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Anti-atherosclerotic action of Ger-Gen-Chyn-Lian-Tang and AMPK-dependent lipid lowering effect in hepatocytes

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ABSTRACT

Ethnopharmacological relevance: The Ger-Gen-Chyn-Lian-Tang (GGCLT), an officially standardized mixture of Chinese herbal medicines, consists of Puerariae Radix, Scutellariae Radix, Coptidis Rhizoma and Glycyrrhizae Radix in a ratio of 8:3:3:2. In this study, we evaluated the benefits of GGCLT in atherosclerotic progression.

Methods: The major constituents of GGCLT were analyzed by HPLC. ApoE$^{-/-}$ mice taken 0.15% cholesterol diet were orally given vehicle or GGCLT (2 g/kg/day) for 12 weeks. Serum levels of lipid and glucose were analyzed, and atherosclerosis was examined by histological analyses. Cultures of vascular smooth muscle cells, hepatocytes and bone marrow-derived macrophages were used to investigate the action mechanisms of GGCLT.

Results: Our quantitation results indicated that GGCLT contains puerarin, daidzin, daidzein, baicalin, baicalein, wogonin, palmatine, coptisine, berberine and glycyrrhizin. GGCLT decreased serum levels of total cholesterol and LDL, but not TG and HDL in ApoE$^{-/-}$ mice. In parallel, GGCLT treatment reduced atherosclerotic lesions and collagen expression in atheroma plaques. In vascular smooth muscle cells, GGCLT could reduce cell migration, but failed to affect cell viability and proliferation. In hepatocytes, GGCLT can reduce lipid accumulation, and this action was accompanied by the activation of AMPK, upregulation of PPARs, and downregulation of FAS. Pharmacological approach indicated that the latter two events contributing to the anti-lipogenesis is resulting from AMPK pathway, and the lipid lowering effect of GGCLT in hepatocytes is mediated by AMPK and PPARα pathways. Meanwhile, two of the major components of GGCLT, berberine and puerarin, also activated AMPK and decreased lipid accumulation in hepatocytes with berberine of higher efficacy. Besides in hepatocytes, AMPK signaling was also activated by GGCLT in vascular smooth muscle cells and macrophages.

Conclusions: These results demonstrate the anti-atherosclerotic action of Chinese medicine mixture GGCLT in ApoE$^{-/-}$ atherosclerotic mouse model. Mechanistic study suggests that activation of AMPK and PPARα in hepatocytes leading to a decrease of lipid formation contributes to the beneficial action of GGCLT in atherosclerosis treatment.

1. Introduction

Ger-Gen-Chyn-Lian-Tang (GGCLT) is a traditional extract mixture of four Chinese medicine herbs, consisting of Puerariae Radix (roots of *Pueraria lobata*, PR), Scutellariae Radix (roots of *Scullellaria baicalensis*, SR), Coptidis Rhizoma (rhizomes of *Coptis chinensis*, CR) and honey-processed Glycyrrhizae Radix (roots of *Glycyrrhiza uralensis*, GR). Based on long term traditional use, the combination ratio of these herbs in GGCLT of 8:3:3:2 has been officially standardized in Taiwan

Abbreviations: AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; BMDM, bone marrow-derived macrophages; FAS, fatty acid synthase; FG, fasting glucose; GGCLT, Ger-Gen-Chyn-Lian-Tang; HCD, high cholesterol diet; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PI, propidium iodide; PPAR, peroxisome proliferator-activated receptor; TC, total cholesterol; TG, triglycerides; VSMCs, vascular smooth muscle cells

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by Chinese Medicine and Pharmacy, Department of Health, Executive Yuan Taiwan, and the suggested dose for human is 0.4–2.0 g/kg/day. In the traditional medicine, GGCLT is used to control inflammatory response (Tang et al., 2004), and this benefit is primarily ascribed to the actions of some major constituents, flavonoids in major, of GGCLT. Puerarin, daidzein and daidzein, isoflavonoids derived from *Pueraria lobata* (Rong et al., 1998; Cherdshewasart and Sutjit, 2008), wogonin, oroxylin A, baicalin and baicalein, active components of *Scutellaria baicalensis* (Hong et al., 2002; Chi et al., 2003; Huang et al., 2006; Woo et al., 2006), berberine, coptisine, and palmatine, the major alkaloids of the *Coptis chinensis* (Ye et al., 2009; Lee et al., 2010, 2012), and glycyrrhizin from *Glycyrrhiza uralensis* (Kasai et al., 2008; Wang et al., 2011a), all exhibit antiinflammatory activity and were shown to ameliorate tissue damage following inflammation. This includes prevention of myocardial infarction, inhibition of ischemic brain injury, endotoxic shock and osteoporosis (Park et al., 2005; Lee et al., 2010, 2012; Wang et al., 2011a). Moreover, anticancer properties of wogonin, baicalein, baicalin, and berberine were also demonstrated (Li-Weber, 2009; Tang et al., 2009).

Atherosclerosis is a chronic inflammatory disease involving endothelial dysfunction, increased total cholesterol, foam cells formation, proliferation and migration of vascular smooth muscle cells (VSMCs). Recently the atherosclero-protective potential of GGCLT is suggested based on some studies of its components. Puerarin was reported to attenuate the increased total cholesterol induced by hypercholesterolemic diet in rats, and this action may be due to the promotion of cholesterol and bile acids excretion in liver (Yan et al., 2006). In animal models, puerarin also can attenuate high glucose- and diabetes-induced VSMCs proliferation (Zhu et al., 2010). Moreover, aglycon form of puerarin could cause lipogenesis via increasing fatty acid synthase (FAS) and peroxisome proliferator-activated receptor γ (PPARγ) in a preadipocyte cell line (Hirota et al., 2010). In addition, wogonin was suggested to modulate atherosclerotic change in vitro (Sieveking et al., 2005), and baicalein was shown to inhibit VSMC proliferation, and suppress intimal hyperplasia after balloon vascular injury in the rat (Peng et al., 2008). Like baicalein, berberine is capable of inhibiting growth of VSMCs after in vitro mechanical injury (Liang et al., 2006). In addition, berberine and Coptidis Rhizoma improve lipid dysregulation, atherosclerosis and obesity in high-fat diet-fed mice (Kim et al., 2009a; Wang et al., 2011b; Xie et al., 2011).

Natural Chinese medicine which has been widely and successfully used for a long history in Chinese society is usually of mixed type ingredients. Comparing to many studies focusing on discovery of therapeutic pure compounds nowadays, application of mixed type Chinese medicine in treating atherosclerosis is still limited and needs more extensive investigation. Given that several pure components of GGCLT have been shown their potential in cardiovascular diseases including atherosclerosis, in this study we like to address whether mix product GGCLT could achieve beneficial efficacy in atherosclerosis, and what the major cellular action contributes to this potential therapy. One advantage for this study is because GGCLT mix product is an officially approved standardized remedy available in Taiwan and widely used as a health medicine in Chinese society. Thus identifying new therapeutic actions of GGCLT would accelerate its beneficial use in human. To this end, anti-atherosclerotic property of GGCLT was evaluated in ApoE-deficient mice, and cellular actions were assessed in VSMCs, hepatocytes and macrophages.

2. Methods

2.1. Chemicals and drugs

Antibodies against AMPK-p, AMPK, ACC-p and ACC were purchased from Cell Signaling (Beverly, MA, USA). Antibodies against PPARα, PPARγ and fatty acid synthase (FAS) were purchased from GeneTex Inc. (Irvine, CA, USA). PPARδ antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against β-actin was from Upstate Biotechnology (Charlottesville, VA, USA). DMEM, RPMI-1640, trypsin-EDTA and 1% penicillin/streptomycin were from Invitrogen Corporation (Rockville, MD, USA). ITS^{TM} Premix (insulin, transferrin, Na_2SeO_3) was from BD Biosciences (San Jose, CA, USA). BAPTA/AM, N-acetylcysteine (NAC) and dexamethasone were from Sigma Aldrich (St. Louis, MO, USA). Ro318220 and compound C were from Calbiochem (La Jolla, CA, USA). The enhanced chemiluminescence reagent was from Perkin Elmer (Wellesley, MA, USA). Berberine, puerarin, bisphenol A diglycidyl ether (BADGE) and ciglitazone were from Cayman Chemical (Ann Arbor, MI, USA) and Enzo Biochem (Butler Pike, PA, USA), respectively. GW6471 was from Tocris (Louis, MO, USA).

2.2. Preparation of GGCLT extract and analysis of major components

GGCLT concentrated powder was prepared by Sun Ten Pharmaceutical Co., Taipei, Taiwan, through extracting the crude drugs with hot water. The filtered decoction was concentrated by spray drying and added starch as an excipient. One gram of GGCLT powder was equivalent to a total amount of 1.71 g of crude drugs of PR, SR, CR and GR at a ratio of 8:3:3:2. To determine constituents, GGCLT (200 μl, 1 g/ml) was diluted with 200 μl of water, and mixed with MeOH (v/v, 3:7). After centrifuged, the supernatant (160 μl) was added with 40 μl of amyl paraben solution (100 μg/ml in methanol as internal standard) and 20 μl was subjected to HPLC analysis, which included a pump (LC-10ATVP, Shimadzu, Japan), a diode array detector (SPD-M10AVP, Shimadzu, Japan) and an automatic injector (SiL-10AF, Shimadzu, Japan). An Apollo C18 column (4.6 × 250 mm, 5 μm) was equipped with a guard column (4.6 × 50 mm, 5 μm) (Alltech Associates Inc., U.S.A.). The mobile phase consisted of acetonitrile (A) and 0.1% phosphoric acid (B). A gradient elution was programmed as follows: A/B: 11/89 (0–10 min), 18/82 (12–25 min), 30/70 (45 min), 43/57 (54 min), 46/54 (59 min), 65/35 (65 min) and 11/89 (70–75 min). The detection wavelength was set at 250 nm and the flow rate was 1.0 ml/min.

2.3. Experimental animals and pharmacological treatments

C57BL/6 mice and ApoE knockout (ApoE$^{-/-}$) mice of C57BL/6 background were purchased from the Animal Center of National Taiwan University, College of Medicine, housed under controlled conditioning (25 ± 1 °C constant temperature, 55% relative humidity, 12 h lighting cycle), and were allowed free access to diet and water ad libitum during the study period. To accelerate the progress of atherosclerosis, 10-week old C57BL/6 mice and ApoE$^{-/-}$ mice were randomly allocated into two groups with receiving normal chow diet or a high 0.15% cholesterol diet (HCD) (Purina Mills, Inc., USA) for 12 weeks during the experiment. At the same time, the HCD-fed ApoE$^{-/-}$ mice were divided into two groups, which were fed orally with either water or GGCLT (2 g/kg/day) for 12 weeks. This orally administered dosage of GGCLT in mouse was designed based on the officially standardized dose of GGCLT (0.4–2 g/kg/day) in human, and previous studies using berberine in obesity mice (200 mg/kg) (Xie et al., 2011) and in hypercholesterolemic mice (1 mM in drinking water) (Wang et al., 2011b). Since berberine content is around 0.5% (w/w) of GGCLT, we herein took 2 g/kg GGCLT in our animal study. In summary, five groups of 10-week old mice were divided in this study: group 1: C57BL/6 mice fed with normal diet, group 2: C57BL/6 mice fed with HCD; group 3: ApoE$^{-/-}$ mice fed with normal diet; group 4: ApoE$^{-/-}$ mice fed with HCD; group 5: ApoE$^{-/-}$ mice fed with HCD and GGCLT. Biochemical data of ten mice for each group were collected and analyzed. All animal