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Screening anti-inflammatory components from Chinese traditional medicines using a peritoneal macrophage/cell membrane chromatography-offline-GC/MS method

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ABSTRACT

We report the development of an analytical method combining cell membrane chromatography (CMC) with gas chromatography/mass spectrometry (GC/MS). This was applied to the purification and identification of anti-inflammatory components from traditional Chinese medicines. The stationary phase of the CMC employed mouse peritoneal macrophage (PM) cell membranes. We investigated the performance of the PM/CMC-offline-GC/MS method using hydrocortisone (HC) and dexamethasone (DM) as standards. The method was then applied to the identification of anti-inflammatory components in extracts of Rhizoma *Atractylodes macrocephala* (RAM) and Rhizoma *Atractylodes lancea* Thunb DC (RALD). The major components from both species retained by CMC were identified as atractylenolide I (AO-I) by GC/MS. Competition experiments' results showed that AO-I and lipopolysaccharide (LPS) bound competitively to cell surface receptors while AO-I and HC had only partly overlapping binding sites on the PM membrane. In vitro experiments revealed that AO-I was able to inhibit LPS-induction of TNF-α, IL-1β and NO production in a dose-dependent manner. IC50 values were 5.3 μg/mL, 5.1 μg/mL and 7.5 μg/mL, respectively. The PM/CMC-offline-GC/MS method is an effective screening system for the rapid detection, enrichment, and identification of target components from complex samples.

1. Introduction

Peritoneal macrophages (PMs) constitute an important class of immune cells. Receptors expressed at the cell surface include the toll-like receptors (TLRs) [1], membrane-bound glucocorticoid receptors (mGCRs) [2,3], leukotriene receptors (LTRs) [4], platelet activating factor (PAF) receptor [5,6], and vascular endothelial growth factor (VEGF) receptor-1 (Flt-1) [7]. The TLRs are perhaps the most important membrane receptors in relation to inflammatory processes in PMs. Binding of gram-negative bacterial endotoxin lipopolysaccharide (LPS) to plasma membrane TLRs leads to an inflammatory response by activation of the nuclear factor κB (NF-κB) pathway and the release of pro-inflammatory cytokines including tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β). Moreover, glucocorticoids such as hydrocortisone act both at plasma membrane mGCRs and at intracellular glucocorticoid receptors, and can reduce the inflammatory response by blocking the NF-κB pathway. These receptors provide important targets for drug development.

Cell membrane chromatography (CMC) offers a powerful approach to the study of ligand–receptor interactions [8,9]. Whereas radioactive ligand assay (RLA) is the standard method for studying these interactions [10,11], a significant correlation between results obtained with CMC and RLA has been reported [11–13]. CMC has previously been applied to the screening of medicinal plants for active components targeting membrane receptors [14–16]. Gas chromatography/mass spectrometry (GC/MS) is most commonly used for separation and identification of unknown components and is particularly applicable to volatile components often encountered in Chinese medicine [17,18]. We have therefore sought to develop a combined PM/CMC-offline-GC/MS method for the efficient detection and identification of active components in complex samples. We report here the development of a combined CMC–GC/MS method based on peritoneal macrophages (Fig. 1). The method was used to analyze two medicinal plants, Rhizoma *Atractylodes macrocephala* (RAM) and Rhizomza *Atractylodes lancea* (RAL), for anti-inflammatory compounds. Hydrocortisone and dexamethasone were used as positive controls. We report the preliminary characterization of pathways mediating the

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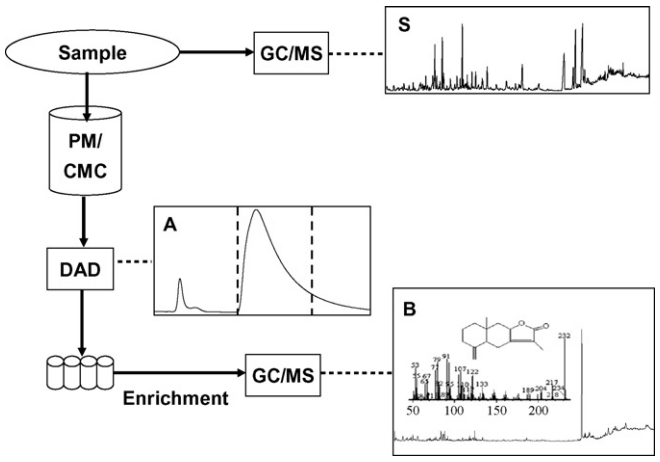

Fig. 1. Schematic outline of the PM/CMC-offline-GC/MS method. (A) CMC chromatography using PM/CMC; GC/MS, the GC/MS system. (B) Total ion current chromatograms and the mass spectra of the retention components. (S) Total ion current chromatograms of the samples analyzed. *Abbreviations*: PM/CMC, peritoneal macrophage (PM) cell membrane chromatography (CMC) column; DAD, diode array detector; GC/MS, gas chromatography with mass spectrometry.

anti-inflammatory effects of active components identified by this method.

2. Materials and methods

2.1. Materials

Silica gel (ZEX-II, 100–200 mesh) was obtained from Qingdao Meigao Chemical Company (Qingdao, PR China). RPMI-1640 medium was purchased from Gibco (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), ethylenediamine tetra-acetic acid (EDTA), lipopolysaccharide, trypan blue dye and trypsin were purchased from Sigma (Saint Louis, MO, USA). The enzyme immunosorbent assay (ELISA) kit for mouse TNF-α and IL-1β were purchased from R&D Systems (Minneapolis, MN, USA). HPLC grade methanol and ethyl acetate were purchased from Fisher Scientific (Pittsburgh, PA, USA). Hydrocortisone (HC), dexamethasone (DM), and atractylenolide I (AO-I) were supplied by the National Institute for the Pharmaceutical and Biological Products of China. Rhizoma *A. macrocephala* and Rhizomza *A. lancea* Thunb DC (RALD) were purchased from the TCM Store (Xi'an, PR China).

2.2. Standard solutions

Standard stock solutions (1 mg/mL each) of HC, DM, AO-I were prepared in ethyl acetate. Mixed standard solution I contained 1 mg/mL of both HC and DM. Mixed standard solution II contained 1 mg/mL of both HC and AO-I.

2.3. Sample preparation

Essential oils of RAM and RALD were extracted using supercritical CO_2. Dried RAM and RALD roots were separately powdered (~60 mesh), 2 kg of powder was placed into a 5 L supercritical extraction vessel and subjected to slow heating. When the temperature of extraction vessel reached 50 °C a compressor pump was employed to maintain pressure and temperature at 20.0 MPa and 50 °C, respectively, in the extraction vessel, and at 10.0 MPa and 30 °C in the separation vessel. Cyclic extraction was performed for 3 h with a CO_2 flow rate of 40 kg/h and generated yellow oil extracts. Extraction yields were 2.5% and 2.1% for RAM and RALD, respectively.

2.4. Preparation of peritoneal macrophage (PM) cell membrane chromatography (CMC) columns

BALB/c mice (25–30 g) were from the Animal Center at Xi'an Jiaotong University (Xi'an, China). Mice were injected (ip) with 2 mL of 3% thioglycollate 4 d before sacrifice. PMs were collected by lavaging the peritoneal cavity with 5 mL of RPMI-1640. Cells were collected by centrifugation, washed, and suspended in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator with 5% CO_2 at 37 °C. Cells were purified by adherence to tissue culture plates for 2 h. The viability of the macrophages was assessed by trypan blue dye exclusion. We routinely measured viabilities of greater than 90% in all preparations. PM cell membranes were prepared as previously described [19]. Cells (7×10^6) were washed 3 times with normal saline solution centrifuging each time ($650 \times g$, 5 min, 4 °C) and resuspended into suspension buffer (50 mM Tris–HCl pH 7.4). The resulting homogenate was centrifuged ($200 \times g$, 5 min), the pellet discarded, and the supernatant was centrifuged at $15{,}000 \times g$ for 20 min at 4 °C. The supernatant was discarded, the membrane pellet was washed (suspension buffer), recentrifuged as before, and the membrane pellet suspended into 5 mM phosphate-buffered solution (PBS pH 7.4). The PM cell membrane stationary phase (PM-CMSP) was prepared as described [9]. Briefly, the membrane suspension was added to 0.05 g activated (105 °C, 30 min) silica carrier under vacuum at 4 °C with gentle agitation. The homogenate obtained was packed into a column by a wet method to generate the PM/CMC column (10 mm × 3.1 mm, 5 μm).

2.5. PM/CMC assay

A HPLC system and a 32 Karat workstation (Beckman Coulter, Fullerton, CA, USA) were used in conjunction with the PM/CMC column. The mobile phase was 5 mM PBS (pH 7.4) with a flow rate of 0.2 mL/min and a column temperature of 37 °C. The detection wavelength ranged from 220 nm to 240 nm. The chromatographic system was stabilized (~1.5 h) before sample injection. 1 μL of standard solutions or RAM or RALD samples were injected. During "recognition analysis", fractions were collected into 96-well plates every 0.3 min using a Model SC-100 fraction collector (Beckman Coulter). Retention fractions from the chromatogram were combined and evaporated with a SpeedVac concentrator (5301, Eppendorf, Germany). After extraction with 100 μL ethyl acetate by vigorous agitation for 5 min, samples were analyzed by GC/MS.

2.6. Gas chromatography with mass spectrometry (GC/MS)

Standard solutions, standard mixed solutions I and II, and all samples retained by PM/CMC were analyzed by GC/MS. A capillary gas chromatography coupled mass spectrometer (GCMS-QP2010 Shimadzu, Kyoto, Japan) with a Rtx-5MS capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness, Restek, CA, USA) was used. Helium (purity 99.999%) was the carrier at a constant column flow of 2.0 mL/min. Initial temperature was 140 °C ramped at 10 °C/min to 280 °C and held for 8 min. Inlet temperature was maintained at 280 °C. For RAM, RALD essential oils and corresponding PM/CMC samples, initial temperature was 120 °C ramped at 5 °C/min to 180 °C and held for 12 min; then ramped at 20 °C/min to 300 °C and held for 5 min. Inlet temperature was maintained at 280 °C. The mass spectrometer was operated in total ion current (TIC) scanning mode, and we got TIC chromatogram. The mass range scanned was from 40 *m*/*z* to 700 *m*/*z*. Electron impact energy was set