INHIBITORY MECHANISM OF COSTUNOLIDE, A SESQUITERPENE LACTONE ISOLATED FROM Laurus nobilis, ON BLOOD-ETHANOL ELEVATION IN RATS: INVOLVEMENT OF INHIBITION OF GASTRIC EMPTYING AND INCREASE IN GASTRIC JUICE SECRETION

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Abstract — Basic inhibitory mechanisms of costunolide and its active component, \( \alpha \)-methylene-\( \gamma \)-butyrolactone (\( \alpha \)-MGBL), on blood-ethanol elevation were investigated in rats. In normal rats, blood-ethanol elevation (30 min later) induced by 20% (v/v) ethanol [5 ml/kg, \textit{per os} (p.o.)] was strongly inhibited by pretreatment (30 min earlier) with costunolide and \( \alpha \)-MGBL (50 mg/kg, p.o.). In pylorus-ligated rats given ethanol, blood-ethanol level (30 min) was barely elevated compared with that of normal rats. Neither costunolide nor \( \alpha \)-MGBL affected the blood-ethanol elevation in pylorus-ligated rats or that induced by intraperitoneal and intraduodenal ethanol administration. Moreover, these compounds given orally induced no irreversible changes in alcohol dehydrogenase activity in rat liver. We continuously investigated the rate of gastric emptying in rats given various test meals. Costunolide and \( \alpha \)-MGBL suppressed gastric emptying in rats given 20% ethanol and 1% sodium carboxymethyl cellulose. \( \alpha \)-MGBL (50 mg/kg), but not costunolide, suppressed gastric emptying in 20% glucose-loaded rats. In an \textit{in vitro} experiment, \( \alpha \)-MGBL contracted the pylorus strip at a high concentration (20 mM), which was the estimated concentration in the stomach when the substance was given orally \textit{in vivo}. These findings suggested that \( \alpha \)-MGBL constricted the pylorus and caused delay of gastric emptying. Moreover, both compounds increased gastric fluid secretion with pepsin and mucus. In conclusion, the inhibitory effects of costunolide and \( \alpha \)-MGBL on blood-ethanol elevation were based on inhibition of gastric emptying and dilution of the ethanol concentration by the increased gastric fluid.

INTRODUCTION

Reduction of the appetite for and addiction to alcohol provides a useful means for the treatment of alcoholism. Experimental applications of herbal and traditional medicines have been used to cure alcohol dependence. For instance, the intraperitoneal administration of daidzin and daidzein, the major constituents of \textit{Puerariae Radix}, suppressed ethanol preference in Sardinian alcohol-preferring rats (sP rats) (Colombo et al., 1999). An acetone-soluble extract from the root of \textit{Salvia miltiorrhiza} was also reported to diminish ethanol preference in Sardinian alcohol-prefering rats (sP rats) (Colombo et al., 1999).

Reduction of ethanol absorption is also thought to be a convenient and useful means of preventing alcohol disorders. Our group has reported on many bioactive saponins from herbal medicines with inhibitory effects on blood-ethanol elevation (Yoshikawa and Yamahara, 1996; Yoshikawa \textit{et al}., 1996\textit{a,b,c,d}, 1997). Recently, sesquiterpenes isolated from the leaves of \textit{Laurus nobilis} (bay leaf, laurel) such as costunolide (Fig. 1) and dehydrocostus lactone were found to potentely inhibit blood-ethanol elevation in ethanol-loaded rats (Matsuda \textit{et al}., 1999\textit{b}; Yoshikawa \textit{et al}., 2000). In addition, costunolide is known to be present in the roots of \textit{Saussurea lappa}, which are used as a stomachic, and the leaves of \textit{Magnolia grandiflora} (el-Feraly and Chan, 1978; Matsuda \textit{et al}., 2000). Investigation of structure–activity relationships revealed that an \( \alpha \)-methylene-\( \gamma \)-butyrolactone (\( \alpha \)-MGBL) moiety of the active constituents was essential for activity. However, it remains unclarified whether the inhibitory effects of the sesquiterpenes and \( \alpha \)-MGBL on blood-ethanol elevation depend on either inhibition of alcohol absorption from the digestive tract or acceleration of alcohol metabolic enzyme activity in the liver. In the present study, we examined the basic inhibitory mechanism of costunolide and \( \alpha \)-MGBL on blood-ethanol elevation in ethanol-loaded rats.

MATERIALS AND METHODS

Animals

Male \textit{Wistar} rats aged 4–6 or 10–11 weeks were purchased from Kiwa Laboratory Animals Co., Ltd (Wakayama, Japan) and maintained in an air-conditioned room at 23 ± 2°C. Standard laboratory chow (MF: Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water were given freely for a week. Animals were starved for 20–22 h or 40 h prior to the experiments, but were allowed free access to water. Test samples were suspended in 5% acacia solution and given orally in a volume of 5 ml/kg in each experiment.

Reagents

Costunolide was isolated from the extract of the leaves of \textit{Laurus nobilis} using the method reported previously (Matsuda \textit{et al}., 1999\textit{b}; Yoshikawa \textit{et al}., 2000). \( \alpha \)-MGBL and pepsin (from porcine stomach mucosa) were purchased from Sigma Co. Ltd (St Louis, MO, USA). NAD* was purchased from Oriental Yeast Co. Ltd and other reagents were purchased from Wako Pure Chemical Industries Co., Ltd (Osaka, Japan).
Measurement of blood-ethanol elevation in several routes of ethanol-loaded rats

Test samples were given orally to starved (20–22 h) rats (120–150 g, body wt). Thirty minutes later, 20% (v/v) ethanol (5 ml/kg) was given orally (p.o.), injected into the abdominal cavity (i.p.), or injected into duodenum (i.d.) after laparotomy under ether anaesthesia. Blood samples were collected from infra-oral venous plexus at 15 (i.p.) and 30 min after loading of ethanol (p.o., i.p., i.d.). Blood was immediately mixed with a 10-fold volume of 0.33 M perchloric acid and centrifuged (4°C, 800 g, 10 min). Ethanol in supernatant was determined by an enzymatic method (F-kit™ ethanol; Boehringer, Mannheim, Germany).

Measurement of blood-ethanol elevation in pylorus-ligated rats

Starved (20–22 h) rats (120–150 g, body wt) were laparotomized under ether anaesthesia, and the pylorus was ligated with suture. Twenty per cent (v/v) ethanol was administered immediately (p.o.) to the pylorus-ligated rats, and blood samples were collected 30 min later. Test samples were given orally 30 min before the operation.

Measurement of liver alcohol dehydrogenase activity

Rats were starved for 40 h to decrease liver glycogen prior to the experiment. Test samples were given orally to the fasted rats (120–140 g, body wt), and 30 min thereafter 20% ethanol was given orally (5 ml/kg) to the rats. Thirty minutes later, the abdominal cavity was opened under ether anaesthesia, and the liver was perfused with 10 to 15 ml of 0.25 M ice-cold sucrose solution. The liver was removed and homogenized with a 3-fold volume of liver weight of a solution (pH 8.4) containing 0.05 M HEPES and 0.33 mM dithiothreitol. The homogenate was centrifuged (4°C, 105 000 g, 60 min), and the supernatant (cytosol fraction) was obtained. The activity of alcohol dehydrogenase in the cytosol fraction was determined using a slightly modified version of the method reported by Lumeng et al. (1979). Briefly, the cytosol fraction (20 μl), the protein concentration of which was determined using Lowry’s method (Lowry et al., 1951), was added to 180 μl of substrate mixture [the substrate mixture was composed of 1 ml of glycine buffer (0.2 M glycine, 0.4 M NaCl, adjusted to pH 10.0 with 1 M NaOH), 0.1 ml of ethanol (660 mM in water), 0.1 ml of NAD+ (48 mM in water) and 0.7 ml of distilled water] in a 96-well microplate (UV plate, Corning, NY, USA). The mixture was incubated at 25°C for 0 to 5 min and absorbance at 340 nm was measured every 1 min. The enzyme activity (nmol/min/mg of protein) was calculated from the reaction curve plotted from the NADH production and the reaction time.

Measurement of gastric emptying

Test samples were given orally to starved (20–22 h) rats (120–150 g, body wt). Thirty minutes later, the test mixture consisting of 20% ethanol, 1% CMC-Na, or 20% glucose and 0.05% Phenol Red was given orally (0.6 ml/rat). Thirty minutes later, the stomach was removed, and the quantity of Phenol Red remaining in the stomach was determined according to the method reported previously (Matsuda et al., 1999b).
pylorus-ligated rats (Fig. 2B). When ethanol was given i.p. (Fig. 2C) or i.d. (Fig. 2D), neither costunolide nor α-MGBL was able to influence blood-ethanol levels.

**Effects on liver alcohol dehydrogenase activity**

Liver alcohol dehydrogenase activity in rats given ethanol tended to increase moderately (18.5%), although not significantly. Costunolide and α-MGBL had no significant effect on enzymatic activity (Fig. 3).

**Effects on gastric emptying**

Figure 4 shows the effects of costunolide and α-MGBL on gastric emptying in rats given several test meals containing Phenol Red as a marker. Costunolide (50 mg/kg) suppressed gastric emptying in rats given 20% ethanol and 1% CMC-Na. However, it did not affect gastric emptying in rats given a typical nutrition, 20% glucose. However, α-MGBL inhibited gastric emptying in all test meals in a dose-dependent manner, and was ~2-fold more active than costunolide.

**Effect of α-MGBL on isolated pylorus preparation**

The pylorus strip gradually contracted 10 min after addition of 20 mM α-MGBL. The contractile force reached ~70% of that induced by 53 mM KCl in 30 min followed by gradual relaxing (Table 1). It was not possible to assess high concentrations of costunolide (10 mM), due to the difficulty encountered in dissolving the latter in the medium at such concentration.

**Effects on gastric fluid volume in normal rats**

The quantity of gastric fluid remaining in the stomach increased dose-dependently in rats given costunolide and α-MGBL (Fig. 5). This increase in gastric fluid was also observed in ethanol-loaded rats pretreated with costunolide and α-MGBL (Fig. 5).
Effects on gastric fluid factors in pylorus-ligated rats

Figure 6 shows the effects of costunolide and α-MGBL on the volume of gastric fluid, pH, acidity, pepsin activity and the quantity of hexosamine. In this experiment, ethanol had no significant influence on gastric fluid secretion. Costunolide and α-MGBL dose-dependently increased the volume of gastric fluid, similar to that observed in normal rats. Both total pepsin activity and the quantity of hexosamine for 30 min were also increased on a par with volume of gastric fluid. However, pH and acidity were not significantly modified under these conditions.

DISCUSSION

In the present study, several inhibitory mechanisms of costunolide and α-MGBL on blood-ethanol elevation in ethanol-loaded rats were revealed. First, to clarify whether inhibition of blood-ethanol elevation by costunolide and α-MGBL is based on inhibition of ethanol absorption from the digestive tract, we examined the effects on blood-ethanol elevation in ethanol-loaded rats through various routes. Both compounds significantly inhibited blood-ethanol elevation caused by oral administration of ethanol, but they did not affect blood-ethanol elevation after i.p. injection of the latter. These findings suggest that the inhibitory effects of costunolide and α-MGBL on blood-ethanol elevation are due to suppression of ethanol absorption from the gastrointestinal tract. Furthermore, costunolide and α-MGBL given orally induced no irreversible changes in liver alcohol dehydrogenase, an important liver metabolic enzyme, in ethanol-loaded rats. This observation also supported the finding that the inhibitory effects of both compounds depend on actions in the gastrointestinal tract.

Table 1. Contractile effect of α-methylene-γ-butyrolactone (α-MGBL) in rat pylorus strip

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (mM)</th>
<th>n</th>
<th>Contraction (% of KCl-induced constriction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (vehicle)</td>
<td>-</td>
<td>3</td>
<td>0.5 ± 1.0</td>
</tr>
<tr>
<td>α-MGBL</td>
<td>10</td>
<td>4</td>
<td>-4.8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4</td>
<td>69.8 ± 11.1**</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM. Significant difference from the control: **P < 0.01.
Ethanol given orally is partly oxidized in the upper digestive tract (Baraona et al., 2000). Ethanol is then absorbed slowly from the stomach and rapidly from the small intestine (Holt, 1981). In the present study, blood-ethanol elevation in pylorus-ligated rats was lower than that in normal rats (7.3% against normal rats) in agreement with data from previous studies. Costunolide and \( \alpha \)-MGBL did not elicit any significant effects on blood-ethanol elevation in the pylorus-ligated rats. These findings suggested that the compounds block absorption of ethanol from the intestine but not from the stomach and may delay the excretion of ethanol from the stomach to the duodenum. However, both compounds lacked significant effects on blood-ethanol elevation after i.d. injection of ethanol; this finding suggests that they delay the excretion of ethanol from the stomach to the duodenum, but do not inhibit ethanol absorption at the small intestine.

Moreover, using the Phenol Red method, in ethanol-loaded rats, both costunolide and \( \alpha \)-MGBL dose-dependently delayed gastric emptying, although the effect of \( \alpha \)-MGBL was more potent than that of costunolide. This difference appears to be reflected in the intensity of inhibitory activity on blood-ethanol elevation. Costunolide and \( \alpha \)-MGBL suppressed gastric emptying in CMC-Na-loaded rats at a high dose (50 mg/kg). In contrast, \( \alpha \)-MGBL, but not costunolide, inhibited gastric emptying in glucose-loaded rats. We previously reported that \( \alpha \)-MGBL suppressed both the increase of blood-ethanol and glucose in both ethanol- and glucose-loaded rats, but costunolide inhibited only blood-ethanol elevation (Matsuda et al., 1999b; Yoshikawa et al., 2000). These findings indicate that the inhibition of blood-ethanol elevation by costunolide and \( \alpha \)-MGBL is due to their inhibition of gastric emptying; moreover, the inhibitory effect of costunolide on gastric emptying appears to be more selective than that observed in rats administered glucose.

Low concentrations of ethanol, HCl and high concentrations of NaCl are known to be mild irritants, thus demonstrating the adaptive cytoprotection of gastric mucosa. Moreover, the cytoprotective mechanism of low concentrations of ethanol (20% ethanol) is reported to differ with respect to those of other mild irritants (0.3 M HCl and 5% NaCl), since only ethanol suppresses gastric emptying (Ko et al., 1995). Since this cytoprotection is known to be inhibited by pretreatment with atropine and lidocaine and vagotomy, the vagal nerve is thought to participate in cytoprotection of ethanol (Ko and Cho, 1996). In the present study, no prominent difference was observed between the rates of gastric emptying in the control groups given 20% ethanol (mild irritant), 1% CMC-Na (non-nutritional meal) and 20% glucose (nutritional meal). However, costunolide and, in particular, \( \alpha \)-MGBL showed strong inhibition against ethanol-induced gastric emptying. Costunolide showed a potent protective effect on acidified ethanol-induced gastric lesions (Matsuda et al., 2000). Several vanilloid analogues such as capsaicin and resiniferatoxin, originated from plants, have been reported to act as irritants (Szallasi and Blumberg, 1989) and show cytoprotective action against gastric lesions at very low concentrations (Abdel-Salam et al., 1999). A similar partial structure can be observed in costunolide, \( \alpha \)-MGBL (\( \gamma \)-lactone) and resiniferatoxin (\( \gamma \)-lactol). Costunolide may, therefore, stimulate the gastric mucus as a mild irritant and be capable of enhancing the inhibition of gastric emptying by ethanol in rats. Since costunolide selectively inhibited gastric emptying in ethanol-loaded rats, the inhibitory mechanisms of costunolide on gastric emptying (e.g. role of capsaicin-sensitive vagal afferent nerves etc.) should be further studied.

However, to investigate the direct effects of \( \alpha \)-MGBL, an active component of costunolide, on pylorus muscle constriction, the effect of the compound on a pylorus strip was investigated using the Magnus method. As a result, a high concentration of \( \alpha \)-MGBL (20 mM), estimated according to the effective dose of \( \alpha \)-MGBL used (12.5 mg/5 ml/kg), proved to be equal to that required for inhibition of gastric emptying, and elicited contraction of the pylorus strip. This finding suggested that

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**Fig. 5.** Effects of costunolide (COS) and \( \alpha \)-methylene-\( \gamma \)-butyrolactone (\( \alpha \)-MGBL) on gastric fluid in normal and ethanol-loaded rats. Samples were administered to starved (20–22 h) rats, and 20% ethanol (5 ml/kg) was given orally 30 min later. Thirty minutes later, rats were killed and the stomach was removed. Each column represents the mean ± SEM (bars) of four to six rats. Significant differences from the control: *\( P < 0.05 \), **\( P < 0.01 \).
α-MGBL directly constricted the pylorus muscle and delayed gastric emptying as one of the mechanisms of action involved. In the course of gastric emptying, remarkable stagnations of gastric fluid were observed in normal rats given costunolide and α-MGBL. Increases in gastric fluid are thought to dilute concentrations of ethanol and to slow down ethanol absorption. We therefore measured the volume of gastric fluid in rats both with and without ethanol administration. In normal rats, costunolide and α-MGBL increased the gastric fluid volume, and the effect of α-MGBL was almost 2-fold stronger than that of costunolide. Increasing effects of both compounds were also observed in ethanol-loaded rats and these effects were well correlated with those in normal rats. Finally, various gastric factors, such as pH, acidity, pepsin activity and the quantity of

Fig. 6. Effects of costunolide (COS) and α-methylene-γ-butyrolactone (α-MGBL) on gastric factors in ethanol-loading, pylorus-ligated rats. Rats administered samples 30 min earlier were laparotomized, and the pylorus was ligated. Immediately, ethanol was administered orally followed by suturing of the abdominal cavity. Thirty minutes later, the stomach was removed and gastric factors were measured. Cont.: 20% ethanol-treated rats; Non.: ethanol-untreated rats. Each column represents the mean ± SEM (bars) of four to six rats. Significant differences from the control: *P < 0.05, **P < 0.01.
hexosamine in pylorus-ligated rats were determined. The pH and acidity did not significantly change in rats given costunolide and α-MGBL. However, pepsin activity and hexosamine contents in rats given costunolide were increased in accordance with the increase in the volume of gastric fluid. α-MGBL also increased both factors, although the increasing effect on hexosamine was not significant. These findings indicate that both compounds accelerate gastric fluid secretion with increases in pepsin and gastric mucus.

In conclusion, the inhibitory effects of costunolide and α-MGBL on blood-ethanol elevation are due to inhibition of gastric emptying and dilution of the ethanol concentration by the increased gastric fluid.

REFERENCES


