

2-Chloro-*s*-Triazine Herbicides Induce Aromatase (CYP19) Activity in H295R Human Adrenocortical Carcinoma Cells: A Novel Mechanism for Estrogenicity?

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Received May 25, 1999; accepted September 7, 1999

There is increasing concern that certain chemicals in the environment can cause endocrine disruption in exposed humans and wildlife. Investigations of potential effects on endocrine function have been limited mainly to interactions with hormone receptors. A need exists for the development of alternate *in vitro* methods to evaluate chemicals for their potential to disturb various endocrine functions via other mechanisms. Our laboratory is using the human H295R adrenocortical carcinoma cell line to examine chemicals for their potential to interfere with the activity and/or expression of several key cytochrome P450 (CYP) enzymes involved in the biosynthesis of steroid hormones. In this report we demonstrated that the commonly used 2-chloro-*s*-triazine herbicides atrazine, simazine, and propazine dose-dependently (0–30 μ M) induced aromatase (CYP19) activity to an apparent maximum of about 2.5-fold in H295R cells. Basal- and triazine-induced aromatase activity was completely inhibited by the irreversible aromatase inhibitor 4-hydroxyandrostenedione (100 μ M). The triazines increased levels of CYP19 messenger ribonucleic acid (mRNA) between 1.5- and 2-fold. The time-response profile of the induction of aromatase activity and CYP19 mRNA by the triazines was similar to that by 8-bromo-cyclic adenosine monophosphate, a known stimulant of the protein kinase-A pathway that mediates the induction of aromatase in these cells. The observed induction of aromatase, the rate-limiting enzyme in the conversion of androgens to estrogens, may be an underlying explanation for some of the reported hormonal disrupting and tumor promoting properties of these herbicides *in vivo*.

Key Words: aromatase; atrazine; simazine; propazine; triazines; induction; adrenocortical carcinoma; CYP19; mRNA.

In recent years, there has been growing concern that certain environmental contaminants and commercial products have the potential to disturb endocrine functions in exposed humans and wildlife. Disturbances by these “endocrine disruptors” may lead to impaired reproductive capacity and other toxicities related to sexual differentiation, growth, and development. Current research has focused on potential interactions with the

sex hormone receptors, particularly the estrogen receptor (Davis *et al.*, 1993; Kavlock *et al.*, 1996; Villeneuve *et al.*, 1998). However, many other mechanisms of potential interference with endocrine functions exist, and the term endocrine disruption, unless more specifically defined, can refer to interactions with an almost unlimited number of physiological functions. We wish to focus on effects on key enzymes involved in steroid synthesis and breakdown. For this purpose, we have evaluated the human H295R adrenocortical carcinoma cell line (Gazdar *et al.*, 1990; Rainey *et al.*, 1994) as an *in vitro* tool for examining potential interferences of chemicals with levels of mRNA and/or catalytic activities of several steroidogenic cytochrome P450 (CYP) enzymes (Sanderson and Van den Berg, 1998).

The H295 and H295R (a subpopulation of H295 that forms a monolayer in culture) human adrenocortical carcinoma cell lines have been characterized in detail and shown to express most of the key enzymes necessary for steroidogenesis (Gazdar *et al.*, 1990; Rainey *et al.*, 1993, 1994; Staels *et al.*, 1993). These include CYP11A (cholesterol side-chain cleavage), CYP11B1 (steroid 11 β -hydroxylase), CYP11B2 (aldosterone synthetase), CYP17 (steroid 17 α -hydroxylase and/or 17,20 lyase), CYP19 (aromatase), CYP21B2 (steroid 21-hydroxylase) and 3 β -hydroxysteroid dehydrogenase. The cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells, with the ability to produce the steroid hormones of each of the three phenotypically distinct zones found in the adult adrenal cortex (Gazdar *et al.*, 1990; Staels *et al.*, 1993). To elucidate an underlying mechanism of action for some of the reported endocrine-disrupting properties of the 2-chloro-*s*-triazine herbicides (Elridge *et al.*, 1994; Stevens *et al.*, 1994), we examined the effects of atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-*s*-triazine), simazine (2-chloro-4,6-bis[ethylamino]-*s*-triazine), and propazine (2-chloro-4,6-bis[isopropylamino]-*s*-triazine) on mRNA expression and catalytic activity of CYP19 in the H295R cell line.

The triazine family of broad-leaved herbicides has been used increasingly since the 1960s to control weeds, particularly in maize crops, in North America and Europe. The estimated use

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of atrazine in the United States was almost 35,000 tons in 1993 (U.S. EPA, 1994). Consequently, it is found in relatively high concentrations in surface waters in large parts of the North American continent (Solomon *et al.*, 1996). It is relatively resistant to abiotic and biotic breakdown (Khan and Foster, 1976; Solomon *et al.*, 1996). Epidemiological studies have associated long-term exposures to triazine herbicides with increased risk of ovarian cancer in female farm workers in Italy (Donna *et al.*, 1989) and increased risk of breast cancer in the general population of Kentucky in the United States (Kettles *et al.*, 1997). In experiments with female F344 rats, atrazine has been shown to induce tumors of the mammary gland and reproductive organs (Pinter *et al.*, 1990). In female Sprague-Dawley rats, atrazine caused lengthening of the estrous cycle and a dose-dependent increase in plasma levels of estradiol (Wetzel *et al.*, 1994). Atrazine also resulted in an earlier onset of the incidence of mammary and pituitary tumors (Wetzel *et al.*, 1994), responses typical of exposure to exogenously administered estrogens (Brawer and Sonnenschein, 1975; Geschickter and Byrnes, 1942). Investigations into the mechanism of the observed pro- and/or antiestrogenic effects of triazine herbicides have been limited to interactions with the estrogen receptor and effects on receptor-mediated responses (Connor *et al.*, 1996; Tennant *et al.*, 1994a,b). These studies have not been able to provide a consistent explanation for the observed responses and any spurious effects on estrogen receptor function occurred at extremely high concentrations. Our study provides a mechanistically plausible explanation for the estrogen-related endocrine-disrupting effects of the family of 2-chloro-*s*-triazine herbicides by demonstrating their common ability to induce the catalytic activity and mRNA levels of CYP19 using the H295R cell line as a steroidogenic model system.

MATERIALS AND METHODS

Cell culture conditions. H295R cells were obtained from the American Type Culture Collection (ATCC # CRL-2128) and grown in 75-cm² flasks (Greiner, Germany) under culture conditions published previously (Rainey *et al.*, 1993, 1994). Cells were cultured in the presence of 2% of the steroid-free serum replacement Ultrosor SF (Soprachem, France). Cells were grown until almost confluent and then trypsinized and suspended in 75 ml of culture medium, sufficient for seeding three 24-well plates. These plates were seeded with 1 ml of cell suspension per well. The culture medium was changed 24 h after plating, during which time attachment of the cells had occurred. Under these conditions, the cells were almost confluent in each well (between 1 and 2 × 10⁵ cells/well). At this time, cells were exposed to the triazine herbicides (Riedel-deHaen, Germany) added to the wells at various concentrations and dissolved in 1 μl of dimethyl sulfoxide (DMSO; D4540, Sigma-Aldrich, U.S.), resulting in a 0.1% or 14 mM concentration of DMSO in the culture medium. Negative control cells received 1 μl of DMSO. Positive control cells were exposed to 100 μM of 8-bromo-cyclic adenosine monophosphate (8Br-cAMP) dissolved in medium containing 0.1% DMSO. 8-Br-cAMP is a synthetic analog of the intracellular signal transducer cAMP, which is known to increase CYP19 gene transcription, resulting in the induction of aromatase activity. Unexposed cells were included as further controls. DMSO at 0.1% had no effect on CYP19 expression or catalytic activity relative to unexposed cells. The triazines did not cause cytotoxicity at any of the test concentrations (0–30

μM), based on visual inspection of the cells, cell attachment, protein content of the wells, and the inability of the triazines to decrease the mitochondrial activity of succinate dehydrogenase (MTT test; Denizot and Lang, 1986). Protein concentrations were determined by the fluorometric method of Udenfriend *et al.*, (1972), using bovine serum albumin (A9647, Sigma-Aldrich, U.S.) as standard. Triazines were added to the cell culture medium at concentrations well below their aqueous solubility limit of 300 μM. Exposures were for 24 h unless otherwise stated.

Isolation and amplification of RNA. RNA was isolated using the RNA Insta-Pure System (Eurogentec, Belgium) according to the manufacturer's instructions and stored at -70°C. RT-PCRs were performed using the Access RT-PCR System (Promega, U.S.). Northern blotting was not considered as an option because of the low basal expression of aromatase, which could not be detected by other investigators using this technique (Staels *et al.*, 1993). RNA preparations were considered acceptable for RT-PCR when their A260 (nm)/A280 (nm) ratios were greater than 1.8. The purity of the RNA preparations was verified by denaturing agarose gel electrophoresis. Suitable primer pairs were obtained by entering the human CYP19 cDNA sequence obtained from the European Molecular Biology Laboratories database into the software program Geneworks (version 2.4; IntelliGenetics, U.S.). The primer pair used for CYP19 mRNA amplification was: 5'-TTA-TGA-GAG-CAT-GCG-GTACC-3'; 5'-CTT-GCA-ATG-TCT-TCA-CGT-GG-3', resulting in an amplification product of 314 base pairs. PCR conditions, such as annealing temperature and Mg²⁺ concentration were optimized empirically. The conditions of the RT-PCR using the Access RT-PCR system kit were adapted as follows: RT-PCR for CYP19 mRNA (100 ng/reaction) was performed in the presence of 0.75 mM MgSO₄. After a reverse transcription step at 48°C for 45 min, the resultant cDNA underwent 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 1 min and extension at 68°C for 2 min. A final extension of 7 min completed the amplification. As reference, RT-PCR was performed on β-actin mRNA using a commercially available primer pair (Promega, U.S.). Beta-actin mRNA (200 ng/reaction) was amplified according to the procedure above with slight modifications. RT-PCR was performed in the presence of 1 mM MgSO₄. The reverse transcription step took place at 55°C and the annealing and extension temperatures were 70 and 72°C, respectively, during the 40 cycles. The final extension took place at 68°C. Serial dilutions of total RNA concentrations were amplified using each primer pair to determine the linear range of the PCR reaction, so semi-quantitative inferences could be made. Furthermore, beta-actin mRNA was found not to be affected by any of the treatments (triazines or 8Br-cAMP) and could be used reliably as a reference amplification response. To further enhance the reproducibility and comparability of the RT-PCR method, we included in each triazine-exposure experiment, apart from a vehicle control (cells exposed to DMSO), also a positive control (cells exposed to 8Br-cAMP). Within a typical experiment, amplification response ratios of CYP19/beta-actin obtained from RT-PCR of 3 different RNA preparations would result in an average value with a standard deviation between 10 and 20%. Triplicate amplification response ratios obtained from single RNA preparations resulted in standard deviation less than 5%. Amplification products were detected using agarose gel electrophoresis and ethidium-bromide staining. Intensities of the ethidium bromide stains were quantified using a FluorImager (Molecular Dynamics, U.S.).

Aromatase assay. The catalytic activity of aromatase was determined based on the method of Lephart and Simpson (1991) with minor modifications. Cells were exposed to 54 nM 1β-³H-androstenedione (New England Nuclear Research Products, U.S.) dissolved in serum-free (Ultrosor SF-free) culture medium and incubated for 1.5 h at 37°C in an atmosphere of 5% CO₂ and 95% air. All further steps were as reported previously (Letcher *et al.*, 1999). The release of tritiated water was quantified by liquid scintillation counting after quench correction using an internal quench correction curve for ³H. The activity of aromatase was corrected for the distribution of the ³H-label on the androstenedione molecule, which was 25.7% at the 1α position and 74.2% at the 1β-position (Krekels *et al.*, 1991). A small volume (20 μl) of reaction medium directly added to liquid scintillation cocktail (total counts) verified the concentration of tritiated androstenedione in the reaction medium. Incubations

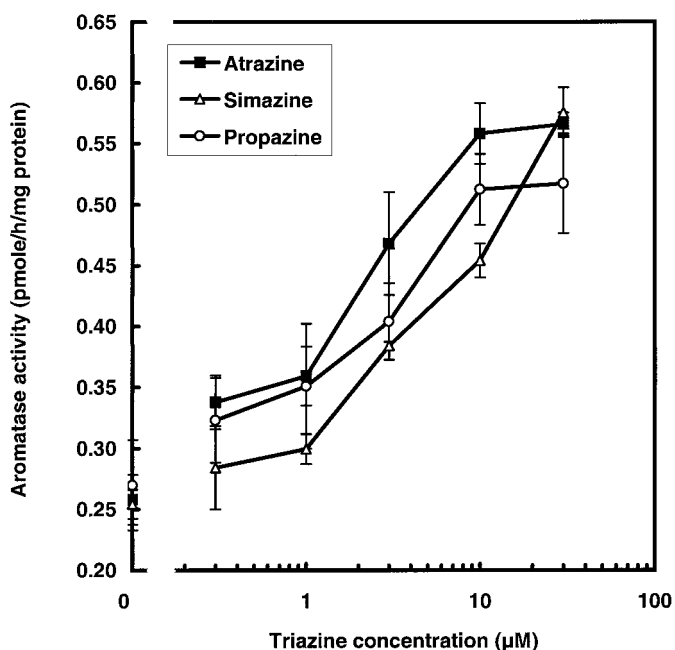


FIG. 1. Examples of dose-response curves for induction of aromatase activity by the 2-chloro-s-triazine herbicides atrazine, simazine, and propazine in H295R human adrenocortical carcinoma cells, after a 24-h exposure. Each concentration was tested in quadruplicate; error bars represent standard deviations. The first statistically significant ($p < 0.05$; t -test) increases in aromatase activity were observed at 300 nM for atrazine and propazine, and at 1 µM for simazine. Erratum: Values on y-axis should be multiplied by 4 for correct aromatase activities. Basal activity was about 1.1 pmole/h/mg protein.

in the absence of cells (blanks or background counts) were included as negative controls. The release of tritiated water by the cells was linear over time and led to about 2–6% conversion of androstenedione after 1.5 h. Aromatase activity was expressed in pmol of androstenedione converted per h per milligram protein. The specificity of the aromatase assay, based on the release of tritiated water, was verified by measuring the production of estrone (the aromatization product of androstenedione), using a 125 I-labeled double-antibody radioimmunoassay kit (DSL-8700; Diagnostic Systems, Inc, U.S.), and by using 4-hydroxyandrostenedione, an irreversible inhibitor of the catalytic activity of aromatase, to block the formation of tritiated water (Brodie *et al.*, 1977).

RESULTS

Atrazine, simazine and propazine induced aromatase activity in H295R cells concentration-dependently after a 24 h exposure, with an apparent maximum of about 2- to 2.5-fold around 30 µM (Fig. 1). A 24-h exposure to as little as 300 nM of atrazine or propazine resulted in a statistically significant increase in aromatase activity. In contrast, direct addition of the triazines to the assay medium used to determine aromatase activity had no effect (Fig. 2), ruling out a direct influence on catalytic activity. The aromatase inhibitor 4-hydroxyandrostenedione completely blocked (95–98%) the basal and triazine- or 8Br-cAMP-induced activity of CYP19 at a concentration of 100 µM (Fig. 2). The induction of aromatase activity by atrazine was further verified by the parallel measurement of tritiated water and estrone in the same cellular incubations.

Estrone production increased dose-dependently in atrazine-treated cells and in cells exposed to 8Br-cAMP (Fig. 3). The amount of estrone produced corresponded well with the amount of tritiated water released, in the expected one-to-one ratio (see correlation equation in legend of Fig. 3).

Reverse-transcriptase polymerase chain reaction analysis of various amounts of RNA isolated from cells exposed to atrazine (30 µM) or 8Br-cAMP (100 µM) demonstrated that CYP19 mRNA was consistently increased relative to control cells by both treatments (Fig. 4). The amplification response to β-actin mRNA, used as a reference, was not affected by the treatments (Fig. 4). The amplification responses to CYP19 and β-actin mRNA were linear with the logarithm of the amount of RNA used in the PCR reaction, indicating the ability of the RT-PCR assay to be used semi-quantitatively. H295R cells exposed to atrazine, simazine, or propazine for 24 h, showed an increase in the amplification response to CYP19 mRNA of 1.5- to 2-fold compared with control cells (Fig. 5). The amplification response to β-actin was not affected by any of these treatments. A 24-h exposure to 100 µM 8Br-cAMP led to an increase in amplification response to CYP19 mRNA of about 2.5-fold (Fig. 5).

The induction of the catalytic activity of aromatase by 30 µM atrazine was measured over several days and compared

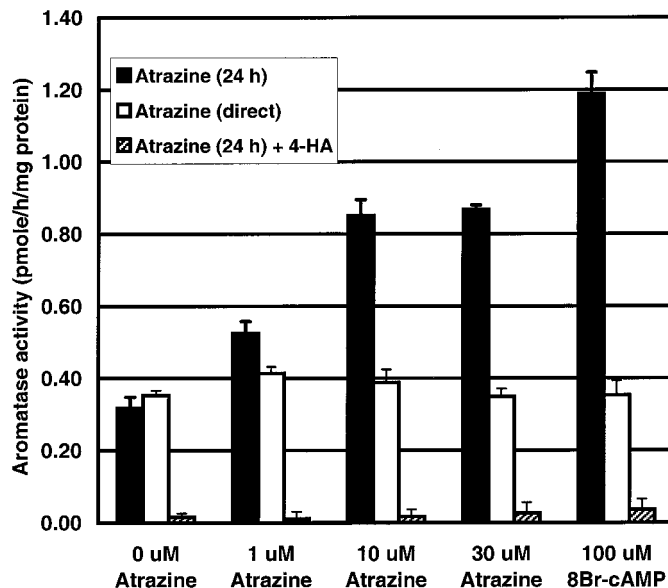


FIG. 2. Aromatase activity in H295R human adrenocortical carcinoma cells exposed to atrazine or 8-bromo-cyclic adenosine monophosphate (8Br-cAMP) 24 h prior to aromatase assay (solid bars), exposed to atrazine or 8Br-cAMP for only the duration of the assay (1.5 h) (these cells were exposed to the test chemicals while they were being incubated in medium containing radiolabeled androstenedione [open bars]), exposed to atrazine or 8Br-cAMP 24 h prior to assay and to 100 µM of the irreversible aromatase inhibitor 4-hydroxyandrostenedione (4-HA) for the duration of the assay (shaded bars). Each concentration was tested in quadruplicate; error bars represent standard deviations. Erratum: Values on y-axis should be multiplied by 4 for correct aromatase activities.

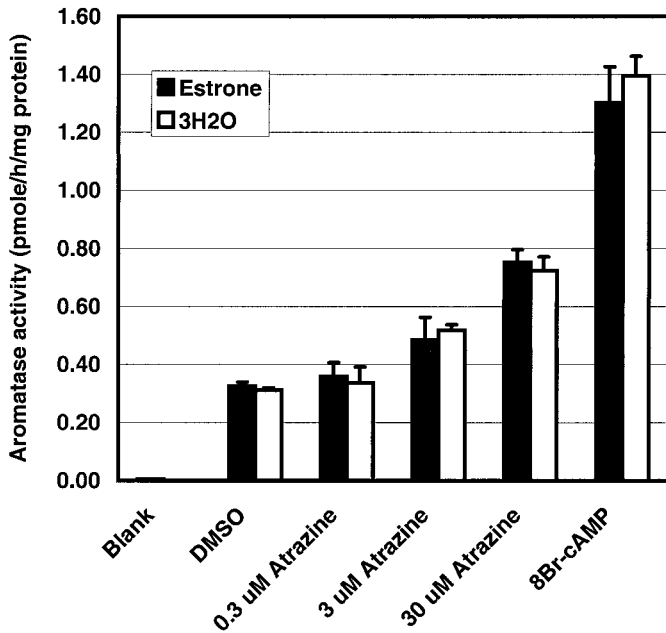


FIG. 3. Comparison of aromatase activity based on estrone production or tritiated water release in H295R human adrenocortical carcinoma cells treated with atrazine or 100 μ M 8Br-cAMP. Each concentration was tested in quadruplicate; error bars represent standard deviations. The correlation between the two measurements resulted in the equation: $1.1 \times (\text{estrone production}) - 0.04 = {}^3\text{H}_2\text{O}$ release ($r = 0.99$; $n = 20$). Erratum: Values on y-axis should be multiplied by 4 for correct aromatase activities.

with the response to 100 μ M 8Br-cAMP (Fig. 6). Atrazine led to induction levels of 2- to 2.5-fold after 72 h, less efficacious than the 6-fold induction by 8Br-cAMP. Over the same time-

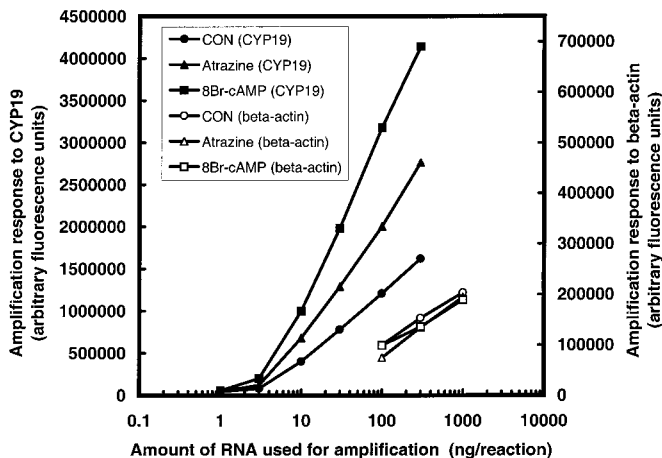


FIG. 4. Relationship between amount of RNA used in the PCR reaction and the amplification response to CYP19 (coding for the aromatase enzyme) (solid symbols) or β -actin messenger RNA (open symbols), after various treatments. The amplification response to the mRNA of both proteins was linear with the logarithm of the amount of RNA under the RT-PCR conditions used (see Materials and Methods). The amplification response to CYP19 mRNA was greater in H295R cells treated with atrazine (30 μ M) or 8Br-cAMP (100 μ M) than in controls (DMSO). The amplification response to β -actin mRNA was not affected by any of the treatments.

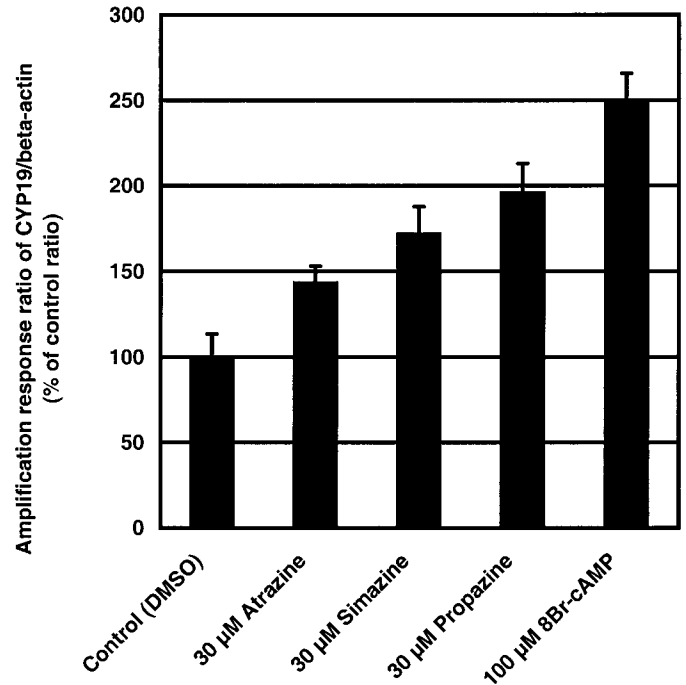


FIG. 5. Levels of CYP19 mRNA in H295R human adrenocortical carcinoma cells exposed to dimethyl sulfoxide (DMSO) vehicle, 30 μ M atrazine, simazine or propazine, or 100 μ M 8Br-cAMP. Each concentration was tested in triplicate; error bars represent standard deviations. Each treatment resulted in an amplification response that was significantly greater than control ($p < 0.05$; t -test).

period, atrazine increased the amplification response to CYP19 mRNA about 1.5-fold in the first 24 h, after which the increase reversed (Fig. 7). 8Br-cAMP-mediated induction of CYP19 mRNA resulted in a similar response, causing a 2.5-fold increase after 24 h, before declining (Fig. 7). The time-response profiles for aromatase and CYP19 mRNA induction by atrazine and 8Br-cAMP were comparable (Figs. 6 and 7), suggesting that the triazines induce aromatase by increasing transcription of the CYP19 gene. However, alternate mechanisms resulting in increased mRNA levels, such as mRNA stabilization, cannot be ruled out at present.

DISCUSSION

The cellular regulation of CYP19 gene induction and that of other steroidogenic CYPs is highly complex and tissue-specific, and poorly understood (Simpson *et al.*, 1993, 1994). In the human adrenal cortex, steroidogenic CYPs are induced following an initial interaction of adrenocorticotrophic hormone (ACTH) with the ACTH receptor in the cell membrane, resulting in increased intracellular cAMP levels and a subsequent cascade of events leading to increased gene transcription (Parker and Schimmer, 1995). H295R cells are poorly responsive to ACTH, suggested to be due to loss of ACTH receptor expression, which is a common phenomenon in cultured adre-

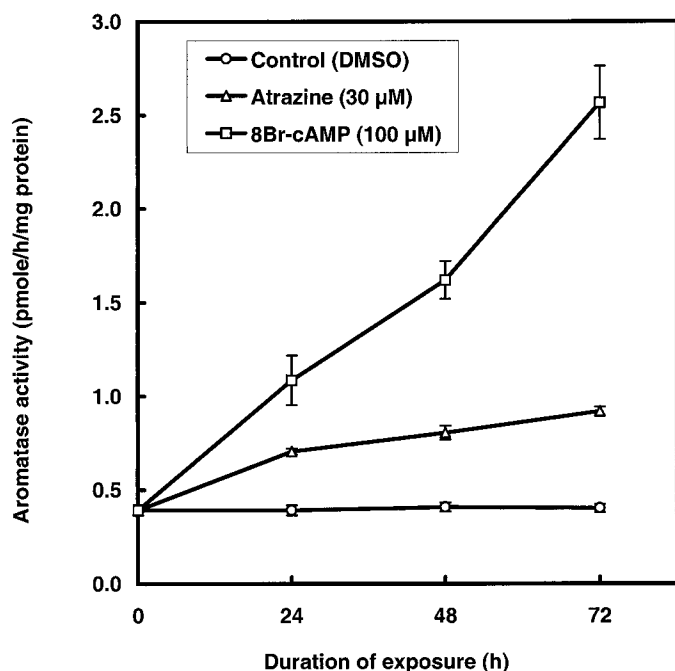


FIG. 6. Time-course of aromatase induction in H295R human adrenocortical carcinoma cells exposed to 30- μ M atrazine or 100- μ M 8Br-cAMP. Each concentration was tested in quadruplicate; error bars represent standard deviations. The triazines and 8Br-cAMP induced aromatase activity significantly above control at each time point ($p < 0.05$; t -test). Erratum: Values on y-axis should be multiplied by 4 for correct aromatase activities.

nocortical cells (Rainey *et al.*, 1993), but they are highly responsive to forskolin and analogs of cAMP, indicating that the cAMP-mediated protein kinase-A pathway is functional (Rainey *et al.*, 1993; Staels *et al.*, 1993). The observed similarities in response between the triazines and 8Br-cAMP suggest that they induce CYP19 by stimulation of this pathway. One possible mechanism of stimulation could be via the inhibition of phosphodiesterase, the enzyme responsible for the breakdown of cAMP to AMP; some compounds containing a triazine-moiety have been reported to be weak inhibitors of this enzyme, causing increased smooth-muscle relaxation in guinea pig tracheal tissue (Leroux *et al.*, 1999). Given the complex and tissue-specific regulation of aromatase, the induction response observed in H295R cells, which appear to be most similar to not-fully-differentiated fetal adrenocortical cells, may not be a general response of triazine herbicides in other aromatase-expressing tissues such as ovaries, brain, and adipose tissue. It is, however, the first consistent *in vitro* response by these herbicides that may provide a plausible explanation for some of their endocrine-disrupting properties *in vivo*. A recent study reported elevated aromatase activity in the gonadal-adrenal mesonephros of male alligator (*Alligator mississippiensis*) hatchlings exposed *in ovo* to atrazine (Crain *et al.*, 1997). Although the increase was not statistically significant, this finding leaves open the possibility that CYP19 induction by triazine herbicides may be a more general response. Further

studies are required to establish whether triazine-mediated aromatase induction can be achieved in other steroidogenic tissues and in other species *in vitro* and *in vivo*.

This is the first report to demonstrate consistently that several members of the class of 2-chloro-*s*-triazine herbicides induce the human aromatase enzyme *in vitro* at relatively low concentrations. Although the observed 2-fold induction is relatively weak, aromatase is the rate-limiting enzyme in the conversion of androgens to estrogens. Induction of this rate-limiting enzyme *in vivo* would be expected to lead to increased local production of estrogens with the potential to cause or contribute to estrogen-mediated pathologies, such as the tumor promotion observed experimentally in atrazine-exposed rats (Elridge *et al.*, 1994; Pinter *et al.*, 1990; Stevens *et al.*, 1994; Wetzel *et al.*, 1994). Furthermore, elevated expression of aromatase has been associated with increased risk of breast cancer (Bulun and Simpson, 1994; Lu *et al.*, 1996; Reed *et al.*, 1993) and endometrial cancer (Bulun *et al.*, 1994) in female humans. A study examining the effects of aromatase inhibition on embryonic development found that exposure *in ovo* of chickens to non-steroidal aromatase inhibitors led to "masculinization" of females (Elbrecht and Smith, 1992). Furthermore, co-administration of exogenous estrogen prevented the observed masculinization of females but led to "feminization" of males, which is the "default" sex in avian species (Elbrecht and Smith, 1992). Estrogens appear to be the key hormones that lead to

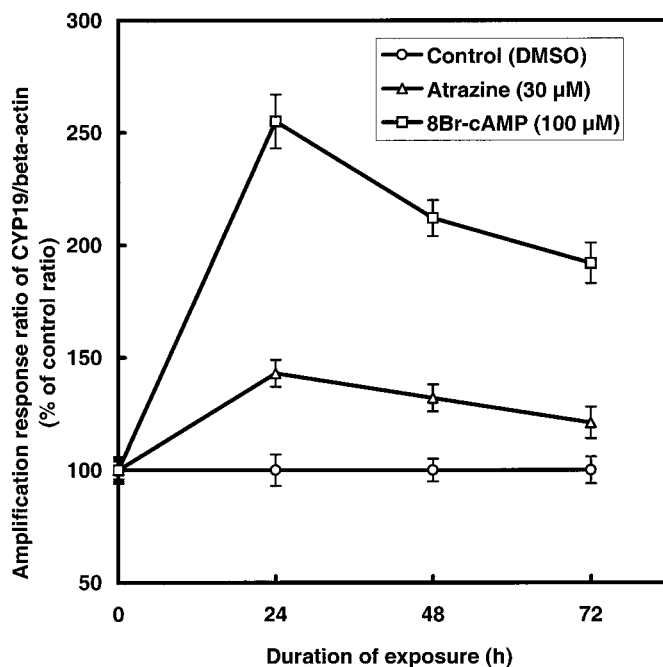


FIG. 7. Time-course of the increase in CYP19 mRNA levels in H295R human adrenocortical carcinoma cells exposed to dimethyl sulfoxide (DMSO) vehicle, 30 μ M atrazine or 100 μ M 8Br-cAMP. Each concentration was tested in triplicate; error bars represent standard deviations. The triazines and 8Br-cAMP increased the amplification response to CYP19 mRNA significantly above control at each time point ($p < 0.05$; t -test).

feminization of the central nervous system in birds, while they lead to defeminization and masculinization of the mammalian central nervous system (Jost, 1983). Thus, it can be suggested that during critical (irreversible) developmental periods, such as embryonic, perinatal, and pubertal development, aromatase induction may result in inappropriate (de)feminizing responses, depending on the tissues in which local estrogen concentrations have been increased. A logical concern would be that exposure of wildlife and humans 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.

The H295R cell system deployed in the present study has proven useful in identifying a potential essential target for the endocrine-disrupting effects of several 2-chloro-*s*-triazine herbicides. If future studies *in vivo* support our hypothesis linking aromatase induction in specific target tissues to the endocrine toxicities of the triazines, this may have implications for the regulation of this class of herbicides as potential endocrine disrupters and tumor promoters.

ACKNOWLEDGMENTS

We thank Marjoke Heneweer for her technical assistance. We are grateful to Bas Defize at the Hubrecht Laboratory for the use of their FluorImager. We are thankful to Dr. Bas Blaauboer and Dr. Gail Bellward for their comments and suggestions. This work was supported financially by European Union Grant ENV4-CT97-0581.

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