Platonin, a Photosensitizing Dye, Improves Circulatory Failure and Mortality in Rat Models of Endotoxemia

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Platonin, a cyanine photosensitizing dye, is a potent macrophage-activating agent and an immunomodulator. In this study, we compare the inhibitory effects of platonin with those of the three clinical drugs minocycline, clindamycin, and cyclosporin, on hypotension, tachycardia, and nitric oxide (NO) formation in a rat model of circulatory shock induced by Escherichia coli lipopolysaccharide (LPS). We also evaluate the effect of drugs on the 6 h survival rate in LPS-treated rats. Administration of LPS (15 mg/kg) caused a rapid drop in mean arterial blood pressure (MAP). Minocycline (10 mg/kg, i.v.) significantly prevented the fall of MAP at 3 h, and platonin (100 µg/kg, i.v.) markedly prevented the fall of MAP within the 0-3 h period after LPS administration. However, neither clindamycin (10 mg/kg, i.v.) nor cyclosporin (15 mg/kg, i.v.) had any effects in this study. On the other hand, an inducible NO synthase inhibitor, N^G-nitro-L-arginine ester (L-NAME), caused a significantly increase in MAP and a moderate bradycardia after LPS administration. In addition, an increase in plasma nitrate formation elicited by endotoxemia was significantly reduced by pretreatment with either minocycline (10 mg/kg) or platonin (100 μ g/kg). However, only platonin (100 μ g/kg) markedly reduced the mortality and prolonged the mean survival time in LPS-treated rats. Minocycline, clindamycin, and cyclosporin had no effects under the same conditions. Further studies using an electron spin resonance (ESR) method were conducted on the scavenging activity of platonin on the free radicals formed. Platonin (10 μ M) greatly reduced the ESR signal intensity of superoxide anion, hydroxyl radical, and methyl radical formation. In conclusion, platonin has beneficial effects on ameliorating endotoxaemia. This protective effect of platonin may be mediated, at least partly, by the reduced drop in MAP and the inhibition of NO and free radical formation in rat models of endotoxemia.

Key words platonin; cyanine photosensitizing dye; sepsis; nitric oxide; free radical

Endotoxins are high-molecular-weight complexes of lipopolysaccharide (LPS) that are major components of the outer membranes of cell walls of gram-negative bacteria, which are shed from bacteria when cell lysis occurs and to a lesser extent during active growth.¹⁾ The most severe septic microvascular inflammatory responses, however, are caused by gramnegative bacteriemia, and these responses can be produced by injection of endotoxin. In addition, clinical and experimental endotoxemia is often associated with intravascular coagulation, such as renal cortical necrosis.²⁾ Escherichia coli LPS has also been shown to cause the clinical syndrome known as septic shock.¹⁾ Endothelial injury, activation of the coagulation cascade, and platelet aggregation have all been shown to contribute to vascular fibrin deposition.¹⁻³ Many substances, including cytokines, prostaglandins, vasoactive substances, and procoagulant factors, have been shown to be stimulated by LPS in vitro and in vivo and may contribute to the clinical effects of LPS.¹⁾

It has been shown that LPS induces the release of cytokines such as interleukin-1, tumor necrosis factor- α (TNF α), and γ -interferon, which contribute to the hypotension and lethality associated with endotoxemia. In addition, the enhanced formation of NO in response to LPS is also associated with the development of hypotension, peripheral vasodilation, and vascular hyporeactivity to vasoconstrictor agents in endotoxic shock.^{4,5)}

Platonin, a cyanine photosensitizing dye, is a potent macrophage-activating $agent^{6,7)}$ and an immunomodulator.⁸⁾ Nakagawa *et al.*⁶⁾ reported that when small amounts of platonin (20–40 ng/mouse) were administered for 14 d, peri-

toneal macrophages exhibited greatly enhanced Fc-receptormediated phagocytic capacities. Furthermore, it is also used as an effective medicine for rheumatoid arthritis.^{8,9)} Kondo et al.⁸⁾ reported that platonin inhibited the immunoglobin production of lymphocytes stimulated by Staphylococcus aureus Cowan I (SAC) or pokeweed mitogen (PWM), and increased the percentage of CD 8+ T cells. They concluded that platonin suppresses Ig production through inhibiting B cells and enhancing CD 8+ T cell formation.⁹⁾ However, the effect of platonin on LPS-induced endotoxemia in vivo has not been evaluated. On the other hand, cyclosporin acts as an immunosuppressant whose major effect on T-helper cell activation is the inhibition of cytokine production, such as interleukin-2, tumor necrosis factor- α , and interferon- γ .¹⁰⁾ In addition, minocycline,¹¹⁾ an antibiotic, significantly inhibited both LPS- and interferon- γ stimulated NO formation in RAW 264.7 cells *in vitro*.^{11,12} Clindamycin is a useful antibiotic for septicemia.¹³⁾ Therefore, we compared the inhibitory effects of platonin with three clinically used agents including minocycline, clindamycin, and cyclosporin in rats treated with endotoxin, and utilized the findings to characterize the mechanisms involved in this influence.

MATERIALS AND METHODS

Materials Lipopolysaccharide (*E. coli* serotype 0127: B8), minocycline, clindamycin, cyclosporin, sodium nitrate, chloral hydrate, urethane, DMSO, DMPO (5,5-dimethyl-1-pyrroline *N*-oxide), L-NAME (N^G-nitro-L-arginine ester), and hydrogen peroxide were purchased from Sigma Chem. Co.

(St. Louis, MO, U.S.A.). Vanadium (III) chloride (VCl₃) was obtained from Aldrich (Milwaukee, WI, U.S.A.). Platonin, 4,4',4"-thrimethyl-3,3',3"-triheptyl-7-(2"-thiazolyl)-2,2'-trimethinethiazolocyanine-3-3"-diiodide was synthesized by Kankohsha Co. (Osaka, Japan), and was obtained from GC Pharma (Tainan, Taiwan). All of minocycline, clindamycin, cyclosporin, and platonin were disolved in normal saline, then stored at 4 °C until use.

In Vivo Experiments Ten-week-old male Wistar-Kyoto (WKY) rats weighing 250-300 g, whose stock originated from the Charles River Breeding Laboratories in Japan, were purchased from the Department of Laboratory Animal Science of the National Defense Medical Center, caged individually in clear plastic cages, and kept in an environmentally controlled room maintained at 24 ± 1 °C, a relative humidity of 55%, and a light-dark cycle of 12 h/12 h. Rats were anesthetized by an intraperitoneal injection of chloral hydrate (0.4 g/kg) with urethane (0.6 g/kg). The trachea was cannulated to facilitate respiration and a thermometer was placed into the rectum to record the rectal temperature (about 37.5 °C) of the rats. The right femoral artery was cannulated with PE-50 tubing and connected to a pressure transducer (P23ID, Statham, Oxnard, CA, U.S.A.) for measurement of mean arterial blood pressure (MAP) and heart rate (HR), which were displayed on a Gould model RS 3400 polygraph recorder (Gould, Valley View, OH, U.S.A.). The left femoral vein was cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 10 min. After recording baseline hemodynamic parameters, rats were given normal saline followed by the addition of E. coli LPS (15 mg/kg, i.v., at time 0), they were then monitored for 3 h. Prior to (*i.e.*, at time 0) and at every hour after LPS administration, 0.3 ml of blood mixed with 3.8% sodium citrate was taken to measure changes in plasma levels of nitrate. Any blood withdrawn was immediately replaced by an injection of an equal amount of normal saline (i.v.). In other experiments, minocycline (10 mg/kg, i.v.), clindamycin (10 mg/kg, i.v.), cyclosporin (15 mg/kg, i.v.), platonin $(100 \mu \text{g/kg}, \text{ i.v.})$ or L-NAME (5 mg/kg, i.v.) was administered at 20 min prior to the injection of LPS.

Mortality and Survival Time Studies LPS (15 mg/kg, i.v.) was injected in the presence of normal saline, minocycline, clindamycin, cyclosporin, and platonin in rats, respectively. Mortality and survival times were monitored every hour up to 6 h. Different groups of rats received normal saline or drugs plus LPS in this study.

Determination of Plasma Nitrate Blood was collected from the femoral artery and centrifuged (10000 rpm) for 3 min to prepare plasma, then was stored at -20 °C.

Nitric oxide (NO) was assayed in the blood sample using a sensitive and specific chemiluminescence detection method. Blood samples were deproteinized by incubation with 95% ethanol at 4 °C for 30 min. Samples were then centrifuged for another 7 min at 13000 rpm. It should be noted that the nitrate concentrations in blood samples depicted in this study actually represent the total of both nitrite and nitrate concentrations in blood samples. This method reduced nitrate to NO *via* nitrite. The amount of nitrate in blood samples (10 μ l) was measured by adding a reducing agent (0.8% VCl₃ in 1 M HCl) to the purge vessel to convert nitrate to NO, which was

stripped from blood samples by a helium purge gas. The NO was then drawn into a Sievers Nitric Oxide Analyzer (Sievers 280 NOA, Sievers, Boulder, CO, U.S.A.). Nitrate concentrations were calculated by comparison with standard solutions of sodium nitrate.

Electron Spin Resonance Spectrometry Electron spin resonance (ESR) spectra were recorded at room temperature on a Bruker EMX ESR spectrometer using a quartz flat cell designed for aqueous solutions. Conditions of ESR spectrometry were as follows: 3456 ± 50 G; power, 0.635 mW; modulation frequency, 100 kHz; frequency, 9.663 GHz; modulation amplitude, 1 G; receiver gain, 6.3×10^{-4} ; time constant, 81.92 ms; and conversion time, 327.68 ms. The ESR spectrum was obtained in the H₂O₂/NaOH/DMSO system as described previously.¹⁴⁾ Briefly, $100 \,\mu$ l of DMSO and the same volume of 25 mM NaOH and platonin solution (aqueous) were mixed in a test tube, followed by the addition of 10 μ l of DMPO and 100 μ l of 30% hydrogen peroxide. The reaction mixture was sucked into the quartz flat cell and set in the ESR apparatus; scanning was begun 10 min after the mixing of all reagents.

Statistical Analysis Experimental results are expressed as the means \pm S.E.M. and are accompanied by the number (*n*) of observations. Data were assessed by the method of analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by the Newman–Keuls method. The Mann–Whitney test was used to determine the significance of differences in mortality. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Effects of Minocycline, Clindamycin, Cyclosporin and Platonin on Changes in Blood Pressure and Heart Rate Caused by LPS in Vivo The mean baseline values of MAP ranged from 104±5 to 109±9 mmHg in all animal groups studied, and did not significantly differ among groups. Figure 1A shows that the administration of LPS (15 mg/kg) caused a rapid drop in MAP, from 104 ± 5 to 78 ± 4 mmHg within 1 h. There was a continuous drop in MAP to 47 ± 4 mmHg at 3 h after LPS administration. In non-treated rats, there was no significant change in MAP during the experimental period (*i.e.*, from 107 ± 6 mmHg at time 0 to 109 ± 5 mmHg at 3 h, n=6) (data not shown). Furthermore, mean baseline values of HR ranged from 301±17 to 317±18 beats/min and did not significantly differ between any of the experimental groups studied. Figure 1B demonstrates that the administration of LPS resulted in an increase in HR (tachycardia), while in the SOP group, there were no significant changes in HR during the experimental period (data not shown).

Pretreatment of rats with minocycline (10 mg/kg), clindamycin (10 mg/kg), cyclosporin (15 mg/kg), or platonin (100 μ g/kg) alone did not exert a significant effect on MAP or HR (data not shown). Furthermore, minocycline (10 mg/kg) and platonin (100 μ g/kg) significantly prevented the drop in MAP observed in LPS-treated rats (Figs. 1A, 2A). Minocycline significantly prevented the drop in MAP 3h after LPS administration (n=8, p<0.05) (Fig. 1A), and platonin markedly prevented the same at 1, 2, and 3 h after LPS administration (Fig. 2A). However, neither clindamycin nor



Fig. 1. Effects of Minocycline, Clindamycin, and L-NAME on (A) Mean Arterial Pressure (MAP) and (B) Heart Rate (HR) in Anesthetized Rats Treated with LPS

Depicted are the changes in (A) MAP and (B) HR during the experimental period in different groups of animals pretreated with normal saline (NS), minocycline (MI, 10 mg/kg), clindamycin (CL, 10 mg/kg), and L-NAME (5 mg/kg), followed 20 min later by the addition of LPS (15 mg/kg, i.v., at time 0). Data are presented as the means \pm S.E.M. (n=8). *p<0.05, **p<0.01, and ***p<0.001 represent significant differences as compared with the normal saline plus LPS group (NS+LPS).

cyclosporin significantly prevented the decrease in MAP induced by LPS (Figs. 1A, 2A). Furthermore, minocycline (at 3 h) and platonin significantly suppressed the increase in HR induced by LPS (Figs. 1B, 2B), whereas clindamycin and cyclosporin had no effect on tachycardia induced by LPS (Figs. 1B, 2B). On the other hand, at 3 h after LPS injection , L-NAME (N^G-nitro-L-arginine ester, 5 mg/kg), an inducible NO synthase inhibitor,¹⁵⁾ caused about a 39 mmHg rise in MAP and moderate bradycardia (388±11 beats/min) in LPStreated rats (Figs. 1A, B).

Effects of Minocycline, Clindamycin, Cyclosporin, and Platonin on Plasma Nitrate Formation Induced by LPS Mean plasma levels of nitrate ranged from 13.7 ± 1.0 to $16.0\pm1.9\,\mu$ M, and did not significantly differ between any of the experimental groups studied. Endotoxemia for 3 h was associated with a 7-fold increase in the plasma level of nitrate (p < 0.001, n=8) (Fig. 3). However, there were no significant changes in plasma nitrate levels during the experimental period in the non-treated group (data not shown). The increase in plasma nitrate elicited by endotoxemia was significantly reduced by pretreatment with either minocycline (10 mg/kg) or platonin $(100 \mu \text{g/kg})$ (Fig. 3). On a molar basis, the inhibitory effect of platonin on plasma nitrate levels was more potent than that of minocycline in LPS-treated rats. In contrast, neither clindamycin (10 mg/kg) nor cyclosporin (15 mg/kg) significantly changed plasma nitrate



Fig. 2. Effects of Cyclosporin and Platonin on (A) Mean Arterial Pressure (MAP) and (B) Heart Rate (HR) in Anesthetized Rats Treated with LPS

Depicted are the changes in (A) MAP and (B) HR during the experimental period in different groups of animals pretreated with normal saline (NS), cyclosporin (CY, 15 mg/kg), or platonin (PL, 100 μ g/kg), followed 20 min later by the addition of LPS (15 mg/kg, i.v., at time 0). Data are presented as the means±S.E.M. (*n*=8). **p*<0.05, ***p*<0.01, and ****p*<0.001 represent significant differences as compared with the normal saline plus LPS group (NS+LPS).



Fig. 3. Effects of Minocycline, Clindamycin, Cyclosporin, and Platonin on Plasma Nitrate in LPS-Treated Rats

Depicted are the changes in plasma nitrate during the experimental period in different groups of animals pretreated with normal saline, minocycline (10 mg/kg), clindamycin (10 mg/kg), cyclosporin (15 mg/kg), or platonin $(100 \mu g/kg)$ 20 min before LPS (15 mg/kg, i.v., at time 0) administration. Data are presented as the means \pm S.E.M. (n=8). *p < 0.05 and **p < 0.01 represent significant differences as compared to the LPS group (normal saline plus LPS).

levels in rats with endotoxemia. This result indicates that platonin and minocycline prevent the decrease in MAP, at least partly, by the inhibition of nitrate formation in rats with bacteriemia.

Table 1. Effect of Platonin on Mortality and Mean Survival Time 6 h afterIntravenous Injection of LPS in Anesthetized Rats

	Total number	Number of deaths	Mortality (%)	Mean survival time
Normal saline	6	0	0	>6 h
LPS (15 mg/kg)				
+normal saline	12	5	41.6	3.9±0.4 h (5)
+minocycline (10 mg/kg)	10	4	40	4.1±0.5 h (4)
+ clindamycin (10 mg/kg)	10	4	40	4.2±0.4 h (4)
+ cyclosporin (15 mg/kg)	10	5	50	3.8±0.6 h (5)
+platonin (100 μ g/kg)	10	2	20	5.7±0.2*h(2)

Data of mean survival time are presented as the means \pm S.E.M. (number of dead rats). * p<0.05 as compared with normal saline plus LPS group.



Fig. 4. Effect of Platonin on Free Radical-Scavenging Activity in the $H_2O_2/NaOH/DMSO$ System

(A) Typical ESR spectrum in the $H_2O_2/NaOH/DMSO$ system. ESR conditions are described in "Materials and Methods". Scanning was started 10 min after the mixing of all reagents. Each free radical-derived signal was assigned, and signal heights were calculated in the figure. The intensity of the hydroxyl radical was much stronger (quarted signal peaks) than the superoxide anion and methyl radical peaks. (B) ESR spectrum of the free radical-scavenging activity of platonin (10 μ M). The inhibition rate of platonin is defined by the following equation: inhibition rate=1-signal height (platonin)/signal height (control). The spectrum is a representative example of five similar experiments.

Effects of Minocycline, Clindamycin, Cyclosporin, and Platonin on Mortality and Survival Time in LPS-Treated The injection of LPS (15 mg/kg, i.v.) caused a much Rats greater lethal effect as compared with the normal salinetreated group in rats (Table 1). Pretreatment with platonin $(100 \,\mu g/kg)$ markedly reduced the mortality and prolonged the mean survival time in LPS-treated rats (Table 1). The injection of LPS caused about 41.6% mortality associated with a mean survival time of about 3.9 ± 0.4 h, whereas pretreatment with platonin caused death in only 20% of the LPStreated rats, associated with a mean survival time of about 5.7 ± 0.2 h. However, neither minocycline, clindamycin, nor cyclosporin significantly reduced the mortality or prolonged the mean survival time in LPS-treated rats (Table 1). This result indicates that platonin can effectively reduce the mortality and prolong the mean survival time in LPS-treated rats.

Free Radical-Scavenging Activity of Platonin The rate of free radical scavenging activity is defined by the following equation: inhibition rate=1-signal height (platonin)/signal height (control).¹⁴⁾ In this study, typical ESR signals of the

superoxide anion, hydroxyl radical, and methyl radical were observed as in Fig. 4A. Platonin (10 μ M) markedly suppressed superoxide anion and hydroxyl radical formation by about 71% and 69% (*n*=5), respectively (Fig. 4B). In addition, platonin also suppressed methyl radical formation by about 41% (*n*=5); however, the suppression rate of platonin against the methyl radical was smaller than those against the superoxide anion and hydroxyl radical. This observation may provide *in vitro* evidence that suggests the usefulness of platonin for its free radical-scavenging activity.

DISCUSSION

Administration of LPS produces many cardiovascular features of septic shock, including hypotension and persistent loss of vascular tone, which is often unresponsive to vasoconstrictors, and is associated with high mortality.^{16,17)} The suggestion that endotoxemia is associated with an overproduction of NO, presumably via inducible NO synthase (NOS II), is based on findings, using electron paramagnetic resonance, of a time-dependent increase in nitrosylated hemoglobin in mice and rats with endotoxic shock.^{18,19} Indeed, the present study demonstrates that plasma nitrate (a final metabolite of NO) levels are increased in rats treated with LPS, suggesting an overproduction of NO in this rodent model. Therefore, this acute rodent model of endotoxemia mimics most of the clinical features of sepsis and, hence, is applicable for investigating the pathophysiology of endotoxemia. This overproduction of NO (1) contributes to the hypotension caused by endotoxin in anaesthetized animals²⁰⁾ and (2) accounts for the vascular hyporeactivity to vasoconstrictors, including noradrenaline and phenylephrine,²¹⁾ just as an intravenous infusion of the NO synthase inhibitor, N^Gmonomethyl-L-arginine (L-NMMA), or the in vitro treatment of blood vessels with L-NMMA can improve vascular hyporesponsiveness.²²⁾ Furthermore, we also found that L-NAME (5 mg/kg) significantly reverses the effect of MAP and HR induced by LPS (Figs. 1A, 1B). Thus, in endotoxic shock, the excessive production of NO stimulated by LPS contributes to the development of profound hypotension and hyporesponsiveness to exogenous vasoconstrictors.

Platonin significantly ameliorated these hemodynamic changes, an effect which was accompanied by the reduction of plasma nitrate levels in this study. Therefore, we suggest that the mechanism of these actions of platonin is associated with the inhibition of the overproduction of NO in rats treated with endotoxin. On the other hand, we found that neither clindamycin nor cyclosporin significantly prevented the decrease in MAP or decreased the overproduction of NO in LPS-treated rats. It has been reported that compounds of the tetracycline group, such as minocycline, block and reverse both spontaneous and interleukin-1 β -induced osteoarthritis-NO synthase activity ex vivo.¹²⁾ In addition, minocycline also inhibits both LPS- and interferon- γ stimulated NO formation in RAW 264.7 cells in vitro.¹²⁾ In this study, we demonstrate that minocycline (10 mg/kg) significantly prevented the drop in MAP and decreased the overproduction of NO in LPStreated rats.

Due to concerns of recent studies which have demonstrated that selective inhibitors of NO formation only prevent circulatory failure, but not organ injury/dysfunction caused by endotoxin,²³⁾ the more directly relevant need is to examine survival in animal models with endotoxemia. Platonin markedly lowered the mortality and prolonged the survival time; however, minocycline did not significantly attenuate either effect under the same circumstances. How can the relatively weak effect of minocycline on survival be reconciled with the marked reduction of plasma NO levels *in vivo*? In addition to NO, many mediators play an important role in the pathophysiology of septic shock; these mediators including cytokines, granulocyte-colony stimulating factor, macrophage-colony stimulating factors, as well as free radicals.²⁴⁾ Thus, a reduction in NO levels may not be the only factor needed to improve survival in animals with endotoxemia.

In recent years, reactive oxygen species (ROS) have been thought to play a critical role in many diseases such as cancer and sepsis.²⁵⁾ In several reports,²⁶⁾ the reduction of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) spin adducts in ESR was applied to the determination of free radical-scavenging activities, but it is difficult to apply this method to water-soluble substances. The H2O2/NaOH/DMSO system has been developed to evaluate the antioxidative ability of both watersoluble and oil-soluble antioxidants.¹⁴⁾ The mechanisms of free radical formation in the H2O2/NaOH/DMSO system assume that the superoxide anion and hydroxyl radical are generated from the degradation of hydrogen peroxide, and that the methyl radical is generated from the degradation of DMSO by the hydroxyl radical. The superoxide anion changes into a hydroxyl radical by the catalytic action of contaminating trace iron, so that the amount of hydroxyl radical is consequently relatively larger than that of the superoxide anion. Using this system, free radical-scavenging activities of the superoxide anion, hydroxyl radical, and methyl radicals can be evaluated at the same time. In this study, we found that platonin can effectively inhibit hydroxyl radical, superoxide anion, and methyl radical formation in vitro (Fig. 4); however, minocycline (100 μ M) had no effect on this, even at a higher concentration (1 mM) (data not shown). Thus, the mechanisms of these actions of platonin involve, at least partly, the inhibition of free radical formation.

In conclusion, platonin has beneficial effects in improving the survival of rodents with endotoxemia or endotoxic shock. This protective effect of platonin may be mediated, at least partly, by the reduced decrease in MAP and inhibition of NO and by free radical formation in anesthetized rats treated with LPS, with a resulting reduction in circulatory failure, thereby leading to prolonged survival time and reduced mortality.

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