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Research Paper

Inhibition of inducible nitric oxide synthesis by *Cimicifuga racemosa* (*Actaea racemosa*, black cohosh) extracts in LPS-stimulated RAW 264.7 macrophages

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Abstract

Objectives *Cimicifuga racemosa (Actaea racemosa,* black cohosh) is used as an anti-inflammatory, antipyretic and analgesic remedy in traditional medicines. The present study focuses on the effects of *C. racemosa* root extracts on inducible nitric oxide synthase (iNOS) in lipopolysaccharide-stimulated murine macrophages (RAW 264.7).

Methods *C. racemosa* rhizome and phosphate-buffered saline extracts were analysed for phenolcarboxylic acids and triterpene glycosides using an HPLC photodiode array/evaporative light-scattering detector system. iNOS was characterised by measurement of iNOS protein (immunoblotting), iNOS mRNA (semiquantitative competitive RT-PCR), nitric oxide production (nitrite levels) and nuclear translocation of nuclear factor-κB (p65 subunit) protein.

Key findings Incubation of lipopolysaccharide-stimulated macrophages with aqueous *C. racemosa* extracts (0–6 mg/ml) inhibited nitrite accumulation in a concentration-dependent manner. *C. racemosa* extracts also reduced iNOS protein expression and iNOS mRNA levels in a dose-dependent manner. *C. racemosa* extracts did not significantly inhibit iNOS activity and did not affect nuclear translocation of nuclear factor-κB (p65 subunit) protein. Incubation with the extract was associated with a concentration-dependent reduction of interferon beta and interferon regulatory factor 1 mRNA. Among the triterpene glycosides, 23-epi-26-deoxyactein was identified as an active principle in *C. racemosa* extracts.

Conclusions Extracts from the roots of *C. racemosa* inhibit nitric oxide production by reducing iNOS expression without affecting activity of the enzyme. This might contribute to the anti-inflammatory activities of *C. racemosa*.

Keywords *Actaea racemosa; Cimicifuga racemosa*; inducible nitric oxide synthase; inflammation; murine macrophages; nuclear factor κB; Ranunculaceae

Introduction

Cimicifuga racemosa (L.) Nutt. (syn. *Actaea racemosa* L., Ranunculaceae), commonly known as black cohosh, is a perennial plant native in North America. It was used by Native American Indians for the treatment of various conditions, including inflammatory diseases such as chronic rheumatism.[1] *C. racemosa* (CR) rhizome has become one of the most important herbal products in the US dietary supplement market and is gaining importance in the European herbal remedy market because of reports that it alleviates menopausal symptoms.[2–4] In addition, extracts of roots and rhizomes of various *Cimicifuga* species (e.g. *C. dahurica, C. heracleifolia, C. foetida, C. iaponica* and *C. acerina*) have been used in traditional Asian medicines for their anti-inflammatory, antipyretic and analgesic activities.[5–7] Inhibition of neutrophil elastase activity by cinnamic acid derivatives from CR has been described.[8] However, the mechanisms underlying the anti-inflammatory activity of CR have not been fully elucidated.

Nitric oxide (NO) is synthesised from L-arginine by the L-arginine–NO pathway.[9] A family of enzymes, termed the NO synthases (NOS), catalyse the formation of NO and citrulline from L-arginine, oxygen and NADPH.[10] The inducible isoform of NOS (NOS-2 or

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iNOS, E.C. 1.14.13.39) generates large amounts of NO over a prolonged period of time through a calcium-independent pathway.[11] iNOS is induced by lipopolysaccharide (LPS) or proinflammatory cytokines such as tumour necrosis factor (TNF) α, interleukin (IL)-1β and interferon (IFN) γ.[12] Expression of iNOS has been observed in many cells, including murine macrophages.[13] Human iNOS is most readily observed in monocytes or macrophages from patients with infectious or inflammatory diseases, and the sustained production of NO endows macrophages with cytostatic or cytotoxic activity against viruses, bacteria, fungi, protozoa, helminths and tumour cells.[14] Thus, NO is recognised as a mediator and regulator of inflammatory responses,[15] and inhibition of NO production might be a useful therapeutic strategy in the treatment of inflammatory diseases.[16,17]

The aim of the present study was to clarify the effects of CR rhizome extracts on inducible NO synthesis as a marker of inflammation, using LPS-stimulated murine macrophages (RAW 264.7).

Materials and Methods

Materials

Rabbit anti-iNOS polyclonal antibody was obtained from Biomol (Hamburg, Germany). Purified iNOS protein was from Calbiochem (San Diego, CA, USA). Anti-rabbit IgG was obtained from Promega (Madison, WI, USA). CR (*A. racemosa*, Ranunculaceae, dried, powdered rhizomes), cimigenol-3-*O*-xyloside, caffeic acid and 23-epi-26-deoxyactein were purchased from ChromaDex (Santa Ana, CA, USA). Ferulic acid was obtained from Carl Roth (Karlsruhe, Germany) and isoferulic acid was from Extrasynthese (Genay, France). Cell culture materials, *Escherichia coli* LPS serotype 055:B5 and all other chemicals were purchased from Sigma (St Louis, MO, USA).

Preparation of *Cimicifuga racemosa* extracts

Aqueous extracts of CR (dried rhizomes, powdered drug) were prepared by dispersing 100 mg in 1 ml phosphate-buffered saline (PBS) at 4°C. The dispersion was homogenised on ice for 4×10 s using an Ultra Turrax (IKA Labortechnik, Staufen, Germany), sonicated with a Branson Sonifier B30 at 50% for 6×10 s, and centrifuged at 10 000*g* for 10 min at 4°C. The soluble fraction of the extract was filtered through a 0.45 μm filter (Sartorius, Goettingen, Germany) to remove solid particles, giving the PBS solution. Concentrations of CR root extracts are expressed as mg of the original amount of *C. racemosa* dried rhizomes per ml solution.

A fraction of the aqueous extract was obtained by *n*-butanol extraction. In brief, equal amounts (500 μl) of *n*-butanol and aqueous CR extract were mixed and shaken for 30 min at room temperature. Thereafter, phases were allowed to separate, the *n*-butanol layer was evaporated to dryness under vacuum and the residue reconstituted in 500 μl PBS.

Cell culture

The mouse monocyte/macrophage cell line RAW 264.7 (ATCC TIB 71) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 25 mM HEPES, 2 mM glutamine, 100 U penicillin per ml and 100 μg streptomycin per ml at 37°C, 5% CO_2 and 95% humidity. Cells were studied between passages 7–30.

Macrophages were seeded into 24-well dishes at a density of 2×10^5 cells per well. LPS (1 μg/ml) and CR extracts (0–6 mg/ml final concentration) were added simultaneously to the culture medium and the cells cultured for 24 hours. The dose of LPS was chosen on the basis of previous findings to achieve a maximum induction of inducible NO synthesis in RAW 264.7 mouse macrophages.

Nitrite analysis

Nitrite concentration in supernatants was determined spectrophotometrically using the Griess reagent (0.5% sulfanilic acid, 0.002% *N*-1-naphthyl-ethylenediamine dihydrochloride, 14% glacial acetic acid). Absorbance was measured at 550 nm with baseline correction at 650 nm; nitrite concentration was determined using sodium nitrite as the standard.[18]

Protein measurement

Protein concentration was determined according to the method of Bradford[19] using bovine serum albumin as standard.

Determination of iNOS activity

LPS-stimulated RAW 264.7 macrophages (25×10^6 cells) were washed with PBS, harvested into medium, transferred into microcentrifuge tubes and centrifuged at 300*g* for 5 min at 4°C. The cell pellet was homogenised in 1 ml 15 mM HEPES containing 500 μM dithiothreitol, 10 μg/ml trypsin inhibitor, pH 7.4, and sonicated for 3×10 s. The homogenate was centrifuged at 20 000*g* for 15 min at 4°C. A 200 μl sample of the soluble fraction was incubated with 200 μl reaction buffer (4 mM NADPH, 120 μM tetrahydrobiopterin, 20 μM FAD, 20 μM FMN, 20 μM calcium chloride, 10 units/ml calmodulin and 500 μM L-arginine). After incubation for 5 h at 37°C, the nitrite concentration was measured as described above.

Western blotting

Cells were lysed in ice-cold buffer containing 25 mM monosodium phosphate (pH 7.4), 75 mM NaCl, 5 mM EDTA and 1% Triton X-100, and centrifuged at 20 000*g* for 15 min at 4°C. The cytosolic proteins (12 μg per lane) were separated by 12% SDS-PAGE. Proteins were transferred to nitrocellulose filters, and then immunoblotted with a rabbit anti-iNOS or a rabbit anti-actin polyclonal antibody at a 1 : 2000 dilution. Anti-rabbit alkaline phosphatase-conjugated antibody was used as a secondary antibody at a dilution of 1 : 7500. Finally, the blots were incubated with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium reagent (Promega) for 10–15 min.

Preparation of nuclear extracts

RAW 264.7 cells (8×10^5) were seeded into six-well plates and incubated with increasing amounts of CR extracts (0–6 μg/μl) for 30 min, followed by LPS (1 μg/ml) stimulation for 1 h. Cells were washed twice, resuspended in 500 μl lysis buffer (10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol and 0.6% Igepal CA-630) and vortex mixed for 30 s. Nuclei were pelleted by centrifugation at 10 000*g* for 2 min at 4°C, resuspended in 30 μl buffer containing 20 mM

Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EGTA, 0.5 mM dithiothreitol and 25% (v/v) glycerol, vortex mixed for 30 s, and incubated on ice for 15 min. Lysates were centrifuged at 20 000*g* for 5 min at 4°C. Aliquots of supernatants were stored at −70°C until analysed by Western blotting as described above using a rabbit anti-nuclear factor (NF)-κB (p65) primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1 : 2000 dilution.

Semi-quantitative competitive RT-PCR

Total RNA was isolated using the guanidinium thiocyanate method.[20] To determine the RNA concentration, the absorption at 260, 280 and 320 nm was measured photometrically. Single-stranded cDNA synthesis was carried out on 0.5 μg total RNA primed with oligo$(dT)_{12–18}$ (Pharmacia, Freiburg, Germany) using murine leukaemia virus reverse transcriptase (MMLV-RT; MBI Fermentas, Vilnius, Lithuania) at 37°C for 60 min. Reactions were stopped by heating at 70°C for 5 min. iNOS cDNA was subjected to DNA amplification by PCR using 0.5 units of Taq DNA polymerase (MBI Fermentas) with oligonucleotide primers complementary to murine iNOS cDNA (MWG-Biotec, Ebersberg, Germany) at a final concentration of 0.25 μM. Oligonucleotide primers against β–actin were used as the competitor at a concentration of 0.03 μM. Reaction mixtures were subjected to the following conditions in a PE 2400 DNA thermal cycler (Perkin-Elmer, Norwalk, CT, USA): denaturing at 94°C for 30 s, annealing at 58°C for 35 s, and extension at 72°C for 35 s. After 32 cycles, the reaction mixture was cooled to 4°C. The primers for iNOS were 5-CTA AGA GTC ACC AAA ATG GCT CCC-3′ (sense) and 5′-ACC AGA GGC AGC ACA TCA AAG C-3′ (antisense). The expected product length was 775 bp. The following primers for the 'housekeeping gene' β-actin were used: 5′-ATG GTG GGA ATG GGT CAG AAG GAC-3′ (sense) and 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′ (antisense). The expected product length was 513 bp. All PCR reactions were in the linear range. Final PCR products were separated on 1% agarose gel and detected by ethidium bromide staining.

The primers for IFN-β were 5′-CTG CGT TCC TGC TGT GCT TC-3′ (sense) and 5′-AAA CAC TGT CTG CTG GTG GAG TTC-3′ (antisense) and the expected product length was 317 bp. The primers for interferon regulatory factor 1 (IRF-1) were 5′-TTC CAA CCA AAT CCC AGG GC TG-3′(sense) and 5′-CAT AAG GTC TTC GGC TAT CTT CCC-3′ (antisense); the expected product length was 667 bp. Oligonucleotide primer concentrations were 1.0 μM against IFN-β and 0.05 μM against IRF-1. PCR conditions were as described above except for an annealing temperature of 57°C. The PCR was stopped after 40 cycles. Actin mRNA was quantified separately.

HPLC analysis of phenolcarboxylic acids

HPLC analysis of phenolcarboxylic acids was performed using a Perkin Elmer series 200 lc pump, 200 autosampler, diode array detector 235 C, PE Nelson 600 series LINK interface, TCNav software, equipped with a Phenomenex Gemini C_{18} 5 μm, 250 × 4.6 mm column, and a Phenomenex Gemini C_{18}, 5 μm, 4 × 4.6 mm precolumn.[2] Gradient elution was performed with a mobile phase consisting of water, adjusted to pH 2.40 with formic acid, (solvent A) and acetonitrile (solvent B) at a flow rate of 1.0 ml/min: 18–33% B (0–35 min), 33–80% B (35.0–35.1 min), 80% B (35.1–38 min), 80–18% B (38.0–38.1 min), 18% B (15 min). After filtration through a 45 μm disposable filter, 25 μl of sample was injected. Detection was at 330 nm at room temperature. Caffeic acid was used as the external standard.

HPLC analysis of triterpene glycosides

Analysis of triterpene glycosides was performed on an HPLC Waters 2690 Alliance system with an evaporative light-scattering detector (PL-ELS 1000, Polymer Laboratories, Amherst, MA, USA) at 0.8 ml/min gas flow rate, 60°C nebulisation temperature and 100°C evaporation temperature, equipped with an Agilent Technologies Zorbax XDB C_{18} (5 μm) column (250 × 4.6 mm ID). Gradient elution was performed with a mobile phase consisting of 0.1% (v/v) solution of formic acid in water (solvent A) and 0.1% (v/v) formic acid in a mixture of equal volumes of acetonitrile and methanol (solvent B) at a flow rate of 1.0 ml/min: 50–80% B (0–40 min), 80–95% B (40–41 min), 95% B (41–44 min), 95–50% B (44–45 min), 50% B (45–55 min). After filtration through a 45 μm disposable filter, 10 μl of sample was injected. Monoammonium glycyrrhizate was used as the external standard. The content of triterpene glycosides was calculated as 23-epi-26-deoxyactein as described previously.[21]

Characterisation of phenolcarboxylic acids in extracts

Samples (25 mg) of the powdered CR rhizome were weighed in centrifuge vessels, suspended in 2 ml 80% methanol (analytical grade), vortex mixed for a few seconds, sealed with Parafilm, ultrasonicated at 40°C for 30 min and centrifuged at 3500 rpm for 10 min. The supernatant was transferred into a 5 ml volumetric flask. The residue was extracted a second time as described above, the supernatant combined with the first extract and the volume made up to 5 ml with 80% methanol. Caffeic acid (0.017%), ferulic acid (0.018%), isoferulic acid (0.056%), fukinolic acid (0.164%), cimicifugic acids A (0.047%), B (0.146%), E (0.008%) and F (0.022%) were detected (Figure 1), giving a total of 0.478% of phenolcarboxylic acids (values are % of the dried rhizome). Their pattern, as well as their ratios, are in good agreement with the literature and confirm the identity of *C. racemosa*.[22]

Characterisation of triterpene glycosides in extracts

Powdered CR root (1.0 g) was weighed into a 200 ml screw-cap bottle, sonicated with 25 ml 50% methanol and shaken for 24 h. According to HPLC analyses, 2.539% triterpene glycosides, expressed as 23-epi-26-deoxyactein, were detected (values represent % of the dried rhizome): unknown triterpene glycoside (0.197%), cimiracemoside F (0.104%), G (0.136%), H (0.073%), 23-epi-26-deoxyactein (0.237%), 23-*O*-acetylshengmanol-3-*O*-arabinoside (0.047%), 23-*O*-acetylshengmanol-3-*O*-xyloside and bugbanoside E or cimicifol (0.325%), cimiracemoside D (0.074%), cimidahuside I (0.102%), 24-acetylhydroshengmanol-3-*O*-xyloside* (0.098%; *values interchangeable), 24-acetylhydroshengmanol-3-*O*-arabinoside*

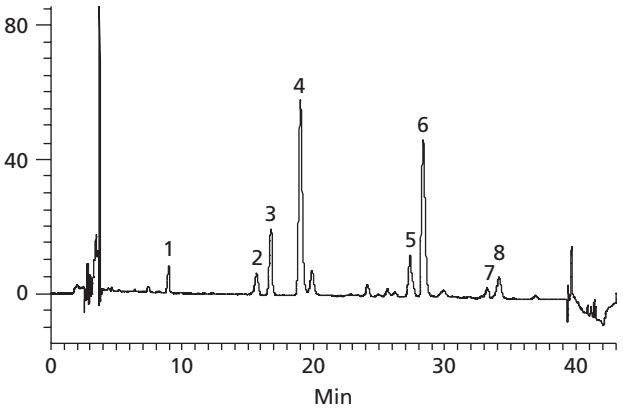

Figure 1 HPLC chromatogram of phenolcarboxylic acids of *Cimicifuga racemosa* rhizome. Peak 1, caffeic acid; 2, ferulic acid; 3, isoferulic acid; 4, fukinolic acid; 5, cimicifugic acid A; 6, cimicifugic acid B; 7, cimicifugic acid E; 8, cimicifugic acid F.

(0.191%), shengmanol-arabinoside (0.052%), shengmanol-xyloside (0.075%), cimigenol-3-*O*-xyloside (0.306%) and cimiracemoside C (0.522%). Among ten different *Cimicifuga* species, 23-*O*-acetylshengmanol-3-*O*-arabinoside was found in CR, exclusively.[22] Its presence in the 50% methanol extract further confirms the identity of *C. racemosa*.

HPLC analysis of the PBS extract

CR PBS extract (12 ml) was prepared as described above and subjected to lyophilisation; 230 mg of dry extract was obtained, resulting in a drug extract ratio of 5.2 : 1.

Analysis of phenolcarboxylic acids

Samples (10 and 20 mg) of the lyophilised CR PBS extract were dissolved in 1 ml distilled water, ultrasonicated for 5 min at room temperature and analysed by HPLC. Caffeic acid (0.010%), ferulic acid (0.028%), isoferulic acid (0.152%) and cimicifugic acids E (0.025%) and F (0.041%) were detected, giving a total of 0.256% of phenolcarboxylic acids (values represent % of the dried PBS extract).

Analysis of triterpene glycosides

Lyophilised CR PBS extract (85 mg) was dissolved in 1.0 ml 50% methanol, sonicated, and shaken for 24 h. According to HPLC analysis, 1.005% triterpene glycosides, expressed as 23-epi-26-deoxyactein, were detected (values represent % of the dried PBS extract): unknown triterpene glycoside (0.081%), cimiracemoside F (0.074%), G (0.081%), H (0.037%), 23-epi-26-deoxyactein (0.107%), 23-*O*-acetylshengmanol-3-*O*-xyloside and bugbanoside E or cimicifol (0.071%), cimiracemoside D (0.038%), 24-acetylhydroshengmanol-3-*O*-xyloside* (0.039%), 24-acetylhydroshengmanol-3-*O*-arabinoside* (0.093%), shengmanol-arabinoside (0.081%), cimigenol-3-*O*-xyloside (0.098%) and cimiracemoside C (0.205%).

Statistical analysis

Statistical analyses were performed using analysis of variance followed by Student's *t*-test for unpaired data. Results and additional repeated measurements were also analysed using the Mann–Whitney–Wilcoxon *U* test and the Kruskal–Wallis test. Statistical significance was defined as $P < 0.05$. When SD is not displayed, it is smaller than the size of the symbol.

Results

Inhibition of inducible NO synthesis

Activated RAW 264.7 cells released large amounts of nitrite into the culture medium (98.8 ± 4.2 nmol nitrite/mg protein within 24 h, vs 1.2 ± 0.3 nmol nitrite/mg protein for unstimulated control incubations). Incubation of stimulated RAW 264.7 macrophages with aqueous CR extracts (1, 2, 4 and 6 mg/ml) was associated with a concentration-dependent reduction in NO production (Figure 2). The extent of inhibition (percent inhibition) was 51.6 ± 1.0% with 1 mg/ml CR extract, 72.4 ± 0.9% with 2 mg/ml, 83.0 ± 1.4% with 4 mg/ml and 84.8 ± 1.1% for 6 mg/ml.

Incubation of macrophages with a butanol fraction of the aqueous CR extract resulted in a comparable decrease in NO production (data not shown). Inhibition of NO production by CR was significant at all concentrations tested.

Effects of *Cimicifuga racemosa* extracts on cell viability

Measurements of cytotoxicity were performed to exclude toxic effects of CR extracts towards RAW 264.7 cells. Cell viability (as measured by trypan blue exclusion) was more than 95% up to a concentration of 6 mg/ml CR. The MTT assay was used to show that CR extracts (1, 2, 4, 6 mg/ml) did not have cytotoxic effects (data not shown).

Effects of *Cimicifuga racemosa* extracts on iNOS protein expression

Figure 3 shows the Western blot analysis of iNOS expression in RAW 264.7 macrophages. Immunoblotting shows a band

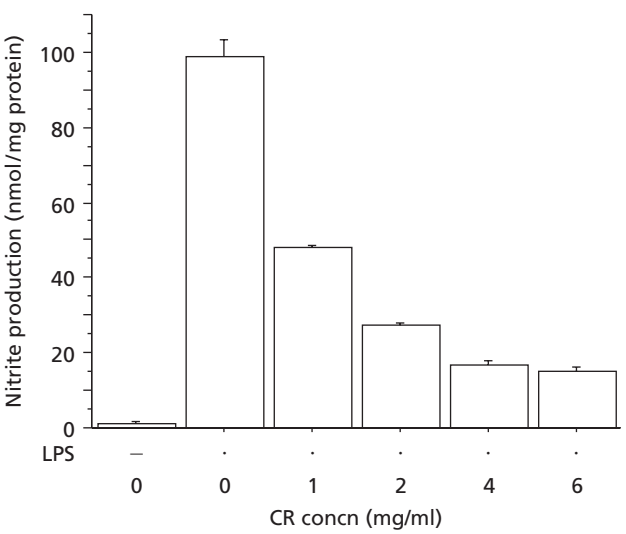

Figure 2 Inhibition of inducible nitric oxide production by *Cimicifuga racemosa* extracts in lipopolysaccharide-stimulated macrophages. Values are means ± SD of triplicate measurements. Data shown are representative for three independent experiments. CR, *Cimicifuga racemosa*; LPS, lipopolysaccharide.

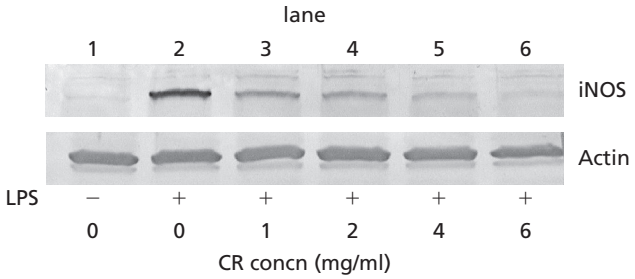

Figure 3 Effect of *Cimicifuga racemosa* extracts on inducible nitric oxide synthase protein expression. Western blotting analysis of inducible nitric oxide synthase (iNOS) protein expression was performed in macrophages incubated with lipopolysaccharide (LPS; 1 μg/ml) and *Cimicifuga racemosa* (CR) extract (1–6 mg/ml) for 24 h. Lane 2 shows a band with an estimated molecular mass of 130 kD (molecular mass of iNOS) Expression of β-actin (43 kD) levels remained unchanged during incubation with CR extracts. Data shown are representative of three independent experiments.

with the estimated molecular mass of 130 kD (the known molecular mass of iNOS) in stimulated cells. An identical molecular mass was found by blotting against purified iNOS protein (not shown). Incubation with CR extract showed a dose-dependent decrease in iNOS protein expression. Levels of β-actin (43 kD) protein remained unchanged. This shows that incubation with CR extracts is not associated with generalised reduction of protein expression.

Effects of *Cimicifuga racemosa* extracts on iNOS mRNA expression

Figure 4 shows the semiquantitative competitive RT-PCR analysis of iNOS mRNA expression in RAW 264.7 cells. iNOS mRNA levels showed a concentration-dependent reduction when macrophages were incubated with CR extracts. Actin mRNA levels remained unchanged. Thus, CR extracts did not cause toxic effects or a generalised decrease in mRNA transcription at the concentrations tested.

iNOS enzymatic activity in the presence of *Cimicifuga racemosa* extracts

To determine the effect of CR extracts on iNOS enzyme activity, we measured cytosolic enzyme activity. CR extracts (0–6 mg/ml final concentration) did not significantly inhibit iNOS enzyme activity compared with cell lysates without CR extracts. In contrast, 300 μl of the NOS inhibitor L-nitro-arginine methyl ester reduced iNOS activity by more than 70% (data not shown).

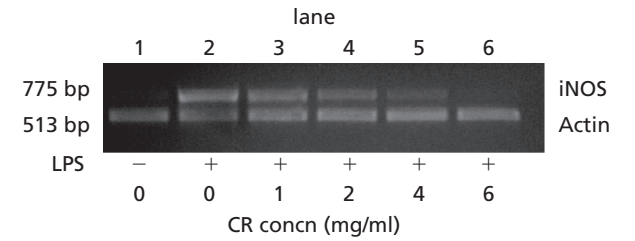

Figure 4 Effects of *Cimicifuga racemosa* extracts on expression of inducible nitric oxide synthase mRNA (775 bp) expression. Macrophages were incubated with lipopolysaccharide (LPS; 1 μg/ml) and 1, 2, 4, 6 mg/ml *Cimicifuga racemosa* (CR) extracts for 24 h, and showed dose-dependent inhibition of inducible nitric oxide synthase (iNOS) mRNA expression. Levels of β-actin mRNA (513 bp) remained unchanged. Data shown are representative for three independent experiments.

Effects of *Cimicifuga racemosa* extracts on nuclear translocation of NF-κB (p65)

Western blotting against NF-κB (p65) identified a band with an estimated molecular mass of 65 kD in nuclear extracts from stimulated RAW 264.7 mouse macrophages (Figure 5, lane 2). Nuclear extracts from unstimulated cells showed a decreased translocation of NF-κB (p65) (lane 1); nuclear NF-κB (p65) protein was not reduced by CR extract.

Effects of *Cimicifuga racemosa* extracts on IFN-β and IRF-1 mRNA expression

IFN-β and IRF-1 mRNA showed a concentration-dependent reduction when macrophages were incubated with increasing amounts of CR extracts (Figure 6). In contrast, β-actin mRNA levels remained unchanged.

HPLC analysis of the PBS extract

The content of phenolcarboxylic acid derivatives and triterpene glycosides was determined for the lyophilised PBS dry extract and used to calculate the concentration in the aqueous PBS CR solution: 3 μg/ml caffeic acid, 9 μg/ml ferulic acid, 47 μg/ml isoferulic acid, 8 μg/ml cimicifugic acid E and 13 μg/ml cimicifugic acid F. Fukinolic acid, cimicifugic acid A and cimicifugic acid B were found in 80% methanolic extracts of CR rhizome but were not detectable in the PBS CR extract. The concentrations of triterpene glycosides in the PBS solution were 308 μg/ml, with 33 μg/ml 23-epi-26-deoxyactein as a major constituent.

Inhibition of inducible nitric oxide synthesis by 23-epi-26-deoxyactein

NO production by LPS-activated RAW 264.7 cells decreased with increasing amounts of 23-epi-26-deoxyactein (5–20 μg/ml) (Figure 7). The percentage inhibition was 23.5 ± 0.1% at

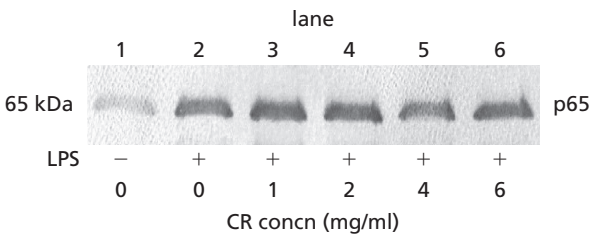

Figure 5 Effect of *Cimicifuga racemosa* extracts on nuclear translocation of nuclear factor-κB. Immunoblotting against nuclear factor (NF)-κB (p65) in macrophages incubated with *Cimicifuga racemosa* (CR) extract (1, 2, 4 and 6 mg/ml) for 30 min, followed by lipopolysaccharide (LPS; 1 μg/ml) stimulation for 1 h identified a band with an estimated molecular mass of 65 kD in nuclear extracts from stimulated macrophages (lane 2). Lane 1 shows nuclear extracts from unstimulated cells. NF-κB (p65) protein was not reduced by CR extracts. Data shown are from one representative experiment.

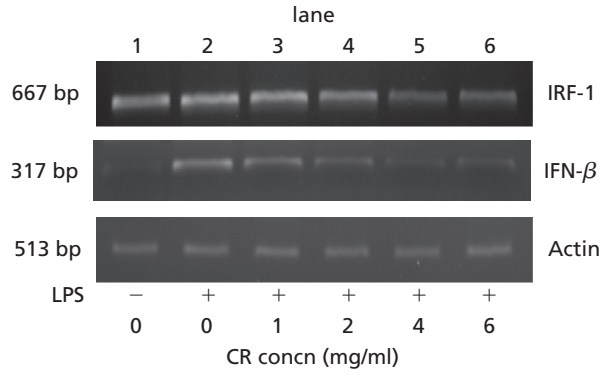

Figure 6 Effect of *Cimicifuga racemosa* extracts on expression of interferon-β and interferon regulatory factor 1 mRNA expression. *Cimicifuga racemosa* (CR) extract showed dose-dependent inhibition of interferon (IFN)-β and interferon regulatory factor 1 (IRF-1) mRNA expression in lipopolysaccharide (LPS)-stimulated macrophages. Control β-actin mRNA levels remained unchanged.

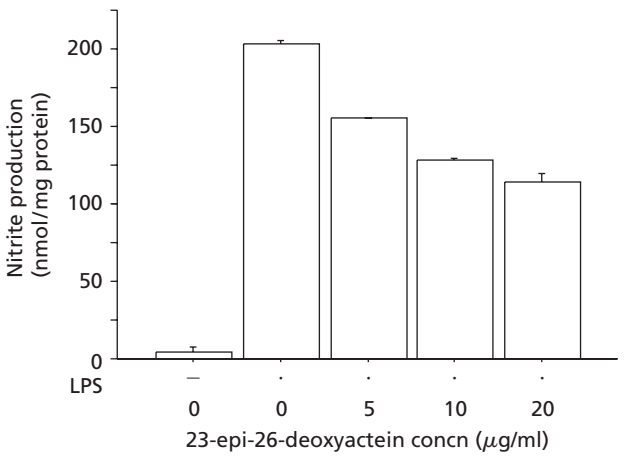

Figure 7 Inhibition of inducible NO production by 23-epi-26-deoxyactein in LPS-stimulated macrophages, showing dose-dependent reduction of nitrite release.

5 μg/ml 23-epi-26-deoxyactein, 36.8 ± 0.7% at 10 μg/ml and 43.9 ± 2.8% at 20 μg/ml (all significant).

Caffeic acid reduced nitrite levels to 61.0% ± 1.95% and 88.1% ± 1.47% at 50 μg/ml and 200 μg/ml, respectively. Ferulic acid (116.5 μg/ml) and isoferulic acid (500 μg/ml) did not significantly inhibit inducible NO synthesis.

Discussion

Incubation of RAW 264.7 macrophages with CR root extracts inhibited LPS-induced NO production; the aqueous CR extracts inhibited NO production by 84.8% (Figure 2). Comparable results were observed when cells were incubated with a butanol fraction of the aqueous CR extract. CR extracts did not significantly inhibit iNOS enzyme activity compared with cells lysates without *C. racemosa*. We therefore investigated the effects of CR extracts on iNOS protein and mRNA expression. Western blot and semiquantitative competitive RT-PCR analyses showed that CR extracts reduced the intracellular concentration of iNOS protein and iNOS mRNA expression (Figures 3 and 4).

Various agents with anti-inflammatory properties such as glucocorticoids,[23] mycophenolate mofetil,[24] ciclosporin[25] and azathioprine[26] have been shown to inhibit inducible NO synthesis. The findings of our study may help to explain possible immunomodulatory effects of CR extracts by showing that inhibition of inducible NO synthesis was comparable to known anti-inflammatory agents by reducing iNOS protein and iNOS mRNA levels.

The inhibition of iNOS expression by plant extracts and immunosuppressive agents such as ciclosporin and FK-506 has been shown to be independent of altered activation of NF-κB in murine macrophages.[27,28] This is in accordance with our finding that CR extracts did not inhibit NF-κB (p65) translocation (Figure 5). Reduced expression of IFN-β and IRF-1 mRNA expression in LPS-stimulated macrophages has been revealed as a mechanism underlying the inhibition of inducible NO synthesis.[29] In our experiments, CR clearly inhibited IFN-β and IRF-1 mRNA expression (Figure 6).

Two major classes of secondary metabolites have been isolated from the rhizomes of CR: triterpene glycosides (e.g. 23-epi-26-deoxyactein, cimigenol-3-*O*-xyloside, 23-*O*-acetylshengmanol-3-*O*-arabinoside)[22,30] and phenolcarboxylic acids (e.g. caffeic, ferulic and isoferulic acids) and their derivatives with fukiic and piscidic acid (cimicifugic acids A, B, E and F and fukinolic acid).[31,32] HPLC analysis of phenolcarboxylic acids (Figure 1) and triterpene glycosides in 80% and 50% methanol extracts confirmed the identity of CR. The presence of caffeic acid, ferulic acid, isoferulic acid and cimicifugic acids E and F in the PBS extract was confirmed. In addition, 23-epi-26-deoxyactein (33 μg/ml) and cimiracemoside C, D, F, G and H were found.

Among other phenolic compounds of CR, ferulic acid and isoferulic acid have been reported to suppress the production of proinflammatory cytokines.[33,34] However, ferulic acid (116.5 μg/ml) and isoferulic acid (500 μg/ml) did not significantly inhibit inducible NO synthesis in our experiments, which is in accordance with previous reports.[35] Caffeic acid reduced inducible NO synthesis in a dose-dependent manner, ranging from 26.1 ± 2.8% inhibition at 40 μg/ml to 70.9 ± 6.9% at 200 μg/ml. According to HPLC analysis, the aqueous PBS solution contained 3 μg/ml caffeic acid, which implies that the final concentration of caffeic acid in the culture medium is 0.18 μg/ml. Therefore, caffeic acid may make a minor contribution to the inhibition of inducible NO synthase by aqueous CR extracts.

Among the triterpene glycosides, 23-epi-26-deoxyactein was found to be a potent inhibitor of inducible NO production in LPS-stimulated macrophages. CR PBS extracts contained 33 μg/ml 23-epi-26-deoxyactein, and the final concentration in the culture medium after addition of 6 mg/ml CR extract was 1.98 μg/ml. The IC50 for 23-epi-26-deoxyactein derived from dose–response curves was 24.7 μg/ml, and polynomial regression analysis determined that the degree of inhibition for 1.98 μg/ml 23-epi-26-deoxyactein was 14.3%. Cimigenol-3-*O*-xyloside did not inhibit inducible NO production.

According to Kim and colleagues, administration of CR extracts caused potent analgesic effects in the acetic acid

writhing and tail flick assays, and also revealed anti-inflammatory properties by inhibiting cyclooxygenase-2-mediated production of 6-keto-prostaglandin F1α in LPS-stimulated macrophages.[36] Inhibition of neutrophil elastase activity by cinnamic acid derivatives from CR has also been reported.[8] Furthermore, CR extracts exhibited anti-allergic activity due to inhibition of histamine release and induction of IL-4, IL-5 and TNFα mRNA in human leukaemia mast cells.[37]

Conclusions

Our data suggest that the inhibitory effect of CR extracts on inducible NO production is due to inhibition of iNOS expression. Among the triterpene glycosides, 23-epi-26-deoxyactein was an inhibitor of inducible NO production in LPS-stimulated macrophages. Inhibition of iNOS by aqueous extracts of CR and our determination of the active principles of CR extracts contribute to understanding of the anti-inflammatory actions of black cohosh.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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