Journal of Ethnopharmacology 125 (2009) 51–58

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Anti-inflammatory effects of the extract of indigo naturalis in human neutrophils

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ARTICLE INFO

Article history:
Received 22 January 2009
Received in revised form 30 April 2009
Accepted 17 June 2009
Available online 25 June 2009

Keywords:
Calcium
Elastase
Human neutrophils
Indigo naturalis
p38 mitogen-activated protein kinase
Superoxide anion

ABSTRACT

Ethnopharmacological relevance: Indigo naturalis is used by traditional Chinese medicine to treat various inflammatory diseases.
Aim of the study: Topical indigo naturalis ointment showed efficacy in treating psoriasis in our previous clinical studies. In this study, we investigated the anti-inflammatory effects of the extract of indigo naturalis (QD) and its main components indirubin, indigo, and tryptanthrin in human neutrophils.
Materials and methods: Superoxide anion ($O_2^{\bullet -}$) generation and elastase release were measured by spectrophotometry. Some important signals including mitogen-activated protein kinase (MAPK), cAMP, and calcium were studied by Western blot analysis, an enzyme immunoassay, and spectrofluorometry.
Results: QD significantly inhibited $O_2^{\bullet -}$ generation and elastase release in formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated human neutrophils in a concentration-dependent fashion, while neither indirubin, indigo, nor tryptanthrin produced a comparable result. QD attenuated the FMLP-induced phosphorylation of extracellular regulated kinase, p38 MAPK, and *c-Jun* N-terminal kinase. Furthermore, QD inhibited calcium mobilization caused by FMLP. However, QD did not affect cellular cAMP levels. On the other hand, neither indirubin, indigo, nor tryptanthrin produced similar changes in human neutrophils.
Conclusions: Taken collectively, these findings indicate that QD, but not indirubin, indigo, or tryptanthrin, inhibited $O_2^{\bullet -}$ generation and elastase release in FMLP-induced human neutrophils, which was at least partially mediated by the inhibition of MAPK activation and regulation of calcium mobilization.

1. Introduction

Psoriasis is a common chronic inflammatory disease of the skin that is characterized by thickened, scaly plaques. Psoriasis affects people of all ages, but there is a strong tendency for disease onset in early adulthood in patients who develop psoriasis due to genetic transmission (Lebwohl, 2003; Bowcock and Krueger, 2005). The chronic inflammatory skin disease, psoriasis, is characterized by prominent skin infiltration by neutrophils and microabscess formation (Terui et al., 2000; Wetzel et al., 2006). The significance of neutrophils in the pathogenesis of psoriasis was emphasized by a study from Toichi et al. (2000) describing rapid improvement in a long-lasting case of psoriasis during agranulocytosis. After neutrophil recovery, psoriatic plaques reappeared (Toichi et al., 2000).

Traditional Chinese medicine is one of the most frequently chosen alternative therapies in China and Taiwan, and psoriasis has been treated for centuries with topical and oral herbal preparations (Koo and Arain, 1998; Bedi and Shenefelt, 2002). Indigo naturalis (Qing Dai) is used by traditional Chinese medicine to treat various inflammatory diseases and dermatosis. Indigo naturalis is taken orally to treat psoriasis in Chinese medicine, and its efficacy was proven in several clinical reports (Yuan et al., 1982). However, long-term systemic use is often associated with adverse gastrointestinal effects and liver damage (Verucchi et al., 2002). Recently, in order to avoid the adverse systemic effects, but retain the demonstrated efficacy of indigo naturalis as an anti-psoriasis medicine, an alternative approach by applying the drug topically to skin lesions was reported by our group (Lin et al., 2006, 2007a,b, 2008). We showed that topical indigo naturalis ointment is a novel, safe, and effective therapy for plaque-type psoriasis (Lin et al., 2008). Our data suggested that the effectiveness of this topical therapy might be mediated by downregulating the proliferating potential in keratinocytes, recovery of epidermal differentiation, and reduction of inflammatory reactions (Lin et al., 2007a). However, no evidence was found to describe anti-neutrophilic inflammation by the extract of indigo naturalis (QD) or its major ingredients, indirubin, indigo, and tryptanthrin, after searching the literature. The aims of this study were to investigate the effects of QD on superoxide anion

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doi:10.1016/j.jep.2009.06.014

($O_2^{\bullet-}$) generation and elastase release in human neutrophils and to elucidate the signaling pathways responsible for the QD-caused inhibition of neutrophil responses. Our data suggest that the suppressive effects of QD on human neutrophil respiratory burst and degranulation are at least partially mediated by inhibition of calcium, extracellular regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), and *c-Jun* N-terminal kinase (JNK) signaling pathways.

2. Methods and materials

2.1. Sample extraction and preparation

Indigo naturalis powder was purchased from Guang Sheng Trading (Taipei, Taiwan), and was prepared from the plant of *Strobilanthes formosanus* Moore (Acanthaceae). The plant was grown in the mountains near Sansia, Taiwan, and was ascertained by the authority of Dr. Rong-Chi Yang who is the chief of the Chinese Herbal Pharmacy at Chang Gung Memorial Hospital, Taoyuan, Taiwan. The fingerprints and quantity analysis of standard samples of indirubin, indigo, and tryptanthrin were established by Dr. Yann-Lii Leu. A voucher specimen (SF-1) is deposited in the herbarium of Chang Gung University, Taoyuan, Taiwan. The powder of indigo naturalis was dissolved in dimethyl sulfoxide (DMSO) in a proportion of 1:10 (w/v), then sterilized by filtration (pore size 0.2 μm), and stored at −20 °C for subsequent bioassay testing. Purified indigo was purchased from Fluka (Buchs, Switzerland), indirubin was purchased from Alexis (Lausen, Switzerland), and tryptanthrin was obtained from Sigma (St. Louis, MO, USA). Indirubin, indigo, and tryptanthrin were dissolved in DMSO to make stock solutions.

2.2. Reagents

Aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Calbiochem (La Jolla, CA, USA). Fura-2 AM was purchased from Molecular Probes (Eugene, OR, USA). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were obtained from Sigma. When drugs were dissolved in DMSO, the final concentration of DMSO in cell experiments did not exceed 0.4% and did not affect the parameters measured.

2.3. Preparation of human neutrophils

Blood was taken from healthy human donors (20–30 years old) by venipuncture, using a protocol approved by the institutional review board at Chang Gung Memorial Hospital. Neutrophils were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes. Purified neutrophils that contained >98% viable cells, as determined by the trypan blue exclusion method, were resuspended in a calcium (Ca^{2+})-free Hank's balanced salt solution (HBSS) buffer at pH 7.4, and were maintained at 4 °C before use.

2.4. Measurement of $O_2^{\bullet-}$ generation

The assay of the generation of $O_2^{\bullet-}$ was based on the SOD-inhibitable reduction of ferricytochrome *c* (Babior et al., 1973). In brief, after supplementation with 0.5 mg/ml ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils (6×10^5 cells/ml) were equilibrated at 37 °C for 2 min and incubated with drugs or an equal volume of vehicle (0.1% DMSO) for 5 min. Cells were activated with 100 nM FMLP during the preincubation of 1 μg/ml cytochalasin B (FMLP/CB) for 3 min. Changes in the absorbance with a reduction in ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan). Calculations were based on differences in the reactions with and without SOD (100 U/ml) divided by the extinction coefficient for the reduction of ferricytochrome *c* (ε = 21.1/mM/10 mm).

2.5. Lactate dehydrogenase (LDH) release

LDH release was determined by a commercially available method (Promega, Madison, WI, USA). Cytotoxicity was represented by LDH release in a cell-free medium as a percentage of the total LDH release. The total LDH released was determined by lysing cells with 0.1% Triton X-100 for 30 min at 37 °C.

2.6. $O_2^{\bullet-}$-scavenging activity

The $O_2^{\bullet-}$-scavenging ability of drugs was determined using xanthine/xanthine oxidase in a cell-free system, based on a previously described method (Tan and Berridge, 2000). After 0.1 mM xanthine was added to the assay buffer (50 mM Tris (pH 7.4), 0.3 mM WST-1, and 0.02 U/ml xanthine oxidase) for 15 min at 30 °C, the absorbance associated with the $O_2^{\bullet-}$-induced WST-1 reduction was measured at 450 nm.

2.7. 1,1-Diphenyl-2-picrylhydrazyl (DPPH)-scavenging activity

An ethanol solution of the stable nitrogen-centered free radical, DPPH (100 μM), was incubated with drugs, α-tocopherol, or an equal volume of vehicle (0.1% DMSO) for 16 min at 25 °C, and the absorbance was measured at 517 nM.

2.8. Measurement of elastase release

Degranulation of azurophilic granules was determined by elastase release as described previously (Hwang et al., 2009). Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide as the elastase substrate. Briefly, after supplementation with MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide (100 μM), neutrophils (6×10^5/ml) were equilibrated at 37 °C for 2 min and incubated with drugs or an equal volume of vehicle (0.1% DMSO) for 5 min. Cells were activated by 100 nM FMLP and 0.5 μg/ml cytochalasin B, and changes in absorbance at 405 nm were continuously monitored to assay elastase release. The results were expressed as the percent of elastase release in the FMLP/CB-activated, drug-free control system.

2.9. Immunoblotting analysis of whole-cell lysates

Neutrophils were incubated with drugs or an equal volume of vehicle (0.1% DMSO) for 5 min at 37 °C before being stimulated with FMLP for another 30 s. The reaction was stopped on ice, and cells were centrifuged at 4 °C. After removing the supernatants, the pellets were lysed in 150 μl buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 2 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1% dilution of Sigma protease inhibitor cocktails, and 1% Triton X-100). Samples were centrifuged at 14,000 × *g* for 30 min at 4 °C to yield whole-cell lysates. Proteins derived from whole-cell lysates were separated by SDS-PAGE using 12% polyacrylamide gels and blotted onto nitrocellulose membranes. Immunoblotting was performed using the indicated antibodies and horseradish peroxidase-conjugated secondary anti-rabbit antibodies (Cell Signaling Technology, Beverly, MA, USA). The immunoreactive bands were visualized by an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK).