Carnitine Alters Binding of Aflatoxin to DNA and Proteins in Rat Hepatocytes and Cell-Free Systems

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ABSTRACT The objective of this study was to determine effects of L-carnitine on aflatoxin B1 (AFB1)-DNA adduct formation in isolated rat hepatocytes, its dose response, specificity and mode of action. All experiments were conducted in either freshly isolated rat hepatocytes or cell-free systems. There was negative linear correlation between the dosage of carnitine and formation of [3H]AFB1-DNA adducts in the hepatocytes; however, the partitioning of AFB1 into cellular compartments was not affected by carnitine. The attenuating effect of carnitine on AFB1-DNA adduct formation was also present in a cell-free system, but there was lack of specificity because acetylcarnitine and γ-aminobutyric acid (GABA) were equally effective. Carnitine appears to interfere with bioactivation of AFB1 and binding of AFB1-epoxide to DNA. On the contrary, carnitine enhanced the binding of AFB1, and its epoxide to microsomal proteins, plasma proteins and bovine serum albumin. These results indicate that carnitine diverts AFB1-epoxide away from DNA by promoting binding to proteins. We conclude that modulation of AFB1, binding to proteins and DNA by carnitine alters the carcinogenic and hepatotoxic potential of AFB1, and poses concerns about the human AFB1-exposure data based on the AFB1-albumin adduct concentrations as a biomarker. J. Nutr. 131: 1903–1908, 2001.

KEY WORDS: • aflatoxin B1 • choline • L-carnitine • γ-aminobutyric acid • rats

Aflatoxins, a group of secondary metabolites of Aspergillus flavus and Aspergillus parasiticus, are commonly found to contaminate food and feed supplies. Aflatoxin B1 (AFB1)3 is the most hepatotoxic (1) and carcinogenic (2) of all naturally occurring aflatoxins. The effects of AFB1 are mediated through its metabolites, AFB1-8,9-epoxide, by covalent binding to cellular proteins and nucleic acids (3). AFB1-DNA adduct concentrations have been correlated to incidences of liver cancer in animals (4) and humans (5). Certain amounts of AFB1-epoxide may be rendered harmless by conjugation to various endogenous and exogenous compounds such as glutathione (6).

After absorption from the small intestine, AFB1 readily binds to plasma albumin, which serves as the major transporter of AFB1 in blood (7). It has been postulated that some bioactivation of AFB1 occurs in the intestinal mucosa (8,9) and in blood (10); therefore, AFB1 metabolites are also bound to albumin. The postabsorption binding of AFB1 to albumin has been proposed to lessen the toxicity of AFB1 (11).

L-Carnitine is a carrier of acyl groups, particularly the long-chain fatty acids, across the cellular compartments (12). It was reported recently that carnitine in combination with coenzyme Q10 offered significant protection against oxygen radicals induced by mycotoxins including AFB1 in bacteria (13). When a carnitine-supplemented diet (4 g/kg) was fed to rats for 6 wk followed by a single oral dose of AFB1 (1 mg/kg), there was significant reduction in the concentrations of AFB1-epoxide adducts of hepatic DNA and RNA, 6 and 24 h after AFB1 dosing (14). This study left a number of unanswered questions about the effects of carnitine including the following: is it dose-dependent? Can it be reproduced by other carnitine-like compounds? Is biotransformation of AFB1 a prerequisite? Is there alteration in intracellular partitioning of aflatoxin? We have attempted to answer these questions using isolated hepatocytes and cell-free systems. We believe that the results reported here bring us one step closer to understanding the mechanism by which carnitine reduces AFB1-DNA adducts.

MATERIALS AND METHODS

Animals. The Animal Care and Use Committee of the University of Tennessee, Knoxville, approved the research protocol. Male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 300–350 g were housed individually in suspended stainless steel cages in a cubicle of the animal facility and were given free access to Teklad 22/5 Rodent Diet (W) 8640 (Harlan, Indianapolis, IN) and water. These rats were used for obtaining hepatocytes, liver microsomes and blood plasma for the studies outlined below.

Liver perfusion and isolation of hepatocytes. The buffers used for preperfusion, collagenase perfusion and incubation were modified Hank’s balanced salt solutions (HBSS) as described by Lotlikar et al. (15). All buffers were saturated with 95% O2:5% CO2 gas before use. The perfusion apparatus and procedures used followed the modified...

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2 To whom correspondence should be addressed. E-mail: dsachan@utk.edu.
3 Abbreviations used: AFB1, aflatoxin B1; BSA, bovine serum albumin; DDW, double deionized water; DMSO, dimethyl sulfoxide; GABA, γ-aminobutyric acid; HBSS, Hank’s balanced salt solutions.
earlier.

**Preparation of liver microsomes.** Rats were anesthetized with Metofane (Pitman-Moore, Mundelein, IL); the portal vein was cannulated and the liver was perfused in situ with 100 mL ice-cold physiologic saline. The perfused liver was removed and homogenized in 0.154 mol/L KCl buffer containing 0.01 mol/L KH₂PO₄ (pH 7.4). The homogenate (250 g/L) was centrifuged at 100,000 g for 60 min at 4°C. The microsomal pellet was suspended in the glycerol/3 mol/L phosphate buffer (1:1) buffer, pH 7.4, and the concentration of protein in microsomes was determined (20).

**[3H]AFB₁ binding to calf thymus DNA mediated by microsomal enzymes.** The DNA-binding method of Allamch et al. (21) was modified by Hasler et al. (22) was used. Calf thymus DNA (Sigma Chemical, St. Louis, MO) was incubated with the [3H]AFB₁ as follows: 1) without microsome and carnitine; 2) without microsome but with carnitine; 3) with microsome but no carnitine; and 4) with both microsome and carnitine. The 1 mL incubation mixtures contained 0.1 mol/L phosphate buffer, microsome equivalent to 1.0 mg protein, 2 mmol/L NADPH, 2 mmol/L [3H]AFB₁, dissolved in DMSO, 0.1 mg calf thymus DNA, 1.2 mmol/L t-carnitine, i-acetylcarnitine, choline, y-aminobutyric acid (GABA) or glycine (pH 7.0), and double-deionized water (DDW). The samples were incubated in triplicate at 37°C for 30 min and replicated five times using microsomes from five rats. At the end of the incubation time, 1 volume of 5 mol/L NaCl was added to produce a mixture containing 1 mol/L NaCl followed by 2 mL of chloroform/isooamyl alcohol (24:1, v/v) and 0.9 mg of calf thymus DNA as a carrier. The tubes were shaken and centrifuged at 10,000 × g for 10 min. The rest of the procedure for DNA extraction and determination of concentration or radioactivity was as described earlier.

**[3H]AFB₁, binding to microsomes.** The incubation medium and procedure were similar to those used in the AFB₁ binding to BSA described above. There were two groups in this experiment, control and carnitine. The medium contained 0.1 mol/L phosphate buffer, microsomes equivalent to 1.0 mg protein, 1.2 mmol/L carnitine (pH 7.0) for the carnitine group, 2 mol/L NADPH, 2 mmol [3H]AFB₁ dissolved in DMSO and DDW to a total volume of 1.0 mL. After the incubation, the mixtures were quickly chilled in ice water and microsomes isolated by centrifugation at 100,000 × g for 1 h at 4°C. The supernatant was removed and microsomes were suspended in 1 mL KOH (1 mol/L) and extracted with 2 mL of chloroform/ethy-
specificity of the carnitine effect on AFB1-DNA adduct formation in the cell free–system with liver microsomes was tested using a few carnitine-like substances (Figure 4). The concentrations of AFB1-DNA adducts (pmol/mg DNA) in the presence of carnitine (170 ± 12), acetylcarnitine (154 ± 8) and GABA (164 ± 9) were significantly lower compared with the control (216 ± 13), choline (203 ± 11) and glycine (212 ± 14) treatments, but were not different from one another.

Binding of AFB1 to BSA in the presence and absence of microsomes and carnitine is presented in Figure 5. There was some binding of AFB1 to BSA (13.4 pmol/mg BSA in group 1, and 16.2 pmol/mg BSA in group 2). These are noncovalent bindings because no microsomes were added to the incubation mixtures to produce the AFB1-epoxide that binds covalently to macromolecules. Carnitine had no significant effect (contrast P-value = 0.171) on the noncovalent binding of AFB1 to BSA (group 1 vs. group 2). In the presence of microsomes, however, there were significantly more AFB1-BSA adducts formed and carnitine enhanced formation (Fig. 5, groups 3 and 4). The BSA adducts formed were 4.8 fold higher in group 3 than in group 1, and 4.5 fold higher in group 4 than in group 2. The concentrations of AFB1-BSA adducts in the presence of microsomes were higher primarily because of the covalent and some noncovalent binding. The average amount of covalent AFB1-BSA bindings in group 3 was 64.1 pmol/mg BSA; in group 4, it was 72.7 pmol/mg BSA (calculated by subtracting the adduct concentrations of group 1 from group 3, and group 2 from 4). Therefore, covalent bindings constituted the higher portion of the AFB1-BSA adduct shown in group 3 and 4. The presence of carnitine in the incubations containing microsomes significantly increased (P = 0.0005) the AFB1-BSA adduct formation (group 4 vs. group 3), clearly indicating that carnitine promoted covalent binding of AFB1-epoxide to BSA.

The concentrations of AFB1 bound to microsomes in the absence or presence of carnitine are shown in Figure 6. There was some binding of AFB1 to the microsomal component, and carnitine significantly increased (P = 0.007) this binding. This

FIGURE 1 Effect of L-carnitine on aflatoxin B1 (AFB1)-DNA adduct formation in freshly isolated rat hepatocytes. Carnitine was preincubated for 15 min before addition of 0.5 μmol/L [3H]AFB1 and incubated for an additional 60 min. Incubations were carried out in triplicate (each point) from each preparation of hepatocytes from five different rats of each group.

FIGURE 2 Effects of L-carnitine on [3H]aflatoxin B1 (AFB1) entry into isolated hepatocytes and distribution into cellular compartments. Carnitine was preincubated for 15 min before addition of 0.5 μmol/L [3H]AFB1, and incubated for an additional 60 min. Incubations were carried out in triplicate for each hepatocyte suspension isolated from 5 different rats. Values are means ± SEM, n = 5.

FIGURE 3 Effects of L-carnitine (1.2 mmol/L) on aflatoxin B1 (AFB1)-calf thymus DNA adduct formation in the absence or presence of microsomal enzymes. Incubations were carried out in triplicate from each microsomal preparation of 5 different rats. Values are means ± SEM, n = 5. Different letters above bars indicate significantly different at P < 0.05.
AFB₁ binding to microsomal protein was about two times the binding of AFB₁ to BSA (Fig. 5; groups 1 and 2). In microsomal systems, it is not easy to separate covalent and noncovalent binding, but the latter is most likely minimal. Noncovalent binding of unmetabolized AFB₁ to plasma proteins and BSA, as measured by the ultrafiltration method, is presented in Figures 7A and B, respectively. The carnitine group had significantly greater binding of AFB₁ to plasma proteins than the control group (P < 0.022). The AFB₁-BSA adduct formation was also greater in the carnitine group than in the control group (P = 0.018). Compared with rat plasma, more protein adducts were formed when the protein was BSA. This is most likely related to the purity of BSA protein because plasma has components other than albumin.

**DISCUSSION**

Carnitine-mediated attenuation of AFB₁-DNA adduct formation seen in the intact rats fed a carnitine-supplemented diet (14) was demonstrated in the isolated hepatocytes from a group of rats fed a nonpurified diet. As shown in Figure 1, in vitro addition of carnitine to the freshly isolated hepatocytes decreased AFB₁-DNA adduct formation in a dose-dependent manner with a strong negative correlation. The attenuation of AFB₁-DNA adducts could not have been due to the effect of carnitine on AFB₁ uptake by hepatocytes or the differential partitioning of AFB₁ among the nuclear and cytosolic fractions (Fig. 2). The formation of AFB₁-RNA adducts in the isolated hepatocytes was not significantly affected by carnitine under these conditions (data not shown); therefore, AFB₁-RNA adducts were no longer pursued.

In the intact rats (6), it was difficult to distinguish whether carnitine was affecting binding of AFB₁ or AFB₁-epoxide to DNA. The speculation was that carnitine may have suppressed bioactivation of AFB₁ in a manner similar to that suggested for crocetin (18). The in vitro system provided an opportunity to evaluate this hypothesis.

**FIGURE 4** Effects of L-carnitine, acetylcarnitine and structurally-related compounds on aflatoxin B₁ (AFB₁)-DNA adduct formation. The concentrations of the compounds were 1.2 mmol/L. The bars represent means ± SEM, n = 5. Different letters above the bars indicate significant differences among groups, P < 0.05. L-CNE, L-carnitine; ACNE, acetylcarnitine; GABA, γ-aminobutyric acid.

**FIGURE 5** Effects of L-carnitine (1.2 mmol/L) with or without microsomes from 5 rats on the binding of aflatoxin B₁ (AFB₁) to bovine serum albumin (BSA). The bars represent means ± SEM, n = 5. Different letters above the bars indicate significant differences among groups, P < 0.05.

**FIGURE 6** Effects of L-carnitine (1.2 mmol/L) on aflatoxin B₁ (AFB₁) binding to rat microsomes. The bars represent means ± SEM, n = 5. Different letters above the bars indicate significant differences between groups, P < 0.05.

**FIGURE 7** Effect of L-carnitine on aflatoxin B₁ (AFB₁) binding to plasma protein (panel A) and BSA (panel B) as determined by an ultrafiltration separation technique. Values are means ± SEM, n = 10 (BSA) or 8 (plasma protein). Different letters above the bars indicate significant differences, P < 0.05.
The hepatic microsomal enzymes metabolize AFB\(_1\) to AFB\(_1\)-epoxide which covalently binds to the electrophilic centers of DNA (6). In the cell-free system, binding of AFB\(_1\) to calf-thymus DNA was not significantly affected by carnitine (Fig. 3). However, when microsomes were added to the cell-free in vitro incubation system containing purified calf thymus DNA, AFB\(_1\)-DNA adducts were formed, and these were significantly decreased in the presence of carnitine (Fig. 3). This indicates that carnitine interferes with AFB\(_1\)-bioactivation in a manner analogous to the inhibition of the formation of oxygen-free radicals (24, 25). The highly reactive nature of epoxides results in rapid formation of AFB\(_1\)-DNA adducts, which have a half-life of only ~12 h. It is also possible that carnitine prevented binding of the epoxide to DNA, resulting in reduced AFB\(_1\)-DNA adducts. Carnitine has the quaternary nitrogen similar to the electrophilic center of guanine of DNA and is available for electrophilic attack by AFB\(_1\)-epoxide. The present data preclude separation of the effect of carnitine on the bioactivation of AFB\(_1\) to AFB\(_1\)-epoxide and the electrophilic binding of epoxide to the guanine of DNA.

The inhibition of AFB\(_1\)-DNA adduct formation by carnitine in the cell-free system was mimicked by acetylcarnitine and GABA but not by choline and glycine (Fig. 4). The important distinction between inhibitors (carnitine, acetylcarnitine, GABA) and noninhibitors (choline, glycine) is the carbon chain length because amino and trimethylamino groups are common to both groups of compounds. The superior effect of acetylcarnitine on AFB\(_1\)-DNA adduct formation is analogous to the effect of this molecule on the inhibition of ethanol metabolism in hepatocytes (26). Acetylcarnitine competitively inhibited binding of NAD\(^+\) to alcohol dehydrogenase in the cell-free system (27). A variety of acylcarnitines, including acetylcarnitine, can be produced in microsomal systems (28). The fact that the magnitude of decrease in AFB\(_1\)-DNA adducts in the presence of GABA was equal to that of carnitine is intriguing and requires additional studies. The common features of carnitine and GABA are the 4-carbon length, carboxyl group on carbon-1 and amino nitrogen on carbon-4. The lack of an effect of choline on AFB\(_1\)-DNA adducts is analogous to that seen in ethanol metabolism (29).

The reports on choline-AFB\(_1\) interactions are conflicting. For example, choline deficiency had no effect on the liver AFB\(_1\)-DNA adduct concentration in rats given a single dose of AFB\(_1\); however, multiple doses of AFB\(_1\) markedly elevated AFB\(_1\)-DNA adduct formation (30). On the other hand, rats fed a diet marginally deficient in lipotropes (methionine, choline and folacin) and given a single dose of AFB\(_1\) showed suppression of hepatic AFB\(_1\)-DNA adduct formation (31). Recently a choline and methionine–deficient diet was shown to have no significant effects on serum biochemistry or liver pathology due to a dose of AFB\(_1\) in rats (32).

AFB\(_1\) and its metabolites, particularly AFB\(_1\)-epoxide, are known to bind to plasma proteins (33), and this binding may be altered by the pH and fatty acid concentrations (34). Carnitine significantly increased the covalent binding of microsomal-activated AFB\(_1\) to BSA, a purified serum protein (Fig. 5). Plasma contains ~7–8% proteins, and albumin constitutes ~50–60% of plasma proteins (35). Albumin has been shown to be the main plasma protein that binds AFB\(_1\) and thus serves as the major transporter of AFB\(_1\) in the blood (7). Carnitine also increased the binding of inactivated (nonmetabolized) AFB\(_1\) to BSA and plasma proteins of rats (Fig. 7). It has been shown that >95% of AFB\(_1\) found in rat plasma proteins was noncovalently bound, and ~80% of it was associated with the albumin fraction (36). The binding of AFB\(_1\) was nearly 5 times higher in the presence of microsomes than in absence of microsomes, suggesting that AFB\(_1\)-metabolites have a higher affinity for BSA; however, the effect of carnitine was significant.

The AFB\(_1\) binds to serum albumin and hepatic DNA in a dose-dependent manner (10) and carnitine attenuates the latter (Fig. 1). AFB\(_1\) has been shown to have the highest affinity for liver microsomes followed by the cytosol, mitochondria and nuclei (36). The binding of AFB\(_1\) to microsomes is increased by carnitine (Fig. 6). It has been suggested that binding of AFB\(_1\) to plasma albumin offers protection to the liver, which has a high capability to draw free AFB\(_1\) from the blood (11). Because carnitine increases the AFB\(_1\) retention by plasma proteins, it is possible that less free AFB\(_1\) will be available for uptake and metabolism by liver cells in the intact animal. We know that under in vitro conditions, carnitine does not significantly alter the entry of AFB\(_1\) into the hepatocytes or its partitioning among the subcellular fractions (Fig. 2). It may be argued that carnitine increases covalent binding of AFB\(_1\) to cellular proteins in a manner similar to that seen for plasma proteins (Figs. 5, 7), resulting in the reduced binding of AFB\(_1\) to nuclear DNA.

AFB\(_1\)-albumin adduct concentration in plasma has been widely used as an acceptable biomarker for evaluating exposure of humans to AFB\(_1\) (37, 38). Our findings indicate that caution should be taken in the interpretation of the AFB\(_1\)-albumin data. Higher concentrations of AFB\(_1\)-albumin adduct in the blood may not always mean a higher intake of AFB\(_1\) but may be a reflection of the modulation by dietary nutrients such as carnitine and perhaps others not yet identified. Dirr (34) reported that the increase in plasma concentrations of long-chain fatty acids considerably increases the AFB\(_1\)-albumin concentration in plasma, which may be modulated by acylcarnitines.

We conclude that carnitine lowers binding of AFB\(_1\) to DNA in isolated hepatocytes as well as to calf thymus DNA in a cell-free system. The carnitine effect is dose dependent but not highly specific because acetylcarnitine and GABA were equally effective. Carnitine enhances binding of AFB\(_1\) and its metabolites to various proteins (BSA, rat serum, microsomal) and thereby reduces the formation of AFB\(_1\)-DNA adducts.

**LITERATURE CITED**


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