The enhancing effect of ethanol on the mutagenic activation of $N$-nitrosomethylbenzylamine by cytochrome P450 2A in the rat oesophagus

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Alcohol consumption is frequently associated with various cancers and the enhancement of the metabolic activation of carcinogens has been proposed as a mechanism underlying this relationship. The ethanol-induced enhancement of $N$-nitrosodiethylamine (DEN)-mediated carcinogenesis can be attributed to an increase in hepatic activity. However, the mechanism of elevation of $N$-nitrosomethylbenzylamine (NMBA)-induced tumorogenesis remains unclear. To elucidate the mechanism underlying the role of ethanol in the enhancement of NMBA-induced oesophageal carcinogenesis, we evaluated the hepatic and extrahepatic levels of the cytochrome P450 (CYP) and mutagenic activation of environmental carcinogens by immunoblot analyses and Ames preincubation test, respectively, in F344 rats treated with ethanol. Five weeks of treatment with 10% ethanol added to the drinking water or two intragastric treatments with 50% ethanol, both resulted in elevated levels of CYP2E1 (1.5- to 2.3-fold) and mutagenic activities of DEN, $N$-nitrosodimethylamine and $N$-nitrosopyrrolidine in the presence of rat liver S9 (1.5- to 2.4-fold). This was not the case with CYP1A1/2, CYP2A1/2, CYP2B1/2 or CYP3A2, nor with the activities of 2-amino-3-methylimidazo[4,5-f]quinoline, 3-amino-1-methyl-5H-pyrido[4,3-b]indole, aflatoxin B1 or other $N$-nitroso compounds (NOCs), including NMBA. Ethanol-induced elevations of CYP2A and CYP2E1 were observed in the oesophagus (up to 1.7- and 2.3-fold) and kidney (up to 1.5- and 1.8-fold), but not in the lung or colon. In oesophagus and kidney, the mutagenic activities of NMBA and four NOCs were markedly increased (1.3- to 2.4-fold) in treated rats. The application of several CYP inhibitors revealed that CYP2A were likely to contribute to the enhancing effect of ethanol on NMBA activation in the rat oesophagus and kidney, but that CYP2E1 failed to do so. These results showed that the enhancing effect of ethanol on NMBA-induced oesophageal carcinogenesis could be attributed to an increase in the metabolic activation of NMBA by oesophageal CYP2A during the initiation phase, and that this occurred independently of CYP2E1.

Introduction

Epidemiological studies have shown that alcohol consumption is associated with cancers of the upper gastrointestinal tract, liver, large bowel and breast. It is suspected that there is also an association with cancers of the lung and pancreas. Approximately 3.6% of cancers worldwide are associated with the chronic consumption of alcohol (1). Ethanol-induced cancers are caused by various molecular mechanisms including the genotoxic effects of acetaldehyde, the production of reactive oxygen species and the inhibition of S-adenosyl-L-methionine synthesis (2). The metabolic activation of exogenous and endogenous carcinogens by cytochrome P450 (CYP) 2E1 is also one of the routes by which ethanol exerts its carcinogenic action. Ethanol is known to be a hepatic CYP2E1 inducer in rodents, and there are a few reports on the induction of other CYP isozymes by ethanol in rats (3). CYP2A, CYP2B and CYP2E are specifically involved in the metabolic activation of environmental N-nitroso compounds (NOCs) to produce carcinogenic end products. Rat CYP2E1 predominantly activates $N$-nitrosodimethylamine (DMN), $N$-nitrosodimethylamine (DEN) and $N$-nitrosopyrrolidine (NPYR), whereas CYP2A and CYP2B1/2 activate $N$-nitrosodialkylamines that possess relatively long alkyl chains (4,5). Administration of ethanol in the drinking water of F344 rats showed an enhancing effect on DEN-induced (6) and $N$-nitrosomethylbenzylamine (NMBA)-induced (7) oesophageal carcinogenesis during the initiation phase. It has been reported that chronic ethanol treatment reduces UDP-glucurononyltransferase (UGT) 2B mRNA and protein levels and UGT activity in the rat liver (8,9). It has also been suggested that UGT2B1 is likely to be the enzyme responsible for the glucuronidation of some NOCs (10). Therefore, we postulated that ethanol increases NOC-induced carcinogenesis by enhancing its metabolic activation or by suppressing its inactivation. Previous reports showed that supplementing drinking water with 10% ethanol for 2 weeks increased the mutagenic activation of DEN, DMN and NPYR by CYP2E1 in rats, but did not affect the mutagenic activation of NMBA by CYP2B1/2 or the activities of the three types of UGT (11). However, the failure to observe any enhancement of NMBA activation might have been attributable to insufficient duration of ethanol treatment.

NMBA is known to be the most potent carcinogen in the rat oesophagus. This is irrespective of its mode of administration and requires metabolic activation to have a mutagenic or carcinogenic effect (12). The metabolism of NMBA is inhibited to >95% by CO and 70% by SKF-525A, which are both typical CYP inhibitors, indicating that there is a relationship between NMBA activation and CYP activity (13). In our previous report we showed that, in a phenobarbital-induced rat liver, NMBA is mutagenically activated by CYP2B1 and CYP2B2, but not by CYP2E1 (11). In contrast to this, other researchers have proposed that CYP2A3 (14) or CYP2E1 (15) contribute to the metabolic activation of NMBA. The activation of NMBA is observed not only in the liver but also in the oesophagus. The total CYP content in oesophageal microsomes is only 7% of that in liver microsomes (16). Oesophageal mucosal microsomes from male Sprague–Dawley rats can produce benzaldehyde and formaldehyde from NMBA at rates of 1/5 and 1/60 of their respective hepatic levels (13). The O$^b$-methylated guanine level in rats treated
with NMBA was six times higher in oesophageal DNA than in hepatic DNA (17). In addition, ethanol is known to increase the total CYP content (18) and CYP2E1 enzyme protein in the rat oesophagus (15,19). This suggests that metabolic activation in target organs may play an important role in ethanol-induced carcinogenesis. Our previous report showed that suppression of NMBA-induced oesophageal carcinogenesis by curcumin could be attributed to a decrease in the metabolic activation of NMBA by oesophageal CYP2B1 during the initiation phase (20). However, only limited data exist about the effect of ethanol on metabolic activation by the CYP protein in extrahepatic tissues such as rat oesophagus.

To elucidate the mechanism(s) underlying the enhancement of NMBA-induced oesophageal tumorigenesis by ethanol, we assayed the hepatic and extrahepatic levels of microsomal CYP enzymes that are known to activate typical environmental carcinogens in F344 rats treated with ethanol and/or NMBA. Furthermore, the mutagenic activation of these carcinogens by tissue S9 and the three types of hepatic UGT was assayed. We also report on the CYP forms that are responsible for the metabolic activation of NMBA in the liver, oesophagus, kidney, lung and colon of ethanol-treated rats.

Materials and methods

Chemicals

Metyrapone, NPYR and orphenadrine were obtained from Aldrich Chemical (Milwaukee, WI, USA) and aflatoxin B (AFB), was obtained from Makor Chemicals (Jerusalem, Israel). Bilirubin, coumarin, DEN, DMN, ethanol, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), methoxsalen, 4-methylpyrazole, 4-nitrophenol, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), SKF-525A, testosterone, 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) acetate and UDP-glucuronic acid were purchased from Wako Pure Chemicals (Osaka, Japan) and NMBA was purchased from Sakai Laboratory Chemicals (Osaka, Japan). All other commercial products were purchased from Oriental Yeast (Tokyo, Japan). UDP-[14C(U)]glucuronic acid was obtained from American Radiolabeled Chemicals (St Louis, MO, USA) and ATP, glucose-6-phosphate (G6P), G6P dehydrogenase (G6PDH), NADH, NADP+ and NADPH were used. In experiments on CYP inhibition, 25 μM methoxsalen, 1.0 mM coumarin and metyrapone, 0.25 mM SKF-525A, 0.2 mM orphenadrine and 0.1 mM 4-methylpyrazole were preincubated with the carcogen substrate and S9 mix (20,22,23).

Animal experiments and tissue preparation

Animal experiments were executed following the guidelines for the care and use of experimental animals set out by Gifu Pharmaceutical University and Osaka City University (Osaka, Japan).

Six-week-old male F344/DuCJ and F344/N Sc rats were purchased from Charles River Japan (Hino, Shiga, Japan) and Japan SLC, Inc. (Hamamatsu, Japan), respectively. The rats were housed in wire cages (2–3 rats/cage) and maintained under standard laboratory conditions. Twenty-eight male F344/DuCJ rats were divided into four groups of seven animals. Rats in groups 1 and 2 were given access to tap water ad libitum; group 1 rats were subcutaneously treated with 20% dimethyl sulfoxide (DMSO) three times per week for 5 weeks and served as the controls; group 2 rats received injections of 0.5 mg/kg NMBA dissolved in 20% DMSO three times per week for 5 weeks. Rats in groups 3 and 4 were treated for 5 weeks with 10% ethanol added to their drinking water and simultaneously treated with vehicle or NMBA, respectively. Rats were decapitated 24 h after the last dose of vehicle or NMBA. In addition, 32 male F344/N Sc rats were divided into two groups. They were twice treated orally with 50% ethanol (0.5 ml/kg) or unsupplemented drinking water (the second dose was given 12 h after the first). Rats were decapitated 24 h after the last administration. Harvested livers and lungs were perfused in situ with ice-cold sterile 1.15% KCl solution and 25% homogenates were prepared in 1.15% KCl. Oesophageal, kidneys, livers and lungs were homogenized in ice-cold 1.15% KCl after harvesting. Oesophageal and colonic mucosae were stripped off the submucosal and muscular tissues.

Results

Western blots

Goat anti-rat polyclonal antibodies against CYP1A1/2, CYP2B1/2 and CYP2E1, rabbit anti-rat antibodies against CYP3A2 (Nossan Co., Yokohama, Japan) and goat anti-human polyclonal antibodies against CYP2A (Santa Cruz Biotechnology, Santa Cruz, CA) were used as the primary antibodies. Gel electrophoresis and blot analyses were performed as described previously (20).

Mutation assay

All tests were performed using the Ames preincubation assay (21). Seven NOCs, BHP, DEN, DMN, NDMM, NMBA, NNK and NPYR were dissolved in 100 μl of water and all other carcinogens in 50 μl of DMSO. The mutagenicity of Trp-P-2 and IQ (0.03 μg/plate), AFB, (1 μg NPYR (0.25 mg), DMN, DEN, NMBA and NNK (1 mg), BHP and NDMM (10 mg) were assayed in the presence of liver, oesophagus, kidney, lung and colon S9 using established procedures (5,11,20). The tissue S9 fraction volume was 10 μl/plate for heterocyclic amines and 150 μl for NOCs and AFB. Salmonella typhimurium tester strains TA100 and TA98 were used for the seven NOCs and the other carcinogens, respectively. The S9 mix contained the following cofactors: 4 mM NADPH, 4 mM NADH, 0.5 μM G6PDH, 5 mM G6P and 5 mM ATP (except for the NOCs, where 4 mM NADP+ and 5 mM G6P were used). In experiments on CYP inhibition, 25 μM methoxsalen, 1.0 mM coumarin and metyrapone, 0.25 mM SKF-525A, 0.2 mM orphenadrine and 0.1 mM 4-methylpyrazole were preincubated with the carcogen substrate and S9 mix (20,22,23).

Assay of UGT activity

UGT activity on bilirubin and 4-nitrophenol in liver microsomes was assayed according to the method described by Heirwegh et al. (24) and Isselbacher et al. (25), respectively. UGT activity on testosterone was determined using UDP-[14C(U)]glucuronic acid as described by Matern et al. (26).

Statistical analyses

Statistical analyses of the two groups were performed using a Student’s t-test. One-way analysis of variance (ANOVA) followed by a Tukey–Kramer test were used for comparing the four groups with the help of the computer package KyPlot version 2.0 (Kyence Inc., Tokyo, Japan). P < 0.05 was considered significant.

Figure 1 shows the immunoblots and levels of microsomal CYP proteins in male F344 rats treated with NMBA and ethanol for 5 weeks. Oesophageal CYP2E1, CYP2B1, CYP1A1, CYP1A2 and CYP3A2 were constitutively detected with an antibody against hepatic CYP in the vehicle group (group 1, lane 1). The hepatic CYP2A1/2 and oesophageal CYP2A proteins were constitutively detected with antibody against human CYP2A in the vehicle group. CYP2A proteins migrated slightly slower than the standard CYP2A6. The hepatic (left) and oesophageal (right) levels of CYP2E1 in rats treated with 10% ethanol in drinking water (groups 3 and 4, lanes 3 and 4) were 2.1- to 2.3-fold and 1.8- to 2.3-fold higher, respectively, than those observed in group 1 rats. The CYP2E1 level was unchanged in rats subcutaneously treated with 0.5 mg/kg NMBA (group 2, lane 2). The level of oesophageal CYP2A was slightly increased in ethanol-treated group, but no significant differences were observed. There were no significant differences in hepatic levels of constitutively detected CYP2A1/2, CYP2B2, CYP1A2 and CYP3A2 and oesophageal levels of CYP2B1, CYP1A1, CYP1A2 and CYP3A2 among the four groups. Neither hepatic CYP2B1 and CYP1A1 nor oesophageal CYP2B2 were expressed in any group of rats. Figure 2 shows the immunoblots of CYP proteins in the lung, kidney and colon of rats in group 1 (V) and 3 (E). CYP2A, CYP2E1, CYP2B1 and CYP1A1 in the lung; CYP2A, CYP2E1 and CYP2B1 in the kidney; and CYP2A, CYP2E1, CYP2B1 and CYP3A2 in the colon were constitutively detected with antibodies against hepatic CYP in the vehicle group. Treatment with ethanol significantly increased renal CYP2E1 1.8-fold compared with that for
Ethanol activates NMBA by oesophageal CYP2A

group 1 rats ($P < 0.01$). In contrast, there were no significant alterations in the levels of CYP2E1 in the lung and colon and other detected CYP isoforms in the three tissues.

To confirm the evidence for CYP induction, the potency of ethanol was further checked in hepatic and extrahepatic microsomes from F344/N Slc rats, which is genetically similar to F344/DuCrj, orally treated with 50% ethanol twice (corresponding to the daily intake in drinking water containing 10% ethanol without acute toxicities). Figure 3 shows the immunoblots of CYP proteins in the liver, oesophagus, kidney, lung and colon. In addition to the results shown in Figures 1 and 2, CYP2E1 levels in the liver, oesophagus and kidney in ethanol-treated rats were 1.5-, 1.7- and 1.4-fold higher, respectively, than those observed in control rats. Oesophageal and renal CYP2A levels in the treated group were significantly higher than those in the vehicle group (1.7- and 1.5-fold, respectively) although there were no significant alterations in the levels of pulmonary and colonic CYP2E1 and CYP2A, hepatic CYP2A1/2 and other detected CYP isoforms in the five tissues.

To clarify the potential of liver S9 to mediate the mutagenic activation of carcinogens, the mutagenicities of NMBA and eight other carcinogens known to be metabolically activated by specific CYP enzymes were tested in Salmonella strains TA100 and TA98. Table 1 shows the mutagenic activities of six NOCs including NMBA, two heterocyclic amines and AFB1 in the presence of liver S9 mix from rats treated with NMBA and ethanol for 5 weeks. Liver S9 from ethanol-treated rats (groups 3 and 4) increased the mutagenic activities of NPYR, DMN and DEN 1.8-, 2.8- and 1.5-fold, respectively, in TA100 strain compared with that from group 1 rats. No significant alterations in the mutagenicity were observed with NMBA, BHP and NDMM, and there were no significant differences between groups 1 and 2 in the mutagenic activities of any NOCs. A significant effect on the mutagenic activities of IQ, Trp-P-2 and AFB1 in TA98 strain was not observed.

Figure 4 shows the mutagenic activities of NPYR, DMN, DEN, NMBA and NNK in the presence of liver, oesophagus, kidney, lung and colon S9 from rats treated with 50% ethanol twice. Mutagenic activities of NPYR, DMN and DEN with liver S9 increased up to 1.5-fold compared with those in the vehicle group by ethanol treatment, although those of NMBA and NNK did not (A). The mutagenic activities of the five NOCs, including NMBA, in the presence of oesophageal and kidney S9 in ethanol-treated rats increased 1.3- to 1.4-fold or 1.4- to 1.9-fold, respectively, compared with those in control rats (B and C). However, no significant alterations in the mutagenicity of the five NOCs in the presence of lung or colon S9 were produced (D and E).

In an attempt to obtain more information about CYP species responsible for NMBA activation, typical selective and non-selective CYP inhibitors were used in mutagenic activation assays. Figure 5 shows the effects of specific and non-specific inhibitors on the mutagenic activity of NMBA in the presence of liver, oesophagus, kidney, lung and colon S9 from 50% ethanol-treated rats.
Coumarin and methoxsalen are used for specific inhibition of CYP2A activity. Metyrapone, orphenadrine and 4-methylpyrazole are specific inhibitors for CYP2B1/2, CYP2B1 and CYP2E1, respectively, whereas SKF-525A is a non-specific inhibitor. SKF-525A inhibited the mutagenic activation of NMBA in the presence of liver, oesophagus, kidney, lung and colon S9 by 56–94%. Metyrapone inhibited mutagenic activation in the presence of liver S9 by 32%, whereas orphenadrine, methoxsalen and 4-methylpyrazole showed no significant inhibition (A). Alternatively, coumarin and methoxsalen decreased the mutagenic activity of NMBA in the presence of oesophagus (37–53%) and kidney S9 (33–47%) (B and C). Metyrapone and orphenadrine also inhibited the mutagenic activity of NMBA in the oesophagus (21–23%) and kidney (47–50%), but 4-methylpyrazole did not show significant inhibition in these tissues. Five selective inhibitors showed no significant alterations in the activity of NMBA with lung or colon S9 (D and E).

**Table II** summarises the effects of NMBA and ethanol treatment on hepatic UGT activities towards 4-nitrophenol, bilirubin and testosterone in liver microsomes (groups 1–4). Treatment with NMBA, ethanol or both for 5 weeks did not produce significant differences in the three types of UGT activities among the four groups. Similarly, no significant alteration in these activities was observed when treated with 50% ethanol.

**Discussion**

Alterations to hepatic xenobiotic-metabolising enzymes reflect the activation of environmental carcinogens and can predict their carcinogenic potential. NMBA treatment produced no obvious effects on the levels of any CYP subfamilies, mutagenic activation by liver S9 or UGT activities. It has been confirmed by our previous findings that no significant effects are observed in these expressions and activities by NMBA, N-nitrosobis(2-oxopropyl)nitrosamine or N-butyl-N-(4-hydroxybutyl)nitrosamine (11,20,27,28). A 5-week treatment with 10% ethanol administered via drinking water enhanced the mutagenic activation of DEN, DMN and NPYR, reflecting an increase in hepatic CYP2E1. These results are in accordance with our previous reports, which demonstrated the mutagenic activation of DMN and DEN by CYP2E1 after a 2-week treatment with ethanol (11). Similar observations were obtained after intragastric treatments twice with 50% ethanol, suggesting that specific CYP induction does not depend on the route of ethanol administration. Interestingly, the levels of other hepatic CYPs and the activation of NMBA were not induced even by a 5-week treatment. The mutagenic activity of NMBA in the presence of liver S9 from ethanol-treated rats was significantly inhibited by metyrapone, but
Ethanol activates NMBA by oesophageal CYP2A not by 4-methylpyrazole. This supports our previous finding that NMBA was mutagenically activated by hepatic CYP2B2, but not by CYP2E1 in phenobarbital (PB)-induced rats (11). Therefore, it is reasonable to suppose that the mutagenic activation of NMBA was not enhanced by ethanol in the rat liver. Conflicting results have been reported for the inductive potentials of UGT activities by ethanol and we have discussed this matter in a previous report (11). NMBA is known to be a substrate for UGT (29), but no alterations to the activities of the three forms of hepatic UGT were observed in either group of ethanol-treated rats. Consequently, ethanol does not affect the hepatic activation or detoxification of NMBA by these enzymes in rats.

It has been reported that ethanol treatment induces not only oesophageal cancer but also pulmonary, renal and colorectal cancers in rodents (30,31). Ethanol caused an increase in the levels of CYP2E1 in oesophageal and renal tissue, but not in the lung or colon. These results are consistent with other findings in which the CYP2E1 level is induced in the oesophagus (15,19) and kidney (32) by feeding rats with liquid diets containing ethanol. In contrast, it has also been reported that pulmonary and colonic CYP2E1 is induced by ethanol treatment in rats (33,34). The induction of CYP2E1 by ethanol can be attributed to protein stabilisation by protection from cytosolic degradation (35). Furthermore, the ethanol induction of renal and hepatic CYP2E1 involves an additional transcriptional component when blood ethanol concentrations exceed a threshold value of 200–300 mg/dl (32). Therefore, this discrepancy may be due to a lower induction of CYP2E1 by ethanol in the lung (1.2- to 3.0-fold) compared with that of the kidney (5.0- to 9.0-fold) (36). Together with reports that ethanol does not produce an increase in colonic CYP2E1 (19), induction in the lung

Table I. Mutagenic activities of NOCs, HCAs and AFB1 in the S. typhimurium tester strains TA100 and TA98 in the presence of liver S9 from rats treated with NMBA, ethanol or both for 5 weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>TA100 revertants/plate</th>
<th>TA98 revertants/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NPYR (0.25 mg)</td>
<td>DMN (1 mg)</td>
<td>DEN (1 mg)</td>
</tr>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>570 ± 64</td>
<td>117 ± 16</td>
</tr>
<tr>
<td>2</td>
<td>NMBA</td>
<td>471 ± 28 (0.8)</td>
<td>118 ± 24 (1.0)</td>
</tr>
<tr>
<td>3</td>
<td>Ethanol</td>
<td>1018 ± 140* (1.8)</td>
<td>324 ± 61* (2.8)</td>
</tr>
<tr>
<td>4</td>
<td>NMBA + EtOH</td>
<td>1039 ± 141* (1.8)</td>
<td>282 ± 47* (2.4)</td>
</tr>
</tbody>
</table>

Each test was carried out in duplicate (4–8 plates) with liver S9 pooled from seven rats. Data are given as means ± SD after subtraction of spontaneous revertants (TA100, 110-135; TA98, 13-19). The values in parentheses show the ratio to the activity obtained with the vehicle group.

*P < 0.01, compared with the vehicle group (one-way ANOVA followed by Tukey–Kramer test).
and colon may be undetectable in these experiments. In rats, CYP2A1 and CYP2A2 are predominantly expressed in the liver, whereas CYP2A3 is expressed in extrahepatic tissues. Oesophageal and renal CYP2A were significantly increased by intragastric treatment with 50% ethanol, supporting previous findings that renal CYP2A was increased 1.9-fold, albeit not significantly, by a combination of ethanol and a low-fat diet (32). However, clear enhancement of CYP2A was not observed in these tissues from rats treated with 10% ethanol in the drinking water. The reason for this discrepancy is entirely unknown, but activation of NMBA in rat oesophagus by ethanol performed on the initiation phase. Although other researchers have reported that CYP2A3 mRNA was not detected in the kidneys of untreated rats (37). We tested CYP2A activity in the rat kidney by using the inhibitory effect of coumarin and methoxsalen on the mutagenic activity of NMBA in the presence of kidney S9. Additionally, this is the first report in which ethanol did not induce hepatic CYP2A1/2, further investigation of the mechanism of tissue-specific ethanol induction of CYP2A is required.

Enhancement of the mutagenic activities of DEN, DMN and NPYR with tissue S9 by ethanol was observed in the oesophagus, kidney and liver. In addition, ethanol increased the mutagenicity of NMBA and NNK in the presence of oesophageal or renal S9. These results are consistent with the finding that an addition of 5% ethanol to drinking water increases the mutagenicity of NMBA in the rat oesophagus (40). They are also in agreement with reports that NMBA-induced oesophageal carcinogenesis is promoted by ethanol in drinking water (7) or liquid diets containing ethanol (19). This enhancement would be associated with increases not in CYP2E1 but in CYP2A because this activation was suppressed by coumarin and methoxsalen but not 4-methylpyrazole. This is in accordance with findings that the CYP2A3 enzyme, expressed in baculovirus, catalyses the conversion of NMBA to benzaldehyde (14) and that the oesophageal CYP2A3 level in the rat can be a determinant of its ability to metabolise NMBA in vivo (39). However, the inhibition by coumarin and methoxsalen was lower than that observed with SKF-525A in the oesophagus or kidney. This supports the finding that a CYP subfamily other than CYP2A3 is responsible for catalysing the methylene hydroxylation of NMBA (39, 41). Metyrapone and orphenadrine produced a small but significant inhibition of the mutagenicities in these tissues. Suppression of NMBA-induced oesophageal carcinogenesis by curcumin can be attributed to a reduction in the metabolic activation of NMBA by oesophageal CYP2B1 (20). The inhibition by SKF-525A was also observed in lung and colon, indicating that other unconfirmed CYP species were associated with activation in these tissues. In conjunction with these findings, multiple CYP species might be relevant
Ethanol activates NMBA by oesophageal CYP2A to activation of NMBA and responsible enzymes depend on the class of the tissue. Anyway, this is the first demonstration that ethanol exerts an enhancing effect on the mutagenic activation of NMBA by an action on oesophageal CYP2A in rats. In addition, activation of NMBA is predominantly associated with multiple CYP proteins including CYP2A and CYP2B, but not in the case for CYP2E.

In conclusion, it has been demonstrated that ethanol administered either to drinking water or intragastrically results in an enhancing effect on the levels of oesophageal and renal CYP2A and CYP2E1 and hepatic CYP2E1, and the mutagenic activation of five NOCs, including NMBA, in the oesophagus and kidney. Consequently, this suggests that this ethanol-mediated enhancement of NMBA-induced oesophageal carcinogenesis in F344 rats can be attributed to an increase in the metabolic activation of NMBA by oesophageal CYP2A. This occurs during the initiation phase without the contribution of metabolic activation or inactivation by glucuronidation in the liver. Furthermore, activation of NMBA is predominantly associated with CYP2A and CYP2B, but not CYP2E. This difference suggests that N-nitrosodialkylamines with bulky groups are activated by CYP2A and CYP2B, whereas those with relatively short chain(s) are activated by CYP2E1 (42). The present data also suggest that treatment with ethanol may enhance the carcinogenesis initiated by DMN (43) and NPYR (4) or the oesophageal and renal carcinogenesis initiated by other carcinogens activated by CYP2A or CYP2E1 (44). Tissue-specific activation of N-nitrosopiperidine and NPYR contributes to organ-specific carcinogenicities (45), suggesting that CYP expression in the relevant organ may play an important role in tumorigenesis by inducing NOCs during the initiation phase.

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**Table II.** UDPGT activities in liver microsomes from rats treated with NMBA, ethanol or both

<table>
<thead>
<tr>
<th>Treatment</th>
<th>UDPGT activity (nmol/min/mg protein)</th>
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<tr>
<td></td>
<td>4-Nitrophenol</td>
</tr>
<tr>
<td>Drinking treatment</td>
<td></td>
</tr>
<tr>
<td>Vehicle (group 1)</td>
<td>25.2±2.1</td>
</tr>
<tr>
<td>NMBA (group 2)</td>
<td>24.2±1.4</td>
</tr>
<tr>
<td>Ethanol (group 3)</td>
<td>22.5±2.0</td>
</tr>
<tr>
<td>NMBA + ethanol (group 4)</td>
<td>26.3±2.1</td>
</tr>
<tr>
<td>Intragastric treatment</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>22.5±2.5</td>
</tr>
<tr>
<td>Ethanol</td>
<td>18.3±1.4</td>
</tr>
</tbody>
</table>

Each test was carried out with liver microsomes pooled from 10 rats. The results are expressed as mean ± SD of between three and six experiments.
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