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UHPLC/Q-TOFMS-based metabolomics for the characterization of cold and hot properties of Chinese materia medica

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ABSTRACT

Ethnopharmacological relevance: The cold/hot property of Chinese materia medica (CMM) and the application of its corresponding knowledge in the diagnosis, differentiation and treatment of diseases have been considered to be the extremely important part of traditional Chinese medicine (TCM). As highly abstracted TCM theory, the cold/hot property of CMMs is still not fully understood and remains to be elucidated by systems biology approach. The cold and hot properties of CMM are mainly defined by the response of the body to a given CMM. Metabolomics is a promising systems biology method to profile entire endogenous metabolites and monitor their fluctuations related to an exogenous stimulus. Thus, a metabolomics approach was applied to characterize the cold and hot properties of CMMs.
Material and methods: Mice were intragastrically administered three selected cold property CMMs (i.e., *Rheum palmatum* L., radix et rhizoma; *Coptis chinensis* Franch, rhizome and *Scutellaria baicalensis* Georgi, radix) and three hot property CMMs (i.e., *Cinnamomum cassia* (L.) J. Presl, cortex; *Zingiber officinale* Roscoe, rhizoma and *Evodia rutaecarpa* (Juss.) Benth., fructus) once daily for one week. The comprehensive metabolome changes in the plasma of mice after treatment with cold or hot property CMMs were characterized by ultra-high performance liquid chromatography/time of flight mass spectrometry (UHPLC/Q-TOF-MS), and the potential biomarkers related to cold and hot properties of CMM were explored.
Results: Metabolites perturbation in plasma occurs after treatment with cold CMMs and hot CMMs in mice, and 15 and 16 differential biomarkers were identified to be associated with the cold and hot properties of CMMs, respectively. Among them, LPC (18:0), LPC (18:1), LPC (20:4) and LPC (20:5) showed decreased trends in the cold property CMM treated groups, but increased in the hot property CMM treated groups.
Conclusions: There is a strong connection between the cold/hot property of CMMs and lysophosphatidylcholines metabolism. This study offers new insight into CMM properties and their clinical application.

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1. Introduction

Ancient TCM has been widely practiced to treat diseases and disorders for thousands of years in China, and play an important role in the current health care system in many countries (Lao et al., 2012; Nestler, 2002). There are many types of treatment associated with TCM, including CMM, acupuncture, moxibustion, cupping and massage. As the most commonly used remedy, CMM can be classified in accordance to their own properties and rules. Each CMM has its own specific characteristics, such as the properties, flavors and channel tropisms, which are important factors for prescribing herbal formulae. The four major properties refer to the cold, hot, warm and cool nature of the CMM and can be summed up as cold and hot (Liang et al., 2013; Long et al., 2011a).The classification of cold and hot properties of CMMs is derived from the responses of the body to the CMM and also the effects of herbs observed by ancient TCM physician. According to the TCM theory, the term of ZHENG (syndrome), as a basic and fundamental concept, has been used to diagnose and treatdiseases for thousands of years in China (Su et al., 2012). ZHENG is also classified into cold

Abbreviations: C, cold; CMM, Chinese material medica; ESI, electrospray ionization; H, hot; LCAT, lecithin-cholesterol acyltransferase; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethano-lamine; PGI_2, prostacyclin; PLA_2, phospholipase; PLS-DA, partial least squares discriminant analysis; QC, quality control; ROS, reactive oxygen species; RSD, relative standard deviation; TCM, traditional Chinese medicine; UHPLC/Q-TOFMS, ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry; VIP, variable importance in the projection

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and hot statuses. Cold ZHENG-related symptoms include coldness, cold pain, tastelessness, and clear abundant urine, whereas hot ZHENG-related symptoms include heat, diaphoresis, flushed face, burning pain,and deep-colored urine (Li et al., 2007). A CMM with cold property is used to treat "hot" ZHENG, in contrast, hot CMMs are used to treat "cold" ZHENG (Li et al., 2007; Su et al., 2012).For example, according to TCM theory and the description stated in the Chinese Pharmacopoeia (2010 edition), Radix et rhizoma rhei (Da Huang in Chinese, *Rheum palmatum* L., radix et rhizoma), Rhizoma coptidis (Huang Lian, *Coptis chinensis* Franch, rhizoma) and Radix scutellaria (Huang Qin, *Scutellaria baicalensis* Georgi, radix) are three representative CMMs with cool property and are commonly used to treat "hot" ZHENG, whereas, Cortex cinnamomi (Rou Gui, *Cinnamomum cassia* (L.) J. Presl, cortex), *Rhizoma zingiberis* (Gan Jiang, *Zingiber officinale* Roscoe, rhizoma), and Fructus evodiae (Wu Zhu Yu, *Evodia rutaecarpa* (Juss.) Benth., fructus) belong to the CMM with hot property and are traditionally used in the treatment of "cold" ZHENG (Editorial Committee of Chinese Pharmacopoeia, 2010).Therefore, it is important to characterize the cold or hot property of CMM using a modern scientific means to ensure their safety and effectiveness in clinical treatment.

Due to the importance of the hot and cold nature of CMMs in both theory and clinical practice, much effort has been made to investigate the biomedical fundaments, including chemical components analysis (Long et al., 2011b), animal thermotropism behavior and biothermokinetic monitoring (Jia et al., 2010; Wang et al., 2009; Zhao et al., 2011), and bioinformatics (Liang et al., 2013). Additionally, several pharmacological approaches have been used to investigate the endocrinological, immunological and neurological alterations related to the cold and hot properties of CMMs (Deng et al., 2009; Liu et al., 2008). However, as highly abstracted TCM theory, CMM properties are not fully understood and remain to be elucidated. Metabolomics is an emerging platform to profile entire endogenous metabolites in biological systems and to monitor their fluctuations related to genetic, biological or environmental perturbation (Zhang et al., 2013). The integral view of metabolomics is consistent with the holistic thinking of TCM and the comprehensive actions of CMM. Numerous metabolomics studies have been performed to provide an in-depth understanding of TCM theory, including the interpretation of TCM syndrome, the mechanism of the therapeutic effect of CMM and acupuncture, and the analysis of TCM formulas (Hu and Xu, 2014; Zhang et al., 2013). Moreover, the cold and hot properties are believed to be defined according to the interactions of the body to a specific CMM, therefore, the metabolomics approach is one of the most plausible tools to uncover CMM properties. We hypothesize that a similar response occurs in the body when treated with CMMs with same property and there are different responses in animals after treatment with cold and hot property CMMs. Therefore, the aim of the present study is to characterize the comprehensive metabolome changes in mice after treatment with cold or hot typical CMMs using UHPLC/Q-TOFMS, and to explore the potential biomarkers for the evaluation of cold and hot properties of CMMs. This study offers new insight into CMM properties and their clinical application.

2. Materials and methods

2.1. Chemicals and herbs

HPLC-grade methanol and formic acid were purchased from Merck KGaA (Darmstadt, Germany); acetonitrile, HPLC-grade, was obtained from Fisher Scientific Corporation (Loughborough, UK); ultra-high purity water (18.2 MΩ) was prepared by a Millipore SAS-67120 (Molsheim, Cedex, France).Three typical CMMs with cold property, i.e., Radix et rhizoma rhei (Da Huang in Chinese, *R. palmatum* L., radix et rhizoma), Rhizoma coptidis (Huang Lian, *C. chinensis* Franch, rhizoma), Radix scutellaria (Huang Qin, *S. baicalensis* Georgi, radix), and three typical CMMs with hot property, i.e., Cortex cinnamomi (Rou Gui, *C. cassia* (L.) J. Presl, cortex), Rhizoma zingiberis (Gan Jiang, *Z. officinale* Roscoe, rhizoma), Fructus evodiae (Wu Zhu Yu, *E. rutaecarpa* (Juss.) Benth., fructus), were selected and purchased from Beijing Tong-Ren-Tang (Macao) Chinese Medicine Co, Ltd (Macao, China).The botanical origin of all materials was authenticated by Dr. Chunfeng Qiao, a pharmacognosist from our institute. Voucher specimens were deposited at the Institute of Chinese Medical Sciences, University of Macau. The pulverized herbs (100 g) were accurately weighed and then decocted with 500 mL boiling water twice for 30 min each. After filtering by gauze, the percolate was combined, and concentrated in a rotary vacuum evaporator at 60 °C followed by lyophilization. The freeze-dried extract was dispersed in distilled water for animal treatment.

2.2. Animals and treatments

C57BL/6 mice, weighting 25–30 g, were housed in an individually ventilated cage (IVC) system at the Institute of Chinese Medical Sciences, University of Macau, and were maintained on a 12 h dark/light cycle in temperature-controlled rooms (22.5 ± 0.5 °C, 50 ± 5% humidity) with access to lab chow and water *ad libitum*. All mice were randomly divided into seven groups (half male and female, n = 10), including control, 3 cold CMM-treated groups and 3 hot CMM-treated groups. After fasting overnight, the treatment groups were intragastrically administered CMM extract once daily for one week, the mice in the control group received an equal volume of distilled water. The doses of CMMs were 80 times as the calculated dosage according to their usage description in China Pharmacopoeia 2005 (Zhao et al., 2011) and are shown in Table 1. At day 8, all mice were euthanized by isoflurane inhalation (Abbott laboratories, IL, USA), and their blood samples were immediately collected in heparinized tubes. Plasma was separated by centrifugation at 3000*g* at 4 °C for 10 min and was frozen immediately at -80°C until metabolomics analysis. All animal protocols were approved by the Animal Ethics Committee, Institute of Chinese Medical Sciences, University of Macau, in agreement with institutional animal care guidelines.

2.3. Sample preparation

Plasma samples were thawed at 4 °C and were mixed before use. A 100 μL plasma sample was deproteinized with 300 μL of methanol. After 30 s of vortexing, the samples were centrifuged at 15,800*g* for 15 min at 4 °C, and the supernatant was transferred to a new centrifuge tube and was lyophilized with a freeze dryer (ilShin Biobase Co., Ltd., Dongducheon, Korea). The residue was reconstituted in 100 μL of water and centrifuged at 15,800*g* for 15 min at 4 °C. An aliquot of 5 μL supernatant was subjected to UHPLC/Q-TOFMS analysis. A pooled "quality control" (QC) sample was prepared by mixing an equal aliquot (10 μL) from all plasma samples for the optimization of the chromatographic and TOF/MS conditions, and method validation.

2.4. LC–MS analysis

Plasma metabolic profiles were acquired using a Waters ACQUITY™ ultra performance liquid chromatography system coupled with a Waters SYNAPT G2-Si Q-TOF mass spectrometer (Waters, Manchester, UK). The chromatographic separation of the plasma sample was achieved using an ACQUITY BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm) at 50 °C. The mobile phase

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