G-Protein–Coupled Receptor 30 and Estrogen Receptor-α Are Involved in the Proliferative Effects Induced by Atrazine in Ovarian Cancer Cells

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Background: Atrazine, one of the most common pesticide contaminants, has been shown to up-regulate aromatase activity in certain estrogen-sensitive tumors without binding or activating the estrogen receptor (ER). Recent investigations have demonstrated that the orphan G-protein–coupled receptor 30 (GPR30), which is structurally unrelated to the ER, mediates rapid actions of 17β-estradiol and environmental estrogens.

Objectives: Given the ability of atrazine to exert estrogen-like activity in cancer cells, we evaluated the potential of atrazine to signal through GPR30 in stimulating biological responses in cancer cells.

Methods and results: Atrazine did not transactivate the endogenous ERα in different cancer cell contexts or chimeric proteins encoding the ERα and ERβ hormone-binding domain in gene reporter assays. Moreover, atrazine neither regulated the expression of ERα nor stimulated aromatase activity. Interestingly, atrazine induced extracellular signal-regulated kinase (ERK) phosphorylation and the expression of estrogen target genes. Using specific signaling inhibitors and gene silencing, we demonstrated that atrazine stimulated the proliferation of ovarian cancer cells through the GPR30–epidermal growth factor receptor transduction pathway and the involvement of ERα.

Conclusions: Our results indicate a novel mechanism through which atrazine may exert relevant biological effects in cancer cells. On the basis of the present data, atrazine should be included among the environmental contaminants potentially able to signal via GPR30 in eliciting estrogenic action.

Key words: 17β-estradiol, atrazine, estrogen receptor, GPR30, ovarian cancer cells. *Environ Health Perspect* 116:1648–1655 (2008). doi:10.1289/ehp.11297 available via *http://dx.doi.org/* [Online 22 July 2008]

Atrazine belongs to the 2-chloro-*s*-triazine family of herbicides (Figure 1) and is the most common pesticide contaminant of groundwater and surface water (Fenelon and Moore 1998; Kolpin et al. 1998; Lode et al. 1995; Miller et al. 2000; Müller et al. 1997; Solomon et al. 1996; Thurman and Cromwell 2000). Among the endocrine-disrupting effects, atrazine interferes with androgen- and estrogen-mediated processes (Babic-Gojmerac et al. 1989; Cooper et al. 1999, 2000; Cummings et al. 2000; Friedmann 2002; Kniewald et al. 1979, 1995; Narotsky et al. 2001; Shafer et al. 1999; Simic et al. 1991; Stoker et al. 1999, 2000). The interference of atrazine with androgen and estrogen action does not occur by direct agonism or antagonism of cognate receptors for these steroids as shown by binding affinity studies (Roberge et al. 2004; Tennant et al. 1994a, 1994b). In this respect, previous investigations have suggested that atrazine reduces androgen synthesis and action (Babic-Gojmerac et al. 1989; Kniewald et al. 1979, 1980, 1995; Simic et al. 1991) and stimulates estrogen production (Crain et al. 1997; Heneweer et al. 2004; Keller and McClellan-Green 2004; Sanderson et al. 2000, 2001, 2002; Spano et al. 2004). The latter ability is exerted through at least two mechanisms that converge on increasing aromatase expression and activity. First, inhibiting phosphodiesterase, atrazine up-regulates cAMP, which induces the expression of SF-1, an important regulator of the PII promoter of aromatase gene *CYP19*. The enhanced transcription of the aromatase gene increases both enzymatic activity of aromatase and estrogen production (Heneweer et al. 2004; Lehmann et al. 2005; Morinaga et al. 2004; Roberge et al. 2004; Sanderson et al. 2000, 2001). Next, atrazine binds to SF-1 and facilitates the recruitment of this factor to the PII promoter of the aromatase gene, further stimulating the biological effects described above (Fan et al. 2007a, 2007b).

Epidemiologic studies have associated long-term exposure to triazine herbicides with increased risk of ovarian cancer in female farm workers in Italy (Donna et al. 1989) and breast cancer in the general population of Kentucky in the United States (Kettles et al. 1997). In addition, atrazine leads to tumor development in the mammary gland and reproductive organs of female F344 rats (Pintér et al. 1990), whereas in Sprague-Dawley rats it causes an earlier onset of mammary and pituitary tumors (Wetzel et al. 1994), a typical response to exogenously administered estrogens (Brawer and Sonnenschein 1975).

Given the potential ability of atrazine to interfere with reproduction and to cause cancer, the European Union banned its use. However, the U.S. Environmental Protection Agency has approved the use of atrazine because of the lack of a clear association between the levels of exposure and cancer incidence in pesticide applicators (Gammon et al. 2005; McElroy et al. 2007; Rusiecki et al. 2004; Sass and Colangelo 2006; Young et al. 2005).

Regarding the apparent estrogenic effects of atrazine, previous studies have demonstrated that triazine herbicides do not bind or activate the classical estrogen receptor (ER) (Connor et al. 1996; Tennant et al. 1994a, 1994b). In recent years, increasing evidence has demonstrated in different experimental models that steroid hormones, including estrogens, can exert rapid actions interacting with receptors located within or near the cell membrane (Falkenstein et al. 2000; Norman et al. 2004; Revelli et al. 1998). The importance of this signaling mechanism is becoming more widely recognized as steroid membrane receptors have been implicated in a large number of physiologic functions. Moreover, it has been suggested that nongenomic estrogen actions, like genomic ones, are susceptible to interference from environmental estrogens (Thomas 2000). Of note, these compounds compete with [^{3}H]17β-estradiol ([^{3}H]E_2) for binding to estrogen membrane receptors (Loomis and Thomas 2000) and exert agonist effects on nongenomic transduction pathways in different cell contexts (Loomis and Thomas 2000; Nadal et al. 2000; Ruehlmann et al. 1988; Watson et al. 1999). However, the precise identity and function of many steroid membrane receptors are still controversial in terms of their specific molecular interactions with endogenous and environmental estrogens.

A seven-transmembrane receptor, G-protein–coupled receptor 30 (GPR30), which is structurally unrelated to the nuclear ER, has been recently shown to mediate rapid actions of estrogens (Filardo et al. 2002; Revankar et al. 2005). Recombinant GPR30 protein, produced in ER-negative HEK-293 cells, exhibited all the steroid binding and signaling

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characteristics of a functional estrogen membrane receptor (Thomas et al. 2005; Thomas and Dong 2006). Our studies and others have also demonstrated that GPR30 mediates the rapid response to E_2 in a variety of estrogen-responsive cancer cells by activating the epidermal growth factor receptor (EGFR)–mitogen-activated protein kinase (MAPK) transduction pathway (Albanito et al. 2007; Bologa et al. 2006; Filardo et al. 2000; Maggiolini et al. 2004; Revankar et al. 2005; Thomas et al. 2005; Vivacqua et al. 2006a, 2006b).

In the present study, for the first time we have demonstrated that atrazine stimulates gene expression and growth effects in estrogen-sensitive ovarian cancer cells through GPR30 and the involvement of ERα. Moreover, we show that GPR30 mediates the stimulatory effects of atrazine in ER-negative SkBr3 breast cancer cells.

Materials and Methods

Reagents. We purchased atrazine [2-chloro-4-(ethylamine)-6-(isopropylamine)-*s*-triazine], 17β-estradiol (E_2), *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89), wortmannin (WM), and PD98059 (PD) from Sigma-Aldrich (Milan, Italy); AG1478 (AG) from Biomol Research Laboratories (DBA, Milan, Italy); ICI 182,780 (ICI) from Tocris Chemicals (Bristol, UK); and GF109203X (GFX) from Calbiochem (VWR International, Milan, Italy). All compounds were solubilized in dimethyl sulfoxide (DMSO), except E_2 and PD, which were dissolved in ethanol.

Cell culture. Human BG-1 and 2008 ovarian cancer cells as well as human Ishikawa endometrial cancer cells were maintained in phenol red–free Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). H295R adrenal carcinoma cells were cultured in DMEM/F12 1:1 supplemented with 1% ITS Liquid Media Supplement (Sigma-Aldrich), 10% calf serum, and antibiotics. Human MCF-7 breast cancer cells were maintained in DMEM with phenol red supplemented with 10% FBS, and human SkBr3 breast cancer cells were maintained in phenol red–free RPMI 1640 supplemented with 10% FBS. Cells were switched to medium without serum the day before experiments for immunoblots and reverse transcription-polymerase chain reaction (RT-PCR).

Plasmids. Firefly luciferase reporter plasmids used were XETL for ERα (Bunone et al. 1996) and GK1 for yeast transcription factor Gal4 fusion proteins (Webb et al. 1998). XETL contains the estrogen response element (ERE) from the *Xenopus* vitellogenin *A2* gene (nucleotides –334 to –289), the herpes simplex virus thymidine kinase promoter region (nucleotides –109 to +52), the firefly luciferase coding sequence, and the SV40 splice and polyadenylation sites from plasmid pSV232A/L-AA5. Gal4 chimeras Gal-ERα and Gal-ERβ were expressed from plasmids GAL93.ER(G) and GAL.ERβ, respectively. They were constructed by transferring the coding sequences for the hormone-binding domain (HBD) of ERα (amino acids 282–595) from HEG0 (Bunone et al. 1996), and for the ERβ HBD (C-terminal 287 amino acids) from plasmid pCMV5-hERβ into the mammalian expression vector pSCTEVGal93 (Seipel et al. 1992). We used the *Renilla* luciferase expression vector pRL-TK (Promega, Milan, Italy) as a transfection standard.

Transfection and luciferase assays. BG-1, MCF-7, Ishikawa, and SkBr3 cells (1×10^5) were plated into 24-well dishes with 500 µL/well DMEM (BG-1, MCF-7, and Ishikawa cells) or RPMI 1640 (SkBr3 cells) containing 10% FBS the day before transfection. We replaced the medium with phenol red–free DMEM or RPMI 1640, both supplemented with 1% charcoal-stripped (CS) FBS, on the day of transfection. Transfections were performed using FuGENE 6 Reagent as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany) with a mixture containing 0.3 µg of reporter plasmid, 1 ng pRL-TK, and 0.1 µg effector plasmid where applicable. After 5–6 hr, the medium was replaced again with serum-free DMEM lacking phenol red and supplemented with 1% CS-FBS; ligands were added at this point, and cells were incubated for 16–18 hr. We measured luciferase activity with the Dual Luciferase Kit (Promega) according to the manufacturer's recommendations. Firefly luciferase values were normalized to the internal transfection control provided by *Renilla* luciferase activity. The normalized relative light unit values obtained from cells treated with vehicle were set as 1-fold induction, from which the activity induced by treatments was calculated.

RT-PCR. Using semiquantitative RT-PCR as described previously (Maggiolini et al. 1999), we evaluated gene expression for *ERα* [GenBank accession no. NM 000125 (National Center for Biotechnology Information 2008)], c-*fos* (NM 005252), progesterone receptor (*PR;* NM 000926), *pS2* (NM 003225), cathepsin D (NM 001909), cyclin A (NM 001237), cyclin D1 (NM 053056), cyclin E (NM 001238), and the acid phosphoprotein *P0* (*36B4*) (NM 001002) used as a control gene. We used the primers 5′-AATTCAGATAATCGACGCCAG-3′ (*ERα* forward) and 5′-GTGTTTCAACATTCTCCCTCCTC-3′ (*ERα* reverse); 5′-AGAAAAGGAGAATCCGAAGGGAAA-3′ (c-*fos* forward) and 5′-ATGATGCTGGGACAGGAAGTC-3′ (c-*fos* reverse); 5′-ACACCTTGCCTGAAGTTTCG-3′ (*PR* forward) and 5′-CTGTCCTTTTCTGGGGGACT-3′ (*PR* reverse); 5′-TTCTATCCTAATACCATCGACG-3′ (*pS2* forward) and 5′-TTTGAGTAGTCAAAGTCAGAGC-3′ (*pS2* reverse); 5′-AACAACAGGGTGGGCTTC-3′ (cathepsin D forward), and 5′-ATGCACGAAACAGATCTGTGCT-3′ (cathepsin D reverse); 5′-GCCATTAGTTTACCTGGACCCAGA-3′ (cyclin A forward) and 5′-CACTGACATGGAAGACAGGAACCT-3′ (cyclin A reverse); 5′-TCTAAGATGAAGGAGACCATC-3′, (cyclin D1 forward) and 5′-GCGGTAGTAGGACAGGAAGTTGTT-3′ (cyclin D1 reverse); 5′-CCTGACTATTGTGTCCTGGC-3′ (cyclin E forward) and 5′-CCCGCTGCTCTGCTTCTTAC-3′ (cyclin E reverse); and 5′-CTCAACATCTCCCCCTTCTC-3′ (*36B4* forward) and 5′-CAAATCCCATATCCTCGTCC-3′ (*36B4* reverse) to yield products of 345, 420, 196, 210, 303, 354, 354, 488, and 408 bp, respectively, with 20 PCR cycles for *ERα*, c-*fos*, *PR*, *pS2*, cathepsin D, *cyclin* A, and cyclin E and 15 PCR cycles for both cyclin D1 and *36B4*.

Western blotting. Cells were grown in 10-cm dishes, exposed to ligands, and then lysed in 500 µL of 50 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 1% sodium dodecyl sulfate (SDS), and a mixture of protease inhibitors containing 1 mmol/L aprotinin, 20 mmol/L phenylmethylsulfonyl fluoride, and 200 mmol/L sodium orthovanadate. We then diluted samples 10 times and determined protein concentration using Bradford reagent according to the manufacturer's recommendations (Sigma-Aldrich). Equal amounts of whole protein extract were resolved on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences, Milan, Italy). Membranes were probed overnight at 4°C with the antibody against ERα (F-10), c-fos (H-125), β-actin (C-2), phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2; E-4), and ERK2 (C-14), all purchased from Santa Cruz Biotechnology, DBA (Milan, Italy), and human P450 aromatase (MCA 2077S; Serotec, Milan, Italy), and then revealed using the ECL Western Blotting Analysis System (GE Healthcare, Milan, Italy).

ER binding assay. BG-1 cells were stripped of any estrogen by keeping them in medium without serum for 2 days. Cells were incubated

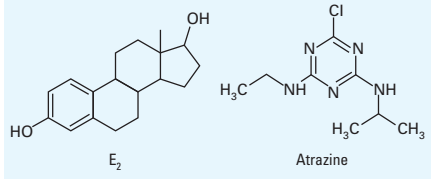

Figure 1. Structures of E_2 and atrazine.

with 1 nM [2,4,6,7-^{3}H]E2 (89 Ci/mmol; Amersham Biosciences) and increasing concentrations of nonlabeled E_2 or atrazine for 1 hr at 37°C in a humidified atmosphere of 95% air/5% CO_2. After removal of the medium, cells were washed with ice-cold phosphate-buffered saline/0.1% methylcellulose twice, harvested by scraping and centrifugation, and lysed with 100% ethanol, 500 µL/60-mm dish, for 10 min at room temperature (Lee and Gorski 1996). We measured the radioactivity of extracts by liquid scintillation counting.

Aromatase assay. In subconfluent BG-1 or H295R cells, we measured aromatase activity in the cell culture medium by tritiated water release using 0.5 µM [1β-^{3}H(*N*)]androst-4-ene-3,17-dione (25.3 Ci/mmol; DuPont NEN, Boston, MA, USA) as a substrate (Lephart and Simpson 1991). The cells were treated in a six-well dish in culture medium in the presence of atrazine or DMSO for 40 hr and then incubated with [1β-^{3}H(*N*)]androst-4-ene-3,17-dione. Incubations were performed at 37°C for 6 hr under a 95%/5% air/CO_2 atmosphere. The results were calculated as picomoles per hour, normalized to milligrams of protein (pmol/hr per 1 mg protein), and expressed as percentages of untreated cells (100%).

GPR30 and ERα silencing experiments. Cells were plated onto 10-cm dishes, maintained in antibiotic-free medium for 24 hr, and then transfected for additional 24 hr before treatments with a mixture containing Opti-MEM, 8 µL/well LipofectAMINE 2000 (Invitrogen, Milan, Italy), and 0.5 µg/well vector or short hairpin GPR30 (shGPR30) (Albanito et al. 2008), control small interfering RNA (siRNA), or ERα siRNA (Sigma-Aldrich).

Proliferation assay. For the quantitative proliferation assay, we seeded 10,000 cells in 24-well plates in regular growth medium. Cells were washed once they had attached and then incubated in medium containing 2.5% CS-FBS with the indicated treatments. Medium was renewed every 2 days (with treatments), and cells were trypsinized and counted in a hemocytometer on day 6. The day before treatments, 200 ng/L of the indicated short hairpin RNA was transfected using FuGENE 6 Reagent as recommended by the manufacturer, and then renewed every 2 days before counting.

Statistical analysis. Statistical analysis was performed using analysis of variance followed by Newman-Keuls testing to determine differences in means. *p*-Values < 0.05 are considered statistically significant.

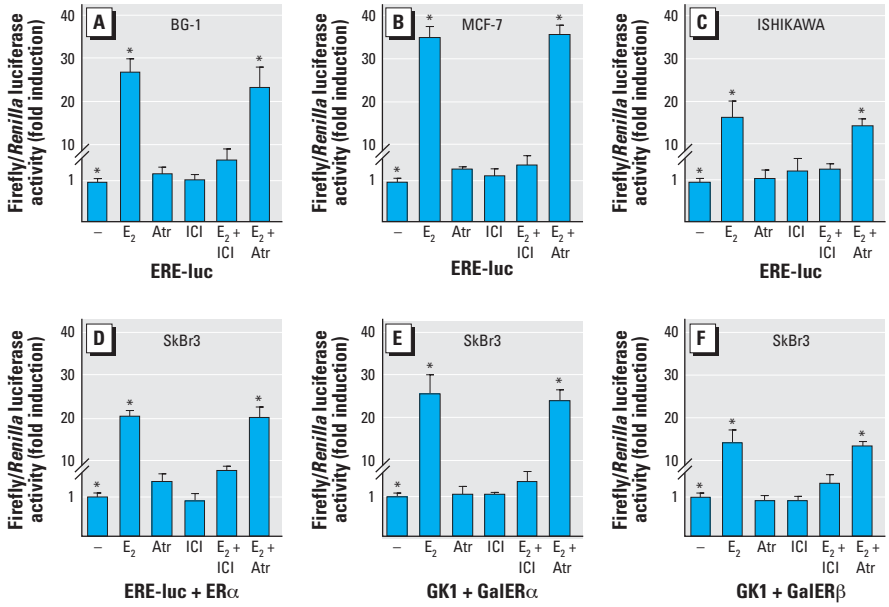

Figure 2. ERα transactivation in BG-1 (*A*), MCF-7 (*B*), and Ishikawa (*C*) cells transfected with the ER luciferase reporter plasmid XETL (ERE-luc) and treated with 100 nmol/L E_2 or 1 µmol/L atrazine (Atr), with and without 10 µmol/L ER antagonist ICI. Luciferase activities were normalized to the internal transfection control, and values of cells receiving vehicle (–) were set as 1-fold induction, from which the activity induced by treatments was calculated. (*D–F*) SkBr3 cells were transfected with ER luciferase reporter gene *XETL* and ERα expression plasmid (*D*) and with Gal4 reporter gene (*GK1*) and the Gal4 fusion proteins encoding the HBD of ERα (GalERα; *E*) and or ERβ (GalERβ; *F*) and treated with 100 nmol/L E_2 or 1 µmol/L atrazine, with and without 10 µmol/L ICI. Values shown are mean ± SD of three independent experiments performed in triplicate.
*$p < 0.05$ compared with vehicle.

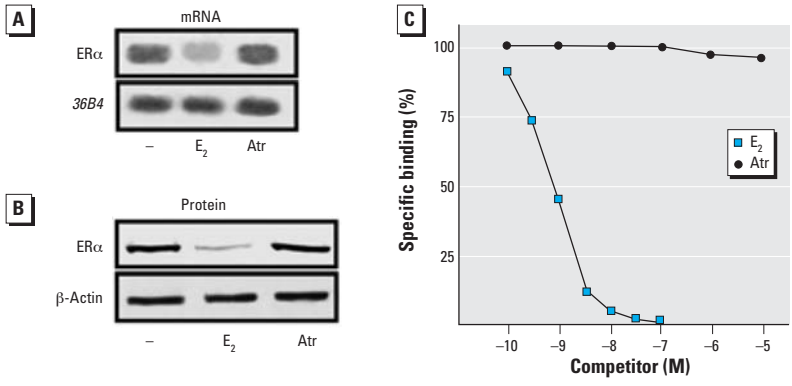

Figure 3. mRNA expression and binding of ERα in BG-1 cells treated for 24 hr with vehicle (–), 100 nmol/L E_2, or 1 µmol/L atrazine (Atr). (*A*) mRNA expression of ERα was evaluated by semiquantitative RT-PCR; the values of housekeeping gene *36B4* were determined as a control. (*B*) Immunoblot of ERα from BG-1 cells, with 100 nmol β-actin serving as a loading control. Results in (*A*) and (*B*) are representative of three independent experiments. (*C*) ERα binding assay using increasing concentrations of atrazine.

Results

Atrazine does not activate ERα in cancer cells. Based on the evidence that atrazine produces early onset and increased incidence of estrogen-sensitive tumors in different experimental models (Cooper et al. 2007), we first evaluated whether atrazine could activate a transiently transfected ER reporter gene in estrogen-sensitive ovarian (BG-1), breast (MCF-7), and endometrial (Ishikawa) cancer cells. Exposure to 100 nM E_2 induced a strong ERα transactivation that was absent in the presence of 10 µM of the ER antagonist ICI in all cell contexts evaluated (Figure 2A–C). In contrast, treatments with 1 µM atrazine and even concentrations ranging from 1 nM to 10 µM (data not shown) failed to stimulate luciferase expression or to block that observed upon addition of E_2 (Figure 2A–C). Moreover, atrazine did not activate an expression vector encoding ERα transiently transfected in ER-negative SkBr3 breast cancer cells (Figure 2D). To confirm that atrazine is not an ERα agonist and to examine whether ERβ could respond to atrazine, we turned to a completely heterologous system. Chimeric proteins consisting of the DNA binding domain of the yeast transcription factor Gal4 and the ERα or ERβ HBD transiently transfected in SkBr3

cells were strongly activated by E_2 but not upon atrazine treatment (Figure 2E,F), further corroborating the aforementioned results.

Atrazine neither regulates ERα expression nor competes with estrogen binding to ERα. Considering that the down-regulation of ERα induced by an agonist has been considered an additional hallmark of receptor activation (Santagati et al. 1997), we further investigated whether atrazine could modulate ERα expression in BG-1 cells, which lack ERβ (data not shown), and express a receptor expression pattern similar to that found in primary ovarian tumors (Bardin et al. 2004; Geisinger et al. 1989). As shown in Figure 3A,B, 100 nM E_2 down-regulated ERα at both mRNA and protein levels, whereas 1 μM atrazine did not produce any modulatory effect. In agreement with these results and those obtained in transfection experiments, atrazine showed no binding capacity for ERα (Figure 3C), as previously reported (Cooper et al. 2007). Altogether, our findings rule out that the estrogen action of atrazine occurs through binding and direct activation of ERα.

Aromatase activity is not induced by atrazine. Given that atrazine is able to up-regulate aromatase expression and function in different cell contexts (Cooper et al. 2007; Fan et al. 2007a, 2007b; Roberge et al. 2004; Sanderson et al. 2000, 2001), we then determined aromatase activity by tritiated water release assays in BG-1 cells. As shown in Figure 4, 1 μM atrazine did not stimulate aromatase activity, which in contrast was strongly induced in human H295R adrenocorticocarcinoma cells previously used as a model system to assess aromatase catalytic activity (Heneweer et al. 2004; Sanderson et al. 2001). In addition, the low aromatase protein expression detected in BG-1 cells did not increase upon exposure to 1 μM atrazine (data not shown). Hence, atrazine is neither an ERα activator nor an aromatase regulator in estrogen-sensitive ovarian cancer cells.

ERK phosphorylation is stimulated by atrazine. In recent years, numerous reports have demonstrated that estrogens and xenoestrogens can generate rapid signaling via second messenger systems such as Ca^{2+}, cAMP, nitric oxide, and G-proteins, which in turn leads to activation of different downstream kinases (Bulayeva and Watson 2004; Watson et al. 2007).

To evaluate whether the potential estrogenic activity of atrazine is exerted through a rapid cellular response, we investigated its ability to produce ERK phosphorylation in BG-1 cells. Interestingly, atrazine stimulated ERK phosphorylation, although a higher concentration and prolonged time period were required to trigger this biochemical response compared with E_2 (Figures 5A,B, 6A). ERK activation was also delayed in the presence of 1 μM atrazine compared with 100 nM E_2 in 2008 ovarian cancer cells (Figure 6D), which present a receptor expression similar to that of BG-1 cells (Safei et al. 2005). To determine the transduction pathways involved in ERK activation by atrazine, cells were exposed to 100 nM E_2 and 1 μM atrazine along with specific inhibitors widely used to pinpoint the mechanisms contributing to ERK phosphorylation (Bulayeva and Watson 2004). Of note, the ER antagonist ICI, the EGFR inhibitor AG and the ERK inhibitor PD prevented ERK activation induced by both E_2 and atrazine, whereas GFX, H89, and WM, inhibitors of protein kinase C (PKC), protein kinase A (PKA), and phosphoinositide 3-kinase (PI3K), respectively, did not (Figure 6B,C,E,F). Considering that in a previous study ICI was able to trigger ERK phosphorylation (Filardo et al. 2000), we exposed SkBr3 cells to increasing concentrations of ICI. We observed no ERK activation after

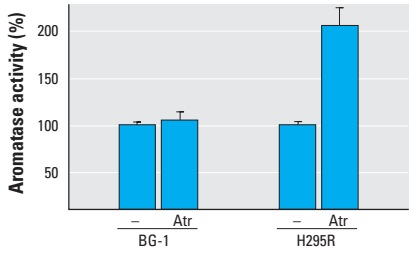

Figure 4. Aromatase activity assessed by tritiated water release in BG-1 and H295R cells treated with vehicle (–) or 1 μmol/L atrazine (Atr). Results are expressed as percentages of untreated cells (100%). Values are mean ± SD of three independent experiments, each performed in triplicate.
*$p < 0.05$ compared with vehicle.

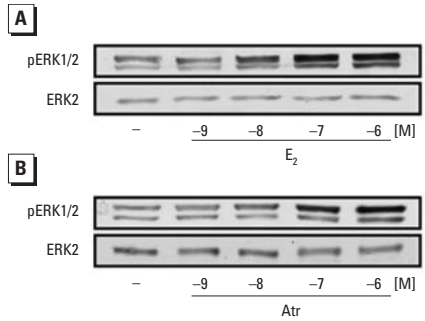

Figure 5. ERK1/2 phosphorylation (pERK1/2) in BG-1 cells exposed to increasing concentrations of E2 or atrazine (Atr) for 20 min.

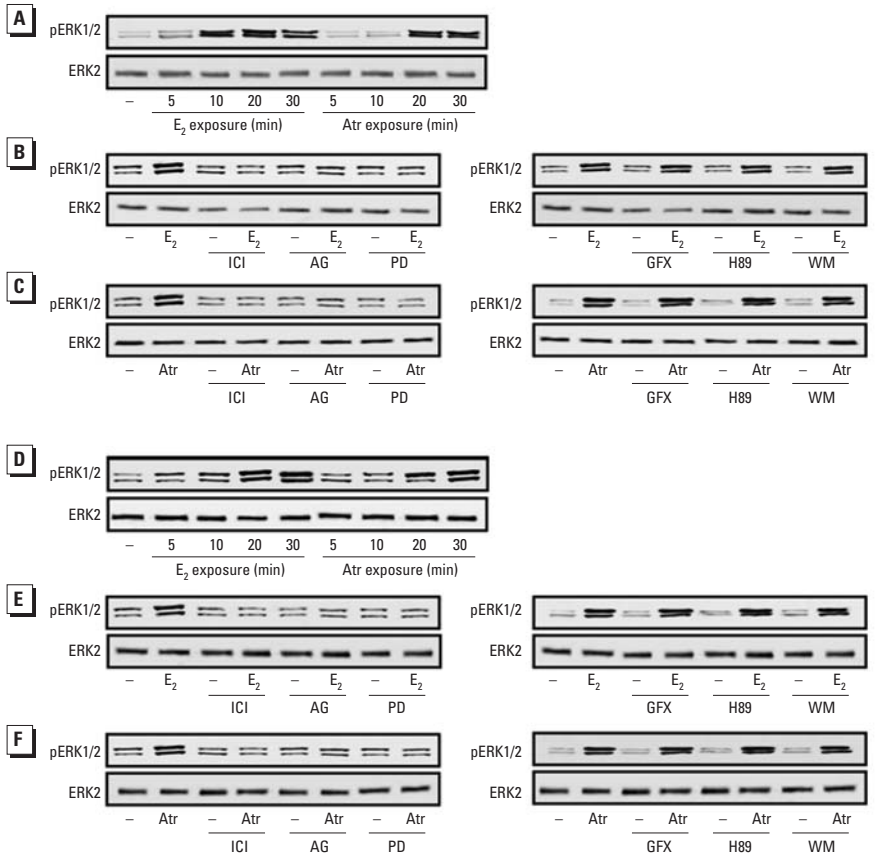

Figure 6. BG-1 (*A–C*) and 2008 (*D–F*) cells treated with vehicle (–) or 100 nmol/L E_2 with or without 1 μmol/L atrazine (Atr) for 5, 10, 20, or 30 min (*A,D*), or for 20 min with vehicle E_2 (*B* and *E*), or 1 μmol Atr in combination with 10 μmol/L ICI, AG, PD, GFX, H89, or WM, inhibitors of ER, EGFR, MEK (MAP/ERK kinase), PKC (protein kinase C), PKA (protein kinase A), and PI3K (phosphoinositide 3-kinase), respectively. pERK1/2, phosphorylated ERK 1/2.

either 5 min (data not shown) or 20 min of treatment (Figure 7). Hence, in our experimental conditions, ICI showed only ERK inhibitor activity.

Atrazine up-regulates the mRNA expression of estrogen target genes. Having determined that atrazine signals through a rapid ERK activation, we evaluated in BG-1 cells its ability to regulate the expression of c-*fos*, an early gene that responds to a variety of extracellular stimuli, including estrogens (Maggiolini et al. 2004; Nephew et al. 1993; Singleton et al. 2003; Vivacqua et al. 2006ab), along with other estrogen target genes. To this end, we performed semiquantitative RT-PCR experiments comparing mRNA levels after standardization with a housekeeping gene encoding the ribosomal protein 36B4. A short treatment (1 hr) with 1 μM atrazine enhanced c-*fos* and cyclin A levels, although to a lesser extent than 100 nM E_2, which also stimulated PR, pS2, and cyclin D1 expression (Table 1). After a 24-hr treatment, atrazine increased PR, pS2, and cyclin A levels, whereas E_2 additionally induced the expression of c-*fos*, cathepsin D, cyclin D1, and cyclin E (Table 1). We obtained results similar to those described above in 2008 cells (data not shown). Hence, atrazine is able to stimulate the expression of diverse estrogen target genes without an apparent activation of ERα.

Transduction pathways involved by atrazine in the up-regulation of* c-fos *protein levels. Using c-*fos* expression as a molecular sensor of atrazine action at the genomic level, we sought to determine whether c-fos protein levels are also regulated by atrazine in a rapid manner and the transduction pathways involved in this response (Figure 8). Interestingly, the up-regulation of c-fos observed in BG-1 and 2008 cells after a short treatment (2 hr) was abolished by the ER antagonist ICI, the EGFR inhibitor AG, or the ERK inhibitor PD (Figure 8). On the contrary, GFX, H89, and WM, inhibitors of PKC, PKA, and PI3K, respectively, did not interfere with c-fos stimulation (Figure 8). Thus, in ovarian cancer cells, atrazine involves ERα and the EGFR/MAPK pathway to trigger c-fos protein increase.

On the basis of these and our previous results showing that c-*fos* stimulation by E_2 occurs through GPR30 and requires ERα and EGFR-mediated signaling in cancer cells expressing both receptors (Albanito et al. 2007; Maggiolini et al. 2004; Vivacqua et al. 2006a, 2006b), we examined whether atrazine could act in a similar manner. Interestingly, both E_2 and atrazine were no longer able to induce c-*fos*

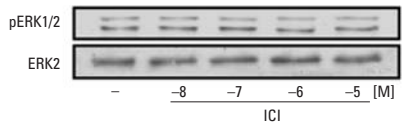

Figure 7. ERK1/2 phosphorylation (pERK1/2) in SkBr3 cells treated for 20 min with vehicle (–) or increasing concentrations of ICI.

Table 1. mRNA expression (mean percent variation ± SD) induced by 100 nM E_2 and 1 μM atrazine in BG-1 cells.

	E_2		Atrazine	
Gene	1 hr	24 hr	1 hr	24 hr
c-*fos*	423 ± 28*	239 ± 17*	269 ± 21*	120 ± 9
PR	228 ± 18*	298 ± 18*	122 ± 18	180 ± 11*
pS2	175 ± 17*	270 ± 21*	99 ± 19	187 ± 20*
Cathepsin D	106 ± 9	217 ± 16*	102 ± 5	109 ± 6
Cyclin A	262 ± 22*	293 ± 23*	220 ± 20*	190 ± 22*
Cyclin D1	258 ± 19*	242 ± 19*	107 ± 4	118 ± 8
Cyclin E	120 ± 11	343 ± 21*	118 ± 8	119 ± 10

The values calculated by optical density in cells treated with vehicle were set at 100%, and the expression induced by treatments is presented as percent variation.
*$p < 0.05$ compared with vehicle.

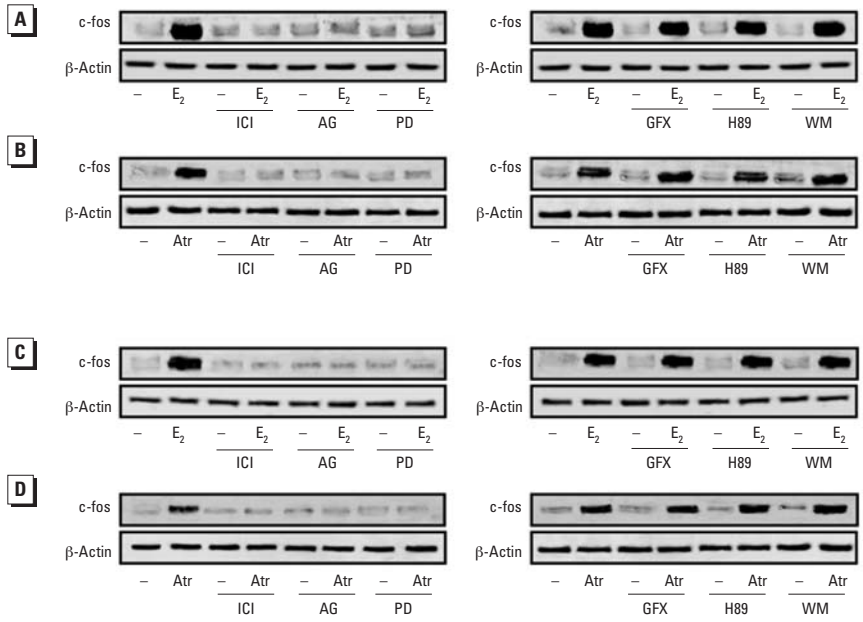

Figure 8. Immunoblots of c-fos from BG-1 (*A,B*) and 2008 (*C,D*) cells treated for 2 hr with vehicle (–), 100 nmol/L E_2, or 1 μmol/L atrazine (Atr) in combination with 10 μmol/L ICI, AG, PD, GFX, H89, or WM, inhibitors of ER, EGFR, MEK, PKC, PKA, and PI3K, respectively. β-Actin served as a loading control.

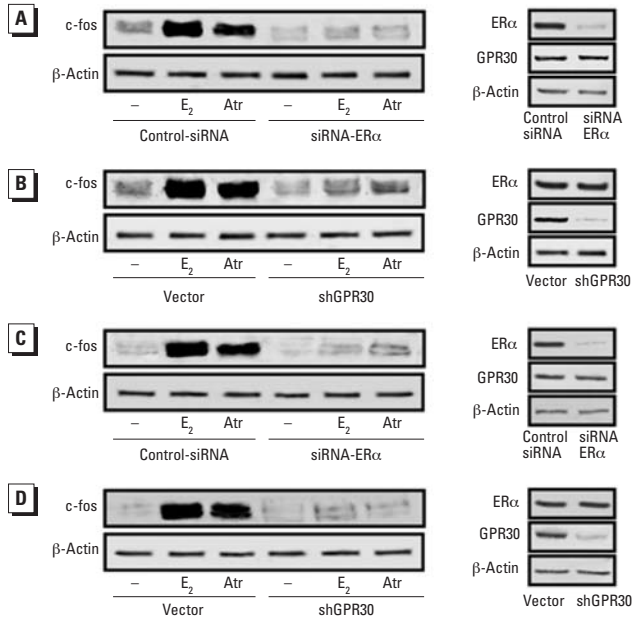

Figure 9. Immunoblots of c-fos from BG-1 (*A,B*) and 2008 (*C,D*) cells after silencing ERα and GPR30 expression. Cells were transfected with control siRNA or siRNA-ERα (*A,C*) or with vector or shGPR30 (*B,D*) and treated for 2 hr with vehicle (–) or 100 nmol/L E_2 or 1 μmol/L atrazine (Atr). Efficacy of ERα and GPR30 silencing was ascertained by immunoblots, as shown in side panels. β-Actin served as a loading control.

expression after silencing either ERα or GPR30 in BG-1 and 2008 cells (Figure 9). To evaluate whether atrazine could induce a rapid response in a cell context expressing GPR30 alone, we turned to ER-negative SkBr3 breast cancer cells. Notably, both ERK phosphorylation and c-*fos* induction stimulated by atrazine were abolished after silencing GPR30 (Figure 10), indicating that the response to atrazine is differentially regulated according to cancer cell type.

The proliferation of ovarian cancer cells induced by atrazine occurs through* GPR30 *and requires both ERα and EGFR/MAPK-mediated signaling. The aforementioned results were recapitulated in a more complex physiologic assay such as cell growth. We observed that both E_2 and atrazine induced the proliferation of BG-1 and 2008 cells in a concentration-dependent manner (Figure 11A,E). Moreover, the growth effects elicited by E_2 and atrazine were no longer evident in the presence of AG and PD (Figure 11B,F) or after silencing the expression of either GPR30 or ERα (Figure 11C,D,G,H), indicating that both receptors, along with the EGFR/MAPK transduction pathway, are involved in the growth effects as well as in the c-*fos* expression profile described above.

Discussion

In the present study, we demonstrated for the first time that atrazine exerts an estrogen-like activity in ovarian and breast cancer cells through GPR30, which is recently of interest because of its ability to mediate rapid estrogen signals (Albanito et al. 2007, 2008; Filardo et al. 2006, 2007; Revankar et al. 2005, 2007).

Previous studies have demonstrated that atrazine elicits estrogen action by up-regulating aromatase activity in certain cancer cells with elevated aromatase levels (Fan et al. 2007a, 2007b; Heneweer et al. 2004; Sanderson et al. 2000, 2001) but not by binding to or activating ERα (Connor et al. 1996; Roberge et al. 2004; Tennant 1994a). Using different tumor cells and reporter genes, we confirmed that atrazine did not interact directly with ERα, yet it did not stimulate aromatase activity in our model system, likely as a consequence of a very low aromatase expression. Nevertheless, atrazine induced the expression of diverse estrogen target genes, recalling previous studies that demonstrated the recruitment of ERα by distinct compounds and growth factors to gene promoter sequences different from the classical estrogen response element (reviewed by Dudek and Picard 2008).

Interestingly, we showed that GPR30 and ERα, together with the EGFR/MAPK pathway, are involved in the biological response to atrazine in ovarian cancer cells, which is in accordance with our recent investigation showing that the selective GPR30 ligand G-1 exerts biological activity similar to that of atrazine without binding or activating ERα (Albanito et al. 2007). Hence, our data indicate that a complex interplay between different ERs and transduction pathways contributes to atrazine activity, which nevertheless is still noticeable in the presence of GPR30 alone, as demonstrated in SkBr3 breast cancer cells. Although E_2 exhibited an exclusive up-regulation of target genes through direct activation of ERα, the GPR30–EGFR transduction pathway was involved in estrogen-induced proliferation of ovarian tumor cells, as evidenced by silencing GPR30 and using specific pharmacologic inhibitors.

A variety of environmental contaminants exhibit binding affinities for GPR30 and agonist activities similar to those for ERs (Thomas and Dong 2006). In the present study atrazine triggered rapid biological responses through GPR30 in both ovarian and breast cancer cells irrespective of ERα expression and despite a low binding affinity for GPR30 ectopically expressed in HEK-293 cells (Thomas and Dong 2006). In line with these findings, an efficient competitor of E_2 for endogenous GPR30 in SkBr3 cells, such as an *ortho*,*para*-dichlorodiphenyldichloroethylene (DDE) derivative, was ineffective in binding to recombinant GPR30 (Thomas et al. 2005; Thomas and Dong 2006). Likely, the interaction of atrazine with GPR30 is facilitated by the relative abundance of this membrane receptor in cancer cells with respect to cells engineered to express recombinant GPR30, and/or yet unknown factors may contribute to the binding to GPR30 by these contaminants.

Regarding the role of ERα, we proved that a complex interplay with GPR30 exists, as previously reported with some growth factor receptors (Migliaccio et al. 2006), but the

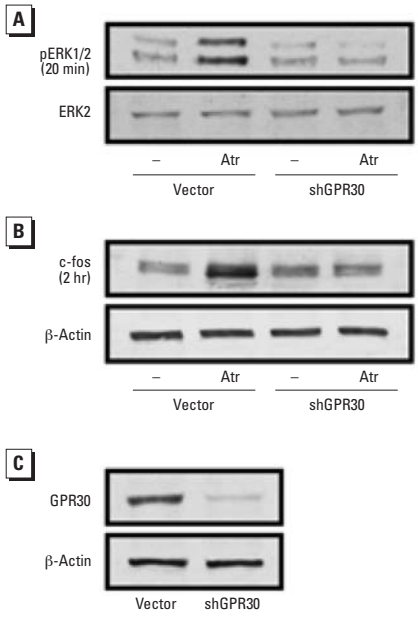

Figure 10. ERK1/2 phosphorylation (*A*) and c-fos expression (*B*) after silencing GPR30 in SkBr3 cells treated with vehicle (–) or 1 μmol/L atrazine (Atr). (*C*) The efficacy of GPR30 silencing was ascertained by immunoblots. β-Actin served as a loading control.

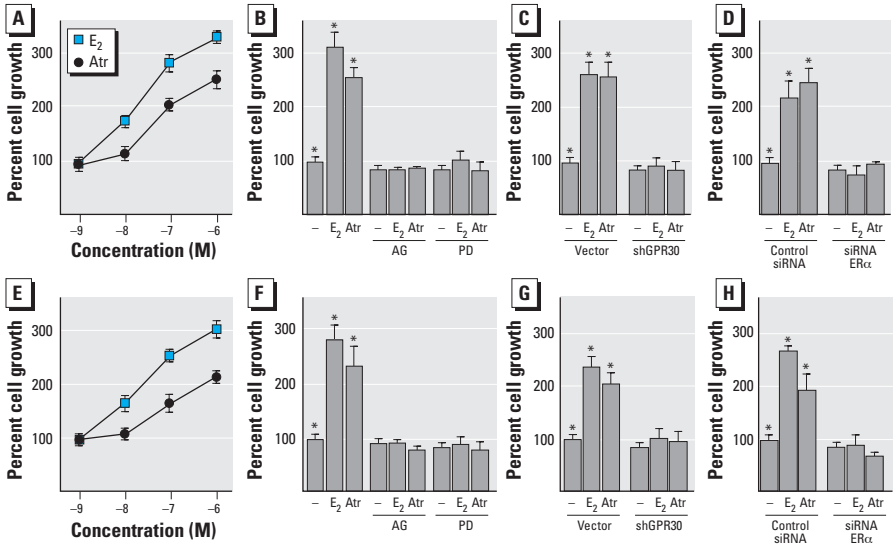

Figure 11. Proliferation of BG-1 (*A–D*) and 2008 (*E–H*) cells exposed to E_2 or atrazine (Atr). (*A,D*) Proliferation of cells in response to increasing concentrations of E_2 or Atr. (*B–H*) Proliferation of cells treated with vehicle (–), 100 nmol/L E_2, or 1 μmol/L Atr with or without 10 μmol/L AG or PD (*B,F*) (*C,D, G, H*) or transfected with vector or shGPR30 (*C,G*) or with control siRNA or siRNA-ERα (*D,H*). See "Materials and Methods" for details of experiments. Proliferation of cells receiving vehicle was set as 100%, and the cell growth induced by treatments was calculated. Values shown are mean ± SD of three independent experiments performed in triplicate; Efficacy of ERα and GPR30 silencing was ascertained by immunoblots (Figure 9).
*$p < 0.05$ compared with treated cells.

molecular mechanisms involved remain to be elucidated. Our study and previous investigations indicate that environmental estrogens exert pleiotropic actions by directly binding to ERα as well as through GPR30–EGFR signaling, which can engage ERα depending on the receptor expression pattern present in different cell types. This mode of action of xenoestrogens fits well with the results obtained after silencing GPR30 or ERα expression in ovarian cancer cells, because silencing each gene prevented the growth response to atrazine.

Our data recall the results of previous studies showing that xenoestrogens mimic rapid estrogen action in several animal and cell models (Bulayeva and Watson 2004; Loomis and Thomas 2000; Nadal et al. 2000; Ruehlmann et al. 1988; Watson et al. 1999, 2007). Particularly, in GH3/B6/F10 pituitary tumor cells, diverse xenoestrogens induced ERK phosphorylation with a temporally distinct activation pattern compared with E_2 (Bulayeva and Watson 2004). In the latter study, on the basis of the inhibitory activity exerted by ICI, the authors hypothesized that an ER localized to the plasma membrane could mediate the ERK phosphorylation response by xenoestrogens, depending on their different ER binding affinities. Moreover, the authors suggested that the signaling cascades leading to ERK activation may involve the nature of membrane ERs and their ability to interact with various signaling partners (Bulayeva and Watson 2004). Interestingly, our findings have provided evidence that ERα may be involved by xenoestrogens without a direct binding activity and produce relevant responses such as ERK phosphorylation, gene expression, and cell growth.

A subset of estrogen-sensitive cell tumors can proliferate independently from ER expression (i.e., ER-negative cells). In this condition, well represented by SkBr3 breast cancer cells, GPR30–EGFR signaling may still allow for environmental estrogen activity as we have shown in the present study as well as in a previous study (Maggiolini et al. 2004). Hence, multiple transduction pathways triggered simultaneously at the membrane level, as well as within each cell type, may contribute to the nature and magnitude of biological responses to distinct estrogenic compounds. These consequently should be examined individually for their complex mechanistic and functional outcomes that result from interaction with a different repertoire of receptor proteins.

Atrazine, a potent endocrine disruptor, is the most common pesticide contaminant of groundwater and surface water. Here, we have provided novel insight regarding the potential role of GPR30 in mediating the action of atrazine in endocrine-related diseases, such as estrogen-sensitive tumors.

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