Biochemical Pharmacology 83 (2012) 139–148

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

A conserved antioxidant response element (*ARE*) in the promoter of human carbonyl reductase 3 (*CBR3*) mediates induction by the master redox switch Nrf2

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ARTICLE INFO

Article history:
Received 6 July 2011
Accepted 28 September 2011
Available online 5 October 2011

Keywords:
Carbonyl reductase 3
Antioxidant response element
Nuclear factor [erythroid-derived 2]-like 2

ABSTRACT

Carbonyl reductase activity catalyzes the two electron reduction of several endogenous and exogenous carbonyl substrates. Recent data indicate that the expression of human carbonyl reductase 3 (*CBR3*) is regulated by the master redox switch Nrf2. Nrf2 binds to conserved antioxidant response elements (*AREs*) in the promoters of target genes. The presence of functional *AREs* in the *CBR3* promoter has not yet been reported. In this study, experiments with reporter constructs showed that the prototypical Nrf2 activator tert-butyl hydroquinone (t-BHQ) induces *CBR3* promoter activity in cultures of HepG2 (2.7-fold; $p < 0.05$) and MCF-7 cells (22-fold; $p < 0.01$). Computational searches identified a conserved *ARE* in the distal *CBR3* promoter region ($_{-2698}$*ARE*). Deletion of this *ARE* from a 4212-bp *CBR3* promoter construct impacted basal promoter activity and induction of promoter activity in response to treatment with t-BHQ. Deletion of $_{-2698}$*ARE* also impacted the induction of *CBR3* promoter activity in cells overexpressing Nrf2. Electrophoretic mobility shift assays (EMSA) demonstrated increased binding of specific protein complexes to $_{-2698}$*ARE* in nuclear extracts from t-BHQ treated cells. The presence of Nrf2 in the specific nuclear protein–$_{-2698}$*ARE* complexes was evidenced in EMSA experiments with anti-Nrf2 antibodies. These data suggest that the distal $_{-2698}$*ARE* mediates the induction of human *CBR3* in response to prototypical activators of Nrf2.

1. Introduction

Carbonyl reductase activity plays a crucial role during the reduction of several endogenous and exogenous carbonyl compounds [1]. In humans, there are three carbonyl reductases, CBR1, CBR3 and CBR4. CBR1 and CBR3 are cytosolic monomeric enzymes, whereas CBR4 forms a mitochondrial heterotetramer with 17β-hydroxysteroid dehydrogenase type 8 (17β-HSD8) [2,3]. The *CBR1* and *CBR3* genes are located in chromosome 21 (*CBR1*, 21q22.13 and *CBR3*, 21q22.22), where they are separated by a relatively short distance of 62 kb. CBR3 catalyzes the reduction of 1,2-naphthoquinone, isatin, oracin, coniferyl aldehyde and acetohexamide [4,5]. The anticancer anthracyclines doxorubicin and daunorubicin are also reduced by CBR3 into their corresponding C-13 alcohol metabolites doxorubicinol and daunorubicinol. Recent studies suggest that genetic polymorphisms in *CBR3* contribute to the variable toxicodynamics of anthracycline drugs in cancer patients [6–9].

CBR3 is expressed in various tissues including heart, liver, kidney, spleen, lung and brain [5]. Variable *CBR3* expression at the mRNA and protein level has been documented in liver [10,11]. Absolute quantification of CBR3 with a new liquid chromatography/mass spectrometry assay showed that protein expression varied by 14-fold (range: 1.3–17.9 ppm) [11]. Recent research has provided the first insights into the molecular mechanisms that dictate variable *CBR3* expression. Several lines of evidence indicate that *CBR3* expression is modulated by the transcription factor Nrf2 (nuclear factor [erythroid-derived 2]-like 2, official symbol: NFE2L2) [12–17]. For example, Hu et al. examined gene expression profiles in livers from *nrf2* wild type and *nfr2* knockout mice after treatment with the Nrf2 activator sulforaphane. The authors documented significant *Cbr3* mRNA induction in livers of nfr2 proficient animals relative to the levels of transcript in nrf2 deficient mice (e.g., 4.0-fold after 3 h treatment) [12]. More recently, Ebert et al. have shown that Nrf2 also mediates the induction of *CBR3* in human cancer cell lines after treatment with prototypical Nrf2 activators (e.g., sulforaphane and diethylmaleate) [14]. It is known that Nrf2 coordinates the induction of a

Abbreviations: *CBR3*, carbonyl reductase 3; *ARE*, antioxidant response element; Nrf2, nuclear factor [erythroid-derived 2]-like 2 (official symbol: *NFE2L2*); RT-PCR, reverse transcription-polymerase chain reaction; DMSO, dimethyl sulfoxide; bp, base pairs; EMSA, electrophoretic mobility shift assay; ROS, reactive oxygen species; *GPXs*, glutathione peroxidases; *NQO1*, NAD(P)H:quinone oxidoreductase-1; *NQO2*, NRH:quinone oxidoreductase 2; *SOD*, superoxide dismutase; *GSTs*, glutathione S-transferases; *GPXs*, glutathione peroxidases; *GCLC*, glutamate-cysteine ligase catalytic subunit; *GCLM*, glutamate cysteine ligase modifier subunit.

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doi:10.1016/j.bcp.2011.09.027

battery of stress-responsive genes including NAD(P)H:quinone oxidoreductase-1 (*NQO1*), superoxide dismutase (*SOD*), glutathione S-transferases (*GSTs*) and glutathione peroxidases (*GPXs*) [18]. Mutational analyses identified a conserved DNA sequence element known as an antioxidant response element (*ARE*) in the promoter of genes regulated by Nrf2. Under conditions of oxidative stress, Nrf2 translocates to the nucleus and forms heterodimers with other transcription factors such as small Maf proteins. The Nrf2 heterodimers bind to functional *AREs* and mediate the transcriptional activation of a wide range of target genes encoding stress-responsive proteins [18–21].

The promoter of human *CBR3* has been recently identified [10]. To the best of our knowledge, the functional role of conserved *AREs* in the *CBR3* promoter region has not yet been elucidated. This study documents the identification and functional characterization of a conserved *ARE* ($_{-2698}$*ARE*) in the promoter region of human *CBR3*.

2. Materials and methods

2.1. Cell culture and reagents

HepG2 (human hepatoblastoma, HB-8065), and MCF-7 (human breast adenocarcinoma, HTB-22) cell lines were obtained from the American Type Culture Collection (Manassas, VA). RPMI 1640 and other cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Cells were routinely cultured in 10 cm^2 dishes or 48-well plates using RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma–Aldrich, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were grown in an incubator at 37 °C, 5% CO_2, and 95% relative humidity. Cultures were maintained at low passage numbers ($n < 12$) and were free of mycoplasma contamination. Tert-butyl hydroquinone (t-BHQ) was purchased from Sigma–Aldrich.

2.2. Quantification of CBR3 mRNA

Total RNA (100 ng) was reverse-transcribed and amplified using one-step QuantiTect SYBR Green RT-PCR kits (Qiagen, Valencia, CA) with the following primers: 5′-GCTTCCACCAACTGGACATC-3′ (forward) and 5′-GGGCATTGGATCATCACTCT-3′ (reverse). Melting curve analyses demonstrated a unique PCR amplification product. Amplification reactions were performed as described [10]. In brief, relative *CBR3* mRNA levels were determined by the comparative quantitation method using individual β-actin mRNA levels as normalizers. Amplification efficiencies for *CBR3* and β-actin mRNAs were similar. Standard curves for both mRNAs were run in parallel (20-fold dynamic range, $r^2 \geq 0.96$). Experimental samples and standards for calibration curves were analyzed in quadruplicate [22].

2.3. Detection of CBR3 protein by immunoblotting

HepG2 cells (80% confluence) were incubated with DMSO (0.1%, v/v) or 50 μM t-BHQ for 18 h. HepG2 cell lysates were prepared with ice-cold Pierce RIPA buffer (Thermo Fisher Scientific, Rockford, IL) supplemented with Halt Protease and Halt Phosphatase inhibitor cocktails (Thermo Fisher Scientific). The concentration of total proteins was determined with the BCA Protein Assay Reagent (Thermo Fisher Scientific). HepG2 cell lysates (50 μg of protein/lane) were loaded into 12% precast polyacrylamide gels (Invitrogen, Carlsbad, CA) and separated by electrophoresis. Protein blots were probed with a specific polyclonal anti-human CBR3 antibody (1:2000; sc-70220, Santa Cruz Biotechnology, Santa Cruz, CA) and a secondary goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:10,000; Sigma–Aldrich). Membranes were also probed with anti-β-actin antibody (1:10,000; Santa Cruz) to correct for differences in protein loading. Immunoreactive bands were visualized with the ECL Plus Western blotting detection system (GE Healthcare, Chalfont St. Giles, UK). CBR3 and β-actin band intensity values (pixels/mm^2) were quantified with a Molecular Imager® Gel Doc™ XR+ System (Bio-Rad Laboratories, Hercules, CA).

2.4. Reporter gene studies

DNA sequence upstream (4212 bp) from the translation start codon of *CBR3* ($A_{+1}TG$) was amplified by PCR from human DNA sample HD17249 (Coriell Institute for Medical Research, Camden, NJ). The amplicon was cloned into a pGL3 basic firefly luciferase vector (Promega, Madison, WI). The *ARE* core sequence (5′-GTGACCCTGC-3′; Fig. 1) was deleted from the $_{-4212}$*CBR3* construct by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA) with the following primers: 5′-GCTGGGTTTGTTGCCAAACTTATAGAGACCCTTCG-3′ (forward) and 5′-CGAAGGGTCTCTATAAGTTTGGCAACAAACCCAGC-3′ (reverse). The human Nrf2 expression plasmid pNrf2-CMV6-XL5 was purchased from OriGene (Rockville, MD). The empty CMV6-XL5 vector was generated by removal of the Nrf2 insert from pNrf2-CMV6-XL5 with the restriction enzyme NotI-HF™ (New England Biolabs, Ipswich, MA). The identity of all constructs and the absence of mutations were verified by direct sequencing.

HepG2 and MCF-7 cells were plated 24 h before transfections in 48-well plates. Cell cultures at 60–80% confluence were co-transfected with *CBR3* reporter constructs (250 ng) and the internal control plasmid pRL-TK (50 ng; Promega). In some experiments, cultures were co-transfected with various amounts of the Nrf2 expression plasmid (1, 10, and 100 ng), and the total amount of co-transfected DNA was adjusted up to 100 ng with the empty pCMV6-XL5 vector. The pGL3-basic empty vector was transfected into control cultures to correct for background luciferase activity. Firefly luciferase activities from each reporter

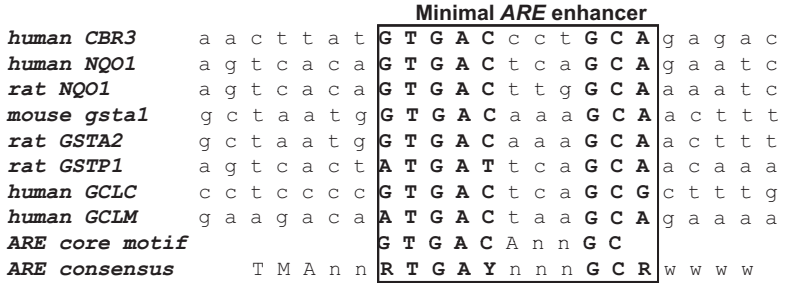

Fig. 1. DNA sequence alignment for $_{-2698}$*ARE* in the promoter of human *CBR3* and *AREs* in the promoters of prototypical target genes. The conserved $_{-2698}$*ARE* encompasses the −2704 to −2695 bp region upstream the translation start codon ($A_{+1}TG$). Nucleotides in bold capitals indicate the consensus *ARE* sequence. *ARE* sequences are taken from Nioi and Hayes [20].