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Time and dose-dependent effects of phenobarbital on the rat liver miRNAome

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ABSTRACT

In a previous study we had shown that treatment of male Fischer rats with exogenous chemicals for three months resulted in prominent, mode-of-action dependent effects on liver microRNA (miRNA) (Koufaris et al., 2012). Here we investigated how the effects of chemicals on liver miRNA in male Fischer rats relate to the length and dose of exposure to phenobarbital (PB), a drug with multiple established hepatic effects. Importantly, although acute PB treatment (1–7 days) had significant effects on liver mRNA and the expected effects on the liver phenotype (transient hyperplasia, hepatomegaly, cytochrome P450 induction), limited effects on liver miRNA were observed. However, at 14 days of PB treatment clear dose-dependent effects on miRNA were observed. The main effect of PB treatment from days 1 to 90 on liver miRNA was found to be the persistent, progressive, and highly correlated induction of the miR-200a/200b/429 and miR-96/182 clusters, occurring after the termination of the xenobiotic-induced transient hyperplasia. Moreover, in agreement with their reported functions in the literature we found associations between perturbations of miR-29b and miR-200a/200b by PB with global DNA methylation and zeb1/zeb2 proteins respectively. Our data suggest that miRNA are unlikely to play an important role in the acute responses of the adult rodent liver to PB treatment. However, the miRNA responses to longer PB exposures suggest a potential role for maintaining liver homeostasis in response to sub-chronic and chronic xenobiotic-induced perturbations. Similar studies for more chemicals are needed to clarify whether the temporal and dose pattern of miRNA–toxicant interaction identified here for PB are widely applicable to other xenobiotics.

1. Introduction

Rodents are routinely used to evaluate the safety of pharmaceutical, industrial, and environmental agents. A substantial fraction of the tested chemicals target the liver due to the metabolic and detoxifying functions of this organ. Such biologically effective agents elicit hepatic responses, which maintain homeostasis (adaptive) or those that disrupt homeostasis (adverse) (Williams and Iatropoulos, 2002). Hepatic responses to xenobiotic exposure include the induction of biotransformation enzymes, activation of anti-oxidative stress response, liver enlargement, increased hepatic necrosis, and altered apoptosis or proliferation (Williams and Iatropoulos, 2002). However, the underlying molecular mechanisms driving hepatic responses to xenobiotics are often not well defined. An improved understanding of these molecular mechanisms would facilitate the extrapolation of exposure outcomes from rodents to humans and improve risk assessment.

It has been suggested that miRNA, small non-coding genes which regulate protein levels at the post-transcriptional stage, could contribute to the cellular responses to toxicant exposures (Taylor and Gant, 2008). Examining miRNA profiles could therefore enhance mechanistic understanding of how xenobiotics elicit their diverse effects on their target cells and tissues. Significantly, regulation by miRNA has been demonstrated to be crucial for the proper functioning of the liver, a complex organ with multiple key functions in physiology and disease. For example liver miRNAs are involved in the regulation of liver regeneration after hepatectomy (Chen et al., 2011; Song et al., 2010), in hepatocyte differentiation and development (Gailhouste et al., 2013), lipid and glucose metabolism (Esau et al., 2006; Trajkovski et al., 2013), and bile

Abbreviations: miRNA, microRNA; PB, phenobarbital; CAR, Constitutive Androstane Receptor; ppm, parts per million; pparα, peroxisome proliferator-activated receptors.

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acid homeostasis (Li et al., 2013). Recently we reported for the first time an investigation into the effects of multiple chemicals with different modes of action on the liver miRNAome after three months of exposure (Koufaris et al., 2012). Importantly, all the tested chemicals affected the liver miRNAome, with the hepatic miRNA profiles being associated with their mode of action. However, reported studies so far have been limited to examining effects of a single dose of xenobiotics on the liver miRNAome at one or few time-points, which limits the ability to interpret the biological significance of observed effects. Additionally, there exists little rationalisation regarding the dosage levels and time-points to be used in studies investigating the contribution of miRNA to toxicological responses.

For this study we evaluated the expression of hepatic miRNAs of male Fischer rats dietary exposed to a control diet; a diet supplemented with a PB dose that induced centrilobular hypertrophy and weak CYP450 induction, but does not induce hepatomegaly or proliferation (50 parts per million (ppm)); or with two PB doses that cause strong CYP450 induction, hepatomegaly, and transient proliferation (500 and 1000 ppm). For each group miRNA profiles were assessed at 1, 3, 7 and 14 days. The effects of these PB treatments on liver growth, morphology, pathology, clinical parameters, metabolism, and expression of liver mRNAs in these animals were reported previously (Waterman et al., 2010). Importantly, by the end of the 14 days alterations on liver growth and proliferation due to PB treatment were complete (Waterman et al., 2010). Our analysis indicates that initially liver miRNA are relatively resistant to acute PB treatment, but more prominent, dose-dependent effects are observed following long-term treatments.

2. Materials and methods

2.1. Animal study

All animal procedures conformed to the Home Office (UK) guidelines for experimentation with animals and were approved by local ethics committee. Groups of six-week old male Fischer (F344) rats were obtained from Harlan Olac (Netherlands), randomly assigned to cages and treatment, and left to acclimatise for seven days prior to commencement of PB treatments. Three animals were examined per group. The rats were kept under controlled lighting (12 h light cycles), humidity (30–70%), air flow (15 changes per hour), and temperature (22 ± 3 °C) conditions. Each group was exposed to different concentrations (0, 50, 500, 1000 ppm in the diet) of the sodium salt of phenobarbital which was added to the standard laboratory diet and milled to homogeneity. Rats were given access to mains water and powdered diet *ad libitum*. At the end of the study animals were killed midway through the light cycle to minimise circadian effects by an overdose of anaesthetic (halothane Ph.Eur.Vapour) followed by exsanguination after 1, 3, 7, 14 days of phenobarbital exposure. Control rats (0 ppm PB diet) were killed at each time point. Liver tissue (left lateral lobe) was obtained from the animals immediately upon sacrifice, weighed, snap frozen and stored at −80 C until needed. For some analyses, samples were also examined from rats treated in a separate study. In this additional study, groups of male Fischer (F344) rats (Harlan Olac) treated with PB (0, 50, 500 and 1000 ppm in the diet) for 28 and 90 days were used.

2.2. RNA extraction

Total RNA was extracted using Trizol (Invitrogen), following the manufacturer's instructions. RNA was used for subsequent analysis only if it had an RNA integrity number, as determined using an Agilent 2100 Bioanalyser and the RNA 6000 nano kit (Agilent), greater than eight and a 260:280 ratio greater than 2.0, as determined with a ND-1000 nanodrop spectrophotometer.

2.3. miRNA microarrays

The hepatic miRNAome was profiled at the MRC Genomics laboratory at Imperial College London using the Agilent microRNA microarray platform as previously described (Koufaris et al., 2012). Livers from three animals were examined per group. The hybridisation data were extracted using the Agilent feature extraction software and normalised to the 75th percentile using Genespring GX (Agilent). For subsequent analysis we retained miRNAs which were flagged as present in at least two out of the three examined animals in any group. Box plots of normalised microarray data for individual samples displayed similar distributions supporting the quality of the hybridisation data. The miRNA microarray data is available at Gene Expression Omnibus (GSE48489). The miRNA profiling data from male Fischer rats treated with 1000 ppm PB for 90 days were generated previously (Koufaris et al., 2012) and is available at Gene Expression Omnibus (GSE48492).

2.4. Analysis of mRNA microarray data

The mRNA microarray data (GSE18753) was processed in BRB-Array tools by MAS5 summarisation, averaging the replicate spots in each array and setting the threshold for each spot to 10, baseline transforming each gene to the median. Genes failing to appear in at least 25% of the samples were excluded for further analysis. Where a gene was represented by more than one probe, the average expression for the probes was used.

2.5. Hierarchical clustering analysis

Hierarchical clustering analysis for mRNAs and miRNAs was performed in Cluster 3.0 using genes that had passed our filtering criteria. The probes were median centred and normalised and the tree was constructed using correlation centred as the similarity metric and average linkage.

2.6. Polymerase chain reaction (PCR)

For quantitative reverse-transcription real time PCR (qRT-PCR), total RNA was reverse transcribed using the miRNA reverse transcription kit (Agilent) and then amplified using the Taqman 2X Universal PCR master mix, No AmpErase UNG (Applied Biosystems), with each PCR reaction performed in triplicate. Mature miRNA Taqman assays were purchased from Applied Biosystems. The qRT-PCR data were analysed in the ABI 7500 Sequence Detection System (Applied Biosystems) using the comparative Ct Method (ΔΔCt Method) to quantify miRNA expression using snoRNA as the endogenous control. For semi-quantitative RT-PCR total RNA was reverse transcribed using MMLV reverse transcriptase (Promega). The cDNA was then amplified using Tfi polymerase (Invitrogen). The primers used were: e-cadherin For ACAGCAAGCATGCCAGTGAA; e-cadherin Rev GCACCAACACACCCAGCATA; cyp2b1 For GGA GAG CGC TTT GAC TAC; cyp2b1 Rev CTC GTG GAT AAC TGC ATC; gapdh For CAT GGA CTG TGG TCA TGAG; gapdh Rev TTC AAC GGC ACA GTC AAGG.

2.7. Immunoblotting

Livers were homogenised with a polytron machine (Labortechnik) in 500 μl of lysis buffer (50 mM Tris–HCl, pH 7.4; 1% IGEPAL (Sigma–Aldrich); 0.25% sodium deoxycholate (Sigma–Aldrich); 150 mM NaCl (Sigma–Aldrich); 1 mM EDTA (Sigma–Aldrich); 100× Halt Protease inhibitor (Pierce)). Homogenisation was followed by sonication and incubation at 4 °C for 30 min. The solutions were then centrifuged for 20 min to remove insoluble debris. The protein extracts were quantified using the BCA (bicinchoninic acid) protein assay (Pierce). Primary antibodies were purchased from Santa Cruz (Heidelberg) (zeb1 1:100, zeb2 1:100) or Sigma–Aldrich (Beta-actin; 1:10000).

2.8. Global DNA methylation analysis

DNA was extracted from liver samples using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's protocol, and using the reagents and buffers included in the kit. Extracted DNA was quantified using the Nanodrop ND-1000 and used for subsequent experiments if its 260:280 ratio was higher than 1.8. The levels of global DNA methylation for extracted DNA were determined using the Methylamp Global DNA Methylation Quantification Ultra Kit (Epigentek) following the manufacturer's instructions. This kit is based on the recognition of the methylated fraction of DNA by a labelled 5-methylcytosine which is subsequently quantified through an ELISA-like reaction.

2.9. Statistical analysis

Differentially expressed mRNAs and miRNAs were identified by one-way fixed-model ANOVA with False Discovery Rate (FDR), the expected percentage of false positives within a given set of predicted differentially regulated genes, set to <0.1 in BRB-Array tools. The identified genes were then examined to determine whether they are over-represented for gene sets defined to be members of common biological pathways in the KEGG database using the ConsensuspathDB tool (Kamburov et al., 2009). Similarly, enriched KEGG pathways for predicted and known targets of miRNAs were identified using miRSystem (Lu et al., 2012) and setting the threshold for a true target as being predicted by at least three software packages or being a verified target, an observed/expressed ratio above or equal to 2, and using pathways containing 25–500 genes. Student's *t*-test or Analysis of variance (ANOVA) was used to test for significance of all other analysis.