PCR purification kit (Qiagen, Valencia, CA). We used 1 μL from a 30-μL DNA extraction for PCR (28 cycles) and primed by sequences as follows: forward, 5′-GGG AAG AAG ATT GCC TAA AC-3′; reverse, 5′-TGT GGA AAT CAA AGG GAC AG-3′; the PCR size was 401 bp. Immunoprecipitated DNA samples were then set to real-time PCR analysis to quantify the relative amount to their corresponding input controls with a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 1 μL immunoprecipitated DNA sample (or H_2O as negative control), was placed into a 20-μL reaction volume containing 1 μL of each primer (10 μM) and 2 μL LightCycler-FastStart DNA Master SYBR Green I (Roche), which includes nucleotides, Tag DNA polymerase, and buffer. Input samples were amplified simultaneously as the internal controls. Real-time PCR data for each immunoprecipitated sample were calculated as a ratio to the corresponding input sample. Briefly, threshold values (crossing line) obtained where fluorescent intensity was in the geometric phase, cycle number at the crossing point of an immunoprecipitated sample (Cip), and the corresponding input sample (Cco) were determined via LightCycler software, version 3.5. The relative amount of the immunoprecipitated sample (Aip) to input sample was calculated by the formula $Aip = 2^{(Cco - Cip)}$.

We also performed deletion–mutation assays on the 4.0 kb ArPII to identify the responsible site for atrazine and simazine stimulation. The promoter was cut down using restriction enzymes (*Sna*BI, *Afl*II, or *Eco*RI) to make three other ArPII reporters with lengths of 3.1 kb, 2.0 kb, and 1.0 kb, respectively. Responsiveness was indicated by 10^{-5} mol/L atrazine-induced multiples of relative luciferase activity (RLA) mediated by each promoter. The 516 bp ArPII luciferase reporter (PGL3-PII-516) and the PGL3-PII-516-SF1-M (of which the SF-1 site was mutated from AGGTCA to ATTTCA) were provided courtesy of E.R. Simpson (Monash University, Melbourne, Australia) (Rubin et al. 2002).

Surface plasmon resonance. We used baculovirus to express the Flag containing SF-1 fusion protein in insect sf21 cells. The baculovirus mouse SF-1 expression vector was established as described previously (Komatsu et al. 2004). Sf21 cells were infected with baculovirus, and extracts were prepared 72 hr postinfection. The Flag-SF-1 fusion protein was purified by affinity chromatography with anti-Flag M2 antibody-agarose (Sigma, St. Louis, MO, USA) and eluted with 150 μg/mL 3 × Flag peptide (Sigma). The binding affinity of atrazine to Flag-SF-1 was measured by Surface Plasmon Resonance (SPR) using a Biocore T100 biosensing system (Biocore, Tokyo, Japan) following the standard manufacturer's protocol. The Biocore T100 can investigate interactions involving binding partners with molecular weight as low as 100 Da. Purified Flag-SF-1 protein (11700–14250 resonance units) was immobilized on a Series S Sensor Chip (CM5; Biocore) by using the Amincoupling kit (Biocore). Chemicals [atrazine as the one of interest; 1,2-dihexadecanoyl-*n*-glycero-3-phosphocholine (16PC) as the positive control; benzophenone and *p*-nitrotoluene as negative controls) were dissolved in PBS (pH 7.4) + 5% DMSO at various concentrations. PBS + 5% DMSO was used as the mobile phase medium (running buffer) at a flow rate of 30 μL/min at 25°C. Binding of chemicals dissolved in the running buffer to immobilized Flag-SF-1 was monitored in real time by measuring changes in resonance units. The sensorgrams for the reference channel (non-SF-1–bearing CM5 chip) were subtracted simultaneously from the sensorgrams for sensing channel (SF-1 bearing CM5 chip). All data were automatically analyzed by Biocore T100 evaluation software (version 1.00).

Quartz crystal microbalance. We examined binding of atrazine to SF-1 using a 27-MHz quartz crystal microbalance (QCM; Initium Co., Tokyo, Japan). SF-1 was immobilized onto a QCM electrode according to the manufacturer's protocol. The electrode was soaked in 8 mL PBS buffer (pH 7.4) and monitored continuously for QCM frequency change at 25°C. After the frequency change was stabilized, chemicals of interest were added to the solution and we assessed the time course of frequency change in response to the addition of chemicals.

Statistical analysis. All data are expressed as the mean ± SD and were evaluated by one-way analysis of variance (ANOVA) or two-tailed Student's *t*-test, followed by post hoc comparisons with Fisher's protected least-significant-difference test. $p < 0.05$ was considered statistically significant.

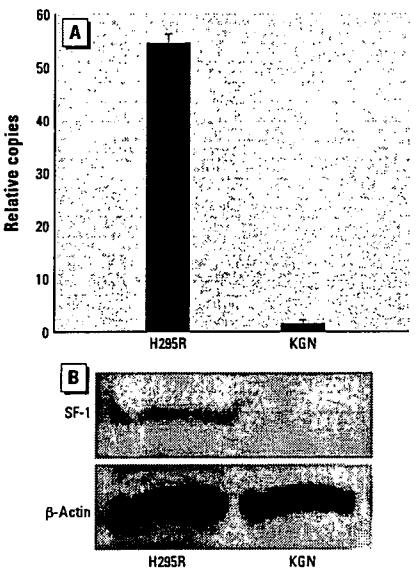

Figure 1. Effects of SF-1 on induction of *CYP19* by 10^{-5} mol/L atrazine. (*A*) SF-1 expression was significantly higher (54-fold; ANOVA, $p < 0.05$) in atrazine-responsive H295R cells compared with atrazine-nonresponsive KGN cells. (*B*) SF-1 protein levels were also higher in H295R cells as determined by Western blot.

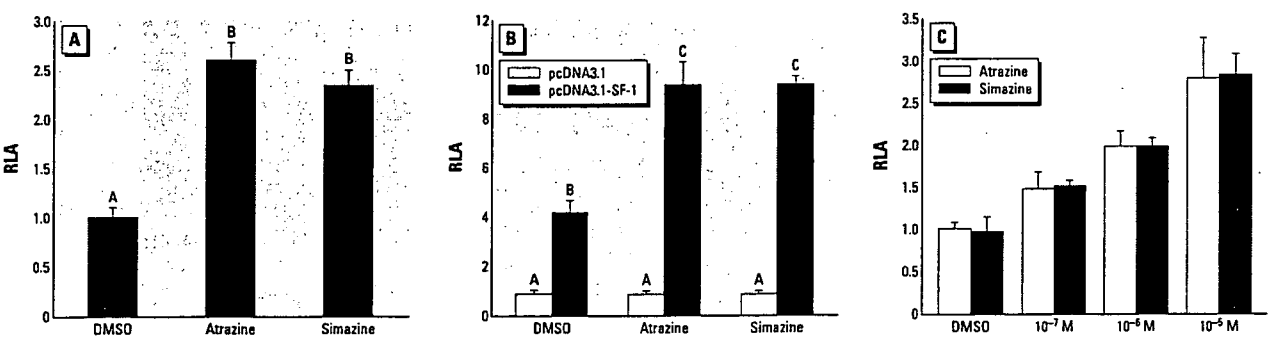

Figure 2. Effects of atrazine and simazine (10^{-5} mol/L each for *A* and *B;* as marked for *C*) of three cell types, measured by RLA. (*A*) Atrazine and simazine stimulated ArPII in H295R cells without exogenous SF-1 supplementation. (*B*) ArPII response to atrazine and simazine in NIH-3T3 cells required coexpression of SF1. (*C*) Atrazine and simazine stimulation of SF-1–mediated ArPII in SF-1–co-transfected NIH-3T3 cells was dose dependent. Both triazines were effective at concentrations as low as 10^{-7} mol/L (ANOVA, $p < 0.05$). Bars show mean ± SD; letters above bars indicate statistical groups (ANOVA, $p < 0.05$).

Results

Experiment 1. Although the nonresponsive KGN granulosa cells expressed SF-1 mRNA, real-time PCR revealed that copy numbers of SF-1 mRNA were 54 times lower in these atrazine nonresponsive cells compared with the atrazine-responsive H295R adrenocortical carcinoma cells (ANOVA, $p < 0.05$; Figure 1A). A concomitant Western-blot analysis revealed markedly higher SF-1 protein levels in atrazine-responsive H295R adrenocortical carcinoma cells compared with the nonresponsive KGN granulosa cells (Figure 1B).

Experiment 2. Atrazine and simazine (a similar triazine herbicide) both induced luciferase activity in H295R adrenocortical carcinoma cells (which express SF-1 endogenously) without co-transfection of SF-1 (ANOVA, $p < 0.05$; Figure 2A). Neither atrazine nor simazine affected ArPII in the absence of SF-1 coexpression in NIH/3T3 fibroblast cells, which lack endogenous SF-1 expression (ANOVA, $p > 0.05$; Figure 2B). Once SF-1 was present (pcDNA3.1-hSF-1–co-transfected cells), however, there was a 4.2-fold elevation in basal activity and increased responsiveness to atrazine and simazine (ANOVA, $p < 0.05$; Figure 2B). Both atrazine and simazine increased SF-1–enhanced ArPII activity 2.25- and 2.26-fold, respectively (ANOVA, $p < 0.05$; Figure 2B) in the pcDNA3.1-hSF-1–transfected NIH/3T3 cells. The 2.2- and 2.3-fold are similar to the chlorotriazine-stimulation of ArPII activity in H295R (SF-1 nontransfected). Thus, the ArPII response to atrazine and simazine is SF-1 dependent. Furthermore, in these SF-1 coexpressing NIH/3T3 cells, the atrazine/simazine stimulation of SF-1–mediated ArPII was dose dependent, with both chemicals effective at concentrations as low as 10^{-7} mol/L (ANOVA, $p < 0.05$; Figure 2C).

Experiment 3. We previously showed that activation of the cAMP-PKA signal potentiates SF-1 transactivation by modifying the interactions between SF-1 and its cofactors, and PKA-induced activation of ArPII requires SF-1 (Fan et al. 2004). We studied whether any other environmental contaminants affected PKA-enhanced SF-1–mediated ArPII expression. In a luciferase reporter system, in which the 4.0-kb human ArPII luciferase reporter and pcDNA3.1-hSF-1 were coexpressed in NIH-3T3 fibroblast cells, 55 known environmental hormone chemicals were screened; among them, atrazine and the related triazine, simazine, stimulated the forskolin-enhanced SF-1–mediated ArPII expression by a factor of two to three (ANOVA, $p < 0.05$; Figure 3). As shown in Figure 2, the results were repeated and confirmed in the absence of forskolin. A third chemical, benzopyrene, also significantly induced luciferase ($p < 0.05$) but was less potent than the two triazines. Nonylphenol, di-*n*-butyl phthalate (DBP), dicyclohexylpthalate (DCHP), fenevalerate, and octylphenol all decreased luciferase activity (ANOVA, $p < 0.05$; Figure 3).

Experiment 4. Adeno-SF-1–infected KGN ovarian granulosa cells had an elevated basal level of aromatase expression (ANOVA, $p < 0.05$) and showed a 3.78- and a 4.94-fold increase in responsiveness to atrazine and simazine, respectively (ANOVA, $p < 0.05$; Figure 4A). The control (adeno-lacZ) vector had no effect (ANOVA, $p > 0.05$; Figure 4A). Thus, exogenous SF-1 conferred aromatase responsiveness to atrazine and simazine in otherwise atrazine-nonresponsive KGN granulosa cells. Furthermore, adeno-SF-1–infected KGN ovarian granulosa cells showed a significant increase in aromatase activity (ANOVA, $p < 0.05$; Figure 4B) as determined by a tritium release assay. Thus, in line with the results of promoter assays shown in experiment 2, the mRNA expression of CYP19, as well as the

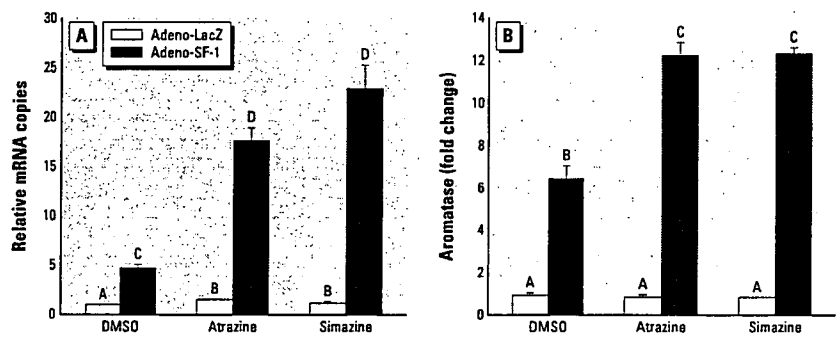

Figure 4. Effects of adeno-SF1 on responsiveness of KGN cells (mean ± SD) to 10^{-5} mol/L atrazine or 10^{-5} mol/L simazine. (*A*) Basal aromatase mRNA (*CYP19*; relative copies) was significantly increased in cells transfected with adeno-SF-1 relative to controls infected with adeno-LacZ. (*B*) Aromatase enzymatic activity (fold change) also increased in response to atrazine or simazine in adeno-SF-1 infected KGN cells, but not in the control adeno-LacZ infected cells. Letters above bars indicate statistical groups (ANOVA, $p < 0.05$).

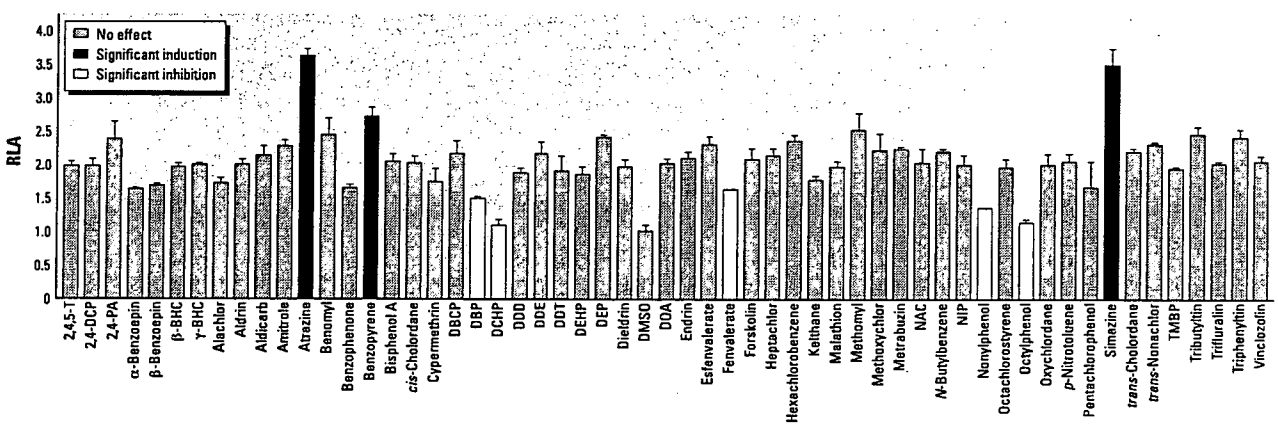

Figure 3. RLA (mean ± SD) of endocrine disruptors that affect forskolin-enhanced SF-1–mediated ArPII presented as chemicals with no effect on luciferase activity, those with significantly induced luciferase activity, or those with significantly inhibited luciferase activity as determined by ANOVA, followed by Fisher's protected least-significant-difference post hoc test ($p < 0.05$). Abbreviations: 2,4-DCP, 2,4-dichlorophenol; 2,4-PA, 2,4-dichlorophenoxy acetic acid; BHC, benzene hexachloride; DBCP, dibromochloropropane; DDD, 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane; DDE, 1,1-dichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethylene; DDT, 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane; DEHP, di-2-(ethyl-hexyl)-phthalate; DOA, dioctyl adipate; NAC, *N*-acetylcysteine; NIP, dinitrophenyl-phosphorothioate; TMBP, 4-(1,1,3,3- tetramethylbutyl) phenol. All chemicals were examined at ecologically relevant concentrations: 10^{-5} mol/L for all chemicals, except tributyltin and triphenyltin, which were examined at 10^{-7} mol/L.

enzymatic activities of aromatase, becomes responsive to both atrazine and simazine when SF-1 is exogenously expressed in KGN cells, which are otherwise atrazine nonresponsive.

Experiment 5. ArPII DNA sequences in the chromatin immunoprecipitates were significantly enriched by either simazine or atrazine, demonstrating that the triazines enhanced binding of SF-1 to ArPII (ANOVA, $p < 0.05$; Figure 5A, B). The responsiveness to atrazine and simazine was well-preserved when ArPII was reduced to 516 bp; however, when the SF-1 binding site (AGGTCA) was mutated to ATTTCA (a treatment that impairs SF-1 binding to ArPII), the responsiveness to atrazine and simazine was eliminated (ANOVA, $p < 0.05$; Figure 5C).

Experiment 6. The known SF-1 ligand 16PC was examined as a positive control for comparison with atrazine and two negative controls (*p*-nitrotoluene and benzophenone). A dose-dependent interaction between the control ligand (16PC) and immobilized SF-1 was observed by SPR (Figure 6A). Of the test ligands, only atrazine caused a significant (Figure 6B) and dose-dependent SPR response (Figure 6C). A quartz-crystal microbalance study confirmed that both 16PC and atrazine bound SF-1, whereas the solvent and negative control *p*-nitrotoluene did not (Figure 6D). The atrazine response was significantly lower than the positive control (16PC), but experiments such as scintillation proximity assays using radioactively labeled atrazine are required to determine the precise dissociation constant.

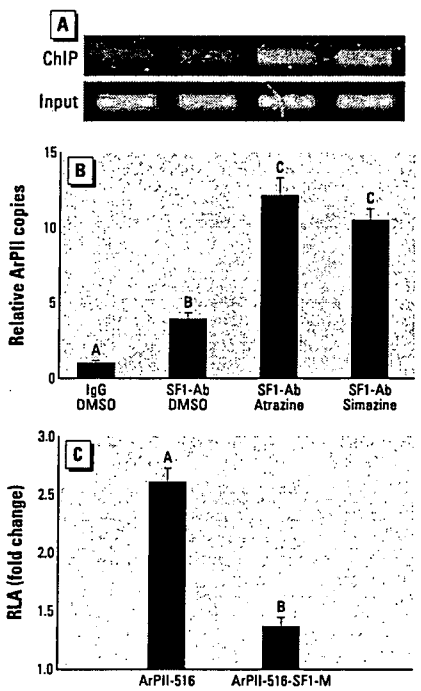

Figure 5. Effects of atrazine and simazine (10^{-5} mol/L) on SF-1 binding to ArPII. Atrazine and simazine enhanced SF-1–ArPII interactions in H295R cells as determined by common PCR (28 cycles; *A*) and as quantified by real-time PCR (*B*); mutation of the SF-1 binding site on ArPII significantly reduced responsiveness to atrazine. The responsiveness to atrazine and simazine was well preserved when ArPII was reduced to 516 bp (ArPII-516), but responsiveness was lost when the SF-1 binding site was mutatated to ATTTCA (ArPII-516-SF1-M) (*C*). In (*B*) and (*C*), bars show mean ± SD. Letters above bars indicate statistical groups (ANOVA, $p < 0.05$).

Discussion

Atrazine increases aromatase by binding to and inhibiting phosphodiesterase (Roberge et al. 2004; Sanderson et al. 2000, 2001), resulting in elevated cAMP. Elevated cAMP results in increased transcription of *CYP19*, increased aromatase activity, and ultimately increased estrogen production. Although the effects of atrazine on aromatase vary between cell lines and tissues, the current study explains this variation. There are six tissue- and cell-specific aromatase promoters in humans. Atrazine affects aromatase expression only in cell and tissue types that use the SF-1–dependent ArPII promoter. Tissue types and cell lines that do not respond to atrazine are those types that do not utilize ArPII or that do not express SF-1.

In addition to elucidating the role of SF-1 in atrazine-induced aromatase expression and explaining variation in responses between cell types, we developed an assay for detecting endocrine disruption via aromatase induction and for distinguishing chemicals that alter aromatase activity from those that alter aromatase expression. We confirmed that atrazine and simazine induce aromatase, consistent with the literature (Heneweer et al. 2004; Sanderson et al. 2000, 2001, 2002). The reduction in aromatase by phthalates (DCHP and DBP) and octylphenol is consistent with their previously reported reduction in estrogen synthesis (Davis et al. 1994; Kim et al. 2003; Lovekamp and Davis 2001). Vinclozolin (which had no effect in the present study) increases aromatase, but at concentrations 10 times higher than used here (Sanderson et al. 2002). The other chemicals examined have not been previously examined for effects on aromatase.

The role of SF-1 we identified in the present study is important. Orphan receptors, such as SF-1, bind their response elements and regulate transcription constitutively, but ligand-binding may enhance their activity. To date, only phospholipids (Krylova et al. 2005; Li et al. 2005) have been identified as endogenous ligands for SF-1. Here, we show that atrazine not only elevates cAMP, which also increases SF-1 expression (Heneweer et al. 2004; Lehmann et al. 2005; Roberge et al. 2004; Sanderson et al. 2000), but also binds SF-1 and increases its interaction with ArPII.

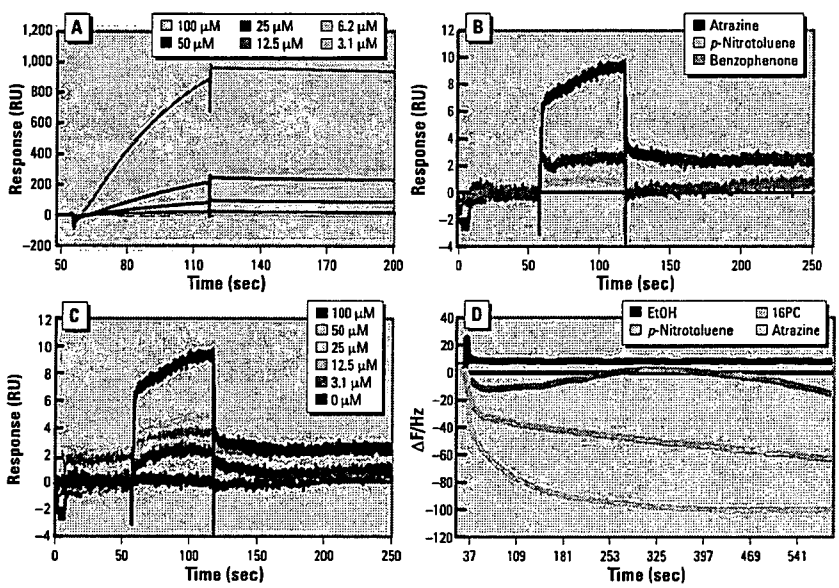

Figure 6. Kinetic analysis of atrazine binding to SF-1 protein as analyzed by SPR [changes in mass concentration are detected as differences in the refractive index and shown in resonance units (RU)]. (*A*) Sensorgrams of six concentrations of 16PC (the positive control ligand) added to SF-1. (*B*) Sensorgrams showing atrazine, benzophenone, or *p*-nitrotoluene (100 µM each) binding to immobilized SF-1 (only atrazine caused a significant response). (*C*) Sensorgrams of six concentrations of atrazine added to the immobilized SF-1 (note the dose-dependent response). (*D*) Sensorgrams showing interactions between atrazine and SF-1 on a quartz-crystal microbalance. 16PC caused a clear decrease in frequency, demonstrating binding between the ligand and SF-1; atrazine also substantially decreased the frequency, but ethanol (EtOH) and the negative control *p*-nitrotoluene did not.

The findings of the present study are important for understanding the negative impact that atrazine contamination has on both environmental and public health. In addition to its vital roles in the reproductive system, the atrazine-responsive ArPII is also critically involved in breast cancer oncogenesis. The role of estrogen in breast cancer and the potential role of atrazine exposure is consistent with increased plasma estrogen levels in several strains of rats when exposed to atrazine (Eldridge and Wetzel 1999; Eldridge et al. 1994a, 1994b; Stevens et al. 1994; Stoker et al. 2000; Wetzel et al. 1994) and the increased incidence of estrogen-dependent mammary cancers in rodents (Eldridge et al. 1994a; Stevens et al. 1994; Wetzel et al. 1994). Further, Ueda et al. (2005) showed that atrazine-induced tumors in rodents are estrogen-receptor positive. The findings in rodents are consistent with increased aromatase expression and activity in human cell lines in the present study and in previous studies (Heneweer et al. 2004, Sanderson et al. 2000, 2001) and in human tissues (Roberge et al. 2004). The estrogen that stimulates breast cancer growth in humans is derived from both ovarian and extraovarian sources. Local estrogen production, which contributes to hormonal stimulation of breast cancers in breast adipose tissue and fibroblasts, is also dependent on ArPII (Bulun et al. 2005). This extraovarian estrogen plays a profound mitogenic role in breast tumors (Bulun et al. 2005): Local estrogen levels in breast tumors can be 10 times higher than that in the circulation of postmenopausal women (Van Landeghem et al. 1985), likely due to a critical shift in promoter usage from I.4 (used in normal adipose tissue) to ArPII (abnormally activated in breast adipose tissue containing a tumor) (Agarwal et al. 1996; Harada et al. 1993). Although normal breast tissue does not typically utilize ArPII, once transformed, breast cancer cells (malignant epithelial cells) induce use of ArPII in adjacent fibroblasts (Agarwal et al. 1996; Harada et al. 1993; Utsumi et al. 1996; Zhou et al. 1996). In this regard, the ability of atrazine to stimulate ArPII is extremely significant. Atrazine increases the incidence of mammary cancer in rodents (Eldridge et al. 1994a; Stevens et al. 1994; Wetzel et al. 1994), and at least one cohort study in humans showed that atrazine is associated with breast cancer in women whose well water is contaminated with atrazine (Kettles et al. 1997).

Atrazine induces prostatitis (Stoker et al. 1999) and prostate cancer in rats (Pintér et al. 1980) and was also associated with an 8.4-fold increase in prostate cancer in men working in an atrazine production facility in San Gabriel, Louisiana (Maclennan et al. 2002; Sass 2003). Although typically considered androgen dependent, prostate cancer is also estrogen dependent and is associated with increased local estrogen production (Christensen and Lephart 2004; Ellem et al. 2004; Härkönena and Mäkelä 2004). Aromatase expression and activity are low in normal prostate cells, but in malignant cells in the prostate, they increase to levels comparable with those observed in breast cancer (Prins and Birch 1997). This aromatase activity is associated exclusively with the atrazine-regulated ArPII and renders the popular antiandrogen treatments for prostate cancer useless (Ellem et al. 2004). Also, low levels of estrogen, when bound to estrogen receptor-α (ER-α), result in proliferation of the prostate (Ellem et al. 2004). Thus, in prostate cancer, induction of aromatase via ArPII in prostate epithelia results in estrogen synthesis that, in turn, affects the prostate epithelia in an autocrine/intracrine fashion via binding to ER-α. Further, elevated estrogens during early development inhibit prostate growth but predispose individuals to prostate disease later in life (Prins and Birch 1997; Pylkkanen et al. 1993).

In addition to atrazine's contribution to adverse health outcomes in humans, the effect of atrazine on cells and tissues that utilize ArPII is also significant, because all vertebrates utilize ArPII during gonadal differentiation and development (Simpson et al. 1994, 2002). In fact, the activity of atrazine and simazine at 10^{-7} M (21.57 mol/L) in the present study is in the range that chemically castrates and feminizes male amphibians (0.1–20 ppb; 1 ppb = 1 mol/L) (Carr et al. 2003; Hayes 2004, 2005; Hayes et al. 2002a, 2002b, 2003, 2006a, 2006b; McKoy et al. 2002; Miyahara et al., unpublished data; Reeder et al. 1998; Tavera-Mendoza et al. 2002) and fish (6 ppb) (Moore and Waring 1998). Thus, the present study is also significant because the effects in wildlife likely occur through the same molecular mechanisms as documented here; however, the cell lines and molecular tools are not available for wildlife species to examine these effects on this same level.

Considering the prevalence of atrazine in the environment, the continued rise of cancer as the leading cause of death in the United States (breast cancer and prostate cancer are the most common cancers in men and women, respectively), the present findings raise concern for the impact of atrazine on environmental and public health. This is especially troubling because African Americans and Hispanic Americans, more likely to be occupationally exposed to pesticides and less likely to have proper access to health care, are two to four times more likely to die from breast and prostate cancer, respectively.

The fact that aromatase inhibitors have proven effective at treating breast cancer induced by adipose aromatase (Brodie et al. 1999) and promise to have similar therapeutic value in endometriosis (Shippen and West 2004) underscores the potential role that atrazine (an aromatase inducer) plays in increasing the risk of these diseases: Aromatase expression and estrogen production is exclusively regulated by ArPII and is SF-1 dependent in endometriosis, a disease that affects 4–6 million women (10% of American women) per year (Bulun et al. 2005). Further, overexpression of aromatase plays a role in other diseases including uterine fibroids (uterine leiomyomata), aromatase overexpression syndrome (Bulun et al. 2005), and polycystic ovarian syndrome (Pierro et al. 1997). The many mammalian tissues and cell types that express SF-1 (and use ArPII) are shown in Table 1. Given the ubiquity of atrazine contamination, atrazine's persistence in the environment, and the concern for effects of

Table 1. Summary of mammalian tissues and cells that show cAMP/SF-1 dependent, ArPII-like expression of aromatase.

Tissue/cell type	References
Rat ovary (granulosa)	Carlone and Richards 1997; Falender et al. 2003; Fitzpatrick and Richards 1994; Lynch et al. 1993
Rat R2C (Leydig cell carcinoma)	Carlone and Richards 1997; Falender et al. 2003; Fitzpatrick and Richards 1994
Rat H540 (Leydig tumor cells)	Young and McPhaul 1997
Human prostate stroma	Ellem et al. 2004
Human prostate tumor (epithelial cells)	Ellem et al. 2004
Human LNCaP (prostate cancer cells)	Ellem et al. 2004
Human Sertoli cells	Gurates et al. 2002
Human endometrial stroma	Gurates et al. 2003
Human corpus luteum	Michael et al. 1995
Human preovulatory follicles	Simpson et al. 1994
Human ovary (granulosa)	Bulun et al. 2005; Sanderson et al. 2000
Human adipose tissue fibroblast	Bulun et al. 2005
Human breast tumor fibroblast	Bulun et al. 2005
Human malignant epithelial cells	Bulun et al. 2005
Human breast cancer adipose tissue	Bulun et al. 2005
Human extra-ovarian endometrium	Bulun et al. 2005
Human ovary-derived endometrial cells	Gurates et al. 2003
Human H295R (adrenal corticocarcinoma)	Sanderson et al. 2000

endocrine-disrupting chemicals in wildlife, especially in amphibian declines (Hayes et al. 2002a, 2002b, 2003, 2006a, 2006b), and in cancer (Kettles et al. 1997; Maclennan et al. 2002; Sass 2003), the findings reported here are quite significant. This concern was voiced several years ago (Sanderson et al. 2000):

> A logical concern would be that exposure to triazine herbicides, which are produced and used in large quantities, and are ubiquitous environmental contaminants, may similarly contribute to estrogen-mediated toxicities and inappropriate sexual differentiation.

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MOLECULAR AND CELLULAR BIOLOGY, July 2007, p. 4807–4814 Vol. 27, No. 13
0270-7306/07/$08.00+0 doi:10.1128/MCB.02039-06
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The Pituitary Function of Androgen Receptor Constitutes a Glucocorticoid Production Circuit[▽]

Junko Miyamoto,[1]† Takahiro Matsumoto,[1,2]† Hiroko Shiina,[1] Kazuki Inoue,[1] Ichiro Takada,[1] Saya Ito,[1] Johbu Itoh,[3] Takeo Minematsu,[4] Takashi Sato,[1] Toshihiko Yanase,[5] Hajime Nawata,[5] Yoshiyuki R. Osamura,[4] and Shigeaki Kato[1,2]*

Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan[1]; ERATO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchisi, Saitama 332-0012, Japan[2]; Teaching and Research Support Center[3] and Department of Pathology,[4] Tokai University School of Medicine, Boseidai, Isehara, Kanagawa 259-1193, Japan; and Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan[5]

Received 1 November 2006/Returned for modification 18 December 2006/Accepted 16 April 2007

Androgen receptor (AR) mediates diverse androgen actions, particularly reproductive processes in males and females. AR-mediated androgen signaling is considered to also control metabolic processes; however, the molecular basis remains elusive. In the present study, we explored the molecular mechanism of late-onset obesity in male AR null mutant (ARKO) mice. We determined that the obesity was caused by a hypercorticoid state. The negative feedback system regulating glucocorticoid production was impaired in ARKO mice. Male and female ARKO mice exhibited hypertrophic adrenal glands and glucocorticoid overproduction, presumably due to high levels of adrenal corticotropic hormone. The pituitary glands of the ARKO males had increased expression of proopiomelanocortin and decreased expression of the glucocorticoid receptor (GR). There were no overt structural abnormalities and no alteration in the distribution of cell types in the pituitaries of male ARKO mice. Additionally, there was normal production of the other hormones within the glucocorticoid feedback system in both the pituitary and hypothalamus. In a cell line derived from pituitary glands, GR expression was under the positive control of the activated AR. Thus, this study suggests that the activated AR supports the negative feedback regulation of glucocorticoid production via up-regulation of GR expression in the pituitary gland.

Sex steroid hormones exert a wide variety of biological actions. They are also involved in pathological events, such as the development of hormone-dependent cancers in reproductive organs (5, 37). In vertebrates, sex hormones play a pivotal role in male reproductive function and metabolic control. Most sex steroid actions are mediated through transcriptional control of target genes by nuclear receptors (NRs). NRs form a gene superfamily and act as transcriptional factors (9, 20). Sex hormone receptors have been shown to transactivate particular sets of target genes in a hormone-dependent manner through direct DNA binding to specific elements in target gene promoters. Hormone receptors activated by hormone binding recruit a number of coregulator-coregulator complexes for transactivation (28). These complexes then affect transcription through chromatin remodeling (12, 17, 22) and histone modification (1, 7). Hormone binding to the receptors may also transrepress target genes. The mechanisms of hormone-dependent transrepression of steroid receptors likely involve protein-protein interactions and are thus more diverse than that of transactivation (8, 10, 13, 21).

The molecular mechanisms behind the regulation of gene transcription by hormones and their NRs are complicated. Gene disruption studies have clarified the role of various NRs in steroid hormone action. By combining a Cre-loxP system with a canonical gene disruption approach, we succeeded in disrupting the androgen receptor (AR) on the X chromosome in mice in a manner that did not result in male infertility (14). Male AR null mutant (ARKO) mice exhibit abnormalities typical of testicular feminization mutants, including female external genitalia with atrophic testis and impaired sex behavior (29). Growth of the male ARKO mice is partially retarded, with impaired bone growth coupled with high bone turnover (16). The male mice also develop late-onset obesity (30). In contrast, no clear phenotypic abnormalities are present in female ARKO mice. However, normal folliculogenesis does require the AR, which suggests that androgen/AR signaling is also physiologically important in females (32).

To study how and why obesity develops in ARKO males, we began by examining the adrenal glands, which were hypertrophic in both males and females. In the present study, we explored the molecular basis of this observation. Dissection of the gland revealed that the layers of the zona fasciculata were thicker and coupled to the remaining layers of the X-zone (fetal zone). The hypertrophy resulted from a hypercorticoid state. Adrenal corticotropic hormone (ACTH) overproduction was driven by impaired negative feedback through the hypothalamus-pituitary-adrenal (HPA) axis. No clear alteration in the numbers of hormone-producing cells in the pituitary glands and hypothalamus was detected, but there were increased proopiomelanocortin (POMC) and decreased glucocorticoid

* Corresponding author. Mailing address: Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. Phone: 81-8-5841-8478. Fax: 81-3-5841-8477. E-mail: uskato@mail.ecc.u-tokyo.ac.jp.

† J.M. and T.M. contributed equally to this work.

▽ Published ahead of print on 30 April 2007.

receptor (GR) expression levels of transcripts in the ARKO pituitary glands. Androgen-induced GR gene activation was further confirmed in a pituitary gland-derived cell line (AtT-20 cells). These findings suggest that androgen/AR signaling in the pituitary gland supports the normal feedback system of glucocorticoid production through the HPA axis.

MATERIALS AND METHODS

Animals. ARKO mice were generated by targeted disruption of the AR gene by means of a Cre-loxP system (19) and maintained as described previously (16, 29, 30, 32). Experiments were performed with 2- to 25-week-old male mice. All mice protocols were approved by the Animal Care and Use Committee of the University of Tokyo (31, 40).

Cell culture. Adherent AtT-20 cells, a murine corticotropic tumor cell line, were cultured in a 5% CO_2 atmosphere at 37°C with Dulbecco's modified Eagle's medium-Ham's F12 at 1:1 containing 15% fetal calf serum (FCS) and penicillin-streptomycin. 3T3-L1 cells, a murine preadipocyte cell line, were cultured with Dulbecco's modified Eagle's medium containing 10% FCS. FCS in the culture media was replaced with charcoal-treated FCS for 1 week prior to the administration of 5α-dihydrotestosterone (DHT). For Northern and Western blot analyses, the cultured AtT-20 cells were subcultured in six-well plates. After incubation for 24 h, DHT (10^{-7} M) was added to the medium.

Histology and immunohistochemistry. Adrenal glands and pituitary glands were fixed by immersion with 4% paraformaldehyde for 24 h at 4°C. They were then embedded in paraffin, sliced into 4-μm sections by standard methods, and mounted onto silane-coated slides. After perfusion by 0.9% saline followed by 4% paraformaldehyde, brains were postfixed in the same fixative for 2 h at 4°C and soaked in phosphate-buffered saline containing 20% sucrose. Frontal sections were cut at 30-μm thickness using a cryostat. Serial sections were divided into four groups and used for single-labeling immunohistochemistry for the GR, corticotropin-releasing hormone (CRH), α-melanocyte-stimulating hormone (α-MSH), or thionin to allow determination of the areas to be measured.

Immunostaining was carried out using antibodies as described below (34). The primary antibodies included rabbit polyclonal anti-human AR (N-20; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-GR (M-20; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human ACTH (DAKO, Carpinteria, CA), mouse anti-human luteinizing hormone β (Immunotech, Marseille, France), mouse anti-human follicle-stimulating hormone β (DAKO, Carpinteria, CA), mouse anti-human thyroid-stimulating hormone β (Advanced Immunochemical Inc., CA), rabbit anti-rat glycoprotein hormone (kindly supplied by A. F. Parlow, the National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK], Bethesda, MD), rabbit anti-human growth hormone (DAKO, Carpinteria, CA), and rabbit anti-rat prolactin (kindly supplied by A. F. Parlow, NIDDK).

After treatment with 0.5% H_2O_2 (30 min) and 5% normal serum (1 h), the sections were incubated for 24 h at 4°C with specific primary antibodies. The sections were then incubated with secondary antibodies and an avidin-biotin complex (Vectastain ABC Elite kit; Vector Laboratories). The signals were visualized with diaminobenzidine and the nuclei were counterstained with hematoxylin.

For dual labeling of ACTH and the AR or GR, a single staining of the AR or GR was first performed as described above. After the primary antibodies were removed by treatment with 0.1 M glycine, sections were incubated with anti-ACTH antibodies followed by alkaline phosphatase-conjugated anti-mouse immunoglobulin G (DAKO, Carpinteria, CA). The signals were visualized with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium.

Detection of proliferation and apoptosis of adrenal gland cells. Eight-week-old mice were injected intraperitoneally (i.p.) with the thymidine analog 5'-bromo-2'-deoxyuridine (BrdU) (30 mg/g body weight [BW]) every 12 h five times (25). Mice were fully anesthetized and their adrenal glands removed 12 h after the last injection. Incorporated BrdU was detected immunohistochemically using a mouse monoclonal anti-BrdU antibody. The proliferative index was defined as the number of BrdU-positive cells per microscopic field. Five fields per mouse were counted for each of three wild-type (WT) and three ARKO mice.

Cells undergoing apoptosis were identified by digoxigenin labeling of the free 3'OH ends of fragmented DNA by use of terminal deoxynucleotidyltransferase (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling [TUNEL] assay). Assays were performed on sections from the same tissue blocks used for BrdU immunohistochemistry. Sections were counterstained with hematoxylin to facilitate cell counting. The fraction of apoptotic cells was defined as the fraction of diaminobenzidine-positive cells per total number of cells. Five fields per mouse were counted for each of three WT and three ARKO mice.

Serum endocrine parameters. A circadian rhythm experiment and dexamethasone suppression tests were performed on 8-week-old male mice as previously described (2). For the circadian rhythm experiment, blood was collected at 08:00 or 18:00 h. For the dexamethasone suppression tests, mice were injected i.p. with different doses of dexamethasone (0, 2, or 5 μg/20 g BW) in 0.3 ml of 0.9% saline. Injections were performed between 08:00 and 08:30 h and blood was collected 6 h later. Mice were fully anesthetized and blood was collected by cardiac puncture. Plasma ACTH and serum corticosterone were measured using radioimmunoassay kits (IRMA; Mitsubishi, Tokyo, Japan) at SRL (Tokyo, Japan), according to the manufacturers' instructions. Measurements were independently duplicated, and interassay variability and buffer dilution were corrected for by using internal correction factors.

RNA extraction and mRNA quantitation. Total mRNA was extracted from pituitary glands with TRIzol (Invitrogen) for reverse transcription-PCR (RT-PCR) and Northern blotting (35). To remove any possible DNA contamination prior to semiquantitative RT-PCR, the DNA was digested with RNase-free DNase. The digested total mRNA (2 μg) was subjected to RT using SuperScript reverse transcriptase (Invitrogen) primed by oligo(dT) primers. After first-strand cDNA synthesis, 1 ml from a 5% reaction mixture was diluted serially (2- to 128-fold). Amplification was performed with *rTaq*DNA polymerase (Takara) using primer pairs for GAPDH as an internal control to allow for concentration estimation (38). Expression levels of transcripts were measured using the standardized cDNA and specific primer pairs. The validity of the PCR products was confirmed by direct sequencing.

Western blot analysis. The lysates of mouse tissue and AtT-20 cells were resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (15, 39). Membranes were probed with rabbit polyclonal anti-GR antibody (M-20; Santa Cruz Biotechnology) and goat polyclonal anti-β-actin antibody (I-19; Santa Cruz Biotechnology) as an internal control. The blots were visualized using peroxidase-conjugated anti-rabbit antibody and anti-goat antibody, together with an ECL detection kit (Amersham Biosciences). The small interfering RNA analysis used AR and control small interfering RNA (Ambion), and transfection was accomplished with the Lipofectamine 2000 system (Invitrogen).

Luciferase reporter assay. GR promoter regions (upstream regions of exons 1B, 1C, and 2) were cloned by PCR and subcloned into a luciferase reporter gene driven by a tk promoter (tk-luc). PCR primers were as follows: for 1B Fw, 5'-GGCATAGTTAGGCCACTAAAGAGA-3'; for 1B Rv, 5'-GGGAGAAGT TGCAAAGCAGA-3'; for 1C Fw, 5'-CTGGAGCAGCAAATGTCAAG-3'; for 1C Rv, 5'-AGCTCGCAAAATGGAGGAG-3'; for 2 Fw, 5'-GGATCTGGCGT CCTTTTC-3'; and for 2 Rv, 5'-CCACATTATCTCTGATCCGATT-3'.

For the luciferase reporter assay, cultured cells were transfected with the indicated plasmids using the Lipofectamine Plus reagent (Invitrogen) into 24-well plates at 40 to 50% confluence. The total amount of DNA was adjusted by supplementing with empty vector up to 1.0 μg/well. Luciferase activity was determined using a dual luciferase assay system (Promega). As a reference plasmid to normalize the transfection efficiency, 1.5 ng/well of pRL-CMV plasmid (Promega) was cotransfected in all experiments.

Statistical analysis. Values are given as the means ± standard deviations. Comparisons between two groups were made by Student's *t* test. *P* values of <0.05 were accepted as statistically significant.

RESULTS

High serum levels of ACTH and corticosterone in male ARKO mice. The male ARKO ($AR^{L-/Y}$) mice exhibited growth retardation in comparison to WT male mice until 10 weeks of age but then showed catch-up growth over the next few weeks. Thereafter, the male ARKO mice weighed more than the WT mice and developed severe obesity (Fig. 1A) as previously reported (6, 30). Obesity to this extent was not seen for female ARKO ($AR^{L-/L-}$) mice. To identify causes for the late-onset obesity in male ARKO mice, serum endocrine parameters were measured. We found that serum corticosterone levels in male ARKO mice were elevated at 8 weeks of age and became significantly higher at 13 and 20 weeks ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 1B).

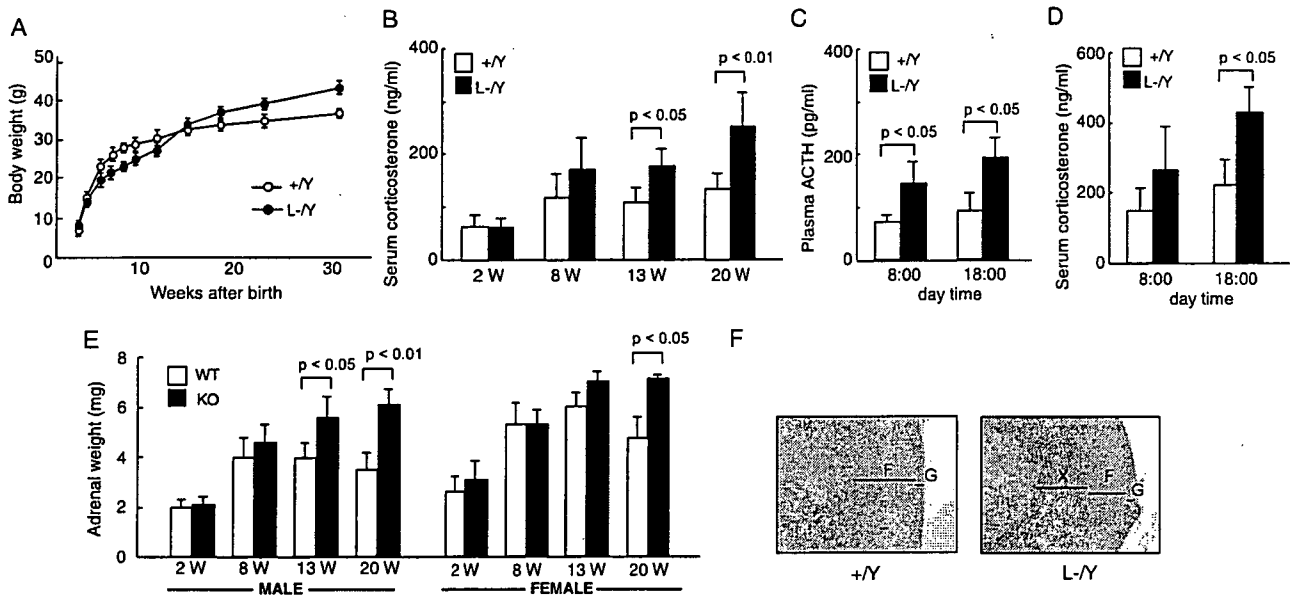

FIG. 1. Hypertrophic adrenal glands with high serum levels of ACTH and corticosterone in ARKO mice. (A) Growth curves of ARKO and WT littermate mice. The floxed AR mice (female, $AR^{L+/L+}$; male, $AR^{L+/Y}$) were crossed with Cre-CMV transgenic mice to generate ARKO male ($AR^{L-/Y}$) and female ($AR^{L-/L-}$) mice (16, 30). (B) Serum corticosterone levels of ARKO and WT mice at 2, 8, 13, and 20 weeks (W) of age. (C) Plasma ACTH levels of ARKO and WT mice measured in the morning (8:00) and evening (18:00). (D) Serum corticosterone levels of ARKO and WT mice in the morning (8:00) and evening (18:00). (E) Adrenal gland weights of male and female ARKO and WT mice at 2, 8, 13, and 20 weeks of age. (F) Histology of ARKO and WT adrenal glands. All sections were stained with hematoxylin and eosin. F, zona fasciculata; G, zona glomerulosa; X, X-zone.

To more carefully examine the hypercorticoid state in male ARKO mice, we measured the serum levels of corticosterone and its upstream hormone ACTH. Measurements were taken both in the morning and in the evening, as these hormones exhibit a circadian rhythm. As expected, the 8-week-old WT and ARKO mice had low levels of both hormones in the morning and higher levels in the evening (Fig. 1C and D). Overall, the ARKO males tended to have high levels of ACTH and corticosterone at any time compared to WT mice. They had significantly high levels of plasma ACTH at both 8:00 and 18:00 and high levels of corticosterone at 18:00 compared to WT mice ($P < 0.05$) (Fig. 1C and D). However, for female ARKO mice, though serum levels of these hormones tended to be higher than in WT littermates, the differences were not statistically significant (data not shown). The obesity seen for these mice was likely the result of their hypercorticoid state, as centripetal obesity is a typical symptom of Cushing's syndrome. In the following experiments, we explored the etiology of the hypercorticoid state in the ARKO mice.

Hypertrophic adrenal glands in ARKO mice. To investigate the hypercorticoid state in the ARKO mice, we first examined the adrenal glands. The adrenal glands in the ARKO males clearly weighed more than the glands of WT mice at 13 weeks of age (Fig. 1E). This coincided with the onset of obesity and the hypercorticoid state. Likewise, in ARKO females, the adrenal glands also increased in size in comparison to what was seen for WT littermate females; however, the growth was not as pronounced as that in ARKO males (Fig. 1E). The adrenal glands of male ARKO mice were then used for subsequent experiments.

The adrenal cortex forms the major part of the gland and is divided into three layers in mammals: the zona glomerulosa, immediately beneath the capsule, followed by the zona fasciculata and the zona reticularis. The zona reticularis is replaced in rodents by the X-zone, which develops prenatally and begins to degenerate at pubertal maturity in males. In mice, corticosterone, the major glucocorticoid in rodents, is produced in the zona fasciculata, while aldosterone, the most potent mineralocorticoid, is formed in the zona glomerulosa. Hematoxylin and eosin staining of adrenal glands in 13-week-old mice revealed that the enlargement of the adrenal glands in ARKO males was caused by cellular hypertrophy of the zona fasciculata as well as by a failure of X-zone (fetal zone) regression (Fig. 1F). Since glucocorticoids are produced in the zona fasciculata, it is likely that the overproduction of corticosterone is the result of the hypertrophy in this area.

Increased proliferation and decreased apoptosis in the adrenal cortex of ARKO males. The failed regression of the X-zone in 13-week-old ARKO males raised the possibility of impaired cell death or decreased apoptosis in the adrenal cortex. Indeed, the percentage of apoptotic cells in the zona fasciculata, detected by TUNEL assay in the ARKO males, was clearly less (19.5%) than that for WT mice (33.1%) (Fig. 2A). When actively proliferating cells of the adrenal glands were counted by BrdU labeling in WT and ARKO males, 2.5 times more BrdU-labeled cells/section were found in ARKO mice. This suggests increased proliferation in the adrenal cortex of ARKO mice (Fig. 2B). Thus, the hypercorticoid state likely results from the overproduction of glucocorticoid by the hypertrophic zona fasciculata. This hypertrophy is caused by chronic exposure to high levels of ACTH.

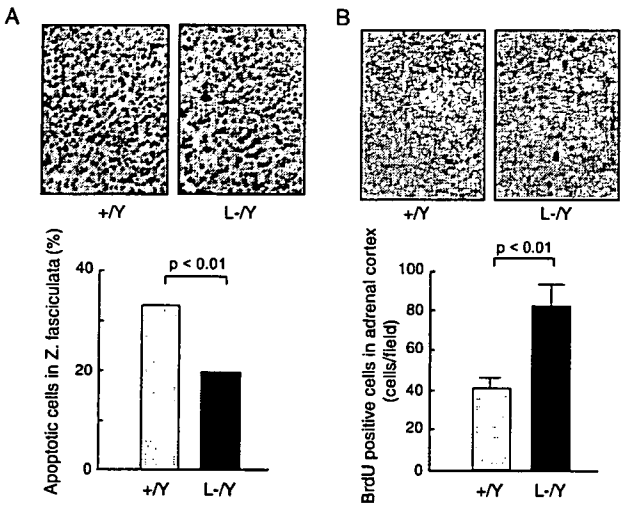

FIG. 2. Increased proliferation and decreased apoptosis in ARKO adrenal glands. (A) Decreased apoptosis in the ARKO adrenal glands. Histogram showing the number of TUNEL-positive cells in the zona fasciculata (Z. fasciculata). (B) Increased proliferation in the ARKO adrenal cortex. Histogram showing the number of BrdU-positive cells.

The HPA negative feedback system for glucocorticoid production is impaired in ARKO males. Glucocorticoid synthesis is regulated by a negative feedback loop via the HPA axis involving CRH and ACTH, produced by the hypothalamus and pituitary glands, respectively. We assessed whether the axis was intact and functioning normally in the ARKO males with a dexamethasone suppression test. As expected, serum corticosterone levels were down-regulated in 8-week-old WT mice 6 hours after i.p. injection of either 2 μg/20 g BW or 5 μg/20 g BW of dexamethasone (Fig. 3A). In ARKO mice, injection with 2 μg/20 g BW of dexamethasone did not suppress the serum levels of corticosterone. However, a dose of 5 μg/20 g BW was effective in lowering the serum levels of corticosterone in ARKO mice (Fig. 3A). This is similar to the high-dose dexamethasone suppression seen for patients with central Cushing's syndrome. Plasma ACTH levels in both the ARKO and WT males were decreased 6 h after dexamethasone injection at both the low and high doses; however, suppression was less sensitive in the ARKO males than in the WT males (Fig. 3B). No statistical difference in ACTH levels was detected between ARKO and WT males in this suppression test.

No overt abnormalities were present in the hypothalami or pituitary glands of ARKO males. The results of the suppression tests suggested that the adrenal hypertrophy of the ARKO males resulted from the hyperfunction of the hypothalami and/or pituitary glands. To address this issue, the hypothalami and pituitary glands of 8-week-old ARKO males were histologically examined. No overt abnormalities were detected in sections of the ARKO mice stained with hematoxylin and eosin (Fig. 4A). Immunohistochemical staining of the pituitary glands demonstrated similar numbers of cells expressing pituitary hormones in WT and ARKO mice (Fig. 4B). AR protein expression was detectable in several types of hormone-producing cells in the WT males but was absent in the ARKO mice (data not shown).

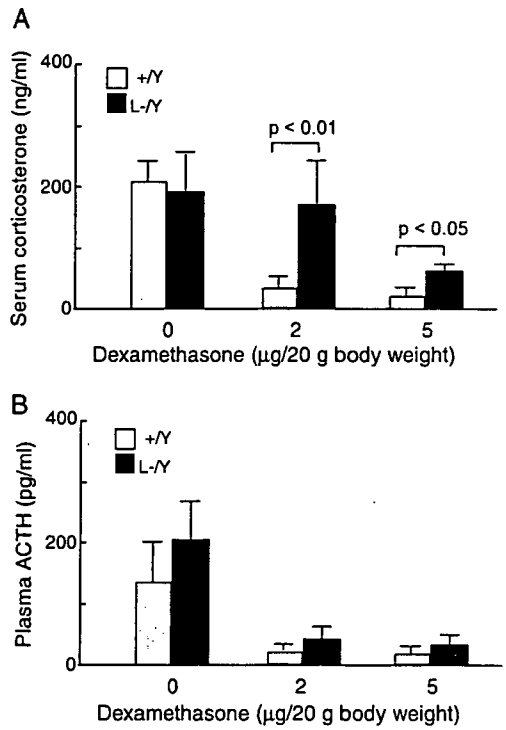

FIG. 3. Impairment of the HPA negative feedback system of glucocorticoid production in ARKO mice. (A) Serum corticosterone levels of ARKO and WT mice in the dexamethasone suppression test. Trunk blood was collected from ARKO and WT mice 6 hours after injection with increasing doses of dexamethasone. (B) Plasma ACTH levels of ARKO and WT mice in the dexamethasone suppression test.

Increased POMC expression and decreased GR expression in the pituitary glands is caused by AR deficiency. To address if hormone production was intact following AR inactivation, we examined the mRNA expression levels of pituitary hormones by RT-PCR. As shown in Fig. 5A, the expression of luteinizing hormone β, follicle-stimulating hormone β, and thyroid-stimulating hormone β, as well as that of the orphan NRs (Nur77 and Nurr1), appeared unaltered by AR deficiency. The POMC mRNA levels, however, were clearly up-regulated in males (Fig. 5A) but not in females (Fig. 5B). The up-regulation of POMC mRNA was confirmed by Northern blot analysis (Fig. 5C). This finding is consistent with the high ACTH levels observed for the ARKO mice. In contrast, pituitary GR expression was decreased at both the mRNA and protein levels (Fig. 5A and D). Decreased GR gene expression in ARKO males was also seen for the spleen but not for the other tested tissues (Fig. 5E), suggesting tissue-specific regulation of GR expression by the AR. Interestingly, a clear decrease in the GR mRNA levels was not detected in the total brain RNA of ARKO males (Fig. 5E). Additionally, there was no alteration in the numbers of GR and CRH immunoreactive cells in the hypothalamic paraventricular nucleus in the male ARKO brain (Fig. 4D). These results suggest that the androgen/AR signaling system affects the negative feedback regulation of glucocorticoid production via pituitary GR expression. This view is further supported by the observation that colocalization of ACTH with the AR and/or GR in the pituitary

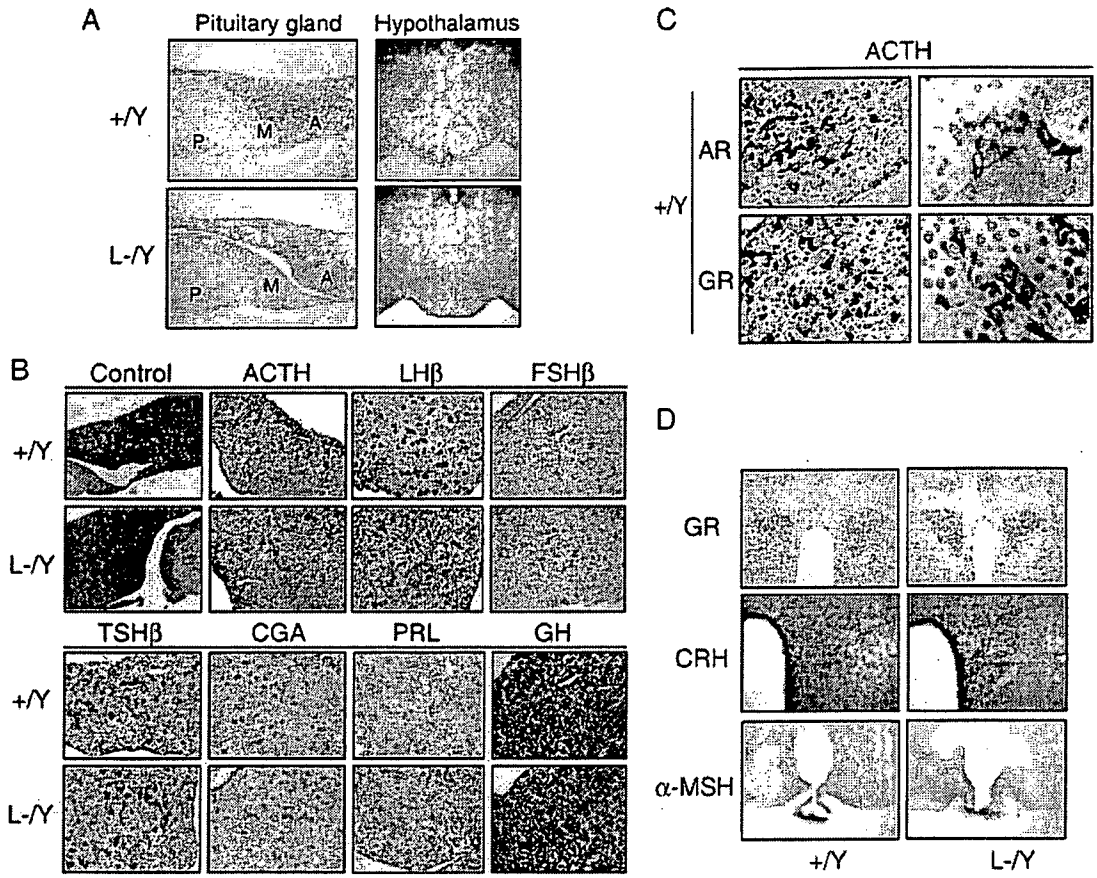

FIG. 4. Histological appearance of the hypothalamus and pituitary gland in ARKO mice. (A) No clear alteration in morphology of the hypothalami or pituitary glands of ARKO mice. Sections of pituitary glands and hypothalami were stained with hematoxylin and eosin. A, anterior lobe; M, intermediate lobe; P, posterior lobe. (B) No overt abnormality in the distribution of cells expressing pituitary hormones in ARKO mice by immunohistochemical staining. LHβ, luteinizing hormone β; FSHβ, follicle-stimulating hormone β; TSHβ, thyroid-stimulating hormone β; CGA, glycoprotein hormone; PRL, prolactin; GH, growth hormone. (C) Pituitary ACTH (black/gray) colocalized with AR (brown) or GR (brown) (left) and its higher magnification (right) in WT mice as detected by immunostaining with specific antibodies. (D) No clear alterations in the GR and CRH (in the paraventricular nucleus) and α-MSH (in the arcuate nucleus) immunoreactive neurons in the hypothalami of ARKO mice.

glands of WT mice was detected by double immunostaining with specific antibodies (Fig. 4C).

Induction of the GR gene by DHT in a pituitary cell line. Finally, to confirm that GR expression levels in the pituitary glands of ARKO mice were reduced, we tested whether DHT induced GR gene expression in cultured cells. DHT treatment of the pituitary cell line AtT-20 for 4 h induced expression of the GR gene (Fig. 6A) and protein (Fig. 6B). Unexpectedly, the expression levels of POMC mRNA (Fig. 6A) and protein (Fig. 6B) were reduced. The AR effect was confirmed following treatment with an AR antagonist (Flutamide) (Fig. 6A and B) and RNA interference (Fig. 6C). Reflecting tissue-specific regulation of GR expression by the AR in intact animals, no response to DHT in the expression levels of either GR or POMC was seen in 3T3-L1 preadipocytes (Fig. 6A to C, lower panels). No consensus androgen response elements or closely related sequences are present up to −3 kb in the GR promoter. However, the intron between exons 1A and 1B of the GR gene (33) was found to counter androgen responsiveness in a transient expression assay in AtT-20 cells (Fig. 6D). This suggests that this element is responsible for androgen-induced GR expression in the pituitary.

DISCUSSION

Hypertrophic and hyperplastic adrenal glands are associated with a hypercorticoid state in male mice deficient for the AR. A hypercorticoid state was observed in sexually mature male mice deficient for the AR. It likely resulted from glucocorticoid overproduction by the hypertrophic and hyperplastic zona fasciculata of the adrenal gland. The ARKO mice demonstrated hyperplasia of the X-zone, which normally regresses by the time sexual maturity is attained (11). TUNEL assays and BrdU labeling confirmed that the hypertrophy and hyperplasia of adrenal glands resulted from decreased apoptosis and increased cell proliferation. Chronic ACTH stimulation causes zona fasciculata cell hypertrophy and hyperplasia, and ACTH is a potent inhibitor of apoptosis in the adrenal cortex (36). Thus, the findings for the ARKO males are consistent with exposure to high levels of ACTH.

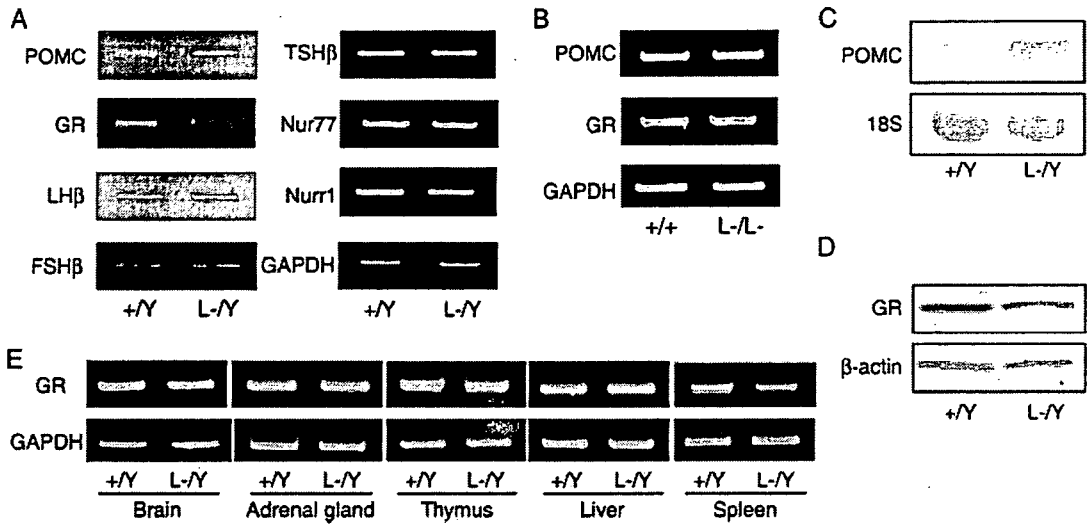

FIG. 5. Altered expression levels of gene transcripts involved in the HPA axis. (A) Increased POMC and decreased GR expression levels of transcripts in ARKO pituitary by semiquantitative RT-PCR. LHβ, luteinizing hormone β; FSHβ, follicle-stimulating hormone β; TSHβ, thyroid-stimulating hormone β. (B) No significant alterations of POMC and GR mRNA levels in the pituitary glands of female ARKO ($AR^{L-/L-}$) mice. (C and D) Northern blot analyses showing clear up-regulation of POMC mRNA levels and down-regulation of GR mRNA levels in the ARKO pituitary. (E) Tissue-specific reduction of GR transcripts in ARKO mice. GR expression levels are down-regulated only in the spleen and pituitary in male ARKO mice.

The hypertrophic and hyperplastic adrenal glands in the ARKO mice probably resulted from high levels of serum ACTH, derived from high POMC transcript levels in the pituitaries of ARKO mice. Studies with transgenic mice expressing antisense RNA against the GR in the brain and anterior pituitary demonstrate that the GR mediates the negative feedback regulation of glucocorticoid production through HPA axis activity (26, 36). Consistent with this observation, the male ARKO mice had low pituitary GR mRNA levels but no difference in the distribution of pituitary hormone-producing cells compared to WT animals. Thus, our findings suggest that the activated AR in the pituitary gland is needed to express pituitary GR at a sufficiently high level to participate in the negative feedback regulation of glucocorticoid production. The X-zone, which is considered a fetal zone, regresses during sexual maturation and reappears after gonadectomy (11). The molecular basis underlying X-zone regression during sexual maturation remains to be investigated. However, our results raise the possibility that the activated AR in adrenal glands induces X-zone repression by the induction of apoptosis. Consequently, the identification of AR target genes expressed in the X-zone is another interesting direction to pursue.

Liganded AR augments GR gene expression in the pituitary gland. We found that GR gene expression was impaired in the pituitary glands of ARKO males. We presumed that the reduced GR levels led to increased expression of the POMC gene, with subsequent high levels of serum ACTH. This idea was supported by the observation that the suppression of ACTH production by exogenous glucocorticoids was partially impaired in the ARKO mice. Moreover, the DHT-activated AR enhanced the GR mRNA levels in a pituitary cell line but not in 3T3-L1 preadipocytes. The effect of DHT was most likely mediated by a response element in an upstream region of the GR promoter exon 1B (33). Thus, the activated AR directly induces the pituitary GR in a cell-specific manner. How this is accomplished on a molecular level remains to be elucidated.

Do androgen/AR signaling disorders link with an ACTH-dependent hypercorticoid state? A hypercorticoid state in humans is well known to cause Cushing's syndrome, in which patients suffer from a number of disorders such as centripetal obesity, facial rounding, glucose intolerance, hyperinsulinemia, and impaired lipid and bone metabolism (23). Most of these lesions are a reflection of glucocorticoid-driven gluconeogenesis. The hypercorticoid state may result from either endogenous disorders or chronic treatment with exogenous glucocorticoid. Endogenous causes of Cushing's syndrome are further classified as ACTH dependent or independent (18). The ACTH-dependent syndrome is characterized by up-regulated levels of ACTH; however, the molecular basis underlying the ACTH overproduction remains to be investigated. It is possible that sex steroids are involved, but this has not yet been fully addressed.

The male ARKO mice exhibited abnormalities similar to those seen for ACTH-dependent Cushing's syndrome patients. Since we detected up-regulation of the pituitary POMC transcript, other POMC-derived peptides might have contributed to the onset of obesity in male ARKO mice. For example, α-MSH in the neurons of the hypothalamus plays a central role in appetite control and energy homeostasis (3, 4). Although we detected no clear alteration in α-MSH immunoreactivity in the arcuate nuclei of the hypothalami of male ARKO mice, it will be of interest in future experiments to examine the melanocortin receptor system in ARKO brain. In contrast to the male ARKO mice, ARKO females did not display some of the abnormalities, such as obesity. It is possible that the lack of obesity in female ARKO mice may result from activation of estrogen receptors (ERs). ERs activated by high physiological

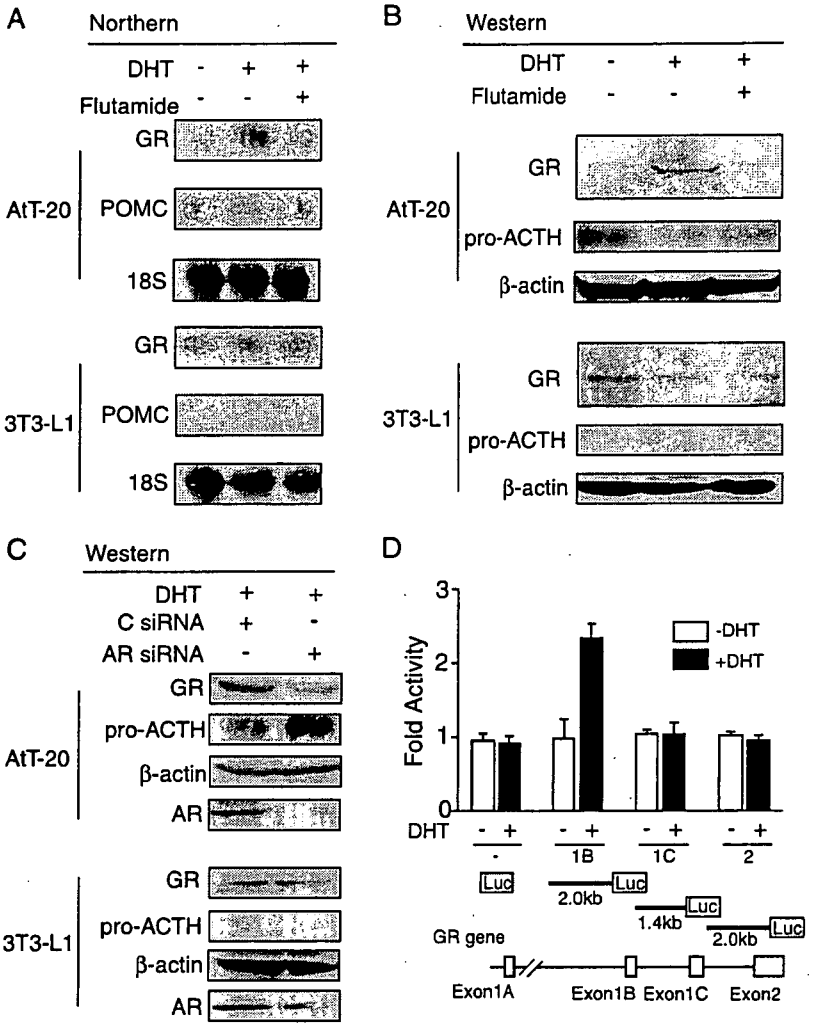

FIG. 6. Cell-type-specific regulation of the GR by activated AR. (A) Regulation of GR and POMC gene expression by treatment with either DHT or an AR antagonist (Flutamide) in the cultured cells as analyzed by Northern blot analysis. (B) Expression of the GR and pro-ACTH proteins was analyzed by Western blot analysis. (C) The significance of AR in the GR gene regulation was tested by AR RNA interference (with small interfering RNA [siRNA]) in the cultured cells. C siRNA, control siRNA. (D) Luciferase assay was performed with a series of the GR promoter regions in AtT-20 cells. After transfection with each of the promoter tk-luciferase vectors, the transfected cells were incubated with or without 10^{-7} M DHT.

levels of endogenous estrogens are effective in maintaining the proper levels of pituitary GR mRNAs needed to control POMC gene expression. This idea is indeed supported by the finding of unaltered levels of GR and POMC transcripts in the pituitary glands of the female ARKO mice. Moreover, estrogen treatment in female rats is shown to suppress serum levels of ACTH (27, 41). The common but gender-specific putative functions of the AR and ER in the brain have already been described in the context of mouse sexual behavior (24, 29). Though the possible ER functions remain to be studied for female ERKO mice, the present study suggests that the activated AR potentiates the negative HPA feedback regulation of glucocorticoid production through up-regulation of GR expression levels. Our study implies that the AR may be a potential therapeutic target for ACTH-dependent Cushing's syndrome.

In conclusion, the present study suggests that the androgen/AR signaling system is a negative pathway for glucocorticoid secretion in adult male mice. ARKO mice showed decreased GR expression in the pituitary glands and increased circulating ACTH and glucocorticoid. Androgens may increase the sensitivity of the HPA negative feedback loop to glucocorticoids by increasing GR expression in the pituitary gland, leading to suppression of adrenal cortical function. Thus, we presume that activated AR in the pituitary gland is a component of the negative feedback system for glucocorticoid production.

ACKNOWLEDGMENTS

We thank members of the KO project team for experimental support, A. F. Parlow (NIDDK, National Hormone and Peptide Program, Torrance, CA) for kindly providing antisera, and H. Higuchi for manuscript preparation.

This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and priority areas from the Ministry of Education, Culture, Sports, Science and Technology (to S.K.).

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Herbicide atrazine activates SF-1 by direct affinity and concomitant co-activators recruitments to induce aromatase expression via promoter II

WuQiang Fan [a], Toshihiko Yanase [a,*], Hidetaka Morinaga [a], Shigeki Gondo [a], Taijiro Okabe [a], Masatoshi Nomura [a], Tyrone B. Hayes [b], Ryoichi Takayanagi [a], Hajime Nawata [c]

[a] *Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan*

[b] *Laboratory for Integrative Studies in Amphibian Biology, Group in Endocrinology, Museum of Vertebrate Zoology, Energy and Resources Group, and Department of Integrative Biology, University of California, Berkeley, CA 94720-3140, USA*

[c] *Graduate School of Medical Science, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan*

Received 8 February 2007
Available online 22 February 2007

Abstract

The popular herbicide atrazine is an endocrine disruptor that demasculinizes and feminizes several species of animals, and co-relates with breast and reproductive disorders in mammalians. We recently reported that atrazine induces human *aromatase* gene expression via promoter II (ArPII) in a steroidogenic factor 1 (SF-1)-dependent manner. Here, we show that knockdown of SF-1 abolishes ArPII induction by atrazine in H295R cells, which harbor high SF-1 expression and are originally atrazine-responsive. Conversely, exogenous SF-1 enables atrazine to induce ArPII in the otherwise non-responsive KGN cells. Atrazine's effect is independent from protein kinase A and LRH-1, a close relative of SF-1. However, it binds directly to the SF-1, and concomitantly, enhances interactions of SF-1 with co-activator TIF2, and renders more SF-1 binding to ArPII chromatin. Intriguingly, LBD mutations do not alter SF-1's ability to mediate atrazine stimulation, suggesting that atrazine interacts with SF-1 via a region(s) other than the ligand binding pocket. These data suggest that atrazine binds to and activates SF-1 to induce ArPII.
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Keywords: Aromatase; Steroidogenic factor 1; Atrazine; Endocrine disruption

Atrazine is one of the most commonly used herbicides in the world, and is the most common contaminant of ground and surface water [1]. Atrazine poses concerns because it is a potent endocrine disruptor. Studies in every vertebrate class examined show that atrazine inhibits androgen-mediated development and simultaneously produces estrogen-like effects in exposed individuals [2]. Atrazine induces feminization in male tadpoles, renders some males infertile, and may thus contribute the recent global amphibians decline [1,3]. It is also reportedly associated with decreased semen quality and fertility in men living in agricultural areas [4]; and increased breast cancer incidence in women whose well water is contaminated with atrazine [5].

Atrazine does not act as an androgen receptor antagonist or an estrogen receptor agonist due to lack of affinity for both hormone receptors [6]. However, it reportedly inhibits phosphodiesterase [6–8], leads to elevated cAMP

Abbreviations: ArPII, aromatase promote II; DMSO, dimethyl sulfoxide; LBD, ligand binding domain; LRH-1, liver receptor homolog-1; PKA, protein kinase A; QCM, quartz crystal microbalance; SF-1, steroidogenic factor 1; SPR, surface plasmon resonance.

* Corresponding author. Fax: +81 92 642 5287.
E-mail address: yanase@intmed3.med.kyushu-u.ac.jp (T. Yanase).

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doi:10.1016/j.bbrc.2007.02.062

levels, which result in elevated transcription of *CYP19* (the gene for human aromatase), increased aromatase activity, and subsequent estrogen production.

Although this mechanism is consistent with the demasculinization and feminization effects of atrazine, some studies suggest that atrazine does not induce aromatase in some species and/or in certain cell lines [9]. For example, although atrazine induces aromatase in human H295R adrenal carcinoma cells, the current authors failed to reproduce this result in ovarian granulosa KGN cells [10]. We recently reported that this apparent discrepancy is due to the requirement of the nuclear receptor, steroidogenic factor 1 (SF-1) in atrazine-enhanced aromatase expression [2].

SF-1, which specifically expresses in steroidogenic tissues, is essential for steroidogenesis, steroidogenic tissues development, and normal sex differentiation [11,12]. SF-1 is a critical factor in the induction of aromatase via the aromatase promoter II (ArPII); and is required for ArPII to be transcriptionally regulated by the cAMP-protein kinase A (PKA) pathway [13]. Our present study confirmed that atrazine induces aromatase expression via promoter II in a SF-1-dependent manner. We also demonstrated that the chemical binds directly to the receptor, and that the affinity is most likely independent of the LBD. Concomitantly, atrazine significantly enhanced interactions of SF-1 with its co-activator, TIF2; and also induces binding of SF-1 to native chromatin of ArPII. These data suggest that the herbicide atrazine is potentially an artificial ligand for SF-1; and highlight the importance of atrazine as a risk factor in endocrine disruption in wildlife and reproductive cancers in rodents and humans.

Materials and methods

Materials. The human ovarian granulosa-like tumor cell line KGN was established and maintained as previously reported [14]. H295R and NIH-3T3 cells were obtained from the American Type Culture Collection. Atrazine and simazine standards were obtained from Wako Pure Chemical Co. (Osaka, Japan). H-89 dihydrochloride hydrate, a selective, potent inhibitor of cAMP-dependent protein kinase, was purchased from Sigma–Aldrich, Inc. (St. Louis, MO). A 4.0 kb human *CYP19* promoter II luciferase reporter (pGL3-ArPII4.0), and a 4.0 kb human SF-1 promoter reporter were constructed previously [15,16]. The adenovirus construct expressing bovine SF-1, Adeno-SF-1 and the one expressing β-galactosidase, Adeno-LacZ, were prepared as previously described [2].

Western blotting and mRNA quantification. The anti-SF-1 antibody, used for both the ChIP assay and Western blotting, was kindly provided by Professor K. Morohashi (National Institution for Basic Biology, Okazaki, Japan). Relative mRNA copy numbers to β-actin of endogenous LRH-1 in H295R and KGN cells were analyzed by real-time PCR. Primers for human LRH-1 were, forward: 5′CTG ATA CTG GAA CTT TTG AA3′; reverse: 5′CTT CAT TTG GTC ATC AAC CTT3′. Those for human β-actin were, forward: 5′AAA CTA CCT TCA ACT CCA TC3′; reverse: 5′ATG ATC TTG ATC TTC ATT GT3′.

Human SF-1 RNA interference. To knockdown endogenous SF-1 expression, a pool of three human SF-1-targeting siRNA oligonucleotides (Santa Cruz, Cat#: sc-37901) or a control scrambled siRNA pool (Santa Cruz, Cat#: sc-36869) was transfected to H295R cells pre-seeded in 6-well plate by X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics GmbH, Germany). The final siRNA concentration in medium was 100 nM. Three, five or seven days after transfection, cells were harvested, and expressions of SF-1 were determined by Western blotting. The anti-SF-1 siRNA substantially lowered SF-1 protein levels from the 5th day, and the effect sustained at the 7th day. For combined luciferase reporter assays, ArPII reporter together with an internal control vector (pRL-CMV) were transfected to H295R cells on the 6th day post siRNA transfection, chemicals applied on the 7th day, and luciferase assays were performed 48 h later. SF-1 knockdown was also achieved by using a pool of four SF-1-targeting siRNA oligonucleotides (ON-TARGET SMART-pool; Cat#: L-003429-00-0005 Dharmacon, Lafayatte, CO) and the subsequent reporter assays were replicated.

Relative luciferase reporter assays and mammalian two-hybrid assays were performed essentially as previously described [17].

In vitro atrazine–SF-1 interaction assay. Baculovirus was used to express the Flag containing SF-1 fusion protein in insect sf21 cells [2]. The binding affinity of atrazine to SF-1 was measured by using a 27-MHz Quartz Crystal Microbalance (QCM) (Initium Co., Tokyo, Japan). Chemical of interest was immobilized onto a QCM electrode, which was soaked in PBS buffer (8 mL, pH 7.4), and monitored continuously for QCM frequency change at 25 °C. After the frequency change was stabilized, recombinant SF-1 protein was added to the solution, and the subsequent time-course of frequency change was assessed.

Statistical analysis. Data are expressed as means ± SD and was evaluated by one-way ANOVA or Student's two-tail *t*-test, followed by post-hoc comparisons with Fisher's protected least significant difference test, as appropriate. A value of $P < 0.05$ was considered statistically significant.

Results and discussion

ArPII responds to atrazine SF-1 dependently

By applying a luciferase reporter system, in which a 4.0 kb human ArPII luciferase reporter and a human SF-1 expression vector are co-expressed in NIH-3T3 fibroblast cells, which lack endogenous SF-1, we previously showed that of 55 known environmental hormone chemicals screened, atrazine and its analog simazine were found to stimulate SF-1-mediated ArPII activity by a factor of 2–3; while neither chemical affected ArPII in the absence of SF-1 co-expression in these cells [2]. As introduced above, atrazine is reportedly able to induce aromatase expression in H295R adrenocortical carcinoma cells, but not in KGN granulosa-like cancer cells. We proposed that the difference is due to an over fiftyfold higher of endogenous SF-1 mRNA expression in H295R cells as compared to KGN cells. Upon exogenous SF-1 over-expression, both aromatase mRNA expressions and enzymatic activities became responsive to atrazine in KGN cells [2]. Here, we show that the ArPII reporter readily responded to atrazine without exogenous SF-1 co-expression in H295R cells (Fig. 1a). The atrazine stimulation was dose-dependant, and effective at concentrations as low as 10^{-7} M (21.6 ppb), which is ecologically relevant [3]. To further confirm that SF-1 is essential for atrazine stimulation, we knocked down endogenous SF-1 in H295R cells by RNAi. Fig. 1b shows that siRNA mediated down-regulation of SF-1 in the cells resulted in a reduced basal promoter activity, and importantly, a compete loss of responsiveness of ArPII to either atrazine or simazine. In KGN cells, consistent with the lack of effect on aromatase activity [10], ArPII, the dominant aromatase promoter the cells use,

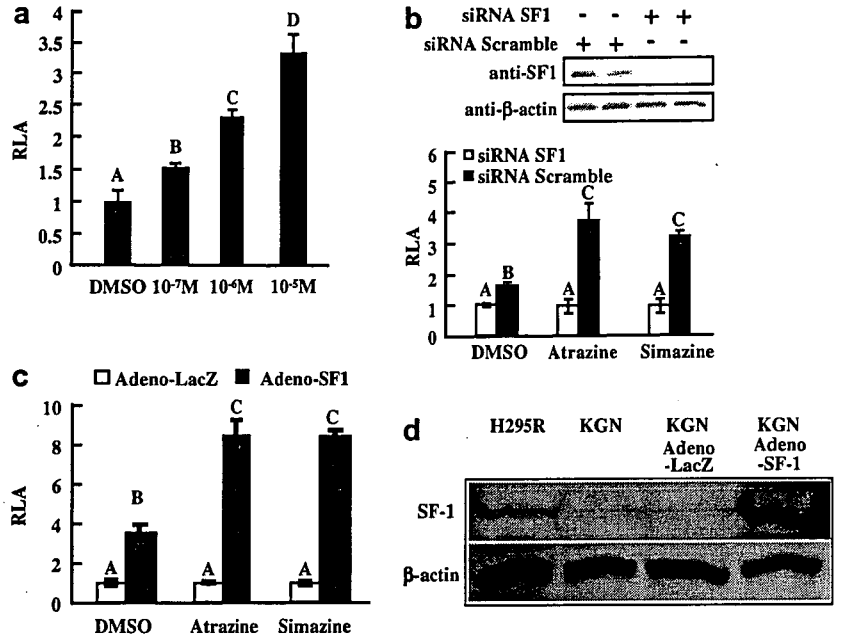

Fig. 1. ArPII responds to atrazine SF-1 dependently. (a) H295R cells with pGL3-ArPII4.0 and pRL-CMV internal control co-transfected were incubated for 48 h in DMSO or increasing concentrations of atrazine before luciferase assay. (b) Anti-SF-1 and anti-β-actin immunoblottings in H295R cells on the 5th day post siRNA transfection (upper panel). siRNA pre-treated H295R cells were co-transfected with pGL3-ArPII4.0 and pRL-CMV, and were subsequently incubated for 48 h in 10^{-5} M of atrazine, simazine or DMSO before relative luciferase assay (lower panel). (c) ArPII reporter were transfected in KGN cells pre-infected with either Adeno-LacZ or Adeno-SF-1, the cells were then incubated for 48 h in 10^{-5} M atrazine, simazine or the solvent DMSO before luciferase assay. (d) Anti-SF-1 immunoblottings in H295R cells, native KGN cells and KGN cells infected with Adeno-LacZ or Adeno-SF-1. Letters above bars show statistical groups (ANOVA, $P < 0.05$). RLA, relative luciferase activity.

was unchangeable by either chemical (Fig. 1c). However, once SF-1 was delivered by adenovirus, there was a near fourfold increase in basal activity and enhanced responsiveness to the chemicals; both atrazine and simazine further increased SF-1-enhanced ArPII activity by two- to threefold (Fig. 1c). Fig. 1d shows markedly higher endogenous SF-1 protein levels in H295R cells compared with KGN cells, and a successful SF-1 over-expression mediated by adenovirus vector in KGN cells. Thus atrazine or simazine induction of ArPII is SF-1 dependent.

Besides its vital roles in the reproductive system, ArPII is also critically involved in oncogenesis of breast cancer [15]. Importantly, in addition to reproduction disrupting effects, atrazine also increases the incidence of mammary cancer in rodents [2]; and is associated with breast cancer in women [5].

PKA and LRH-1 is not essential for atrazine stimulation

Both chemicals at concentration of 10^{-5} M were not cyto-toxic (Supplementary Fig. 1a), and didn't affect *SF-1* gene expression (Supplementary Fig. 1b and c).

SF-1 is required for conveying cAMP-PKA-dependent *CYP19* transcription via ArPII [13]. PKA directly enhances SF-1 transactivation by recruiting co-activators such as GCN5, TRRAP [13] and p300 [18], and disassociations of repressor DAX-1 [13]; PKA also reportedly removes an inhibitory ligand, sphingosine, from SF-1 [19]. On the other hand, Atrazine increases cellular cAMP levels by inhibiting phosphodiesterase as above-mentioned. Thus, activation of PKA is likely a mechanism by which atrazine may enhance SF-1 function. Consistently, a CRE (cAMP responsive element) luciferase reporter was found activated by either atrazine or simazine in H295R cells (ANOVA < 0.05, Fig. 2a); suggesting that the herbicides truly activate PKA in these cells. We then elucidated whether atrazine is still effective when PKA signaling is eliminated by H89. Fig. 2b shows that 10^{-5} M atrazine-induced PKA signaling was sufficiently blocked by 10^{-5} M H89. Fig. 2c shows that the basal levels of ArPII activities were dose-dependently decreased by H89 as expected, but surprisingly, atrazine stimulation of the promoter was not significantly changed by either 10^{-6} or 10^{-5} M H89. In an independent experiment, we also found that while responsiveness of ArPII to 10^{-6} M forskolin (a specific PKA agonist) in H295R cells was dose-dependently reduced by H89 as expected, responsiveness to atrazine was not altered (Fig. 2d). These data suggest that PKA signaling is not the only mechanism by which atrazine induces aromatase. Actually, neither chemical altered intracellular cAMP production or CRE-Luc activities in KGN cells (Supplementary Fig. 2a and b), even with Adeno-SF-1 infection.

Given that liver receptor homolog-1 (LRH-1), a close relative of SF-1, also regulates ArPII, and is also linked

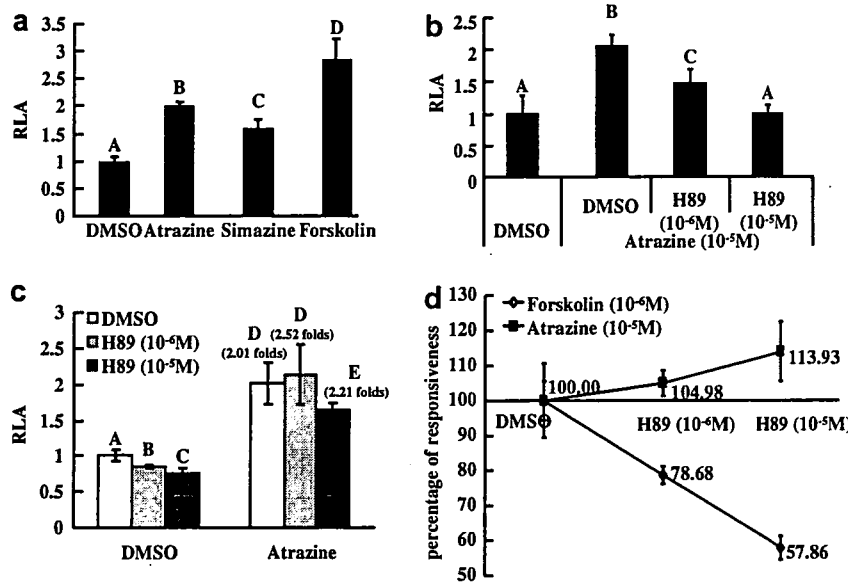

Fig. 2. PKA is not essential for atrazine stimulation. (a) CRE-Luc was transfected to H295R cells to sensitively monitor intracellular PKA signaling induced by 48 h of atrazine, simazine or forskolin. (b) CRE-Luc transfected H295R cells were treated with 10^{-5} M atrazine together with increasing concentrations of H89 as indicated. (c) pGL3-ArPII4.0 was transfected to H295R cells, which were subsequently treated with 10^{-5} M of atrazine together with increasing concentrations of H89, as indicated. (d) H295R cells were transfected with pGL3-ArPII4.0, and were subsequently exposed to 10^{-6} M forskolin or 10^{-5} M atrazine in combination with increasing concentrations of H89. Folds induction (responsiveness) of ArPII by forskolin or atrazine in the absence of H89 (DMSO) was set to 100. Note that H89 dose-dependently reduced ArPII responsiveness to forskolin but not that to atrazine. Letters above bars show statistical groups (ANOVA, $P < 0.05$). RLA, relative luciferase activity.

to the PKA-mediated induction of ArPII [20]. We therefore analyzed the role LRH-1, if any, in mediating the atrazine stimulation of ArPII. NIH-3T3 cells pre-cotransfected with ArPII reporter and expression vectors for SF-1 or LRH-1 or blank pcDNA3.1, were exposed to 48 h of 10^{-5} M atrazine, 10^{-6} M forskolin or the solvent DMSO. Fig. 3a shows that although SF-1 rendered ArPII responding to both forskolin and atrazine, the promoter was responsive to only forskolin, but not atrazine, in the presence of LRH-1. Furthermore, the endogenous mRNA expression of LRH-1 in the atrazine-non-responsive KGN cells was several hundredfold higher than that in the atrazine-responsive H295R cells (Fig. 3b), further suggesting a lack of LRH-1 involvement in atrazine stimulation of ArPII. Given that LRH-1 is sufficient to mediate PKA stimulation over ArPII, the abundant expression of the receptor in the atrazine non-responsive KGN cells serves as indirect evidence that atrazine stimulation is PKA independent.

Physical interaction between atrazine and SF-1 in vitro

We then studied whether atrazine binds to recombinant SF-1 protein *in vitro* using 27-MHz QCM. Atrazine, phospholipid 16PC (a known ligand for SF-1 [21]), or 2,4,5T (a chemical that dose not affect SF-1 function [2] were first fixed to individual QCM electrodes. Interactions were monitored by the linear decrease of the emitted fre-

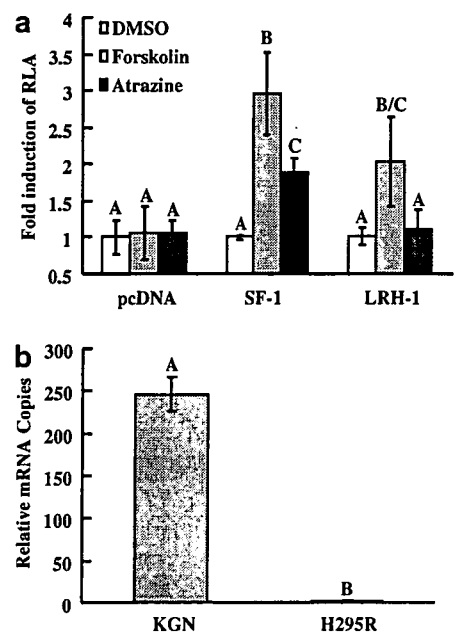

Fig. 3. Lack of role of LRH-1 in mediating atrazine responsiveness. (a) Responsiveness of ArPII to 48 h of 10^{-5} M atrazine, 10^{-6} M forskolin or the solvent DMSO in NIH-3T3 cells co-transfected with either pcDNA3.1, pcDNA3.1-hSF-1, or pcDNA3.1-hLRH-1. RLA, relative luciferase activity. (b) Real-time PCR quantification of endogenous LRH-1 mRNA in H295R and KGN cells. The relative copies of LRH-1 to that of β-actin in H295R cells were set to 1. Letters above bars show statistical groups (ANOVA, $P < 0.05$).

quency (ΔF) with increasing mass present on the QCM electrode. As shown in Fig. 4a, the frequency of 16PC immobilized on the QCM electrode decreased when SF-1 was added to reaction solution. SF-1 also decreased atrazine frequency, while exhibited no effect on the negative control 2,4,5T, indicating that there was a direct and specific interaction between atrazine and SF-1. The affinity data are significant because SF-1 has, for a long time since its discovery, been considered as an orphan nuclear receptor, until the recent discovery of phospholipids as endogenous ligands for SF-1 and the closely related receptor LRH-1 [21,22]; although the physiological significance and functional role of phospholipids in the regulation of receptor activity remains to be elucidated and is seems to be confounded by the absence of a lipid in the pocket of the mouse LRH-1 [23].

Atrazine enhances SF-1–TIF2 interaction

To address whether the affinity between SF-1 and atrazine is followed by co-activator recruitment, we further performed mammalian two hybrid assay-based interaction assays in which full length human TIF2 (an established co-activator for SF-1 [21] was fused to VP16 activation domain to produce pACT-TIF2 and full length of human SF-1 was fused to DBD of GAL4 to produce pBIND-SF-1, respectively. As shown in Fig. 4b, transfection of pACT-TIF2 or pBIND-SF-1 had either no or very mild effects on the pG5-Luc reporter in KGN cells; co-transfection of both pACT-TIF2 and pBIND-SF-1 significantly stimulated the reporter, suggesting that the two proteins interacted and the system worked effectively. In cells co-transfected with both fusion proteins, atrazine was found to further stimulate the reporter activities in a dose-dependent manner (Fig. 4c), suggesting that the chemical enhanced the interaction between SF-1 and its co-activator TIF2. We also made alternative fusion constructs of pACT-SF-1 and pBIND-TIF2 and similar results were obtained (Supplementary Fig. 3a and b). While being less dramatic, atrazine was also enhanced SF-1–SRC-1 interactions (Supplementary Fig. 3c). Thus the atrazine–SF-1 affinity is coupled with enhanced receptor–co-activators interactions. Functional augmentation of SF-1 by atrazine is also evidenced by enhanced SF-1–ArPII chromatin association [2].

Mutations in DBD, but not LBD of SF-1 alter atrazine stimulation

To address the corresponding functional domain of SF-1 in mediating atrazine stimulation over ArPII, we introduced mutations to the DBD (G35E, DBD mutation, abolishes SF-1 transactivation dramatically [13]) or LBD (G341E and Y436F-K440A, pocket mutations that abolish

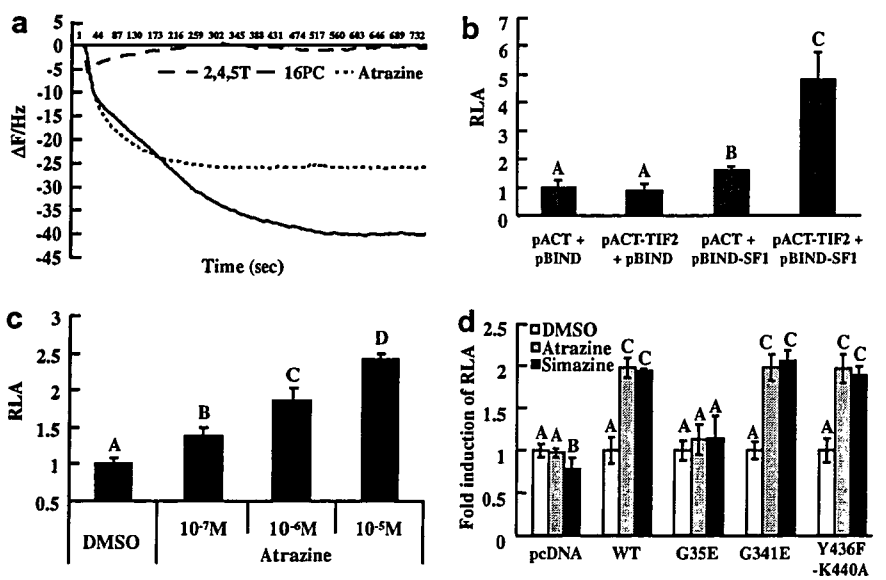

Fig. 4. Atrazine is potentially a ligand for SF-1. (a) Detection of direct atrazine–SF-1 interaction by quartz-crystal microbalance. Chemicals of interest were immobilized on the electrode individually and purified SF-1 was then added to the reaction solution. (b) Mammalian two-hybrid assays for in vivo SF-1–TIF2 interaction. KGN cells were co-transfected with a DNA mixture consisting of pG5-LUC together with equimolar cross-combinations of pACT or pACT-TIF2 and pBIND or pBIND-SF-1 as indicated. The co-existence of pACT-TIF2 and pBIND-SF-1 significantly induced the reporter, suggesting interaction. (c) KGN cells co-transfected with pG5-LUC, pACT-TIF2, and pBIND-SF-1 were treated with increasing concentrations of atrazine for 48 h before the luciferase assay. (d) Effects of domain specific mutations of SF-1. Responsiveness of ArPII to 48 h of 10^{-5} M atrazine, simazine or the solvent DMSO in NIH-3T3 cells co-transfected with either pcDNA3.1 or variants of pcDNA3.1-hSF-1 (WT, wild type; G35E, DBD mutation that abolishes SF-1 transactivation dramatically; G341E and Y436F-K440A, pocket mutations that abolishes SF-1-phospholipid interaction partially or near-completely, respectively). Letters above bars show statistical groups (ANOVA, $P < 0.05$). RLA, relative luciferase activity.

interaction of SF-1 and its endogenous phospholipid ligand partially and near-completely, respectively [22]). The effects of these mutants in mediating the responsiveness of ArPII to atrazine were analyzed by an experiment similar to Fig. 3a. Fig. 4d shows that, while G35E SF-1 lost its ability to convey the responsiveness, effects of both LBD pocket mutants were indistinguishable from that of wild type. These data indicate that functional LBD is not critically required for SF-1 to mediate the atrazine stimulation, suggesting the binding is unlikely to be within the hydrophobic ligand binding pocket, and atrazine may interact with SF-1 via regions other than the LBD.

While the precise domain(s) responsible for the affinity awaits further study, our data suggest an alternative mechanism by which atrazine induces *CYP19* expression. Atrazine may not only elevate cellular cAMP levels as a result of phosphodiesterase inhibition; but also binds to and activates the receptor SF-1, and thus induces *CYP19* expression via promoter II (a proposed model is depicted in Supplementary Fig. 4). This may serve as a common mechanism for the various adverse effects reported for atrazine (including reproductive toxicity, sexual differentiation disruption and carcinogenicity in wildlife, laboratory animals, and humans) and may also have important impacts on the current debate over atrazine.

Acknowledgments

The study was supported by a Grand-in-Aid for "mechanism of sex differentiation" and a grand for the 21st century COE program from the Ministry of Education, Culture, Sports, Science and Technology of Japan. WuQiang Fan is supported by the Japan Society for the Promotion of Science (JSPS).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.062.

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