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INHIBITORY EFFECT OF BISBENZYLISOQUINOLINE ALKALOIDS ON NITRIC OXIDE PRODUCTION IN ACTIVATED MACROPHAGES

YOSHIKAZU KONDO,* FUMIHIDE TAKANO and HIROSHI HOJO
Pharmaceutical Institute, Tohoku University, Aobayama, Aoba-ku, Sendai 980, Japan

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Abstract—Bisbenzylisoquinoline (BBI) alkaloids are anti-inflammatory constituents of plants of the families Menispermaceae and Ranunculaceae, which have been used as folk remedies in Japan and China. Five BBI alkaloids (cepharanthine, chondocurine, cycleanine, isotetrandrine and tetrandrine) were tested for suppressive effect on *in vitro* nitric oxide (NO) production by lipopolysaccharide-stimulated peritoneal macrophages, which were induced with thioglycollate or bacillus Calmette-Guerin in mice. All these BBI alkaloids significantly suppressed NO production at 5 μg/mL. Cepharanthine, isotetrandrine and cycleanine were slightly more inhibitory than tetrandrine and chondocurine. The suppression persisted for at least 48 hr. As NO is one of the critical mediators in inflammation, these results may explain some aspects of the anti-inflammatory mechanisms of BBI compounds.

Several isoforms of nitric oxide (NO) synthase have been identified [1]. Ca^{2+}-independent NO synthase is induced with bacterial lipopolysaccharide (LPS†) or some cytokines in macrophages, neutrophils, Kupffer cells and hepatocytes [2–4]. In particular, macrophages can express large amounts of this type of synthase in the cytosol [5]. On the one hand, NO formed by the Ca^{2+}-independent NO synthase plays a role in non-specific immune defences against tumor cells, parasitic fungi, bacteria and protozoa [6]. On the other hand, NO is known to be responsible for the hypotension observed in endotoxin shock [7–9]. Recently, it was proposed that the lysis of pancreatic islet cells by NO produced in activated macrophages is involved in the development of type-1 (insulin-dependent) diabetes [10].

We recently demonstrated that bisbenzylisoquinoline (BBI) alkaloids are immunosuppressive [11] and protect against lethal toxicity [12] and hepatic damage [13] induced by LPS in bacillus Calmette-Guerin (BCG)-treated mice. Activation of macrophages, which may result in the production of large amounts of NO, is thought to be critical for lethal toxicity. Anti-inflammatory and immunosuppressive agents, glucocorticoids, have been established to be strong inhibitors of NO production [14–16]. In this context, we investigated the suppressive effect of BBI alkaloids on NO production by LPS-stimulated macrophages and found that the BBI alkaloids, cepharanthine, isotetrandrine, cycleanine, tetrandrine and chondocurine, inhibited NO production in macrophage cultures.

MATERIALS AND METHODS

Animals. C3H/HeN male mice were purchased from Japan SLC (Shizuoka, Japan) and used for experiments at 5–7 weeks of age. Mice were maintained on water and routine chow *ad lib.*

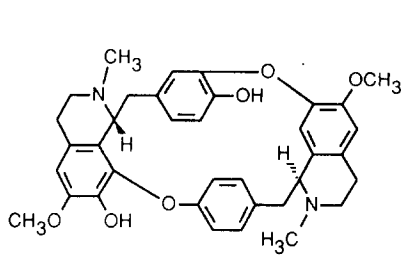
Reagents. Cepharanthine was kindly donated by Dr M. Akasu, the Kaken Drug Co. (Tokyo, Japan). Chondocurine and cycleanine were obtained from E. Merck, isotetrandrine was from Kaken, and tetrandrine was from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). LPS (*Escherichia coli* 055:B5), dexamethasone (DEX) and aprotinin were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Leupeptin and soybean trypsin inhibitor were from Wako (Tokyo, Japan) and NADPH was from Oriental (Tokyo, Japan). Oxyhemoglobin was prepared from bicristallized bovine hemoglobin (Sigma) according to the method of Fleelish and Noack [17].

Culture medium. Phenol red-free RPMI1640 medium (Gibco, Grand Island, NY, U.S.A.) was supplemented with 100 U/mL penicillin, 100 U/mL streptomycin and 5% heat-inactivated fetal calf serum (Gibco).

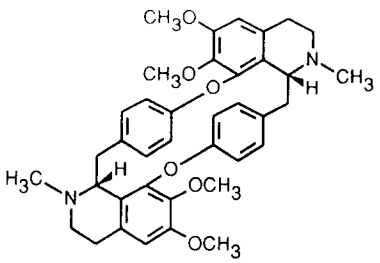
Assay for NO production by macrophages. Four days after an i.p. injection of 2 mL of 3% thioglycollate solution (Difco, Detroit, MI, U.S.A.) or 1 mg of BCG (Nippon BCG, Tokyo) into C3H/HeN mice, peritoneal exudate cells were washed out with phosphate-buffered saline (pH 7.4) through the anterior abdominal wall. After peritoneal exudate cells suspended in culture medium at 5–10×10^5 cells/mL were incubated for 2 hr in 24-well tissue culture plates (Becton Dickinson, Oxnard, CA, U.S.A.) in a humidified CO_2 incubator, non-adherent cells were washed out twice with warm phosphate-buffered saline. Remaining adherent cells (macrophages) were cultured in the same medium containing 10 μg/mL LPS with or without BBI alkaloids for 24 or 48 hr. In order to measure the amount of NO_2^- derived from NO in the culture, 100 μL of culture supernatants were mixed with

* Corresponding author.

† Abbreviations: BCG, bacillus Calmette-Guerin; BBI, bisbenzylisoquinoline; DEX, dexamethasone; LPS, lipopolysaccharide.



chondocurine



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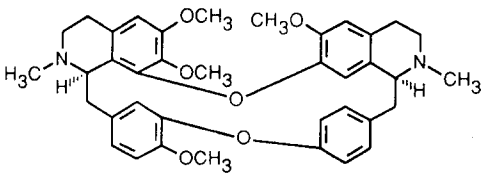
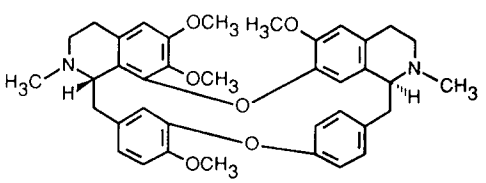


isotetrandrine



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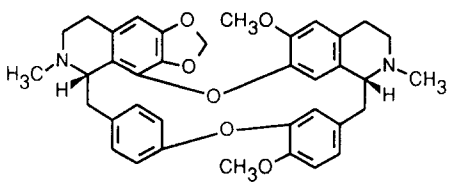

cepharanthine

Structure 1. Structure of BBI alkaloids.

150 μL of Griess reagent (1% sulfanilamide/0.1% naphthyl ethylenediamine dichloride/3% H_3PO_4) [18]. After chromophore was formed at room temperature for 10 min, absorbance was determined at 555 nm using Immunoreader (InterMed, Tokyo).

Preparation of cytosol fractions and assay for NO synthase. Macrophage cytosol fractions containing Ca^{2+}-independent NO synthase were prepared as reported previously [15]. Macrophages were stimulated with LPS in the presence or absence of BBI alkaloids and/or dexamethasone for 24 hr. After incubation, the cells were scraped out, mixed with homogenization buffer containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1 mM DL-dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor and 4 μg/mL aprotinin (pH 7.0), and extracted at 4° by a Dounth type homogenizer. The homogenates were centrifuged at 105,000 *g* for 1 hr to obtain the cytosol fraction. Cytosolic NO synthase activities were determined spectrophotometrically using the oxidation of oxyhemoglobin by nitric oxide radical [19]. The cytosol fraction (200 μL) was added to 400 μL of reaction mixture which consisted of 5 μM oxyhemoglobin, 1 mM $MgCL_2$, 200 μM $CaCL_2$, 100 μM L-arginine, 100 μM NADPH and 50 mM L-valine dissolved in 40 mM potassium phosphate buffer (pH 7.2). For measuring Ca^{2+}-independent NO synthase activity, 800 μM EGTA was added to the reaction mixture. Change in difference between the absorbance at 401 and 421 nm was monitored by a two wavelength spectrophotometer (Hitachi UV-3200) with a band pass of 5 nm (extinction coefficient of 77,200 $M^{-1}\,cm^{-1}$) at 37° for 10 min. Protein content was determined colorimetrically [20].

RESULTS

Inhibition of NO production in activated macrophages by BBI alkaloids

We investigated the effect of BBI alkaloids (Structure 1) on NO production in cultured LPS-stimulated peritoneal macrophages induced with BCG and thioglycollate in mice. Macrophages were cultured with 5 μg/mL BBI alkaloids in addition to LPS for 24 hr and then the supernatants were analysed for the amount of NO as NO_2^- level, since

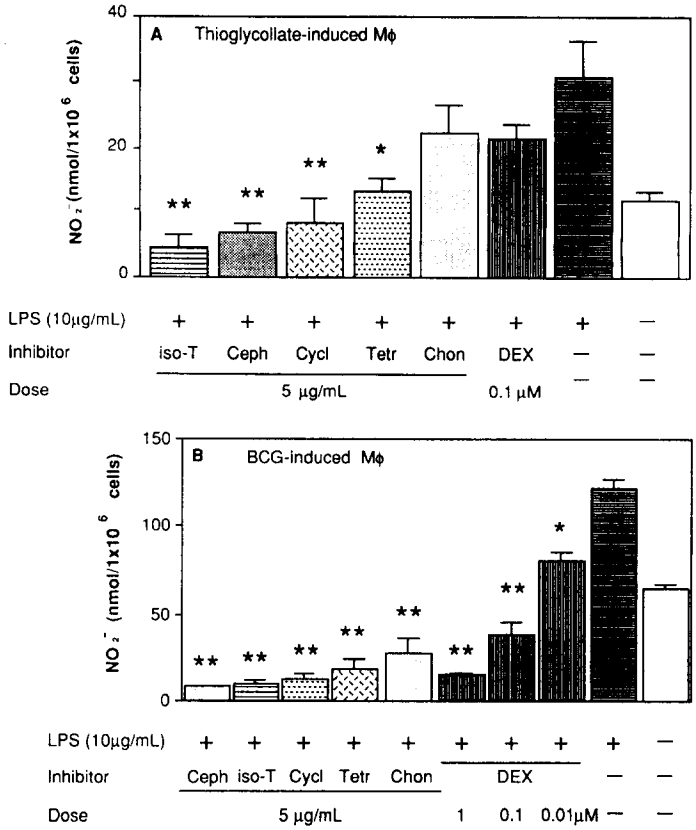

Fig. 1. Effect of BBI alkaloids and DEX on NO production by BCG- or thioglycollate-induced macrophages. BCG (A)- or thioglycollate (B)-induced macrophages were cultured with 10 μg/mL LPS together with BBI alkaloids or DEX for 24 hr, and NO_2^- content in the culture supernatants was determined. Results are expressed as the means ± SD of quadruplicate cultures. Significantly different from control, *P < 0.01, **P < 0.001.

the major part of NO produced in the cultures was oxidized to NO_2^- [6]. Thioglycollate-induced macrophages produced 31.0 ± 5.4 nmol of NO_2^-/mL by stimulation with 10 μg/mL LPS. BCG-induced macrophages produced a much greater amount of NO_2^- upon stimulation with 10 μg/mL LPS (128.2 ± 2.0 nmol/mL). All the five BBI alkaloids tested significantly suppressed NO production at 5 μg/mL in the LPS-stimulated cultures of thioglycollate-induced macrophages (Fig. 1A) and of BCG-induced macrophages (Fig. 1B). Of these the BBI alkaloids, isotetrandrine, cepharanthine and cycleanine, seemed to be more suppressive than chondocurine and tetrandrine (Fig. 1A and B). NO-suppressive doses of the BBI alkaloids (10 μg/mL) did not enhance lactate dehydrogenase (LDH, EC 1.1.1.27) during incubation for 48 hr (less than 5% of the total LDH activity), which was used as a quantitative indicator of cytotoxicity. DEX greatly suppressed NO production in the LPS-stimulated culture of BCG-induced macrophages at 10^{-8} and 10^{-6} M. A dose-dependent suppression by cepharanthine and chondocurine of NO production by the LPS-stimulated cultures of BCG-induced macrophages is shown in Fig. 2. There was no

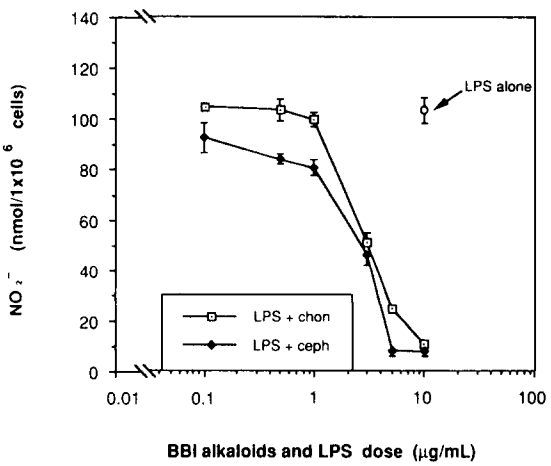

Fig. 2. Dose-dependent inhibition by cepharanthine and chondocurine on NO production by activated macrophages. BCG-induced macrophages were cultured with cepharanthine or chondocurine at doses of 0.01–10 μg/mL together with 10 μg/mL LPS for 24 hr, and then NO_2^- content in the culture supernatants was determined. Results are expressed as the means ± SD of quadruplicate cultures.

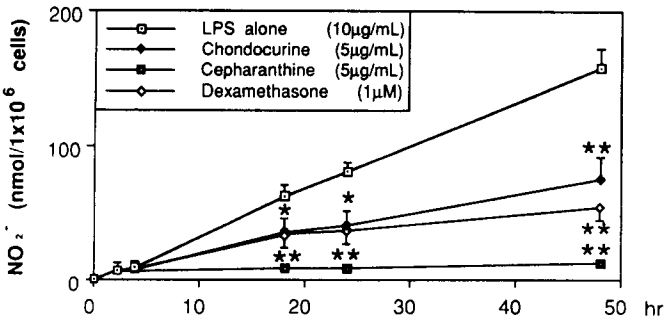

Fig. 3. Time course of suppression of NO production by cepharanthine, chondocurine and DEX in culture of activated macrophages. BCG-induced macrophages were cultured with LPS alone or together with 5 μg/mL cepharanthine, 5 μg/mL chondocurine or 1 μM DEX, and then NO_2^- content in the culture supernatants was determined at the indicated time. Results are expressed as the means ± SD of quadruplicate cultures. Significantly different from control, *P < 0.01, **P < 0.001.

Table 1. Effect of BBI alkaloids and DEX on NO synthase activity in the cytosol of BCG-induced macrophages

Inhibitor	Dose	LPS (10 μg/mL)	NO synthase activity (pmol/min/mg protein)	N
—		−	14.8 ± 4.1	3
—		+	23.4 ± 6.1	6
Chondocurine	1 μg/mL	+	14.3 ± 5.2*	4
	5 μg/mL	+	12.6 ± 1.2†	4
Cepharanthine	1 μg/mL	+	12.1 ± 5.3*	3
	5 μg/mL	+	9.4 ± 2.1†	4
DEX	0.01 μM	+	10.1 ± 1.8†	4
	0.1 μM	+	8.4 ± 3.7†	4

BCG-induced macrophages were cultured with LPS in combination with inhibitors for 24 hr and then homogenized. Supernatants post-centrifugation at 105,000 *g* for 30 min were assayed for NO synthase activity.

Results are expressed as the means ± SD of three experiments. Significantly different from control, *P < 0.05, †P < 0.01.

evidence that BBI alkaloids and DEX reacted directly with NO or Griess reagent (data not shown).

Figure 3 shows the time course of suppression of NO production caused by cepharanthine, chondocurine and DEX. Suppressive activity of cepharanthine (5 μg/mL) and chondocurine (5 μg/mL) continued significantly for at least 48 hr.

Effect of BBI alkaloids on NO synthase

In order to examine whether the decrease in NO production by BBI alkaloids was due to reduction of the enzyme, the cytosol fraction of macrophages was extracted and assayed for enzyme activity. As shown in Table 1, LPS stimulation of the BCG-induced macrophages resulted in a significant increase in NO synthase in the cytosol. When LPS-stimulated macrophages were cultured with 5 μg/mL cepharanthine, 5 μg/mL chondocurine or 0.01 μM DEX, the cytosol NO synthase activity was reduced to 46.3%, 59.8% and 43.1%, respectively.

Additive suppression of NO production by cepharanthine and dexamethasone

The additive inhibitory effect of cepharanthine and dexamethasone on NO production was examined. Neither 0.01–1.0 μg/mL cepharanthine nor 0.01 μM DEX significantly suppressed NO production in the LPS-stimulated cultures of thioglycollate (Fig. 4A)- or BCG (Fig. 4B) -induced macrophages; however, 0.5–1.0 μg/mL cepharanthine showed significant suppression in combination with 0.01 μM DEX (Fig. 4A and B).

DISCUSSION

More NO was produced by the BCG-induced peritoneal macrophages than by those induced by thioglycollate, irrespective of stimulation by LPS (Fig. 1). The high level of NO-producing activity in the activated macrophages was observed for at least 48 hr (Fig. 3). It was suggested that the NO production was dependent on Ca^{2+}-independent NO synthase, since this type of enzyme was reported to be induced for longer than 48 hr in macrophages activated by interferon-γ and LPS [21]. Previous reports showed that cytotoxicity toward murine tumor cells was expressed in BCG-induced macro-

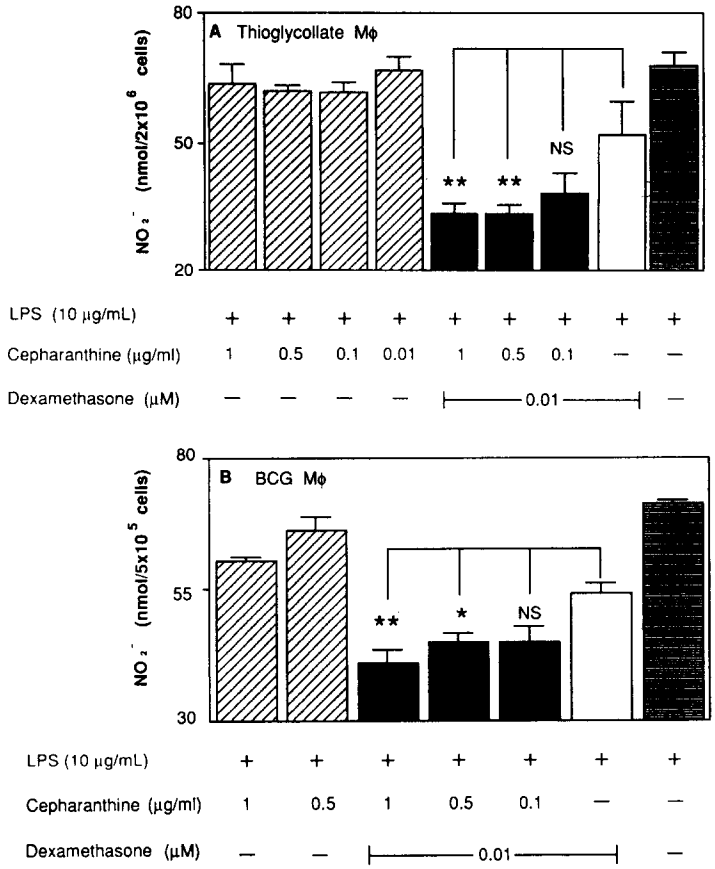

Fig. 4. Additive effects of cepharanthine and dexamethasone on NO production by activated macrophages. BCG-induced macrophages were cultured with cepharanthine without or in combination with a suboptimal dose of DEX (0.01 μM) in the presence of 10 μg/mL LPS for 24 hr. NO_2^- content in the culture supernatants was determined. Results are expressed as the means ± SD of quadruplicate cultures. Significantly different from control, *P < 0.01, **P < 0.001.

phages but not in thioglycollate-induced macrophages [22], and recently it was demonstrated that NO was a cytotoxic mediator released from activated macrophages which impaired ribonucleotide reductase in tumor cells [23].

In the present study, all the five BBI alkaloids were shown to inhibit NO production by LPS-stimulated macrophages prepared from thioglycollate- or BCG-primed mice. Cepharanthine, isotetrandrine and cycleanine appeared more potent than chondocurine and tetrandrine. The inhibition by cepharanthine was approximately three times stronger than that by chondocurine (Fig. 1). We reported previously that chondocurine and cycleanine protected mice from the lethal toxicity triggered by LPS in BCG-primed mice [12], in which activation of macrophages was thought to play a critical role. Some investigators have proposed that NO is responsible for the hypotension in endotoxin shock [8, 9]. Although cepharanthine was demonstrated here to be inhibitory to NO production by activated macrophages, we had not obtained any positive evidence that cepharanthine protected mice from LPS/BCG-induced lethal toxicity (unpublished data). It is possible to speculate that inhibiting NO generation is necessary but not sufficient to prevent the lethal toxicity.

In order to examine whether BBI alkaloids affect the level of NO synthase, cytosol fractions were extracted from LPS-stimulated macrophages cultured with cepharanthine and assayed for Ca^{2+}-independent NO synthase activity. The Ca^{2+}-independent NO synthase activity was shown to be decreased (Table 1). Together with the fact that the BBI alkaloids did not affect the enzyme activity itself (data not shown), they seem to suppress NO production due to inhibition of *de novo* biosynthesis of NO synthase.

BBI alkaloids are anti-inflammatory constituents of plants of the families Menispermaceae and Ranunculaceae [24], which were used as folk remedies for rheumatism in Japan and China. Of the BBI alkaloids, tetrandrine has been used clinically as an agent for lung silicosis [25]. Inhibition by BBI alkaloids of excessive formation of NO might provide the basis of the anti-silicosis action. More recently, it was reported that the combined use of glucocorticoid and cepharanthine for the treatment

of erythema nodosum had been successful. In combination with cepharanthine, the dose of glucocorticoid could be reduced gradually [26]. The present evidence that a small amount of cepharanthine causes significant suppression of NO production in combination with a non-effective level of DEX (Fig. 4) might explain the combined clinical use of glucocorticoid and cepharanthine.

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