Synergistic Activation of the Nrf2-ARE Oxidative Stress Response Pathway by a Combination of Botanical Extracts

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Abstract

Many fruits and vegetables have been tested for their ability to scavenge free radicals. It is more likely, however, that the therapeutic effect of dietary fruits and vegetables on oxidative stress may be due to the induction of free radical defense mechanisms via activation of the Nrf2-ARE signaling pathway. In this study, we screened botanical extracts for the ability to activate the Nrf2 pathway using an antioxidant response element luciferase reporter cell line. Extracts of turmeric, pagoda tree seed pod, and rosemary exhibited activity in the reporter assay, and a combination (1:3:5 w/w/w) stimulated a synergistic response greater than expected for an additive effect of the individual extracts. The results suggest a reduction in oxidative stress with botanical extracts selected for their ability to induce the Nrf2-ARE signaling pathway may be possible in healthy individuals who consume a synergistic blend of turmeric, pagoda tree seed pod, and rosemary extracts designed to upregulate the Nrf2-ARE signaling pathway.

Key words
Curcuma longa · Zingiberaceae · Rosmarinus officinalis · Lamiaaceae · Styphnolobium japonicum · Fabaceae · antioxidant response element · Nrf2

Results and Discussion

The initial goal of this study was to identify fruit and vegetable extracts that induce the endogenous Nrf2-mediated antioxidant defense pathway. Turmeric, pagoda tree seed pod, and rosemary were three botanical extracts that showed luciferase activity in an Nrf2-dependent reporter assay. A concentration-response curve for these extracts is shown in Fig. 1. Quercetin containing pagoda tree extract induced the most robust response with a peak response of 80% of the positive control at a concentration of 33 µg/mL. The peak luciferase response for the turmeric extract was 30% of the positive control at 10 µg/mL, but higher concentrations were cytotoxic in this assay (data not shown). Rosemary extract had a minimal concentration-dependent response. The response of the cells to sulforaphane, the positive control for the assay, is denoted at 100% as a dashed line.

Representative chromatograms of the botanical extracts used in this study are shown in Fig. 2. All chromatograms were obtained by monitoring absorbance at 280 nm. For rosemary extract, a potential interference in the quantification of rosmarinic acid was eliminated by subtracting the absorbance at 270 nm from that at 280 nm. Analyses of quercetin in pagoda tree extract and of curcuminoids in turmeric extract were sufficiently well separated that subtraction was not required for accurate quantification.

Since the botanical extracts may act on the Nrf2-antioxidant response element (ARE) pathway through different mechanisms, we next tested different combinations of the three extracts to determine whether mixtures of extracts induce a greater response in the ARE luciferase reporter assay compared to each extract alone. The ARE luciferase response for five different combinations of turmeric extract, quercetin containing pagoda tree extract, and rosemary extract (TQR) are shown in Fig. 3. The most robust luciferase response seen was with the 1:3:5 TQR combination and was sevenfold greater than that seen for the sulforaphane positive control. A dose-response curve for the 1:3:5 TQR combination in the luciferase reporter assay is presented in Fig. 4, with an EC₅₀ of 30 µg/mL.

The free radical theory of aging [6] postulates that organisms age as they accumulate oxidative damage over time, and that much of the pathology that develops as humans age is linked to long-term exposure to oxidative stress. As dietary fruits and vegetables contain high levels of phytochemicals with antioxidant properties, thousands of botanical samples, extracts, and phytochemicals have been tested for their ability to scavenge reactive oxygen in chemical tests such as the oxygen radical absorbance capacity assay (ORAC and DPPH antioxidant assays) [7,8]. Investigators have therefore postulated that the health benefits of dietary fruits and vegetables may be due to the ability of some phytochemicals to reduce the level of oxidative stress [4,9,10]. However, clinical studies based on high ORAC interventions have mostly failed to provide evidence for the reduction of disease outcomes [1,2]. These results have led many investigators to suggest that free radical scavenging is not the primary mechanism responsible for the observed health benefits of a plant-based diet, and the United States Department of Agriculture’s Nutrient Data Library has removed the ORAC database from their website [11]. An alternative hypothesis as to how these health benefits occur might be due, in part, to phytochemical-induced stimulation of endogenous antioxidant defense pathways via activation of the Nrf2-ARE path-
way of gene expression, and a large number of phytochemicals have been shown to upregulate the expression of proteins involved in mitigating oxidative stress through this pathway [12]. Based on this proposed mechanism for the effect of dietary fruits and vegetables on the reduction of oxidative stress, we screened a variety of plant extracts for their ability to upregulate the Nrf2-ARE signaling pathway using an ARE luciferase reporter assay. Of the extracts found to activate the ARE luciferase reporter assay, we selected a high curcumin turmeric extract, a high quercetin-containing extract, and a rosemary extract for further study. All three extracts showed a concentration-dependent response in the reporter assay and when combined, the ARE-mediated luciferase response was greater than expected by simply an additive response of each extract alone. The apparent synergistic effects of the three botanical extracts tested suggest that each of the botanicals may act on a different part of the Nrf2 activation pathway. It also suggests that consuming a variety of plant foods may synergistically activate the Nrf2 pathway to increase the likelihood of healthy longevity.

Materials and Methods

Botanical extracts and reagents

Turmeric (*Curcuma longa* L.; Zingiberaceae) rhizome extract (Batch# OCL3EG1301C01), standardized to 85% total curcuminoids, was purchased from Verdure Sciences; rosemary (*Rosmarinus officinalis* L.; Lamiaceae) leaf extract (Batch# 610036338), standardized to 6% rosmarinic acid, was from Naturex; and pagoda tree (*Strychnos japonicus* L.; Schott, syn. *Sophora japonica* L.; Fabaceae) seed pod extract (Batch# 0100199904), standardized to 95% anhydrous quercetin, was purchased from Novel Ingredient Services. All extracts were authenticated through vendor certification, examination of HPLC chromatograms for phytochemical content, and comparison to published reports. Myrosinase (> 100 U/g) and DL-Sulforaphane (synthetic, > 90%) were purchased from Sigma-Aldrich. All solvents were HPLC grade from Fisher Scientific. MEM, penicillin-streptomycin were purchased from Sigma-Aldrich. All solvents were HPLC certified, examination of HPLC chromatograms for phytochemicals were standardized to 95% anhydrous quercetin, was purchased from Novel ingredient services. Myrosinase (> 100 U/g) and DL-Sulforaphane (synthetic, > 90%) were purchased from Sigma-Aldrich. All solvents were HPLC grade from Fisher Scientific.

Antioxidant response element luciferase reporter assay

To assess activation of Nrf2 in the ARE luciferase reporter assay, a synthetic oligonucleotide containing four repeats of the ARE DNA sequence (5’-GTGACTCAGCA-3’) was hybridized to its complementary oligonucleotide, and the resulting DNA fragment ligated at the SacI/BglII site of a pGL4.27/luc2P/minP/Hygro plasmid (Promega). A stable cell line was generated by transfecting human hepatocellular carcinoma HepG2 cells (ATCC) with the vector using Fugene 6 (Roche) according to the manufacturer’s instructions.

To test the botanical samples, ARE luciferase reporter HepG2 cells were plated (1 × 10⁴ cells/well) in white-walled, clear-bottom, 96-well plates and incubated for 48 h in a humidified, 37°C, 5% CO₂ incubator. The cells were treated with samples at specified concentrations for an additional 48 h. Sulforaphane (10 µM) was used as a positive control. Following incubation, luciferase activity was quantified using a luciferase assay kit (Biotium, Inc.) according to the manufacturer’s instructions. Light emission was read on a SpectraMax M5 spectrophotometer ( Molecular Devices). Relative luminescence values were obtained for all samples and results are expressed as percent response relative to the sulforaphane control.

Statistical analysis

The results of most experiments described here are expressed as mean ± SD values and are representative of three independent experiments. Statistical analysis was carried out by Student’s t-test using PRISM version 6.01 statistical analysis software (GraphPad Software, Inc.).

Chromatographic analysis of botanical extracts

Quercetin (95.5%), rosmarinic acid (99.4%), and curcumin (99.0%) reference standards were obtained from the United States Pharmacopeia. Standards were dissolved in methanol:DMSO (4:1) solution and covered a linear range of approximately 6–500 ppm. Total curcuminoids were calculated as the sum of bis-demethoxycurcumin, demethoxycurcumin, and curcumin. Botanical extract samples were prepared for UPLC analysis by adding approximately 0.4 g of rosemary extract, 0.1 g of pagoda tree seed pod extract, or 0.1 g of turmeric extract to 60 mL of methanol:DMSO (4:1) solution, sonicating for 30 min, and diluting the samples to a final volume of 100 mL. The turmeric and pagoda tree extracts were further diluted 1:3.33 with solvent. Samples were mixed, filtered using a 0.22-µm GV-PVDF Millipore syringe filter (Millipore Corp.), and directly injected into the UPLC system.

Chromatographic separation was performed on a Waters Acquity H-Class UPLC equipped with an Acquity eλ photodiode array detector, monitoring at 280 nm (Waters Corp.). The column used was a Waters UPLC HSS T3, 1.8 µm, 2.1 × 100 mm. The mobile phases used were: (A) 0.2% o-phosphoric acid in water (v/v), (B) methanol, and (C) acetonitrile. The ternary mobile phase gradient for analysis was as follows: initial, 60% A, 25% B, 15% C; 3.2 min, 45% A, 35% B, 20% C; 4.8 min, 20% A, 40% B, 40% C; 5.8 min, 10% A, 45% B, 45% C; 5.81–7.0 min, 50% B, 50% C; 7.01–10.0 min, 60% A, 25% B, 15% C. The column temperature was 25°C and the sample temperature was 20°C.

**Fig. 1** Effect of turmeric, pagoda tree seed pod, and rosemary extracts on ARE responsive luciferase reporter activity. HepG2 cells expressing an ARE responsive luciferase reporter were treated with select concentrations of turmeric (0–8 µg/mL), pagoda tree seed pod (0–30 µg/mL), or rosemary extract (0–40 µg/mL) for 48 h. The level of luminescence produced in response to sulforaphane (10 µM), the positive control, is considered 100% and the sample responses are calculated relative to the sulforaphane response. Results of a representative experiment are reported as mean % of sulforaphane response.
Fig. 2 UPLC chromatograms of rosemary, pagoda tree, and turmeric extracts. Peak identifications are A rosmarinic acid, B quercetin, C bis-demethoxycurcumin, D demethoxycurcumin, and E curcumin.

Fig. 3 Optimization of turmeric, quercetin, and rosemary extract ratio. HepG2 cells expressing an ARE responsive luciferase reporter were treated with select ratios of turmeric, pagoda tree seed pod, and rosemary extracts at a combined concentration of 50 µg/mL for 48 h. The level of luminescence produced in response to sulforaphane (10 µM), the positive control, is considered 100% and the sample responses are calculated relative to the sulforaphane response. Results of a representative experiment are reported as mean % of sulforaphane response.

Fig. 4 Concentration-response curve for the optimized turmeric, quercetin, and rosemary extracts (TQR) botanical blend. HepG2 cells expressing the ARE responsive luciferase reporter were treated with select concentrations of the TQR botanical blend (1:3:5) for 48 h. The level of luminescence produced in response to sulforaphane (10 µM), the positive control, is considered 100% and the sample responses are calculated relative to the sulforaphane response. Results from a representative experiment are reported as mean % response.
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Conflict of Interest

We wish to draw the attention of the Editor to the following fact which may be considered as a potential conflict of interest and to significant financial contributions to this work. All authors were paid employees of Amway Corporation which wholly funded this work.

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