POTENTIATION OF AFLATOXIN B\textsubscript{1} INDUCED HEPATOTOXICITY IN MALE WISTAR RATS WITH ETHANOL PRETREATMENT

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Accepted November 22, 1985

Abstract.....The interaction of ethanol and aflatoxin B\textsubscript{1} (AFB\textsubscript{1})-induced hepatotoxicity was studied in male Wistar rats using the activity of plasma GOT and GPT, liver triglyceride and histopathologic changes of liver necrosis as indices. Pretreatment of four oral doses of ethanol (4.0 g/kg BW each) at 48, 45, 24 and 21 hrs prior to AFB\textsubscript{1} (0.5 to 2.0 mg/kg BW) single i. p. administration caused a significant increase in the activity of PGOT (6 folds) and PGPT (5 folds), liver triglycerides (2 folds) and severity of liver necrosis at 48 hrs after AFB\textsubscript{1} administration. Ethanol pretreatment potentiated AFB\textsubscript{1}-induced hepatotoxicity by increasing MFO enzymes, aniline hydroxylase and \(p\)-nitroanisole-O-demethylase activity and lipid peroxidation, and decreasing in cytochrome b\textsubscript{5}, epoxide hydrolase activity and hepatic glutathione content. However, it did not cause any significant change in the activity of NADPH-cytochrome c reductase and glutathione-S-transferase and cytochrome P-450. These results suggest that potentiation of ethanol pretreatment on AFB\textsubscript{1}-induced hepatotoxicity may due to an increase in the metabolic formation of AFB\textsubscript{1}-2, 3oxide and subsequent binding to DNA.

Key words: Ethanol, aflatoxin B\textsubscript{1}, liver injury, hepatic drug-metabolizing enzyme activity, lipid peroxidation, glutathione.
INTRODUCTION

AFB₁ is a potent hepatotoxic, hepatocarcinogenic, teratogenic and mutagenic mycotoxin in a variety of in vivo and in vitro assay systems (Wogan, 1973; Campbell and Hayes, 1976). Acute aflatoxicosis occurred in many human populations and the epidemiological evidences suggested that it might be a contributing factor in the etiology of human liver cancer (Bourgeois et al., 1969; Krishnamachari et al., 1975; Shank et al., 1977). AFB₁ is frequency found in peanuts (Dickens, 1977; Glinsukon et al., 1980). Hence, there is a possibility of having an intoxication of AFB₁ in peanuts as a snack with ethanol in beer or whisky. As expected, it has been recently reported that ethanol pretreatment enhanced hepatotoxicity of AFB₁ in male and female Fischer rats (Glinsukon et al., 1978; Toskulkao et al., 1982; Toskulka and Glinsukon, 1983). It was proposed that ethanol induced an increase in metabolic activation of AFB₁ to active metabolite AFB₁-2, 3-oxide (previously reported by Swenson et al., 1977) which in turn attacked cellular nucleophiles (proposed by Campbell and Hayes, 1976) or a decrease in the conjugation of active metabolite with reduced glutathione (GSH) as described by Degan and Neumann (1978).

Therefore, in this study, we report the effects of ethanol pretreatment and simultaneous treatment on the hepatotoxicity of AFB₁ in male Wistar rats with the possible mechanism of action of ethanol.

MATERIALS AND METHODS

Materials: AFB₁ was purchased from Makor Chemical (Israel) and ethanol from Wako (Japan) and E. Merck (Germany). Trioleine, GSH, DNA and other chemicals were purchased from Sigma Chemical (U.S.A.) and Wako (Japan). All other chemicals were reagent grade.

Animals: Male Wistar rats, weighing 170-200 g, were used throughout the study and supplied by Nippon Seibutu Zairyo, Tokyo, Japan (used for microsomal enzyme assay) and the National Animal Center, Mahidol University, Thailand. All animals were housed in an air-conditioned room at 25±2°C with relative humidity of about 65%, and were provided with commercial rat chow and with water ad libitum.

Treatments: Rats were treated with four oral doses of 4.0 g/kg ethanol (40% aqueous solution) by gavage at 48, 45, 24 and 21 hrs prior to i. p. administration of various doses of AFB₁ in 0.5 mg/kg DMSO. Control rats were simultaneously given equal volumes of water (p. o.) and DMSO (i. p.). The rats were anesthetized with ether and blood was collected with heparinized syringe at 48 hrs after AFB₁ administration. A portion of liver was removed and frozen for triglyceride analysis (Mendez et al., 1975) and fixed in 10% buffered-formalin for histopathologic examination. Plasma glutamic-oxaloacetic transaminase (PGOT) and glutamic-pyruvic transaminase (PGPT) activities were measured with Transaminase (GOT and GPT) Test Kit (Sigma Chemical, U.S.A.). Simultaneously treatment, rats were treated with a single dose of ethanol
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(40% aqueous solution) and i. p. administration of AFB, in 0.5 mg/kg DMSO. Control rats were simultaneously given equal volumes of water (p. o.) and DMSO (i. p.). The rats were anesthetized with ether and blood was collected at 48 hrs after AFB administration. The determinations were carried out as in the case of pretreatment above.

Ethanol on the liver constituents and microsomal mixed function oxidase system (MFO): Rats were treated with four oral doses of ethanol 4.0 g/kg (40% ethanol) by gavage at 48, 45, 24 and 21 hrs prior to sacrifice. Control rats were simultaneously given equal volume of water (p. o.). Rats were sacrificed by decapitation and exanguination at 48 hrs after the first dose of ethanol. Blood samples were collected for PGOT and PGPT activity and blood ethanol (Sigma Kit) determination. Then the livers were perfused with cold 0.9% NaCl solution via the portal vein, removed, weighed and homogenized with 4 volumes of 1.15% KCl by using a glass homogenizer with a teflon pestle. Aliquots of the homogenate were removed for the assay noted later; the remaining portion was used to isolate microsomes. All tissue preparations were carried out at 0-5°C.

Determination of liver constituents: Aliquots of the liver homogenate were used for assay of triglyceride, DNA by the method of Hubbard et al. (1970), RNA by the method of Ceriotti (1955), GSH by the method of Boyland and Chasseaud (1970). Lipid peroxide was measured as the amount of accumulation of malondialdehyde (MDA) in total by the method of Ohgawa et al. (1979), and total protein by the method of Lowry et al. (1951).

MFO assays: The following measurements on MFO enzyme activities were carried out on microsomal suspensions of the liver homogenate. For measurement of these activities aniline hydroxylase by the method of Imai et al. (1966), p-nitroanisole-0-demethylase by the method of Conney and Burns (1962), NADPH-cytochrome c reductase according to the method described by Mazel (1979), epoxide hydrolase by the method of Watabe et al. (1984), cytochrome p-450 and cytochrome b, content by the method of Omura and Sato (1964).

Cytosolic glutathione-S-transferase activity was determined by the method of Habig and Jacobsky (1981) by using 1, 2-dichloro-4-nitrobenzene as a substrate.

RESULTS

Blood ethanol concentrations: At the time of AFB, administration, the actual blood ethanol concentration was less than 1 mg/100 ml.

Potentiation of hepatotoxicity of AFB: Dose-response studies on plasma enzyme activities of GOT (Fig. 1), GPT (Fig. 2) and liver triglyceride (Fig. 3) in rats pretreated with four oral doses of ethanol (4.0 g/kg) and then treated with a single i. p. dose of AFB, are shown. Ethanol pretreatment by itself did not cause any highly significant change in PGOT and PGPT from those of water. However, it significantly increased the AFB-induced hepatotoxicity at all doses of AFB, (0.5-2.0 mg/kg) studied by 2-6 times in the activities of plasma GOT and GPT and approximately 1-2 times in the level
Fig. 1  Dose-response curves for aflatoxin B$_1$ induced elevations in plasma GOT activity in rats pretreated with four oral doses of water or four oral doses of ethanol. Plasma GOT was measured 48 hrs after the administration of aflatoxin B$_1$. Each value is mean ± S. E. of 5 rats (*p<0.05, **p<0.01 and ***p<0.001).

Fig. 2  Dose-response curves for aflatoxin B$_1$ induced elevations in plasma GPT activity in rats pretreated with four oral doses of water or four oral doses of ethanol. Plasma GPT was measured 48 hrs after the administration of aflatoxin B$_1$. Each value is mean ± S. E. of 5 rats (*p<0.05, **p<0.01 and ***p<0.001).
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Fig. 3  Dose-response curves for aflatoxin B₁-induced elevations in liver triglyceride content in rats pretreated with four oral doses of water or four oral doses of ethanol. Liver triglyceride content was measured 48 hrs after the administration of aflatoxin B₁. Each value is mean ± S. E. of 5 rats (*p<0.05, **p<0.01 and ***p<0.001).

Fig. 4  Effect of single dose of ethanol on the activities of plasma GOT and GPT and liver triglyceride measured 48 hrs after treatment simultaneously with aflatoxin B₁ in rats. Figures in parentheses are the doses of ethanol (g/kg) and aflatoxin B₁ (mg/kg). Values are means ± S. E. of 5-10 rats (*p<0.05).
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of liver triglyceride. At a higher dose of AFB, 2.0 mg/kg, ethanol (4.0 g/kg) pretreatment induced a significant increase in the activities of plasma GOT and GPT from 1390±248 to 5670±640 I. U./ml (p<0.001) and from 1150±140 to 4975±480 I. U./ml (p<0.001) respectively and in liver triglyceride from 16.6±3.5 to 29.6±2.1 mg/g liver (p<0.01). In addition, potentiation was also supported by microscopically examination (severity of necrosis and fatty degeneration in liver). In hepatic lesions revealed the periportal zone necrosis with mild fatty infiltration in the hepatocytes of the rats treated with AFB, whereas most severe necrosis and fatty infiltration was also observed in the rats pretreated with ethanol prior to AFB, administration.

The activities of plasma GOT and GPT and liver triglyceride of rats treated simultaneously with single oral dose of ethanol and AFB, are illustrated in Fig. 4. In this case, ethanol could not increase the hepatotoxicity induced by AFB, even through the doses of ethanol, 6.0 g/kg, and AFB, 4.0 mg/kg were increased.

Ethanol on the liver constituents: The liver constituents of rats treated with four oral doses of ethanol (4.0 g/kg) are shown in Table 1. The rats were sacrificed at 48 hrs after the first dose of ethanol or at the time of AFB, administration. Ethanol treatment caused a slight decrease in body weight and an increase in liver weight but not significant. Neither total protein, DNA, nor RNA content was altered by ethanol treatment. Liver triglyceride and lipid peroxide content were significantly increased from 5.5±6.0 to 31.8±4.0 mg/g liver (p<0.001) and from 0.08±0.0 to 0.11±0.01 mg/g liver (p<0.01) respectively whereas GSH significantly decreased from 1.6±0.1 to 0.9±0.1 mg/g liver (p<0.001) in ethanol treated group.

Ethanol on the hepatic MFO enzyme system: The hepatic microsomal MFO enzyme system of rats treated with four oral doses of ethanol (4.0 g/kg) are shown in Table 2. The rats were sacrificed at 48 hrs after the first dose of ethanol or at the time of AFB, administration. Microsomal protein, cytochrome P-450 content and cytochrome c reductase activity were not altered by ethanol treatment. Cytochrome b5 was significantly reduced from 0.31±0.02 to 0.23±0.03 nmol/mg microsomal protein (p<0.05) in the ethanol treated group. Ethanol treatment caused a significant increase in the activities of aniline hydroxylase from 0.68±0.02 to 1.23±0.06 nmol p-aminophenol/mg microsomal protein/min (p<0.001) and p-nitroanisole-O-demethylease from 0.86±0.06 to 1.14±0.10 nmol p-nitrophenol/mg microsomal protein/min (p<0.05) whereas epoxide hydrolase significantly decreased from 52.7±2.5 to 35.6±3.4 nmol DHC/mg microsomal protein/min (p<0.01). Cytosolic GSH-S-transferase activity was slightly increased, but not significant in ethanol treated group.
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**Table 1** Effect of pretreatment with four oral doses of ethanol on the liver composition, PGOT and PGPT activity in rats.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Pretreatment of rats&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>185.6±7.5</td>
</tr>
<tr>
<td>Liver weight (g/100 g body weight)</td>
<td>6.5±0.4</td>
</tr>
<tr>
<td>Total protein (mg/g liver)</td>
<td>179.3±12.4</td>
</tr>
<tr>
<td>DNA (mg/g liver)</td>
<td>3.2±0.2</td>
</tr>
<tr>
<td>RNA (mg/g liver)</td>
<td>7.8±0.1</td>
</tr>
<tr>
<td>Triglyceride (mg/g liver)</td>
<td>5.5±0.6</td>
</tr>
<tr>
<td>Lipid peroxide content (mg/g liver)</td>
<td>0.08±0.0</td>
</tr>
<tr>
<td>GSH (mg/g liver)</td>
<td>1.6±0.1</td>
</tr>
<tr>
<td>PGOT (I. U./ml)</td>
<td>56.6±1.0</td>
</tr>
<tr>
<td>PGPT (I. U./ml)</td>
<td>18.2±0.6</td>
</tr>
</tbody>
</table>

Ethanol 4.0 g/kg, p. o. in a 40% aqueous solution (v/v) or water were given 48, 45, 24 and 21 hrs prior to sacrifice, rats were sacrificed at 48 hrs after the first dose of ethanol.

1) Values are mean ± S. E. of 5-10 rats. Statistical significance (Student's t-test): *p<0.05; **p<0.01; ***p<0.001.

**Table 2** Effect of pretreatment with four oral doses of ethanol on the hepatic microsomal mixed function oxidase system and glutathione-S-transferase activity in rats.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Pretreatment of rats&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal protein (mg/g liver)</td>
<td>18.8±1.1</td>
</tr>
<tr>
<td>Cytochrome P-450 (nmol/mg protein)</td>
<td>0.62±0.02</td>
</tr>
<tr>
<td>Cytochrome b&lt;sub&gt;5&lt;/sub&gt; (nmol/mg protein)</td>
<td>0.31±0.02</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase (nmol cytochrome c reduced/mg protein/min)</td>
<td>158.1±10.9</td>
</tr>
<tr>
<td>Aniline hydroxylase (nmol p-aminophenol/mg protein/min)</td>
<td>0.68±0.02</td>
</tr>
<tr>
<td>p-Nitroanisole-O-demethylase (nmol p-nitrophenol/mg protein/min)</td>
<td>0.86±0.06</td>
</tr>
<tr>
<td>Epoxide hydrolase (nmol DHC/mg protein/min)</td>
<td>52.7±2.5</td>
</tr>
<tr>
<td>Glutathione-S-transferase (nmol DCNB conjugated/mg protein/min)</td>
<td>0.95±0.07</td>
</tr>
</tbody>
</table>

Ethanol 4.0 g/kg, p. o. in a 40% aqueous solution (v/v) or water were given 48, 45, 24 and 21 hrs prior to sacrifice, rats were sacrificed at 48 hrs after the first dose of ethanol.

1) Values in parentheses represent percentage of control. Values are mean ± S. E. of 5 rats. Statistical significance (Student's t-test): *p<0.05; **p<0.01; ***p<0.001.
DISCUSSION

In the present study, it was found that pretreatment with ethanol had an enhanced hepatotoxic effects of AFB$_1$, and an additive effect on triglyceride accumulation in liver of AFB$_1$ in male rats. These findings are similar to those of female rats (Toskulkao and Glinsukon, 1983). Our results also support the data of previous findings on the potentiation of ethanol to hepatotoxicity of several agents in experimental animals (Maling et al., 1975; Strubelt et al., 1978; Toskulkao and Glinsukon, 1983).

At present, the mechanism that responsible for the potentiation of ethanol on AFB$_1$-induced hepatotoxicity is not quite understood. However, two possible mechanisms were proposed as follows: i) an increase in the formation of active metabolite AFB$_1$-2, 3-oxide as the result of the increased MFO enzyme activity and/or ii) a decrease in the inactivation of AFB$_1$-2, 3-oxide by conjugation to GSH with cytosolic glutathione S-transferases (Degan and Neuman, 1978) and could indeed inhibit microsome mediated AFB$_1$ binding to DNA (Lotlikar, 1984), and the decreased hydrolysis of microsomal epoxide hydrolase, a pathway usually associated with detoxification of this reactive metabolites (Adekunle et al., 1977). Second possibility is more likely as there was a depletion of hepatic GSH in rats treated with ethanol in this experiment, while there was only slight increase in glutathione-S-transferase activity. In addition, microsomal epoxide hydrolase activity was also decreased and this would lead to an increase in the amount of AFB$_1$-2, 3-oxide available for binding to DNA and other macromolecules. In fact, it was recently observed that ethanol pretreatment induced an increase in $^3$H- AFB$_1$ binding to hepatic DNA in vivo in our laboratory (published elsewhere). However, the possibility of increased formation of AFB$_1$-2, 3-oxide can not be ruled out as it was found that MFO enzyme namely aniline hydroxylase was increased and this enzyme may possibly involve in the formation of AFB$_1$-2, 3-oxide. Although, there is no change in the content of microsomal proteins, cytochrome P-450 and the activity of NADPH-cytochrome c reductase which resembled those report by Maling et al. (1975). Therefore, further studies are needed to clarify whether the detoxified mechanism of AFB$_1$-2, 3-oxide by depletion of hepatic GSH and reduction of epoxide hydrolase activity is more important than those activation mechanism of AFB$_1$ to AFB$_1$-2, 3-oxide.

Our results also indicated that ethanol treatment induced an elevation of hepatic lipid peroxidation in a manner similar to those results from other investigators (Sippel, 1983; Kocak-Toker et al., 1983). It is not yet known whether or not this lipid peroxidation involves in the potentiation of AFB$_1$-induced hepatotoxicity. Several lines of evidence suggest that peroxidation of membrane lipids leads to the loss of membrane structure and membrane-bound enzyme activities (Hogberg et al. 1973; Pasquali-Roucetti et al., 1980). Thus, it is speculated that loss of the the integrity of plasma and mitochondrial membrane for controlling calcium transport and intracellular ATP production may probably increase AFB$_1$-induced hepatotoxicity to further extent. This hypothesis is subjected for further studies in our laboratory.
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Etanol treated simultaneously with AFB, did not increase the hepatotoxicity of AFB, in male rats which different from the female rats (unpublished observation). From this experiment in male rats, ethanol need more time to stimulate mixed function oxidases enzymes before AFB, administration.

ACKNOWLEDGEMENT

The authors would like to thank Miss Suthada Homjun for her typing. Financial support was provided by Showa University, Japan; Mahidol University and The National Research Council of Thailand, Thailand.

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