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# Protection by sinomenine against endotoxin-induced fulminant hepatitis in galactosamine-sensitized mice

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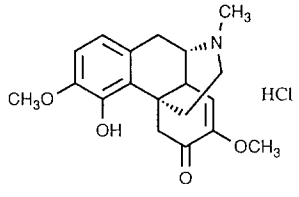
**Abstract**—Sinomenine, an epimorphinan alkaloid, was tested for protecting hepatitis induced by lipopolysaccharide (LPS) in galactosamine (GalN)-sensitized mice. Sinomenine protected against the hepatic injuries in the dose range of 10–100 mg/kg in a dose-dependent manner and suppressed the production of tumor necrosis factor (TNF), which appeared in serum earlier than aminotransferases in GalN/LPS-treated mice. Sinomenine significantly suppressed the *in vitro* production of superoxide anion and hydrogen peroxide in the macrophage cultures stimulated with phorbol 12-myristate acetate. It is discussed that sinomenine prevents GalN/LPS-treated hepatic failure by suppressing TNF production and/or reactive oxygen generation.

*Key words:* sinomenine; hepatitis; endotoxin; galactosamine; tumor necrosis factor; oxygen radical

Sinomenine, an epimorphinan alkaloid isolated from *Sinomenium acutum* Rehder et Wilson has been used as an analgesic and anti-inflammatory drug in Japan and China. The suppressive effect of sinomenine on granuloma formation was reported in connection with its histamine-releasing activity [1]. Recently, we found that sinomenine suppressed the immune responses dependent on T cells and macrophages [2]. In the present study we evaluated the pharmacological activity of sinomenine on the endotoxin (LPS*)-induced hepatic injuries mediated through macrophage activation in GalN-sensitized mice.




Structure 1. Chemical structure of sinomenine–HCL.

## *Materials and Methods*

*Reagents.* Sinomenine was isolated from *Sinomenium acutum* and its hydrochloride was used throughout the experiment (see Structure 1) [3]. LPS *Escherichia coli* 055:B5, Cyt. c (type IV), PMA and HRP (type II) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). BCG was obtained from Nippon BCG Co. (Tokyo, Japan). GalN HCL and transaminase assay kit were obtained from Wako Pure Chemicals Ltd (Tokyo, Japan). GalN HCL and transaminase assay kit were obtained from Wako Pure Chemicals Ltd (Tokyo, Japan). Sinomenine was administered in pyrogen-free aqueous solution.

*Animals.* Male ICR mice were purchased from Japan SLC (Shizuoka, Japan) and they were used for experiments at 5–6 weeks of age.

*Induction of hepatic failure.* ICR mice received i.p. 700 mg/kg GalN, and 1 hr later, 33 μg/kg LPS was given i.v.

*Assay for aminotransferase and TNF activities.* Activities of GPT and GOT were measured using a commercial transaminase assay kit. TNF activity was measured using TNF-sensitive LM cells [4, 5]. In brief, serially diluted serum samples and $10^4$ LM cells suspended in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 0.5% FBS (Gibco) were cultured in the wells of a 96-well microtest plate in a $CO_2$-incubator at 37° for 48 hr. The plate was washed with PBS and the remaining viable cells were stained with 0.5% methylene blue solution at room temperature for 15 min. After washing the stained cells were solubilized with detergent. Absorbance of dye was read at 590 nm by Immunoreader (Japan Intermed, Tokyo, Japan). The titer of TNF in sera was defined as a reciprocal dilution of serum causing 50% cell toxicity.

*Macrophage preparation and assay for production of superoxide and hydrogen peroxide.* Male ICR mice were injected i.p. with 1 mg of BCG suspended in pyrogen free saline. Peritoneal exudate cells were collected in Krebs-Ringer phosphate buffer containing 5.4 mM glucose 4 days after BCG injection and cultured in RPMI medium supplemented with 5% FBS for 1 hr in plastic culture dishes. Adherent cells (macrophages > 90%) were recovered by pipetting. The cell suspension containing $10^6$ cells/mL was dispensed into a glass cuvette (final volume 2 mL) with or without test compound and preincubated at 37° for 15 min and then, added with 100 ng/mL PMA. For determing superoxide ($O_2^-$) production reduction of Cyt.c (80 μM) was measured by monitoring the difference of the absorbances at 540 and 550 nm for 15 min by a two wavelength photometer (Hitachi U-3200) and amount of superoxide was calculated using the molecular extinction coefficient ($\varepsilon_{550} = 19.1 \times 10^3$/cm/M) [6]. For determining hydrogen peroxide ($H_2O_2$) production, the formation of HRP–$H_2O_2$ complex, resulting in a shift of the HRP absorption maximum (403–417 nm) was measured. Briefly, macrophages ($1 \times 10^6$ cells/mL) were treated with $Ca^{++}$-free Krebs–Ringer phosphate buffer containing 5 mM glucose and 2.5 μM HRP, and absorption maximum was monitored in a double beam spectrophotometer for 10 min. Hydrogen peroxide released was calculated using the molar absorption coefficients ($\varepsilon_{417} = 47 \times 10^3$/cm/M) [7].

All values were expressed as means ± SE and data were analysed according to Student's *t*-test.

* Abbreviations: BCG, bacillus Calmette-Guérin; Cyt. c, cytochrome c; FBS, fetal bovine serum; GalN, D-galactosamine; GOT, aspartate aminotransferase; GPT, alanine aminotransferase; HRP, horseradish peroxidase; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor.

Table 1. Suppression by sinomenine of serum GOT and GPT activities in GalN/LPS-treated mice

| GalN | LPS | Sinomenine (mg/kg) | Transaminase activities (U/mL) GOT | GPT | N |
|---|---|---|---|---|---|
| − | − | — | 96.1 ± 10.0 | 19.8 ± 2.3 | 5 |
| − | + | — | 74.2 ± 6.4 | 10.7 ± 0.6 | 5 |
| + | + | — | 1120.3 ± 135.4 | 1501.8 ± 169.4 | 6 |
| + | + | 100 | 396.5 ± 222.1† | 233.4 ± 162.7† | 5 |
| + | + | 30 | 780.7 ± 305.5* | 451.4 ± 200.1* | 5 |
| + | + | 10 | 697.1 ± 128.4* | 528.2 ± 151.6† | 4 |

Sinomenine was injected i.p. 1 hr before GalN treatment. Eight hours after LPS injection, blood samples were obtained to measure serum GOT and GPT activities.

Data are expressed as means ± SE. Significantly different from GalN/LPS control group, *$P < 0.05$ and †$P < 0.01$.

Table 2. Suppression by sinomenine of LPS-induced TNF in serum of GalN-sensitized mice

| Sinomenine (mg/kg) | TNF activity (U/mL) | Inhibition (%) | N |
|---|---|---|---|
| — | 288.1 ± 51.9 | — | 8 |
| 100 | 143.0 ± 39.2† | 50.4 | 7 |
| 30 | 215.6 ± 40.3* | 25.2 | 5 |
| 10 | 278.0 ± 109.5 | 3.5 | 6 |

Sinomenine was injected i.p. 1 hr before GalN treatment into the mice. Blood samples were collected 1 hr after LPS injection. TNF activity was undetectable in serum of mice treated GalN alone.

Data are expressed as means ± SE. Significantly different from control group, *$P < 0.05$ and †$P < 0.01$.

*Results and Discussion*

LPS is well known to induce lethal shock in animals and humans, which is mediated through macrophage activation accompanied by many kinds of inflammatory and immunological mediators [8]. It was reported that administration of a small dose of LPS in combination with non-hepatotoxic dose of GalN induced lethal toxicity with fulminant hepatitis in mice [9].

Sinomenine was tested for protecting activity on this hepatitis model. Activities of GOT and GPT in sera of GalN-sensitized mice were not changed from 0 to 5 hr (data not shown) and significantly increased at 8 hr after an i.v. injection of LPS and, when these mice received sinomenine 2 hr before LPS injection, the elevation of transaminase activities was significantly suppressed by 10–100 mg/kg sinomenine in a dose-dependent way (Table 1). Administration of GalN, LPS or sinomenine alone did not increase the hepatic markers during observation (data not shown).

TNF$\alpha$ is a major inflammatory cytokine released from macrophages in response to LPS and other agents. Since passive immunization against TNF$\alpha$ partially protected mice from lethal effects of LPS in normal mice [10] and a small amount of TNF$\alpha$ causes lethal toxicity with severe hepatitis in GalN-sensitized mice [11], TNF$\alpha$ was considered to be responsible for GalN/LPS-induced toxicity. Therefore, we tested whether sinomenine could suppress TNF production in the GalN/LPS mice. After LPS injection into GalN-sensitized mice, TNF activity in serum was increased within 60 min, and sinomenine administration suppressed the increase of TNF level at the doses of 30 and 100 mg/kg (Table 2). Furthermore, sinomenine was tested for suppressing the hepatitis induced by a combination of GalN and TNF$\alpha$ (15 $\mu$g/kg i.v.), however, there was no evidence that sinomenine affected the elevation of GOT and GPT levels in serum (data not shown). These findings suggest that sinomenine inhibits TNF$\alpha$ production but not TNF$\alpha$ action.

Niehorster *et al.* [12] recently reported that pretreatment with the radical scavengers such as catalase and superoxide dismutase protected against liver injury and suppressed TNF$\alpha$ production in GalN/LPS-treated mice, and suggested that the formation of extracellular reactive oxygen species is linked with TNF$\alpha$ production. Hence, we examined the effect of sinomenine on the formation of reactive oxygens by macrophages. Amounts of $O_2^-$ and $H_2O_2$ produced in

Table 3. Inhibitory effect by sinomenine on the production of oxygen radicals by PMA-stimulated macrophages *in vitro*

| Sinomenine ($\mu$g/mL) | $O_2^-$ (pmol/min) | Inhibition (%) | $H_2O_2$ (pmol/min) | Inhibition (%) | N |
|---|---|---|---|---|---|
| 0 | 1581 ± 68 | — | 1579 ± 85 | — | 5 |
| 100 | 433 ± 32* | 72.6 | 644 ± 99* | 59.2 | 4 |
| 50 | 644 ± 19* | 59.3 | 911 ± 70* | 42.3 | 4 |
| 10 | 837 ± 102* | 47.1 | 1631 ± 99 | −3.3 | 3 |

Data are expressed as means ± SE. Significantly different from non-treated control, *$P < 0.01$.

the culture of macrophages stimulated with PMA were significantly reduced by preculture with sinomenine at 50 and 100 μg/mL (Table 3). However, when sinomenine was added to the macrophage cultures after PMA stimulation, any suppression was not observed (data not shown). If events associated with reactive oxygen species are localized upstream to the action of TNFα in the sequence of the some pathogenic mediators in GalN/LPS-induced hepatitis as demonstrated by the comparison of various inhibitors [13], sinomenine might primarily suppress the production of reactive oxygen species, leading to the decrease of TNFα production. Additional basic and preclinical investigations will be required to confirm the usefulness of sinomenine for hepatitis.

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