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Phenylpropanoid glycosides from plant cell cultures induce heme oxygenase 1 gene expression in a human keratinocyte cell line by affecting the balance of NRF2 and BACH1 transcription factors

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ABSTRACT

Phenylpropanoids have several highly significant biological properties in both plants and animals. Four phenylpropanoid glycosides (PPGs), verbascoside (VB), forsythoside B (FB), echinacoside (EC) and campneoside I (CP), were purified and tested for their capability to activate NRF2 and induce phase II cytoprotective enzymes in a human keratinocyte cell line (HaCaT). All four substances showed similar strong antioxidant and radical-scavenging activities as determined by diphenylpicrylhydrazyl assay. Furthermore, in HaCaT cells, FB and EC are strong activators of NRF2, the nuclear transcription factor regulating many phase II detoxifying and cytoprotective enzymes, such as heme oxygenase 1 (HMOX1). In HaCaT cells, FB and EC (200 μM) induced nuclear translocation of NRF2 protein after 24 h and reduced nuclear protein levels of BACH1, a repressor of the antioxidant response element. FB and EC greatly *HMOX1* mRNA levels by more than 40-fold in 72 h. Cytoplasmic HMOX1 protein levels were also increased at 48 h after treatment. VB was less active compared to FB and EC, and CP was slightly active only at later times of treatment. We suggest that hydroxytyrosol (HYD) could be a potential bioactive metabolite of PPGs since HYD, in equimolar amounts to PGGs, is able to both activate HO-1 transcription and modify Nrf2/Bach1 nuclear protein levels. This is in agreement with the poor activity of CP, which contains a HYD moiety modified by an *O*-methyl group. In conclusion, FB and EC from plant cell cultures may provide long-lasting skin protection by induction of phase II cytoprotective capabilities.

1. Introduction

High levels of reactive oxygen species (ROS) cause damage to skin cells and are involved in several human dermatological pathologies [1] and aging [2]. To deal with excessive ROS levels, the body has endogenous defense mechanisms such as antioxidative enzymes and nonenzymatic antioxidative molecules to protect it from free radicals. Phase II detoxifying genes provide a major mechanism by which cells reduce ROS toxicity and their induction is highly effective for protecting cells against oxidative stress [3]. Keratinocytes constitutively express ROS-detoxifying enzymes, including superoxide dismutase, catalase, glutathione peroxidase and heme oxygenase 1 (HMOX1), but also contain substantial levels of the antioxidants tocopherol and ubiquinol [4,5]. Unfortunately, these endogenous defense mechanisms decrease as part of the natural aging process, while ROS production increases, resulting in accelerated skin damage [6].

Isoenzymes HMOX1 and -2 catalyze the stepwise degradation of heme to release free iron and equimolar concentrations of carbon monoxide and biliverdin, which is converted to bilirubin. HMOX1 is a phase II enzyme that is transcriptionally regulated by a large variety of stimuli. These include its substrate, heme [7,8], oxidative stress [9], the signaling proteins nerve growth factor, tumor necrosis factor-α, interleukin-1β and interferon-γ [10], and exogenous substances such as curcumin [11]. Many reports have demonstrated the potent antioxidant activity of heme-derived metabolites generated by HMOX1 catalysis (biliverdin and bilirubin) and the cytoprotective actions of carbon monoxide on vascular endothelium and nerve cells [12]. Therefore, it is now widely accepted that induction of HMOX1 expression represents an adaptive response that increases cell resistance to oxidative injury.

The nuclear transcription factor NRF2 controls the orchestrated expression of most phase II enzymes and genes [13,14]. When cells are exposed to oxidative stress, electrophiles or chemopreventive

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agents, NRF2 escapes KEAP1-mediated repression in the cytoplasm, translocates to the nucleus and activates antioxidant responsive element (ARE)-dependent gene expression to maintain cellular redox homeostasis. In addition to this canonical mechanism of NRF2 activation, several protein kinases, such as MAPK [15], PI3K [16] and CK2 [17], regulate nuclear accumulation or transcriptional activity of NRF2. The constitutive basal activity of NRF2 in the skin is crucial for normal wound repair and prevention of skin carcinogenesis [18].

The ARE sequence in the promoter regions of genes such as *HMOX1* and NADP(H) quinone reductase is constitutively repressed by BACH1–MAF heterodimers [19]. In the presence of oxidative stress, BACH1 is inactivated [20], which allows transcriptional activation of these genes by NRF2 and other activators. Understanding of *HMOX1* gene regulation in terms of the balance between BACH1 as repressor and NRF2 as activator is important for the development of therapeutic intervention strategies for the skin.

Phenylpropanoid glycosides (PPGs) are a class of more than 200 plant-derived polyphenols acting as potent antioxidants by direct scavenging of ROS and reactive nitrogen species [21]. Verbascoside (VB), also called acteoside and one of the best-studied PPGs, is structurally characterized by the presence of caffeic acid (PP moiety), 4,5-hydroxyphenylethanol (hydroxythyrosol, HYD), glucose, and rhamnose. Lee et al. reported that the anti-inflammatory effect of acteoside involved inhibition of lipopolysaccharide-inducible nitric oxide synthase expression in a mouse cell line system [22]. Several reports have indicated that VB can suppress important pro-inflammatory mediators [23,24].

Echinacoside (EC) is the main phenolic compound found in *Echinacea angustifolia* and *Echinacea pallida*, both of which have long been used in traditional medicine to treat infectious and traumatic diseases [25], with strong antioxidant and anti-inflammatory properties [26,27]. Forsythoside B (FB) and VB, isolated from dried fruits of *Forsythia* species, inhibited the formation of 5-hydroxy-6,8,11,14-eicosatetraenoic acid from arachidonic acid in rat peritoneal cells [28]. Moreover, campneoside (CP), VB, EC and FB from plants of the genus *Buddleja*, used in traditional Chinese medicine for their anti-inflammatory properties, were tested for their activity against enzymes of the arachidonate cascade [29].

Korkina et al. demonstrated that PPGs such as teupolioside and VB possess skin-regenerative properties and exert a powerful inhibitory effect on the expression of cytokines and chemokines in normal human keratinocytes [30].

The identification of natural antioxidants for effective photoprotection and suppression of inflammatory reactions in the skin is now a major focus in investigative dermatology [31]. The results presented here strongly suggest that the PPGs FB, EC and VB, and to some extent CP, may represent natural substances of special dermatological interest for skin protection against environmentally induced oxidative stress and inflammatory insults. An understanding of their molecular mechanism in terms of NRF2 activation and HMOX1 expression could promote innovative use of these compounds in dermatology to slow down aging or alleviate diseases in which oxidative stress is a primary cause.

2. Materials and methods

2.1. Plant cell lines

Syringa vulgaris L., also known as the common lilac, and *Marrubium vulgare* plants from the Botanical Gardens of the University of Bologna (Bologna, Italy) were used by Istituto di Ricerche Biotecnologiche to generate plant cell lines. Stabilized cell lines were obtained from dissected young *S. vulgaris* and *M. vulgare* leaves according to standard procedures [32] and was selected to maximize the synthesis of secondary metabolites.

E. angustifolia plants were collected from *Il Giardino delle Erbe* (botanical garden) of Casola Valsenio (Ravenna, Italy) and were processed as previously described for *S. vulgaris* to generate stable selected plant cell lines. *M. vulgaris* plant cell lines were selected to produce FB and CP, *S. vulgaris* cell lines were used to produce VB, and *E. angustifolia* cells were selected and optimized to produce EC.

2.2. Extract preparation

S. vulgaris cell cultures obtained at the end of the fermentation process were collected and homogenized. The cell remnants were separated by centrifugation at 5000 rpm for 10 min, the sediment was discarded and the supernatant was collected. The VB yield was approximately 1 g per liter of cell culture suspension. To purify VB, the supernatant was loaded onto a chromatographic column containing solid phase extraction XAD4 resin. Following extensive washing of the resin with water, VB was recovered by elution with 80:20 ethanol/water (v/v). The eluted VB was then concentrated under reduced pressure and lyophylized. The final powder extract contained VB at a purity of >80% (w/w), together with minor components (<10% w/w) of other VB analogs, mainly iso-VB. VB was further purified by repeated column chromatography on C18 silica gel and Sephadex LH20 and subsequent crystallization yielded VB of >97% purity.

Similar procedures were used for purification of FB and CP from *M. vulgare* cell cultures and EC purification from *E. angustifolia* cultures. The chromatographic purity of all phenylpropanoids used in the study was >97% w/w.

2.3. HPLC analysis

HPLC analysis was performed on an Agilent system (series 1100 DAD, Hewlett–Packard) consisting of an autosampler, a high-pressure mixing pump and a Phenomenex C18 column (4.6 × 150 mm). The gradient mobile phase consisted of water/0.01 N phosphoric acid (phase A) and acetonitrile/0.01 N phosphoric acid (phase B). The gradient elution program was as follows:

Time (min)	% Phase A	% Phase B
0.0	88	12
0.5	88	12
3.0	80	20
4.0	60	40
4.1	88	12
5.0	88	12

The flow rate was 1 mL/min and the UV detector measured absorbance at 330 nm. The retention time was 3.16 min for VB, 2.72 min for EC, 3.03 min for FB and 2.56 min for CP (Supplementary Fig. 1).

2.4. Antioxidant DPPH assay

A diphenylpicrylhydrazyl (DPPH) assay was performed according to Molyneux [33]. In brief, a working solution of 100 μM DPPH in methanol was freshly prepared (A_{515nm} = 0.5–0.6). PPGs were diluted in methanol to double the final assay concentration. The reaction mixture comprised 500 μL of PPG sample and 500 μL of 100 μM DPPH in a cuvette. The negative control consisted of 500 μL of MeOH and 500 μL of DPPH. Triplicate samples were incubated in the dark at room temperature for 15 min before reading the absorbance at 515 nm on a Shimadzu UV-1601 spectrophotometer. The percentage radical reduction is expressed as: