Alcohol & Alcoholism Vol. 35, No. 5, pp. 424–426, 2000

RAPID COMMUNICATION

EFFECT OF METHYLENE BLUE ON THE DISPOSITION OF ETHANOL

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(*Received* 24 *February* 2000; *in revised form* 19 *May* 2000; *accepted* 23 *May* 2000)

Abstract — The effect of methylene blue on the disposition of ethanol was studied in rats and humans. Methylene blue increased the metabolism of [^{14}C]ethanol to $^{14}CO_2$ in isolated hepatocytes and in intact rats by 75% and 30%, respectively. In healthy volunteers, methylene blue did not affect the pharmacokinetics of ethanol and did not alleviate the ethanol-induced NAD redox changes as reflected by the increase in the [lactate]/[pyruvate] ratio.

INTRODUCTION

Many of the consequences of excessive alcohol consumption are the result of redox changes occurring in the course of the metabolism of ethanol. Oxidation of ethanol to acetaldehyde and subsequently to acetate leads to an increase in the [NADH]/[NAD$^+$] ratio which contributes to the accumulation of fat in the liver of alcoholics (Lieber, 1994). An increased [NADH]/[NAD$^+$] ratio may also stimulate the mitochondrial respiratory chain. The resulting increase in electron flow along the respiratory chain generates reactive oxygen species that have been implicated in ethanol-associated cell injury (Bailey *et al*., 1999). Consistent with this hypothesis, interventions to decrease the concentration of NADH during metabolism of ethanol decrease the generation of reactive oxygen species and the toxicity of ethanol (Bailey and Cunningham, 1998).

The rate-limiting step in alcohol metabolism is controversial. A number of experiments suggest that the activity of alcohol dehydrogenases is the key determinant (Lumeng *et al*., 1980; Braggins and Crow, 1981). Other data indicate that the rate of re-oxidation of NADH and thus the [NADH]/[NAD$^+$] ratio and the intracellular concentration of acetaldehyde influence the rate of disposition of alcohol (Cheema-Dhadli *et al*., 1987; Cronholm *et al*., 1988; Zorzano and Herrera, 1990). In support of this hypothesis, fructose in part alleviates the ethanol-induced redox shift and increases the rate of disappearance of ethanol from blood (Ylikahri *et al*., 1976; Rawat, 1977), an effect which is thought to be due to facilitation of intramitochondrial re-oxidation of NADH (Bode and Thiele, 1975; Rawat 1977; Crownover *et al*., 1986). Other investigators, however, have not been able to show a reduction of the [NADH]/[NAD$^+$] ratio by fructose (Mascord *et al*., 1991) nor a correlation between the elimination of ethanol and the [NADH]/[NAD$^+$] ratio (Ryle *et al*., 1985*a*; Morgan *et al*., 1989).

Methylene blue, which is able to accept electrons from pyrimidine nucleotides and non-enzymatically transfers them to oxygen, has also been shown to prevent ethanol-induced redox changes and fat accumulation in isolated hepatocytes and HeLa cells (Cronholm, 1993; Galli *et al*., 1999). Redox changes, but not fat accumulation, are prevented by methylene blue in rats after long-term feeding of ethanol (Ryle *et al*., 1985b). Whether methylene blue modifies the metabolism of ethanol in humans is not known. Therefore, the aim of the present investigation was to evaluate the effect of methylene blue on the disposition of ethanol and ethanol-induced redox changes in humans and to compare it with the effects of methylene blue in isolated hepatocytes and intact rats.

MATERIALS AND METHODS

Human studies

Eleven healthy male volunteers participated in the study that had been approved by the local ethics committee. The average age was 23 years and the average body mass index 23.9 (range 19.1–30.8). They all consumed <30 g ethanol per day and did not take any medication. The volunteers were studied on two occasions, once without pretreatment and once at least 1 week after the first experiment, after oral administration of one gelatine capsule containing 50 mg methylene blue 24, 18, 12 and 1 h prior to ingestion of ethanol.

The volunteers were admitted to the clinical research unit after an overnight fast. There they received a standardized breakfast and a cannula was placed in a vein of a forearm. One hour later, a 0.5 g/kg body weight dose of ethanol was ingested in 400 ml of orange juice. Blood samples were obtained before and 15, 30, 45, 60, 90, 120, 180 and 240 min after administration of ethanol for the determination of blood ethanol and before, 30, 130 and 240 min after ingestion of ethanol for the determination of lactate and pyruvate.

Ethanol in plasma was measured by headspace gas liquid chromatography using 1-propanol as internal standard (Beyeler *et al*., 1985). Blood for the determination of lactate was collected in tubes containing EDTA and fluoride. For the determination of pyruvate, blood was immediately added to ice-cold perchloric acid. Following centrifugation, lactate and pyruvate were measured enzymatically (Richterich, 1965).

Metabolism of ethanol by isolated rat hepatocytes

Hepatocytes were isolated from male Sprague–Dawley rats (Stewart and Inaba, 1979). The cells (12×10^6 cells/ml) were incubated in stoppered flasks at 37°C for 30 min with and

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without methylene blue at a final concentration of 15 μM. The reaction was started by adding [^{14}C]ethanol (0.3 μCi/mmol) (final concentration 4 mM) and stopped by adding H_2SO_4 through the side arm of the flask. The $^{14}CO_2$ generated by the metabolism of ethanol and liberated by the acid was trapped in a well containing a filter paper that had been dipped in 1 M NaOH (Stewart and Inaba, 1979).

Metabolism of ethanol in intact rats

Two groups of four male Sprague–Dawley rats each, weighing 230–250 g, were kept in a climatized room and had free access to food and water. Methylene blue was added to the drinking water of one group at a concentration of 1 mM for 4 days. On the morning of the experiments, all rats received 0.5 g ethanol-1-^{14}C (0.3 μCi/mmol) in 5 ml 0.9% (w/v) NaCl per kg body weight intraperitoneally (i.p.) whereupon breath was collected as described previously (Lauterburg and Bircher, 1976). Trapped $^{14}CO_2$ was then measured by liquid scintillation spectroscopy.

Statistics

The data are presented as means ± SD, unless mentioned otherwise. Statistical significance of the differences between treatment groups was assessed by Student's *t*-test. The time-courses of ethanol in blood and the [lactate]/[pyruvate] ratio were analysed by repeat measure analysis of variance followed by Dunnett's test for multiple comparisons.

RESULTS

As shown in Fig. 1, hepatocytes incubated in the presence of 15 μM methylene blue metabolized significantly more ethanol to CO_2 than hepatocytes incubated without methylene blue ($P < 0.01$).

This observation *in vitro* was confirmed *in vivo*. Rats who had received methylene blue in their drinking water exhaled a significantly larger fraction of the administered dose of ethanol as CO_2 in 60 min than did control animals (means ± SD: 23.1 ± 1.2% vs 17.8 ± 2.0% of dose, $P < 0.01$)

In healthy volunteers, the concentration of ethanol in whole blood increased following administration of ethanol and subsequently decreased at an average rate of 4.1 and 3.9 mmol/l/h (0.19 and 0.18 g/l/h) in control and methylene blue-treated subjects, respectively (Fig. 2). In contrast to the animal experiments, there was no effect of methylene blue on the ethanol-induced increase ($P < 0.05$) in the [lactate]/[pyruvate] ratio (Fig. 3).

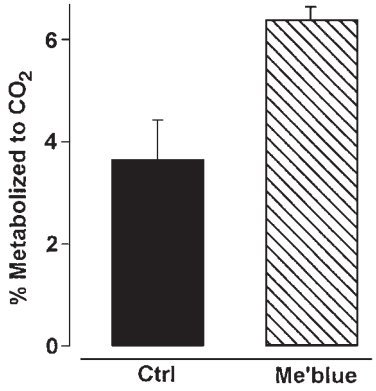

Fig. 1. Metabolism of [^{14}C]ethanol to $^{14}CO_2$ by rat hepatocytes incubated with methylene blue (Me'blue) and without (control, Ctrl). Values are means ± SD (bars) for $n = 3$.

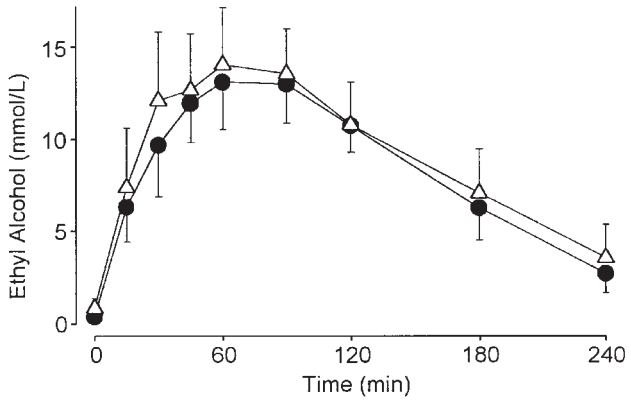

Fig. 2. Time course of blood ethanol concentration in 11 healthy volunteers following administration of ethanol with and without pretreatment with methylene blue.

Closed circles represent the session with methylene blue, triangles the session without methylene blue. Values are means ± 95% confidence intervals. All values are significantly ($P < 0.05$) higher than at baseline, but there was no difference between the two groups.

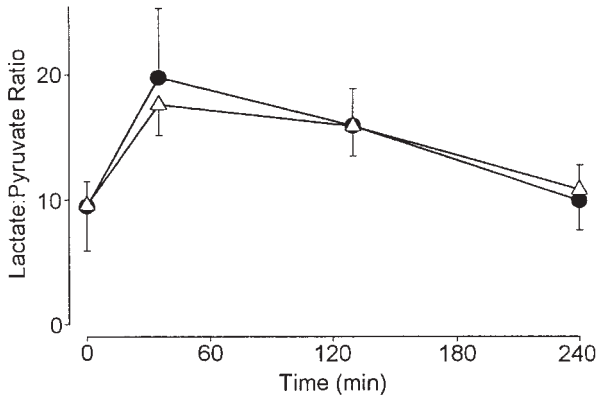

Fig. 3. Time course of the [lactate]/[pyruvate] ratio in 11 healthy volunteers following administration of ethanol with and without pretreatment with methylene blue.

Closed circles represent the session with methylene blue, triangles the session without methylene blue. Values are means ± 95% confidence interval. The values at 35 and 130 min are significantly ($P < 0.05$) higher than at baseline in both groups, but there was no difference between the two groups.

DISCUSSION

In hepatocytes and intact rats, methylene blue significantly increased the metabolism of ethanol to CO_2, indicating that this electron acceptor can speed up the elimination of ethanol.

Although methylene blue has been shown to inhibit acetaldehyde dehydrogenase *in vitro* with a Ki in the low micromolar range (Helander *et al*., 1993), this effect does not appear to be relevant in intact cells and *in vivo*, as indicated by the increased generation of CO_2 from ethanol in the presence of methylene blue.

In humans, no effect on the disposition of ethanol or the [lactate]/[pyruvate] ratio was observed. It is possible that a stimulation of the disposition of ethanol would also have been observed if the volunteers had taken a larger dose of methylene blue. With a 1 mM methylene blue concentration in drinking water, rats consumed ~100 μmol/kg body wt per day, compared to the 10 μmol/kg ingested by the volunteers experiment in the 24 h preceding the study. The chosen dosing regimen in the healthy volunteers was based on the experience with methylene blue in the treatment and prevention of ifosfamide-associated encephalopathy, where this particular dose was effective (Küpfer *et al*., 1994). Similar doses have also been utilized in the treatment of urolithiasis (Smith, 1975). Higher doses might have been toxic. Following oral administration of a 50 mg dose of methylene blue, its concentration in blood reaches ~0.05 μM between 60 and 180 min after dosing (Peter *et al*., 2000), which is much lower than the concentration used in the experiments *in vitro*. The concentration in blood, however, may not be relevant, since experiments in rats have shown that, after oral administration, the concentration of methylene blue is much higher in the liver where most of the metabolism of ethanol takes place (Peter *et al*., 2000).

In summary, whereas methylene blue stimulates the oxidation of ethanol to CO_2 in isolated hepatocytes and intact rats, no effect of methylene blue on the disposition of ethanol and its metabolic consequences could be demonstrated in humans, possibly because the dose of methylene blue that can be safely administered to humans is too low to be effective.

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