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Molecular alterations underlying the enhanced disruption of spermatogenesis by 2,5-hexanedione and carbendazim co-exposure

Sarah N. Campion[a,1], Natasha Catlin[a], E. Andres Houseman[b], Janan Hensley[c], Yunxia Sui[b], Kevin W. Gaido[c], Zhijin Wu[b], Kim Boekelheide[a,*]

[a] Department of Pathology and Laboratory Medicine, 70 Ship St., Brown University, Providence, RI 02903, USA
[b] Department of Community Health, 121 South Main St., Brown University, Providence, RI 02912, USA
[c] The Hamner Institutes for Health Sciences, 6 Davis Drive, P.O. Box 12137, Research Triangle Park, NC 27709, USA

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ABSTRACT

The current study investigated the co-exposure effects of 2,5-hexanedione (HD) and carbendazim (CBZ) on gene expression underlying the enhanced pathology previously observed. Adult male rats were exposed to HD (0.33 or 1%) followed by CBZ (67 or 200 mg/kg), and testis samples were collected after 3 and 24 h. Microarray analysis at 3 h revealed that CBZ and HD interact in an agonistic, or synergistic, way at the gene level. Further analysis of candidate genes by qRT-PCR at both 3 and 24 h after co-exposure, revealed that Loxl1 and Clca2/Clca4l were both decreased in expression. Immunohistochemical analysis of Loxl1 at 24 h revealed that Loxl1 is localized to the seminiferous tubules, with the most intense staining in the basement membrane, blood vessels, and acrosomes, with the relative intensity reflecting the gene level changes at 3 h. These findings provide candidate genes for further investigation of the testicular response to damage.

1. Introduction

The cellular and molecular targets and dose level of each toxicant within a chemical mixture all play a role in determining the biological responses following exposure. There is an emerging need for improved methods for risk assessment and a better understanding of toxicological consequences of mixed exposures, given that most real world exposures involve more than one chemical. Recent work has begun to elucidate how critical the dose and cellular and subcellular targets are during co-exposure to testicular toxicants. This work utilized an established co-exposure paradigm, which involves an 18 day priming exposure of adult male rats to the Sertoli cell toxicant 2,5-hexanedione (HD) followed by acute exposure to either a direct-acting germ cell toxicant or a second Sertoli cell toxicant. Utilizing this exposure paradigm to study co-exposure responses, it was determined that co-exposure to the Sertoli cell toxicant (HD) and the germ cell toxicant X-radiation (X-ray) results in an attenuation of germ cell toxicity when compared with X-ray exposure alone [1]. In this co-exposure scenario we hypothesize that HD induces an adaptive response of the seminiferous epithelium, which helps render the germ cells resistant to X-ray-induced damage. In contrast to this co-exposure response, co-exposure to two Sertoli cell toxicants, HD and carbendazim (CBZ), results in synergistic effects on testicular injury, much greater than the single toxicant exposures, manifested as enhanced seminiferous tubule diameter, vacuolization, sloughing, and germ cell apoptosis [2].

While HD and CBZ share the same target, Sertoli cell microtubules, these two toxicants have opposing effects on microtubules. HD, a metabolite of the commonly used solvents *n*-hexane and methyl *n*-butyl ketone (2-hexanone), causes Sertoli cell dysfunction by promoting rapid assembly and enhanced stability of microtubules [3]. Consequences of HD exposure include spermatid head retention, Sertoli cell vacuolization, and decreased seminiferous tubule fluid, ultimately resulting in germ cell loss and sloughing [3,4]. HD-induced testicular toxicity requires at least 2 weeks of exposure before the manifestation of pathology [3], which differs from the more rapid onset of pathology following CBZ exposure.

Abbreviations: HD, 2,5-hexanedione; X-ray, X-radiation; CBZ, carbendazim; ms, milliseconds; h, hours; FDR, false discovery rate; ANOVA, one-way analysis of variance.

* Corresponding author at: Brown University, Box G-E5, Providence, RI 02901, USA. Tel.: +1 401 863 1783; fax: +1 401 863 9008.
E-mail address: kim_boekelheide@brown.edu (K. Boekelheide).
[1] Present address: Pfizer Worldwide Research and Development, Eastern Point Road, MS 8274 1260, Groton, CT 06340, USA.

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A single high dose of CBZ results in testicular alterations within a few hours, including increased testis weights, increased seminiferous tubule diameter, and sloughing of the seminiferous epithelium [2,4]. CBZ is the toxic metabolite of the benzimidazole fungicide benomyl, which elicits testicular toxicity by inhibiting, rather than promoting microtubule polymerization [5,6].

Mechanistic investigations into the molecular changes underlying the phenotypic consequences of HD and X-ray co-exposure led to the establishment of a methodology for the analysis and interpretation of microarray data from animals co-exposed to multiple dose levels of each toxicant [7]. This same approach was applied in the current study to investigate the dose and co-exposure effects on gene expression following combined HD and CBZ exposure and to determine the molecular mechanisms underlying the pathologic changes. It was hypothesized that HD and CBZ co-exposure would result in synergistic or agonistic effects at the molecular level, reflective of the agonistic phenotypic effects of this co-exposure. The log2-expression values obtained by microarray analysis performed with testis tissue of co-exposed adult male rats were analyzed using LIMMA and summarized across all treatment groups to determine the effect of HD in excess of CBZ. These summarized linear contrasts were examined to identify individual genes and biological pathways where HD modification of CBZ-induced gene alterations was the greatest. Several genes of interest were further analyzed by qRT-PCR and immunohistochemistry to begin to better understand and mechanistically explain the synergistic toxicity elicited by HD and CBZ co-exposure. This is an important area of investigation, which has provided valuable information regarding the molecular profiles of toxicants with synergistic phenotypic effects as compared to the molecular profiles of toxicants with antagonistic phenotypic effects. These are significant pieces of information in the field of mixtures research.

2. Materials and methods

2.1. Animals

Adult male Fischer 344 rats weighing 200–250 g were purchased from Charles River Laboratories (Wilmington, MA). Upon arrival, rats were acclimated for 1 week prior to use and maintained in a temperature and humidity controlled environment with a 12 h alternating dark-light cycle. All rats were housed in community cages with free access to water and Purina Rodent Chow 5001 (Farmer's Exchange, Framingham, MA). The Brown University Institutional Animal Care and Use Committee approved all experimental animal protocols in compliance with National Institute of Health guidelines.

2.2. Toxicant exposure

HD was administered in drinking water ad libitum for 18 days at concentrations of 0.33% and 1% using a previously established treatment protocol [2]. On day 17, animals ($n = 4$ for each treatment group) were administered CBZ by gavage, at a dose of 67 mg/kg or 200 mg/kg in corn oil at a dose volume of 2 ml/kg. At either 3 h or 24 h after treatment with CBZ, following continued HD drinking water exposure, rats were euthanized by CO_2 asphyxiation and half of the right testis was homogenized in Tri Reagent (Sigma–Aldrich, St. Louis, MO), snap frozen in liquid nitrogen, and stored at −80 °C. The remaining testis tissue was fixed in neutral buffered formalin for histological examination.

2.3. RNA isolation and microarray hybridization

Using tissues collected at 3 h after treatment with CBZ, RNA was isolated from testes homogenized in Tri Reagent using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Complementary (cDNA) was synthesized from 2.5 μg total RNA and purified using the Affymetrix One-Cycle Target Labeling and control reagents kit (Affymetrix, Santa Clara, CA) according to manufacturer's protocol. Equal amounts of purified cDNA per sample were used as the template for subsequent in vitro transcription reactions for complementary RNA (cRNA) amplification and biotin labeling using the Affymetrix GeneChip IVT labeling kit (Affymetrix) included in the One-Cycle Target Labeling kit (Affymetrix). cRNA was purified and fragmented according to the protocol provided with the GeneChip Sample Cleanup module (Affymetrix). All GeneChip arrays (Rat Genome 230 2.0 arrays) were hybridized, washed, stained, and scanned using the Complete GeneChip Instrument System according to the Affymetrix Technical Manual.

2.4. Microarray data analysis

Affymetrix CEL files were pre-processed by GCRMA background correction [8], quantile normalization and Robust Microarray summarization, resulting in a single log2-transformed expression measure for each of 31,099 genes. The expression measures were analyzed as previously described [7] to facilitate the detection of nonlinear effects of exposure and interactions of co-exposure on mRNA expression. This method of analysis resulted in the generation of a summary statistic with the interpretation of an estimated aggregate HD effect in excess of CBZ, e.g. up-regulation (positive) or down-regulation (negative) by HD. The overall linear trend in CBZ was also summarized by fitting the equivalent saturated model reparameterized using polynomials in exposure dose (i.e. linear and quadratic terms for each exposure together with their interactions), and extracting the linear CBZ term. To control for multiple comparisons, q-values representing false discovery rates (FDR) were computed from the collection of all 31,099 p values using the *qvalue* package in R [9].

2.5. Quantitative real time-polymerase chain reaction (qRT-PCR) analysis

Using tissues collected at both 3 h and 24 h after treatment with CBZ, Stat-60 reagent (Tel-Test, Friendswood, TX) was used to extract total RNA from whole testis tissue according to the manufacturer's protocol. RNA concentrations were determined using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA quality was determined using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. cDNA was synthesized from total RNA isolated from each sample using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. For the detection of MACRO domain containing 1 (Macrod1), dipeptidylpeptidase 7 (Dpp7), SH3 and multiple ankyrin repeat domains 3 (Shank3), chloride channel calcium activated 2/chloride channel calcium activated 4-like (Clca2/Clca4l), lysyl oxidase-like 1 (Loxl1), and tubulin beta 3 (Tubb3) the cDNA templates were amplified using QuantiTect® Primer Assays (Qiagen, Valencia, CA). These primers are pre-optimized and bioinformatically validated. Each sample was run in triplicate in 25 μl reactions. Relative mRNA levels of each target gene were normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Log_2-transformed relative expression ratios were calculated using the ddCt method.

2.6. Immunofluorescent staining and densitometric analysis

Rat testes (control, 1% HD, 200 mg/kg CBZ, and 1% HD + 200 mg/kg CBZ; $n = 6$) were collected 24 h after treatment and cryopreserved in Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, CA). Sections (8 μm) were fixed in acetone for 10 min, air dried for 5 min and then washed in phosphate buffered saline (PBS). Endogenous activity was blocked with 6% goat serum for one hour and then incubated overnight at 4 °C with rabbit anti-Loxl1 primary antibody (0.5 μg/ml) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Alexa Fluor 568 goat anti-rabbit (2.5 μg/ml) (Invitrogen, Carlsbad, CA) was applied to detect the anti-Loxl1 antibody. Sections were counterstained and coverslipped with Vectashield® Hard Set mounting medium with DAPI (Vector Laboratories Inc., CA, USA). Images of Stage IV seminiferous tubules were captured on an Axio Imager.M1 microscope, with an AxioCam MRm camera and Axio Vision 4.8 Software, (Carl Zeiss, Inc, Germany) at 40× magnification and 60 millisecond (ms) exposure for densitometric quantification of the fluorescent staining. The 60 ms exposure time was determined following an exposure time course study that was performed to determine the linear range capabilities of the gray scale.

Images of the anti-Loxl1 staining in Stage IV seminiferous tubules were blinded and uploaded into Image J (NIH, Bethesda, MD) as black and white JPEGs for densitometric quantification of the fluorescent staining. Using the Image J software, a set of intersecting lines was drawn over each seminiferous tubule to separate each cross-section into quadrants. Within each quadrant, two circles (with standard areas) were drawn over the basement membrane and over the closest acrosome to the basement membrane. The mean gray value of the area of each circle was measured and recorded. The mean gray values for both the basement membrane and the acrosome from the four quadrants were averaged together. The averaged value for the acrosome was then divided by the averaged value for the basement membrane to create a ratio of mean gray value, which acts as a control to compensate for staining differences between sections. The ratios of mean gray value for each stage IV seminiferous tubule were then averaged, resulting in one ratio of mean gray value per animal.

2.7. Statistical analysis

qRT-PCR data were analyzed using a one-way analysis of variance (ANOVA) with Bonferroni post hoc analysis. The analyses were performed separately for each gene, comparing the expression data among all treatment groups (control, HD, CBZ, HD + CBZ) for each individual gene. Immunofluorescent quantification data was also analyzed by one-way ANOVA with Bonferroni post hoc analysis. p values <0.05 were considered significant.