RESEARCH ARTICLE

Coffee, broccoli and spices are strong inducers of electrophile response element-dependent transcription *in vitro* and *in vivo* – Studies in electrophile response element transgenic mice

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Scope: Cytoprotective gene products, *e.g.* phase II – and antioxidant enzymes, are important in cellular redox homeostasis. A common feature of these genes is binding sites for transcription factor nuclear factor erythroid-2-related factor 2 (Nrf2), named electrophile response elements (EpREs) within their promoters.

Methods and results: To identify dietary bioactive compounds and foods with Nrf2/EpRE inducing properties in an intact organism, we utilized transgenic mice encoding luciferase under control of EpRE from the thioredoxin promoter. We found that 18 of 31 phytochemicals and 10 of 14 dietary plant extracts induced EpRE activity in liver HepG2 cells. Surprisingly, some dietary plant extracts showed profound inducing capability as compared to pure compounds indicating combinatorial effects of compounds found in whole foods. Furthermore, intraperitoneal injections of carnosol, curcumin and *tert* benzohydroquinine induced EpRE-dependent promoter activity in transgenic mice. In further experiments with curcumin, we found highly induced EpRE activity in intestine, liver, kidney and spleen. Finally, a combination extract made of coffee, thyme, broccoli, rosemary, turmeric and red onion fed orally, induced EpRE mediated luciferase in lung and adipose tissue.

Conclusion: These results show that plant-based foods contain compounds that can be absorbed and induce the antioxidant defence in a living organism in an organ-specific manner.

Keywords:
Cytoprotective proteins / Electrophile response element / *In vivo* imaging / Phytochemicals / Transgenic mice

1 Introduction

A diet rich in plant-based food protects against various chronic diseases such as cancer [1], but the biochemical processes involved in the protective effects are not completely understood. In addition to important nutrients, dietary plants contain non-nutrient compounds such as phytochemicals [2]. The biological actions of phytochemicals have been attributed to their antioxidant properties, either through their reducing capacity *per se* or their influence on intracellular redox status. Antioxidant activity is, however, unlikely to explain all cellular effects as phytochemicals are conjugated and metabolized *in vivo*, leading to alteration of their redox potential [3]. Phytochemicals may also exert their effect by modulating cell signaling pathways and gene expression [4]. In recent years,

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Abbreviations: AUC, area under the curve; **EpRE,** electrophile response element; **EpRE-luc,** EpRE-luciferase; **i.p.,** intraperitoneally; **luc,** luciferase; **Nrf2,** nuclear factor erythoid-2-related factor 2; **Trx,** thioredoxin; ***t*-BHQ,** *tert* benzohydroquinine

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evidence has accumulated indicating that the beneficial action of phytochemicals is due, at least in part, to induction of cytoprotective proteins (also referred to as phase II enzymes) [5]. Induction of a number of these genes is controlled primarily by nuclear factor erythroid-2-related factor 2 (Nrf2), a leucine zipper (bZIP) transcription factor mediating induction of detoxification and antioxidant genes that contain an electrophile response element [EpRE, 5′-(A/G)TGACNNNGC(A/G)-3′] within their promoters [6]. The activation of Nrf2 is normally repressed by the inhibitory factor Kelch-like ECH-associated protein 1 which facilitate degradation of Nrf2 [7]. Electrophile agents modify Kelch-like ECH-associated protein 1 and prevent it from targeting Nrf2 for degradation [8]. Accumulation of Nrf2 enhance heterodimer formation with other bZIP proteins and binding to *cis*-elements known as EpRE thereby transactivating target genes such as GST, NAD(P)H:quinone oxidoreductase 1 (NQO1), catalase and thioredoxin (Trx) [9].

We have previously shown that phytochemicals can increase GSH by increasing the expression of the GSH synthesizing enzyme, γ-glutamylcysteine synthetase, through potential EpRE motifs [10]. Trx plays an important role in cellular processes *via* redox regulation of the two cysteine residues in its active site. The GSH and Trx systems have overlapping functions in thiol/disulfide redox control in both the cytoplasm and the nucleus [11]. Both are essential for mammalian life as evidenced by embryonic lethality in γ-glutamylcysteine synthetase – and Trx – knockout mice [12, 13].

Throughout the years, we have developed numerous transgenic mice for optical imaging in the exploration of gene regulation [14–18]. In this report, we utilize EpRE-luciferase (EpRE-luc) mice, a valuable tool to study EpRE-dependent transcription of antioxidant defense genes in a living organism.

We have investigated the ability of phytochemicals with different chemical structures and dietary plant extracts to regulate EpRE-dependent transcription in cell culture and in transgenic reporter mice *in vivo*. Our selection of dietary plant extracts is based on their content of phytochemicals from our cell screening of pure compounds.

Induction of EpRE-regulated signaling may contribute to protection against oxidative stress. Thus our study can strengthen the knowledge of how dietary phytochemicals and plant-based foods work at a mechanistic level, with emphasis on their ability to regulate gene expression.

2 Materials and methods

2.1 Cell culture

HepG2, human hepatocellular carcinoma cells were purchased from American Type Culture Collection (ATCC No. HB-8065) (Rockville, MD). Cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 U/mL), streptomycin (50 mg/mL), L-glutamine (2 mM), non-essential amino acids (0.01%) and sodium pyruvat (1 mM). Cells were cultured at 37°C in a humidified incubator containing 5% CO_2. For transfection, a previously described EpRE-luc plasmid was used, showing a nearly 30-fold increase in luc activity when cotransfected with Nrf1 expression vector, indicating functional EpRE elements within the construct [19]. Cells were plated in 22 mm tissue culture wells the day before transfection at a density of approx. 70% confluence, and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) with DNA concentrations of 1.6 μg in 100 μL Lipofectamine according to manufacturer's instruction. Following the transfection, the culture medium was replaced with fresh growth medium before adding phytochemicals, plant extracts or corresponding controls. Luc activity was measured after 17 h. When testing kinetics of EpRE activity, cells were harvested and luc activity was measured at 0, 2, 6, 10, 17 and 24 h. For the RNA analysis, cells were incubated with indicated phytochemicals or 0.1% DMSO (control cells) for 17 h and RNA was extracted for quantitative real-time PCR analysis.

2.2 Quantitative mRNA analysis

Total RNA was extracted from cells with Magnapure RNA Isolation Kit, High Performance and cDNA synthesis was performed with Omniscript kit (Qiagen, Hilden, Germany) according to the manufacture's instructions. Quantitative real-time PCR was performed according to Myhrstad *et al.* [10]. The PCR conditions were 4 mM $MgCl_2$ for Trx and GAPDH (endogen control), 10 pmol of each primer and annealing at 60°C (Trx primers: F; 5′-CTG CTT TTC AGG AAG CCT TG-3′ R; 5′-TTG GCT CCA GAA AAT TCA CC-3′, GAPDH primers: F; 5′-TCA TCA ACG GGA AGC CCA TCA CCA TCT TC–3′ R; 5′-GTC TTC TGG TTG GCA GTA ATG GCA TGG ACT-3′). Gene expression values were quantified based on the ΔΔCt method with data normalized to DMSO-treated cells (controls). Briefly, Ct values for the target gene were normalized against Ct values of GAPDH housekeeping gene (= ΔCt) in both phytochemical- and DMSO-treated cells. ΔΔCt was then calculated as ΔCt for phytochemical-treated cells minus ΔCt for control cells. The fold increase in gene expression was calculated as 2^ΔΔCt.

2.3 Luc activity

Luc activity was measured in the cell lysates according to the manufacturer's protocol (Promega, Madison, WI, USA). Briefly, the cell medium was removed and 300 μL lysis buffer was added, and cells were incubated in 4°C for 20 min. The lysate was then centrifuged to remove cell debris. Luc activity was measured by adding 100 μL Luc assay solution (Promega) to 20 μL of the lysate and the luminescence was detected in a TD 20/20 luminometer (Turner Design, Sunnyvale, CA, USA). Luc activity in

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homogenates of organs was measured and related to protein content as described previously [14].

2.4 EpRE-luc reporter mice and fibroblasts

The mice were housed in accordance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA), and the permission number for these experiments at the University of Oslo is 01.04. All animal experiments were performed according to national guidelines for animal welfare, and the mice had access to water and regular chow *ad libitum*, unless otherwise stated. Transgenic reporter mice were generated as described previously [14] using the EpRE-luc plasmid containing two EpRE elements from the Trx promoter in front of the luc gene [19]. A *Kpn*I fragment from pNI-CD containing two copies of b-globin ''core'' fragment in AseI site of pNI were inserted into both *Kpn*I site and blunted *Bam*HI site of the pEpRE-luc to make transgene flanked with insulator core element as described elsewhere [20, 21].

The transgenic mice are inbred to be homozygote for the transgene. The mice used were fasted 3 h prior to oral gavage of 300 μL extract or vehicle control. Male mice were used in all experiments except carnosol given *per os*.

In order to obtain skin fibroblasts from EpRE-luc mice, a small piece was cut off from the outer ear of an anaesthetized mouse and cleansed with DMEM and then further in 75% ethanol. The ear pieces were cultivated in 1:1 DMEM containing L-glutamine (2 mM), and penicillin (50 U/mL) and streptomycin (50 mg/mL) and fetal calf serum in a cell culture flask for proliferation of fibroblasts from the skin fragments. After 2 to 3 weeks, fibroblasts covered a significant portion of the culture flask. The ear pieces were removed and the medium changed to DMEM supplemented with L-glutamine, penicillin and streptomycin (concentrations as above), and 10% FCS. At confluence, the cells were split 1:3 and for the experiments described, primary fibroblasts between passages 3 and 10 were used. EpRE-luc activity in the skin fibroblasts was measured 17 h after treatment with phytochemicals using the IVIS 100 Imaging System (Caliper Life Sciences, Hopkinton, MA, USA). Light emission was collected for 1 min, 4 min after addition of 0.2 mg D-luciferin. The number of photons emitted from each well was calculated using Living Image Software version 2.50 (Caliper Life Sciences) and quantified using Living Image Software version 2.50 (Caliper Life Sciences) and expressed as photons/s/cm^2/steradian (Sr).

2.5 *In vivo* imaging

Mice were anesthetized with isoflurane (2.5%) and placed in a light sealed imaging chamber. Throughout the imaging period isoflurane anesthesia was maintained inside the imaging chamber. D-luciferin (Biosynth, Staad, Switzerland) (4 mg (~150 mg/kg)) in 200 μL PBS was injected intraperitoneally (i.p.). After, 7 min the mice were imaged for 1 min on the ventral side. Imaging and quantification was performed as described above.

2.6 Dietary phytochemicals

All phytochemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) except carnosol (Cayman Chemical, Ann Arbor, MI, USA), α-tocopherol (Supelco, Bellafonte, PA, USA) and γ-tocopherol (Calbiochem, San Diego, CA, USA).

Stock solutions were prepared in DMSO for cell experiments except α-tocopherol and γ-tocopherol, which were dissolved in ethanol. Cells were treated with the indicated concentration of agent, with matching controls containing an equivalent volume of DMSO (0.1% v/v). Phytochemicals for mice experiments were dissolved in corn oil (curcumin; 12.5 mg/mouse i.p., *tert* benzohydroquinine (*t*-BHQ); 5 mg/mouse i.p., carnosol; 5 mg/mouse, i.p and gavage).

2.7 Dietary plant extracts

Dietary plants were obtained from local grocery stores in Oslo. All dietary plants were pulverized and concentrated by use of water/methanol (50:50, v/v) as described previously [22]. Concentrated extracts were diluted in water/corn oil (for mice) or DMSO and/or PBS ($\leq$0.2% DMSO) (for cells), and thereafter sterile filtered (for cells) and stored under argon gas in airtight tubes at −70°C.

2.8 Presentation of data and statistical analysis

The effects of the treatments were expressed relative to the control, which was set as 1. The fold inductions are presented as means ± SEM of independent cell experiments each run in triplicates. One-way ANOVA was used to examine possible effects of phytochemicals or extracts on EpRE activity in HepG2 cells. Differences were identified using Dunnett's comparisons. Data from RNA analysis and fibroblasts were compared to their controls by Student's *t*-test. Area under curve (AUC) for each mouse was calculated and compared between the two groups, using Mann–Whitney *U* test for all experiments in Figs. 5 and 6. For comparison of AUC in Fig. 7, Student's *t*-test was used due to a larger *n* and normally distribution was observed.

3 Results

3.1 Phytochemicals induce EpRE activity *in vitro* – time- and dose-dependent induction

In our attempt to identify and compare bioactive plant compounds with EpRE-inducing properties, we initially

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tested a number of inducers of EpRE-dependent transcription for their kinetics. HepG2 cells transiently transfected with an EpRE-luc construct were incubated with the indicated phytochemicals (final concentration 10 μM). As shown in Figs. 1A and B, the EpRE activity was induced in a time-dependent manner with significant increases from 10 h of incubation. Sulforaphane and carnosol increased EpRE activity exponential up to 24 h, whereas curcumin and quercetin reached a maximum EpRE activity at 10 and 17 h, respectively. Based on these experiments, 17 h incubations were used for later experiments.

We next tested whether some of these phytochemicals could also influence the mRNA level of Trx in HepG2 cells (Fig. 1C). We observed increased relative mRNA levels of Trx using RT-PCR as related to the housekeeping gene GAPDH, after 17 h of stimulation with quercetin (average fold induction 2.0 ± 0.8, $p = 0.19$), curcumin (3.63 ± 1.13, $p = 0.02$), genistein (2.75 ± 0.67, $p = 0.01$), sulforaphane (3.04 ± 1.03, $p = 0.04$) and synthetic *t*-BHQ (3.74 ± 0.93, $p = 0.03$). This indicates that these phytochemicals can also activate the intact promoter of Trx.

For comparison of bioactive plant compounds with EpRE-inducing properties, we screened a number of plant compounds and dietary plant extracts. HepG2 cells transfected with EpRE-luc construct were stimulated for 17 h, and as shown in Fig. 2, there was a large variation in the different compounds' ability to induce EpRE-dependent transcription. Of the 31 phytochemical tested, 18 compounds showed inducing effect by at least one concentration. Over all, the best inducers were carnosol, curcumin and sulforaphane, inducing by 14.2 ± 3.4, 10.9 ± 1.9, 9.0 ± 0.9-fold compared to control cells at 10 μM, respectively. Moreover, the synthetic phenolic compound *t*-BHQ also showed a strong inducing ability. Furthermore, quercetin, genistein, eugenol and the coffee diterpenes kahweol and cafestol also potently induced EpRE-luc. Interestingly, the carotenoid lycopene was also among the strong inducers.

In addition, a wide range of phytochemicals that we tested did not show the ability to induce EpRE-luc activity at any of the concentration tested. Resveratrol somewhat induced EpRE, but failed to reach statistical significance (ANOVA $p = 0.064$, Dunnets *post-hoc* test $p = 0.034$ for 2.0-fold induction at 25 μM).

Extracts of different food items were also tested for their abilities to induce EpRE-dependent transcription. Of the 14 dietary extracts tested, ten significantly induce EpRE activity at one or more of the concentrations tested (Fig. 3). Extract of coffee (medium roasted Arabica beans) was an exceptionally potent inducer of EpRE activity with 23.6 ± 3.0 and 17.7 ± 2.0 fold change at 30 and 15 mg/mL, respectively, as compared to control cells. Also extracts of broccoli, cocoa and red onion significantly induced EpRE-luc activity. In addition, spices like turmeric, rosemary, thyme, oregano and clove exhibited a remarkably strong ability to induce EpRE-dependent transcription. Dietary plants generally contain ~90% water, except dried samples such as spices. Thus, a final concentration of 30 mg/mL broccoli and red onion corresponds to approximately 3 mg/mL spices, in terms of dry weight.

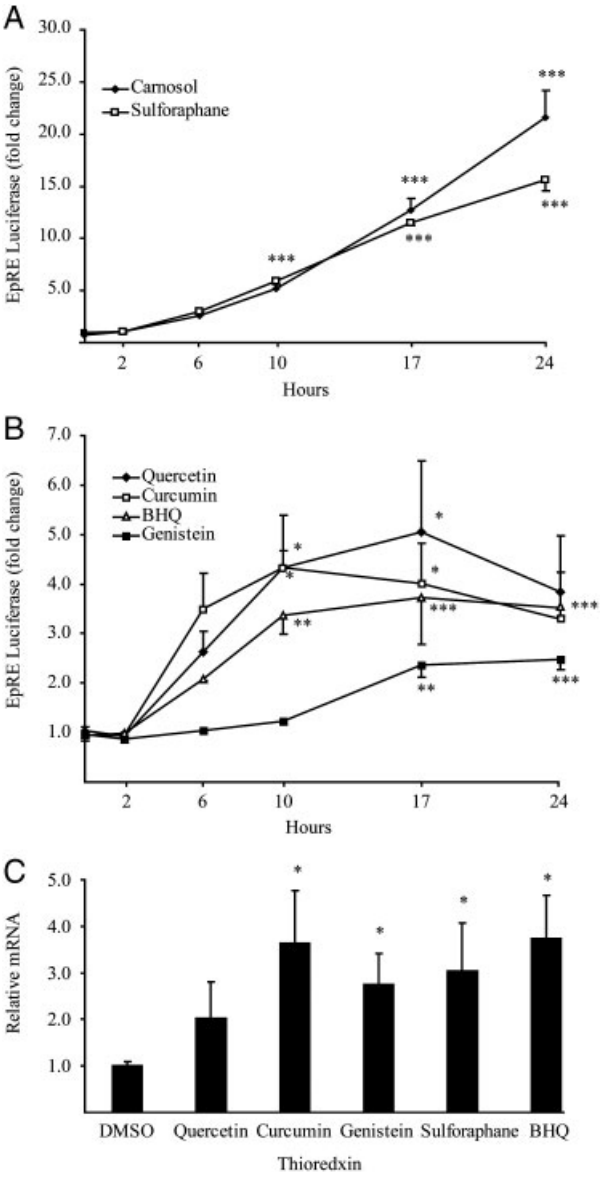

Figure 1. Time-course induction of EpRE-dependent transcription of phytochemicals. (A) HepG2 cells transiently transfected with EpRE-luc were incubated with 10 μM of carnosol, sulforaphane or 0.1% DMSO (control), and (B) 10 μM of quercetin, curcumin, *t*-BHQ and genistein or 0.1% DMSO (control) for indicated time periods and luc activity was measured at 0, 2, 6, 10, 17 and 24 h. (C) mRNA levels of Trx were measured in HepG2 cells after 17 h incubation with 10 μM of indicated phytochemicals or DMSO using RT-PCR and related to that of GAPDH housekeeping gene. Both points or bars represents the mean values of three experiments (in a few exceptions two experiments) each performed in triplicates ± SEM. Data is given as fold change related to that of 0.1% DMSO (control). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

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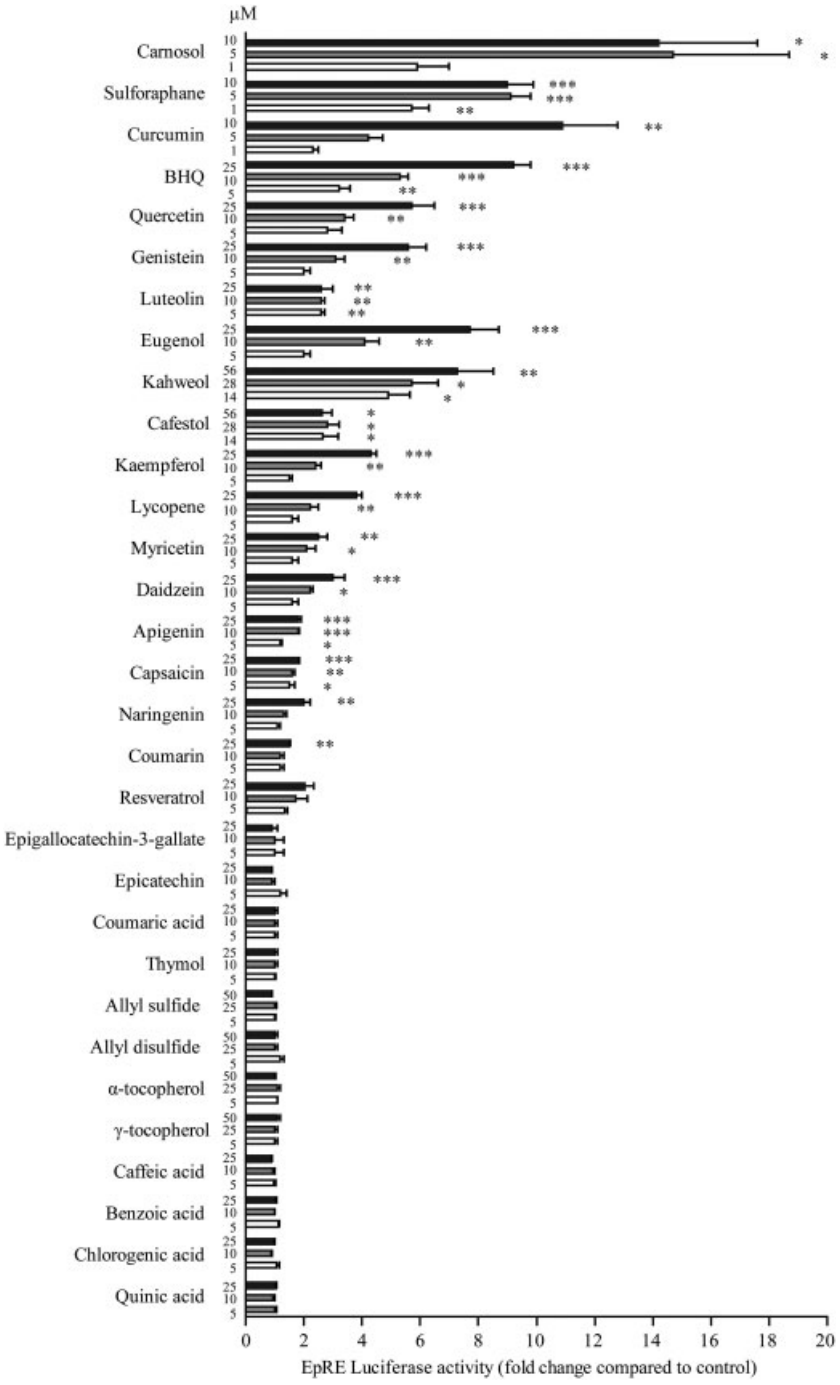

Figure 2. Effect on EpRE-dependent transcription by various phyochemicals. HepG2 cells transiently transfected with EpRE-luc were incubated with three different concentrations of phytochemicals or 0.1% DMSO for 17 h and luc activity was measured. Each bar represents the mean values of three experiments (in a few exceptions two experiments) each performed in triplicates ± SEM. Data is given as fold change related to that of 0.1% DMSO (control) set as one. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$. The values presented for the highest concentration kahweol, cafestol, caffeic acid, benzoic, chlorogenic acid and quinic acid have also been included in a separate study [60].

3.2 Phytochemicals induce EpRE activity *in vivo*

We also tested the ability of dietary phytochemicals and food extracts to induce EpRE-dependent transcription in transgenic EpRE-luc reporter mice which allow us to monitor the *in vivo* gene expression longitudinally.

Skin fibroblasts harvested from transgenic mice carrying the EpRE-luc transgene were used to validate the activity of the EpRE-luc reporter construct in response to curcumin, carnosol, sulforaphane, *t*-BHQ and genistein, known to induce EpRE-driven genes (Fig. 4). At 10 µM, curcumin (7.2 ± 0.2 fold change, $p<0.001$), carnosol (6.1 ± 0.6 fold change, $p=0.013$), sulforaphane (5.5 ± 1.0 fold change $p=0.046$), *t*-BHQ (2.0 ± 0.1 fold change, $p=0.007$) induced EpRE-luc, as compared to 0.1% DMSO (control). Genistein showed a slight but not statistically significant increased EpRE-luc activity at 10 µM (1.4 ± 0.1 fold change, $p=0.060$), but significantly induced EpRE activity at 5 µM (1.6 ± 0.1 fold, $p=0.047$).

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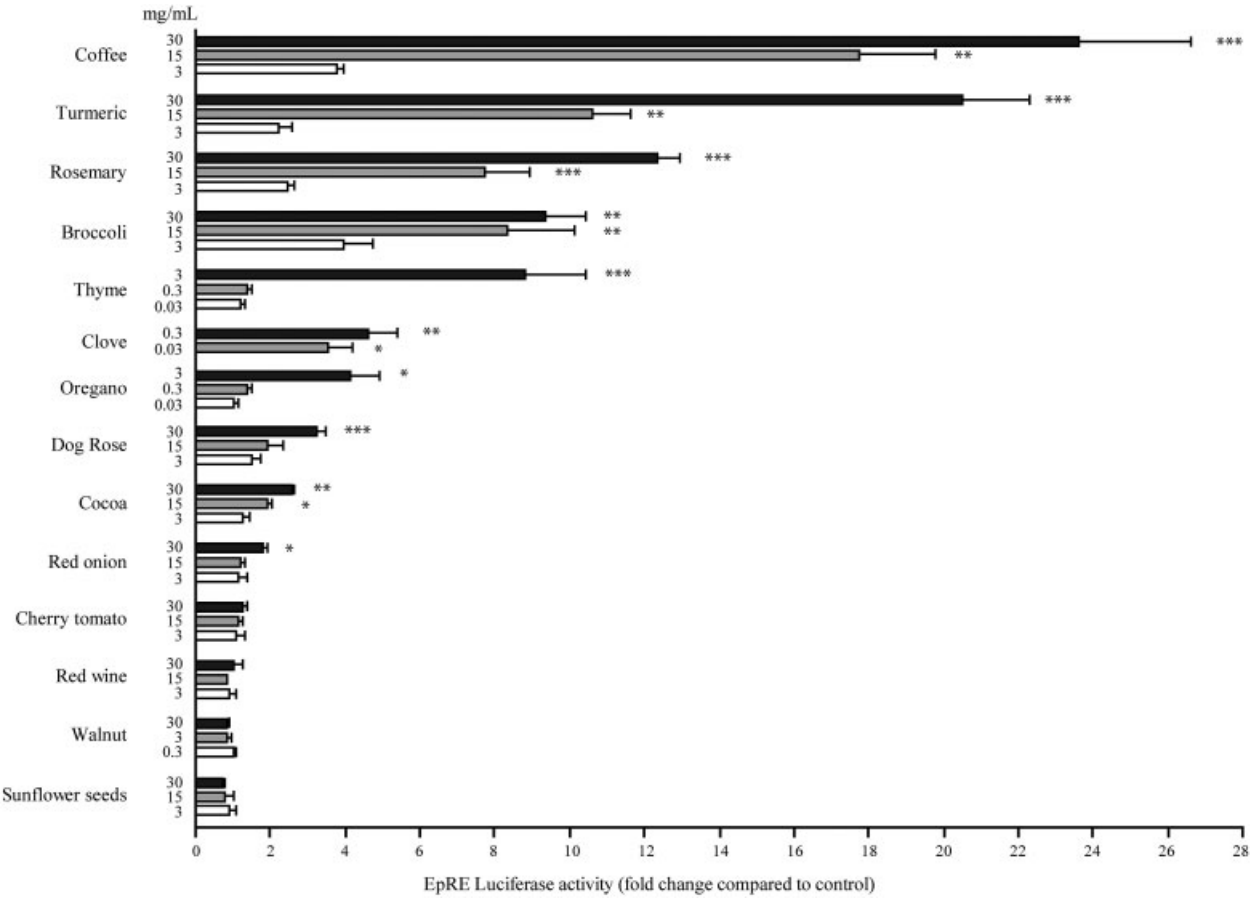

Figure 3. Induction of EpRE-dependent transcription by various dietary plant extracts. HepG2 cells transiently transfected with EpRE-luc were incubated with three different concentrations of food extracts, DMSO and/or PBS for 17 h and luc activity was measured. Each bar represents the mean values of at least three experiments performed in triplicates ± SEM. Data is given as fold change related to ≤0.2% DMSO and/or PBS (control) set as one. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$.

We next administrated curcumin, carnosol or *t*-BHQ to the mouse *via* i.p. injections to study *in vivo* effects on EpRE activity. Mice were given a single dose of phytochemicals, and imaged at the indicated time points (Fig. 5). We found maximum luc activity 24 h after administration and based on AUC from each mouse, curcumin ($p = 0.012$), carnosol ($p = 0.049$), and *t*-BHQ ($p = 0.014$) significantly induced EpRE-luc activity, compared to mice given vehicle only. Furthermore, 24 h following i.p. injection of curcumin mice were euthanized and organs were imaged *ex vivo*. The EpRE activity in small intestine from mice receiving curcumin was increased by 9.9-fold, as compared to intestine from control mice (Figs. 6A and B). Surrounding tissues of the intestine also showed significant induction of EpRE activity in the curcumin group; liver (8.6-fold, $p = 0.043$), kidney (4.7-fold, $p = 0.043$) and spleen (5.7-fold, $p = 0.021$) as shown in Fig. 6C. Also the skin had increased EpRE activity in the mice receiving curcumin (1.7-fold, $p = 0.021$).

In addition, we wanted to test whether these compounds could also alter whole body and tissue-specific EpRE activity after administration *per os*. Since curcumin was already known to be very poorly absorbed [23], carnosol was chosen. A single dose of carnosol was given by gavage feeding, mice were euthanized and luc activity was measured in organs by *ex vivo* imaging 24 h after administration (Figs. 6D and E). Average EpRE activity in the liver was twice as high in mice receiving carnosol compared to controls ($p = 0.049$) and also significant increased in spleen ($p = 0.049$). Furthermore, the activity in the kidneys of two of the three mice in the carnosol group was extremely high compared to control mice although this was overall not significantly different between the two treatments ($p = 0.275$).

3.3 Combination extract induce EpRE activity *in vivo*

Finally, we tested a combination extract made from good candidates from *in vitro* experiments; coffee, red onion, broccoli, turmeric, rosemary and thyme. Initially, the combination extract was tested in HepG2 cells for its ability to induce EpRE-luc activity. Cells were incubated with a final concentration of 3 mg/mL broccoli, coffee and red onion

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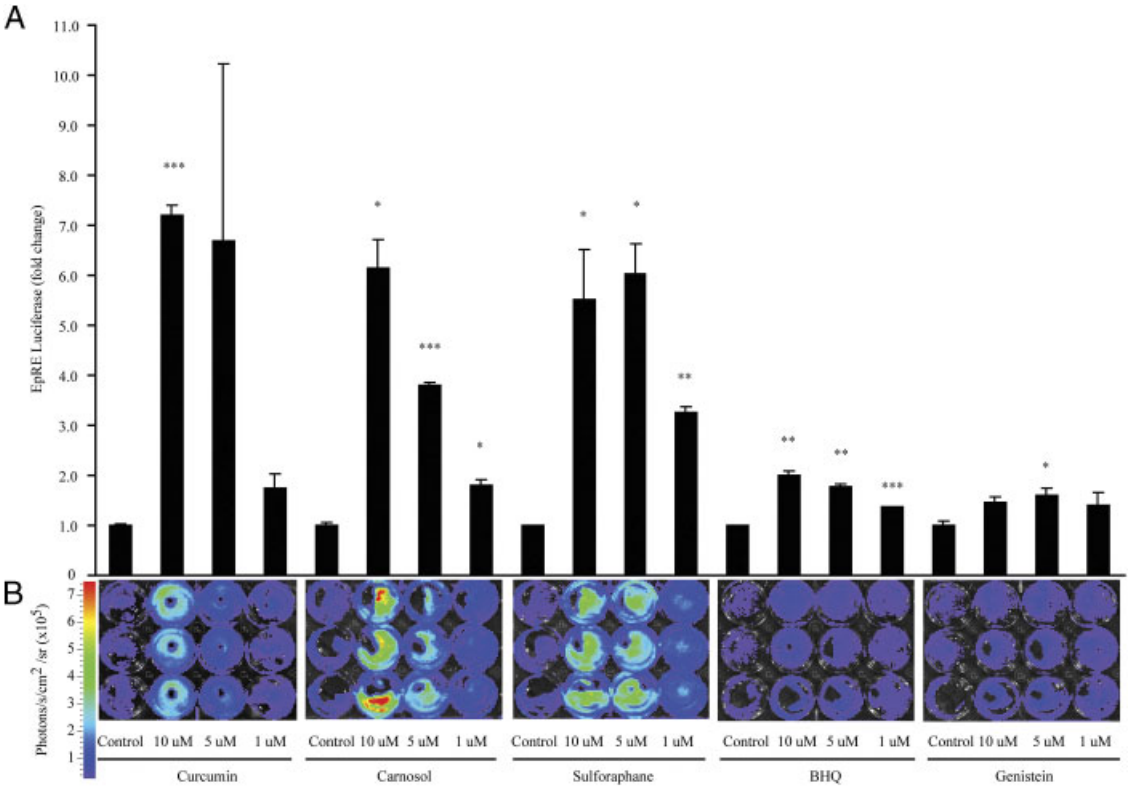

Figure 4. Validation of EpRE activity in fibroblasts from transgenic mice. Skin fibroblasts from the EpRE-luc mice were incubated with 10 μM of the indicated phytochemicals or 0.1% DMSO (control). After 17 h luc activity was measured by imaging in an IVIS 100 Imaging System. (A) Each bar represents the mean values of two experiments performed in triplicates ± SEM related to control. (B) Luminescence from cells from one experiment (as quantified in A) is shown. Data is given as fold change related to 0.1% DMSO (control) set as one. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$.

and 0.3 mg/mL turmeric, rosemary and thyme for 17 h and induced EpRE-dependent transcription by 8.7 ± 1.2-fold ($p = 0.003$) compared to control. The effect of the combination extract was large as compared to the effects of the single extracts, as the combination contains the lowest concentration of broccoli, coffee and red onion and ten times lower concentration of spices (except thyme) than we used in the testing of single extracts. This indicates additive or synergistic effect of the combination.

Mice were fed, by gavage feeding, a single dose of 300 μL combination extract or vehicle control. Based on AUC, luc activity was significantly higher in mice receiving the extract compared to controls ($p = 0.043$) (Figs. 7A and B). Surprisingly, the average luc activity in the abdominal region reached a maximum 2 h after administration, of 1.8-fold higher in the mice fed extract compared to mice given vehicle only. Thus in a subset of the mice, following imaging at 2 h, mice were euthanized and luminescence was measured in tissue homogenates. Lung (2.96-fold, $p = 0.047$) and adipose tissue (1.94-fold, $p = 0.047$) showed increased EpRE activity in mice receiving the combination extract compared to controls (Fig. 7C).

The total content of redox active compounds was measured in the combination extract, using the ferric reducing ability of plasma assay [24]. The average content of redox active compounds in the combination extract was 7.5 ± 1.7 mmol/100 mL corresponding to 63.16 ± 14.4 mmol for a 70 kg person. This is about twice the average daily intake in a Norwegian population (data not shown). Transferred to a human (70 kg), the dose given to the mice corresponds to about 14–23 g each of turmeric, rosemary and thyme, and 140–233 g each of coffee, red onion, broccoli, taken into account that mice have a metabolic rate six to ten times higher than humans [25, 26].

4 Discussion

The regulation of gene expression by plant phytochemicals can help explain the molecular biological mechanisms by which dietary intake of fruits and vegetables has favorable impact on pathogenesis of many diseases. EpRE-mediated activation of gene expression is considered an important aspect of the chemopreventive action of phytochemicals [27]. This study utilized EpRE elements which present direct expression of the luc transgene, in both cells and transgenic mice.

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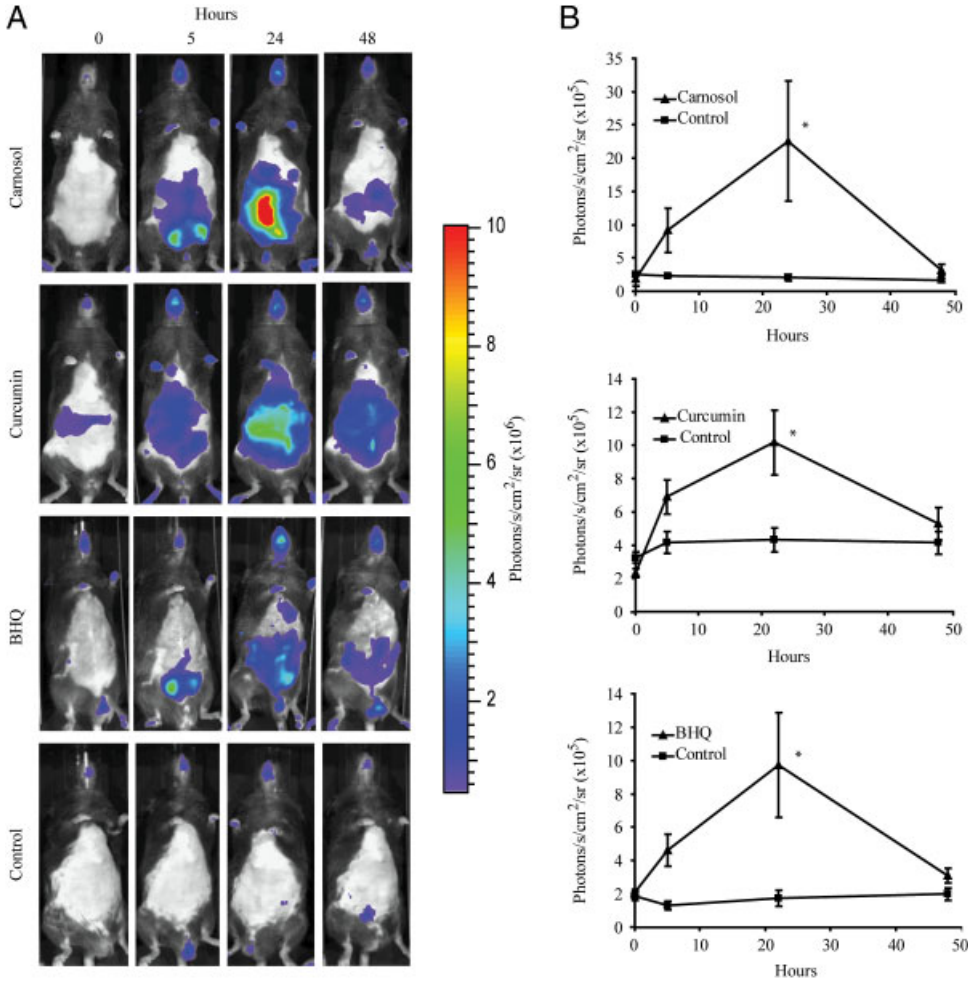

Figure 5. *In vivo* imaging of EpRE-luc mice given phytochemicals. (A) Transgenic EpRE-luc mice were injected with a single dose of indicated phytochemicals i.p. or control only and imaged at different time points. Luminescence was measured prior to administration (0 h), and at 5, 24 and 48 h. Representative images from one mouse from each treatment are shown. Carnosol: $n = 3$, controls $n = 3$. Curcumin: $n = 9$, controls $n = 8$. *t*-BHQ: $n = 5$, controls $n = 4$. (B) Average photons/s/cm^2/sr ($\times 10^5$) ($\pm$SEM) from the abdomen area is presented, and AUC was measured for each mice. $^*p < 0.05$.

In this study, we have screened 31 phytochemicals and 14 plant extracts for their ability to modulate EpRE-dependent transcription in cells. This screening facilitates comparison between compounds and shows that among phytochemicals tested, carnosol (*e.g.* rosemary, salvie), sulforaphane (cruciferous vegetables) and curcumin (*e.g.* turmeric) were the most potent inducers of EpRE-dependent transcription. To our knowledge, this is the first screening and comparison of EpRE-inducing properties of phytochemicals with structural diversity including polyphenols such as flavonoids, monophenols, isoflavones, isothiocyanates, carotenoids, tocopherols and organosulfur compounds tested in the same model system. Some of the best inducers such as carnosol, curcumin, *t*-BHQ and eugenol have a monophenolic structure, but this is also the case for non-inducers such as coumaric acid, thymol and benzoic acid indicating varying ability to induce EpRE-mediated gene expression among compounds with similar classifications based on the chemical structure and functional characteristics. Previous studies have shown phytochemicals to induce EpRE-dependent transcription, with sulforaphane and curcumin being some of the most studied [28, 29]. More surprisingly was the strong effect observed by carnosol. Carnosol and myricetin have been shown to induce EpRE-dependent transcription through Nrf2 [30, 31], but are less studied compared to sulforaphane and curcumin. To the best of our knowledge, eugenol has not been shown to regulate EpRE-dependent transcription in the previously published studies.

Among the plant extracts, coffee, turmeric, thyme, clove, broccoli and oregano exhibit strong inducing effect of EpRE activity. The effects of whole food extracts were surprisingly high, as the content of *e.g.* curcumin in turmeric, sulforaphane in broccoli, carnosol in rosemary is much lower than the concentration of the pure compounds tested in this report. Curcumin content varies from ~1 to 6% among batches of turmeric powder [32], thus the final concentration of curcumin in the cell wells can be ~1–5 μM in 30 mg/mL turmeric. Interestingly, curcumin can then only account for maximum 25% of the 20-fold induction of 30 mg/mL turmeric, indicating that other compounds with EpRE-inducing properties are present in turmeric. As for broccoli, the average sulforaphane content is about 10 μmol/200 g broccoli [33], giving a final concentration of ~1.5 μM sulforaphane in 30 mg/mL broccoli which can contribute to

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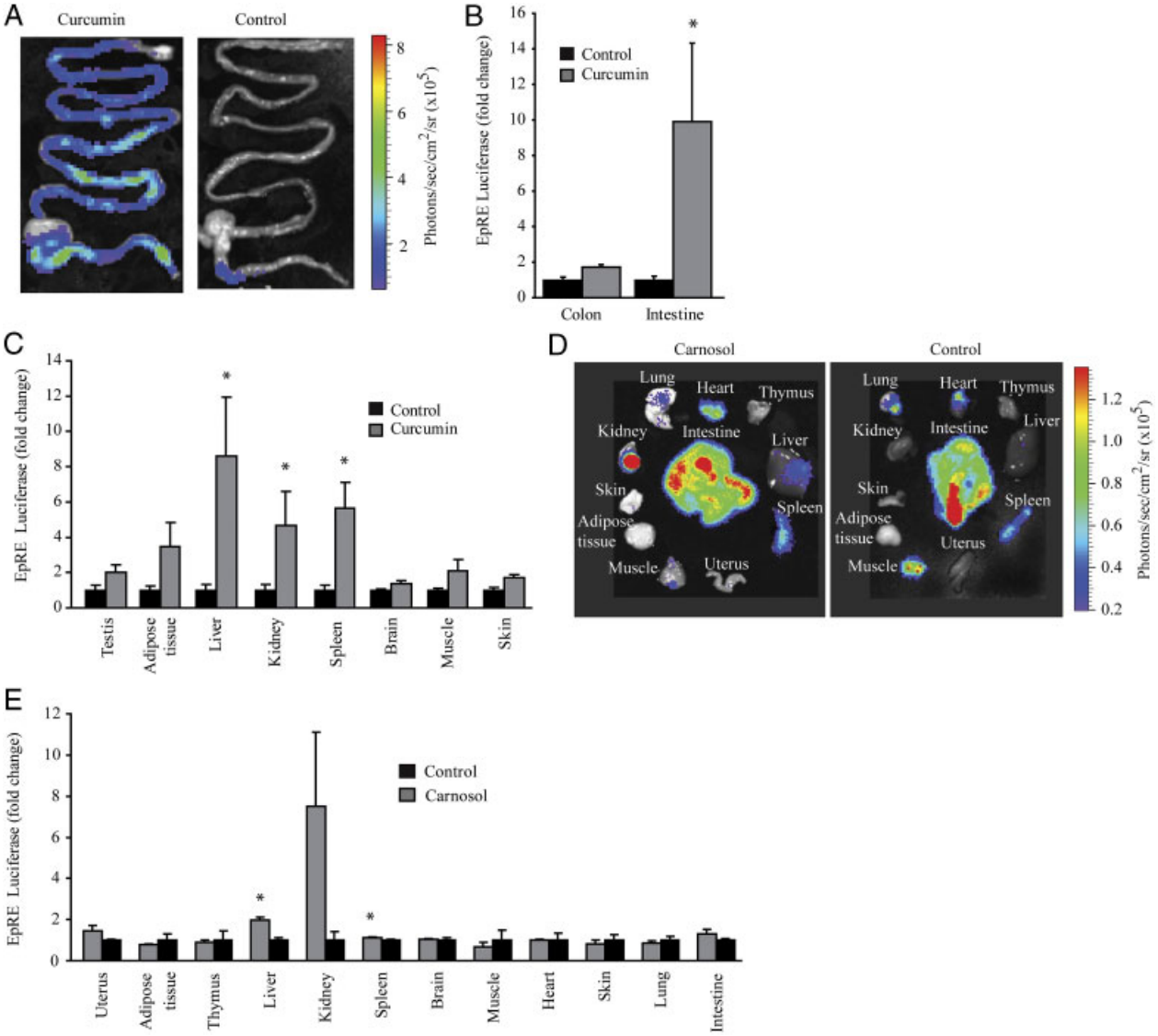

Figure 6. *Ex vivo* imaging of organ-specific effect on EpRE activity in mice given phytochemicals. EpRE-luc mice were given a single dose of indicated phytochemicals or control only and e*x vivo* imaging of organs was measured. (A) *Ex vivo* imaging of intestine from mice after a single dose of curcumin i.p. measured after 24 h, and(B) quantified showing average photons (±SEM) compared to controls (set as one). (C) The average photons/s/cm^2/sr ($\times 10^5$) emitted (±SEM) in *ex vivo* quantification of other tissues is shown. $n = 4$ for both groups.(D) *Ex vivo* imaging of organs after a single dose of carnosol *per os* measured after 24 h, and (E) quantified showing average photons/s/cm^2/sr ($\times 10^5$) (±SEM)in tissues is shown. $n = 3$ for both groups (except uterus $n = 2/2$) $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$.

only ~50% of the EpRE inducing effect when compared to the effect of sulforaphane alone. This indicates that other compounds or, even more interestingly, a combinatorial effect (additive or synergistic) of several compounds can come into play with these whole food extracts.

We tested a variety of food extracts and our selection of dietary plants is partly based on their content of some pure phytochemicals tested in the screening. In addition, several of these phytochemicals and plant extracts have been shown to be strong modulators of the activity of the transcription factor nuclear factor κ B [22], a transcription factor implicated in the pathogenesis of many inflammation-associated disorders such as cancer [34]. Potential crosstalk between the Nrf2-EpRE and the nuclear factor κ B signaling pathways has been suggested [35, 36], as both pathways involve redox-sensitive cysteine thiols [37]. In addition to its role in orchestrating carcinogen detoxification and cellular antioxidant defense, Nrf2 also has anti-inflammatory functions [38, 39] and Nrf2's role in inflammatory disorders has recently been extensively reviewed [40].

In addition, the screening of phytochemicals and dietary plant extracts was utilized to identify candidates for further testing and validation of our EpRE-luc transgenic mice. When utilizing EpRE transgenic mice, carnosol, curcumin

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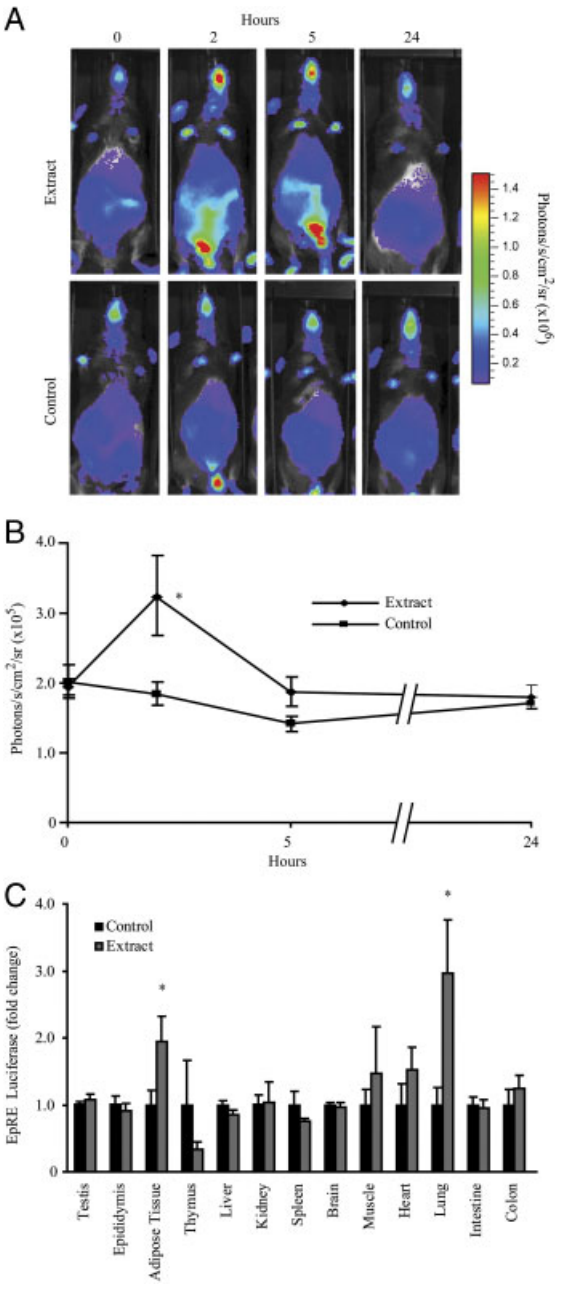

Figure 7. Increased EpRE-luc activity in mice fed a combination exctract. (A) Transgenic EpRE-luc mice were given a single dose of a combination extract of coffee, thyme, broccoli, rosemary, red onion and turmeric or vehicle control by oral gavage. Luminescence was measured prior to administration (0 h), and at 2, 5 and 24 h. Representative image from one mouse from each treatment is shown. (B) Average photons/s/cm^2/sr ($\times 10^5$) ($\pm$SEM) from the abdominal area is presented and AUC was measured for each mouse. (C) Directly following imaging at 2 h, the mice were euthanized, and organs excised. Luc activity was measured in tissue homogenates and normalized to the average of controls (mean $\pm$ SEM). Luc activity of intestine and colon was performed using *ex vivo* imaging. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$. (A) and (B) $n = 20$ extract and $n = 14$ controls. (C) $n = 5$ for both groups.

and *t*-BHQ administrated by i.p. injection, a maximum EpRE-luc activity was observed at 24 h after baseline.

In vivo bioluminescence does not have the resolution to precisely define the anatomic location of the luc activity to identify organs or cell types. Therefore, the EpRE-luc activity was also measured in tissues following the whole body imaging. Curcumin given i.p. strongly increased EpRE-luc activity in intestine, liver, kidney and spleen, and carnosol given by gavage feeding also significantly increased EpRE activity in liver and spleen. These findings indicate EpRE inducing effects in our transgenic mice by different ways of administration, and in addition, an intestinal absorption or metabolism of carnosol leading to induction of antioxidant defense in these organs.

Finally, we found that a dietary extract high in redox-active phytochemicals, induced EpRE-dependent transcription in mice fed a single dose of extract. Interestingly, the maximum effect was observed already at 2 h after oral administration and lung and white adipose tissue had a significantly higher EpRE activity as compared to their controls.

The liver is the main organ for detoxification and drug metabolism; however, xenobiotic responses are also observed in extra-hepatic tissues. Nrf2 is expressed ubiquitously and is abundant in tissues such as intestine, lung and kidney where detoxification occur routinely [41–43]. In the lung, Nrf2 plays an essential role since this is the first point of contact with inhaled oxidants and toxins [44]. A study by Kaspar *et al.* showed a pronounced translocation of Nrf2 from cytoplasm to the nucleus in a human bronchial epithelial cell line compared to liver hepatoma cells upon the same stimuli [45], indicating differences in the dynamics of EpRE regulation between cells and organs. In general, the liver seems to respond at a later time point, to inducers of detoxifying enzymes [46]. This may be explained by a higher expression of Phase I enzymes in the liver [47], leading to extensive modification before induction of Phase II enzymes can occur. The knowledge about the role of Nrf2 in adipose tissue is limited, but the Nrf2-EpRE pathway is highly inducible in adipose tissue [48], targeting detoxification and elimination of lipophilic compounds from the body. Nrf2 also inhibit lipid accumulation and oxidative stress in adipose tissue indicating a functional role in lipid metabolism [48].

In this study, we observed differences between inducible expression of EpRE regulation in organs, which may be explained by their physiological conditions and differential responses among genes and cell types. Cellular action of phytochemicals requires uptake and to be retained by the cells and this varies greatly among cell types [49]. Nevertheless, different inducing agents, doses of inducers and routes of administration will also have great impact on the response. Thyme, rosemary, turmeric, broccoli, onion and coffee are all rich in phytochemicals [24, 50]; however, there is generally limited knowledge about tissue distributions of dietary phytochemicals. Coffee diterpenes has been shown to enhance GST activity in liver, kidney and lung of rats [51] and also increase GST levels in plasma in humans [52],

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whereas thyme has been shown to induce xenobiotic enzymes in liver [53]. Furthermore, the consumption of cruciferous vegetables such as broccoli has been associated with reduced risk of various cancer [54], and a recent clinical study by Riedl *et al.* demonstrated an enhanced Phase II antioxidant enzyme expression in human airway cells by a oral intake of broccoli sprouts [55]. The protective effects have been attributed to the content of sulforaphane, by the induction of Nrf2-controlled cytoprotective genes [56], which may contribute to the increased levels of EpRE activity that we found in lung.

Quercetin, found in among others onion, has been traced in most organs with the highest concentration found in lungs and lowest in adipose tissue of rats [57]. The high levels of quercetin found in lung tissue may also contribute to the rapid EpRE induction observed in lungs of our mice. In terms of EpRE activity in adipose tissue, curcumin has been shown to suppress obesity-induced inflammation in adipocytes [58], and studies with carnosol inhibit differentiation of preadipocytes possibly through regulation of GSH by Nrf2 [59]. This indicates that phytochemicals can have an effect on the homeostasis of adipose tissue and, taken together, this is pointing out a possible organ specific effect by different phytochemicals. Further study should be done to trace phytochemicals and bioactive metabolites of food components in different organs.

We have utilized transgenic mice which makes it possible to study dynamics of EpRE-dependent transcription over time, and in many organs simultaneously. In this study, we demonstrated that phytochemicals and plant extracts can regulate EpRE-dependent transcription both *in vivo* and *in vitro*. Future nutrition research on the health effects of dietary plants should focus on whole foods rather than single compounds as the induction of endogenous antioxidant defense by dietary plant extracts may be an important contributor to the chemopreventive effects of a diet rich in plant-based food.

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R. B. and H. C. have interests in Cgene A/S, which was established by Birkeland Inovation (the Technology transfer office at the University of Oslo), and holds rights to the transgenic luciferase reporter mice. T.R.B. was previously employed by Cgene. The other authors have declared no conflict of interest.

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