N-Allylsecoboldine as a novel agent prevents acute renal failure during endotoxemia
Chin-Wei Chiaoa, Shoei-Sheng Leeb, Chin-Chen Wuc, Ming-Jai Sua,⁎

⁎ Corresponding author. Tel.: +886 2 23123456x8317; fax: +886 2 23971403.
E-mail address: mjsu@ha.mc.ntu.edu.tw (M.-J. Su).

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Abstract
Blockades of cytokine and oxygen radicals release are considered to be beneficial in reducing multiple organ injury and increasing the survival rate in sepsis/septic shock. Thus, we examined the protective efficacy of N-allylsecoboldine, a secoaporphine derivative with antioxidant and α1-adrenoceptor blocking activities, in rats treated with endotoxin (E. coli lipopolysaccharide, LPS). Pretreatment of LPS-treated rats with N-allylsecoboldine significantly attenuated the late-phase hypotension, hypoglycemia and incremental plasma tumor necrosis factor (TNF-α). Overproduction of plasma nitrate in endotoxemia was not changed but the continuous decrease of urinary nitrate appeared to be partially ameliorated by N-allylsecoboldine. However, N-allylsecoboldine inhibited the inducible nitric oxide synthase (iNOS) protein expression in the renal cortex of endotoxemic rats. N-allylsecoboldine also improved the endotoxemia-induced organ injury as demonstrated from the conspicuous recovery of marker enzymes in the LPS-treated rats. Endotoxemia was associated with renal dysfunctions as indicated by decreases in renal blood flow, urinary potassium excretion, and renal nitrate clearance. However, pretreatment with N-allylsecoboldine showed significant alleviation of these renal dysfunctions. In addition, a lower dose of N-allylsecoboldine ameliorated the mortality of LPS-treated mice. This study demonstrates N-allylsecoboldine’s ability to avail against acute renal failure and increase survival rate during endotoxemia. These beneficial effects may be attributed to the inhibition of iNOS expression, TNF-α production, and free radical scavenging activities. However, the role of α1-adrenoceptor antagonism for N-allylsecoboldine in sepsis remains unclear.

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Keywords: N-Allylsecoboldine; Acute renal failure; Endotoxemia; Nitric oxide synthase; α1-adrenoceptor antagonist; Tumor necrosis factor-α

1. Introduction
During the onset of sepsis, the inflammatory system, including macrophages, neutrophils and endothelial cells, becomes hyperactive, to produce powerful pro-inflammatory mediators, especially tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1 and IL-8. Large amounts of granular enzymes are released, and free radicals, such as reactive oxygen species and reactive nitrogen species, are overproduced responding to the bacterial stimulus in the hyperactive immune phase (Harlan, 1987). Production of these excessive mediators may cause edema, hypotensive shock, tissue damage, and multiple organ failure, leading to a hyporeactive immune response (Riedemann et al., 2003). According to the cascade of septic procession, many strategies for therapeutic intervention of sepsis have been reported. Since 1986, it has been demonstrated that TNF-α blockade is beneficial in models of shock after infusion of endotoxin (Tracey et al., 1986). Free radical scavenging (Goldfarb and Szabo, 2005) or treatment with antioxidants (Pinsky, 2003) diminishes the endotoxin toxicity and contributes to the therapy of sepsis. In addition, inhibition of immune response and/or antagonism of platelet-activating factor (PAF), IL-1, arachidonic acid metabolites (e.g. prostaglandin (PG) E1, thromboxane), coagulation, neutrophil activation, bradykinin, phosphodiesterase or even the complement system were also proposed as therapeutic strategies in sepsis (Riedemann et al., 2003). However, the organ failure
always occurs too quickly leading to serious septic shock and death. The pathogenesis of organ dysfunction is multi-factorial and incompletely understood. Until now, the recombinant human active protein C product (drotrecogin alpha, Xigris) has been the only approved agent for treatment of severe sepsis in clinics because of an observed increase in survival rate (Bernard et al., 2001).

Various studies in lipopolysaccharide (LPS)-treated animals have provided evidence that vascular hyporeactivity to vasoconstrictors in vitro and vasopressor-resistant hypotension in vivo are related to overproduction of nitric oxide (NO·) by inducible nitric oxide synthase (iNOS) (Thiemermann and Vane, 1990; Gunnett et al., 1998). NO· can also react further with reactive oxygen species (e.g. superoxide anion (O2·−)) to form the more potent metabolite peroxynitrite anion (ONOO−) (Beckman et al., 1990), which results in severe oxidative damage leading to multiple organ failure and septic shock with high mortality despite advances in cardiovascular support and antibiotic therapy (Rackow and Astiz, 1991). Thus, treatment with antioxidants is considered effective in reducing mortality or preventing organ damage in septic animal models (Wu et al., 2001; Liaw et al., 2005), and useful in protecting cardio-functions in patients with septic shock (Galley et al., 1997).

From chemical classification of antioxidants, many of them are non-alkaloids such as resveratrol, vitamin C, vitamin E or flavones. Recently, some agents were found to have antioxidant activity and exert a protective effect in septic rats, e.g. melatonin (Wu et al., 2001), tempol (Liaw et al., 2005) and thaliporphine (Chiao et al., 2005).

N-allylsecoboldine is a secoperaporphine alkaid with free radical-scavenging activity, antioxidant activity (Teng et al., 1996), and anti-arrhythmic activity via its inhibition of Na+ current and prolongation of effective refractory period of myocardium in Langendorff perfused heart (Wu et al., 1994). Recently, it was found to exert α1a-adrenoceptor antagonist activity in the human hyperplastic prostate (Guh et al., 1999). In this study, we examined whether N-allylsecoboldine exerted a protective effect in endotoxemic animals. Also, we investigated possible mechanisms by which N-allylsecoboldine ameliorated endotoxin-induced circulatory failure, organ dysfunction, and mortality.

2. Materials and methods

2.1. Materials

N-allylsecoboldine was synthesized from boldine with allylbromide under reflux (Lee et al., 1992). Bacterial LPS (E. coli serotype 0.127:B8), norepinephrine bitartrate, phenylephrine and p-aminohippuric acid (PAH) were all obtained from Sigma Chemical Co., St. Louis, MO, USA.

All solutions were made in saline (in vivo experiment) or distilled water (in vitro experiment). In the in vivo experiments, N-allylsecoboldine was dissolved in saline or 3 mg/ml respectively; the saline solutions were then infused intravenously to the rats at a volume of 1 ml/kg. For survival rate study in mice, N-allylsecoboldine was dissolved in saline and then subcutaneously injected into the mice at a volume of 0.1 ml/mouse/time.

2.2. Organ bath in vitro experiments

Thoracic aortas were obtained from normal ten-week-old male Wistar rats. The thoracic aortas were cleared of adhering perivascular fat and cut to 3–4 mm in length. The constriction of the aorta with phenylephrine (0.1 μM), followed by relaxation with acetylcholine (1 μM) was used as evidence of endothelial intactness. The tissue was incubated with warmed (37 °C), oxygenated (95% O2/5% CO2) physiological salt solution (PSS, pH 7.4) consisting of (mM): NaCl 118.2, KCl 4.7, KH2PO4 1.2, MgSO4 1.2, CaCl2·2H2O 1.9, NaHCO3 25 and glucose 11.7. One end of the segment was fixed to the organ bath chamber while the other end was connected to a force transducer (Force transducer-Type BG 25, Gould Inc. Cleveland, Ohio, USA). Tension was recorded on a Gould model RS 3400 recorder (Gould Inc., Valley View, Ohio, USA). The preparations had equilibrated for at least 60 min under a passive tension of 2 g. Cumulative response to phenylephrine (1 nM–30 μM) was examined at 20 min after 1 or 3 μM N-allylsecoboldine addition to the organ bath.

2.3. In vivo experiments

2.3.1. General surgical procedures

This study was approved by the local Institutional Review Board according to the Helsinki recommendations and internationally accepted principles for the care and use of experimental animals. Ten-week-old male Wistar rats were purchased from the National Laboratory Animal Center of Taiwan and maintained under a 12-h light/dark cycle at a controlled temperature (21±2 °C) with free access to food and tap water. Rats were anaesthetized by intraperitoneal injection of urethane (0.4 g/kg i.p.) followed by thiobutabarbitral (80 mg/kg i.p.). The trachea was cannulated to facilitate respiration. Since the response to LPS is strongly dependent on ambient temperature (Romanovsky et al., 2002), the environmental temperature was maintained at 24 °C in the room with an air-conditioning system. The rectal temperature was measured from the anus during the experiment. The left carotid artery was cannulated and connected to a pressure transducer (P23XL, Spectramed, Oxnard, CA, USA) for the measurement of pulsatile blood pressure and mean arterial pressure as well as heart rate, which were displayed on a polygraph software monitor (Power Lab/4sp, Chart v4.2.2 for Windows, ADInstruments, USA). The right jugular vein was cannulated for the administration of drugs. Also, both of the ureters were cannulated by PE-10 for the collection of urine. Twenty-five milligrams per milliter PAH was infused intravenously at the rate of 0.8 ml/h during all experiments. The rats were not treated with any other agents until at least 1 h after PAH infusion.

2.3.2. Drugs administration and plasma or urine collection during 240 min

After recording baseline hemodynamic parameters, the pressure responses of rats were assessed by giving norepinephrine (1 μg/kg i.v.), and 20 min later the rats received
saline or E. coli LPS (5 mg/kg i.v.) and were monitored for 240 min. The pressure responses to norepinephrine were reassessed every hour after vehicle or LPS injection. Prior to (i.e., at 20 min before LPS injection) and every hour after vehicle or LPS injection, 0.5 ml of blood was taken to measure the changes in TNF-α, biochemical values, and nitrate (an indicator of NO·). Any blood withdrawn was immediately replaced by the injection of an equal volume of saline (i.v.) in order to maintain the blood volume. Urine was also collected and weighed during 30 min durations at the same times as blood. N-allylsecoboldine (0.3, 1, and 3 mg/kg) were intravenously infused by 20 μl/min at 20 min prior to the injection of LPS. All hemodynamic, biochemical values, and urinary parameters were recorded for 240 min. Renal blood flow was determined using the p-aminohippurate-clearance method in all animal groups.

2.4. Determination of blood glucose

Before the blood sample was centrifuged to prepare plasma, 15 μl of whole blood was taken to measure the blood levels of glucose by means of a “SureStep” complete blood glucose monitoring system (Johnson and Johnson Co., Milpitas, CA, USA). The rest of the plasma was kept in a freezer at −20 °C for use in following experiments.

2.5. Measurement of TNF-α in plasma and urine

Plasma and urinary samples were obtained at −20, 60, 120, 180, and 240 min after the injection of saline or LPS. These samples were collected from a catheter placed in the carotid artery and both ureters, respectively, and were centrifuged at 7200 g for 5 min in order to obtain the plasma and urine for measuring the levels of TNF-α and nitrate (as described below). The samples of plasma, but not urine, were diluted 5 times and measured with an enzyme-linked immunoadsorbent assay (ELISA) kit (BioSource International, Inc., Camarillo, CA, USA) as previously described (Wu et al., 2001).

2.6. Determination of plasma and urinary nitrate levels

Thirty microliters of plasma and urine were thawed and deproteinized by incubating them with 60 μl 95% ethanol (4 °C) for 30 min. The samples were subsequently centrifuged for an additional 6 min at 14,000 g. It should be noted that the nitrate concentration depicted in the study is actually the total nitrite and nitrate concentration in plasma and urine. With this method, nitrate is reduced to NO· via nitrite. The amounts of nitrate (6 μl) were measured by adding a reducing agent (0.8% VCl₃ in 1 N HCl) to the purge vessel to convert nitrate to NO·, which was stripped from the plasma or urine by using a helium purge gas. The NO· was then drawn into the Sievers Nitric Oxide Analyzer (Sievers 280 NOA, Sievers Inc., Boulder, CO, USA). Nitrate concentrations were determined from a curve constructed from standard solutions of sodium nitrate.

2.7. Western blot analysis of iNOS protein expression in rat renal cortex

At 240 min after the injection of saline or LPS, the renal cortex was obtained from sham-treated controls, as well as from endotoxemic rats treated with vehicle or N-allylsecoboldine and frozen at −70 °C before assay. Frozen samples were homogenized on ice with a PolyTron PT-MR 6100 homogenizer (Kinematics AG, Switzerland) in a T-PER tissue protein extraction reagent (Pierce Biotechnology, IL, USA) and proteinase inhibitor mixture. After a 30-min centrifugation at 14,000 g, the supernatants were centrifuged further for 60 min at 100,000 g and the final supernatants were then stored at −70 °C until further analysis. Aliquots of final supernatants were used for protein assay (with BCA protein assay reagent A and B; Pierce Biotechnology, IL, USA) and Western blot analysis. Forty micrograms of protein were eluted from the supernatant and loaded directly into the sodium dodecyl sulfate (SDS) sample buffer for 7.5% SDS-polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred onto 0.45 μm PVDF (Immobilon-P Transfer Membrane; Millipore Corporation, Bedford, MA, USA). The membranes were blocked with 5% defatted milk in Tris buffer solution (TBS) containing 0.1% Tween-20 for 1 h and then incubated with 1:1000 dilution of specific polyclonal antibody against iNOS (Transduction Laboratories, Lexington, KY) in TBS containing 0.1% Tween-20 for 1 h. The membranes were washed and finally incubated with 1:4000 dilution of goat anti-mouse IgG-horseradish peroxidase (HRP) anti-body (Santa Cruz Biotechnology, CA, USA) for 1 h. After successive washes as before, the immunocomplexes were developed using an enhanced peroxidase/luminol chemiluminescence reaction (ECL Western blotting detection reagents; Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to X-ray film (Eastman Kodak Company, Rochester, NY, USA) for 3−5 min. The relative expression of iNOS protein was quantified by densitometric scanning of the Western blots using ImageQuant Analysis Software v 5.0 (Molecular Dynamics, Inc., Sunnyvale, CA).

2.8. Quantification of kidney, liver and heart injury

One hundred microliters of plasma was used to analyze enzymes relative to kidney, liver or heart damage. The following marker enzymes measured in the plasma were regarded as biochemical indicators of multiple organ injury. Kidney damage and failure were assessed by measuring the rises in serum levels of creatinine (an indicator of reduced glomerular filtration rate and renal failure). Liver damage and failure were assessed by measuring the serum levels of aspartate aminotransferase (a nonspecific marker for hepatic parenchymal injury), alanine aminotransferase (a specific marker for hepatic parenchymal injury), and albumin (as a marker for protein metabolism alteration). Heart damage and failure were assessed by measuring the serum levels of lactate dehydrogenase (an indicator of myocardial infarction) and creatine phosphate kinase muscle-brain (a specific marker for heart injury). All of these plasma biochemical parameters were
evaluated by the biochemical colorimetric analyzer (DRI-CHEM 3000, Fujifilm, Japan) (Chiao et al., 2005).

2.9. Analysis of urinary chemical parameters

Plasma and urine were collected, respectively, during 240 min. Both plasma and urine of PAH levels were measured by the colorimetry, and the hematocrit was determined to calculate the renal blood flow. The urinary Na$^+$ and K$^+$ concentration were measured by flame photometry (FCM 6341, Eppendorf, Hamburg, Germany). The renal blood flow, urinary flow rate, and urinary Na$^+$, K$^+$ excretion rate were showed in per gram of both kidneys weight (Chen et al., 1996).

2.10. Survival studies

Survival studies were performed in ICR mice (28–35 g), purchased from the National Laboratory Animal Center of Taiwan, and maintained under a 12-h light/dark cycle at a controlled temperature (21±2 °C) with free access to food and tap water. LPS (60 mg/kg i.p.) was injected in three groups of mice: group one, LPS injection only; group two, N-allylsecoboldine (0.1, 1 or 10 mg/kg s.c.) at 0 and 6 h after LPS injection; group three, normal controls. Survival was monitored every 6 h until 72 h.

2.11. Histological studies

Parts of the kidney sections were obtained from surviving mice in each group after the survival study and these tissues were fixed in 10% formaldehyde for histopathological examination. The fixed kidney tissues were dehydrated in graded ethanol and embedded in paraffin. Each paraffin block was processed into 5-μm-thick slices that were stained with hematoxylin and eosin.

2.12. Statistical analysis

All values in the figures and text are expressed as mean±S.E.M. of $n$ observations, where $n$ represents the number of animals studied. Statistical evaluation was performed by using analysis of variance (ANOVA) followed by a multiple-comparison test (Bonferroni’s test), except biochemical values and renal parameters, which were analyzed by unpaired Student’s $t$-test. The Chi-square test was used for determining the significance of differences in survival rate between LPS and drug-treated groups. A $P$ value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. N-Allylsecoboldine antagonizes $\alpha_1$ receptor in rat thoracic aorta

Contractions of rat aortic strips were induced by phenylephrine at concentrations ranging from 1 nM to 30 μM in the control group. The presence of N-allylsecoboldine (1 and 3 μM) did not affect the maximal contraction induced by phenylephrine, but shifted concentration–response curves of phenylephrine to the right (Fig. 1).

Fig. 1. Effects of N-allylsecoboldine on cumulative phenylephrine-induced contractile responses in rat thoracic aortas. Preparations were incubated for 20 min with vehicle ($n=6$) or N-allylsecoboldine (1 μM; $n=6$ and 3 μM; $n=6$). Data are expressed as mean±S.E.M. of $n$ animals.

Fig. 2. Effects of N-allylsecoboldine on (A) mean arterial pressure, and (B) heart rate in rats treated with endotoxin. The changes in mean arterial pressure and heart rate during the experimental period in different groups of animals receiving saline (Sham; $n=12$), lipopolysaccharide (LPS; 5 mg/kg, $n=14$), or LPS plus N-allylsecoboldine (N-allylsecoboldine/LPS; 0.3 mg/kg N-allylsecoboldine, i.v. infusion for 20 μl/min at 20 min prior to LPS; $n=12$) are depicted. Data are expressed as mean±S.E.M. of $n$ animals. *$P<0.05$ represents significant differences between the LPS group and the Sham group. †$P<0.05$ represents significant differences between endotoxemic rats pretreated with and without N-allylsecoboldine.
Urinary nitrate concentration (μmol/min/g) Sham 1144.90±210.36 N/A 208.36±43.39 N/A 958.55±417.65 10

Table 1
Effects of N-allylsecoboldine on plasma nitrate concentration, urinary nitrate concentration and urinary nitrate excretion rate in rats treated with endotoxin

<table>
<thead>
<tr>
<th></th>
<th>−20 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
<th>240 min</th>
<th>n</th>
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<tbody>
<tr>
<td><strong>Plasma nitrate concentration (μM)</strong></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Sham</td>
<td>16.73±1.57</td>
<td>17.39±1.54</td>
<td>18.46±2.17</td>
<td>20.10±2.41</td>
<td>20.29±2.46</td>
<td>13</td>
</tr>
<tr>
<td>LPS</td>
<td>18.70±1.96</td>
<td>24.57±2.53</td>
<td>49.44±3.72</td>
<td>120.86±8.45</td>
<td>229.56±15.97</td>
<td>19</td>
</tr>
<tr>
<td>N-allylsecoboldine/LPS</td>
<td>18.32±1.29</td>
<td>22.88±0.95</td>
<td>45.01±2.71</td>
<td>116.12±5.94</td>
<td>233.15±12.97</td>
<td>13</td>
</tr>
</tbody>
</table>

| **Urinary nitrate concentration (μM)** |         |        |         |         |         |     |
| Sham                     | 373.18±85.49 | 297.31±55.49 | 324.95±47.15 | 352.18±50.30 | 404.43±49.31 | 5   |
| LPS                      | 375.81±54.62 | 233.08±41.38 | 55.26±12.51 | 28.86±8.07 | 41.32±9.66 | 6   |
| N-allylsecoboldine/LPS    | 377.87±57.29 | 241.07±33.85 | 63.52±21.22 | 205.99±76.52 | 229.56±15.97 | 19  |

| **Urinary nitrate excretion rate (μmol/min/g)** |         |        |         |         |         |     |
| Sham                     | 947.00±262.16 | N/A | 754.54±174.62 | N/A | 1134.51±316.84 | 5   |
| LPS                      | 1047.78±284.58 | N/A | 273.89±58.60 | N/A | 115.75±31.61 | 6   |
| N-allylsecoboldine/LPS    | 1144.90±210.36 | N/A | 208.36±43.39 | N/A | 958.55±417.65 | 10  |

Data are expressed as mean±S.E.M. of n observations. Values from LPS-treated rats that are significantly different from sham-operated ones at same time are shown:

*P<0.05.
120 min. The treatment with N-allylsecoboldine failed to prevent the transient bradycardia after LPS, nor did it affect the late tachycardia induced by LPS. Infusion of normal control rats with N-allylsecoboldine alone had no significant effects on mean arterial pressure or heart rate (data not shown).

3.3. N-Allylsecoboldine has no effects on the vascular hyporeactivity to norepinephrine in rats with endotoxemia

We measured the vascular reactivity to intravenous norepinephrine. The mean baseline values for the pressor responses to norepinephrine (1 μg/kg i.v.) were normalized in all groups studied. The injection of LPS resulted in a substantial, time-dependent attenuation of the pressor response elicited by norepinephrine (100% at −20 min, 19.55 ± 1.87% at 60 min, 18.09 ± 1.30% at 120 min, 15.77 ± 1.84% at 180 min, and 15.02 ± 1.21% at 240 min). In contrast, the injection of saline for the sham group had no significant effect on norepinephrine-induced pressor responses during the experimental period. Pretreatment of rats with N-allylsecoboldine had no significant effect on norepinephrine-induced pressor responses during the experimental period. Pretreatment of rats with N-allylsecoboldine had no significant effects on the vascular hyporeactivity to norepinephrine between 60 and 240 min after LPS injection (100% at −20 min, 22.70 ± 1.32% at 60 min, 18.88 ± 1.51% at 120 min, 18.71 ± 2.04% at 180 min, and 19.63 ± 1.03% at 240 min).

3.4. N-Allylsecoboldine attenuates hypoglycemia caused by LPS

Baseline values of blood glucose in all experimental animal groups were between 108 ± 2 and 113 ± 3 mg/dl, which were not significantly different among the groups. Administration of LPS to rats caused a significant increase in blood glucose within 60 min, which thereafter started to decline. From 180 to 240 min, the blood glucose level was significantly lower than that in the sham group. The LPS-induced hypoglycemia at 240 min was attenuated by pretreatment with N-allylsecoboldine (Fig. 3A).

3.5. N-Allylsecoboldine suppresses the plasma TNF-α level in rats with endotoxemia

The basal plasma levels of TNF-α were not significantly different between any of the experimental groups. LPS caused a significant increase of plasma level, which reached a peak at 60 min after LPS injection and subsequently decreased slowly (Fig. 3B). In the sham group, no significant increase in plasma TNF-α during the experimental period indicates that the surgical procedure alone did not cause an increase in the plasma TNF-α level.

Pretreatment with N-allylsecoboldine significantly inhibited the LPS-induced increase in plasma TNF-α level at 180–240 min (Fig. 3B). Because there was little urinary TNF-α activity, we could not detect any activity in all urinary experimental groups during 240 min.

3.6. N-Allylsecoboldine recovers the urinary nitrate level partly, but decreases the renal cortex iNOS expression, while having no effect on the plasma nitrate level in endotoxemic rats

NO produced in plasma and urine of rats were measured at −20, 60, 120, 180 and 240 min after saline or LPS administration. The mean plasma levels of nitrate were not...
significantly different between any of the experimental groups studied. Endotoxemia for 240 min was associated with an almost 12-fold rise (from 19±2 to 230±16 μM) in the plasma level of nitrate. However, there was no significant change during the 240 min study in the sham group. The increase in plasma nitrate level induced by LPS was unchanged by pretreatment with N-allylsecoboldine (Table 1). In urinary NO· productions, the basal mean levels of nitrate were also not significantly different between each group, whereas these values (between 330±82 and 406±55 μM) were higher than NO· concentrations in plasma. There was no significant change during 240 min in the sham group. Pretreatment of LPS-treated rats with N-allylsecoboldine seemed to recover the urinary nitrate levels (nitrate concentration and nitrate excretion rate) at 240 min, but did not reach significance (Table 1). In addition, the iNOS protein expression in the renal cortex was observed in the LPS-treated group. The pretreatment with N-allylsecoboldine significantly attenuated LPS-induced iNOS protein expression in the kidney cortex (Fig. 4).

3.8. N-Allylsecoboldine reduces the polymorphonuclear neutrophil infiltration in the kidneys and liver of endotoxemic mice

In the time-control group, light microscopy did not show infiltration of polymorphonuclear neutrophil (Fig. 6A and D). In contrast, at 72 h after the injection of mice with LPS, there was an overt infiltration, partial penetration, and nuclear necrosis of polymorphonuclear neutrophils (arrow) in both the kidneys and livers. We found interstitial edema and apparent absence of individual tubular epithelial cells (arrow head) in the kidneys of LPS-treated mice (Fig. 6B and E). In LPS-injected mice treated twice with N-allylsecoboldine 0.1 mg/kg s.c. at 0 and 6 h after LPS, the histopathological changes by LPS were reduced in both the kidneys and livers (Fig. 6C and F).

3.9. N-Allylsecoboldine reduces the kidneys and liver injury in endotoxemic rats

Endotoxemia caused significant rises in the plasma activities of creatinine, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and creatine phosphate kinase muscle-brain, whereas it caused a decrease in albumin (Table 2). The pretreatment with N-allylsecoboldine significantly ameliorated the changes in levels of creatinine, albumin, aspartate aminotransferase and lactate dehydrogenase induced by LPS. However, the amelioration of LPS-induced increase of creatinine was the most significant (P<0.005) (Table 2).

3.10. N-Allylsecoboldine alleviates kidney dysfunctions in endotoxemic rats

As shown in Table 3, the renal blood flow was significantly decreased at 120 and 240 min after LPS. Pretreatment with N-allylsecoboldine significantly increased the renal blood flow.
in endotoxemic rats, whereas the hematocrit was not significantly different among the groups. The urinary rate was increased to a maximum (4.84 ± 0.73 μl/min/g) at 120 min but decreased to 2.66 ± 0.27 μl/min/g at 240 min after LPS. However, this urinary rate was not significantly different between sham and LPS groups at 240 min. Pretreatment with N-allylsecoboldine significantly increased urinary rate in endotoxemic rats at 240 min (Table 3). Both urinary sodium and potassium excretion rates were significantly decreased in endotoxemic rats. Pretreatment with N-allylsecoboldine significantly improved the urinary potassium excretion rate in endotoxemic rats. Pretreatment with N-allylsecoboldine, partly but not significantly, increased the nitrate clearance in endotoxemic rats (P = 0.066). However, the basal values of renal blood flow, hematocrit, urinary flow rate, urinary Na+, K+ excretion rate, and nitrate clearance were not significantly different among the groups.

4. Discussion

This study demonstrates that N-allylsecoboldine, both α1-adrenoceptor antagonist and antioxidant, prevents acute renal failure in endotoxemia. The general acute renal failure in endotoxemia occurred near the late phase time of our experiment. We found N-allylsecoboldine attenuated the delayed circulatory failure (e.g. mean arterial pressure and blood glucose, etc.), improved the kidney dysfunctions (e.g. renal blood flow, urinary K+ excretion and creatinine, etc.), and increased the survival rate in LPS-treated animals. These protective effects of N-allylsecoboldine seem to be associated with inhibition of the iNOS protein expression in the renal cortex and TNF-α production in plasma.

Endotoxin shock, immune system activation and neutrophil infiltration induce the overgeneration of free radicals (Harlan, 1987), such as NO· via enhanced iNOS expression. The overgeneration of NO· is believed to cause circulatory failure such as hypotension and vascular hyporeactivity to vasoconstrictor agents in endotoxic shock (Szabo et al., 1993). In this study, we clearly showed that N-allylsecoboldine significantly reduced the iNOS protein expression in the kidney during endotoxemia. However, the NO· levels in plasma remained unaffected, since N-allylsecoboldine has been known to possess O2· scavenging activity (Teng et al., 1996). This activity, which would reduce the ONOO− formation from combining NO· with O2·, resulted in an accumulation of NO· in plasma. This accumulation would lead to an elevation of NO· in plasma. However, the inhibition of iNOS expression by N-allylsecoboldine in the kidney cortex (Fig. 4) would counterbalance the increase of plasma NO·, which results in unchanged plasma NO· levels (Table 1).

Endotoxin decreases glomerular filtration rate, renal blood flow, and induces lipid peroxidation, which are all associated with reactive oxygen species. Reactive oxygen species has been

<table>
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<tr>
<th>Sham (n=12–14)</th>
<th>LPS (n=18–20)</th>
<th>N-allylsecoboldine/LPS (n=13)</th>
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<tr>
<td>CRE (mg/dl)</td>
<td>0.34±0.03</td>
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<tr>
<td>ALB (g/dl)</td>
<td>3.26±0.09</td>
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<td>GOT (U/l)</td>
<td>99.64±19.72</td>
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<td>GPT (U/l)</td>
<td>24.42±4.01</td>
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<td>LDH (U/l)</td>
<td>107.25±13.84</td>
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<td>Nitrate Cr (μmol/min/g)</td>
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<td>Urinary K+ (mmol/min)</td>
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</tbody>
</table>

Data are expressed as mean±S.E.M. of n observations. aP<0.05, represents values from sham-operated rats are significantly different at 20 and 240 min. bP<0.05 and dP<0.005, represent values from LPS-treated rats injected with N-allylsecoboldine are significantly different from those from LPS-treated ones at same time point.

Table 4

<table>
<thead>
<tr>
<th>Sham (n=5–6)</th>
<th>LPS (n=7–8)</th>
<th>N-allylsecoboldine/LPS (n=8–10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KW/BW (%)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.49±0.01</td>
<td>0.45±0.01</td>
</tr>
<tr>
<td>RBF (ml/min/g)</td>
<td>5.22±0.49</td>
<td>5.37±0.48</td>
</tr>
<tr>
<td>UV (μl/min/g)</td>
<td>2.72±0.38</td>
<td>2.75±0.34</td>
</tr>
<tr>
<td>UaNaV (μmol/min/g)</td>
<td>0.16±0.04</td>
<td>0.27±0.07</td>
</tr>
<tr>
<td>UcK+ (μmol/min/g)</td>
<td>1.10±0.14</td>
<td>1.21±0.11</td>
</tr>
<tr>
<td>Nitrate Cr (μmol/min/g)</td>
<td>53.67±17.25</td>
<td>37.55±11.66</td>
</tr>
</tbody>
</table>

Data are expressed as mean±S.E.M. of n observations. KW/BW, kidney weight-to-body weight ratio; RBF, renal blood flow; UV, urinary flow rate; UaNaV and UcK+, urinary sodium and potassium excretion rate, respectively; Nitrate Cr, nitrate clearance. *P<0.05 and **P<0.01, represent values from LPS-treated rats are significantly different from those from sham-operated ones at same time point. †P<0.05 and ‡P=0.066, represent values from LPS-treated rats injected with N-allylsecoboldine are significantly different from those from LPS-treated ones at same time point.
found to also cause renal failure, hepatotoxicity, and heart dysfunction (Sugino et al., 1987; Khador et al., 2002; Schrier and Wang, 2004). In the present biochemical analysis, N-allylsesecoboldine ameliorated organ injury of endotoxemic rats, especially in the protection of the kidneys against injury (bio-chemical test in creatinine, \( P<0.005 \)) (Table 2). We also demonstrated that the levels of renal blood flow, urinary flow rate, and urinary K⁺ excretion rate in endotoxemic rats seemed to return to normal levels by the application of N-allylsesecoboldine. Agents with antioxidant activity have been shown to prevent the reduction of endogenous superoxide dismutase (SOD), glomerular filtration rate, and renal blood flow during endotoxemia (Wang et al., 2003). Although we did not measure the change of SOD levels with N-allylsesecoboldine in endotoxemia, the antioxidant activity of this agent (Teng et al., 1996) probably contributed to its preservation of renal functions. LPS increases plasma TNF-α release, which peaks at 60–90 min, followed by increases of NO⁻ and O₂⁻ formation (possibly induced by TNF-α (Saito and Nakano, 1996; Wang et al., 1999). Even though the peak increase of TNF-α release by LPS was unchanged by N-allylsesecoboldine, the late increment of TNF-α release by LPS between 120 to 240 min was significantly decreased by N-allylsesecoboldine. This effect may contribute to the improvement of renal functions. Indeed, a recent study reveals that TNF-α causes renal failure in iNOS knockout endotoxemic mice (Knotek et al., 2001). Since LPS-induced increase in plasma NO⁻ level was unchanged by N-allylsesecoboldine, we propose that the decrease of TNF-α level by N-allylsesecoboldine during 120–240 min after LPS treatment may contribute to the improvement of renal functions. The cytokine-mediated induction of iNOS occurring in sepsis decreases systemic vascular resistance (Landry and Oliver, 2001), causes systemic hypotension, induces the increase of sympathetic outflow and the release of arginine vasopressin from the central nervous system, and activates the renin–angiotensin–aldosterone system, leading to vasoconstriction and a predisposition to acute renal failure (Schrier and Wang, 2004). Therefore, the mechanism of renal blood flow falling in endotoxemia is due to the systemic hypotension and the consequent increase of renal vascular resistance. Furthermore, intravenous LPS infusion in rat, α₁-adrenoceptor stimulation produces weaker than normal constriction of isolated aorta and mesenteric artery, but reactivity of the renal artery remains normal (Farmer et al., 2003). This implies that the renal microcirculation is distinct from other non-renal circulation systems in endotoxemia. Here, we showed that N-allylsesecoboldine failed to prevent the early phase decrease of renal blood flow, but significantly ameliorated the late phase of renal blood flow in this experimental endotoxemia (Table 3). However, this effect might not be mediated by the α₁-adrenoceptor antagonistic activity of N-allylsesecoboldine.

In normal condition, the glomerular filtrate is concentrated by the kidneys. Thus, the urinary nitrate level is higher than the plasma nitrate level in the sham group or in the rats before the challenge with LPS (Table 1). The plasma nitrate levels significantly increased within 240 min after LPS treatment, but the urinary nitrate declined and maintained low levels during endotoxemia (Table 1). This finding suggests that the urine concentrated function is impaired during the progress of LPS-induced endotoxemia. In addition, the osmotic diuretic action, which was mediated by the early phase increase of blood glucose during endotoxemia (Fig. 3A), may contribute to the dilution of urine nitrate. The treatment with N-allylsesecoboldine failed to prevent the acute increase of urination and the decrease of urine nitrate concentration and excretion at 120 min. However, a slight but significant recovery of urine nitrate excretion was observed in N-allylsesecoboldine-treated LPS rats at 240 min. In addition, the renal blood flow at 240 min, but not at 120 min, in the LPS rats treated with N-allylsesecoboldine (N-allylsesecoboldine/LPS) was higher than that in rats treated with LPS alone (Table 3). The recovery of urine nitrate excretion in N-allylsesecoboldine/LPS rats at 240 min could not be due to the increase of renal blood flow, but a recovery of renal tubular function including potassium excretion function was noted in the LPS rats treated with N-allylsesecoboldine (N-allylsesecoboldine/LPS) was higher than that in rats treated with LPS alone (Table 3). This implies that the renal preservation of renal functions by N-allylsesecoboldine in the LPS rats may be attributed to its suppression of a late inflammatory response to LPS. In fact, this was supported by the reduction of TNF-α secretion, neutrophils infiltration, and iNOS expression.

In addition, it should be emphasized that one must be cautious when using a large dose of N-allylsesecoboldine. This was based on our survival rate study showing that the large dose could not attenuate the mortality in mice treated with a lethal dose of LPS. We also observed that the use of N-allylsesecoboldine over 2 mg/kg (separated two times during 240 min) had no beneficial effects in most cardiovascular and renal parameters of endotoxemic rats (data was not shown). Although we did not know why the larger dose of N-allylsesecoboldine failed to protect animals against endotoxemia, it may be due to severe hypotension derived from the α₁-adrenoceptor antagonistic action (Fig. 1) as reported in human hyperplastic prostate (Guh et al., 1999). However, this remains to be clarified. This is also a major reason that we chose a lower dose of N-allylsesecoboldine in the experiment.

In conclusion, acute renal failure secondary to sepsis is a highly prevalent diagnosis in the ICU setting and continues to be associated with a high rate of morbidity and mortality. Our findings for the first time provided novel information regarding N-allylsesecoboldine, an α₁-adrenoceptor antagonist and antioxidiant, which effectively prevented acute renal failure and improved the survival rate in endotoxemic animals. The protection by N-allylsesecoboldine in animals with sepsis/septic shock seems to be associated with the suppression of iNOS, the reduction of TNF-α, the free radical-scavenging activity (e.g. scavenging NO⁻ and O₂⁻), and antioxidant capacity (Teng et al., 1996). In addition, N-allylsesecoboldine improved urinary potassium excretion rate in endotoxemia, suggesting that the renal tubule function is preserved by this agent in sepsis. Many bio-chemical values and renal parameters also indicated a better prognosis for endotoxemia with N-allylsesecoboldine. Thus,
N-allylsecoboldine seems to have a beneficial effect toward acute renal failure in sepsis. Collectively, the main action of N-allylsecoboldine, the amelioration of acute renal failure, contributes to the alleviation of sepsis/septic shock. However, additional studies are needed to elucidate the molecular mechanisms of renal dysfunction relief by N-allylsecoboldine, and to further comprehend its application for multiple terrible syndromes associated with sepsis/septic shock.

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References


