A potential role of YC-1 on the inhibition of cytokine release in peripheral blood mononuclear leukocytes and endotoxemic mouse models

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Summary

To evaluate the anti-sepsis potential of YC-1, we have examined the effect of YC-1 on the regulation of cytokine production in human leukocytes and endotoxemic mice. The data demonstrated that YC-1 showed a preferential inhibition on proinflammatory cytokine production without inhibition of cell growth or induction of cytotoxicity in human leukocytes. On the other hand, in the septic mouse model, treatment with an intraperitoneal application of LPS caused a cumulative death within 27 hours. The post-treatment administration of YC-1 significantly increased the survival rate in endotoxemic mice. Furthermore, several mediators were detected and the data showed that YC-1 profoundly blocked LPS-induced NO as well as TNF-α production, and prevented lung damage by histological examination. Samples from the animal model showed that LPS-induced NF-κB/DNA binding activity and consequent up-regulation of iNOS expression in tissues were abolished by post-administration of YC-1. Furthermore, YC-1, by itself, did not modify cGMP content while significantly inhibit LPS-induced cGMP formation, suggesting that YC-1-mediated effect was not through a cGMP-elevating pathway. Taken together, it is evident that the post-treatment administration of YC-1 after LPS application significantly inhibits NF-κB activation, iNOS expression, NO over-production, and cytokine release reaction resulting in an improved survival rate in endotoxemic mice. It is suggested that YC-1 may be a potential agent for the therapeutic treatment of sepsis.

Keywords

YC-1, lipopolysaccharide, nuclear factor-κB

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Introduction

Septic shock is the most common cause of death in intensive care units despite recent progress in antibiotics and critical care therapy. Numerous patients throughout the world suffer from sepsis each year, and its incidence and mortality rate continue to increase (1). Multiple organ failure (MOF) accounts for most of the poor outcomes in sepsis (2). Recently, several action mechanisms have been focused on, and treatment strategies have been suggested for sepsis therapy, such as antioxidant, antiinflammation, and inhibition of massive nitric oxide (NO) synthesis (3–5). Clinical trials of these innovative therapies have been conducted; however, to date, there still have been no documented improvements in outcome (6). Therefore, other action targets and strategies are needed to improve therapeutic effectiveness.

Nitric oxide and guanosine 3':5'-cyclic monophosphate (cGMP) regulates several functions responding to stimuli, including the regulation of vascular tone and cell growth. The endothelium synthesizes picomolar amounts of NO under normal physiological conditions. However, after several pathological attacks, such as trauma and sepsis, the exaggerated synthesis of much larger amounts of NO occurs (7). Cells respond to bacterial products (such as lipopolysaccharide [LPS]) or inflammatory cytokines (such as interleukin-1β and tumor necrosis factor-α [TNF-α]) and up-regulate the inducible NO synthase (iNOS), which produces micromolar amounts of NO (8). There are nu-

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merous lines of evidence that the large quantities of NO are the important mediators of inflammation and contribute to the pathogenesis of vascular failure in sepsis (9). It is also evident that the cytotoxic effect is due to a combined action of oxygen- and nitrogen-derived free radicals and oxidants. The cytotoxic oxidant, peroxynitrite ($ONOO^-$), formed from the reaction of NO and superoxides, account for this oxidative injury (10). It is conceivable that the inhibition of cytotoxic oxidant generation and prevention of the organ and vascular failure induced by endotoxins may be a potential strategy in the treatment of septic shock and death, although antioxidant therapy has shown only modest benefit in preventing septic death in clinical trials (5).

Bacteria and immune responses are known to strongly trigger the release of cytokines, which initiate a cascade of events that lead to activation of the coagulation system, vasodilatation, increase of vascular permeability, cellular collapse, and intensive organ damage. In the search for anti-sepsis strategies, the immune basis of cytokine production and release should be taken into consideration. Several proinflammatory and antiinflammatory cytokines have been investigated with respect to their effects on MOF and mortality in patients with sepsis. There are numerous lines of evidence that up-regulated production of interleukins-1β, –6, –8, and TNF-α serve as proinflammatory mediators and exaggerate the progression of sepsis (11, 12). The major antiinflammatory factor is interleukin-10 (13). New therapies have been sought and examined to inhibit the biologic function of two major proinflammatory cytokines, interleukin-1β and TNF-α. Unfortunately, the anticytokine therapies did not show a statistically significant reduction in a 28-day mortality (14). Accordingly, it has been suggested that systemic inflammation of sepsis requires more than single anticytokine therapy to significantly improve the outcome.

NF-κB, a transcription factor that induces the expression of many genes for cytokine production, plays a central role in the modulation of the inflammatory and immune response. It is well evident that bacterial endotoxin is able to stimulate immuno-responsive cells to activate NF-κB, thereby increasing the production and release reaction of TNF-α and iNOS, and the subsequent production of other mediators (15). Therefore, NF-κB has been identified as a potential target for the development of new antiinflammatory strategy.

In our laboratory, we have defined the pharmacological characterization of YC-1 (3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazol) being a novel NO-independent type of soluble guanylyl cyclase (sGC) activator (16). It has been suggested that YC-1 and NO activate sGC in a synergistic manner. Furthermore, YC-1 may potentiate sGC activation mediated by exogenous as well as endogenous treatment of carbon monoxide (17). In this study, the effect of YC-1 on LPS-induced proinflammatory and antiinflammatory cytokine release in human peripheral blood mononuclear leukocytes has been examined. The animal survival following post-LPS administration of YC-1 in LPS-induced endotoxemic mice was monitored and the regulation of NF-κB activity, iNOS expression, NO production, and cytokines release were also examined in an *in vivo* model to evaluate the therapeutic potential of YC-1 in the treatment of sepsis.

Materials and methods

Examination of cytokine release in human peripheral blood mononuclear leukocytes

Approximately 450 ml of blood was obtained from healthy human volunteers. PBMC were prepared by centrifugation at 500 *g* for 20 min at 15°C from diluted blood 1:2 in RPMI 1640 culture medium over Ficoll gradient (MSL; Eurobio, les Ulis, France). Cell pellets were resuspended at a final concentration of 6 x 10^6 cells/ml. Aliquots of 500 µl of cell suspension were dispensed into each well of a 24-well plate and incubated at 37°C in the presence of YC-1 in a 5% CO_2 air incubator in a humidified atmosphere. 25 ng/ml LPS (smooth *E. coli* 0111:B4) (induces IL-1β, IL-5, IL-6, IL-8 and TNF-α cytokines production) or 20 µg/ml Concanavalin A (induces IL-2, IL-4, IL-10 and interferon-γ cytokines production) combine with YC-1 were incubated for 16 h. At the end of incubation, the supernatants were collected, centrifuged at 400 *g* for 10 min at 15°C and stored at –20°C until cytokine determination. All cytokines were assayed using the commercial kits provided by R & D Systems (Abingdon, UK).

Induction of endotoxic shock and histological examination

In these experiments, mice (25–30 g, ICR strain) were separated into three groups of ten mice in each group and injected intraperitoneally with LPS (60 mg/kg). The Animal Care Committee of the College of Medicine, National Taiwan University, conducted the study in accordance with the guideline for the care and use of laboratory animals. After a two- or six-hour reaction, vehicle, YC-1 (10 mg/kg) or ODQ (30 mg/kg), a sGC inhibitor, in 0.5% carboxymethyl cellulose was orally administered. The survival rate was monitored every three to six hours after LPS injection. Before histological examination, all mice were euthanatized with intraperitoneal administration of pentobarbital after a 48-h treatment with LPS. Tissues were fixed with 4% paraformaldehyde and embedded in paraffin. The embedded tissues were sectioned at 5-µm thick, stained with monoclonal antibody NF-κB p65 (Chemicon; formerly Roche 32N) and analyzed using a microscope. In a parallel experiment, blood samples (1 ml) were collected before animals died for determination of plasma NO, cGMP, and TNF-α concentrations.

Measurement of hemodynamic parameters

Male Wistar rats (250–300 g) were anesthetized with urethane (1.0 g/kg, i.p.). The trachea was incubated to keep the airway patent. Femoral arterial blood pressure was measured through PE50 tubing filled with heparin solution (25 units/ml) connected to a polygraph (Lectromed, Jersey Channel Islands, UK) via a transducer. The femoral vein was cannulated for drug administration. Heart rate was derived by means of a cardiotachometer that was triggered by the arterial pressure pulse. The mean arterial blood pressure (MBP) and heart rate were monitored.

Western blot analysis

All tissues were homogenized in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1%

Table 1: Effect of YC-1 on the cytokine production in human peripheral blood mononuclear leukocytes. Human peripheral blood mononuclear leukocytes were exposed to lipopolysaccharide (LPS, 25 ng/ml) or Concanavalin A (20 μg/ml) in the absence or presence of YC-1 for 16 h. Then, the cytokine production was detected using enzyme-linked immunosorbent assay (ELISA) assay as described in the Methods section.

Cytokines	Inducer	IC_{50} (μM)	Potency ratio (compared with IL-4)	Potency ratio (compared with IL-10)
IL-1 β	LPS	2.5	18.6	16.4
IL-2	Concanavalin A	24.2	1.9	1.7
IL-4	Concanavalin A	46.4	1.0	0.9
IL-5	Concanavalin A	>100		
IL-6	LPS	2.4	19.3	17.1
IL-8	LPS	2.4	19.3	17.1
IL-10	Concanavalin A	41.1	1.1	1.0
Interferon-γ	Concanavalin A	23.4	2.0	1.8
TNF-α	LPS	3.0	15.5	13.7

Triton X-100). The homogenate was centrifuged at 17,500 g for 30 min, and the supernatant was collected for the Western blot analysis. Protein (60 μg/lane) was separated on a 7.5% SDS-PAGE. The nitrocellulose membrane was immuno-reacted with the primary antibody to iNOS or α-tubulin (1:1000 dilution) (Santa Cruz Biotechnology) for overnight incubation at 4°C. After four washings with PBS/0.1% Tween 20, the secondary antibody (dilute 1:2000) was applied to the membranes for 1 h at room temperature. The antibody-reactive bands were performed with an enhanced chemiluminescence kit (Amersham, Bucks, UK).

Determination of NO

The amount of NO was determined from the accumulation of the stable NO metabolite nitrite by adding Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamide in 5% phosphotic acid) to plasma samples, and the absorbance was measured at 550 nm.

Assays for cGMP and cytokine

Plasma cGMP and cytokine concentrations were determined by commercial enzyme immuno-assay kits (Amersham).

Electrophoretic mobility shift assay

DNA binding activities of NF-κB were determined using electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared and applied to gel shift assay as described previously (18). Briefly, 2 μg of nuclear extracts were incubated with a 35-base pair double-stranded ^{32}P-labeled probe encoding the κB consensus sequence (5'-AGT TGA GGG GAT CCC CCC AGG C-3') in the binding buffer at room temperature for 30 min. Then, samples were applied to native 5% polyacrylamide gels and analyzed on autoradiography.

NF-κB p65 transcription factor assay

Analysis of p65 binding to its consensus oligonucleotide was performed in lung, spleen and kidney tissues using an ELISA-based Trans-AM™ NF-κB p65 kit (Active Motif Europe, Rixensart, Belgium).

Immunohistochemistry assay

NF-κB was also characterized by immunohistochemistry on paraffin sections. In brief, 5-μm paraffin sections were deparpaffinized and endogenous peroxidase was quenched with 0.3% H_2O_2 in 100% methanol. Antigen was unmasked by heating the sections for 15 min in 10 mM citrate buffer pH6.0 using a microwave, followed by 60 min cooling in the same buffer. Tissues were incubated with a monoclonal antibody that recognizes the active form of NF-κB p65 (Chemicon; formerly Roche 32N) for 45 min at room temperature. A standard LSAB Technique (Dako, Glostrup, Denmark) was used to detect the reaction products. Sections were counterstained with hematoxylin and mounted.

Statistics

Data are presented as the means plus or minus SEM for the indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance (ANOVA) and Bonferroni method followed by a *t*-test and *P*-values less than 0.05 were considered significant.

Results

Effect of YC-1 on the regulation of cytokine release in human peripheral blood mononuclear leukocytes

To investigate the effect of YC-1 on the regulation of proinflammatory and antiinflammatory cytokine release, several cytokines were detected in human peripheral blood mononuclear leukocytes. As demonstrated in Table 1, YC-1 exhibited different inhibitory effects on LPS (25 ng/mL)-induced and Concanavalin A (20 μg/mL)-induced cytokine release. It is worth noting that YC-1 showed a preferential inhibition (15.5 to 19.3 fold) on proinflammatory cytokine release, such as TNF-α and interleukins 1β, 6 and 8, than those on antiinflammatory cytokines (interleukins 4 and 10). However, YC-1 showed weak potency on the inhibition of interleukin-5 release (Table 1).

In a parallel experiment, we used alamarBlue fluorometric assays to determine the activity of mitochondrial enzymes, which reduce alamarBlue to a fluorescent form, in human peripheral blood mononuclear leukocytes. This assay method can quantitatively detect the cell proliferation as well as cytotoxic effect in cells. However, neither LPS nor Concanavalin A induced a significant effect on the mitochondrial reduction activity in these cells, indicating that YC-1-mediated inhibition on cytokine production was not through the influence on cell growth or cytotoxic effect (data not shown).

Effect of YC-1 on LPS-induced animal death

To investigate the therapeutic potential of YC-1 in endotoxemic animals, an LPS-induced endotoxemic death in the mouse model was used. As showed in Figure 1, the intraperitoneal application of LPS (60 mg/kg) caused a cumulative animal death within 27 hours. However, oral administration of YC-1 at both 2 and 6 hours post-LPS injection significantly increased the survival rate of endotoxemic mice (Fig. 1A). The data demonstrated that ani-

mal survival was better with the administration of YC-1 6 hours after LPS injection than that of the administration of YC-1 at 2 hours. These survival mice remained vigorous more than 1 month after the initiation of LPS. We also demonstrated that ODQ, a sGC inhibitor, did not reverse YC-1-mediated animal survival rate (Fig. 1A). For histological examination, the lung, liver and kidney tissues were fixed, embedded and stained. Massive leakages of blood and inflammatory cells in the lung, liver and kidney tissues were observed in the LPS group (Fig. 1B). Furthermore, in our present study, YC-1 only induced a small and transient decrease in mean arterial blood pressure (MAP) in both normal and endotoxemic rat models (Table 2).

Examination of LPS-induced NO and TNF-α production

It is well evident that large quantities of NO and TNF-α production contribute to the pathogenesis of vascular failure and severe inflammation progression in sepsis. In response to intraperitoneal LPS administration, a marked elevation of plasma TNF-α and NO levels was detected in a time-dependent manner (Fig. 2). YC-1 alone had little influence on NO and TNF-α productions, while post-treatment administration of YC-1 after LPS injection for 6 hours significantly inhibited the production of these 2 mediators induced by LPS (Fig. 2).

Effect of YC-1 on LPS-induced iNOS expression

To examine the effect of YC-1 on iNOS protein expression, several tissues were collected in each group for Western blot analysis. As demonstrated in Figure 3, LPS (60 mg/kg) application more than 12 hours induced a profound increase of iNOS protein expression in spleen (Fig. 3A), kidney (Fig. 3B), and lung tissues (Fig. 3C). YC-1 (10 mg/kg) alone had little influence on iNOS expression (lanes 7 and 8 in Fig. 3) but significantly inhibited the LPS-induced effect when administered after LPS application for 6 hours, suggesting that YC-1 regulates upstream signals of the iNOS expression pathway.

Effect of YC-1 on NF-κB DNA binding activity

After the analysis of the above data, we further examined the effect of YC-1 on the regulation of NF-κB activation in the *in vivo* mouse model. We detected the DNA binding activities of NF-κB using electrophoretic mobility shift assay in nuclear extracts of lung tissues. As shown in Figure 4, LPS caused a significant increase in the level of NF-κB/DNA complex after 6 hours of treatment with YC-1, returning to baseline thereafter. In addition, a densitometric evaluation of binding data was showed in Figure 4B, group 3 and 4 still increase NF-κB activities 3-fold and 2-fold than control group, separately. And YC-1 attenuated NF-κB activity to basal level was also observed in group 5 and 6. Furthermore, in Figure 4C, we also observed the post-administration of YC-1 (2 h after LPS induced) was able to significantly abolish NF-κB activation. It demonstrated that YC-1 completely abolished the LPS-induced effect.

We then examined and quantified the effect of YC-1 on LPS-mediated NF-κB binding to DNA in the lung, spleen, and kidney tissues. In this assay, an oligonucleotide containing the NF-κB consensus site was attached to plate and the p65 binding to κB oligonucleotide in tissue samples was performed and detected.

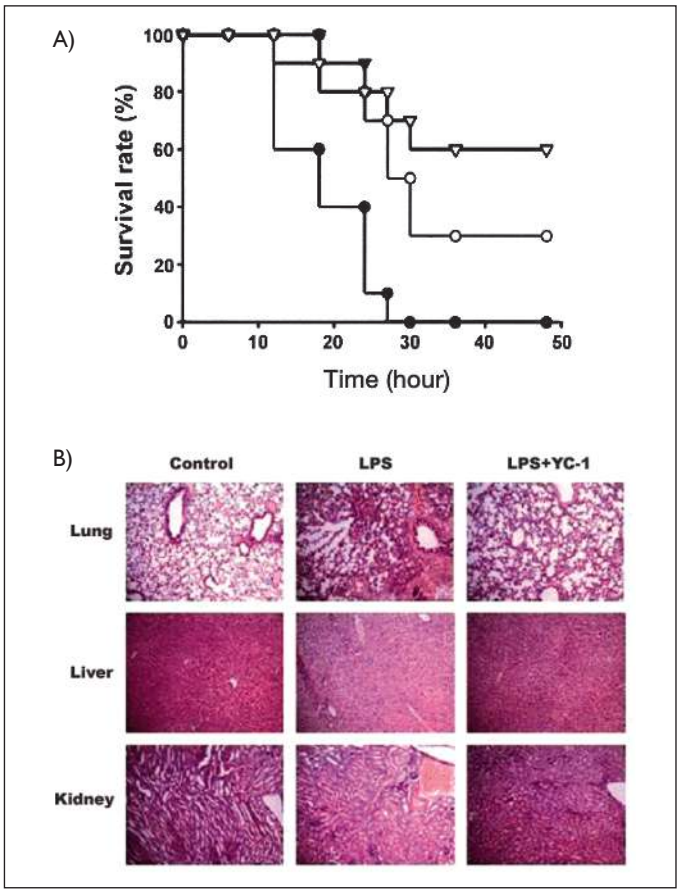

Figure 1: Effect of YC-1 on LPS-induced animal death. (A) Mice (ICR strain) were injected intraperitoneally with LPS (60 mg/kg), and vehicle (○) (0.5% carboxymethyl cellulose, p.o.) or YC-1 (10 mg/kg, p.o.) was post-administered after two (Δ) or six (❑) hours of LPS injection. On the other hand, YC-1 combined with ODQ (30 mg/kg, p.o.) (▽) were post-administered after six hours of LPS injection. The survival rate was monitored every three to six hours. Each group had 10 mice. (B) Histological examination of lung, liver and kidney tissues in mice. Mice (ICR strain) were injected intraperitoneally with vehicle (control) or 60 mg/kg of LPS. After 6 h treatment of LPS, vehicle or YC-1 (10 mg/kg) was orally post-administered in mice. For histological examination, the lung, liver and kidney tissues were fixed, embedded and stained. Massive leakages of blood and inflammatory cells in the lung, liver and kidney tissues were observed in the LPS group. (magnification ×Ñ 100).

Table 2: Effect of YC-1 on mean arterial blood pressure (MAP) in normal and endotoxemic rats. MAP changes in comparison to baseline values (0 min) are presented as ΔMAP (mmHg). Each value represents the mean ± S.E. mean of five experiments. * $P<0.05$ versus LPS-treated group.

Time/ΔMAP/Group	Control	LPS	LPS+YC-1
5 mins	0	–5.2 ± 0.7	---
10 mins	0	–12.5 ± 1.3	---
15 mins	0	–18.7 ± 1.7	---
1 h	0	–30.0 ± 2.2	---
2 h	0	–35.3 ± 2.5	---
6 h	0	–45.4 ± 1.8	–50.1 ± 1.2
12 h	0	–55.7 ± 3.7	–8.2 ± 1.1*

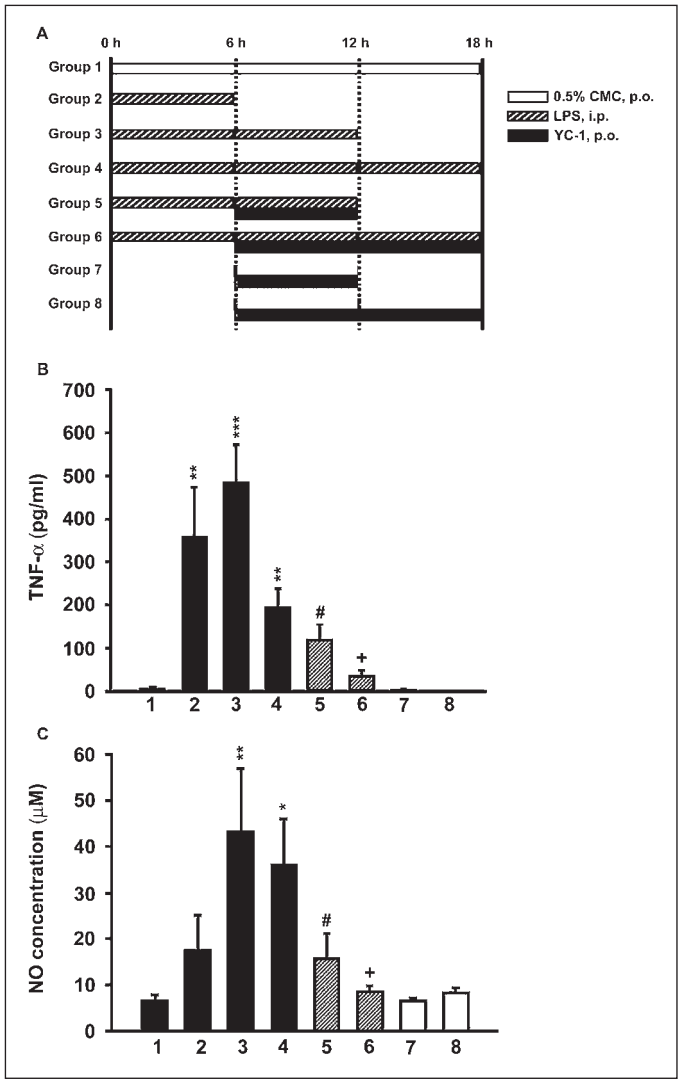

Figure 2: Effects of YC-1 on LPS-induced TNF-α and NO production. Mice were separated into several groups. Each mouse was injected intraperitoneally with LPS (60 mg/kg) except for the first group. After a six-hour exposure to LPS, 0.5% carboxymethyl cellulose (CMC) or YC-1 (10 mg/kg) in 0.5% CMC was orally administered as the indicated time schedule (A). Before blood collection in each group, mice were euthanatized with intraperitoneal administration of pentobarbital and blood samples were collected before animals died for determination of TNF-α (B) and NO (C) as described in the Methods section. Data are expressed as means plus or minus SEM of six determinations in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.01$ versus group 1; $^{\#}P < 0.05$ versus group 3; $^{?}P < 0.05$ versus group 4.

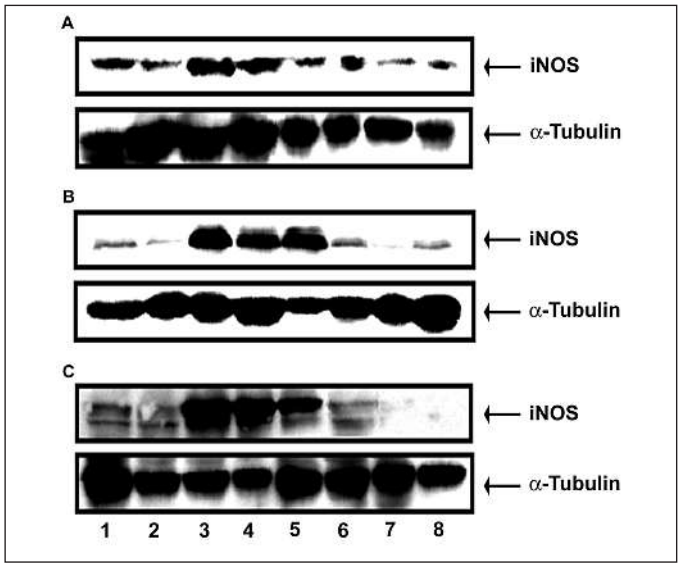

Figure 3: Effect of YC-1 on LPS-induced iNOS protein expression in mice. Mice were separated into several groups. Each mouse was injected intraperitoneally with LPS (60 mg/kg) except for the first group. After a six-hour exposure to LPS, YC-1 (10 mg/kg) in 0.5% carboxymethyl cellulose (CMC) was orally administered as the indicated time schedule in Figure 2A. Mice were euthanatized with intraperitoneal administration of pentobarbital and spleen (A), kidney (B), and lung tissues (C) were separated for the detection of iNOS protein expression by Western blotting as described in the Methods section.

The data showed that the p65 binding to κB sites was maximally increased after 6 hours of LPS administration, and then down-regulated after that (Fig. 5). Furthermore, LPS induced the most increase in p65 binding activity in the lungs compared with the other two tissues. Again, administration of YC-1 following LPS treatment completely inhibited the LPS-induced effect (Fig. 5).

Effect of YC-1 on LPS-induced NF-κB expression in lung tissues

To determine whether expressed NF-κB (RelA/p65) is present in the biopsies of pigmented lesions at various stages of LPS treatment, immunohistochemical staining of paraffin-embedded lung specimens was performed using a RelA/p65 monoclonal antibody. As shown in Figure 6, the active RelA/p65 was displayed by LPS treatment for 6, 12 and 18 hours (Figs. 6B, 6C, and 6D, respectively). The administration of YC-1 following LPS treatment significantly reduced the expression of active RelA/p65 in mice treated with LPS (Figs. 6E and 6F). However, there was little active RelA/p65 being detected in animals treated with YC-1 alone (Figs. 6G and 6H).

Effect of YC-1 on LPS-induced cGMP formation

We examined the effect of YC-1 on LPS-induced plasma cGMP formation in an *in vivo* mouse model. The data demonstrated that the injection of LPS for 12 to 18 hours induced a profound increase in plasma cGMP formation (Fig. 7A). The administration of YC-1 (10 mg/kg) following LPS treatment significantly inhibited LPS-induced effect (Fig. 7A). We also demonstrated that ODQ did not suppress the YC-1-mediated cGMP formation in *in vivo* assay (Fig. 7B). The data are consistent with those on NO formation demonstrated in Figure 2C. In this study, YC-1, by itself, did not modify cGMP content while significantly inhibit LPS-induced cGMP formation, suggesting that YC-1-mediated effect was not through a cGMP-elevating pathway.

Discussion

Cytokines are endogenous mediators that play an important role in coordinating the inflammatory signaling in the human body (19). The generation of proinflammatory (TNF-α, interferon-β,

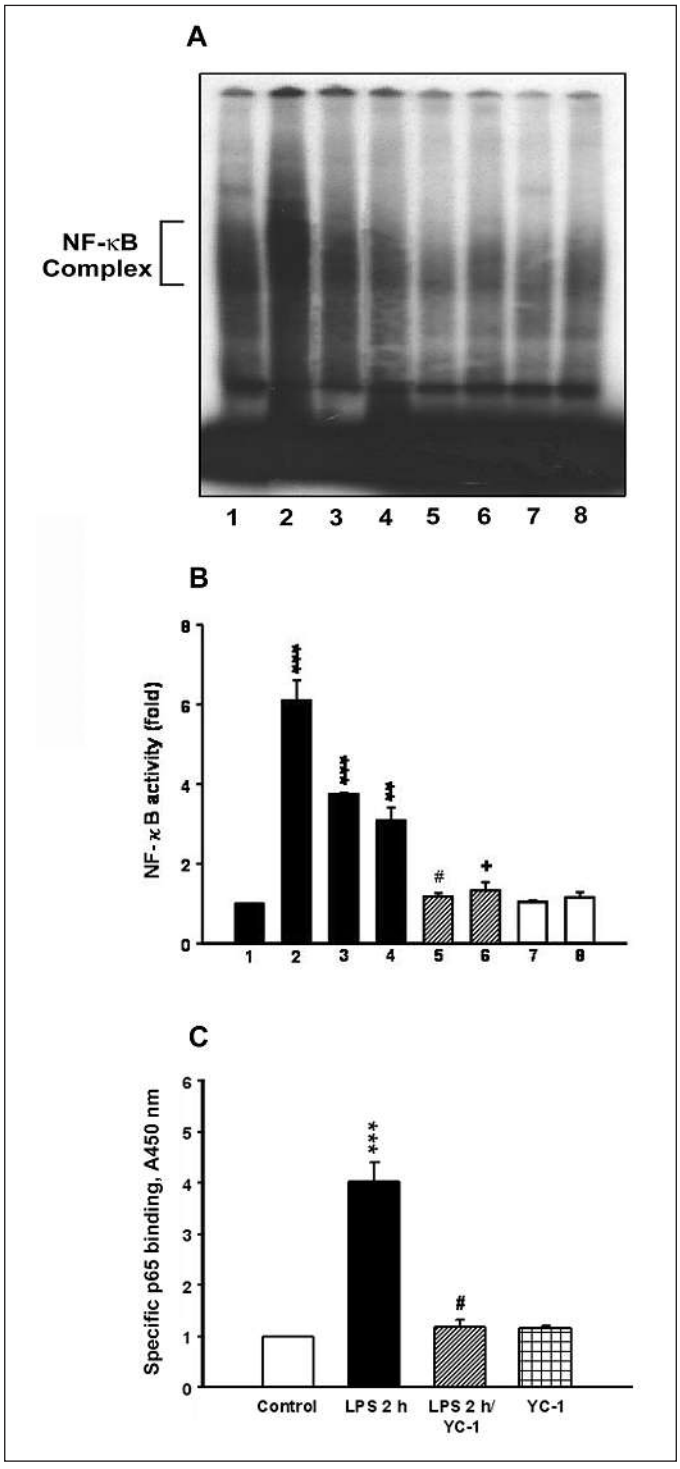

Figure 4: Effect of YC-1 on LPS-induced nuclear translocation of NF-κB in lung tissues. (A) Mice were separated into several groups. Each mouse was injected intraperitoneally with LPS (60 mg/kg) except for the first group. After a six-hour exposure to LPS, YC-1 (10 mg/kg) in 0.5% CMC was orally administered as the indicated time schedule in Figure 2A. Mice were euthanized with intraperitoneal administration of pentobarbital and lung tissues were separated for the detection of NF-κB nuclear translocation by electrophoretic mobility shift assay as described in the Methods section. (B) Quantitative data are represented the mean±SEM from three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus group 1; $^{\#}P < 0.05$ versus group 3; $^{\dagger}P < 0.05$ versus group 4. (C) Effect of YC-1 on LPS-induced nuclear translocation of NF-κB in lung tissues. Mice were separated into several groups. Each mouse was injected intraperitoneally with LPS (60 mg/kg) except for the first group. YC-1 (10 mg/kg) in 0.5% CMC was orally administered after LPS treated 2 hr in mice. Mice were euthanized with intraperitoneal administration of pentobarbital and tissues were separated for the detection of NF-κB binding to κB oligonucleotide by means of the Trans-AM™ p65 transcription factor assay kit. Data are expressed as means plus or minus SEM of ten determinations in each group. $^{***}P < 0.001$ versus control group; $^{\#}P < 0.05$ versus LPS-treated group.

and interleukins 1β, 2, and 8) and antiinflammatory (interleukins 4 and 10) cytokines is strictly regulated by complicated mechanisms in several diseases (20–22). The antiinflammatory cytokines are generated for the cessation or attenuation of inflammatory progression to retain the function of vital organs (20, 21, 23). These studies provide evidence that the target on cytokine production, either the inhibition on proinflammatory cytokines or the potentiation on antiinflammatory cytokines, might be a potential strategy for the prevention of MOF and septic death. In this study, LPS and Concanavalin A were used to stimulate the production and release of cytokines in human peripheral blood mononuclear leukocytes. The data demonstrated that YC-1 showed a more potent inhibition on the proinflammatory cytokines than on the antiinflammatory cytokines. Furthermore, it has been reported that a good correlation exists between high proinflammatory cytokine/antiinflammatory cytokine ratio and poor outcomes in patients with systemic inflammatory response such as sepsis (23, 24). In this study, YC-1 showed a 15.5-fold to 19.3-fold preferential inhibition on proinflammatory cytokine release. It is suggested that YC-1 may play a beneficial role on the prevention of inflammatory progress.

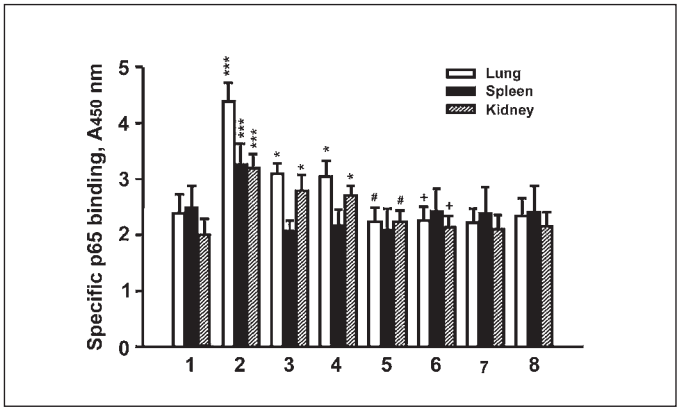

Figure 5: Effect of YC-1 on LPS-mediated DNA binding activity of NF-κB in several tissues. Analysis of p65 binding to its consensus oligonucleotide was performed in lung, spleen and kidney tissues. Mice were separated into several groups. Each mouse was injected intraperitoneally with LPS (60 mg/kg) except for the first group. After a six-hour exposure to LPS, YC-1 (10 mg/kg) in 0.5% CMC was orally administered as the indicated time schedule in Figure 2A. Mice were euthanized with intraperitoneal administration of pentobarbital and tissues were separated for the detection of NF-κB binding to κB oligonucleotide by means of the Trans-AM™ p65 transcription factor assay kit as described in the Methods section. Data are expressed as means plus or minus SEM of ten determinations in each group. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus group 1; $^{\#}P < 0.05$ versus group 3; $^{\dagger}P < 0.05$ versus group 4.

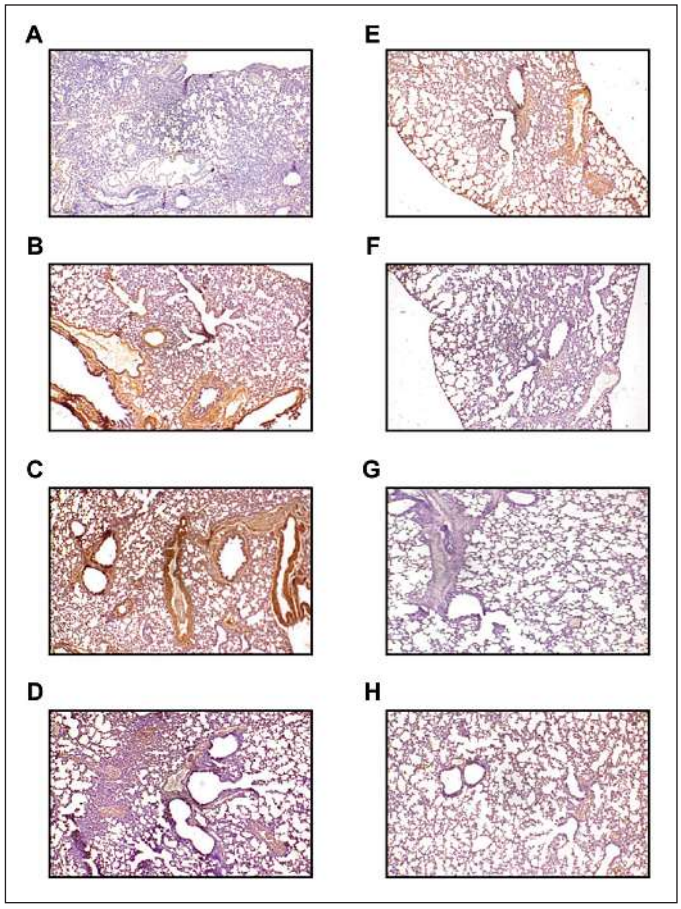

Figure 6: Effect of YC-1 on LPS-induced p65/NF-κB expression in lung tissues. Mice were separated into several groups. Each mouse was injected intraperitoneally with LPS (60 mg/kg) except for the first group. After a six-hour exposure to LPS, YC-1 (10 mg/kg) in 0.5% CMC was orally administered as the indicated time schedule in Figure 2A. Mice were euthanatized with intraperitoneal administration of pentobarbital and lung tissues were separated for the detection of p65/NF-κB expression as described in the Methods section. Figures a to h are representative for groups 1 to 8, respectively. Brown color is positive staining of p65/NF-κB expression (magnification ×40).

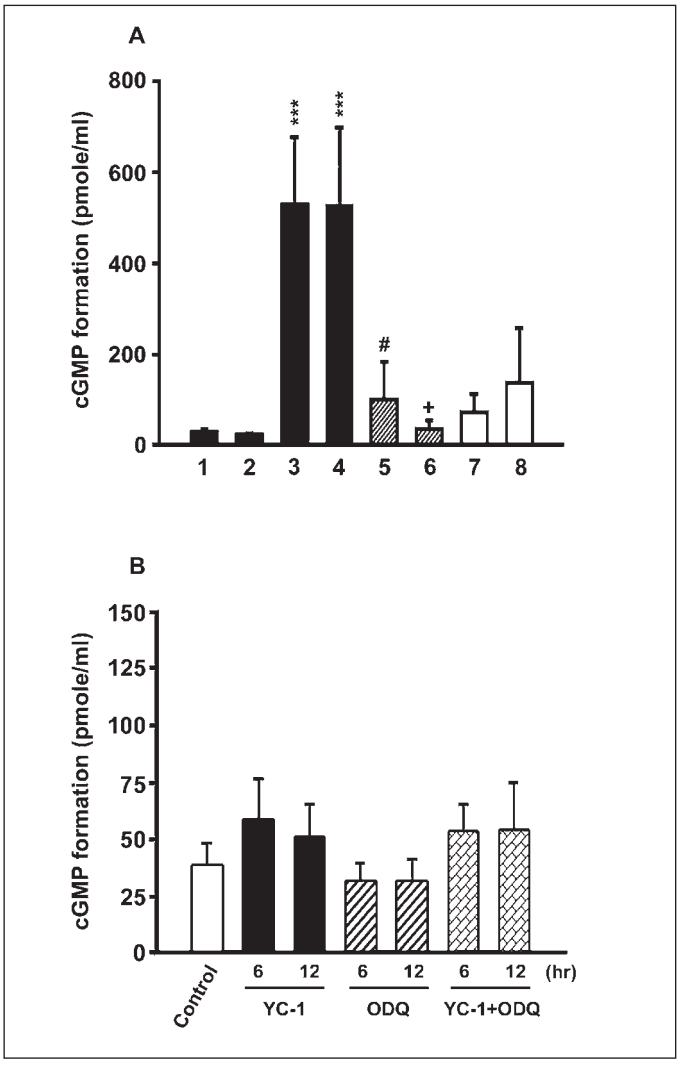

Figure 7: Effect of YC-1 on LPS-induced cGMP formation in mice. Mice were separated into several groups. (A) Each mouse was injected intraperitoneally with LPS (60 mg/kg) except for the first group. After a six-hour exposure to LPS, YC-1 (10 mg/kg) in 0.5% CMC was orally administered as the indicated time schedule in Figure 2A. Data are expressed as means plus or minus SEM of six determinations in each group. ***$P < 0.01$ versus group 1; #$P < 0.05$ versus group 3; †$P < 0.05$ versus group 4. (B) Each mouse was orally administrated with 0.5% CMC (control group), YC-1 (10 mg/kg), and ODQ (30 mg/kg), respectively. YC-1 and ODQ in 0.5% CMC was orally administered as the indicated time. Before blood collection in each group, mice were euthanatized with intraperitoneal administration of pentobarbital and blood samples were collected before animals died for determination of cGMP formation as described in the Methods section. Data are expressed as means plus or minus SEM of ten determinations in each group. ***$P < 0.001$ versus control group; #$P < 0.05$ versus LPS-treated group.

The endotoxin LPS is a major component of the outer membranes of Gram-negative bacteria. It has been shown that patients with Gram-negative septicemia elicit more proinflammatory cytokine production and have more illness than those with Gram-positive septicemia (23). To determine the therapeutic potential of YC-1 in sepsis, the LPS-induced endotoxemic death in the mouse model was used. The data showed that the post-administration of YC-1 after LPS application significantly increased the survival rate of endotoxemic mice. Furthermore, the survival rate was better by post-administration of YC-1 at 6 hours than at 2 hours. It might be explained by the pharmacokinetic property since orally applied YC-1 had a rapid onset (less than 10 minutes) but short half-life (about one hour). It was evident in this study that all of the LPS-mediated events, such as NF-κB activation, iNOS expression, as well as NO over-production, and cytokine release reaction, occurred maximally after 6 to 12 hours of LPS application in mice. However, the 6th hour post-administration of YC-1 after LPS injection could optimize YC-1 action.

Advances in the realization of complicated biological processes responsible for the clinical syndrome of sepsis have led to the development of numerous therapeutic targets, such as endotoxin, inflammatory cytokines, bioactive lipid mediators, NO, and downstream coagulation response (25). A lot of studies directed against these targets have demonstrated marked effect in septic animal models; however, in humans, the outcomes have been only modest (25). One of the most convincing explanations

is that the pathogenesis of sepsis is multifactorial and it requires more than single therapy against the mentioned targets. The most prominent effect of NO is the activation of guanylate cyclase and the following cGMP production, which results in the vasodilatation that is characteristic of septic shock. In this study, the NO production and cGMP synthesis occurred maximally after 12 hours of LPS application in endotoxemic mouse models. The post-administration of YC-1 significantly inhibited LPS-induced effects. In a further investigation, YC-1 dramatically inhibited LPS-induced iNOS expression in spleen, kidney, and lung tissues in mice, indicating that YC-1 is effective on this NO target. However, it is worth noting that YC-1 is a sGC activator and may activate sGC in a synergistic manner in combination with NO donors (16). One hypothesis might be that YC-1 deteriorates the hypotensive effect during septic shock. In this study, YC-1 alone induced a moderate but insignificant increase of cGMP synthesis and ODQ, a sGC inhibitor, did not reverse YC-1-mediated animal survival rate and nor suppress the YC-1-mediated cGMP formation in *in vivo* assay, whereas profoundly inhibited LPS-mediated cGMP production suggesting that YC-1 induced the effect in this study was not through a cGMP-elevating mechanism. Furthermore, in our study, YC-1 only induced a small and transient decrease in mean arterial blood pressure (MAP) in both normal and endotoxemic rat models. These data are similar to those reported by Rothermund and colleagues that YC-1 causes a short-term (less than 10 min) decrease of MAP in both the hypertensive and normotensive rats (26). This may partly exclude the possibility of YC-1 on the deterioration of septic shock.

According to the diverse effects of YC-1 on the inhibition of both iNOS expression and cytokine production, a common upstream signaling of transcription was examined. There are many lines of evidence suggesting that the engagement of endotoxin on immune-related cells induces numerous signaling cascades that cause new gene expressions through NF-κB activation. iNOS and TNF-α are two of the prominent NF-κB-mediated cellular products that are responsible for vascular failure and MOF in septic shock (15, 22, 23, 25). We further examined the effect of YC-1 on the regulation of NF-κB activation in the *in vivo* mouse models. The data showed that YC-1 completely abolished LPS-induced p65 binding to κB oligonucleotide in the lung, spleen, and kidney tissues, although LPS induced the most increase in p65 binding activity in lung tissues in these endotoxemic mice. For further convincing determination, an immunohistochemical staining of paraffin-embedded lung specimens was performed using a RelA/p65 monoclonal antibody. The data also demonstrated that the post-administration of YC-1 after LPS treatment significantly reduced the expression of active RelA/p65 in LPS-treated mice. It is suggested that YC-1 inhibits iNOS expression and proinflammatory cytokine production through the suppression on NF-κB activation and protein expression.

One potential treatment strategy in septic shock has been focused on coagulation target (27–29). In our previous studies, YC-1 has been examined to show an antithrombotic effect in several *in vivo* models and a decrease in mortality in fatal pulmonary thromboembolism model (30). Furthermore, in the study by Cauwels (31), the pretreatment with methylene blue can efficiently prevent TNF-induced cGMP accumulation, bradycardia, hypotension, and mortality. Our data are not in conflict with their study since YC-1 significantly inhibits LPS-induced cGMP formation, although this effect is not yet identified to be attributed to the potential benefit of YC-1 or not. However, we could not rule out any other potential mechanism of YC-1 since it has been suggested that YC-1 inhibits fMLP-induced superoxide anion O_2^- generation in rat neutrophils (32) and inhibits thrombotic effect in mice (30). These biological activities of YC-1 can also contribute to the potential benefit in the treatment of sepsis.

In summary, the data demonstrates that YC-1 preferentially blocks the proinflammatory cytokine release in human peripheral blood mononuclear leukocytes. The administration of YC-1 following LPS treatment significantly inhibits NF-κB activation, iNOS expression, NO over-production, and cytokine release reaction in an *in vivo* model, and furthermore, increases the survival of endotoxemic mice. In combination with its antithrombotic and profibrinolytic properties, we suggest that YC-1 may be a potential small molecule agent for the therapeutic treatment of sepsis.

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