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Immunomodulatory Activity of Oenothein B Isolated from Epilobium angustifolium

Igor A. Schepetkin,* Liliya N. Kirpotina,* Larissa Jakiw,* Andrei I. Khlebnikov,† Christie L. Blaskovich,* Mark A. Jutila,* and Mark T. Quinn2*

Epilobium angustifolium has been traditionally used to treat a number of diseases; however, not much is known regarding its effect on innate immune cells. In this study, we report that extracts of E. angustifolium activated functional responses in neutrophils and monocyte/macrophages. Activity-guided fractionation, followed by mass spectroscopy and NMR analysis, resulted in the identification of oenothein B as the primary component responsible for phagocyte activation. Oenothein B, a dimeric hydrolysable tannin, dose-dependently induced a number of phagocyte functions in vitro, including intracellular Ca2+ flux, production of reactive oxygen species, chemotaxis, NF-κB activation, and proinflammatory cytokine production. Furthermore, oenothein B was active in vivo, inducing keratinocyte chemoattractant production and neutrophil recruitment to the peritoneum after intraperitoneal administration. Biological activity required the full oenothein B structure, as substructures of oenothein B (pyrocatechol, gallic acid, pyrogallol, 3,4-dihydroxybenzoic acid) were all inactive. The ability of oenothein B to modulate phagocyte functions in vitro and in vivo suggests that this compound is responsible for at least part of the therapeutic properties of E. angustifolium extracts. The Journal of Immunology, 2009, 183: 6754–6766.

Enhancement of innate immunity by immunomodulators can increase host resistance to pathogens (1), and a number of innate immunomodulators have been identified, including cytokines (2), substances isolated from microorganisms and fungi (3), and substances isolated from plants (4, 5). However, many of these substances are high molecular mass carbohydrates (6) or lectins (7), and only a few plant-derived compounds with a relatively low molecular mass are known to modulate phagocyte functions, e.g., taxol (8), phenylpropanoid glycoside acetoside (9), and alkylamides from Echinacea purpurea (10). Thus, there is a significant amount of interest in identifying low molecular mass compounds with potential medicinal properties.

The genus Epilobium is widely distributed around the world and consists of over 200 species, with the most common being Epilobium angustifolium L. Many species of the genus Epilobium have been used in folk medicine to treat a variety of diseases and enhance wound healing (reviewed in Ref. 11). Indeed, extracts from Epilobium taxa have been shown in both in vitro and in vivo studies to exhibit many therapeutic properties, including anti-inflammatory (12), antidiagnostic (13), antiinflammatory (14, 15) fungifungal (16, 17), antimicrobial (18, 19), antitumor activity and that this may be due to enhancement of the host-immune system via induction of IL-1β (27). However, little else has been reported on the effects of oenothein B on innate immunity. Thus, we evaluated the effects of E. angustifolium extracts on phagocyte function.

In this study, we report that E. angustifolium extracts can activate phagocyte functional responses. Furthermore, fractionation of the extracts indicated that the active component was oenothein B. Oenothein B activated monocyte/macrophages and neutrophils, resulting in increased intracellular Ca2+ flux, production of reactive oxygen species (ROS)3 and cytokines, and chemotaxis. Thus, part of the observed therapeutic effects of oenothein B and Epilobium extracts is due to modulation of innate immune function.

Materials and Methods

Reagents

Corilagin (1-O-galloyl-3,6-hexahydroxydiphenol-β-D-glucopyranose) was from Toronto Research Chemicals; 1,2,3,4,6-pentakis-O-galloyl-β-D-glucose

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2 Address correspondence and reprint requests to Dr. Mark T. Quinn, Veterinary Molecular Biology, Montana State University, Bozeman, MT 59717. E-mail address: mquinn@montana.edu

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fractions were stored at phase, resulting in 180 mg of the final product. All lyophilized extracts and for 10 min), the zymosan particles were washed and resuspended in /H11022 ml/min and detection at 270 nm, at 30°C over 15 min (34). Purity was /H11003 Invitrogen. RPMI 1640 medium was purchased from Mediatech. Percoll /H9262 Acetone-d6 was from Cambridge Isotope Laboratories. HPLC grade /H11001 dismutase, percoll, HEPES, Histopaque 1077, Histopaque 1119, LPS from /H11003 E. angustifolium Fully blossomed Extraction and isolation of active compound

Fully blossomed E. angustifolium were collected and identified by Robyn A. Klein (Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT). The dried plant material (400 × g) was extracted with 80% methanol at room temperature for 3 days. The combined extracts were concentrated, and any precipitates were removed by filtration through a 0.22-µm filter. The filtrate was lyophilized to obtain the crude extract or subjected to concentration and fractionation on a Sephadex LH-20 column (2.8 × 33 cm) using 80% methanol as an eluent. The relevant fractions were pooled and evaporated to dryness. For analysis of biological activity, the crude extract and pooled fractions were dissolved in DMSO. Bioactive subfraction S-3 was rechromatographed twice on a biological activity, the crude extract and pooled fractions were dissolved in DMSO. Bioactive subfraction S-3 was rechromatographed twice on a

Cell culture

Human monocyte THP-1Blue cells obtained from InvivoGen were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 100 µg/ml streptomycin, 100 U/ml penicillin, 100 µg/ml Zeocin, and 10 µg/ml blastidin. THP-1Blue cells are stably transfected with a secreted embryonic alkaline phosphatase gene that is under the control of a promoter inducible by NF-κB.

Human leukemia HL-60 cells were cultured in RPMI 1640 supplemented with 10% (v/v) heat inactivated FCS, 10 mM HEPES, 100 µg/ml streptomycin, and 100 U/ml penicillin. HL-60 cells were differentiated to macrophage-like cells by treatment with 10 nM PMA for 3 days (35). All cultured cells were grown at 37°C in a humidified atmosphere containing 5% CO2. Cell number and viability were assessed microscopically using trypan blue exclusion.

Isolation of murine bone marrow leukocytes and neutrophils

All animal use was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Montana State University. Murine bone marrow cells were flushed from tibias and femurs of BALB/c mice with HBSS using a 27-gauge needle. The cells were dextran sedimentation, followed by Histopaque 1077 gradient separation and hypotonic lysis of RBC, as described previously (38). Isolated neutrophils were collected and identified by Robyn A. Klein (Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT). The dried plant material (400 × g) was extracted with 80% methanol at room temperature for 3 days. The combined extracts were concentrated, and any precipitates were removed by filtration through a 0.22-µm filter. The filtrate was lyophilized to obtain the crude extract or subjected to concentration and fractionation on a Sephadex LH-20 column (2.8 × 33 cm) using 80% methanol as an eluent. The relevant fractions were pooled and evaporated to dryness. For analysis of biological activity, the crude extract and pooled fractions were dissolved in DMSO. Bioactive subfraction S-3 was rechromatographed twice on a

Isolation of human neutrophils and mononuclear cells

Blood was collected from healthy donors in accordance with a protocol approved by the Institutional Review Board at Montana State University. Neutrophils and mononuclear cells were purified from the blood using dextran sedimentation, followed by Histopaque 1077 gradient separation and hypotonic lysis of RBC, as described previously (38). Isolated neutrophils were washed twice and resuspended in HBSS−. Neutrophil preparations were routinely ≥95% pure, as determined by light microscopy, and ≥98% viable, as determined by trypan blue exclusion. PBMC were isolated from blood using dextran sedimentation and Histopaque 1077 gradient separation (39).

Analysis of phagocyte ROS production

ROS production was determined by monitoring t-012-ECL, which represents a sensitive and reliable method for detecting superoxide anion (O2−) production in vitro (40). In brief, phagocytes were aliquoted into wells (103 cells/well) of 96-well flat-bottom white microplates, and test extracts or lyophilized fractions diluted in DMSO were added (final DMSO concentration of 1%). After preincubation at 37°C for the indicated times, an equal volume of 0.05% BSA in HBSS− was added to each well, the plates were centrifuged, the medium was removed, and fresh HBSS− supplemented with 40 µg/ml L-012 and 8 µg/ml HRP was added. In some experiments, the plates were read directly without replacing the medium to evaluate PMN or zymosan-stimulated ROS production in the presence of the oenothein B or total E. angustifolium extract. Luminescence was monitored for 60 min (2-min intervals) at 37°C using a Fluoroscope Ascent FL microplate reader (Thermo Electron). The curve of light intensity (in relative luminescence units) was plotted against time, and the area under the curve was calculated as total luminescence.

Xanthine/xanthine oxidase system

O2− was generated in an enzymatic system consisting of 500 µM xanthine, 500 µM NBT, 3.75 mM/mL xanthine oxidase, and 0.1 M phosphate buffer (pH 7.5), and O2− production was determined by monitoring reduction of NBT to monoformazan dye at 560 nm in the presence or absence of oenothein B. The reactions were monitored with a SpectraMax Plus microplate spectrophotometer at 27°C. To evaluate whether oenothein B affected the generation of O2− by direct interaction with xanthine oxidase,
enzyme activity was evaluated by spectrophotometric measurement of uric acid formation from xanthine at 295 nm (41). The reaction mixture contained 500 μM xanthine, 5 mM/ml xanthine oxidase, and 0.1 M phosphate buffer (pH 7.5), and the reaction was monitored in the presence or absence of oenothein B at 27°C.

**Ca²⁺ mobilization assay**

Changes in intracellular Ca²⁺ were measured with a FluoScreen II scanning fluorometer using a FLIPR Calcium Assay Kit (Molecular Devices). Human and murine neutrophils or HL-60 cells, suspended in HBSS containing 10 mM HEPEs, were loaded with FluoScreen Calcium 3 dye following the manufacturer’s protocol. After dye loading, Ca²⁺ was added to the cell suspension (2.25 mM final), and cells were aliquotted into the wells of a flat-bottom black microplate (2 x 10⁵ cells/well). The compound source plate contained dilutions of E. angustifolium extract or test compounds in HBSS or DMSO. Changes in fluorescence were monitored (λ_ex = 485 nm, λ_em = 525 nm) every 5 s for 120 s at room temperature after automated addition of compounds to the wells. Maximum change in fluorescence, expressed in arbitrary units over baseline signal observed in cells treated with vehicle (HBSS or DMSO), was used to determine agonist response. Curve fitting and calculation of median effective concentration values (EC₅₀) were performed by nonlinear regression analysis of the dose-response curves generated using Prism 5 (GraphPad Software).

**Chemotaxis assay**

Human neutrophils were suspended in HBSS containing 2% (v/v) FBS (2 x 10⁵ cells/ml), and chemotaxis was analyzed in 96-well ChemoTx chemotaxis chambers (NeuroProbes), as described previously (42). In brief, lower wells were loaded with 30 μl of HBSS containing 2% (v/v) FBS and E. angustifolium extract, the indicated concentrations of oenothein B vehicle control (DMSO or HBSS), or IL-8 and FMLP as positive controls. The number of migrated cells was determined by measuring ATP in lysates of transmigrated cells using a luminescence-based assay (CellTiter-Glo; Promega), and luminescence measurements were converted to absolute cell numbers by comparison of the values with standard curves obtained with known numbers of neutrophils. The results are expressed as percentage of negative control and were calculated as follows: (number of cells migrating in response to test compounds/spontaneous cell migration in response to control medium) x 100. EC₅₀ values were determined by nonlinear regression analysis of the dose-response curves generated using Prism 5 (GraphPad Software).

**Cytokine analysis**

Cells were incubated for 24 h in culture medium supplemented with 3% (v/v) endotoxin-free FBS, with or without compounds or LPS as a positive control. Human PBMCs and THP-1 cells were plated in 96-well plates at a density of 2 x 10⁴ cells/well. A human cytokine MultiAnalyte ELISArray Kit (SABiosciences) was used to evaluate differences in cytokine subfractions was evaluated in the NF-κB A reporter assay. Only subfraction S-3 activated NF-κB as reported in Figure S1. Based on these results, we fractionated the extract in efforts to identify the active component in this crude mixture. Concentrated methanol extract from E. angustifolium was fractionated by preparative Sephadex LH-20 chromatography and sixty 10-ml fractions were collected (Fig. 2A). These fractions were pooled into three subfractions, designated as S-1 to S-3, and activity of these subfractions was evaluated in the NF-κB reporter assay. Only subfraction S-3 activated NF-κB in this assay (Fig. 2B).

**Results**

**Effect of E. angustifolium extracts on ROS production and NF-κB activity**

To evaluate the effects of E. angustifolium extracts on phagocyte function responses, we analyzed the effects of methanol extracts from this plant on phagocyte ROS production and NF-κB activation. As shown in Fig. 1, extracts of E. angustifolium dose-dependently activated ROS production in murine bone marrow leukocytes and induced NF-κB in human THP-1 monocytes.

**Identification of the phagocyte-activating component in E. angustifolium extract**

Based on these results, we fractionated the extract in efforts to identify the active component in this crude mixture. Concentrated methanol extract from E. angustifolium was fractionated by preparative Sephadex LH-20 chromatography and sixty 10-ml fractions were collected (Fig. 2A). These fractions were pooled into three subfractions, designated as S-1 to S-3, and activity of these subfractions was evaluated in the NF-κB reporter assay. Only subfraction S-3 activated NF-κB in this assay (Fig. 2B).

The pooled subfraction S-3 was rechromatographed twice more on a Sephadex LH-20 to obtain the final sample, which was concentrated to dryness and analyzed by HPLC, mass spectroscopy, and ¹H- and ¹³C-NMR (supplemental Table S1). Based on comparison of mass spectroscopy and NMR data with those in the literature (34, 43–45), we found that the active component present in subfraction S-3 isolated from E. angustifolium was oenothein B (Mₑ = 1568; structure of the compound is shown in supplemental Figure S1).

As shown in supplemental Figure S2, the mass spectrum was characterized by the presence of monosodium (M plus Na) at m/z 1592.1 (1 m/z unit separation between isotopic peaks) and doubly charged disodium (M plus 2Na)²⁻ at m/z 807.1 (0.5 m/z unit separation between peaks) adducts of oenothein B. The prominent series at m/z 1068.7 is apparently a noncovalent dimeric aggregate of the monosodium and disodium adducts. It should be noted that time-of-flight mass spectroscopy of polyphenols, including ellagitannins, tends to favor association with sodium ions because naturally occurring Na⁺ ions are abundant in these samples (46, 47).
The 1H-NMR spectrum of our sample in D$_2$O contained six 1H-singlets and two 2H-singlets in the aromatic region (supplemental Table S1), which is in agreement with the presence of two galloyl and two valoneoyl moieties in oenothein B. Two glucopyranose residues gave well-resolved signals of sugar protons, with characteristic coupling similar to that of the oenothein B spectrum in acetone (44). Anomeric proton doublets appeared at $\delta^{9254} 4.47$ (J 9 Hz) and 5.47 (J 3.4 Hz), indicating that the anomeric hydroxyls of both glucose residues were nonacylated. Although an equilibrium occurs between the $\alpha$- and $\beta$-forms of each of the glucopyranose rings, the 1H-NMR spectrum showed that the $\alpha$-form dominates one ring, whereas the $\beta$-form dominates the other glucopyranose moiety. This is in contrast to oenothein F, an isomer of oenothein B, where a mixture of anomeric forms for both rings results in a more complex 1H-NMR spectrum (43). Because previous NMR analysis of oenothein B has been performed in acetone-d$_6$, we obtained additional 1H-NMR spectra of our sample in acetone-d$_6$ with 2% (v/v) D$_2$O. Although some of the signals were overlapped by the broad singlet of residual water protons, many signals characteristic of oenothein B were observed. For example, resonances of $\alpha$-glucose H-1 at $\delta^{6.17}$ (d, J 3 Hz) and of $\beta$-glucose H-5 at $\delta^{4.11}$ (dd, J 1 5, J 2 10 Hz) are very similar to the corresponding signals of oenothein B reported previously [$\delta^{6.18}$ and $\delta^{4.12}$, respectively, in acetone-d$_6$–D$_2$O (43); $\delta^{6.24}$ and $\delta^{4.14}$, respectively, in acetone-d$_6$ (44)]. These values are quite distinct from any glucose chemical shifts in the 1H-NMR spectrum of oenothein D (43). Eight singlets of galloyl and valoneoyl aromatic rings in the spectrum of our isolated sample were also very close (rms deviation of 0.07 ppm) to the corresponding signals of oenothein B reported previously [8 6.18 and 8 4.12, respectively, in acetone-d$_6$–D$_2$O (43); 8 6.24 and 8 4.14, respectively, in acetone-d$_6$ (44)]. These values are quite distinct from any glucose chemical shifts in the 1H-NMR spectrum of oenothein D (43). Eight singlets of galloyl and valoneoyl aromatic rings in the spectrum of our isolated sample were also very close (rms deviation of 0.07 ppm) to the corresponding signals reported for oenothein B in acetone-d$_6$ (44). Note that chemical shifts of aromatic moieties differ significantly between oenothein isomers, and galloyl and valoneoyl NMR signals for oenothein D and oenothein F are in quite different positions than for oenothein B (43). Taken together, our data demonstrate that the primary phagocyte-activating component of E. angustifolium extract is oenothein B.

**Effect of oenothein B on ROS production**

In studies described above, the E. angustifolium extract and subfractions were removed from the cells and replaced with fresh...
medium before analysis of ROS production by treated phagocytes. We used this approach to avoid antioxidant effects of potential plant-derived compounds, as it is known that *E. angustifolium* extract contains flavonoids, such as myricetin, kaempferol, and quercetin, which have antioxidant activity (45, 48, 49). To directly evaluate this issue, we analyzed potential scavenging effects of crude *E. angustifolium* extract or oenothein B on ROS produced by zymosan- and PMA-stimulated murine bone marrow leukocytes and human neutrophils. We found that the ROS signal was dose-dependently decreased when crude extract (5–20 μg/ml; data not shown) or oenothein B were present. Indeed, the ROS signal was completely lost when oenothein B was present in the assay at concentrations ≥2–3 μM (Fig. 3, A and B). IC₅₀ values were 50 and 90 nM oenothein B for scavenging ROS produced by PMA-stimulated human neutrophils and murine bone marrow leukocytes, respectively, and 110 and 505 nM oenothein B for scavenging ROS produced by zymosan-stimulated human neutrophils and murine bone marrow leukocytes, respectively. Because ROS are generated extracellularly by PMA-stimulated cells, whereas zymosan-stimulated cells generate intracellular ROS, which then can diffuse out of the cell, it is not surprising that oenothein B had lower IC₅₀ values for scavenging ROS in the PMA-stimulated cell system.

To verify oenothein B was an effective ROS scavenger, we analyzed scavenging activity in an enzymatic, O₂⁻-generating system. As shown in Fig. 3C, oenothein B (1–50 μM) effectively scavenged O₂⁻ in a xanthine/xanthine oxidase assay. To confirm the effect of oenothein B was due to ROS scavenging and not direct inhibition of xanthine oxidase itself, we measured xanthine oxidase-dependent production of uric acid from xanthine and found no effect of oenothein B over the entire concentration range tested (Fig. 3C). Thus, it is clear that, in addition to its phagocyte-activating properties, oenothein B is an effective scavenger of phagocyte-derived ROS, which is consistent with previous reports demonstrating oenothein B has antioxidant properties (26).

To eliminate or decrease antioxidant effects of the compounds/extract under investigation, the medium containing test samples was removed and replaced with fresh medium before subsequent analysis of ROS production. As shown in Fig. 4A, the kinetics of murine bone marrow leukocyte ROS production induced by *E. angustifolium* extract and isolated oenothein B were similar, and ROS production was induced in a time-dependent manner by oenothein B (Fig. 4B). Likewise, purified murine neutrophils dose-dependently generated ROS in response to oenothein B (Fig. 4C). At concentrations of 10–50 μM oenothein B, the response plateaued, which may be due to competing antioxidant activity of compounds remaining even after medium replacement. SOD (50 U/ml) completely (>95%) inhibited ROS production in oenothein B-stimulated neutrophils (data not shown), indicating the response was primarily due to NADPH oxidase-generated O₂⁻.

To evaluate the role of oenothein B structural components on phagocyte activation, we evaluated ROS production by phagocytes treated with substructures of oenothein B, including pyrocatechol, gallic acid, pyrogallol, 3,4-dihydroxybenzoic acid, and two related tannins, PGG and corilagin (structures of the compounds are shown in supplemental Figure S3). Only oenothein B and PGG activated ROS production over the concentration range tested (10–100 μM). Fig. 5 shows the response to these compounds at selected concentrations, as compared with that induced by 25 μM oenothein B.

**Effect of *E. angustifolium* extract and purified oenothein B on phagocyte Ca²⁺ mobilization and chemotaxis**

The ability of *E. angustifolium* extract and oenothein B to induce an intracellular Ca²⁺ flux in neutrophils was examined. Crude *E. angustifolium* extract induced a dose-dependent increase in [Ca²⁺]i in human neutrophils (Fig. 6A), with an EC₅₀ of 53 μg/ml. Likewise, oenothein B induced a rapid and dose-dependent Ca²⁺ flux in human (Fig. 6A) and murine neutrophils (Fig. 6B), with EC₅₀ values of 25.5 and 18.3 μM, respectively. The peak levels of intracellular Ca²⁺ were reached within 60 s of exposure to oenothein B and then decreased. Note however, that [Ca²⁺]i were still higher than the basal levels at 3 min postexposure, which is...
similar to the response observed in cells treated with fMLF (Fig. 6). If cells were treated in the absence of extracellular Ca\(^{2+}\), no Ca\(^{2+}\) flux was observed, suggesting that oenothein B treatment induced influx of extracellular Ca\(^{2+}\) (data not shown).

Treatment of PMA-differentiated human HL-60 with oenothein B resulted in an intracellular Ca\(^{2+}\) flux, whereas no significant changes in [Ca\(^{2+}\)]\(_i\) were observed in undifferentiated HL-60 cells (Fig. 7, upper panel). In comparison, the Ca\(^{2+}\) ionophore ionomycin increased [Ca\(^{2+}\)]\(_i\) in both nondifferentiated and differentiated HL-60 cells (Fig. 7, lower panel). These findings suggest that oenothein B does not act as an ion channel and that phagocyte activation by this compound depends on phagocyte differentiation and/or level of receptor expression.

Similar to the results observed for ROS production, pyrocatechol, gallic acid, pyrogallol, 3,4-dihydroxybenzoic acid, and corilagin failed to induce an intracellular Ca\(^{2+}\) flux in human and murine neutrophils, while PGG treatment resulted in increased [Ca\(^{2+}\)]\(_i\), although with a higher EC\(_{50}\) (31.3 \(\mu\)M) than oenothein B.

Previous reports indicated that Ca\(^{2+}\) mobilization is associated with chemotactic activity of various agents (50). Thus, we examined the effect of E. angustifolium extract and oenothein B on human neutrophil chemotaxis. Both the extract (data not shown) and purified oenothein B exhibited neutrophil chemotactic activity and dose-dependently induced neutrophil migration, with EC\(_{50}\) values of 46 \(\mu\)g/ml and 11.8 \(\mu\)M, respectively (Fig. 8). Note, however, that the magnitude of this response was generally lower than that induced by the positive controls, fMLF and IL-8, which had EC\(_{50}\) values of \(~1\) and 12 nM, respectively (Fig. 8).

**Effect of oenothein B on phagocyte NF-\(\kappa\)B activity**

To evaluate possible signaling pathways involved in the immunomodulatory activity of oenothein B, we used a transcription factor-based bioassay for NF-\(\kappa\)B activation in human THP-1 monocytes. NF-\(\kappa\)B transcription reporter activity was time-dependently induced by oenothein B (25 \(\mu\)M) and LPS (100 ng/ml), but with slightly different kinetics for these stimuli (Fig. 9A). For example,
The data are representative of three independent experiments. The level of NF-κB activation by oenothein B at concentrations of 25–50 μM oenothein B was just as active as untreated sample (supplemental Fig. S4), again confirming the absence of endotoxin contamination.

**Effect of E. angustifolium extract and purified oenothein B on phagocyte cytokine production**

Previous studies showed that oenothein B stimulated release of IL-1β from murine and human macrophages (28); however, the effect of this compound on other cytokines has not been evaluated. To address this issue, condition medium from THP-1Blue monocytes and human PBMCs was analyzed using a cytokine ELISA semiquantitative array. Among the 12 cytokines analyzed, five were consistently induced in PBMCs (>10-fold) by 10 μM oenothein B, as compared with control cells. These included IFN-γ (fold increase (FI) = 11), IL-1β (FI = 13), GM-CSF (FI = 15), TNF-α (FI = 31), and IL-6 (FI = 34) (Fig. 1A). IL-8 production was inconclusive because of high background production by PBMCs (data not shown).
not shown), a problem which has also been documented previously (e.g., Ref. 53). Human monocytic THP-1Blue cells, treated with 25 μM oenothein B produced high levels of TNF-α (FI = 24), IL-6 (FI = 94), and IL-8 (FI = 98) (Fig. 10A).

To quantify dose-dependent effects of *E. angustifolium* extract and oenothein B on cytokine production, levels of TNF-α and IL-6 were determined by ELISA of supernatants from treated cells. Untreated cells produced negligible amounts of TNF-α and IL-6, whereas incubation of THP-1Blue monocytes and PBMCs with crude extract (Fig. 10, B and D) or purified oenothein B (Fig. 10, C and E) enhanced TNF-α and IL-6 production in a dose-dependent manner. Note, however, that pretreatment of oenothein B with endotoxin-removing gel no effect on its activity, indicating that the induction of TNF-α and IL-6 production was not due to endotoxin contamination (data not shown).

**In vivo analysis of oenothein B**

To evaluate the effect of oenothein B in vivo, mice were treated by intraperitoneal injection of oenothein B, and neutrophil recruitment to the peritoneum was evaluated. As shown in Fig. 11, oenothein B significantly induced neutrophil recruitment (~10-fold increase over saline controls). Because the mice were analyzed 4-h postinjection, neutrophils were the primary phagocyte recruited. As expected, little change in monocyte/macrophage levels was observed in this short period of time (data not shown). The primary neutrophil chemotactic agent at inflammatory sites is IL-8 (KC in mice). Thus, we evaluated KC levels in mice treated with oenothein B and found that oenothein B dose-dependently induced significant levels of serum KC, which correlated with the observed recruitment of neutrophils into the peritoneum (Fig. 12). Thus, these studies verify our in vitro experiments and confirm that this oenothein B is active in vivo.

**Discussion**

Extracts from *E. angustifolium* have been reported to be beneficial for treating a variety of medical problems, such as gastrointestinal, and prostate diseases, and to improve the healing of wounds (11). However, little is known regarding the effects of *E. angustifolium* on innate immune functions. In this study, we demonstrate that *E. angustifolium* extracts can induce or enhance phagocyte functional responses and that the active principle in these extracts is oenothein B. Because oenothein B is a major component of *Epilobium* (24), we propose that the effects of oenothein B on innate immune function likely contribute to the therapeutic efficacy of *Epilobium* extracts.

Oenothein B was first isolated from *Oenothera erythrosepala* (Onagraceae) (54) and was subsequently found in Eucalyptus, Eugenia species, and Lythraceae species (31). Previous studies on oenothein B have shown that it exhibits significant antioxidant (26), antitumor (27, 28, 30, 55), antibacterial (56), and antiviral
activities, although the mechanisms involved are not well defined. Most studies on oenothein B have focused on its antitumor activity, and it has been shown to inhibit poly-(ADP-ribose) glycohydrolase, uridine 5'-O-(2-Thiophenyl)riboside-reductase, and aromatase (24, 57, 58), and also to induce a neutral endopeptidase in prostate cancer cells (34). Miyamoto et al. (27) reported that oenothein B had potent antitumor activity upon intraperitoneal administration to mice before tumor inoculation and suggested that this may be due to enhancement of the host-immune system via macrophage activation. However, essentially nothing else has been reported on the effects of oenothein B on innate immunity. We show in this study that oenothein B activates phagocytic cells, including monocyte/macrophages and neutrophils, resulting in increased intracellular Ca²⁺, production of ROS and cytokines, and chemotactic activity. Additionally, we demonstrated that oenothein B induced IL-8 production and neutrophil recruitment to the peritoneum of treated mice. Given the critical role played by phagocytes in innate immunity against pathogens and their contribution to tumor cell destruction (reviewed in Ref. 59), our data support the possibility that at least part of the observed therapeutic effects of oenothein B and Epilobium extracts in general are due to enhancement of innate immune responses.

In addition to enhancement of innate immune function, oenothein B also was found to scavenge ROS generated by activated phagocytes or by an enzymatic system, which confirms previous reports on the antioxidant activity of this compound determined using a radical scavenging assay (26, 45). Thus, oenothein B is able to stimulate local innate immunity but may also protect tissues from excessive ROS production. Although the antioxidant activity of polyphenols has been assumed to be the primary therapeutic property, recent studies indicate that many polyphenols directly impact cellular signaling events, which is independent of their antioxidant activity (e.g., see Ref. 33). Because antioxidant capacity is often diminished or even lost during absorption in vivo, the primary therapeutic properties of oenothein B and other polyphenols may indeed be due to direct modulation of cellular activity, such as the modulation of innate immune functions shown in this study.
The nonspecific nature of immunomodulators makes them attractive because they can be used to treat a broad-spectrum of infections and are not susceptible to antibiotic resistance (60). In general, immunomodulators mimic the natural mechanisms used by pathogens to stimulate innate immunity and thus are potentially beneficial in preventing infection (60). Thus, the balance between therapeutic and proinflammatory properties is important to consider when evaluating immunomodulators, and the goal is to enhance or prime local host defense without inducing excessive or systemic inflammation. This balance is dependent on pharmacodynamic and pharmacokinetic properties of the compound and must be determined empirically. For example, CpG DNA, an immunomodulator with therapeutic promise, induces a range of phagocyte inflammatory responses via TLR9, which leads to beneficial Th1-type responses (60, 61). Conversely, excessive activation of TLR9 can contribute to detrimental inflammatory states (e.g., Ref. 62). Likewise, we suggest that therapeutic concentrations of oenothein B could prime or enhance innate immune cells without inducing adverse inflammatory responses, which is supported by our in vivo experiments. In contrast, our in vitro data suggest that treatment with high concentrations of oenothein B could lead to excessive inflammation if sufficient local concentrations were achieved. Thus, further analyses are clearly needed to further evaluate the specific pharmacological properties of oenothein B in vivo.

Initiation of intracellular Ca\(^{2+}\) flux is one of the earliest events associated with phagocyte receptor activation and plays a central role in receptor-mediated intracellular signaling events (50). Furthermore, Ca\(^{2+}\) mobilization is required for ROS production in phagocytes, mainly through the activation of NADPH oxidase (e.g., Ref. 63). We found that oenothein B induced a transient elevation of [Ca\(^{2+}\)]\(_i\) in neutrophils, indicating this compound is a phagocyte agonist; however, compounds structurally related to the building blocks of oenothein B (pyrocatechol, gallic acid, pyrogallol, and 3,4-dihydroxybenzoic acid) had did not induce Ca\(^{2+}\) mobilization or other phagocyte responses. In contrast, one of the two tannins tested (PGG) did induce a Ca\(^{2+}\) and ROS production in neutrophils. Although various tannins and other compounds with galloyl groups, including galloylpunicalagin, woodfordin, and cotton-derived tannins, have been reported previously to activate phagocytes, mainly through the activation of NADPH oxidase (e.g., Ref. 63).

**FIGURE 11.** Neutrophil recruitment to the peritoneum in response to oenothein B. BALB/c mice were injected i.p. with oenothein B (100 µg) or control saline, as indicated. After 4 h, peritoneal cells were collected and stained for flow cytometric analysis. *Upper panel,* Representative examples of two-color dot plots comparing CD11b and GR-1 staining on CD45\(^+\) cells from saline- and oenothein B-treated mice. The quadrant containing the neutrophil population is indicated (arrow), which was used to determine the percentage of the overall CD45\(^+\) cell population in the peritoneum. *Lower panel,* Pooled results from three mice in each test group, as analyzed above. The data are presented as mean ± SEM of three mice. Representative of two independent experiments. Statistically significant difference (**, p < 0.01) vs saline control is indicated.

**FIGURE 12.** Oenothein B-induced recruitment of neutrophils in vivo is associated with induction of KC production. BALB/c mice were injected i.p. with either 100 or 400 µg of oenothein B or control saline, as indicated. After 4 h, serum was collected for KC analysis by ELISA (*A*), and peritoneal cells were collected and stained for flow cytometric analysis of percent neutrophils (RB6-8C5 bright, CD45\(^+\) cells) in the peritoneal washes, as described under Fig. 12 (*B*). The data are presented as mean ± SD of four mice. Representative of two independent experiments. Statistically significant differences (**, p < 0.01) vs saline control are indicated.
also induced in human monocyte/macrophages. Additionally, we found that intraperitoneal administration of oenothein B induced a significant level of KC in treated mice and that KC induction directly correlated with neutrophil influx into the peritoneum, demonstrating that cytokine induction defined in this report is relevant in vivo. Thus, the ability of oenothein B to induce these cytokines may also play an important role in the microbicial, vircidal, and antitumor effects of this compound. For example, IL-1β is capable of upregulating the activity of tumoraludic NK cells and inducing antitumor reactivity in the regional lymph nodes and spleen (reviewed in Ref. 72). Among the proinflammatory cytokines, IL-6 is one of the most important mediators of fever and the acute-phase response (73). TNF-α has direct in vitro and in vivo cytotatic and cytokidal effects and, together with IL-6, is also considered as a major immune and inflammatory mediator (73). One of the most prominent characteristics of TNF-α is its ability to cause apoptosis of tumor cells, resulting in tumor necrosis (74). TNF-α also plays a pivotal role in host defense and can act on macrophages in an autocrine manner to enhance various functional responses and induce the expression of a number of other immunoregulatory and inflammatory mediators (75).

NF-κB is activated in response to stimulation by inflammatory agents, including LPS, and activation of NF-κB is an essential step in inducing proinflammatory cytokines, chemokines, inflammatory enzymes, adhesion molecules, receptors, and inhibitors of apoptosis (reviewed in Ref. 76). Treatment of phagocytes with oenothein B resulted in the activation of NF-κB. In addition, treatment of cells with both oenothein B and LPS resulted in an even greater NF-κB response, suggesting a synergistic effect. Therefore, the ability to activate phagocyte NF-κB signaling provides further evidence that oenothein B possesses immunomodulatory properties. The synergistic effect of oenothein B and LPS is consistent with studies of Feldman (25), who suggested that dimeric tannins could mimic the lipid A moiety of LPS. In contrast with our data, Chen et al. (77) reported that oenothein B modestly inhibited LPS-induced NF-κB activity in Bcl-2-overexpressing murine RAW264.7 macrophages. Although the reasons for this difference are not clear, it is possible that this may be due to differences in the cell lines used. Alternatively, NF-κB activation by oenothein B could be indirect and mediated by cytokines induced during the assay, as NF-κB reporter activity only began to increase at after 12 h incubation with oenothein B and was not significant until 18 h. Thus, further studies are now in progress to evaluate this issue and determine which receptor(s) and intercellular pathway(s) are in NF-κB activation and expression of various cytokines induced by oenothein B.

Oenothein B and a related tannin, PGG, induced ROS production and Ca2+ mobilization in phagocytes, whereas, the monomeric polyphenols pyrocatechol, gallic acid, pyrogallol, and 3,4-dihydroxybenzoic acid were inactive. On the other hand, only oenothein B, which has a dimeric macrocyclic structure (see supplemental Fig. S1), induced NF-κB activity. In addition, HL-60 cells responded to oenothein B only after differentiation. These findings suggest oenothein B may be activating phagocytes through a specific receptor or cellular target, which is yet to be identified. Tannins bind to a wide range of targets, including phospholipids, carbohydrates, and proteins (78–80). For example, Teng et al. (81) reported that the ellagitannin rugosin E activated platelet Ca2+ flux by acting as an ADP receptor agonist. Thus, it is possible that oenothein B could be interacting with a number of extracellular membrane targets, such as receptors or lipid rafts involved in regulating phagocyte activation. Nevertheless, further work is necessary to identify the target of oenothein B.

As reported previously, oenothein B is a major component of Epilobium extracts and can be present at concentrations up to 14% (~9 μM in 100 μg/ml extract), although the level varies between species and is reported to be 4.5% in E. angustifolium (24). However, it has not yet been determined whether these concentrations vary in different regions of the world or between different lots of plants. We found that E. angustifolium extracts induced intracellular Ca2+ flux, ROS production, chemotaxis, and cytokine production in qualitatively similar patterns as purified oenothein B, supporting the conclusion that oenothein B is indeed the active component in these extracts. However, it is also apparent that the extracts were more potent than oenothein B if we use the estimated concentration of 4.5% in our extracts (~3 μM oenothein B in 100 μg/ml extract). The reasons for this difference are not clear; however, this observation is supported by studies from Kiss et al. (34) who showed that E. angustifolium extract was ~10-fold more potent that pure oenothein B in inducing neutral endoproteinase activity in prostate cancer cells. One possibility is that other constituents in the extract may help to increase oenothein B bioavailability or stability. For example, the solubility of the active component in a crude extract can often be reduced when the component is purified. It is also possible that some reactive groups are altered during purification, thus affecting activity. These issues are currently under investigation.

Overall, our studies demonstrate that oenothein B activates a number of phagocyte functions, including Ca2+, NADPH oxidase activity, chemotaxis, and cytokine production. In addition, we established that oenothein B can modulate phagocyte activity in vivo. The ability of this compound to modulate innate immune functions suggests that at least part of the reported effects of Epilobium extracts and purified oenothein B on wound healing and inhibition of tumor growth is through modulation of macrophage function. These studies suggest that oenothein B may serve as a promising lead for further therapeutic development.

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Disclosures

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References


