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Anti-inflammatory activity of *Barleria lupulina*: Identification of active compounds that activate the Nrf2 cell defense pathway, organize cortical actin, reduce stress fibers, and improve cell junctions in microvascular endothelial cells

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ABSTRACT

Ethnopharmacological relevance: Hot aqueous extracts of the plant *Barleria lupulina* (*BL*) are used for treating inflammatory conditions and diabetic vascular complications.
Aim of the Study: The goal was to identify active compounds in hot aqueous extracts of *BL* (HAE-*BL*) that are consistent with a role in reducing inflammation and reducing the vascular pathology associated with diabetes. In particular, we examined activation of the Nrf2 cell defense pathway because our initial findings indicated that HAE-*BL* activates Nrf2, and because Nrf2 is known to suppress inflammation. Activation of Nrf2 by HAE-*BL* has not been described previously.
Materials and methods: Human endothelial cells, real-time PCR, western blotting, cytoskeletal analyses, and assay-guided fractionation with HPLC were used to identify specific compounds in HAE-*BL* that activate the Nrf2 cell defense pathway and reduce markers of inflammation in vitro.
Results: HAE-*BL* potently activated the Nrf2 cell defense pathway in endothelial cells consistent with its traditional use and reported success in reducing inflammation. Assay guided fractionation with HPLC identified three alkyl catechols: 4-ethylcatechol, 4-vinylcatechol, and 4-methylcatechol, that are each potent Nrf2 activators. In addition to activating Nrf2, HAE-*BL* and akyl catechols each profoundly improved organization of the endothelial cell actin cytoskeleton, reduced actin stress fibers, organized cell-cell junctions, and induced expression of mRNA encoding claudin-5 that is important for formation of endothelial tight junctions and reducing vascular leak.
Conclusions: HAE-*BL* contains important alkyl catechols that potently activate the Nrf2 cell defense pathway, improve organization of the endothelial cell cytoskeleton, and organize tight cell junctions. All of these properties are consistent with a role in reducing inflammation and reducing vascular leak. Because activation of the Nrf2 cell defense pathway also prevents cancers, neuro-degeneration, age-related macular degeneration, and also reduces the severity of chronic obstructive pulmonary disorder and multiple sclerosis, HAE-*BL* warrants additional consideration for these other serious disorders.

1. Introduction

Barleria lupulina Lindl. (Acanthaceae family), also known as hop-head Philippine violet and Vishallakarani, is a well-known historical herb used in folklore medicine of Southeast Asia, India, and South China. The plant is cultivated both as an ornamental and medicinal plant; and, for medicinal use, a hot aqueous extract of the aerial parts of the plant is taken orally. *Barleria lupulina* (*BL*) is traditionally used for insect and snakebites, inflammation, allergic responses, rheumatoid arthritis, and diabetes (Mazumder et al., 2012; Suba et al., 2004, 2005; Wanikiat et al., 2008). In particular, the Thailand Ministry of Public Health has categorized *BL* as an essential medicinal plant for primary healthcare (Wanikiat et al., 2008). Phytochemical studies on this plant have focused on a number of iridoid glycosids as main components (Kanchanapoom et al., 2001; Kim et al., 2015; Suksamrarn et al., 2003). However, the compounds in *BL* that provide medicinal benefits towards reducing inflammation and the vascular complications of diabetes have not been defined.

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An important possibility, not considered previously, is that *BL* exerts its medicinal benefits through activation of the Nrf2 (NFE2L2) cell defense pathway (Kensler et al., 2007; Motohashi and Yamamoto, 2004). The Nrf2 transcription factor, through binding to antioxidant response elements in ~200 genes, induces expression of anti-oxidant and detoxifying enzymes that protect against oxidative damage and also provide protection against toxic foreign chemical substances through phase II enzyme modification (Dinkova-Kostova and Abramov, 2015; Holmstrom et al., 2013). Compelling evidence for the importance of the Nrf2 pathway for disease prevention and control of tissue damage associated with inflammation comes from numerous studies with mice lacking the Nrf2 gene (reviewed in Senger et al. (2016)). Also, consistent with folklore use of *BL* for diabetic complications, Nrf2 has been implicated in protection from diabetic pathology (de Haan, 2011; Evans and Goldfine, 2016; Li et al., 2012; Long et al., 2016). Consequently, in studies described here, we investigated regulation of the Nrf2 pathway by *BL* in human microvascular endothelial cells, which are pivotal "gatekeepers" of inflammation (Dejana, 2004; Granger and Senchenkova, 2010) and closely involved in diabetic complications (Avogaro et al., 2011; Brownlee, 2005). In addition, we investigated *BL* activity towards the microvascular endothelial cytoskeleton that, at the sub-cellular level, exerts critical barrier control over blood vessel leakiness, tissue edema, and the extravasation of inflammatory cells (Bogatcheva and Verin, 2008; Lampugnani, 2010; Lum and Malik, 1994; Millan et al., 2010).

2. Materials and methods

2.1. Plant material

The aerial parts of *B. lupulina* Lindl. (the accepted name, see: http://www.theplantlist.org/tpl1.1/record/kew-2670049) were purchased from Vung Tau City, Ba Ria – Vung Tau Provence, Vietnam (Coordinates: 10 °35′N 107 °15′E), from March 2012 to March 2014. Voucher specimens (Nos. 101-103) are deposited at Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA.

2.2. Preparation of hot aqueous extract of Barleria lupulina (HAE-BL)

200 g of dried, chopped, and uniformly mixed aerial portions of *Barleria lupulina*, consisting of wood and leaf fragments, was boiled in purified, deionized water (1.2 L) for 6 h in a stainless steel teapot on an electric hot plate with surface temperature of 550 °F (288 °C). After 6 h, aqueous extract typically was condensed to a final volume of 100 mL. Boiling time or final volume was adjusted, as necessary, to achieve final volume of 100 mL. However, boiling time never differed from 6 h by more than 30 min. The condensed extract was filtered through a funnel lined with fluted filter paper and then centrifuged at 15,000 × *g* for 30 min. Finally, it was filter-sterilized and stored under sterile conditions at 4 °C. Aliquots of the extract were evaporated to dryness, and dry weight of the extract was determined to be 150 mg/mL.

2.3. Analytical and preparative HPLC

Agilent analytical HPLC 1100 system: Column=C18, 100 Å, 100 × 4.6 mm, 5 µm; Flow-rate=0.7 mL/min; Solvents: from 100% water to 100% acetonitrile in 20 min, and then 100% acetonitrile for 10 min. Agilent preparative HPLC 1100 system: Column=C18, 100 Å, 100 × 21.2 mm, 5 µm; Flow-rate=10 mL/min; Solvents= from 100% water from to 100% acetonitrile in 20 min, and then 100% acetonitrile for 10 min.

2.4. LC/MS condition for the analysis of 4-EC (4-ethylcatechol), 4-MC (4-methylcatechol), 4-vinylcatechol (4-VC) and HAE-BL

Agilent 1290 uHPLC coupled to a Bruker Impact HD ESI-q-TOF mass spectrometer in negative mode. Column: Phenyl, 150 Å~4.6 mm, 5 µm; Flow-rate: 0.5 mL/min; Solvent A: water 0.1% formic acid, Solvent B: methanol 0.1% formic acid, 0–2 min loading at 1% B, then increase gradient over 20 min to 100% B, re-equilibrate over 7 min to 1% B.

2.5. Chemicals

Shanzhiside methyl ester was purchased from ChromaDex, Y-27632 from Tocris, 4-ethycatechol and 4-methylcatechol from Sigma Aldrich, and 4-vinylcatechol (from Toronto Research Chemicals). 4-Vinylcatechol is highly unstable and was preserved with 1% w/w butylated hydroxytoluene. In control experiments, we determined that this proportion of butylated hydroxytoluene had no affect on results.

2.6. Antibodies

The following antibodies (Abcam, Inc., Cambridge, MA) were used for western blotting: heme oxygenase-1 (rabbit monoclonal, clone EP1391Y), Nrf2 (rabbit monoclonal, clone EP1808Y), CD31 (rabbit monoclonal, clone EPR3094). Secondary antibody used for western blotting was polyclonal goat anti-rabbit (H+L), conjugated to horseradish peroxidase (Life Technologies, cat. # G-21234). The following antibody was used for immunohistochemical staining of Nrf2 in cells (Paupe et al., 2009): Nrf2 (H-300), sc-13032 rabbit polyclonal raised against amino acids 37-336 of human Nrf2 (Santa Cruz Biotechnology Inc.). Secondary antibody used for immunohistochemical staining was goat anti-rabbit IgG (H+L) Alexa Fluor 488 conjugate (ThermoFisher).

2.7. Cells and cell culture experiments, cell viability assays, RNA isolation from cells, immunohistochemical staining of cells, and cell lysates for western blotting

Primary human dermal microvascular endothelial cells were purchased from Lonza Inc, Walkersville MD, and cultured in Lonza EGM-2MV medium. For short-term experiments lasting 4 h, cells were shifted to serum-free EBM-2 (Lonza), containing 25 ng/mL human VEGF165 (R&D Systems). In experiments lasting 24 h, cells were fed complete EGM-2MV medium at time zero. RNA was isolated from cells cultured in 6 well plates with the Qiagen RNeasy Plus Mini Kit that provides on-column removal of genomic DNA. For immunohistochemical staining of Nrf2, cells were fixed and stained exactly as described (Paupe et al., 2009). In addition, cells were co-stained for F-actin with Alexa Fluor 488 phalloidin (Molecular Probes, #A12379) or Alexa Fluor 594 phalloidin (Molecular Probes, #A12381), as indicated. All images used in comparisons were captured with identical exposures. For western blotting applications, cells were cultured in 24 well plates, treated as described in Figure legends, washed 3 × with ice cold PBS, and harvested in 120 µL of 1.5 × Laemmli SDS sample buffer containing protease inhibitor cocktail (Sigma-Aldrich, #P8340).

2.8. Western blotting

Cell lysates were subjected to SDS polyacrylamide electrophoresis on pre-cast 4–20% gradient gels (GenScript) and separated proteins transferred to PVDF immunoblotting membrane (Bio-Rad). PVDF membrane was blocked in 5% w/v skim milk (from powder, EMD Millipore), and stained with primary antibodies and secondary horseradish peroxidase-conjugated antibody (see