Toxicology Letters 206 (2011) 166–171

Contents lists available at ScienceDirect

Toxicology Letters

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

Gene expression of biomarkers of nephrotoxicity in F344 rats co-exposed to melamine and cyanuric acid for seven days

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ARTICLE INFO

Article history:
Received 13 May 2011
Received in revised form 6 July 2011
Accepted 7 July 2011
Available online 18 July 2011

Keywords:
Melamine
Cyanuric acid
Kidney
Gene expression
Biomarkers

ABSTRACT

A number of studies have demonstrated that co-exposure to low levels of melamine and cyanuric acid elicits renal toxicity due to the formation of melamine cyanurate crystals in the kidney nephrons. In this work, we investigated if co-exposure of rats to these compounds leads to alterations in the expression of the genes encoding kidney injury molecule 1 (KIM-1), metallopeptidase inhibitor 1 (TIMP1), clusterin, osteopontin, and neutrophil gelatinase-associated lipocalin/lipocalin 2 (NGAL), which have been proposed as urinary biomarkers for nephrotoxicity. Six-week-old male and female F344 rats were fed *ad libitum* a diet fortified with 0 (control), 7, 23, 69, 229, or 694 ppm melamine and cyanuric acid (co-exposure groups), 1388 ppm melamine, or 1388 ppm cyanuric acid for seven days. Histopathology and clinical chemistry examination indicated marked toxicity only in the animals exposed to the two highest combined doses of melamine and cyanuric acid. Consistent with these observations, quantitative real-time polymerase chain reaction analysis of kidney tissue indicated increased expression of all genes analyzed relative to the control in both male and female rats fed daily with 229 or 694 ppm melamine and cyanuric acid. Exposure to lower levels of both compounds or to the individual compounds did not induce gene expression changes. These data indicate that quantifying the expression levels of the selected biomarker genes constitutes a useful endpoint to assess the combined toxicity of melamine and cyanuric acid in both male and female rats.

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1. Introduction

Nephrotoxicity has been traditionally assessed by histopathology and clinical chemistry. More recently, an effort has been made by the scientific community to identify biomarker proteins and genes, whose levels are specifically affected by nephrotoxicants (Davis and Kramer, 2006). This effort has been mainly driven by the evidence that urinary levels of proteins, such as KIM-1, osteopontin, NGAL, and clusterin, are often more sensitive and/or occur at earlier stages of toxicity than histopathological or clinical chemistry changes (Dieterle et al., 2010; Ozer et al., 2010). Given the non-invasive nature of these biomarkers, their potential use in the clinical environment has been considered by the U.S. Food and Drug Administration and the European Medicines Agency, which recently qualified several of these proteins as biomarkers for the detection of tubular or glomerular kidney injury in preclinical studies (Dieterle et al., 2010).

Several studies have also demonstrated renal expression changes in the genes encoding the proteins noted above in response to insult by a variety of nephrotoxicants (Ichimura et al., 2004; Kondo et al., 2009; Rached et al., 2008; Wang et al., 2008; Zhou et al., 2008). While the evaluation of the gene expression levels in kidney tissue is an invasive procedure, hampering, in principle, its translational potential, measuring changes in gene expression as a terminal endpoint presents great potential in animal studies. Compared to histopathology, gene expression assays offer several advantages, including the potential for being an earlier and/or more sensitive endpoint, quantitative over a wide dynamic range, suitable for high-throughput, and relatively inexpensive.

The triazines melamine and cyanuric acid, components of the industrial by-product "scrap melamine", were found in adulterated wheat and rice gluten used in pet food that led to the illness and death of a large number of cats and dogs in 2007. While, individually, these triazines present very low toxicities, reports in the literature indicate that, when animals are exposed to mixtures of melamine and cyanuric acid, the compounds are absorbed in the GI tract, distributed systemically, precipitate in the kidney, and form nephrotoxic melamine cyanurate crystals (Dobson et al.,

Abbreviations: BMD, benchmark dose; BMDL, benchmark dose (lower 95% confidence limit); BUN, blood urea nitrogen; Ct, cycle threshold; CYA, cyanuric acid; KIM-1, kidney injury molecule 1; MEL, melamine; NGAL, neutrophil gelatinase-associated lipocalin/lipocalin 2; NOAEL, no-observable-adverse-effect level; PCR, polymerase chain reaction; TIMP1, metallopeptidase inhibitor 1.

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0378-4274/$ – see front matter. Published by Elsevier Ireland Ltd.
doi:10.1016/j.toxlet.2011.07.009

2008; Puschner et al., 2007); hence, the mechanism of nephrotoxicity due to co-exposure to melamine and cyanuric acid seems to be very distinct from those of other well characterized nephrotoxicants (Balakumar et al., 2010; Pabla and Dong, 2008; Dirheimer and Creppy, 1991). A correlation of the renal expression levels of known candidate biomarker genes of nephrotoxicity with the kidney toxic response induced by melamine and cyanuric acid would, thus, reinforce the usefulness of genomic biomarkers of nephrotoxicity and expand their application.

In a previous study, we reported the effect of a seven-day combined exposure to melamine and cyanuric acid in F344 rats on body and kidney weights, kidney histopathology, and serum creatinine and blood urea nitrogen (BUN) levels (Jacob et al., 2011). We found that, while dietary exposure to 1388 ppm melamine or cyanuric acid failed to induce any significant changes on the endpoints analyzed, co-exposure to mixtures of 229 or 694 ppm melamine and cyanuric acid led to a decrease in body weight, enlarged and pale-yellow kidneys, multiple kidney tubular histopathological lesions, deposition of melamine cyanurate crystals in the renal tubules, and elevated serum creatinine and BUN levels compared to control animals. The no-observable-adverse-effect level (NOAEL) was established at 69 ppm melamine and cyanuric acid, equivalent to 8.6 mg/kg bw/day of each compound. In the present study, we expanded the list of endpoints to include the analysis of the expression levels of biomarker candidate genes of nephrotoxicity. We aimed to (1) determine if these biomarker genes could be used to assess nephrotoxicity induced by a combined exposure to melamine and cyanuric acid, and (2) determine if the changes in gene expression levels are more sensitive endpoints than those previously assessed.

2. Materials and methods

2.1. Experimental design

The kidney tissue used in the current study was obtained from the same rats used to analyze the endpoints reported in Jacob et al. (2011). Briefly, six-week-old F344 rats (6 males and 6 females per dose group) were fed *ad libitum* for seven days NIH-41 irradiated meal fortified with 0 (control), 7, 23, 69, 229, or 694 ppm melamine and cyanuric acid (co-exposure groups), 1388 ppm melamine only, or 1388 ppm cyanuric acid only. This dosing regimen resulted in the exposure of the animals to, respectively, *ca.* 0, 0.9, 2.8, 8.6, 17.6, or 29.8 mg melamine and cyanuric acid/kg bw/day, 123.7 mg melamine/kg bw/day, or 167.5 mg cyanuric acid/kg bw/day (Jacob et al., 2011). At necropsy, both kidneys were removed, weighed, sectioned longitudinally, and one half of each kidney was flash-frozen and stored at -80 °C until further processing. All procedures involving care and handling of animals were reviewed and approved by the National Center for Toxicological Research Institutional Animal Care and Use Committee.

2.2. Total RNA isolation and reverse-transcription

Total RNA was isolated from half of a kidney per animal. The frozen kidney tissues were macerated in liquid nitrogen using a mortar and pestle, and 10–20 mg samples of frozen tissue powder were used to isolate total RNA using an RNeasy Mini kit, with on-column DNase I digestion, following the manufacturer's protocol (Qiagen, Valencia, CA). RNA purity and concentration were assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) and the RNA integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All RNA samples had an absorption 260 nm/280 nm ratio greater than 2.0 and the RNA integrity number (RIN) was 9.19 ± 0.59 (mean ± standard deviation (SD); n = 96). One microgram of total RNA was reverse transcribed using random hexamer primers and an Advantage RT-for-PCR kit (Clontech, Palo Alto, CA), following the manufacturer's protocol. The cDNA was diluted 1:40 with nuclease-free water and stored at −20 °C.

2.3. Quantitative gene expression analysis

The gene expression levels of the biomarkers of nephrotoxiticy were quantified using quantitative real-time polymerase chain reaction (PCR). cDNAs were amplified in duplicate using TaqMan assays and a 7900HT real-time PCR detection system (Applied Biosystems, Foster City, CA), following the manufacturer's protocol. The TaqMan assays used were Rn00597703_m1 (*Kim-1/Havcr1*, NM_173149; encodes KIM-1), Rn01430875_g1 (*Timp1*, NM_053819; encodes TIMP1), Rn01449972_m1 (*Spp1*, NM_012881; encodes osteopontin), Rn00562081_m1 (*Clu*, NM_053021; encodes clusterin), Rn00590612_m1 (*Lcn2*, NM_130741; encodes NGAL). A FastStart master mix (Roche Diagnostics, Indianapolis, IN) was used, and the quantitative real-time PCR cycling conditions were set at 95 °C for 10 min for the first cycle and 15 s at 95 °C followed by 1 min at 60 °C for the remaining 40 cycles. The coefficient of variation between technical replicates was <1%. Data were normalized for the endogenous control *Gapdh* (NM_017008; assay part # 4352338E) and analyzed using the ΔΔCt method (Livak and Schmittgen, 2001). To express the relative gene expression levels as a %GAPDH, the cycle threshold (Ct) values of the gene of interest were subtracted from that of *Gapdh* (ΔCt) and the $2^{-\Delta Ct} \times 100$ value was calculated. *Gapdh* levels had been determined in preliminary absolute quantitation experiments to be unchanged with treatment (data not shown). Data are expressed as mean ± SD.

Table 1
Summary of the results reported in Jacob et al. (2011). Animals were exposed to feed supplemented with 0 (control), 7, 23, 69, 229, or 694 ppm melamine and cyanuric acid (co-exposure groups), 1388 ppm melamine, or 1388 ppm of cyanuric acid for seven days. All endpoints analyzed were affected by combined melamine and cyanuric acid at a dose level greater than 69 ppm (NOAEL).

Endpoint	Observations per dose group
Body weight	↔ 0, 7, 23, 69 ppm MEL+CYA ↓ 229, 694 ppm MEL+CYA ↔ 1388 ppm MEL ↔ 1388 ppm CYA
Kidney weight	↔ 0, 7, 23, 69 ppm MEL+CYA ↑ 229, 694 ppm MEL+CYA ↔ 1388 ppm MEL ↔ 1388 ppm CYA
Kidney crystals	−0, 7, 23, 69 ppm MEL + CYA + 229, 694 ppm MEL + CYA + 1388 ppm MEL[a] −1388 ppm CYA
Urinary bladder uroliths and crystals	−0, 7, 23, 69 ppm MEL + CYA + 229, 694 ppm MEL + CYA −1388 ppm MEL −1388 ppm CYA
Serum creatinine and BUN	↔ 0, 7, 23, 69 ppm MEL + CYA ↑ 229, 694 ppm MEL + CYA ↔ 1388 ppm MEL ↔ 1388 ppm CYA

Note. Abbreviations: ↔ No change; ↓ Decreased; ↑ Increased (relative to control). − Absent; +Present. MEL+CYA: co-exposure to melamine and cyanuric acid. MEL: exposure to melamine only. CYA: exposure to cyanuric acid only.

[a] A very small number of dispersed crystals was observed in 5 of the 12 rats, when the kidneys were examined by wet mount, but not by histopathology (Jacob et al., 2011).

2.4. Benchmark dose modeling

Benchmark doses (BMDs) and the lower 95% confidence limits (BMDLs) were calculated using Environmental Protection Agency Benchmark Dose Software (version 2.1.1; http://www.epa.gov/ncea/bmds). The calculations were conducted using Hill, linear, polynomial, and power models to fit the relative gene expression levels of *Kim-1/Havcr 1*, *Timp1*, *Clu*, *Lcn2*, and *Spp1* (mean ± SD; n = 6) and the mean doses of melamine and cyanuric acid from the entire seven-day study. The BMD was defined as the dose corresponding to a change in the mean response equal to one control standard deviation from the control mean.

2.5. Statistical analysis

Statistical significance between male and female control animals was assessed by t-tests. Statistical significance between dose groups within each sex was assessed by one-way ANOVA, followed by Dunnett's test to compare treated groups to the matching control (SigmaStat v3.11). In order to maintain an equal variance and normal data distribution, the data were natural log transformed before conducting the analyses. A p-value < 0.05 was considered statistically significant.

3. Results and discussion

Table 1 summarizes the results previously reported by our group in the same set of animals used for the current study (Jacob et al., 2011). All endpoints analyzed, which included body weight, kidney weight, histopathology, and blood chemistry, indicated that feeding six-week-old male and female F344 rats 229 or 694 ppm melamine and cyanuric acid for seven days resulted in alterations

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