Ethanol enhanced the genotoxicity of acrylamide in human, metabolically competent HepG2 cells by CYP2E1 induction and glutathione depletion

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Abstract

In the present study, the genotoxicity of acrylamide (AA) was investigated in HepG2 cells using SCGE. Additionally, the influence of ethanol on the modulation of AA-induced DNA migration caused by CYP2E1-upregulation and/or GSH-depletion was examined in the same cell line. For the ethanol/AA combination assays, the cells were treated with ethanol for 24 h prior to exposure to 5 mM AA for another 24 h. 1.25 to 10 mM AA-induced DNA migration (OTM) in HepG2 cells in a concentration-dependent manner, e.g., exposure to 10 mM AA, resulted in an 8-fold increase of DNA migration compared to the negative control. Treatment with 120 mM ethanol prior to exposure to 5 mM AA increased the level of DNA migration more than 2-fold as compared to cells treated with 5 mM AA alone. Immunoblotting showed a clear ethanol-induced increase of CYP2E1, which plays a pivotal role in AA toxification. Additionally, intracellular GSH levels were significantly reduced after ethanol or AA treatment. In the ethanol/AA combination experiments, GSH depletion was comparable to the additive effect of the single compounds. No induction of apoptosis (ssDNA assay), but necrosis was identified as responsible for the reduction of viability with increasing compound concentration. The data clearly show a higher genotoxic potential of ethanol/AA combination treatment compared to AA treatment alone. In conclusion, both the ethanol-mediated induction of CYP2E1 and the depletion of GSH provide a mechanistic explanation for the over-additive effects of ethanol and AA. Even though the concentrations used in this study were rather high, consequences for the dietary intake of AA-containing food and alcoholic beverages should be discussed.

Keywords: Acrylamide; CYP2E1; Comet assay; SCGE; ssDNA apoptosis assay; Glutathione depletion; HepG2

Introduction

Acrylamide (AA) is an important industrial high volume chemical used in wastewater treatment, adhesives and grout, cosmetics and in laboratories. Therefore, human exposure to monomeric AA, the toxic form of the compound, either during production or use is...
possible. However, carbohydrate-rich foods heated above 175 °C present the main exposure source for the general public. Relevant amounts of AA have been identified in several processed food items such as French fries, baked potatoes etc. including coffee (Zyzak et al., 2003; Andrzejewski et al., 2004). Also epidemiological studies have been carried out to examine the risk of cancer as a result of consumption of food with a high concentration of AA (Mucci et al., 2003, 2004, 2005, 2006; Pelucchi et al., 2006). However, these studies failed to give proof to a clear connection between AA consumption and an increased cancer risk in humans so far.

In contrast, accumulating evidence indicates that AA is genotoxic in vitro and in vivo in both somatic cells and germ cells. AA induced chromosomal aberrations, micronuclei formation, sister chromatid exchanges, unscheduled DNA synthesis, single-strand DNA breaks, polyploidy, aneuploidy and other mitotic disturbances, and dominant lethal and specific locus mutations (WHO, 2002; Dearfield et al., 1995). In bacterial mutation assays, however, AA consistently exhibited negative results with and without metabolic activation whereas glycldiamide (GA), the epoxide metabolite of AA, induced a positive response in Salmonella TA100 and TA1535 (Hashimoto and Tanii, 1985). Chronic administration of AA was identified as a multisite carcinogen in mice and rats, but underlying mechanisms of action are not fully understood so far (Bull et al., 1984a, b; Dearfield et al., 1988; Friedman et al., 1995; Johnson et al., 1986). After considering all these results, the International Agency for Research on Cancer (IARC) and the US Environmental Protection Agency (US EPA) classified the compound as a “probable human carcinogen (group 2A)” and the National Toxicology Program (NTP) considers AA as “reasonably anticipated to be a human carcinogen”.

The genotoxicity and carcinogenicity of AA is attributed to three mechanisms: (i) radical mediated polymerisation, (ii) “Michael reactivity”, i.e. the addition of carbanions to a,b-unsaturated chemicals, and (iii) oxidation to GA by cytochrome P450 enzymes (Dearfield et al., 1995).

In a recent in vivo study, pretreatment with 1-aminobenzotriazole, a non-specific P450 inhibitor, abrogated or reduced the dominant lethal effect of AA in mice suggesting that GA is the cause of germ cell mutation in mouse spermatids (Adler et al., 2000). Sumner et al. (1999) and Ghanayem et al. (2005b) investigated the role of cytochrome P450 in the metabolism of AA using mice differing in CYP2E1 expression (wild type CYP2E1 /+ and CYP2E1 −/− knock-out mice). Both studies demonstrated that CYP2E1 plays a pivotal role in the epoxidation of AA to GA. Additionally, Ghanayem et al. (2005a) confirmed that the AA to GA transformation leads to the formation of GA-DNA adducts in liver, lung and testes. In another study, AA did not exhibit genotoxicity in organs of CYP2E1-null vs. wild-type mice using the comet assay and micronucleus test.

In the present study, we investigated the genotoxicity of AA in human HepG2 hepatoma cells using the alkaline version of the single-cell gel electrophoresis assay (SCGE). Moreover, we studied the combined effect of ethanol and AA to shed light on the possible increased health risk of simultaneous consumption of AA-containing foods and alcoholic beverages caused by ethanol-induced CYP2E1-induction and GSH-depletion.Mechanisms of action are discussed.

Materials and methods

Chemicals

Dimethyl sulfoxide (DMSO) (purity >99%), benz[α]pyrene (BaP) (purity >99%) were purchased from Sigma Aldrich (Steinheim, Germany). AA (CAS No. 79-06-1; purity >99.9%) and normal melting point agarose (NMA) were from Merck (Darmstadt, Germany). Ethanol (purity >99.8%) was from Roth (Karlsruhe, Germany). Low melting point agarose (LMA) and gentamycine were obtained from Serva (Heidelberg, Germany). Dulbeccos Minimal Essential Medium (DMEM) and fetal calf serum (FCS) were from PAA (Pasching, Austria), BaP and AA were dissolved in sterile DMSO and sterile distilled water, respectively.

HepG2 cells

HepG2 cells were kindly provided by Dr. Firouz Darroudi, Leiden University Medical Center “LUMC”, The Netherlands. The cells were cultured in low glucose DMEM supplemented with 15% fetal calf serum and 50μg/ml gentamycine in a 5% CO2 atmosphere at 37 °C.

Determination of cell viability

HepG2 cells were plated onto multiwell plates at a density of 1.5 × 10³cells/ml culture medium. After 24 h growth the cells were exposed to AA (1.25–20 mM) for 24 h at 37 °C and 5% CO2. In combination experiments with ethanol the cells were pretreated with ethanol (16, 30, 60, 120 and 240 mM; control: aqua bidest) for 24 h before exposure to AA (5 mM) for another 24 h. After treatment, the viability of the cells was determined by erythrosin B.
SCGE assay

The SCGE assay, also known as comet assay, was carried out as described by Singh et al. (1988) according to the guidelines developed by Tice et al. (2000) and Klaude et al. (1996) with slight modifications as described earlier (Lamy et al., 2004). The olive tail moment (OTM) was calculated as indicator of DNA damage. One hundred and two systematically screened cells per concentration were evaluated and the test was carried out three times. The reported OTM is mean ± standard deviation (SD) of three independent experiments. For AA genotoxicity tests and AA/ethanol combination experiments the significance was calculated in comparison to the negative control (DMSO) and AA 5 mM, respectively, using the Student’s t-test (p ≤ 0.05).

Western blot

HepG2 cells were seeded on culture flasks at a density of 1 x 10^5, grown overnight and treated with ethanol (25, 50 or 100 mM) or 5 mM AA for 24 h. Afterwards, the cells were harvested with a cell scraper, sonicated and centrifuged (16 000 g, 15 min, 4 °C). The supernatant was again centrifuged (100 000 g, 1 h, 4 °C) and the sediment containing the microsomes was kept at −80 °C until used for immunoblotting. Anti-human CYP2E1 monoclonal antibodies of mouse were used as primary antibodies followed by treatment with horseradish peroxidase (HRP)-labeled anti-mouse secondary antibodies. Protein levels were assayed as described by Bradford (1976). Liver microsomes of isoniazid-treated rats (0.5 or 1 µg) were used as positive control.

Determination of reduced glutathione (GSH)

GSH content of HepG2 cells was determined using a Cayman GSH assay kit (Cayman Chemical Co., Grünberg, Germany) according to the manufacturer’s instructions. Cells (1 x 10^6) were collected by scraping and centrifugation. The cell pellet was resuspended in cold PBS w/o Ca^2+ /Mg^2+ and lysed by sonication. After centrifugation (16 000 g, 20 min, 4 °C) the supernatant was deproteinated with an equal volume of 10% metaphosphoric acid and again centrifuged (2000 g, 2 min, 4 °C). Before adding the assay cocktail from the kit, 50 µl triethanolamine (4 M) was added per ml supernatant. The absorbance was subsequently measured at 410 nm on a 96-well plate reader in 5 min intervals for 30 min. Samples and standards were assayed three times in duplicate. The concentration of GSH was determined by producing a standard curve of known GSH concentrations.

ssDNA apoptosis assay

Apoptosis was measured with the ssDNA apoptosis ELISA kit (Chemicon, Germany) based on the specific reaction of a monoclonal antibody with formamide denatured single stranded apoptotic DNA. The assay was carried out according to the manufacturer’s instructions. Absorbance was measured at 410 nm and calculated with reference to the negative control.

Results

DNA damaging effect of AA on HepG2 cells

A concentration between 1.25 and 10 mM AA-induced DNA migration (SCGE) in HepG2 cells in a concentration-dependent manner (Fig. 1). For example, a concentration of 2.5 mM AA induced a significant (OTM 1.94 ± 0.54) increase in DNA migration compared to the negative control (OTM 0.6 ± 0.22). The highest AA concentration (10 mM) resulted in an 8-fold increase of DNA migration (OTM 4.98 ± 0.47) compared to the negative control.

DNA damaging effect of ethanol on HepG2 cells

Ethanol-treatment of HepG2 cells in concentrations of 15–240 mM resulted in a marginal, concentration-independent induction of DNA migration (Fig. 2). The second highest ethanol concentration used in these experiments (120 mM) induced an OTM of 0.95 ± 0.16 compared to an OTM of 0.53 ± 0.09 of the control.

![Image](https://example.com/image.png)

Fig. 1. Effect of 0–10 mM AA (24 h) treatment on DNA-migration in HepG2 cells using SCGE (negative control: aqua dest.; positive control: 50 µM BaP). The bars represent the mean values ± standard deviation of the OTM in three independent experiments (n = 3). *p < 0.05; **p < 0.01 compared to the negative control. The displayed line represents the cell viability. Standard deviation was less than ±5%.

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than represents the cell viability. Standard deviation was less compared to the negative control. The displayed line represents the cell viability. Standard deviation was less than ±5%.

Enhancement of AA-induced DNA damage by ethanol pretreatment of HepG2 cells

Pretreatment of HepG2 cells with ethanol increased the genotoxicity of AA in a concentration-dependent manner. As shown in Fig. 3, pretreatment of HepG2 cells with 120 mM ethanol for 24 h followed by exposure to 5 mM AA for another 24 h increased the level of DNA migration more than 2-fold as compared to cells treated with AA alone (OTM of 4.32 ± 0.44 vs. 1.90 ± 0.32). The solvent control and BaP control induced an OTM of 0.59 ± 0.11 and 3.80 ± 0.62, respectively. After treatment of HepG2 cells with 240 mM ethanol and subsequently 5 mM AA, a slight decrease in DNA migration (OTM 3.8 ± 0.59) was observed.

Effect on cell viability and measurement of apoptosis

With increasing concentration of AA a continuous reduction in viability could be observed. The viability of AA-treated cells was reduced by 8.7% after incubation with 1.25 mM AA; 10 mM AA reduced the viability by 26.8% compared to the negative control. 15 mM ethanol reduced the viability of HepG2 cells by 6.4% whereas a treatment of HepG2 cells with 120 and 240 mM ethanol reduced the viability by 25.5% and 27.8%, respectively, compared to the negative control.

In pretreatment experiments, AA (5 mM) reduced the vitality by 13.5% compared to the control; pretreatment of HepG2 cells with 15 and 240 mM ethanol and subsequent AA-treatment reduced the vitality by 16.76% and 27.03%, respectively compared to control (see Figs. 1–3). (Fig. 4)

To elucidate whether the origin of this observed cytotoxic effect was due to apoptosis we measured the formation of ssDNA as a marker of apoptosis.

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induction. The results are presented in Figs. 5a and b, displayed as a multiple of the negative control aqua dest. No elevated induction of apoptosis compared to control cells could be observed after AA treatment (1.25–10 mM) or ethanol (15–240 mM)/AA (5 mM) combination treatment.

Induction of CYP2E1 by ethanol in HepG2 cells

Fig. 4 shows the effect of ethanol or AA treatment on the expression of CYP2E1 in HepG2 cells as determined by western blot technique. Microsomes of isoniazide (INH)-treated rats showed a clear band at 54 kDa for CYP2E1 (1, 2 and 7), no CYP2E1 enzyme expression could be detected in microsomes from 5 mM AA treated (9) or untreated (3 and 8) HepG2 cells (negative control) or HepG2 cells treated with 25 mM ethanol (4). A 54 kDa band identical to the one generated by INH-treated rat microsomes was detected in microsomes from HepG2 cells after 50 mM (5) and 100 mM (6) ethanol treatment, indicating the expression of CYP2E1 after ethanol exposure in HepG2 cells.

Modulation of GSH level by ethanol and AA treatment in HepG2 cells

The results of experiments on ethanol and AA treatment on the intracellular GSH level is shown in Table 1. In untreated HepG2 cells a GSH content of 4.86 μM ± 0.98/10⁶ cells was found. Compared to that, treatment of HepG2 cells with 240 mM ethanol reduced the GSH level by 75% to 1.25 μM ± 0.56/10⁶ cells. Exposure to 5 mM AA resulted in a decrease of GSH to 2.08 μM ± 0.77/10⁶ cells. After pretreatment with 240 mM ethanol and subsequent 5 mM AA exposure GSH was depleted by about 90% compared to the control.

Discussion

Numerous studies have been carried out recently to assess the genotoxic potency of AA in vitro and in vivo (Baum et al., 2005; Glatt et al., 2005; Maniere et al., 2005; Puppel et al., 2005). Most of the data suggested that the genotoxic potential of AA could be mainly

<table>
<thead>
<tr>
<th>Concentration (μM/10⁶ cells)</th>
<th>Control</th>
<th>EtOH 15 mM</th>
<th>EtOH 60 mM</th>
<th>EtOH 240 mM</th>
<th>Acrylamide 5 mM</th>
<th>EtOH 15 mM + acrylamide 5 mM</th>
<th>EtOH 60 mM + acrylamide 5 mM</th>
<th>EtOH 240 mM + acrylamide 5 mM</th>
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<tr>
<td></td>
<td>4.865 ± 0.989</td>
<td>3.892 ± 1.005</td>
<td>2.216 ± 0.541</td>
<td>1.254 ± 0.558</td>
<td>2.086 ± 0.776</td>
<td>1.077 ± 0.385</td>
<td>1.214 ± 0.602</td>
<td>0.408 ± 0.378</td>
</tr>
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The results represent mean values ± standard deviation of GSH in three independent experiments (n = 3).
attributed to GA, the CYP2E1-mediated epoxide metabolite of AA. Therefore, in the present study we raised the question whether the genotoxic potential of AA might be enhanced by ethanol, a known CYP2E1 inducer. CYP2E1-linked synergisms of ethanol and AA regarding DNA damaging effects may be crucial because of the consumption of AA-containing food as potato chips and alcoholic beverages at the same time. For the present study we used the human hepatoma cell line HepG2. As a biological endpoint DNA migration was monitored in the alkaline version of the comet assay as an indicator for DNA damage. Since liver represents the organ with the highest capacity of phase I and II enzyme activities, this cell line, which is known to possess inducible CYP activities, seemed to be a suitable model especially for our combination studies.

We observed a concentration-dependent DNA-migration in HepG2 cells after AA treatment, although no induction of CYP2E1 could be detected under these experimental conditions. After ethanol treatment and subsequent AA exposure, DNA-migration was almost two-fold increased compared to cells treated with AA alone. As expected, ethanol-exposure of HepG2 cells resulted in an increase of CYP2E1. This suggests that CYP2E1-mediated transformation of AA to GA might play a certain role in the present study. However, CYP2E1-induction was only weak and therefore cannot fully explain the ethanol-induced increase of the genotoxic potency of AA. The treatment of HepG2 cells with AA, ethanol and their combination led to a significant depletion of GSH, indicating an increase in oxidative stress level. We assume that this observed decrease in total cellular GSH levels plays a crucial role in the DNA-damaging effect after AA or AA/ethanol-treatment. Being the most abundant cellular antioxidant, GSH represents a critical point in the defence against cell injury by oxidative stress. AA is a strong nucleophil and as such known to react quite easily with thiol-groups like GSH (Sumner et al., 1992). This nucleophil and as such known to react quite easily with thiol-groups like GSH (Sumner et al., 1992). This suggestion is in accordance with other published data (Park et al., 2002; Puppel et al., 2005), where the authors assumed that AA itself, and not its metabolite GA possesses cytotoxic and genotoxic properties by influencing the oxidative defense systems. Park et al. (2002) described an AA-entailed reduction of GSH in Syrian hamster embryo (SHE) cells. In that study, GSH reduction was inhibited by the sulfhydryl-group donor N-acetyl-l-cysteine (NAC); but no prevention of GSH depletion was seen after treatment with an unspecific CYP P450 inhibitor. Thus the authors assumed that AA itself and not its metabolite (e.g., GA) seemed to be involved in cellular transformation and that cellular thiol status was involved in it.

Puppel et al. (2005) investigated the impact of CYP2E1 expression and intracellular GSH depletion by DL-buthionine-[S,R]-sulfoximine (BSO) on the DNA damaging properties of AA and GA in cells differing in their CYP2E1 status (V79, Caco-2 cells and primary rat hepatocytes) with the comet assay. They concluded that the strand breaking effect was not necessarily correlated with the CYP2E1 status of the cells but with a depletion of GSH, which also lead to a substantial enhancement of cytotoxicity. However, the origin of this observed cytotoxic effect was not determined. Blasiak et al. (2004) concluded that double strand breaks observed in human lymphocytes after treatment with 50 μM AA were the result of apoptosis possibly caused by AA-induced ROS-formation. In the present study an increase in cytotoxicity after AA or AA/ethanol treatment of up to 30% was detected. Since it was reported that in the comet assay the onset of apoptotic fragmentation could appear as “comets” and lead to false positive results (Chourcoun et al., 2001), we investigated the origin of this cytotoxicity. Since no induction of apoptosis could be observed in the ssDNA apoptosis assay (even with higher concentrations), we suggest the reason of the viability decrease in HepG2 cells to be an unspecific effect rather than programmed cell death. Interestingly, no DNA-migration could be observed in primary rat hepatocytes in the comet assay in concentrations comparable to those used in the present study. Only after severe GSH depletion by BSO, a significant increase in DNA damage and cytotoxicity could be detected (Puppel et al., 2005). AA was found to be carcinogenic in various organs of mice and rats but not in the liver (Rice, 2005). Although place of biotransformation and CYP2E1 production in the body, the liver represents obviously no target organ for AA-induced carcinogenicity and only a limited one for genotoxicity.

Most studies investigating AA in vivo did only find genotoxicity in the liver in very high concentrations. As reported by Butterworth et al. (1992) AA induced unscheduled DNA synthesis in rat spermatocytes in vivo but apparently not in rat hepatocytes after single or repeated exposure. Maniere et al. (2005) did not find any significant increase in DNA damage (SCGE) after single dose treatment with 18, 36 or 54 mg/kg b.w. in the liver of mice after 24 h, only after 5 h with the highest dosage (54 mg/kg b.w.) of AA. Manjanatha et al. (2006) could not find significant mutagenic responses in the liver of big blue mice until application of 500 mg/l of AA to the drinking water. In this dosage, however, animals already developed signs of toxicity. A possible explanation for these results could be the high efficiency of liver-mediated AA- and/or GA-clearance as indicated in a study from Doerge et al. (2005) who found continuously low levels of GA in the liver of AA-exposed Fischer 344 rats despite high circulating levels of GA. Conjugation of GA with GSH represents the primary elimination route of AA in rodents, irrespective of the route of exposure (Sumner et al., 2003) and a pharmacokinetic model in the rat suggests that liver glutathione levels
would not become appreciably depleted at doses lower than 10 mg/kg b.w. (Kirman et al., 2003). The concentrations applied to the cell cultures in the present study were also rather high, and this has to be taken into consideration when further discussing the consequences for the dietary intake of AA-containing food and alcoholic beverages.

In conclusion, AA was found to be genotoxic in HepG2 cells without detectable amounts of CYP2E1. We showed furthermore an over-additive DNA-damaging effect of ethanol and AA-treatment determined by the comet assay. Ethanol-pre-treatment resulted in a slight, but clear increase in CYP2E1 expression, which might account partly for the observed effect. However, the observed depletion of cellular GSH seems to play a pivotal role in the genotoxicity caused by AA alone and ethanol pre-treatment of HepG2 cells. The in this study applied in vitro model gives first hints of a possible increased health risk entailed by simultaneous consumption of AA-containing foods and a high amount of alcoholic beverages. In further studies this question has to be examined more closely.

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