Arch Toxicol (2008) 82:717–727
DOI 10.1007/s00204-008-0296-8

TOXICOKINETICS AND METABOLISM

Human CYP2E1 mediates the formation of glycidamide from acrylamide

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Received: 6 December 2007 / Accepted: 12 March 2008 / Published online: 17 April 2008

Abstract Regarding the cancer risk assessment of acrylamide (AA) it is of basic interest to know, as to what amount of the absorbed AA is metabolized to glycidamide (GA) in humans, compared to what has been observed in laboratory animals. GA is suspected of being the ultimate carcinogenic metabolite of AA. From experiments with CYP2E1-deficient mice it can be concluded that AA is metabolized to GA primarily by CYP2E1. We therefore examined whether CYP2E1 is involved in GA formation in non-rodent species with the focus on humans by using human CYP2E1 supersomes™, marmoset and human liver microsomes and in addition, genetically engineered V79 cells expressing human CYP2E1 (V79h2E1 cells). Special emphasis was placed on the analytical detection of GA, which was performed by gas chromatography/mass spectrometry. The results show that AA is metabolized to GA in human CYP2E1 supersomes™, in marmoset and human liver microsomes as well as in V79h2E1 cells. The activity of GA formation is highest in supersomes™; in human liver it is somewhat higher than in marmoset liver. A monoclonal CYP2E1 human selective antibody (MAB-2E1) and diethyldithiocarbamate (DDC) were used as specific inhibitors of CYP2E1. The generation of GA could be inhibited by MAB-2E1 to about 80% in V79h2E1 cells and to about 90% in human and marmoset liver microsomes. Also DDC led to an inhibition of about 95%. In conclusion, AA is metabolized to GA by human CYP2E1. Overall, the present work describes (1) the application and refinement of a sensitive methodology in order to determine low amounts of GA, (2) the applicability of genetically modified V79 cell lines in order to investigate specific questions concerning metabolism and (3) the involvement, for the first time, of human CYP2E1 in the formation of GA from AA. Further studies will compare the activities of GA formation in genetically engineered V79 cells expressing CYP2E1 from different species.

Keywords Acrylamide · Glycidamide · Human CYP2E1 · Metabolism · Risk assessment

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Introduction

Acrylamide (AA) is an important industrial chemical. Recently it was discovered that AA was formed in high levels after frying or baking of a variety of carbohydrate-rich foods (Tareke et al. 2000). Hence, there is concern that health risks may arise from dietary exposure to AA. Neurotoxic effects of AA have been observed in laboratory animals and are well known in humans as a consequence of occupational and accidental exposures. Besides, AA has been shown to be also a reproductive toxicant in rodents, main concern however relates to its carcinogenic effects. There is evidence from long-term

studies in AA-exposed laboratory animals for an increased incidence of tumors in certain tissues. The recent EU risk assessment report on AA assessed all the evidence present in published and unpublished studies and came to the conclusion that AA is a genotoxic carcinogen which should be classified as Carc Cat 2 according to the EU classification scheme (EU 2002). IARC (1994) has classified AA as a "probable human carcinogen". According to the present toxicological paradigm a safe dose cannot be established. A dietary intake assessment performed by FAO/WHO (2005) leads to a value of 0.001 mg/kg bw representing the average intake of the general population. When this value was compared with the BMDL (95% lower confidence limit for the benchmark dose of 10% extra risk of tumors) of 0.30 mg/kg bw/day for induction of mammary tumors in rats the resulting MOE (margin of exposure) of 300 was considered to indicate a possible human health concern. However, the question as to whether the dietary uptake of AA may cause cancer in humans is still a matter of controversy (Ruden 2004). In one recent prospective study, increased risks of postmenopausal endometrial and ovarian cancer with increasing dietary AA intake were observed (Hogervorst et al. 2007). But several retrospective epidemiological studies performed in workers and in unselected adult populations were not able to demonstrate an increased cancer risk in subjects higher exposed to AA.

Consequently, similar to the situation with most other chemicals, the risk assessment of AA has to be undertaken on the basis of findings in laboratory animals, which may have relevant limitations. Major uncertainties are possible differences in toxicokinetics between rodents and humans. Whereas the metabolism and the metabolizing enzymes involved are known in rodents information on the human metabolism of AA would be of paramount importance in order to overcome the uncertainty in species difference.

In experimental animals, following oral administration, AA is rapidly and extensively absorbed from the gastrointestinal tract and is widely distributed to the tissues, as well as to the foetus (Callemann 1996; Kadry et al. 1999; Bjellaas et al. 2007). A certain fraction is converted metabolically to the chemically reactive epoxide GA. Kinetic findings suggest that exposure to low levels of AA could promote the more efficient formation of GA (Barber et al. 2001; Doerge et al. 2005a). Furthermore, both AA and GA can be conjugated with glutathione. AA and its metabolites are rapidly eliminated via the urine, primarily as mercapturic acid conjugates of AA and GA. The conjugation of AA and GA with glutathione in blood proceeds mainly via uncatalysed reactions. The percentage of AA, which is conjugated with glutathione or oxidized to GA varies from species to species (Paulsson et al. 2005; Sumner et al. 1992, 2003; Twaddle et al. 2004).

While there is some evidence that the mechanism of AA neurotoxicity involves the reaction of AA with protein sulfhydryl groups in the nervous system (LoPachin 2004; LoPachin et al. 2007), it is not completely clear whether AA or GA or both are responsible for the carcinogenic activity. For instance AA itself appears to be involved in AA-induced cellular transformation in vitro (Park et al. 2002). AA is both clastogenic and mutagenic in mammalian cells in vitro and in vivo. However, while GA is clearly mutagenic in the Ames Salmonella Assay, AA is not. In addition GA dosing produced comparable or greater increases in mutant frequencies at the hprt and cell loci in Big Blue transgenic mice compared with AA (Manjanatha et al. 2006). AA and GA were also studied in human lymphoblastoid TK6 cells by examining three different parameters: DNA damage (comet assay), clastogenesis (micronucleus test) and gene mutation [thymidine kinase (TK) assay]. In a 4-h treatment without metabolic activation, AA was mildly genotoxic in the micronucleus and TK assays at high concentrations (>10 mM), whereas GA was significantly and concentration-dependently genotoxic at all endpoints at concentrations $\geq$0.5 mM. Molecular analysis of the TK mutants revealed that AA predominantly induced loss of heterozygosity (LOH) mutation while GA primarily induced point mutations. These results indicate that the genotoxic characteristics of AA and GA were distinctly different: AA was clastogenic and GA was mutagenic (Beseratinia and Pfeifer 2003; Koyama et al. 2006). GA is much more reactive with DNA than AA. Several purine base adducts have been identified in laboratory animals (Segerbäck et al. 1995; Gamboa da Costa et al. 2003; Doerge et al. 2005b). However, there are no reports about binding of AA metabolites to human DNA in vivo. In this respect it is worth noting that the tissue and organ distribution of GA-DNA adducts do not correlate with AA-induced tumors in rat organs (Segerbäck et al. 1995). A single oral administration of AA to rats induced DNA adducts, which were evenly distributed among all tissues examined. Organ-specificity cannot therefore be explained by a selective accumulation of GA-DNA adducts in the target organs (Calleman 1996; Maniere et al. 2005). Based on the pattern of neoplastic development [e.g. mammary gland fibroadenomas (females only) and thyroid follicular-cell adenomas (males and females) in F344 rats], it appears that AA is targeting endocrine sensitive tissues (Klaunig and Kamendulis 2005). The possibility of hormonal imbalance, cell proliferation or interaction with motor protein systems was raised. Alterations of thyroid-stimulating hormones, prolactin, and testosterone levels have been observed in rats following AA treatment (Ali et al. 1983). Also, results from GA-treated human epithelial cells suggest a hormonal dysregulation due to the observed up-regulation of aldo-keto reductases, which might link AA exposure to the development of

tumors in steroid-dependent tissues (Clement et al. 2007). In this connection it may be important that carcinogenic effects occur only at or just below the range of toxic dosing as was stated by Bolt (2003).

Data from mice demonstrated that CYP2E1 is responsible for the conversion of AA to GA. Whereas metabolites derived from GA could be detected in the urines from AA-treated wild-type mice, no GA-derived metabolites could be determined in the urines of CYP2E1 null mice or in mice pretreated with the CYP2E1 inhibitor aminobenzotriazole (Adler et al. 2000; Sumner et al. 1999). In addition, studies with CYP2E1-deficient mice and wild-type mice showed that CYP2E1-mediated oxidation is the predominant pathway leading to GA-DNA adduct formation. Administration of AA to wild-type mice caused a large increase in N7-GA-guanine and N3-GA-adenine adducts in the liver, lung and testes. Only traces of N7-GA-guanine adducts were measured in the tissues of AA-treated CYP2E1-null mice, which were 52- to 66-fold lower than in wild-type mice. These comparative data demonstrate that CYP2E1 is the primary enzyme responsible for the epoxidation of AA to GA (Ghanayem et al. 2005a). Furthermore, significant dose-related increases in micronucleated erythrocytes and DNA damage in somatic cells were induced in AA-treated wild type, but not in CYP2E1-null mice (Ghanayem et al. 2005b). However, in another study with primary rat hepatocytes no DNA fragmentation, detected by the COMET assay, was induced by AA despite substantial expression of CYP2E1 protein demonstrating that the expression of CYP2E1 protein per se does not concomitantly implicate enhanced genotoxicity of AA in these cells (Puppel et al. 2005).

While there is sufficient evidence that GA is also generated in humans as was first shown, for instance, by samples of haemoglobin-GA adducts taken from workers exposed to high levels of AA (Bergmark et al. 1993), up to now, the enzyme involved in GA formation in humans has still not been identified. It is therefore of interest to know whether CYP2E1 is responsible for GA formation also in species other than the mouse, e.g. rats, monkeys and humans. CYP2E1 is of clinical and toxicological importance; it is constitutively expressed in the liver and in many other tissues (Bernauer et al. 1999, 2000, 2002, 2003a, 2006; Tanaka et al. 2000). Consequently, it was the aim of the study to clarify whether CYP2E1 is the enzyme responsible for GA formation in species other than mice, especially humans.

We therefore investigated the metabolization of AA to GA by using human CYP2E1 supersomes™, human and marmoset liver microsomes, and genetically modified V79 cells stably expressing human CYP2E1 as well as specific antibodies against human CYP2E1. Special emphasis was placed on the analytical detection of GA.

Materials and methods

Materials

Genetically modified V79 cells expressing human CYP2E1 (V79h2E1) were obtained from the German Institute for Human Nutrition (Nuthetal, Germany). Human CYP2E1 supersomes™ (microsomes from insect cells expressing human CYP2E1), human liver microsomes and a monoclonal mouse raised inhibitory antibody targeting human CYP2E1 (MAB-2E1) were purchased from BD Biosciences (Woburn, MA, USA). Acrylamide (AA), nicotinamide adenine dinucleotide ($NADP^+$), and diethyldithiocarbamate (DDC) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Glycidamide (GA), ^{13}C-glycidamide were obtained from Toronto Research Chemicals Inc. (North York, Canada). D_3-acrylamide and methacrylamide (MA) were obtained from Polymer source, Inc. (Quebec, Canada).

Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Roche Diagnostics GmbH (Mannheim, Germany). Chemicals for cell culture (e.g., PBS, DMEM, foetal calf serum, penicillin, streptomycin) were purchased from Biochrom (Berlin, Germany), respectively, from Pan-Biotech (Aidenbach, Germany).

All other reagents were of the highest grade commercially available and were obtained from Merck (Darmstadt, Germany), MP Biomedicals (Eschwege, Germany) and LGC Promochem GmbH (Wesel, Germany).

The extraction columns were manufactured with a mixture of 47% activated charcoal, 33% aluminium oxide 90 active neutral and 20% celite 545. A column contains 3 g of this mixture. Activated charcoal and aluminium oxide 90 active neutral were purchased from Merck (Darmstadt, Germany). Celite 545 was obtained from Serva (Heidelberg, Germany). Column reservoirs (15 ml) and frits were obtained from Alltech (Unterhaching, Germany).

Supersomes™ and microsomes

Supersomes™

According to the manufacturer, the human CYP2E1 supersomes™ contained 2,000 pmol Cytochrome P450/ml, 700 pmol Cytochrome b5/mg protein and a Cytochrome c reductase activity of 4,100 nmole/(mg protein × min). They exhibited a *p*-nitrophenol hydroxylase activity of 11 pmol product/(pmol P450 × min). The protein content was 5.4 mg/ml in 100 mM potassium phosphate (pH 7.4).

Human liver microsomes

Ready-to-use human liver microsomes from two different donors were used. Information on donors was supplied by

the manufacturer. The samples were tested for pathogens and characterized with respect to protein content and the expression of xenobiotic-metabolizing enzymes. One sample was from a 30-year-old Caucasian female, who died from glioblastoma and who was on dopamine and lidocaine as medication. The other sample was from a 56-year-old female, who died of brain haemorrhage. No data on medication were available but there was a medical history of hypertension.

Preparation of liver microsomes from marmoset monkeys (Callithrix jacchus)

Livers were removed from marmoset monkeys (which were killed in the course of an experiment in which livers were not required) and microsomes were prepared according to standard preparation methods by differential centrifugation (Siekevitz 1962). All steps were performed at 4°C. The final 100,000×*g* microsomal pellet was resuspended in 0.1 M potassium phosphate buffer pH 7.4, divided into aliquots and kept frozen at −70°C.

Cell culture and isolation of cell protein

V79h2E1 cells were maintained in DMEM (Dulbeccos's modified Eagle medium), supplemented with 10% foetal calf serum, penicillin (110 IE/ml), streptomycin (110 µg/ml) at 37°C, 5% CO_2, and 90% saturated atmospheric humidity. The cells were routinely tested for the absence of mycoplasm contamination. For the isolation of cell protein, cells were grown in 80 cm^2 plastic flasks in the presence of 40 mM ethanol in the culture medium until confluence, because this treatment has been shown to increase CYP2E1 content (Schmalix et al. 1995). At confluency, cells were harvested with all procedures performed on ice. Culture medium was removed from the cells and the cells were washed twice with ice cold phosphate-buffered saline (PBS). Cells were scraped off with a rubber policeman and collected in PBS. Cell suspensions were combined and centrifuged at 2,000 rpm for 5 min at 4°C. Supernatants were removed and the cell pellet was re-suspended in ice cold PBS. After a second centrifugation step at 2,000 rpm, supernatant was discarded and the resulting cell pellet was stored at −70°C until further use. Prior to performing any experiments (activity determination, western blotting), the cells were thawed and disintegrated applying 5 × 10 pulses at 20 kHz using a Branson B15 cell disruptor (Carouge-Geneva, Switzerland).

Protein determination

Total protein contents in homogenates of the pelleted and resuspended cells were determined in a microplate assay according to the method of Bradford (1976) using bovine serum albumin (BSA) as the protein standard.

Determination of chlorzoxazone (CLX) hydroxylase activity

CYP2E1 activity was determined by formation of 6-hydroxychlorzoxazone (6-OH-CLX) from CLX according to the method of Peter et al. (1990) with slight modifications as described by Bernauer et al. (1999). Briefly, CLX [stock solution (25 mM) dissolved in 60 mM KOH] was incubated at 37°C with protein (supersomes™: 350 µg, human liver microsomes: 200 µg, marmoset liver microsomes: 150 µg, V79h2E1 cell protein: 500 µg) and a nicotinamide adenine dinucleotide phosphate- (NADPH, reduced form) generating system consisting of glucose-6-phosphate, $NADP^+$ and glucose-6-phosphate dehydrogenase in 0.1 M Tris, pH 7.4 (final volume: 1 ml). For supersomes™, human liver microsomes, marmoset liver microsomes and V79h2E1 cell protein the substrate (CLX) concentration was: 250 µM CLX. After 20 min, the reaction was stopped by the addition of 50 µl phosphoric acid (H_3PO_4) (43% [w/v]). Phenacetin was added as an internal standard (42 µM) and the reaction product was purified by solid phase extraction (SPE) using 100 mg/1 ml tube ENVI-18 endcapped columns (Supelco, Deisenhofen, Germany). After elution with acetonitrile and evaporation of the solvent, the residues were dissolved in 100 µl acetonitrile/water (10:90 [v/v]) and analysed by HPLC (Agilent Series 1100, Waldbronn, Germany) using a 2.1 × 100 mm 5 µm Hypersil ODS C18 column (Agilent, Waldbronn, Germany), equipped with 2 × 30 mm 5 µm Hypersil ODS precolumn (Knauer, Berlin, Germany). The injection volume was 10 µl. Isocratic conditions (acetonitrile/ 0.5% H_3PO_4 (10:90 [v/v]) at a flow rate of 0.45 ml/min and a column temperature of +40°C were applied for elution. Effluents were monitored at 297 nm using a Diode Array Detector (Agilent Series 1100, Hewlett-Packard, Waldbronn, Germany). Formed 6-OH-CLX was identified by its characteristic UV-spectrum and quantified by comparison with a standard curve obtained from commercially available 6-OH-CLX (limit of determination of 6-OH-CLX: 0.001 µg/ml [5.4 nM]). Two independent incubations were performed, for each sample.

Incubation of supersomes™, liver microsomes and V79h2E1 cells with AA and sample preparation for GA determination

The incubation mixture contained 1 mg protein/ml from V79h2E1 cells, human or marmoset liver microsomes or 0.6 mg protein/ml from supersomes™ and 2 mM AA in a final reaction volume of 2 ml buffer (0.1 M Tris, pH 7.4). The enzymatic reaction was started by the addition of a NADPH-generating system at 37°C as described under "Determination of chlorzoxazone (CLX) hydroxylase activity". After different incubation periods varying

between 5 and 60 min, the enzymatic reaction was stopped with 3 ml 1-propanol. After the addition of ^{13}C-labelled glycidamide (0.6 µg/ml[6.7 µM]) and D_3-labelled acrylamide (6 µg/ml[81 µM]) as internal standards, the mixture was centrifuged at 4,000 rpm for 15 min. The supernatant was cleaned up by passing the solution through an activated charcoal-celite-aluminium oxide-column. The elution was performed by using 7 ml of a 1-propanol/water mixture (8:2, v/v). After the addition of 100 µl 1-octanol, the samples were evaporated to dryness under a stream of nitrogen. The residues were resolved in acetonitrile. Methacrylamide (12 µM) was added and this was followed by two extraction steps with 1 ml *n*-hexane, respectively. After centrifugation at 15,000 rpm for 10 min, the acetonitrile phase was used for analysis.

Determination of GA by GC-MS

The determination of GA was performed according to a procedure, which was originally elaborated for the determination of AA in food and feed (Klaffke et al. 2005; Pabst et al. 2005). This method was extended here for the simultaneous determination of AA and GA.

The prepared samples were analysed using a double-focussing GC-MS system. A Hewlett Packard Series 5890 gas chromatograph (Waldbronn, Germany) equipped with a split-splitless injector and a DB-WAX 60 meter column (i.d. 0.32 mm, 0.25 µm film) was used to separate the analytes. Helium 4.9 was used as the carrier gas. A Finigan MAT 95 from Thermoquest (Bremen, Germany) was used for mass-spectrometric analysis. Detection was performed using the MID (multiple-ion detection)-technique and negative chemical ionization (NCI) with ammonia as the reactant gas. For quantification the quasi-molecular ions of native GA ($m/z = 86 = M-1$) and of the ^{13}C-GA ($m/z = 89 = M-1$) were selected. Methacrylamide was used as the recovery standard for recording the quasi-molecular mass ($m/z = 84 = M-1$). ^{13}C-GA was used as an internal standard for quantification. The concentration of the internal standard was higher in relation to the native compound GA in all samples. Repeatability was checked by the repeated injection of a standard mixture.

For each sample, two independent incubations were performed and samples were analysed at least twice. The amount of GA, formed during the enzymatic incubation, was quantified by comparison with the internal standard ^{13}C-GA. Figure 1a and b demonstrate typical GC-MS chromatograms. The injection volume was 2 µl. The detection limit of this method for GA was 0.006 µg GA/ml. This is equivalent to 0.1378 pmol GA.

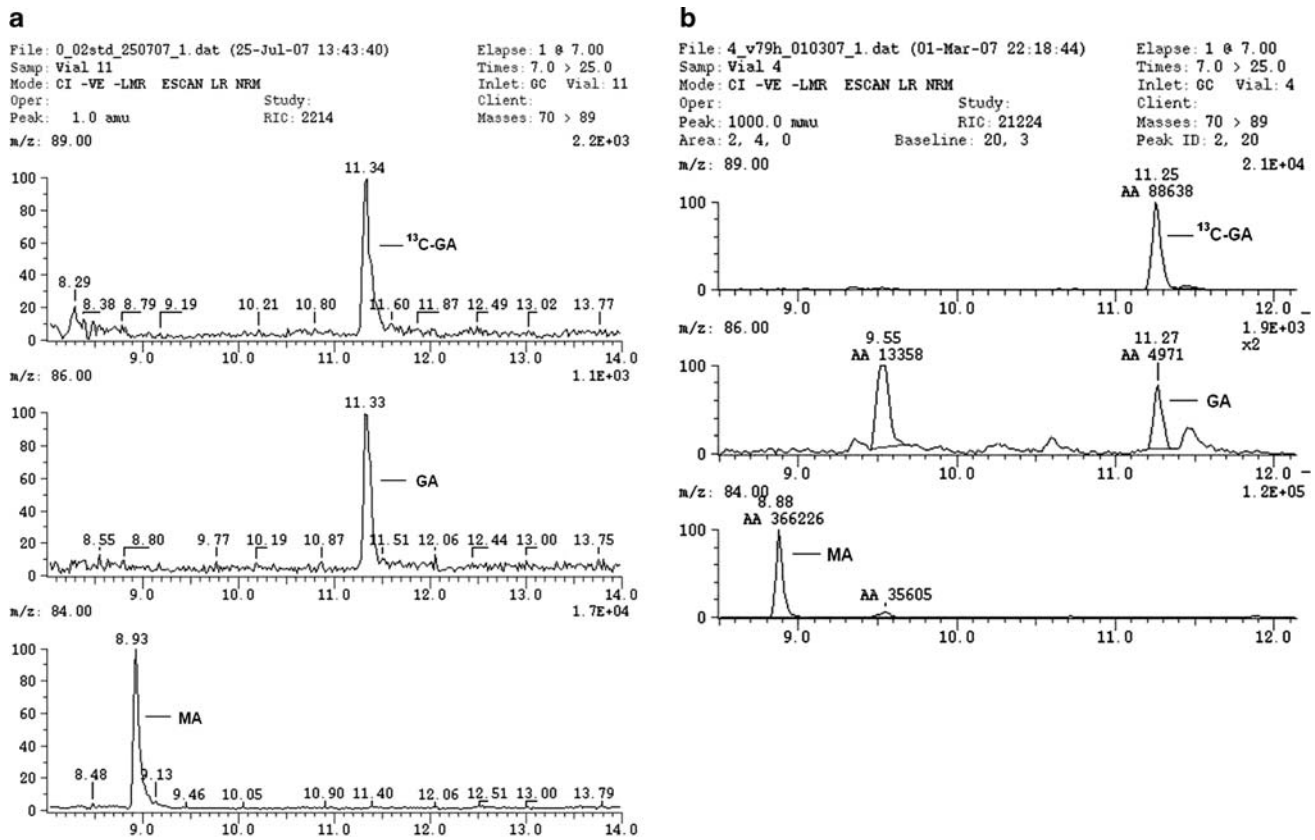

Fig. 1 **a** Chromatogram of a standard mixture. **b** Chromatogram of a real sample

Inhibition experiments

For immunoinhibition, a monoclonal, mouse-raised inhibitory antibody targeting human CYP2E1 was used. 20 µl of antibody (0.1 mg protein/ml) and a solution containing 1 mg protein/ml (from human liver microsomes, marmoset liver microsomes or V79h2E1 cells) in a final volume of 2 ml 0.1 M Tris, pH 7.4 were incubated for 15 min on ice. Afterwards, enzymatic incubation, extraction and analysis were performed as described in "Incubation of supersomes™, liver microsomes and V79h2E1 cells with AA and sample preparation for GA determination" and "Determination of GA by GC-MS".

For chemical inhibition, DDC was used as the inhibitor. 2 mM DDC and a solution containing 1 mg protein/ml (from human and marmoset liver microsomes or V79h2E1 cells) in a final volume of 2 ml buffer (0.1 M Tris, pH 7.4) were incubated for 15 min at 37°C, extraction and analysis of GA were performed as described in "Incubation of supersomes™, liver microsomes and V79h2E1 cells with AA and sample preparation for GA determination" and "Determination of GA by GC-MS".

Results

CLX hydroxylase activity

The metabolization of CLX to 6-OH-CLX as an established test system for CYP2E1 activity was used in order to compare the findings of GA formation in this study with results of this assay. Figure 2 shows the CYP2E1-dependent activity of CLX hydroxylation. Supersomes™ exhibit the highest activity [4.952 ± 0.045 nmol/(mg protein × min)]. Liver microsomes from humans [1.862 ± 0.106 nmol/(mg protein × min)] and liver microsomes from marmosets [0.984 ± 0.033 nmol/(mg protein × min)] show a significant lower activity. V79h2E1 cells demonstrate the lowest activity [0.069 ± 0.002 nmol/(mg protein × min)]. The activity of this system (more than 20-fold lower than the activity in human liver microsomes) has already been demonstrated previously for other CYP450-dependent reactions (Bernauer et al., 2003b).

GA formation from AA

The formation of GA from AA was determined according to the method described in "Materials and methods". The procedure was originally elaborated for the detection of AA in food and feed (Klaffke et al. 2005; Pabst et al. 2005). By using this method, it can be demonstrated that supersomes™, human and marmoset liver microsomes as well as genetically modified V79 cells expressing CYP2E1 metabolize AA to GA. The lowest formation rate of GA is seen in the genetically modified cells and the highest in supersomes™. The activities in human and marmoset liver microsomes are somewhere in between (Fig. 3). Due to the low detection limit of the elaborated method for the determination of GA we were able to determine the formation of GA from AA in the genetically modified V79h2E1 cells despite their low enzymatic activity. This enables the conclusion that CYP2E1 is involved in the metabolism of AA to GA. The activities of CLX hydroxylation and GA forma-

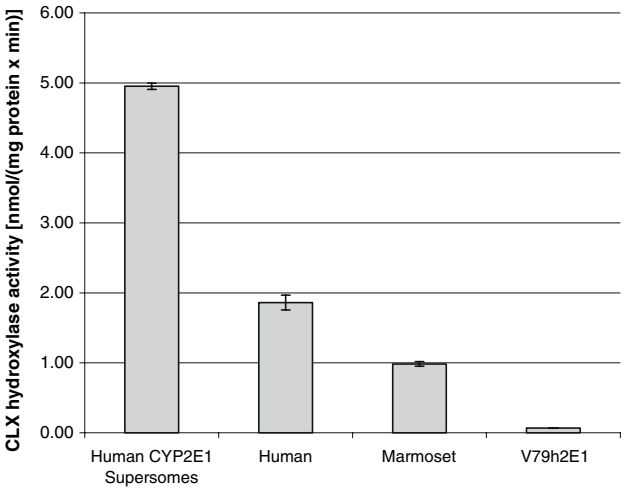

Fig. 2 Chlorzoxazone (*CLX*) hydroxylase activities measured in human CYP2E1 supersomes™, human and marmoset liver microsomes and in genetically modified V79 cells expressing human CYP2E1. Incubation time was 20 min. The data are the average of three separate incubations with two measurements of each sample. Standard deviation was calculated from six determinations.

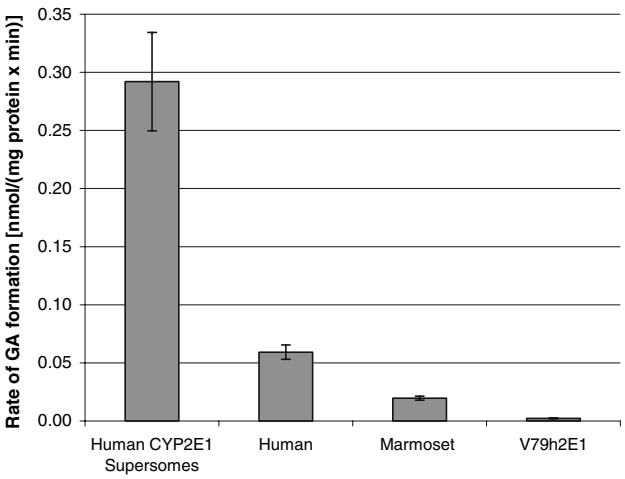

Fig. 3 Enzymatic activities of GA formation from AA in human CYP2E1 supersomes™, human and marmoset liver microsomes and in genetically modified V79 cells expressing human CYP2E1. Incubation period was 20 min. The data are the average of three separate incubation samples with two measurements of each sample. Standard deviation was calculated from six determinations

tion run parallel to each other as it is demonstrated in Fig. 4. This may further support the conclusion that human CYP2E1 is involved in the epoxidation of AA to GA.

Time dependency

It is well known that both AA and GA react with proteins by covalent binding and this is probably also the case with microsomal proteins in the incubation mixture. Hence, it was of basic interest to investigate if there is interference between the formation of GA and its disappearance by reaction with microsomal proteins. Therefore, also the time dependency of the rate of GA detection was investigated in V79h2E1 cells and in liver microsomes from marmosets and humans. This time dependency is demonstrated for human liver microsomes in Fig. 5, while the time-dependent formation of GA in liver microsomes from three different marmoset monkeys is shown in Fig. 6. Here a considerable intraspecies variability was observed between these three marmosets showing a nearly 15-fold difference. Also in V79h2E1 cells an increase of GA formation could be determined with increasing incubation time despite the fact that only very low activities could be measured (Fig. 7). The fact that the 60-min time point is low in the cases of liver microsomes from marmorset monkeys and the V79h2E1 cells may be explained by the reaction of GA with proteins lowering its detectable concentration. Other possibilities however, like the metabolic transformation of GA by e.g., microsomal epoxide hydrolase cannot be excluded.

Inhibition experiments

Figure 8 presents some inhibition experiments. Inhibition of GA formation was performed using an inhibitory anti-

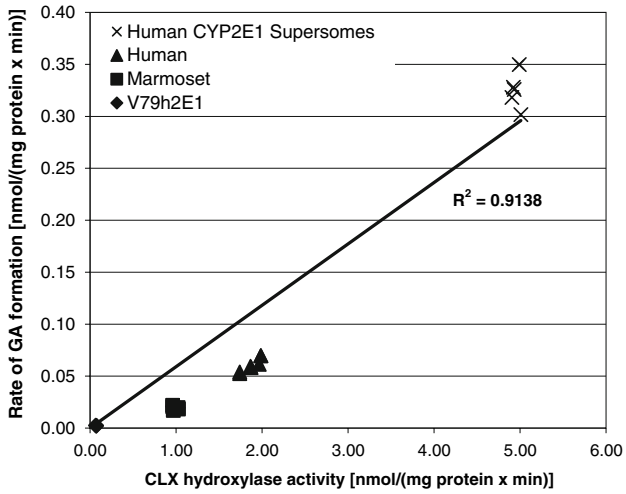

Fig. 4 Correlation between CYP2E1-dependent CLX hydroxylase activity and the formation of GA from AA in human CYP2E1 supersomes™, human and marmoset liver microsomes and V79h2E1 cells

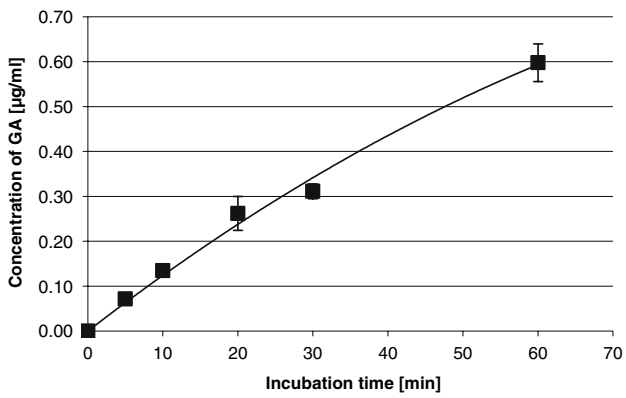

Fig. 5 Time dependency of the formation of GA from AA in human liver microsomes. The data are the average of two incubations with two measurements of each sample. Standard deviation was calculated from four determinations

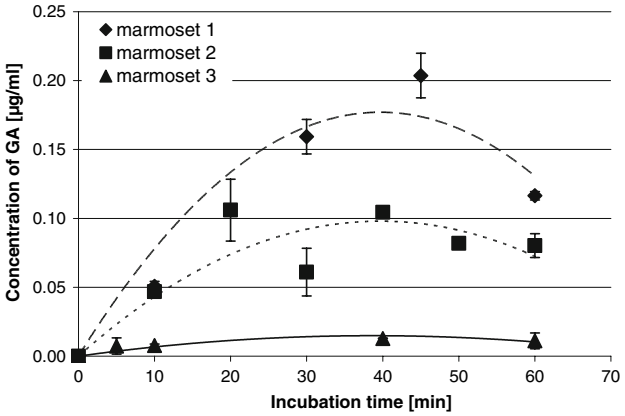

Fig. 6 Time dependency of the formation of GA from AA in liver microsomes of three different marmosets. The data are the average of two incubations with two measurements of each sample. Standard deviation was calculated from four determinations

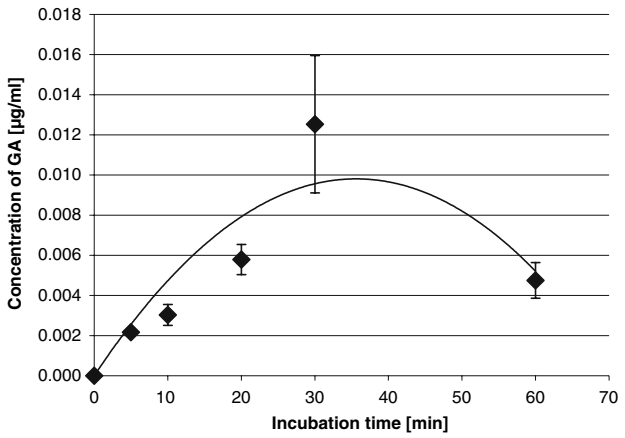

Fig. 7 Time dependency of the formation of GA from AA in genetically modified V79 cells expressing human CYP2E1. The data are the average of three separate incubations with two measurements of each sample. Standard deviation was calculated from six determinations

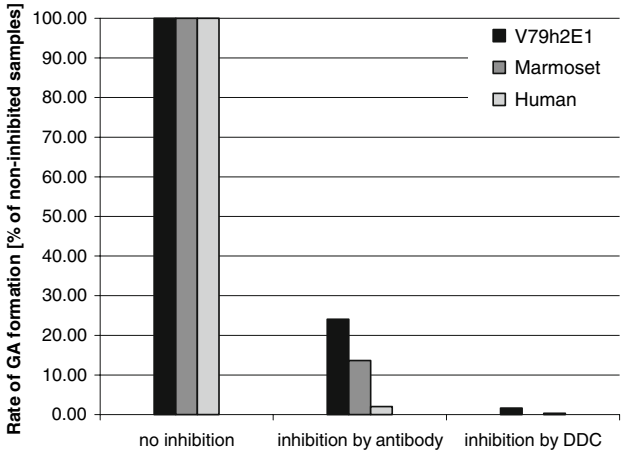

Fig. 8 Inhibition of GA formation from AA by antibody MAB-2E1 and diethyldithiocarbamate (*DDC*) in human and marmoset liver microsomes and genetically modified V79 cells expressing human CYP2E1. The data are the average of three separate incubation samples with two measurements of the samples without inhibition. The incubation period for each sample was 20 min. The data of the samples with inhibition by antibody are the average of two separate incubations with two measurements. The data of the samples with inhibition by DDC are the average of one incubation samples with two measurements

body that specifically targeted human CYP2E1. In addition DDC was used as a chemical inhibitor, which is also a rather specific inhibitor of CYP2E1-dependent reactions (Court et al. 1997). All the inhibition experiments were quite effective. When using the specific antibody MAB-2E1 inhibition was approximately 80% in V79h2E1 cells, 90% in marmoset liver microsomes and 95% in human liver microsomes. With DDC, the extent of inhibition was even more pronounced. For instance, the inhibition in marmoset liver microsomes was almost 100%. These experiments also demonstrate an unequivocal contribution of CYP2E1 to the epoxidation of AA to GA.

Discussion

Regarding the risk assessment of AA, it is of paramount importance to investigate the metabolism of AA to GA in different species as has been described in the introduction. Hepatic CYP2E1, one of the most conserved enzymes of the xenobiotic-metabolizing CYP-450 family, metabolizes a large number of low-molecular-weight compounds including compounds of toxicological and carcinogenic significance. Relevant substrates are for instance certain *N*-nitrosamines, benzene and a wide range of important industrial chemicals (Amelizad et al. 1989; Bolt et al. 2003).

Up to now, it has only been demonstrated for mice, that CYP2E1 is the enzyme involved in the conversion of AA to GA (Sumner et al. 1999; Ghanayem et al. 2005a, b). Therefore, our investigations were aimed at identifying, whether human CYP2E1 is also able to generate GA from AA. In order to address this question very specifically, we used several approaches. First we determined the extent of GA formation from AA in genetically modified V79 cells expressing human CYP2E1. As CYP2E1 is the only CYP enzyme expressed in these cells, this system allows investigating the ability of CYP2E1 to metabolize a specific substrate (Bernauer et al. 2003b). Second, to come closer to the natural situation, the findings in this system were supplemented by the use of human liver microsomes to determine whether in a system in which an array of CYPs is expressed, CYP2E1 is the most important one responsible for the formation of GA from AA. We answered this question by performing experiments in which the CYP2E1 activity was inhibited by using a specific inhibitory antibody targeting human CYP2E1 and by using DDC as a chemical inhibitor. In addition, we used so-called supersomes™ containing human CYP2E1 with a content of ~370 pmol/mg protein. In these supersomes™ AA was metabolized to GA very efficiently.

From the results obtained we were able to show that human CYP2E1 is the most relevant CYP enzyme for the metabolism of AA to GA in the in vitro systems used (Czech et al. 2007).

One major challenge was to elaborate a sensitive method for the detection of GA, which enabled us to measure the very low CYP2E1 activity present in the genetically modified V79 cells. The new method presented is an advanced development of the method used for the determination of AA in food and feed established by Pabst et al. (2005) and Klaffke et al. (2005).

Various investigations have demonstrated that the hydroxylation of CLX can be used as a specific indicator of the activity of CYP2E1, both in vivo and in vitro (Peter et al. 1990). For instance this method was used to investigate interracial differences (Kim et al. 1996) or the increased CYP2E1-activity in obese patients (Lucas et al. 1998). Therefore we used this method to support the findings that the metabolism of AA to GA is catalysed by CYP2E1. A good correlation could be demonstrated between the CLX hydroxylase activity and the epoxidation of AA.

Several studies have been undertaken to elucidate the metabolic fate of AA in vivo. The results indicate that GA is a metabolite of AA not only in rodents but also in humans (Fennell et al. 2005, 2006). For instance, Fennell and Friedman (2005) evaluated AA metabolites in the urine of volunteers who drank water containing 3 mg AA/kg bw. Approximately 34% of the administered dose was recovered in the first 24 h and 86% of the metabolites were derived from GSH-conjugation (72% *N*-acetyl-*S*-(3-amino-3-oxopropyl)cysteine and 14% of its *S*-oxide metabolite. GA, conjugated GA or its hydrolysis metabolites were

13.5% of the total metabolites. Free GA represented less than 3% of the total urinary metabolites.

In a second study with six healthy volunteers having consumed a meal, which contained 0.94 mg of AA, unchanged AA, its mercapturic acid metabolite (AAMA), GA and the respective metabolite of GA (GAMA) were quantified. Overall 60.3 ± 11.2% of the dose was recovered in the urine. GA was not found, AA, AAMA and GAMA accounted for urinary excretion of 4.4 ± 1.5%, 50 ± 9.4% and 5.9 ± 1.2% of the dose, respectively (Fuhr et al. 2006).

From the results of our study it can now be concluded that also in humans CYP2E1 is the main metabolizing CYP enzyme involved in the conversion from AA to GA which is probably the relevant metabolite with regard to an initiating potential.

To infer from the data in rodents to the situation in man, it has to be evaluated whether the same mechanisms take place in different species in order to draw qualitative conclusions. This has been elucidated by the results of studies on the metabolism of AA in humans cited above (Fennel and Friedman 2005; Fuhr et al. 2006). Therefore, one could compare the formation rate of GA in laboratory animals with those in humans. Furthermore, it may be feasible to replace the toxicokinetic portion of the intraspecies default assessment factor by a pathway-specific factor given the known variability of CYP2E1 expression in the liver in the human population (Lipscomb et al. 2003; Lipscomb 2004; Snawder and Lipscomb 2000).

In conclusion, our work shows that AA is metabolized by human CYP2E1 to GA. GA is considered to be the relevant metabolite of AA with respect to the carcinogenic activity of AA. Although CYP2E1 is highly conserved between species, this enzyme is not identical in different species (Bernauer et al. 2003b). So it can be assumed that species differences in GA formation are caused by species differences in CYP2E1 activities. The characterization and quantification of species-dependent differences with regard to GA formation will be addressed by our further work. This will be performed by further experiments with genetically modified V79 cells expressing CYP2E1 from rat and mouse to make the various enzymatic activities from various species comparable.

Interestingly, results of our study show that interspecies differences exist even between humans and a species, which is nearer to man than rodents. In this case, microsomes from marmoset monkeys were metabolically less active than microsomes from humans. Hence, one would conclude that this species was less sensitive than humans. It is important to consider this fact when deriving risk estimates from animal studies.

In addition, based on the results of our study, a pathway-specific assessment factor accounting for the toxicokinetic portion of the species variability can be derived and can replace the commonly used default factor, a procedure which has been proposed by IPCS (2005). In summary, our work is a major contribution to an improved quantitative risk assessment of AA.

Acknowledgments We thank Prof. Dr. Ralf Stahlmann (FU Berlin) for providing livers from marmoset monkeys. The most excellent technical assistance of Mrs. Almstadt, Mrs. Henschke and Mrs. Storm is gratefully acknowledged.

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