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Effects of intravenous infusion of highly purified vitamin B_2 on lipopolysaccharide-induced shock and bacterial infection in mice

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Abstract

We investigated the effect of an i.v. infusion of highly purified vitamin B_2 (riboflavin 5′-sodium phosphate: purity >97%) on lipopolysaccharide-induced shock and bacterial infection in mice. Six hours after lipopolysaccharide injection or 1 h after bacterial infection, vitamin B_2 or human activated protein C (APC) was administered by 6-h i.v. infusion. Vitamin B_2 at 10 mg/kg/6 h and up to 80 mg/kg/6 h significantly improved lipopolysaccharide-induced endotoxin shock. APC was also effective at low doses, but was deleterious at higher doses. Moreover, vitamin B_2 at 80 mg/kg/6 h significantly reduced the lethality of *Escherichia coli* and *Staphylococcus aureus* infection, whereas APC at up to 600 units/kg/6 h was ineffective. The i.v. infusion of vitamin B_2 reduced the elevations of proinflammatory cytokines and nitric oxide induced by lipopolysaccharide. These results suggest that i.v. infusion of vitamin B_2 represents a promising strategy for the treatment of sepsis and septic shock.
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Keywords: Vitamin B_2; I.v. infusion; Proinflammatory cytokine; Nitric oxide

1. Introduction

Sepsis and septic shock, which are characterized by fever, disseminated intravascular coagulation and multiple organ failure, are intractable conditions with a high mortality rate (Parrillo, 1993; Cohen, 2002). Excessive host responses, aggravated by mobilizing leukocytes, disturb the coagulation and complement cascade (Cohen, 2002; Esmon, 2001) and promote the production of various proinflammatory cytokines (Bossink et al., 1995; Dinarello, 1997; Marty et al., 1994) and excessive NO (nitric oxide) (Gross and Wolin, 1995); these are continuous processes, so systemic multiple organ dysfunctions usually evolve after a latent period of damaging reactions. It is therefore conceivable that long-term i.v. infusion would be a suitable approach for the treatment of patients with sepsis and septic shock.

We have recently revealed that i.v. administration of highly purified vitamin B_2 (riboflavin 5′-sodium phosphate: purity >97%) ameliorated both toxin-induced shock and bacterial infection, even when administered after the onset of endotoxemia, and also reduced excessive proinflammatory cytokines and NO (Toyosawa et al., 2004). These results indicated that highly purified vitamin B_2 might be an effective treatment for patients with sepsis and septic shock; however, the effectiveness of long-term i.v. infusion for its delivery and suitable clinical surrogate markers remain to be established.

The purposes of the present study are to investigate the therapeutic effects of long-term i.v. infusion of vitamin B_2 on lipopolysaccharide-induced shock, and *Escherichia coli* and *Staphylococcus aureus* infection in mice and to compare these effects with those of human activated protein C (APC), which is clinically used for the treatment of sepsis and septic shock (Lolis and Bucala, 2003). We also examined the effects of i.v. infusion of vitamin B_2 on proinflammatory cytokines and NO levels in lipopolysaccharide-challenged mice to identify clinical surrogate markers.

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2. Materials and methods

2.1. Animals

Male ICR mice aged 5 or 6 weeks were obtained from Japan SLC (Shizuoka, Japan). Mice were provided with pellet food (MF, Oriental Yeast, Tokyo, Japan) and sterilized tap water ad libitum. The animal room was set at a temperature of 23 °C (± 3 °C) with 55% relative humidity (± 15%), and lighting was artificial with a sequence of 12-h light/12-h dark. After 1 week of acclimatization, mice were used for experiments. All procedures were approved by the Animal Care and Use Committee of Eisai.

2.2. I.v. infusion in toxin shock model

The procedure followed that of our previous study (Toyosawa et al., 2004). Mice were given an i.v. bolus injection of lipopolysaccharide at 12 mg/kg through the tail vein. Six hours after lipopolysaccharide injection, mice were treated with saline (control), vitamin B_2 (2.5, 5, 10, 20, 40 and 80 mg/kg) or APC (37.5, 75, 150, 300, 600 and 1200 units/kg) by i.v. infusion for 6 h at a speed of 0.58 ml/h via the tail vein using an infusion pump (Natsume, Tokyo, Japan). The experimental protocol is presented in Fig. 1A.

2.3. I.v. infusion in infection model

A clinical isolate of *E. coli* E01292 and *S. aureus* E311122 maintained in our laboratory are used for infection models (Araki et al., 1987; Toyosawa et al., 2004). The strain was grown overnight on brain heart infusion (Difco, Detroit, MI, USA) with 1.5% agar (Difco) at 37 °C. After being harvested, the bacterial cells were suspended in physiological saline, frozen in acetone with dry ice, and stored in a deep freezer at − 80 °C. An appropriate dilution of the bacterial suspensions in physiological saline from stock cells was used as inoculation.

One hour after i.v. inoculation of *E. coli* (2.0×10^8 CFU/mouse) or *S. aureus* (1.7×10^8 CFU/mouse) through the tail vein, mice were given with saline (control), vitamin B_2, APC or heparin by 6-h i.v. infusion at the speed of 0.58 ml/h through the tail vein. Vitamin B_2 (80 mg/kg), APC (150, 300 and 600 units/kg) or heparin (500 units/kg) were infused in *E. coli* infection model (Fig. 1B), and vitamin B_2 (20, 40 and 80 mg/kg) were infused in *S. aureus* infection (Fig. 1C). The survival rates of *E. coli* and *S. aureus* infection were calculated after 7 and 14 days, respectively.

2.4. Measurement of plasma parameters

Firstly, we investigated the protective effect of vitamin B_2 in terms of the plasma biochemical parameters in lipopolysaccharide-challenged mice. One hour after the beginning of i.v. infusion of saline (control) or vitamin B_2 at 20 mg/kg/7 h (2.9 mg/kg/h), lipopolysaccharide at 4 mg/kg was intravenously injected. Then, 2, 4 and 7 h after the start of saline or vitamin B_2 infusion, blood samples were collected (Fig. 2A).

Secondly, we investigated the effects of vitamin B_2 on the plasma biochemical parameters in lipopolysaccharide-challenged mice. The i.v. infusion of saline or vitamin B_2 at

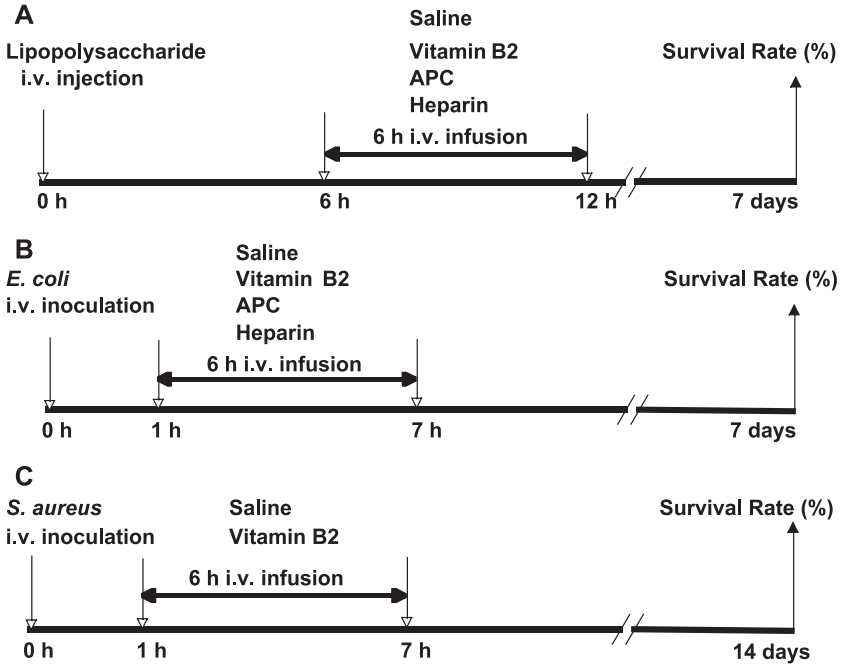

Fig. 1. Experimental protocols of toxin shock and bacterial infection model in mice. (A) Lipopolysaccharide-induced shock model; (B) *E. coli* infection model; (C) *S. aureus* infection model.

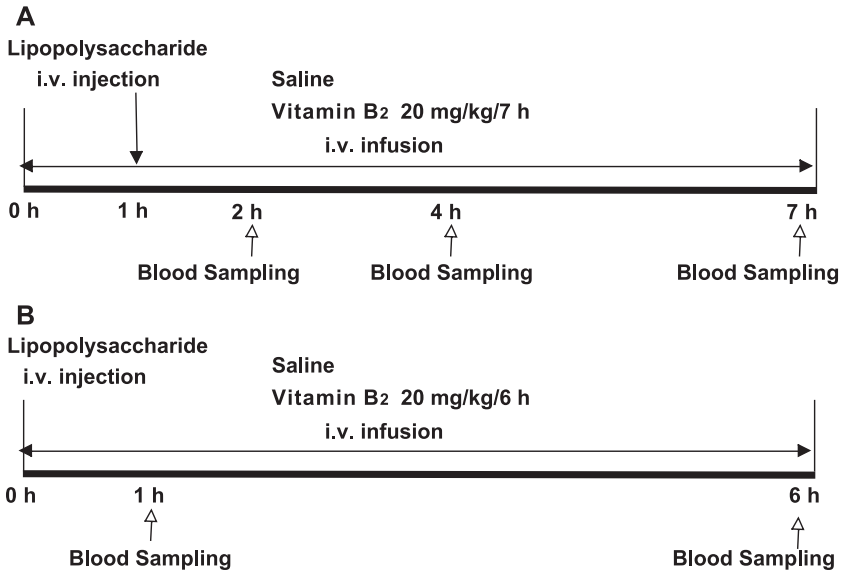

Fig. 2. Experimental protocol for measurement of plasma parameters. (A) Preventive effect of vitamin B_2; (B) therapeutic effect of vitamin B_2.

20 mg/kg/6 h (3.3 mg/kg/h) was commenced immediately after the i.v. injection of lipopolysaccharide at 4 mg/kg. At 1 and 6 h after lipopolysaccharide injection, blood sampling was performed (Fig. 2B).

Each blood sample was drawn from the abdominal vein of animals under ether anesthesia, into a plastic syringe containing EDTA. Then the blood was centrifuged at 3000 rpm for 10 min at 4 °C to isolate plasma and stored at − 80 °C until used for the measurements of cytokines and NO concentration. Enzyme-linked immunosorbent assay kits for interleukin-1β, interleukin-6, tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) were purchased from Biosource International (Camarillo, CA, USA). A nitrate/nitrite colorimetric assay kit was purchased from Dojindo Laboratories (Kumamoto, Japan). The plates were read with a plate reader (Spectra max 250; Molecular Devices, Sunnyvale, CA, USA) and the data were analyzed using SOFT max PRO 1.1 (Molecular Devices). Detection limits were < 3 pg/ml for interleukin-6, < 7 pg/ml for interleukin-1β, < 3 pg/ml for TNF-α, < 9 pg/ml for MCP-1. Values below the detection limit was expressed as 0 pg/ml.

2.5. Drugs

Highly purified vitamin B_2 (riboflavin 5′ -sodium phosphate; purity >97%) was synthesized at Eisai Kashima Plant. Anact C® (human activated protein C; APC) and heparin were obtained from Teijin (Tokyo, Japan) and Aventis (Tokyo, Japan), respectively. Lipopolysaccharide (*E. coli* O111:B4) was purchased from Sigma (St. Louis, MO, USA). Vitamin B_2, APC and lipopolysaccharide were each dissolved in physiological saline before use. The vitamin B_2 solution was passed through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA) before use. Heparin was also diluted with physiological saline before use.

2.6. Statistical analysis

The differences of survival rates and plasma parameters were analyzed by use of the Steel test and Student's *t*-test, respectively. Statistical analysis was conducted using the software package SAS 6.12 (SAS Institute Japan, Tokyo, Japan). A value of $P < 0.05$ (two-sided) was considered statistically significant.

3. Results

3.1. Effects of vitamin B_2, APC and heparin on lipopolysaccharide-induced shock

The therapeutic effects of i.v. infusion of vitamin B_2, APC and heparin on lipopolysaccharide-induced shock are

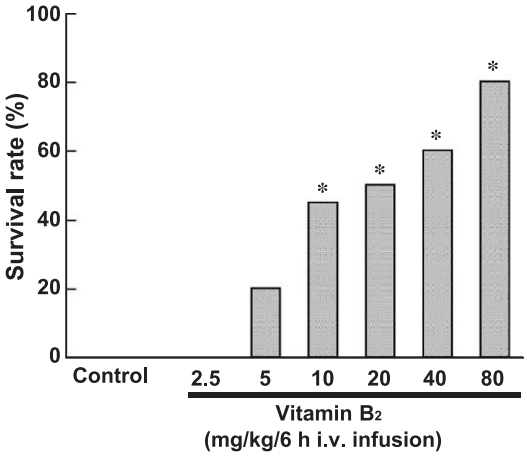

Fig. 3. Therapeutic effects of vitamin B_2 on the mortality of lipopolysaccharide-induced shock in mice. *$P < 0.05$ vs. control (Steel test).

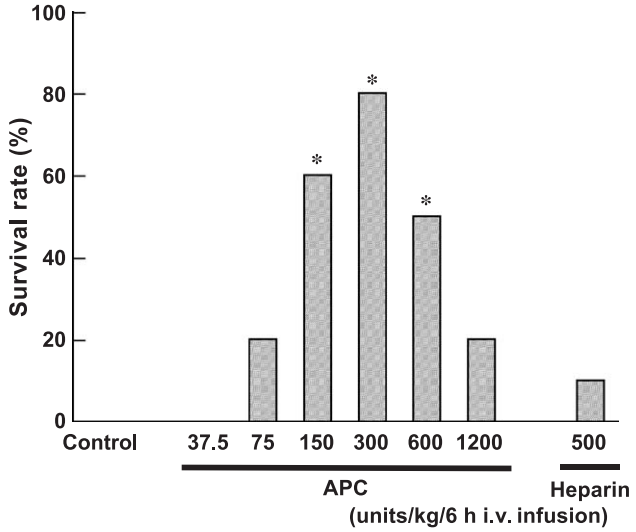

Fig. 4. Effects of activated protein C and heparin on mortality of lipopolysaccharide-induced shock in mice. Activated protein C (APC). *$P<0.05$ vs. control (Steel test).

presented in Figs. 3 and 4. All 20 mice treated with saline died within 2 days. The survival rates in the groups treated with vitamin B_2 at 2.5, 5, 10, 20, 40 and 80 mg/kg/6 h were 0% (0 of 10), 20% (2 of 10), 45% (9 of 20: $P<0.05$ vs. Control), 50% (5 of 10: $P<0.05$), 60% (6 of 10: $P<0.05$) and 80% (8 of 10: $P<0.05$), respectively (Fig. 3). Those of the groups treated with APC at 37.5, 75, 150, 300, 600 and 1200 units/kg/6 h were 0% (0 of 10), 20% (2 of 10), 60% (6 of 10: $P<0.05$ vs. control), 80% (8 of 10: $P<0.05$), 50% (5 of 10: $P<0.05$) and 20% (2 of 10), respectively. Thus, APC at doses up 600 units/kg/6 h ameliorated mortality, while APC at 1200 units/kg/6 h was deleterious (Fig. 4). The survival rate of heparin-treated group was 10% (1 of 10, Fig. 4).

3.2. Effects of vitamin B_2, APC and heparin on E. coli infection

The therapeutic effects of vitamin B_2, APC and heparin on *E. coli*-induced infection are summarized in Fig. 5. All 30 mice treated with saline (control) died within 1 day, whereas the survival rate of mice given vitamin B_2 at 80 mg/kg/6 h was 80% (8 of 10: $P<0.05$ vs. control). The survival rates of mice given APC at 150, 300 and 600 units/kg/6 h were 0% (0 of 10), 10% (1 of 10) and 0% (0 of 10), respectively. Heparin, likely APC, was ineffective against *E. coli* infection (0%, 0 of 10).

3.3. Effects of vitamin B_2 on S. aureus infection

The survival rate of saline-treated group (control) gradually decreased, and all 10 mice were died 8 days after inoculation. The survival rate of vitamin B_2 at 20, 40 and 80 mg/kg/6 h were 10% (1 of 10), 40% (4 of 10) and 60% (6 of 10: $P<0.05$ vs. control), respectively (Fig. 6).

3.4. Effects of vitamin B_2 on plasma parameters in lipopolysaccharide-challenged mice

The preventive effect of i.v. infusion of vitamin B_2 on levels of plasma proinflammatory cytokines and NO are shown in Fig. 7. The plasma TNF-α level increased immediately after lipopolysaccharide administration, and then gradually decreased. Vitamin B_2 did not affect the early-phase elevation of TNF-α, but lowered the TNF-α level at 6 h after lipopolysaccharide injection (11.5 ± 8.1 vs. 119.6 ± 24.7 pg/ml, $P<0.05$). interleukin-1β, MCP-1, interleukin-6 and NO gradually increased after lipopolysaccharide challenge. Vitamin B_2 significantly inhibited these increases. The inhibitory effects of vitamin B_2 on interleukin-1β and inter-

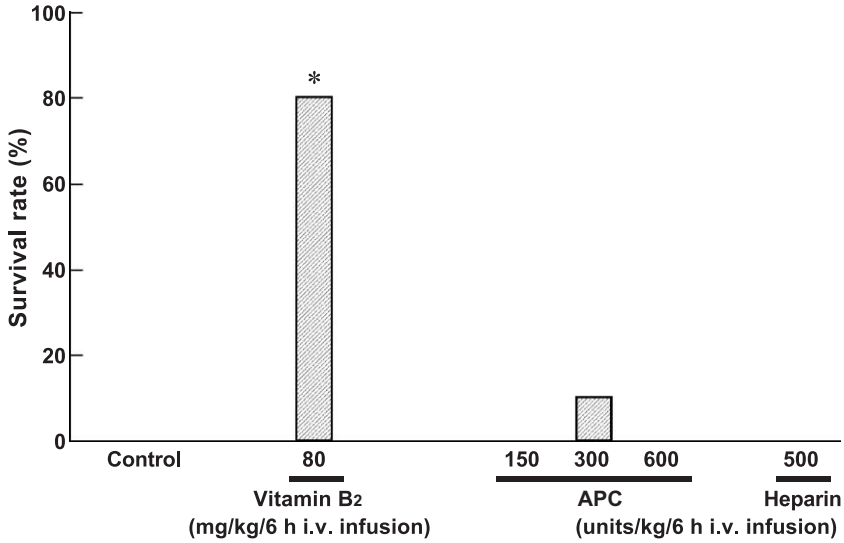

Fig. 5. Therapeutic effects of vitamin B_2, activated protein C and heparin on mortality of *E. coli* infection in mice. Activated protein C (APC). *$P<0.05$ vs. control (Steel test).

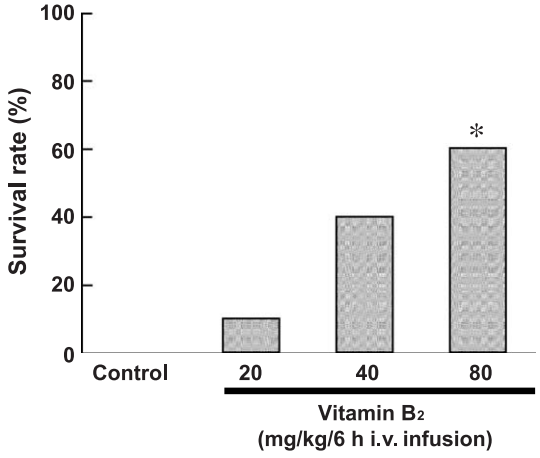

Fig. 6. Therapeutic effects of vitamin B_2 on mortality of *S. aureus* infection in mice. *$P<0.05$ vs. control (Steel test).

leukin-6 levels were long-sustained. Vitamin B_2 reduced the interleukin-1β levels at both 4 h (121.3 ± 25.5 vs. 264.8 ± 45.2 pg/ml, $P<0.05$) and 7 h (127.2 ± 11.4 vs. 274.5 ± 48.8 pg/ml, $P<0.05$). Interleukin-6 was decreased at 2 h (15.6 ± 1.6 vs. 91.9 ± 23.8 ng/ml, $P<0.05$), 4 h (74.8 ± 0.1 vs. 122.7 ± 5.9 ng/ml, $P<0.05$) and 7 h (15.6 ± 1.6 vs. 91.9 ± 23.8 ng/ml, $P<0.05$). MCP-1 and NO were decreased at 7 h (3.4 ± 0.8 vs. 7.8 ± 1.0 ng/ml, $P<0.05$) and at 4 h (76.6 ± 15.5 vs. 143.9 ± 22.1 μM, $P<0.05$), respectively.

The therapeutic effects of i.v. infusion of vitamin B_2 on plasma proinflammatory cytokines and NO levels are summarized in Fig. 8. Because plasma blood sampling was carried out at the same time after lipopolysaccharide injection in both experiments, the changes in proinflammatory cytokines and NO in the second study were similar to those in the first experiments. Vitamin B_2 did not affect the elevations of plasma biochemical parameters at 1 h after

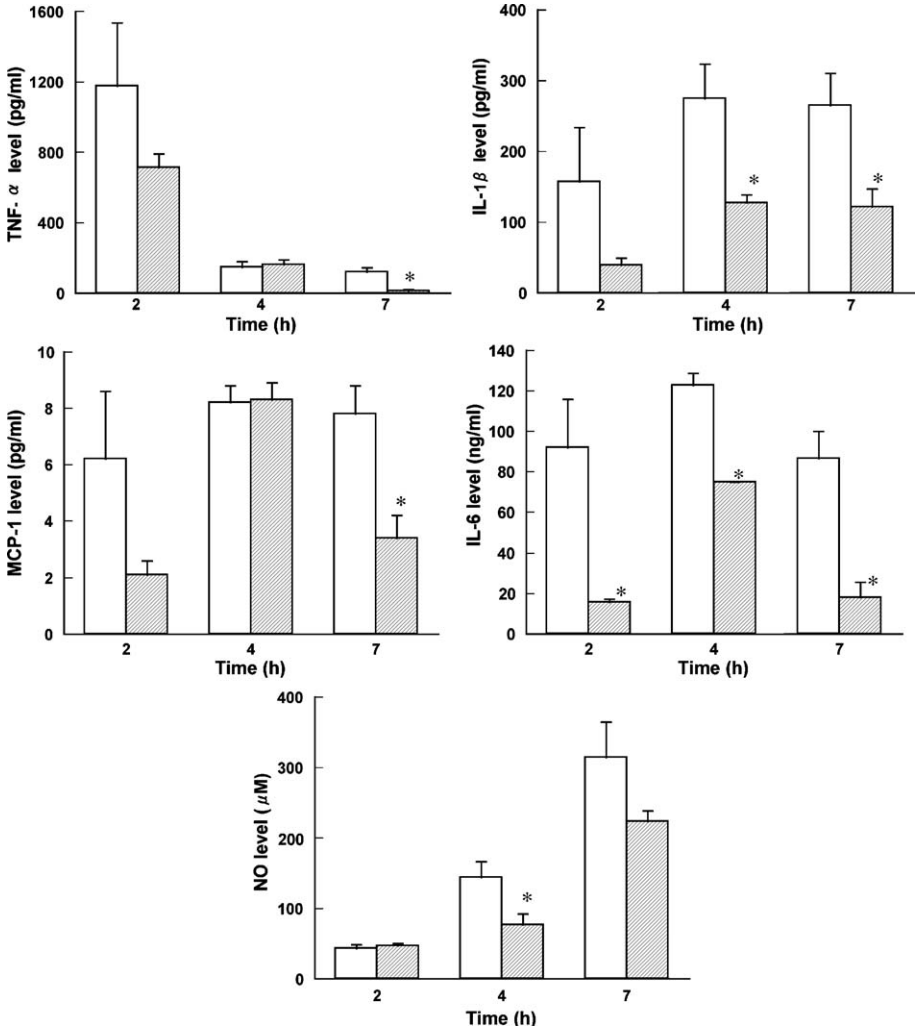

Fig. 7. Preventive effect of intravenous infusion of vitamin B_2 on plasma inflammatory cytokines and NO levels in lipopolysaccharide-challenged mice. Open columns and hatched columns indicate saline-treated group and vitamin B_2-treated group, respectively. Tumor necrosis factor-α, TNF-α; interleukin-1β, IL-1β; monocyte chemoattractant protein-1, MCP-1; interleukin-6, IL-6; nitric oxide, NO. Each value was expressed as mean ± S.E.M. ($n=7$). *$P<0.05$ vs. saline-treated group (Student's *t*-test).

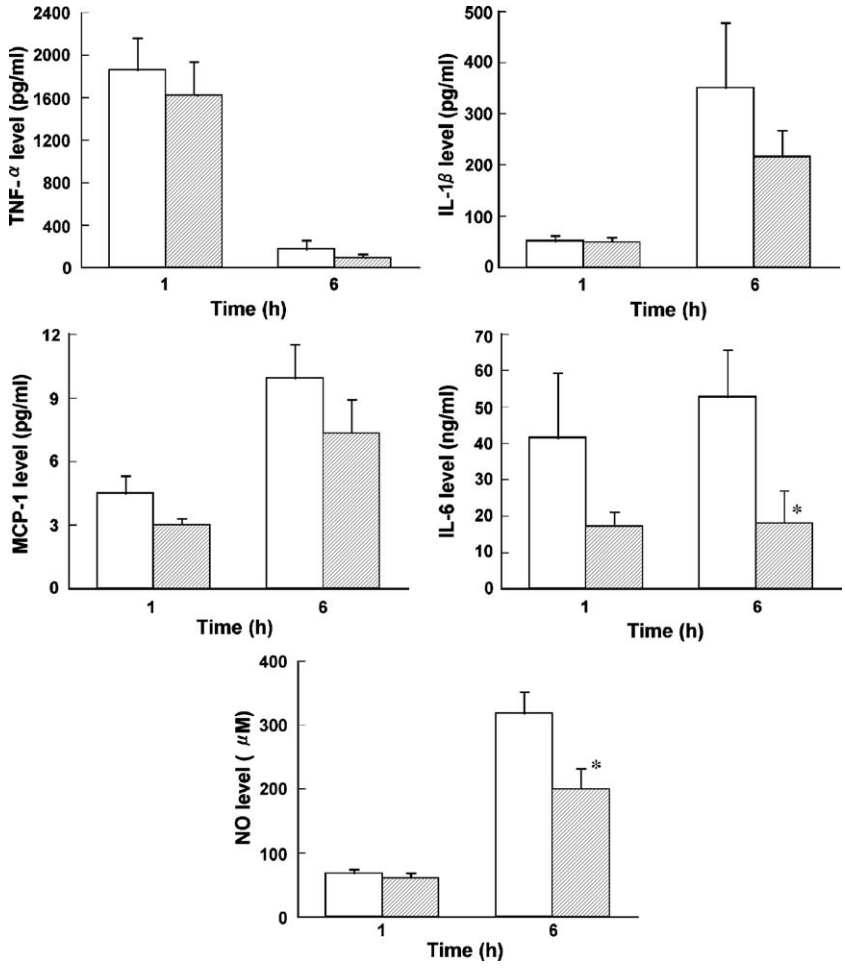

Fig. 8. Therapeutic effect of intravenous infusion of vitamin B_2 on plasma inflammatory cytokines and NO levels in lipopolysaccharide-challenged mice. Open columns and hatched columns indicate saline-treated group and vitamin B_2-treated group, respectively. Tumor necrosis factor-α, TNF-α; interleukin-1β, IL-1β; monocyte chemoattractant protein-1, MCP-1; interleukin-6, IL-6; nitric oxide, NO. Each value was expressed as mean ± S.E.M. ($n = 7$). *$P < 0.05$ vs. saline-treated group (Student's t-test).

lipopolysaccharide injection, while it decreased the elevation of interleukin-6 (18.0 ± 8.9 vs. 52.7 ± 12.9 ng/ml, $P < 0.05$) and NO (199.6 ± 32.1 vs. 318.4 ± 33.4 μM, $P < 0.05$) levels at 6 h.

4. Discussion

The major findings of this study are that, even when i.v. infusion was commenced after *E. coli* and *S. aureus* inoculation or the injection of a lethal dose of lipopolysaccharide, highly purified vitamin B_2 reduced the mortality of bacterial infection and toxin-induced shock. APC reduced the lipopolysaccharide-induced mortality, but failed to protect against *E. coli* infection.

Vitamin B_2 (riboflavin, 5′ -riboflavin sodium phosphate, etc.), a well-known oral nutritional supplement, has been widely used to treat localized inflammatory diseases, such as angulus infectiosus, chilitis and glossitis. Further, we have previously reported that vitamin B_2 enhanced host resistance to infection (Araki et al., 1995), stimulated neutrophil functions (Osame et al., 1995), and boosted macrophage function when it was administered prophylactically (Kimura et al., 1996). These findings raised the possibility that vitamin B_2 would ameliorate the inflammation and improve the regulation of the host defense system in sepsis, although its therapeutic effects still remain to be established. Moreover, we have ascertained that highly purified vitamin B_2 (purity >97%), which has not been used for medical purposes to date, markedly improved toxin-induced shock in mice compared with commercially available vitamin B_2, whose purity is less than 90% (as riboflavin 5′ -sodium phosphate). Based on these results, we considered that highly purified vitamin B_2 might be a promising treatment for sepsis and septic shock. However, orally administered vitamin B_2 shows limited absorption in human (Zempleni et al., 1996), and the physiological characteristics of vitamin B_2 intravenously administered at doses above those given to treat deficiency have not been sufficiently investigated, nor has i.v. injection been clinically applied, despite the general use of vitamin B_2.

On the basis of a toxicological study, a high dose of intraperitoneally administered riboflavin can cause death (LD_{50}: 560 mg/kg) due to obstruction of the kidneys by concretions (Unna and Gresln, 1942). The renal dysfunction is commonly induced by septic shock (Brivet et al., 1996). Vitamin B_2 given as a bolus i.v. injection reduced the mortality of mice with septic shock (Toyosawa et al., 2004), though we would expect i.v. infusion to be safer. Moreover, the pathogenetic networks in sepsis are so complicated that the blockade of individual early detrimental mediators, e.g., with anti-TNF-α antibody (Dinarello, 1997) or interleukin-1β receptor antagonist (Dinarello, 1997), offers little benefit. It seems, therefore, that long-term i.v. infusion of vitamin B_2 may be the best approach to protect against the increase of adverse mediators in sepsis.

Human APC prevents thrombin-induced thromboembolism in mice (Gresele et al., 1998), so the therapeutic effect of APC in this toxin shock model can be explained in terms of anticoagulation action (Esmon, 2001; Taylor et al., 1987). In addition, the therapeutic effect was also partly attributed to its immunosuppressive effect (Okajima, 2001). However, a higher dose of APC worsened the mortality induced by lipopolysaccharide, suggesting that the safety range may be narrow. In a clinical study, the incidence of bleeding was higher in an APC-treated group than in the vehicle-treated group (Bernard et al., 2001).

Vitamin B_2 at 10, 20 and 40 mg/kg/6 h ameliorated the morbidities evoked by *E. coli* infection, in agreement with this study (80 mg/kg/6 h). Moreover, i.v. infusion of vitamin B_2 at 40 and 80 mg/kg/12 h also reduced the mortality of *S. aureus* infection. Vitamin B_2 enhances both clearance of *E. coli* from the blood (Toyosawa et al., 2004) and macrophage phagocytosis (Araki et al., 1995; Kimura et al., 1996). We consider that the beneficial effect of vitamin B_2 against bacterial infection might be mediated through enhancement of the host immune system. Combined therapy with vitamin B_2 and APC was effective against endotoxin- and exotoxin-induced shock (Toyosawa et al., 2004), so it is worth considering such combination therapies for the treatment of patients with sepsis and septic shock.

The i.v. infusion of vitamin B_2 also reduced the elevations of plasma proinflammatory cytokines and NO, as did i.v. bolus injection. Elevated plasma proinflammatory cytokine levels are known to increase the morbidity of septic shock (Barriere and Lowry, 1995; Bossink et al., 1995; Dinarello, 1997) and these levels progressively decrease from admission to recovery (Endo et al., 1996). Consequently, the inhibitory effects of i.v. infusion of vitamin B_2 on the elevated plasma cytokine levels should be useful as sensitive and quantitative surrogate markers.

Elevation of interleukin-6 is also involved in age-related diseases, such as anemia, dementia, osteoporosis, etc. (Ershler and Keller, 2000). The expression of interleukin-6 is inhibited by estrogen and testosterone, and therefore decrease of these hormones may allow increased interleukin-6 production, leading to disorders similar to chronic inflammatory syndrome after menopause or andropause. Therefore chronic vitamin B_2 treatment might improve the frailty of aged people. Further investigations, including research to develop orally active derivatives, will be necessary to establish the value of vitamin B_2 for many diseases.

In conclusion, long-term i.v. infusion of highly purified vitamin B_2, even when commenced after toxemia and bacterial infection, reduced the mortality in mouse models of sepsis and septic shock. These results suggest that clinical trials of highly purified vitamin B_2 would be worthwhile.

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