

CIPROFLOXACIN ADMINISTRATION DECREASES ENHANCED ETHANOL ELIMINATION IN ETHANOL-FED RATS

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Abstract — Many colonic aerobic bacteria possess alcohol dehydrogenase (ADH) activity and are capable of oxidizing ethanol to acetaldehyde. Accordingly, some ingested ethanol can be metabolized in the colon *in vivo* via the bacteriocolonial pathway for ethanol oxidation. By diminishing the amount of aerobic colonic bacteria with ciprofloxacin treatment, we recently showed that the bacteriocolonial pathway may contribute up to 9% of total ethanol elimination in naive rats. In the current study we evaluated the role of the bacteriocolonial pathway in enhanced ethanol metabolism following chronic alcohol administration by diminishing the amount of gut aerobic flora by ciprofloxacin treatment. We found that ciprofloxacin treatment totally abolished the enhancement in ethanol elimination rate (EER) caused by chronic alcohol administration and significantly diminished the amount of colonic aerobic bacteria and faecal ADH activity. However, ciprofloxacin treatment had no significant effects on the hepatic microsomal ethanol-oxidizing system, hepatic ADH activity or plasma endotoxin level. Our data suggest that the decrease in the amount of the aerobic colonic bacteria and in faecal ADH activity by ciprofloxacin is primarily responsible for the decrease in the enhanced EER in rats fed alcohol chronically. Extrahepatic ethanol metabolism by gastrointestinal bacteria may therefore contribute significantly to enhanced EER.

INTRODUCTION

Chronic administration of ethanol to experimental animals (Misra *et al.*, 1971; Nomura *et al.*, 1983) and chronic alcohol abuse in man (Misra *et al.*, 1971; Salaspuro and Lieber, 1978; Lindros *et al.*, 1980) increase the ethanol elimination rate (EER). The biochemical background of this phenomenon, however, remains under discussion. We have demonstrated that many facultative aerobic intestinal bacteria possess marked alcohol dehydrogenase (ADH, EC 1.1.1.1) activity (Jokelainen *et al.*, 1996b; Nosova *et al.*, 1998) and are capable of oxidizing ethanol to acetaldehyde both *in vivo* and *in vitro* (Jokelainen *et al.*, 1996a,b). This implies that some of the ingested ethanol is metabolized to acetaldehyde in the colon via the bacteriocolonial pathway during normal alcohol metabolism (Jokelainen *et al.*, 1996a,b; Salaspuro, 1996, 1997). By

diminishing the number of intestinal aerobic bacteria and lowering ADH activity of the colonic contents with the selective antibiotic ciprofloxacin, we recently found that the bacteriocolonial pathway for ethanol oxidation may contribute up to 9% of the total EER in rats (Jokelainen *et al.*, 1997). Furthermore, ciprofloxacin treatment has been shown to almost totally block the alcoholic fermentation pathway and acetaldehyde production by the gut flora of rats (Visapää *et al.*, 1998).

The purpose of this study was to examine whether the bacteriocolonial pathway for ethanol oxidation also contributes to the enhanced EER caused by chronic alcohol feeding. To that aim, we studied whether the diminution of aerobic gut flora by ciprofloxacin treatment influences EERs in chronically alcohol-fed and control rats. In addition to EERs, we also determined the effect of chronic alcohol feeding and ciprofloxacin addition to ethanol on the composition of faecal flora, on faecal ADH activity, on hepatic microsomal ethanol-oxidizing system (MEOS) and ADH

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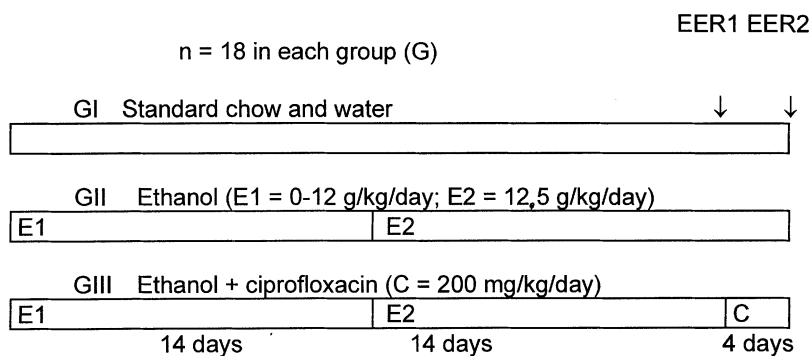


Fig. 1. Study protocol used for the determination of the ethanol elimination rates before (EER 1) and after (EER 2) ciprofloxacin treatment (200 mg/kg body wt daily for 4 days).

activities as well as on plasma endotoxin levels.

MATERIALS AND METHODS

Animals

Fifty-four male Wistar rats, weighing 190 ± 6 g (mean \pm SEM) were housed in groups of three animals in plastic cages under conventional conditions. All rats had free access to standard rat chow (Altromin 1324-pellets, Altromin, Lage, Germany) until the start of the ciprofloxacin treatment. Thirty-six rats (group II and group III) received increasing ethanol doses starting from 5% (v/v) in drinking fluid for the first 14 days. They then received 12.5 g/kg/day of ethanol as 21% ethanol solution over the next 18 days (Fig. 1). To increase the palatability of drinking fluids, 2% (w/v) sucrose was added to the ethanol solutions (Batey and Patterson, 1991). Ciprofloxacin mixed with standard chow was administered additionally to 18 ethanol-fed rats (group III) during the last 4 days at a daily dose level of 200 mg/kg body wt (Fig. 1). Eighteen control rats (group I) received tap water and standard chow during the experimental period. The volume of ethanol solution consumed each day was measured in order to calculate the daily ethanol intake. Twelve hours before the determination of the EER the ethanol–water–sucrose mixture was replaced by tap water.

In vivo determination of the EER

The EERs were determined in all rats after 28

days of ethanol administration (EER 1), and again on the fourth day after the beginning of the ciprofloxacin treatment (EER 2) (Fig. 1). Ethanol (1.5 g/kg body wt) was injected i.p. as a 16% (v/v) solution in saline. Duplicate blood samples (50 μ l each) were drawn from the tail vein before injection and at 30-min intervals up to 180 min, and thereafter once an hour up to 300 min. Blood samples were haemolysed in 450 μ l of ice-cold water and blood ethanol was determined by head-space gas chromatography as described earlier (Jokelainen *et al.*, 1997). The EER was calculated by extrapolating the linear part of the ethanol elimination curve to zero. The area under the curve (AUC) (0–5 h) was calculated using the trapezoidal rule.

Faecal samples

In a preliminary study, we found that faecal ADH activity corresponds well to the ADH activity determined from fresh contents obtained from the caecum of rats. Faecal samples were collected before EER 2, immediately put into dry ice and stored at -80°C . Faecal samples were lyophilized (Micro Modulyo, Edwards) for 18 h and homogenized in 8 vol 100 mM potassium phosphate buffer (pH 7.4), then sonicated for 8×30 s at 4°C . This was followed by centrifugation at 100 000 *g* for 60 min to obtain the supernatant. Faecal ADH activity was then assayed immediately (see below).

Subcellular fractionation of tissues

After the EER 2 determination was completed,

the rats were anaesthetized with phenobarbital. The abdomen was opened and the whole liver was removed and weighed. A piece of liver was then placed into ice-cold medium containing 0.25 M sucrose, 5 mM Tris, and 0.5 mM EDTA (pH 7.2) to make the tissue proportion from 15 to 20% of the total. The samples were homogenized with a Potter S-homogenizer (B. Braun, Germany) using maximum speed. Subcellular fractions were then prepared by differential centrifugation. The homogenate was first centrifuged at 700 *g* for 15 min to remove unbroken cells, nuclei, and debris. Mitochondria were isolated thereafter by centrifugation at 12 000 *g* for 15 min. Cytosolic fraction and microsomes were then separated by centrifugation at 120 000 *g* for 70 min. Cytosols and microsomes were stored separately at -80°C until assayed.

Enzyme determinations

ADH activity was determined spectrophotometrically at 340 nm by measuring the reduction of nicotinamide adenine dinucleotide (NAD^+ , 2.5 mM final concentration) in 100 mM glycine buffer (pH 9.6) at 37°C by using 25 mM ethanol (final concentration). Protein concentrations were determined using the BIORAD method with bovine serum as a standard. ADH activities were then calculated as nmol of NADH produced per mg of protein/min. To calculate the total hepatic ADH activity, protein concentrations were determined in hepatic cytosolic fractions. Total hepatic ADH activity was then calculated by multiplying the hepatic ADH activity by the amount of cytosolic proteins/g of liver and the liver weight, and expressed as μmol of NADH produced by the liver/min. Hepatic MEOS activity was determined according to Ardies *et al.* (1987). Sequential addition of 0.3 M barium hydroxide and 0.3 M zinc sulphate was used to stop the MEOS reaction during the assay.

Quantitative bacterial cultures

To determine the influence of ethanol feeding and of ciprofloxacin addition on bacterial culture quantities, faecal samples of 18 rats were collected before ethanol feeding, and before the EER 1 and EER 2 studies. The faecal samples were thawed, weighed, suspended, and serially diluted (10-fold) from 10^{-1} to 10^{-7} in peptone yeast extract (PY) broth. An aliquot of 100 ml of the appropriate dilutions was inoculated and spread with a bent

sterile rod on several selective and non-selective agar media for the enumeration and isolation of aerobic and anaerobic bacteria and yeasts. The aerobic plates were incubated at 36°C in an atmosphere containing 5% CO_2 for 2–4 days and anaerobic plates in anaerobic jars filled with mixed gas (90% N_2 :5% CO_2 :5% H_2) for 7 days using the evacuation replacement method. The bacteria were enumerated and identified by established methods.

Endotoxin determination

Blood was collected by heart puncture in pyrogen-free tubes containing heparin (Chromogenix). Plasma was immediately separated in a refrigerated centrifuge at 800 *g* for 10 min. The supernatant was stored in pyrogen-free storage tubes (BioWhittaker) at -80°C until analysis. Plasma endotoxin levels were measured within 2 weeks of collection using the Limulus Amebocyte Lysate (LAL)-based chromogenic assay (BioWhittaker QCL 1000) by the microplate (Costar) method using a Multiscan RC microplate reader (Labsystems, Finland). Increased sensitivity of the test was achieved by extending the incubation of the samples with LAL up to 30 min.

Statistical methods

All results are expressed as means \pm SEM. Statistical analysis was performed using one-way ANOVA, followed by the Tukey multiple comparison test, the *t*-test and paired *t*-test using the Sigma Stat 2.0 program. The EERs were determined by linear regression.

Ethical considerations

The study was conducted in accordance with the principles of the declaration of Helsinki and approved by the Local Committee on Animal Experimentation.

RESULTS

Chronic ethanol administration enhanced EER 1 in both ethanol-fed rat groups by 14% [372 ± 9 (group I) vs 423 ± 8 mg/kg/h (group II + group III)]. Accordingly, the mean AUC decreased significantly [53.3 ± 3.1 (group I) vs 42.3 ± 1.6 mmol/l/h (group II + group III), $P < 0.002$]. The enhancement in the EER was abolished by ciprofloxacin treatment in group III ($332 \pm$

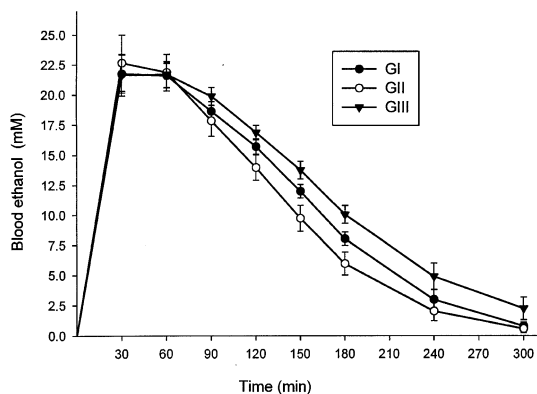


Fig. 2. Blood-ethanol concentration after acute administration to control rats and to rats chronically treated with ethanol or ethanol + ciprofloxacin.

Ethanol (1.5 g/kg) was injected i.p. to control rats (group I) and to rats chronically treated with ethanol (group II) or ethanol + ciprofloxacin (group III) according to the protocol in Fig. 1. Values are means \pm SEM (bars) for 18 rats/group.

11 mg/kg/h, EER 2), and was even lower than that of control rats (group I; Fig. 2). Accordingly, the mean AUC (60.5 ± 3.9 mmol/l/h) increased significantly ($P < 0.001$) in comparison to group II after the addition of ciprofloxacin to ethanol feeding (Fig. 3). However, the maximal ethanol concentration in the blood did not differ between the groups (Fig. 2) indicating that the absorption of ethanol from the peritoneal cavity was equal in all groups.

Chronic alcohol feeding to rats (group II + group III) resulted in a significant decrease ($P < 0.001$) in the number of *Lactobacillus* spp. ($2.3 \pm 0.4 \times 10^9$ vs $3.8 \pm 1.3 \times 10^8$ CFU/g) in the rat faecal samples (before ethanol vs after 4 weeks of ethanol feeding, respectively). Accordingly, the total amount of anaerobic bacteria decreased significantly ($P < 0.05$) in faecal samples of these rats ($9.2 \pm 3 \times 10^9$ vs $4.2 \pm 0.8 \times 10^9$ CFU/g). However, the aerobic bacterial counts were not significantly affected by ethanol feeding. Ciprofloxacin treatment for 4 days produced a clear decline ($P < 0.02$) in the total number of faecal aerobic bacteria in group III, compared to group II rats. Moreover, the number of *E. coli*, *Enterococcus*, and *Proteus mirabilis* in rat faecal samples decreased to zero with ciprofloxacin treatment. Additionally, the mean ADH activity

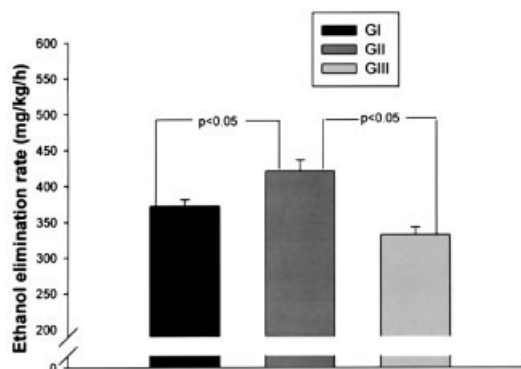


Fig. 3. Effect of ethanol feeding, and addition of ciprofloxacin on the ethanol elimination rates.

Details are as described in Figs 1 and 2 and in the Materials and methods section. Values are means \pm SEM (bars) for 18 rats/group.

of the faecal samples was significantly ($P < 0.01$) lower in group III, compared to group II rats (Table 1).

However, ciprofloxacin treatment affected neither hepatic MEOS nor ADH activity in comparison to the ethanol-fed group II rats (Table 2). No significant differences either in the mean total hepatic ADH activity or in the mean body weight among rat groups were found. Plasma endotoxin levels also remained below the detection limit in all rat groups.

DISCUSSION

We have recently shown that ciprofloxacin treatment (200 mg/kg/day for 4 days) decreased EER in rats by 9% (Jokelainen *et al.*, 1997). This was associated with a marked decrease in the number of coliforms and Enterococcae and in the total aerobic bacteria count in rat faecal samples. The drug had no influence on either hepatic MEOS or ADH activities. In contrast, acute administration of ciprofloxacin had no effect on the EER (Jokelainen *et al.*, 1997). These data indicate that the decrease in the EER caused by ciprofloxacin treatment results from the reduction of the number of gut aerobic bacteria. The current study shows that the reduction of the aerobic gastrointestinal flora by similar ciprofloxacin treatment completely abolishes alcohol-induced enhancement in EER in rats.

Table 1. Aerobic bacterial counts and alcohol dehydrogenase activity in faecal samples of control rats, ethanol-fed rats and ethanol + ciprofloxacin-treated rats

Parameter	GI	GII	GIII
Total count (CFU/g)	$9.8 \pm 6 \times 10^8$	$8.7 \pm 4 \times 10^7$	$5 \pm 3 \times 10^{4**}$
<i>E. coli</i> sp. (CFU/g)	$1.6 \pm 0.9 \times 10^6$	$7.3 \pm 3 \times 10^5$	0**
<i>Enterococcus</i> sp. (CFU/g)	$2.2 \pm 0.5 \times 10^7$	$3.3 \pm 0.8 \times 10^6$	0**
<i>Proteus mirabilis</i> sp. (CFU/g)	$1.8 \pm 1.6 \times 10^4$	$3.5 \pm 2.2 \times 10^5$	0**
ADH activity (nmol/min/mg protein)	3.26 ± 0.9	0.98 ± 0.4	0**

GI (control group); GII (ethanol-treated group 2); GIII (ethanol + ciprofloxacin-treated group 3). ADH, alcohol dehydrogenase. Values are means \pm SEM for each group of 18 rats. * $P < 0.01$ vs chronic ethanol (GII), ** $P < 0.02$ vs chronic ethanol (GII).

In the present study, the decrease in the enhanced EER after ciprofloxacin treatment was associated with diminished total amounts of gut aerobic bacteria and of *E. coli*, *Enterococcus* and *Proteus mirabilis*, as well as with a decreased total faecal ADH activity. However, neither hepatic MEOS nor ADH was affected by ciprofloxacin treatment. Recently, the sterilization of the intestinal tract with antibiotics has been shown to prevent the elevation of plasma endotoxin levels and the increase in the EER in chronically alcohol-fed rats (Adachi *et al.*, 1995). The latter effect was related to the inhibition of the activation of hepatic Kupffer cells (Adachi *et al.*, 1994, 1995). In the current study, despite the induction of the EER, plasma endotoxin levels remained below the detection limit in all rat groups. Therefore, it is unlikely that the changes observed in the EER could be explained by an effect of endotoxin on hepatic Kupffer cells. Accordingly, the decrease in the amount of the aerobic colonic bacteria and in faecal ADH activity may be primarily responsible for the

decrease in the enhanced EER caused by ciprofloxacin treatment.

Bacterial overgrowth and the increased number of aerobic and anaerobic bacteria have been reported in the small intestinal microflora of chronic alcoholics (Bode *et al.*, 1984) and especially among patients with alcoholic liver disease (Bode *et al.*, 1993). However, it remains to be established whether chronic alcohol feeding might lead to bacterial overgrowth in the stomach or the small intestine of rats. In the current study, chronic alcohol feeding of rats, however, increased neither the amount of colonic aerobic bacteria nor faecal ADH activity. Accordingly, the lack of increase in ADH activity by chronic alcohol feeding implies that faecal ADH activity could not be the rate-limiting step in the bacteriocolonial pathway for ethanol oxidation. However, it may become rate-limiting after treatment with ciprofloxacin. In this respect, the situation may be comparable to the regulation of the rate of ethanol metabolism, demonstrated previously in experimental animals, when hepatic

Table 2. Faecal alcohol dehydrogenase (ADH) activity, hepatic microsomal ethanol-oxidizing system (MEOS), and ADH activity, total hepatic ADH activity and plasma endotoxin levels in the controls (GI), the ethanol-fed (GII) and the ethanol and ciprofloxacin-treated rats (GIII)

Parameter	<i>n</i>	GI	GII	GIII
Hepatic MEOS activity (nmol/min/mg protein)	36	2.0 ± 0.3	2.5 ± 0.4	2.8 ± 0.7
Hepatic ADH activity (nmol/min/mg protein)	18	23.3 ± 3.6	23.8 ± 1.8	22.5 ± 2.7
Total hepatic ADH activity (μ mol/min/liver)	18	26.7 ± 4.2	21.6 ± 2.2	20.1 ± 2.3
Plasma endotoxin level (IU/ml)	18	<0.01	<0.01	<0.01

Values are means \pm SEM for the numbers (*n*) shown. GI–GIII as Table 1.

ADH activity has not been shown to be a rate-limiting step in ethanol oxidation (Salaspuro, 1991).

We did not find any evidence in the current literature that ciprofloxacin could directly inhibit hepatic ethanol metabolism and, as a consequence, decrease the EER. Earlier studies have shown that ciprofloxacin administration inhibits neither MEOS (Jokelainen *et al.*, 1997) nor the hepatic cytochrome P450s (Schulz *et al.*, 1995) that participate in ethanol metabolism in rats (Kunitoh *et al.*, 1993). In the current study, a similar finding was obtained by using chronically ethanol-fed rats. Furthermore, a short pre-treatment with ciprofloxacin has been shown to have no significant effect on the EER in man (Kamali, 1994). Accordingly, it is unlikely that ciprofloxacin treatment could lead to such metabolic changes in the liver that might affect the EER and thus influence the conclusions of this study.

In conclusion, the reduction of aerobic gastrointestinal flora and faecal ADH activity in rats by ciprofloxacin treatment completely abolishes the alcohol-induced enhancement in the EER. In contrast, hepatic MEOS and ADH activities and plasma endotoxin levels were not affected by ciprofloxacin. Accordingly, our results suggest that the bacteriocolonial pathway for ethanol oxidation significantly contributes to the enhanced EER following chronic alcohol administration to rats. The participation of the bacteriocolonial pathway in ethanol oxidation in the enhanced ethanol metabolism during chronic alcohol administration further supports the possible role of gut flora in the pathogenesis of alcohol-related symptoms and organ damage and diseases.

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