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Potentiation by amino acid of the therapeutic effect of highly purified vitamin B_2 in mice with lipopolysaccharide-induced shock

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Abstract

The aim of this experiment was to clarify whether an amino acid supplement could enhance the therapeutic effect of vitamin B_2 (riboflavin 5'-sodium phosphate; purity>97%) in mice with lipopolysaccharide-induced shock. Six hours after injection of a lethal dose of lipopolysaccharide, treatment (6-h i.v. infusion) was commenced. All mice died in the groups treated with saline or aminolevane® (an amino acids mixture used to treat hepatopathy); however, the survival rates in the vitamin B_2 (10 mg/kg/6 h) and vitamin B_2 plus aminolevane® groups were 45% ($P<0.05$) and 80% ($P<0.05$), respectively. Valine (200 mg/kg/6 h) alone had little effect on the survival rate (10%), but the combination of vitamin B_2 (10 mg/k/g/6 h) and valine was highly effective (80%, $P<0.05$). Clinical trials of vitamin B_2 plus amino acids for the treatment of patients with sepsis would appear to be warranted.

Keywords: Vitamin B_2; Amino acid; Mortality; Macrophages; Sepsis

1. Introduction

Sepsis is characterized by hypermetabolism, increased energy expenditure, and augmented peripheral protein catabolism caused by the excessive inflammatory responses (Braga and Gianotti, 2002). Interleukin-6, tumor necrosis factor-α (TNF-α), oxygen radicals and catecholamines appear to be directly implicated in the pathophysiology of muscle proteolysis and protein wasting (Chang and Bistrian, 1998). The amino acids released from peripheral tissues are shunted to the liver, where they are used for gluconeogenesis and synthesis of acute-phase proteins. During this hypermetabolic state, the flux and utilization of a variety of substances are altered, resulting in increased skeletal muscle proteolysis, tissue mobilization of fatty acids, and gluconeogenesis (Vary, 1998). The plasma amino acids levels are decreased due to higher clearance in patients with sepsis (Druml et al., 2001). The long-lasting losses of constitutive and structural tissue proteins lead to delayed tissue repair, impaired wound healing and compromised immune function, thereby increasing the risk of the multiple organ dysfunction and failure (Braga and Gianotti, 2002).

We have previously indicated that highly purified vitamin B_2 (riboflavin 5′-sodium phosphate; purity>97%) would be a promising agent for the treatment of patients with sepsis and septic shock (Toyosawa et al., 2004). Considering the high mortality of sepsis, it is worthwhile to consider combination therapy to improve the clinical outcome. We considered that an amino acid supplement might reduce the organ damage evoked by hypermetabolism in sepsis. There is clinical evidence that supplementation of amino acids reduces morbidity (Gianotti et al., 1993) and reduces intensive care unit and hospital costs (Jones et al., 1999), though its efficacy has not been well studied.

The purpose of this study was to examine the effect of supplementary amino acids on the therapeutic effect of vitamin B_2 in a mouse endotoxin shock model. We used aminolevane® solution (an injectable solution of several amino acids used to treat encephalopathy associated with chronic hepatopathy) and individual amino acids.

Besides hypermetabolism, sepsis involves a failure of the circulatory systems to supply adequate oxygen to the tissue from the standpoint of energy metabolism. Insufficient oxygen availability in the face of continued energy demand

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forces the cell into anaerobic glycolysis, followed by lactic acidosis (Morgan and Machiedo, 2002). Thus, sepsis causes disruption of cellular oxygen metabolism, as indicated by the plasma elevation of lactic acid concentration and other signs of accelerated anaerobic metabolism. Vitamin B_2 regulates macrophage functions such as phagocytosis (Kimura et al., 1996) and bacterial clearance (Toyosawa et al., 2004). Since vitamin B_2 may ameliorate the disturbed energy balance in sepsis, we also studied the effect of vitamin B_2 on activated macrophage function evoked by lipopolysaccharide to clarify the nature of its beneficial effect on toxin-induced mortality.

2. Material and methods

2.1. Animals

Male ICR mice aged 5 weeks were obtained from Japan SLC (Shizuoka, Japan) and housed at a room temperature of 23 °C (± 3 °C) and a relative humidity of 55% (± 15%) with a 12-h light/dark cycle (lights on at 7:00 a.m., light off at 19:00 p.m.). Mice had free access to tap water and a laboratory diet (MF, Oriental Yeast, Tokyo, Japan). After 1 week of acclimation, mice were used for experiments. All experiments were approved by The Animal Care and Use Committee of Eisai.

2.2. Lipopolysaccharide-induced shock model

Mice were given an i.v. bolus injection of lipopolysaccharide (12 mg/kg) through the tail vein. Six hours after lipopolysaccharide injection, treatment was administered by i.v. bolus injection or by 6-h continuous infusion via the tail vein (Toyosawa et al., 2004). The therapeutic effect of vitamin B_2 is presented as the survival rate (percent survival) after 7 days. In the first experiment, vitamin B_2 dissolved in saline or aminolevane® solution was intravenously infused into mice for 6 h at a speed of 0.58 ml/h by an infusion pump (Natsume, Tokyo, Japan). The experimental protocol is summarized in Fig. 1A.

Secondly, the effect of individual amino acids present in aminolevane® solution on the therapeutic effect of vitamin B_2 was examined. Six hours after lipopolysaccharide injection, mice were intravenously administered vitamin B_2 with or without an amino acid (components of aminolevane® solution: tryptophane, isoleucine, proline, threonine, alanine, serine, methionine, leucine, phenylalanine, arginine, cysteine, histidine and valine). The experimental protocol is shown in Fig. 1B.

Thirdly, we investigated the effect of valine on the therapeutic effect of vitamin B_2. Six hours after lipopolysaccharide injection, vitamin B_2 with or without valine was administered by 6-h i.v. infusion (Fig. 1C).

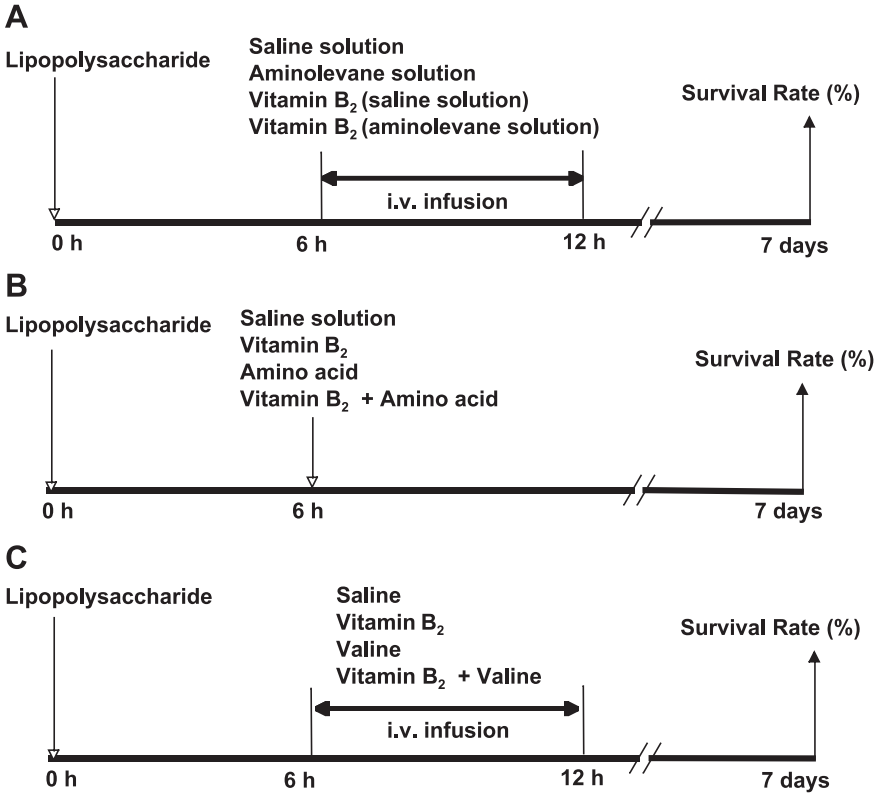

Fig. 1. Experimental protocol. (A) Effect of aminolevane® solution on the therapeutic effect of i.v. infusion of vitamin B_2, (B) Effect of amino acids on the therapeutic effect of i.v. infusion of vitamin B_2, (C) Effect of valine on the therapeutic effect of i.v. infusion of vitamin B_2.

2.3. Effect of vitamin B_2 on the function of mouse peritoneal macrophages

This experiment was performed according to a reported method (Kimura et al., 1996). Mice were killed by exsanguination via the carotid artery under ether anesthesia and peritoneal macrophages were harvested after the intraperitoneal injection of 4.5 ml/mouse of cold Hank's balanced salt solution (Gibco Laboratories Grand Island, NY, USA). The cell suspension obtained from the peritoneal cavity was washed with cold Hank's balanced salt solution by centrifugation for 10 min at 1000 rpm, and the resultant cells consisted exclusively of macrophages. These cells were suspended at 1×10^6 cell/ml in Eagle's minimal essential medium (Nissui Pharmaceutical, Tokyo, Japan) containing 15% heat-inactivated murine serum and were cultured in Lab-Tek chambers (Nalgene Nunc International, Naperville, IL, USA) at 37 °C with 5% CO_2.

To elucidate the effect of vitamin B_2 on the macrophage activities, we evaluated the effects of vitamin B_2 on interleukin-6 and lactic acid production, and glucose consumption in lipopolysaccharide-stimulated macrophages. The mice peritoneal macrophages were stimulated with 0.01 μg/ml of lipopolysaccharide in the absence or presence of various concentrations of vitamin B_2 (1.57, 3.15, 6.3, 12.5 and 25 μg/ml) and were incubated for 24 h. The levels of interleukin-6 (Biosource International, Camarillo, CA, USA), glucose (Wako Pure Chemical Industries, Osaka, Japan) and lactic acid (Kyowa Medex, Shizuoka, Japan) 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).

2.4. Drugs

All agents were dissolved in physiological saline. Vitamin B_2 (riboflavin 5′-phosphate sodium; purity>97%) was synthesized at Kashima Plant, Eisai (Ibaraki, Japan). Aminolevane®, lipopolysaccharide from *Escherichia coli* O111:B4 and amino acids such as tryptophan, isoleucine, proline, threonine, alanine, serine, methionine, leucine, phenylalanine, arginine, cysteine, histidine and valine was obtained from Otsuka Pharmaceutical (Tokyo, Japan), Sigma (St. Louis, MO, USA) and Wako Pure Chemical Industries, respectively. Vitamin B_2 and amino acids were passed through a 0.22-μm membrane filter (Millipore Bedford, MA, USA) before use.

2.5. Statistical analysis

The differences of survival rate and macrophage functions were analyzed by use of the Steel test and Dunnett's multiple range 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. Influence of amino acids on therapeutic effect of vitamin B_2 in lipopolysaccharide-induced shock in mice

In the saline- and aminolevane® solution-treated groups, there were no survivors at 7 days. The beneficial effect of i.v. infusion of vitamin B_2 at 10 mg/kg/6 h on lipopolysaccharide-induced mortality was higher using aminolevane® solution compared with using saline solution (Table 1).

The results of combination therapy of vitamin B_2 with each of 13 amino acids at 200 mg/kg (all the component amino acids of aminolevane® solution) on lipopolysaccharide-induced mortality are summarized in Table 2. Vitamin B_2 at 2.5 mg/kg did not significantly improve the survival rate by itself, but in combination with tryptophan, isoleucine, proline, threonine, alanine or valine, it was significantly superior to the saline-treated group. The combination therapy of vitamin B_2 (2.5 mg/kg) with 100 mg/kg tryptophan, isoleucine, proline, threonine and valine (all those which had improved the survival rate to more than 60% in the i.v. treatment) was further examined by i.v. bolus administration. Only valine was significantly effective in combination with vitamin B_2 (Table 3).

3.2. Effect of valine on therapeutic effect of vitamin B_2 in lipopolysaccharide-induced shock in mice

Neither valine at 100 or 200 mg/kg by itself nor the combination therapy of valine at 12.5, 25 or 50 mg/kg with vitamin B_2 at 2.5 mg/kg by i.v. bolus administration improved the survival rate of lipopolysaccharide-injected mice. However, valine at 100 mg/kg in combination with vitamin B_2 at 2.5 mg/kg was effective (Table 4).

The effect of combination therapy of valine with vitamin B_2 administered by i.v. infusion in mice with lipopolysaccharide-induced shock is summarized in Fig. 2. All mice treated with saline ($n=20$) died within 2 days after lipopolysaccharide injection. Valine at 200 mg/kg/6 h ($n=10$) did not significantly improve the mortality by itself (10%), but combination therapy of valine at 200 mg/kg/6 h with vitamin B_2 at 10 mg/kg/6 h ($n=10$) did improve the survival rate compared with saline-treated group 80%, $P<0.05$).

Table 1
Effect of vitamin B_2 on lipopolysaccharide-induced shock in mice

Treatment	Survival	
	No. of survivors/total	Rate (%)
Saline solution	0/20	0
Aminolevane solution	0/10	0
Vitamin B_2 10 mg/kg/6 h (saline solution)	9/20	45*
Vitamin B_2 10 mg/kg/6 h (aminolevane solution)	8/10	80*

* $P<0.05$ vs. saline solution (Steel test).

Table 2
Effect of amino acid on lipopolysaccharide-induced mortality in mice

Treatment	Survival	
	No. of survivors/total	Rate (%)
Saline solution	0/10	0
Vitamin B_2 2.5 mg/kg	3/10	30
Vitamin B_2 2.5 mg/kg + Tryptophane 200 mg/kg	6/10	60*
Vitamin B_2 2.5 mg/kg + Isoleucine 200 mg/kg	6/10	60*
Vitamin B_2 2.5 mg/kg + Proline 200 mg/kg	7/10	70*
Vitamin B_2 2.5 mg/kg + Threonine 200 mg/kg	7/10	70*
Vitamin B_2 2.5 mg/kg + Alanine 200 mg/kg	5/10	50*
Vitamin B_2 2.5 mg/kg + Serine 200 mg/kg	3/10	30
Vitamin B_2 2.5 mg/kg + Methionine 200 mg/kg	3/10	30
Vitamin B_2 2.5 mg/kg + Leucine 200 mg/kg	4/10	40
Vitamin B_2 2.5 mg/kg + Phenylalanine 200 mg/kg	4/10	40
Vitamin B_2 2.5 mg/kg + Arginine 200 mg/kg	3/10	30
Vitamin B_2 2.5 mg/kg + Cysteine 200 mg/kg	1/10	10
Vitamin B_2 2.5 mg/kg + Histidine 200 mg/kg	1/10	10
Vitamin B_2 2.5 mg/kg + Valine 200 mg/kg	8/10	80*

* $P < 0.05$ vs. saline solution (Steel test).

3.3. Effect of vitamin B_2 on the activity of mouse peritoneal macrophages stimulated by lipopolysaccharide

The effects of vitamin B_2 on interleukin-6 and lactic acid production, and glucose consumption in mouse peritoneal macrophages are summarized in Fig. 3. A significant increase in medium interleukin-6 level (13954.3 ± 1348.2 vs. 103 ± 48.8 pg/ml, $P < 0.05$) was observed after lipopolysaccharide stimulation, and vitamin B_2 at 12.5 (7966.1 ± 587.2 pg/ml, $P < 0.05$) and 25 μg/ml (3769.5 ± 314.1 pg/ml, $P < 0.05$) reduced the interleukin-6 production. Lipopolysaccharide induced prominent elevation of lactic acid (58.0 ± 1.4 vs. 19.9 ± 1.6 mg/dl, $P < 0.05$) and evoked a significant reduction of glucose levels (25.4 ± 1.3 vs. 87.3 ± 6.1 mg/dl, $P < 0.05$) compared to the control. Vitamin B_2 at 25 μg/ml inhibited the elevated lactic acid production (31.0 ± 1.5 mg/dl, $P < 0.05$) and improved the excessive glucose consumption (64.1 ± 1.9 mg/dl, $P < 0.05$).

Table 3
Effect of amino acid and vitamin B_2 on lipopolysaccharide-induced mortality in mice

Treatment	Survival	
	No. of survivors/total	Rate (%)
Saline solution	0/10	0
Vitamin B_2 2.5 mg/kg	2/10	20
Vitamin B_2 2.5 mg/kg + Tryptophane 100 mg/kg	3/10	30
Vitamin B_2 2.5 mg/kg + Isoleucine 100 mg/kg	4/10	40
Vitamin B_2 2.5 mg/kg + Proline 100 mg/kg	4/10	40
Vitamin B_2 2.5 mg/kg + Threonine 100 mg/kg	4/10	40
Vitamin B_2 2.5 mg/kg + Valine 100 mg/kg	6/10	60*

* $P < 0.05$ vs. saline solution (Steel test).

Table 4
Effect of valine and vitamin B_2 on lipopolysaccharide-induced mortality in mice

Treatment	Survival	
	No. of survivors/total	Rate (%)
Saline solution	0/10	0
Vitamin B_2 2.5 mg/kg	3/10	30
Valine 100 mg/kg	0/10	0
Valine 200 mg/kg	2/10	20
Vitamin B_2 2.5 mg/kg + Valine 12.5 mg/kg	3/10	30
Vitamin B_2 2.5 mg/kg + Valine 25 mg/kg	3/10	30
Vitamin B_2 2.5 mg/kg + Valine 50 mg/kg	4/10	40
Vitamin B_2 2.5 mg/kg + Valine 100 mg/kg	6/10	60*

* $P < 0.05$ vs. saline solution (Steel test).

4. Discussion

Although aminolevane® solution showed no improvement in the lipopolysaccharide-induced mortality, the therapeutic effect of vitamin B_2 on endotoxin-induced shock was significantly enhanced by this amino acid supplement. Among the amino acid constituents of aminolevane® solu-

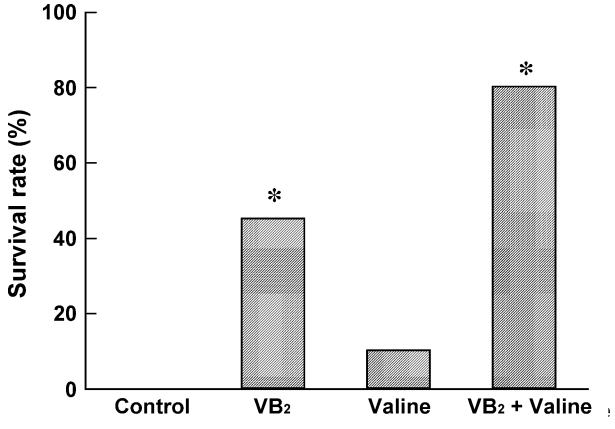

Fig. 2. Effect of i.v. infusion of vitamin B_2 and valine on lipopolysaccharide-induced shock in mice. * $P < 0.05$ vs. control (Steel test).

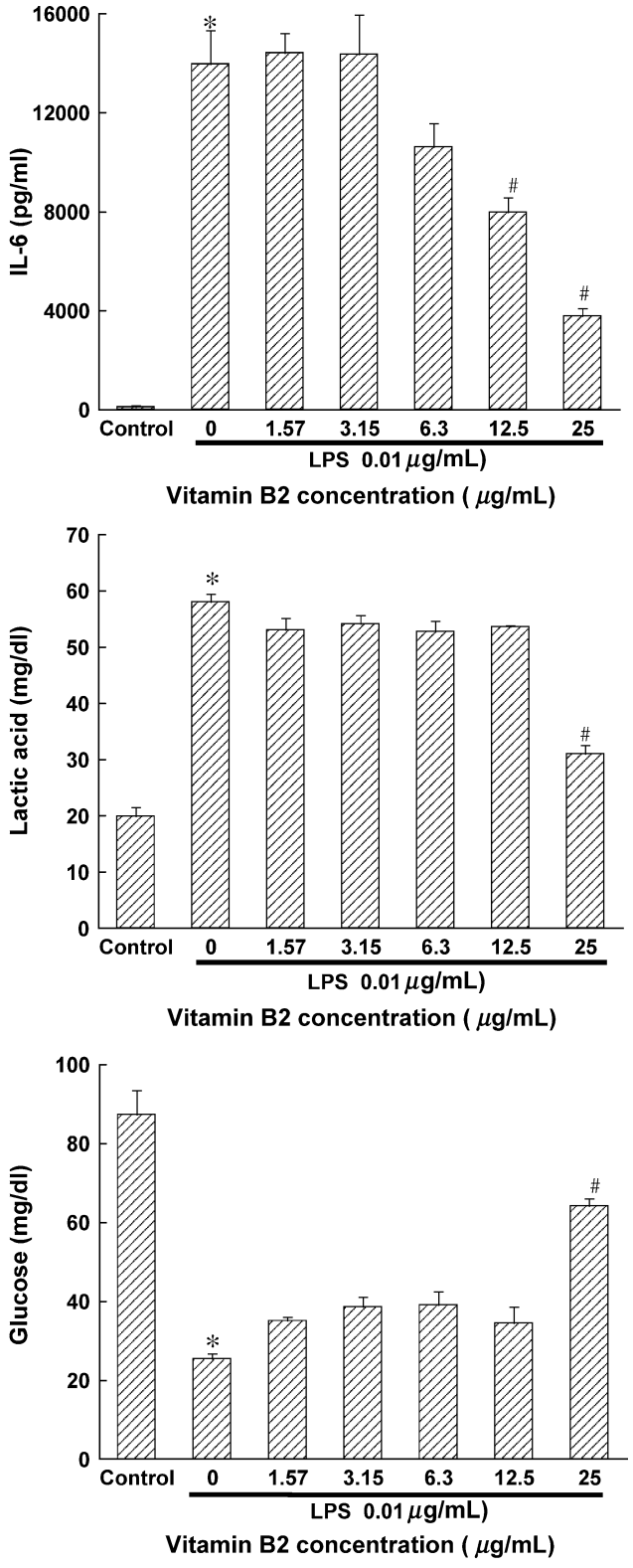

Fig. 3. Effects of vitamin B_2 on interleukin-6 and lactic acid production, and glucose consumption in mouse peritoneal macrophages stimulated by lipopolysaccharide. LPS: lipopolysaccharide, IL-6: interleukin-6. Each value is the mean ± S.E.M. ($n=3$). *$P<0.01$ vs. Control, $^{\#}P<0.01$ vs. lipopolysaccharide 0.01 μg/ml (Dunnett's multiple range test).

tion, valine was the most effective in combination therapy of vitamin B_2.

Vitamin B_2 diminished the elevations of interleukin-6 and lactic acid, and ameliorated the increased glucose consumption in lipopolysaccharide-stimulated mouse peritoneal macrophages. The rate of glucose utilization in peritoneal macrophages has been used as an index of lipopolysaccharide-induced activation (Ryan et al., 1979). We speculated that vitamin B_2 could play a role in normalizing the energy production of macrophages. Lactic acidosis was reported to increase TNF-α production by rat peritoneal macrophages (Jensen et al., 1990). Further, TNF-α-induced excessive interleukin-6 production is well-known to occur, so lactic acidosis might have induced the excessive production of interleukin-6 observed in our experiment. The survivors had significantly lower initial lactate levels than nonsurvivors, and were able to significantly decrease their lactate levels over the course of their treatment (Morgan and Machiedo, 2002).

Clinical research has indicated that branched amino acids (isoleucine, leucine, valine)-rich formulas improve protein balance (Echenique et al., 1984) and the mortality of patients with sepsis (Garcia-de-Lorenzo et al., 1997). Valine, like vitamin B_2, also inhibited interleukin-6 and TNF-α production by lipopolysaccharide-stimulated mouse peritoneal macrophages (data not shown). Since proinflammatory cytokines activate muscle proteolysis and promote organ dysfunction in sepsis (Chang and Bistrian, 1998), the combination therapy of vitamin B_2 with valine should reduce these changes and moderate the hypermetabolism. However, other groups have found that branched amino acids-rich solution failed to improve sepsis (Vente et al., 1991; von Meyenfeldt et al., 1990; Bower et al., 1986). An unbalanced amino acid solution may increase the risk of deterioration of sepsis, so the establishment of the optimal composition of amino acids for the combination therapy with vitamin B_2 is very important. Simultaneous administration of vitamin B_2 with human activated protein C also reduced the mortality of toxin-induced shock (Toyosawa et al., 2004). Therefore, combination therapy using vitamin B_2 with agents having other properties could be an effective approach to treat sepsis.

In severe human sepsis, the mitochondrial membrane potential, an index of the capacity for energy generation, is reduced (Adrie et al., 2001). Because mitochondria play a crucial role in energy-producing processes mediated by the respiratory chain, mitochondrial injury causes impaired respiratory chain function and leads to decreased production of ATP. We have demonstrated that vitamin B_2 immediately entered into cells and restored the decreased mitochondrial membrane potential of mouse neutrophils exposed to lipopolysaccharide by using flow cytometer, and blocked excessive cytokine production from macrophages exposed to inhibitors of mitochondrial electron transporter (data not shown). Accordingly, we expected that vitamin B_2 would alleviate the excessive macrophage activation induced by

lipopolysaccharide and improve mitochondrial dysfunction in sepsis, thereby leading to improved energy production. However, further investigations are necessary to clarify the mechanism(s) involved.

In conclusion, combination treatment with amino acids and vitamin B_2 appears to have considerable potential for the treatment of sepsis and septic shock.

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