Diabetic Wound Healing and Activation of Nrf2 by Herbal Medicine

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Nrf2 defense is a very important cellular mechanism to control oxidative stress, which is implicated in wound healing. Nrf2 can induce many cytoprotective genes, including HO-1, NQO1 and G6PD. Among many natural products that have been reported as Nrf2 activators, sulforaphane and curcumin have been studied more widely than any others, and both are in clinical trials for non-cancerous disorders. Recently, we reported 4-ethyl catechol and 4-vinyl catechol as Nrf2 co-factors that can induce Nrf2 as potently as sulforaphane and curcumin. These new Nrf2 co-factors were identified in hot aqueous extract of an herbal medicine Barleria lupulina, and fermented Noni (Morinda citrifolia) juice, which are used traditionally for diabetic wound healing.

Nrf2 | diabetic wound healing | herbal medicine | Barleria lupulina | Morinda citrifolia | alkyl catechols

The Nrf2 defense pathway
In mammals, the master regulator of antioxidant defense is the Nrf2 pathway. The Nrf2 transcription factor controls expression of anti-oxidant and detoxifying enzymes that maintain a healthy cellular redox state and protect against toxic foreign chemical substances through phase II modification, which neutralizes dangerous species, generating less reactive and more soluble substances that are readily eliminated. The network of cytoprotective genes regulated by Nrf2 is more than two hundred, accounting for more than 1% of the genome. In the absence of stress, Nrf2 with a half-life of about 20 minutes is sequestered within the cytosol by the actin-binding protein Kelch-like ECH-associated protein 1 (KEAP1) and Cullin 3, which degrade Nrf2 within the cytosol by the actin-binding protein Kelch-like ECH-associated protein 1 (KEAP1) and Cullin 3, which degrade Nrf2 through ubiquitination. Under conditions of oxidative stress, Nrf2 is released from Keap1 and rapidly moves to the nucleus to induce transcription of anti-oxidant and detoxifying enzymes. The “redox sensor” mechanism that releases Nrf2 from Keap1, resulting in Nrf2 transport to the nucleus, involves oxidation-sensitive sulfhydryl groups in cysteine residues of Keap1.

Nrf2 induction of cytoprotective genes and diabetic wound healing
Compelling evidence for the importance of the Nrf2 pathway comes from numerous studies with mice lacking the Nrf2 gene. These Nrf2 null mice exhibit increased sensitivity to a multitude of chemical toxins, resulting in increased inflammation and damage to brain, lung, and kidney. Similarly, Nrf2 null mice are considerably more sensitive to chemical carcinogens, with increased incidence of cancers demonstrated in skin, stomach, colon, and bladder. Also, mice engineered with a dominant negative Nrf2 mutant transgene develop skin cancer at three times the frequency of control mice in a classical two-stage model of chemical carcinogenesis. Additional problems for Nrf2 null mice include impaired liver regeneration, accelerated UVB-induced photo-ageing of skin, increased rheumatoid arthritis, development of lupus-like autoimmune nephritis, and development of age-related retinopathy. Thus, the protective importance of the Nrf2 pathway is well established.

Diabetes often causes slow-healing wounds that can worsen rapidly. The Keap1-Nrf2 system is a critical target for preventing the onset of diabetes mellitus. Nrf2 transcription factor, a novel target of keratinocyte growth factor action, regulates gene expression and inflammation in the healing skin wound. In a streptozotocin-induced diabetes mouse model, Nrf2 null mice have delayed wound closure rates compared with Nrf2+/+ mice. Hence, Nrf2 can be activated to potentially heal diabetic wounds and improve overall health.

Nrf2 small molecule co-factors were first discovered as “cancer-protective” compounds, which could be utilized for diabetic wound healing
Long before the discoveries of Nrf2 and the antioxidant response element (ARE) to which Nrf2 binds, a variety of small chemical compounds were observed to protect rodents from chemically induced carcinogenesis. Remarkably, these “cancer-protective” compounds were from distinctly different chemical classes, but they all shared the critical property of high susceptibility to oxidation-reduction reactions. The simplest of these active cancer-protective compounds were identified as 1,4-diphenol (hydroquinone) and 1,2-diphenol (catechol), and more complex examples include the isothiocyanate sulforaphane isolated from broccoli and curcumin from the turmeric plant (see Figure 1). With the discovery of Nrf2, it became clear that these previously identified redox-sensitive, cancer-protective compounds worked as co-factors for Nrf2 activation. Collectively, these findings suggested that support of Nrf2 activation by redox-sensitive co-factors, particularly dietary factors such as sulforaphane and curcumin, could be employed as an effective anti-cancer strategy. This provided impetus for clinical trials, which are continuing. Sulfuraphane and curcumin are also currently in clinical trials for non-cancer disorders in which the Nrf2 pathway has been implicated. Current challenges with the application of sulfuraphane and curcumin for clinical benefit appear to involve bioavailability of these compounds. Acrolein (CH=CHO-H) induces Nrf2 translocation and ARE-luciferase reporter activity. Cinnamaldehyde (Ph-CH=CHO-trans double bond) inhibits thioredoxin reductase and induces Nrf2.

Recent studies have demonstrated the protective role of Nrf2 and the potential therapeutic effect of Nrf2 activators, sulfuraphane and cinnamaldehyde in a diabetic nephropathy animal model.

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The alkyl catechols, 4-ethyl catechol and 4-vinyl catechol, potent Nrf2 co-factors, from *Barleria lupulina* and *Morinda citrifolia*, both of which are used traditionally for diabetic wound healing

*Barleria lupulina* (BL): Recently, our work with a traditional Vietnamese medicine (*Barleria lupulina*, BL) has identified 4-ethyl catechol and 4-vinyl catechol as potently active, natural Nrf2 co-factors (Figure 2) in the hot water extract of *Barleria lupulina*. Although these compounds had been reported, they had not been recognized as important Nrf2 co-factors. Nonetheless, these compounds each satisfy the well-defined structural criteria for “oxidation-reduction lability” that is required for a compound to induce protective enzymes. Interestingly, catechol (Figure 2) was among the first compounds recognized as an Nrf2 co-factor, but 4-ethyl catechol and 4-vinyl catechol had not received attention. Importantly, we found that 4-ethyl catechol and 4-vinyl catechol are much more potent Nrf2 co-factors than catechol and that they are comparably potent to sulforaphane and curcumin. Thus, apart from sulforaphane and curcumin, we believe that 4-ethyl catechol and 4-vinyl catechol may be the most potent of the naturally occurring Nrf2 co-factors. Moreover, the relatively small size and simple structure of these compounds, suggests the likelihood of better bioavailability than sulforaphane and curcumin. We have reported the activities of these alkyl catechols recently. Besides inducing Nrf2, BL, 4-EC and 4-VC significantly improved the organization of the endothelial cell actin cyto-skeleton, 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.

*Morinda citrifolia* (Noni): Encouraged by the discovery of new Nrf2 co-factors, 4-MC, 4-EC, and 4-VC from the hot water extract of *Barleria lupulina*, we screened many herbs for their activity of Nrf2 activation. Only fermented Noni juice (Order Number 809979, Virgin Noni juice, http://www.virginnonijuice.com) showed Nrf2 activation, which was as strong as BL.

The traditional medicinal plant *Morinda citrifolia* L. (Rubiaceae) is believed to have the origin in Southeast Asia and later on distributed to Polynesia. It is called Indian mulberry in India, ba ji tian (巴戟天) in China, nono in Tahiti, and noni in Hawaii (Noni) is now widely cultivated in tropical areas of the South Pacific, including Hawaii. Traditionally, noni bark and roots were used as dye or clothing, while medicinal usage of all plant parts, including leaves and fruits, were mostly restricted to treat wounds, infections, menstrual cramps, bowel irregularities, diabetes, high blood pressure or as a purgative. Pacific Islanders and Native Hawaiians consume fresh fruits or noni juice prepared by fermenting the fruits. Claims of its ‘healing powers’ have fuelled much of the commercial interest in noni and promoting a worldwide market for noni-based dietary supplements including fruit juice, in North America, Mexico, Australia, and Asia.
Young Noni fruits are green, and will turn yellow before ripe, but harvested Noni fruits are mainly whitish (Figure 3), and will turn dark after fermentation. We tested FNJ from ripe Noni fruits, and juices from both green and whitish Noni fruits for their activity of Nrf2 activation. Results showed that juice from young green Noni fruits was inactive, and both juice from ripe whitish Noni fruits and FNJ exhibited Nrf2 activation (Figure 4, Figure 5).

Using the same assay-guided separation method as described in our previous publication, we have identified 4-MC, 4-EC, and 4-VC (Figure 2, Figure 6) in FNJ, which partially accounted for the Nrf2 activation. We are in progress of identifying other Nrf2 activators. At the same time, questions remain why juice from ripe whitish Noni fruits with a fouling smell was active while juice from young green Noni fruits was not, and why people prefer FNJ to juice from ripe whitish Noni fruits.

Figure 4. FNJ induces nuclear translocation of Nrf2 in MVECs. MVECs were incubated with FNJ at 1:25 dilution. Staining of Nrf2 in human MVECs incubated with FNJ (right), in comparison with control (left). Green = Nrf2, red = F-actin. Note increased nuclear staining for Nrf2 in cells incubated with FNJ. Overall intensity of Nrf2 staining is also increased, because Nrf2 activation typically involves Nrf2 stabilization in combination with nuclear translocation.

Figure 5. Induction of Nrf2 target gene RNAs by FNJ in MVECs, as measured with RT-PCR. Y-axis = (mRNA copies)/(10^6 18S rRNA copies). Nrf2 target genes = heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), glucose 6-phosphate dehydrogenase (G6PD). Control, non-NRF2 target mRNAs = CD31 (PECAM-1) and VE-cadherin (cadherin-5). FNJ was added to a final dilution of 1:25, and cells were harvested at 24 hours. For all panels, error bars = ± standard deviation (S.D.); n > 4 for each data point. Statistical significance: For HO-1, NQO1, and G6PD panels: FNJ vs. vehicle Ctrl = all extremely significant (p<0.001); for CD31 and VE-cadherin panels: no significant differences.

Figure 6. Agilent prep-HPLC HPLC chromatogram of the aqueous acetonitrile eluent of FNJ. Sample: FNJ (2 mL/injection, about 150 mg); Flow-rate: 10 mL/min; Solvents: 100% water (0.1% formic acid) for 10 min (0-10 min), then to 100% acetonitrile (0.1% formic acid) in 20 min (10-30 min), finally 100% acetonitrile for 10 min (30-40 min). Y-axis = absorbance at 254nm (mAU), X-axis = minutes. Fraction 11 (t_R = 29-31 min) was active and Nrf2 activators 4-MC, 4-EC and 4-VC (minor compounds) were identified.
Discussion

Nrf2 is a master regulator that can modulate many cytoprotective genes. Many small molecules, including natural products, were reported as Nrf2 activators, but only a few showed strong Nrf2 induction. Sulforaphane and curcumin are in clinical trials for non-cancer disorders, and dimethyl fumarate (CH$_2$O-CO-CH=CH-O-CO.CH$_3$, trans), an Nrf2 activator, is also in phase III clinical trials for multiple sclerosis. Many traditional herbal medicines have been used for diabetic wound healing, but most of the active compounds and mechanisms of actions are unknown. We have studied BL and more recently Noni, and we have identified 4-MC, 4-EC, and 4-VC as Nrf2 co-factors. 4-EC and 4-VC demonstrated Nrf2 induction, which were as potent as sulforaphane and curcumin. Since Nrf2 activation by Noni has not been fully characterized, it is worthy of further investigation.

Abbreviation

BL, Barleria lupulina; Noni, Morinda citrifolia; FNJ: fermented Noni juice; 4-MC, 4-methyl catechol; 4-EC, 4-ethyl catechol; 4-VC, 4-vinyl catechol.

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2. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annual review of pharmacology and toxicology. 2007;47:89-116.
the JNK pathway by sublethal concentrations of acrolein. Toxicology and applied pharmacology. 2006;214(3):244-252.