Extracts of Fruits and Vegetables Activate the Antioxidant Response Element in IMR-32 Cells

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Abstract

Background: The biological effects of antioxidant nutrients are mediated in part by activation of antioxidant response elements (AREs) on genes for enzymes involved in endogenous pathways that prevent free radical damage. Traditional approaches for identifying antioxidant molecules in foods, such as total phenolic compound (TP) content or oxygen radical absorption capacity (ORAC), do not measure capacity to activate AREs.

Objectives: The goal of this study was to develop an assay to assess the ARE activation capacity of fruit and vegetable extracts and determine whether such capacity was predicted by TP content and/or ORAC activity.

Methods: Fruits and vegetables were homogenized, extracted with acidified ethanol, lyophilized, and resuspended in growth medium. Human IMR-32 neuroblastoma cells, transfected with an ARE-firefly luciferase reporter, were exposed to extracts for 5 h. Firefly luciferase was normalized to constitutively expressed Renilla luciferase with tertiary butylhydroquinone (tBHQ) as a positive control. TP content and ORAC activity were measured for each extract. Relations between TPs and ORAC and ARE activity were determined.

Results: A total of 107 of 134 extracts tested significantly activated the ARE-luciferase reporter from 1.2- to 58-fold above that of the solvent control (P < 0.05) in human IMR-32 cells. ARE activity, TP content, and ORAC ranked higher in peels than in associated flesh. Despite this relation, ARE activity did not correlate with TP content (Spearman r = 0.05, P = 0.57) and only modestly but negatively correlated with ORAC (Spearman r = −0.24, P < 0.01). Many extracts activated the ARE more than predicted by the TP content or ORAC.

Conclusions: The ARE reporter assay identified many active fruit and vegetable extracts in human IMR-32 cells. There are components of fruits and vegetables that activate the ARE but are not phenolic compounds and are low in ORAC. The ARE-luciferase reporter assay is likely a better predictor of the antioxidant benefits of fruits and vegetables than TP or ORAC.

Keywords: IMR-32 cells, Nrf2, ORAC, antioxidant response element, polyphenols, total phenolic compounds

Introduction

The transcription factor Nuclear factor erythroid 2–related factor 2 (NRF2) and its negative regulator Kelch-like ECH-associated protein 1 (KEAP1) modulate the activation of cis-acting regulatory antioxidant response elements (AREs) in genes coding for enzymes involved in endogenous pathways that protect against free radical damage, such as glutathione peroxidase, superoxide dismutase (SOD), catalase, thioredoxin, heme oxygenase 1 (HMOX1), NAD(P)H:quinone oxidoreductase (NQO1), and glutamate cysteine ligase, modifier subunit (GCLM) (1). Antioxidants in foods are traditionally thought to act by trapping free radicals. However, the structures and properties of many of these molecules suggest that they are poor free radical scavengers and may even function as weak

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3 Supplemental Tables 1 and 2 and Supplemental Figure 1 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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pro-oxidants (electrophiles) that can activate the ARE pathway in cells. 

The binding of NRF2 to the ARE is redox sensitive. Cellular concentrations of NRF2 are regulated by a cytosolic protein complex [Cullin3 (CUL3)–KEAP1–ubiquitin E3 ligase] that binds to NRF2 and facilitates the CUL3-mediated ubiquitylation of NRF2, which marks NRF2 for proteasome-dependent degradation, thereby limiting cellular concentrations of NRF2 (2). However, when the reactive cysteine residues of KEAP1 are oxidized by electrophiles, KEAP1 dissociates from CUL3, and NRF2 is not ubiquitylated. NRF2 accumulates and translocates to the nucleus, where it heterodimerizes with the musculoaponeurotic fibrosarcoma oncogene homolog and binds to and transactivates ARE-containing genes (3). Many of the biological effects of what are designated antioxidant nutrients are mediated in part by their ability to oxidize cysteine residues on Keap1, thereby leading to activation of the ARE, with the resulting activation of endogenous antioxidant defense enzymes.

Traditional approaches for identification of antioxidants in foods have not directly assessed the ability to activate the ARE, but rather have identified a certain structure (e.g., polyphenol) or assessed ability to absorb oxygen radicals [oxygen radical absorption capacity (ORAC)]. The protective effects of polyphenols in human health were traditionally ascribed to their free radical scavenging properties, which prevent cellular damage to organelles, membranes, and DNA (4). A number of polyphenols also activate the ARE in mammalian cells and animal models (5–13), but polyphenols are not the only food constituents with the capacity to activate the ARE. At this time, it is not clear whether the ability to scavenge free radicals or the ability to activate ARE-dependent endogenous antioxidant responses is the property of an antioxidant that is more relevant to human health. We suggest that it is more appropriate to measure the ability to activate the ARE when attempting to identify the antioxidant properties of foods.

We tested the potential efficacy of multiple fruit and vegetable cultivar extracts for activation of an ARE-firefly luciferase reporter in transiently transfected human IMR-32 neuroblastoma cells. We also assessed total phenolic compound (TP) content and ORAC of these extracts and determined whether there was a correlation.

**Methods**

Efficiency of extraction of polyphenols from fruits and vegetables is affected by temperature, pH, solvent polarity, and the percentage modifier (alcohol) used (14). Because no single solvent is optimal for all of the fruits and vegetables we studied, we used an ethanol/HCl solvent that worked well on a variety of matrices, and was least toxic to cultured cells should traces remain. Extraction of TPs into 35% ethanol/HCl was 88–90% as efficient as extraction into 70% ethanol/HCl. One part of fresh, washed fruit or vegetable flesh or peel was diced into small pieces, added to 2 parts 35% ethanol/67.5 mM HCl (0.33 g/mL), and homogenized at medium speed in a Waring LB10 blender (Waring Commercial) for 2–5 min. Homogenates were centrifuged at 14,000 × g for 10 min and the supernatant was subjected to centrifugation at 14,000 × g for 10 min and the supernatant was retained for assay. Polyphenols and tertiary butylhydroquinone (tBHQ) activate endogenous antioxidant genes and also activate ARE reporter constructs in human IMR-32 neuroblastoma cells (11, 15–17). IMR-32 cells (ATCC) were grown and maintained in MEM (Life Technologies) containing 10% heat-inactivated FBS (hiFBS; Sigma), 1% nonessential amino acids (MEM NEAA; Life Technologies), 1 mM pyruvate (Hyclone), and 100 U/mL penicillin-streptomycin (Life Technologies). Cells were grown to ~100% confluence, trypsinized, washed twice with OptiMEM/5% hiFBS/1% NEAA, and resuspended in the same. Cells were counted and plated onto 96-well plates (BD Falcon; 353377) at 1.2 × 10^4 viable cells per well in a 100 µL volume of OptiMEM/5% hiFBS/1% NEAA. Plates were incubated at 37°C/5% CO2 overnight for 5 h and then transfected overnight with 100 ng/well (5 µL) of a reporter construct containing an ARE driving expression of a firefly luciferase reporter gene (Cignal Reporter Assay Kit; CCS-5020L; Qiagen) with the use of Fugene 6 (Promega) at a ratio of 3:1 (microliter Fugene 6 per microgram DNA). Triplicate wells of IMR-32 cells were treated for 5 h with 5 µL volumes of a solvent blank and the positive control tBHQ at concentrations ranging from 0.1 to 20 µM. tBHQ induces accumulation of NRF2 and activates the ARE-firefly luciferase reporters in these cells (15, 16). Treatment of cells at a 1× concentration is roughly equivalent to a 0.33 serving of original fruit or vegetable in humans, assuming an average serving of ~225 g, a plasma volume of 5 L, and 100% absorption and exposure, the plasma concentration would be 45 g/L. We delivered 5 µL of 1× solution (0.33 gm/mL) in a 110 µL final volume (15 g/L). Extracts were tested in triplicate at 1× concentrations (see above) in a 5 h incubation period. Extracts exhibiting toxicity at 1× were retested at higher dilutions. After treatment, cells were lysed with Dual-Glo (Promega) reagent for measurement of firefly luciferase activity, which emits a luminescent signal read, on a Synergy 2 plate reader (BioTek) with the use of Gen5 software. The transfected cells constitutively expressed a Renilla luciferase that was unaffected by ARE activators and was used to normalize for well-to-well variability in cell number and transfection efficiency. A standard curve of normalized firefly luciferase luminescence vs. micromolar tBHQ was constructed, from which normalized firefly luminescence signals for each extract were interpolated to picomole equivalents of tBHQ and expressed per microliter extract. This value is referred to as the ARE activity. Testing of all 134 extracts, with triplicate solvent controls conducted on each 96-well plate, was performed in 8 separate experiments. This was an exploratory study designed to determine which extracts would activate reporter expression vs. the solvent control, and how that expression might correlate with TPs or ORAC. The study was not designed to compare efficacy of extract-induced ARE activities. Data analyses were performed on log-transformed data to account for unequal variances as determined by a Bartlett’s test. A 1-factor ANOVA was used to compare the ARE activity of each individual extract to the solvent-only controls on each plate, and a Dunnett’s multiple comparisons test was performed to compare each treatment to the solvent control. A paired t test was done to compare ARE activities of triplicates vs. the corresponding solvent control, P ≤ 0.05 was considered to be significant. Paired t tests were done in Microsoft Excel (v14.4.8), whereas ANOVA analyses and Dunnett’s tests were performed with GraphPad Prism 5 software (v5.04).

For gene expression assays, IMR-32 cells were grown to ~100% confluence, trypsinized, washed twice with OptiMEM/5% hiFBS/1% NEAA, and resuspended in the same. Cells were counted and plated onto 12-well plates (BD Falcon; 353377) at 3 × 10^4 viable cells per well in a 1 mL volume of OptiMEM/5% hiFBS/1% NEAA. Plates were incubated at 37°C/5% CO2 overnight, and then treated for 5 h with 1× concentrations of solvent control, 10 µM tBHQ, or 1× concentrations of pineaple, green pear peel, or green lettuce extract in triplicate wells. After 5 h, the medium was aspirated and total RNA isolated with the use of an RNeasy Mini Kit (catalog no. 74104; Qiagen). cDNA was prepared from 300 ng total RNA with the use of a High Capacity Reverse Transcription Kit (part no. 4368813; Life Technologies). Gene expression was measured by real-time PCR with the use of TaqMan assays from Life Technologies for NQO1 (Hs01045994_m1), HMOX1 (Hs0110250_m1), and others.
FIGURE 1 Extracts of fruits and vegetables activate AREs in human IMR-32 cells. Transfected human IMR-32 cells were treated for 5 h with extract and then analyzed for activation of ARE reporter activity. The top 20 active extracts included a variety of fruits and vegetables with ARE-activating activities ranging from 21 to 70 pmol tBHQ equivalents/µL extract (equivalent to 1.67 mg original cultivar) (Figure 1, inset). Avocado peel, carrot, red pear peel, pineapple, lemon flesh, green pear peel, and red delicious apple peel gave the highest responses. The rank order is a rough approximation because extraction efficiency is a function of solvent polarity and hence could vary with the cultivar, and because only a single cultivar was used for each extract. However, the goal here was to determine whether ARE activation correlates with TP content and ORAC within a given extract. In 9 of 12 cases, peel extracts were more active than the corresponding flesh extracts, as indicated by activity ratios >1 for peel vs. flesh from among the top 20 (Figure 2). ARE activities of peels and the corresponding flesh were significantly different (P ≤ 0.05) for all extracts shown except for Granny Smith apple (not significantly different).

**Gene expression.** Pineapple, green lettuce, green pear peel extracts, and tBHQ activated expression of the endogenous ARE-driven genes NQO1 (P ≤ 0.001), HMOX1 (P ≤ 0.01), and GCLM (P ≤ 0.05) relative to control cells treated with 0.125× solved these issues in most cases, and data were normalized to a 1× concentration. The 1× concentration represents ~0.33 serving, assuming 100% exposure in humans, which is not always the case because of differences in absorption, distribution in the body, metabolism, and excretion properties. Because only metabolism would be an issue in vitro, the cell exposure in this study is likely higher than the actual human exposure would be.

**ARE activation.** Of 134 extracts tested, 107 significantly activated the ARE (P ≤ 0.05) vs. the solvent control (1.2 ± 0.1 pmol tBHQ equivalents/µL, mean ± SEM) (Supplemental Table 1). The top 80 extracts significantly stimulated ARE activity (P ≤ 0.01) vs. the solvent control (Figure 1). Figure 1 displays the collated results from several independent experiments and the mean solvent control activity from all experiments. Supplemental Figure 1 displays an additional 27 extracts that activated ARE activity (P ≤ 0.05), whereas 27 extracts were found to be inactive relative to solvent control (Supplemental Table 2). The top 20 active extracts included a variety of fruits and vegetables with ARE-activating activities ranging from 21 to 70 pmol tBHQ equivalents/µL extract (equivalent to 1.67 mg original cultivar) (Figure 1, inset). Avocado peel, carrot, red pear peel, pineapple, lemon flesh, green pear peel, and red delicious apple peel gave the highest responses. The rank order is a rough approximation because extraction efficiency is a function of solvent polarity and hence could vary with the cultivar, and because only a single cultivar was used for each extract. However, the goal here was to determine whether ARE activation correlates with TP content and ORAC within a given extract. In 9 of 12 cases, peel extracts were more active than the corresponding flesh extracts, as indicated by activity ratios >1 for peel vs. flesh from among the top 20 (Figure 2). ARE activities of peels and the corresponding flesh were significantly different (P ≤ 0.05) for all extracts shown except for Granny Smith apple (not significantly different).

**Results**

**Toxicity of extracts.** A tBHQ concentration-response curve was conducted on each plate, yielding a mean half maximal effective concentration of 0.8 ± 0.2 µM (22% CV). Ideally, half maximal effective concentrations would have been determined for each extract; however, cells would invariably detach at higher concentrations before a maximal effect was achieved. Extracts were tested at a concentration of 1×, and then retested at a lower dose if there were any apparent toxic or inhibitory effects. Toxicity was evident in highly viscous extracts, and in some cases highly pigmented extracts quenched the luminescence signal. Some extracts caused the cells to clump together in the 5 h incubation period, suggesting that the cells were stressed. Retesting of extracts diluted to between 0.5× and 3× solved these issues in most cases, and data were normalized to a 1× concentration. The 1× concentration represents ~0.33 serving, assuming 100% exposure in humans, which is not always the case because of differences in absorption, distribution in the body, metabolism, and excretion properties. Because only metabolism would be an issue in vitro, the cell exposure in this study is likely higher than the actual human exposure would be.

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HMOX1

Green pear peel–induced expression was on the order of correlation between TP content and ARE stimulation activity, TPs, and ORAC, there was no (Tables 1 and 2). Despite similar peel vs. flesh relations among extracts ranked higher in peels than in flesh (Supplemental activity (micromole trolox equivalents per 100 grams) of the (milligrams gallic acid equivalents per 100 grams) and ORAC TP content. ORAC activity (micromole trolox equivalents per 100 grams) of the activation in IMR-32 cells.

Interestingly, the target genes varied in response to treatments, although significant and negative correlation of ARE stimulation (Figure 3A). There was a weak albeit significant and negative correlation of ARE stimulation activity with ORAC (Spearman $p = -0.24, P < 0.01$) (Figure 3B). It was evident that there were extracts with low TPs and ORAC but with high ARE stimulation activity. These data strongly suggest that TPs and ORAC are poor predictors of the cellular antioxidant response to fruit and vegetable extracts.

Discussion

We used a standard solvent system designed to extract activating molecules from a variety of cultivars. Because changing solvent polarity could affect the yield of activating molecules, and because a single cultivar was used in preparation of each extract, we are unable to definitively compare the activating potential of the fruits and vegetables we studied. However, the goal of the study was to determine whether TP content and ORAC correlate to the ARE activation activity within each extract. We identified a large number of extracts of fruits and vegetables that activated the ARE reporter in human IMR-32 neuroblastoma cells. A few extracts and tBHQ were tested alongside a solvent control in untransfected IMR-32 cells to demonstrate expression of endogenous ARE-driven genes. NQO1, HMOX1, and GCLM were activated to different extents by tBHQ and pineapple, green lettuce, and green pear peel extracts, suggesting that regulation is somewhat gene dependent (Table 1). These data suggest that there are other promoter elements besides the ARE that regulate expression of these genes. Although the ARE reporter lacks these other promoter elements, it appears to be a useful proxy for predicting endogenous responses to these extracts. In addition, the ARE reporter assay had a larger dynamic range than the endogenous gene expression, making it more useful for discriminating among the various extracts.

ARE reporter activity was significantly higher in peels than in flesh ($P < 0.02$), and TP content and ORAC activity also ranked higher in peels than in flesh. This is consistent with the protective role of these molecules and agrees with the higher TP and flavonoid concentrations reported in, e.g., apple peels vs. flesh (19). Interestingly, traditional TP content and ORAC assays failed to predict significant activation of the ARE in IMR-32 cells in response to many of the extracts. Our data are consistent with the previous observation that chemical antioxidant capacity indexes poorly predict biological antioxidant performance in cells (20). The ORAC activity assay we used measures the ability

![FIGURE 2](https://example.com/figure2.png)

**FIGURE 2** Peel-to-flesh ratio of ARE activities from the top 20 extracts in human IMR-32 cells. Ratios were obtained by dividing the mean ARE activity of the peel by the mean ARE activity of the corresponding flesh ($n = 3$ independent measurements for each). ARE stimulation activity in peel and flesh differed, $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$. The dash indicates a ratio of $\leq 1$; n.s. indicates peel not significantly different from flesh, $P > 0.05$. ARE, antioxidant response element.

**TABLE 1** Endogenous gene expression and ARE activity in human IMR-32 cells treated with extracts or tBHQ

<table>
<thead>
<tr>
<th>Extract</th>
<th>NQO1 $\pm$ SEM</th>
<th>HMOX1 $\pm$ SEM</th>
<th>GCLM $\pm$ SEM</th>
<th>ARE activity $\pm$ SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent control</td>
<td>1.0 $\pm$ 0.1a</td>
<td>1.0 $\pm$ 0.2a</td>
<td>1.0 $\pm$ 0.1a</td>
<td>1.0 $\pm$ 0.1</td>
</tr>
<tr>
<td>Pineapple</td>
<td>2.2 $\pm$ 0.2***</td>
<td>2.0 $\pm$ 0.1***</td>
<td>1.5 $\pm$ 0.1**</td>
<td>45 $\pm$ 6***</td>
</tr>
<tr>
<td>Green lettuce</td>
<td>8.3 $\pm$ 1.2***</td>
<td>13 $\pm$ 2***</td>
<td>2.7 $\pm$ 0.3***</td>
<td>28 $\pm$ 4***</td>
</tr>
<tr>
<td>Green pear peel</td>
<td>8.4 $\pm$ 1.3***</td>
<td>17 $\pm$ 1***</td>
<td>3.5 $\pm$ 0.3***</td>
<td>41 $\pm$ 1***</td>
</tr>
<tr>
<td>10 $\mu$M tBHQ</td>
<td>13 $\pm$ 1***</td>
<td>51 $\pm$ 6***</td>
<td>7.7 $\pm$ 0.4***</td>
<td>190 $\pm$ 3***</td>
</tr>
</tbody>
</table>

$^1$ Values are means ± SEMs, $n = 3$ per treatment. Labeled means in a row without a common letter differ significantly, $P \leq 0.05$. Means in a column labeled with asterisks differ significantly from the solvent control, $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$. ARE, antioxidant response element; GCLM, glutamate-cysteine ligase, modifier subunit; HMOX1, heme oxygenase 1; NQO1, NAD(P)H:quinone oxidoreductase 1; tBHQ, tertiary butylhydroquinone.
of an extract to absorb peroxyl radicals generated by a free radical initiator in vitro. Thus, whereas ORAC is a measure of chemical antioxidant capacity, it does not reflect the ability of weak pro-oxidants to oxidize the sulfhydryl residues of KEAP1 cysteines, leading to the release of NRF2 from the CUL3–KEAP1 ubiquitin E3 ligase complex, and subsequent activation of ARE-containing antioxidant genes. The same is true for TP content, which measures a mixture of compounds variously classified as antioxidants, weak pro-oxidants, or both, as in the case of resveratrol (21).

Pro-oxidant molecules may not be the only components of fruits and vegetables that are capable of activating ARE-stimulated pathways. There are KEAP1-independent mechanisms that regulate NRF2, including phosphorylation by protein kinases (22). Several NRF2 activators, including tBHQ, have been shown to stimulate kinases, which activate the MAPK/extracellular signal-regulated kinase pathway, leading to phosphorylation and stabilization of NRF2 (23). The phytochemical nordihydroguaiaretic acid stabilizes NRF2 via activation of extracellular signal-regulated kinases 1/2, p38, c-Jun NH2-terminal protein kinase, and phosphoinositide-3 kinase pathways and by inhibition of glycogen synthase kinase-3β (10). Baicalein, a flavanoid found in the traditional Chinese medicinal herb Scutellaria baicalensis, induced NRF2 accumulation and expression of HMOX1 via the protein kinase Co and phosphoinositide-3 kinase/protein kinase B signaling pathway (24). In the present study, the ARE reporter activity of any particular plant extract would represent the sum of KEAP1-mediated and KEAP1-independent effects, and it is therefore a more relevant measure of the beneficial effects of these extracts than TP or ORAC activity. The importance of this pathway for human health is borne out in numerous rodent- and cell-based studies. For example, curcumin attenuated liver injury induced by dimethylnitrosamine via NRF2-mediated upregulation of heme oxidae 1 gene expression (25). A pomegranate emulsion reduced hepatocarcinogenesis in rats exposed to the carcinogen diethylnitrosamine by upregulation of the NRF2 protein and several of its target genes, including Nqo1 and glutathione S-transferase α2 (Gsta2) (26). Also, NRF2 has a role in protection against alcohol-induced liver failure, as demonstrated in Nrf2 knockout mice (27). The ARE pathway activator sulforaphane, which is high in several cruciferous vegetables that were tested (red cabbage, 3.2; green cabbage, 1.4; broccoli, 4.3; Brussels sprouts, 2.6 pmol tBHQ equivalents/µL ARE stimulation activity), and cinnamic aldehyde conferred significant renal protection in streptozotocin-diabetic mice but not in Nrf2 knockout mice (28). Overexpression of Nrf2 in primary cortical neurons prevented ethanol-induced depletion of glutathione and prevented ethanol-related apoptotic death (29), suggesting an important role for this pathway in the brain.

Future studies are planned to determine whether this in vitro assay predicts ARE activation in humans after oral administration of extracts with high in vitro capacity to activate the ARE.

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