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Time course investigation of PPARα- and Kupffer cell-dependent effects of WY-14,643 in mouse liver using microarray gene expression

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Abstract

Administration of peroxisome proliferators to rodents causes proliferation of peroxisomes, induction of β-oxidation enzymes, hepatocellular hypertrophy and hyperplasia, with chronic exposure ultimately leading to hepatocellular carcinomas. Many responses associated with peroxisome proliferators are nuclear receptor-mediated events involving peroxisome proliferators-activated receptor alpha (PPARα). A role for nuclear receptor-independent events has also been shown, with evidence of Kupffer cell-mediated free radical production, presumably through NAPDH oxidase, induction of redox-sensitive transcription factors involved in cytokine production and cytokine-mediated cell replication following acute treatment with peroxisome proliferators in rodents. Recent studies have demonstrated, by using *p47*^{phox}-null mice which are deficient in NADPH oxidase, that this enzyme is not related to the phenotypic events caused by prolonged administration of peroxisome proliferators. In an effort to determine the timing of the transition from Kupffer cell-to PPARα-dependent modulation of peroxisome proliferator effects, gene expression was assessed in liver from *Pparα*-null, *p47*^{phox}-null and corresponding wild-type mice following treatment with 4-chloro-6-(2,3-xylidino)-pyrimidynylthioacetic acid (WY-14,643) for 8 h, 24 h, 72 h, 1 week or 4 weeks. WY-14,643-induced gene expression in *p47*^{phox}-null mouse liver differed substantially from wild-type mice at acute doses and striking differences in baseline expression of immune related genes were evident. Pathway mapping of genes that respond to WY-14,643 in a time- and dose-dependent manner demonstrates suppression of immune response, cell death and signal transduction and promotion of lipid metabolism, cell cycle and DNA repair. Furthermore, these pathways were largely dependent on PPARα, not NADPH oxidase demonstrating a temporal shift in response to peroxisome proliferators. Overall, this study shows that NADPH oxidase-dependent events, while detectable following acute treatment, are transient. To the contrary, a strong PPARα-specific gene signature was evident in mice that were continually exposed to WY-14,643.

Keywords: Peroxisome proliferators; PPARα; Kupffer cells; Toxicogenomics; Microarrays

Introduction

Peroxisome proliferators (PPs) are a chemical class comprised of a wide range of industrial, pharmaceutical and endogenous compounds. These compounds have been the subject of debate for several decades because of their carcinogenicity in rodents (Lalwani et al., 1981) and uncertain risk to humans (Klaunig et al., 2003; Rusyn et al., 2006). A number of peroxisome proliferator-induced events leading up to carcinogenesis, including increased cell replication, oxidative damage and tumorigenesis itself require activation of nuclear receptor peroxisome proliferators-activated receptor alpha (PPARα) (Peters et al., 1997; Rusyn et al., 2004). This receptor is largely responsible for lipid metabolism through transcriptional regulation of fatty acid oxidation enzymes, apolipoproteins

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and transporters (Peters et al., 2005). Among species there are substantial structural differences in the DNA binding element, peroxisome proliferator response element (PPRE) along with differences in basal PPARα expression, with humans exhibiting a tenth of the levels observed in rodents (Palmer et al., 1998).

A wide range of nuclear receptor-independent effects of PPARa agonists in rodent liver have been reported and some of these were attributed to activation of the Kupffer cells (Rusyn et al., 2006). Therefore, delineating the relative contribution of receptor-independent and -dependent molecular events is important for human health risk assessment of these agents, which is currently based largely on the interspecies differences in PPARα activation and signaling (Peters et al., 2005). Studies performed *in vivo* and *in vitro* demonstrated that peroxisome proliferators stimulate superoxide and cytokine production by liver macrophages (Hasmall et al., 2000b). Studies using NADPH oxidase-deficient (*p47^{phox}*-null) and *Pparα*-null mice demonstrated that acute effects of peroxisome proliferators seem to be largely mediated by oxidant production in Kupffer cells (Peters et al., 2000; Rusyn et al., 2001). WY-14,643-induced cell proliferation is abrogated in *p47^{phox}*-null mice or when mice are pre-treated with NAPDH oxidase inhibitor, diphenyliodonium. Despite evidence demonstrating a role for Kupffer cells and mitogens in the early events in rodent liver associated with peroxisome proliferators, other studies conducted in TNFα (Lawrence et al., 2001)- and TNF receptor (TNFR)-null mice (Anderson et al., 2001) suggest that WY-14,643-induced cell proliferation may not be mediated by TNFα. It is clear, however, that PPARα mediates key events in rodent liver following sub-chronic and chronic treatment of peroxisome proliferators (Peters et al., 1997; Woods et al., 2007b).

Microarray technology has served as a valuable tool for gathering mechanistic information regarding toxicants molecular targets and temporal progression of toxicity leading to specific disease states. Genomic studies investigating the effects of peroxisome proliferators in rodent liver have provided critical insight into the molecular mechanisms responsible for liver-specific effects of peroxisome proliferators in rodents and supported a non-genotoxic mechanism of their action (Cherkaoui-Malki et al., 2001; Hamadeh et al., 2002; Currie et al., 2005). However, the gap in our knowledge remains on the temporal relationship between peroxisome proliferator-modulated effects and molecular mediators of these effects, especially at the early time points where PPARα-independent events are also known to occur. To address this, gene expression analysis was conducted in livers from *p47^{phox}*-null, *Pparα*-null and wild-type mice treated from 8 h to 4 weeks with a potent peroxisome proliferator, 4-chloro-6-(2,3-xylidino)-pyrimidynylthioacetic acid (WY-14,643). We show that NADPH oxidase-dependent events, while detectable following acute treatment, are short-lived. To the contrary, a strong PPARα-specific gene signature was evident in mice that were continually exposed to WY-14,643.

Methods

Animals, diets and tissue collection. *p47^{phox}*-null male mice (C57BL/6J background; Jackson et al., 1995), *Pparα*-null male mice (SV129 background; Lee et al., 1995), and corresponding wild-type counterparts (6–8 weeks of age at the beginning of treatment) were used in these experiments. All animals used for this study were housed in sterilized cages in a facility with a 12-h night/day cycle. Temperature and relative humidity were held at 22±2 °C and 50±5%, respectively. The UNC Division of Laboratory Animal Medicine maintains these animal facilities, and veterinarians were always available to ensure animal health. All animals were given humane care in compliance with NIH and institutional guidelines and studies were performed according to protocols approved by the appropriate institutional review board. Prior to experiments, animals were maintained on standard lab chow diet and purified water *ad libitum*. WY-14,643 was obtained from Aldrich (Milwaukee, WI). Acute doses were administered by a single oral gavage of 0 (control), 5 or 50 mg/kg of WY-14,643 in olive oil. Mice ($n=3$) were sacrificed 8 h, 24 h or 72 h post dosing. Sub-chronic doses of WY-14,643 were administered in the diet *ad libitum*. NIH-07 was used as the base for the powdered diet containing either 0 (control), 50 or 500 ppm of WY-14,643. Mice ($n=3$) were sacrificed after either 1 week or 4 weeks of dietary treatment. Animals had free access to water throughout the study and the health status of the animals was monitored every other day. At sacrifice, mice were anesthetized with pentobarbital (100 mg/kg) and following exsanguination livers were removed and weighed. A section from the left lateral lobe was fixed in 10% formalin. The remaining tissue was snap frozen in liquid nitrogen. The samples were stored at 80 °C until assayed.

RNA isolation. While frozen, a small fragment (approximately 30 mg) was removed for each liver sample and homogenized for 30 s in 600 μl RLT buffer (Qiagen, Valencia, CA) containing 1% β-mercaptoethanol. The lysate was centrifuged for 3 min at 13000 rpm. From the resulting supernatant, total RNA was isolated using an RNeasy kit (Qiagen) as per the manufacturer's protocol. Total RNA integrity and quantification were assessed using RNA 6000 nano assay LabChips® (Agilent Technologies, Santa Clara, CA) and analyzed on a 2100 Bioanalyzer (Agilent Technologies) as per the manufacturer's protocol.

cDNA preparation and microarray hybridization. Preparation of cDNA, labeling and hybridizations were performed using reagents from the low RNA input fluorescent linear amplification kit (Agilent Technologies) based on the manufacturer's protocol. A pooled mouse RNA sample (Cogenics, RTP, NC) derived from equal amounts of RNA from kidney, spleen, lung, brain and liver was used as a reference and prepared in parallel to the samples of interest. Samples were analyzed using an Agilent Mouse Oligo Microarray (21,000 features, catalogue# G4121). The hybridized microarrays were washed and scanned using an Agilent G2565BA scanner. Data were extracted from the scanned image using Agilent Feature Extraction software version 6.1. Raw data are available from the UNC Microarray database (genome.unc.edu).

Microarray data analysis. Array quality was assessed using Agilent Feature Extraction software and genes with fewer than 70% present data across all arrays were excluded from further analysis. A total of 16,030 probes passed this data quality filter. Removal of control oligos and RIKENs, for which little or no functional data were available, reduced the list to 11,421 genes. These transcripts comprised our working data set. LOWESS normalization was performed to eliminate dye bias. A second normalization was performed to correct for basal differences in gene expression between SV129 and C57BL/6J mice or between wild-type and knockout mice on both backgrounds. This normalization involved dividing the Cy5/Cy3 ratio for a given gene by the mean Cy5/Cy3 ratio for the same gene from time and strain-matched controls. Missing data points were calculated using K-nearest neighbor imputation method (Troyanskaya et al., 2001). Average linkage, hierarchical clustering was performed using Cluster software on median centered (by genes) data, and visualization was facilitated by Treeview (Eisen et al., 1998).

Differentially expressed genes were identified using either Significance Analysis of Microarrays (SAM) or EDGE software (Tusher et al., 2001; Leek et al., 2006). SAM was performed in cases where statistical significance across only one variable (strain or dose) was being assessed. EDGE was used for identifying differentially expressed genes over time within a single condition or among several conditions. *Q*-values, which represent the false discover rate (FDR) of less than 0.05 for SAM and EDGE, were selected as thresholds for differential expression. Once the list of significant genes was generated by EDGE, a *t*-statistic was calculated for each gene at each strain/time combination to determine statistical difference between high dose and control expression. For each strain/time combination,

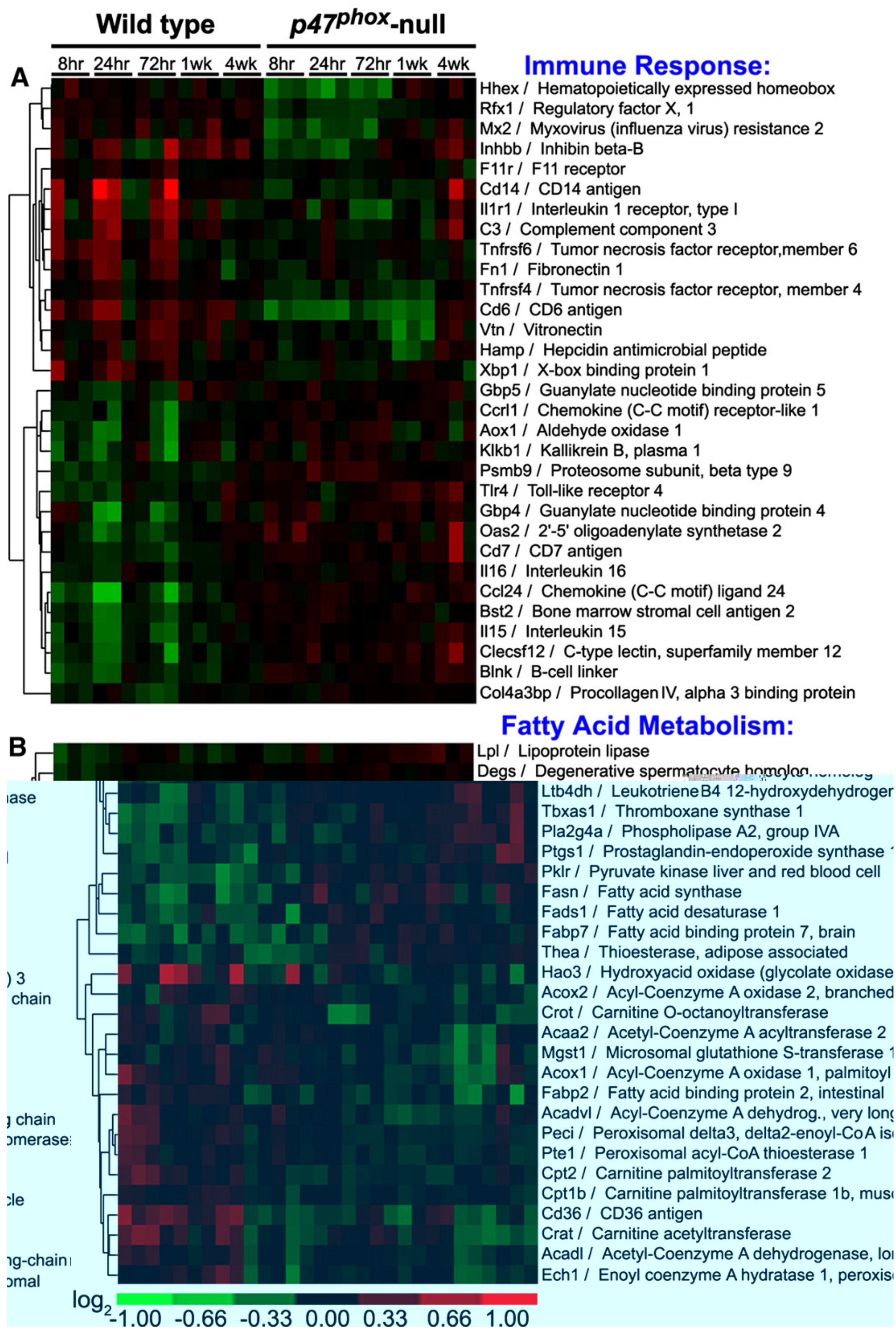

Fig. 1. Immune response and fatty acid metabolism are affected in liver of *p47*^{phox}-null mice. Differentially expressed genes were identified between untreated wild-type and *p47*^{phox}-null mice across all time points. Biological pathway analysis identified (A) immune response and (B) fatty acid metabolism as significantly enriched categories. Hierarchical clustering of the genes contributing to these biological processes is shown.