The effects of heavy metal ions, phthalates and ochratoxin A on oxidation of carcinogenic aristolochic acid I causing Balkan endemic nephropathy

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Abstract

OBJECTIVES: Balkan endemic nephropathy (BEN) is a chronic progressive fibrosis associated with upper urothelial carcinoma (UUC). Aetiology of BEN is still not fully explained. Although carcinogenic aristolochic acid I (AAI) was proven as the major cause of BEN/UUC, this nephropathy is considered to be multifactorial. Hence, we investigated whether other factors considered as potential causes of BEN [a mycotoxin ochratoxin A (OTA), Cd, Pb, Se and As ions and organic compounds (i.e. phthalates) released from lignite deposits in BEN areas] can influence detoxication of AAI, whose concentrations are crucial for BEN development.

METHODS: Oxidation of AAI to 8-hydroxyaristolochic acid I (AAIa) in the presence of Cd, Pb, Se, As ions, dibutylphthalate (DBP), butylbenzylphthalate (BBP), bis(2-ethylhexyl)phthalate (DEHP) and OTA by rat liver microsomes was determined by HPLC.

RESULTS: Only OTA, cadmium and selenium ions, and BBP inhibited AAI oxidation by rat liver microsomes. These compounds also inhibited activities of CYP1A1 and/or CYP2C6/11 catalysing AAI demethylation in rat livers. Therefore, these CYP inhibitions can be responsible for a decrease in AAIA formation. When the combined effects of these compounds were investigated, the most efficient inhibition was caused by OTA combined with BBP and selenium ions.

CONCLUSION: The results show low effects of BBP, cadmium and selenium ions, and/or their combinations on AAI detoxication. No effects were produced by the other metal ions (Pb, As) and phthalates DBP and DEHP. This finding suggests that they do not influence AAI-mediated BEN development. In contrast, OTA might influence this process, by inhibition of AAI detoxication.
INTRODUCTION

Balkan endemic nephropathy (BEN) is a chronic progressive renal fibrosis affecting rural population in Bulgaria, Bosnia and Herzegovina, Romania, Croatia and Serbia along Danube river basin (Stefanovic 1983; Radanovic 2002). The unique feature of this disease is that BEN seems to be a familial, but not inherited occurring in several endemic areas (Toncheva et al. 1998; Radanovic 2002; Grollman 2013). Moreover, this serious disease is closely associated with upper urothelial carcinomas (UUC) of the renal pelvis and ureter (Stefanovic 1983; Jankovic et al. 2002). Although BEN has been studied for more than 50 years, aetiology of this nephropathy is still a matter of debate. There are several hypotheses suggesting that BEN/UUC is the multifactorial disease which might be occurring in several endemic areas (Ivic 1969). This plant alkaloid found in Aristolochia species was found in wheat used for home-prepared bread (Ivic 1969; Jelakovic et al. 2012; Gokmen et al. 2013). The plant extract of AA is a mixture of structurally related nitrophenanthrene carboxylic acids whose major components are aristolochic acid I (AAI) and aristolochic acid II (AAII). AAI is supposed to be the predominant compound responsible for BEN development. AA and herbal products derived from genera Aristolochia have been classified by International Agency for Research on Cancer (IARC) as carcinogenic to human (Group 1) (Grosse et al. 2009). Furthermore, this nephrotoxic and carcinogenic agent was found to cause another disease similar to BEN, aristolochic acid nephropathy (AAN) (Cosyns et al. 1994; Arlt et al. 2002b, 2007; Debelle et al. 2008; Schmeiser et al. 1996, 2009; Gokmen 2013).

In contrast to the finding that AAI might directly cause interstitial nephropathy, metabolic activation of this alkaloid to species forming DNA adducts is a necessary step for AAI-induced malignant transformation (Cosyns et al. 1994; Arlt et al. 2002b; Schmeiser et al. 1996, 2009; Chen et al. 2012). In organisms, AAI can be either reductively activated to N-acyltrimetnium ion leading to AAI-DNA adduct formation or oxidatively detoxified to an O-demethylated product, 8-hydroxyaristolochic acid (aristolochic acid Ia, AAI; Figure 1) (Arlt et al. 2002b, 2007; Grollman et al. 2007; Stiborova et al. 2014a, 2014b). The AA-DNA adducts formed from activated AAI with adenosine and guanosine residues in DNA were found in BEN and AAN patients (Schmeiser et al. 1996; Bieler et al. 1997; Arlt et al. 2002a). The most persistent DNA adduct, 7-(deoxyadenosin-N6-yl)-aristolactam I (dA-AAI), is proposed to cause a characteristic AT→TA transversion mutations which have been detected in the TP53 tumour suppressor gene in tissues of patients from the endemic areas. Such AT→TA transversions are responsible for tumour development in patients suffering from BEN and AAN (Arlt et al. 2007; Grollman et al. 2007; Stiborova et al. 2008; Hollstein et al. 2013). These findings indicate that the concentration of AAI in organisms is essential for both renal injury and induction of UUC initiated by activated AAI. The effective concentration of AAI in organism is dictated by its metabolism. Since AAI can be both bio-activated to reactive species forming AAI-DNA adducts resulting in cancer development and detoxified to AAIs, these reactions might significantly modulate the AAI toxic/genotoxic potential (Stiborova et al. 2008, 2011a, 2012, 2013b; Arlt et al. 2011).

A common feature of BEN/UUC is that not all individuals exposed to AAI suffer from these diseases (Arlt et al. 2002b, 2011; Stiborova et al. 2008, 2012, 2013b, 2015; Jelakovic et al. 2012). This phenomenon might be explained by different efficiencies of enzymes participating in metabolism of AAI and by genetic sensitivity of individuals (Stiborova et al. 2001, 2003, 2008, 2013b; Toncheva et al. 2004; Grollman 2013). Therefore, detailed understanding of enzymes involved in AAI
metabolism (activation and/or reduction) is crucial for risk assessment of AA exposure.


However, it is still not exactly known whether the other compounds suggested to be responsible for BEN development might influence the AAI-induced BEN. Hence, in this work, we investigated whether OTA, heavy metal ions and organic chemicals released from lignite deposits in the endemic areas can influence the detoxication of AAI, whose concentrations are crucial for BEN development. Heavy metal ions and organic compounds were selected according to data from epidemiologic studies (Radic et al. 1997; Karmaus et al. 2008; Pfohl-Leszkowicz 2009; Yordanova et al. 2010; Maharaj et al. 2014).

**MATERIALS AND METHODS**

**Chemicals**

AAI sodium salt, CdCl2, Pb(CH3COO)2, Na2SeO3, Na2HASO4·7H2O, dibutylphthalate (DBP), butylbenzylphthalate (BBP) and bis(2-ethylhexyl)phthalate (DEHP) as well as other chemicals were purchased from Sigma Chemical Co. (St. Louis MO, USA). All chemical were of 97% purity or better.

**Preparation of rat hepatic microsomal fraction**

Microsomes were prepared from liver of untreated Wistar rats by differential centrifugation as described previously (Stiborova et al. 2013a; Indra et al. 2014).

**AAAla formation in the presence of heavy metal ions/phthalates/OTA**

Incubation mixtures, in a final volume 500 μL, consisted of 100 mmol.L⁻¹ potassium buffer (pH 7.4), NADPH-generation system (1 mmol.L⁻¹ NADP⁺, 10 mmol.L⁻¹ MgCl₂·6H₂O, 1 U/mL glucose-6-phosphate dehydrogenase), 1–100 μmol.L⁻¹ of heavy metal ions/phthalates/OTA, 0.25 mg rat hepatic micro-

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**Fig. 1.** Scheme of detoxication and bio-activation of AAI in organisms. dA-AAI, 7-(deoxyadenosin-N⁶-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-N²-yl)aristolactam I; UGT, UDP glucuronosyl transferase; SULT, sulfotransferase.
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Some microsomes and 10 μmol.L⁻¹ AAI, CdCl₂, Pb(CH₃COO)₂, Na₂SeO₃, Na₂HASO₄·7H₂O were dissolved in distilled water and OTA was dissolved in 0.1 mol.L⁻¹ NaHCO₃ (pH 7) whereas phthalates were prepared in acetonitrile. Incubations with microsomes were carried out at 37 °C for 10 min and AAI oxidation to AAla was linear up to 25 min (Levova et al. 2011; Stiborova et al. 2012). Control incubations were carried out (i) without microsomes, (ii) without NADPH-generating system or (iii) without AAI. AAI and AAla were analysed by high performance liquid chromatography (HPLC) as described (Sistkova et al. 2008; Levova et al. 2011; Stiborova et al. 2012).

**HPLC analysis of AAla formation**

AAI and its O-demethylated metabolite (AAla) were extracted from incubations with ethyl acetate (2 x 1 mL), the extracts were evaporated to dryness and the residues redissolved in 30 μL of methanol and subjected to reverse-phase HPLC. HPLC was performed with a reversed phase column (Nucleosil 100-5 C₁₈, 25 x 4.0 mm, 5 mm; Macherey-Nagel) preceded by a C-18 guard column, using a linear gradient of acetonitrile (20–60% acetonitrile in 55 min) in 100 mmol.L⁻¹ triethylammonium acetate with a flow rate of 0.5 mL.min⁻¹. A Dionex HPLC pump P580 with UV/VIS UVD 170S/340S spectrophotometer detector was set at 250 nm and CHROMELEON™ 6.01 integrator was used for integration of peaks. AAla and AAI eluted with retention times (r.t.) of 24.5 and 37.7 min, respectively. The product eluting at 24.5 min was identified as AAla by mass spectrometry previously (Sistkova et al. 2008; Levova et al. 2011; Stiborova et al. 2011b).

**Determination of CYP1A1/2 and CYP2C6/11 enzyme activities**

In rat hepatic microsomes, CYP1A1/2 was determined by ethoxyresorufine-O-deethylation (EROD) (Burke et al. 1994). Enzyme activity of CYP1A1 was measured as capability of Sudan I oxidising (Stiborova et al. 2002). CYP2C6/11 activities in rat microsomes were characterised as well: CYP2C6 was measured with diclofenac as a marker substrate (Kaphalia et al. 2006) and CYP2C11 activity was determined as testosterone 16α-hydroxylation (Yamazaki et al. 2006). The effect of heavy metal ions/phthalates/OTA on the above mentioned enzyme activities was carried out by addition of compounds tested to incubation mixtures in a final concentration of 100 μmol.L⁻¹.

**Statistical analyses**

For statistical data analysis we used Student’s t-test. All p-values are two-tailed and considered significant at the 0.001 level.

**RESULTS AND DISCUSSION**

**Oxidation of AAI to AAla in the presence of the heavy metal ions, phthalates and ochratoxin A**

In rat microsomes, AAI was oxidised to one metabolite eluted by HPLC at r.t. of 24.5 minutes (Figure 2). This metabolite was previously identified by positive MALDI-TOF-TOF analysis as AAla (Levova et al. 2011; Stiborova et al. 2011b). Because of a low toxicity, AAla was considered to be the detoxication metabolite of AAI (Shibutani et al. 2010). The effects of compounds, which were suggested that might contribute to development of BEN/UUC, namely the heavy metal ions, phthalates and OTA, on detoxication of AAI to AAla catalysed by rat liver microsomes are shown in Figures 3–5. Cadmium and selenium ions inhibited AAla formation (Figures 3A,C) whereas no such effect was found in the presence of Pb²⁺ and arsenate ions (Figures 3B,D). However, the 10-times higher concentration of cadmium and selenium ions than the concentration of AAI was necessary for the significant decrease in AAI oxidation. Of phthalates examined, only butylbenzylphthalate (BBP) inhibited oxidation of AAI to AAla.
The other two studied phthalates (DBP and DEHP) that are known as important toxic environmental pollutants (Ferguson et al. 2014; Yan et al. 2015) were without this effect (Figures 4A,C). In the case of OTA, the significant inhibition of AAI demethylation by this mycotoxin was found. The 10 and 100 μmol.L⁻¹ OTA led to a 23% and 42% decrease in AAIa formation, respectively (Figure 5). These findings are consistent with the results found in our former study which demonstrated that OTA is capable of inhibiting AAI oxidation to AAIa in vivo (Stiborova et al. 2015).

Because human population living in the endemic areas might be exposed not only to each of these compounds individually but also to their combination, in the next step of this study we investigated a combined effect of the substances that inhibited AAI oxidation, namely, cadmium and selenium ions, BBP and OTA (Figure 6). Interestingly, although oxidation of AAI to AAIa was not influenced by a combination of BBP and OTA, all four contaminants (cadmium and selenium ions, BBP and OTA) added to incubation mixtures led to the significant inhibition of AAIa formation, by 34% (Figure 6B). In addition, the most efficient inhibition of AAI demethylation was caused by OTA combined with BBP and selenium ions, by 37% (Figure 6B). Using the other combinations, no additive effects of these combinations compared to inhibition caused by individual compounds were found. This phenomenon is now difficult to be explained. One can speculate that compounds might compete against each other, thereby decreasing the inhibition of enzymes involved in AAI oxidation or inter-molecular interactions between the
tested compounds might decrease their actual concentrations. These suggestions need, however, to be explored in further studies.

Effect of the heavy metal ions, butylbenzylphthalate and ochratoxin A on enzymatic activities of cytochromes P450.

In order to evaluate the mechanisms of inhibition of AAI oxidation in rat microsomes, the effects of the compounds found to inhibit this reaction (Figures 3–5) on activities of the major enzymes participating in AAI detoxication were analysed. Namely, the effects of cadmium and selenium ions, BBP and OTA on activities of CYP1A and 2C6/11 enzymes were tested. The enzyme activities were determined utilising a marker substrates (see the Material and Methods section). The data shown in Table 1 demonstrate that CYP1A1 activity was inhibited mainly by Cd^{2+} and BBP; a 54 and 75% decrease in a CYP1A1 marker activity (Sudan I oxidation) was found, respectively, whereas the other tested compounds did not influence CYP1A activity. In the case of CYP2C, BBP significantly inhibited activity of CYP2C6, whereas OTA decreased activity of CYP2C11, the CYP enzyme that is predominantly expressed in rat liver (Zachaňová et al. 2012). Based on these results, the decreased oxidation of AAI to AAIa caused by tested
The effects of cadmium and selenium ions, BBP, and OTA on AAI detoxication to AAIa. Incubations with CdCl₂, Na₂SeO₃ and OTA (A) and in combination of these compounds with BBP (B) were carried out. Values are given as means ± standard deviations of three experiments (n = 3). Values significantly different from incubation with buffer/acetonitrile only: *p<0.1, **p<0.01, ***p<0.001 (Student’s t-test). The incubation mixtures contained 0.5 mg.mL⁻¹ microsomal protein, 10 μmol.L⁻¹ CdCl₂, Na₂SeO₃, dissolved in distilled water, OTA dissolved in 0.1 mol.L⁻¹ NaHCO₃ (pH 7) or BBP dissolved in acetonitrile, the NADPH-generating system containing 1 mmol.L⁻¹ NADP⁺, and 10 μmol.L⁻¹ AAI dissolved in distilled water (see Materials and Methods). Numbers above the columns ("F") indicate fold changes in amounts of AAIa compared to incubations without the above mentioned compounds.

Tab. 1. The effects of cadmium and selenium ions, BBP, and OTA on enzyme activity of rat CYP1A and 2C.

<table>
<thead>
<tr>
<th>Pollutant</th>
<th>EROD activity</th>
<th>Sudan I oxidation</th>
<th>Diclofenac 4'-hydroxylation</th>
<th>Testosterone 16α-hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(CYP1A)</td>
<td>(CYP1A1)</td>
<td>(CYP2C6)</td>
<td>(CYP2C11)</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>83±0.09*</td>
<td>46±4.27***</td>
<td>89±0.44*</td>
<td>100±6.43</td>
</tr>
<tr>
<td>Na₂SeO₃</td>
<td>95±0.83</td>
<td>100±2.62</td>
<td>94±5.59</td>
<td>82±1.01**</td>
</tr>
<tr>
<td>OTA</td>
<td>100±7.50</td>
<td>99±4.93</td>
<td>NEb</td>
<td>18±1.24***</td>
</tr>
<tr>
<td>BBP</td>
<td>83±1.24*</td>
<td>25±1.27***</td>
<td>7±0.26***</td>
<td>68±12.48**</td>
</tr>
</tbody>
</table>

Data are expressed as % of control without pollutants. Values in the table are averages ± standard deviations of three experiments (n = 3). The incubation mixtures contained 0.5 mg.mL⁻¹ microsomal protein, 10 μmol.L⁻¹ AAI (dissolved in distilled water), the NADPH-generating system containing 1 mmol.L⁻¹ NADP⁺, and 100 μmol.L⁻¹ pollutant [heavy metal ions dissolved in distilled water, OTA dissolved in 0.1 mol.L⁻¹ NaHCO₃ (pH 7) or BBP dissolved in acetonitrile] (see Materials and Methods). Values significantly different from control incubations without pollutants: *p<0.1, **p<0.01, ***p<0.001 (Student’s t-test). bIsomers of CYP whose enzyme activity is measured are shown in brackets.
increase actual concentrations of AAI by inhibition of its detoxication, may enhance the development of AAI-induced UUC in BEN patients.

CONCLUSIONS

The results found in this study demonstrate that AAI detoxication to AAla is inhibited by OTA, phthalate BBP and partially also by cadmium and selenium ions. Such inhibition is caused by inhibition of the CYP1A1 and 2C enzymes that catalyse this reaction. Even though these inhibitions are not fatal, their contributions to the AAI-mediated development of BEN/UUC, considering mainly the chronic exposure of population living in the endemic areas to these pollutants, cannot be ruled out.

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