NUTRITION AND CANCER, *54*(1), 94–101

Bilberry Extracts Induce Gene Expression Through the Electrophile Response Element

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Abstract: *A number of genes important for detoxification and antioxidant defense induced by mild stress generated by, for example, physical activity/exercise, caloric restriction, or alcohol may provide health benefits by causing the organism to mount such a defense response. More recently, induction of these defenses has also been attributed to phytochemicals or secondary metabolites from dietary plants. Many polyphenols, which constitute a large fraction of these phytochemicals, increase cellular levels of antioxidants, such as glutathione and other components of the detoxification systems, via the transactivation of genes containing electrophile response elements (EpREs) within their promoters. One such gene, γ-glutamylcysteine synthetase, has previously been shown to be positively regulated by quercetin, a flavonoid found in high concentrations in onions, apples, and bilberries through EpRE transactivation. As a further step, we have investigated whether bilberries and quercetin have the ability to induce transcription of Fos-related antigen 1 (Fra-1), which contains two EpREs in its promoter. Fra-1 is a member of the activator protein 1 (AP-1) family of transcription factors and, due to the lack of transactivation domain Fra-1, can suppress activation of AP-1. We present results demonstrating that extracts from bilberries, and the flavonoid quercetin, abundant in bilberries, induce the fra-*1 *promoter and the cellular content of Fra-1 mRNA. We further provide evidence that this induction is mediated through EpREs.*

Introduction

All organisms respond to various types of stress by activating transduction pathways or genes that counteract the stressful situation. This stress response includes cell cycle control, protein chaperoning and repair, DNA and chromatin stabilization and repair, removal of reactive molecular species, and induction of antioxidant defense and detoxification mechanisms (1). Recent studies suggest that mild stress generated by, for example, physical activity/exercise, caloric restriction, or alcohol may provide health benefits by causing the organism to mount such a defense response (2,3). Thus, mild stress, not harmful in itself, may activate a number of protective and beneficial mechanisms that promote better health and longer life (4).

A diet rich in fruit, vegetables, and some other plants may also reduce the incidence of major chronic diseases and increase longevity (5,6). Several mechanisms are most likely involved in the health beneficial effects of dietary plants but "mild stress" caused by phytochemicals resulting in an increased defense against environmental stress is likely also to mediate some of these effects (7). The molecular mechanisms mediating these beneficial effects of mild stress may include sirtuin deacetylases as well as the NF-E2–related factor-2 (Nrf2), activator protein 1 (AP-1), NF-κB, and Forkhead box class O (FOXO) families of transcription factors (8,9).

A number of different phytochemicals exist in dietary plants, exhibiting a vast array of activities in plant cells as well as in animal cells. A large fraction of the phytochemicals found in dietary plants are polyphenols (10). Polyphenols are a versatile group of plant constituents, with more than 8,000 compounds divided into different classes (that is, phenolic acids, flavonoids, stilbenes, and lignans). All the polyphenols are antioxidants (that is, redox reactive molecules) by virtue of the hydroxyl group, but they may also modulate a number of cellular mechanisms independent of their antioxidant properties (11). The health beneficial effects of polyphenols are also supported by observations suggesting that polyphenols have significant qualities related to amelioration of carcinogenesis, cardiovascular disease, and neurodegenerative disease (12).

One of the best-studied polyphenols is the flavonoid quercetin, found mainly in onions, tea, apples, and red wine (13). However, in Nordic countries, where berries are commonly consumed, berries are a more important source of

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quercetin than, for instance, red wine (14). In European blueberries (bilberries, *Vaccinium myrtillus*), the main polyphenols are anthocyanins (3,000–5,000 mg/kg) (15), phenolic acids (~3,000 mg/kg) (16), and the flavonol quercetin (70 mg/kg) (17). Anthocyanins are in general poorly absorbed, whereas quercetin is reasonably well absorbed and can reach concentrations from 0.3 to 0.75 mmol/l in plasma after ingestion of quercetin-rich plants (18–20).

In addition to having antioxidant properties, quercetin and other polyphenols modulate a wide range of enzymes and cellular receptors (21), for instance, by inducing genes encoding phase II detoxification, and endogenous antioxidant enzymes (22–25) through modulation of Nrf2, AP-1, NF-κB, and FOXO transcription factors.

A common characteristic for many phase II detoxification genes and genes crucial for antioxidant defense is the presence of electrophile responsive elements (EpREs, also called electrophile response element) within their promoters. Transcription factor Nrf2 is initially associated with the cytosolic protein Keap1, but, following appropriate stimuli, Nrf2 is released from Keap1. In the nucleus, they can dimerize with other factors such as Maf-proteins, bind EpRE, and activate transcription. It is not clear how polyphenols mediate EpRE-dependent transcription. However, it has been suggested that polyphenols, after entering the cell, are metabolized to an active metabolite, which either directly (26) and/or indirectly through protein kinase C activation (27) interferes with the association between Keap1 and Nrf2, thus permitting Nrf2 to enter the nucleus.

We have previously observed that quercetin and other flavonoids such as kaempferol and apigenin can transactivate the γ-glutamylcysteine synthetase heavy subunit (GCS_h), the rate-limiting enzyme in glutathione synthesis, through EpRE with a resultant increase in intracellular glutathione levels (23). We have also shown that mice fed juices or homogenates from berries modulate GCS_h promoter activity in brain muscle and liver (28).

In the present study, we have investigated the effect of bilberry juice and quercetin on fos-related antigen 1 (Fra-1) promoter activity, which contains EpRE. Fra-1 is a member of the AP-1 family of transcription factors, which can bind Jun-proteins, but, due to the lack of transactivation domain in Fra-1, Jun-Fra heterodimers have low transcriptional potential through the AP-1 binding site (29) and could potentially act as a repressor of tumor-promoting genes induced by AP-1. Fra-1 expression is affected in several human tumors (30), but the role of Fra-1 in carcinogenesis seems to be complex and varies between tumor types.

To provide evidence for a diet-mediated induction of Fra-1 transcription by polyphenols, cells transfected with a *fra-1* promoter-luciferase reporter gene were treated with flavonoid-rich extracts of bilberry and the flavonol quercetin. HepG2 cells were used because hepatocytes are central for metabolism and function of dietary polyphenols, whereas the kidney epithelial cell line COS-1 was used as a typical target cell line. Our experiments demonstrate that, indeed, the *fra-1* promoter is significantly increased by bilberry extract and quercetin. Quercetin also increased levels of Fra-1 mRNA. To test whether this effect was mediated through the EpREs, we first truncated the promoter to eliminate a xenobiotic response element (XRE), which has been demonstrated to be regulated by quercetin, and finally we used a synthetic reporter construct containing pure EpREs coupled to luciferase. Our results demonstrate that the increased *fra-1* promoter activity induced by bilberry and quercetin is mediated largely through EpREs.

Materials and Methods

Cell Culture

COS-1 cells and HepG2 cells, purchased from LLG Promochem (Boras, Sweden), were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM l-glutamine, 5 units/ml penicillin, 50 μg/ml streptomycin (Sigma, St. Louis, MO), and 10% fetal calf serum (Integro b.v., Zaandam, The Netherlands). The cell cultures were contained in a humidified atmosphere with 5% CO_2 at 37°C.

Plasmid Constructs

MatInspector (31) was used with the Matrix Family Library (version 2.4; MatInspector, Genomatix, Munich, Germany) to identify consensus XREs in the 5′-flanking sequence of the human *fra-1* gene. A plasmid containing a 442-bp fragment of the 5′-flanking region of fra-1 inserted into the pGEM-4 vector (fra-1-CAT) was kindly provided by Dr. Masahiro Fujii (32). The *fra-1*-LUC promoter construct was obtained by amplification of a 480-bp fragment containing the 5′-flanking region of the *fra-1* gene in the fra-1 CAT vector by using the primers 5′-GGT ACC TTG GGT GGC GGT TGG CGT and 5′-GCT AGC GCC AAG CTG ACT CTA GAG G-3′. The fra-1-ΔXRE -LUC promoter construct was obtained by amplification of a 428-bp fragment of the *fra-1* promoter from the fra-1-CAT construct by using the primers 5′-GGT ACC GTG GGA GCA GAA ACG GAG G-3′ and 5′-GCT AGC GCC AAG CTG ACT CTA GAG G-3′. The polymerase chain reaction (PCR) products were cloned into a pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA). The plasmids were digested with the enzymes *Kpn*I and *Nhe*I, and the resulting fragments were ligated into a pGL3-basic vector digested with *Kpn*I and *Nhe*I (Promega, Madison, WI), resulting in the *fra-1*-LUC and *fra-1*-ΔXRE -LUC promoter plasmids.

The EpRE LUC construct was made by ligating oligonucleotides containing a consensus EpRE sequence into the Bgl II site of the pTAL-LUC vector (BD Biosciences, Franklin Lakes, NJ). The sequences were as follows: 5′-GATCTGGTCACCGTTACTCAGCACTTTGTGGGGT TCACA-3′ and 5′-GATCTGTGAACCCCACAAAGTGC TGAGTAACGGTGACCA-3′. The resulting construct contained two EpREs.

Transient Transfection of COS-Land HepG2 Cells

The cells were cultured in 35-mm tissue culture wells the day before transfection at a density of approximately 60% confluence and transfected using a standard dextran-chloroquin method as described previously (33) with 0.7 μg (COS-1 cells) or 1.0 μg (HepG2 cells) DNA in each well. The cells were subjected to a dimethyl sulfoxide (DMSO) shock [Sigma, 10% in phosphate-buffered saline (PBS)], after which they were incubated overnight in cell medium. At the end of this incubation period, the medium was replaced with fresh cell medium containing bilberry juice (Helios, Slemmestad, Norway), quercetin, kaempferol, or cyanidin-Cl (Sigma) dissolved in DMSO. The lowest concentrations of quercetin used (that is, 1–10 μM) are likely to reflect concentrations that can be obtained in tissues as demonstrated recently by de Boer and colleagues (34).

Plant Extracts

Bilberry juice prepared by hot vapor extraction (Helios) was sterile filtered before added to the cell medium as indicated in the figure legends. The bilberry juice contained 65.9 mM total reductants as determined by the ferric-reducing ability of plasma assay (23).

Luciferase Measurements

Luciferase activity was measured in cell lysates according to the manufacturer's protocol (Promega). Briefly, the cells were washed in PBS without Ca^{2+} or Mg^{2+} prior to the addition of 300 μl lysis buffer. The cells were incubated at room temperature for 20 min before collection by scraping and subsequent lysis by vigorous vortexing. The lysate was then briefly centrifuged to remove cell debris. Luciferase activity was measured by adding 100 μl luciferase assay solution to 20 μl of the lysate, and luminescence was detected in a TD 20/20 luminometer (Turner Design, Sunnyvale, CA). Protein concentration was measured in the cell lysates using Coomassie brilliant blue reagent from Bio-Rad Laboratories (Hercules, CA).

Quantitative mRNA Analysis

The cells were plated in 35-mm tissue culture wells 2 days before mRNA isolation at a density of approximately 60% confluence. mRNA was isolated by oligo (dT)16 beads according to the manufacturer's protocol (Genovision, Oslo, Norway). mRNA was eluted from the beads in 20 μl diethyl pyrocarbonate-treated water, and cDNA synthesis was performed with an Omniscript kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA from each sample was analyzed by PCR amplification using a LightCycler™ (Roche Diagnostics, Ottweiler, Germany) with Fast Start DNA Master Sybrgreen I kit (Roche Diagnostics). The PCR conditions were 4 mM $MgCl_2$ for fra-1 or 3 mM $MgCl_2$ for β-actin, 10 pmol of each PCR primer (fra-1 primers: 5′-AGC TGC AGA AGC AGA AGG AG-3′ and 5′-GTG GTC AGT GCC TCA GGT T-3; β-actin primers: 5′-TCG TGC GTG ACA TTA AGG AG-3′ and 5′-AGC ACT GTG TTG GCG TAC AG-3′), 2 ml of LightCycler DNA Master mix, and cDNA to a final volume of 20 ml. After 10-min preincubation at 95°C, 45 PCR cycles were performed with 15-s denaturation at 95°C, 5-s annealing at 55 or 60°C, and 12-s extension at 72°C. cDNA obtained by reverse transcriptase (RT)-PCR of fra-1 and β-actin was cloned into a pCR 2.1-TOPO vector (Invitrogen) to obtain a plasmid used as the standard to calculate the number of cDNA copies in a sample. After amplification, data analyses were performed using the second-derivative maximum method. The second-derivative maximum values were used to plot cycle number versus log concentration of the standard plasmid. The actual number of fra-1 cDNA copies was related to that of β-actin in each sample. Contamination of mRNA samples with genomic DNA was ruled out by omitting the reverse transcriptase in control reactions. The identity of the PCR products was confirmed by melting-curve analyses and size determination.

Results

Promoter Analyses of the 5 -Flanking Region of the Human *fra-1* Gene

It was noted by Yoshioka et al. (29) that the 5′-flanking region of the human *fra-1* gene contains a potential EpRE. Therefore, we used MatInspector (31) to analyze ~600 bp upstream of the translation start of the human *fra-1* gene. Two EpRE motifs were found, the proximal motif at position -94 to -104 and a more distal motif located at position -242 to -250 (both relative to the *fra-1* initiation codon, Fig. 1A). These two EpRE elements completely match the consensus EpRE core sequence (TGAC/GnnnGC) (35) and the distal of the two overlaps with a c-fos AP-1 binding site (32). When cells were cotransfected with fra-1-LUC and an expression vector containing Nrf1, luciferase activity increased approximately 2.5-fold, confirming that at least one of the elements is a functional EpRE (Fig. 1B). To test whether Nrf1 overexpression induced luciferase activity through EpRE, we contransfected cells with the synthetic EpRE LUC construct together with the Nrf1 expression vector. This led to a nearly 30-fold increase of luciferase activity compared with the control (Fig. 1C). In addition, we found an XRE as defined by Fujisawa-Sehara et al. (36) at position -420 to -425 (Fig. 1A).

Transcriptional Activation of *fra-1* by Bilberry Extract

Bilberries are rich in flavonoids with antioxidant properties that could possibly influence responsive elements in the *fra-1* promoter. Therefore, we incubated both COS-1 and HepG2 cells transfected with the fra-1-LUC plasmid, with

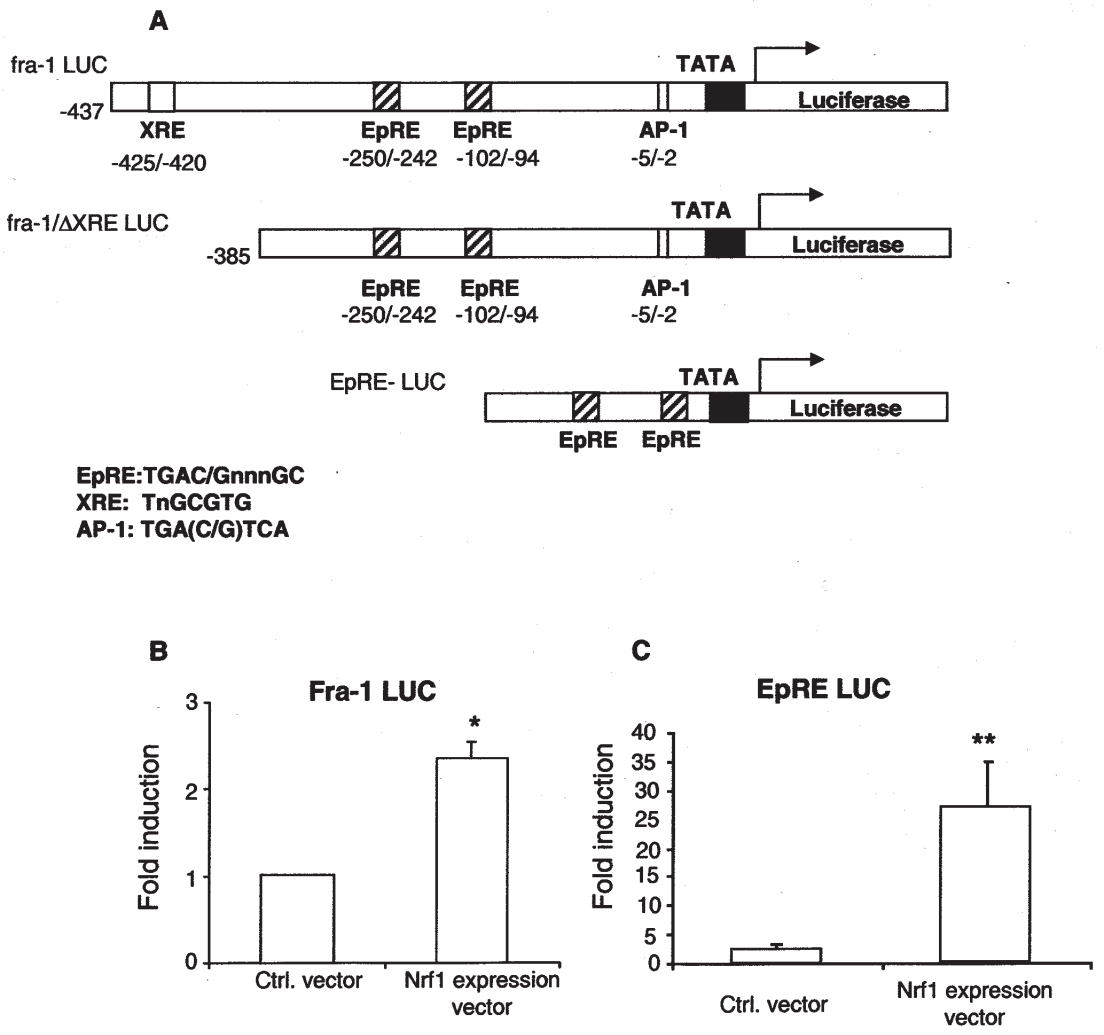

Figure 1. (A) Schematic illustration and (B) Nrf1 induction of fra-1- and EpRE-reporter constructs. (A) The outline shows the position of *cis*-acting elements relative to the translation initiation codon of the human *fra-1* gene depicted by boxes. COS-1 cells were transfected with 0.5 μg of the (B) fra-1 LUC reporter construct or (C) EpRE LUC together with 0.25 μg of the empty expression vector pcDNA3 (control) or pcDNA3-Nrf1. The cells were subsequently washed in phosphate-buffered saline, lysed, and scraped from the cell wells. Cell lysates were collected and luciferase activity was measured. Luciferase activity was related to protein concentrations in each well. Data are given as fold increase related to transfection with the corresponding amount of the empty expression vector pcDNA3. Each bar represents the mean value ± SD. $*P < 0.05$; $**P < 0.01$ ($n = 9$).

various amounts of bilberry juice in the cell culture medium. We observed in both cell types a positive dose-response relationship between the concentration of bilberry treatment and luciferase activity after 17 h of incubation (Fig. 2A), reaching a significant increase with a maximum of ~2-fold induction in HepG2 cells and 1.6-fold induction in COS-1 cells. We also measured the relative amount of concentration of Fra-1 mRNA in HepG2 cells after treatment with different volumes of bilberry juice for 17 h. We found that 1% bilberry juice infusion in the medium increased the level of Fra-1 mRNA by a factor of 2.8 (Fig. 2 B).

Transactivation of *fra-1* by Quercetin

Bilberries are known to be rich in quercetin (17). We have already demonstrated that quercetin has the ability to transactivate GCS_h through EpRE in its promoter (23). To demonstrate whether the *fra-1* promoter can be transactivated by dietary polyphenols, quercetin was added to HepG2 or COS-1 cells transfected with the luciferase reporter construct comprising the complete *fra-1* promoter. Figure 3A shows that quercetin induced *fra-1* promoter activity in a concentration-dependent manner after 17-h incubation in both HepG2 and COS-1 cells. In COS-1 cells, there is a significant increase at the lowest quercetin concentration (1 μM) with a peak activation of threefold at 10 μM. In HepG2 cells, 5 μM quercetin is the lowest concentration of which luciferase is significantly induced with the highest induction at 25 μM (threefold).

mRNA assessments of Fra-1 verified the observations made using the luciferase reporter. As shown in Fig. 3B, the mRNA levels of Fra-1 in HepG2 cells were increased ap-

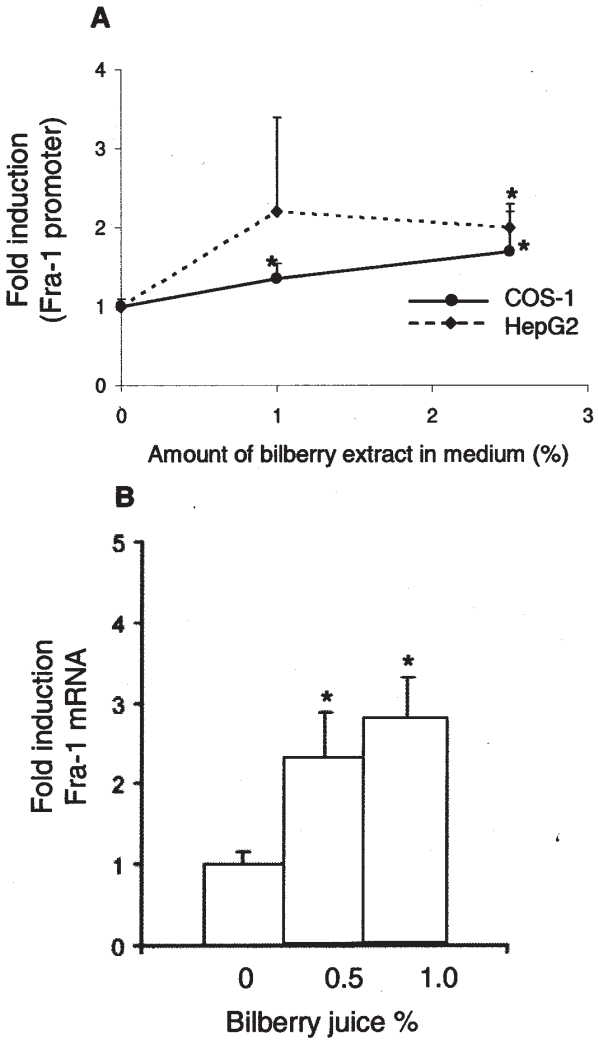

Figure 2. Induction of (A) *fra-1* promoter activity or (B) Fra-1 mRNA levels after addition of flavonoid-rich extracts in cell medium. (A) Cells transiently transfected with fra-1 LUC were incubated with increasing volumes of bilberry juice extract for 17 h. The cells were subsequently washed in phosphate-buffered saline and lysed, and cell lysates were measured for luciferase activity. Luciferase activity was related to protein concentrations in each well. Data are given as fold increase compared with control levels and represent the mean values ± SD. $*P < 0.05$ ($n = 9$). (B) HepG2 cells were incubated with 0, 5 (0.5%), or 10 µl (1%) of bilberry juice per milliliter of cell culture medium for 17 h. The cells were harvested, and quantitative RT-PCR was performed as described. The mRNA level of fra-1 is related to the mRNA level of β-actin in each sample. The results are given as fold increase compared with control levels and represent the mean values ± SD. $*P < 0.05$ ($n = 9$).

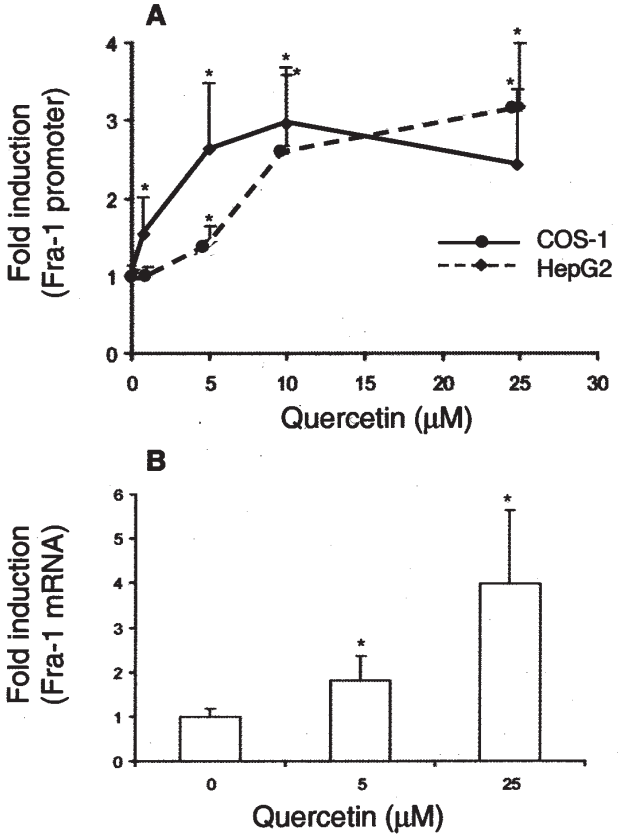

Figure 3. Quercetin activates the *fra-1* promoter and increases the Fra-1 mRNA level. (A) HepG2 and COS-1 cells transiently transfected with fra-1 LUC were incubated with increasing concentrations of quercetin for 17 h. The cells were thereafter washed in phosphate-buffered saline, lysed, and scraped from the cell wells. Cell lysates were then collected and assessed for luciferase activity, which was related to protein concentrations in each well. (B) HepG2 cells were incubated with 0 [0.1% dimethyl sulfoxide (DMSO), control], 5, or 25 µM quercetin for 17 h. The cells were harvested, and quantitative RT-PCR was performed as described. The mRNA level of fra-1 is related to the mRNA level of β-actin in each sample. Data are given as fold increase related to 0.1% DMSO (control) and represent the mean value ± SD. $*P < 0.05$ ($n = 9$).

proximately two- and fourfold by 5 µM and 25 µM quercetin, respectively.

We also tested the effect of cyanidin, which is together with delphinidin the most abundant anthocyanin in bilberries (15). Cyanidin had no effect on the *fra-1* reporter construct (data not shown).

Role of Responsive Elements in the *fra-1* Promoter

As outlined in Fig. 1A, the *fra-1* promoter contains at least two types of responsive elements that may respond to phytochemicals, two EpREs and one XRE. Quercetin has previously been shown to transactivate XRE-containing promoters when bound to the aryl hydrocarbon receptor (37). To determine which of the two types of elements is responsible for regulation of promoter activity by this flavonoid, we made a second reporter construct lacking the XRE consensus sequence. When HepG2 cells were transfected with the fra-1-ΔXRE-LUC construct and treated with quercetin, the effect was similar to that obtained with the complete promoter (Fig. 4).

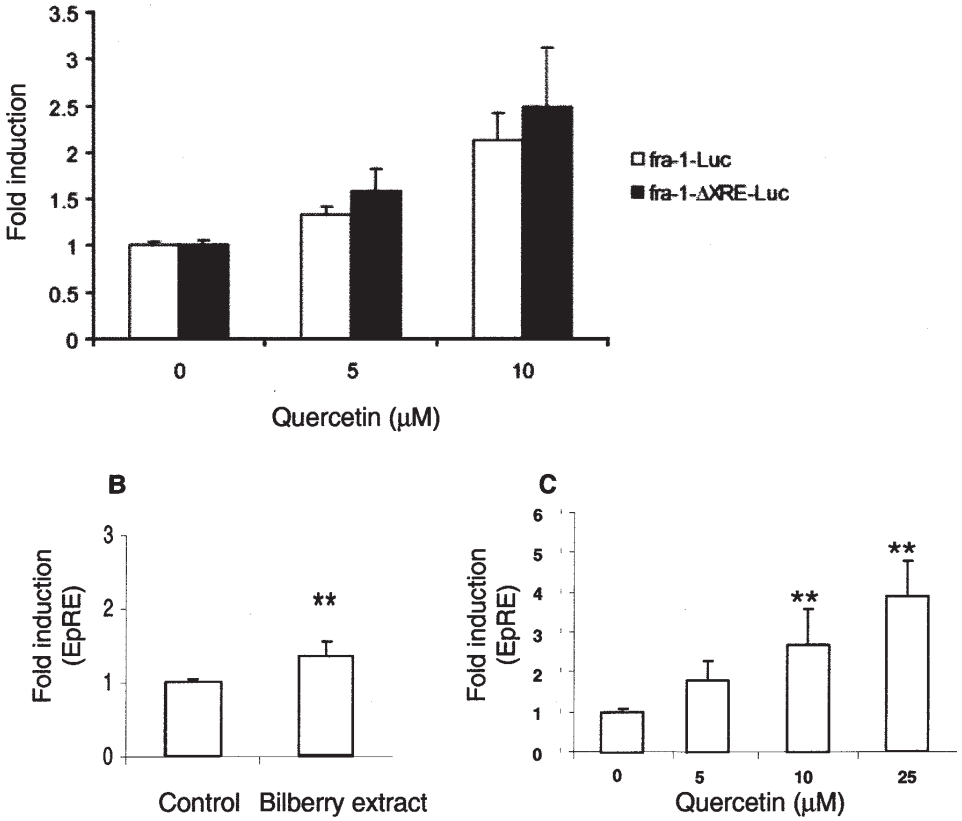

Figure 4. Identification of polyphenol responsive elements in the *fra-1* promoter. (A) HepG2 cells transiently transfected with fra-1-LUC or fra-1 ΔXRE-LUC were incubated with 0 [0.1% dimethyl sulfoxide (DMSO), control], 5, or 10 μM quercetin for 17 h. (B) COS-1-cells were transfected with an electrophile responsive element LUC construct and treated with a bilberry extract or (C) quercetin (0, 5, 10, or 25 μM) for 17 h. The cells were thereafter washed in phosphate-buffered saline, lysed, and scraped from the cell wells. Cell lysates were collected and luciferase activity was measured. Luciferase activity was related to protein concentrations in each well. Data are given as fold increase related to 0.1% DMSO. Each bar represents the mean value ± SD. *$P < 0.05$; **$P < 0.01$ ($n = 9$).

To verify that the effect of bilberry extract and quercetin is in fact mediated through EpRE, COS-1 cells were transfected with a reporter construct containing only a consensus EpRE coupled to luciferase. Luciferase activity was increased 1.4-fold by the bilberry extract and dose dependently by quercetin with a 2.5-fold induction at 10 μM and a 4-fold induction with 25 μM quercetin.

Discussion

Important genes involved in cellular defense, detoxification, and antioxidant synthesis are known to be regulated by dietary components (23,25), providing a plausible explanation for the prevention of oxidative stress–related diseases by diets high in fruit and vegetables. In this particular study, we asked whether a bilberry extract and one of its main polyphenol components, quercetin, could activate transcription of Fra-1, which contains EpRE binding elements in its promoter. In the two cell types we used (COS-1 and HepG2), we find a significant and dose-dependent increase in *fra-1* promoter activity by both the bilberry extract and quercetin. Furthermore, when using a truncated version of the promoter coupled to luciferase (lacking the XRE) or a synthetic EpRE LUC construct, we find that the induction is similar to the induction driven by the complete *fra-1* promoter. These results thus indicate that EpREs are responsible for the bilberry- and quercetin-mediated increase of Fra-1 transcription.

In a previous study using the GCS_h promoter, we observed that the flavonoid myricetin did not induce GCS_h promoter activity (23) or EpRE-mediated luciferase expression (unpublished results). The only difference between quercetin and myricetin, for example, is one additional hydroxyl group in the flavonoid backbone structure, indicating that the activation of transcription through EpRE by flavonoids is structure dependent. We also observed that neither anthocyanins such as cyanidin, petunidin, pelargonidin, malvidin, and peonidin nor delphinidin induced the *fra-1* promoter or the EpRE (unpublished results). These results may suggest that such compounds are less efficiently taken up by the cells, that they are more prone to degradation, or that they are less efficient EpRE activators as such. The stability of anthocyanins is highly dependent on pH, and the neutral pH in the medium may cause spontaneous degradation of cyanidin. This was previously shown by Seeram and co-workers, who detected at least three different benzoic acid degradation derivatives

after incubating cyanidin in a standard cell culture medium at pH 7.4, with a subsequent loss of bioactivity (38).

Previous reports have demonstrated that Fra-1 is up-regulated by phenolic antioxidants (29,39) or by pyrrolidine dithiocarbamate, a known activator of Nrf2 (40). These studies showed that Fra-1 induction is associated with a repression of AP-1–regulated transcription. In similar studies using resveratrol, the authors find that resveratrol induces Fra-1 levels in metastatic melanoma cells, with a resulting beneficial alteration in cell phenotype, such as differentiation and reduced proliferation (39). Therefore, it is tempting to speculate that increased Fra-1 levels induced by polyphenols could be one of many activities of these compounds referred to as chemopreventive actions. However, a number of reports also demonstrate that high Fra-1 levels are associated with tumor transformation and metastasis. Furthermore, increased AP-1 activity has been observed using other polyphenols such as epigallocatechin gallate (EGCG). Balasubramanian and co-workers have shown that EGCG from green tea modulates AP-1 positively through a mitogen activated protein kinase-dependent increase in Fra-1 levels (41). Therefore, it is reasonable to assume that both positive and negative regulation of Fra-1, mediated by kinases, is affected by polyphenols, adding additional complexity to the regulation of Fra-1 and other EpRE-containing promoters.

Acknowledgments and Notes

This work was supported by grants from the Norwegian Research Council and The Johan Throne Holst Nutrition Research Foundation. We thank Dr. Masahiro Fujii for the kind gift of the construct containing the human *fra-1* promoter and Dr. George Alexander for reading of the manuscript. Address correspondence to R. Blomhoff, Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, P.O. Box 1046 Blindern, 0316 Oslo, Norway. E-mail: rune.blomhoff@basalmed.uio.no.

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