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Expression of human carbonyl reductase 3 (CBR3; SDR21C2) is inducible by pro-inflammatory stimuli

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ABSTRACT

Until today, the physiologic role of human carbonyl reductase 3 (CBR3; SDR21C2), a member of the short-chain dehydrogenase/reductase superfamily remains obscure. Since the transcriptional regulation is closely related to the function of a protein, elucidation of the regulation of CBR3 should help to understand its physiologic role. We recently identified CBR3 as a novel target gene of Nrf2, a cellular sensor of oxidative stress. In this study, we provide for the first time evidence that pro-inflammatory stimuli induce the expression of the *CBR3* gene. Treatment of human cancer cells HT-29 (colon) and HepG2 (liver) with TNF-α, IL-1β, and LPS induced CBR3 expression differentially. While TNF-α (50 ng/ml) or IL-1β (1 and 10 ng/ml), induced CBR3 mRNA expression in HT-29 cells (up to 10-fold) and HepG2 cells (up to 20-fold), LPS activated the *CBR3* gene only in HepG2 cells. Furthermore, overexpression of the NFκB subunits p65 and p50 alone or in combination elevated CBR3 mRNA levels (3.9-fold) in HT-29 cells. According to our results, CBR3 is a novel target gene of inflammatory stimuli, and elucidation of its detailed role in inflammation deserves further investigation.

1. Introduction

As carbonyl groups often govern the biologic activity of endo- and xenobiotics, enzymes mediating carbonyl reduction drive numerous physiologic processes (e.g., steroid biotransformation) as well as the detoxification of potentially harmful exogenous (e.g., drugs, environmental pollutants) and endogenous (e.g., lipid peroxidation products) compounds [1].

So far, three carbonyl reductases (CBRs) have been identified in humans (CBR1 [SDR21C1], CBR3 [SDR21C2] and CBR4 [SDR45C1]) all of which belong to the short-chain dehydrogenase/reductase (SDR) superfamily [2].

Since the identification of CBR3 in 1998 [3] some progress has been made with regard to its tissue-specific distribution, but still the understanding of its molecular function is incomplete [4]. Although being considered an enzyme in the reductive metabolism of anthracyclines such as doxorubicin and daunorubicin [5,6], the poor catalytic efficiencies for other tested carbonyl compounds imply that the expected function of CBR3 in xenobiotic carbonyl metabolism is very unlikely [7,8]. Therefore, the physiologic role of CBR3 in the human body remains obscure. Elucidation of its transcriptional regulation may shed light on the physiologic role of CBR3, since molecular mechanisms that regulate an enzyme's expression are often closely related to its function.

In a recent study, we could identify CBR3 as a novel Nrf2 (nuclear factor-erythroid 2 related factor 2)-target gene [9], which was later confirmed by others [10]. Since Nrf2 mediates the transcriptional activation of genes in response to oxidative stress [13], CBR3 might fulfill antioxidant functions. However, as the promoter of CBR3 contains putative binding sites for various other transcription factors, some additional environmental conditions may be involved in the regulation of CBR3 as well.

Recently, two potential NFκB (nuclear-factor kappa-B) consensus motifs were identified in the 5′-UTR of the *CBR3* gene which are located −1160 and −593 basepairs upstream of the transcriptional start site [11]. Moreover, our preliminary promoter analysis revealed the existence of even a third putative NFκB binding site (nt −364: 5′-GGGGTTTTCCC-3′). Meanwhile, it is widely accepted that oxidative stress and inflammation are two cellular events which are closely connected. For instance, NFκB attenuates the production of reactive oxygen species (ROS) via increased expression of antioxidant proteins. By contrast, transcriptional targets of NFκB itself promote the production of ROS, especially enzymes important for the inflammatory response, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [12].

Abbreviations: CBR3, carbonyl reductase 3; IL-1, interleukin-1; LPS, lipopolysaccharides; NFκB, nuclear-factor kappa-B; qPCR, quantitative real-time RT-PCR; sqPCR, semi-quantitative RT-PCR; TNF-α, tumor necrosis factor-α.

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NFκB comprises a family of transcription factors [e.g., p65 (RelA) and p50 (NFκB1) among others] that control a wide range of critical physiologic processes in the organism including inflammation, immune response and cell survival [13]. These various subunits form homodimers and heterodimers with each other [14,15]. In their inactive form, NFκB dimers are associated with inhibitory proteins such as IκBα that retains them in the cytoplasm. IκBα itself is regulated by NFκB, thereby providing an autoregulatory feedback loop allowing for the control of the inflammatory response [13]. A great variety of biologic factors (e.g., pathogens, cytokines) and environmental conditions (e.g., UV irradiation, oxidative stress) trigger the formation of NFκB dimers that translocate to the nucleus where they bind to NFκB response elements of target genes [16].

There are two main signaling pathways activating NFκB, referred to as "classical" (canonical) and "alternative" (non-canonical) pathway [17]. The former is activated by endogenous inflammatory stimuli (e.g., pro-inflammatory cytokines such as tumor necrosis factor-α [TNF-α] and interleukin-1 [IL-1]), and in response to invading microorganisms or their products (e.g., lipopolysaccharides [LPS]).

The aim of this study was to infer whether CBR3 expression is regulated via pro-inflammatory stimuli in the model cell lines HT-29 and HepG2. According to our findings, this is the first report providing clear evidence that the expression of the *CBR3* gene is induced under inflammatory conditions.

2. Materials and methods

2.1. Cell culture materials and cell lines

Cell culture media and supplements were purchased from PAA Laboratories GmbH (Cölbe, Germany). The human colon cancer cell line HT-29 was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and the human hepatoma cell line HepG2 was from Cell lines service (CLS, Eppelheim, Germany).

2.2. Plasmids

Expression plasmids for NFκB subunits p65 and p50 were constructed by Warner Greene [18] and obtained from Addgene (Addgene Inc., Cambridge, MA, USA) (pCMV4 p65: Addgene plasmid 21966 and pCMV4 p50: Addgene plasmid 21965). Plasmids were purified from bacterial cultures with the QIAGEN plasmid Midi kit (Qiagen, Hilden, Germany).

2.3. Cell culture

HT-29 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose) supplemented with 2 mM L-glutamine, 1% of non-essential amino acids and 10% heat-inactivated fetal calf serum (FCS). HepG2 cells were cultured in DMEM/Ham's F12 (1:1) supplemented with 2 mM L-glutamine and 10% FCS. All cells were routinely cultured without antibiotics in a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.4. Gene expression experiments

Cells were seeded in 60 mm Petri dishes or six-well plates (9.3 cm^2) and grown until they reached 60–90% of confluence. Recombinant TNF-α and IL-1β were purchased from Cell Systems (Troisdorf, Germany) and LPS was from Sigma–Aldrich (Deisenhofen, Germany). Stock solutions were prepared in sterile H_2O (IL-1β and LPS) or sterile H_2O containing 0.1% BSA (TNF-α) and stored as aliquots at −20 °C until used. After serum-starvation for 18–24 h, the cells were incubated with test compounds freshly dissolved in serum-free medium for times as indicated in the figures. The medium was replaced with fresh serum-free medium and test compounds every 24 h. All experiments were performed 2–5 times.

2.5. Transient transfections

Cells were seeded in six-well plates and allowed to recover for 24 h. Expression plasmids pCMV4 p65 and pCMV4 p50 were diluted in 250 µl of OptiMEM I (Invitrogen, Karlsruhe, Germany), combined with 250 µl of OptiMEM I containing 10 µl of Lipofectamine 2000™ (Invitrogen, Karlsruhe, Germany), and, after incubation for 20 min at room temperature, the mixture was added to the cells. The medium was replaced after 7 h with fresh culture medium. Transfected cells were harvested for RNA isolation after 24 h incubation.

2.6. RNA-isolation and cDNA synthesis

RNA was isolated using the MasterPure™ RNA Purification Kit (Epicentre Biotechnologies, Madison, Wisconsin, USA). The cell-monolayer was washed with phosphate buffered saline (PBS) and RNA isolation was performed following the manufacturer's protocol including removal of contaminating DNA and with an additional ethanol wash step. cDNA synthesis was carried out as described before [9].

2.7. Semi-quantitative RT-PCR (sqPCR)

Semi-quantitative RT-PCR (sqPCR) was performed using Phire Hot-start DNA polymerase (Biozym Scientific, Hessisch Oldendorf, Germany) as described in detail elsewhere [9]. β-Actin served as the house-keeping gene. The following primers were used: β-Actin (fwd: 5'-ACTCTTCCAGCCTTCCTTCCT, rev: 5′-AGGTTTTGTCAAGAA AGGGTGT), CBR3 (fwd: 5′-GCTCAACGTACTGGTCAACAAC, rev: 5′-ATCCTCGATAAGACCGTGACC), IκBα (fwd: 5′-CTACACCTTGCCTGT-GAGCA, rev: 5′-GCTCGTCCTCTGTGAACTCC), COX-2 (fwd: 5′-GA ATGGGGTGATGAGCAGTT, rev: 5′-GGTCAATGGAAGCCTGTCAT), p50 (fwd: 5′-TGCCAACAGCAGATGGCCCAT, rev: 5′-AAACATGAG CCGCACCACGCT), p65 (fwd: 5′-AAGTTCCTATAGAAGAGCAGCG, rev: 5′-TGCTCTTGAAGGTCTCATATG).

Densitometric analyses were carried out with GIMP2.0 software.

2.8. Quantitative real-time RT-PCR (qPCR)

Real-time PCR (qPCR) experiments were run on an AB 7500 Fast Real-time PCR system (Life Technologies, Darmstadt, Germany). Two micrograms of RNA were reverse transcribed into cDNA in a total volume of 35 µl. Each cDNA sample was diluted 2-fold and 2 µl of this dilution were used for qPCR analyses. One 20 µl reaction contained 1 µl of TaqMan Gene expression Assay Hs01025918_m1 (CBR3) or Hs99999903_m1 (ATCB, β-Actin), 10 µl of TaqMan Fast Universal PCR Mastermix, 2 µl cDNA and 7 µl sterile H_2O. The thermal cycling conditions included an initial activation-step at 95 °C for 20 s, followed by 40 cycles of denaturation (95 °C, 3 s) and annealing/extension (60 °C, 30 s). Samples and no-template controls were run in triplicate. The fold-changes in the mRNA expression levels were calculated by the comparative quantitation ($2^{-\Delta\Delta Ct}$) method by using the instrument's software.

2.9. Statistical analysis

Statistical analyses were performed with the GraphPad Prism5 software. Differences between mean values were determined by a two-tailed paired Student's *t*-test or a one-way ANOVA followed by a Tukey's post-test. Statistically significant differences were set at $P \leqslant 0.05$, $P \leqslant 0.001$, and $P \leqslant 0.0001$ indicated highly significant data.

3. Results and discussion

The existence of putative NFκB binding sites in the promoter region of the *CBR3* gene [11] was the basis for our working hypothesis that CBR3 may be up-regulated during inflammation. This idea was supported by data from high-throughput experiments available from the public database GEO ("Gene Expression Omnibus", http://www.ncbi.nlm.nih.gov/geo/). For example, elevated levels of CBR3 mRNA were detected in colon biopsy samples from patients suffering from ulcerative colitis, a type of inflammatory bowel disease (GEO profiles: **GDS3119**) and in bronchial epithelial cells treated with interferon gamma (IFN-γ), a prototypical inflammatory cytokine (GEO profiles: **GDS1256**). Although these microarray data provide valuable information about the regulation of a certain gene, a PCR-based validation of these data is always essential.

In our study, the cell lines HT-29 and HepG2 were chosen as suitable models because of their low constitutive level of CBR3 expression.

3.1. Pro-inflammatory stimuli increased CBR3 expression in HT-29 cells

To investigate the role of inflammation in the up-regulation of CBR3, HT-29 cells were incubated with TNF-α (25 and 50 ng/ml), IL-1β (10 and 20 ng/ml) and LPS (100 and 1000 ng/ml) for 4, 8 and 24 h. While IL-1β treatment (20 ng/ml) led to a slight increase in the expression of CBR3 mRNA after 24 h (2.2-fold; Fig. 1A), TNF-α caused the strongest increase in the expression of CBR3 mRNA from 4 h through 24 h (see Fig. 2A, B). The effect of LPS after 8 h was marginal (1.5-fold, 1000 ng/ml; not shown).

Since the NFκB activators used in this experiment mediate their effects through binding to specific receptors (e.g., TNF receptors [TNF-α], IL-1 receptors [IL-1β], and Toll-like receptors [LPS]), our findings might result from a cell-specific expression of these cognate receptors. Most interestingly, mature colon cells per se show a relatively low inflammatory response upon exposure to LPS, a fact which results from a micro RNA (miRNA-146)-mediated repression of the NFκB-signaling pathway [19], thereby preventing an unwanted chronic inflammation in response to the normal gut flora. In our study, the low effect of LPS on CBR3 expression in HT-29 colon cells, which is in contrast to the strong response of HepG2 liver cells to LPS (see below) might be explained by this miRNA-based mechanism.

3.2. Time- and concentration-dependent up-regulation of CBR3 by TNF-α in HT-29 cells

Because TNF-α had the strongest effect in HT-29 cells, we examined the effect of this cytokine on CBR3 expression in more detail. HT-29 cells were incubated with 50 ng/ml of TNF-α under serum-free conditions. After 2, 4, 8, 12, 24 and 48 h of treatment, the amounts of CBR3, COX-2 and IκBα mRNA were determined. As presented in Fig. 2A, TNF-α increased the expression of CBR3 throughout the whole time course of the incubation. Densitometric analysis (Fig. 2B) of four independent cell culture experiments that

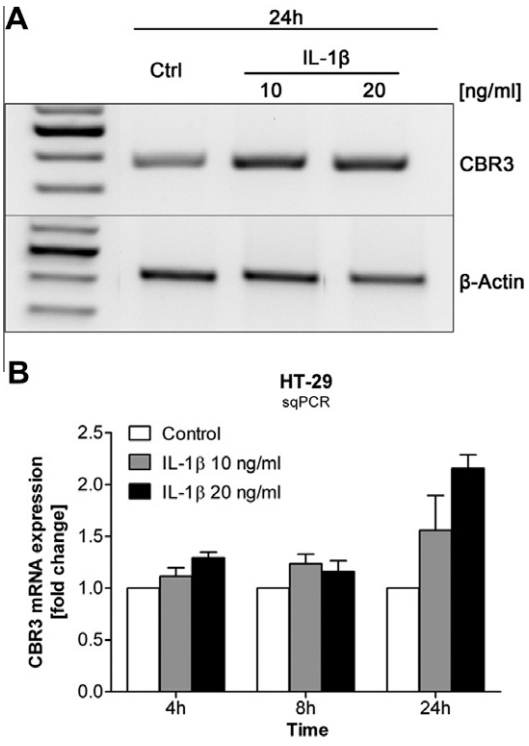

Fig. 1. CBR3 expression in HT-29 cells after exposure to IL-1β. HT-29 cells were treated with IL-1β (10 and 20 ng/ml) or serum-free medium only for 4, 8 and 24 h and changes in the mRNA expression were assayed by means of sqPCR. One representative gel of the treatment with IL-1β for 24 h (A) is shown. Panel (B) shows the densitometric analysis of two representative experiments of the IL-1β treatment. Bars represent means of $n = 2$ experiments ± the standard deviation. The CBR3 mRNA expression was normalized to that of β-actin, and the untreated control ("Ctrl") was set to 1.

were subjected to semi-quantitative RT-PCR (sqPCR) revealed that CBR3 mRNA levels continuously increased from the 2 h-treatment on (2-fold increase vs. control, $P < 0.05$) until it reached a peak after 48 h (9.2-fold vs. control, $P < 0.05$). Also, the mRNA levels of two established NFκB-target genes, COX-2 and IκBα that served as controls for the activation of the NFκB signaling pathway, were induced, indicating that TNF-α successfully activated NFκB in HT-29 cells.

Next, to investigate a possible concentration-dependency in response to TNF-α, HT-29 cells were exposed to 10, 25, 50 and 100 ng/ml of TNF-α for 24 h. Indeed, TNF-α was shown to increase CBR3 mRNA expression in a concentration-dependent manner (Fig. 2C, D). sqPCR analysis (Fig. 2D) revealed that CBR3 mRNA was inducible even by the lowest concentration of 10 ng/ml of TNF-α (2.3-fold, $P < 0.05$), and this effect was continuously growing with increasing concentrations of TNF-α up to 6.6-fold compared to the control ($P < 0.001$) when 100 ng/ml of TNF-α was applied. Again, the exposure to TNF-α led to an elevated level of the NFκB-driven control genes COX-2 and IκBα in HT-29 cells (Fig. 2C).

3.3. Overexpression of the NFκB subunits p65 and/or p50 increased the level of CBR3 mRNA in HT-29 cells

To directly activate the NFκB pathway, HT-29 cells were transfected with the expression vectors pCMV4 p65 and pCMV4 p50 encoding the NFκB subunits p65 and p50, respectively. In this experiment, either two different concentrations (2 μg and 6 μg) of each pCMV4 p65 or pCMV4 p50, respectively, were transfected into the cells alone or in combination (2 μg each). The increased amounts of p65 mRNA (lanes 2, 3, and 6 in Fig. 3A) or p50 mRNA

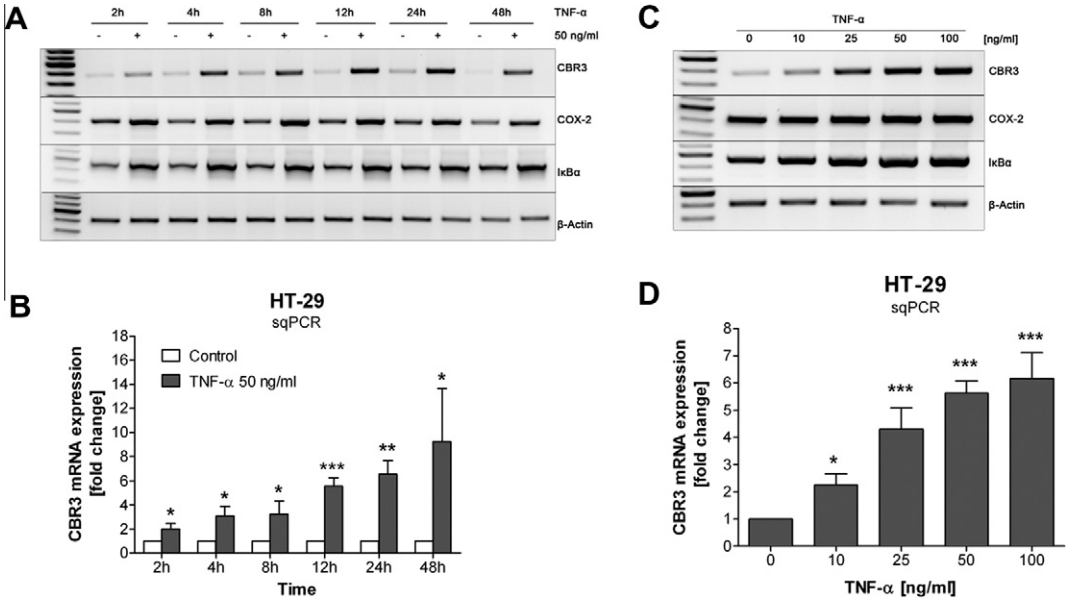

Fig. 2. Time- and concentration-dependent effect of TNF-α on CBR3 mRNA expression in HT-29 cells. HT-29 cells were incubated for 2, 4, 8, 12, 24 and 48 h with (+) or without (−) TNF-α (50 ng/ml) and sqPCR was performed with β-actin as the house-keeping gene. (A) CBR3 mRNA as well as the NFκB-regulated control genes COX-2 and IκBα were induced by TNF-α during the whole time-course. (B) Densitometric analyses of four independent experiments show changes in the CBR3 mRNA expression relative to untreated cells. Bars represent means of $n = 4$ experiments ± the standard deviation (three asterisks denote $P < 0.001$; two asterisks denote $P < 0.01$; one asterisk denotes $P < 0.05$; two-tailed paired Student's t-test). (C, D) Different concentrations of TNF-α (0, 10, 25, 50, and 100 ng/ml) were applied for 24 h and sqPCR was performed. (C) A representative gel shows the effect on CBR3 and NFκB-regulated control genes COX-2 and IκBα. (D) Densitometric analysis of five independent experiments. Bars represent means of $n = 5$ experiments ± the standard deviation (three asterisks denote $P < 0.001$; one asterisk denotes $P < 0.05$; ANOVA).

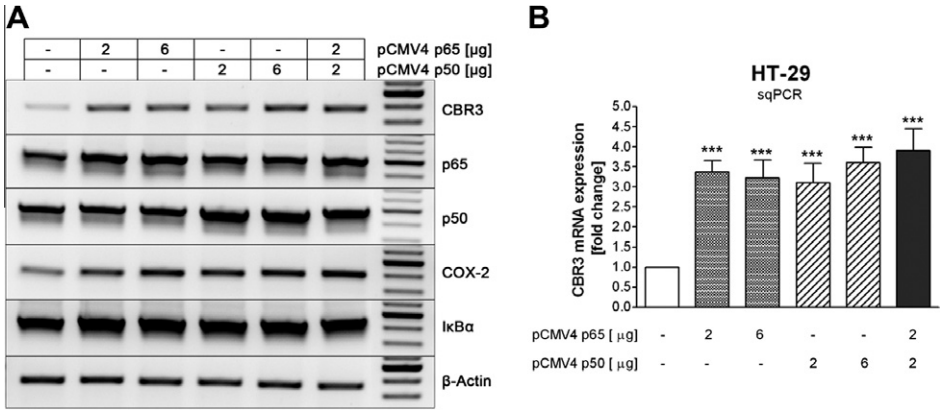

Fig. 3. Transfection of HT-29 cells with NFκB subunits p65 and/or p50 induced CBR3 expression. HT-29 cells were transfected with different amounts of expression plasmids *pCMV4 p50* ("p50") and/or *pCMV4 p65* ("p65") and the changes in the expression of CBR3, p65, p50, as well as of the control genes COX-2 and IκBα were determined by sqPCR. Amounts (in µg) of transfected plasmid DNA are indicated in the table above or below the figures, respectively. (A) One representative gel is shown. (B) The graph shows the densitometric analyses of $n = 3$ independent experiments ± the standard deviation (three asterisks denote $P < 0.001$; ANOVA). The non-transfected control was set to 1.

(lanes 4–6) 24 h post-transfection indicated that the plasmids had been successfully delivered into the cells. Moreover, the activation of the NFκB signaling pathway after transfection of p65 and/or p50 was monitored by the induction of the NFκB target genes COX-2 and IκBα.

As shown in Fig. 3B, sqPCR analysis of four individual experiments revealed that all transfections elevated the levels of CBR3 mRNA in a range from 3.1-fold to 3.9-fold compared to non-transfected cells. Interestingly, the transfection of 2 µg of only one of both expression vectors was sufficient to cause a 3.4-fold (pCMV4 p65) or 3.1-fold (pCMV4 p50) induction of the *CBR3* gene ($P < 0.0001$). A similar effect on CBR3 mRNA expression was observed after co-transfection of both the NFκB subunits p65 and p50 (3.9-fold vs. control, $P < 0.0001$).

Activation of the classical NFκB pathway provokes the translocation of p65 and p50, which act in most of the cases as a p65:p50 heterodimer [14]. However, p65:p65 or p50:p50 homodimers are able to activate gene transcription as well [15]. In HT-29 cells, overexpression of either p65 or p50 already had an equivalent inducing effect on CBR3 mRNA expression, whereas co-transfection with both expression plasmids further enhanced the CBR3 gene transcription only slightly. If both homodimers (p65:p65 and p50:50) and heterodimers (p65:p50) were involved in the transcription of CBR3 mRNA, one would expect a much larger amount of CBR3 mRNA in p50 and p65 co-transfected cells. Thus, the same effectiveness of overexpressed subunits alone and in combination indicates the involvement of p65:p50 heterodimers rather than homodimers. In our case it seems that the plasmid-expressed

p50 or p65 subunits form dimers with the p65 and p50 subunits that were already endogenously present in HT-29 cells.

3.4. CBR3 mRNA expression is inducible by pro-inflammatory stimuli in HepG2 cells and IL-1β regulates CBR3 in a time- and concentration-dependent manner

In order to test, whether the results obtained with HT-29 cells are reproducible in a cell line originating from another tissue, HepG2 cells were incubated with TNF-α, IL-1β, and LPS. All tested NFκB-activators strongly induced CBR3 mRNA expression in HepG2 cells. As shown in Fig. 4A, IL-1β (10 and 20 ng/ml) was the most effective inducer, followed by TNF-α (50 ng/ml) and LPS (1000 ng/ml). Therefore, IL-1β was chosen to study its effect on CBR3 mRNA expression in HepG2 cells in more detail.

From our preliminary experiments it was obvious that HepG2 cells responded more promptly than HT-29 cells with regard to CBR3 up-regulation by pro-inflammatory stimuli. Accordingly, for HepG2 cells, a shorter period of time (2–8 h) was chosen. HepG2 cells were exposed to two different concentrations of IL-1β (1 ng/ml and 10 ng/ml) under serum-free conditions and the changes in CBR3 mRNA expression levels were determined after 2, 4, and 8 h by means of sqPCR (Fig. 4B, C). Again, to monitor the activation of the NFκB-signaling pathway, IκBα served as a control gene that exhibited an expression pattern similar to that of CBR3 when HepG2 cells were subjected to IL-1β-treatment (Fig. 4B). Noteworthy, the second NFκB-regulated control gene, COX-2 was undetectable in this cell line.

As presented in Fig. 4C, even after the shortest incubation time of 2 h, IL-1β-treatment (1 ng/ml and 10 ng/ml) could provoke a strong elevation of CBR3 mRNA expression (4.2-fold and 10.5-fold, respectively; $P < 0.05$ and $P < 0.001$). Interestingly, treatment with 10 ng/ml of IL-1β beyond 2 h did not further increase the amount of CBR3 mRNA, but rather decreased the CBR3 mRNA (to 7.8-fold; $P < 0.001$).

Two out of four individual experiments were additionally verified by quantitative real-time PCR (qPCR) (Fig. 4D). Clearly, due to the two different methods, values of fold-inductions differed between sqPCR and qPCR, especially when the mRNA levels reach the point of saturation in the sqPCR. However, the results turned out to be comparable: IL-1β-treatment for 2 h (1 ng/ml and 10 ng/ml) clearly induced *CBR3* gene expression to 3.7- and 20.7-fold, respectively. A slight further increase was observed after 4 h only at a concentration of 1 ng/ml of IL-1β (5.5-fold vs. control). As seen upon sqPCR after 8 h of incubation with IL-1β (1 ng/ml and 10 ng/ml) the CBR3 mRNA expression declined to 1.5- and 4.1-fold vs. control.

Taken together, our results clearly demonstrate that in HepG2 cells *CBR3* gene expression is up-regulated by IL-1β in a time- and concentration-dependent manner.

Comparing the results obtained with both cell lines, the most striking difference between HT-29 and HepG2 towards pro-inflammatory cytokines was the very prompt response of HepG2 cells followed by a rapid decline in the mRNA levels of both CBR3 and the control gene IκBα (cf. Fig. 4B). The corresponding effect in HT-29 cells (cf. Fig. 2A) was comparatively slow but more persistent. In addition, CBR3 mRNA inducibility was more pronounced in HepG2 cells than in HT-29 cells. Different IκBα activities in colonic cells and hepatocytes may explain the high and rapid increase in CBR3 mRNA in HepG2 cells in contrast to the low but prolonged effects in HT-29 cells. For HT-29 cells as well as for some other human colonic epithelial cells it is known that IκBα-degradation is incomplete after NFκB stimulation, however, without preventing the NFκB binding activity [20]. In contrast, NFκB activation in HepG2 cells caused a strong and rapid proteolysis of IκBα followed by a prompt re-synthesis of IκBα, resulting in an inhibition of NFκB-mediated gene transcription [21]. In addition, different mRNA-stabilities may also contribute to the observed effects. In summary, our findings imply a cell-specific regulation of CBR3 by pro-inflammatory stimuli.

In conclusion, we provide for the first time evidence that the *CBR3* gene is activated under inflammatory conditions. Treatment of cancer cell lines with either TNF-α (HT-29) or IL-1β (HepG2) regulated the expression of CBR3 mRNA in a time- and

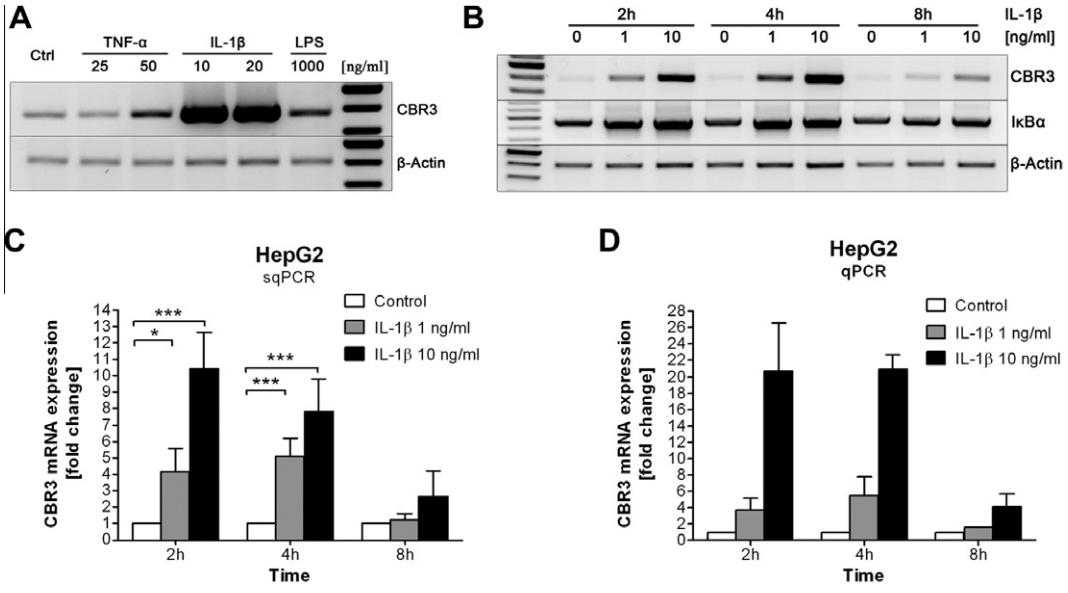

Fig. 4. Time- and concentration-dependent effect of IL-1β on CBR3 mRNA expression in HepG2 cells. (A) HepG2 cells were treated with TNF-α (25 and 50 ng/ml), IL-1β (10 and 20 ng/ml), LPS (1000 ng/ml) or serum-free medium only (control; "Ctrl") for 4 h. Expression levels of CBR3 mRNA were examined by sqPCR. One representative gel is shown. (B) HepG2 cells were incubated for 2, 4 and 8 h with 1 ng/ml and 10 ng/ml of IL-1β or serum-free medium only (0 ng/ml) and the expression of CBR3 mRNA and that of the NFκB-regulated control gene IκBα was determined by sqPCR. (C) The densitometric analysis of four independent experiments is shown. Bars represent means of $n = 4$ experiments ± the standard deviation (three asterisks denote $P < 0.001$; one asterisk denotes $P < 0.05$; ANOVA). (D) Two representative experiments were analyzed by real-time RT-PCR (qPCR). Bars represent means of $n = 2$ experiments ± the standard deviation.

concentration-dependent manner. However, there were striking differences between both cell lines, pointing to a possible cell-specific regulation of CBR3. The identification of the functional NFκB binding sites is currently on-going in our lab by means of reporter gene assays and chromatin immuno-precipitation (ChIP) analyses. Nevertheless, still the greatest challenge is the identification of physiologic substrates that will help to decipher the exact role of CBR3 in the human body.

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