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Gene expression changes in human prostate carcinoma cells exposed to genotoxic and nongenotoxic aryl hydrocarbon receptor ligands

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ABSTRACT

Carcinogenic polycyclic aromatic hydrocarbons (PAHs) are known as efficient mutagens and ligands of the aryl hydrocarbon receptor (AhR), which has been suggested to play an important role in prostate carcinogenesis. In order to evaluate the complex relationship between the genotoxicity and the AhR-mediated activity of PAHs in prostate cells, we selected benzo[*a*]pyrene (BaP) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), as model genotoxic and nongenotoxic AhR ligands, respectively, to explore global changes in gene expression in LNCaP cells by microarray analysis. We identified 112 genes that were differentially expressed in cells treated for 24 h with BaP, TCDD or both compounds. Our data indicated that the impacts of BaP and TCDD on transcriptome of LNCaP cells significantly overlap, since over 64% of significantly up-regulated genes and 47% of down-regulated genes were similarly affected by both AhR ligands. This suggested that the activation of AhR played a prominent role in the nongenotoxic effects of BaP in the prostate carcinoma cell model LNCaP. Both AhR ligands suppressed expression of genes associated with cell cycle progression, DNA replication, spindle assembly checkpoint or DNA repair, which probably occurred secondary to inhibition of cell cycle progression. In contrast, we identified Wnt5a, an important regulator of prostate cancer progression, to be induced as early as 6 h after exposure to both AhR ligands. The AhR ligand-induced Wnt5a upregulation, together with other observed alterations of gene expression, may further contribute to enhanced cell plasticity of prostate carcinoma cells.

1. Introduction

The prostate carcinoma is presently one of the most prevalent forms of cancer in the developed world (ACS, 2009). Epidemiologic studies suggest a possible link between diet and prostate carcinogenesis, and a recent study indicated that red and processed meat may be positively associated with prostate cancer via mechanisms involving, among other factors, benzo[*a*]pyrene (BaP) (Sinha et al., 2009). BaP has been listed by the International Agency for Research on Cancer (IARC) as a compound carcinogenic to humans (IARC, 2010). Similar to other genotoxic polycyclic aromatic hydrocarbons (PAHs), BaP is known to produce stable DNA adducts and further DNA damage, resulting in activation of cellular DNA damage response (Binková et al., 2000; Luch et al., 1999; Ramet et al., 1995; Solhaug et al., 2004; Tekpli et al., 2010; Topinka et al., 2008). BaP is also an efficient ligand of the aryl hydrocarbon receptor (AhR), which plays a key role in the regulation of expression of enzymes, which metabolize PAHs to mutagenic dihydrodiol epoxides, such as cytochromes P450 1A1 (CYP1A1), P450 1A2 (CYP1A2) and P450 1B1 (CYP1B1) (Nebert et al., 2004; Xue and Warshawsky, 2005). However, a number of studies have indicated that AhR itself is directly involved in the regulation of carcinogenesis. The constitutively active AhR mutant has been shown to produce tumors of stomach and liver (Andersson et al., 2002; Moennikes et al., 2004), while the unliganded AhR has been suggested to function as a tumor suppressor (Fan et al., 2010). The activation of AhR might thus play a much wider role in carcinogenesis than a simple transcriptional control of CYP enzymes.

Interestingly, recent studies of Fritz and colleagues have indicated that AhR and/or its selective ligands may inhibit prostate carcinogenesis in TRAMP mice (Fritz et al., 2007, 2009). Among other effects, the activated AhR has been suggested to inhibit the androgen receptor (AR) activity and cell cycle progression in prostate carcinoma models (Barnes-Ellerbe et al., 2004; Kizu et al., 2003; Morrow et al., 2004). Nevertheless, AhR activity may interfere also with the regulation of numerous other cellular processes closely related to tumor promotion and progression, such as cell differentiation, inhibition of apoptosis, cell motility and invasiv-

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ity, senescence or intercellular communication (Dietrich and Kaina, 2010; Diry et al., 2006; Kung et al., 2009; Puga et al., 2009; Ray and Swanson, 2009). Therefore, in order to understand the whole spectrum of toxic effects of AhR ligands in prostate cells, it is vital to analyze the whole spectrum of changes in gene expression, not only those related directly to AR or cell proliferation.

The LNCaP cell line is a well-characterized androgen-sensitive model of human prostate carcinoma cells, which has been successfully used to analyze the impact of both PAHs and nongenotoxic AhR ligands, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), on AR and/or the AR-dependent proliferation (Barnes-Ellerbe et al., 2004; Jana et al., 1999; Kizu et al., 2003; Morrow et al., 2004). Importantly, our previous study suggested that despite forming significant levels of DNA adducts, the genotoxic PAHs, such as BaP, fail to induce the p53-dependent DNA damage response, including transcriptional activation of p53 target genes, induction of apoptosis or cell cycle arrest, in LNCaP prostate carcinoma cells (Hrubá et al., 2010). These results have indicated that activation of AhR might play a more prominent role in the toxic effects of BaP than its genotoxic impact. Unlike TCDD, BaP and/or its principle genotoxic metabolite, *anti*-BaP-*trans*-7,8-dihydrodiol-9,10-epoxide (BPDE) have been shown to produce unique changes in expression of genes involved in cell cycle regulation, apoptosis and DNA repair in human cell lines (Hockley et al., 2007). Therefore, in order to better understand the complex relationship between genotoxicity and AhR-mediated activity of PAHs in prostate cells, we selected BaP and TCDD, as model genotoxic and nongenotoxic AhR ligands, respectively, in order to evaluate global changes in gene expression in LNCaP cells by microarray analysis.

2. Materials and methods

2.1. Reagents

TCDD was purchased from Cambridge Isotope Laboratories (Andover, MA) and BaP was from Fluka (Buchs, Switzerland). Both compounds were dissolved in DMSO (Merck, Darmstadt, Germany) and stock solutions were stored in dark. All other reagents were from Sigma–Aldrich (Prague, Czech Republic), if not indicated otherwise. The sources of other specific chemicals and kits are indicated below.

2.2. Cells

A human prostatic carcinoma cell line LNCaP, originally derived from a lymph node metastasis, was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Cells were grown in RPMI 1640 growth medium with L-glutamine (Gibco BRL, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Sigma–Aldrich), sodium bicarbonate (2 g/l), penicillin (100 U/ml) and streptomycin (100 mg/l). For the treatment, LNCaP cells were maintained in modified RPMI-1640 without phenol red (Sigma–Aldrich) supplemented with 5% charcoal-treated (CT) serum. Cells were maintained in 5% CO_2 at 37 °C.

2.3. Sample processing for microarray analysis

LNCaP cells were grown in growth medium on 60-mm-diameter cell culture dishes at concentration 20,000 cells/cm^2 for 48 h. Cells were then maintained in medium with charcoal-treated serum for one day and for duration of following 24 h-exposure to 10 μM BaP, 5 nM TCDD and DMSO (0.1%) as a solvent control. Medium was removed, the samples were washed with PBS and total RNA was isolated from cells using the NucleoSpin RNA II kit (Macherey-Nagel). Quantity of the RNA was measured on NanoDrop ND-1000 (NanoDrop Technologies LLC, USA). RNA integrity was assessed with Agilent 2100 Bioanalyser (Agilent Technologies, USA). All RNA samples had RIN above 8.

2.4. Microarray analysis

Illumina HumanRef-8 v2.0 Expression BeadChip (Illumina Inc., USA) was used for the microarray analysis following the standard protocol (250 ng of total RNA was amplified with Illumina TotalPrep RNA Amplification Kit (Ambion Inc., USA) and 750 ng of amplified RNA was hybridized on the chip according to the manufacturer procedure). Hybridized slides were scanned on Illumina Beadstation and images were analyzed using Illumina BeadScan. Bead level data were summarized by Illumina BeadStudio Software.

Bead summary data were imported into R statistical environment (http://www.r-project.org) and normalized by quantile method in the lumi package. Only probes with detection p-value $p < 0.01$ on at least 2 arrays in group were included for further analyses. Differential expression analysis was performed using the limma package (Smyth, 2004). Linear model corrected for batch effect was fitted for each gene given a series of arrays using lmFit function. Multiple testing correction was performed using the Benjamini and Hochberg method. Hierarchical clustering analysis of the samples and genes was performed using the average linkage and Euclidean distance.

2.5. Functional analysis of microarray data

Since the fold change of expression of the individual genes across expression data can be relatively modest, the gene set enrichment analysis (GSEA) was employed to classify microarray data at the level of groups of genes that share common biological function. GSEA evaluates the distribution of genes belonging to a biological category in a given sorted list of genes by computing running sum statistics (Subramanian et al., 2005). Enrichment analysis was performed for the treatment groups vs. control using GSEA implemented in java GSEA application, version 2.07. Gene sets tested were taken from Molecular Signature Database v 3.0 curated from the KEGG online database that contained genes present on the used microarray platform. Number of permutations and permutation type was set to 100 and gene set, respectively.

2.6. Real-time RT-PCR

In addition to the 24 h-exposure microarray samples, total RNA samples were also prepared from LNCaP cells after 6, 12 and 24 h exposure to 10 μM BaP, 5 nM TCDD or DMSO (0.1%). The amplifications of the samples were carried out in a final volume of 20 μl using QuantiTect Probe RT-PCR kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's specifications with primers from Generi Biotech (Hradec Králové, Czech Republic) and specific TaqMan probes from the Universal Probe Library (Roche Diagnostics GmbH, Mannheim, Germany) that are listed in Table 1. The amplifications were run on the LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) according the following program: reverse transcription at 50 °C for 20 min and initial activation step at 95 °C for 15 min, followed by 40 cycles at 95 °C for 0 s and 60 °C for 60 s. The changes in gene expression were calculated using the comparative threshold cycle method with B2M as a normalizing gene (Livak and Schmittgen, 2001). The primers and probes for B2M were purchased as qPCR kits from Generi Biotech.

2.7. Western blotting

LNCaP cells were grown and exposed to treatment compounds for 24 h, as described above. After the exposure, cells were harvested with lysis buffer (1% SDS, 10% glycerol, 100 mM Tris and protease/phosphatase inhibitors) and the samples were sonicated. Protein concentrations were determined using bicinchonic acid and copper sulfate (Sigma–Aldrich). For Western blot analyses, equal amounts of total protein lysates were separated by SDS-polyacrylamide gel electrophoresis on 10% gel and electrotransferred onto PVDF membrane Hybond-P (GE Healthcare, Little Chalfont, UK). Pre-stained molecular weight markers (Fermentas GmbH, St. Leon-Rot, Germany) were run in parallel. The membranes were blocked and incubated with primary antibodies against Wnt5a (AF645, R&D Systems, MN, USA) and E2F1 (sc-251, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then with secondary peroxidase-conjugated anti-goat IgG (A 5420, Sigma–Aldrich) or anti-mouse IgG (A 9044, Sigma–Aldrich) antibodies. As a positive control for Wnt5a, we used recombinant human/mouse Wnt5a protein (R&D Systems), or cell lysate from human Burkittís lymphoma (sc-2234, Santa Cruz) for E2F1. Expression of β-actin was used to verify equal loading. To visualize peroxidase activity, the ECLPlus Western blotting detection system (GE Healthcare, Little Chalfont, UK) was used.

2.8. Statistical analysis

Quantitative RT-PCR data were expressed as means ± S.D. and analyzed by Student's t-test, P value of less than 0.05 was considered significant.

3. Results

3.1. Microarray analysis

In order to analyze the impact of BaP and TCDD on global gene expression in LNCaP cells, we selected cultivation conditions based on both our own and previously published studies evaluating the impact of AhR ligands and carcinogenic PAHs on AR activity, cell proliferation or induction of DNA damage (Barnes-Ellerbe et al., 2004; Endo et al., 2003; Hrubá et al., 2010; Kizu et al., 2003; Morrow et al., 2004). Cells were cultivated in the growth medium with