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Protective Effect of Neurotropin Against Lipopolysaccharide-Induced Hypotension and Lethality Linked to Suppression of Inducible Nitric Oxide Synthase Induction

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ABSTRACT—Neurotropin is a non-protein extract from the dermis of rabbits inoculated with vaccinia virus and has been clinically used as an analgesic in Japan. We present in the current report evidence for its potential therapeutic value against endotoxin shock. Administration of this compound prior to lipopolysaccharide (LPS) challenge resulted in a reversal of a decrease of the mean arterial pressure in rats and also amelioration of lethality in mice. Anti-inducible nitric oxide synthase (iNOS) Western blotting of tissue extracts from LPS-treated mice revealed almost complete suppression of iNOS induction by Neurotropin. The findings in vivo were reproduced in in vitro experiments in which cultured human umbilical vascular endothelial cells were challenged with LPS. Simultaneous treatment of the cells with Neurotropin resulted in complete suppression of iNOS induction and significant reduction of cell death. These results suggested a therapeutic value of Neurotropin in the treatment of endotoxin shock that was linked, at least in part, to suppression of iNOS induction and reduced cell damage in vascular endothelial cells.

Keywords: Neurotropin, Lipopolysaccharide, Hypotension, Inducible nitric oxide synthase, Endothelial cell

Several lines of evidence indicated that excess release of nitric oxide (NO) by vascular endothelial cells is the primary mechanism responsible for systemic hypotension in septic shock and that the excess release is caused primarily, if not exclusively, by lipopolysaccharide (LPS) that resides in the outer membrane of bacteria (1 – 4). Release of NO and its stable derivatives, nitrites and nitrates, is enhanced in rats treated with LPS and also in patients with septic shock (5 – 7). As in immunocytes, LPS induces expression of inducible NO synthase (iNOS) in vascular endothelial cells (1, 8, 9). As a consequence, arteries from LPS-treated animals contained a high concentration of cGMP and showed diminished contractile responses to norepinephrine and other vasocontrictors (4, 10). This hyporeactivity of the vessels was abolished in animals treated with antisense oligonucleotides against iNOS prior to LPS challenge (11). Accordingly, iNOS gene knockout mice were resistant to LPS-induced mortality (12).

Depending on these observations, NOS inhibitors N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl ester (L-NAME) have been tested both in animal models of septic shock (1) and in patients (13, 14) and shown to have protective effects against systemic hypotension. These drugs, however, are at present still in the phase of clinical trial.

Neurotropin is a non-protein extract from the dermis of rabbits inoculated with vaccinia virus. Neurotropin exerts immunomodulatory effects in several animal models with compromised immune functions (15 – 18). It also has anti-neuropain effects (19 – 22) and has been used clinically as an anti-allergic and analgesic drug (23). The analgesic effect of this compound, however, cannot be explained by its effect on the immune system alone. We demonstrated previously that in patients with Fabry disease, Neurotropin caused a strong and selective improvement of blood flow to painful extremities and decreased local levels of hypoxanthine (24). Restoration of the hypoxic state of extremities in this disease suggested its potential effect on the vascular endothelial cells. In support of this idea, Neurotropin has been shown to modulate the binding and metabolism of Factor XII and kininogen in cultured vascular endothelial cells (25).

The hypothesis to be addressed is that Neurotropin, with its potential effects on vascular endothelial cells, may have a therapeutic value in septic shock. To test the hypothesis,

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we examined the effects of Neurotropin against LPS toxicity in three experimental models: hypotension in anesthesized rats, lethality and iNOS induction in mice, and cell death of cultured human umbilical vascular endothelial cells (HUVEC).

MATERIALS AND METHODS

Materials

Male Fisher rats (230 – 250 g) and male std;ddy mice (25 – 30 g) were obtained from Japan SLC (Shizuoka). The animals had free access to water and standard laboratory diet. LPS (*Escherichia coli* 055:B5) was from Difco Laboratories (Surry, UK). Neurotropin was from Nippon Zoki Pharmaceutical Co. (Osaka). Medium 199 was from Gibco (Berkeley, CA, USA). Fetal bovine serum was from M.A. Bioproducts (Walkersville, MA, USA). Endothelial growth supplement was from Collaborative Reseach, Inc. (Lexington, MA, USA). A rabbit polyclonal antibody against the carboxy-terminus of mouse macrophage iNOS was from Affinity Bioreagents, Inc. (Golden, CO, USA). All other chemicals were of reagent grade and were obtained commercially.

Monitoring of arterial blood pressure in rats

The animals were anesthetized with pentobarbital (40 mg/kg, i.p.) and catheters were inserted into the femoral artery for drug delivery and measurement of blood pressure. Thirty minutes after the administration of Neurotropin (60 mg/kg) or saline, LPS (15 mg/kg) was administrated. Blood pressure was monitored every 10 min up to 3 h.

LPS-induced lethality of mice

Thirty mice in each group were injected intraperitoneally (i.p.) with Neurotropin (40 mg/kg) or PBS just before the injection of LPS (0.75 mg/mouse). The mortality of mice was monitored twice a day for 5 days.

Cell culture

HUVEC were obtained from umbillical cord vein and cultured in medium 199 containing 10% fetal bovine serum, 90 μg/ml heparin, 50 μg/ml endothelial growth supplement, 500 U/ml penicillin G and 100 μg/ml streptomycin (26). The number of cells was counted by a hemocytometer. The viability of cells was examined by a trypan blue dye exclusion method.

Measurement of NO

HUVEC were cultured on 96-well plates (Corning, Corning, NY, USA) at a density of 2×10^4/well. The cells were incubated in fresh medium containing LPS (0.01 – 100 μg/ml) in the presence or absence of either Neurotropin (10 or 100 μg/ml) or dexamethasone (1 μM). Twenty-four hours later, the level of NO in the medium was determined by a calorimetric assay (27). In brief, a medium aliquot (100 μl) was mixed with the same volume of Griess reagent (1% sulfanilamide and 0.1% *N*-1 naphthylethylenediamine dihydrochroride in 2% phosphoric acid). The mixture was incubated at 25°C for 10 min and optical densities at 540 nm were measured in a microplate reader (MPR-A4i; Tosoh, Tokyo). The concentration was determined using sodium nitrite as a standard.

NADPH-diaphorase activity

Cells cultured on a coverglass were exposed for 24 h to 0.1 μg/ml of LPS with or without Neurotropin (100 μg /ml) or dexamethasone (1 μM). The cells were washed twice with tris-buffered saline (TBS) and fixed in 4% paraformaldehyde/TBS for 10 min. They were incubated at 37°C for 30 min in a reaction mixture (0.1 M phosphate-buffer, pH 7.4, 0.3% Triton X-100, 0.1 mg/ml nitroblue tetrazolium, 1 mg/ml NADPH) (28). Following serial dehydration in ethanol and xylene, the sections were mounted on glass slides and photographed.

Immunoblotting of iNOS

Mice were injected intraperitioneally with LPS (0.75 mg /mouse, i.p.) simultaneously with Neurotropin (40 mg/kg, i.p.), dexamethasone (3 mg/kg, i.p.) or saline (i.p.). Twenty-four hours later, the animals were sacrificed by exsanguination, and the tissues were removed and frozen at −80°C. The frozen tissues were thawed and put in a lysis buffer (10 mM Tris-HCl, 1 mM Na orthovanadate, 150 mM NaCl, 0.2 mM PMSF and 0.5% Triton X-100). They were homogenized with a Dounce homogenizer and further incubated at 4°C for 20 min. HUVEC were scraped off in ice-cold PBS, collected by centrifugation, resuspended in the lysis buffer and incubated at 4°C for 20 min. The tissue or cell lysates were clarified by centrifugation at $2{,}000 \times g$ for 15 min at 4°C and the protein concentrations were determined using a BCA assay kit (Bio-Rad). Proteins were separated on SDS 7.5%-PAGE and electro-transferred to nitrocellulose membranes. The membranes were probed with anti-iNOS antibody and developed using HRP-anti-rabbit IgG and an ECL detection system (Amersham, UK).

Statistical analyses

Results are expressed as the mean ± S.E.M. for each experiment and analyzed by the unpaired Student's *t*-test and two-way ANOVA to determine the significant difference between means, and a P value of <0.05 was taken as significant.

RESULTS

Reversal of LPS-induced hypotension by Neurotropin in anesthetized rats

Intra-arterial administration of LPS (15 mg/kg) to anesthetized rats caused a significant decrease of the mean arterial pressure that manifested with a 1-h time lag and lasted at least for additional 1 h (29). In the group of rats pretreated with Neurotropin (40 mg/kg), LPS did not cause the decrease and rather increased the mean arterial pressure slightly above the control levels (Fig. 1). While four out of six rats died within 3 h following LPS administration, all of the six Neurotropin-treated rats survived this period. This dose of Neurotropin was equivalent to those applied to mice in experimental models of cerebral inflammation (30) and allergic reaction (18). There was no significant change of the mean arterial pressure in rats treated with Neurotropin alone (data not shown).

Neurotropin rescues mice from LPS-induced lethal toxicity

Given the acute effect of Neurotropin against LPS-induced hypotension, we investigated the effect of Neurotropin on LPS-induced lethal toxicity in a longer term using mice. At a dose of 0.75 mg per mouse, i.p. injection of LPS induced 77% lethality within 5 days. Simultaneous administration of Neurotropin (40 mg/kg, i.p.) reduced the lethality to 40% (Fig. 2). Both groups of the animals were immobile and had diarrhea during the first 2 days. Thereafter, more than half of the animals that had been treated with Neurotropin gradually recovered, and on the fifth day, they appeared healthy. There were no visible toxic manifestations in mice treated with Neurotropin alone. The animals of both groups that survived the initial 5 days were followed for additional several weeks; no life shortening or toxic effects were noted in either group.

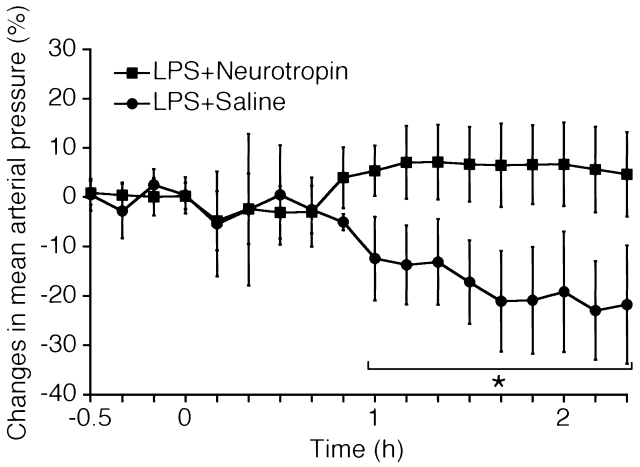

Fig. 1. Effect of Neurotropin on LPS-induced hypotension in anesthetized rats. Neurotropin (60 mg/kg) or saline was administrated 30 min before administration of LPS (15 mg/kg). Each point represents the mean ± S.D. of six experiments. *P<0.05, significantly different from the value at 30 min before LPS administration.

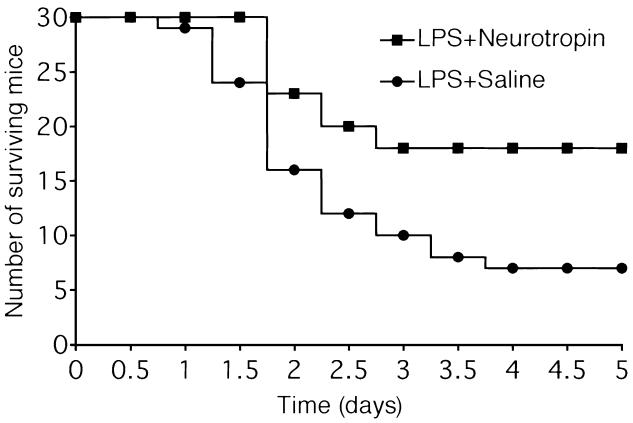

Fig. 2. Effect of Neurotropin on LPS-induced lethal toxicity in mice. LPS (0.75 mg/mouse) was i.p. injected with Neurotropin (40 mg/kg) or saline to 30 mice in each group.

Neurotropin inhibits induction of iNOS by LPS in mice

LPS-induced expression of iNOS and resultant excess release of NO is supposed to be the primary cause of systemic hypotension and multi-organ dysfunction. Therefore, we examined whether Neurotropin caused any change in the effect of LPS on the iNOS level in mice. On the western blotting of tissue proteins, LPS treatment (15 mg/kg, i.p. for 24 h) caused an obvious increase in the levels of iNOS in all of the three tissue examined (lung, liver, kidney) (Fig. 3). This increase was not detected in lung and liver samples from mice that were simultaneously treated with Neurotropin (40 mg/kg, i.p.). LPS failed to induce expression of iNOS protein in mice simultaneously treated with dexamethasone (3 mg/kg, i.p.) as reported previously (31).

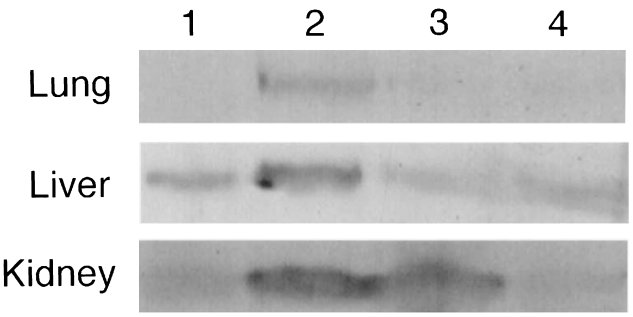

Fig. 3. Immunoblot analysis of iNOS synthase in mice. Tissue extracts were prepared from mice treated for 24 h with saline (lane 1), LPS (15 mg/kg, i.p.) plus saline (lane 2), LPS plus Neurotropin (40 mg/kg) (lane 3) or LPS plus dexamethasone (3 mg/kg) (lane 4). A sample of 30 μg protein was loaded on each lane.

Neurotropin inhibits induction of iNOS by LPS in HUVEC

The in vivo experiments described above suggested a protective effect of Neurotropin against LPS toxicity. To confirm this, we examined the effects of Neurotropin on iNOS induction by LPS in HUVEC. First, expression of iNOS was analyzed by anti-iNOS immunoblotting. The immunoreactive band of iNOS was not detected in the lysate from unstimulated cells, whereas a band with a molecular mass of 130 kDa clearly appeared in that from cells exposed to LPS (0.1 μg/ml) for 24 h (32). Simultaneous application of Neurotropin (100 μg/ml) prevented the LPS-induced expression of iNOS protein (Fig. 4). Dexamethasone (1 μM) also inhibited the LPS-induced expression (Fig. 4). These findings with anti-iNOS Western blotting were reproduced by in situ visualization of the NADPH-diaphorase activity. A faint NADPH-diaphorase activity was detected in untreated cells, whereas a large amount of NADPH-diaphorase positive cytoplasmic granules were detected in cells exposed to LPS (0.1 μg/ml for 24 h) (Fig. 5). Simultaneous application of Neurotropin (100 μg/ml) as well as that of dexamethasone (1 μM) prevented the appearance of the NADPH-diaphorase positive granules in LPS-treated cells (Fig. 5). We have also examined NO release by cells by Griess methods that detect NO metabolites in solution (27). Exposure of cells to LPS for 24 h caused a dose-dependent increase in NO release. Neurotropin at a dose of 10 μg/ml or 100 μg/ml almost completely abolished the effect of LPS. Again, the same effect was obtained with dexamethasone (1 μM) (Fig. 6).

Neurotropin inhibits cytotoxicity of LPS on HUVEC

We and others have demonstrated that LPS reduces viabilities of cultured endothelial cells and caused cell death in a long-term exposure (33). Given the suppression of iNOS induction by Neurotropin, we examined whether this compound could ameliorate the cytotoxicity of LPS (Fig. 7). Exposure to LPS for 24 h caused a dose-dependent increase of the number of dead cells as judged by a dye exclusion test. Simultaneous application of Neurotropin (10 or 100 μg/ml) caused a significant reduction of the number of dead cells. Dexamethatsone (1 μM) also reduced the number of the dead cells. At high concentrations of LPS (>10 μg/ml), Neurotropin (10 μg/ml) was more efficient in preventing cell death than dexamethasone (1 μM).

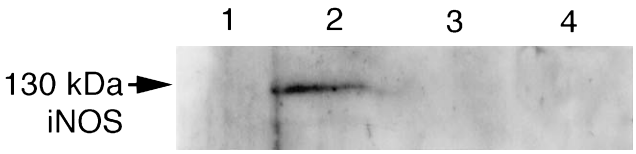

Fig. 4. Immunoblot analysis of iNOS synthase in HUVEC. Cell lysates were prepared from cells without drug treatment (lane 1) or from those treated for 24 h with LPS (0.1 μg/ml) (lane 2), LPS plus Neurotropin (100 μg/ml) (lane 3), LPS plus dexamethasone (1 μM) (lane 4). A sample of 30 μg protein was loaded on each lane.

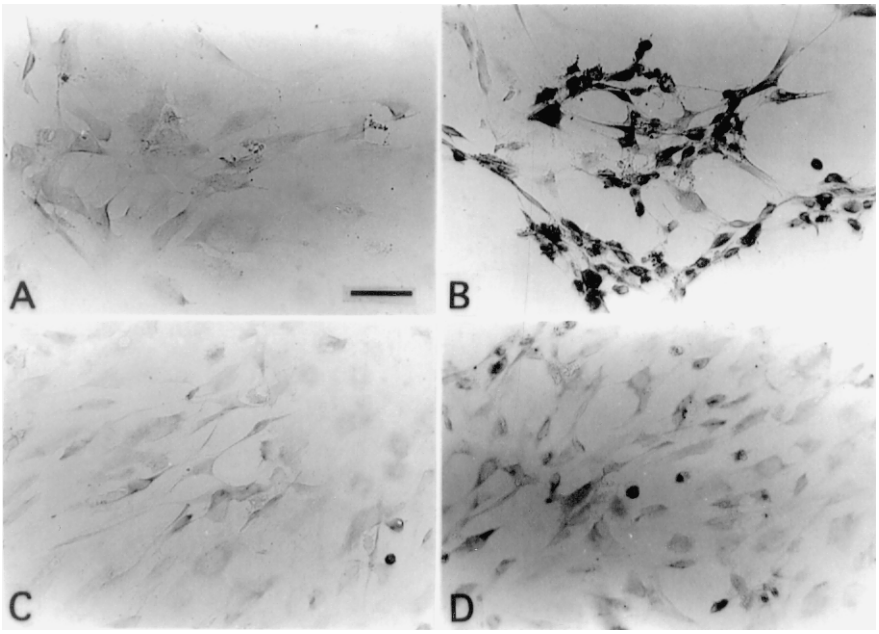

Fig. 5. Expression of NADPH-diaphorase in HUVEC. A: control. B – D: Cells were treated for 24 h with 0.1 μg/ml of LPS (B), LPS plus 100 μg/ml of Neurotropin (C), or LPS plus 1 μM of dexamethasone (D). Dark granules contain a NADPH-diaphorase activity. Scale bar = 50 μm.

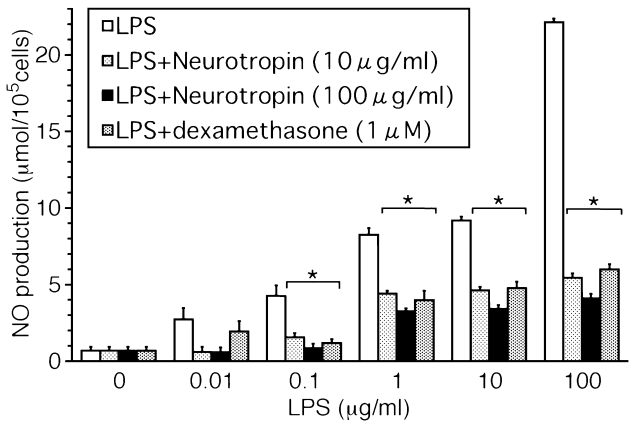

Fig. 6. Effect of LPS on NO production by HUCVEC and its modulation by Neurotropin and dexamethasone. The content of NO metabolites is expressed in μmol/10^5 viable cells. Values are the mean ± S.D. of 5 independent cultured determinations. *P<0.05, significantly different from the values from cells treated with LPS alone.

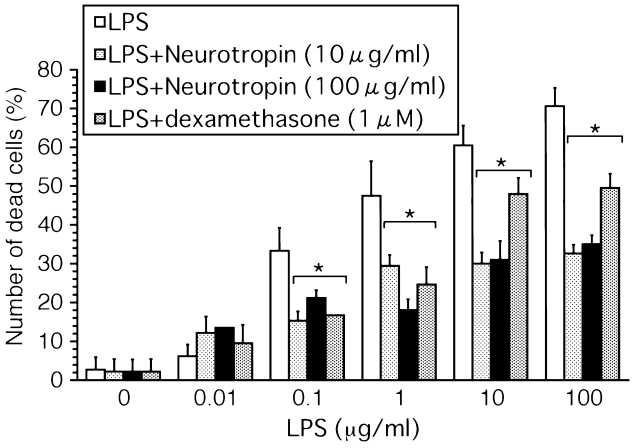

Fig. 7. Effect of LPS on viability of HUVEC and its modification by Neurotropin and dexamethasone. The number of dead cells was counted at 24 h after the application of the drugs and expressed as percentage of the total number (1×10^3) of cells. Values are the mean ± S.D. of 5 independent determinations. *P<0.05, significantly different from the values from cells treated with LPS alone.

DISCUSSION

In this study, we demonstrated protective effects of Neurotropin against LPS in two in vivo experimental models of endotoxin shock. Prior or simultaneous administration of this compound caused a remarkable effect in both systems. Neurotropin, at the dose that manifested no side-toxic effect, reversed the LPS-induced hypotension in rats (Fig. 1) and caused obvious reduction of lethality in mice (Fig. 2). LPS-induced shock is a complex pathological process that involves multiple systems including immune and cardiovascular systems (31, 34, 35). Previous reports suggest that not only prevention of hypotension but also iNOS suppression by NOS inhibitors lead to amelioration of tissue-damages and lethality in septic shock (36, 37). In accordance with this notion, the protective effect of Neurotropin against LPS lethality in mice correlated with suppression of iNOS induction in liver and kidney (Fig. 3). The suppression of iNOS induction was reproduced in in vitro experiments in HUVEC (Figs. 4 and 5). These observations suggested a therapeutic value of Neurotropin in septic shock and indicated that the in vivo protective effects of Neutropin were, at least partly, due to blockade of iNOS induction in vascular endothelial cells.

In addition to the induction of iNOS, the well-documented phenotypic change of endothelial cells caused by LPS is apoptosis, which is supposed to be one of the major causes of the multi-organ failure (38). In our experiments on HUVEC, Neurotropin was as effective as dexamethasone to suppress LPS-induced cell death, further supporting its therapeutic value in septic shock.

Neurotropin is a deproteinized extract from inflamed skin of rabbit inoculated with vaccinia virus and obviously contains multiple biologically active substances. The fact that both of the inhibitory effects of Neurotropin in HUVEC against LPS-induced iNOS induction and cell death were mimicked by dexamethasone may simply imply that Neurotropin contains glucocorticoids. This is, however, unlikely because of the lack of prominent side-effects of Neurotropin; that is, Neurotropin is administrated orally or by direct injection did not cause digestive ulcers (39). Neurotropin is not a single substance but a compound, and it is impossible to measure the blood concentration. Identification of the active substance contained in this compound and clarification of the mechanism of action must await further studies.

Septic shock is a major cause of death among patients in intensive care units. Except for supportive cares, no specific therapy is known, although a limited success has been reported for the use of antibodies against endotoxin (40). The therapeutic effects of NO synthase inhibitors such as L-NMMA (1, 13) and tyrosine kinase inhibitors such as tyrphostine and herbimycin A (41, 42) have been demonstrated in septic shock models. These drugs, however, have yet to undergo formal toxicological studies. When one considers the drug therapy of septic shock, an obvious advantage of Neurotropin over the NOS and tyrosine kinase inhibitors is that it has been used in Japan as an injectable drug in patients for over 35 years with few side effects.

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