (*11*). Chlorometabolite concentrations of ~2 µM are reported in serum with less than 1 µM of parent compound following administration of 200 mg/kg ATR or an equimolar SIM or PRO dose in male Wistar rats (*36*). Although, these human and rodent urine and serum levels encompass the concentrations used in our concentration-response studies, the effects on steroidogenesis appear to occur at triazine levels not encountered by humans, even in occupational environments.

Responses in BLTK1 cells are consistent with *in vivo* studies indicating ATR, PRO, DEA, DIA and DACT induce dose-dependent increases in serum P levels in male Wistar rats within 15 min to 3 hrs with no significant induction in response to SIM (*36*). SIM treatment for 41 days delayed vaginal opening and the onset of puberty in female rats (*35*) suggesting more modest disruption of steroidogenesis compared to other triazines and chlorometabolites in agreement with its effects in BLTK1 cells. ATR and DIA were reported to be equally efficacious on eliciting changes in plasma progesterone levels in male Wistar rats (*36*), consistent with their equipotent induction of P and T levels in BLTK1 cells. Additionally, DACT was described to be the least efficacious in male Wistar rats (*36*), also consistent with T induction in the current study where it had no effects at concentrations <600 µM. In addition, the CMG categorization of ATR, PRO,

SIM, DEA, DIA and DACT is in agreement with their P and T induction effects in BLTK1 cells as demonstrated by their similar EC_{50} values (*33*). Furthermore, TBA elicits comparable effects including P and T induction as well as differential steroidogenic gene expression. Moreover, the effects of all triazines and their cholormetabolites, including TBA, are similar to effects reported in rat primary Leydig cells at doses as low as 20 μM ATR (*52*). Despite differences in the differential expression of specific steroidogenic genes, the effects on P and T levels were comparable across all treatments suggesting ATR, PRO, SIM and TBA as well as their major chlorometabolites alter the regulation of steroidogenesis using a common mode of action.

Activation of Leydig cell steroidogenesis exhibits (1) an acute phase initiated by gonadotropin binding to LHCGR and induction of intracellular cAMP and PKA signaling events, and (2) a longer term effect involving differential gene expression and increased steroidogenic enzyme expression that affects the steroidogenic maintenance (*53*). While the acute phase induces immediate T levels, sustained production requires both phases. ATR alters basal T levels in BLTK1 cells and alters *Star*, *Hsd3b*, *Cyp17a1* and *Hsd17b* expression in rat primary Leydig cells (*51*). The current study extends the effects on P and T levels to other triazines and their major chlorometabolites to include time- and dose-dependent effects on the long-term phase of steroidogenesis. Most ATR-elicited differential gene expression occurred after 12 hrs suggesting effects on long-term steroidogenesis. For example, while *Star* and *Cyp17a1* showed maximum induction at ~8 hrs, repression at later time points (24-48 hrs) is consistent with the feedback inhibition exerted by androgens mediated by the AR (*54, 55*). These genes contributed to the prediction of AR activation at later time points (24-48 hr), most likely due to increasing T levels at these time points. In addition, the induction of several cholesterol biosynthetic genes and

predicted activation of Srebf1 and Srebf2 are consistent with steroid biosynthesis and longer term maintenance of steroidogenesis.

The early induction of *Star* also coincides with immediate increases in P and T levels. StAR mediates the rate-limiting transport of cholesterol to the inner mitochondrial membrane required for steroidogenesis and is one of the few genes with significant induction at earlier time points. *Star* expression is largely cAMP-mediated, with cAMP levels regulated not only by production but also by phosphodiesterase (PDE) turnover (*56*). ATR is reported to inhibit PDE4 in rat Leydig and anterior pituitary cells (*57*), human recombinant PDE4A1 (*58*), and cAMP-specific PDEs in swine (*59, 60*). Biologically Multiplexed Activity Profiling (BioMAP; a primary, human, cell-based assays covering a diverse array of regulatory networks with a broad range of target mechanisms relevant to human toxicity) also suggests ATR, SIM and PRO increase cAMP levels, consistent with PDE inhibition (*61*). These data suggest that the late induction of *Pde10a*, *Pde4b* and *Pde4dip* in BLTK1 cells at 48 hrs may be a compensatory response to PDE inhibition that warrants further investigation.

Collectively, the current study demonstrates the effects of the CMG triazines, as well as TBA, and their primary chlorometabolites on steroidogenesis in BLTK1 Leydig cells. Our data suggest that altered gene expression may underlie the long-term steroidogenic effects of triazines and their chlorometabolites, although additional studies are needed to elucidate their acute effects on P and T levels. Although our results are consistent with other reports of triazines inducing P and/or T levels (*34, 36, 52, 62*), other *in vivo* studies show triazines antagonize serum T levels (*63-66*). Preliminary studies suggest triazines antagonize rhCG-stimulated steroidogenesis (*40*), and ongoing co-treatment studies with triazine and rhCG are

examining potential triazine targets that underlie the disruption of steroidogenesis in BLTK1 Leydig cells.

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CHAPTER 5

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ATRAZINE-MEDIATED DISRUPTION OF STEROIDOGENESIS IN BLTK1 LEYDIG CELLS

ABSTRACT

Atrazine (ATR), a widely used broad-spectrum triazine herbicide, is an endocrine disruptor altering steroidogenesis resulting in reproductive and developmental toxicity. BLTK1 Leydig cells were used as a steroidogenesis model to investigate the effects of ATR on testosterone (T) biosynthesis. Induction of steroidogenesis by 3 ng/ml recombinant human chorionic gonadotropin (rhCG) induced intracellular 3',5' cyclic adenosine monophosphate (cAMP) ~20-fold and T ~3-fold at 4 hrs. Co-treatment with 300 µM ATR potentiated cAMP levels 100-fold yet antagonized rhCG-induction of T by ~20% at 4 hrs, the same time point. ATR inhibited cAMP-specific phosphodiesterase (cPDE) with an EC_{50} of ≥98 µM, suggesting potentiation of cAMP levels is a result of cPDE inhibition. However, concentrations of up to 3 mM db-cAMP did not antagonize T levels suggesting super-induction of cAMP is not sufficient to disrupt T steroidogenesis. Evaluation of cAMP-activated protein kinase A (PKA) activity by western blot revealed ATR dose-dependent changes in target protein phosphorylation. These results suggest ATR inhibits cPDEs, but that decreases in rhCG-induced T levels are likely due to changes in phosphorylation of key steroidogenic regulatory proteins.

INTRODUCTION

Atrazine (ATR; 2-chloro-4,6-bis(isopropylamino)-*s*-triazine), a broad-spectrum herbicide used on agricultural crops and grasses for pre-emergent weed control, is one of the most widely distributed herbicides in North America since the early 1960s. ATR exposure remains a concern as high volume use and mobility in soil and groundwater result in ATR equivalent levels (comprised of ATR and its chlorinated metabolites) above U.S. maximum contaminant levels in 75% of streams and 40% of groundwater samples tested in agricultural and urban areas in the United States between 1992 and 2001 (*1-4*).

ATR-elicited reproductive and developmental toxicity has been consistently observed across fish, amphibian and mammalian species (*5-8*), with endocrine disruptive effects targeting the hypothalamic-pituitary-gonadal (HPG) axis (*7, 8*). In male rodents these effects include altered testicular morphology with instances of testicular atrophy (*9*), decreased androgen-dependent organ development such as decreased seminal vesicle and ventral prostate weights (*10*), delayed perputial separation (*11*), and decreased sperm number and motility (*12*). These effects have been attributed to ATR-mediated decreases in testosterone (T) levels in the testicular interstitium and in serum (*9-15*). Several studies also noted increases in circulating 17β-estradiol (E2) levels in the serum of male rodents (*9, 13*). These results have contributed to the hypothesis that ATR-mediated effects are estrogenic; however, there is negligible interaction of ATR with the estrogen receptor (ER) and on ER activity across species (*16-19*).

To better understand ATR's effects on steroid hormone biosynthesis and metabolism, *in vitro* evaluation ATR effects on steroidogenesis in H295R adrenocortical carcinoma cells has been conducted. H295R cells, unlike adult differentiated adrenal cells, have the characteristics

of zonally undifferentiated fetal adrenal cells producing T and E2 in addition to glucocorticoids and mineralocorticoids (*20*). In H295R cells ATR increases E2 production (*21-24*). However, reports are inconsistent regarding T production being increased (*24*) or decreased (*21, 22*). Consequently, the induction of aromatase (P450c19), the enzyme responsible for the aromatization of T to make E2, is a proposed mechanism for ATR (*13, 21, 22, 25*).

Leydig cells are the primary site for T biosynthesis and are a more physiologically relevant system in which to evaluate the effects of ATR on steroidogenesis. Leydig cells are required for proper male development and reproduction and are capable of *de novo* steroidogenesis, producing both T and E2 from cholesterol. In rat primary Leydig cells ATR induces basal T production, and increases cAMP levels (*26*). The secondary messenger cAMP was also increased in ATR-treated H295R cells (*21, 23*), leading to the investigation of ATR as a cAMP-specific phosphodiesterase (cPDE) inhibitor (*27-29*). While cPDE inhibition by ATR has been demonstrated (*21, 27-29*), it remains to be elucidated how elevated cAMP levels relate to disruption of hormone production in Leydig cells.

This study examines the mode of action by which ATR disrupts steroidogenesis in BLTK1 murine Leydig cells. BLTK1 cells are a subclone of BLT-1 murine Leydig cells isolated from a testicular tumor in a inhibin α/SV40Tag transgenic mouse (*30, 31*). Unlike other murine Leydig cell lines such as BLTC-1 (*32*), MA-10 (*33*) and mLTC-1 (*34*), BLTK1 cells produce low basal P and T levels, which are inducible by recombinant human chorionic gonadotropin (rhCG, a luteinizing hormone analog) and forskolin (*31*). Previous studies in BLTK1 cells have demonstrated ATR-elicits partial agonism of steroidogenesis relative to rhCG (*35*). The effects of ATR on rhCG-induced steroidogenesis were evaluated, revealing antagonism of T induction, consistent with

in vivo studies reporting decreased T in serum. Furthermore, our results support ATR-mediated inhibition of cPDE, but are the first to demonstrate that elevated intracellular cAMP levels do not underlie antagonism of rhCG induction of T production. Evaluation of protein phosphorylation reveals that ATR likely affects T production in BLTK1 Leydig cells by altering the phosphorylation status of key steroidogenic regulatory proteins.

MATERIALS AND METHODS

CHEMICALS

Culture medium composed of phenol-red free Dulbecco's modified eagle medium nutrient mixture F-12 (DMEM/F-12 media; Invitrogen, Carlsbad, CA) was supplemented with 10% fetal bovine serum (FBS; Thermo Scientific HyClone, Logan, UT), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen). Recombinant human chorionic gonadotropin (rhCG) was obtained from A.F. Parlow, NIDDK's National Hormone & Peptide Program, Harbor-UCLA Medical Center (Torrance, CA). Dimethyl sulfoxide (DMSO), Atrazine (ATR), 3-isobutyl-1-methyl-xanthine (IBMX), forskolin (FSK), and dibutyryl-3',5' cyclic adenosine monophosphate (db-cAMP) were obtained from Sigma-Aldrich (St. Louis, MO). Primary antibodies for phosphorylated PKA substrate (RRXS*/T*; #9624) and phosphorylated ERK1/2 (p44/42 MAPK phospho-Thr202/Tyr204; #9101) were obtained from Cell Signaling Technology (Danvers, MA), and for actin (sc-1616) from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody goat anti-rabbit IgG (#170-6515) and donkey anti-goat (sc-2056) conjugated with horseradish

peroxidase were obtained from Bio-Rad Laboratories (Hercules, CA) and Santa Cruz, respectively.

CELL CULTURE AND TREATMENT

Cell culture and treatment were conducted as previously described (*35, 36*). Briefly, BLTK1 murine Leydig cells were maintained under standard conditions (5% CO_2, 37^{o}C), grown to 80% confluency, transferred into 24-well tissue culture plates (Sarstedt, Newton, NC), and incubated overnight prior to treatment. Following 4 hr treatment, media was collected for progesterone (P) and testosterone (T) measurement while cells were harvested for intracellular cAMP measurement and protein quantification.

ENZYME IMMUNOASSAYS

Intracellular cAMP was measured by EIA (Cayman Chemical Company, Ann Arbor, MI) following hydrochloric acid extraction as described by vendor. Levels of P and T from media samples were also measured by commercially available EIA kits for T (Cayman Chemical Company) and P (ALPCO Diagnostics, Salem, NH), used according to the manufacturer's instructions. Spectrophotometric detection was conducted at 420 nm for cAMP and T or 405 nm for P using an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA). Standards (0.3 – 750 pmol/ml cAMP, 0 - 60 ng/ml P, or 3.9 - 500 pg/ml T) were used to generate a standard curve allowing quantification.

WESTERN BLOTTING

Lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton) containing protease inhibitor (Roche Diagnostics, Indianapolis, IN). Protein

concentrations were determined spectrophotometrically using Bradford reagent (Cayman). Ten µg of protein was resolved by 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad Laboratories) and transferred to Immobilon membrane (Millipore, Billerica, MA). Membranes were incubated with primary antibody at 4^{o}C overnight, washed in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST; Sigma) and incubated with secondary antibody for 1 hr at room temperature. Enhanced Chemiluminescence (Thermo Scientific Pierce, Rockford, IL) was used to visualize detected protein.

PHOSPHODIESTERASE INHIBITION ASSAY

PDE inhibition was evaluated using a commercially available kit (Enzo Life Sciences, Farmingdale, NY) with purified PDE enzyme from bovine brain. Briefly, 3',5'-cAMP substrate is metabolized by cAMP-specific PDE (cPDE) into 5'-nucleotides and subsequent cleavage into nucleoside and phosphate by a 5'-nucleotidase is detected using BIOMOL Green^{TM} reagent. Assays are conducted in 96-well plates with a 50 µL reaction volume containing 200 µM 3',5'-cAMP as substrate, 20 U PDE enzyme, 50 kU 5'-nucleotidase and 0.03 – 300 µM ATR or 0.02 – 200 µM IBMX. Plates were incubated at 30^{o}C, reactions were stopped by the addition of BIOMOL Green^{TM} reagent followed by 20 min incubation at room temperature to allow color to develop, and measured at 620 nm using an Emax Precision microplate reader. Quantification was carried out using a standard curve generated with known quantities of 5'-AMP substrate incubated with 5'-nucleotidase alone. Controls were included using 5'-AMP as substrate to ensure that ATR did not inhibit the activity of the 5'-nucleotidase (data not shown). The cPDE-specific inhibitor, IBMX, was used as positive control. All determinations were conducted in triplicate.

DOSE-RESPONSE MODELING AND STATISTICAL ANALYSES

Dose-response modeling was performed using the ToxResponse modeler (*37*) whereby particle swarm optimization identifies the best-fit across five model classes: sigmoidal, exponential, linear, quadratic and Gaussian. Data fitting sigmoidal dose-response curves were used to calculate EC_{50} values. All statistical analyses were carried out using SAS v9.1 (SAS Institute, Cary, NC) by analysis of variance (ANOVA), with Dunnett's or Tukey's *post hoc* tests for concentration-response and time course data, respectively. Differences between treatment groups were considered significant when $p < 0.05$ relative to time-matched DMSO control.

RESULTS

ATR-MEDIATED DISRUPTION OF rhCG-ELICITED INDUCTION OF cAMP, P AND T LEVELS

The temporal effects of 300 µM ATR, 3 ng/ml rhCG and their co-treatment on cAMP, P and T levels were evaluated in BLTK1 cells (Figure 26). Upon stimulation of Leydig cells by rhCG, a rapid ~200-fold induction of intracellular cAMP was observed within 1hr and persisted to 4hrs. Meanwhile, ATR only resulted in a maximal 2-fold induction at 1hr. Interestingly, co-treatment of ATR and rhCG potentiated the induction of intracellular cAMP levels to ~400-fold at 1 hr with similar potentiation at all time points where intracellular cAMP was detected (≤ 4 hrs). P and T levels were induced by both ATR and rhCG although ATR only resulted in partial agonism relative to rhCG. Concomitantly, co-treatment resulted in antagonism of rhCG-mediated induction of both P and T at all time points where these steroid hormones were detected (≥2 hrs).

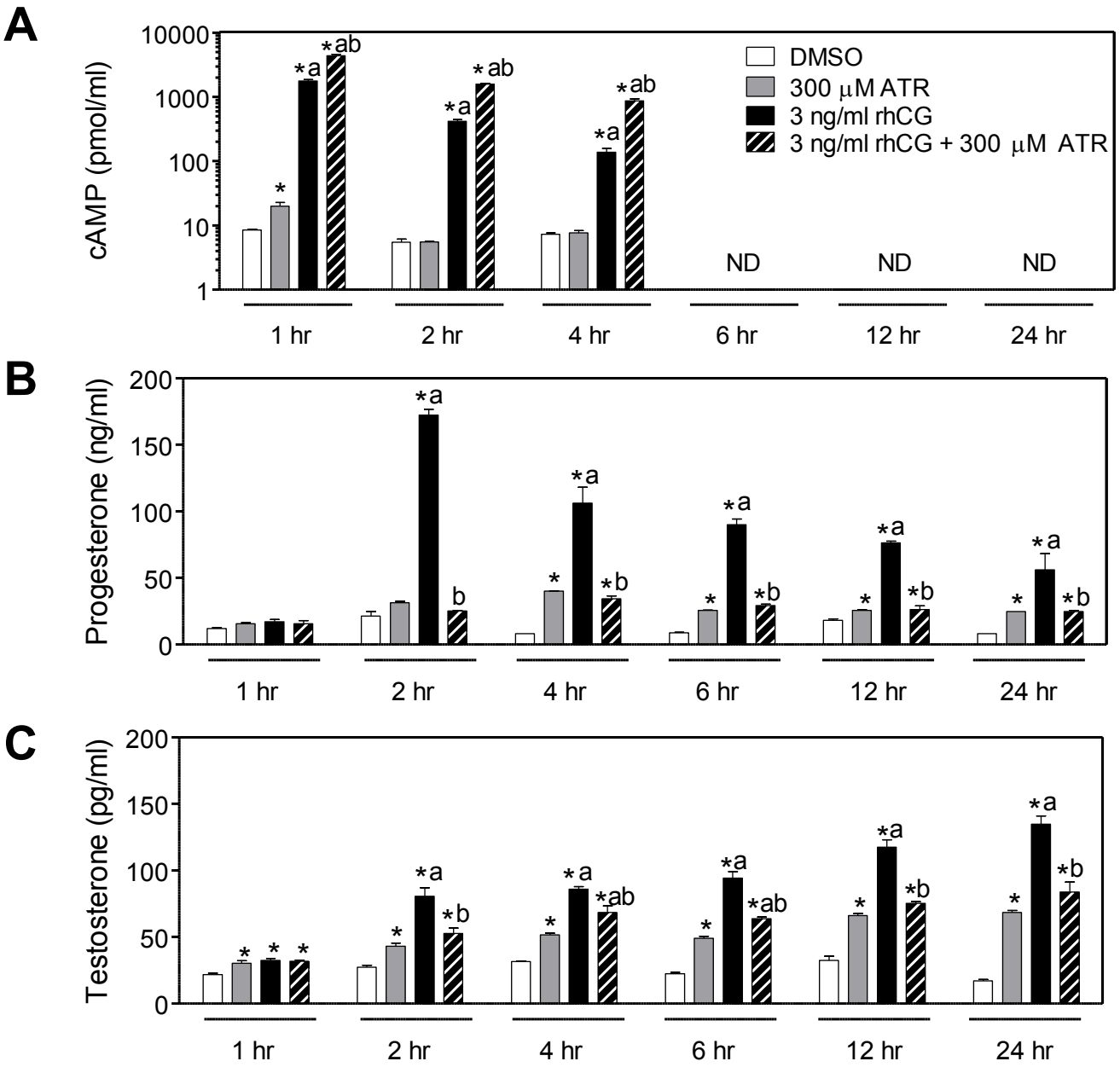

FIGURE 26. TEMPORAL EFFECTS OF ATR ON rhCG- INDUCTION OF STEROIDOGENESIS.
Intracellular cAMP **(A)**, P **(B)** and T **(C)** levels in media were evaluated upon treatment with DMSO vehicle (white bars), 300 μM ATR (grey bars), 3 ng/ml rhCG (black bars), or their co-treatment (striped bars) from 1-24 hrs. Data are plotted as mean ± standard error, n=3, and were analyzed by ANOVA followed by Tukey's *post hoc* test where *p<0.05 vs. time-matched DMSO, [a]p<0.05 vs. time-matched ATR, [b]p<0.05 vs. time-matched rhCG. ND: not detected.

Dose-response evaluation of ATR's effects on rhCG-mediated induction of cAMP and T levels was evaluated at 4 hrs (Figure 27). Only cAMP and T were evaluated as cAMP is an essential secondary messenger required for the stimulation of steroidogenesis and T is the primary product of Leydig cells; P was not pursued as treatment effects were comparable to T. Concentration-dependent potentiation of rhCG-induced intracellular cAMP levels was observed at ≥100 µM ATR with a maximal ~10-fold super-induction at 600 µM ATR. Meanwhile, ATR co-treatment resulted in up to ~35% antagonism of rhCG-elicited induction of T. EC_{50} values of ~100 µM were consistent for both cAMP potentiation and T antagonism with ATR /rhCG co-treatment.

ATR EFFECTS ON cAMP INDUCTION

Elevated intracellular cAMP levels can be the result of increased production or decreased turnover. To evaluate whether ATR affects G-protein-coupled receptor (GPCR) signaling underlying adenylyl cyclase activation, and hence cAMP production, the effect of ATR on rhCG- and FSK-mediated cAMP induction and T production was compared (Figure 28). Induction of steroidogenesis with rhCG is initiated by GPCR signaling via LHCGR to activate adenylyl cyclase, whereas FSK-mediated induction of steroidogenesis directly targets adenylyl cyclase (Figure 5). Both rhCG and FSK induced cAMP and T levels (Figure 28). ATR co-treatment resulted in potentiation of cAMP levels ~10-fold on both rhCG- and FSK-mediated induction and decreased T levels ~20% relative to rhCG or FSK induction alone. These data suggest that disruption of GPCR signaling likely does not underlie ATR's effects on cAMP or T levels since the same effects were observed when inducing steroidogenesis with and without LHCGR.

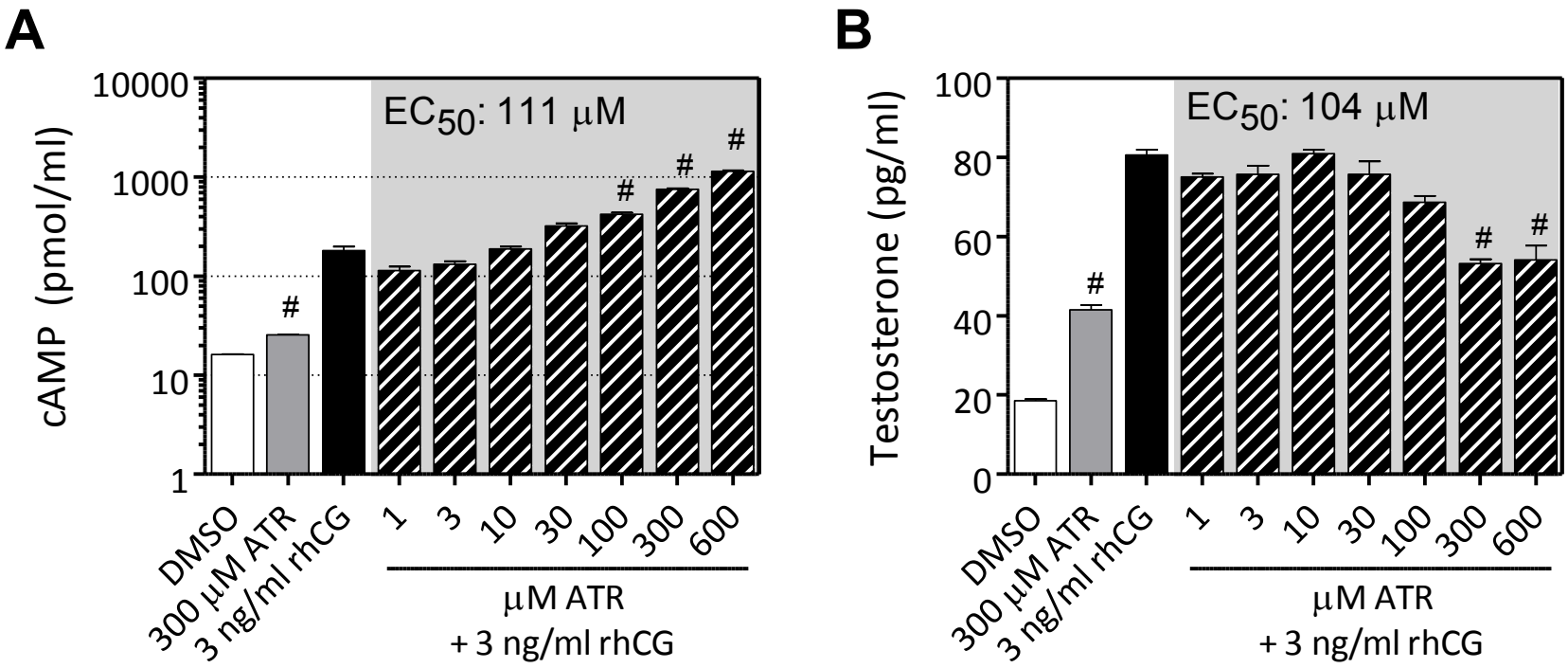

FIGURE 27. CONCENTRATION-DEPENDENT EFFECT OF ATR ON rhCG-MEDIATED INDUCTION OF STEROIDOGENESIS.

Intracellular cAMP **(A)** and T levels in media **(B)** were evaluated after 4 hrs of treatment. EC_{50} determined by sigmoidal dose-response curves fit by the ToxResponse Modeler for the co-treatment of 3 ng/ml rhCG and 1-600 μM ATR (in shaded area). Data are plotted as mean ± standard error, n=3, and were analyzed by ANOVA followed by Dunnet's *post hoc* test where $^{\#}p<0.05$ vs. rhCG. The ToxResponse modeler was used to determine EC_{50} values based on sigmoidal dose-response curve modeling.

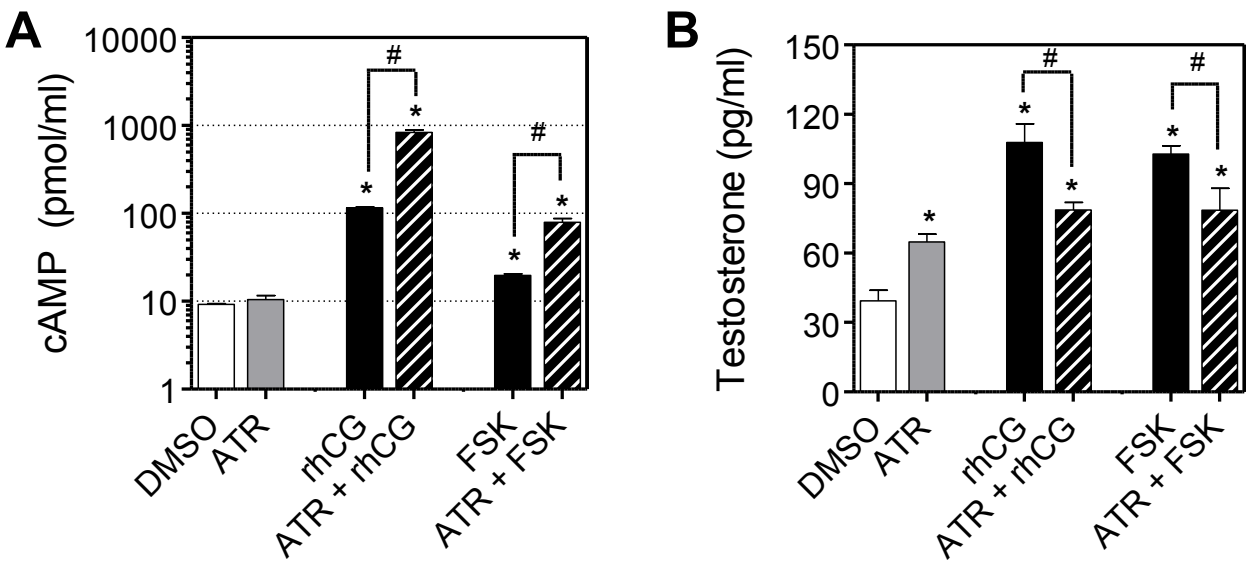

FIGURE 28. ATR-MEDIATED EFFECTS ON rhCG- OR FSK-INDUCED STEROIDOGENESIS. Intracellular cAMP **(A)**, and T levels in media **(B)** were evaluated after 4 hrs with DMSO, rhCG (3 ng/ml), FSK (10µM), ATR (300 µM), or the combinations of these treatments as indicated. Data are plotted as mean ± standard error, n=3, and were analyzed by ANOVA followed by Tukey's *post hoc* test where $^{*}p<0.05$ vs. DMSO, $^{\#}p<0.05$ vs. rhCG or FSK as indicated.

Inhibition of cAMP turnover by cPDE could also lead to elevated cAMP levels, thus the effects of ATR on intracellular cAMP levels were compared to the effects elicited by the cPDE-specific inhibitor IBMX in BLTK1 cells (Figure 29). The potentiation of rhCG-induced intracellular cAMP levels was comparable with ATR or IBMX co-treatment. However, the IBMX/rhCG co-treatment also resulted in increased T levels in contrast to the antagonism observed with ATR/rhCG co-treatment. The co-treatment of IBMX with ATR did not change T levels compared to ATR alone (Figure 29). These data suggest that cPDE inhibition elicits comparable effects as ATR on rhCG-induction of intracellular cAMP levels. However, the super-induction of

intracellular cAMP in response to rhCG co-treatment with the cPDE inhibitor IBMX does not to correlate with the antagonism of T levels observed with ATR co-treatment.

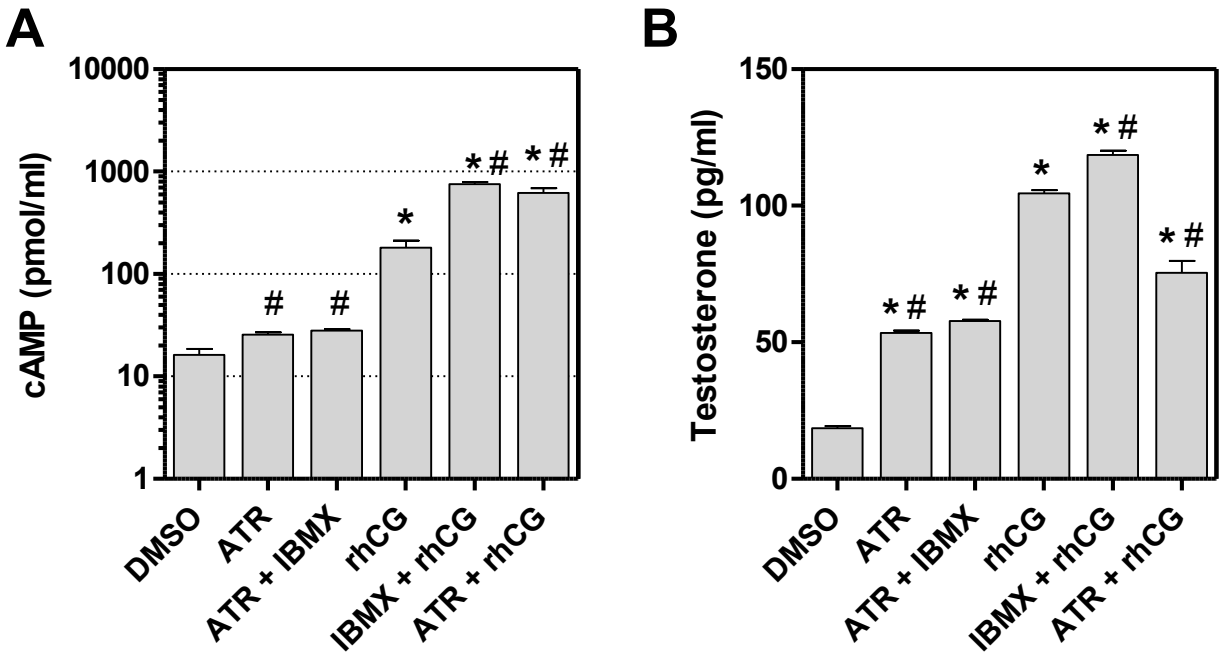

FIGURE 29. ATR AND IBMX EFFECTS ON rhCG-INDUCTION OF cAMP AND T.

Intracellular cAMP **(A)**, and T levels in media **(B)** were evaluated after 4 hrs of treatment with DMSO, rhCG (3 ng/ml), IBMX (0.2 mM), ATR (300 µM), or the combinations of these treatments as indicated. Data are plotted as mean ± standard error, n=3, and were analyzed by ANOVA followed by Tukey's *post hoc* test where *p<0.05 vs. DMSO, $^{\#}p$<0.05 vs. rhCG.

To confirm ATR inhibits cPDEs purified bovine PDEs were incubated with ATR or IBMX positive control (Figure 30). Temporal evaluation using 300 μM ATR or 30 μM IBMX for 5-60 min in the presence of cAMP substrate revealed ATR-mediated inhibition of PDE activity is most robust at early time points inhibiting cAMP turnover nearly entirely. However, at ≥30 min ATR only elicited ~60% inhibition while IBMX inhibited cAMP turnover >90% at all time points with nearly no 5'-AMP produced throughout the time course. Dose-dependent inhibition of PDE was conducted with 0.01-300 μM ATR or 0.01-200 μM IBMX for 10 min. This early time point was selected based on ATR having more robust inhibition at early time points in the time course. IC_{50}s for PDE inhibition by ATR and IBMX at 10 min were ≥98 μM and 21 μM, respectively. The highest concentration of ATR did not maximally inhibit cAMP turnover, as seen with ≥100 μM IBMX. However greater concentrations were not evaluated due to solubility limit of ATR.

To confirm that super-induction of cAMP does not underlie antagonism of T production, BLTK1 cells were treated with db-cAMP up to millimolar concentrations, comparable to intracellular cAMP levels achieved with ATR/rhCG co-treatment. Concentration-dependent db-cAMP-mediated induction of T levels reached 5-fold at 3 mM, comparable to the levels achieved with 3 ng/ml rhCG (Figure 31). These results demonstrate that millimolar concentrations of cAMP are not sufficient to antagonize T production.

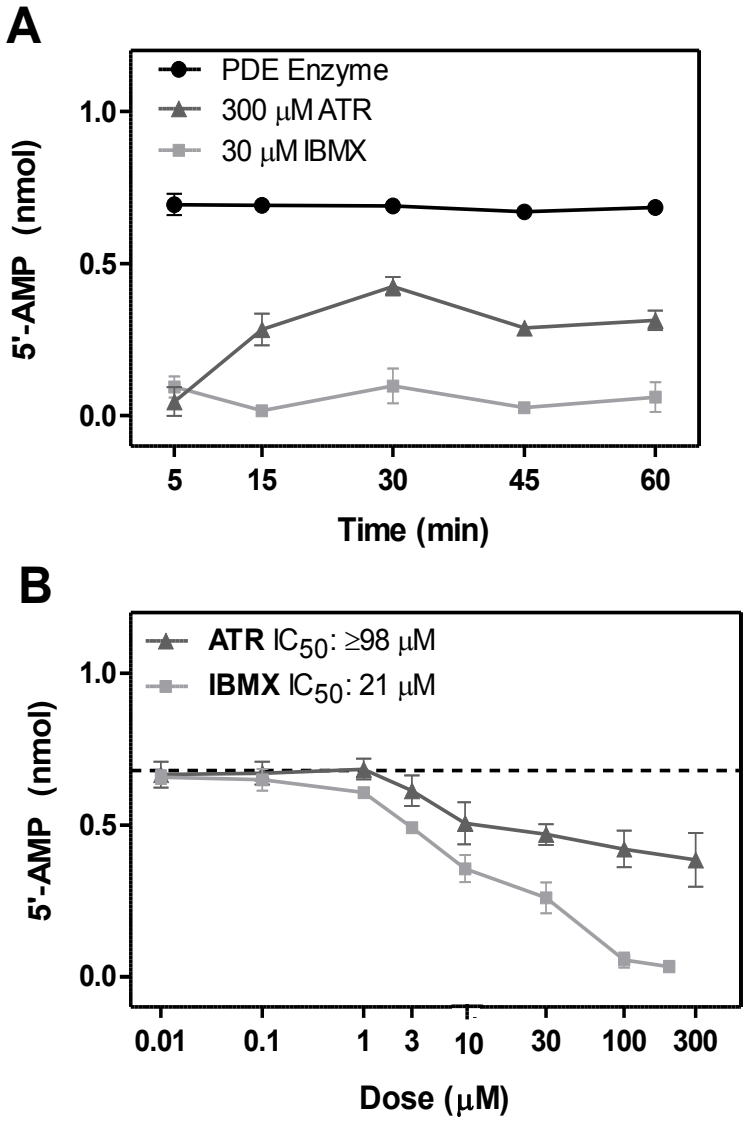

FIGURE 30. ATR-MEDIATED INHIBITION OF cPDE.

Temporal effects on PDE activity **(A)** were assessed by quantification of 5'-AMP produced upon incubation of PDE enzyme and 3',5'-cAMP substrate alone (black circles) or with the addition of 300 μM ATR (dark grey triangles) or 30 μM IBMX (light squares) for up to 60 min. Dose-dependent effects of 0.01 – 300 μM ATR (dark grey triangles) or 0.01 – 200 μM IBMX (light grey squares) were evaluated after 10 min **(B)**, with amount of 5'-AMP produced with enzyme alone (ie. 100% enzyme activity) depicted as the black hashed line. The ToxResponse modeler was used to determine IC_{50} values based on sigmoidal dose-response curve modeling.

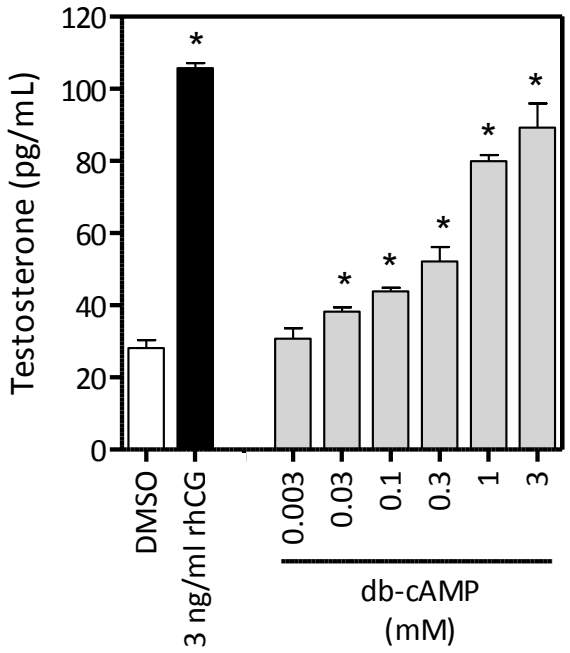

FIGURE 31. EFFECT OF db-cAMP ON T LEVELS.

Concentration-dependent induction of T levels by db-cAMP at 4 hrs in BLTK1 cells demonstrates that high mM db-cAMP concentrations do not antagonize T production. Data are plotted as mean ± standard error, n=3, and were analyzed by ANOVA followed by Dunnett's *post hoc* test where *p<0.05 vs. DMSO.

ATR-MEDIATED CHANGES IN PROTEIN PHOSPHORYLATION

Many regulators of steroidogenesis including the transcription factors steroidogenic factor 1 (SF-1) and cAMP response element binding protein (CREB), the cholesterol transporter StAR, and PDEs are activated by PKA phosphorylation (*38*). Given the critical role of PKA in the induction of steroidogenesis, the effect of ATR on PKA activity was visualized by western blot using an antibody that detects PKA consensus motif phosphorylation. Whole cell extracts from BLTK1 cells treated with DMSO, 3, 30, or 300 µM ATR for 1 hr were evaluated (Figure 32). The

results illustrate the promiscuity of PKA revealing many proteins with this phosphorylated motif. While the identities of the proteins visualized on the western blot are unknown, ATR resulted in dose-dependent effects on phospho-protein abundance. For example at ~35 kDa and ~25 kDa the abundance of phospho-proteins appears to dose-dependently increase or decrease, respectively.

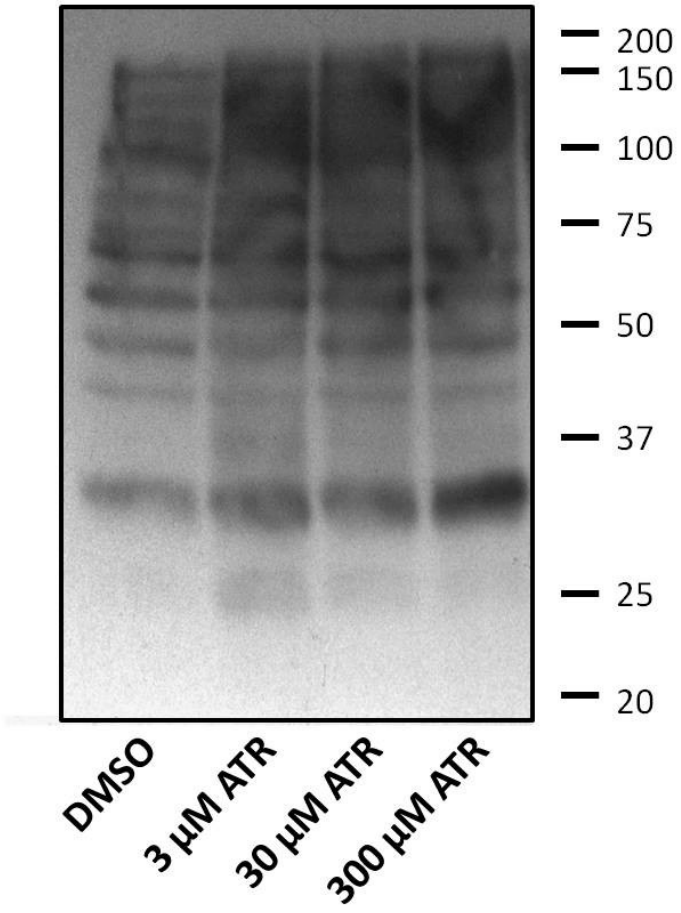

FIGURE 32. ATR EFFECTS ON PKA SUBSTRATE PHOSPHORYLATION LEVELS.
Western blot detection of phosphorylated PKA motif phospho-Ser/Thr residue with Arg at the -3 and -2 positions ((K/R)(K/R)X(S*/T*)) from whole cell BLTK1 extracts after 1 hr of treatment with DMSO or 3-300 µM ATR. Protein size (kDa) is labeled on the right. n=1.

The mitogen-activated protein kinase (MAPK) phosphorylation cascade interacts with the PKA phosphorylation network, with many shared target proteins including PDEs and StAR (39-45). More specifically, the MAPK ERK1/2 has been implicated in the regulation of steroidogenesis, by phosphorylation that activates StAR (41, 42, 44) and inhibits PDEs (40, 43). To evaluate the role of ERK1/2 phosphorylation in BLTK1 T biosynthesis, the MAPK kinase (MEK1/2) responsible for activating ERK1/2 was inhibited using U0126 (Figure 33A). U0126 completely abolished rhCG-induced T production at ≥10 μM, confirming the importance of the MEK/ERK phosphorylation cascade in BLTK1 steroidogenesis. Levels of phospho-ERK1/2 in BLTK1 cells treated with 3 ng/ml rhCG or 300 μM ATR were similar to DMSO, while the rhCG/ATR co-treated BLTK1 cells had significantly more phospho-ERK1/2 (Figure 33B). The induction of phospho-ERK1/2 in co-treated BLTK1 cells further evaluated upon co-treatment of 3-300 μM ATR with 3 ng/ml rhCG (Figure 33C). Although no dose-dependent effect was observed, it is possible that lower concentrations of ATR need to be evaluated.

DISCUSSION

Atrazine is a reproductive toxicant shown to disrupt androgen-mediated reproductive development and antagonize serum T levels in male rodents (*9-15*). With Leydig cells as the primary site of androgen steroidogenesis, the current study characterized the effects of Atrazine on BLTK1 murine Leydig T biosynthesis. The *in vitro* BLTK1 cell line is a novel model system amenable to mechanistic evaluation of steroidogenesis (*36*). Upon stimulation of Leydig cells by gonadotropin (ie. rhCG or LH) rapid increases in the intracellular secondary messenger cAMP (within an hour) and in P (~1-2 hrs) are expected, followed by increased T at later time

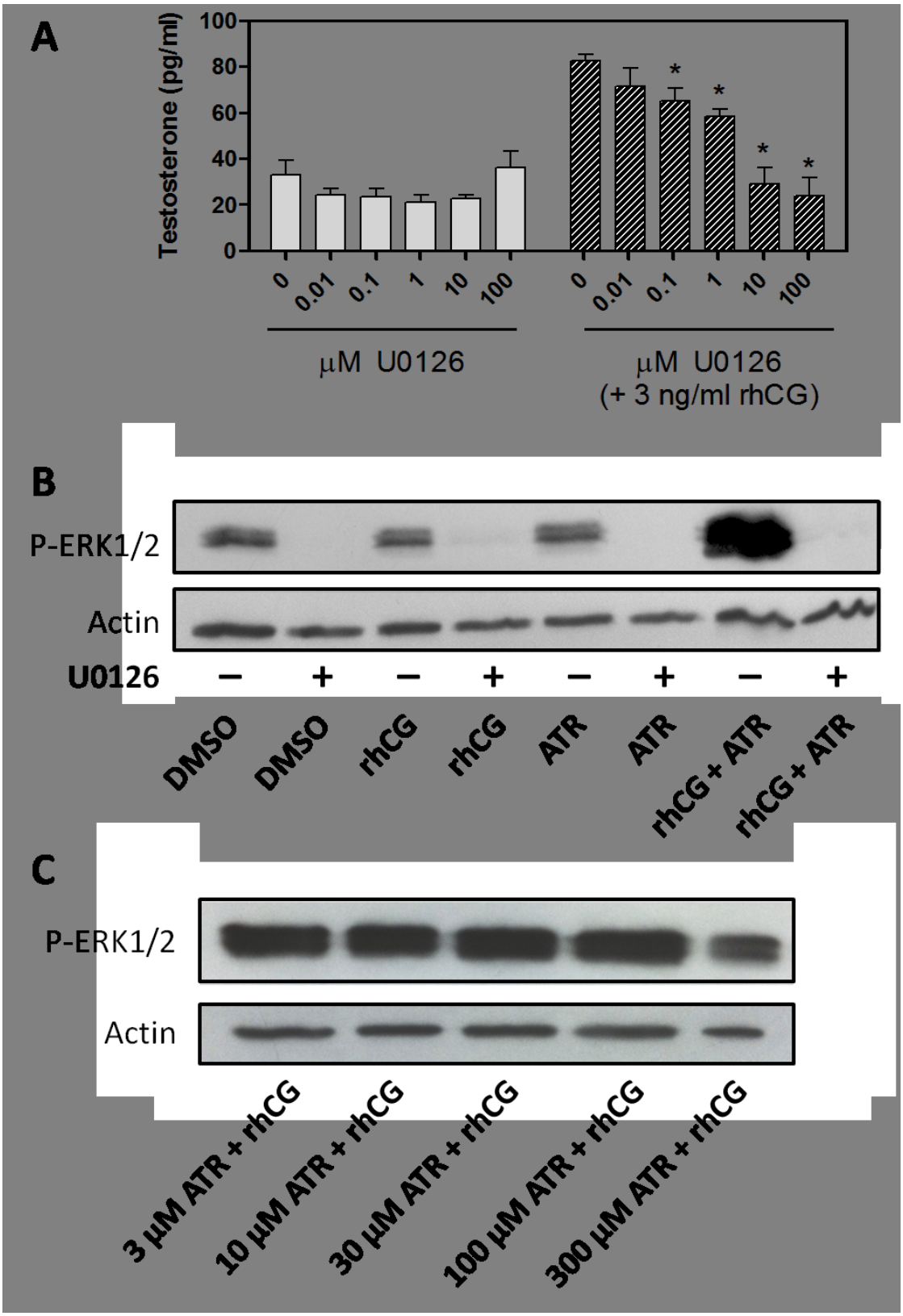

FIGURE 33. EFFECTS OF U0126 AND EVALUATION OF ERK1/2 PHOSPHORYLATION.

FIGURE 33 (cont'd)

(A) The effects of MEK1/2 inhibitor U0126 on T in BLTK1 cells was measured by EIA at 4 hrs reveal no effects on basal T (grey bars) but dose-dependent inhibition of rhCG-elicited T induction (hashed bars). Western blot detection of phosphorylated ERK1/2 (42/44 kDa) from whole cell BLTK1 extracts after 1 hr of treatment. Data are plotted as mean ± standard error, n=3, and were analyzed by ANOVA followed by Dunnet's *post hoc* test where $^{*}p<0.05$ vs. rhCG. **(B)** Comparison of DMSO, 3 ng/ml rhCG, 300 µM ATR and rhCG/ATR co-treatment with and without 10 µM U0126. **(C)** Dose-response evaluation of 3-300 µM ATR with 3 ng/ml rhCG. Membranes were stripped and probed for actin (37 kDa) as a loading control. N=1.

points (>4 hrs) (*46*). These kinetics were observed in response to rhCG treatment in BLTK1 cells, as well as in response to ATR albeit with lower efficacy, as shown previously (*35*). Interestingly, cAMP levels were potentiated when rhCG-stimulated cells were treated with ATR while both P and T levels were antagonized. The induction of P and T levels in un-stimulated (non-rhCG treated) BLTK1 cells is similar to other *in vitro* studies such as induction of T in H295R cells (*23, 24, 26*). H295R cells do not have functional GPCR-inducible steroidogenesis, as such evaluating the effect of ATR on gonadotropin stimulated steroidogenesis is not possible. Though our results suggest ATR does not target GPCR-mediated activation of adenylyl cyclase, the ability to make such determinations is unique to BLTK1 cells. In BLTK1 cells, antagonism of gonadotropin-induced T biosynthesis is consistent with most *in vivo* reports describing ATR-mediated decreases in interstitial testicular and circulating T levels (*9, 13*).

Several studies have suggested ATR is a cPDE inhibitor (*26-29, 47*). We have confirmed ATR-mediated cPDE inhibition using isolated bovine PDEs. Furthermore, in BLTK1 cells the effects on intracellular cAMP levels were comparable with ATR/rhCG and IBMX/rhCG co-treatments suggesting ATR elicits a similar cAMP induction profile as the cPDE inhibitor IBMX.

ATR-mediated cAMP accumulation in rat primary Leydig cells treated *ex vivo* was shown to be PDE4-specific (*27*). PDE4, along with PDE8, is the highest abundance cPDE in Leydig cells (*40*). In BLTK1 cells the gene expression of PDE4 isoform B (*Pde4b*) and PDE4 isoform D interacting protein (*Pde4dip*) were induced nearly 2-fold after 12-48 hrs of ATR treatment, supporting ATR-mediated effects on PDE4 in BLTK1 cells (*35*).

The results in BLTK1 cells suggest potentiated intracellular cAMP levels do not antagonize T steroidogenesis. This was confirmed by IBMX/rhCG-elicited cAMP potentiation and by db-cAMP treatment both increasing T production. Thus, accumulation of cAMP does not underlie ATR-mediated antagonism of T levels in BLTK1 Leydig cells. These data are contradictory to previous studies in primary Leydig cells where cPDE inhibition was assumed to cause decreases in T production (*26, 27*). The study by Pogrmic-Majkic *et al*. (*26*) noted cAMP accumulation and altered T production with ATR treatment; they abolished this effect with the addition of a PKA inhibitor (H-89). Since PKA activation requires cAMP binding to the regulatory subunit, the authors assumed cAMP signaling, via PKA, caused the changes in T levels. PKA activity was affected by ATR in BLTK1 cells as shown by western blot, consistent with the previous study suggesting a critical role of PKA in ATR-mediated disruption of Leydig cell steroidogenesis (*26*). However, more studies are needed to identify the targets of PKA involved. Furthermore, not all phosphorylated PKA substrate peptides increased in abundance as a function of ATR concentration, rather there appears to be at least one peptide that decreases. These data demonstrate that ATR dose-dependently affects PKA activity, and that evaluating changes in the phospho-proteome is likely important for understanding how ATR disrupts steroidogenesis. predicted activation of CREB as well as the differential expression of several SF-1 target genes

using whole-genome microarrays (*35*), supporting the hypothesis that PKA target protein activity is affected by ATR in BLTK1 cells.

Of particular interest is the interaction between PKA and PDE4, as these two enzymes have both been previously implicated in ATR-elicited disruption of steroidogenesis in Leydig cells (*26, 27*). In fact, both PKA and PDE4 are found bound to the same scaffold protein, A kinase anchor protein (AKAP; Figure 34) (*40-43, 48, 49*). Scaffolding by AKAP keeps PKA near its target proteins at specific subcellular locations to optimize spatiotemporal regulation of signaling pathways in one complex (*41, 42, 48*). Furthermore, MAPK association with AKAP complexes, namely MEK and ERK association, has also been demonstrated (*39, 41, 48, 50*). As a result, the tightly controlled activity of regulatory enzymes is accomplished by a complex of AKAP-scaffolded proteins. For example, PDE4 is phosphorylated at two unique sites: activated by PKA-mediated phosphorylation in the up-stream conserved region near the N-terminus but inhibited by ERK1/2-mediated phosphorylation in the catalytic region near the C-terminus (*40, 43, 45, 49, 51*). Additionally, the rate limiting step of steroidogenesis, mobilization of cholesterol to the mitochondria by StAR, also requires phosphorylation by PKA, which is proposed to be facilitated by interactions between PKA, StAR and AKAP at the mitochondria in Leydig cells (*42, 52*). Activation of several other important steroidogenic regulatory proteins such as CREB, SF-1, and StAR are also mediated by PKA and/or MAPK phosphorylation (*41, 44, 52-54*). We have shown

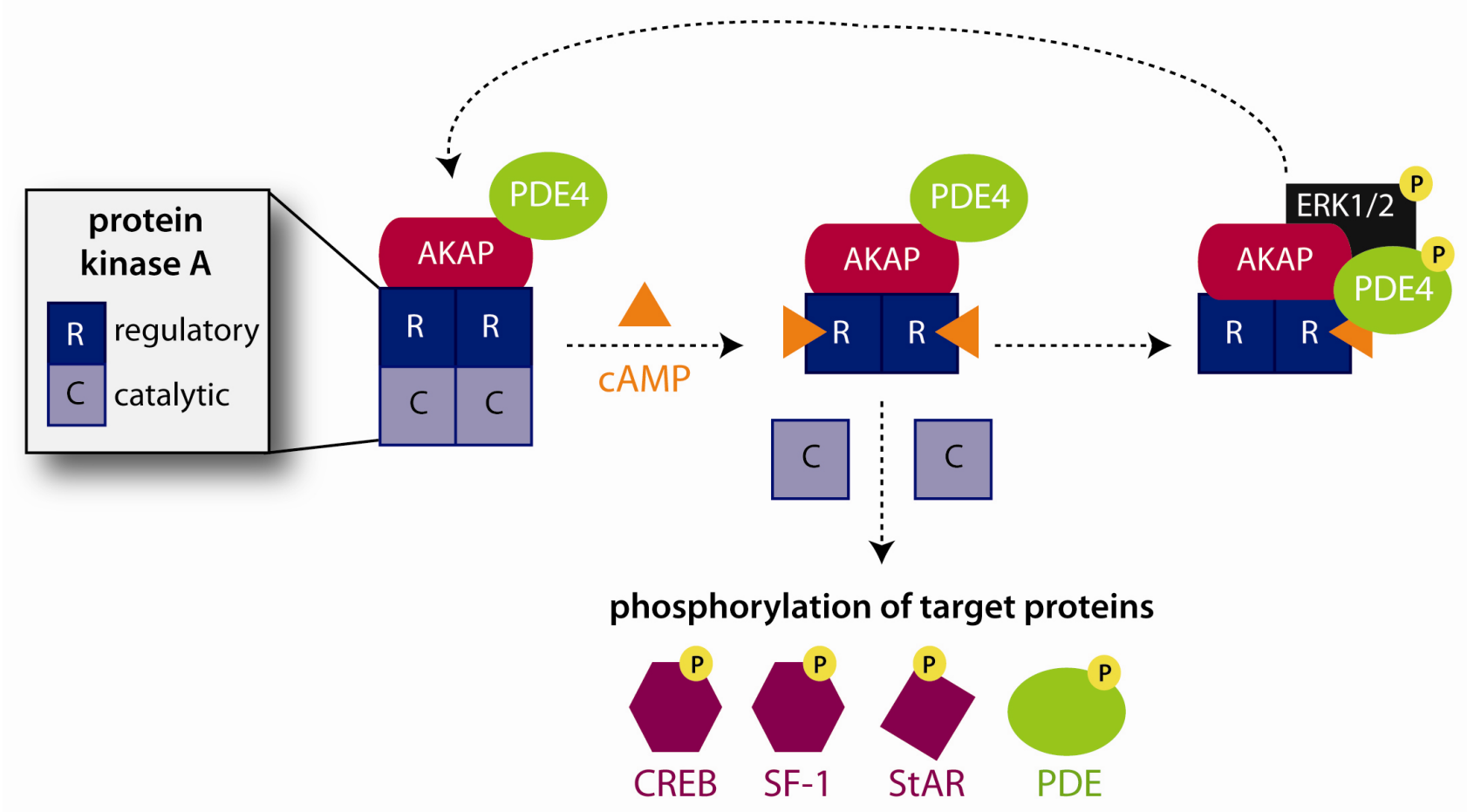

FIGURE 34. SIMPLIFIED SCHEME OF PKA ACTIVATION AND PKA INTERACTING PROTEINS.

Inactive PKA, comprised of two regulatory (R) and two catalytic (C) subunits, exists in the cytosol where it is in complex with AKAP which serves as a scaffolding protein. PKA is activated upon binding of cAMP to (R) and subsequent release of (C) which phosphorylates target proteins including key steroidogenic regulators including CREB, SF-1, StAR and cPDEs. PKA-mediated phosphorylation of PDE4, also known to interact with the AKAP scaffold, activates PDE4 resulting in the turnover of cAMP and deactivation of PKA. PDE4 can also be phosphorylated by phospho-ERK1/2 at an alternate site, resulting in inhibition of its activity.

In summary, the current study presents the novel hypothesis that ATR elicits disruption of steroidogenesis in Leydig cells by altering PKA- and MAPK-mediated phosphorylation. Specific target proteins of interest include StAR and PDE4, both of which can be phosphorylated by PKA and ERK1/2 (*42, 44, 45, 51*). Our results support previous suggestions of ATR-mediated cPDE inhibition and consequently accumulation of intracellular cAMP. However, the results presented herein demonstrate that potentiation of cAMP levels does not cause antagonism of T production in BLTK1 Leydig cells. Future studies will seek to identify the target proteins of PKA showing altered phosphorylation status in an ATR-dependent manner and evaluate AKAP-interacting proteins that may affect PKA and ERK activity contributing to the disruption of steroidogenesis.

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CHAPTER 6

CHAPTER 6

CONCLUSIONS AND FUTURE RESEARCH

The preceding studies describe the establishment, characterization, and application of a novel BLTK1 murine Leydig cell model for the evaluation of EDC-mediated effects on steroidogenesis. BLTK1 Leydig cells offer a physiologically relevant, gonadotropin-inducible *in vitro* system amenable to mechanism determination. Evaluation of the triazine CMG chemicals ATR, PRO, SIM, DEA, DIA and DACT confirmed their equipotency for sterodogenic disruption, but highlighted differences in their efficacy. Furthermore, evaluation of ATR as the representative triazine revealed effects on both the short and long-term phases of steroidogenesis. The short-term effects include potentiation of rhCG-induced intracellular cAMP levels resulting from inhibition of cPDEs and changes in PKA-mediated phosphorylation. The long-term effects include the differential expression of genes associated with cholesterol metabolism, regulation of hormone levels and tissue morphology as well as the predicted activation of AR and CREB. The work embodied in this dissertation presents a new model for the evaluation of steroidogenesis that can be of great value to screen for reproductive and developmental toxicants and novel findings regarding a suggested mode of action for triazine-mediated disruption of steroidogenesis.

BLTK1 MURINE LEYDIG CELL MODEL FOR STEROIDOGENESIS

BLTK1 Leydig cells were characterized as a novel steroidogenic model for the evaluation of reproductive and developmental toxicants. BLTK1 cells maintain the enzymes and regulatory proteins necessary for steroidogenesis, biosynthesizing androgens and estrogen from cholesterol in response to gonadotropin stimulus. Having a physiologically relevant *in vitro* model for evaluating steroidogenesis allows for mechanism of action elucidation by using molecular approaches that are not feasible *in vivo*. Though *in vitro* models are routinely used to investigate mechanism of action, endocrine disruption is rarely limited to one cell type or tissue. For example, triazines target the HPG axis by not only altering gonadal steroidogenesis but also by disrupting LH surge in rodents. Studies evaluating ATR-mediated effects in the hypothalamus or pituitary should also be evaluated to further characterize the effects of triazine on the HPG. Similar cAMP- and MEK-mediated phosphorylation cascades regulate both GnRH and LH release(*1, 2*), with PDE4 expression throughout the brain ATR may elicit potentiation of cAMP in hypothalamus, however PDE10 is the only characterized cPDE in the pituitary (*3*).

TRIAZINE EFFECTS ON PROTEIN PHOSPHORYLATION

Triazines may alter T biosynthesis in BLTK1 Leydig cells by disrupting PKA and MAPK phosphorylation. However, the specific target proteins involved have not yet been identified. The delineation between cAMP accumulation and disruption of T production described in Chapter 5 is a novel finding that requires further examination. The hypothesis that ATR interferes with AKAP-tethered protein interaction complexes affecting the phosphorylation of

interacting proteins can be evaluated as antibodies are readily available for specific phospho-proteins including PDE4. Additionally, mass spectrometry-based proteomic approaches can be used to identify the PKA-targeted proteins. For example, the antibody used to visualize proteins with PKA motif phosphorylation by western blot in Chapter 5 could be used for immunoprecipitation to isolate target proteins with PKA phosphorylation. The extracted proteins can be analyzed by mass spectrometry to attempt protein identification. Comparison of proteins found in ATR-treated BLTK1 extracts compared to controls or rhCG-treatment can reveal treatment-mediated effects. The integration of protein phosphorylation data with the whole-genome microarray data in Chapter 4 and the characterized time- and dose-dependent effects on P and T levels measured by EIA in Chapter 5 would provide a more complete characterization of ATR's mechanism underlying disruption of BLTK1 steroidgenesis.

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