Detection of Novel Endocrine Disrupting Chemicals in the Water

D. A. Stavreva, L. Varticovski and G. L. Hager

Laboratory of Receptor Biology and Gene Expression, National Cancer Institute, NIH, Bethesda, MD 20892

ABSTRACT

There is a growing interest in the health risk posed by endocrine-disrupting chemicals (EDCs). Steroidal EDCs can interfere with the normal function of the endocrine system, and have been associated with developmental defects, metabolic disorders and cancer. Identification of EDCs relies on a laborious analysis of chemical structures. Considering that many natural steroids are rapidly metabolized, their derivatives are not present in the currently existing libraries, and thus cannot be identified by chemical methods. We developed an inexpensive high-throughput assay for biological testing of EDCs using mammalian cells that express GFP-tagged nuclear steroid receptor constructs [1]. This assay is based on translocation of a fluorescent fusion protein from the cytoplasm to the nucleus in the presence of a ligand. Using this assay we screened water samples collected from 14 states in the US and found androgen activity in 35% of samples, and a previously unrecognized glucocorticoid (GC) activity in 27% of the samples. Widespread contamination with the two classes of steroidal EDCs represents a potential health hazard not only for the aquatic ecosystems, but also for humans. Our automated, highly reproducible, and low cost assay detects biologically active steroidal EDCs and is suitable for wide application in testing water samples.

Keywords: water contamination, endocrine disruptors, nuclear receptors, high-throughput screening

INTRODUCTION

Increasing evidence links adverse health impacts in animals and humans to environmental contaminants [2-5]. An endocrine disruptor is an exogenous substance or a mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny, or its subpopulations [6]. Contamination of environmental water sources with EDCs poses a serious concern for human and animal health [5;7]. There is evidence that EDCs impair normal metabolic processes via interaction with endocrine hormone receptors [8]. A Scientific Statement of the Endocrine Society [7] postulates that EDCs have effects on reproduction, breast development and cancer, prostate cancer, neuroendocrinology, thyroid metabolism, obesity, and cardiovascular dysfunction. Harmful effects of synthetic progestogens [9;10] and estrogenic water contaminants [11-13] on fish reproduction have been documented. Other abnormalities such as an increased susceptibility to infections, have been associated with fish kills from the Potomac river watershed [14;15], suggesting a possible contamination with additional classes of EDCs.

Variety of methods including laborious chemical methods of identifications by combination of HPLC, liquid or gas chromatography and mass spectroscopy, as well as "omic" approach (genomics, transcriptomics, proteomics, and/or metabolomics on fish and other affected organisms) have been described for detection of EDCs in the water [16]. However, because of high cost and lack of uniform quantitation, the levels of steroidal EDCs in the environment are not efficiently monitored and/or regulated.

Here we report development and validation of automated high-throughput live cell assay based on sub-cellular localization of two nuclear receptors, GFP-tagged glucocorticoid and androgen receptors (GFP-GR and GFP-AR). This assay can be expanded to screening activation of other nuclear receptors by steroidal EDCs.

RESULTS

The unbound glucocorticoid and androgen receptors are largely retained in the cytoplasm in a large multi-protein complex by interaction with heat-shock proteins and immunophilins [17;18]. Upon hormone binding, these receptors dissociate from this complex and translocate to the nucleus (Fig. 1A and B), where they interact with appropriate regulatory elements in DNA and with other nuclear proteins to elicit hormone-specific effects [19;20]. We exploited their translocation property by generating cell lines that express fusion proteins containing GFP-tagged GR (Fig. 1B) and AR (Fig. 1C) under tetracycline-repressible promoter [18;21]. In these cells, tetracycline suppresses the expression of the receptors, and removal of the drug results in protein expression. We used these cell lines and the Perkin Elmer Opera automated Image Screening System to study GFP-GR and GFP-AR translocation from cytoplasm to the nucleus in response to glucocorticoid and androgen contamination in water samples (Fig. 1C). We discovered GC activity in 28% and androgen activity in 37% of all samples tested (Fig. 2). One of the samples (SS97) which was highly positive for glucocorticoid activity by GFP-GR translocation assay (Fig. 3A) also induced GR-mediated transcriptional activation of Per1 gene, a regulator of circadian oscillator, and a direct GR-target [22] (Fig. 3B). To identify the active constituent in this sample, known GC, such as dexamethasone, corticosterone, and 20 other synthetic compounds were analyzed using HPLC by retention time on a C18 column [23].

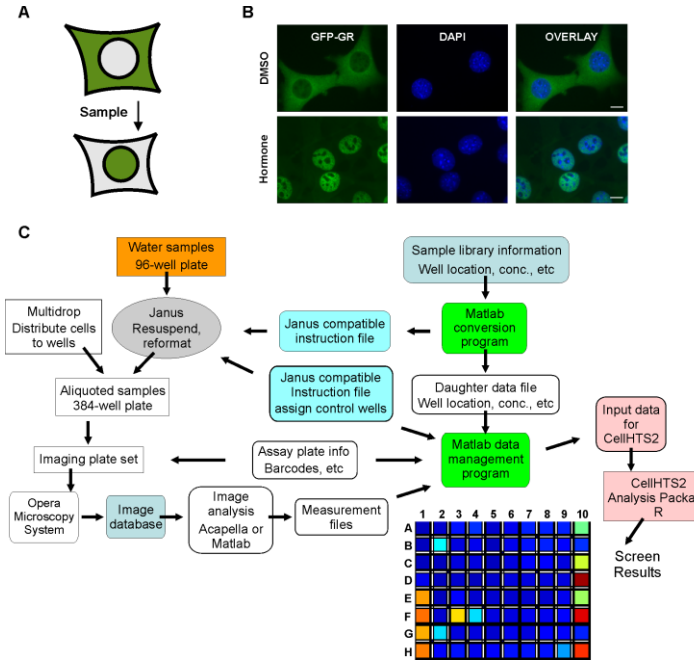

Figure 1. Analysis of water samples for contamination with endocrine disrupting chemicals. (A) Schematic representation of green fluorescent protein (GFP)-tagged nuclear receptor translocation in response to a hormone. (B) GFP-tagged glucocorticoid receptor (GFP-GR) translocation in mammalian cells exposed to dexamethasone for 30 min. Nuclei were stained with DAPI. Scale bar, 5 µm. (C) Workflow for image-based screening of environmental contaminants with GC activity using Perkin Elmer Opera Image Screening System. Sample of the screen using a 96-well plate is shown as Screen Results.

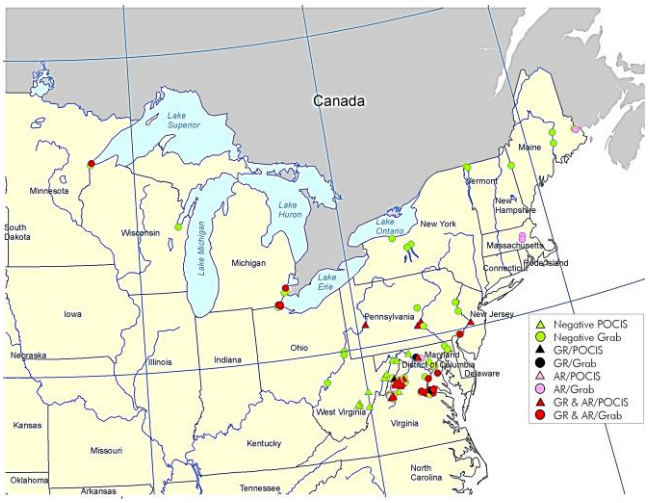

Figure 2. Geographic locations of the collection sites and their contamination with glucocorticoid and androgenic activity. Positive samples are indicated in the insert.

HPLC fractions from SS87 positive for biological activity were analyzed by ultra performance liquid chromatography/mass spectrometry (UPLC/MS) and gas chromatography/MS (GC/MS) [24]. The mass spectra were searched in the NIST/EPA/NIH Mass Spectral Library, and the Wiley Mass Spectra Database of Androgens, Estrogens, and other Steroids from 2010. In spite of this extensive analysis, sample SS97 had no evidence of any known GC. However, comparison of the mass spectra of chromatographic peaks 1-3 (Fig. 3B) with standard spectra from the AES 2010 database suggested similarities to known androstane-class compounds.

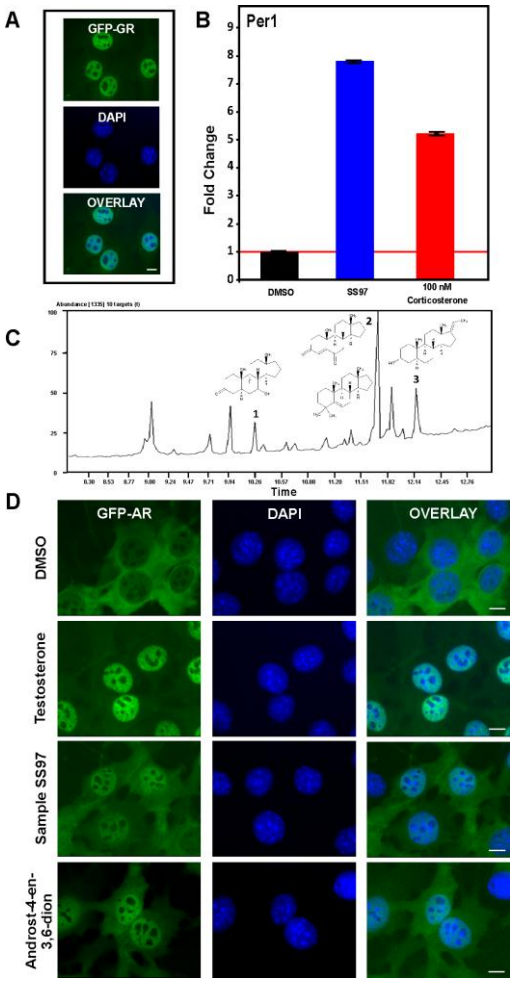

Figure 3. Analysis of water sample SS97 for glucocorticoid and androgen contamination
(A) Sample SS97 induced GFP-GR translocation. Scale bar, 5 µm. (B) Transcriptional activation of the GR-regulated Per1 gene by sample SS97 collected by a polar organic chemical integrative sampler (POCIS) is compared to corticosterone. Data is normalized to DMSO alone. Error bars represent the mean ± s.e.m., n=3. (C) GC/MS total ion chromatogram of one of the HPLC fractions revealed the presence of a complex mixture of volatile hydrocarbons, as indicated by the peaks. Database searching of the extracted MS spectra corresponding to peaks 1-3 showed structural similarity to known androstane-type steroids. (D) Representative images of GFP-AR nuclear translocation in response to 100 nM of testosterone, androst-4-ene-3,6-dione, and sample SS97 (100x). Scale bar, 5 µm.

One of the known androstane-class compounds, androst-4-en-3,6-dione (Fig. 3C, peak 2), was synthesized [25] and its translocation activity tested in cell lines expressing GFP-GR as well as GFP-AR constructs (Fig. 3D and data not shown). We confirmed that the androst-4-en-3,6-dione induced GFP-AR, but no GFP-GR translocation. In addition, sample SS97 also induced translocation of GFP-AR to the nucleus (Fig. 3D). Thus, SS97 induces translaocation of both, glucocorticoid and androgen receptors.

These results unambiguously demonstrate that our translocation-based assay detects biologically active glucocorticoid and androgenic activity in water sources. This assay can be easily automated and is superior to the currently used chemical methods.

METHODS

Samples: Water samples were provided by U.S. Geological Survey (USGS) projects collected between 2005 and 2010 from different geographic locations in the United States [1]and (Fig. 2). Samples included grab water samples subjected to solid phase extraction [26], or collected via polar organic chemical integrative samplers (POCIS) [12].

Cell lines and translocation assay: The cell lines that express green fluorescent protein (GFP)-tagged GR (GFP-GR) and AR (GFP-AR) under control of the tetracycline-repressible promoter were derived from 3134 mouse mammary adenocarcinoma cell line [18;21]. Cells were plated in 96 or 384 well plates overnight in DMEM medium with 10% charcoal stripped serum without tetracycline to allow the expression of the GFP-tagged receptors. Cells were exposed to vehicle control, hormones (up to 100 nM) or reconstituted water samples for 30 min at 37^{o}C, fixed in 4% paraformaldehyde for 15 min, and washed 3 times with PBS. Cells were stained with DRAQ5 (BioStatus Limited) for 15 min and imaged on the Perkin Elmer Opera Image System.

Automated imaging and analysis: Fully automated collection of images was performed using 40x water immersion objective. To automatically segment the nucleus and the cytoplasm, we customized Acapella image analysis software (Perkin Elmer). Nuclear translocation was calculated as a ratio of each compartment to the mean total GFP. Each value was further normalized to the value for the DMSO control.

Statistical Analyses: Data were analyzed using the statistical functions of IBM SPSS Statistics 19 and SigmaPlot 11 (SPSS Inc., Chicago, IL).

DISCUSION

At present, the prevalence of GC activity in US water is unknown. However, using chemical methods, a few reports on water contamination have demonstrated detectable levels of GC in the Netherlands and China [27;28]. The anti-inflammatory properties of GCs make them highly prescribed pharmaceuticals which could readily enter water sources. Moreover, wastewater treatment plants (WWTP) are not capable of efficiently removing GCs and it is well documented that anti-inflammatory chemicals are among the most resistant to such treatments with only 30-40% removal rates [5]. Epidemiological and animal studies suggest that exposures to stress hormones, including GC, during the prenatal period, have programming effects on the hypothalamic-pituitary-adrenal axis, brain neurotransmitter systems, cognitive abilities and the immune system of the offspring [29;30]. Prenatal exposure of guinea pigs and monkeys to androgens is also associated with irreversible changes in sexual and social behaviors later in life [31;32]. Thus, contamination of water sources with both glucocorticoid and androgenic activity could have long-term consequences. Largely unrestricted human activity with respect to pharmaceuticals and other potential endocrine disruptors is of concern, and represents one of the main reasons for these widespread contaminations. In addition, limited methods for the detection of EDCs in the environment impair their efficient screening.

Our studies highlight the prevalence of water contamination in 14 states of the US with glucocorticoid and androgen activities. This assay introduces a novel quantitative approach for screening and monitoring water quality. This approach allows detection of biologically relevant EDCs without the need for specific chemical identification. It is important to expand this screening program, and develop a global strategy for monitoring water contamination with steroidal EDCs.

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