Flavonoids protect mice from two types of lethal shock induced by endotoxin


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Abstract

The protective effect of flavonoids on two types of lethal endotoxic shock was studied. A lethal endotoxic shock was induced by administration of lipopolysaccharide (LPS) into d-galactosamine (d-GalN)-sensitized mice and another one was done by administration of a high dose of LPS into normal mice. Pretreatment with a series of flavonoids protected mice from two types of endotoxin lethality. Flavonoid pretreatment reduced the serum tumor necrosis factor-α (TNF-α) level in mice injected with d-GalN and LPS, but not in mice injected with a high dose of LPS. TNF-α-induced lethal shock in d-GalN-sensitized mice was also protected by pretreatment with flavonoids, suggesting that flavonoids augmented the resistance to TNF-α lethality. On the other hand, flavonoids reduced the plasma level of lipid peroxides in mice injected with a high dose of LPS, but not in d-GalN-sensitized mice. Taken together, these results indicated that flavonoids might protect mice from two types of endotoxin lethality. The protective mechanism of flavonoids in each endotoxin lethality is discussed. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Flavonoid; Lipopolysaccharide; Endotoxin; Endotoxic shock; Anti-oxidant

1. Introduction

Bacterial lipopolysaccharide (LPS) has been implicated as a major factor in the pathogenesis of Gram-negative septic shock, a clinical problem with significant morbidity and mortality [1]. Many experimental animal models of endotoxin lethality have been established with the objectives of understanding not only the pathogenic mechanisms involved, but also potential therapeutic approaches to prevent lethal shock induced by endotoxin. A number of agents have been examined in terms of addressing the lethal effects of endotoxin, including glucocorticoids, antibody to endotoxin, LPS analogues, antibody to proinflammatory cytokine, inhibitor of tumor necrosis factor (TNF) production, and anti-oxidant [2,3].

Flavonoids, one of the large families of plant constituents, have various biological actions including anti-inflammatory, anti-oxidant, anti-tumor and anti-virus action [4,5]. A series of flavonoids have been reported to inhibit the production of proinflammatory mediators, which shows cytotoxicity and tissue damage [6–9]. Therefore, flavonoids may have potential therapeutic value for prevention of endotoxin lethality. However, there are few reports on the in vivo protective action of flavonoids against endotoxin lethality. In the present study we investigated the effect of a series of flavonoids on the protection of endotoxin-induced lethal shock in mice. Two types of endotoxin lethality were induced by administration of a small dose of LPS into d-galactosamine (d-GalN)-sensitized mice or a high dose of LPS into normal mice.

2. Materials and methods

2.1. Animals

Female BALB/c mice, 8–10-week-old, were obtained from Japan SLC (Hamamatsu, Japan). Male and female inbred SMA mice were supplied by the Laboratory Ani-
mal Research Center, Aichi Medical University. All experiments were conducted with sex- and age-matched mice.

2.2. Reagents

LPS was prepared from *Klebsiella* O3 as described previously [10]. Flavonoids used in this study are as follows: quercetin (Wako Pure Chemical, Osaka, Japan) and rutin hydrate (Aldrich, Milwaukee, USA) in flavonol, baicalein and baicalin (Aldrich) in flavone, daidzein (Wako) in isoflavone, catechin (Sigma Chemical, St. Louis, MO, USA) in flavanol, and hesperetin (Sigma) in flavanone. Flavonoids were suspended in 100 μl of dimethyl sulfoxide. The water-soluble α-Grutin was a gift of Toyo Seito, Tokyo, Japan. Recombinant murine TNF-α was obtained from Sigma. d-GalN was purchased from Wako. Snake venom from *Agkistrodon naltysblomho⁄i* and *Crotalus atrox* was obtained from Wako and Sigma, respectively.

2.3. Lethality studies

A LPS-induced lethal shock was induced by an intraperitoneal (i.p.) injection with the mixture of LPS (5 μg) and d-GalN (20 mg) and another one was done by an i.p. injection of a high dose of LPS (400 μg) into mice. A series of flavonoids were injected i.p. before or after the challenge of LPS. Mice were mock-treated with phosphate-buffered saline. Flavonoid pretreatment was performed 3 h before LPS challenge unless otherwise stated. The lethality was monitored until 4 days after LPS challenge.

2.4. Measurement of serum TNF-α

The sera were obtained 3 h after LPS challenge. The concentration of circulating TNF-α in the sera was determined by an enzyme-linked immunosorbent assay kit (Genzyme, Cambridge, MA, USA) according to the manufacturer’s instruction. More than three mice were used for each experimental group. The data represent the mean ± S.D.

2.5. Measurement of lipoperoxide

In order to measure lipid peroxidation, the lipoperoxide concentration in sera was determined by a lipoperoxide assay kit (Wako) using thiobarbituric acid. Briefly, the sera were obtained 6 h after LPS challenge and mixed with sulfonic acid and phosphotungstic acid. The precipitate was treated again with sulfonic acid and phosphotungstate. The precipitate was washed with the thiobarbituric acid reagent, and boiled for 60 min. The solution was mixed with n-butanol, and the fluorescence intensity in the butanol fraction was measured in absorbance at 553 nm. More than three mice were used for each experimental group. The data represent the mean ± S.D.

### 3. Results

#### 3.1. Effect of flavonoids on endotoxin lethality in d-GalN-sensitized mice

We tried to assess the effect of a series of flavonoids in preventing endotoxin lethality in d-GalN-sensitized mice. Various flavonoids (100 μg) suspended in dimethyl sulfoxide (100 μl) were injected i.p. before or after the challenge of d-GalN and LPS and the survival rate of the mice was determined (Table 1). The pretreatment with a series of flavonoids 3 h before the challenge of LPS and d-GalN definitely protected mice from lethal shock. Partial protection was found in mice pretreated with flavonoids 24 h before LPS challenge. Simultaneous administration of some flavonoid with LPS and d-GalN was also effective in protection from endotoxin lethality. The protective action of rutin and quercetin was stronger than that of hesperetin. On the other hand, the post-treatment of flavonoids did not exhibit the protective action against the lethal shock. A higher dose (500 μg) of flavonoids did not necessarily exhibit the stronger protective action against the lethal shock (data not shown). Pretreatment with less than 10 μg of flavonoids did not prevent d-GalN-sensitized mice from endotoxin lethality.

#### Table 1

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flavonoids injected (100 μg)</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
</tr>
<tr>
<td>−24</td>
<td>8/16 (50)</td>
</tr>
<tr>
<td>−3</td>
<td>14/17 (82)</td>
</tr>
<tr>
<td>0</td>
<td>0/9 (0)</td>
</tr>
<tr>
<td>3</td>
<td>1/17 (6)</td>
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</tbody>
</table>

*Flavonoids were injected i.p. at 24 or 3 h before LPS challenge, the same time (0) or 3 h after LPS challenge.*

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3.2. Effect of flavonoids on the lethality in normal mice injected with a high dose of LPS

We assessed the effect of flavonoids on preventing endotoxin lethality in normal mice injected i.p. with a lethal dose of LPS. LPS (400 µg) was injected i.p. various hours after flavonoid pretreatment. The survival rate of LPS-injected mice was determined (Table 2). The pretreatment with flavonoids 24 h before LPS challenge significantly protected mice from the lethal shock. The pretreatment of flavonoids 3 h before LPS challenge and the post-treatment did not prevent mice from endotoxin lethality.

Further, the time-dependent effect of flavonoid pretreatment on protection of the endotoxin lethality was followed by using rutin (Table 3). The pretreatment of rutin 24 and 12 h before LPS challenge was effective in protection from the lethal endotoxic shock. The injection of rutin 2, 3 and 5 days before LPS challenge did not protect mice from the lethal shock. The time-dependent effect of rutin was applied to other flavonoids, such as quercetin, baicalein and catechin. Since hydrophobic rutin might exhibit the protective action through affecting the cell membrane, we compared the protective effect between rutin and water-soluble αGrutin in the endotoxin lethality. Water-soluble αGrutin did not exhibit a protective action, although mice receiving water-insoluble rutin did survive against LPS challenge. The hydrophobic property of rutin was suggested to be involved in the protection of endotoxic lethality.

3.3. Effect of rutin pretreatment on serum TNF-α level in two types of lethal shock

A major feature of LPS-induced hepatic injury of n-GaIN-sensitized mice is increased sensitivity to TNF-mediated effects [11,12]. The effect of flavonoid pretreatment on the systemic release of TNF-α in LPS challenge was studied (Fig. 1). Administration of n-GaIN and LPS into rutin-pretreated mice resulted in marked reduction of the serum TNF-α level, although a high level of circulating TNF-α was induced in untreated control mice. However, there was no significant difference in serum TNF-α level between rutin-pretreated and untreated mice in the administration of a high dose of LPS.

3.4. Effect of rutin on TNF-α-induced lethality in n-GaIN-sensitized mice

In the Section 3.3 we found that rutin pretreatment might prevent n-GaIN-sensitized mice through reduced serum TNF-α level. It was not determined whether rutin pretreatment augmented the resistance to TNF-α in normal mice.

Table 2

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids injected (100 µg)</td>
<td>Quercetin</td>
</tr>
<tr>
<td>−24</td>
<td>5/6 (83)</td>
</tr>
<tr>
<td>−3</td>
<td>0/11 (0)</td>
</tr>
<tr>
<td>0</td>
<td>0/11 (0)</td>
</tr>
<tr>
<td>3</td>
<td>0/11 (0)</td>
</tr>
</tbody>
</table>

*Flavonoids were injected i.p. at 24 or 3 h before LPS challenge, the same time (0) or 3 h after LPS challenge.
The time-dependent effect of rutin pretreatment on the survival rate of D-GalN-sensitized mice by challenge of LPS

<table>
<thead>
<tr>
<th>Pretreatment time (h) before LPS challenge</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>72</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>48</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>24</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>12</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>6</td>
<td>0/3 (0)</td>
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<tr>
<td>3</td>
<td>0/3 (0)</td>
</tr>
</tbody>
</table>

3.5. Effect of rutin pretreatment on lipid peroxidation in endotoxin lethality

Production of lipoperoxide in rutin-pretreated and untreated mice was examined by challenge of D-GalN and LPS or a high dose of LPS (Fig. 2). Rutin pretreatment caused the reduction of the serum lipoperoxide level in the challenge of a high dose of LPS. On the other hand, there was no significant difference in the serum lipoperoxide level between rutin-pretreated or untreated mice in the challenge of D-GalN and LPS.

3.6. Effect of flavonoids on the lethality in mice injected with snake venom

The effect of rutin on the lethality induced by snake venoms was examined by using five mice per group (data not shown). Rutin-pretreated mice were injected i.p. with 50 or 200 μg of A. nalyshkoi or C. atrox. The administration of snake venoms killed both rutin-pretreated and untreated mice. Rutin pretreatment did not prevent mice from the lethal shock induced by snake venoms.

4. Discussion

In this study, we have studied the effect of flavonoids on two murine models of endotoxin lethality. Our results demonstrate that flavonoids protected mice from two types of lethal shock induced by a high dose of LPS or the combination of LPS and D-GalN. We for the first time demonstrated that a series of flavonoids exhibit a protective action on in vivo endotoxin lethality. Flavonoids prevented LPS-induced death of D-GalN-sensitized mice when given 3 h before LPS administration, whereas it protected mice receiving a high dose of LPS when given 24 h before LPS administration. Furthermore, flavonoids did not exhibit a protective action against the lethal shock induced by snake venom, suggesting that the protective action of flavonoids might be selective on LPS-induced lethality.

Flavonoids down-regulated LPS-induced systemic release of TNF-α in D-GalN-sensitized mice. Flavonoids have been reported to attenuate LPS-induced TNF-α production in macrophages [9,13–16]. The protective action of flavonoids on LPS-induced lethal shock in D-GalN-sensitized mice might be due to reduced TNF-α production since the lethal endotoxic shock in D-GalN-sensitized mice is mainly due to excessive release of TNF-α [11,12]. Surprisingly, flavonoids protected TNF-α-induced lethal shock in D-GalN-sensitized mice as well. This indicated that flavonoid pretreatment might confer the resistance to TNF-α-induced lethal shock on D-GalN-sensitized mice. It is of particular interest to clarify the exact mechanism how flavonoids confer the resistance to TNF-α-induced lethal shock. In conclusion, the protective action of flavonoids on LPS-induced death of D-GalN-sensitized mice was due to the augmented resistance to TNF-α as well as its attenuated production.

Flavonoids did not inhibit systemic release of TNF-α in mice receiving a high dose of LPS. The protective action of flavonoids in mice receiving a high dose of LPS appeared to be different from that in D-GalN-sensitized mice. Rather, it seemed to be related to the inhibition of lipid peroxidation, suggesting that the preventive action of flavonoids might be dependent on its anti-oxidant action. A number of flavonoids possess anti-oxidant activity [4,5], although there is a difference in the intensity of in vitro anti-oxidant activity among flavonoids [17,18]. There was no significant difference in the protective action between baicalein and baicalin, although there is a marked difference in anti-oxidant action between them [17,18]. Therefore, the protective effect of flavonoids did not seem to correlate with their in vitro anti-oxidant activity. At present, it was unclear how the protective action of flavonoids was related to their anti-oxidant activity. Furthermore, water-soluble α-rutin possessing anti-oxidant activity was ineffective on protection against lethal shock whereas water-insoluble rutin was effective. The hydrophobic property of flavonoids might be important for exhibition of the protective action, although the exact mechanism was unclear.

In the present study a number of flavonoids protect experimental endotoxic shock in mice. Endotoxin has been implicated as a major factor in the pathogenesis of Gram-negative septic shock with a clinical problem with significant morbidity and mortality. Flavonoids might be used as a potential therapeutic agent to prevent lethal shock induced by endotoxin.
Acknowledgements

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References