Published in final edited form as:

J Ethnopharmacol. 2009 February 25; 122(1): 76–85. doi:10.1016/j.jep.2008.11.028.

***Echinacea* increases arginase activity and has anti-inflammatory properties in RAW 264.7 macrophage cells indicative of alternative macrophage activation**

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Abstract

The genus *Echinacea* is a popular herbal immunomodulator. Recent reports indicate that *Echinacea* products inhibit nitric oxide (NO) production in activated macrophages. In the present study we determined the inhibitory effects of alcohol extracts and individual fractions of alcohol extracts of *Echinacea* on NO production, and explored the mechanism underlying the pharmacological anti-inflammatory activity. The alcohol extracts of three medicinal *Echinacea* species, *E. angustifolia*, *E. pallida* and *E. purpurea*, significantly inhibited NO production by lipopolysaccharide (LPS)-activated the RAW 264.7 macrophage cell line, among them *E. pallida* was the most active. The *Echinacea*-mediated decrease in NO production was unlikely due to a direct scavenging of NO because the extracts did not directly inhibit NO released from an NO donor, sodium nitroprusside. An immunoblotting assay demonstrated that the extract of *E. pallida* inhibited inducible nitric oxide synthase (iNOS) protein expression in LPS-treated macrophages. The enzymes iNOS and arginase metabolize a common substrate, L-arginine, but produce distinct biological effects. While iNOS is involved in inflammatory response and host defense, arginase participates actively in anti-inflammatory activation. Arginase activity of RAW 264.7 cells stimulated with 8-bromo-cAMP was significantly increased by alcohol extracts of all three *Echinacea* species. The polar fraction containing caffeic acid derivatives enhanced arginase activity, while the lipophilic fraction containing alkamides exhibited a potential of inhibiting NO production and iNOS expression. These results suggest that the anti-inflammatory activity of *Echinacea* might be due to multiple active metabolites, which work together to switch macrophage activation from classical activation towards alternative activation.

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Keywords

Echinacea; Alcohol extract; Anti-inflammatory; Macrophage; Nitric oxide; Inducible nitric oxide synthase; Arginase

1. Introduction

The genus *Echinacea*, one of the top-selling botanical supplements, has been widely used for centuries in North America and later in Europe for many therapeutic purposes. Although there are nine known species of *Echinacea*, three of them, *E. angustifolia* (EA), *E. pallida* (EPA) and *E. purpurea* (EP), are of similar and important medicinal values in the modulation of the immune system (Borchers et al., 2000). These *Echinacea* species are rich in bioactive metabolites of which lipophilic alkamides, water-soluble phenolic compounds (mainly caffeic acid derivatives) and polysaccharides are the most recognized for their immunomodulatory properties (Barnes et al., 2005). Although historically polysaccharides were considered critical for stimulation of nonspecific immune responses (Borchers et al., 2000; Percival, 2000), recent research focuses on the alkamides and caffeic acid derivatives. Caffeic acid derivatives are good antioxidants in cell-free free radical generation systems (Hu and Kitts, 2000; Pellati et al., 2004; Dalby-Brown et al., 2005) and echinacoside, the main caffeic acid derivative in EA and EPA, has been functionally linked to anti-inflammatory and wound healing properties of *Echinacea* when applied topically (Speroni et al., 2002; Rousseau et al., 2006).

Pharmacokinetic studies demonstrate that natural or synthesized caffeic acid derivatives (e.g. caftaric acid, chlorogenic acid and echinacoside) are quickly absorbed in the rat stomach, but rapidly eliminated from circulation (Jia et al., 2006; Lafay et al., 2006; Vanzo et al., 2007). Matthias et al. (2004) suggests a low bioavailability for caffeic acid derivatives of *Echinacea* based on their poor permeability through Caco-2 monolayers, a model for the intestinal epithelial barrier. These data indicate that there may be a limited pharmacological role for caffeic acid derivatives when consumed orally. In this regard, lipophilic alkamides, which are considered orally bioavailable, may be more important phytochemicals (Matthias et al., 2005; Woelkart et al., 2005a).

Echinacea-derived alkamides appear to have immunomodulatory and anti-inflammatory activity (for review see Woelkart and Bauer, 2007). Both alkamide-containing *Echinacea* extracts and purified alkamides inhibit production of proinflammatory tumor necrosis factor- α (TNF- α) and nitric oxide (NO) in an activated murine macrophage cell line (Chen et al., 2005; Matthias et al., 2007) with low cytotoxicity *in vitro* (Zhai et al., 2007a). Orally administered alcohol extracts of *Echinacea* significantly decreased production of TNF- α and interleukin (IL)-1 β by activated splenocytes (Zhai et al., 2007b). Although *Echinacea* extracts containing alkamides exhibit suppressive effects on these inflammatory mediators, the underlying cellular mechanism is unclear.

Upon induction by certain inflammatory stimuli, e.g., bacterial lipopolysaccharide (LPS), the NF- κ B signal pathway activates and regulates the expression of a wide variety of genes involved in inflammatory responses, e.g., the cytokines TNF- α and IL-1 β , and the enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (McKay and Cidlowski, 1999; Yamamoto and Gaynor, 2001). The enzyme iNOS catalyzes the conversion of amino acid L-arginine to NO, which is cytotoxic and cytostatic (Meurs et al., 2003). Although NO as an active free radical and inflammatory mediator is part of the host defense, excessive production of NO and its reactive nitrogen intermediates is involved in the pathogenesis of many chronic diseases, such as inflammatory bowel disease, arthritis, atherosclerosis and tumors (Chan et al., 2000; Yamamoto and Gaynor, 2001). Selective inhibition of the iNOS pathway is an important strategy for control of many chronic inflammatory diseases (Chan et al., 2000).

L-arginine metabolism can be redirected toward the synthesis of L-ornithine catalyzed by the enzyme arginase. The subsequent products from L-ornithine, such as polyamines and L-proline, are involved in cell growth, collagen synthesis and wound healing (Meurs et al., 2003). Thus, arginase and iNOS are the two enzymes that catalyze a common substrate but produce divergent biological effects. They are generally recognized to be functionally competitive because the stimulation of one enzyme will negatively regulate the other one due to the limited availability of the same substrate (Wang et al., 1995; Gotoh and Mori, 1999). These two cross-regulated metabolic processes may reflect opposite functional states of macrophages. It is well known that macrophages can be either classically activated or alternatively activated, and are therefore termed as inflammatory or anti-inflammatory cells (Munder et al., 1998; Porcheray et al., 2005). A given macrophage may switch from one activated state to another upon a specific signal (Porcheray et al., 2005). Inflammatory mediators and cytokines induce classical activation involving iNOS expression while anti-inflammatory cytokines induce alternative activation with upregulated arginase activity (Modolell et al., 1995; Munder et al., 1998; Yamamoto et al., 1998). Oral administration of alcohol extracts of *Echinacea* to mice increases production of anti-inflammatory cytokines IL-4 and IL-10, but decreases production of TNF- α and IL-1 β in activated spleen cells (Zhai et al., 2007b), suggesting that *Echinacea* may modulate macrophage function to be anti-inflammatory via a decreased proinflammatory/anti-inflammatory cytokine ratio. Thus, we hypothesized that *Echinacea* can perturb the iNOS/arginase balance in macrophages in favor of increasing arginase activity for alternative anti-inflammatory activation.

In this study, we examined the effects of *Echinacea* extracts and their fractions on the production of NO in activated macrophage cell line as well as the cellular mode of action underlying the biological activity. We found that alcohol extracts of *Echinacea* had opposite effects on NO production and arginase activity, and these differential effects were induced by different fractions (or chemical constituents), which could work coordinately to drive macrophages to alternative activation.

2. Materials and methods

2.1. Preparation of alcohol extracts

Dried powdered roots of EA, EPA and EP harvested in 2003 were provided by Iowa State University Botanical Supplements Research Center; and by the USDA North Central Regional Plant Introduction Station in Ames, Iowa, with accession numbers PI 631285, PI 631293, and PI 631307, respectively. Alcohol extracts from the root powders of these plants were prepared as previously described (Zhai et al., 2007b). The extracts were diluted to 20 mg/ml in PBS containing 5% ethanol. Aliquots of the dilutions were stored at -80°C for up to 2.5 years during which no obvious herbal efficacy was reduced.

2.2. Fractionation of alcohol extracts

For fractionation, the dried powdered roots of the plants harvested in 2005 (EA) or 2006 (EPA and EP) were used to prepare new alcohol extracts using the Soxhlet extraction. The dry residues were redissolved in 75% ethanol to obtain ≤ 0.6 g/ml. Five milliliters were injected into a semi-preparative high performance liquid chromatography (HPLC). The starting conditions of the semi-preparative HPLC was 90% Pump A, which was 0.1% acetic acid, and 10% Pump B, which was acetonitrile at a flow rate of 3 ml/min. Eluent was collected over time with increasing solvent B as outlined in Table 1A. The gap of 1–2 minutes between fractions indicated the time of stopping the flow of the mobile phase for the change of the collection flasks. The fractions were evaporated to remove the mobile phase and lyophilized. Each lyophilized fraction was dissolved and diluted to 4 mg/ml in PBS containing 25% ethanol, and aliquots were kept at -80°C and used within one year.

2.3. Phytochemical analysis

The phytochemical analysis was performed to detect bioactive metabolites of alkamides and caffeic acid derivatives in the crude alcohol extracts with the use of HPLC as described previously (Wu et al., 2004). We did not identify ketones as no internal standards for ketones were used. However, ketones are expected to be present as lipophilic compounds and concomitantly appear in alkamide fractions of EPA (Chicca et al., 2008).

Fractions were identified as described by Liu and Murphy (2007). The mobile phase used for the analytical HPLC method was, Pump A: 0.1% acetic acid; and Pump B, acetonitrile. At a flow rate of 1 ml/min, a UV scan was collected in the range of 190–600 nm. Alkamides were viewed at 260 nm, while caffeic acid derivatives were viewed at 330 nm.

Peak identification was based on relative retention time, similarity of UV spectra, internal standards, spiking and chromatogram fingerprinting (Wu et al., 2004; Liu and Murphy, 2007). Individual standards were run to check for relative retention times and UV spectra. An EP sample with identified peaks was also run to obtain the finger print of the chromatogram, the relative retention times of the alkamides and caffeic acid derivatives, and to obtain the UV spectra of identified compounds.

2.4. Determination of endotoxin in herbal preparations

All glassware used in the extraction and fractionation procedure was baked at 185°C overnight prior to use in order to minimize endotoxin contamination. Endotoxin-free Nanopure water was used for preparation and dilution of crude alcohol extracts and fractions. The endotoxin levels were evaluated in aliquots of the herbal preparations using the Limulus Amebocyte Lysate Test (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) according to the manufacturer's specifications for a microplate assay, and were found to be below the limit of detection (< 0.1 EU/ml).

2.5. Macrophage cell line

The murine peritoneal macrophage cell line RAW 264.7 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in RPMI 1640 medium (GIBCO, Invitrogen Corporation, Grand island, NY, USA) supplemented with 2 mM glutamine, 25 mM Hepes, 50 µg/ml gentamicin and 10% heated-inactivated iron-fortified bovine calf serum (JRH Biosciences, Lenexa, KS, USA) at 37°C in a 7% CO₂ incubator. Cells between passage 5 and 20 (Kierner et al., 2002) were used in this study.

2.6. NO and TNF-α assays

RAW 264.7 cells (8×10^4 cells/well) were seeded in flat bottom 96-well tissue culture plates (Corning Inc., Corning, NY, USA) in the presence or absence of various concentrations of *Echinacea* preparations (as indicated in each graph) and/or LPS (*E. coli* 055:B5; Sigma, St. Louis, MO, USA). Final concentration of ethanol used as a solvent of lipophilic metabolites in each well was 0.25% (v/v). Ethanol (0.25%) control wells were tested in parallel. After 23 h incubation, culture supernates were collected for NO and TNF-α assays, and the attached cells were evaluated for viability (see below).

In a second set of experiments, *Echinacea* extracts (100 µg/ml) were either added simultaneously with LPS (1 µg/ml) or up to 6 h after LPS addition (Kierner et al., 2002). Culture supernates from cells activated with LPS for 22 h were collected for NO assay.

Nitrite, a stable NO metabolite, was determined by the method of Griess reaction (Park et al., 2005). A trace amount of nitrite present in cell-free culture medium was subtracted from each value obtained with cells. Sodium nitrite (0.39–100 µM) was used as a standard. It was

confirmed that *Echinacea* extracts at 200 µg/ml did not interact with nitrite and interfere with its detection (data not shown).

TNF- α was assayed by BD OptEIA ELISA set (BD Biosciences, San Diego, CA, USA). The cytokine levels were calculated by using a purified recombinant mouse cytokine as a standard.

2.7. Scavenging of NO production from NO donor

Sodium nitroprusside (SNP), an NO donor, in aqueous solution at physiological pH spontaneously liberates NO, which rapidly interacts with oxygen to produce nitrite (Sreejayan and Rao, 1997; Chan et al., 2000; Kiemer et al., 2002). In order to determine if *Echinacea* metabolites directly interact with NO, SNP (final concentration 2.5 mM; Sigma) was mixed with the Griess reagent (Park et al., 2005) in the presence of 0.25% ethanol or various concentrations of alcohol extracts, and then incubated at room temperature for a set of time points. NO released from SNP in this reaction system was immediately captured by Griess reagent via nitrite, which was sequentially monitored on a plate reader (Bio-Tek Instruments, Winooski, VT, USA). A series of sodium nitrite standard concentrations instead of SNP was set up and measured at the same time. The reading of the standard curve remained stable throughout the observational period.

2.8. Determination of arginase activity

Arginase activity was determined according to a microplate method (Corraliza et al., 1994; Munder et al., 1998) with slight changes. RAW 264.7 cells (4×10^5 cells/ml) were incubated in 96-well tissue culture plates in the presence or absence of LPS (1 µg/ml), 8-bromo-cAMP (0.25 mM; Sigma) and/or *Echinacea* preparations. After 23–24 h incubation, supernates were collected for NO assay, the cells rinsed with PBS, and then lysed with a CellLytic M lysis solution (Sigma). Following centrifugation at $2,500 \times g$ for 5 min at 4°C, 50 µl of the lysate was mixed with 40 µl of 25 mM Tris-HCl (pH 7.4) and 10 µl of 10 mM MnCl₂. The arginase was activated by heating for 10 min at 56°C. Arginine hydrolysis to urea was conducted by addition of 100 µl of 0.5 M L-arginine (Sigma), pH 9.7, with incubation at 37°C for 60 min. The reaction was stopped with 800 µl of H₂SO₄ (96%)/H₃PO₄ (85%)/H₂O (1/3/7, v/v/v). The urea concentration was measured at 550 nm after addition of 40 µl of 9% (w/v) α -isotonitrosopropiophenone (Sigma) dissolved in 100% ethanol and heating at 95°C for 90 min. A standard curve was performed with 2-fold dilutions of urea (0.03–4 mM) followed by mixing with the stop reagent and heating. One unit of arginase activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol urea per min at 37°C. Protein concentrations in cell lysate were determined using a micro BCA (Bicinchoninic Acid) protein assay (Pierce Labs, Rockford, IL, USA).

2.9. Western blotting analysis

RAW 264.7 cells (5×10^5 cells/well) were seeded in 24-well plates in the presence or absence of LPS (1 µg/ml) and various *Echinacea* preparations. After 24 h incubation, cells were rinsed with ice-cold PBS and lysed with the CellLytic M lysis solution containing 1% (v/v) protease inhibitor cocktail (Sigma). Cell debris was removed by centrifugation ($12,000 \times g$ for 15 min), and the resultant supernates were stored at –80°C until use. The thawed cell lysates were mixed with 4 $\times$ NuPAGE SDS sample loading buffer (Invitrogen). Equal amounts of cellular protein (25 µg/lane) were separated on 10% Tris-HCl ready gel (Bio-Rad Laboratories, Hercules, CA, USA), followed by electrotransfer onto a PVDF plus membrane. The immunoblot was performed by incubation with rabbit polyclonal anti-iNOS (1:500 dilution) (sc-8310, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in 5% non-fat milk in PBS/0.1% Tween-20 (PBST) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:2500 dilution) (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. The bands were visualized using an amplified Opti-4CN Substrate kit (Bio-Rad

Laboratories). The blots were photographed and the band density quantified with Kodak Image Station 440 CF and Kodak 1D image analysis software (Eastman Kodak Company, Rochester, NY, USA). To assess the changes of iNOS expression and to normalize protein loading, membranes run in parallel were incubated with mouse monoclonal antibody against β -actin (1:5000 dilution) (Santa Cruz Biotechnology Inc.).

2.10. Cell viability assay

Mitochondrial reduction of MTS, a tetrazolium compound, to a colored formazan was used as an indicator of cell viability (Cory et al., 1991). Briefly, after removing of 100 μ l of culture supernates (i.e. for NO and TNF- α assays), 15 μ l of MTS (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega Corporation, Madison, WI, USA) was added to the remaining 100 μ l of culture medium incubation continued for 1 h. The extent of formazan formation was determined photometrically at absorbance 490 nm on a plate reader. The absorbance of cell-free culture medium with or without vehicle or *Echinacea* preparations was used as a blank (self-control) and subtracted from the value of the corresponding treatment groups.

2.11. Statistical analysis

Statistix software (version 8.0, Analytical Software, Tallahassee, FL, USA) was used for the statistical analysis. Differences between the vehicle and other groups were tested by two-way analysis of variance (group $\times$ experiment). A value $P < 0.05$ was considered significant.

3. Results

3.1. Phytochemical analysis

Table 2 lists the amounts of total alkaloids and several important caffeic acid derivatives in crude alcohol extracts of three *Echinacea* species.

The chemical constituents in the fractions are shown in Table 1B. Fraction 1 was rich in polar caffeic acid derivatives. Fraction 3 contained lipophilic constituents including alkaloids (in all three species) and ketones (in EPA). Other hydrophobic compounds were found in fractions 4 and 5. For fractions 3–5, the later fractions contained compounds which are more hydrophobic than the earlier ones.

3.2. Inhibition of TNF- α production by alcohol extracts

As TNF- α production was regulated by *Echinacea in vivo* (Zhai et al., 2007b), we measured this cytokine in *Echinacea*-treated RAW 264.7 cells. In the absence of LPS, very low amounts (or lower than limit of detection, 15 pg/ml) of TNF- α were measured in the supernates from RAW 264.7 cells incubated with or without alcohol extracts of *Echinacea* overnight. In the presence of LPS, the cells secrete high levels of TNF- α . Vehicle (0.25% ethanol) alone had no effect on the cytokine production, however, all three alcohol extracts at a concentration of 100 μ g/ml significantly inhibited TNF- α production (P values < 0.001 , Fig. 1A). The inhibitory effects of *Echinacea* extracts in the presence of 0.1 μ g/ml LPS were dose-dependent as indicated by a significant linear effect of dose (P values < 0.05) (Fig. 1B). Alcohol extracts of *Echinacea* at the test concentration range of 10–200 μ g/ml had little effect on cell viability based on the MTS assay (Fig. 1B), indicating that the inhibitory effect of *Echinacea* extracts on TNF- α is unlikely due to cytotoxicity.

3.3. Inhibition of NO production by alcohol extracts

In the absence of LPS, less than 0.5 μ M of NO was detectable in culture medium of RAW 264.7 cells incubated *in vitro* for 23 h. In the presence of increasing concentrations of LPS,

cells produce NO, which reached a maximum with LPS at 1 $\mu\text{g/ml}$ (Fig. 2A). Exposure to vehicle had no effect on NO production, however, the three *Echinacea* extracts at 100 $\mu\text{g/ml}$ reduced NO production. The inhibitory effect of *Echinacea* extracts on NO production was dose-dependent as indicated by a significant linear effect of dose (P values <0.008) (Fig. 2B). At 100 $\mu\text{g/ml}$, the inhibitory potential of three species extracts in the presence of 1 $\mu\text{g/ml}$ LPS is generally $\text{EPA} > \text{EP} > \text{EA}$. All three extracts at tested concentrations had no effect on cell viability (Fig. 2B).

3.4. Direct scavenging of NO generated by alcohol extracts

NO production was estimated by measuring nitrite using the Griess reaction system. NO derived from SNP increased in a linear time-dependent manner (Fig. 3A). Vehicle alone had no any effect on the NO production, but alcohol extracts of EA and EPA at a final concentration of 50 $\mu\text{g/ml}$ significantly inhibited nitrite accumulation beginning 120 min after initiating of the reaction (Fig. 3A) (P values <0.018 at 120-min time points). However, the inhibitory rates of NO production by these two extracts throughout the 3-h observation period were small ($<15\%$), and were not concentration-dependent (Fig. 3B). EP extracts at lower concentrations of 5–25 $\mu\text{g/ml}$, but not at higher concentrations (50–200 $\mu\text{g/ml}$), significantly reduced nitrite accumulation (P values <0.015).

3.5. Inhibition of iNOS induction by alcohol extracts

Effects of alcohol extracts on iNOS induction were measured by an indirect method described by Kiemer et al. (2002). Once macrophages are activated, iNOS gene transcription is initiated about 1 h after activation, whereas its protein expression occurs some 3–4 h later. If the *Echinacea*-mediated decrease in NO production is through interference with the transcriptional induction process, earlier treatment (i.e. 0.5 h after LPS stimulation) with *Echinacea* extracts should have a more profound effect on NO production than adding the extracts later. Therefore, in these experiments, alcohol extracts (100 $\mu\text{g/ml}$) were either added simultaneously with LPS (1 $\mu\text{g/ml}$) or up to 6 h after addition of LPS. The results showed that after LPS activation, the earlier addition of alcohol extracts resulted in more inhibition of NO production (Fig. 4), indicating that *Echinacea* inhibits the transcriptional induction of iNOS. Of three alcohol extracts, EPA extract was the most active, in agreement with its strongest inhibitory potential of $\text{TNF-}\alpha$ and NO production.

3.6. Opposite regulation of NO production and arginase activity by alcohol extracts

Raw 264.7 cells were incubated with alcohol extracts in the presence or absence of LPS to stimulate iNOS and/or 8-bromo-cAMP to induce arginase activity (Morris et al., 1998). The induction of arginase activity by 8-bromo-cAMP was further enhanced by the addition of LPS, which itself is a weak stimulant of arginase. In the presence of LPS alone, alcohol extracts of EP and EPA at 100 $\mu\text{g/ml}$ significantly inhibited NO production (P values <0.05) (Fig. 5A), but their influence on arginase activity did not reach statistical significance. Note that 8-bromo-cAMP alone did not induce NO production, but decreased the NO production when added with LPS to RAW 264.7 cells. Moreover, all three alcohol extracts induced additional suppression of NO production in conjunction with 8-bromo-cAMP. In the presence of 8-bromo-cAMP, the three *Echinacea* extracts at 100 $\mu\text{g/ml}$ significantly increased arginase activity up to 2-fold (P values <0.001) (Fig. 5B). The enhancing effect of *Echinacea* extracts on arginase activity was also seen when both 8-bromo-cAMP and LPS were present in the culture medium (Fig. 5B). When the concentrations of *Echinacea* extracts were reduced to 10 $\mu\text{g/ml}$, an enhancing effect was still seen when cells were exposed to *Echinacea* extracts (Fig. 5C). The test dose range (10–200 $\mu\text{g/ml}$) of *Echinacea* extracts had no effect on cell viability (Fig. 5C).

3.7. Differential regulation of NO production and arginase activity by fractions

In order to understand if the same fractions that inhibited NO production are responsible for increased arginase activity, five fractions of alcohol extracts were prepared (Table 1). Generally, the HPLC fractions of the three *Echinacea* extracts exhibited very similar patterns of effects on NO production and arginase activity. Note that the opposite effects of *Echinacea* extracts on NO production and arginase activity resulted from different fractions. For all three species of *Echinacea*, fraction 3 containing abundant alkamides had robust inhibition of NO production. For EA, NO was also suppressed by fraction 4 with greater suppression by fraction 5, and for EP, there is some NO suppression by all fractions with the greatest suppression by fractions 2, 3, and 5 (Fig. 6A). On the other hand, the polar fractions (fractions 1) induced increased arginase activity (Fig. 6B). However for EPA, the greatest enhancement of arginase activity was seen with fraction 4 which contains unidentified hydrophobic compounds. The HPLC fractions of alcohol extracts showed no cytotoxicity as indicated by the MTS assay (data not shown).

3.8. Inhibition of iNOS protein expression

Decreased NO production might be relative to decreased iNOS protein expression as indirectly indicated in Figure 4. To that end, we carried out Western blot analysis of lysates of cells exposed to alcohol extracts of three *Echinacea* species at 100 µg/ml or their fractions at 25 µg/ml for 24 h in the presence of LPS. For EA, iNOS expression was significantly inhibited by fractions 3, 4 and 5 (P values <0.05). For EPA, the unfractionated alcohol extract and its fraction 3 had a robust inhibiting activity (P values <0.05). The unfractionated alcohol extract of EP and its fractions all showed, to some extent, an inhibiting effect, but they did not reach statistical significance except for fraction 5 ($P = 0.046$).

4. Discussion

NO and TNF- α are two key mediators in host defence and inflammatory response. They positively regulate each other (Yamamoto and Gaynor, 2001; Wu et al., 2003) and therefore amplify inflammatory signals. However, improper upregulation of these inflammatory players are implicated in a pathological role in inflammatory processes (Yamamoto and Gaynor, 2001; Kiemer et al., 2002). Our results demonstrated the suppressive effects of *Echinacea* extracts and certain individual fractions on production of NO and TNF- α in an activated macrophage cell line, in accordance with previous *in vitro* observations (Chen et al., 2005; Matthias et al., 2007; Zhai et al., 2007a). In order to understand the cellular mechanism as well as the biological implication underlying the herbal anti-inflammatory activity, we carried out additional experiments. We focused our interest on the mechanistic basis for decreased NO production because NO is an important effector molecule in the classical activation of macrophages (MacMicking et al., 1997; Munder et al., 1998).

Macrophages metabolize arginine to produce either NO through iNOS or ornithine through arginase. These two metabolic processes have important yet divergent roles in the cellular function. NO functions primarily with cytotoxic and cytostatic activity while the products of arginase pathway promote cell proliferation (Meurs et al., 2003). The predominance of one pathway over the other may rest on the presence of desirable inducers in the microenvironment. Macrophages express two arginase isoforms with distinct subcellular localizations: cytosolic type I and mitochondrial type II (Louis et al., 1999). LPS co-induces iNOS and arginase II, but the arginase II expression lags behind iNOS expression (Wang et al., 1995; Morris et al., 1998; Gotoh and Mori, 1999). The delayed expression of arginase II helps control NO production, and prevent NO-induced cell apoptosis (Munder et al., 1998; Gotoh and Mori, 1999). We hypothesized that an *Echinacea*-mediated decrease in NO production implies an increased activity of arginase in activated macrophages. As expected, *Echinacea* extracts

oppositely regulated NO production and arginase activity even though the determinations of these two parameters were performed with different inducers (LPS or 8-bromo-cAMP). This differential regulation of two cross-regulated metabolic processes by *Echinacea* species may have important implications in the anti-inflammatory activity.

Although we predicted that one fraction responsible for decreased NO production would also be a strong stimulant of arginase, we observed the opposite regulatory effects on NO production and arginase activity were due to different fractions. Lipophilic fraction 3 was found to be the most effective in suppressing NO production while the polar fraction containing caffeic acid derivatives was an inducer of arginase activity, though the polar metabolites from EPA only showed an increasing trend in arginase activity. It is not surprising that the lipophilic fractions mediated a decrease in NO production, since an anti-inflammatory effect of alkamides has been reported (Chen et al., 2005; Matthias et al., 2007). Rather, it is noteworthy that caffeic acid derivatives (fraction 1) from EA and EPA have no effects on NO production considering that they are well demonstrated antioxidants (Hu and Kitts, 2000; Pellati et al., 2004; Dalby-Brown et al., 2005). Some natural antioxidants (e.g., flavonoids, resveratrol and curcumin) have been found to have strong inhibitory effects on iNOS expression and NO production in activated macrophages (Surh et al., 2001; Yamamoto and Gaynor, 2001).

Although caffeic acid derivatives have been determined to penetrate poorly across Caco-2 monolayers (Matthias et al., 2004), *in vitro* they enter into cells to accumulate high enough concentrations and consequently affect arginase activity. Cyclic AMP-stimulated arginase expression is through a complicated signal pathway involving intracellular activation of protein kinase A (Chang et al., 2000), and it has been proposed that Th2 cytokine-induced increase in arginase activity is cAMP-dependent (Corraliza et al., 1997; Wei et al., 2000). Although we can not answer based on the present data if caffeic acid derivatives directly mediate the activation of protein kinase A pathway, they may affect the signal pathway by upregulating Th2 cytokines, such as IL-4 and IL-10, as oral administration of *Echinacea*-mediates an increase in Th2 cytokines (Zhai et al., 2007b).

The lipophilic fraction of EPA includes two classes of phytochemicals, alkamides and ketones. While alkamides are widely investigated, ketones are almost completely neglected. Ketones are, in fact, the main lipophilic constituents in EPA roots, and ketone 23 is recognized as the diagnostic marker for hydrophobic metabolites (Kraus et al., 2005). The biological activity of ketones from EPA is little known. It has been suggested that ketones have antifungal activity (Binns et al., 2000). Obviously, like other bioactive metabolites of *Echinacea* (Awang, 1999), the antifungal activity of ketones may be through modulation of immune cell function rather than through a direct fungal killing. For lipophilic fraction 3 of EPA, it is possible that in addition to alkamides, ketones play a role in the regulation of NO production.

NO reacts rapidly with oxygen to form nitrogen dioxide, a gas capable of inducing cell damage (Meurs et al., 2003). It has been suggested that the polar caffeic acid derivatives of *Echinacea* possess an antioxidant and radical scavenging nature (Hu and Kitt, 2000; Pellati et al., 2004; Dalby-Brown et al., 2005), which could be enhanced by lipophilic alkamides (Dalby-Brown et al., 2005). However, *Echinacea* extracts (a mixture of caffeic acid derivatives and alkamides) did not scavenge or interact with NO and nitrogen dioxide species directly. Moreover, *Echinacea* extracts did not strongly inhibit nitrite accumulation by competing with oxygen to react with NO generated from SNP. Additionally, *Echinacea* extracts could not effectively protect RAW 264.7 cells from SNP-induced cell damage or cytotoxicity via the release of high levels of NO (data not shown). Based on these collected data, we could conclude that *Echinacea*-mediated decrease in NO production by macrophages is mainly via the modulation of the production yield of NO, but not direct scavenging of NO.

Echinacea present at the onset of LPS-induced iNOS expression resulted in a stronger inhibitory effect on NO production, suggesting that *Echinacea* could block the LPS-initiated upstream signaling pathway. Sharma et al. (2006) demonstrated that an alcohol tincture of EP roots significantly inhibited the nuclear expression of multiple pro-inflammatory transcription factors including NF- κ B and STATs in a viral infection model. Others showed that in stimulated macrophages, certain individual alkamides could inhibit NF- κ B activity (Matthias et al., 2007). Down-regulation of NF- κ B expression will lead to decreased iNOS expression and NO production. Our results suggested that the *Echinacea*-mediated decrease in NO production was generally associated with decreased protein expression of iNOS as indicated by Western blotting assay. The control protein β -actin was not affected under the same conditions suggesting that *Echinacea*-mediated inhibition of iNOS expression did not result simply from a broad decrease in protein expression. Note that several *Echinacea* preparations showed relatively weak inhibition of iNOS protein expression when compared with their effects on NO production, this may suggest other mechanisms in the context of decreased NO production, i.e. a direct inhibition of iNOS enzyme activity. However, NO production correlates with iNOS activity and is commonly used as an indirect indicator of iNOS activity (Wang et al., 1995; Munder et al., 1998). In addition, decreased NO production might be in part due to a switch of macrophage function to alternative activation, leading to less availability of the substrate for the iNOS pathway.

Echinacea-derived alkamides have been found recently to regulate cellular signaling and immune responses involving the endocannabinoid system. Interestingly, alkamides have a structural similarity with anandamide, an endogenous cannabinoid (CB) receptor ligand. Alkamides showed anandamide-like activities by binding to CB2 receptors highly expressed on immune cells and CB1 receptors predominantly expressed in the brain (Gertsch et al., 2004; Woelkart et al., 2005b; Raduner et al., 2006). This is the first molecular target identified for *Echinacea* so far. The endocannabinoid system involves many pathophysiological activities including anti-inflammation and analgesic action. The finding of the interaction of alkamides with CB receptors may help explain the traditional use of *Echinacea* in phytotherapy for wound healing, pain relief and alleviation of cold symptoms. Naturally occurring alkamides may have other therapeutic targets besides the endocannabinoid system (Gertsch et al., 2008; Raduner et al., 2006).

Taken together, *Echinacea* affects macrophage immune function at multiple levels, resulting in inhibition of NO production and increased arginase activity. Observations on fractions of alcohol extracts of *Echinacea* indicate that *Echinacea*-mediated decrease in NO production is due to bioactive alkamides. Such *in vitro* effects may be directly applicable to *in vivo* administration as alkamides are bioavailable. Moreover, the anti-inflammatory activity of *Echinacea* might be dependent on a synergistic action of both alkamides and caffeic acid derivatives, which act together to drive macrophages to alternative activation. These noteworthy findings will help define the mechanisms behind the success of traditional use of *Echinacea* in phytotherapy for inflammatory diseases.

Acknowledgments

This publication or project was made possible by the National Institute of Environmental Health Sciences (P01ES012020), the Office of Dietary Supplements, and the National Center for Complementary and Alternative Medicine (9 P50 AT004155-06), at the National Institutes of Health, and was performed as part of the Center for Research on Dietary Botanical Supplements at Iowa State University and the University of Iowa. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIEHS, ODS, NCCAM or the NIH. We thank Dr. Manuel Modolell (Max-Planck-Institut für Immunbiologie, Germany), Dr. Nair Sreejayan (University of Wyoming), and Dr. Jodi McKay (Iowa State University) for technical help.

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Fig. 1.

Alcohol extracts of *Echinacea* inhibit production of TNF- α by LPS-stimulated macrophages. RAW 264.7 cells were incubated *in vitro* for 23 h in the presence of indicated *Echinacea* extracts 100 μ g/ml plus different concentrations of LPS (A), or incubated with different concentrations of *Echinacea* extracts (0–200 μ g/ml) in the presence of 0.1 μ g/ml LPS (B). Culture supernates were assayed for TNF- α by ELISA (A and B (solid lines)). The attached cells were evaluated for cell viability by MTS assay (B (dashed lines)). The data are expressed as mean $\pm$ standard error of the mean of 3 independent experiments performed in triplicate. * $P < 0.001$ vs the vehicle control at the same concentration of LPS.

Fig. 2.

Alcohol extracts of *Echinacea* inhibit production of NO by LPS-stimulated macrophages. Cells were incubated *in vitro* for 23 h in the presence of *Echinacea* extracts 100 μg/ml plus different concentrations of LPS (A), or incubated overnight with different concentrations of indicated *Echinacea* extracts (0–200 μg/ml) in the presence of 1 μg/ml LPS (B). After removing supernates for NO assay (A and B (solid lines)), cell viability was evaluated by addition of MTS (B (dashed lines)). The data are expressed as mean ± standard error of the mean of 3 independent experiments performed in triplicate.

Fig. 3.

Scavenging of NO derived from SNP by alcohol extracts of *Echinacea*. A. SNP was mixed with Griess reagent in the presence of vehicle (0.25% ethanol) or 50 $\mu\text{g/ml}$ of *Echinacea* extracts and incubated for 6 h. Nitrite accumulation was monitored at 550nm at various time points and calculated based on the absorbance of sodium nitrite standards manipulated in the same way with Griess reagent. B. SNP was mixed with Griess reagents containing various concentrations of *Echinacea* extracts and then incubated for 150 min. The data are expressed as mean $\pm$ standard error of the mean of 3 independent experiments performed in triplicate.

Fig. 4.

Time course of inhibition of NO production by alcohol extracts of Echinacea. *Echinacea* extracts (100 µg/ml) were either added simultaneously with LPS (1 µg/ml) (0 h) or up to 6 h after addition of LPS. The nitrite accumulation by RAW 264.7 cells was determined 22 h after LPS stimulation by the Griess reaction. The data are expressed as mean $\pm$ standard error of the mean of 4 experiments performed in triplicate. * P <0.05 and ** P <0.001 vs 0.25% ethanol in the presence of LPS (control). + P <0.05 and ++ P <0.001 vs respective group in the presence of LPS at time point 0 h.

Fig. 5. Regulation of NO production and arginase activity of activated RAW 264.7 cells by alcohol extracts of *Echinacea*. Cells were incubated in the presence or absence of indicated concentrations of *Echinacea* extracts, LPS (1 µg/ml) and/or 8-bromo-cAMP (abbreviated cAMP, 0.25 mM) for 23–24 h, then supernates were collected for NO assay (A). The cell lysates were assayed for arginase activity (B and C (solid lines)) as described in Materials and Methods. Cell viability was determined by MTS assay (C (dashed lines)). Data are mean $\pm$ standard error of the mean of 4–5 experiments performed in triplicate (for NO and cell viability) or duplicate (arginase). * P <0.05 and ** P <0.001 vs 0.25% ethanol as control in respective treatment group.

Fig. 6.

Regulation of NO production and arginase activity of activated RAW 264.7 cells by fractions of alcohol extracts of *Echinacea*. NO production was determined in supernates of cells exposed to LPS (1 $\mu\text{g/ml}$) plus individual fraction (25 $\mu\text{g/ml}$) for 24 h (A). Arginase activity was assayed in cells treated with LPS (1 $\mu\text{g/ml}$), 8-bromo-cAMP (0.25 mM) and individual fraction (25 $\mu\text{g/ml}$) for 24 h (B). Data are expressed as mean $\pm$ standard error of the mean of 4–5 experiments performed in triplicate (NO) or duplicate (arginase). * P <0.05 and ** P <0.001 vs 0.25% ethanol as vehicle as in respective treatment group.

Fig. 7.

Regulation of iNOS expression by alcohol extracts and fractions of *Echinacea*. Cells were incubated with or without LPS (1 $\mu\text{g/ml}$) plus *Echinacea* extract (100 $\mu\text{g/ml}$) or individual fraction (25 $\mu\text{g/ml}$) for 24 h. The iNOS expression was analyzed by Western blotting and digitally quantitated using image analysis software. The data represent the mean of three independent experiments with different cell preparations. Representative bands are shown. The net intensity of iNOS bands was compared to that of corresponding β -actin with the same treatment, and the ratio was expressed as a percent of the ratio for the vehicle- and LPS-treated cells. A. EA; B. EPA; C. EP. * $P < 0.05$ vs vehicle plus LPS.

Table 1**A. Fractionation procedure using semi-preparative HPLC**

Retention time (min)	Solvent A ^a	Solvent B ^b	Fraction
0–31	90–70%	10–30%	1
33–42	70–60%	30–40%	2
43–83	60–20%	40–80%	3
84–94	20–0%	80–100%	4
95–115	0%	100%	5

B. *Echinacea* fractions and their chemical constituents

Fraction	<i>Species</i>		
	<i>E. angustifolia</i>	<i>E. pallida</i>	<i>E. purpurea</i>
1	Caffeic acid derivatives	Caffeic acid derivatives	Caffeic acid derivatives
2	Unknown	Unknown	Unknown
3	Alkamides	Alkamides and ketones	Alkamides
4	Alkamide 11 and other very hydrophobic compounds	Very Hydrophobic compounds	Very hydrophobic compounds
5	Most hydrophobic compounds	Most hydrophobic compounds	Most hydrophobic compounds

^aSolvent A: 0.1% acetic acid^bSolvent B: acetonitrile

Table 2Concentrations of known metabolites in alcohol extracts of *Echinacea*

Metabolites	Species		
	<i>E. angustifolia</i>	<i>E. pallida</i>	<i>E. purpurea</i>
Amides	1.71 ^a	1.04	2.61
Caftaric acid	ND	0.06	0.06
Chlorogenic acid	0.09	0.21	ND
Cichloric acid	ND ^b	0.06	0.46
Echinacoside	0.39	1.26	ND

^a % of dried extract^b ND = not detectable