Temporal and dose-dependent hepatic gene expression patterns in mice provide new insights into TCDD-mediated hepatotoxicity

Darrell R Boverhof[1], Lyle D Burgoon[1], Jack R Harkema[1], Colleen Tashiro[2], Brock Chittim[3], Tim R Zacharewski[1]

[1]Michigan State University
[2]Wellington Laboratories Inc
[3]Wellington Laboratories Inc.

Introduction

Although the mechanisms of AhR/ARNT-mediated changes in gene expression are fairly well established, TCDD elicited modulation of gene expression and pathways associated with toxicity remains poorly understood. Global gene expression technologies provide a comprehensive strategy whereby critical AhR-regulated target genes can be identified and used to elucidate target pathways involved in the etiology of TCDD and related compound toxicity. Sustained activation of the AhR and its target genes has been hypothesized as a prerequisite for toxicity that typically requires days or weeks to develop. Alternatively, activation of the AhR may initiate a cascade of secondary and tertiary gene expression changes leading to the compromised physiological state. Hepatotoxicity is a classical endpoint of TCDD exposure characterized by hepatomegaly accompanied by hepatocyte hypertrophy, fat accumulation, immune infiltration, necrosis and alterations in liver enzymes which likely contribute to tumor promotion and hepatocarcinogenesis. In order to identify gene expression changes causal to hepatotoxicity and carcinogenesis and to further characterize the spectrum of AhR/ARNT responsive transcripts, temporal and dose-dependent effects of TCDD on hepatic gene expression were examined in the context of complementary histological and clinical chemistry endpoints. This integrative approach has provided a powerful strategy to comprehensively assess the *in vivo* effects of TCDD.

Materials and Methods

Comprehensive temporal and dose response microarray analyses were performed on hepatic tissue from immature ovariectomized C57BL/6 mice treated with TCDD. For temporal analysis, mice were gavaged with 30 µg/kg of TCDD or vehicle and sacrificed after 2, 4, 8, 12, 18, 24, 72 or 168 hours. Dose response mice were gavaged with 0, 0.001, 0.01, 0.1, 1, 10, 100 or 300 µg/kg of TCDD and sacrificed after 24 hours. Hepatic gene expression profiles were monitored using custom cDNAmicroarrays containing 13,362 cDNA clones. Detailed protocols for microarray preparation, labeling of the cDNA probe, sample hybridization and washing can be found at http://dbzach.fst.msu.edu/interfaces/microarray.html. These gene expression analyses identified 443 and 315 microarray features which exhibited a significant change at one or more doses or time points, respectively, as determined using an empirical Bayes statistical analysis approach. Complementary histopathology (H&E and Oil Red O stains), clinical chemistry (i.e. ALT, TG, FFA, cholesterol) and high resolution gas chromatography/mass spectrometry assessment of hepatic TCDD levels (conducted at Wellington Laboratories Inc. Guelph, ON, Canada) were also performed in order to phenotypically anchor changes in gene expression to physiological endpoints. To verify the responses detected by the microarray, 24 genes were selected for verification by Sybr Green quantitative real-time PCR.

Results and Discussion

Tissue Results

TCDD treatment resulted in a significant ($p<0.05$) dose-dependent increase in relative liver weights at 100 and 300 µg/kg in the dose response study. In the time course study, relative liver weights were significantly ($p<0.05$) increased at 24, 72 and 168 hours with maximal increases observed at 168 hours. The principle TCDD related histological alteration was a minimal to moderate cytoplasmic vacuolization of hepatocytes primarily in the periportal and midzonal regions of the liver. This effect was absent or minimal in mice from the 0.001-0.1µg/kg groups, whereas in mice from the 1-300µg/kg dose groups, mild to moderate cytoplasmic vacuolization was consistently

observed. In the time course study, cytoplasmic vacuolization was observed in the periportal and midzonal regions with extension into the centriacinar regions at later time points. Minimal vacuolization was first observed at 18 hours with severity progressing from mild to moderate at 24 and 72 hours, respectively. Marked cytoplasmic vacuolization was noted at 168 hours and was accompanied by individual cell apoptosis and immune cell accumulation. Oil Red O staining confirmed that the dose- and time-dependent vacuolization was due to lipid accumulation. Analysis of liver lipid extracts by thin layer chromatography revealed increases in triglycerides, free fatty acids and cholesterol in TCDD treated mice. Significant treatment related alterations were noted in serum ALT, cholesterol, FFAs, and triglycerides. ALT levels increased steadily after 24 hours to a maximum of 2.6-fold at 168 hours, indicative of mild liver injury in TCDD-treated mice. Serum cholesterol was significantly ($p<0.05$) decreased by 33 and 28% at 72 and 168 hours, respectively. Serum FFAs were increased 33, 16 and 28% at 24, 72 and 168 hours, respectively. Triglyceride levels were also elevated by 24, 15 and 40% in TCDD treated mice at 24, 72 and 168 hours, respectively.

Hepatic TCDD levels were determined in dose-response and time course studies in order to relate tissue concentrations to molecular responses. TCDD levels were significantly ($p<0.05$) increased in a dose dependent manner at doses as low as 0.1 µg/kg. In the time course study, significant ($p<0.05$) increases in hepatic TCDD content were noted at all time points with a gradual increase between 2 and 72 hours followed by a 60% decrease at 168 hours. Hepatic TCDD levels in this study are comparable to other reports using similar exposure regimens. The accurate determination of hepatic TCDD levels is essential in order to elucidate correlations between gene expression and physiological effects.

Gene Expression analysis

Empirical Bayes analysis of the dose response data identified 443 microarray features, representing 374 annotated clones and 349 unique genes, which were differentially expressed relative to vehicle controls, at one or more doses. A dose dependent increase in the number of active genes was observed which began to plateau at 100 and 300µg/kg TCDD. Based on these results, a dose of 30µg/kg was chosen for temporal studies as it represents the approximate ED_{50} for overall gene responses and would avoid overt toxicity and lethality in a longer term study.

Analysis of the time course data identified 315 microarray features, representing 269 annotated clones corresponding to 255 unique genes, which were differentially expressed, relative to the time-matched vehicle controls at one or more time points. The 2 hour time point displayed the fewest number of active genes followed by a large increase at 4 hours which was largely stable through 18 hours and followed by an additional increase between 24 and 168 hours. This temporal pattern indicates that a majority of the gene expression responses are preceding the observed histological alterations. In addition, the later increases in active features coincide with the appearance and severity of hepatotoxicity indicating that these responses may be a result of the emerging toxicity. The gene lists obtained from these initial stringent filtering criteria were used for data clustering, organization and the identification of functional pathways affected by TCDD.

Functional annotation extracted from public databases revealed that many of the transcriptional responses were associated with metabolizing enzymes, development and differentiation, fatty acid uptake and metabolism, gluconeogenesis, immune signaling and apoptosis. Metabolizing enzymes included oxidoreductases, monooxygenases and xenobiotic metabolizing enzymes such as the well characterized TCDD inducible genes, Cyp1a1 and Nqo1. Novel responsive oxidoreductase and xenobiotic metabolizing genes included abhydrolase domain containing 6 (Abhd6), carbonyl reductase 3 (Cbr3), dehydrogenase/reductase (SDR family) member 3 (Dhrs3), epoxidehydrolase 1 (Ephx1) and UDP-glucose dehydrogenase (Ugdh). Glutathione S-transferases alpha2, alpha4 and pi2 (Gsta2, a4 and p2) as well as glutamate-cysteineligase (Gclc) and glutathione synthetase (Gss) were also regulated by TCDD, which is consistent with the induction of both phase I and II metabolizing enzymes by TCDD, commonly referred to as the AhR gene battery.

Genes involved in development and differentiation were also induced or repressed in response to TCDD treatment including Notch1, tumor necrosis factor, alpha-induced protein 2 (Tnfaip2), hairy and enhancer of split 6 (Hes6), growth arrest and DNA-damage-inducible 45 beta (Gadd45b) and growth arrest specific 1 (Gas1), all of which have not been previously reported to be regulated by TCDD. AhR-mediated dysregulation of these genes may play a role in mediating the effects of TCDD on cellular differentiation or in AhR signaling which has been implicated in the differentiation and development of various organ systems. TCDD also repressed transcripts encoding enzymes involved in gluconeogenesis including Pck1, which has been previously reported. In addition, Got1 and glycerol phosphate dehydrogenase 2 (Gpd2), two additional enzymes involved in the gluconeogenic pathway, were also

down regulated. These results suggest that TCDD may affect multiple steps in gluconeogenesis, although clinical chemistry did not detect any alterations in circulating glucose.

Effects on fatty acid uptake and metabolism, immune signaling and apoptosis are consistent with the observed hepatic histological findings. H&E and Oil Red O staining revealed marked fatty vacuolization of hepatocytes at 24 hours with maximal effects at 168 hours. Numerous genes involved in fatty acid transport including fatty acid binding protein 4 and 5 (Fabp4 and 5), CD36 antigen (Cd36), solute carrier family 27, member 2 (Slc27a2) and lipoprotein lipase (Lpl), were significantly induced and may mediate the fatty accumulation. Induced apoptotic genes included receptor (TNFRSF)-interacting serine-threoninekinase 1 (Ripk1), caspase 6 (Casp6), BCL2-like 11 (Bcl2l11) and huntingtin interacting protein 1 (Hip1), which is also consistent with the histopathologic identification of hepatocyte apoptosis at 168 hours. In general, these gene expression responses preceded or paralleled the observed histopathology for each functional category. In contrast, the induction of immune signaling genes was largely confined to168 hour coincident with the histology. Consequently, these gene expression changes are likely due to the infiltration of immune cells as opposed to changes in hepatocyte gene expression.

QRTPCR was used to verify changes in transcript levels for a selected subset of active genes representing different responses and functional categories. In total 24 genes were verified by QRTPCR, all of which displayed temporal expression patterns comparable with the microarray data

Summary

The present study represents the first comprehensive *in vivo* examination of the acute transcriptional response of the liver to TCDD. Alterations in gene expression were directly related to physiological outcomes demonstrating the importance of phenotypic anchoring when interpreting microarray data. Integration of gene expression, histological and clinical chemistry endpoints facilitated the development of a response network that further elucidates potential mechanisms involved in TCDD mediated hepatotoxicity. The comprehensive time course analysis also allowed for the identification of gene expression responses that precede and may mediate subsequent physiological/toxicological responses. Early and sustained induction of ROS-generating oxidoreductase enzymes likely contribute to later liver damage, as indicated by mild increases in ALT levels, and the subsequent accumulation of immune cells. Changes in gene expression and histopathology also indicated the occurrence of apoptosis which may be due to direct transcriptional responses or may be a secondary response to oxidative stress. Dysregulation of gene expression responses involved in fatty acid uptake and metabolism concomitant with serum TRIG and FFA increases and inhibition of glyceroneogenesis, suggests a putative mechanism for mediating the subsequent fatty liver response. Additional studies are required to more fully delineate these responses and determine if other hepatotoxicants use common pathways to elicit comparable steatotic effects. Furthermore, examination of additional target tissues and animal models will reveal whether these responses are tissue- and/or species-specific which will aid in development of accurate models of toxicity for TCDD and related compounds as well as human risk assessments.