Anti-Inflammatory Effects of LK-3, on LPS-Induced Sepsis in Rats

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Abstract

Dextromethorphan (DM), an antitussive agent, has been shown to have anti-inflammatory and immunomodulatory effects in vitro. Thus, the aim of this study was to evaluate the effects of LK-3, an analog of DM, on sepsis induced by intravenous (i.v.) administration of lipopolysaccharide (LPS; 10 mg/kg) in anesthetized Wistar rats. Results demonstrated that post-treatment with LK-3 (4 mg/kg, i.v.) significantly attenuated the deleterious hemodynamic changes (e.g., hypotension and bradycardia) in rats treated with LPS. Meanwhile, LK-3 (4 mg/kg) significantly inhibited the elevation of plasma tumor necrosis factor-α, as well as values of glutamate-oxalacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) caused by LPS. The induction of inducible NO synthase and the overproduction of NO and superoxide anions by LPS were also reduced by post-treatment of LK-3. Moreover, infiltration of neutrophils into the lungs and liver of rats 8 h after treatment with LPS was also reduced by post-treatment with LK-3. In conclusion, the beneficial effects of LK-3 on LPS-induced sepsis resulted from its anti-inflammatory and antioxidant effects.

Key Words: dextromethorphan, reactive oxygen species, sepsis, TNF-α, nitric oxide, circulatory failure

Introduction

Sepsis is a generalized systemic inflammatory condition that can be defined as a progressive failure of the circulation, clinically characterized by systemic hypotension, hyporeactiveness to vasoconstrictors, and subsequent organ perfusion and function abnormalities followed by multiple organ failure (4). Septic shock is a serious clinical problem with high mortality (2, 4). According to Bone et al. (4), the progression from septicemia to septic shock involves several steps. First, local inflammation may provoke the release of proinflammatory mediators, such as tumor necrosis factor (TNF) and interleukins. Then, proinflammatory mediators cause migration of leukocytes, lymphocytes, and platelets to infected areas. Systemic pathologic
changes include endothelial damage, increased microvascular permeability, platelet aggregation, local blood flow reduction, and ischemia/reperfusion injury. Excessive inflammatory responses may result in tissue injury. Finally, continuous inflammatory responses lead to multiple organ injury (5, 19, 20).

Recently, several investigators have linked the excessive generation of nitric oxide (NO) to lipopolysaccharide (LPS)-induced hypotension, vascular hyporeactivity, and death, suggesting that overproduction of NO plays an important role in septic shock (21, 33). LPS-dependent induction of inducible NO synthase (iNOS) is responsible for the overproduction of NO in circulatory shock (25). This enzyme is expressed in many types of cells, such as neutrophils, macrophages, endothelial cells, vascular smooth muscle cells, mesangial cells, and chondrocytes (13). The triggers for iNOS include LPS, interferon-γ, and many kinds of proinflammatory cytokines (e.g., TNF-α) (12).

LPS activates macrophages causing the generation of free radicals, including the superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (·OH), leading to oxidative damage in many tissues such as the liver (22). NO can react with O$_2^-$ to form the peroxynitrite anion (ONOO$^-$), which results in severe oxidative damage, leading to multiple organ failure and septic shock with high mortality (3, 8). A previous study finds that the inhibition of NOS activity by a selective iNOS inhibitor can attenuate the hepatic, renal, and pancreatic dysfunction associated with LPS-induced endotoxemia in rats (32). Therefore, the overproduction of NO and NO-dependent free radicals seems to be related to development of multiple organ failure in endotoxic shock.

Dextromethorphan (DM), a dextrorotatory morphinan, is widely used clinically as a nonopioid antitussive (29). However, the exact mechanism of its cough suppression action remains unclear. DM has been shown to be neuroprotective in several animal neurodegenerative models through inhibition of N-methyl-D-aspartate (NMDA) receptors (7, 9). Nevertheless, recent studies demonstrate that micromolar concentrations of DM reduce the inflammation mediated degeneration of dopaminergic neurons through the inhibition of microglial activation (18). The neuroprotective effect elicited by femtomolar concentrations of DM is mediated through the inhibition of LPS-induced proinflammatory factors, especially superoxide (16). DM is effective in protecting mice against LPS/GaIN-induced hepatotoxicity, and the mechanism is likely through a faster TNF-α clearance, and the decrease of superoxide production and inflammation and cell-death related compound (17). These studies suggest that the anti-inflammatory property of DMs may underlie its protective effect. Thus, the aim of this study is to evaluate the anti-inflammatory effect of (+)-3-methoxymorphinan hydrochloride (LK-3) (Fig. 1), an analog of DM, on LPS-induced sepsis in vivo.

### Materials and Methods

#### Animal Preparation

Male Wistar-Kyoto rats (250-300 g) were purchased from National Laboratory Animal Breeding and Research Center of National Science Council, Taiwan. All these rat were housed at an ambient temperature of 23 ± 1°C and moisture of 55 ± 5%. The rats were anesthetized by intraperitoneal injection of urethane (1.2 g/kg). The trachea was cannulated to facilitate respiration. The left femoral artery was cannulated for administration of drugs. After the surgical procedure was completed, all of the cardiovascular parameters were allowed to stabilize for 30 min.

#### Drug Administration

The rats were randomly assigned into the following groups: (I) control group: rats were treated with vehicle (saline; i.v.); (II) LK-3 group: Rats were treated with LK-3 (2 or 4 mg/kg, i.v.); (III) LPS group: Rats were treated with E. coli lipopolysaccharide 3129 (LPS) 10 mg/kg (i.v.) (30); (IV) LK-3-posttreatment group (LPS/LK-3): Rats were treated with LK-3 (2 or 4 mg/kg, i.v.) at 30 min after LPS (10 mg/kg; i.v.) administration.

After recording the baseline hemodynamic parameters, the rats were injected with vehicle or LPS and were monitored for 8 h. Prior to (at time 0) and every hour after vehicle or LPS administration,
0.5 ml of blood was withdrawn to measure the level of TNF-α and nitrate. Any blood withdrawn was immediately replaced by an injection of an equal volume of saline (i.v.) to maintain the blood volume. Blood samples were centrifuged for 5 min at 12,000 g. Plasma samples were stored at -80°C until analysis. At the end of the 8 h experiment, thoracic aortas of each group were dissected out for vascular responses or superoxide analysis.

**Organ Bath Experiments**

The blood vessels were cleared of adhering periadventitial fat and cut into sections (3-4 mm long). The segments were mounted in 20 ml organ baths filled with 95% O_2/5% CO_2 oxygenated Kreb's solution (pH 7.4) at 37°C, consisting of (in mM): NaCl 118, KCl 4.7, NaHCO_3 25, KH_2PO_4 1.2, MgSO_4 1.2, CaCl_2 2.5, glucose 11. Isometric force was measured using Grass FT03 type transducers (Grass instruments, Quincy, MA, USA) and recorded on a MacLab Recording and Analysis System (ADInstrument, Castle Hill, Australia). In the segments, 2 g passive tension was applied, and the preparations were equilibrated for 60-90 min. The presence of functional endothelium was confirmed when the vessels, which were pre-contraction with norepinephrine (NE, 0.1 µM), by a minimum of 80% relaxation with acetylcholine (ACh, 1 µM). The Kreb's solution in the organ baths was changed every 15 min, 2-3 times.

After the tested intact segments were proven stable at the baseline tension level the next steps were performed. Concentration-response curves for NE were performed by adding varying concentrations of NE (10 nM to 10 µM) to the organ bath; any changes in tension were monitored using the force displacement transducer, and recorded on a computer. Kreb's solution was used to wash the preparations twice every 15 min for the next step. Concentration-response curves for ACh were carried out by adding NE (1 µM) to obtain maximum contraction, then adding ACh cumulatively from 10 nM to 10 µM and recording relaxation. The Kreb's solution was changed twice every 15 min for the next step. Concentration-response curves for L-arginine were performed by adding NE (1 µM) to obtain maximum contraction, then adding L-arginine (10 nM to 10 µM) cumulatively and recording relaxation.

**Quantification of Organ Function and Injury**

At 0 and 8 h after the injection of LPS, blood samples were collected. The blood samples were centrifuged 12,000 g to prepare serum. All serum was analysed within 24 h. Eighty microliters of plasma were used to analyze liver functions. The following marker enzymes measured in the plasma were regarded as biochemical 6060 indicators of multiple organ dysfunction or failure. Liver dysfunction and failure were assessed by measuring the rises in plasma levels of glutamate-pyruvate transaminase GPT (a specific marker for hepatic parenchymal injury), glutamate-oxalacetate transaminase GOT (a nonspecific marker for hepatic parenchymal injury). All of these biochemical parameters were analyzed within 15 min by Fuji DRI-CHEM 3030 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

**Histopathological Studies**

The vital organs, including the lung, liver, were harvested the 8th h for histopathological studies, as previously described (6). These organ tissues were fixed in buffered formaldehyde (10% in phosphate-buffered saline) for more than 8 h. The fixed organs were dehydrated in graded ethanol and embedded in paraffin (Tissue-processor, Tokyo Japan). Four-micrometer sections were cut (sliding microtome, Leica Jung SM 2000, Leica, Nussloch, Germany) and removed paraffin by xylene. Then, the tissue sections were stained with the hematoxylin and eosin reagent for light microscopy. This histologic alteration was quantitatively analyzed as an index of the severity of tissue injury. The index was determined by counting the numbers of polymorphonuclear neutrophil (PMN) in 10 randomly selected high-power fields and by the histologic changes (e.g., interstitial edema and/or congestion) evaluated by a pathologist in a blinded fashion.

**Measurement of Plasma TNF-α Concentration**

The blood sample (0.5 ml) was collected at 0, 1, 2 and 4 h after the injection of LPS for measurement of the TNF-α level in plasma by an enzyme-linked immunosorbent assay (mouse TNF-α ELISA Kit, Genzyme Co., Cambridge, MA, USA), as previously described (31).

**Plasma Nitrite/Nitrate Determination**

A sample of 30 µl thawed plasma was de-proteinated with 100 µl 95% alcohol for 30 min (4°C). Subsequently, these serum samples were centrifuged for 6 min at 12,000 g. The supernatant (6 µl) was injected into a collection chamber containing 5% VCL_3. In this strong reducing environment, both nitrate and nitrite were converted to NO. A constant stream of helium gas carried NO into a NO analyzer (Sievers 280NOA; Sievers Instruments Inc., Boulder, CO, USA), where the NO reacted with ozone (O_3), resulting in the emission of light. Light emission is proportional to the NO formed. Standard amounts of sodium nitrate were used for calibration (Sigma Chemical Co., St. Louis, MO, USA).
Western Blot Analysis of iNOS Protein Expression in Lungs

At the 8th h after the injection of saline or LPS, the rats were sacrificed. The lung tissues were obtained from rats in control, LK-3, LPS, and LK-3-post-treatment groups, and frozen at -80°C before assay. Frozen samples were ground in a mortar containing liquid nitrogen. The powdered tissue was suspended in 1 ml of lysis buffer containing protease inhibitors, as previously described (6). Lung protein were submitted to 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). The membranes were incubated with antibodies against iNOS (1:1000 dilution, Stressgen Biotechnologies Co., Victoria, BC, Canada) or β-actin (1:2000 dilution, Sigma-Aldrich, St. Louis, MO, USA). Immunodetection was performed with the use of enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). Protein quantities were measured by densitometric scanning of the blots using Image-Pro software (Media Cybernetics, Inc, Washington, DC, USA).

Aortic Superoxide Anion Detection by Chemiluminescence

Detection of superoxide anions was performed as described previously (10). Thoracic aorta was cut into 3-4 mm rings and incubated in 95% O₂/5% CO₂ oxygenated modified Kreb’s/HEPES solution (37°C) for 30 min. Then the aorta sections were put into a 96 well plate in which every well was filled with 200 µl modified Kreb’s/HEPES solution, and placed in a luminescence measurement system (Microplate Luminometer LB 96 V, Berthold, Germany). It can perform auto-injection of 250 µM lucigenin (final volume of 250 µl) into the vessels for interacting with superoxide. Counts were obtained at 15 min intervals at room temperature. After recording was complete, the vessel ring was dried in a 95°C oven for 24 h. The results were expressed as relative units of luminescence (RUL) per 15 min per milligram dry weight vessel (i.e., RUL/15 min/mg).

Statistical Analysis

Data are expressed as means ± SE. One-way ANOVA was performed in the statistical analysis of data. When group comparisons showed a significant difference, the Student–Newman Keuls test was used. A value of P < 0.05 was accepted to indicate statistical significance.

Results

Effects of LK-3 on MAP and HR in Rats with Endotoxemia

The baseline MAPs of five groups were about 120 ± 3 mmHg and did not show significant difference among groups. The MAP of the control group is not significantly changed during the period of the experiment. The injection of LPS resulted in a rapid decrease in MAP within 15 min. The MAP then slowly returned to 108 ± 2 mmHg at 1 h, and gradually decreased to 59 ± 2 mmHg at the end of the experiment (8 h). Results are shown in Fig 2A; the MAP of post-treatment with LK3 (4 mg/kg) group was significantly greater than that of the LPS group.

Fig. 2B illustrates the time course of changes in HR. The mean baseline values of HR in five groups were about 371 ± 1 to 406 ± 16 beats/min and there was no significant difference among groups. In the LPS group, HR progressively increased and peaked at 4 h, and then decreased lasting to the end of the experiment. However, posttreatment with LK-3 (4 mg/kg) prevent LPS-induced bradycardia at 6-8 h (Fig. 2B).

Effects of LK-3 on NE-Induced Vasoconstriction, ACh- and L-Arginine-Induced Vasorelaxation, In Vitro

Results in Fig. 3 shows that vascular hy-
poreactivity of NE and ACh were found in the LPS group. This hyporesponse to NE and ACh were significantly reversed by posttreatment with LK-3 (4 mg/kg, i.v.) \( (P < 0.05, \text{Fig. 3A, B}) \). However, LPS significantly enhanced L-arginine-induced vasorelaxation, and LK-3 post-treatment significantly reversed the effect of LPS (Fig. 3C).

**Effect of LK-3 on Liver Function (GOT and GPT)**

Baseline values of GPT and GOT were not significantly different among groups (Fig. 4, A and B). LPS caused a significant increase in the plasma

levels of GPT and GOT at the late stage (8 h). The rise in plasma levels of GPT and GOT caused by LPS were reduced by the post-treatment of rats with LK-3.

**Effect of LK-3 on Plasma TNF-α Level**

The basal plasma levels of TNF-α were not significantly different among groups. LPS caused a significant increase in plasma TNF-α level, which reached a peak at 1-2 h after LPS injection and subsequently decreased slowly. LK-3 alone did not cause an increase in the plasma TNF-α level. However, post-treatment with LK-3 (4 mg/kg) significantly decreased the LPS-induced increase of plasma TNF-α level at 1-2 h as compared with the LPS group (Fig. 5A).

**Effect of LK-3 on Superoxide Anion Formation**

The content of superoxide anions in thoracic aorta after 8 h LPS treatment was significantly higher than that of the control group \( (5112 \pm 658 \text{ vs. } 1002 \pm 27 \text{ RLU, } P < 0.05) \). However, post-treatment with
LK-3 (4 mg/kg) significantly reduced superoxide anion formation as compared with the LPS group (1676 ± 134 vs. 5112 ± 658 RLU, P < 0.05) (Fig. 5B).

Effect of LK-3 on Plasma Nitrite/Nitrate Content

In the control and LK-3 alone groups, the level of plasma nitrite/nitrate did not significantly change throughout the experimental period. LPS treatment significantly induced the elevation of plasma nitrite/nitrate content at 4-8 h, as compared with the control group (P < 0.05). However, post-treatment of LK-3 (4 mg/kg) significantly suppressed this LPS-induced increase in plasma nitrite/nitrate levels (P < 0.05) (Fig. 5C).

PMN Infiltration

In the control group, light microscopy showed no infiltration of PMNs in the liver (Fig. 6A) or lungs (Fig. 7A). In contrast, increased infiltration of PMNs in both liver (Fig. 6B) and lungs (Fig. 7B) was noted at the 8th h after injection of LPS. In rats post-treated with LK-3, infiltration of PMNs in liver (Fig. 6C) and lungs (Fig. 7C) was significantly reduced.

Effect of LK-3 on Expression of iNOS Protein in Lungs

As shown in Figure 8, iNOS protein expression was low in lung homogenates obtained from the control rats, whereas a significant induction of iNOS protein was observed in rats treated with LPS for 8 h. Post-treatment of rats with LK-3 (4 mg/kg) significantly reduced the induction of iNOS challenged with LPS.

Discussion

In the present study, we demonstrate that post-treatment with LK-3, an analog of DM, possesses the protective effect on LPS-induced septic shock in rats. LK-3 improved circulatory function evidenced by preventing hypotension as well as bradycardia, and preserving vascular contracture ability as well as endothelial function in late stage. It also improved hepatic and lung function during sepsis, accompanied with attenuation of pulmonary PMN infiltration. The protective effects of LK-3 could be due to the inhibition of LPS-induced oxidative stress, reduction of TNF-α and superoxide anion, prevention of neutrophils infiltration, and the suppression of inflammatory gene.

Sepsis is a systemic response to infection, and septic shock is one of the most common causes of death in intensive care units (28). The most common cause of sepsis is an exposure to the structural component of a gram-negative bacterial membrane LPS and key symptoms include hypotension and vasoplegia, which may lead to multiple organ dysfunction and ultimately death (26). Bacterial LPS in the bloodstream induces the overexpression of various inflammatory mediators, such as interleukin-1β, TNF-α, NO, and prostaglandin E₂ (PGE₂). A large amount of inflammatory mediators produced in the body are thought to contribute to the LPS-induced symptoms of septic shock and mortality (1). Among these inflammatory mediators; NO is known to be closely associated with hypotension and hyporesponsiveness to vasoconstrictor stimuli in
Fig. 6. Histopathological studies by light microscope showing morphologically normal liver tissues from rat in the control group (A), infiltration of PMN (arrows) in liver from LPS-treated group (B), and only minimal infiltration by PMN (arrows) in liver from LPS-treated rats treated with LK-3 (C), and the statistical analysis of the PMN index in the liver (D). Tissue sections were stained with hematoxylin and eosin and viewed by light microscopy (400×). Data represent as mean ± SE (n = 6). *P < 0.05: LPS vs. control, #P < 0.05: LPS/LK-3 vs. LPS.

Fig. 7. Histopathological studies by light microscope showing morphologically normal lung tissues from rat in the control group (A), infiltration of PMN in lung from LPS-treated group (B), and only minimal infiltration by PMN in lung from LPS-treated rats treated with LK-3 (C), and the statistical analysis of the PMN index in the lung (D). Tissue sections were stained with hematoxylin and eosin and viewed by light microscopy (400×). Data represent as mean ± SE (n = 6). *P < 0.05: LPS vs. control, #P < 0.05: LPS/LK-3 vs. LPS.
endotoxin-induced sepsis (26, 28). In the present study, LK-3 increased NE-induced vasocontraction, and ACh-induced vasorelaxation (Fig. 3A and B), indicating that it improves vascular contracture ability and preserve endothelium function in sepsis. Moreover, LK-3 reduced the L-arginine-induced relaxation after LPS treatment (Fig. 3C), implying that it could suppress the activity of iNOS. This point was further supported by the results in which LK-3 reduced plasma nitrate/nitrite concentration and iNOS protein expression in lung tissue challenged by LPS (Fig. 5C)(8).

Production of NO by iNOS is beneficial in fighting bacteria, but its overproduction can be harmful as shown in endotoxic shock (22). There is increasing evidence that overproduction of TNF-α during infection also leads to severe systemic toxicity and even death (15). Evidence supporting this hypothesis comes from reports indicating that mediators (e.g., TNF-α and interleukins) produced by endotoxin challenge can induce iNOS expression and produce large amounts of NO (24, 27). In the present study, the elevation of plasma TNF-α level by LPS was downregulated by LK-3 treatment (Fig. 5, A and C), indicating that this anti-inflammatory activity of LK-3 may contribute to improve hyporeactivity and circulatory function in endotoxic rats.

In sepsis, abundant reactive oxygen species are produced, and several sources of oxygen radical species have been proposed as being the cause of tissue damage. Following transmigration and activation, infiltrating neutrophils produce abundant oxygen radicals via oxidative bursts. Other sources of oxygen radical species include activated macrophages and various extracellular molecular processes such as arachidonic acid metabolism and xanthine dehydrogenase oxidation (11). NO may combine with O_2^- to form the more-potent reactive oxygen metabolite, the peroxynitrite anion (ONOO^-), which decomposes to form 'OH (3). Both ONOO^- and 'OH are responsible for cellular lipid peroxidation, protein oxidation, and mitochondrial impairment function, which cause further damage to tissues and can induce cell death (14). Results in this study demonstrated that post-treatment with LK-3 significantly suppressed the superoxide anion production in blood vessels induced by LPS (Fig. 5B). Moreover, LPS induced multiple organ injuries/dysfunctions, which were further evidenced by histologic findings of PMN infiltration in the lung and liver. Treatment with LK-3 not only ameliorated the deterioration of hemodynamic changes (hypotension and bradycardia) but also attenuated liver and lung abnormalities (Figs. 6B and 7B) caused by LPS. These results indicate that some of the beneficial effects of LK-3 may be associated with its antioxidant properties (as shown in Fig. 5B). In addition, gram-negative-related sepsis is frequently associated with acute renal failure, which is associated with the overproduction of ROS (30). The cellular mechanisms of action responsible for the inhibitory effects of LK-3 on TNF-α release, NO synthesis, and superoxide anion generation need to be further investigated.

In conclusion, LK-3 is capable of reducing circulatory failure and improves multiple organ dysfunction in animals with endotoxic shock. These finding may provide insights into the potential novel effect of LK-3 as a therapeutic agent against septic shock in the future. As for the detail of anti-inflammatory molecular mechanism of LK-3 on sepsis will further designed experiments to approach.

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