Inhibitory Effects of Herbal Alkaloids on the Tumor Necrosis Factor-α and Nitric Oxide Production in Lipopolysaccharide-Stimulated RAW264 Macrophages

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It is beneficial to treat chronic inflammatory condition in patients through diets that inhibit the production of proinflammatory cytokines and mediators such as tumor necrosis factor-α (TNF-α) and nitric oxide (NO). Since less attention has been paid to alkaloids in the diets than to polyphenols in this regard, we aimed at investigating anti-inflammatory activity of herb-derived alkaloids through suppression of TNF-α and NO production in lipopolysaccharide (LPS)-stimulated mouse RAW264 and/or human THP-1 cells. A harmala alkaloid, harmine, an opium alkaloid, papaverine, and *Lycoris* alkaloids, lycorine and lycoricidinol, showed TNF-α suppressive activities stronger than or comparable to that of a reference polyphenol, butein, in RAW264 cells (IC_{50}=4, 10, 2.1, 0.02, and 8 μM, respectively). Other alkaloids showed no or marginal to moderate inhibitory activities. Similar tendency of inhibition was found for NO production in RAW264 cells and TNF-α production in THP-1 cells. In addition, harmine was found to suppress interleukin-6 (IL-6) production in RAW264 cells. The above four inhibitory alkaloids had essentially no antioxidative property in the superoxide anion scavenging assay. Western blotting and reverse transcriptase-polymerase chain reaction (RT-PCR) showed that harmine caused neither prevention of nuclear factor-κB (NF-κB) translocation into the nucleus nor inhibition of p38 mitogen activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) phosphorylation, while that the LPS-induced transcription of TNF-α and inducible NO synthase was dose-dependently attenuated by harmine. This result suggests that the molecular mechanism of harmine action is different from those of many other anti-inflammatory phytochemicals. In conclusion, some herbal alkaloids like harmine, in spite of lacking antioxidative property, have potential as anti-inflammatory agents that strongly suppress TNF-α and NO production by a unique mechanism.

Key words alkaloid; anti-inflammatory; harmine; tumor necrosis factor-α; nitric oxide

Uncontrolled production of proinflammatory cytokines is the primary cause of chronic inflammatory diseases such as rheumatoid arthritis, psoriasis, and ulcerative colitis.[1] Among the above cytokines, tumor necrosis factor-α (TNF-α) plays important multiple roles in the pathobiology of inflammation, and induces itself as well as other inflammatory cytokines. Nitric oxide (NO) is another potent proinflammatory mediator. NO at low concentrations is the essential regulator of physiological homeostasis in cardiovascular system, but its overproduction by inducible nitric oxide synthase (iNOS) in the inflammation exerts harmful effects on the bodies.[2] Although many synthetic drugs and biologics have been developed for suppressing TNF-α[3] and NO,[2] application of natural products of the same activity in dietary plants without or with other functional food factors like polyunsaturated fatty acids[4] is an attractive alternative, especially for ameliorating long-lasting inflammatory condition. The benefit of this approach is a better quality of life for the patients and reduction of medical costs.

The anti-inflammatories, that is, TNF-α suppressing phytochemicals so far reported are mainly phenolic compounds with antioxidative property (*e.g.*, flavonoids and stilbenoids).[5] Another major class of natural products, alkaloids, has received little attention in this regard. The situation might be partly due to the relatively weak antioxidative property of alkaloids, because reactive oxygen species and NO (a radical) are involved in the inflammatory process.[6]

We decided to investigate fourteen alkaloids from herbal plants (Fig. 1) for their effect on TNF-α and NO production. Five β-carboline alkaloids (**1**—**5**), four indole alkaloids (**6**—**9**) and three quinoline alkaloids (**10**—**12**) were arbitrarily chosen from textbooks of pharmacognosy[7] and catalogues of commercial reagents, and two *Lycoris* alkaloids (**13**, **14**) were included to confirm the previous report on their anti-TNF-α activity.[8] These compounds are known to have various pharmacological activities (*e.g.*, monoamine oxidase inhibition for **3**—**5**,[9] intellectual capacity improvement for **7**, anti-

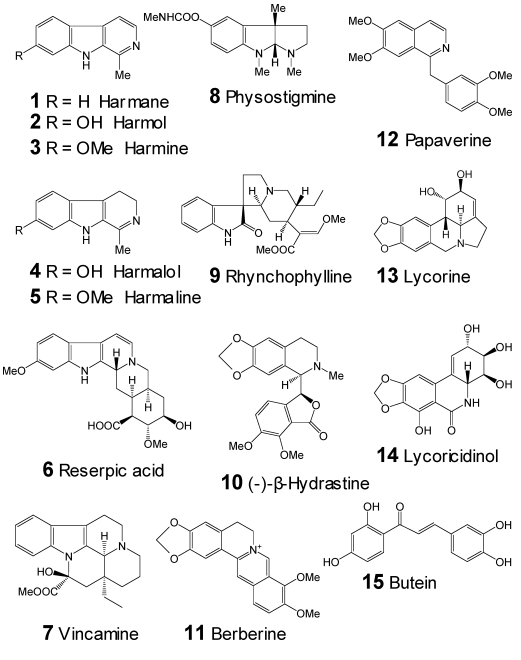

Fig. 1. Chemical Structures of the Compounds

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cholinergic activity for **8**, uterine hemostatic and antiseptic activity for **10**, antimalarial activity for **11**, and smooth muscle relaxing activity for **12**[10]), but information about their effect on inflammatory cytokine production has been sparse and limited to a few reports on harmine (**3**),[11] rhyncophylline (**9**),[12] and berberine (**11**),[13] and that on **13** and **14** as described above.[8] A polyphenol, butein (**15**),[14] was used as a reference compound.

Experimental

Materials **1**, Hydrochloride dihydrate salts of **2**, **4**, and **5**, **12** (hydrochloride), and Isogen were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and **3** (hydrochloride) was from Tokyo Chemical Industries Ltd. (Tokyo, Japan), **10**, **11** (chloride salt), **13** (hydrochloride), Griess reagent, human interferon (IFN)-γ, anti-β-actin (AC-15), anti-iNOS, and anti-α-tubulin (AA13) were from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.), **7** (dihydrochloride) was from MP Biochemicals LLC (Solon, OH, U.S.A.), **8** was from Alexis Biochemicals (San Diego, CA, U.S.A.), **9** was from Tokiwa Phytochemical Co., Ltd. (Sakura, Japan), **6** (hydrochloride) and **15** were from Extrasynthése (Genay, France), and lipopolysaccharide (LPS, 055:B5) was from List Biological Laboratories, Inc. (Campbell, CA, U.S.A.). Lycoricidinol (narciclasine, **14**) was extracted from bulbs of *Lycoris radiata* (obtained from a planter in Ibaraki Prefecture, July 2009) with ethanol and the crude extract was purified by silica gel column chromatography (methanol/ethyl acetate) followed by reverse phase column chromatography (Wakogel 100C18) with 0—50% methanol in water as the solvent. The fractions of 20% methanol/water were concentrated to give 20 mg **14** as fine needles, mp 224—235 °C dec. and $[\alpha]_D^{28}$ +120° (c=0.69, methanol) [lit. mp 246 °C dec. and $[\alpha]_D$ +126.5° (c=0.5, methanol)[15]], MS m/z 307.0673 (M^+) (Calcd $C_{14}H_{13}NO_7$=307.0691). Enzyme-linked immunosorbent assay (ELISA) kits for mouse and human TNF-α and mouse interleukin (IL)-6 were purchased from Thermo Scientific (Rockford, IL, U.S.A.). Anti-nuclear factor-κB (NF-κB) p65 (C-20), anti-inhibitor of κB (IκB)-α (C-21), anti-*p*-IκB-α (B-9), and anti-*p*-c-Jun N-terminal kinase (JNK) (G-7) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.), anti-phospho-p38 mitogen activated protein kinase (MAPK) (28B10) was from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.), and horseradish peroxidase-conjugated secondary antibodies were from Zymed Laboratories Inc. (San Francisco, CA, U.S.A.).

Cell Culture and Assay of Cytokines and NO Mouse RAW264 macrophage cells were obtained from Riken Cell Bank (Tsukuba, Japan), and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Precultured cells were suspended in the medium at a rate of 2×10^5 cells/ml and 0.2 ml suspension was added to each well of 96-well plates. After 2 d incubation, the medium was changed to a fresh medium (0.2 ml/well) and ethanol or ethanol solutions (2 μl) containing 0.01—10 mM test compounds were added to the culture in triplicate wells. After incubation for 3 h, a LPS solution (100 μg/ml in the medium) containing 10 μg/ml arginine and 1.4 μg/ml IFN-γ was added by 6 μl to each well, and the plates were further incubated for 18 h before harvesting the medium. NO and TNF-α in the medium were determined by Griess reagent (absorption at 540 nm) and ELISA, respectively. The suppression of IL-6 production was studied with 0.1—10 μM harmine and butein in the same way as above, but using plate cultures incubated for 1 d and without IFN-γ. The cell viability was assayed with a tetrazolium reagent (CellTiter 96®, Promega Corp., Madison, WI, U.S.A.). Assay with human THP-1 monocytic cells (Dai Nippon Sumitomo Pharmaceutical Co., Osaka, Japan) was done essentially in the same way as above, but the medium was RPMI 1640 containing 10% FBS and the plate culture was performed in the presence of 0.1 μM 12-*O*-tetradecanoylphorbol 13-acetate for 2 d before changing the medium and adding the test compounds.[4]

Antioxidant Test Superoxide anion scavenging by the compounds was determined according to the published method[16] with some modifications.

Western Blot Analysis RAW264 cells were precultured and 1.8×10^6 cells in 3 ml medium were put into each of dishes (3 cm in diameter). After incubation for 1 d, the medium was changed to a fresh medium (1 ml per dish) and ethanol (as control) or 10, 30, or 100 mM harmine solution in ethanol (1 μl each) was added to each culture. After 1 h incubation, LPS solution (1 mg/ml, 1 μl each) was added to the cultures. After 30 min incubation (or 18 h for iNOS detection), the medium was aspirated off, and cells were scraped with 2 ml cold phosphate buffered saline (PBS) by a scraper and collected by centrifugation. Cells from each dish were extracted with the cell lysis buffer (30 μl each) and 5% NP-40 [polyoxyethylene(9) octylphenyl ether] solution (3.4 μl each) to afford the cytoplasmic extracts, and then with the nucleus extraction buffer (20 μl each) to afford the nuclear extracts according to the published method.[17] Proteins in these extracts were determined by bicinchoninic acid method with bovine serum albumin as standard. The extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10 or 20 μg protein per lane) and Western blotting by the usual procedure.[18]

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Experiment RAW264 cells (8×10^5 per well in 12-well plates) were incubated for 1 d. The medium was changed to a fresh medium (1 ml per well) and ethanol (as control) or 10, 30, or 100 mM harmine solution in ethanol (1 μl each) was added to each culture. After 80 min incubation, LPS solution (1 mg/ml, 1 μl each) was added to the cultures. After incubation for 4 or 6 h, the medium was collected for TNF-α determination by ELISA. Cells were immediately washed with cold PBS and treated with Isogen (0.4 ml per well). The extracted RNA was used to detect gene expression of TNF-α, iNOS, and β-actin by RT-PCR system (Invitrogen Co., Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. Primers and cycling conditions were as follows: TNF-α—sense strand 5′-ATG AGC ACA GAA AGC ATG ATC-3′ and anti-sense strand 5′-TAC AGG CTT GTC ACT CGA ATT-3′,[19] and 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C for 34 cycles; iNOS—sense strand 5′-TTT GGA GCA GAA GTG CAA AGT CTC-3′ and anti-sense strand 5′-GAT CAG GAG GGA TTT CAA AGA CCT-3′,[20] and 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C for 34 cycles; β-actin—sense strand 5′-GAC GAG GCC CAG AGC AAG AGA-3′ and anti-sense strand 5′-TAG ATG GGC ACA GTG TGG GTGA-3′,[18] and 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C for 30 cycles.

Results and Discussion

Nine alkaloids of the panel suppressed the TNF-α production in LPS-stimulated RAW264 cells, among which harmine (**3**), papaverine (**12**), lycorine (**13**), and lycoricidinol (**14**) were more potent than or comparable to butein (**15**) from the IC_{50} values. The data are summarized in Table 1. Typical dose dependency curves for **3** and **13**—**15** are shown in Fig. 2. The strong activity of **14** was consistent with the previous report on mouse peritoneal macrophages.[8] Harmol (**2**) and harmaline (**5**), and harmalol (**4**) and rhyncophylline (**9**) exhibited moderate and the marginal activities, respectively. Other compounds, harmane (**1**), reserpic acid (**6**), vincamine (**7**), physostigmine (**8**), and hydrastine (**10**), were essentially inactive. Berberine (**11**) was active, but this activity was accompanied by the cytotoxicity. The above active alkaloids were not toxic at the concentrations around the IC_{50} for TNF-α suppression, except for **11** and **13**, as evidenced by ED_{50} in cell viability test (Table 1). These TNF-α suppressive alkaloids also inhibited NO production in RAW264 cells and the IC_{50} values were apparently parallel to those for TNF-α suppression (Table 1). In addition, IL-6 production in LPS-stimulated RAW264 cells was found to be suppressed by harmine (**3**) (IC_{50}=0.4±0.2 μM) and butein (**15**) (IC_{50}=0.9 μM). Furthermore, **3**, **9**, **12**, **13**, **14**, and **15** were tested with LPS-stimulated human THP-1 cells and showed the same tendency of TNF-α inhibitory activity as that found with RAW264 cells [IC_{50} (mean±S.D.)=4±2, >100, 40±12, 0.9±0.2, 0.03±0.02, and 7±2 μM, respectively (n=3)].

Several alkaloids except for berberine (**11**) showed only marginal superoxide anion scavenging activities at high concentrations (0.5—2 mM), while butein (**15**) showed a strong activity with IC_{50} of 43 μM (Table 1). This result indicates that there is no correlation between the anti-inflammatory activity and the antioxidative property for these alkaloids. The strong TNF-α inhibitors **3** and **12** have a methoxylated nitrogen-heteroaromatic ring as the common structural feature, but it does not apply in the more potent inhibitors **13** and **14**.

Table 1. Suppression of TNF-α and NO Production in LPS-Stimulated Mouse RAW264 Macrophages and Antioxidative Property

Compound	RAW264 cells			Superoxide anion scavenging	Typical herb
	TNF-α production	NO production	Cell viability		
	IC_{50} (μM)[a]	IC_{50} (μM)[a]	ED_{50} (μM)[b]	IC_{50} (mM)[b]	
Harmane (**1**)	—[c]	>100	>100	—	Passion flower
Harmol (**2**)	16±10	53±13	80	1.1	Passion flower
Harmine (**3**)	4±1	21±5	70	>1[e]	Passion flower
Harmalol (**4**)	>100	—	—	>2	Passion flower
Harmaline (**5**)	34±12	—	>100	>2	Passion flower
Reserpic acid (**6**)	—	—	—	>0.5	Rauwolfia[f]
Vincamine (**7**)	—	—	>100	—	Periwinkle
Physostigmine (**8**)	—	—	—	—	Calabar bean
Rhyncophylline (**9**)	>100	—	>100	—	Cat's claw
Hydrastine (**10**)	—	—	>100	—	Goldenseal
Berberine (**11**)	73±10[d]	89±17[d]	44	0.18	Goldenseal
Papaverine (**12**)	10±3	59±11	>100	>0.75[e]	Opium
Lycorine (**13**)	2.1±0.4	1.2±0.4	8	>1	*Lycoris radiata*
Lycoricidinol (**14**)	0.020±0.003	0.010±0.003	0.3	>1	*Lycoris radiata*
Butein (**15**)	8±4	5±2	>30	0.043	Cashews

a) Values are means±standard deviation (S.D.) (n=4). *b*) Means of three experiments. *c*) —, inactive or too large to be determined. *d*) n=3. *e*) Measurement at higher concentrations was disturbed by precipitation. *f*) **6** is a hydrolysis product of reserpine in Rauwolfia.

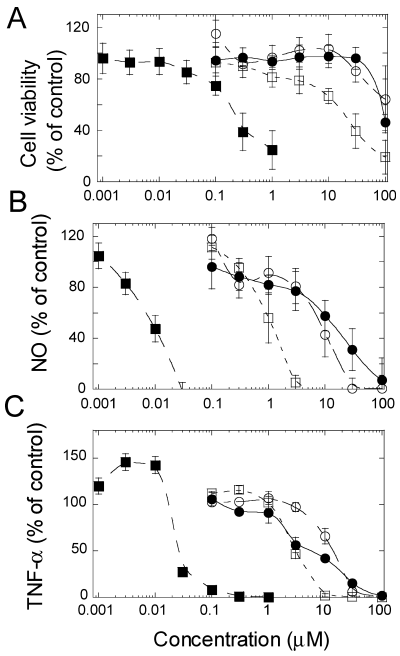

Fig. 2. Dose Dependency of the Inhibitory Effects of Harmine (**3**), Lycorine (**13**), Lycoricidinol (**14**), and Butein (**15**) on Cell Viability (A), NO Production (B), and TNF-α Production (C) in LPS-Stimulated RAW264 Cells

Data are means±S.D. (n=3) represented as % of control. Symbols: ●, **3**; □, **13**; ■, **14**; and ○, **15**.

The structure–activity relationship of alkaloids suppressing TNF-α production is presently not clear and warrants further study with a greater diversity of compounds.

The TNF-α suppression by **14** was very strong, but was no doubt complicated by the inhibition of protein biosynthesis.[8] As to **13**, its effect on the IκB/NF-κB system in human promyelocytic leukemia HL-60 cells was recently reported.[21] Here, we investigated the mechanism of TNF-α and NO suppression by harmine (**3**) by Western blotting and RT-PCR. The expression of TNF-α and iNOS is mainly controlled by the transcription factor NF-κB. The signal by LPS is transduced to phosphorylation of an inhibitor IκB-α that blocks NF-κB in cytoplasm. The phosphorylated IκB-α is rapidly degraded and the liberated NF-κB moves into the nucleus to activate the gene expression.[1,17] The LPS signal is also transduced to the expression of TNF-α and iNOS *via* MAP kinase pathway through phosphorylation of protein kinases such as p38 MAPK and JNK.[19] In our experiments, the nuclear NF-κB p65 increased due to the treatment with LPS, and it was not affected by the pretreatment with 10—100 μM **3** (Fig. 3A). In addition, α-tubulin was hardly detected in the nuclear extract lanes on the reproved membrane, indicating that the NF-κB p65 bands for the nuclear extracts were not due to contamination of cytoplasmic proteins. The level of IκB-α decreased due to the LPS treatment and was not restored by the pretreatment with **3**. Phosphorylated IκB-α increased due to the LPS treatment, whereas the increase was slightly suppressed by the pretreatment with **3** (Fig. 3B). Phosphorylation of p38 MAPK and JNK was also stimulated by LPS, but it was not suppressed by the pretreatment with **3** (Figs. 3C, D). On the other hand, the LPS-induced gene expression of TNF-α and iNOS was attenuated in a dose-dependent manner by the pretreatment with **3** at the mRNA level (Fig. 3E) as well as at the protein level (Figs. 3F, G). These results suggest that **3** inhibited neither the NF-κB nuclear translocation pathway nor the MAP kinase activation pathway for the proinflammatory gene expression, although many anti-inflammatory phytochemicals have been reported to block either or both of these pathways.[12—14,17,19—21] The target molecule of **3** is possibly involved in the process within nucleus including the further activation of NF-κB[22] and construction of transcriptional machinery,[23] but its elucidation is to be studied in future.

Harmine (**3**) is contained in several plants such as *Peganum harmala*,[9] *Banisteriopsis caapi*,[24] and *Passiflora incarnata*,[25] which have been used as traditional medicines and beverages.[26] **3** has been recognized as the psychoactive constituent in the plants through its monoamine oxidase A

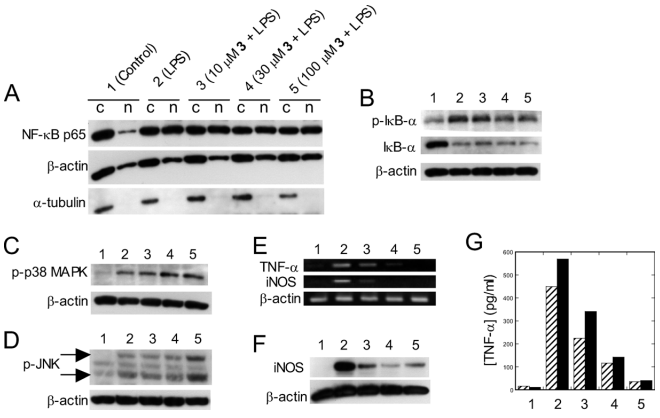

Fig. 3. Western Blot for the Proteins Related to the Expression of TNF-α and iNOS, RT-PCR of the mRNA, and ELISA of TNF-α in RAW264 Cells Stimulated or Not Stimulated by LPS without or with Pretreatment with Harmine (**3**)

Western blot: cytoplasmic (c) and nuclear (n) NF-κB p65 (A), cytoplasmic phosphorylated IκB-α and IκB-α (reproved) (B), phosphorylated p38 MAPK (C), phosphorylated JNK (D), and iNOS (F). RT-PCR: detection of mRNA of TNF-α, iNOS, and β-actin (E). ELISA: determination of TNF-α in the medium (G). The number given to each lane or column represents the treatment: 1, control (without LPS and **3**); 2, with LPS only; 3, with 10 μM **3** and LPS; 4, with 30 μM **3** and LPS; and 5, with 100 μM **3** and LPS. Membranes of A—D and F were reproved for β-actin and/or α-tubulin. Cells for E were extracted after the treatment with LPS for 4 h. In G, values are means of 2 or 3 wells. The medium was collected 4 h (hatched bars) or 6 h (closed bars) after the addition of LPS.

inhibition.[9] Recently, **3** was found to be a very specific inhibitor of DYRK1A (dual-specificity tyrosine-phosphorylated and -regulated kinase 1A) whose elevated expression might be involved in Down's syndrome.[27] Furthermore, **3** was found to act as a regulator of transcription factor peroxisome proliferator-activated receptor γ (PPARγ) expression and to suppress TNF-α in diabetic mice.[11] The report is interesting because the closely related transcription factor PPARα does transrepression against NF-κB and AP-1 (a downstream factor in MAP kinase pathway).[28] Although it is not clear whether and how the above activities of **3** are linked to the inflammatory system, our study revealed that **3** suppresses the TNF-α and NO production in LPS-stimulated immune cells, demonstrating one new aspect for the various pharmacological functions of **3**. Since **3** suppresses not only TNF-α but also IL-6 that plays an important role in chronic inflammation,[29] this compound would be a promising lead in developing new agents for treatment of chronic inflammatory diseases such as rheumatoid arthritis.

In conclusion, some alkaloids like harmine (**3**) that have not been recognized as anti-inflammatory agents nevertheless exhibit the ability to strongly suppress TNF-α and NO production by a unique mechanism. This anti-inflammatory activity is independent of antioxidative property.

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