Influence of aflatoxin B₁ on mRNA levels of acute-phase proteins and oncoproteins in albino rat liver

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SUMMARY

Background: The effect of aflatoxin B₁ (AFB₁) administration on expression of genes coding for acute-phase proteins, and nuclear protooncogenes c-fos and c-jun, and alpha-fetoprotein gene has been studied in rats.

Methods: AFB₁ was administered to male Albino Oxford (AO) rats as a single intraperitoneal dose (1 mg/kg body weight). The expression of genes for albumin, cystein protease inhibitor, fibrinogen, haptoglobin, α₁-acid glycoprotein and for c-Fos, c-Jun and alpha-fetoprotein in rat liver was measured by Northern hybridization.

Results: The mild increase in the levels of mRNA for acute-phase proteins after AFB₁ administration was observed during the first 24 hours. The exceptions were the mRNA levels in liver for cystein protease inhibitor, which were 50%, decreased as compared to the control values. In addition, mild increase of the expression of c-fos protooncogene with two peaks were noted at three (1.3 fold) and 72 hours (1.5 fold) after injection AFB₁ to rats. The expression of nuclear protooncogene c-jun at 1 hour and 72 hour after acute poisoning was 2.6 fold and 3.7 fold increased as compared to control values, respectively. The mRNA levels in liver for the alpha-feto-protein reached a maximum at 1 hour after AFB₁ injection and it was 1.8 times higher than the levels in the livers of nontreated animals.

Conclusion: Single administration of AFB₁ induced increased transcription of c-jun and c-fos genes while typical acute-phase response was not found.

Key words: Aflatoxin B₁; Acute-Phase Reaction; Oncogenes; Gene Expression; Acute-Phase Proteins; Rats

INTRODUCTION

Aspergillus flavus and A. parasiticus, both members of the Aspergillus group, are aflatoxin-producing strains. These fungi are widespread and have been isolated from a host of different materials. Optimal conditions for toxin formation are prevalent in areas with high humidity and temperature. Out of the four major aflatoxins (B₁, B₂, G₁, G₂), B₁ is usually found in the greatest concentrations. Aflatoxin B₁ (AFB₁), chemically classified as a furoucoumarin, is known to be the most potent hepatocarcinogen in numerous animal species and humans (1,2). The mutagenic and toxic properties have also been attributed to AFB₁ (3). The carcinogenic activity of AFB₁ derives from metabolically activated reactive intermediates that covalent binds to hepatocellular DNA, which leads to mutations in the host genome. The liver is responsible for many vital and complex functions in an organism (such as detoxification, bile formation, carbohydrate and fat metabolism, urea formation and inactivation of polypeptide hormones), and it is the target organ for the toxic effects of different compounds (4). In addition, liver plays a major role in the acute phase response (5).

The maintenance of physiologic homeostasis is assured by a number of physiologic mechanisms. Infection, poisoning by different compounds including AFB₁, mechanical or thermal tissue injuries can induce a complex early general reaction of an organism, which is known as the acute-phase response (APR) (6). APR helps surviving during the period immediately after injury. Prominent among all changes after the exposure of an organism to different traumas (7,8) is the increased synthesis of a group of plasma proteins that are synthesized in the liver, the so-called acute-phase proteins (APPs). During the acute-phase response, increased synthesis of APPs in the liver is preceded by an increased transcription of APP genes, and followed by increase of their serum concentrations (9). The expression of genes coding for APP in the liver is mostly controlled at the transcriptional level (10).

Actions of carcinogenic agents (among these are aflatoxins) are usually directed at macromolecules such as DNA. The results are point mutations (transversion), frame-shift, and gross chromosomal alterations, which lead to lethal lesions (11). Since the liver is often the primary target organ for carcinogens of diverse chemical structures, characterization of oncogene activation in liver tumors is important (12). Some of oncogenes, classified as „immediate early” genes, such as c-jun and c-fos, increased transcription at early stages of response to a variety of extracellular stimuli (13). These oncogenes code two subunits of a dimer for the transcription factor named activating protein-1 (AP-1), which activate genes whose promoters or enhancers have an AP-1 binding site (14). c-Fetoprotein (AFP) is glycoprotein, a product of an oncofetal gene, which has been considered as one of the most reliable diagnostic markers of hepatocellular carcinomas (HCC) (15). AFP gene is normally repressed in adult quiescent hepatocytes. This gene is also an example of a gene activated in the early prereplicative phase of hepatocyte proliferation during liver regeneration or in the hepatocellular carcinoma (16). AFB₁, besides cancerogenic, has also toxic effects on the live. Therefore, the goal of this study was to measure the expression of: (a) APP genes whose products primary have protective role such as: cystein protease inhibitor (CPI), fibrinogen (Fb), haptoglobin (Hp), α₁-acid glycoprotein (AGP), albumin (Al), (b) transcription factors, whose products are important in regulation of APP gene expression and in process of cancerogenesis (c-fos and c-jun), and (c) AFP gene, in rat liver after administration of AFB₁.

MATERIALS AND METHODS

Experimental animals. Male rats of the inbred Albino Oxford (AO) strain (6-8 weeks old) weighing approximately 150 g were used for all experiments. They were kept in wire-bottomed cages under standardized conditions of humidity, light, and temperature at the Institute for Medical Research of the Military Medical Academy. Food and water were given ad libitum.

AFB₁ treatment. Rats were divided into three groups. Each experimental group consisted of at least six animals. AFB₁ (Sigma Chemical Co., St Louis, MO, USA)
freshly dissolved in dimethyl sulfoxide (DMSO) was given intraperitoneally to rats in the single dose of 1 mg/kg body weight. Group II and III served as non-treated and solvent (DMSO) control, respectively. Animals were sacrificed after 1, 3, 6, 12, 24, 72, and 96 hours after administration of the AFB1. The rat livers were removed under anesthesia and the remainder of the liver was rapidly frozen in liquid nitrogen for later RNA extraction. All experiments were performed following the ethics of using animals regulated by the institutional guidance, which is in concordance with the NIH guidelines for the use of experimental animals.

Isolation of RNA. Total RNA was extracted with guanidine hydrochloride method (17). A piece of liver (0.5-1 g) was disrupted at room temperature by manual homogenization in a buffer containing 8 mol/L and 6 mol/L guanidine HCl, respectively, 50 µmol/L Tris HCl (pH 7.5) and 10 mol/L EDTA. RNA was precipitated with ethanol and resuspended in diethyl pyrocarbonate (DEPC)-treated water. Polyadenylated RNA was obtained by one cycle of oligo (dT)-cellulose chromatography (Serva) of total RNA using method of Aviv and Leder (18).

Plasmids. Plasmids carrying the cDNA inserts for rat AGP (pIRL-21), Hp (pIRL-25), CPI (pIRL-28), α-fibrinogen (Fb) (pIRL-14), c-fos (pSRT), c-jun (pBR322) mRNAs were kindly donated by Dr. H. Baumann, while probes for Al (pRSA 57) and AFP (pRAF 87) mRNAs were obtained from Dr. Nada Urošević currently at the University of West (WA) Australia, Perth, Australia. β-globin probe was used for internal control.

Northern blot analysis of mRNA. For Northern analysis, 5 µg of poly (A)+RNAs were separated in 1% agarose gel containing formaldehyde, and transferred to Hybond-N membrane (Amersham, UK) by capillary blotting. For dot blots, 1, 2.5, and 5 µg of total RNAs were spotted onto membranes to Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Richmond CA). Blots were hybridized with plasmid cDNA probes and labeled using the Random Primer DNA labeling kit (Takara Bio, Otsu, Japan) to a specific activity of 4x10⁶ cpm/µg cDNA. Prehybridization and hybridization (6x10⁶ cpm/ml) of membranes was performed at 42°C overnight according manufacture’s instructions (Amersham, UK). Stringent washings were made twice in 2 x standard saline-phosphate-EDTA buffer (SSPE), 3.6 mol/L NaCl, 0.2 mol/L NaH₂PO₄, pH 7.4 and 0.02 mol/L ethylene diamine tetra-acetate (EDTA) and 0.1% sodium dodecyl sulfate (SDS) at room temperature, once in 1 x SSPE and 0.1% SDS at 42°C and once in 0.1xSSPE and 0.1% SDS at 52°C. Blots were exposed to Kodak-X-Omat AR films (Kodak) at -70°C for 24 - 72 hours. The areas of the filters that hybridized with the cDNA probes were identified by autoradiography and LKB Ultro Scan XL Laser Densitometer was used to quantify the amounts of hybridized cDNA probes.

Statistical analysis. Statistical analysis was performed using Student’s t test.

RESULTS

The mRNA levels for Al were similar to the control values at all monitored time points indicating that after administration of single dose of AFB1, the expected acute phase mediated reduction in albumin mRNA was not observed (Figure 1A). The changes of AGP mRNA concentrations varied in the range of control values, with the mild increase 24 hour after AFB1 treatment (Figure 1B). The changes in mRNA levels in liver after AFB1 administration for Hp reached maximum increase of 1.4 fold at 12th and 24th hour compared to controls and remained enhanced even at 72nd and 96th hour after AFB1 injection (Figure 1C). The level of Fb mRNA in liver was significantly increased (p<0.05) at the first and 6th hour after AFB1 administration in respect to the control values. It decayed more rapidly towards the control values at 12th and 24th hour with slight increase at 72nd and 96th hour after administration of AFB1 (Figure 1D). The mRNA level in liver for CPI was decreased at all examined time points with the most significant decay (46% compared to control, p<0.05), one hour after AFB1 administration (Figure 1E).
Increase of 1.3 fold of the expression of c-fos protooncogene was noted three hours after AFB1 injection in acute treated rats and was followed by a second peak at 72h hour (Figure 2A). The similar trend of two peaks was evident in expression of AFP, an oncofetal gene normally repressed in adult quiescent hepatocytes, from third hour after AFB1 treatment (Figure 2C). After acute treatment of rats with AFB1, early increase of the expression of c-jun at the first and 12th hour was found but the most significant increase (3.7 fold) was found at 72h hour (Figure 2B).

**DISCUSSION**

In this study, we have investigated acute-phase response through expression of the genes for AGP, Fb, Hp, CPI, and Al in the rat liver after treatment with a single dose of 1 mg AFB1/kg body weight. The observed changes in mRNA levels of the major APPs were different from those obtained by other carcinogenic agents (19). The increases of mRNA levels for AGP, Fb, and Hp, were lower than after administration of soman, a typical organophosphate. Soman induced 4-fold increase in AGP and Hp mRNA levels, and 6-fold increase in Fb and CPI mRNA levels (7). As compared to turpentine (6), soman induced increase in mRNA levels for APPs appeared to be 30%-40% lower. The results of earlier experiments showed that even after administration of direct DNA damaging agents such as lethal total body irradiation, the concentrations of the APP mRNAs displayed tendency to increase over a period of 3 days (8). We did not obtain significant decrease in albumin mRNA, as previously reported, while mRNA levels for CPI were decreased at all examined time points after AFB1 administration. The inhibition of the expression CPI gene as well as absence of increased concentrations for positive acute-phase proteins indicated that acute-phase protein gene expression in rat liver was probably overcome by toxic effect of AFB1.

The early-response genes, c-fos and c-jun, encode proteins c-Fos and c-Jun, that are constituents of the transcription factor named activator protein 1 (AP-1). AP-1 binds by high affinity to AP-1 binding sequence of promoter many genes (14) and regulates the expression of these genes as response to different extracellular stimuli. AP-1 like binding sequences are found in the promoter elements of genes as cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and APPs (20). Although c-jun is expressed in a variety of tissues, it seems to be especially important for hepatocytes. The precise function of c-jun in hepatocytes seems to depend on their differentiation stage. Fetal hepatocytes require c-jun for cell survival while differentiated hepatocytes, rather, require c-jun for cell-cycle progression (21). In unstimulated liver, small amounts of the c-fos and c-jun transcripts were present. Activation of c-jun and c-fos in hepatocytes has been linked to the passage of these cells from the G0 non-proliferative stage to the first phase of the replicating cycle (G1). Subsequent progression to the S phase (DNA synthesis) and M phase (mitosis) is not an obligatory event. AFB1 induced significant increase in the level of mRNA for c-jun (more than for c-fos), and the highest values were observed 72 hours after AFB1 treatment (3.7 fold compared to controls). Uncoupling of c-fos and c-jun induction has already been reported after acute inflammation (16). Our earlier study showed that hepatectomy (30%-40%) caused quicky regeneration of the liver accompanied by activation of the genes for CPI, Hp and inhibition of Al gene transcription 24 hours after hepatectomy. The elevated expression of these genes preceded by early activation (during first hours) nonspecific transcriptional factors c-fos and c-jun (22). AP-1 proteins by binding to the receptors of glucocorticoid hormones can suppress their transcription activity. Among other functions in organism, glucocorticoids also have inhibitory effect to the cell proliferation. In that way, AP-1 complex can contribute to the cell proliferation and neoplastic transformation (23-25).

Not all animals are equally susceptible to carcinogenic and toxic effect of mycotoxins (26). The effects of mycotoxins are related to the amount, length of exposure, and natural sensitivity of the host to the mycotoxins (27). Glutathione-S-transferase (GST) activity may be a key factor in determining individual or species susceptibility to AFB1, and at least in rodents is the major route of detoxification. Our earlier investigation showed that administration of both single and multiple doses of AFB1 lead to long time increase of GST activity in the rat plasma and liver (28). Among laboratory animals, the rat is most susceptible to AFB1, hepatocarcinogenicity. Since carcinogenesis is a complex process, which involves multiple stages, mutations of DNA, do not always mean initiation of carcinogenesis. The activation of cellular protooncogenes is also an important step in the initiation, progression, or maintenance of the malignant cell. Activated oncogenes have been identified in a wide variety of tumor types and chemically induced tumors of the liver are of particular interest since this organ is often the primary target organ (12). Much research has
been carried out in attempts to elucidate molecular and cellular mechanisms through which aflatoxins induce their carcinogenic effects. Modali and Yang (11) have shown that AFB1, induced the oncogenicity of PM-1 proto-oncogene that has been cloned DNA derived from hepatocellular carcinoma. Mc Mahon et al., (29), using DNA extracted from 11 AFB1-induced liver tumors in male Fischer rats, detected activated ras gene (c-Ki-ras) in only two rats. Sinha et al., (30) detected activated all three ras oncogenes in all the aflatoxin-induced tumors in male Fischer 344 rats and in two lines generated from such tumors. N-ras activation was most frequent.

AFP and GST-P are widely used as biomarkers of HCC and have been used to evaluate effects of various carcinogens including AFB1-induced hepatocarcinogenesis. AFP is now considered the most representative carcinomembronic protein. This oncologenic gene is activated during the early proliferative response of hepatocytes. We obtained the increase of AFP mRNA at 1st hour after acute poisoning by AFB1, Bernuau et al., (16) observed biphasic accumulation of the two AFP mRNA transcripts, the first at 4th hour and second at 24th hour after acute inflammation.

Better understanding of the role of AFB1 in modulating liver gene expression, such as oncogenes, tumor suppressor genes, repair genes and APPs genes, should provide better insight regarding mechanisms of AFB1-induced carcinogenesis.

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Conflict of interest

We declare no conflicts of interest.

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