Reduced Lipopolysaccharide (LPS)-Induced Nitric Oxide Production in Peritoneal Macrophages and Inhibited LPS-Induced Lethal Shock in Mice by a Sugar Cane (Saccharum officinarum L.) Extract

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A sugar cane extract (SCE) has been found to have an immunostimulating effect in several animals. Lipopolysaccharide (LPS) is known to induce endotoxin shock via the production of inflammatory modulators such as tumor necrosis factor (TNF-α) and nitric oxide (NO). We examined in the present study the effects of SCE on the TNF-α and NO production in LPS-stimulated mice peritoneal cells and the endotoxin shock in mice. The supplementation of SCE to peritoneal macrophages cultured with LPS resulted in a significant decrease in NO production. All the mice injected intraperitoneally with LPS and D-galactosamine (LPS+GalN) died within 24 h. However, a peritoneal injection, but no intravenous or oral administration, of SCE (500–1,000 mg/kg) at 3 to 48 h before the LPS+GalN-challenge resulted in a significantly improved survival rate. These results suggest that SCE had a protective effect on LPS-induced endotoxin shock via one of possible mechanisms involving the suppression of NO production in the mouse peritoneal cavity.

Key words: endotoxin shock; lipopolysaccharide; nitric oxide; sugar cane extract

The extract of sugar cane, one of the natural products of the sugar cane industry, has displayed a wide range of biological effects including anti-stress,1) antioxidative activity,2) and modulation of acetylcholine release.3) We have also demonstrated that our sugar cane extract (SCE) had an adjuvant effect on the activation of antibody and cell-mediated immune responses,4,5) a protective effect against Eimeria tenella infection,6) a radioprotective effect on immune functions and intestinal damage in X-ray irradiated chickens,7,8) and a reconstituting effect on the bursa-dependent immune system in the immunosuppression experimentally induced by injecting cyclophosphamide into chickens.9)

Tumor necrosis factor (TNF)-α induces various biological responses including tissue injury, shock and apoptosis.10) Nitric oxide (NO) is a highly reactive molecule as a potent macrophage-derived effector against a variety of bacteria, parasites and tumors.11) An elevated serum level of NO has been implicated in the pathogenesis of such inflammatory diseases as rheumatoid arthritis and septic shock.12) It is well recognized that endotoxin or lipopolysaccharide (LPS), which is a structural component of the outer membrane of Gram-negative bacteria, plays a cardinal role in septic shock.13) The released LPS activates macrophages, endothelial cells and fibroblasts to produce and release potent inflammatory mediators, including TNF-α, interleukin (IL)-1β, IL-6 and NO.14) Sensitization with α-galactosamine (GalN) greatly increases the sensitivity of animals to LPS and augments...
the lethal activity of LPS. The lethal effect of LPS on GalN-sensitized mice is usually considered an experimental model for clinical endotoxic shock or septic shock.

We demonstrated in the previous study that SCE had a preventive effect against LPS-induced mortality in this mouse model when administered intraperitoneally with SCE 3 h before injecting LPS+GalN at a dose of 500 mg/kg. Therefore, in this present work, we studied the effects of SCE on TNF-α and NO production by mouse peritoneal macrophages cultured with LPS and extended to determine the optimal conditions eliciting the protective effect of SCE on LPS-induced endotoxin shock in mice.

Materials and Methods

SCE. SCE was kindly provided by Mitsui Sugar Co., Ltd. (Kanagawa, Japan). The original materials, including cane juice produced from sugar cane (Saccharum officinum L.) in the raw sugar manufacturing process, were used to prepare SCE. Dried SCE finally prepared by synthetic absorbent chromatography consisted of crude protein (16.9%), crude fat (0.5%), ash (36.1%) and a nitrogen-free extract (46.5%).

Animals. Five-week-old female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). All mice were kept in an air-conditioned room and fed on standard laboratory food pellets and water ad libitum. The present study was done according to the Guideline for Animal Experiments of National Institute of Animal Health (Tsukuba, Japan).

Effects of SCE on TNF-α and NO production by mouse peritoneal cells cultured with LPS. BALB/c mice were injected intraperitoneally with 2 ml of a 4% Brewer thiglycolate medium (Difco Laboratories, Detroit, MI, USA). Peritoneal cells were collected 3 d after this injection, and then washed with phosphate buffered saline (PBS). A cytolical examination of the adherent cells on a Giemsa-stained smear showed that almost all of them were morphologically macrophages. The cells in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 100 μg/ml of streptomycin (Meiji Seika Co., Tokyo, Japan) and 100 U/ml of penicillin (Banyu Pharmaceutical Co., Tokyo, Japan) were seeded at 2 × 10^5 cells/well in 96-well culture plates. After incubating at 37 °C in 5% CO₂ for 2 h, the nonadherent cells were removed by washing twice with PBS, and the remaining adherent cells were stimulated with *Salmonella abortusequii* LPS (Sigma) at different concentrations in RPMI 1640 containing 1% fetal calf serum in the absence or presence of SCE. The experiments were carried out in triplicate. The amount of TNF-α in a 3-h culture supernatant was determined by a TNF-α ELISA kit (Biosource International, Camarillo, CA, USA). The NO concentration in a 24-h culture supernatant was assessed by measuring the amount of the metabolic nitrite product by the Griess reaction as described elsewhere.

**LPS challenge and survival analysis.** An intraperitoneal injection of *Salmonella abortusequii* LPS (5 μg/kg) and GalN (1 g/kg; Wako Pure Chemical, Osaka, Japan) induced 100% lethality in 5-week-old female BALB/c mice. Therefore, this protocol was used as an endotoxic shock mouse model and observed for 7 d after challenging with LPS+GalN. To determine an effective dose of SCE which elicited a protective effect on LPS-induced lethality, the mice were injected intraperitoneally with SCE at doses of 100, 250, 500 and 1,000 mg/kg 3 h before being challenged with LPS+GalN. Subsequently, to determine an appropriate injection time for SCE, which would be the interval between the treatment with SCE and challenge with LPS+GalN to elicit a protective effect on LPS-induced lethality, the mice were injected intraperitoneally with SCE at a dose of 500 mg/kg 0, 3, 6, 12, 24, 48, 72 and 168 h before the challenge with LPS+GalN and 3 h after the challenge with LPS+GalN. Furthermore, to determine an effective administration route for SCE which would elicit a protective effect on LPS-induced lethality, the mice were injected intravenously with SCE at the doses of 250 and 500 mg/kg, or orally administered SCE at a dose of 500 mg/kg/d for 3, 7, 10 and 14 consecutive days before the LPS challenge. The survival rate was determined 7 d after the LPS+GalN challenge.

Statistical analysis. Each value is expressed as the mean ± standard error, and then compared by using the unpaired Student’s t-test. The survival rate was compared by using Fisher’s exact test. P < 0.05 is considered to be statistically significant.

Results

Effects of SCE on TNF-α and NO production by mouse peritoneal macrophages cultured with LPS. As shown in Fig. 1, the TNF-α concentration in the culture supernatant of mouse peritoneal macrophages cultured with LPS was not affected by the addition of SCE. In contrast, NO released into the same supernatant of mouse peritoneal macrophages cultured with LPS was significantly reduced by adding SCE at a dose of 500 μg/ml when compared to the control at doses of 1, 10 and 100 ng/ml of LPS (Fig. 2).

Protective effect of SCE on LPS-induced lethal shock in mice. The effects of an intraperitoneal injection of SCE on the survival rate of mice injected with LPS+GalN are summarized in Tables 1 and 2. All of mice died within 24 h after injecting LPS+GalN (Table 1). Interestingly, all of mice treated with SCE at doses of 500 and 1,000 mg/kg remained alive for up to 7 d of observation.
suggesting a protective effect of SCE. There was no or a negligible protective effect on the mice treated with SCE at doses of 100 and 250 mg/kg. Almost all of the mice treated with SCE at 500 mg/kg 3 to 48 h before the challenge with LPS+GalN were alive for a 7-d observation period (Table 2). However, no or a negligible protective effect was apparent in the mice treated with SCE 0 and 3 h after the challenge with LPS+GalN and 72 and 168 h before the challenge with LPS+GalN when evaluated 7 d after the challenge.

The effects of an intravenous injection and oral administration of SCE on the survival rate of mice injected with LPS+GalN are summarized in Table 3. Only one in five of the mice injected intravenously with SCE at a dose of 250 mg/kg died by day 2 after the challenge with LPS+GalN, but all of the mice died by day 3. Four out of five of the mice injected intravenously with SCE at the dose of 500 mg/kg died by day 4. Of the mice injected intravenously with SCE, sudden death occurred at a dose of more than 1,000 mg/kg. In addition, an oral inoculation of SCE offered no protective effect on the mice injected with LPS+GalN. Therefore, an intraperitoneal injection of SCE resulted in complete protection against LPS-induced lethality in mice. However, neither an intravenous injection nor oral administration of SCE provided complete protection in the lethal endotoxin shock model, offering only a prolonged survival time and decreased mortality.

Discussion

SCE used in the present study, which is a byproduct

Table 1. Effect of Different Doses of SCE on the Survival Rate of Mice Challenged Intraperitoneally with LPS+GalN

<table>
<thead>
<tr>
<th>Injected dose (mg/kg)</th>
<th>No. of live mice/No. of total mice</th>
<th>Days after challenge with LPS</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>5/5</td>
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<tr>
<td>100</td>
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<td>500</td>
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<tr>
<td>1000</td>
<td>5/5</td>
<td>5/5 &lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

Mice (BALB/c, female, 5-week-old) were injected intraperitoneally with SCE (0–1,000 mg/kg) 3 h before being challenged with LPS (5 mg/kg) and GalN (1 g/kg).

<sup>a</sup>p < 0.05, when compared to mice without SCE on day 1 after the challenge with LPS+GalN.

<sup>b</sup>p < 0.05, when compared to mice without SCE on day 2–7 after the challenge with LPS+GalN.

Table 2. Effect of SCE Injection Time on the Survival Rate of Mice Challenged Intraperitoneally with LPS+GalN

<table>
<thead>
<tr>
<th>SCE injection time before challenge (h)</th>
<th>No. of live mice/No. of total mice</th>
<th>Days after challenge with LPS</th>
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<tbody>
<tr>
<td>—&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>—&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0</td>
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<td>0/10</td>
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<tr>
<td>3</td>
<td>10/10</td>
<td>10/10&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>48</td>
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<td>5/5&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>72</td>
<td>5/5</td>
<td>1/5</td>
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<tr>
<td>168</td>
<td>5/5</td>
<td>0/5</td>
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</table>

Mice (BALB/c, female, 5-week-old) were injected intraperitoneally with SCE (500 mg/kg) at different times before or after the challenge with LPS (5 mg/kg) and GalN (1 g/kg).

<sup>a</sup>Mice not treated with SCE were challenged with LPS+GalN.

<sup>b</sup>Mice were injected intraperitoneally with SCE 3 h after being challenged with LPS+GalN.

<sup>c</sup>p < 0.05, when compared to mice challenged with LPS+GalN on day 1.

<sup>d</sup>p < 0.05, when compared to mice challenged with LPS+GalN on day 2–7.
from the process for producing sugar, has been familiar to people from ancient times, suggesting its high safety by oral administration. In fact, SCE has been practically used as a chicken feed additive. In addition, it has been reported to have many biological activities in chickens used as a chicken feed additive. In addition, it has been treated and challenge and dose of SCE might have been due to the different modes of absorption and metabolism of the injected SCE.

It is considered that SCE may have been degraded gradually within 48 h and not 72 h in the mouse peritoneal cavity where an effective dose inducing a protective effect on mouse peritoneal cells cultured with LPS and GalN had been exceeded. The reason for this phenomenon is considered that SCE may be hard to come directly into contact with LPS in the mouse peritoneal cavity. Therefore, the difference in protective effect from the various treatments of mice with SCE such as the route, interval between the treatment and challenge and dose of SCE might have been due to the different modes of absorption and metabolism of the injected SCE.

NO contributes to the cytotoxic or cytostatic actions of macrophages activated by various immunological stimuli. Many studies have linked the production of NO to endotoxin-induced hypotension, vascular hyporesponsiveness and death, suggesting that the excess production of NO plays an important role in the development of septic shock. Several natural products inhibiting NO production have recently been investigated. It has been reported that Sho-saiko-to, one of the most frequently prescribed Kampo medicines and a form of phytotherapy, conferred protection against oxygen toxicity, liver plasma membrane damage during endotoxin shock, and NO production by endotoxin-activated J774A.1 cells. In the present study, there was no effect of SCE intraperitoneally injected on the serum NO concentration in mice challenged with LPS+GalN (data not shown); however, LPS-induced NO release by mouse peritoneal cells was reduced by the addition of SCE. The in vitro experiment showed a small amount of NO production by mouse peritoneal cells cultured with SCE alone, suggesting that some potential substances in the SCE stimulated the cells slightly. SCE suppressed the NO production by mouse peritoneal cells cultured with LPS at a dose of more than 10 ng/ml, suggesting that some substances in SCE worked to decrease the LPS-induced NO release when a threshold value to NO production had been exceeded.
There are many reports of natural components having protective effects against endotoxin shock.21–23 The protective effects of almost all of these products are considered to be due to modulating TNF-α overproduction.24–26 Interestingly, this present study has shown that LPS-induced TNF-α production by mouse peritoneal macrophages cultured with LPS was not affected by the addition of SCE, consistent with the previous study that demonstrated no inhibitory effect of SCE on LPS-induced TNF-α production.27 One of the explanations for the intraperitoneal injection of SCE preventing LPS-induced liver injury in spite of the high concentration of LPS-induced TNF-α might be the preventive effect of SCE on TNF-α-related hepatocyte injury such as apoptosis induced by the TNF receptor family members. In addition, the caspase-independent TNF-receptor 1-mediated injury, as well as necrotic hepatotoxicity, has been reported to be entirely blocked under a condition of glutathione depletion.27 In that report, the plasma peak concentrations of TNF-α and IL-1β were not significantly altered in the glutathione-depleted phorone-treated mice; however, hepatoprotection by a phorone treatment entirely prevented lethality in the endotoxin shock model. Furthermore, it has reported that pretreatment with diethylmaleate (DEM), which is a glutathione-depleting agent, did not suppress the production of TNF-α, suggesting that the mechanism by which it prevented endotoxin shock remains open to speculation. Therefore, SCE could be one of the possible promising candidates as a leading substance for the development of preventive agents against endotoxin shock, if the biologically active components of SCE can be identified.

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