Suppression of NO Production in Activated Macrophages *in Vitro* and *ex Vivo* by Neoandrographolide Isolated from *Andrographis paniculata*

Javzan Batkhuu,[a] Koichi Hattori,[a] Fumihide Takano,[a] Shinji Fushiya,*[,a] Ko-ichi Oshiman,[b] and Yoshiaki Fujimiya[b]

[a] *Experimental Station for Medicinal Plant Studies, Graduate School of Pharmaceutical Sciences, Tohoku University; Aoba, Aramaki, Aoba-ku, Sendai 980–8578, Japan: and* [b] *Tsukuba Research Institute, Sumitomo Forestry Co., Ltd.; 3–2 Midorigahara, Tsukuba 300–2646, Japan.* Received November 8, 2001; accepted April 24, 2002

In this study, we investigated the *in vitro* and *ex vivo* suppressive effects of *Andrographis paniculata* on nitric oxide (NO) production in mouse peritoneal macrophages elicited by bacillus Calmette-Guéin (BCG) and stimulated by lipopolysaccharide (LPS). Incubation of BCG-induced macrophages with the methanol extract of *A. paniculata* reduced LPS stimulated NO production. The diterpene lactones andrographolide and neoandrographolide were isolated as active components from the extract. These compounds suppressed NO production in a concentration-dependent manner in the concentration range from 0.1 to 100 μM and their IC_{50} values were 7.9 and 35.5 μM. Neoandrographolide also suppressed NO production by 35 and 40% when the macrophages were collected after oral administration of neoandrographolide at doses of 5 and 25 mg/kg/d and LPS stimulated NO production was examined. However, andrographolide did not reduce NO production on oral administration at the same doses. These results indicate that neoandrographolide, which inhibited NO production both *in vitro* and *ex vivo* may play an important role in the use of *A. paniculata* as an anti-inflammatory crude drug.

Key words neoandrographolide; andrographolide; nitric oxide; macrophage; inflammation; *Andrographis paniculata*

Inflammatory macrophages play a central role in the inflammatory process by secreting large amounts of mediators that control the initiating process of inflammation.[1)] Nitric oxide (NO) is one of the critical mediators continuously produced by the inducible isoform of NO synthase (iNOS) in inflammatory macrophages when stimulated by lipopolysaccharide (LPS) and some cytokines.[2)] NO generated by iNOS is important in non-specific immune defense against tumor growth and infections by parasites, bacteria and protozoa.[2)] NO is also known to be responsible for the hypotension observed in sepsis.[3)] Glucocorticoides, representative anti-inflammatory agents, are well known for strongly reducing NO production in LPS-activated bacillus Calmette-Guéin (BCG) primed macrophages.[4)] Therefore, it is a beneficial method for estimating anti-inflammatory agents to examine the suppressive effects on NO production in LPS-activated macrophages. We have evaluated the suppressive effects on NO production of various crude drugs that are claimed to be immunosuppressents, and from these, *Andrographis paniculata* Burm. f. Nees (Acanthaceae) was selected for this study.

A. paniculata is one of the Chinese herbs reputed to be effective in the treatment of cold, diarrhea, fever, and inflammation.[5)] Earlier work showed that andrographolide, an active component of this plant, has multiple pharmacological activities such as protozoacidal activity,[6)] inhibitory activity on platelet aggregation,[7)] inhibition of protein convertases-1, -7 and furin,[8)] stimulation of cell differentiation,[9)] anti-hepatotoxic activity,[10—12)] and immunostimulative activity.[13)] Chiou *et al.*[14,15)] recently reported that andrographolide inhibited LPS-induced NO production in an interferon-γ-primed RAW 264.7 macrophage cell line through a post-transcriptional mechanism and improves the hypotension caused by LPS in rat.

In this paper, we report the inhibitory effects of andrographolide and neoandrographolide, the major constituents of *A. paniculata*, on LPS-induced NO production in bacillus Calmette-Guéin primed mouse peritoneal macrophages, after oral administration to mice and by direct addition to the cultured macrophages.

MATERIALS AND METHODS

Experimental Animals Male ICR mice (5 to 6 weeks of age) were purchased from Japan SLC (Hamamatsu, Japan). The mice were housed in plastic cages at a constant temperature (25±1 °C) with a light–dark rhythm of 12 h–12 h and relative humidity (55 to 60%) with free access to water and standard diet. An adoption period to these conditions of at least one week was used for the experiments.

Reagents LPS (*Escherichia coli* 055: B5) and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). BCG was from Nippon BCG (Tokyo, Japan). Phosphate buffered-saline (PBS), phenol red-free RPMI 1640 medium and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY, U.S.A.). Cell culture medium was supplemented with 50 μg/ml penicillin, 50 U/ml streptomycin and 5% FBS. All other reagents, whose suppliers are not indicated, were purchased from Wako Pure Chem. Co. (Tokyo, Japan).

Extraction of *A. paniculata* and Isolation of Andrographolide and Neoandrographolide *Andrographis paniculata* Burm, f. Nees (Acanthaceae) was collected in Yogyakarta Jawa, Indonesia in 1999. The dried leaves (300 g) were macerated in methanol (4.0 l×2 times) and kept at room temperature for 3 d. After filtration, the methanol solution was evaporated under reduced pressure to give the methanol extract (20.5 g). The extract was partitioned between ethyl acetate and water to give an ethyl acetate soluble portion (8.0 g) and a water soluble portion. The latter portion was extracted with *n*-butanol to give the *n*-butanol soluble portion (4.6 g). Filtration of the precipitate formed in the ethyl acetate soluble portion gave crude neoandrographolide (264 mg), which was chromatographed on a silica gel column (25 g) using chloroform/methanol (20/1) as a solvent to yield

* To whom correspondence should be addressed. e-mail: fushiya@mail.pharm.tohoku.ac.jp

pure neoandrographolide (212 mg, MW: 480). Filtration of the precipitate formed in the *n*-butanol soluble portion gave crude andrographolide (1.96 g), a part of which (770 mg) was chromatographed on a silica gel column (50 g) using chloroform/methanol (20/1) as a solvent to yield pure andrographolide (619 mg, MW: 350). The identification of andrographolide (Fig. 1A) and neoandrographolide (Fig. 1B) was carried out by comparison of their spectral data with those in the literature[16] and by direct comparison with authentic samples.

Assay for NO Production in BCG-Induced Mouse Peritoneal Macrophages Four days after intraperitoneal injection of 1 mg/mouse of BCG (suspended in pyrogen-free saline, 0.2 ml/mouse) into ICR mice ($n=5$ to 8), peritoneal elicited cells (PEC) were washed out with PBS (pH=7.4) through the anterior abdominal wall. PEC suspended in culture medium at 1.0×10^6 cells/ml were seeded on 96 well tissue culture plates (Becton Dickinson, Oxnard, CA, U.S.A.) (200 μl/well) and were incubated for 2 h in a humidified CO_2 incubator. Then non-adherent cells were gently removed twice with fresh medium. Remaining adherent cells (>95% macrophages, judged by nonspecific esterase stain) were cultured with the same medium containing 1 to 10 μg/ml LPS for 24 h.[17] To measure the amount of NO_2^- derived from NO in culture, 100 μl of culture supernatant was taken from each well and transferred into a 96 well plate (Becton Dickinson), and mixed with 100 μl of Griess reagent (1 w/v% sulfanilamide, 0.1 w/v% *N*-(1-naphthyl)ethylenediamine dihydrochloride, 3 v/v% H_3PO_4).[18] After chromophore was formed at room temperature for 10 min, absorbance was determined at 510 nm using an Immunoreader (InterMed, Tokyo, Japan).

Macrophage Cell Viability Cell viability was analyzed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium Br (MTT) method.[19] In brief, MTT solution (10 μl of 5 mg/ml in PBS) was added to each well after removal of 100 μl supernatant for NO assay (2.0×10^5 cells/100 μl in 96 well plate). The macrophages were incubated for 4 h in a CO_2 incubator at 37 °C. The reduced form of MTT-formazan was solubilized with 100 μl of detergent (10 w/v% SDS in 0.05 N HCl) at 37 °C for 18 h, and the absorbance was read at 590 nm using an Immunoreader.

Test Sample Treatment Methanol extract, ethyl acetate soluble portion, andrographolide, and dexamethasone were dissolved in PBS containing ethanol. The *n*-butanol soluble portion and neoandrographolide were dissolved in PBS. For *in vitro* assay, macrophages were cultured in the medium containing LPS in the presence or absence of test samples (final ethanol concentration: 0.3%). Ethanol (0.3%) in PBS did not show any significant effect on NO production. For *ex vivo* assay, each sample was orally administered to mice once daily for five consecutive days before and after BCG-immunization (the final treatment was done 2 d before harvesting the macrophages). Four days after BCG-injection, PEC were collected and cultivated, and the LPS-stimulated NO production was examined by the above method. The control group received the vehicle (PBS–ethanol or PBS) instead of the test sample.

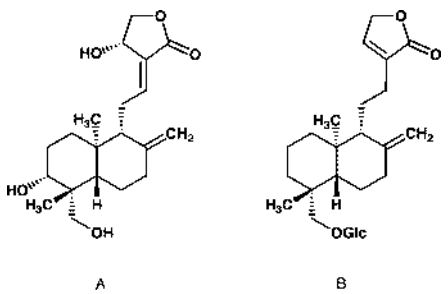

Fig. 1. Chemical Structures of Andrographolide (A) and Neoandrographolide (B)

Statistics All data are expressed as mean±S.E. The percent of inhibition was calculated as follows: inhibitory ratio of NO production (%)=[1−{(NO production (μM) in macrophages with test sample in the presence of LPS−NO production (μM) in macrophages with test sample alone)/(NO production (μM) in macrophages cultured with LPS alone−NO production (μM) in macrophages alone)}]×100. NO production by macrophages stimulated with LPS alone (LPS control) under the individual conditions was indicated as NO_2^- amounts (μM) in figure legends. Differences in data for NO production inhibitory activity or cell viability between control and experimental groups were analyzed by using Dunnett's multiple test after analysis of variance (ANOVA) test, and the differences were considered significant if $p<0.05$.

RESULTS

Suppression of NO Production in Cultured Macrophages by Andrographolide and Neoandrographolide Activated macrophages isolated from BCG-primed ICR mice produced a large amount of NO (60.5±3.2 μM/2×10^5 cells) upon stimulation with 10 μg/ml of LPS. It is well known that BCG produces interferon-γ in T lymphocytes, and this lymphokine leads macrophages to enhance the biosynthesis of iNOS in response to LPS.[20—22] The methanol extract of *A.*

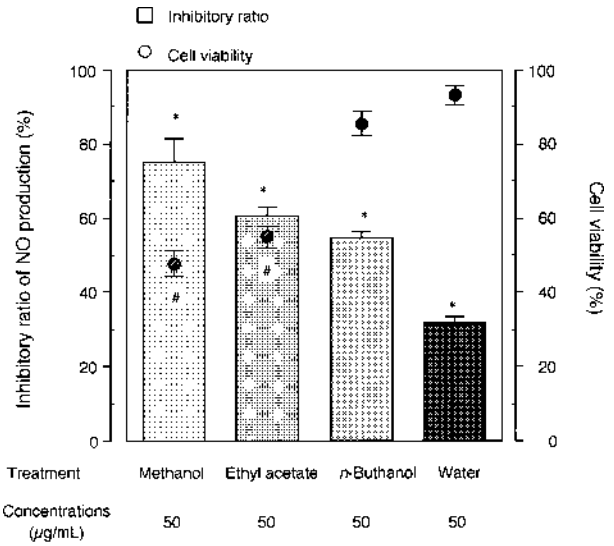

Fig. 2. Effects of the Fractions of *A. paniculata* on NO Production in BCG-Elicited Macrophages Stimulated with LPS

Cells were cultured with 10 μg/ml LPS together with test samples and NO_2^- contents in the supernatants of the cultured macrophages were measured at 24 h after LPS triggering. Data for inhibition of NO production in response to LPS are expressed as mean (%)±S.E. (NO production, LPS alone: 60.5±3.2 μM/2×10^5 cells). Data for cell viability are indicated as percentage of absorbance of test sample-treated macrophages to that of non-treated macrophages. Statistically significant from control group, $*, {}^{\#}p<0.05$.

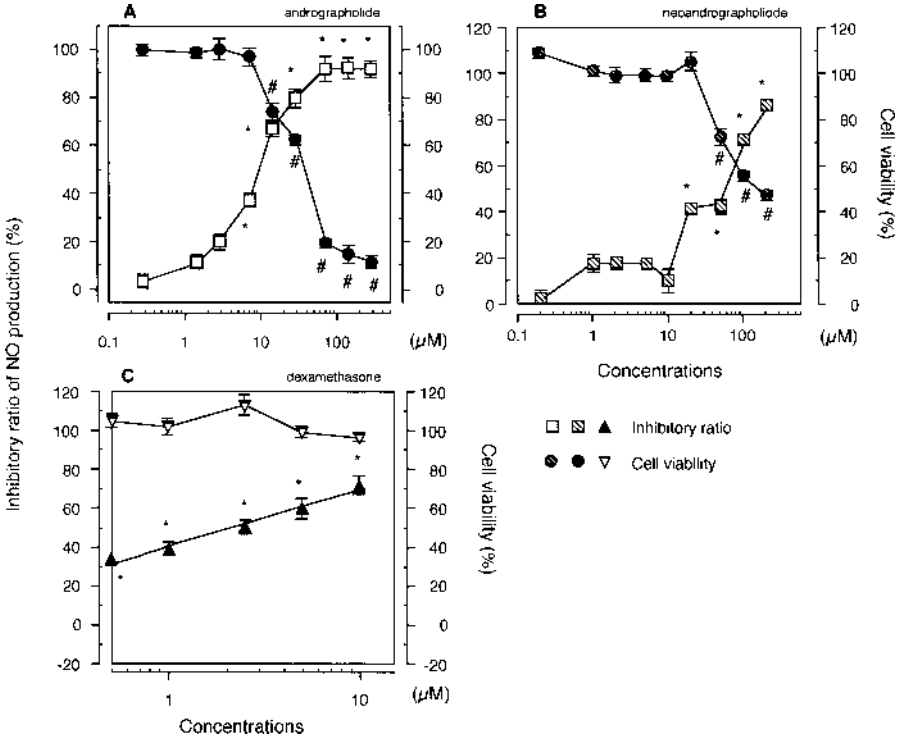

Fig. 3. Effects of Andrographolide (Panel A), Neoandrographolide (Panel B), and Dexamethasone (Panel C) on NO Production and Cell Viability

Cells were cultured with various concentrations of andrographolide, neoandrographolide, or dexamethasone together with 10 μg/ml LPS for 24 h, and NO_2^- content and cell viability were measured. Data for inhibition of NO production in response to LPS are expressed as mean (%)±S.E. (NO production, LPS alone: 56.4±1.8 μM/2×10^5 cells). Data for cell viability are indicated as percentage of absorbance of test sample-treated macrophages to that of non-treated macrophages. Statistically significant from control group, *,$^{\#}$ $p<0.05$.

paniculata reduced NO production by 75.1±6.3% for 24 h cultivation at a concentration of 50 μg/ml (Fig. 2). Among the three portions separated from the methanol extract, the ethyl acetate and *n*-butanol soluble portions reduced NO production. That is, the ethyl acetate soluble portion inhibited NO production by 60.6±2.2% and the *n*-butanol soluble one inhibited it by 54.6±1.6% at a concentration of 50 μg/ml (Fig. 2). Since neoandrographolide and andrographolide were isolated as main constituents from the ethyl acetate and *n*-butanol soluble portions, their effects on NO production were examined. In this experiment, the test samples showed no influence on the Griess reaction and caused no NO production in the cultured macrophages in the absence of LPS (data not shown). As shown in Fig. 3, andrographolide reduced NO production in a concentration dependent manner with an IC_{50} value of 7.9 μM. This result is in accord with the report that andrographolide suppressed LPS-induced NO production in the RAW 264.7 murine macrophage cell line.[14,15] Neoandrographolide showed the same inhibitory pattern with andrographolide and the IC_{50} value was 35.5 μM. Both compounds, however, showed cytotoxic activities to macrophages at concentrations ranging from 10 to 300 μM (Figs. 3A, B). In contrast, dexamethasone, a representative anti-inflammatory glucocorticoid suppressed NO production without cytotoxicity.

To determine if andrographolide and neoandrographolide interfere with the LPS triggering process, 10 μM andrographolide or 40 μM neoandrographolide was added to the cultured macrophages and the culture was pre-incubated for 0.5, 1, 2, 4, or 8 h. After removal of the test sample by washing with fresh medium, the macrophages were cultivated in fresh medium with LPS for a further 24 h. The inhibition of NO production by andrographolide was increased in proportion to the pre-incubation time (Fig. 4A). That is, andrographolide inhibited the NO production by 61.3±2.7%, 75.5±1.0% and 83.3±1.6% for 2, 4, and 8 h pre-incubation, respectively. Neoandrographolide showed the same pattern of inhibition, but the effect was weaker than that of andrographolide (Fig. 4B). The pattern of reduction of the NO production by dexamethasone (5 μM) was different from those by andrographolide and neoandrographolide. That is, the reduction by dexamethasone slowly increased in the later stage of incubation (Figs. 4A—C).

Effects of Oral Administration of Andrographolide and Neoandrographolide on NO Production Since andrographolide and neoandrographolide inhibited NO production when directly added to cultured macrophages with LPS, we next examined the effect of these compounds on NO production upon oral administration. Thus, andrographolide, neoandrographolide and dexamethasone were injected orally into mice once a day for five consecutive days before (day −2) and after (day +2) BCG-immunization (day 0). Macrophages were collected on the fourth day after BCG-injection. Cultivation of the macrophages and measurement of LPS-stimulated NO production were carried out by the same method used in the *in vitro* experiment. Neoandrographolide suppressed NO production in a characteristic manner. That is, neoandrographolide reduced NO production by 35 and 40% at doses of 5 and 25 mg/kg/d, respectively (Fig. 5A), but did not reduce it at doses of 1 and 50 mg/kg/d (data not shown in the case of 1 mg/kg/d). On the other hand, andrographolide inhibited NO production at a dose of 5 mg/kg/d by 13.3±5.5%, but did not inhibit it at any other dose (Fig. 5A). Re-

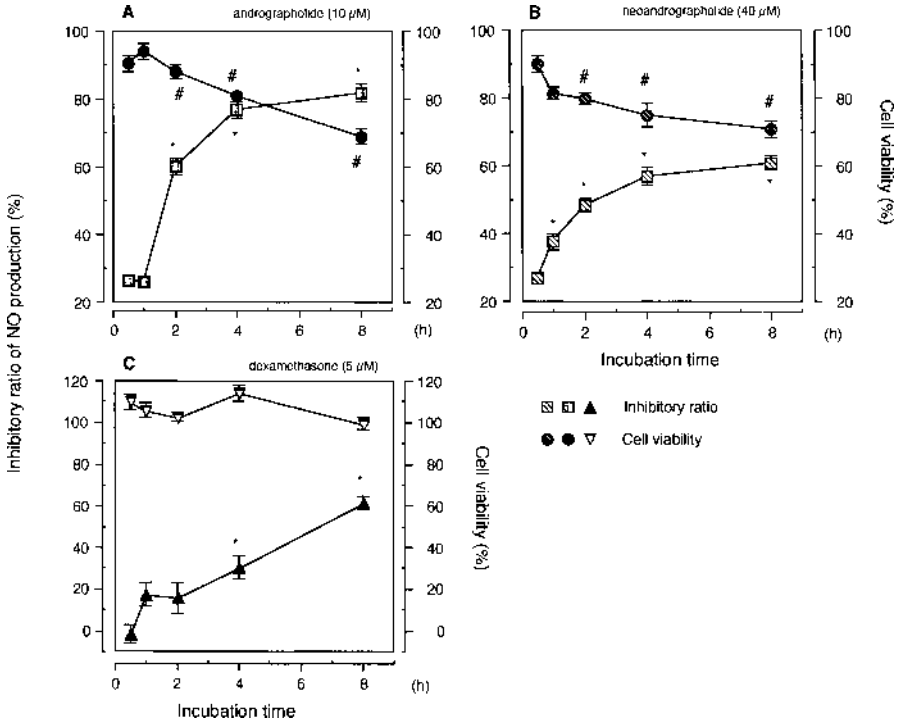

Fig. 4. Effects of Andrographolide (Panel A), Neoandrographolide (Panel B), and Dexamethasone (Panel C) on NO Production in Macrophages Treated for Different Time Periods

Macrophages were treated with 10 μM andrographolide, 40 μM neoandrographolide, or 5 μM dexamethasone for 0.5, 1, 2, 4, or 8 h before LPS challenge. Cells were then washed out twice with warmed PBS and 10 μg/ml LPS was added to each well. NO_2^- content and cell viability in the presence of LPS were measured. Data for inhibition of NO production and cell viability for 24 h after LPS triggering are expressed as mean (%)±S.E. of each sample compared with LPS alone-treated control culture (NO production, LPS alone: 64.1±3.8 μM/2×10^5 cells for 0.5 h, 55.5±2.3 μM for 1 h, 56.0±1.9 μM for 2 h, 37.7±4.8 μM for 4 h, and 17.6±1.5 μM for 8 h, respectively). Statistically significant from control group, $*, {}^{\#}p<0.05$.

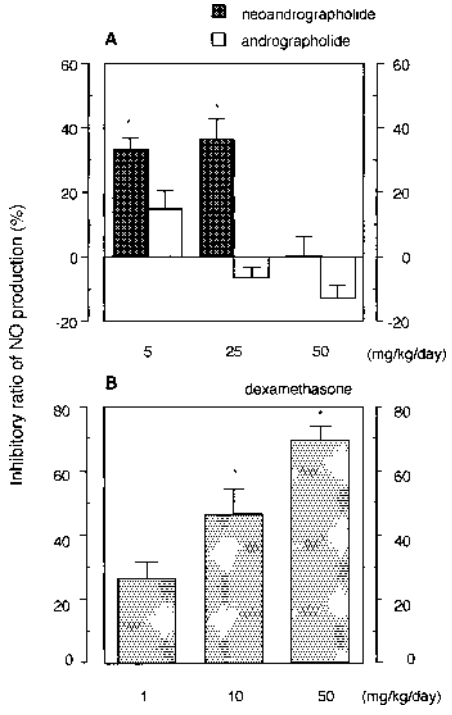

Fig. 5. Effects of Orally Administrated Andrographolide (Panel A), Neoandrographolide (Panel A), and Dexamethasone (Panel B) on NO Production

Test samples were administered orally once daily for five consecutive days before and after (day −2 to day +2) BCG-injection (as day 0). Peritoneal macrophages from test sample or vehicle-administrated mice were collected at day 4 after BCG-immunization. NO_2^- content in the macrophage culture supernatant was measured at 24 h after LPS stimulation. Data for inhibition of NO production for 24 h after LPS stimulation are expressed as mean (%)±S.E. (NO production, LPS alone: 58.0±6.6 μM). Statistically significant from control mice, $*p<0.05$. Number of mice=9 to 10.

Table 1. Effects of Orally Administrated Andrographolide, Neoandrographolide and Dexamethasone on BCG Primed Mouse Peritoneal Inflammatory Cell Numbers and Cell Viabilities

Treatment	Dose (mg/kg/d)	Cell No. ($\times10^6$ cells/mouse)	Cell viability (%)	n
Control	—	11.9±3.2	98.3±0.6	4
Andrographolide	5	10.2±1.1	94.2±0.9	4
	25	11.7±3.6	96.2±0.4	4
	50	12.6±2.2	97.9±0.4	4
Neoandrographolide	5	12.0±1.9	94.4±1.1	4
	25	14.2±3.4	93.3±0.4	4
	50	10.1±2.0	97.1±0.5	4
Dexamethasone	5	6.3±1.0*	90.6±2.7	4

ICR mice were injected intraperitoneally with 1 mg of BCG, and 4 d later, peritoneal exudate cells were collected and counted by hemocytometer with trypan blue stain. All test samples were administrated orally once daily for five consecutive days before and after (day −2 to day +2) BCG-injection (as day 0). Data are expressed as mean±S.E. Statistically significant from control mice, $*p<0.05$. n=number of mice.

duction of NO production by dexamethasone was dose dependent in the dose range from 1 to 50 mg/kg/d. Andrographolide and neoandrographolide did not affect either the number of peritoneal exudate cells or the cell viability in this experiment (Table 1), whereas dexamethasone decreased both the cell number and the cell viability at a dose of 5 mg/kg/d.

We next evaluated the effect of neoandrographolide and andrographolide on the NO production in macrophages stimulated with various concentrations of LPS. That is, BCG-primed macrophages isolated from neoandrographolide- or andrographolide-administered mice were exposed to serially diluted LPS ranging from 0.001 to 10 μg/ml for 24 h. As

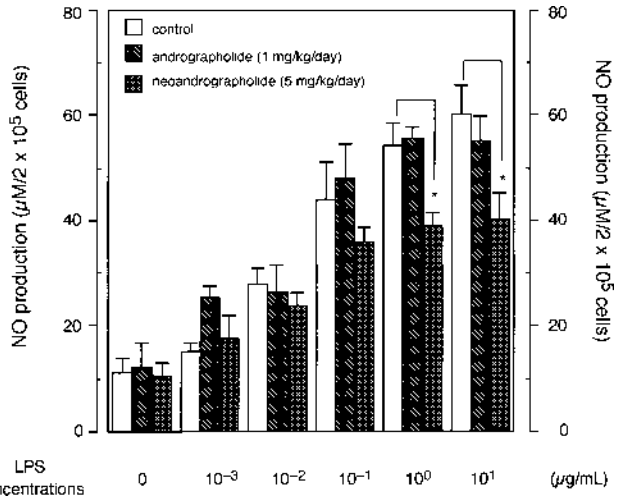

Fig. 6. Effects of Orally Administrated Andrographolide and Neoandrographolide on NO Production at Various Concentrations of LPS

Andrographolide (1 mg/kg/d) or neoandrographolide (5 mg/kg/d) was administrated orally once daily for five consecutive days before and after (day −2 to day +2) BCG-injection (as day 0). Peritoneal macrophages from test sample or vehicle-administrated mice were collected at day 4 after BCG-immunization. NO_2^- content in the macrophage culture supernatant was measured at 24 h after LPS challenge. Data are expressed as mean (μM)±S.E. Statistically significant from control mice, $*p<0.05$. Number of mice=5 to 8.

shown in Fig. 6, the NO production was enhanced with increase of the LPS concentration in the control study. When orally administrated at a dose of 5 mg/kg/d, neoandrographolide inhibited the NO production and the effect was potent at LPS concentrations of 1.0 and 10 μg/ml. On the other hand, andrographolide (1 mg/kg/d) did not restrain NO production at any LPS concentration.

DISCUSSION

In this study, the suppressing effect of *A. paniculata* on NO production was evaluated by using a cell culture system of BCG-primed mouse peritoneal macrophages, which produced a large amount of NO in response to LPS. NO production was inhibited by the methanol extract *in vitro* (Fig. 2), and two diterpenoid lactones, andrographolide and neoandrographolide (Figs. 1A, B), were isolated as active constituents from the extract. Inhibition of NO production by andrographolide and by neoandrographolide was concentration dependent (Figs. 3A, B). Andrographolide has been reported to inhibit LPS-induced NO production in interferon-γ-primed RAW 264.7 macrophages with an IC_{50} value of 17.4 μM and to have no toxic effect toward the macrophages during 24 h incubation.[14,15] In contrast, andrographolide inhibited NO production in mouse peritoneal macrophages with an IC_{50} value of 7.9 μM but showed a cytotoxic action to the macrophages after 24 h cultivation (Fig. 3B). These discrepancies might be attributable to the origin of the macrophages. In this study, we first revealed that neoandrographolide, an analogue of andrographolide having a glucose moiety, inhibited the NO production in activated macrophages. The inhibition by neoandrographolide was concentration dependent but weaker than that of andrographolide. Neoandrographolide showed a moderate cytotoxic effect (Fig. 3A). Accordingly, the suppression of NO production in cultured BCG-induced mouse peritoneal macrophages by andrographolide and neoandrographolide is attributed in part to their cytotoxic effects.

The cytotoxic effects of andrographolide and neoandrographolide were weakened when they were pre-incubated with the macrophages. That is, 10 μM andrographolide inhibited NO production by 61.3±2.7% on 2 h incubation prior to LPS treatment and the cell viability was 89.4±2.3%. Further, 40 μM neoandrographolide inhibited NO production by 56.9±2.0% upon 4 h pre-incubation and the cell viability was 74.2±1.1% (Figs. 4A, B). On the other hand, dexamethasone needed a longer pre-incubation time for inhibition. Thus, 5 μM of dexamethasone inhibited NO production by 31.5±1.6% and 55.6±1.6% on 4 h and 8 h pre-incubation, respectively (Figs. 4A—C). Dexamethasone is well known to suppress NO production in activated macrophages by decreasing iNOS gene (mRNA) transcription.[23] As mentioned above, Chiou *et al.* has demonstrated that andrographolide inhibited NO synthesis in RAW 264.7 cells by reducing the expression of iNOS protein at a post-transcriptional stage, not by altering the transcription of iNOS mRNA.[15] It can therefore be presumed that the apparent discrepancy between dexamethasone and andrographolide *in vitro* might be due to the difference in their intracellular mechanisms.

In the *ex vivo* experiment, andrographolide, neoandrographolide and dexamethasone were orally administrated to mice before isolating macrophages which were then cultivated and stimulated by LPS. Andrographolide slightly decreased NO production only at a dose of 5 mg/kg/d (not significant) and did not affect it at doses of 1 mg/kg/d (data not shown) and 50 mg/kg/d (Fig. 5A). Chiou *et al.*[14] showed that intravenous injection of andrographolide (1 mg/kg) before or after LPS injection significantly reversed LPS-induced hypotension in rats. They reported that this effect might be due to the inhibition of NO production and iNOS induction. It is not clear why oral administration of andrographolide did not reduce NO production in our experiment, however, it may be attributable, in part, to differences in the administration mode and the animal species, since these will affect drug metabolism and pharmacokinetics. Further studies are required to clarify the discrepancy. On the other hand, neoandrographolide apparently reduced NO production by 33.1±2.0 and 36.0±4.7% upon oral administration at doses of 5 and 25 mg/kg/d, respectively. The inhibition of NO production by neoandrographolide, however, was abolished at a dose of 50 mg/kg/d. Therefore, neoandrographolide may require the optimal dose(s) for the inhibition of NO production. The same phenomenon has been observed in the case of the anti-malarial compound febrifugine, which needed an optimal dose to enhance NO production. This enhancement of NO production was well correlated with the anti-malarial activity of febrifugine.[24] Inhibition of NO production by orally administrated neoandrographolide (5 mg/kg/d) was enhanced with an increase in the LPS concentration (Fig. 6). It is well known that the production of inflammatory mediators including NO in activated macrophages responds to LPS concentration and the sensitivity to LPS.[25—27] Thus these results indicate that neoandrographolide decreases the sensitivity of the macrophages to LPS.

In this experiment, no significant toxic effect was observed by oral administration of andrographolide and neoandrographolide to mice (50 mg/kg/d, once daily for five consecu-

tive days), which was judged by the fact that the weights of the mice did not change and the activities of the serum hepatic marker enzyme (L-alanine aminotransferase: ALT, EC 2.6.1.2.) did not increase (ALT levels, control: 18.3±3.5 U/l, $n=3$, andrographolide: 16.6±2.0 U/l, $n=4$, neoandrographolide: 18.6±4.6 U/l, $n=4$).

It is worth noting that neoandrographolide inhibited NO production in LPS-activated macrophages on oral administration. As NO is a critical mediator in inflammation, these results suggest that neoandrographolide may be one of the effective constituents of *A. paniculata*, which has been used as an anti-inflammatory crude drug.

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