Examination of the Nitric Oxide Production-Suppressing Component in *Tinospora tuberculata*

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The component of aqueous *Tinospora tuberculata* extract that inhibits nitric oxide (NO) production was examined using macrophages activated by the addition of lipopolysaccharide. The aqueous extract was partitioned with ethyl acetate. The aqueous layer was fractionated with a Diaion column. The residue of the aqueous extract was extracted with methanol, and partitioned with ethyl acetate. The ethyl acetate layer was found to be associated with a distinct decrease in the NO level and inducible NO synthase. On further fractionation, the subfraction of E-3 showed high anti-NO activity. *N-trans*-Feruloyltyramine isolated from E-3 was identified as exhibiting strong anti-NO activity. This compound is the most active component of *Tinospora tuberculata* with respect to the suppression of NO production.

Key words *Tinospora tuberculata*; *N-trans*-feruloyltyramine; nitric oxide; inducible nitric oxide synthase; macrophage

Tinospora tuberculata, a plant belonging to the family Menispermaceae, is distributed from the southwestern part of China to Southeast Asia including Vietnam, Thai, Malaysia, Indonesia and India, and is used widely as a folk medicine. This galenical has been used for its antipyretic, abdominal pain-relieving, antidiarrheal, detoxifying, anthelmintic and tonic actions.[1)] The pharmacological mechanisms of this agent, however, are mostly unknown. Focusing on nitric oxide (NO), which mediates various physiological events including defense reactions of the body and whose increase leads to the development of various diseases,[2)] we previously studied the effect of *Tinospora tuberculata* in mice and found that an aqueous extract inhibited the production of NO.[3,4)] The present study was designed to investigate the inhibitory activity of the aqueous extract of *Tinospora tuberculata*, using lipopolysaccharide (LPS)-stimulated macrophages.

MATERIALS AND METHODS

Reagents Thioglycollate broth was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Aminoguanidine, LPS, pepstatin A, chymostatin, aprotinin, phenylmethylsulfonyl fluoride, β-nicotinamide adenine dinucleotide phosphate (reduced, NADPH), flavin adenine dinucleotide (FAD), tetrahydrobiopterin, dithiothreitol (DTT), lactate dehydrogenase (LDH) and sodium pyruvate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Fractionation and Purification of *Tinospora tuberculata* As shown in Fig. 1, dried stems of *Tinospora tuberculata* (1 kg) were powdered and extracted with water (3 l) at 60—70 °C three times. The residue was further extracted with methanol (3 l) three times at room temperature. The aqueous extract was concentrated and partitioned between ethyl acetate and water five times to give ethyl acetate extract (fraction A, 7.7 g). The acqueous layer was further separated into three fractions [fractions B (110 g), C (29.6 g), D (7.1 g)] by Diaion HP-20SS column chromatography with water containing increasing proportions of methanol. The methanol extract was also partitioned between ethyl acetate and water to afford ethyl acetate extract (fraction E, 15.2 g) and an aqueous layer (fraction F, 16.6 g). The biologically active fraction E (14.3 g) was further separated by silica gel column chromatography (chloroform–methanol–water) into five fractions [fractions E-1 (1.39 g), E-2 (4.42 g), E-3 (2.73 g), E-4 (3.01 g), E-5 (3.25 g)]. The major constituent of fraction E-3 (1.7 g) was purified by Sephadex LH-20 (80% methanol) followed by silica gel column chromatography (hexane–ethyl acetate, 1 : 2) to yield *N-trans*-feruloyltyramine (370 mg), which was identified by spectral comparison.

Spectral Data of *N-trans*-Feruloyltyramine A white powder, FAB-MS *m/z*: 314 $(M+H)^+$, IR (diffusive reflection) ν_{max} cm^{-1}: 3300, 1652, 1587, 1510. UV λ_{max}^{EtOH} nm (ε): 292 (13600), 319 (16600). ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 7.987 (1H, t, J=5.7 Hz, NH), 7.311 (1H, d, J=15.7 Hz, H-7′), 7.112 (1H, d, J=1.8 Hz, H-2′), 7.016 (2H, br d, J=8.5 Hz, H-2, 6), 6.983 (1H, dd, J=1.8, 8.1 Hz, H-6′), 6.789 (1H,

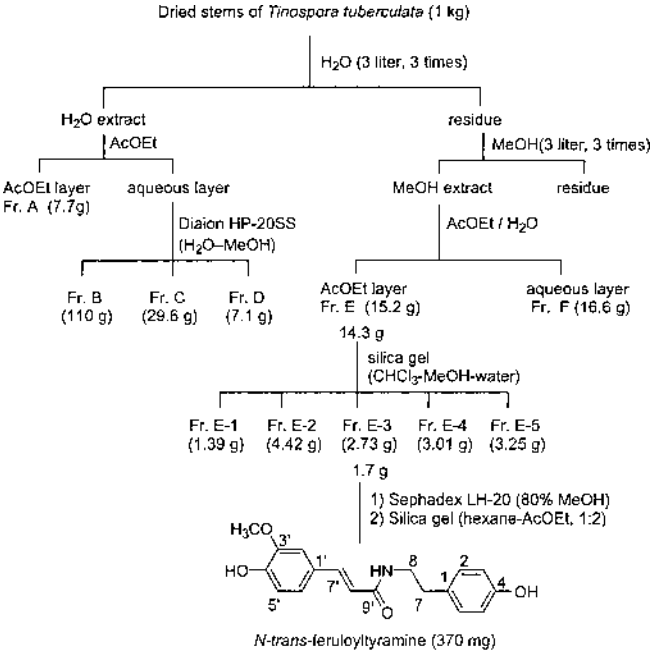

Fig. 1

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d, J=8.1 Hz, H-5′), 6.683 (2H, br d, J=8.5 Hz, H-3, 5), 6.341 (1H, d, J=15.7 Hz, H-8′), 3.808 (3H, s, OCH_3), 3.357 (2H, dt, J=5.7, 7.4 Hz, H-8), 2.647 (2H, t, J=7.4 Hz, H-7). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 165.7 (C-9′), 155.6 (C-4), 148.2, 147.8 (C-3′, 4′), 138.9 (C-7′), 129.5 (C-1), 129.4 (C-2, 6), 126.4 (C-1′), 121.5 (C-6′), 119.0, 115.6, 110.8 (C-2′, 5′, 8′), 115.0 (C-2, 6), 55.5 (OCH_3), 40.7 (C-8), 34.4 (C-7).

Animals Male BALB/c strain mice (Japan SLC, Inc., Hamamatsu, Japan), 6 weeks of age, were used. This study complied with the "Guidelines for Animal Experimentation" approved by Toyama Medical and Pharmaceutical University. They were kept in an air-conditioned room with lighting from 7 a.m. to 7 p.m. and given commercial pellet chow (Clea Japan Inc., Tokyo, Japan; comprising 24.0% protein, 3.5% lipid and 60.5% carbohydrate) and water *ad libitum*.

Cell Culture and Assay According to the method of Lefkowitz *et al*.,[6] mice were injected intraperitoneally with 1.5 ml of a 4% thioglycollate broth. After 4 d, the animals were sacrificed by cervical dislocation. Peritoneal lavage was performed using cold RPMI 1640 medium. Peritoneal macrophages were centrifuged at 1000×***g*** for 2 min, and the sedimented cells were resuspended at a density of 1×10^6/ml in 5% fetal calf serum (FCS)-supplemented RPMI 1640 medium. The cell suspension was added to each well of a 6-well or 96-well tissue culture plate. After 2 h of incubation in a humidified 5% CO_2 atmosphere at 37 °C, the medium was removed and cells were washed with phosphate buffered saline (PBS) to remove those that were nonadherent. The *Tinospora tuberculata* sample or aminoguanidine with 10 μg/ml LPS was then added to each well. The plates were incubated in a humidified 5% CO_2 atmosphere at 37 °C for 24 h. Aliquots of the medium were used to measure NO and cell viability. Nitrite was used as an indicator of NO production and measured by a microplate assay method based on the Griess reaction.[7] Cell viability was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay.[8] For the assay of cell lysates, cells were washed three times with PBS, scraped into cold PBS, and centrifuged at 500×***g*** for 10 min at 4 °C. The cell pellet obtained was resuspended in 0.5 ml of 40 mM Tris–buffer (pH 8.0) containing 5 μg/ml pepstatin A, 1 μg/ml chymostatin, 5 μg/ml aprotinin and 100 μM phenylmethylsulfonyl fluoride, and lysed by three cycles of freeze-thawing. Aliquots of the lysate were used for the determination of inducible NO synthase (iNOS) activity, and protein assay. iNOS activity was measured as described previously.[9] Briefly, 10—20 μg of cell lysate protein was incubated in 20 mM Tris–HCl (pH 7.9), containing 4 μM FAD, 4 μM tetrahydrobiopterin, 3 mM DTT, and 2 mM each of L-arginine (L-Arg) and NADPH. The reaction was carried out in duplicate for 180 min at 37 °C in 96-well plates. Residual NADPH was oxidized enzymatically with 10 units/ml LDH and 5 mM sodium pyruvate in a final volume of 130 μl, with incubation for a further 5 min at 37 °C, and the Griess assay was performed as above.[7] Protein was determined by the micro-biuret method,[10] with bovine serum albumin as a standard.

Statistics Results were assessed by analysis of variance, followed by Dunnett's test. Significance was accepted at $p<0.05$.

RESULTS

NO Production Exposure to LPS induced the production of a large amount of NO, about 6.9-fold that observed in the blank. When macrophages were cultured under identical conditions, but with different doses of *Tinospora tuberculata* extract, NO production was substantially inhibited in a concentration-dependent manner, as shown in Table 1. After separation of the extract, a significant difference in inhibitory activity was observed among the various fractions. The inhibitory activity of fractions C and F was weaker than that of the extract as a whole. In contrast, fraction E showed an apparent activity that was stronger than the extract. We further separated fraction E. Of the subfractions obtained, E-3 had the highest activity, inducing the production of 9.08 μM of NO at 50 μg/ml and 6.39 μM at 100 μg/ml. Fractions E-1 and E-5, by contrast, had no ameliorative effect on production. We next examined *N-trans*-feruloyltyramine isolated from fraction E-3 and found that it inhibited NO production in a concentration-dependent manner, as shown in Table 1. The inhibition induced by 50 μg/ml *N-trans*-feruloyltyramine resembled that induced by 100 μM aminoguanidine.

iNOS Activity In activated macrophages, enhanced formation of NO was associated with the expression of iNOS activity. As shown in Table 1, exposure of macrophages to LPS caused a significant increase in iNOS activity from 6.89 to 27.66 pmol/mg protein/min. Treatment of the cells with *Tinospora tuberculata* extract inhibited iNOS activity in a concentration-dependent manner. iNOS activity was decreased approximately to 86, 72 and 66% of LPS-stimulated control values, in 50, 100 and 200 μg/ml of *Tinospora tuberculata* extract-treated cells, respectively. Examination of its fractions showed that fraction E possessed higher inhibitory activity than the extract itself. Among the subfractions obtained from fraction E, fraction E-3 appeared to most strongly inhibit iNOS activity, while such activity was passed into *N-trans*-feruloyltyramine isolated from fraction E-3. The inhibition induced by 50 μg/ml *N-trans*-feruloyltyramine resembled that induced by 100 μM aminoguanidine.

Cell Viability To test and verify that the inhibitory effect of the *Tinospora tuberculata* extract, fraction and component on NO production was not due to cytotoxicity, we examined their effect on the viability of activated macrophages. As shown in Table 1, LPS impaired cell viability significantly as compared with nontreated cells. However, it was found that the cytotoxic effect of LPS was attenuated by incubation with *Tinospora tuberculata* extract and its fractions except B, C and E-1. *N-trans*-Feruloyltyramine was also able to protect cells that had been exposed to LPS. Although *N-trans*-feruloyltyramine improved the cell viability, aminoguanidine appeared to have no ameliorative effect on the cytotoxicity due to LPS.

DISCUSSION

Living organisms utilize the chemical properties of NO for the intracellular and intercellular processes of cGMP-mediated communication. NO itself is cytotoxic, however, and produces secondary active substances through a reaction with oxygen and active oxygen species. Therefore, the production of a substantial amount of NO due to increased im-

Table 1. NO Production, iNOS Activity and Cell Viability of Macrophages Incubated with LPS

Group	NO (μM)	iNOS (pmol/mg protein/min)	Cell viability (%)
None	5.79±0.32	6.89±0.68	100.0±1.1
LPS-treatment			
Control	39.93±0.36[c]	27.66±0.88[c]	79.6±1.8[c]
Extract (50 μg/ml)	36.26±0.77[c,f]	23.78±1.01[c,f]	81.6±0.9[c]
Extract (100 μg/ml)	22.59±0.49[c,f]	19.92±0.71[c,f]	82.4±1.7[c,d]
Extract (200 μg/ml)	14.67±0.58[c,f]	18.13±0.54[c,f]	83.9±0.9[c,f]
Fraction A (50 μg/ml)	32.69±0.70[c,f]	23.01±0.95[c,f]	80.8±2.7[c]
Fraction A (100 μg/ml)	28.84±0.43[c,f]	21.90±0.55[c,f]	81.7±0.4[c]
Fraction A (200 μg/ml)	17.09±0.74[c,f]	19.98±1.12[c,f]	82.8±0.4[c,d]
Fraction B (50 μg/ml)	57.79±2.28[c,f]	39.50±2.90[c,f]	79.6±1.4[c]
Fraction B (100 μg/ml)	33.46±1.62[c,d]	22.12±1.09[c,f]	79.7±3.4[c]
Fraction B (200 μg/ml)	22.84±1.20[c,f]	19.00±0.79[c,f]	80.7±0.9[c]
Fraction C (50 μg/ml)	54.17±4.67[c,f]	37.69±2.71[c,f]	78.6±0.4[c]
Fraction C (100 μg/ml)	52.40±3.41[c,f]	36.01±1.98[c,f]	76.3±2.7[c]
Fraction C (200 μg/ml)	42.99±4.63[c]	28.71±1.47[c]	78.2±2.4[c]
Fraction D (50 μg/ml)	66.45±1.67[c,f]	47.09±3.25[c,f]	79.1±0.9[c]
Fraction D (100 μg/ml)	34.95±2.73[c,f]	25.50±1.14[c]	82.9±3.8[c]
Fraction D (200 μg/ml)	16.62±0.81[c,f]	19.00±0.86[c,f]	86.4±1.3[c,e]
Fraction E (50 μg/ml)	23.86±0.83[c,f]	20.16±0.59[c,f]	95.4±0.7[f]
Fraction E (100 μg/ml)	15.03±0.70[c,f]	18.71±0.78[c,f]	113.8±3.6[c,f]
Fraction E (200 μg/ml)	8.57±0.21[a,f]	16.29±0.87[c,f]	121.9±3.7[c,f]
Fraction F (50 μg/ml)	73.20±4.76[c,f]	53.08±3.06[c,f]	78.5±1.7[c]
Fraction F (100 μg/ml)	49.00±2.27[c,f]	33.15±2.11[c,f]	82.6±2.6[c]
Fraction F (200 μg/ml)	33.81±2.90[c,e]	20.77±2.59[c,f]	91.1±1.2[c,f]
LPS-treatment			
Control	44.63±0.71[c]	29.91±0.82[c]	79.3±1.7[c]
Fraction E-1 (50 μg/ml)	44.72±1.43[c]	30.90±0.49[c]	77.6±1.1[c]
Fraction E-1 (100 μg/ml)	34.91±0.97[c,f]	26.71±0.39[c,f]	79.5±1.2[c]
Fraction E-2 (50 μg/ml)	23.68±0.43[c,f]	24.97±0.54[c,f]	79.5±0.7[c]
Fraction E-2 (100 μg/ml)	9.66±0.53[c,f]	18.73±1.00[c,f]	82.8±1.4[c,d]
Fraction E-3 (50 μg/ml)	9.08±0.85[c,f]	17.98±0.71[c,f]	85.3±2.0[c,f]
Fraction E-3 (100 μg/ml)	6.39±0.22[f]	12.53±0.55[c,f]	112.1±3.2[c,f]
Fraction E-4 (50 μg/ml)	28.94±0.56[c,f]	26.01±0.77[c,f]	103.9±1.7[a,f]
Fraction E-4 (100 μg/ml)	13.12±1.43[c,f]	18.49±1.02[c,f]	132.6±2.0[c,f]
Fraction E-5 (50 μg/ml)	39.33±0.83[c,f]	28.09±1.23[c,d]	88.3±1.2[c,f]
Fraction E-5 (100 μg/ml)	26.60±0.75[c,f]	26.11±0.48[c,f]	95.5±1.3[b,f]
LPS-treatment			
Control	43.46±0.62[c]	29.65±0.58[c]	78.0±1.2[c]
N-trans-Feruloyltyramine (10 μg/ml)	9.22±0.46[c,f]	17.66±0.72[c,f]	89.0±2.7[c,f]
N-trans-Feruloyltyramine (20 μg/ml)	8.48±0.12[c,f]	16.06±1.01[c,f]	91.1±1.9[c,f]
N-trans-Feruloyltyramine (50 μg/ml)	6.87±0.16[c,f]	12.88±1.13[c,f]	99.3±1.7[f]
N-trans-Feruloyltyramine (100 μg/ml)	3.87±0.19[c,f]	6.05±0.59[f]	112.2±2.7[c,f]
Aminoguanidine (100 μM)	7.07±0.20[c,f]	11.08±0.49[c,f]	81.0±1.3[c]

Significance: [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$ *vs.* non-treatment values, [d] $p<0.05$, [e] $p<0.01$, [f] $p<0.001$ *vs.* LPS-treatment control values.

munological activity can cause disease. Thus, the NO produced at the site of infection by inflammatory cells such as macrophages and neutrophils, or by epithelial cells (bronchus, bowel), is attracting attention as a mediator of inflammatory responses. Macrophages are known to produce NO through the metabolism of L-Arg by NOS promptly in response to stimulation at the site of infection, inflammation, and immune responses.[2] However, excessive production of NO may cause shock and injury to living tissue. In light of this, an effort is now being made to develop a specific NOS inhibitor effective in macrophages.[11—13]

We have previously reported that the aqueous extract of *Tinospora tuberculata* inhibited NO production in macrophages in a dose-dependent manner.[3] The effects of *Tinospora tuberculata* were also evident in a cell-free system; *Tinospora tuberculata* inhibited the production of NO from L-Arg and hydrogen peroxide, and dose-dependently suppressed the release of NO donors from sodium nitroprusside.[4] In the present study, we investigated the active component of *Tinospora tuberculata*, using macrophages.

Macrophages stimulated by LPS produced a large quantity of NO, with increased iNOS activity and decreased cell viability. In macrophages treated with a stepwise dose of *Tinospora tuberculata* extract, the level of NO and the iNOS activity were suppressed in a dose-dependent manner, while cell viability was increased. *Tinospora tuberculata* was then examined to establish the component responsible for the activity found in its aqueous extract. Six fractions were obtained from aqueous *Tinospora tuberculata* extract and its residue. The residue was then extracted with methanol, and the methanol extract was partitioned with ethyl acetate. The ethyl acetate layer thus obtained was found to have the most activity. Fractions having a bitter taste characteristic of this galenical had no effect, suggesting that borapetosides A, B, C and D, and borapetol B have no activity to inhibit the production of NO. The ethyl acetate layer, containing a large quan-

tity of *N-trans*-feruloyltyramine, was divided into 5 fractions with a silica gel column. The fraction richest in *N-trans*-feruloyltyramine proved to have the highest level of activity to inhibit the NO production. It became apparent that the component responsible for the inhibitory activity of *Tinospora tuberculata* was *N-trans*-feruloyltyramine isolated from this fraction. The present study revealed a new effect of this component, the suppression of NO production from macrophages, in parallel with the suppression of iNOS activity. This anti-NO activity was comparable to the effect of aminoguanidine, a specific inhibitor of iNOS.

N-trans-Feruloyltyramine was found in the stem of dried *Tinospora tuberculata* by Fukuda *et al*.,[5] and has been detected in eggplants[14] and leeks.[15] However, little is known about the physiological activity of *N-trans*-feruloyltyramine, though a marked inhibitory effect of this compound on platelet aggregation was reported by Okuyama *et al.*[15] The inhibition of NO production *via* iNOS is a new effect of *Tinospora tuberculata* demonstrated in the present study. However, it is presumed that the NO produced by macrophages through metabolism of L-Arg protects the body in cooperation with the cytokine network centering on interferon-γ and other defense factors (active oxygen, *etc.*), rather than independently by itself.[16,17] On the other hand, Tracey *et al.*[18] demonstrated that purified NOS from brain (neuron NOS), macrophages (iNOS) and endothelium (endothelial NOS) all showed NADPH-diaphorase activity; the relative activities were: macrophage>endothelium>brain. These data indicate that all known NOSs are NADPH-diaphorases. Mitchell *et al.*[19] have also published data showing that in macrophages, both NADPH-diaphorase and NOS activities can be induced by LPS. Moreover, NADPH-diaphorase activity appeared in the peritoneal macrophages of LPS-pretreated mice after the induction of pancreatitis, and the elevated NO was inhibited by N^G-nitro-L-arginine.[20] Although further studies are needed to elucidate the mechanisms by which *N-trans*-feruloyltyramine exerts its inhibitory actions, *Tinospora tuberculata* extract inhibited NADPH-diaphorase activity dose-dependently.[3] *N-trans*-Feruloyltyramine seems to suppress NO production due to inhibition of NADPH-diaphorase.

The ideal NO inhibitor would block only the harmful effects of NO, while maintaining or enhancing the beneficial effects. Recent studies have yielded several compounds which have very high activity and relatively high selectivity. Aminoguanidine is among these compounds and attracting attention as a specific iNOS inhibitor.[21] *N-trans*-Feruloyltyramine at 50 μg/ml was found to have a level of activity equivalent to 100 μM of aminoguanidine. However, unlike aminoguanidine, the cell viability decreased by LPS was restored in the presence of this compound, demonstrating the very low toxicity of this iNOS inhibitor.

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