Nrf2 Mediates the Induction of Ferritin H in Response to Xenobiotics and Cancer Chemopreventive Dithiolethiones*

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Ferritin is a ubiquitous intracellular iron storage protein that consists of 24 subunits of the H and L type. The ability to sequester iron from participation in oxygen free radical formation is consistent with a cytoprotective role for ferritin. Here we demonstrate that ferritins H and L are induced in cells treated with β-naphtoflavone (β-NF) and chemopreventive dithiolethiones. Induction of ferritin H by β-NF and the dithiolethiones oltipraz and 1,2-dithiole-3-thione (D3T) occurs via a transcriptional mechanism that is mediated by the ferritin H electrophile/antioxidant-responsive element (EpRE/ARE). The murine ferritin H gene contains five potential xenobiotic-responsive element (XRE) sequences in its 5′-promoter region. However, deletion analysis demonstrates that these XRE sequences are not functional in inducing ferritin H in response to β-NF. Electrophoretic mobility shift assays demonstrate that the ferritin H EpRE/ARE binds Nrf2. Transfection of chimeric ferritin H reporter genes with Nrf2 expression vectors and Nrf2 dominant-negative mutants indicate that Nrf2 functions at the EpRE/ARE to mediate transcriptional activation of ferritin H. Induction of ferritin H and L was not seen in Nrf2 knockout cells, demonstrating that this transcription factor is required for the induction of ferritin in response to polycyclic aromatic xenobiotics and chemopreventive agents. Nrf2 may also play a role in basal transcription of both ferritin H and L. These results provide a mechanistic link between regulation of the iron storage protein ferritin and the cancer chemopreventive response.

Ferritin is a 480-kDa intracellular protein that can store up to 4500 atoms of iron (1). The protein consists of 24 subunits of the H and L chain type (2). The ratio of subunits within the ferritin protein varies widely by tissue type; the ratio can be further modulated by environmental signals, including cytokine stimulation, stress signals, and disease state (3, 4). The H chain has ferroxidase activity (5), whereas the L subunit is responsible for iron nucleation and protein stabilization (6). Because iron functions as a catalyst in the formation of oxygen free radicals, storage of iron by ferritin may serve a cytoprotective role for ferritin. Here we demonstrate that ferritins H and L are responsible for iron nucleation and protein stabilization (6). The H subunit is further modulated by environmental signals, including cytokine stimulation, stress signals, and disease state (3, 4). The H chain has ferroxidase activity (5), whereas the L subunit is responsible for iron nucleation and protein stabilization (6). Because iron functions as a catalyst in the formation of oxygen free radicals, storage of iron by ferritin may serve a cytoprotective role for ferritin. Here we demonstrate that ferritins H and L are responsible for iron nucleation and protein stabilization (6).

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‡‡‡ The abbreviations used are: t-BHQ, tert-butylinhydroquinone; Ah receptor, aromatic hydrocarbon receptor; AP1, activator protein 1; ARE, antioxidant-responsive element; β-NF, β-naphtoflavone; D3T, 1,2-dithiole-3-thione; FH, ferritin H; FL, ferritin L; hGH, human growth hormone; EpRE, electrophile responsive element; Keap-1, Kelch-like cystein-rich protein with cap ‘n collar homology (ECH)-associated protein 1; Luc, luciferase; NF-κB, nuclear factor κ B; Nrf2, NF-E2 related factor 2; oltipraz, 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione; nt, nucleotide(s); XRE, xenobiotic-responsive element; GST, glutathione S-transferase; NQO1, NADPH:quinone oxidoreductase 1; SOD, superoxide dismutase; GCS, γ-glutamylcysteine synthetase; DMEM, Dulbecco’s modified Eagle’s medium.

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phile-responsive element (EpRE (26); also known as ARE (27), antioxidant-responsive element) (28). Compounds eliciting this response are chemically diverse and overlap those that induce the Ah receptor-dependent pathway. They include oxidants (28, 29), redox cycling agents (30), and electrophiles (i.e. Michael reaction acceptors) (28, 31) as well as polycyclic aromatic hydrocarbons, such as β-NF (28). Functional EpRE/ARE sequences have been identified in GST Ya (20, 26), NQO1 (23), γ-glutamylcysteine synthetase heavy and light subunit (GCS\(_h\), and GCS\(_s\), respectively) (32, 33), heme oxygenase (34), glutathione S-transferase P1 (35), thioredoxin (36), and NF-E2-related factor 2 (Nfr2) (37).

Regulation of transcription at the EpRE/ARE is incompletely understood. Early studies focused on members of the AP1 transcription factor family (38) as regulators of EpRE/ARE-dependent transcription (39–42). More recently, compelling evidence for the involvement of members of the cap and collar family of transcription factors (43), particularly Nrf2, has been presented. For example, induction of cytoprotective enzymes, such as GST and NQO1, by the phenolic antioxidant butylated hydroxyanisole was lost in cells isolated from Nfr2 knockout mice (44). In addition, forced expression of cap and collar family members, such as Nr1f1 and Nfr2, resulted in the induction of EpRE/ARE-dependent reporter gene expression (45–48). Activation of Nfr2 following stimulation of cells with an inducer requires its dissociation from a cytosolic actin binding protein, Keap-1, and subsequent translocation to the nucleus (49, 50). Release of Nfr2 from Keap-1 may be triggered by modification of reactive cysteine residues in Keap-1 (51) and/or post-translational modification of Nfr2 by protein kinases (52).

Our laboratory has identified an EpRE/ARE in the ferritin H gene that mediates the induction of ferritin H transcription in response to H\(_2\)O\(_2\) and t-BHQ (7). The ferritin H EpRE/ARE is 75 bp in length and is located ~4.1 kb from the transcription start site (7). It is comprised of the ferritin H basal enhancer (53), FER-1, and an AP1/NF-E2 consensus sequence located 8 bp 3′ of FER-1. The basal enhancer, FER-1, is in turn composed of an element with close sequence similarity to both AP1 and NF-E2 consensus sequences (previously termed AP1-like element (53, 54) and referred to in this report as AP1/NF-E2-like element), and a recognition sequence for the SP1/3 transcription factors (53, 54). The AP1/NF-E2-like and the AP1/NF-E2 consensus sequence of the ferritin H EpRE/ARE are arranged in inverse repeat, and both of these elements are necessary for full induction of ferritin H by H\(_2\)O\(_2\) and t-BHQ (7). An EpRE/ARE has also been identified in the murine ferritin L promoter (55). Ligation of this element to a luciferase reporter gene demonstrated that the ferritin L EpRE is functional as an enhancer element in HepG2 cells treated with t-BHQ (55).

Collectively, these results suggest that ferritin may constitute a component of the cytoprotective response induced by xenobiotics (electrophiles or polycyclic aromatic hydrocarbons) and candidate chemopreventive agents. However, the mechanism of ferritin induction by these agents is unknown. Here, we demonstrate that ferritins H and L are induced by oltipraz, D3T, and β-NF in fibroblasts and hepatic cells. Furthermore, we show that induction of ferritin occurs via an EpRE/ARE-dependent mechanism that requires Nfr2. These results link ferritin induction mechanistically to the chemopreventive response.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3, Hepa1–6, and HepG2 cell lines and Nfr2 wild type (+/+ ) and Nfr2 knockout (−/−) primary mouse embryonic fibroblasts were maintained at 37 °C in a humidified atmosphere containing 5% CO\(_2\), NIH3T3 (ATCC) cells were grown in Dulbecco’s modified eagle medium (DMEM, Invitrogen) supplemented with 10% bovine calf serum (HyClone), 100 units/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate. The Nfr2+/+ and Nfr2−/− primary mouse embryo fibroblasts were grown in DMEM/F-12 (Invitrogen) supplemented with 15% fetal bovine serum, 2 mM glutamine, 0.1 mM non-essential amino acids, 150 µM β-mercaptoethanol, and 100 units/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate.

Chemicals—Oltipraz and D3T were provided by the Division of Cancer Prevention Repository of NCI, National Institutes of Health, and β-NF was obtained from Sigma. All compounds were dissolved in Me\(_2\)SO and final Me\(_2\)SO concentration in all treatment conditions was 0.04%.

Plasmids—The following ferritin human growth hormone reporter gene constructs have been previously described (7): −4.8kbFH-hGH, −4.13kbFH-hGH (referred to as −4.0kbAP1+hFH-hGH in Ref. 7), −4.0kbFH-hGH, −0.32kbFH-hGH, and 107bpEpREFH-hGH (referred to as 107bpFER-1+AP1 FH-IhGH in Ref. 7). To construct ferritin H luciferase reporter gene constructs −4.8kbFH-CAT (53) was digested with HindIII to release a fragment that contains the 5′-promoter region of the ferritin H gene, from nucleotide −4819 to +24. The HindIII fragment was ligated into the HindIII multiple cloning site of pGL3 (Promega) to create −4.8kbFH-Luc. To construct −3.2kbFH-Luc, −4.0kbFH-Luc, and −0.25kbFH-Luc, −4.8kbFH-Luc was digested with SacI, BglII, and Smal to release 616-, 789-, and 4618-bp fragments, respectively. The 4618-bp fragment without the 99-bp 5′-AP1 region was gel-purified and religated to form −4.2kbFH-Luc, −4.0kbFH-Luc, and −0.225kbFH-Luc, respectively. The −8.4kb3.5kbFH-Luc internal deletion construct was made by removing an internal EcoRI fragment from −4477 to −941 from −4.8kb FH-Luc by digestion with EcoRI and religation. The 75bpEpREFH-Luc insertion construct was made as follows. Sense and antisense oligonucleotides corresponding to the 75-bp ferritin H EpRE/ARE were synthesized by the Wake Forest University School of Medicine Comprehensive Cancer Center DNA synthesis center laboratory, PAGE-purified, phosphorylated using T4 Polynucleotide kinase, annealed, and ligated into the Smal site of 0.225kbFH-Luc. The Nfr2 dominant negative mutant expression plasmid (pEF/Nfr2dm) was made by digesting the empty expression plasmid (pEF/Nfr2) with BglII and cloning it into the SmaI site of pEF. This plasmid was a kind gift of Dr. Jawed Alam (56). The Nfr2 expression plasmid (pEF/Nfr2−/−) has been described previously (57).

Northern Blot Analysis—Total RNA was isolated from cells treated for 24 h with vehicle, oltipraz, D3T, and/or β-NF as described by Chirgwin et al. (58) or utilizing the TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. 10–15 µg of RNA were size-fractionated on 1.1% agarose/6.6% formaldehyde gels and transferred to an Immobilon Ny+ nylon membrane (Millipore) by capillary transfer. DNA probes for both ferritin H (59) and L (60) were generated by random prime labeling and subsequently hybridized to the UV-cross-linked RNA blot in Quick Hyb solution (Stratagene) according to the manufacturer’s protocol. Membranes were subjected to autoradiography; quantitation was performed using a phosphorimager analyzer (model 445SI, Amersham Biosciences).

Western Blotting of Ferritin Induction—To assess ferritin H and β-actin protein levels, cytosolic extracts were prepared as previously described by Schreiber et al. (61). 50 µg of protein was fractionated on 12%SDS-polyacrylamide gels, transferred to nitrocellulose, blocked with 5% nonfat dry milk in phosphate-buffered saline, washed, and incubated with a 1:1,000 dilution of polyclonal rabbit anti-ferritin H peptide antibody (Biosource International) followed by a 1:200 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad). The blots were developed using the Enhanced Chemiluminescence System (Amersham Biosciences). To demonstrate equal protein loading, blots were washed and re-blotted using a 1:5,000 dilution of a 1:1,000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Calbiochem).

Transfection of Ferritin H-human Growth Hormone Reporter Gene Constructs and RNase Protection Assay—NIH3T3 cells were transfected in duplicate with 2 or 3 µg of FH-hGH reporter gene constructs using LipofectAMINE Reagent (Invitrogen) according to the manufacturer’s protocol. Cells were allowed to recover for 20–24 h and treated with 70 µM Oltipraz, 70 µM D3T, or vehicle (Me2SO). RNA was isolated after 24 h, and RNase protection analysis (RPA) was performed as described previously (3). The fold induction was calculated based on means and standard errors of three to eight independent experiments.

Transfection of Ferritin H-luciferase Reporter Gene Constructs and Luciferase Assay—Hepa1–6 cells were transfected for 4 h with a total of 500 ng of DNA (FH-Luc reporter gene constructs, β-galactosidase trans-
RESULTS

Xenobiotics and Chemopreventive Agents Induce Ferritin mRNA—β-NF is a polycyclic aromatic hydrocarbon that has been used to study activation of phase 2 enzymes by both XRE- and EpRE/ARE-dependent mechanisms (23, 24, 32, 63). Oltipraz is an electrophilic dithiolethione that represents a widely studied class of candidate chemopreventive agents. To create a model system in which to explore the mechanism of ferritin induction by these agents, we treated cultured liver cells for varying periods of time with β-NF or oltipraz. As shown in Fig. 1, both β-NF and oltipraz induced ferritin H and L mRNA in Hepa1–6 cells in a time-dependent manner. We also performed Northern blot analysis of NIH3T3 fibroblasts treated with oltipraz and a second dithiolethione, D3T. As shown in Fig. 2, both these agents induced ferritin H and L mRNA in NIH3T3 cells. Induction of ferritin H as well as ferritin L mRNA by oltipraz in NIH3T3 cells was time-dependent and occurred as early as 3 h after treatment, with induction peaking at 24 h. mRNA induction was accompanied by an increase in ferritin protein of similar magnitude (Fig. 3). Induction of ferritin mRNA by oltipraz, D3T, and β-NF was also seen in HepG2 cells (data not shown). Taken together, these results demonstrate that β-NF, oltipraz, and D3T induce both ferritin H and L in a variety of cells, including murine and human hepatocytes and fibroblasts.

Induction of Ferritin Is Mediated by the EpRE/ARE—To assess the mechanism of ferritin induction, we transiently transfected NIH3T3 cells with a chimeric ferritin H-human growth hormone (FH-hGH) gene construct that spans 4.8 kb of the murine ferritin H promoter region fused to the human growth hormone reporter gene (−4.8kbFH-hGH). Subsequently, cells were treated with 70 μM oltipraz or 70 μM D3T. After 24 h, RNA was isolated and RNase protection analysis was performed to assess the induction of the reporter gene as well as the endogenous ferritin H gene. Fig. 4 demonstrates that both oltipraz and D3T induce the endogenous ferritin H mRNA by dithiolethiones. NIH3T3 cells were treated with 70 μM D3T for 24 h or 70 μM oltipraz for 0, 3, 6, 9, 12, and 24 h. Total RNA was isolated from cells, size-fractionated on denaturing agarose gels, transferred to nylon membranes, and allowed to hybridize with cDNA probes specific for ferritin H and L. Ethidium bromide staining was done to assure equal RNA loading. Shown are the average ± S.E. values for two experiments.

Oltipraz and D3T induce ferritin H protein levels. Cell lysates were obtained 24 (D3T-treated cells) or 48 (oltipraz-treated cells) h after treatment of NIH3T3 cells with vehicle (Me2SO), 70 μM oltipraz, or 70 μM D3T. Cell lysates were electrophoresed on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blotted with antibody to ferritin H and β-actin (loading control) as described under “Experimental Procedures.”

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Gene, confirming the Northern blot analysis shown in Fig. 2. In addition, induction of the −4.8kbFH−hGH reporter gene construct by oltipraz and D3T was observed. These results indicate that induction of ferritin H by oltipraz and D3T is mediated by a transcriptional mechanism.

To delineate the element responsible for transcriptional activation of ferritin H, deletion analysis was performed. FH−hGH 5′ deletion constructs containing 4.8, 4.13, or 4.0 kb of the murine ferritin H 5′ flanking region were transiently transfected into NIH3T3 cells, treated with 70 μM oltipraz, and analyzed by RNase protection assay. As shown in Fig. 5, a region located between −4.13 and −4.0 kb of the ferritin H promoter is responsible for activation of ferritin H transcription. Thus, both the −4.8kbFH−hGH and −4.13kbFH−hGH constructs were induced by oltipraz, whereas the −4.0kbFH−hGH construct was not. Because the 75-bp ferritin H EpRE/ARE is indicated by the black rectangle.

To determine whether the induction of ferritin H by β-NF utilized a similar mechanism, we first considered the potential role of XRE sequences. β-NF can activate transcription via both XRE- and EpRE/ARE-dependent mechanisms (16). The XRE consensus sequence has been defined as 5′-T(A/T)GCGTG-3′ (18), and functional XRE sequences have previously been genetically identified in the cytochromes P450 1A1 and 1A2 (18, 19, 64), GST Ya (20), UDP-glucoronyl transferase 1A1 and 1A6 (21, 22), NQO1 (23), and Cu/Zn-SOD gene (24, 25). Inspection of the ferritin H 5′ promoter region revealed five potential consensus XRE sequences, one on the sense strand and four on the antisense strand. Their specific locations and sequences are as follows. The XRE sequence present in the sense strand is located between −3089 and −3083 (5′-CTGCGTG-3′). The XRE sequences in the antisense strand are located between −4579 and −4573 (5′-CACGCAC-3′), −2811 and −2817 (5′-CAGCCAC-3′), −413 and −407 (5′-CAGCTT-3′), and −351 and −345 (5′-CACGCAC-3′). To determine if these elements are functional, we prepared ferritin H-luciferase constructs (luciferase was used to simplify assessment of reporter gene expression). These constructs spanned −4.8, −4.2, and −4.0 kb of the murine ferritin H 5′ promoter region. In addition, to determine the involvement of the XRE sequence located from −4579 to −4573 independent of the ferritin H EpRE/ARE sequence (−4117 to −4043), an internal deletion construct was made in which 3.5 kb of the ferritin H promoter were removed from the full-length −4.8kbFH−Luc construct spanning a region from −4477 to −941. The ferritin H-luciferase 5′ deletion and internal deletion constructs were transiently transfected into Hepa1–6 cells. Subsequently, cells were treated with 25 μM β-NF for 24 h, and luciferase activity was determined. The constructs −4.8kbFH−Luc and −4.2kbFH−Luc demonstrated luciferase induction in response to β-NF, whereas −4.8kbΔ3.5kbFH−Luc and −4.0kbFH−Luc did not (Fig. 7).

**Fig. 5. Ferritin H transcription by dithiolethiones is mediated by an enhancer element located between −4.13 and −4.0 kb.** A, schematic of the ferritin H-human growth hormone (FH−hGH) 5′ deletion constructs used in defining the dithiolethione-responsive element. The location of the ferritin H EpRE/ARE is indicated by the black rectangle. B, FH−hGH reporter gene constructs were transiently transfected into NIH3T3 cells. 20–24 h after transfection, cells were treated with vehicle (MeSO), 70 μM oltipraz, or 70 μM D3T for 24 h. Total RNA was isolated, and 10 μg of RNA was used for RNase protection analysis (RPA). For the RPA, a probe was used that encompasses the ferritin H EpRE/ARE. C, a transcriptional mechanism. Thus, both the −4.8kbFH−hGH and −4.13kbFH−hGH constructs were induced by oltipraz, whereas the −4.0kbFH−hGH construct was not. Because the 75-bp ferritin H EpRE/ARE is indicated by the black rectangle.
Hence, induction of ferritin H by β-NF is mediated by an enhancer element located between 4.2 and 4.0 kb, a region that contains the ferritin H EpRE/ARE but none of the XRE sequences. To confirm that activation of ferritin H by β-NF occurred via the EpRE/ARE, the 75-bp EpRE/ARE element was inserted into a minimal promoter construct, which contains 225 bp of the ferritin H promoter (~0.225kbFH-Luc). As shown in Fig. 7, 75bpEpREFH-Luc mediated induction of luciferase activity, whereas –0.225kbFH-Luc did not. Thus, ferritin H is transcriptionally activated by β-NF via a mechanism that depends on the EpRE/ARE and not the XRE sequences.

Elements in the Ferritin H EpRE/ARE Bind Nrf2—Nrf2 is a member of the NF-E2 family of transcription factors that has been shown to mediate EpRE/ARE-dependent transcription of a variety of cytoprotective genes (36, 47, 56, 65). The ferritin H EpRE/ARE contains two elements with sequence similarity to the NF-E2 consensus sequence. The AP1 consensus sequence of the ferritin H EpRE/ARE is embedded in a canonical NF-E2 site; the AP1-like sequence in the ferritin H EpRE/ARE also possesses considerable sequence similarity to the NF-E2 consensus (9/11 residues) (Fig. 8). To examine the ability of Nrf2 to bind to the ferritin H EpRE/ARE, we performed electrophoretic mobility shift assays (EMSAs). The results are shown in Fig. 8A. Figure 8B shows the AP1/NF-E2 consensus sequence that is present in the ferritin H EpRE/ARE, as well as the NF-E2 consensus sequence that is present in the ferritin H EpRE/ARE. The NF-E2 consensus sequence (5′-CCGAGCGCGCAGCACGAGGTC-3′) is also present in the ferritin H EpRE/ARE, as shown in Fig. 8B. The NF-E2 consensus sequence is located at the 5′-end of the ferritin H EpRE/ARE, and it is important for the binding of Nrf2 to the ferritin H EpRE/ARE.
mobility shift assays. We assessed binding to both the AP1/NF-E2-like and AP1/NF-E2 consensus elements of the ferritin H EpRE/ARE. Nuclear extracts were isolated from HepG2 cells that had been treated with vehicle (Me2SO), 70 μM oltipraz, or 25 μM β-NF for 6 h. 10–20 μg of extract was incubated with 32P-labeled AP1/NF-E2-like or AP1/NF-E2 consensus oligonucleotide (100,000–150,000 cpm), and 100-fold molar excess of specific (SC) and nonspecific (NC) competitors where indicated. A NFκB binding element (Promega) was used as a nonspecific competitor. Following a 20-min incubation at room temperature, an antibody specific for Nrf2 or normal rabbit serum (c) was added to the indicated samples for a 30-min room temperature incubation, and supershifted Nrf2 is marked by the small arrows.

**FIG. 9.** Nrf2 binds to the AP1/NF-E2-like and AP1/NF-E2 consensus elements of the ferritin H EpRE/ARE. Nuclear extracts were isolated from HepG2 cells that had been treated with vehicle (Me2SO), 70 μM oltipraz, or 25 μM β-NF for 6 h. 10–20 μg of extract was incubated with 32P-labeled AP1/NF-E2-like or AP1/NF-E2 consensus oligonucleotide (100,000–150,000 cpm), and 100-fold molar excess of specific (SC) and nonspecific (NC) competitors where indicated. A NFκB binding element (Promega) was used as a nonspecific competitor. Following a 20-min incubation at room temperature, an antibody specific for Nrf2 or normal rabbit serum (c) was added to the indicated samples for a 30-min room temperature incubation, and supershifted Nrf2 is marked by the small arrows.

**FIG. 10.** Nrf2 is required for activation of ferritin H and L by oltipraz, D3T, and β-NF. Total RNA was isolated from Nrf2+/+ and Nrf2−/− cells that had been treated with vehicle (Me2SO), oltipraz, D3T, and β-NF for 24 h. RNA was size-fractionated on denaturing agarose gels, transferred to nylon membranes, and allowed to hybridize with cDNA probes for ferritin H and L. Ethidium bromide staining was done to assure equal RNA loading. The -fold induction was calculated with Nrf2+/+ cells treated with vehicle defined as 1. Shown are the average ± S.E. values for two β-NF or four (oltipraz and D3T) independent experiments.

Several laboratories have demonstrated that ferritin H and L are induced in response to oxidants and pro-oxidant xenobiotics (7–11), and a transcriptional mechanism has been impli-
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Activation of ferritin in response to these agents is thought to serve a cytoprotective function from iron-catalyzed oxidative damage. Previously, we have genetically defined the DNA element responsible for inducing ferritin H transcription in response to oxidants (Fig. 8). This element contains a cis-acting DNA-enhancer located ≈4.1 kb from the transcriptional start site and possesses sequence similarity to the consensus electrophile/antioxidant-responsive element (EpRE/ARE) (7). Sequence searches and reporter assays have also identified a functional EpRE/ARE in the ferritin L gene (55). In this report we demonstrate that ferritin H and L mRNAs are induced by β-NF and by chemopreventive dithiolethiones in fibroblasts and hepatic cells. Elevation of ferritin H mRNA by the dithiolethiones oltipraz and D3T is the result of a transcriptional mechanism. Similarly, Primiano et al. (15) provided evidence for transcriptional activation of ferritin H and L in the livers of rats treated with D3T. Using ferritin H deletion constructs fused to the human growth hormone gene, the results presented in this study identify the EpRE/ARE as the mediator of transcriptional activation of ferritin H in response to β-NF, oltipraz, and D3T. These results implicate ferritin in the chemopreventive response and suggest that ferritin, together with other EpRE/ARE-mediated cytoprotective proteins, contributes to the chemoprotected phenotype in cells in which these proteins have been induced. Classes of proteins induced by chemopreventive agents include both conjugating enzymes (glutathione S-transferases and UDP-glucuronosyltransferase) involved in detoxification and export and antioxidant enzymes (superoxide dismutase and NAD(P)H:quinone oxidoreductase 1). Ferritin, with its ability to sequester iron and prevent the formation of oxygen free radicals, has some characteristics of this latter class. In fact, we and others (66, 67) have shown that repression of ferritin synthesis is correlated with enhanced sensitivity to oxidative stress; conversely, induction/overexpression of ferritin has been linked to enhanced cellular protection against oxidant-induced cytotoxicity (68, 69).

A sequence search of 4.8 kb of the murine ferritin H 5′-flanking sequence revealed the presence of five XRE sequences that were potential candidates for regulating ferritin H transcription in response to polycyclic aromatic hydrocarbons, such as β-NF. However, deletion analysis of the ferritin H 5′-promoter region using ferritin H-luciferase (FH-Luc) reporter gene constructs demonstrated that these elements are non-functional in ferritin induction by β-NF, because reporter constructs containing these putative XRE sequences but lacking the EpRE/ARE were not induced in response to β-NF (Fig. 7). Rather, the EpRE/ARE controls ferritin H transcription in response to β-NF (Fig. 7). Because the five XRE sequences closely resemble the consensus XRE sequence (5′-T(A/T)(G/C)(T/G)-3′) (18), it was surprising that none of these elements in the ferritin H promoter region conferred inducibility of the gene in response to β-NF. In particular, comparison of the ferritin H XRE sequences to XRE sequences identified previously in various genes showed that the ferritin H XRE sequences at nucleotides (nt) −4579 to −4573 and nt −351 to −345 are identical to the functional XRE identified in the rat UDP-glucurononitransferase IA1 (21) and human CYP IA2 (19) gene. Likewise, the XRE sequence at nt −3089 to −3083 and nt −2817 to −2811 are identical to the XREs present in the rat and human Cu/Zn-SOD genes, respectively (24, 25). It has been suggested that the XRE core sequence itself is insufficient to confer transcriptional induction, and that additional nucleotides flanking the core sequence exert an important influence on the ability of the Ah receptor to bind to the core XRE sequence (70). These contextual requirements may contribute to the inactivity of the XRE elements in the ferritin H promoter.

The electrophile/antioxidant-responsive element consensus sequence resembles the recognition sequence for transcription factors of the AP1 and NF-E2 family of DNA binding proteins, allowing for a wide variety of transcription factors to bind to this element and mediate basal as well as inducible transcription. Several laboratories have demonstrated the involvement of the transcription factor Nrf2 in inducing cytoprotective proteins in response to a variety of agents, including β-NF and dithiolethenes (47, 65, 71). Some genes require Nrf2 for basal as well as D3T-inducible transcription, whereas others showed induction by D3T independent of Nrf2 status (65). Results presented here demonstrate that treatment of Nrf2+/+ and
Nrf2−/− primary mouse embryo fibroblasts results in induction of ferritin H and L mRNA in wild type but not Nrf2 knockout cells, indicating that Nrf2 is necessary for dithiolethione- and β-NF-induced transcription of both ferritin H and L. Gel shift and transfection experiments indicate that Nrf2-mediated induction of ferritin H targets the ferritin H EpRE/ARE (Figs. 9, 11, and 12). Although we did not quantitate nuclear Nrf2 following treatment with oltipraz, D3T, or β-NF, the increasing intensity in band shift seen in Fig. 9 is consistent with increased binding of Nrf2 to the ferritin H EpRE/ARE following stimulation with xenobiotics. This would be concordant with demonstrations of nuclear translocation of Nrf2 following treatment with inducing agents (36, 49).

Our experiments showed not only that Nrf2 is involved in activating transcription of ferritin H and L in response to dithiolethiones and β-NF, but also suggested the involvement of Nrf2 in basal transcription of these genes, since expression of ferritin H and L mRNA was decreased in Nrf2−/− cells when compared with the wild type cells (Fig. 10). Effects on ferritin H basal transcription were confirmed by cotransfecting 75bpEpREFH-Luc with a dominant negative mutant of Nrf2, which both suppressed EpRE/ARE-dependent induction of ferritin H in response to an inducer and decreased basal expression. A similar involvement of Nrf2 in basal transcription has been reported for other genes, including glutathione S-transferases (72) and γ-glutamylcysteine synthetase (57).

The ferritin H EpRE/ARE has two sites with which Nrf2 interacts, namely the AP1/NF-E2 consensus sequence and the AP1/NF-E2-like element of FER-1 (Fig. 9). Although we did not examine the interaction of the ferritin L EpRE/ARE with Nrf2, inspection of the ferritin L promoter reveals a NF-E2 consensus sequence embedded in the ferritin L EpRE/ARE, suggesting that Nrf2 may be involved in the coordinate regulation of both ferritin subunits through targeting of the EpRE/ARE.

Nrf2 has been demonstrated to be important in the regulation of several EpRE/ARE-dependent genes. However, Nrf2-independent mechanisms of gene regulation at the EpRE/ARE also exist. For example, it was recently reported that the ability of the model chemopreventive agent sulforaphane to induce ferritin h and l mRNA was decreased in Nrf2−/− cells when compared with the wild type cells (Fig. 10). Effects on ferritin H basal transcription were confirmed by cotransfecting 75bpEpREFH-Luc with a dominant negative mutant of Nrf2, which both suppressed EpRE/ARE-dependent induction of ferritin H in response to an inducer and decreased basal expression. A similar involvement of Nrf2 in basal transcription has been reported for other genes, including glutathione S-transferases (72) and γ-glutamylcysteine synthetase (57).
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