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Transcriptomics analysis and hormonal changes of male and female neonatal rats treated chronically with a low dose of acrylamide in their drinking water

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ABSTRACT

Acrylamide is known to produce follicular cell tumors of the thyroid in rats. RccHan Wistar rats were exposed *in utero* to a carcinogenic dose of acrylamide (3 mg/Kg bw/day) from gestation day 6 to delivery and then through their drinking water to postnatal day 35. In order to identify potential mechanisms of carcinogenesis in the thyroid glands, we used a transcriptomics approach. Thyroid glands were collected from male pups at 10 PM and female pups at 10 AM or 10 PM in order to establish whether active exposure to acrylamide influenced gene expression patterns or pathways that could be related to carcinogenesis. While all animals exposed to acrylamide showed changes in expected target pathways related to carcinogenesis such as DNA repair, DNA replication, chromosome segregation, among others; animals that were sacrificed while actively drinking acrylamide-laced water during their active period at night showed increased changes in pathways related to oxidative stress, detoxification pathways, metabolism, and activation of checkpoint pathways, among others. In addition, thyroid hormones, triiodothyronine (T3) and thyroxine (T4), were increased in acrylamide-treated rats sampled at night, but not in quiescent animals when compared to controls. The data clearly indicate that time of day for sample collection is critical to identifying molecular pathways that are altered by the exposures. These results suggest that carcinogenesis in the thyroids of acrylamide treated rats may ensue from several different mechanisms such as hormonal changes and oxidative stress and not only from direct genotoxicity, as has been assumed to date.

Abbreviations: ADA, adenosine Deaminase; ADRB2, adrenergic; ASF1B, anti-Silencing Function 1B Histone Chaperone; BRIP1, BRCA1 Interacting Protein C-Terminal Helicase 1; BUB1B, BUB1 Mitotic Checkpoint Serine/Threonine Kinase B; C1QTNF3, C1q and Tumor Necrosis Factor Related Protein 3; C5, complement Component 5; CALCR, calcitonin receptor; CARD9, caspase recruitment domain family; CCNA2, cyclin A2; CCNG1, cyclin G1; CD45, protein tyrosine phosphatase; CD46, CD46 molecule; CDC45, cell division cycle 45; CDCA2, cell division cycle associated 2; CDCA5, cell division cycle associated 5; CENPT, centromere protein T; CFB, complement factor B; CGA, glycoprotein hormones; CTLA4, cytotoxic T-lymphocyte-associated protein 4; DAD1, defender against cell death 1; DCTPP1, DCTP pyrophosphatase 1; DNMT3A, DNA (cytosine-5-)-methyltransferase 3 alpha; DUOX2, dual oxidase 2; GCG, glucagon; GCLC, glutamate-cysteine ligase; GOLGA3, golgin A3; GSTM1, glutathione S-transferase Mu 1; GSTP1, glutathione S-transferase Pi 1; HPSE, heparanase; HSPA5, heat shock 70 kDa protein 5; HSPB1, heat shock 27 KDa protein; HSPB2, heat shock 27 kDa protein 2; HSPH1, heat shock 105 kDa/110 kDa protein 1; HTATIP2, HIV-1 tat interactive protein 2; ID1, inhibitor of DNA binding 1; IGF2, Insulin-like growth factor 2 (somatomedin A); IL1B, interleukin 1; INHBA, inhibin; IYD, iodotyrosine deiodinase; KIF20B, kinesin family member 20B; KIF22, kinesin family Member 22; KLK1, kallikrein 1; LAMA2, laminin, alpha 2; MCM8, minichromosome maintenance complex component 8; MIF, macrophage migration inhibitory factor; MIS18A, MIS18 kinetochore protein A; NDC80, NDC80 kinetochore complex component; NPPC, natriuretic peptide precursor C; NPY, neuropeptide; NUBP1, nucleotide binding protein 1; ORC1, origin recognition complex; PDE3A, phosphodiesterase 3A; PINK1, PTEN induced putative kinase 1; PLCD1, phospholipase C; PLK1, polo-like kinase 1; POMC, proopiomelanocortin; PRL, prolactin; PTGIS, prostaglandin I2 (prostacyclin) synthase; PTGS1, prostaglandin-endoperoxide synthase 1; PRKAA2, protein kinase; PRODH, proline dehydrogenase; RAB5A, RAB5A; RAN, ras-related nuclear protein; RRM2, ribonucleotide reductase M2; SCL5A5, solute carrier family 5 (sodium iodide symporter); SELP, selectin P (granule membrane protein 140 kDa; SPAG8, sperm associated antigen 8; TACC3, transforming; TBCB, tubulin folding cofactor B; TFRC, transferrin receptor; TOP2A, topoisomerase (DNA) II alpha; TPO, thyroid peroxidase; TSHR, thyroid stimulating hormone receptor; TSN, translin; VWF, Von Willebrand Factor.

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1. Introduction

Acrylamide (AA) is a monomer used in the manufacture of polymers for mining, oil and natural gas processing, paper manufacture, waste processing, hospital laboratories, among other uses. Adverse health effects from worker exposure to AA have been extensively studied [44]. No adverse effects have been reported with daily human exposure up to 2.1 mg/kg/day [19]. Exposure to AA in foodstuffs has become a worldwide concern because of its generation in a variety of carbohydrate rich foods when these are cooked at temperatures exceeding 120 °C. At these temperatures, AA is made from the Maillard reaction of sugars with asparagine residues [24,55].

The World Health Organization (WHO) and Food and Agriculture Organization [22], the Environmental Protection Agency [18], and the European Food Safety Authority [16] have classified AA as a "probable human carcinogen" by virtue of its conversion to glycidamide, as the ultimate carcinogen in rodents. *In vivo*, AA is metabolized by CYP 2E1 to glycidamide (an epoxide), which also is very reactive toward nucleophiles and has been implicated in adduct formation at active site cysteines of proteins and the amino terminus of hemoglobin and also with nucleic acids [24,47]. Only a portion of the AA gets converted to glycidamide. The classification as a probable human carcinogen is based primarily upon reproducible carcinogenicity studies in Fischer rats [2,26,34]. Tumors were observed in the mammary gland (fibroadenomas), thyroid (follicular tumors) and tunica vaginalis testes in rats [2,26,33]. These tumor sites have well documented rat-specific modes of action, which are not relevant to humans [3,31,56,59].

Thyroid follicular tumors can arise in rats specifically from chemical disturbance of thyroid-pituitary homeostasis, or from a combination of genotoxicity and hormonal alterations. External factors that change thyroid hormone metabolism result in a marked change in thyroid behavior in rats [36]. AA treatment for 7, 14 or 28 days results in increased mitotic figures and Dfigures and DNA synthesis in the thyroids of Fischer rats [43]. The Fischer rat has a unique hormonal milieu that may be responsible for these tumors. However, specific changes in thyroid transcriptomics have yet to be documented. It has been suggested that alteration of the dopamine receptor is responsible for the change in thyroid metabolism [65]. AA also causes oxidative stress in rats [72] and the thyroid has a very high oxidation level [72,73].

AA is mutagenic in rats and mice [12]. This mutagenic profile does not fit the pattern of tumors observed in Fischer rats, as the specificity cannot be explained [27,52,65,74]. AA is a very weak carcinogen and mutagen [74]. Since mutagenicity is the regulatory default assumption as a mechanism for carcinogenicity, use of other mechanistic data for regulatory purposes has been limited. In contrast to the Fischer rat, the only significant tumors observed in male Wistar rats were in the thyroid gland [51]. While thyroid tumors in rats are not considered to be relevant to humans, mechanisms for genotoxicity in the thyroid still represent a possible significant risk. This study was designed to build upon the large volume of data published to date about AA exposure of rats to determine whether tumors formed in the thyroid after chronic exposure to AA were produced only through a genotoxic component.

The research conducted here determines the effect of AA on gene expression in weanling Wistar rat thyroids following in utero exposure to a carcinogenic dose of AA. This research is intended to differentiate between DNA reactivity, oxidative stress and direct action on hormone receptors. A transcriptomics approach (microarrays and bioinformatics) was used to investigate changes in gene expression and the association with physiological responses (changes in plasma hormone levels) in rats treated with AA from gestational day 6 to post-natal day 35. We hypothesized that 3 mg AA/Kg bw/day exposure would produce changes in plasma hormone levels associated with key genes and biochemical pathways involved with molecular actions of AA.

2. Materials and methods

2.1. Test material

AA (C3H3NO, CAS no 79-06-1, 1,2-propenamide; >99.9% pure; Sigma Aldrich) was dissolved in tap water and evaluated for stability at room temperature at 6, 13, 20, and 27 test days after preparation. Recovery ranged from 96.9% to 102.6%.

2.2. Animal exposures

The methods used to conduct this study have been previously published [51]. The *in vivo* phase of this study was conducted under GLP guidelines and was externally audited. It was approved by the responsible local government office according to the German animal welfare law "Tierschutzgesetz" (TierSchG). AA solutions were prepared weekly and concentrations were adjusted for body weight. Water bottles were changed weekly. AA concentration in the drinking water was determined at test week 4 and 10.

Sperm positive female Wistar Han™/RccHan™:WIST rats were obtained from Harlan Laboratories GmbH, Serumweg 48, 27324 Eystrup, Germany in multiple deliveries. At gestation day 6, dams were provided AA in their drinking water. Exposures continued in F1 offspring through postnatal day (pnd) 35 ± 3. Rats were housed 1 per cage in MACROLON cages with granulated wood bedding (Brandenburg, 49424 Goldenstedt/Arkeburg). Animal rooms were alternately lit (about 150 lx at approximately 1.50 m room height) and darkened in a 12-hour lighting cycle. Cage side observations were conducted twice per day during the week and once per day on weekends.

On day 4 after birth, the weights of the pups were determined. The size of each litter was adjusted by eliminating extra pups to yield, as nearly as possible, five males and five females per litter and remaining animals remained with the dams until day 21 of lactation (weaning). On lactation day 21, the F_1 animals were randomized using a computer randomization program to assign the animals to the subsets within each group.

A separate cohort of the animals was allowed to continue on the same regimen for two years. At the end of the two years, mammary gland fibroadenomas were identified in females and thyroid follicular cell tumors were identified in both sexes [51].

2.3. Plasma TSH, T3 and T4 analysis

On pnd 35 ± 3, at approximately 10 AM and 10 PM, respectively, as much blood as possible was withdrawn from 5 male and 5 female F1 rats/dose. The thyroids were removed and frozen. Blood samples were divided into 5 aliquots. Four aliquots of at least 75 μL were frozen and stored at −20 °C. Two aliquots were analyzed using the rat pituitary panel from Millipore for adrenocorticotropic hormone (ACTH), brain-derived neurotrophic factor (BDNF), growth hormone (GH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin. Then two aliquots were analyzed using the rat thyroid milliplex kit from Millipore for T3, T4 and TSH.

2.4. Extraction of total RNA and RNA quality control

Total RNA was extracted from the thyroid gland of rats (n = 4; 4 controls and 4 AA-treated per tissue) using RNA STAT-60 reagent (Tel-Test Inc., Friendswood, TX, USA) according to the manufacturer's instructions. A total of 500 ng of RNA was DNase-treated with Turbo DNA-free (Ambion Austin, TX) following the manufacturer's protocol. RNA quantity for microarray analysis was