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Transcriptional profiling of male F344 rats suggests the involvement of calcium signaling in the mode of action of acrylamide-induced thyroid cancer

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ABSTRACT

Acrylamide (AA) exposure in 2-year cancer bioassays leads to thyroid, but not liver, adenomas and adenocarcinomas in rats. Hypothesized modes of action (MOAs) include genotoxicity/mutagenicity, or thyroid hormone dysregulation. To examine the plausibility of these two or any alternative MOAs, RNA-sequencing was performed on the thyroids and livers of AA-exposed rats, in parallel with measurement of genotoxicity (blood micronucleus and Pig-a mutant frequency) and serum thyroid hormone levels, following the exposure of male Fischer 344/DuCrl rats to 0.0, 0.5, 1.5, 3.0, 6.0, or 12.0 mg AA/kg bw-day in drinking water for 5, 15, or 31 days. Differentially expressed genes in both tissues provided marginal support for hormonal and genotoxic MOAs, which was consistent with negative/equivocal genotoxicity assay and marginal changes in thyroid hormone levels. Instead, there was a pronounced effect on calcium signaling/cytoskeletal genes in the thyroid. Benchmark dose modeling of RNA-sequencing data for the calcium signaling pathway suggests a point of departure (POD) of 0.68 mg/kg bw-day, which is consistent with a POD of 0.82 mg/kg bw-day derived from the thyroid 2-year cancer bioassay data. Overall, this study suggests a novel MOA for AA-induced thyroid carcinogenicity in male rats centered around perturbation of calcium signaling.

1. Introduction

Acrylamide (AA) is an important industrial chemical, but also a probable human carcinogen (International Agency for Research on Cancer (IARC), 1994). Renewed interest in the toxicology of AA was sparked by the demonstration that AA is found at low levels in a variety of common foods and thus poses a hazard for widespread human exposure; elevated AA levels in food are related to carbohydrate content and cooking temperature (Tareke et al., 2002). Currently, the mode of action (MOA) of AA-induced carcinogenicity in rats is not firmly established, and the debate on the potential carcinogenic effects of dietary AA in humans remains open (Pelucchi et al., 2014).

To date, four 2-year cancer bioassays on AA have been conducted. These used F344 rats (Johnson et al., 1986; Friedman et al., 1995), B6C3F1 mice and F344/N rats (Beland et al., 2013), and Wistar Han rats (Maronpot et al., 2015). These studies reported thyroid follicular neoplasms in both male and female rats, in two different strains of rats. It has been suggested that thyroid follicular cell neoplasms arise either through a genotoxic MOA induced by glycidamide (GA, a reactive metabolite of AA (Klaunig, 2008)), or through thyroid hormone (TH) dysregulation, both of which are proposed to alter thyroid follicular cell growth (Fig. 1; reviewed in (Dourson et al., 2008)). The proposed TH-associated MOA is consistent with the MOA for phenobarbital-induced rat thyroid cancer (reviewed in (Meek et al., 2003)). In addition, a "mixed" MOA has been proposed (i.e., "an increased mutagenic burden in hormonally-sensitive tissues with or without disruption of the hormonal pathways" (US Environmental Protection Agency (EPA), 2010)). Therefore, there is a need for additional time-series and dose-response studies to evaluate the weight-of-evidence supporting these MOAs for AA-induced thyroid carcinogenicity in rats.

Several studies suggest that AA itself is a weak carcinogen that

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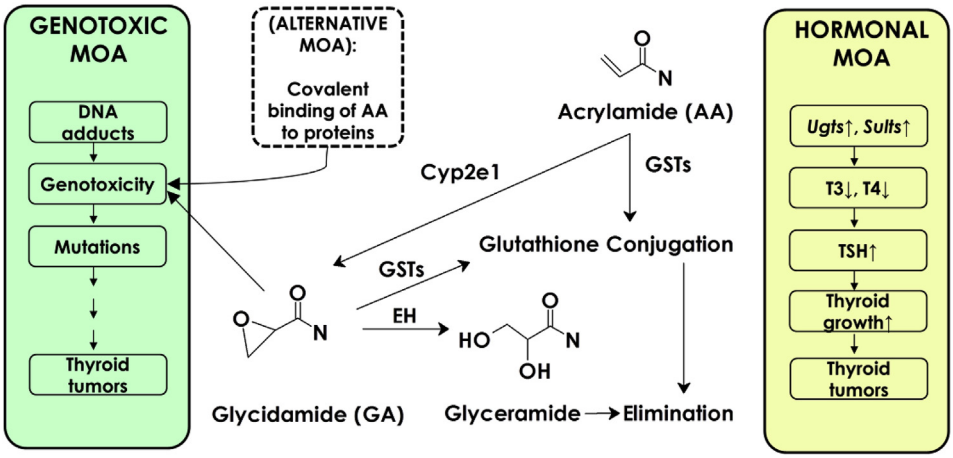

Fig. 1. The proposed genotoxic and hormonal modes of action (MOAs) of AA-induced thyroid tumors in male rats. The genotoxic MOA involves AA metabolism to its genotoxic metabolite GA by cytochrome P450 family 2, subfamily e, polypeptide 1 enzyme (Cyp2e1). GA forms adducts with DNA, leading to genotoxicity and mutations, and thyroid tumors. Alternatively, indirect-acting genotoxicity can stem from AA binding covalently to form adducts with cytoskeletal proteins, including those comprising microtubules and microfilaments (e.g., (Sickles et al., 2007)); this mechanism has not been elucidated in detail (indicated by a dashed arrow). GA can be further metabolized to glyceramide by epoxide hydrolase (EH) or to glutathione (GSH) conjugates by phase II metabolism enzymes GSH transferases (GSTs). In the hormonal MOA, increased hepatic metabolism by phase II enzymes in response to AA exposure (aimed at AA metabolism and removal) also leads to clearance of T3 and T4. Depletion of T3 and T4 elicits compensatory increases in thyroid-stimulating hormone (TSH) release from the pituitary gland that stimulates the thyroid gland to produce more T4. This chronic growth stimulation may lead to thyroid gland enlargement, accompanied by increased cell proliferation and hyperplasia, as seen in rats in response to phenobarbital (reviewed in (Meek et al., 2014a)). The formation of DNA adducts with AA or GA can lead to mutagenesis. Alternatively, AA may produce genotoxicity indirectly, through forming protein adducts or interacting with proteins.

needs to be metabolized by cytochrome P450 family 2, subfamily e, polypeptide 1 enzyme (Cyp2e1) to GA in order to have genotoxic and carcinogenic effects (Ghanayem et al., 2005a, 2005b; Martins et al., 2007; Maronpot et al., 2015). Both GA and AA are electrophiles that react with DNA to form adducts (Fennell et al., 2015); however, GA does so at a faster rate than AA *in vitro* (Solomon et al., 1985), and produces a linear, dose-dependent response in genetic toxicity studies (Mei et al., 2008). In contrast to GA, the genotoxicity of AA is only apparent at high concentrations and shows a sublinear response (Zeiger et al., 2009; Mei et al., 2008; Dobrovolsky et al., 2016). Overall, comparison of GA and AA-mediated genotoxicity profiles suggests that these two compounds are both clastogenic, but cause DNA breakage by different mechanisms. These mechanisms may involve direct GA-DNA adduct formation versus indirect DNA damage through oxidative stress (e.g., due to the production of reactive oxygen species (ROS) that takes place during Cyp2e1 metabolism (reviewed in (Linhart et al., 2014)) or other mechanisms producing sublinear dose-responses, such as covalent cross-linking of chromosomes.

The proposed TH-associated MOA (or "hormonal" MOA) involves increased metabolism of THs, as happens in the case of phenobarbital (reviewed in (Meek et al., 2003)). Decreased circulating levels of T3 and T4 release the hypothalamus-pituitary-thyroid axis from negative-feedback inhibition, leading to increased secretion of thyroid-stimulating hormone (TSH) by the pituitary gland. Chronic stimulation of the thyroid gland by TSH increases cell proliferation, leading to tumor formation (reviewed in (Meek et al., 2003)). Several studies have provided evidence to support this hormonal MOA for AA thyroid carcinogenicity in rats. For example, a significant decrease in serum T4 was shown in male F344 rats following 14-days of exposure to 50, but not 10 or 2.5 mg AA/kg bw-day in drinking water (Bowyer et al., 2008). In contrast, no changes were measured in serum T4 or TSH levels in male F344 rats exposed to 2.0 or 15 mg AA/kg bw-day for two or seven days (Khan et al., 1999). Overall, the data are conflicting but some evidence suggests that the hormonal MOA is plausible, especially at high AA doses (Dourson et al., 2008).

Another MOA for AA-induced rat thyroid cancer that has been proposed (Dourson et al., 2008) involves the interaction of AA with cytoskeletal proteins leading to compromised integrity of microfilaments and microtubules (Lapadula et al., 1989; Sickles et al., 1995, 1996, 2007). Compromised cytoskeletal proteins may interfere with the development of thyroid follicles that normally undergo large changes in volume over the first 120 days of life in rats, and such structural impairment may also lead to compromised hormonal release (Dourson et al., 2008). Interestingly, such interactions of AA with structural proteins have also been hypothesized to play a role in the indirect genotoxicity of AA, through cross-linking of chromosomes or chromosome-associated proteins, as noted above.

Given the controversy that still surrounds the MOA of AA in thyroid cancer, well-designed mechanistic studies to assess each proposed MOA are necessary. Many studies have shown the power of transcriptional profiling in target tissues following sub-chronic chemical exposure (i.e., toxicogenomics) to provide mechanistic support for MOAs and estimate the doses at which adverse effects begin to occur and will be manifested in longer-term studies (e.g., (Thomas et al., 2011, 2012, 2013; Moffat et al., 2015; Chepelev et al., 2015; Webster et al., 2015; Chepelev et al., 2016)). An additional advantage of a whole-genome toxicogenomics approach is the ability to reveal potentially alternative hypotheses (i.e., hypothesis generating experiments) in parallel with hypothesis-directed inquiry.

One previous study explored the use of toxicogenomics in discerning the AA-induced thyroid cancer MOA following sub-chronic exposure of male F344 rats to AA in drinking water for 14 days (Bowyer et al., 2008) The study used a single time point, and spanned a broad dose range, but only had three exposure groups. The work applied an older version of microarrays to measure gene expression in the hypothalamus and pituitary, but not in the thyroid. The authors concluded that AA was thus more likely to operate through a genotoxic rather than a hormonal MOA based on the lack of effect of AA on the expression of certain key genes related to TH transport, metabolism and function, and marginal changes in the serum TH levels (i.e., no change in serum T3, rT3, or TSH, but decreased T4 levels at the high dose only (Bowyer et al., 2008)). With more advanced tools available today, a detailed analysis using RNA deep sequencing of both target and non-target tissue response