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 (IC50=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 e-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. 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. Although many synthetic drugs and biologics have been developed for suppressing TNF-α and NO, application of natural products of the same activity in dietary plants without or with other functional food factors like polyunsaturated fatty acids 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-inflammatoryities, that is, TNF-α suppressing phytochemicals so far reported are mainly phenolic compounds with antioxidative property (e.g., flavonoids and stilbenoids). 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.

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 text books of pharmacognosy and catalogues of commercial reagents, and two Lycoris alkaloids (13, 14) were included to confirm the previous report on their anti-TNF-α activity. These compounds are known to have various pharmacological activities (e.g., monoamine oxidase inhibition for 3–5, intellectual capacity improvement for 7, anti-...
cholinergic activity for 8, uterine hemostatic and antiseptic activity for 10, antimalarial activity for 11, and smooth muscle-relaxing activity for 12), but information about their effect on inflammatory cytokine production has been sparse and limited to a few reports on harmine (3), narcissine (9), and berberine (11), and that on 13 and 14 as described above. A polyphenol, butein (15), was used as a reference compound.

Experimental

Materials. Hydrochloride dihydrate salts of 2, 4, and 5, 12 (hydrochloride), and isoquin were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 11-Beta (hydrochloride) was from Tokyo Chemical Industry Co., 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 Extrasythése (Genay, France), and lipopolysaccharide (LPS, 055:B5) was from List Biological Laboratories, Inc. (Campbell, CA, U.S.A.). Lycorinidol (narciclasine, (ELISA) kits for mouse and human TNF-α, IFN-γ, and IL-6 were purchased 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). Prelabeled cells were suspended in the medium at a rate of 2×10⁵ 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) containing 10% FBS and the plate culture was performed in the presence of 0.1 μM LPS. NO and TNF-α production was studied with 0.1—10 μM IFN-γ and 10, 30, or 100 μM harmine solution in each well of 96-well plates. After 2 d incubation, the medium was changed to a fresh medium (1 ml/well) and 0.2 ml suspension was added to each well. 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 LPS (0.4 ml/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 AGA GAA AGC ATG ATC-3' and anti-sense strand 5'-TAC AGG CTT GCT ACT CGA ATT-3', anti-sense strand 5'-TAC AGG CTT GCT ACT CGA ATT-3', and iNOS—sense strand 5'-GAT CAG GAG GGA TTT CAA AGA CCT-3' and anti-sense strand 5'-GGT CAG GAG GGA TTT CAA AGA CCT-3'.

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 IC₅₀ 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. Harmol (2) and harmaline (5), and harmalol (4) and rhynphylline (9) exhibited moderate and the marginal activities, respectively. Other compounds, harmalone (1), reserpine 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 IC₅₀ for TNF-α suppression, except for 11 and 13, as evidenced by ED₅₀ in cell viability test (Table 1). These TNF-α suppressive alkaloids also inhibited NO production in RAW264 cells and the IC₅₀ 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) (IC₅₀=0.4±0.2 μM) and butein (15) (IC₅₀=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 [IC₅₀ (mean±S.D.)=2±0.2, >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 suprasoxide anion scavenging activities at high concentrations (0.5—2 mM), while butein (15) showed a strong activity with IC₅₀ 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>RAW264 cells</th>
<th>Typical herb</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IC_{50} (μM)^a</td>
<td>IC_{50} (μM)^a</td>
</tr>
<tr>
<td>Harmane (1)</td>
<td>—c</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Harmol (2)</td>
<td>16±10</td>
<td>53±13</td>
</tr>
<tr>
<td>Harmine (3)</td>
<td>4±1</td>
<td>21±5</td>
</tr>
<tr>
<td>Harmalol (4)</td>
<td>&gt;100</td>
<td>—</td>
</tr>
<tr>
<td>Harmaline (5)</td>
<td>34±12</td>
<td>—</td>
</tr>
<tr>
<td>Reserpic acid (6)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Vincamine (7)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Physostigmine (8)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Rhyncophylline (9)</td>
<td>&gt;100</td>
<td>—</td>
</tr>
<tr>
<td>Hydrastine (10)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Berberine (11)</td>
<td>73±10^{10}</td>
<td>89±17^{10}</td>
</tr>
<tr>
<td>Papavereine (12)</td>
<td>10±3</td>
<td>59±11</td>
</tr>
<tr>
<td>Lycorine (13)</td>
<td>2.1±0.4</td>
<td>1.2±0.4</td>
</tr>
<tr>
<td>Lycoricidinol (14)</td>
<td>0.020±0.003</td>
<td>0.010±0.003</td>
</tr>
<tr>
<td>Butein (15)</td>
<td>8±4</td>
<td>5±2</td>
</tr>
</tbody>
</table>

a) Values are means±standard deviation (S.D.) (n=3). 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.

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. As to 13, its effect on the 1xB/NF-κB system in human promyelocytic leukemia HL-60 cells was recently reported. 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. The signal by LPS is transduced to the expression of TNF-α and iNOS via MAP kinase pathway through phosphorylation of protein kinases such as p38 MAPK and JNK. 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. The target molecule of 3 is possibly involved in the process within nucleus including the further activation of NF-κB and construction of transcriptional machinery, but its elucidation is to be studied in future.

Harmine (3) is contained in several plants such as Peganum harmala, Banisteriopsis caapi, and Passiflora incarnata, which have been used as traditional medicines and beverages. 3 has been recognized as the psychoactive constituent in the plants through its monoamine oxidase A...
inhibition.\(^9\) Recently, 3 was found to be a very specific inhibitor of DYRK1A (dual-specificity tyrosine-phosphorylated -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 \(\gamma\) (PPAR\(\gamma\)) expression and to suppress TNF-\(\alpha\) in diabetic mice.\(^11\) The report is interesting because the closely related transcription factor PPAR\(\alpha\) does transrepression against NF-\(\kappa\)B 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-\(\alpha\) and iNOS, RT-PCR of the mRNA, and ELISA of TNF-\(\alpha\) in RAW264 Cells Stimulated or Not Stimulated by LPS without or with Pretreatment with Harmine (3).

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

Western blot: cytoplasmic (c) and nuclear (n) NF-\(\kappa\)B p65 (A), cytoplasmic phosphorylated 1kB-\(\alpha\) and 1kB-\(\alpha\) (reproved) (B), phosphorylated p38 MAPK (C), phosphorylated JNK (D), and iNOS (F). RT-PCR: detection of mRNA of TNF-\(\alpha\), Stimulated or Not Stimulated by LPS without or with Pretreatment with Harmine (E), 10 \(\mu\)M of 3 or DMSO as a control (F), and ELISA: determination of TNF-\(\alpha\) in RAW264 Cells Stimulated or Not Stimulated by LPS without or with Pretreatment with Harmine (G).

References