The Effects of Curcumin on Aflatoxin B1-Induced Toxicity in Rats

SARANYA POAPOLATHEP¹, KANJANA IMSILP¹, KENJI MACHII³, SUSUMU KUMAGAI² AND AMNART POAPOLATHEP¹*

¹Department of Pharmacology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand
²Research Center for Food Safety, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 1138657, Japan
³Division of Biomedical Food Research, National Institute of Health Sciences, Tokyo 1588501, Japan

Received 23 November, 2014/Accepted 8 February, 2015

To evaluate the potential of curcumin on toxic and carcinogenic effects of Aflatoxin B1 (AFB1) in relation to AFB1 metabolism, we studied the effects of curcumin on hepatic AFB1-DNA adduct formation and glutathione S-transferase (GST) activity, and the toxic effects of AFB1 in male Fischer 344 rats. Oral administration of curcumin to 5-week-old male rats at a dose of 8 or 80 mg/kg for five consecutive days for three weeks resulted in reduction of AFB1-DNA adduct formation mediated by both liver microsomal and postmitochondrial fractions. The activity of liver GST toward a universal substrate, CDNB, was increased in curcumin-administered rats. As for the acute toxicity of AFB1, curcumin was orally administered to rats for 3 weeks and then AFB1 was given by intragastric intubation. The result showed a decrease of plasma AST and ALT activities in curcumin-treated rats compared with those which received AFB1 alone. Moreover, we have observed that curcumin also reduced glutathione S-transferase placental form positive single cells and foci caused by AFB1 treatment. These results demonstrate the potential of curcumin to reduce the toxic and carcinogenic effects of AFB1 by modulating hepatic drug metabolizing enzymes responsible for AFB1 metabolism.

Key words: Curcumin / Aflatoxin B1 / Toxicity / Hepatocarcinogenicity / Rats.

INTRODUCTION

Aflatoxin B1 (AFB1) is a potent hepatotoxic and hepatocarcinogenic mycotoxin mainly produced by Aspergillus flavus and Aspergillus parasiticus. Human exposure to AFB1 has been associated with an increased risk of hepatotoxicity and particularly of hepatocellular carcinoma (JECFA 1998; IARC, 1987). AFB1 is activated by the hepatic cytochrome P450 to produce an AFB1-8,9-epoxide, which is believed to be a principal metabolite of cellular injury leading to hepatotoxic and hepatocarcinogenic effects via binding to guanine residues in DNA to form DNA adducts (Choy, 1993; McLean and Dutton, 1995). Curcumin is a polyphenolic compound extracted from the root of Curcuma longa Linn which has profound impact on pharmacological activities and the suppression of carcinogenesis (Doll and Peto, 1981; Kelloff et al., 1994). This compound is considered to be one of the chemicals studied mostly in relation to their ability to reduce toxicity as cancer chemopreventive agents. There has been extensive evidence showing that curcumin possesses anti-inflammatory, antioxidant, free radical scavenging, chemotherapeutic, radio-protective, and carcinogen-induced tumorigenesis-inhibitory effects in laboratory animals and humans (Satoska et al., 1986; Sharma, 1976; Azuine and Bhide 1992; Kelloff et al., 1992; Rao et al., 1993; Smith et al., 2001). As it is a natural compound present worldwide, it could serve as an alternative cancer chemo-preventive agent (Murakami et al., 1999). Meanwhile, many publications have described that curcumin can reduce risk of a variety of diseases,
including myeloma, pancreatic cancer, colon cancer, psoriasis and Alzheimer’s disease (Chin et al., 2013; Gupta et al., 2013), but there is limited information on the potential effect of curcumin on AFB1-induced toxicity and hepatocarcinogenicity. The aim of this research is to investigate the effect of curcumin on AFB1-induced toxicity, hepatocarcinogenicity and the possible mechanisms involved in its action in rats.

**MATERIALS AND METHODS**

**Toxins and chemicals**

AFB1, curcumin, glucose-6-phosphate (G-6-P), G-6-P dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADPH), ethylenediaminetetraacetic acid (EDTA) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Uniformly labeled [3H] AFB1 (sp. activity, 18.6 Ci/mmol; 99.6% purity) was purchased from Moravek Biomedicals (Brea, CA, USA). ACS II (Amersham Pharmacia Biotech Co., Ltd., Chalfont St. Giles, Bucks, UK) was used as scintillation cocktails for DNA adduct determination. Other chemicals and reagents were of analytical grade.

**Animals**

Four-week-old (F344/DuCrj) male Fischer rats were purchased from Nippon Bio-Supp. Center (Tokyo, Japan), and they were acclimatized for 1 week under controlled conditions (23 ± 2 °C, 14-h light and 10-h dark cycle) fed commercial pellets (Oriental Yeast Co., Tokyo, Japan) and provided with water ad libitum.

**Experimental design**

**Exp. 1: Effect of curcumin on the formation of AFB1-DNA adducts.** Fifteen 5-week-old male Fischer rats were divided into three groups each of 5 rats (Figure 1a). After overnight fasting, the treatment groups were orally administered curcumin dissolved in corn oil at the dose of 8 and 80 mg/kg body weight (bw), once daily, five days per week for three consecutive weeks. Rats were sacrificed by heart puncture under ether anesthesia on the last day of curcumin treatment. The remaining 5 rats of the control group were orally given an equal volume of corn oil in the same way and killed. The liver tissues were flash frozen in liquid nitrogen, and stored at -80°C until in vitro determinations of the tissue activity to form AFB1-DNA adducts and GST activity toward AFB1

**Exp. 2: Effect of curcumin on AFB1-induced toxicity.** Thirty 5-week-old male Fischer rats were separated into six groups each of 5 rats (Figure 1b). For Group I, the vehicle control group, rats were orally administered corn oil five times per week for 3 consecutive weeks. For Group II, the treatment control group, rats were orally administered curcumin dissolved in corn oil at a dose level of 8 mg/ml/kg bw five times per week for 3 consecutive weeks. For Group III, the positive control group, rats were orally administered corn oil five times per week for 3 consecutive weeks. Group IV, V and VI rats were orally administered curcumin at dose levels of 0.8, 8 and 80 mg/kg bw, respectively, five times per week for 3 consecutive weeks. The rats from group I-II were later challenged with 10% DMSO in phosphate buffer saline (PBS) by oral administration at 2 h after the last time of corn oil/curcumin administration. The rats from group III-VI were later challenged with AFB1 dissolved in 10% DMSO in phosphate buffer saline (PBS) at a dose level of 1.75 mg/ml/kg bw at 2 h after the time of the last corn oil/curcumin administration. All rats were sacrificed under ether anesthesia at 48 h after AFB1 administration. Blood was collected by heart
puncture using heparinized syringes. Plasma was separated by centrifugation at 3,500 rpm for 15 min for determination of enzyme activity. The liver was taken for histopathological examination.

Exp. 3: Effect of curcumin on AFB1-induced GST-P positive foci formation. Twenty 5-week-old male Fischer rats were separated into two groups (Figure 1c). For positive control, a group of 10 rats were orally administered AFB1 dissolved in DMSO at a dose level of 0.2 mg/kg bw five times per week for 2 consecutive weeks from 6 weeks of age. The other group of rats were orally treated with curcumin dissolved in corn oil at a dose of 80 mg/kg bw five times per week for 3 consecutive weeks from 5 weeks of age, followed by AFB1 at a dose level of 0.2 mg/kg bw for 5 times per week for 2 weeks from 6 weeks of age. All the animals were then kept without any treatment until 24 weeks of age, and sacrificed under ether anesthesia in compliance with ethical guidelines. Blood and livers were taken from them.

Preparation of postmitochondrial, microsomal and cytosolic fractions

The rat livers were taken from animals under ethyl ether anesthesia and perfused with ice-cold saline to remove blood from the liver tissues. The tissues were flash-frozen in liquid nitrogen, and stored at -80 °C until use. Postmitochondrial (S-9), microsomal and cytosolic fractions were prepared as described by Bammler et al. (2000) except that a teflon glass homogenizer instead of a blender was used for tissue homogenization (Esaki and Kumagai, 2002). Protein concentrations in these fractions were measured with a spectrophotometer (U-1500, Hitachi Co., Tokyo, Japan) using bovine serum albumin (Bio-Rad Protein Assay, Bio-Rad Lab., CA) as a standard.

Determination of AFB1-DNA adducts

The microsomal activity to form AFB1-DNA adducts was determined according to the method of Kono and Kumagai (1995). Briefly, 1 mg of protein of the S-9 or microsomal fractions prepared from rat livers was pre-incubated for 5 min at 37 °C with 0.1 mg of calf thymus DNA dissolved in 0.8 mL of 0.1 M potassium phosphate buffer solution (pH 7.4) containing 2 mM NADPH, 5.6 mM G-6-P and 0.5 units of G-6-P dehydrogenase. The pre-incubated mixture was mixed with 250,000 dpm of [3H]-AFB1 dissolved in 10 µL dimethyl sulfoxide and incubated for 30 min. Radioactivity in DNA precipitated with cold ethanol was quantified in a liquid scintillation counter (LCS-5100, Aloka Co., Tokyo, Japan) using ACS II as a scintillation cocktail to determine the amount of [3H]-AFB1-DNA adducts.

Determination of glutathione S-transferase (GST) activity toward the substrates other than AFB1

The GST activity toward various substrates was determined according to the method of Habig et al. (1974). 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (EA), trans-4-phenyl-3-buten-2-one (t-PBO) and cumene hydroperoxide (CPH) were used as substrates. The changes in absorbance were measured with a spectrophotometer (U-1500, Hitachi Co., Tokyo, Japan). The specific activity of GST was expressed in terms of nmol/min/mg protein.

Determination of plasma enzymes

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined as indicators of liver cell damage and the carcinogenicity of AFB1, respectively. Clinical biochemical analyses of plasma samples were carried out with the Fuji DRI-CHEM 7000 system (FUJIFILM Corporation, Tokyo, Japan).

Histopathology

The liver was taken at necropsy, and fixed in 10% neutral buffered formalin for histopathological examinations. Paraffin sections (4 µm) were stained with hematoxylin and eosin (HE). Some of them were subjected to immunohistochemical staining for glutathione S-transferase placental form (GST-P) as mentioned below.

Immunohistochemical staining of glutathione S-transferase placental form (GST-P)

The histochemical detection of GST-P foci was carried out on the paraffin sections of rat liver by a modified method of that first proposed by Hsu et al. (1981) using an ABC kit (Vector Laboratories, Burlingame, CA, USA). Briefly, after deparaffinization with xylene, quenched with 3% hydrogen peroxide in methanol and blocked with normal goat serum, the liver tissue sections were incubated overnight with rabbit anti-rat GST-P polyclonal antibodies, followed by anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). The paraffin sections were then incubated with the avidin-biotin-peroxidase complex (ABC) using the ABC kit. GST-P positive foci were visualized by the peroxidase-diaminobenzidine (DAB) reaction (DAB substrate kit, Vector Laboratories, Burlingame, CA, USA). Counterstaining was performed with hematoxylin for microscopic examination. The area of GST-P positive foci was analyzed by Scion Image software (http://scion-image.software.informer.com/, Informer Technologies, Inc.).
Statistical analysis

Results are expressed as the mean value ± standard deviation (SD). The differences between groups were analyzed using Student’s t-test or one-way analysis of variance (ANOVA) with the Tukey test (GraphPad Software, Inc. CA, USA). Differences among groups were considered statistically significant if \( p < 0.05 \).

RESULTS

AFB1-DNA adduct formation

The levels of AFB1-DNA adduct formation mediated by liver postmitochondrial and microsomal fractions were significantly lower in rats treated with curcumin at either dose of 8 or 80 mg than those in control rats (Figures 2a and 2b).

GST activity towards the marker substrates

The specific activity toward CDNB was significantly increased in the livers of rats treated with curcumin at either dose of 8 or 80 mg (Table 1). However, the activity toward \( t \)-PBO was elevated by treatment with curcumin only at a dose of 80 mg. There was no significant change in the activity of the liver toward cumene hydroperoxide and ethacrylic acid by any doses of curcumin (Table 1).

Effect of curcumin on AFB1-induced acute toxicity

The levels of AST and ALT were significantly increased by the treatment with AFB1 (Figure 3). Curcumin at a dose of 8 mg/kg bw decreased slightly, but significantly, the AFB1-induced AST and ALT levels (Figure 3). Curcumin at a dose of 0.8 mg/kg bw also decreased significantly the AST level (Figure 3). However, curcumin at a dose of 80 mg/kg was ineffective in reducing the AST and ALT levels (Figure 3).

FIG. 2. Effect of curcumin on AFB1-DNA adduct formation in liver postmitochondrial (a) and microsomal (b) fractions of rats. Data are expressed as the mean ± SD of 5 rats in each group and analyzed using student t-test. * \((p<0.05)\) and ** \((p<0.01)\) indicate significant differences compared to control group.

FIG. 3. Effect of curcumin on plasma AST and ALT activities in rats. Data are shown as the mean ± SD of 5 rats in each treatment group and compared to the group receiving AFB1 only. (* * \( p < 0.01 \))

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Liver (nmol/mL/mg protein)</th>
<th>control</th>
<th>8 mg/kg</th>
<th>80 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNB</td>
<td>158 ± 6.4</td>
<td>17.3 ± 12.9*</td>
<td>257.7 ± 13.0**</td>
<td></td>
</tr>
<tr>
<td>EA</td>
<td>102 ± 8.6</td>
<td>112.1 ± 5.6</td>
<td>107.1 ± 6.4</td>
<td></td>
</tr>
<tr>
<td>(t)-PBO</td>
<td>7.7 ± 0.9</td>
<td>9.0 ± 2.8</td>
<td>14.4 ± 2.0*</td>
<td></td>
</tr>
<tr>
<td>CPH</td>
<td>240.8 ± 25.4</td>
<td>240.7 ± 12.9</td>
<td>256.9 ± 12.9</td>
<td></td>
</tr>
</tbody>
</table>

CDNB; 1-chloro-2,4-dinitrobenzene, EA; ethacrylic acid, \( t \)-PBO; trans-4-phenyl-3-buten-2-one, CPH; cumene hydroperoxide

Data are shown as the mean ± SD of 5 rats in each treatment group and analyzed using student t-test. (* \( p < 0.05 \)) and (** \( p < 0.01 \)) indicate significant differences compared to control.
Effects of curcumin on AFB1-induced GST-P positive foci formation and histopathological changes

As for histopathological changes, we found severe hepatocellular damage with diffuse hemorrhage and cell necrosis in the liver of rats treated repeatedly with AFB1. AFB1 treatment induced the increases in the number of GST-P positive single cells (Figure 4b, Table 2), and the number of positive foci (Figure 4a, Table 2). The number of GST-P positive cells and foci was significantly decreased by curcumin treatment (Table 2).

DISCUSSION

The hepatotoxic and hepatocarcinogenic effects of AFB1 have been well documented in various animal species (Wogan, 1999). The liver is the main target organ of AFB1 toxicity, because AFB1 is metabolized to the reactive 8, 9-epoxide most efficiently in the liver. AFB1-induced acute toxicity is mainly attributed to the binding of the epoxide to DNA to form AFB1-DNA adducts. High levels of DNA adduct formation lead to a cellular injury (McLean and Dutton, 1995; Ellis, 2009). Many chemoprotective agents have been demonstrated to reduce AFB1-DNA adduct formation via inhibiting cytochrome P450-mediated AFB1 activation and/or enhancing GST-mediated AFB1 detoxification (Guegerich et al., 1998; Hayes et al., 1998). The increase in levels of plasma AST is an indicator of hepatic injury in rats, while ALT elevation is more associated with the necrotic state (Navarro and Senior, 2006; Plaa and Hewitt, 1986). The GST-P foci, proteins for preneoplastic liver foci, are well known as a marker for hepatocarcinogenesis (Tatematsu et al., 1987).

The results of this study showed that the levels of AFB1-DNA adduct formation mediated by liver postmitochondrial and microsomal fractions were significantly lower in rats treated with curcumin at either dose of 8 or 80 mg than in control rats. Increases in the activities of cytosol GST toward substrates, 1-chloro-2,4 dinitrobenzene (CDNB) and tran-4-phenyl-3-buten-2-one (t-PBO), were observed in the curcumin treated rats. These results demonstrate that curcumin reduces the liver microsomal CYP450 enzyme activity to form AFB1-DNA adducts in rats in association with the increase in the cytosol GST activity, although the possibility of the involvement of the changes in glutathione concentration in the liver (El-Agamy, 2010) and that in cytochrome P450 enzyme activities toward AFB1 metabolism cannot be excluded.

Consistent with other studies (Kalengayi and Desmet, 1975; Preetha et al., 2006; Yin et al., 1980), we observed the increase in the activities of ALT and AST in plasma after AFB1 exposure, indicating AFB1-induced damages in the hepatic cells. However, the levels of ALT and AST were significantly reduced by the treatment with curcumin although the range of effective doses was limited. The liver lesions in rats treated with AFB1 was also confirmed by histopathological findings, which showed severe hepatocellular damages with diffuse hemorrhage and cell necrosis in the liver. Curcumin also reduced the severity of the AFB1-induced histopathological lesions. These results suggest the potential of curcumin to prevent hepatotoxic effects of AFB1. The effect of curcumin on AFB1-DNA formation as well as their antioxidant effects (Sudharsan et al., 2005; Nagaraj et al., 2000; Soto et al., 2003; Franschini et al., 2002) may contribute to the

TABLE 2. Effect of curcumin on AFB1-induced GST-P foci formation in rat livers.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Liver GST-P foci formation</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of single cells (N/cm²)</td>
<td>No. of foci (N/cm²)</td>
<td>Area of foci (mm²/cm²)</td>
</tr>
<tr>
<td>I</td>
<td>AFB1</td>
<td>4.67 ± 2.46</td>
<td>3.70 ± 1.38</td>
<td>0.032 ± 0.01</td>
</tr>
<tr>
<td>II</td>
<td>Curcumin &amp; AFB1</td>
<td>2.00 ± 1.05**</td>
<td>2.08 ± 1.1**</td>
<td>0.023 ± 0.02</td>
</tr>
</tbody>
</table>

Data are shown as the mean ± SD of 10 rats in each treatment group and analyzed using student t-test. **indicates significant difference compared to control (p < 0.01).
observed changes in AFB1-induced acute toxicity.

Glutathione S-transferases in the placental form (GST-P) are an accurate marker enzyme for the detection of initiated neoplastic cells during carcinogen-induced hepatocarcinogenesis (Tatematsu et al., 1987; Bannasch et al., 1992). AFB1 administration induced the formation of GST-P positive single cells and foci in rats. However, the number of AFB1-induced GST-P single cells and foci were markedly decreased by curcumin treatment, indicating the potential of curcumin to protect against AFB1-induced carcinogenesis. GSH and GST play a critical role in the protection of tissue from deleterious effects of AFB1 intoxication including carcinogenic effects (Larsson et al., 1994). Several lines of evidence have suggested that curcumin might be used in cancer chemoprevention (Kaur et al., 2006; Iqbal et al., 2003; Kaul et al., 1997; Reddy and Lokesh, 1996). Thus, this study demonstrates the potential of curcumin to ameliorate AFB1-induced hepatotoxicity and hepatocarcinogenicity by modulating hepatic drug metabolizing enzymes.

ACKNOWLEDGEMENTS

This study was supported by the University of Tokyo, Japan.

REFERENCES


