BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2010,** *49,* 8499–8511 8499
DOI: 10.1021/bi100814d

Regulation of Human Carbonyl Reductase 3 (CBR3; SDR21C2) Expression by Nrf2 in Cultured Cancer Cells[†]

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Received May 21, 2010; Revised Manuscript Received August 31, 2010

ABSTRACT: Carbonyl reduction is a central metabolic process that controls the level of key regulatory molecules as well as xenobiotics. Carbonyl reductase 3 (CBR3; SDR21C2), a member of the short-chain dehydrogenase/reductase (SDR) superfamily, has been poorly characterized so far, and the regulation of its expression is a complete mystery. Here, we show that CBR3 expression is regulated via Nrf2, a key regulator in response to oxidative stress. In human cancer cell lines, CBR3 mRNA was expressed differentially, ranging from very high (A549, lung) to very low (HT-29, colon; HepG2, liver) levels. CBR3 protein was highly expressed in SW-480 (colon) cells but was absent in HCT116 (colon) and HepG2 cells. CBR3 mRNA could be induced in HT-29 cells by Nrf2 agonists [sulforaphane (SUL, 7-fold) and diethyl maleate (DEM, 4-fold)] or hormone receptor ligand Z-guggulsterone (5-fold). Aryl hydrocarbon receptor agonist B[*k*]F failed to induce CBR3 mRNA after incubation for 8 h but elevated CBR3 levels after 24 h, most likely mediated by B[*k*]F metabolites that can activate Nrf2 signaling. Inhibition of Nrf2-activating upstream kinase MEK/ERK by PD98059 weakened DEM-mediated induction of CBR3 mRNA. Proteasome inhibitors MG-132 (5 μM) and bortezomib (50 nM) dramatically increased the level of CBR3 mRNA, obviously because of the increase in the level of Nrf2 protein. While siRNA-mediated knockdown of Nrf2 led to a decrease in the level of CBR3 mRNA in A549 cells (30% of control), Keap1 knockdown increased the level of CBR3 mRNA expression in HepG2 (9.3-fold) and HT-29 (2.7-fold) cells. Here, we provide for the first time evidence that human CBR3 is a new member of the Nrf2 gene battery.

In addition to disease progression and the pathogenesis of different chronic diseases such as arthritis, cancer, and Alzheimer's disease, oxidative stress contributes to normal cell aging. Cellular oxidative stress is associated with increased levels of oxidized proteins and phospholipids and can produce toxic lipids bearing reactive carbonyl functions. Detoxification of such toxic lipids can occur by trapping processes and/or metabolism, among which enzymatic reduction of carbonyl functions is of major significance (*1*, *2*).

Therefore, carbonyl reduction is not only a central physiological process that regulates the concentration of critical signaling molecules such as steroid hormones, prostaglandins, and retinoids in the human body. Similar to the oxidative metabolism, being mediated by cytochrome P_{450}-dependent monooxygenases (CYP), the reductive metabolism of exogenous and endogenous toxicants like toxic lipid aldehydes derived from lipid peroxidation is increasingly recognized as an important part of the detoxification system.

To date, three human carbonyl reductases are known; CBR1 (SDR21C1) and CBR3 (SDR21C2) are exclusively expressed in the cytosolic compartment of the cell, whereas the recently identified CBR4 (SDR45C1) has been localized to the mitochondrial membrane (*3*) (for systematic SDR[1] nomenclature, see ref *4*).

CBR1 and CBR3 both map to chromosome 21 and share a high degree of amino acid sequence identity (77%), which is in contrast to the general low level of amino acid sequence identity found among other members of the short-chain dehydrogenase/reductase (SDR) superfamily.

CBR1, the best characterized carbonyl reductase so far, is also known as prostaglandin (PG) 9-keto reductase and is thought to be involved in endogenous prostaglandin metabolism, in addition to exhibiting a broad specificity for xenobiotic carbonyl compounds. By contrast, CBR3 has no significant PG-9 reductase or 15-hydroxy PG dehydrogenase activity, pointing to distinct physiological roles of both enzymes.

[†]This work was supported by a DFG (Deutsche Forschungsgemeinschaft) grant (MA 1704/5-1).

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[1]Abbreviations: AhR, aryl hydrocarbon receptor; AKR, aldo-keto reductase; AP-1, activator protein-1; AR, androgen receptor; ARE, antioxidant response element; Bach1, broad-complex, tramtrack and bric à brac and cap 'n' collar homology 1 protein; B[*k*]F, benzo[*k*]fluoroanthrene; CBR1, carbonyl reductase 1; CBR3 (SDR21C2), carbonyl reductase 3; CYP1A1, cytochrome P_{450}-dependent monooxygenase 1A1; FXR, farnesoid X receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; γ-GCS (GCLC), γ-glutamylcysteine synthetase, catalytic subunit; GR, glucocorticoid receptor; GS, guggulsterone; GSH, glutathione; GST, glutathione *S*-transferase; HO-1, heme oxygenase-1; HRP, horseradish peroxidase; Keap1, Kelch-like ECH-associated protein 1; MAPKK, MEK, mitotic activated protein kinase kinase/ERK, extracellular signal-regulated kinase; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; MRP2, multidrug resistance-associated protein 2; NFκB, nuclear factor κ-B; NQO1, NADP(H):quinone oxidoreductase; siRNA, short interfering RNA; Nrf2, nuclear factor-erythroid 2 related factor 2; PAH, polycyclic aromatic hydrocarbon; PD98059, 2′-amino-3′-methoxyflavone; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PXR, pregnane X receptor; ROS, reactive oxygen species; SDR, short-chain dehydrogenase/reductase; SUL, sulforaphane; DEM, diethyl maleate; TBHQ, *tert*-butyl hydroquinone; XRE, xenobiotic response element.

Published on Web 08/31/2010

As reported recently, CBR3 mRNA is ubiquitously distributed in several human normal tissues, with its strongest expression in ovary, pancreas, intestine, and lung (*5*). However, very little information about the biochemical characteristics of CBR3 has been found, and neither the physiological role nor the molecular regulation of its expression is known. Although the promoter region of CBR3 has been published very recently (*6*), the mechanisms that control the expression of CBR3 in human cells are still unknown.

Because some carbonyl reducing enzymes from the aldo-keto reductase (AKR) superfamily have been shown to be involved in the cellular defense system against oxidative stress (*7*, *8*), the aim of this study was to elucidate if CBR3 expression is regulated by Nrf2 antioxidant response element signaling pathways.

The Nrf2 (nuclear factor-erythroid 2 related factor 2)/Keap1 (Kelch-like ECH-associated protein 1) system plays a key role in the cell's response to oxidative stress (*9*). To investigate the possible involvement of Nrf2 in the regulation of CBR3, the Nrf2 signaling pathway was experimentally modulated at different levels. These approaches included, on one hand, induction of Nrf2 expression and/or activity (e.g., by chemicals such as SUL or by inhibition of proteasomal degradation of Nrf2) and, on the other hand, a repression or inhibition of Nrf2 activity [by small interfering RNA (siRNA)-mediated knockdown of Nrf2 and Keap1 expression or indirect inhibition of Nrf2 activity by upstream kinase inhibitor PD98059].

Several models have been proposed to explain the repression of Nrf2 by Keap1 (*10*). According to one model, Keap1 serves as a substrate adaptor for Cullin-3 (Cul3) that binds to ring-box 1 (Rbx1) to form the E3 ubiquitin ligase complex that ultimately leads to ubiquitination and proteasomal degradation of Nrf2. An alternative model describes Keap1 as an anchoring protein that is associated with the cytoskeleton and retains Nrf2 in the cytoplasm. Keap1 functions as a sensor of oxidative stress because the oxidation of critical cysteine residues within Keap1 triggers the release of Nrf2, followed by its translocation to the nucleus where Nrf2 forms a heterodimer with proteins that belong to the small musculoaponeurotic fibrosarcoma (Maf) family of transcription factors or with members of the activator protein-1 (AP-1) family (e.g., Jun and Fos). The heterodimer formed then binds to the antioxidant response element (ARE) [5′-$^{A}/_{G}$TGA$^{C}/_{T}$NNNGC$^{A}/_{G}$-3′] in the promoter region of target genes, thereby activating a battery of genes that catalyze the detoxification of xenobiotics and provide direct antioxidants such as glutathione (GSH) (*9*, *11*). Because dissociation of Nrf2 from Keap1 requires phosphorylation of Nrf2, the inhibition of upstream kinases involved, such as protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), mitotic activated kinase kinase (MAPKK, MEK)/extracellular signal-regulated kinase (ERK), and p38 MAP kinase, provides a valuable tool for the investigation of Nrf2-controlled gene transcription (*12*). For example, PD98059 (2′-amino-3′-methoxyflavone) is a MEK/ERK inhibitor, has been successfully used to block Nrf2-mediated gene transcription (*13*, *14*), and therefore has been employed in our study as a diagnostic tool to prove the Nrf2 dependency of CBR3 regulation.

Inducers of xenobiotic metabolizing enzymes are formally divided into monofunctional and bifunctional inducers (*15*). Monofunctional enzyme inducers [e.g., D,L-sulforaphane (SUL) and diethyl maleate (DEM)] regulate gene expression by recruiting Nrf2 to AREs in the upstream regulatory region of responsive genes. Bifunctional inducers (e.g., polycyclic aromatic hydrocarbons and flavonoids) induce gene expression through both ARE and xenobiotic response elements (XRE, 5′-$^{T}/_{G}$N**GCGTG**$^{A}/_{C}$$^{G}/_{C}$A-3′), the latter requiring transactivation by the aryl hydrocarbon receptor (AhR) (*16*). Both monofunctional and bifunctional enzyme inducers have been tested here for their effect on CBR3 expression and to differentiate between the involvement of either the Nrf2/ARE or the AhR/XRE signaling pathway (see Figure 10).

In this investigation, we provide for the first time clear evidence that CBR3 is regulated via Nrf2-dependent signaling pathways, a finding that implies CBR3 plays an important role in the cellular response to oxidative stress.

MATERIALS AND METHODS

Chemicals and Materials. Benzo[*k*]fluoranthene (B[*k*]F), D,L-sulforaphane (SUL), diethyl maleate [maleic acid diethyl ester (DEM)], PD98059 (2′-amino-3′-methoxyflavone), Pefabloc sc, *tert*-butyl hydroquinone (TBHQ), and Z-guggulsterone (GS) were purchased from Sigma (Deisenhofen, Germany). MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) was obtained from Calbiochem (Darmstadt, Germany). Bortezomib (PS-431) was obtained from LC Laboratories (Woburn, MA). Stock solutions of all test compounds were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C until they were used. GelRed nucleic acid stain was obtained from Biotest (Mannheim, Germany). Nrf2 siRNA, Keap1 siRNA, and control siRNA-A were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Cell Culture and Cell Lines. Cell culture media and supplements were purchased from PAA (Coelbe, Germany). Chinese hamster lung fibroblast cell line V-79 and human cell lines A549, HT-29, Caco-2, and SW-480 were obtained from the Deutsche Sammlung für Mikroorganismen and Zellkulturen (DSMZ, Braunschweig, Germany). HCT116 cells were generously provided by J. Abel (IUF, University of Duesseldorf, Duesseldorf, Germany). Cell lines HepG2, PANC-1, and A431 were purchased from Cell lines service (CLS, Eppelheim, Germany).

Cell Culture. Human colon adenocarcinoma cell lines Caco-2, HT-29, HCT116, and SW-480, human lung cancer cell line A549, and V-79 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose) supplemented with 2 mM L-glutamine, 1% nonessential amino acids, and 10% heat-inactivated fetal calf serum (FCS). PANC-1 (pancreas) and A431 (skin) cells were cultured in DMEM supplemented with 4 mM L-glutamine and 10% FCS. HepG2 (liver) cells were maintained in a DMEM/Ham's F12 (1:1) mixture supplemented with 2 mM L-glutamine and 10% FCS. All cells were cultured without antibiotics in a humidified atmosphere of 5% CO_2 in air at 37 °C.

Gene Expression Experiments. Cells were cultured in 60 mm Petri dishes or six-well plates (9.3 cm^2). After reaching 90−100% confluency, cells were treated with test compounds freshly dissolved in culture medium. After incubation times as indicated, medium was removed, the cell-monolayer was washed with phosphate-buffered saline (PBS), and cells were harvested for RNA isolation. All experiments were performed in duplicate or triplicate.

RNA Isolation and Reverse-Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol with an additional 70% ethanol wash. Two micrograms of RNA was reverse transcribed using RevertAid H minus Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (fermentas, St. Leon-Rot, Germany) with 100 pmol of oligo$(dT)_{18}$ as a primer for 60 min at 42 °C.

Table 1: RT-PCR Primers and Amplicon Sizes

gene	GenBank accession number	forward primer/reverse primer	amplicon size (bp)
AKR1B1	NM_001628	CCCATGTGTACCAGAATGAGAA CTGGAGATGGTTGAAGTTGGAG	362
β-actin	NM_001101	ACTCTTCCAGCCTTCCTTCCT AGGTTTTGTCAAGAAAGGGTGT	394
CBR3	NM_001236	GCTCAACGTACTGGTCAACAAC ATCCTCGATAAGACCGTGACC	372
CYP1A1	NM_000499	TGATTGAGCACTGTCAGGAGA GGTTGATCTGCCACTGGTTTA	393
GAPDH	NM_002046	TGG AAG GAC TCA TGA CCA CA TTC TAG ACG GCA GGT CAG GT	239
γ-GCS (GCLC)	NM_001498	CCCATGGAGGTGCAATTAAC TGCGATAAACTCCCTCATCC	465
Keap1	BC002930.1	AGGCCATGTTCACCAACGGGCT GGAAGACCTCGGACTCGCAGCG	417
Nrf2	NM_006164	GAGAGCCCAGTCTTCATTGC ACTGGTTGGGGTCTTCTGTG	343
NQO1	NM_001025433	GGTATCTTTCCAGGCTTCCC TCTGAGCAATTCCCTTCTGC	353
MRP2	NM_000392.3	CCGTATCAGGTTTGCCAGTT CAACAGCCACAATGTTGGTC	312

To avoid the co-amplification of genomic DNA, prior to reverse transcription, RNA was subjected to DNase I digestion (Promega, Heidelberg, Germany). Moreover, all primers were designed to span at least one exon−exon boundary.

Hot-start PCR was performed using Phire Hot-start DNA polymerase (Biozym Scientific, Hessisch Oldendorf, Germany) in a total volume of 20 μL containing 2 μL of cDNA, 500 nM RT-Primer, 200 μM dNTPs, and 1.5 mM $MgCl_2$.

The thermal cycling comprised an initial denaturation step at 98 °C for 30 s, followed by 19−34 cycles of denaturation at 98 °C for 10 s, annealing at 59 °C for 15 s, and extension at 72 °C for 15 s and a final extension step at 72 °C for 2 min. GAPDH or β-actin served as the housekeeping gene.

Amplified PCR products were separated electrophoretically on 1.5% agarose, visualized by staining with GelRed, and documented digitally with an Intas Gel-imaging system (Intas, Goettingen, Germany). Primer sequences and amplicon sizes are listed in Table 1.

Construction of CBR3 Expression Plasmid pCI-neoCBR3. Human CBR3 cDNA was purchased from RZPD (Deutsches Ressourcenzentrum für Genomforschung, Berlin, Germany), amplified by PCR, and cloned into the EcoRI and NotI restriction sites of the pCI-neo mammalian expression vector (Promega, Mannheim, Germany) using the "Rapid Ligation Kit" from fementas. This vector contains the neomycin phosphotransferase gene, which allows for the selection of cells stably expressing CBR3 by treatment with G-418 (Biochrom AG, Berlin, Germany). Restriction enzymes were purchased from New England Biolabs (Frankfurt am Main, Germany).

Transfection of Small Interfering RNA (*siRNA*). A549, HT-29, or HepG2 cells were seeded in six-well plates and grown to 50−60% confluency. Prior to transfection, medium was replaced with 2 mL of fresh growth medium containing FCS. The specific siRNAs targeting either Nrf2 (sc-37030) or Keap1 (sc-43878) or nontargeting control siRNA-A (sc-37007) was diluted in 250 μL of OptiMEM I (Invitrogen, Karlsruhe, Germany), combined with 250 μL of OptiMEM I containing 10 μL of Lipofectamine 2000 (Invitrogen), and after incubation for 20 min at room temperature, the mixture was added to the cells. The final amount of both siRNAs was 100 pmol. To reduce cytotoxic reactions, the medium was replaced after 8 h with fresh culture medium and cells were harvested after incubation for 48 and 72 h for total RNA isolation.

Western Blotting. Cells were cultured in 100 mm Petri dishes and treated with different test compounds or DMSO [0.1% (v/v)] as indicated in the figures. To obtain whole cell lysates, cells were harvested by being scraped into a small volume of ice-cold PBS containing 50 μM Pefabloc sc protease inhibitor, pelleted by centrifugation, and passively lysed in RIPA buffer [50 mM Tris, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium desoxycholate, and 0.1% SDS (pH 7.5)] supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) for 30 min on ice. Soluble fractions were obtained by centrifugation, and protein contents of samples were determined by the bicinchoninic acid (BCA) method (*17*) with bovine serum albumin (BSA) as a standard. Proteins (60−80 μg) were separated on NuPAGE Novex Bis-Tris gels (4 to 12% acrylamide, Invitrogen) and electrotransferred onto PVDF membranes. After samples had been blocked overnight in blocking buffer (5% milk in PBS-T) at 4 °C, probing was performed for 1 h with anti-CBR3 antibody (Santa Cruz; sc-70218, 1:500 dilution in PBS-T containing 2.5% milk). Blots were then incubated with the secondary anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:4000) for 1.5 h at room temperature. Bands were visualized with an enhanced chemiluminescence detection kit (ECL) following the manufacturer's instructions (GE Healthcare, Heidelberg, Germany). Blots were stripped using standard protocols and reprobed for β-actin [anti-β-actin primary antibody from NeoMarkers (Fremont, CA), 1:100 dilution; anti-rabbit HRP-conjugated secondary antibody, 1:10000 dilution]. All experiments were repeated at least once.

Statistical Analysis. Statistical analyses were performed with GraphPad Prism4. Differences between mean values were determined by a two-tailed unpaired Student's *t* test or a one-way ANOVA followed by a Tukey−Kramer post-test. Statistically significant differences were set at $P \leq 0.05$, and $P \leq 0.001$ indicated highly significant data.

RESULTS

Constitutive Expression of CBR3, NQO1, and Nrf2 in Different Human Cell Lines. To identify an appropriate model cell line for studying the regulation of CBR3, we first

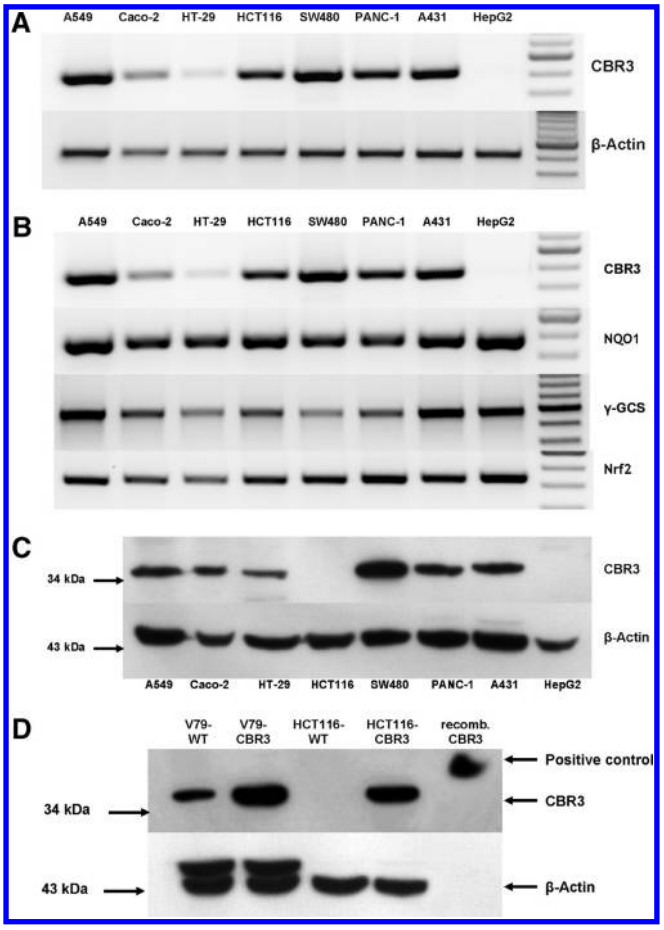

FIGURE 1: (A) Constitutive expression of CBR3 mRNA in different human cancer cell lines derived from lung (A549), colon (Caco-2, HT-29, HCT116, and SW-480), pancreas (PANC-1), skin (A431), and liver (HepG2). Total RNA (2 μg) was reverse transcribed, and gene specific DNA fragments were amplified by 34 (CBR3) and 19 (β-actin) PCR cycles. (B) Constitutive expression of Nrf2-regulated genes NQO1, γ-GCS, and Nrf2 in different human cancer cell lines. Total RNA (2 μg) was reverse transcribed, and gene specific DNA fragments were amplified by 34 (CBR3), 28 (NQO1), and 26 (γ-GCS and Nrf2) PCR cycles. (C) Basal expression levels of CBR3 protein in different human cancer cell lines. Whole cell lysates were prepared from different human cancer cell lines, and proteins (70 μg) were analyzed by Western blotting using the rabbit anti-CBR3 primary antibody (Santa Cruz, sc-70218; 1:500 dilution). β-Actin served as the loading control. (D) Expression of CBR3 protein in cells stably transfected with CBR3 expression plasmid pCI-neo*CBR3*. V-79 cells or human colon carcinoma HCT116 cells were stably transfected with CBR3 expression vector pCI-neo*CBR3*. Whole cell lysates (80 μg per lane) were subjected to Western blot analysis using the anti-CBR3 antibody (Santa Cruz, sc-70218). Recombinant CBR3 containing a His tag served as a positive control (*48*).

screened a variety of cell lines for constitutive CBR3 expression. CBR3 mRNA and protein expression were detected in several human cell lines originating from different tissues, including colon, lung, skin (keratinocytes), pancreas, and liver. As shown in Figure 1A, the largest amounts of CBR3 mRNA were found in A549 and SW-480 cells followed by HCT116, PANC-1, A431, and Caco-2 cells. By contrast, HT-29 and HepG2 cells expressed a strikingly small amount of CBR3 mRNA, requiring a relatively large number of PCR cycles (34) to become detectable. Because of the low basal level of expression of CBR3 in HT-29 cells, this cell line was chosen as a suitable model for most of the experiments to investigate the regulation of CBR3.

Malignant transformation of tumor cells is often accompanied by inappropriate regulation of signaling pathways leading to an overexpression of genes that enhance cell survival and promote tumor growth. Such a constitutive activation of Nrf2 signaling has been found in colon tumors (*18*). Given that Nrf2 signaling is constitutively active in some cancer cell lines, a concerted overexpression of several Nrf2-regulated genes, including Nrf2 itself, would be the result. Therefore, to substantiate our hypothesis of Nrf2 being involved in the regulation of CBR3, we analyzed the expression of Nrf2 and Nrf2-regulated genes NADP(H):quinone oxidoreductase (NQO1) and γ-GCS in those cell lines. As shown in Figure 1B, mRNA amounts of Nrf2, NQO1, and γ-GCS correlated quite well with that of CBR3 in most cases but not all. Very striking is the expression pattern of those genes in HepG2 cells, which express the lowest levels of CBR3 but large amounts of NQO1, γ-GCS, and Nrf2 mRNA (see Discussion). As shown in Figure 1C, protein levels correlated well with CBR3 mRNA expression levels for almost all cell lines, with the exception of HCT116 cells that expressed high levels of CBR3 mRNA but no CBR3 protein. Even when as much as 160 μg of protein was loaded onto the Western blot, no CBR3 protein could be detected [not shown (see Discussion)].

Transfection of Human HCT116 and Chinese Hamster V-79 Cells with CBR3. We stably transfected human HCT116 cells with CBR3 expression vector pCIneo-*CBR3* and assayed whole cell lysates obtained from those cells for CBR3 expression. As shown in Figure 1D, CBR3 protein was undetectable in HCT116 WT (wild type) cells, but CBR3-transfected HCT116 cells showed a strong immunoreactive band of approximately 34 kDa. It should be noted here that the CBR3 bands occurred at around 34 kDa throughout our studies, which is more than the size of 31 kDa as predicted by the UniProtKB/Swiss-Prot database (CBR3_HUMAN, 075828).

To conclusively demonstrate that the 34 kDa band corresponds to CBR3, we stably transfected Chinese hamster lung fibroblast V-79 cells with the same vector (pCIneo-*CBR3*) and performed Western blotting. For this experiment, anti-CBR3 antibody sc-70218 (Santa Cruz) was used to detect CBR3. This antibody is directed against the C-terminus of human CBR3 and has a known cross-reactivity with CBR3 orthologues from other species (cow, rat, and mouse). As seen in Figure 1D, this antibody also detects Chinese hamster CBR3 (CHR3) in V-79 WT (wild type) cells, which shares 86% identity with human CBR3 and has the same predicted size of 31 kDa (*19*). Overexpression of human CBR3 in V-79 cells (V-79-CBR3) strengthened the CHR3 signal in V-79 WT cells at ~34 kDa, showing that both proteins share the same molecular mass. This indicates that the immunoreactive band of approximately 34 kDa obtained in our studies unambiguously corresponds to human CBR3.

Effects of the AhR Agonist B[k]F and the Nrf2 Activator TBHQ on CBR3 mRNA Expression in HCT116 Colon Cancer Cells. Addressing the question of whether CBR3 expression is dependent on AhR/XRE or the Nrf2/ARE signaling pathway, we incubated HCT116 cells with either the AhR agonist B[*k*]F or the Nrf2 activator *tert*-butyl hydroquinone (TBHQ). Compared to the untreated control, no increase in the level of CBR3 mRNA expression could be observed in HCT116 cells treated for 8 h with 5 and 10 μM B[*k*]F (Figure 2A,B). As a control for the activation of the AhR/XRE signaling pathway, we monitored the expression of CYP1A1 (30 PCR cycles), a prototypical AhR target gene. The increased level of expression of CYP1A1 mRNA after incubation for 8 and 24 h with B[*k*]F indicates that the AhR signaling pathway is activated. By contrast, TBHQ, a well-established Nrf2 activator clearly elevated the level

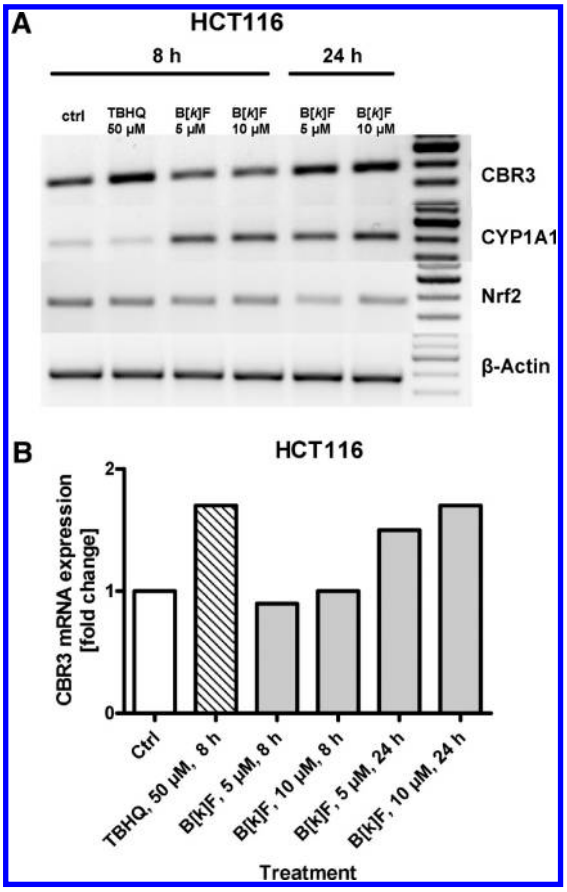

FIGURE 2: Effect of TBHQ and B[*k*]F on CBR3 mRNA expression. In HCT116 cells, Nrf2 activator TBHQ increases CBR3 mRNA levels after only 8 h, whereas treatment with AhR agonist B[*k*]F requires a longer period of time to induce CBR3 expression, most likely because of a secondary effect mediated by B[*k*]F metabolites and/or ROS produced by CYP1A1 activity (ctrl denotes control, treated with vehicle, 0.1% DMSO). The bar graph (B) represents the densitometric analysis of the gel shown in panel A.

of CBR3 mRNA expression (1.7-fold) after incubation for 8 h but failed to induce CYP1A1 (Figure 2B). Interestingly, application of B[*k*]F for a longer period of time (24 h) also led to an increase in the level of CBR3 expression (1.7-fold). Note that in this cell line the inducibility of CBR3 is relatively low in comparison with that of HT-29 cells, because of the higher level of constitutive CBR3 mRNA expression.

CBR3 mRNA Expression in HT-29 Colon Cancer Cells after Long-Term Incubation with B[k]F. On the basis of the observation that induction of CBR3 by B[*k*]F requires a relatively long incubation time of 24 h, it was interesting to see whether a longer incubation of 48 and 72 h would result in a further increase in the level of CBR3 mRNA expression. For this experiment, HT-29 cells were chosen, because of their lower basal level of CBR3 mRNA (see Figure 1A). As shown in Figure 3A, compared to HT-29 cells treated with B[*k*]F (5 μM) for 24 h (1.3-fold increase over control), no substantial further elevation of the level of CBR3 mRNA could be seen after treatment for 48 h (1.7-fold increase). By contrast, a 72 h incubation led to a downregulation of CBR3 mRNA expression. As expected, the AhR-regulated control gene CYP1A1 maintained a high level of mRNA expression after incubation with B[*k*]F for 24, 48, and 72 h.

Effect of Proteasome Inhibitors on CBR3 mRNA and Protein Expression in HT-29 Cells. The proteasome machinery is known to degrade damaged or misfolded proteins, but also to control the levels of key regulatory proteins such as activated transcription factors.

Under basal conditions, Nrf2 has a short half-life of approximately 13−20 min (*20*) because of its constitutive ubiquitin-dependent proteasomal degradation (*21*, *22*). Consequently, inhibition of proteasome function by specific inhibitors of 26S proteasomes such as MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) increases levels of Nrf2 (*20*). To test whether the inhibition of 26S proteasomes by the specific proteasome inhibitor MG-132 would lead to a further increase in the level of CBR3 mRNA, HT-29 cells were co-incubated with B[*k*]F and MG-132 (20 μM). Interestingly, cotreatment with B[*k*]F and MG-132 resulted in a dramatic upregulation of CBR3 mRNA [7-fold over control (Figure 3A,B)], a fact that could not be observed for CYP1A1.

Because of this increase in the level of CBR3 mRNA expression in cells treated with a combination of B[*k*]F and MG-132, we next tested the effect of the proteasome inhibitors MG-132 (20 μM) and bortezomib (0.05 μM) alone. Both compounds strongly elevated the extent of CBR3 mRNA expression (Figure 3C). Importantly, the boronic acid derivative bortezomib had a very strong effect already at a remarkably low concentration of 50 nM. This result clearly indicates that the strong increase in the level of CBR3 mRNA by co-incubation of HT-29 cells with B[*k*]F and MG-132 was attributable to the inhibition of proteasomal turnover of an activated transcription factor, which in this case was most likely Nrf2. However, as shown in Figure 3D, on the protein level, only a marginal elevation in the level of CBR3 could be observed after 8 h, which was further reduced after incubation with bortezomib for 24 h.

Regulation of CBR3 mRNA Expression upon Exposure of HT-29 Cells to the ARE Activator Diethyl Maleate (DEM), the Proteasome Inhibitor MG-132, and the MAPK Kinase (MEK)/ERK Inhibitor PD98059. To substantiate our hypothesis that CBR3 is regulated by Nrf2-dependent signaling pathways, we incubated HT-29 cells with the prototypical ARE activator diethyl maleate (100 μM) (Figure 4A). As expected, DEM treatment led to a large increase in the level of CBR3 mRNA expression after 8 h (4.1-fold compared to control), which declined after 24 h (1.9-fold) (Figure 4B). A slight further increase could be observed by cotreatment with MG-132 after 8 h (5.4-fold). Interestingly, proteasome inhibition for 24 h by MG-132 resulted in a stabilization of the high level of CBR3 mRNA that had been achieved upon coexposure to DEM. PD98059, a substituted flavonoid, is an established inhibitor of MEK that, by this action, blocks Nrf2-mediated gene expression. Co-incubation of HT-29 cells with DEM (100 μM) and PD98059 (10 μM) clearly diminished the DEM-mediated CBR3-inducing effect after 8 h (from 4.2- to 3.6-fold) and even reduced CBR3 mRNA amounts to nearly basal levels after 24 h (to 1.2-fold).

These results strongly confirm our hypothesis that CBR3 expression is regulated via Nrf2-dependent pathways in HT-29 cells.

Time- and Concentration-Dependent Upregulation of CBR3 mRNA by the ARE Activator Sulforaphane (SUL) in HT-29 Cells. D,L-Sulforaphane (SUL), a classical activator of ARE-dependent gene expression, was applied on HT-29 cells at a concentration of 5 μM for 2, 4, 8, and 24 h (Figure 5A,B). CBR3 levels were substantially elevated after 4 and 8 h (2.3-fold compared to control) but declined after incubation for 24 h (to 1.2-fold), consistent with the results obtained with the ARE activator DEM (cf. Figure 4). Because CBR3 levels peaked 4 and 8 h post-treatment, we chose a 6 h incubation to test whether the upregulation of CBR3 by SUL was concentration-dependent. A pronounced induction of CBR3 could be observed after treatment with 5 μM SUL (3.6-fold),

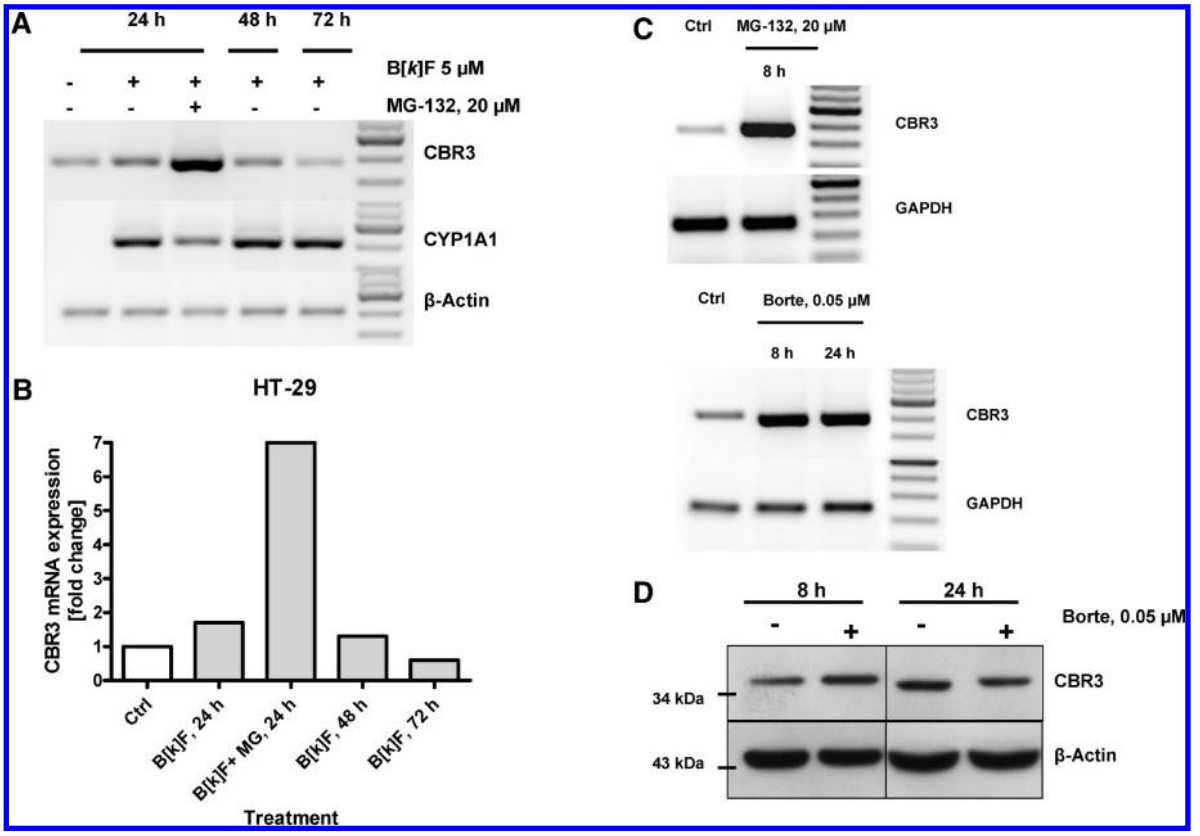

FIGURE 3: (A and B) CBR3 mRNA expression after exposure of HT-29 cells to B[*k*]F and MG-132. The level of CBR3 mRNA expression in HT-29 colon cancer cells treated with B[*k*]F (5 μM) peaked at 24 h and continuously decreased thereafter, although levels of AhR and the target gene CYP1A1 remained elevated throughout the whole time course. Cotreatment with B[*k*]F and the proteasome inhibitor MG-132 (20 μM) resulted in a dramatic upregulation of CBR3 mRNA but weakened the expression of CYP1A1 as compared to that of cells treated with B[*k*]F alone. The bar graph (B) represents the densitometric analysis of the gel shown in panel A. (C) CBR3 mRNA expression after exposure of HT-29 cells to MG-132 or bortezomib. Treatment of HT-29 cells with proteasome inhibitor MG-132 (20 μM) or bortezomib (0.05 μM) led to a pronounced overexpression of CBR3 mRNA, most likely based on increased levels of Nrf2. Noteworthy is the strong effect of bortezomib on CBR3 mRNA expression at a remarkably low concentration of 0.05 μM (ctrl denotes control, treated with vehicle, 0.1% DMSO; Borte denotes bortezomib). (D) Effect of the proteasome inhibitor bortezomib on CBR3 protein expression. After treatment of HT-29 cells with bortezomib (0.05 μM) for 8 and 24 h, whole cell lysates were prepared and 80 μg of protein was analyzed for CBR3 content by Western blotting. β-Actin served as a loading control. In contrast to CBR3 mRNA expression, bortezomib elevated the amount of CBR3 protein only marginally.

which was further enhanced by incubation with 10 and 20 μM SUL (6.9-fold). The simultaneous upregulation of the Nrf2-driven control genes γ-glutamylcysteine synthetase (γ-GCS, catalytic subunit; GCLC) and aldo-keto reductase 1B1 (AKR1B1) (*23*) by SUL is indicative of the fact that CBR3 gene regulation occurs via Nrf2 signaling. Note that the enzyme γ-GCS (glutamate cysteine ligase, GCL) consists of two subunits, the calalytic subunit (GCLC) and the regulatory subunit (GCLM), both of which are regulated by Nrf2. For the purpose of this publication, GCLC will be termed "γ-GCS".

Effect of SUL and B[k]F Treatment on CBR3 Protein Expression in HT-29, A549, and HCT116 Cells. While SUL treatment clearly increased the level of CBR3 protein in HT-29 cells after 72 h, B[*k*]F had no effect (Figure 5C). The same held true for A549 cells, in which SUL treatment elevated the level of CBR3 protein (however, only marginally), whereas B[*k*]F did not change CBR3 protein expression. In HCT116 cells, CBR3 protein was not detectable, in treated cells or in untreated controls.

Nrf2 Knockdown in A549 Cells Decreased the Basal Level of CBR3 mRNA Expression. To finally confirm that CBR3 is regulated via Nrf2, we performed Nrf2 knockdown studies with siRNA (Figure 6A,B). For an initial experiment, A549 cells were chosen, because of all the cell lines tested, they expressed the highest levels of both CBR3 and Nrf2 mRNA. As presented in Figure 6, transfection of A549 cells with Nrf2 siRNA (100 pmol) led to a pronounced decrease in the level of CBR3 mRNA expression after incubation for 48 h (to 35% of control siRNA; $P < 0.001$) and 72 h (to 27% of control siRNA; $P < 0.001$). Clearly, the level of Nrf2 mRNA itself and that of the Nrf2-regulated control gene γ-GCS decreased as well (Figure 6A). These findings unambiguously demonstrate the involvement of Nrf2 in the constitutive expression of CBR3 in A549 cells.

Keap1 Knockdown Increased the Level of CBR3 mRNA Expression in HT-29 and HepG2 Cells. To further substantiate our results obtained in the Nrf2 knockdown experiments, two cell lines with very low levels of CBR3 mRNA expression, HT-29 and HepG2, were transfected with 100 pmol of siRNA directed against Keap1 (Figure 6C,D). As expected, knockdown of Keap1 strongly increased the level of CBR3 mRNA expression in both HT-29 (2.7-fold; $P < 0.05$) and HepG2 (9.3-fold compared to control siRNA; $P < 0.001$) cells.

Nrf2 Knockdown in HT-29 Cells Decreased the Level of SUL-Induced Expression of CBR3 mRNA. Because CBR3 could be strongly induced in HT-29 cells by SUL, this cell line was chosen for investigation of the involvement of Nrf2 in the inducible expression of CBR3. For this experiment, cells were transfected with either Nrf2 siRNA or control siRNA and treated with SUL 48 h later (5 μM, for 4 h). As shown in Figure 7, in

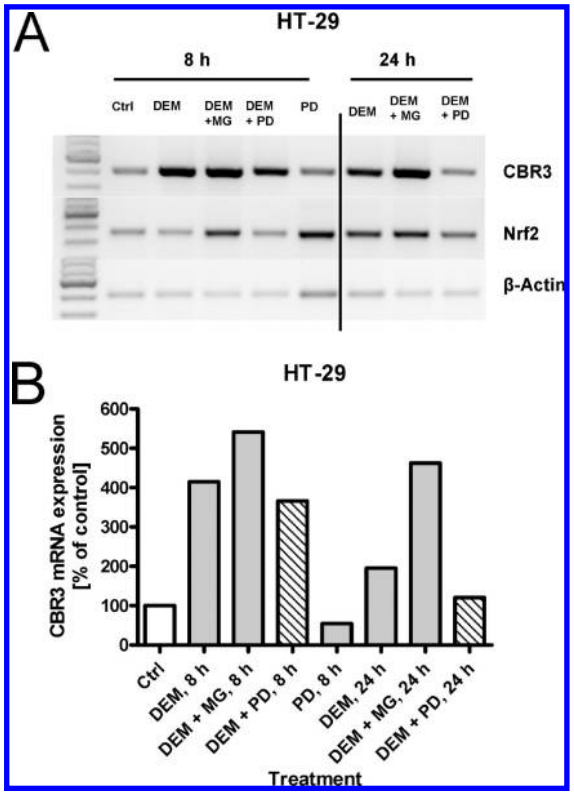

FIGURE 4: Changes in CBR3 and Nrf2 mRNA expression upon exposure of HT-29 cells to the ARE activator DEM, the proteasome inhibitor MG-132, and the MAPK kinase inhibitor PD98059. CBR3 mRNA expression was induced in HT-29 cells upon incubation with the Nrf2 ARE activator and GSH-depleting agent diethyl maleate (100 μM) after 8 h, an effect that subsided after 24 h. Cotreatment with DEM (100 μM) and MG-132 (20 μM) led to increased levels of CBR3 after 8 h and even 24 h, most likely as a result of elevated Nrf2 levels derived from proteasome inhibition by MG-132. Furthermore, cotreatment of HT-29 cells with DEM and the MEK/ERK inhibitor PD98059 (PD; 10 μM) clearly slowed DEM-mediated upregulation of CBR3 mRNA after both 8 and 24 h, thereby providing further evidence of the involvement of Nrf2 in the regulation of CBR3 expression (ctrl denotes control, treated with vehicle, 0.1% DMSO). (A) One representative gel is shown. (B) Densitometric analysis of a representative gel. CBR3 expression was normalized to that of β-actin, and the untreated control was set to 100%.

response to SUL treatment, nontransfected cells showed a strong increase in the level of CBR3 mRNA expression. This level of SUL-induced expression was, however, substantially decreased by siRNA-mediated knockdown of Nrf2 (to 63% of that of control siRNA-transfected cells treated with SUL), which indicates that upregulation of CBR3 mRNA by SUL requires Nrf2. Note that for this experiment CBR3 was amplified for only 30 (instead of 34) cycles. Therefore, the CBR3 bands in untreated and transfected cells (Nrf2 siRNA or control siRNA only) are hardly visible.

Time-Dependent Effect on the Expression of Nrf2 and Nrf2-Regulated Genes of SUL, DEM, Z-Guggulsterone (GS), and MG-132 in HT-29 Cells. Because the Nrf2 agonists SUL and DEM caused a strong induction of CBR3 expression after 8 h that, however, declined after 24 h, it was interesting to determine whether this phenomenon was a general or a specific effect, i.e., dependent on the test compound. For this experiment, we chose the lowest possible concentrations of the previously tested CBR3 modulators SUL (5 μM), DEM (25 μM), and MG-132 (5 μM). Another compound that, because of preliminary results, strongly induced CBR3 expression was the steroidal compound Z-guggulsterone (GS).

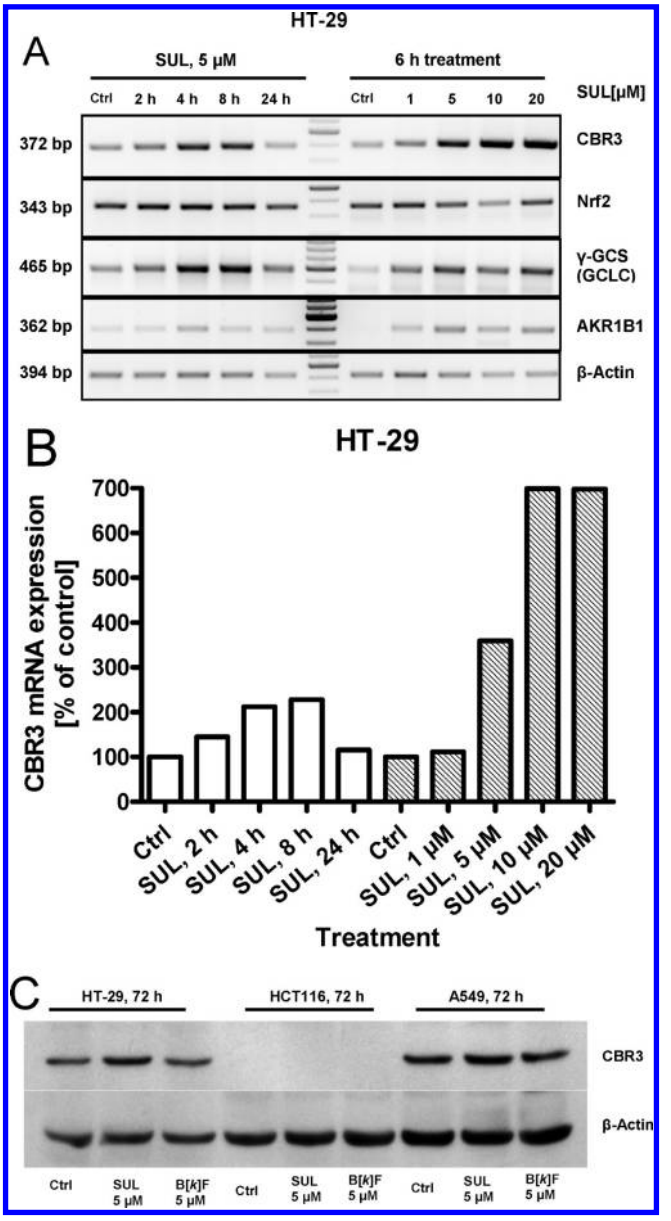

FIGURE 5: (A and B) Time- and concentration-dependent effect of the ARE activator SUL in HT-29 cells. The isothiocyanate sulforaphane (SUL) increased the level of CBR3 mRNA in a time-dependent (left) and concentration-dependent (right) fashion in HT-29 cells. Both Nrf2-regulated control genes γ-GCS and AKR1B1 were upregulated in parallel, supporting the hypothesis that inducible expression of CBR3 is controlled by Nrf2 (Ctrl denotes control treated with vehicle, 0.1% DMSO). The densitometric analysis (B) of a representative gel (A) shows the increase in the level of CBR3 mRNA expression normalized to β-actin over untreated controls (controls set to 100%). (C) Effect of SUL and B[*k*]F treatment (72 h) on CBR3 protein expression in HT-29, HCT116, and A549 cells after treatment with either the Nrf2 agonist SUL or the AhR agonist B[*k*]F for 72 h. Each lane contained 60 μg of protein (whole cell lysate).

Guggulsterone (GS) is a plant sterol and the active substance in guggulipid, an extract obtained from the resin from the guggul tree, *Commiphora mukul*. The resin has been used in Ayurvedic medicine for centuries to treat a variety of disorders such as dyslipidemia, internal tumors, liver disorders, arthritis, obesity, and inflammation (*24*). GS is able to interact with a wide range of transcriptional pathways, including various steroid receptors [farnesoid X receptor (FXR), pregnane X receptor (PXR), androgen receptor (AR), and glucocorticoid receptor (GR)] as well as with nuclear factor κ-B (NFκB) and signal transducers and activators of transcription-3

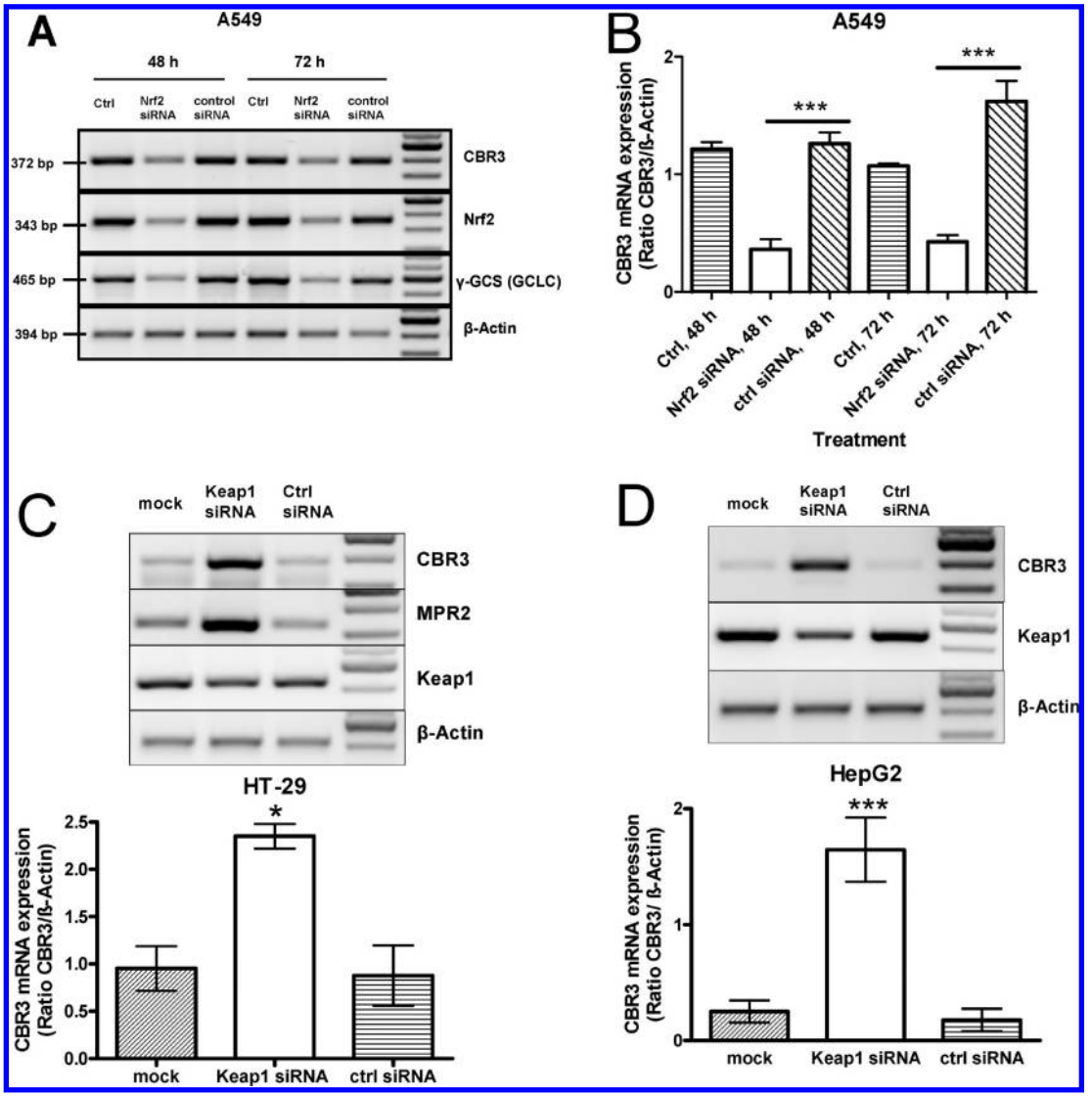

FIGURE 6: Effect of knockdown of Nrf2 or Keap1 on CBR3. Transfection of A549 cells with Nrf2 siRNA substantially decreased mRNA levels of CBR3, Nrf2, and the Nrf2 target gene γ-GCS. Nrf2-targeting siRNA (100 pmol) or nontargeting control siRNA-A ("ctrl siRNA", 100 pmol) was transfected into A549 cells and harvested for total RNA isolation 48 and 72 h post-transfection. (A) One representative gel is shown. (B) Densitometric analysis of three independent experiments. Bars represent means ± the standard deviation (asterisks denote $P < 0.001$). (C and D) Keap1 knockdown in HT-29 (C) and HepG2 (D) cells greatly increased the level of expression of CBR3 mRNA after 72 h. Nrf2-regulated control gene MRP2 is induced in HT-29 cells as well. The densitometric analysis of three independent experiments shows means ± the standard deviation (three asterisks denote $P < 0.001$; one asterisk denotes $P < 0.05$).

(STAT-3), thereby modulating cell proliferation, angiogenesis, and apoptosis (*25*). According to its multiple functions, we used GS in terms of its possible inducing effect on CBR3 expression.

As shown in Figure 8A, CBR3 gene induction by SUL and DEM was strongest after 8 h and then continuously declined after 24 and 48 h. At 48 h, almost baseline levels were reached. However, when the relative level of expression (control set to 100%) is calculated from the CBR3:β-actin ratio, the level of expression of CBR3 remained around 2.5-fold from 8 to 48 h in cells treated with SUL or DEM. Most interestingly, GS and MG-132 provoked the strongest induction of CBR3 mRNA after 24 h (4.7- and 4.8-fold vs control) and 48 h (5.2- and 6.9-fold) of all compounds tested. In general, the expression of the Nrf2-regulated control gene MRP2 correlated well with that of CBR3. However, GS was the most potent inducer of MRP2 expression that was upregulated 13-fold after treatment for 24 and 48 h (Figure 8C). The second control gene for Nrf2-mediated gene transcription, γ-GCS, was induced as well, but to a much lower extent (up to 2-fold) during the whole experiment (Figure 8D). Regulation of Nrf2 expression (Figure 8B) itself did not parallel the expression pattern observed for CBR3, γ-GCS, and MRP2, a fact that might be explained by its central role in the cell's response to oxidative stress (see Discussion).

Effect of SUL, B[k]F, and GS on CBR3 Protein Expression in HT-29 Cells. While CBR3 mRNA levels responded very promptly (4 h) upon treatment with SUL (see Figure 5A), a clear increase in the amount of CBR3 protein could be seen only after a longer incubation time of 72 h (Figure 9A,B). GS led to the greatest elevation of the level of CBR3 protein in HT-29 cells, thereby reflecting the high levels of CBR3 mRNA upon exposure to this compound (see Figure 8A). The AhR agonist B[*k*]F induced CBR3 protein to a relatively low extent after 72 h.

DISCUSSION

In normal cell physiology, the amounts of reactive oxygen species (ROS) and antioxidants are kept in balance. A variety of

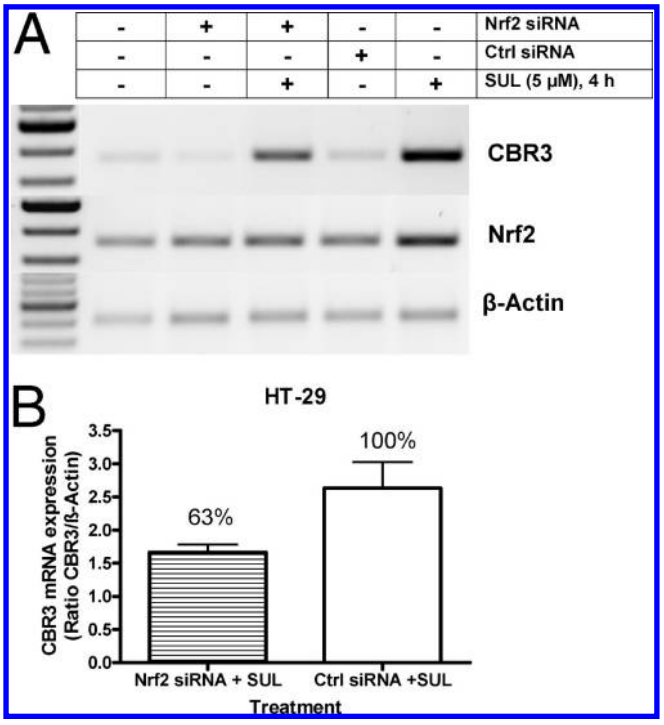

FIGURE 7: siRNA-mediated knockdown of Nrf2 decreases the level of SUL-induced expression of CBR3 mRNA in HT-29 cells. HT-29 cells were grown to 60% confluence, transfected with 100 pmol of either Nrf2-targeting siRNA or nontargeting control siRNA, and treated with SUL (5 μM, for 4 h) 48 h post-transfection. (A) One representative gel is shown. Transfection of HT-29 cells with Nrf2 siRNA reduces the SUL-mediated increase in the level of CBR3 mRNA to 63% of that of control siRNA-transfected cells. (B) Densitometic analysis of three independent experiments. Bars represent means ± the standard deviation. Changes were not statistically significant ($P = 0.07$).

endogenous (such as metabolic activity and production of free electrons and H_2O_2) or exogenous (e.g., exposure to electrophilic xenobiotics and drugs) stresses can result in an overabundance of ROS and thereby produce oxidative stress. Eukaryotic cells have developed a variety of cellular defense systems for adapting to a changing environment and for surviving under conditions of oxidative stress. Redox sensitive transcription factors are believed to play the role of "redox sensors" in these cellular defense systems.

Nrf2 is one major player in the adaptive response to oxidative stress that is known to act as an important regulator in the expression of antioxidant enzymes. Nrf2-regulated genes include enzymes of GSH synthesis (γ-GCS), antioxidant enzymes such as heme oxygenase-1 (HO-1), NADP(H):quinone oxidoreductase (NQO1), phase II drug-metabolizing enzymes [glutathione *S*-transferases (GST)], and drug efflux transporters (MRP2) that export conjugated metabolites across cell membranes. Of the enzymes involved in the reductive metabolism, several members of the aldo-keto reductase (AKR) superfamily have been shown to be regulated by monofunctional inducers (*26*). Among them, AKR1C2 (*27*), AKR1C1, AKR1C3, and AKR1B10 (*28*) have been identified as Nrf2 targets.

The results presented in this study provide for the first time strong evidence that expression of the human carbonyl reducing enzyme CBR3, a member of the SDR superfamily, is regulated via the Nrf2/ARE signaling pathway. (1) CBR3 expression was induced by the monofunctional inducers SUL and DEM after short incubation times of 4 and 8 h, whereas the bifunctional

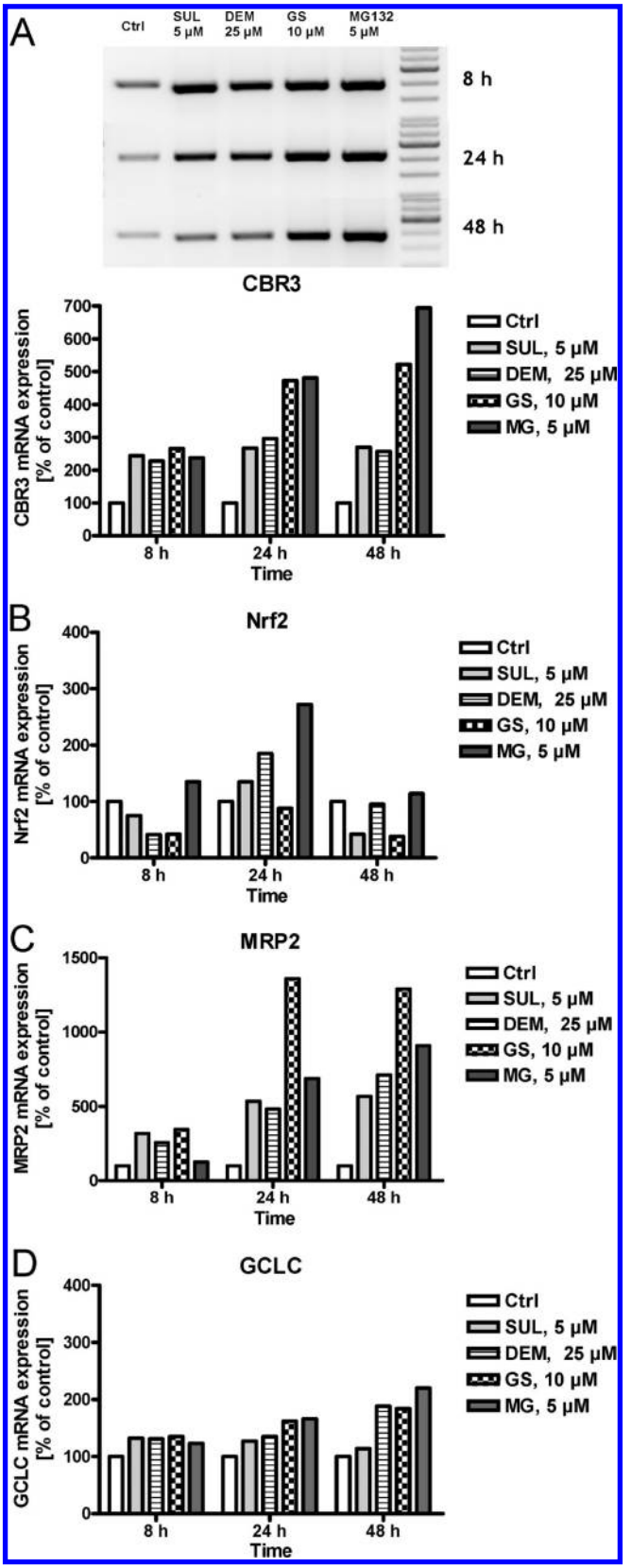

FIGURE 8: Time course of mRNA expression of CBR3, Nrf2, MRP2, and γ-GCS after treatment of HT-29 cells with SUL, DEM, GS, and MG-132. CBR3-inducing compounds SUL, DEM, GS, and MG-132 cause different time-dependent patterns of mRNA expression of CBR3 (A), Nrf2 (B), MRP2 (C), and γ-GCS (D) in HT-29 cells. MRP2, a Nrf2- and PXR-regulated efflux pump, was regulated in a manner quite similar to that of CBR3, except for cells treated with MG-132. Densitometric analysis of a representative gel is shown (controls set to 100%).

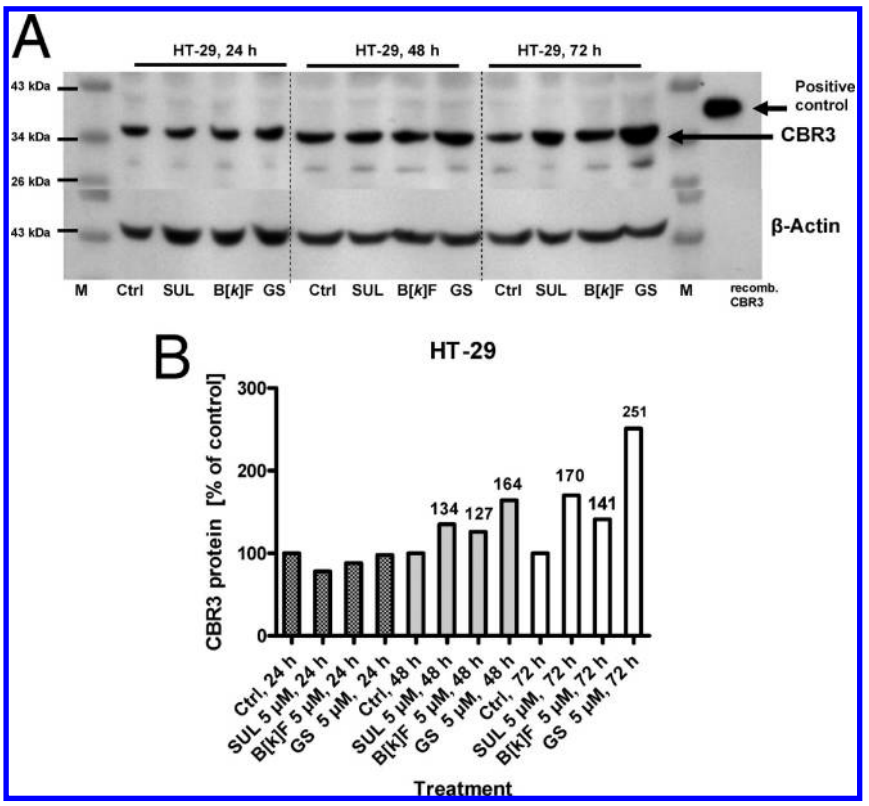

FIGURE 9: CBR3 protein expression in HT-29 cells upon exposure to SUL, B[*k*]F, and GS. (A) HT-29 cells were treated with either vehicle (0.1% DMSO), sulforaphane (SUL, 5 μM), benzo[*k*]fluoranthene (B[*k*]F, 5 μM), or Z-guggulsterone (GS, 10 μM) for the indicated time periods. Aliquots of whole cell lysates corresponding to 80 μg of protein were subjected to Western blot analysis. Recombinant CBR3 protein (containing a His tag) served as the positive control. Ctrl denotes control. (B) For the densitometric analysis of a representative blot, the expression of CBR3 protein was normalized to that of β-actin. Values are given as the percentage of untreated controls.

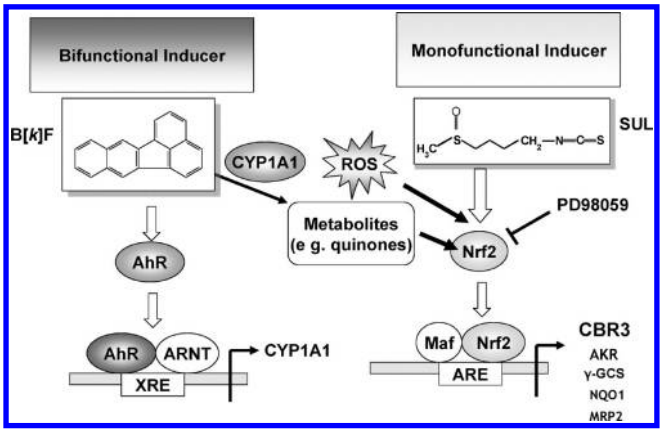

FIGURE 10: Regulation of AhR- and Nrf2-mediated gene transcription by bifunctional and monofunctional inducers of xenobiotic metabolizing enzymes (*49*). Monofunctional inducers mediate gene transcription by interaction with Nrf2 that, upon activation, translocates to the nucleus where it hereodimerizes with other transcription factors (e.g., small Maf) and binds to ARE enhancer sequences in the upstream region of target genes. These include members of the AKRs (e.g., AKR1B1 and AKR1C1) and other proteins (NQO1, γ-GCS, and MRP2). Bifunctional inducers such as PAHs (e.g., B[*k*]F) can, as unaltered compounds, directly transactivate AHR−ARNT (aryl hydrocarbon receptor nuclear translocator) complexes and trigger transcription of genes harboring XREs in their promoter region. Moreover, phase I metabolism by CYPs generates metabolites and ROS, both of which can induce Nrf2-regulated genes. Because the transcriptional activation of Nrf2 requires phosphorylation, MEK/ERK inhibitors such as PD98059 can block Nrf2-mediated gene transcription.

inducer B[*k*]F needed at least 24 h to induce CBR3 mRNA. (2) Co-incubation with DEM and the MEK/ERK inhibitor PD98059 decreased the level of DEM-mediated induction of CBR3 mRNA to almost basal levels. (3) Treatment of HT-29 cells with proteasome inhibitors dramatically increased the amounts of CBR3 mRNA. (4) Knockdown of Nrf2 by siRNA in A549 cells downregulated basal levels of CBR3 mRNA and decreased the level of SUL-mediated induction of CBR3 mRNA in HT-29 cells. (5) Knockdown of Keap1 increased the level of CBR3 mRNA expression in HT-29 and HepG2 cells.

Human CBR3 mRNA exhibits a very unequal expression pattern among different normal organs that implies a tissue specific regulation of its gene (*5*). By analyzing several human cancer cell lines originating from different tissues, we also found CBR3 mRNA being expressed differentially, ranging from a relatively high level of expression in lung cancer cells (A549) to very low levels in colon cancer (HT-29) and hepatoma cellular carcinoma (HepG2) cells. Interestingly, even among four colon cancer cell lines (HT-29, HCT116, Caco-2, and SW-480), CBR3 mRNA expression differed tremendously. Furthermore, via comparison of CBR3 mRNA levels with that of CBR3 protein in the different cell lines investigated, there was one very striking discrepancy: HCT116 colon cancer cells expressed relatively large amounts of CBR3 mRNA, but no CBR3 protein. It might be thought that a mutation within the gene results in a misfolded protein that undergoes subsequent degradation. This hypothesis is currently under investigation in our lab.

We found the largest amounts of CBR3 mRNA in A549 lung cancer cells. A549 cells have been reported to constitutively overexpress HO-1, a stress response gene known to be regulated via Nrf2-dependent gene transcription (*29*). As reported recently by Homma et al. (*30*), a dysfunctional mutation of Keap1 is

responsible for the constitutive activation of Nrf2 in A549 cells. Consequently, this overexpression of Nrf2 causes an induction of all Nrf2-regulated genes, including that of Nrf2 itself. The high levels of CBR3 found in Nrf2-overexpressing A549 cells led us raise the first idea about Nrf2 being involved in the regulation of CBR3 and prompted us to determine whether other Nrf2-regulated genes such as NQO1 and γ-GCS would correlate with basal CBR3 expression in the different cancer cell lines. As expected, in almost all cell lines, except for HepG2 cells, the expression of CBR3, NQO1, and γ-GCS correlated quite well with the abundance of Nrf2 mRNA (see Figure 1B). This striking discrepancy between high levels of Nrf2-regulated genes (Nrf2, NQO1, and γ-GCS) observed on one hand and very small amounts of CBR3 mRNA on the other can possibly be explained by silencing (e.g., promotor hypermethylation or histone modification) of the *CBR3* gene in HepG2 cells. However, this remains speculative and requires further investigation that is currently ongoing in our lab. The fact that CBR3 could be strongly induced by siRNA-mediated knockdown of Keap1 in HepG2 cells shows that at least one mechanism that causes the low CBR3 expression level in this cell line is associated with Keap1 mRNA expression.

To further confirm our hypothesis of Nrf2 being involved in the regulation of CBR3, we treated HCT116 cells with either the Nrf2 agonist TBHQ or the AhR agonist B[*k*]F for different periods of time. B[*k*]F is the so-called bifunctional enzyme inducer. As an unaltered compound, it can directly (within less than 8 h) activate AhR/XRE-driven genes (e.g., CYP1A1). On the other hand, metabolites generated by oxidative metabolism of B[*k*]F, such as quinones, are themselves bifunctional inducers (see Figure 10) that can activate Nrf2-dependent gene expression (*26*). Additionally, CYP1A1 activity produces free electrons and H_2O_2, both of which are origins of oxidative stress that triggers Nrf2-mediated gene expression. The fact that TBHQ is able to induce CBR3 mRNA after incubation for only 8 h, whereas B[*k*]F showed a first effect on CBR3 expression after a minimum of 24 h, indicates that this effect requires the metabolism of B[*k*]F. From the results presented in Figure 2, we can conclude that CBR3 expression is most likely regulated by the Nrf2-mediated but not the AhR-mediated signaling pathway.

Further support that CBR3 expression is controlled by Nrf2 is provided by the strong effect of DEM and SUL on CBR3 mRNA expression in HT-29 cells. Both compounds are prototypical Nrf2/ARE activators without any AhR agonistic activity (*9*).

SUL, a natural isothiocyanate derived from broccoli, has been investigated intensively because of its protective effect against carcinogen-induced tumorigenesis in rodents (*31*). SUL itself can generate ROS (*32*). Thereby, it produces mild oxidative stress that triggers the expression of detoxifying enzymes that elevate cellular defense mechanisms against oxidative damage.

By activating several upstream kinases, including PI3K/Akt or MEK/ERK, SUL has been shown to elevate the level of mRNA expression of several genes harboring ARE enhancers in their promoter such as NQO1, UGT1A1, and MRP2 in Caco-2 cells (*33*). As presented here, SUL is able to induce CBR3 mRNA in HT-29 cells in a time- and concentration-dependent manner (Figure 5A).

Furthermore, DEM induced CBR3 mRNA in HT-29 cells at a concentration as low as 25 μM. In line with the induction of CBR3 upon treatment with SUL and DEM, blocking of Nrf2 phosphorylation by MEK/ERK inhibitor PD98059 prevented DEM-mediated CBR3 induction, even when higher concentrations of DEM (100 μM) were used.

Interestingly, altered Nrf2 levels upon these treatments did not always correlate with the level of CBR3 expression in all cases. Even though PD98059 alone had no effect on CBR3, it induced Nrf2 mRNA levels (Figure 4). Moreover, upon cotreatment with DEM and PD98059, the latter prevented an increased level of CBR3 mRNA expression, although Nrf2 was still induced.

Nrf2 forms heterodimers predominantly with small Maf proteins and also with members of the activator protein-1 (AP-1) family, including Jun and Fos (products of oncogenes c-jun and c-fos, respectively). In contrast to MafG and MafF that activate gene transcription in cooperation with Nrf2 (*34*), it has been shown that Jun and Fos act as both positive and negative regulators of Nrf2-driven gene expression. ARE-driven reporter genes displayed an activation of ARE-mediated gene transcription upon overexpression of both Jun and Nrf2, whereas overexpression of both Fos and Nrf2 repressed the activity of ARE-containing reporter genes (*35*, *36*). Another protein that is known to repress ARE-driven genes such as HO-1 is broad- complex, tramtrack and bric à brac and cap 'n' collar homology 1 (Bach1) protein (*37*). Bach1 is believed to negatively regulate Nrf2-controlled genes by preventing the binding of Nrf2 to ARE-like sequences in their regulatory region. Considering all these facts, high levels of Nrf2 alone may not directly be translated into high levels of Nrf2-mediated gene transcription. Moreover, it should be noted that mRNA levels of Nrf2 do not necessarily correlate with protein levels and provide no information about the nuclear localization and transcriptional activity of Nrf2.

Because Nrf2 regulates its own expression (*38*), Nrf2 levels may, on one hand, indicate the status of Nrf2-mediated signaling pathways. On the other hand, rapid changes in Nrf2 expression dynamics may explain the different "kinetics" observed for Nrf2, CBR3, MRP2, and γ-GCS mRNA expression. Most probably, under conditions of mild oxidative stress, intracellular pools of antioxidants (GSH and thioredoxin) and basal levels of Nrf2 are able to protect cells from oxidative insults, but prolonged stress would require de novo synthesis of Nrf2 to maintain cellular homeostasis.

HT-29 cells exhibited a very distinct time-dependent upregulation of CBR3 in response to different compounds tested in this study (Figure 8). While the CBR3 mRNA levels upon treatment with DEM (25 μM) and SUL (5 μM) was more transiently and were gradually reduced via incubation for 48 h, GS (10 μM) and MG-132 (5 μM) induced CBR3 mRNA even for 48 h. Possible reasons could have been the activation of more than one upstream kinase that transactivated Nrf2 and/or differences in the metabolic clearance of the inducing agents. Moreover, treatment with antioxidants such as SUL is known to induce the expression of proteasomes in mice (*39*). This may have led to the enhanced turnover of Nrf2 protein in cells treated with SUL or DEM and may explain the more transient upregulation of CBR3 in response to these substances.

Interestingly, GS had no effect on Nrf2 expression itself but induced MRP2 strongly and most persistently of all the compounds tested. MRP2 is a known Nrf2 target and is also regulated via PXR-dependent pathways (*40*). Induction of γ-GCS, however, shows that GS is also able to transactivate Nrf2. GS is a known PXR agonist, but no evidence for the regulation of ARE-dependent genes mediated by GS is available in the literature. The strong effect of GS on MRP2 may therefore result from activation of both PXR- and Nrf2-mediated signaling. Because GS has been shown to interact with several other nuclear receptors, it remains to be seen whether the effect of GS on CBR3 is at least in

part attributable to the involvement of other signaling pathways. So far, our preliminary experiments with HT-29 and Caco-2 cells showed no response of CBR3 mRNA expression toward cortisol, testosterone, and rifampicin (data not shown), activators of GR-, AR-, and PXR-mediated gene expression, respectively.

CBR3 mRNA expression responded dramatically to treatment with proteasome inhibitors. Proteasome inhibitors such as MG-132 have been reported to induce an overexpression of other genes such as the AhR target gene CYP1A1, a phenomenon commonly termed "superinduction" (*41*). In this case, the ligand-activated AhR protein complex is released from the nucleus into the cytosol, ubiquitinated, and subsequently degraded by 26S proteasomes. Proteasomal activity therefore controls the levels of key regulatory proteins, including activated transcription factors. Because inhibition of 26S proteasomes increases levels of Nrf2 (*20*), the induction of CBR3 mRNA by MG-132 and bortezomib is most likely dependent on Nrf2-mediated signaling pathways. Moreover, MG-132 has been reported to produce ROS, another trigger of ARE-regulated genes such as heme oxygenase-1 (*42*, *43*). In this study, cotreatment of HT-29 cells with MG-132 and the AhR/XRE activator B[*k*]F resulted in a very large increase in the level of CBR3 mRNA expression (Figure 3A). However, it is very unlikely that CBR3 transcription is controlled via AhR-dependent pathways, because the AhR-regulated control gene CYP1A1 was induced after treatment with B[*k*]F for only 8 h, while CBR3 induction required longer incubation times of 24 h.

AhR-regulated genes must respond very promptly to confer protection from xenobiotic insults. This idea is supported by the work of Song et al. (*44*), who found that CYP1A1 protein in murine Hepa 1c1c7 cells was upregulated after incubation for only 4 h with the AhR agonist TCDD.

The final proof for our hypothesis that CBR3 expression is regulated via the antioxidant Nrf2/ARE network is provided by knockdown studies targeting either Nrf2 or Keap1. It could be clearly shown that transfection of A549 cells with Nrf2 siRNA downregulated not only Nrf2 expression itself but also that of CBR3 and the Nrf2-regulated control gene γ-GCS. Moreover, CBR3 expression is dependent on Keap1, as shown by the strong induction of CBR3 mRNA in HepG2 and HT-29 cells upon knockdown of Keap1.

The microarray analyses of different tissues of nrf2-disrupted mice have evidenced the important role of nrf2-controlled gene expression in the susceptibility to a range of diseases and facilitated the identification of nrf2-regulated genes (*45*, *46*). Because murine Cbr3 has been reported to be upregulated in mouse liver in response to SUL ~4-fold (*47*), one can assume that the mechanism of *CBR3* regulation may be evolutionarily conserved.

Moreover, Zhang and Blanco (*6*) have identified an AP-1 binding site in the promoter region of CBR3. Because an AP-1 binding site represents a half-site of an ARE, this finding strongly supports our results presented here. However, it still remains to be shown whether this putative binding site is occupied by the Nrf2/Maf transcription factor complex in vivo.

In conclusion, we provide for the first time clear evidence that CBR3 is another gene of the human ARE gene battery that is controlled via Nrf2-dependent signaling pathways. Future work will perhaps identify additional pathways and possible cross-talk between them. As the regulation of a gene is closely related to its physiological role, this study sheds some light on the hitherto unknown physiological function of CBR3 in humans, which appears to be a component of the antioxidant stress response.

ACKNOWLEDGMENT

We thank Prof. Dr. J. Abel (University of Duesseldorf) for kindly providing the HCT116 cells.

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