INTRODUCTION

After oral intake, alcohol is absorbed into the portal circulation from the stomach and upper part of the small intestine. Thereafter, it is rapidly transported to other organs, including the large bowel. Ethanol is evenly distributed in the water phase of all organs and, accordingly, after the distribution phase, ethanol levels in the terminal ileum (Halstedt et al., 1973) and colon (Levitt et al., 1982; Jokelainen et al., 1996a) are equal to those of the blood and liver.

It has been demonstrated that normal human colonic contents (Jokelainen et al., 1994), especially its aerobic bacteria (Jokelainen et al., 1996b), are capable of producing significant amounts of acetaldehyde when incubated aerobically or microaerobically with ethanol in vitro (Salaspuro et al., 1999).

Moreover, high levels of acetaldehyde have been demonstrated in the colon during normal ethanol metabolism in vivo (Jokelainen et al., 1996a). Intracolonic acetaldehyde accumulation can be prevented by ciprofloxacin (Visapää et al., 1998), which decreases intestinal aerobic and facultative anaerobic flora, faecal alcohol dehydrogenase (ADH; EC 1.1.1.1) activity and acetaldehyde production (Tillonen et al., 1999).

On the other hand, metronidazole induces an increase in the ADH-containing aerobic and facultative anaerobic flora thus leading to high intracolonic acetaldehyde concentrations (Tillonen et al., 2000).

Colonic mucosal ADH and aldehyde dehydrogenase (ALDH; EC 1.2.1.3) activities are low, as compared to those of the liver (Koivisto and Salaspuro, 1996). It has been proposed that colonic mucosal ALDH activity may be sufficient for the removal of acetaldehyde produced by colonic mucosal ADH, but insufficient for the removal of the acetaldehyde produced by intracolonic bacteria (Koivisto and Salaspuro, 1996). Recent studies on ALDH2-deficient Orientals indicate that human parotid glands can significantly contribute to the local production of acetaldehyde in the saliva (Väkeväinen et al., 2002). When this information is combined with earlier epidemiological data, they provide strong evidence for the local carcinogenic action of acetaldehyde in the upper digestive tract of man (Yokoyama et al., 1998). ALDH2 gene deficiency results in a 3.4-fold risk of colon cancer among Japanese drinkers (Yokoyama et al., 1998; Murata et al., 1999). If colonic mucosal ALDH contributes to the regulation of intracolonic acetaldehyde levels, its genetic or pharmacological inactivation could lead to enhanced local production of acetaldehyde in the large intestine as well.

The aim of this study was therefore to examine the possible inhibition of colonic mucosal ALDH activity by disulfiram and its consequent changes in intracolonic acetaldehyde levels after ethanol challenge in rats. The second aim was to find out how this is affected by metronidazole treatment, which is known to increase intracolonic microbial acetaldehyde production.

MATERIALS AND METHODS

Animals and study protocol

Forty male Wistar rats, weighing 363 ± 3.5 g (mean ± SEM), were used. They were housed in groups of five animals in plastic cages under conventional conditions. The room temperature was maintained at 22 ± 2°C and a 12-h light/12-h dark cycle was applied. All rats had free access to standard chow (Altromin 1324 pellets; Altromin, Lage, Germany) and water until the time of the experimental procedure. Throughout the whole experiment, the general condition and behaviour of the animals as well as their daily intake of food was recorded.

Ten rats (M) received a diet containing 400 mg of metronidazole mixed with 175 ml of water and homogenized with 200 g of standard chow for 5 consecutive days. Another 10 rats (D) received 400 mg of disulfiram mixed in their food.
as above. Ten rats (MD) received both the metronidazole and the disulfiram, mixed as above. The daily dose of each medication was 30 mg per day per rat (100 mg/day/kg). Ten rats served as controls (C) and received only standard chow without any medication. The animals were weighed on the first and last days of the treatment and faecal samples were taken simultaneously for the determination of faecal ALDH activities. After weighing, an ethanol dose of 1.5 g/kg body weight (as a 16% v/v solution in saline) was administered i.p. to all of the rats. Two hours after the ethanol injection, the rats were anaesthetized with sodium pentobarbital (60 mg/kg body weight, i.p.). Thereafter liver and colonic mucosal biopsies for ADH and ALDH activity determinations and colonic content and blood samples by heart puncture for ethanol and acetaldehyde determinations were obtained.

### Intracolonic ethanol and acetaldehyde levels

The samples in which intracolonic ethanol and acetaldehyde concentrations were to be measured were taken 2 h after ethanol administration, during anaesthesia, by dissecting the samples straight from the caecum. To prepare these caecal samples for intracolonic acetaldehyde and ethanol determinations, the samples were diluted (1:6) with distilled ice-cold water and then mixed carefully. An aliquot of 450 µl was pipetted into ice-cold gas chromatography vials and mixed with 50 µl of ice-cold 6 M perchloric acid. The colonic acetaldehyde was analysed by head-space gas chromatography (Visapää et al., 1998).

To control for possible non-enzymatic artefactual acetaldehyde formation from ethanol during protein precipitation, perchloric acid was added simultaneously with ethanol to incubation vials derived from three additional control rats. The revealed values at the ethanol concentration of 22 mM (mean 66 µM of acetaldehyde) were subtracted from in vivo acetaldehyde values.

### Blood ethanol and acetaldehyde analysis

The blood samples were taken by heart puncture and processed for ethanol and acetaldehyde determinations according to the semicarbazide method described by Stowell (1979). Acetaldehyde and ethanol concentrations were determined by head-space gas chromatography at 65°C as previously described (Eriksson et al., 1977). Conditions for analysis were as follows: column 60/80 Carbopack C/0–2% Carbowax 1500, 2 m x 1/8” (Supelco, Inc., Bellefonte, PA, USA); oven temperature 85°C; dosing line and detector temperature 200°C; carrier gas (N₂) flow rate 20 ml/min.

### Preparation of tissues

The faecal samples for ALDH activity determination were collected before the i.p. ethanol injection by gently pressing the rats’ rectal area, and stored immediately at –80°C. After anaesthesia, livers were perfused in situ with saline, removed, and liver samples were placed immediately in liquid nitrogen. The colons were washed with cold saline and the mucosal layers were collected by gentle scraping and placed in liquid nitrogen. Within 30 min, samples were transferred to –80°C until analysis. Ice-cold medium containing 0.25 M sucrose, 5 mM Tris, and 0.5 mM EDTA (pH 7.2) was then added until the tissue constituted 15–20% of the total volume, and the samples were homogenized with a Potter S-homogenizer (B. Braun, Darmstadt, Germany) using maximum speed. This was followed by sonication for 9 x 5 s, as has been recommended for the disruption of intestinal cells (Lindeskog et al., 1986). The homogenate was centrifuged at 700 g for 15 min to remove unbroken cells and nuclei and the supernatant was used for all enzyme assays.

### Determination of ALDH and ADH activities

Faecal ALDH activities were determined spectrophotometrically as previously described (Nosova et al., 1998). Hepatic and mucosal ALDH and ADH activities were determined spectrophotometrically by measuring the formation of NADH at 340 nm at 25°C. ALDH activities of supernatants were assayed in 60 mM sodium pyrophosphate buffer (pH 8.8) containing 0.5 mM of NAD⁺, 0.1 mM of 4-methylpyrazole, 2 µM of rotenone, and either 100 µM (low Kₘ activity) or 5 mM (total activity) of acetaldehyde. High Kₘ activities were calculated by subtracting low Kₘ activities from the total. Before ALDH assays, sodium deoxycholate was added to the samples at a concentration of 0.3% followed by incubation at 4°C for ≥20 min. ADH activities of cytosolic fractions were measured in 100 mM glycine buffer (pH 9.6) containing 1 mM of NAD⁺, 2 µM of rotenone, and 25 mM of ethanol. ALDH and ADH activities are expressed as specific activities. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as standard protein. One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 µmol of NADH/min.

### Statistical analysis

Differences between the groups were analysed by one-way analysis of variance, followed by the Tukey–Kramer multiple comparison test. Logarithmic transformation was performed when appropriate. If two groups were compared, the Mann–Whitney U-test was used. All calculations were derived from two-sided tests. P < 0.05 was regarded as significant. All values are expressed as mean ± SEM.

### RESULTS

### Animals and their body weights

All animals tolerated well the 5-day treatment period. The body weights were not significantly affected by treatments. Thus, rat weights were as follows (initial – final): group 1 (C, control) 378 ± 3 – 379 ± 3 g, group 2 (D, disulfiram) 350 ± 5 – 350 ± 5 g, group 3 (M, metronidazole) 367 ± 4 – 368 ± 4 g, group 4 (MD, metronidazole + disulfiram) 357 ± 7 – 356 ± 7 g.

### Blood ethanol and acetaldehyde levels

Significant differences in blood-ethanol concentrations between the groups could not be detected. They ranged from 23.4 ± 0.9 mM in the disulfiram + metronidazole-treated group to 26.7 ± 2.7 mM in the non-premedicated group. Blood acetaldehyde level was significantly elevated in the disulfiram and disulfiram + metronidazole groups (59.4 ± 11.1 and 29.4 ± 6.5 µM, respectively) as compared to the controls (13.3 ± 1.4 µM) (P < 0.05). Metronidazole treatment did not increase blood acetaldehyde (8.4 ± 3.3 µM) as compared to the controls.
Intracolonic acetaldehyde activities

Metronidazole, disulfiram and the combination of these two treatments markedly increased intracolonic acetaldehyde levels, as compared to those of controls (Fig. 1) \( P < 0.001 \). The differences between the premedicated groups were not statistically significant.

Liver and colonic mucosal ADH and ALDH activities

In general, ADH and ALDH activities were significantly lower in the colon than in the liver (Table 1). ADH activities of liver and colonic samples were \( 15.7 \pm 1.2 \) and \( 3.9 \pm 0.4 \) mU/mg, respectively. Disulfiram treatment reduced both hepatic and colonic mucosal low \( K_M \) ALDH activities, whereas metronidazole treatment did not. The high \( K_M \) ALDH activity was reduced markedly only in colonic mucosae by both disulfiram and metronidazole (Table 2).

Faecal ALDH activities

There were no detectable ALDH activities in faecal samples of any of the rat groups (data not shown).

DISCUSSION

The main finding of this study was that, in addition to microbes, colonic mucosal cells also contribute to the regulation of intracolonic acetaldehyde concentration during ethanol challenge. Earlier, it has been shown that colonic mucosal ALDH activities are relatively low as compared to that of the liver (Koivisto and Salaspuro, 1996) and our present observations are consistent with these findings. Even so, in the light of this study, the local mucosal enzyme activity seems to have more importance in the regulation of local intracolonic acetaldehyde level than has previously been thought.

The human microflora consists of hundreds of bacterial species and subspecies and \( \sim 10^{14} \) individual bacteria (Goldin, 1990). In the colonic lumen, oxygen tension is so low that mainly anaerobes predominate (Hill, 1995). At the colonic mucosal surface, however, oxygen diffusion is sufficient to maintain significant \( O_2 \) pressure, capable of inhibiting the growth of strict anaerobes. Accordingly, aerobes on the colonic mucosa are equally or more numerous than anaerobes (Hill, 1995).

The microbial production of acetaldehyde from ethanol is mediated mainly by aerobic or facultative anaerobic bacteria (Jokelainen et al., 1996b). Reduction of these bacteria by ciprofloxacin reduces the rate of ethanol elimination by 9% both in rats and humans with the subsequent decrease in faecal ADH activity and acetaldehyde-producing capacity (Jokelainen et al., 1997; Tillonen et al., 1999). In chronically ethanol-fed rats, metronidazole treatment, in turn, increases intracolonic acetaldehyde production up to 5-fold without inhibiting ALDH-activity of colonic mucosa or liver (Tillonen et al., 2000). Thus, this increase in intracolonic acetaldehyde

![Fig. 1. Intracolonic acetaldehyde levels in the four study groups. Values are means + SEM (bars). \( * P < 0.001 \) vs disulfiram, metronidazole, disulfiram + metronidazole, \( P > 0.05 \) (not significant) when comparing premedicated groups with each other.]

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>ADH</th>
<th>ALDH ( (high K_M) )</th>
<th>ALDH ( (low K_M) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C)</td>
<td>10</td>
<td>16.8 ± 1.1</td>
<td>35.7 ± 2.5</td>
<td>9.3 ± 0.7</td>
</tr>
<tr>
<td>Disulfiram</td>
<td>10</td>
<td>14.3 ± 0.9</td>
<td>32.7 ± 1.2</td>
<td>5.4 ± 0.5(^a)</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>10</td>
<td>17.0 ± 1.2</td>
<td>33.1 ± 2.7</td>
<td>8.4 ± 0.8</td>
</tr>
<tr>
<td>Metronidazole + disulfiram</td>
<td>10</td>
<td>14.7 ± 1.6</td>
<td>33.1 ± 3.0</td>
<td>5.8 ± 1.3(^b)</td>
</tr>
</tbody>
</table>

\(^a\) \( P < 0.05 \) compared with C; \(^b\) \( P < 0.05 \) compared with C.

Table 2. Colonic mucosal alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) activities in the four study groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>ADH</th>
<th>ALDH ( (high K_M) )</th>
<th>ALDH ( (low K_M) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C)</td>
<td>10</td>
<td>4.1 ± 0.5</td>
<td>7.8 ± 1.1</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>Disulfiram (D)</td>
<td>10</td>
<td>3.5 ± 0.3</td>
<td>3.7 ± 0.4(^a)</td>
<td>1.0 ± 0.2(^b)</td>
</tr>
<tr>
<td>Metronidazole (M)</td>
<td>10</td>
<td>4.8 ± 0.2</td>
<td>4.5 ± 0.2(^a)</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Metronidazole + disulfiram</td>
<td>10</td>
<td>3.2 ± 0.3</td>
<td>3.3 ± 0.3(^a)</td>
<td>1.0 ± 0.2(^d)</td>
</tr>
</tbody>
</table>

\(^a\) \( P < 0.001 \) compared with C; \(^b\) \( P < 0.01 \) compared with C and \( P < 0.01 \) compared with M; \(^d\) \( P < 0.01 \) compared with C; \(^d\) \( P < 0.001 \) compared with C and \( P < 0.001 \) compared with M.
production from ethanol can most probably be explained by the replacement of intestinal aerobes by ADH-containing facultative Enterobacteriaceae. In the present study, metronidazole inhibited markedly the colonic mucosal high $K_M$ ALDH activity, which may also have its own influence on accumulation of intracolonic acetaldehyde.

There is experimental evidence supporting the role of high intracolonic acetaldehyde concentration in ethanol-related rectal co-carcinogenesis (Seitz et al., 1990), and a strong correlation between colonic mucosal acetaldehyde and colonic crypt cell production rate has been observed (Simonowski et al., 1994). Moreover, ALDH2 gene deficiency seems to be associated with markedly increased risk of digestive tract cancers, not only in alcoholics, but also in non-alcoholic drinkers (Yokoyama et al., 1996a,b; Murata et al., 1999). During ethanol challenge, ALDH2-deficient subjects have two to three times higher salivary acetaldehyde levels than those with the normal ALDH2 enzyme (Väkevääinen et al., 2000). The salivary glands, in addition to oral microbes of ALDH2-deficient subjects, are able to produce acetaldehyde from ethanol (Väkevääinen et al., 2000). Accordingly, while drinking, ALDH2-deficient subjects are always exposed to abnormally high local acetaldehyde levels.

In this study, we found that both metronidazole and disulfiram treatment effectively increases intracolonic acetaldehyde levels. The possibility that this effect could be blood-derived can be excluded by the fact that intracolonic acetaldehyde levels were almost 10-fold higher than in the blood. As stated earlier, metronidazole favours the growth of ADH-containing aerobic bacteria in the colonic lumen, which results in enhanced microbially mediated local acetaldehyde production. Disulfiram acts by inhibiting low $K_M$ aldehyde dehydrogenase both in the liver and in colonic mucosa, having only negligible antibiotic properties (Phillips et al., 1991). This leads to high blood and intracolonic acetaldehyde concentrations, when used together with ethanol. There were no detectable ALDH activities in faecal samples of the animals and neither of the medications had any effect on that. Therefore, blocking faecal ALDH cannot explain the measured high intracolonic acetaldehyde concentrations. It has also been shown that disulfiram treatment alone, even in the absence of ethanol, raises endogenous plasma and red blood cell acetaldehyde concentrations, possibly due to diminished catalysis of endogenously generated acetaldehyde (Eriksson, 1985; Rosman et al., 2000).

It has been shown previously that intracolonically produced acetaldehyde is rapidly metabolized to acetate by the first-pass metabolism of the liver (Matsiak-Budnik et al., 1996). This may explain why the high intracolonic levels of acetaldehyde are not reflected in elevated blood acetaldehyde concentrations of metronidazole-treated rats.

Why the combination of metronidazole and disulfiram treatments did not have a synergistic effect is most probably explained by the inhibition of the same colonic mucosal high $K_M$ ALDH by both metronidazole and disulfiram. Another possible explanation could be disulfiram’s non-specific inhibitory effect on many other enzymes besides ALDH. It has been shown that disulfiram, for instance, inhibits ADH enzyme (Carper et al., 1987; Langeland and McKinley-McKee, 1997), and therefore could decrease the bacterial ADH-related acetaldehyde formation. A third possibility is that these two drugs have some as-yet-unknown pharmacological interactions when used together. This phenomenon is supported by the early findings of possible enhanced toxicity of the combination (Goodhue, 1969; Rothstein and Clancy, 1969).

In conclusion, this study points out that colonic mucosal cells, in addition to colonic bacteria, have a more important role in regulating intracolonic acetaldehyde than has previously been thought. In the present study, we were able to show for the first time that disulfiram effectively blocks colonic mucosal low $K_M$ aldehyde dehydrogenase which leads to very high local intracolonic acetaldehyde concentrations during alcohol challenge. This may be important, since the local carcinogenic properties of acetaldehyde have recently been well established. The genetic inactivation of colonic mucosal ALDH among ALDH2-deficient persons may thus explain the 3.4-fold increased risk for colorectal cancer in that population.

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REFERENCES


