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Identifying panaxynol, a natural activator of nuclear factor erythroid-2 related factor 2 (Nrf2) from American ginseng as a suppressor of inflamed macrophage-induced cardiomyocyte hypertrophy

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ABSTRACT

Ethnopharmacological relevance: American ginseng is capable of ameliorating cardiac dysfunction and activating Nrf2, a master regulator of antioxidant defense, in the heart. This study was designed to isolate compounds from American ginseng and to determine those responsible for the Nrf2-mediated resolution of inflamed macrophage-induced cardiomyocyte hypertrophy.

Materials and methods: A standardized crude extract of American ginseng was supplied by the National Research Council of Canada, Institute for National Measurement Standards. A bioassay-based fractionization of American ginseng was performed to identify the putative substances which could activate Nrf2-mediated suppression of pro-inflammatory cytokine expression in macrophages and macrophage-mediated pro-hypertrophic growth in cardiomyocytes.

Results: A hexane fraction of an anti-inflammatory crude extract of American ginseng was found to be most effective in suppressing the inflammatory responses in macrophages. Preparative, reverse-phase HPLC and a comparative analysis by analytical scale LC–UV/MS revealed the hexane fraction contains predominantly C_{17} polyacetylenes and linolenic acid. Panaxynol, one of the major polyacetylenes, was found to be a potent Nrf2 activator. Panaxynol posttranscriptionally activated Nrf2 by inhibiting Kelch-like ECH-associated protein (Keap) 1-mediated degradation without affecting the binding of Keap1 and Nrf2. Moreover, panaxynol suppressed a selected set of cytokine expression via the activation of Nrf2 while minimally regulating nuclear factor-kappa B (NF-κB)-mediated cytokine expression in macrophages. It also dramatically inhibited the inflamed macrophage-mediated cardiomyocyte death and hypertrophy by activating Nrf2 in macrophages.

Conclusions: These results demonstrate that American ginseng-derived panaxynol is a specific Nrf2 activator and panaxynol-activated Nrf2 signaling is at least partly responsible for American ginseng-induced health benefit in the heart.

Abbreviations: Nrf2, nuclear factor erythroid-2 related factor 2; Am. G, American ginseng; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MIP-1β, macrophage inflammatory protein-1 beta; IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNFα, tumor necrosis factor alpha; ARE, antioxidant response element; Atg7, autophagy related gene 7; CSF, colony-stimulating factor; FACS, fluorescence activated cell sorting; LDH, lactate dehydrogenase; NQO-1, NAD(P)H dehydrogenase, quinone 1; WT, wild type; LC–UV DAD, Liquid chromatography with UV diode array detection; LC–MS, Liquid chromatography mass spectrometry

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1. Introduction

Ginseng, the root of genus *Panax* of the family Araliaceae, has been used in Asian countries as a folk medicine for thousands of years (Gillis, 1997). Emerging evidence has suggested that regular use of ginseng is helpful in the treatment of human illnesses including cardiovascular disease (Wang et al., 2007; Zhou et al., 2004). However, the underlying cellular and molecular mechanisms remain largely unknown.

Recently, we have found that American ginseng is capable of suppressing lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) expression independent of NF-κB in macrophages (Ichikawa et al., 2009b) and oxidative stress-mediated cell death in H9C2 cardiomyocytes via its ability to activate Nrf2 (Li et al., 2010). On the other hand, we have demonstrated that Nrf2 activation suppresses a selected set of pro-inflammatory cytokines including iNOS, monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein-1 beta (MIP-1β) while minimally regulating NF-κB activity and its downstream cytokine expression, such as interleulin-6 (IL-6), IL-1β, and tumor necrosis factor alpha (TNFα) in macrophages (Li et al., 2014). These results suggest that American ginseng contains substances which may activate Nrf2-mediated resolution of inflammatory responses in macrophages.

Nrf2 is a key transcription factor that binds to cis-acting enhancer sequence known as the antioxidant response element (ARE) with a core nucleotide sequence of 5′-RTGACnnnGC-3′ to control the basal and inducible expression of more than 200 genes. These genes are functionally grouped into several categories including antioxidants, phase II detoxifying enzymes, transcriptional factors, transporters, scavenger receptors, and chaperone proteins (Kensler et al., 2007; Kobayashi and Yamamoto, 2005; Li et al., 2009a; Suzuki et al., 2013). As a result, Nrf2 appears to be a major transcription factor of the cellular defense system against a variety of environmental or intrinsic insults in different organs including lung, liver, gastrointestinal tract, bladder, kidney, brain, skin, and ovary, and heart (Li et al., 2009a; Li et al., 2009b; Wang et al., 2014). Of note, we have demonstrated that knockout of Nrf2 results in the earlier onset of cardiac maladaptive remodeling and dysfunction while cardiac specific overexpression of Nrf2 is cardioprotective (Li et al., 2009b; Wang et al., 2014). These findings indicate that Nrf2 is a potential drug target for the prevention and/or treatment of cardiovascular diseases such as heart failure. Although there is a large number of Nrf2 activating small molecules that are naturally occurring or chemically synthesized (Kumar et al., 2014; Liby et al., 2007), a therapeutic Nrf2 activator for cardiovascular disease remains to be established.

Given the historically verified safety of American ginseng and the emerging evidence of American ginseng-induced Nrf2 activation for cardioprotection, identifying the natural Nrf2 activating molecules from American ginseng may provide valuable insight into the development of novel Nrf2 activators to treat cardiac disease. Therefore, in the present study, we performed a bioassay based fractionation of American ginseng, aiming at the isolation of Nrf2 activating single compounds which are capable of specifically driving Nrf2-mediated health benefit in the heart.

2. Materials and methods

2.1. Animals

Breeding pairs of heterozygous Nrf2 knockout ($Nrf2^{+/-}$/C57BL/6J) mice were purchased from Riken BioResource Center, Japan, and housed under standard conditions in the Institution's AAALAC approved animal facility. Littermates of wild type (WT; $Nrf2^{+/+}$) and homozygous Nrf2 knockout ($Nrf2^{-/-}$) mice were generated using the $Nrf2^{+/-}$ breeding pairs as previously described (Itoh et al., 1997). Genotypes ($Nrf2^{+/+}$, $Nrf2^{-/-}$, and $Nrf2^{+/-}$) of the animals were determined by polymerase chain reaction (PCR) amplification of genomic DNA obtained from the tail using Tissue-Direct™ PCR KIT (Cat#D300-1000, LAMDA BIOTEC, USA). The PCR products were resolved on a 1% agarose gel. The genotypes of mice were verified by examining the size of the PCR products: $Nrf2^{+/+}$ (734 bp), $Nrf2^{-/-}$ (400 bp), $Nrf2^{+/-}$ (734 and 400 bp). Primers for Genotypes: 5′-TGGACGGGACTATTGAAGGCTG-3′ (sense for $Nrf2^{+/+}$ and $Nrf2^{-/-}$), 5′-GCCGCCTTTTCAGTAGATGGAGG-3′ (antisense for $Nrf2^{+/+}$), and 5′-GCGGATTGACCGTAATGGGATAGG-3′ (antisense for LacZ). All of the animal protocols were conducted in accordance with the Guideline for Care and Use of Laboratory Animals (National Institute of Heath, USA) and were approved by the Institutional Animal Care and Use Committee at Shandong University, China, and the University of South Carolina, USA.

2.2. Preparation of crude extract of American ginseng

A standardized American ginseng crude extract reference material (GINX-1) was prepared by the National Research Council of Canada, Institute for National Measurement Standards (NRCC-INMS) as previously described (Ichikawa et al., 2009b; Li et al., 2010). This extract was derived from four year old, cultivated, ginseng roots grown by Chai-Na-Ta Farms Ltd. (Kamloops, British Columbia, Canada) and processed by Canadian Phytopharmaceuticals Corporation (Richmond, British Columbia, Canada). The identity of the roots was independently confirmed by Agriculture and Agri-Foods Canada and a voucher specimen of the *Panax quinquefolius* used in this study deposited with the University of Ottawa herbarium (UO 19908). Plant morphology conformed to that as previously described for *P. quinquefolius* (Small and Catling, 1999) and the presence of a marker compound ginsenoside F11, unique to *P. quinquefolius*, confirmed by liquid chromatography–mass spectrometry. Following thorough homogenization, 4000 1 g lots of the extract were bottled under argon, irradiated (5 kGy), and stored under cryogenic conditions (-80 °C). Periodic analyses of the extract over 4 years have shown no significant change in ginsenoside content which is 10.5% w/w measured as the sum of: Rg1 3.5 (0.1), Re 21.2 (0.4), Rb1 45.1 (1.6), Rc 15.5 (0.7), Rb2 2.2 (0.1), Rd 17.8 (0.4) mg/g $\pm$ one standard deviation, respectively.

2.3. Fractionation of American ginseng crude extract

The crude extract of American ginseng was further fractionated as previously described (Poudyal et al., 2012). Briefly, 10 g of American ginseng extract was dissolved in 150 mL of water and sequentially partitioned against 3×50 mL aliquots of hexane, dichloromethane, ethyl acetate, water, and butanol. The fractions were reduced to near dryness on a vacuum centrifuge, freeze dried, and their respective dry weights determined: water fraction, 7.320 g (i.e., 73% of the original material); butanol fraction, 1.544 g; ethyl acetate fraction, 0.064 g; dichloromethane fraction, 0.062 g; and hexane fraction, 0.044 g. Each fraction was then redissolved in a small volume of solvent to facilitate blending with the appropriate amount of maltodextrin to give a final weight of 10 g after a second round of evaporation by vacuum centrifuge and freeze drying. Thus, the original extract was subdivided on the basis of polarity and reconstituted with maltodextrin to give an equivalent weight as the starting material for bioassay. All fractions were thoroughly vortexed to give a free flowing powder and split into two: one set was retained at National Research Council (Ottawa, ON, Canada) as a reference and the other used for bioassay. Neat maltodextrin was used as a negative control.