Toxicology 361–362 (2016) 49–61

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Functional and cellular consequences of covalent target protein modification by furan in rat liver

Susanne Ramm[1], Elisabeth Limbeck, Angela Mally*

Department of Toxicology, University of Würzburg, 97078 Würzburg, Germany

ARTICLE INFO

Article history:
Received 10 May 2016
Received in revised form 27 June 2016
Accepted 27 June 2016
Available online 9 July 2016

Keywords:
Furan
Covalent protein binding
Enzyme activity
Metabolic pathways
Liver toxicity

ABSTRACT

Furan hepatotoxicity is thought to be linked to covalent binding of its reactive metabolite, *cis*-2-butene-1,4-dial, to hepatic proteins critical for cell homeostasis and survival. We previously identified 61 putative furan target proteins, which participate in various cellular processes including carbohydrate metabolism, fatty acid β-oxidation, adenosine triphosphate (ATP) synthesis, protein folding and maintenance of redox homeostasis. To further investigate the biological significance of target protein modification, this study was designed to determine the impact of furan on the activity of key target enzymes involved in glycolysis, β-oxidation, ATP synthesis, and redox regulation in rat liver, and to link these functional changes to alterations in cellular processes. While *cis*-2-butene-1,4-dial inhibited thioredoxin 1 (Txn1) in a cell-free assay, in livers of rats treated with a single high dose of furan Txn1 activity was markedly increased due to rapid up-regulation of Txn1 mRNA expression. Significant inhibition of glyceraldehyde-3-phosphate dehydrogenase and metabolic changes consistent with blocked glycolytic breakdown of glucose were observed in rat liver in response to a single high dose of furan. In contrast, furan treatment resulted in increased activity of enoyl-CoA hydratase and enhanced production of ketone bodies, indicative of increased utilization of fatty acids as energy source. Consistent with changes in TCA cycle metabolites, furan treatment resulted in a reduction of succinate dehydrogenase activity, supporting mitochondrial dysfunction as a critical event in furan toxicity. No significant changes in target protein function were observed following repeated administration of furan at lower dose (0.1 and 0.5 mg/kg bw for 4 weeks) closer to estimated human exposure to furan via food. Although the relative contribution of furan mediated alterations in metabolic pathways and antioxidant defense to the overall toxic response to furan, including considerations of dose and time, remains to be established, our work contributes to mapping biological processes and toxicity pathways modulated by reactive electrophiles.

1. Introduction

Furan, an industrial solvent and food contaminant, is a potent hepatotoxin and liver carcinogen in rodents (Crews et al., 2007; NTP, 1993). Hepatotoxicity of furan in rodents is characterized by centrilobular and subcapsular hepatocellular degeneration, hepatic inflammation, and compensatory processes including hepatocellular cytomegaly, mitosis, and regenerative hyperplasia (Gill et al., 2010; Hamadeh et al., 2004; Hickling et al., 2010a; NTP, 1993; Wilson et al., 1992). In focal areas of more extensive degeneration, furan induced liver injury may extend into portal regions, leading to early and persistent biliary cell proliferation and cholangiofibrosis (Hickling et al., 2010a). Both hepatocellular and biliary lesions have been reported to progress into tumors (hepatocellular adenoma/carcinoma and cholangiocarcinoma) upon sustained exposure to furan (Elmore and Sirica 1993; Maronpot et al., 1991; NTP, 1993). While expansion of biliary ducts and differentiation of the expanding biliary ductular cells to hepatocytes in furan treated rat liver is considered to be part of a repair process to replace necrotic tissue in areas of severe hepatocellular injury (i.e. a secondary event) (Hickling et al., 2010a), it is well established that the initial insult to hepatocytes is linked to cytochrome P450 2E1 dependent bioactivation of furan to *cis*-2-butene-1,4-dial (BDA) (Carfagna et al., 1993; Chen

Abbreviations: Arg1, arginase 1; ATP5b, ATP synthase β subunit; BDA, cis-2-buten-1,4-dial; Echs1, enoyl-CoA hydratase 1 mitochondrial; Eno1, α-enolase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SDH, succinate dehydrogenase; TCA cycle, tricarboxylic acid cycle; Txn1, thioredoxin-1; Txnrd1, thioredoxin reductase-1.

* Corresponding author at: Department of Toxicology, University of Würzburg, Versbacher Str. 9, 97078 Würzburg, Germany.
E-mail address: mally@toxi.uni-wuerzburg.de (A. Mally).
[1] Present address: Laboratory of Systems Pharmacology, Harvard Medical School, Boston 02115, MA, United States.

http://dx.doi.org/10.1016/j.tox.2016.06.018
0300-483X/

et al., 1995), a chemically reactive α,β-unsaturated dialdehyde, which readily reacts with amino- and sulfhydryl groups of free or protein-bound amino acids (Chen et al., 1997; Lu and Peterson 2010; Lu et al., 2009). Consistent with its chemical reactivity, extensive binding to hepatic proteins was shown to occur after administration of ^{14}C-labeled furan to rats (Burka et al., 1991; Moro et al., 2012). Thus, it appears that furan cytotoxicity results from toxic electrophile insult to proteins critical for cell homeostasis and survival. However, despite an apparent link between covalent protein binding of reactive intermediates and cytotoxicity, there is as yet a paucity of information on the proteins and pathways affected by reactive electrophiles that ultimately cause cell death.

Using separation of hepatic proteins by two-dimensional gel electrophoresis and subsequent detection and identification of proteins carrying radiolabel by fluorography and mass spectrometry, we previously identified 61 putative target proteins of furan in livers of male F344/N rats administered a single dose of [3,4-^{14}C]-furan (20 mCi/mmol) (Moro et al., 2012). The identified proteins represent enzymes, transport proteins, structural proteins, and chaperones which predominantly localize to the cytosol and mitochondria and participate in various cellular processes including carbohydrate metabolism, mitochondrial β-oxidation, adenosine triphosphate (ATP) synthesis, protein folding, and maintenance of redox homeostasis. Pathway mapping of putative furan target proteins using the web-based annotation tool DAVID (Dennis et al., 2003; Huang da et al., 2009) revealed fatty acid, amino acid, and glucose metabolism as significantly enriched KEGG pathways with 7/61 (11.5%), 7/61 (11.5%), and 8/61 (13.1%) proteins assigned to these annotation terms, respectively. Specifically, several enzymes involved in glycolysis (α-enolase, fructose-bisphosphate aldolase B, glyceraldehyde 3-phosphate dehydrogenase, L-lactate dehydrogenase A chain, phosphoglycerate kinase 1, triosephosphate isomerase), the urea cycle (arginase 1, ornithine carbamoyltransferase, argininosuccinate synthase), fatty acid degradation (short/long-chain–specific acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-ketoacyl-CoA thiolase, acetyl-CoA acetyltransferase), and ATP synthesis (ATP synthase beta subunit) were identified as putative targets of furan (Moro et al., 2012). Based on these data, we speculated that protein adduct formation by furan may interfere with metabolic pathways involved in mitochondrial energy production, which would be consistent with data demonstrating that ATP-depletion and uncoupling of hepatic oxidative phosphorylation present early events in furan-induced cell death (Mugford et al., 1997). However, our analytical target proteomics approach provided no quantitative information on the extent of target protein modification, and therefore we were unable to predict if covalent protein binding by furan would be sufficient to cause changes in individual target protein function. Interestingly, however, integrated analysis of furan target proteins and liver gene expression profiles obtained from rats treated with furan at 40 mg/kg per day for up to 14 days, which were dominated by genes responsive to nutrients, revealed a significant overlap between target proteins and genes deregulated by furan (Huang et al., 2004; Moro et al., 2012). In particular, enzymes involved in lipid transport and metabolism were repressed at the transcriptional level, whereas key enzymes involved in glycolysis (and antioxidant defense) were induced. These data provide some evidence that covalent binding of furan to target proteins may indeed result in altered target protein function and perturbation of cellular metabolism.

The main objective of the present study was to determine the biological significance of covalent binding of furan to individual target proteins by studying the impact of a single hepatotoxic dose of furan on the activity of key target enzymes involved in glycolysis/gluconeogenesis (alpha enolase, GAPDH), β-oxidation (enoyl-CoA hydratase), ATP synthesis (ATP synthase beta subunit), and redox regulation (thioredoxin 1) in rat liver, and by linking these functional changes to alterations in cellular processes, i.e. metabolic pathways, ATP production, and anti-oxidative defense. In view of the fact that dietary exposure to furan is considered a potential risk to human health, we were also interested to understand if changes evident after an acutely hepatotoxic dose also occur after repeated low dose treatment closer to human exposure to furan via food.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany). The *cis*-2-buten-1,4-dial (BDA) precursor 2,5-diacetoxy-2,5-dihydrofuran (purity ≥99%, confirmed by ^{1}H-NMR analysis) was prepared as described elsewhere (Holzapfel and Williams 1995). A 50 mM stock solution of BDA was prepared by solving 4.65 mg 2,5-diacetoxy-2,5-dihydrofuran in 500 ml dest. H_2O. After 24 h at room temperature hydrolysis to two equivalents of acetic acid and one equivalent of *cis*/*trans*-2,5-dihydroxy-2,5-dihydrofuran was completed at >97% (confirmed by ^{1}H-NMR analysis). Purified human recombinant thioredoxin 1 (kTRX-01) was purchased from IMCO Corp Ltd AB, Sweden.

2.2. Animal experiments

We used archived liver samples collected from previous studies to determine if a single high dose of furan (30 mg/kg bw) or repeated low dose exposure to furan affects enzyme activity of known target proteins (Mally et al., 2010; Moro et al., 2012). Briefly, male Fischer F344/N rats (aged 5–7 weeks on arrival; Harlan-Winkelmann GmbH) were housed under standard laboratory conditions in groups of four rats per cage. After 10 days of acclimatization, animals (n = 4 per dose) were administered a single dose of furan dissolved in corn oil (4 ml/kg b.w.) at 0 and 30 mg/kg b.w. by oral gavage (Moro et al., 2012). After 24 h, rats were anesthetized with CO_2 and sacrificed by cardiac puncture. In another experiment, rats were treated with low doses of furan (0, 0.1, 0.5 mg/kg b.w. dissolved in corn oil, oral gavage) for up to 4 weeks (5 d/week) (Mally et al., 2010). The livers were removed, aliquoted, flash frozen in liquid nitrogen and stored at −80 °C for up to 5 years until further analysis. Blood samples were collected in centrifuge tubes (Sarstedt, Nümbrecht, Germany), allowed to clot at room temperature for 30 min in the dark, and used for determination of clinical chemistry parameters in serum.

2.3. mRNA expression by quantitative real-time PCR

Total RNA from frozen rat liver samples was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA (1 μg) was reverse transcribed using the Verso cDNA Kit (Thermo Fisher Scientific, Hamburg, Germany). Primers were purchased from Biomol (Hamburg, Germany) or Biomers (Ulm, Germany) and sequences of oligonucleotides are listed in Table 1. The reaction mixture (20 μl) consisted of 2× Mastermix with SYBR Green I (Thermo Fisher Scientific, Hamburg, Germany), 2 μl cDNA and 100 nM of each primer. The level of gene expression was measured by real time SYBR Green PCR on a Roche LightCycler480 (Roche, Mannheim, Germany) using the following cycling conditions: 95 °C for 10 min, 45 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. Specificity of PCR products was