Inhibitory Effect of Dehydroevodiamine and Evodiamine on Nitric Oxide Production in Cultured Murine Macrophages

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Possible antiinflammatory effects of dehydroevodiamine (**1**) and evodiamine (**2**) were examined by assessing their effects on NO production in the murine macrophage-like cell line RAW 264.7. The results indicated that both **1** and **2** inhibited the IFN-γ/LPS-stimulated NO production in a concentration-dependent manner. However, **1** appeared to inhibit NO production by interfering not only with the priming signal initiated by IFN-γ but also with iNOS protein synthesis, while **2** affected the former only.

"Wu-Chu-Yu", the unripe fruit from *Evodia rutaecarpa*, has long been utilized in traditional Chinese medicine for the treatment of inflammation-related disorders such as eczema and ulcerative stomatitis.[1] Wu-Chu-Yu and some of its bioactive principles have been demonstrated to possess antiinflammatory and analgesic effects.[2] In controlled clinical trials it has also been confirmed that topical administration of Wu-Chu-Yu powder markedly and effectively improved the conditions in 64% of patients suffering from tissue injury and pain of recurrent ulcerative stomatitis following a two-day treatment.[3,4] The underlying mechanisms accounting for its antiinflammatory actions have, however, remained unelucidated.

It is believed that the activation of macrophages plays an important role in the inflammatory process.[5] Increasing evidence also suggests that nitric oxide (NO) is centrally involved.[6] Thus, NO production may reflect the degree of inflammation and provides a measure by which the effects of drugs on the inflammatory process can be assessed. Both interferons, such as interferon-γ (IFN-γ), and bacterial endotoxins, such as lipopolysaccharide (LPS), are known activators of macrophages. Furthermore, it appears that these agents can act cooperatively to bring about full activation, with interferons acting as priming agents and bacterial endotoxins as triggering agents.[7−11] IFN-γ is believed to regulate positively LPS-induced transcriptional activation of the iNOS gene. In vitro studies in our laboratories had indicated that dehydroevodiamine (**1**) and evodiamine (**2**) could inhibit the LPS-induced NO production in a chicken macrophage cell line (MQ-NCSU). In the present study, **1** and **2**, two of the major bioactive quinazoline alkaloids isolated from Wu-Chu-Yu, were examined for antiinflammatory properties by studying their effects on NO production in a murine macrophages-like cell line (RAW 264.7) when stimulated by the inducers IFN-γ/LPS.

Having established that NO production in the RAW 264.7 cells were inducible by IFN-γ/LPS (from the basal 2.9 ± 1.7 μM to 54.3 ± 4.6 μM), a known NOS inhibitor L-N^{ω}-nitroarginine methyl ester (L-NAME) was used to test the inhibitability of NO production in such a system. Results indicated that not only NO production was significantly inhibited by L-NAME, but the inhibited was also effective regardless of the order in which L-NAME was added in the IFN-γ/LPS induction sequence.

The next concern was whether the dissolution vehicle might have effects of its own or in some ways affect the experimental results, as the alkaloids had to be dissolved in DMSO. Test results indicated that DMSO at concentrations of up to 0.1% was neither cytotoxic nor had any insignificant effects on NO production. However, DMSO at concentrations higher than 0.1% exhibited cytotoxicity. Tests of effects of these alkaloids at concentrations requiring dissolution in DMSO higher than 0.1% were thus precluded.

The effects of alkaloids **1** and **2** on unstimulated RAW cells were studied next. The results indicated that neither **1** nor **2** had any significant effects on the NO synthesis in unstimulated cells. The next series of experiments were designed simply to study the overall effects of alkaloids **1** and **2** on NO production in macrophage cells stimulated by sequential IFN-γ/LPS treatments. The alkaloids were added to the medium 30 min prior to treatment with IFN-γ for 3 h. LPS was added only after the removal of IFN-γ by washing the cells twice and having them incubated in fresh culture medium for a further 30 min (Figure 1, Scheme 1). The effects of **1** and **2** on NO production are shown in Fugures 2 and 3. Treatment with **1** (10, 50, 100 μM) inhibited NO production in a concentration-dependent manner from the vehicle-control level of 55.1 ± 2.4 μM (100%) to 44.1 ± 2.3 μM (80.6 ± 4.2%), 40.8 ± 3.4 μM (74.2 ± 6.2%), and 24.5 ± 2.9 μM (44.0 ± 5.3%), respectively (Figure 2A, left panel). In the case of **2** (Figure 3A, left panel), NO production was also suppressed in a concentration-dependent manner (10, 30, 50 μM) from the vehicle-control level of 53.5 ± 2.4 μM (100%) to 42.5 ± 2.1 μM (79.5 ± 4.2%), 36.5 ± 3.2 μM (68.3 ± 6.0%), and 27.0 ± 2.8 μM (50.6 ± 5.2%), respectively. The inhibitory effects in both cases were significant when compared to vehicle (DMSO) treatment

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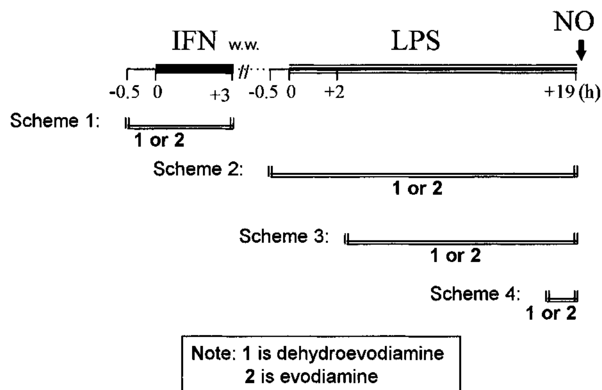

Figure 1. Experimental design for the evaluation of effects of dehydroevodiamine (**1**) and evodiamine (**2**) on NO production in stimulated macrophages.

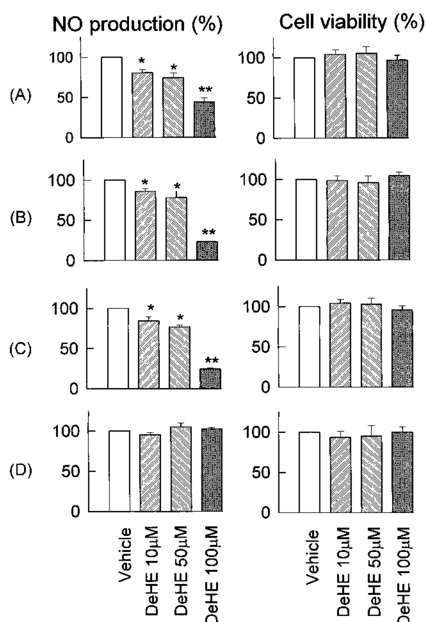

Figure 2. Effect of dehydroevodiamine (**1**) on NO production (left panel) and cell viability (right panel) in stimulated macrophages. Dehydroevodiamine (**1**) was added to RAW 264.7 cells before stimulation by IFN-γ (A) or at -0.5 (B), $+2$ (C), and $+18$ h (D) relative to the adddition of LPS (0 h). In vehicle groups, DMSO up to a maximal final concentratioin of 0.1% was added at correponding time intervals. Each point and vertical bar represents the mean $\pm$ SE ($n = 9-13$ in each group). Degrees of statistical significance were indicated by $^*p < 0.05$, $^{**}p < 0.01$.

values. The inhibitory efficacies between **1** and **2** were, however, different, with **2** being more potent, achieving the same degree of inhibition at the lower concentrations of 10, 30, 50 μM as compared to 10, 50, 100 μM for **1**. With regard to cell viability, MTT-reduction assay indicated that neither **1** nor **2** had any significant suppressive effects (Figures 2A and 3A, right panels). Regrettably, complete definition of the dose–response relationships were not possible as dissolution of higher concentrations of the alkaloids would have required a cytotoxic concentration of DMSO of higher than 01%. Mechanistically, since the test alkaloids were added before IFN-γ and the cells were thoroughly washed subsequently for the removal of IFN-γ, the inhibitory effects observed were unlikely due to lingering direct actions of the alkaloids but rather due to indirect consequences of their effects on IFN-γ-related actions.

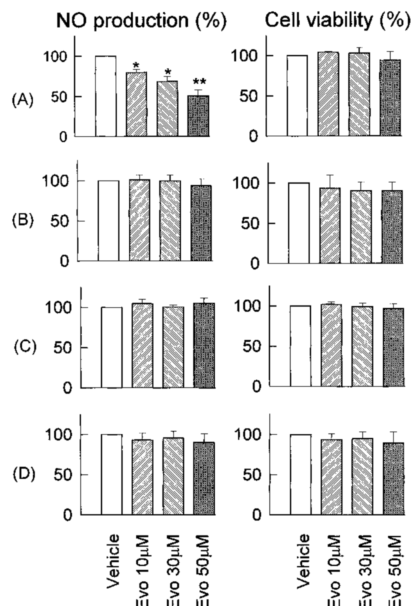

Figure 3. Effects of evodiamine (**2**) on NO production (left panel) and cell viability (right panel) in stimulated macrophages. Evodiamine (**2**) was added to RAW 264.7 cells before stimulation by IFN-γ (A) or at -0.5 (B), $+2$ (C), and $+18.5$ h (D) relative to the adddition of LPS (0 h). In vehicle groups cells were cultured in the presence of DMSO (maximal final concentration 0.1%). Each point and vertical bar represents the mean $\pm$ SE ($n = 10-13$ in each group). Degrees of statistical significance were indicated by $^*p < 0.05$, $^{**}p < 0.01$.

The fact that in some of the experiments no inhibitory effects were seen when **2** was added only after the removal of IFN-γ also lent support to this hypothesis. Possible loci of action might include inhibition of IFN-γ-receptor activation or the subsequent synthesis-induction signal transduction process.

Having established that these alkaloids had overall inhibitory effects, subsequent experiments were designed to define the mechanism of actions of these alkaloids. This process mainly involved the assessment of the inhibitory effectiveness when the alkaloids were added at various points in the cascade of events in IFN-γ/LPS-induced iNOS and NO production. To determine if **1** and **2** interfered with the LPS's triggering process, the alkaloids were added to the medium after the removal of IFN-γ but 30 min before LPS (Figure 1, scheme 2). As shown in Figure 2B, NO production was inhibited in a concentration-dependent manner (10, 50, 100 μM) by **1** from the vehicle-control level of 57.5 $\pm$ 3.0 μM (100%) to 49.5 $\pm$ 4.2 μM (86.1 $\pm$ 3.6%), 44.9 $\pm$ 5.0 μM (78.1 $\pm$ 8.3%), and 13.3 $\pm$ 0.3 μM (23.2 $\pm$ 0.6%), respectively. It appeared that, in addition to inhibiting the actions of IFN-γ, **1** also inhibited LPS-induced actions. In contrast, NO production was not affected by **2** when it was administed 30 min before LPS (Figure 3B), suggesting that **2** acted only at the level of IFN-γ-induced priming action. Neither **1** nor **2**, administered in this fashion, had any cytotoxic effect.

It has been reported that sequential treatment with IFN-γ and LPS results in an increase in iNOS–mRNA until a steady state is reached, in about 2 h, as reflected by a constant NO production rate over the following 16–18 h.[12] According to this view, inhibitors affecting this stage of the process would be most effective if they were introduced within 2 h of the stimulation by IFN-γ/LPS. To test this hypothesis, **1** and **2** was added to the cell

culture 2 and 18.5 h after LPS treatment (Figure 1, schemes 3 and 4). Significant suppression was observed when **1** was added in the initial 2 h of the cell stimulation (Figure 2C). NO production was suppressed from 51.8 ± 2.4 μM (100%) to 43.7 ± 2.8 μM (84.3 ± 5.5%), 39.5 ± 0.9 μM (77.0 ± 1.8%), and 12.5 ± 0.8 μM (24.2 ± 1.6%) by treatment with **1** (10, 50, and 100 μM), respectively. By contrast, addition of **1** 18.5 h after LPS produced no significant inhibition (Figure 2D). These results suggested that **1** might inhibit the translation of iNOS−mRNA, but had little effect once the induction of iNOS had been complete. As indicated in the right panels of Figure 2C and 2D, **1** did not exert any cytotoxic effects. On the other hand, **2** affected neither NO production nor cell viability significantly when administered under the same conditions (Figure 3C and 3D).

It has been reported that co-stimulation of RAW 264.7 macrophages with IFN-γ and LPS resulted in an increase in the rate of transcription of the iNOS gene several times higher than that obtained with LPS alone.[12] The effect of IFN-γ on iNOS induction is transcriptionally mediated. Although transcription of the iNOS gene is detectable earlier in macrophages treated with IFN-γ and LPS than in cells stimulated with LPS alone, the overall kinetics of iNOS transcription are comparable in the two groups, with peak transcription of the iNOS gene occurring at approximately 2 h. However, a previous report indicated that at each time point examined, the extent of transcriptional activation was much greater in cells stimulated with both stimuli than in macrophages treated with LPS alone.[12] In the present study we found that stimulating the RAW cells with LPS alone induced only a modest increase in NO production as compared to background levels (19.1 ± 2.4 μM vs. 2.9 ± 1.7 μM). This, however, was sufficient to produce a detectable cytotoxic effect on the macrophages themselves (cell viability suppressed from 100% to 86.3 ± 5.7%). In contrast, when the cells were first challenged with IFN-γ (3 h) and then LPS, there was a marked augmentation of NO production (54.3 ± 4.6 μM). The cytotoxic effect on cell viability was not significantly enhanced further as compared with LPS treatment alone (cell viability suppressed from 100% to 80.9 ± 4.1%).

The induction by IFN-γ of NO production in RAW macrophages involves *de novo* protein biosynthesis and is transcriptionally based. The synthesis of NO requires multiple signals delivered to the macrophage in a defined sequence of which IFN-γ is the major priming signal.[13,14] The sequential exposure to IFN-γ and LPS is probably the best-studied model.[13] It is predictable that the molecular pathways activated by each of these two compounds for the regulation of iNOS gene expression are not only different from each other, but also apt to allow integration of these pathways, with consequent efficient cooperation between the two signals.[7]

In summary, the kinetics and the loci at which NO synthesis was affected by **1** and **2** were assessed by having them introduced to the medium at different activating stages (priming, triggering, and/or iNOS−mRNA translational stages). Compound **1** was found to inhibit NO production in a concentration-dependent and almost equipotent manner, whether added before IFN-γ or before or after LPS application, indicating that **1** suppressed the activities of iNOS at multiple levels, those related to activation by IFN-γ as well as events subsequent to it. By contrast, the NOS inhibitor L-NAME was equally effective at any time of application.

Taken together, it is believed that, in addition to an antagonistic effect on IFN-γ's priming signal, **1** might also act on the final common pathway resulting in iNOS protein synthesis. Because the suppression of iNOS expression is probably as important in its regulation as in its induction, the complex biochemistry of NO production affords many potential sites for regulatory action. One such site is the interference with the post-transcriptional or translational pathway of iNOS−mRNA.[15] Other possibilities might include acting as a protein-synthesis inhibitor. Whether **1** actually suppressed NO synthesis by decreasing the amount of iNOS protein awaits further study.

In conclusion, our results demonstrated that **1** and **2** inhibited NO production in IFN-γ/LPS-stimulated RAW macrophages in a concentration-dependent manner. Dehydroevodiamine (**1**) inhibited NO production by interfering with IFN-γ's priming signal as well as the subsequent activities leading to the induction of iNOS but apparently not the action of iNOS itself. On the other hand, evodiamine (**2**) affected only the IFN-γ-related actions. It is possible that these two quinazoline alkaloids might account for or contribute to the antiinflammatory properties of Wu-Chu-Yu. Possible underlying mechanisms include general impairment of the macrophage activation processes, leading to a further reduction of the release of some of the inflammatory mediators such as several cytokines and eicosanoids.

Experimental Section

Cell Culture. RAW 264.7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL) and maintained at 37 °C, 5% CO_2 in a humidified chamber. Prior to experiments, cells were harvested and seeded in 96-well plates at 2×10^5 cells/well in FBS-free DMEM. Cells were then allowed to adhere for 60 min at 37 °C. Unadhered cells were removed by aspiration, and the adhered cells were incubated with fresh medium containing 10% FBS.

General Experimental Procedures. Since it has been reported that full activation of the macrophage can be achieved via the ordered sequence of intermediary reactions triggered by different inducers in a cooperative manner,[14] the desired activated macrophages for these studies were obtained by first priming the cells with IFN-γ, followed by stimulation with a second inducer (LPS). Briefly, cells were initially treated with IFN-γ (50 U/mL) for 3 h (first inducer). After two washes and a 30-min incubation of the cells with fresh culture medium, LPS (0.5 μg/mL) was then added and allowed to remain in the medium throughout the culture period. Supernatants were collected overnight (starting at 19 h) for nitrite analysis.

To define the loci of action in the inhibitory actions of these test alkaloids on NO synthesis, the effects were studied separately at the levels of priming, triggering, and protein synthesis. As shown in scheme 1 of Figure 1, the vehicle (DMSO as a control) or the test alkaloids (**1** and **2**) were added to the cell cultures 30 min before IFN-γ. After a further incubation for 3 h, test drugs

were removed by washing twice and then incubating with fresh medium for another 30 min. LPS, the second inducer, was then introduced to the medium, and the cells were then incubated overnight (about 18−19 h).

In another series of experiments as described in schemes 2−4 of Figure 1, cells were first challenged with IFN-γ (50 U/mL). After washing twice, the vehicle DMSO or various concentrations of the alkaloids (**1** or **2**) were added at −0.5, +2, or +18.5 h relative to the addition of LPS (0 h). Following the addition of LPS, the cells were incubated at 37 °C, 5% CO_2 in humidified air for an additional 18−19 h. An aliquot of the medium was removed for assay of NO production.

NO production was estimated by measuring a stable metabolite of NO, nitrite (NO_2^-), using the Griess method.[16,17] At the end of each experiment, 100 μL of supernatant was taken from each well and added to an equal volume of Griess reagent (1:1, v/v, of 0.1% 1-*N*-naphthylethylenediamine HCl in distilled H_2O and 1% sulfanilamide in 5% H_3PO_4) on a 96-well flat-bottom plate. Absorbance at 550 nm was measured using a Bio-Tek EL311 microplate reader. Mean readings from three measurements per sample were used. Nitrite concentration was calculated from a precalibrated standard curve using $NaNO_2$ as standard. The results were expressed as the mean ± SE for *n* separate experiments, and NO productions were indicated as either absolute concentrations in μM or as a percentage of the control. Drug effects were compared with their corresponding controls for statistical significance using a one-way analysis of variance (ANOVA) followed by Student's *t*-tests; *p* values of less than 0.05 were considered to be statistically significant.

Cell viability at the end of each experiment was analyzed by a MTT-reduction assay as described by Mosmann.[18] Briefly, 10 μL of stock MTT solution (5 mg/mL in PBS) was added to each well of macrophage cultures following the removal of 100 μL medium for nitrite analysis. After 4 h of incubation at 37 °C, 100 μL of acid-SDS solution (10% SDS in 0.04 N HCl) were added to each well to stop the reaction. The reduced MTT−formazan product was dissolved by incubating the SDS−MTT medium mixture at 37 °C overnight. Quantitation of MTT reduction was accomplished by measuring absorbance at 570 nm against a 650-nm reference using the EL-311 microplate reader. Results were represented as the mean optical density values from triplicate cultures and were converted to percentages of control.

Effects of IFN-γ and/or LPS on NO Production. Macrophages were stimulated with IFN-γ (50 U/mL) and LPS (0.5 μg/mL) sequentially. Resulting cytotoxicity and NO production were assayed in parallel cultures. Because time-course analyses of the IFN-γ/LPS-induced release of NO had indicated a time lag of about 12 h for a significant amount of NO (>40 μM) to be released or detectable, an overnight collection of about 18−19 h of activation was adopted. This resulted in the generation of about 50−60 μM of NO using the vehicle-control group as the standard.

Chemicals. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and penicillin−streptomycin solution were purchased from GIBCO Laboratories (Grand Island, NY). Low-endotoxin fetal bovine serum was purchased from HyClone Laboratories (Logan, UT). MTT powder, L-glutamine, lipopolysaccharide (LPS, from *E. coli* strain 0127:B8), interferon-γ, 1-*N*-naphthylethylenediamine HCl, sulfanilamide, H_3PO_4, and $NaNO_2$ were from Sigma Chemical Co. (St. Louis, MO).

Isolation of Alkaloids. Compounds **1** and **2** were isolated and identified as previously described from the dried, unripened fruit of *Evodia rutaecarpa* in our institute.[19]

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