Human cytochromes P450 1A1 and 1A2 participate in detoxication of carcinogenic aristolochic acid

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Abstract

OBJECTIVES: A carcinogenic and nephrotoxic plant alkaloid, aristolochic acid (AA), causes the development of aristolochic acid nephropathy, which is characterized by chronic renal failure, tubulointerstitial fibrosis and urothelial cancer. AA may also cause a similar type of kidney fibrosis with malignant transformation of the urothelium, the Balkan endemic nephropathy. The aim of the study was to resolve which cytochromes P450 (CYP) detoxicate the major component of AA, aristolochic acid I (AAI), to its *O*-demethylated metabolite, aristolochic acid Ia (AAIa).

METHODS: High performance liquid chromatography (HPLC) was employed for separation and characterization of AAI metabolites generated by CYPs.

RESULTS: Human, rat and mouse hepatic CYPs oxidize AAI into its detoxication metabolite AAIa. Most of the detoxication of AAI in human hepatic microsomes is mediated by CYP1A2 and 1A1, while other CYPs play a minor role.

CONCLUSIONS: The data are the first report on identification of human CYP enzymes detoxicating AAI.

Abbreviations

AA	- aristolochic acid
AAI	- aristolochic acid I
AAIa	- aristolochic acid Ia
AAII	- aristolochic acid II
AAN	- aristolochic acid nephropathy
α-NF,	- α-naphtoflavone
β-NF	- β-naphtoflavone
BEN	- Balkan endemic nephropathy
CYP	- cytochrome P450
HPLC	- high performance liquid chromatography
IC_{50}	- 50% inhibitory dose
$NADP^+$	- nicotine amide dinucleotide phosphate
NADPH	- nicotine amide dinucleotide phosphate reduced
PB	- phenobarbital
v/v	- volume in volume

ORIGINAL ARTICLE

INTRODUCTION

The plant extract of *Aristolochia* species, aristolochic acid (AA) (Figure 1), was proven to be the cause of so-called Chinese herbs nephropathy, a unique type of rapidly progressive renal fibrosis associated with the prolonged intake of Chinese herbal remedies during a slimming regimen, observed for the first time in Belgium in 1991 [12,23]. The observed nephrotoxicity has been traced to the ingestion of herbal preparation *Aristolochia fangchi* containing nephrotoxic AA inadvertently included in slimming pills [23]. Therefore, this disease is now called aristolochic acid nephropathy (AAN) [3,4]. Recently, a high prevalence of urothelial cancer was found in the cohort of AAN patients [1,10]. These findings highlight the carcinogenic potential of AA to humans. Indeed, AA is among the most potent 2% of known carcinogens [3,8].

It is also noteworthy that AA consumption may be a cause for the development of a similar type of kidney fibrosis with malignant transformation of the urothelium, the Balkan endemic nephropathy (BEN) [2,9,20,22], which is widely found in certain areas of Romania, Croatia, Bosnia, Serbia and Bulgaria along the Danube river basin [13,20,22]. Recent experimental data shows that AA might be one of the most important etiologic factors in BEN and associated urothelial cancer [2,6,13]. AA exposure is associated with chronic dietary uptake of seeds of *Aristolochia clematitis* by the population living in BEN regions [2,7,9].

One of the common features of AAN and BEN is that not all individuals exposed to AA suffer from nephropathy and tumor development. We have suggested earlier that one cause for these different responses may be individual differences in the activities of the enzymes catalyzing the detoxication and/or activation of AA [14,15,18]. Therefore, understanding which enzymes are involved in AA activation and/or detoxication is important in the assessment of an individual's susceptibility to this carcinogen. AAI, the major component of AA, is activated by simple nitroreduction to form a cyclic *N*-acylnitrenium ion as the ultimate carcinogenic species binding to DNA [3]. The most important human and rat enzyme activating AAI *in vitro* is hepatic and renal cytosolic NAD(P)H:quinone oxidoreductase, followed by hepatic microsomal cytochrome P450 (CYP) 1A1/2 and renal microsomal NADPH:CYP reductase, besides prostaglandin H synthase (cyclooxygenase), which is highly expressed in urothelial tissue (for a summary, see [14]).

The oxidation of AAI to aristolochic acid Ia (AAIa, Figure 2) has been suggested to be a detoxication pathway of AAI [3,14,15,18,24]. In contrast to the enzymes activating AAI, those participating in AAI oxidation to AAIa have not been extensively studied so far. Our preliminary studies indicated that CYP enzymes can generate this oxidative metabolite [14]. Indeed, Xiao et al. [24] showed novel data concerning the enzymes detoxicating AAI. The authors' results indicate that mouse hepatic CYPs detoxify AAI by its demethylation to AAIa, and thereby protect the kidney from AAI-induced injury. CYP1A enzymes were suggested to be the most important for such a process [24]. However, the experiments used by these authors, evaluating CYP1A participation in formation of AAIa did not bring unambiguous results. Therefore, here we further investigated whether CYP1A and/or other CYP enzymes oxidize AAI to AAIa. Human hepatic microsomes and microsomes of model organisms (rat and mouse) were utilized for such a study.

MATERIAL AND METHODS

Chemicals and enzymes

The natural mixture consisting of 65% AAI and 34% aristolochic acid ii (AAII) was a gift from Madaus (Cologne, Germany). AAI was isolated from the mixture by preparative HPLC; its purity was 99.7% as estimated by HPLC [16,17]. Supersomes™, microsomes isolated from insect cells transfected with baculovirus constructs containing the cDNA of human CYP enzymes and expressing NADPH:CYP reductase and human male and female hepatic microsomes were obtained from Gentest Corp (USA).

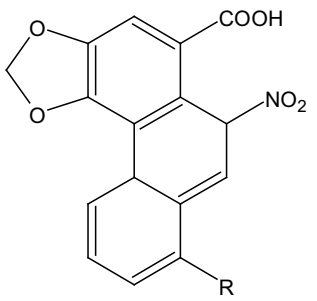

Figure 1. Aristolochic acid I (R = OCH3) and II (R = H)

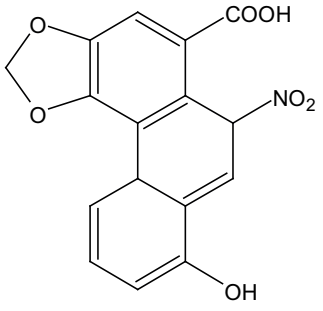

Figure 2. Aristolochic acid Ia

Animal experiments and preparation of microsomes

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which complies with Declaration of Helsinki. Microsomes from livers of three male C57BL/6 mice, those from livers of ten male untreated Wistar rats and those of rats pretreated with β-nafthoflavone (β-NF), Sudan I and phenobarbital (PB) were prepared by the procedure described previously [5,17,19]. Mouse and rat liver microsomes contained 0.1 and 0.6 nmol CYP/mg protein, respectively. Hepatic microsomes of rats induced with β-NF, Sudan I and PB contained 1.3, 1.2 and 2.7 nmol CYP/mg proteins, respectively.

Incubations

The incubation mixtures in a final volume of 500 µl, consisted of 0.1 mM phosphate buffer (pH 7.4), 1 mM NADPH, human, rat or mouse microsomes containing 0.5 mg protein and 11 µM AAI. Incubations with human CYPs in Supersomes™ in a final volume of 250 µl, consisted of 0.1 M phosphate buffer (pH 7.4), 1 mM NADPH generating system (10 mM $MgCl_2$; 10 mM glucose-6-phosphate; 1 mM $NADP^+$; 1 unit/ml glucose-6-phosphate dehydrogenase), 1 µM CYP in Supersomes™ and 11 µM AAI. All incubations were carried out at 37 °C for 20 min. The formation of AAIa from AAI was linear up to 30 min of incubation (data not shown). After extraction of incubation mixtures with ethyl acetate (2 × 1 ml), residual AAI and its metabolites were evaporated to dryness and dissolved in 30 µl methanol. AAI and AAIa were identified using HPLC and mass spectroscopy. HPLC was performed with a reversed phase column (Nucleosil 100-5 C18, 250 × 4 mm, Macherey-Nagel) using a linear acetonitrile-triethylamonium acetate gradient of 0% acetonitrile rising to 50% in 53 min (flow rate of 1 ml/min, detection at 250 and 318 nm). AAI and AAIa were eluted with retention times of 36.7 and 28.8 min, respectively.

RESULTS

Human, rat and mouse hepatic microsomes oxidize AAI to AAIa

The efficiency of hepatic microsomes from three species (humans, rats and mice) to oxidize AAI to AAIa was examined. In the presence of NADPH, a cofactor of NADPH:CYP reductase- and CYP-dependent enzyme systems, AAI is *O*-demethylated by hepatic microsomes of all species to AAIa, which was separated from AAI with HPLC (see Figure 3 for rat hepatic microsomes). No oxidation of AAI was detectable when NADPH was omitted from incubations. This finding suggests that CYP enzymes are responsible for AAI oxidation. Human and rat hepatic microsomes have similar efficiencies to oxidize AAI, while mouse hepatic microsomes were less effective (Figure 4).

Evaluation of CYP enzymes oxidizing AAI in rat hepatic microsomes

In order to evaluate the role of the rat hepatic CYP-mediated oxidation of AAI, formation of AAIa by hepatic microsomes of rats treated with inducers of several CYPs (β-NF and Sudan I as inducers of CYP1A1/2 and PB as an inducer of CYP2B) was compared. All microsomes were capable of oxidizing AAI to form AAIa. Under the experimental conditions used, the highest levels of AAIa were formed by microsomes of untreated (control) rats, whereas those of rats treated with inducers of CYP1A (β-NF, Sudan I) and CYP2B (PB) were less effective (Figure 5).

Evaluation of CYP enzymes oxidizing AAI in human hepatic microsomes

The role of human hepatic CYP enzymes in AAI oxidation was investigated by modulation of this reaction by selective inhibitors of individual CYPs. Male human hepatic microsomes were used for such experiments. Furafylline and α-NF, selective inhibitors of CYP1A2

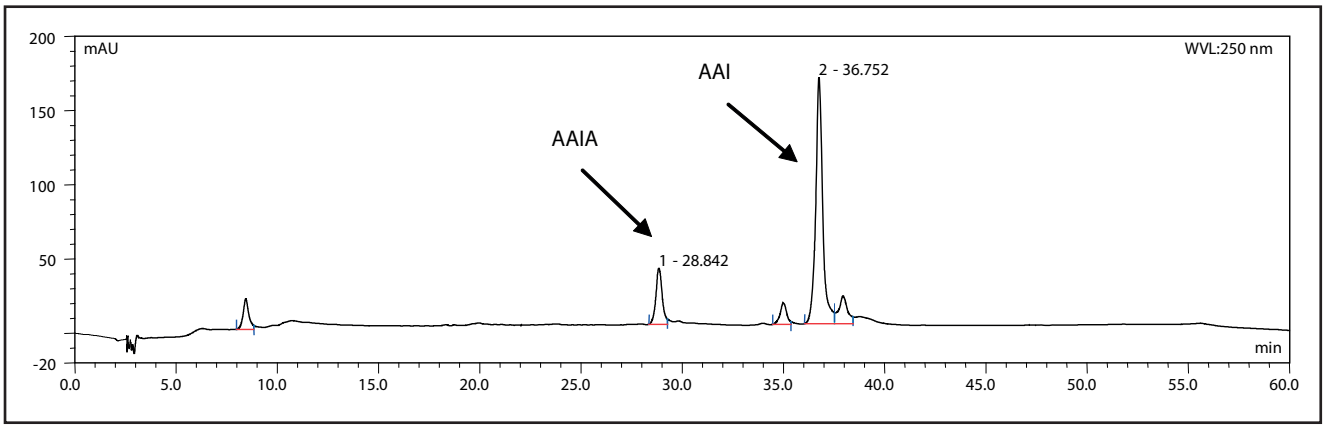

Figure 3. HPLC separation of AAI and AAIa formed by rat hepatic microsomes (0.5 mg protein, 1 mM NADPH and 11 µM AAI)

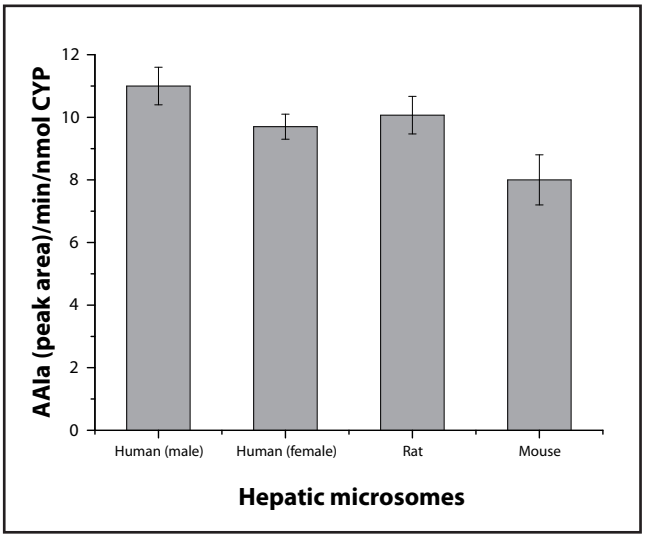

Figure 4. AAIa formation from AAI by human, rat and mouse hepatic microsomes

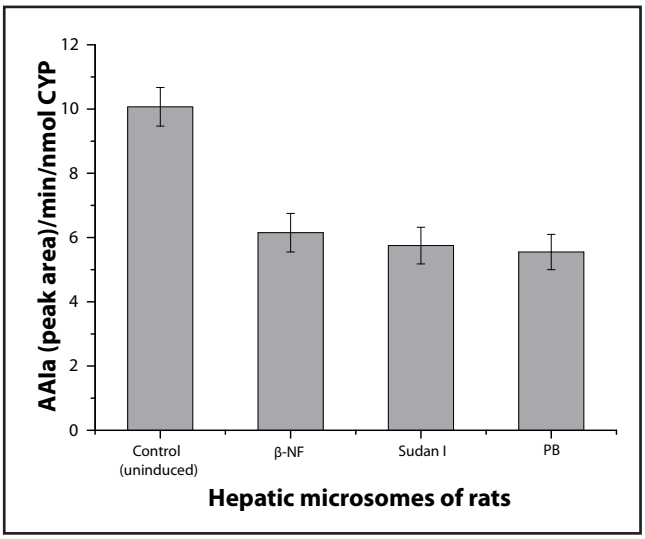

Figure 5. AAIa formation from AAI by rat hepatic microsomes

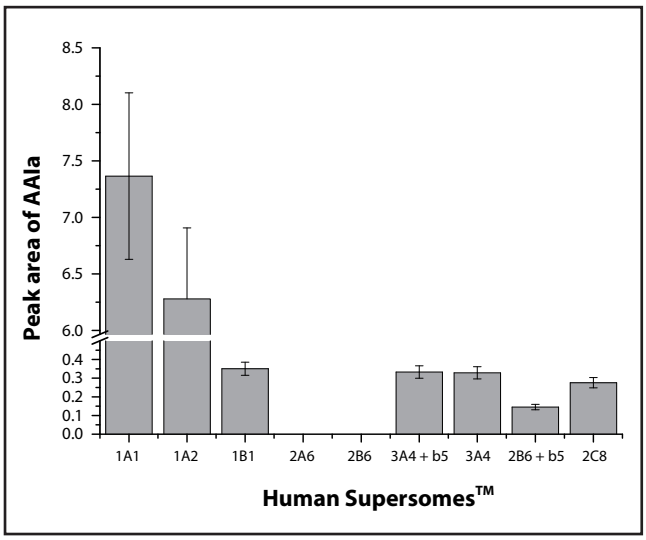

Figure 6. AAIa formation from AAI by human CYPs

and CYP1A1/2, inhibited AAI oxidation, exhibiting the IC_{50} values of 3.1 and 6.2 μM, respectively. On the contrary, inhibitors of CYP2B, 2C and 3A4, diamantane, sulfaphenazole and ketoconazole, respectively, were without effect. This finding suggests a major role of CYP1A enzymes in AAI oxidation to AAIa in human liver microsomes.

Oxidation of AAI by human recombinant CYPs

To further investigate the role of human CYP enzymes in AAI oxidation, Supersomes™ containing human recombinant CYPs were used in additional experiments. Human CYP1A1, followed by CYP1A2, were the most effective human enzymes oxidizing AAI to AAIa. Other CYPs such as CYP1B1, 2C8, 3A4 and CYP2B6 with cytochrome b_5, also oxidize AAI, but with efficiencies more than one order of magnitude lower than CYP1A (Figure 6).

DISCUSSION

Understanding which enzymes are involved in metabolism (detoxication and/or activation) of toxicants is important in the assessment of an individual's susceptibility to such xenobiotics. In the case of the compound studied in this work, carcinogenic and nephrotoxic AAI, the enzymes catalyzing the reductive activation of AAI have already been identified (for a summary, see [14]). Here, we increased our knowledge on the enzymes detoxicating this carcinogen. Even though the experiments of Xiao and coworkers [24] suggest that mouse hepatic CYP1A might generate the AAI detoxication metabolite, AAIa, the exact proof for this finding was missing. The results of this work demonstrate that CYP enzymes present in microsomes of human, rat and mouse livers are capable of oxidizing AAI to AAIa. Using inducers of CYP1A1 and 1A2 in rats we found that CYP1A1 and 1A2 seem not to be the predominant enzymes oxidizing AAI in rat livers. However, this finding needs to be confirmed by further experiments. The detailed study utilizing individual rat CYPs to confirm or exclude this finding is under way in our laboratory. On the contrary, human CYP1A are the major CYP enzymes responsible for AAI oxidation in human liver microsomes. Here, we show that selective inhibitors of these enzymes, α-NF and furafylline, effectively inhibited AAI oxidation by human hepatic microsomes. In addition, human recombinant CYP1A1 and 1A2 were the most efficient CYPs oxidizing AAI. Recently, we have found that under the anaerobic conditions, human CYP1A enzymes also reductively activate AAI to species forming DNA adducts [21]. Therefore, the oxygen concentration of tissues may affect the relative extents of AAI activation by nitroreduction and its detoxication by *O*-demethylation. In addition, orientation and moving of the AAI molecule in the active sites of CYP1A1 and 1A2 should also influenced the pathways of AAI metabolism, namely, AAI *O*-demethylation *ver-*

sus reduction of the nitro group of AAI. The *in silico* docking of AAI to the active sites of CYP1A1 and 1A2 employing soft-soft (flexible) docking procedure [11] is planned to be utilized for further studies to explain the molecular mechanisms of oxidation and reduction of AAI by human CYP1A1 and 1A2.

ACKNOWLEDGMENT

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