MODULATION OF ESTROGEN SYNTHESIS THROUGH ACTIVATION OF PROTEIN KINASE A IN H295R CELLS BY EXTRACTS OF ESTUARY SEDIMENTS

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Abstract—Sediments from two estuaries within Liaodong Bay, China, were examined for the effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Total extracts (TE) isolated from sediments by Soxhlet extraction were separated into three fractions (F1, F2, and F3) using Florisil columns. After exposing H295R cells to each TE and fractions, the expressions of six steroidogenic genes (cytochrome P450 cholesterol side-chain cleavage [CYP11A], 3β-hydroxysteroid dehydrogenase type 1 [3β-HSD1], 3β-hydroxysteroid dehydrogenase type 2 [3β-HSD2], cytochrome P450 17-hydroxylase/17-20 lyase [CYP17], cytochrome P450 aromatase [CYP19], 17β-hydroxysteroid dehydrogenase [17β-HSD]), and the production of six steroid hormones (progesterone [PGT], 17-hydroxyprogesterone [17-HPT], testosterone [TTR], androstenedione [ADD], estrone [E1], and 17β-estradiol [17β-E2]) were measured. The gene expressions of CYP11A, CYP17, 3β-HSD2, and CYP19, and hormone productions of PGT, 17-HPT, TTR, ADD, E1, and 17β-E2 were significantly increased after exposure to F3 extracts from the Daliao River. In particular, greater concentrations of E1 (up to 48-fold) and 17β-E2 (up to 20-fold), as well as up-regulation of CYP19 gene expression (up to tenfold), were caused by exposure to the F3 fraction from the Daliao River, but not from the Daling River. Insight into the mechanism of action was obtained by use of principal component analysis (PCA), the results of which were consistent with unidentified constituents in F3 from the Daliao River activating the protein kinase A (PKA) pathway. This hypothesis was confirmed by reversal of the effects caused by F3 through a co-exposure of a PKA inhibitor (H89) and F3 extract. The H89 down-regulated CYP19 messenger RNA (mRNA) expression with concomitant lesser production of E1 and 17β-E2 in the co-exposure group, indicating unidentified constituents that could modulate estrogen synthesis, primarily through a mechanism of PKA activation. Environ. Toxicol. Chem. 2011;30:2793–2801. © 2011 SETAC

Keywords—H295R assay   Steroidogenesis   Estrogen synthesis   Toxicity identification

INTRODUCTION

Increasing evidence indicates that wild populations of both estuarine and freshwater fish are exposed to endocrine-disrupting chemicals that may modulate physiological responses [1]. In particular, occurrences of altered gonadal histology, such as intersex, have been observed in fish exposed to sewage effluents [2,3]. Toxicity identification and evaluation procedures employing chromatographic fractionation coupled with in vitro assays have identified that the estrogens, including 17β-estradiol (17β-E2), estrone (E1), and the synthetic ethinylestradiol (EE2), are the most potent and also most prevalent estrogenic compounds present in sewage effluents [4–7]. These estrogens and other weakly estrogenic synthetic chemicals such as 4-*tert*-octylphenol and 4-nonylphenol exert their endocrine-disrupting effects mainly by interacting directly with the estrogen receptor. However, some chemicals such as atrazine, ethinylestradiol (EE2), and vinclozolin can modulate the endocrine system through non–receptor-mediated mechanisms [8–11].

Some xenobiotics can alter development and reproduction and modulate endocrine function of wildlife and humans by altering the relative rates of synthesis and degradation of crucial steroid hormones or activities of steroidogenic enzymes [12–15]. The cytochrome P450 (CYP) enzymes, which are responsible for catalyzing specific reactions in the steroid biosynthetic pathways, are of particular interest as potential nonreceptor targets of endocrine-disrupting chemicals [16]. Screening for aromatase-disrupting properties has been included in recent endocrine disruptor screening initiatives, such as the Endocrine Disruptor Screening Program of the U.S. Environmental Protection Agency (U.S. EPA) and the Organization for Economic Cooperation and Development [17]. Although the U.S. EPA has promoted the human recombinant microsomal aromatase assay that measures direct effects of chemicals on aromatase activity, H295R adenocarcinoma cells have been developed to assess potential effects of steroidogenic enzymes responsible for biosynthesis of progestogens, mineralocorticoids, glucocorticoids, and sex hormones [18,19]. The H295R assay has been shown to be useful to assess effects of chemicals such as pharmaceuticals, pesticides, and manmade chemicals on human steroidogenesis pathways [20–23]. For example, amoxicillin, erythromycin, and the β-blocker salbutamol can promote or inhibit production of 17β-E2 [21], and the mechanism of the triazine-containing herbicides atrazine, simazine, propazine, and the fungicide vinclozolin as inducers of aromatase activity have been clarified by studies with H295R cells [11]. This assay has also been used to assess the potential of steroidogenesis modulation by emerging chemicals such as methoxylated polybrominated diphenyl ethers [22,23].

Therefore, this assay has been applied for assessing the effects of environmental samples on steroidogenesis modulation. To our knowledge, three papers have reported the effects of environmental samples on the steroidogenesis using H295R cells [24–26]. The significant increase of 17β-E2 production based on the enzyme-linked immunoassay was observed in sediments from the Upper Danube River in Germany [26]. Although the present study provided a clue that some chemicals in the environment affect the steroidogenesis process, no evidence concerning the potential chemicals or categories is available. Currently, there is a lack of mechanism exploration, including integral analysis of gene expression of steroidogenic enzymes and production of a broad range of steroid hormones, as well as a lack of mechanism verification. From the point of view of the mechanism of steroid hormone biosynthesis, cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) is one of the important signaling pathways contributing to the regulation of steroidogenesis [27]. Therefore, an assay co-exposed to a specific cAMP-dependent PKA inhibitor and environmental samples can verify the estrogenic effects of environmental samples via the cAMP/PKA pathway.

In the present study, an ultraperformance liquid chromatography tandem mass spectrometry method was developed to simultaneously determine production of six steroid hormones, including progesterone (PGT), 17-hydroxyprogesterone (17-HPT), testosterone (TTR), androstenedione (ADD), E1, and 17β-E2. To understand the mechanism of hormone production comprehensively, the six target genes that are related to hormone production (cytochrome P450 cholesterol side-chain cleavage [CYP11A], 3β-hydroxysteroid dehydrogenase type 1 [3β-HSD1], 3β-hydroxysteroid dehydrogenase type 2 [3β-HSD2], cytochrome P450 17-hydroxylase/17-20 lyase [CYP17], cytochrome P450 aromatase [CYP19], and 17β-hydroxysteroid dehydrogenase [17β-HSD]) were also measured by quantitative reverse-transcription polymerase chain reaction. These data were then used for a multivariate statistical analysis to predict the potential mode of action of residues in extracts of sediment based on the patterns of gene expression changes of H295R cells exposed to four typical classes of model chemicals. Finally, an assay that co-exposed H295R cells with a PKA inhibitor, H89 (10^{-5} M), was developed to identify further the potential categories that affect the estrogen synthesis in the sediment extracts.

MATERIALS AND METHODS

Chemicals and reagents

Progesterone, 17-HPT, TTR, ADD, E1, and 17β-E2 were purchased from Sigma-Aldrich. Progesterone-d_9, 17β-E2-d_3, E1-d_2, and TTR-$^{13}C_2$ were purchased as powders from Wako. Methanol, acetone, ethyl acetate, hexane, diethyl ether, and dichloromethane were all high-performance liquid chromatography grade obtained from Fisher Chemical. Ultrapure water (resistivity over 18.2 MΩ·cm) was prepared using a Milli-Q water system (Millipore).

Sediment collection

Surface sediments (0–30 cm depth) were collected within Liaodong Bay, China, from the estuaries of the Daliao River and Daling River, respectively. Sediments were freeze-dried (*Eyela DRC-1000, Tokyo* Rikakikai) and then were ground and sieved through a 0.2-mm mesh before further processing. Two individual samples were stored at −20 °C until extraction.

Sediment extraction and estrogen analysis in F3 fraction

Dried and sieved sediment (20 g) was mixed with 20 g anhydrous Na_2SO_4, and Soxhlet extracted for 20 h with a mixture of dichloromethane and hexane (3:1, v/v, 300 ml). The extracts were treated with acid-activated Cu granules to remove residual S and were rotary-evaporated to approximately 5 ml; the extracts then were dried under a gentle stream of nitrogen and afterward were reconstituted with 2 ml of hexane/dichloromethane (1:1, v/v). The final extract was then divided into two portions. One milliliter of the total extract (TE) was used for the H295R assay. The remaining 1 ml of extract was passed through a preconditioned Florisil cartridge (35 ml, 10 g) for cleanup and further fractionation. Three fractions were eluted from the Florisil cartridge with organic solvents in the following sequence: 100 ml hexane (F1), 100 ml 20% dichloromethane in hexane (F2), and 100 ml 10% acetone in dichloromethane (F3). The TE and three fractions (F1, F2, and F3) were evaporated to approximately 1 ml by rotary evaporation, reduced to dryness by nitrogen stream, and then redissolved in 400 μl methanol, which was divided into two equal aliquots for H295R cell assay and instrumental analysis for hormones. The methanol solvent in the extracts and fractions for use in bioassays was replaced with 200 μl stripped culture medium containing 0.1% dimethylsulfoxide (DMSO), and then extracts and fractions were stored at −20 °C until used for the exposures. Another 200 μl F3 fraction was used for chemical analysis of targeted hormones (E1, 17β-E2, 17α-estradiol [17α-E2], estriol [E3], EE2). The method detection limits were 0.004 ng/g for E1, 0.008 ng/g for 17β-E2, 0.006 ng/g for 17α-E2, 0.008 ng/g for E3, and 0.012 ng/g for EE2. Chemical analysis was performed using liquid chromatography electrospray ionization tandem mass spectrometry based on a method published by Chang et al. [28].

H295R cell assay

The H295R cell line was obtained from the American Type Culture Collection (ATCC CRL-2128), and the cells were cultured as described in a previous paper [8]. Cells were seeded at a density of approximately 1×10^6 cells/ml per well in a six-well cell culture plate. After at least 24 h of growth, old culture medium was removed, and cells were exposed to 2 ml dosing solutions, which were prepared in medium by adding 20 μl dissolved extracts or fractions and its serial dilutions (1:10 diluting step) to 2 ml medium to make DMSO concentrations of 0.1%. After 48 h of exposure, viability of H295R cells treated with sediment extracts (TE, F1, F2, and F3) was evaluated by use of the 3-(4,5-dimethylthiahiazo (-z-y1)-3,5-di-phenytetra-zoliumromide assay, and only the noncytotoxic doses of sediment extracts were used to eliminate possible cell toxicity and nonspecific effects. Exposure concentrations ranging from ≤2.5 mg sediment equivalents (dry wt)/ml for TE and ≤250 mg sediment equivalents (dry wt)/ml for F1, F2, and F3 were studied, respectively. Each total extract or fractions of extract and its two serial dilutions (1:10 diluting step) exposure were performed in triplicate. The concentration of DMSO in each test was kept constant at 0.1%. Cells treated with DMSO and untreated cells served as a solvent control and a negative control, respectively, in triplicate.

After 48 h exposure, cell viability was determined by visual inspection under a microscope. After inspection, medium was removed from those treatments that exhibited no cytotoxicity and was stored at −80 °C for quantification of hormones. Total RNA was then extracted from the adherent cells in each well of

six-well plates by use of 1 ml of Trizol reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. Isolated RNA was quantified spectrophotometrically and then diluted to a final concentration of 50 ng RNA/μl. Then complementary DNA was prepared from RNA (500 ng) by use of the Moloney murine leukemia virus First-Strand cDNA Synthesis System (Invitrogen) according to the manufacturer's guidelines. The resulting complementary DNA was diluted tenfold, and the amounts of complementary DNA for six target genes (CYP11A, 3β-HSD1, 3β-HSD2, CYP17, CYP19, and 17β-HSD) plus the endogenous control gene (β-actin) were quantified by an ABI Prism$^{\circledR}$ 7000 Sequence Detection System (Applied Biosystems) with SYBR$^{\circledR}$ Green PCR master mix (Applied Biosystems). The thermal cycling reaction conditions, primer sequences and concentrations, and quantification of target genes have been described previously by Zhang et al. [18]. Gene expression was determined in triplicate based on the three individual samples.

Sample preparation and hormone measurement in cell medium

Before extraction, surrogate standards, PGT-d$_9$, 17β-E2-d$_3$, E1-d$_2$, and TTR-^{13}C$_2$ (100 ng/ml, 20 μl) were spiked into the cell culture supernatant collected from exposure wells. Steroid hormones were extracted three times from 1 ml aliquots of the cell culture supernatant with 2 ml hexane/ethyl acetate (1:1, v/v). The aqueous phase was discarded, and then the organic layers were pooled and evaporated at room temperature under a gentle stream of nitrogen. The dried extracts were redissolved in 200 μl methanol. One hundred μl of methanol containing the hormones was used to analyze PGT, 17-HPT, TTR, and ADD by ultraperformance liquid chromatography tandem mass spectrometry. Another 100 μl methanol solution was dried and redissolved in 50 μl NaHCO$_3$ buffer (pH = 10.5, 100 mM/L) and 50 μl dansyl chloride (1 mg/ml in acetone) for the derivation of estrogens according to the method reported in Anari et al. [29]. The mixture was vortex-mixed for 1 min and incubated at 60 °C for 5 min, then cooled to room temperature for liquid chromatography tandem mass spectrometry analysis.

The liquid chromatography (LC) apparatus was an ACQUITY ultraperformance LC (Waters). An ACQUITY ultraperformance liquid chromatography BEH C18 column (100 × 2.1 mm, 1.7 μm particle size) (Waters) was used to separate PGT, 17-HPT, TTR, and ADD. An ACQUITY ultraperformance liquid chromatography BEH phenyl column (100 × 2.1 mm, 1.7 μm particle size) (Waters) was used to separate the derivatives of E1 and 17β-E2. For all analytes, methanol and ultrapure water containing 0.1% formic acid were used as mobile phases, and the column was maintained at 40 °C at a flow rate of 0.3 ml/min. For analyzing PGT, 17-HPT, TTR, and ADD, methanol was initially increased linearly from 10 to 60% in 0.5 min, to 65% in the next 2.5 min, to 70% in the next 3.5 min, then sharply to 100% and kept for 1.0 min. For analyzing E1 and 17β-E2, methanol was initially increased linearly from 50 to 65% in 0.5 min, to 75% in the next 4.0 min, then sharply to 100% and kept for 3.5 min. Methanol was finally returned to the initial composition and equilibrated for 2.0 min before the next injection. The injection volume for all analytes was 10 μl.

Mass spectrometry was performed using a Quattro Premier XE tandem quadrupole mass spectrometer (Waters) equipped with a Z-Spray ionization source. The Z-Spray ionization with tandem mass spectrometry detections were performed in the positive ion mode for all analytes. Common mass sectrometry parameters were as follows: capillary voltage, 3.5 kV; source

temperature, 110 °C; desolvation temperature, 450 °C; source gas flow, 50 L/h; and desolvation gas flow, 800 L/h. The two most abundant multiselected reaction monitoring transitions, cone voltages, and collision energies were optimized for each steroid by infusing standard solutions in the mass spectrometer (Supplemental Data, Table S1). All data were acquired and processed with Excel software (Microsoft$^{\circledR}$) and SPSS 11.5 (Applied Bioscience).

Co-exposure assay

The H295R cell was cultured as described previously. After the attachment period of approximately 24 h, old culture medium was removed, cells were initially pretreated with a PKA inhibitor, H89 (10^{-5} M), for 1 h. Then cells were co-exposed with H89 and F3 extracts (250 mg sediment equivalents dry wt/ml) for 48 h from Daliao River sediments. After 48 h of exposure, medium was removed and saved for estrogen analysis. The RNA was extracted and used for analysis of CYP19 gene expression. Solvent control (0.1% DMSO), a negative control, and all exposures were repeated three times.

Statistical procedures

Gene expression and hormone production were calculated as fold-change relative to the mean expression or concentration of the solvent control group (fold induction = 1 for the solvent control). The statistical program SPSS 11.5 (Applied Bioscience) was used to analyze all the collected data. Before using parametric statistics, the normality of each sample set was assessed with the Kolomogrov-Smirnov one-sample test with Lillifor's transformation. Variance homogeneity was determined with Levene's test. Differences in gene expression were evaluated by analysis of variance followed by Tukey's test. Differences with $p < 0.05$ were considered significant.

Principal component analysis (PCA) was performed to elucidate the molecular mechanisms of the potential agents in sediment extracts. The messenger RNA (mRNA) expression pattern of steroidogenic genes in H295R cells exposed to sediment extracts were compared with the mRNA expression patterns caused by the model chemicals that have been reported previously [18]. Four classes of model chemicals were selected based on their known molecular mechanisms of action in H295Rs, which included class 1: forskolin and 8 Br-cyclic adenosine monophosphate (8-Br-cAMP), Class 2: phorbol-12-myristate-13-acetate, Class 3: 17-alpha-ethinyestradiol (EE2) and daidzein, and Class 4: ketoconazole and DL-aminoglutethimide (Supplemental Data, Table S2). This dataset consisted of relative mRNA expression of 11 steroidogenic genes, including steroidogenic acute regulatory protein (StAR), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), CYP11A, 3β-HSD1, 3β-HSD2, CYP21, CYP11B1, CYP11B2, CYP17, CYP19, 17β-HSD, in H295R cells caused by these four classes of chemicals. To fully evaluate the steroidogenic pathway, besides fold changes of the six genes mentioned previously, relative mRNA expression of StAR, CYP21, CYP11B1, CYP11B2, and HMGR were also measured for the exposure groups of F3 fraction at a concentration of 250 mg sediment equivalents (dry wt)/ml. Relative fold changes of steroidogenic mRNA expression by both the model chemicals and extracts of sediments were simplified using criteria described in the Supplemental Data, Table S3. Principal component analysis discriminate analysis was then applied to the data matrix generated by the previously described procedure, and the first two principal components, which were responsible for 83% of the total variance, were used to ordinate the model chemicals

and sediment extracts. Scores of the first two component values of the sediment samples were determined by calculating the product of the fold change of mRNA expression and the first two principal components.

RESULTS AND DISCUSSION

Gene expression changes

Expression of six major genes (CYP11A, 3β-HSD1, 3β-HSD2, CYP17, CYP19, 17β-HSD) in H295R cells was determined after exposure to TE, F1, F2, and F3 of organic extracts of sediments from the Daliao or Daling rivers. No significant differences were found between the blank (nontreated cells) and solvent control for any of the genes (Supplemental Data, Table S4). Of the six genes, only CYP19 gene expression was significantly induced by TE from the Daliao River (up to fivefold induction) at a concentration of 2.5 mg sediment equivalents (dry wt)/ml, but extracts from the Daling River did not significantly affect expression of any of the genes relative to the solvent control. This is the first time that significantly greater expression of CYP19 has been known to be caused by exposure to environmental extracts. Similar extracts, such as TE from freshwater pond sediments in the Czech Republic, significantly up-regulated expression of CYP11B2 mRNA as much as tenfold and down-regulated expression of 3β-HSD2 and CYP21, but did not modulate the gene expression of CYP19 [24]. Effects of organic extracts of marine water near Hong Kong on the steroidogenesis in H295R cells have also been evaluated; however, down-regulation of expression of CYP19 and CYP17 and up-regulation of expression of CYP11β2 mRNA were observed in exposures to some water extracts [25]. Therefore, the results obtained from the present study suggested that specific chemicals could affect estrogen synthesis via up-regulation of the CYP19 expression in the extracts from the estuary sediment.

In the present study, considering the interactions between different chemicals and potential cytotoxcity in TE, TE was further separated into three fractions (F1, F2, and F3) based on their polarity on a Florisil column.To characterize further the potential causative agents of the observed effects, the expression variations of six genes, including CYP11A, 3β-HSD1, 3β-HSD2, CYP17, CYP19, and 17β-HSD, were determined (Fig. 1). Expression of CYP11A mRNA was significantly up-regulated by F3 extract of Daliao River sediments, with a maximum 3.63-fold greater expression. Because CYP11A is an enzyme that catalyzes side-chain cleavage of cholesterol into pregnenolone, which is the starting point of steroid synthesis, the significant up-regulation of CYP11A indicates that F3 extract of Daliao River sediments could affect the subsequent steps of steroid biosynthesis. Alternatively, fractions F2 and F3 of Daling River extracts resulted in down-regulation of CYP11A expression. The 3βHSD is crucial to the biosynthesis of all steroid hormones, including androgens, estrogens, mineralocorticoids, and glucocorticoids. The mRNA expression of 3β-HSD1 and 3β-HSD2 were also greater in cells exposed to fraction F3 of Daling River sediment. This result was different from that of the same fraction extract of Daliao River sediments, in which only 3β-HSD2 mRNA expression was significantly up-regulated. The CYP17 gene is involved in key steps of androgen production, especially for dehydroepiandrosterone and ADD in adrenal glands [30]. Exposure of H295R cells to fractions F2 and F3 of Daliao River sediments resulted in significant up-regulation of CYP17 mRNA expression. Alternatively, expression of CYP17 mRNA was down-regulated by

exposure to F2 or F3 fractions of Daling River sediments. CYP19 is a rate-limiting step that catalyzes aromatization of the androgen TTR to the estrogen 17β-E2. Of the six genes studied, CYP19 was significantly up-regulated in H295R cells when exposed to 2.5, 25, or 250 mg sediment equivalents (dry wt)/ml of F2 and F3 of Daliao River sediments. At the greatest exposure concentration of F1, F2, and F3 extracts of Daliao River sediments, expression of CYP19 mRNA was up-regulated by 1.26-, 3.97-, and 6.86-fold, respectively. No significant alterations on CYP19 gene expression were observed in the exposure groups from the Daling River. The 17β-HSD was not significantly changed by any of the fractions from either river, although 17β-HSD is involved in the final step of sex steroid biosynthesis (Supplemental Data, Fig. S1). Overall, CYP11A, CYP17, 3β-HSD2, and CYP19 gene expressions were significantly induced by F3 extract of Daliao River sediments, which may have remarkable effects on the biosynthesis of sex steroid hormones such as TTR, ADD, E1, and 17β-E2, because these genes are crucial to biosynthesis of steroid hormones.

Hormone production changes

Steroid hormones are arguably the most functional endpoint for the alterations of steroidogenesis process induced by chemicals. We first developed a method for investigating the production changes of six hormones induced by organic extracts of sediments by H295R cells. Because the concentrations of estrogens (E1 and 17β-E2) in the cell medium were usually lower than 50 pg/ml, the instrumental sensitivities of the traditional liquid chromatography tandem mass spectrometry method would not be adequate for such low concentration levels. In the present study, a derivatization method of estrogens with dansyl chloride was applied to enhance electrospray ionization, and the instrumental sensitivities were largely improved with the instrumental detection limits of 1 pg/ml for E1 and 1.7 pg/ml for 17β-E2. However, the extraction solvent can strongly affect extraction efficiency of target analytes, and the matrix interferences introduced by extraction solvents may cause significant ion suppression when Z-Spray ionization source was used, especially for dansyl-estrogens [31]. Therefore, three solvents, including diethyl ether, dichloromethane, and hexane/ethyl acetate (v/v, 1:1), were compared in the present study. Although the absolute recoveries of all target analytes were 88 to 93% and 73 to 88% when using hexane/ethyl acetate (1:1, v/v) and dichloromethane as extraction solvent, respectively, no estrogens were recovered when using diethyl ether as the extraction solvent (Supplemental Data, Table S5). Thus, hexane/ethyl acetate (1:1, v/v) was finally selected as extraction solvent, and the method detection limits were 2 pg/ml for ADD, 4 pg/ml for TTR, 2.5 pg/ml for PGT, 3 pg/ml for 17-HPT, 0.2 pg/ml for E1, and 0.4 pg/ml for 17β-E2. This method is very simple and quick without a cleanup procedure during sample preparation.

Based on this analytical method, production changes of six steroid hormones were studied, and the results were shown in Figure 2. No significant differences were seen between the blank (nontreated cells) and solvent control for any of the hormones (Supplemental Data, Table S4). Concentrations of steroid hormones, including PGT, 17-HPT, TTR, ADD, E1, and 17β-E2, were greater in the medium of H295R cells after exposure to the F3 extract of Daliao River sediments. Concentrations of PGT, 17-HPT, ADD, TTR, E1, and 17β-E2 exhibited dose-dependent positive associations when exposed to media containing 2.5, 25, and 250 mg sediment equivalents of F3 (dry wt)/ml. Concentrations of PGT, 17-HPT, ADD, TTR, E1, and
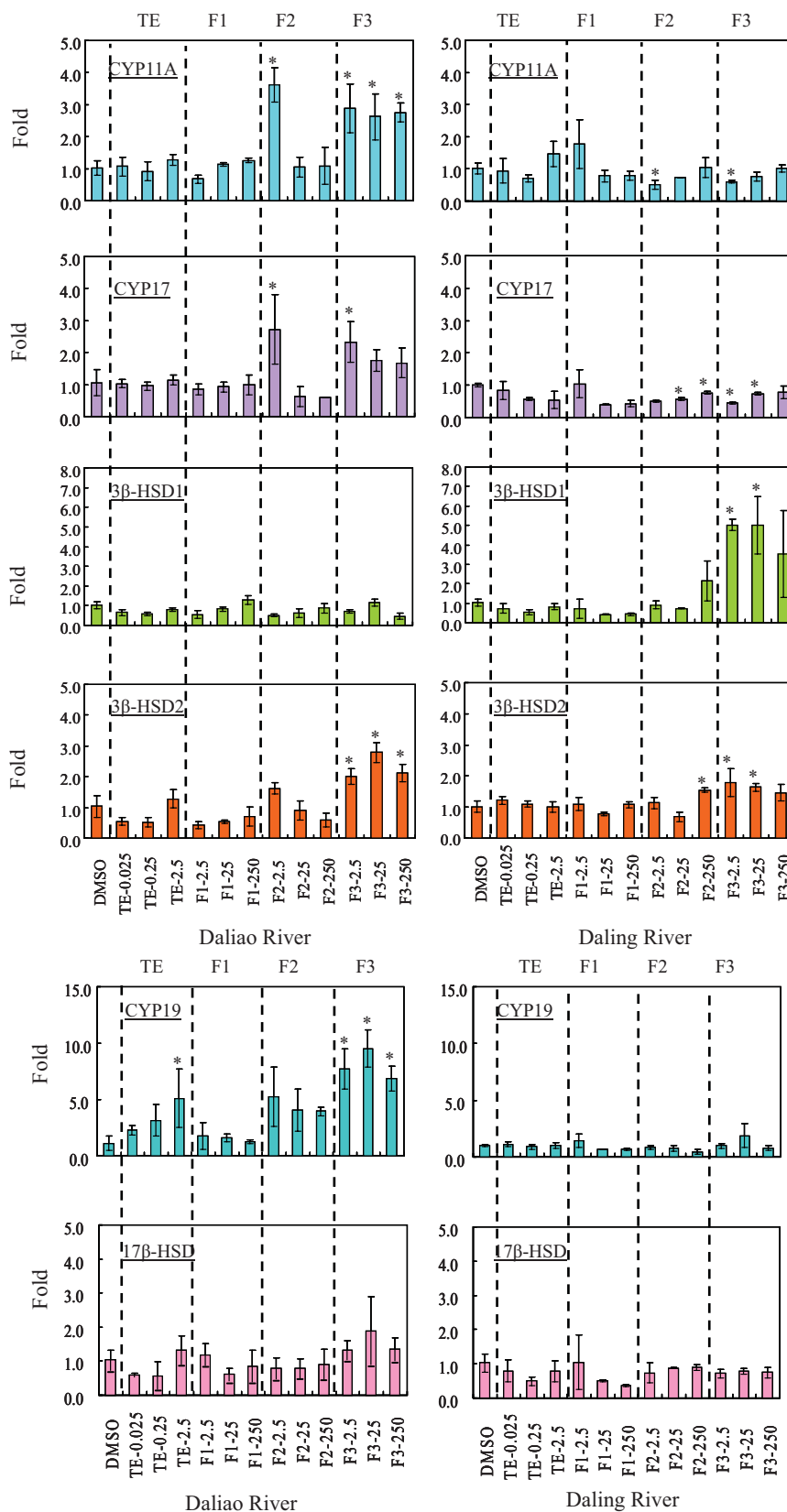
Fig. 1. Fold changes of six key genes (cytochrome P450 cholesterol side-chain cleavage [CYP11A], 3β-hydroxysteroid dehydrogenase type 1[3β-HSD1], 3β-hydroxysteroid dehydrogenase type 2 [3β-HSD2], cytochrome P450 17-hydroxylase/17-20 lyase [CYP17], cytochrome P450 aromatase [CYP19], and 17β-hydroxysteroid dehydrogenase [17β-HSD]) H295R cells were exposed to TE (total extract), F1, F2, or F3 from sediments of the Daliao and Daling rivers within Liaodong Bay, China, at three concentrations (TE: 0.025, 0.25, 2.5 mg sediment equivalents [dry wt]/ml; F1, F2, F3: 2.5, 25, 250 mg sediment equivalents [dry wt]/ml) for 48 h. Data are expressed as fold change (mean ± standard deviation, $n = 3$) relative to the solvent control (dimethylsulfoxide, DMSO). The asterisk (*) indicates statistically different from control ($p < 0.05$). [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]
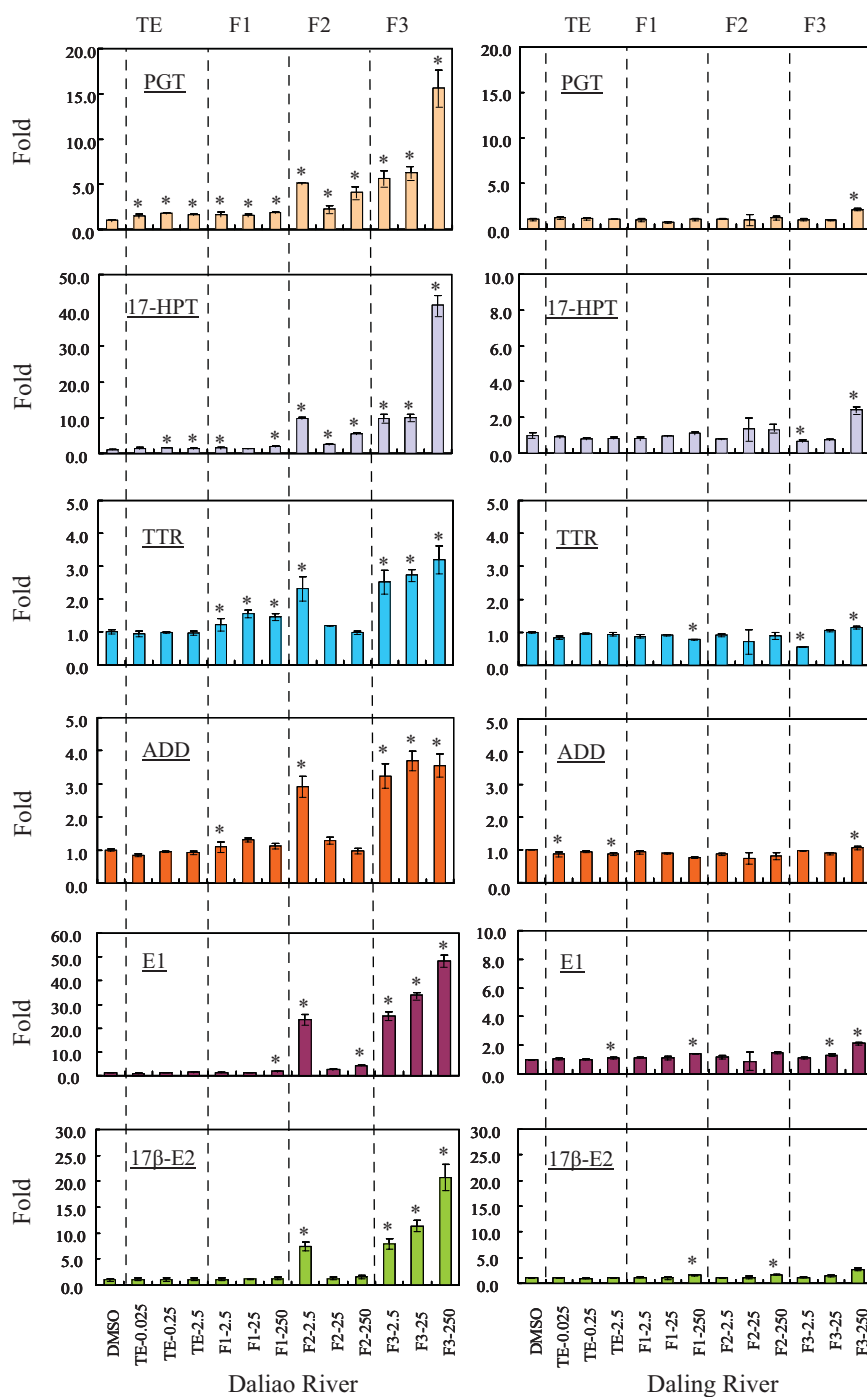
Fig. 2. Fold change of production of progesterone (PGT), 17-hydroxyprogesterone (17-HPT), testosterone (TTR), androstenedione (ADD), estrone (E1), and 17β-estradiol (17β-E2). H295R cells were exposed to TE (total extract), F1, F2, or F3 from sediments of the Daliao and Daling rivers within Liaodong Bay, China, at three concentrations (TE: 0.025, 0.25, 2.5 mg sediment equivalents [dry wt]/ml; F1, F2, F3: 2.5, 25, 250 mg sediment equivalents [dry wt]/ml) for 48 h. Data are expressed as fold change (mean ± standard deviation, $n = 3$) relative to the solvent control (dimethylsulfoxide, DMSO). The asterisk (*) indicates statistically different from control ($p < 0.05$). [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

17β-E2 in medium containing the greatest concentration of sediment equivalents (250 mg [dry wt]/ml) of F3 extracts were 15.61-, 41.32-, 3.19-, 3.55-, 48.34-, and 20.69-fold greater than that of the control, respectively. Fold changes of the 17β-E2 production was much more obvious than that in the sediment from the Danube River [26]. In the study of Grund et al. [26], only 17β-E2 and TTR productions were targeted, and up-regulated 17β-E2 production (4.1-fold) was also observed, whereas TTR production was observed to be slightly down-regulated. The remarkably elevated PGT 17β-HPT, TTR, and ADD production in H295R cells exposed to F3 extract from the Daliao River could be related to an increase in the expression of a sequence of genes such as CYP11A, CYP17, and 3β-HSD2 mRNA that are responsible for progestogens and androgens biosynthesis. The greater concentrations of E1 and 17β-E2 are consistent with the significant up-regulation of the CYP19 mRNA expression and concomitant increase of the CYP17 and the 3β-HSD2 gene expression.

With respect to hormone changes induced by F2 extract from the Daliao River, F2 extract also caused significantly greater concentrations of steroid hormones, including PGT, 17-HPT, ADD, TTR, E1, and 17β-E2. However, concentrations of these hormones in the medium of cells exposed to the greater concentrations of the F2 extract (25 and 250 mg [dry wt]/ml) were less than those in the medium of cells exposed to the lowest concentration of F2 extract (2.5 mg [dry wt]/ml). The inverse concentration dependence at the steroid level, particularly for the most downstream steroid products, such as TTR, ADD, E1, and 17β-E2, parallels the expression of CYP11A and CYP17 in the cells exposed to F2 extract. Elevation of CYP11A and CYP17 transcription at the 2.5 mg/ml concentration, compared with the 25 and 250 mg/ml concentrations provides a plausible explanation for the steroid hormone results. Compared with F2 and F3 extracts from the Daliao River, steroid production of ADD, TTR, E1, and 17β-E2 in TE and F1 extracts had smaller fold changes except for PGT and 17-HPT. As for the Daling River sediments, exposure to the greatest concentrations (250 mg sediment equivalents [dry wt]/ml) of F3 extracts from the Daling River sediments resulted in 2.11-fold, 2.42-fold, 2.11-fold, and 2.72-fold greater concentrations of PGT, 17-HPT, E1, and 17β-E2, respectively.

Through the comprehensive comparison of the gene expression and hormone production changes, different information was presented in this two estuary sediments. The F3 extracts of the Daliao River had an obvious trend of increasing changes in steroid hormone production, indicating that the active chemicals would be present in the Daliao River. Considering the potential effects of estrogens such as EE2 on the estrogen biosynthesis, their concentrations in 200 μl F3 extracts of both the Daliao River and Daling River were analyzed. The concentrations were 3.09 μg/L for E1 and 1.35 μg/L for 17β-E2 in 200 μl F3 extract of the Daliao River, 17α-E2, E3, and EE2 were under the detection limits. Similar results were obtained in 200 μl F3 extract of the Daling River (3.18 μg/L For E1 and 2.25 μg/L for 17β-E2). Considering 100 times dilution when 20 μl F3 fraction was exposed to H295R cells (2 ml cell medium), the concentration levels of E1 and 17β-E2 in the cell medium derived from F3 extracts were similar to the basal levels in the control cells (E1: 0.028 μg/L; 17β-E2: 0.0119 μg/L). Thus, the estrogens detected in F3 fractions could not be a contributing factor because of the significant difference of steroidogenesis modulation between these two rivers, and further study of the mechanism for exploring the possible chemical category that enhanced the estrogen biosynthesis in the H295 cell is necessary.

Possible mechanism of Daliao River sediment extracts

The mRNA expression pattern caused by the Daliao River sediment extracts displayed a pattern similar to that caused by 8-Br-cAMP, which acts through activating the PKA pathway in H295R cells (Fig. 3). The PCA found that forskolin, 8-Brc-AMP, and phorbol-12-myristate-13-acetate clustered along principal component 2 (PC 2) separately from all other chemical classes (Fig. 3). Along principal component 1 (PC 1), some differentiation occurred between forskolin and phorbol-12-myristate-13-acetate relative to 8-Brc-cAMP, where the score plot showed that sample D, which is the F3 extract of the Daliao River sediments (250 mg sediment equivalents [dry wt]/ml), displayed a pattern that was similar to that of the class 1 model, which acts through activating the PKA pathway. The patterns of mRNA expression of H295R cells exposed to other
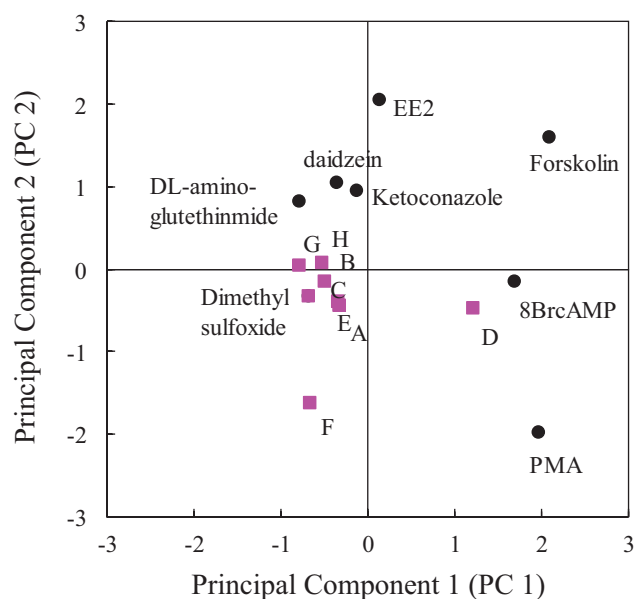

Fig. 3. Plot of four classes of model chemicals and sediment from the Daliao and Daling rivers within Liaodong Bay, China. Solvent control is dimethylsulfoxide (DMSO). Letter encodes sediment samples. (**A**) Total extracts (TE) of the Daliao River (2.5 mg sediment equivalents [dry wt]/ml); (**B**) F1 of Daliao River (250 mg sediment equivalents [dry wt]/ml); (**C**) F2 of the Daliao River (250 mg sediment equivalents [dry wt]/ml); (**D**) F3 of the Daliao River (250 mg sediment equivalents [dry wt]/ ml); (**E**) TE of the Daling River (2.5 mg sediment equivalents [dry wt]/ml); (**F**) F1 of the Daling River (250 mg sediment equivalents [dry wt]/ml); (**G**) F2 of the Daling River (250 mg sediment equivalents [dry wt]/ml); (**H**) F3 of the Daling River (250 mg sediment equivalents [dry wt]/ml). PMA = phorbol 12-myristate 13-acetate. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

organic extracts of sediments were similar and grouped together with the solvent control.

To validate the prediction that the unknown causal agents in fraction F3 of the Daliao River sediments act through the activation of PKA pathway, which results in significant upregulation of the CYP19 gene (aromatase enzyme), an aromatase inhibition assay was conducted. In the present study, H295R cells were co-exposed to 250 mg sediment equivalents (dry wt)/ml of F3 from the Daliao River sediments extract and the chemical H89, which is a selective inhibitor of the PKA pathway. The up-regulation of CYP19 mRNA expression caused by exposure to F3 was significantly (77%) less when H295R cells were co-exposed to F3 and H89 (Fig. 4). In the co-exposure of F3 and H89, concentrations of 17β-E2 and E1 in the medium were 59% and 37% less, respectively, than when exposed to F3 only (Fig. 4). These results are consistent with the hypothesis that the unknown agents in extracts of the Daliao River sediments that up-regulated CYP19 mRNA act through a PKA-mediated processes. The unknown causative agents in F3 of the Daliao River sediments acted through up-regulation of the CYP19 gene. In recent years, considerable emphasis has been focused on the regulation of the CYP19 gene. That CYP19 plays an important role in sex differentiation and reproduction development and in teleosts is the target site of some endocrine-disrupting chemicals has been acknowledged [32]. The occurrence of imposex is associated with the expression of CYP19 in brain of medaka (*Oryzia latipes*) and zebrafish (*Danio rerio*) [33]. Therefore, future studies will attempt to identify the causative agents responsible for the increases of E1 and 17β-E2 production elicited by CYP19 through the PKA pathway in the environmental samples.

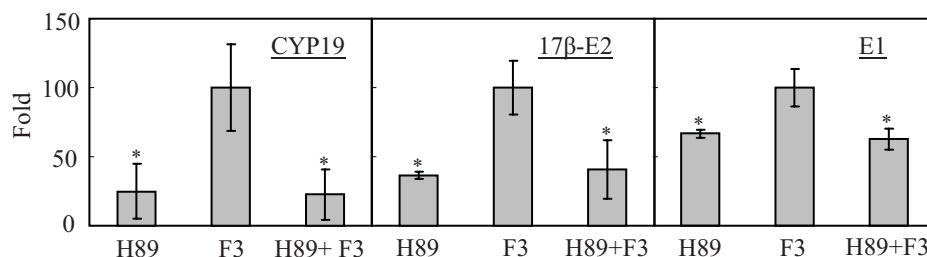Fig. 4. Down-regulation of cytochrome P450 aromatase (CYP19) mRNA expression and 17β-estradiol (17β-E2) and estrone (E1) hormone production after co-exposure of H89 and F3 extract (250 mg sediment equivalents [dry wt]/ml) from Daliao River sediment within Liaodong Bay, China. H89 was used as an inhibitor of the protein kinase A (PKA) pathway. Data are expressed as fold changes relative to the F3 extract of the Daliao River sediment (250 mg sediment equivalents [dry wt]/ml) group (=100). The asterisk (*) indicates statistically different from the F3 extract of the Daliao River sediment (250 mg sediment equivalents [dry wt]/ml) group control ($p < 0.05$).

CONCLUSION

The present study demonstrated that organic extracts of sediments from Liaodong Bay, China, can modulate synthesis of steroid hormones, especially estrogen, in human H295R cells. To the best of our knowledge, this is the first report of significant endocrine-disrupting potencies of complex environmental samples modulated through PKA in H295R cells, although further study is needed to explore the exact causative agents in our future work. The results obtained in the present study suggest that more attention should be given to the occurrence of chemicals that can disrupt the biosynthesis of steroid hormones, especially estrogens.

SUPPLEMENTAL DATA

Fig. S1. Steroidogenic pathways in H295R cells.

Table S1. Multi-selected Reaction Monitoring (MRM) Conditions for Target Steroid Hormones.

Table S2. Molecular mechanisms of four classes of model chemicals used in principalcomponent analysis.

Table S3. Fold changes simplified criteria applied to steroidogenic gene expression.

Table S4. Results of gene expression and hormone production for the blank and the solvent control.

Table S5. Recoveries of target steroids by using different extraction solvents for UPLC-MS-MS. (25 KB DOC)

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