Charles university in Prague
Faculty of pharmacy in Hradec Králové
Department of biochemical sciences

Diploma thesis

The regulation of human carbonyl reductase 3 (CBR3) in epithelial cell lines

Regulace lidské karbonylreduktasy 3 v buněčných liniích epithelu

Petra Malátková

2009

Supervisors:
Prof. Dr. Edmund Maser
Dr. Bettina Ebert
Christian Albrechts University in Kiel
Faculty of Medicine
Department of Toxicology and Pharmacology for Natural Scientists

Prof. Ing. Vladimír Wsól, Ph.D.
Charles University in Prague
Faculty of Pharmacy in Hradec Králové
Department of Biochemical Sciences

Declaration

I declare that this thesis is my original author's work which I have worked out independently. All literature and other sources, from which I gather during elaboration, are listed in the bibliography and properly cited in the work.

4th May 2009

Acknowledgements

First, I would like to express sincere thanks to Dr. Bettina Ebert for teaching me new techniques and performing cell culture experiments. I thank you for lending your time and providing detailed comments.

Also, I would like to thank to Prof. Dr. Edmund Maser and Prof. Ing. Vladimír Wsól, Ph.D. for offering useful advice and, in particular, giving me chance to do the diploma thesis at Department of Toxicology and Pharmacology in Kiel.

Next, I would like to thank to Yasser El-Hawari, who helps me with LaTex and contributes to improving my German skills. I would also like to thank to Michael Kisiela for his helpful assistance with any occurred problems.

Finally, I would like to extend a big thank to my parents for their love and support.

Contents

List of Tables

List of Figures

Abbreviations

4ONE	4-oxonon-2-enal
11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
A	adenine
AhR	aryl hydrocarbon receptor
AhRR	aryl hydrocarbon receptor repressor
AKR	aldo-keto reductase
AR	androgen receptor
ARE	antioxidant response element
ARNT	aryl hydrocarbon nuclear translocator
Asp	Asparagine
B[k]F	benzo[k]fluoranthene
B[a]P	benzo[a]pyrene
BCA	bicinchoninic acid
CBR1	carbonyl reductase 1
CBR2	carbonyl reductase 2
CBR3	carbonyl reductase 3
CBR4	carbonyl reductase 4
cDNA	complementary DNA
DBM	dibenzoyl methane
DCXR	dicarbonyl/L-xylulose reductase
DEM	diethyl maleate
DEPC	diethyl pyrocarbonate
DEX	dexamethasone
DHRS2	dehydrogenase/reductase 2
DHRS4	dehydrogenase/reductase 4
DMEM	Dulbecco's modified Eagle medium
dNTP	deoxyribonucleotide triphosphate
DOXO	doxorubicin
DOXol	doxorubicinol

DRC	daunorubicin
DRCol	daunorubicinol
E_2 α	estradiol
EDTA	Ethylenediaminetetraacetic acid
E-GS	E-guggulsterone
ERα	estrogen receptor α
E. coli	Escherichia coli
FCS	fetal calf serum
FXR	farnesoid X receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GEO	Gene Expression Omnibus
Gly	glycine
GR	glucocorticoid receptor
GS	guggulsterone
HAH	halogenated aryl hydrocarbons
His	histidine
HMEC	human microvascular endothelial cells
HRP	horseradish peroxidase
HSD	hydroxysteriod dehydrogenase
HUVEC	macrovascular human umbilical vein endothelial cells
IKK	IκB kinase
kDa	kilo dalton
Keap1	Kelch ECH associating protein 1
Lys	Lysine
MG-132	carbobenzoxy-L-leucyl-L-leucyl-leucinal
MR	mineralocorticoid receptor
NAD^+/NADH	nicotinamide adenine dinucleotide (oxidised/reduced)
$NADP^+$/NADPH	nicotinamide adenine dinucleotide phosphate (oxidised/reduced)
NF-κB	nuclear factor κB
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
Nrf2	nuclear factor erythroid 2-related factor 2
PAH	polycyclic aromatic hzdrocarbon
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2

$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PR	progesterone receptor
PXR	pregnane X receptor
RA	retinoic acid
RIF	rifampicine
rCBR3	recombinant CBR3
ROS	reactive oxygen species
RU 486	mifepristone
SDR	short-chain dehydrogenase/reductase
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Ser	serine
SUL	D,L-sulforaphane
T	thymine
TBHQ	tert-butylhydroquinone
TNF-α	tumor necrosis factor α
Tris	tris(hydroxymethyl)aminomethane
Tyr	tyrosine
UV	ultraviolet
XRE	xenobiotic response element
Z-GS	Z-guggulsterone

1 Introduction

Biotransformation is a process by which lipid-soluble xenobiotic or endobiotic compound is enzymatically transformed into polar, water-soluble, and excretable metabolites [77]. Therefore, biotransformation serves as an important protection against the potential harmful exposure to xenobiotics from the environment as well as certain endobiotics. In order to minimize the potential injury caused by these compounds, most of the tissues and organs are well equipped with diverse and various xenobiotic metabolizing enzymes, which are present in abundance either at the basal uninduced level, and/or inducible at elevated level after xenobiotics exposure [135]. However, biotransformation can also result in bioactivation, it means, the metabolites are more toxic than the parent substance. Knowledge of biotransformation enzymes is crucial for understanding the potential health risks upon exposure to xenobiotics. Xenobiotic metabolism is divided into two phases (phase I and phase II), which usually occur sequentially. Phase I reaction is reaction which modifies xenobiotic by adding a functional structure or alteration existing one. In phase II reaction, xenobiotics or their phase I metabolites undergo conjugation with endogenous building blocks and can be readily excreted from the body [122].

The three main phase I reactions are oxidation, reduction, and hydrolysis. Redox reactions are clearly the most important ones in xenobiotic metabolism, because the biotransformation of a xenobiotic often begins with redox reactions and moreover, a large diversity of metabolites may be produced from a single substrate [123]. The well studied enzyme system involved in redox reactions of xenobiotics is the cytochrome P450 enzyme system that mediates particularly oxidation reactions. Carbonyl-reducing enzymes initially received less attention [42]. Several carbonyl reductases from the SDR superfamily are not well characterized and further studies are necessary to elucidate their catalytic activity and roles in xenobiotic as well as endogenous metabolism.

Two monomeric carbonyl reductases, namely carbonyl reductase 1 (CBR1) and carbonyl reductase 3 (CBR3), have been found in humans [131]. Whereas substrates and gene regulation of CBR1 have been described, CBR3 is poorly characterized. Until now, gene regulation of CBR3 remains obscure. Elucidation of CBR3 regulation would help to better understand role of this enzyme. Considering to known regulation of CBR1 and available information of CBR3

inducibility from microarray analysis, it has been suggested to investigate effect of NF-κB, AhR- and Nrf2-ligands on CBR3 regulation. Based on CBR3 catalytic function as a carbonyl reductase, this enzyme could play role in xenobiotic metabolism. Respiratory and gastrointestinal tract are widely exposed to xenobiotic compounds. Especially many cells in lungs and also epithelial cells in small intestine expressed biotransformation enzymes involved in xenobiotic metabolism. Therefore, in the present study, experiments were carried out with colon cell lines (Caco-2, HCT-116, HT-29, SW-480, TC-7) or lung cell line (A-549). The colon cell lines used are established lines developed from human colon adenocarcinoma. When cultured under standard conditions, only Caco-2 cells undergo spontaneously an enterocytic differentiation and display features of small intestinal epithelial cells [13, 48]. The lung cell line A-549 is an established lines development from lung carcinoma.

2 Review of the literature

2.1 Carbonyl reducing enzymes

Carbonyl reduction is a significant step in the phase I biotransformation of a great variety of compounds. The chemical carbonyl function (aldehyde or ketone group) occurs frequently in xenobiotics and can also be generated endogenously [42]. Therefore, substrates of carbonyl reducing-enzymes are xenobiotics such as food ingredients, drugs, or environmental pollutants, and, furthermore, endogenous compounds such as hormones, mediators, cofactors, neurotransmitter precursors, and lipid aldehydes derived from oxidative stress and their metabolites [100]. In generally, carbonyl reduction means the formation of hydroxy group from a aldehyde or ketone group. Because the resulting alcohol is easier to conjugate and to eliminate, carbonyl reduction is regarded as an inactivation or detoxification step [42]. The carbonyl moiety is often a determining factor for the biological activity of a molecule, for example reduction of the 3-oxo group to 3α or 3β-hydroxyls in steroid hormones leads to reduced binding properties [98]. Consequently, carbonyl reducing enzymes can affect endogenous processes of both xenobiotic and endogenous ligands [100].

Enzymes mediating carbonyl reduction are ubiquitous in nature. They have been described in plants, bacteria, yeast, teleosts, and insects; however, the most investigated enzymes are the human and rodent members [100]. Human carbonyl-reducing enzymes occur in many different tissues. The differences in tissue and intracellular distribution suggest that the intracellular multiplicity of enzymes may have some relation to their physiological function [42].

Enzymes involved in carbonyl reduction belong to two fundamentally distinct enzyme superfamilies, namely the aldo-keto reductase (AKR) and the short-chain dehydrogenase/reductase (SDR) superfamily. This classification was established on the basis of sequence comparison. The AKRs comprise aldehyde reductases and aldose reductases while several forms of carbonyl reductases are members of the SDR superfamily. Both the AKR and SDR superfamily involve pluripotent hydroxysteriod dehydrogenases (HSDs) [42].

HSDs are pyridine nucleotid-dependent oxidoreductases that mediate the interconversion of secondary alcohols and ketones. They play pivotal roles in the biosynthesis and inactivation of steroides hormones [42]. An interesting feature of several hydroxysteroid dehydrogenases is that they are capable of catalyzing the carbonyl reduction of great variety of non-steroidal carbonyl compounds, and, on the other hand, exhibit high specificity to their physiological steroid substrates [70]. HSDs participate in drug metabolism and play a significant role in the defence against the deleterious effects of endogenous and exogenous toxicants [42]. These pluripotent HSDs belong to either AKR or SDR superfamily.

2.2 Aldo-keto reductases

Aldo-Keto Reductases (AKRs) are a superfamily of NAD(P)H linked oxidoreductases that play fundamental roles in carbonyl metabolism and utilize both natural and exogenous substrates [103]. The AKR superfamily is divided into 15 families. The majority of AKRs are monomeric proteins of 34-37 kDa. The cofactor and the substrate/inhibitor bind in two different regions of the protein and converge at the active site. The cofactor binding site and the active site are highly conserved across the superfamily. The active site contains a conserved catalytic tetrad (Tyr, Asp, Lys, and His) [51]. AKRs catalyze an ordered bi sequential reaction mechanism in which the binding of the obligatory cofactor NAD(P)H precedes the binding of the substrate carbonyl. The reduction occurs in the central complex, followed by the release of the alcohol and $NAD(P)^+$ products in that order [103]. Thirteen human AKRs belong to the five AKR subfamilies, namely 1A, 1B, 1C, 6A, and 7A.

Aldehyde reductase (AKR1A1) catalyzes the reduction of methylglyoxal, certain xenobiotics such as the anthracycline daunorubicin (DRC), or bioactive aldehydes derived from phospholipids or products from lipid peroxidation, e.g. 4-hydroxynon-2-enal and its 4-keto derivate 4-oxo-non-2-enal. Aldose reductase (AKR1B1) reduces many of the substrates identified for AKR1A, however, with lower efficiency. Numerous physiological aldehydes, including glucose, were identified as endogenous substrates for AKR1B1. AKR1B1 is the first enzyme of the polyol/sorbitol pathway [100]. Human AKR1C-enzymes (AKR1C1-AKR1C4) have dual roles in endogenous steroid or eicosanoid regulation and in xenobiotic metabolism. Members of AKR1C subfamily along with AKR1A1 are implicated in the metabolic activation of polycyclic aromatic hydrocarbons (PAHs). PAHs are suspected human carcinogens and implicated in the causation of lung cancer. PAHs require metabolic activation to exert their deleterious effects [51]. AKR1D1, the only human enzymes from the subfamily AKR1D, acts as steroid

5 β-reductase. The AKR16 family does not contain enzymes, but β-subunits of the voltage-dependent shaker potassium channels. Members of the AKR7A subfamily are involved in the metabolic inactivation pathways of aflatoxin B1, a potent hepatocarcinogen [103].

Based on various physiological and xenobiotic functions of AKRs, the regulation of human AKR genes could have a profound effect on the metabolism of endogenous mediators of nuclear receptor signaling and/or detoxication of chemical carcinogens. Several major *cis*-elements affect gene transcription of AKRs; these are the osmotic response element, the phorbol ester response element, and the antioxidant response element [103].

2.3 Short-chain dehydrogenases/reductases

The enzymatic reactions carried out by enzymes from the short-chain dehydrogenase/reductase (SDR) superfamily can be grouped mainly as NAD(P)(H)-dependent oxidoreductions acting on a highly diverse set of substrates, including alcohols, sugars, steroids, aromatic compounds and xenobiotics [105]. The SDR superfamily has several members capable mediating carbonyl reduction. SDRs exist as soluble, or in some cases, membrane-bound proteins, mostly homodimers or tetramers [42]. Pairwise sequence identities between members of the SDR superfamily are typically as low as 15-30 %. However, several sequence motifs conserved across the majority of the members have been identified [9]. The general primary structure of SDRs is composed of a coenzyme-binding site at the N-terminal part, a catalic active site in the central part, a substrate-binding region, and a C-terminal extension important for oligomerization.

The SDR superfamily consists of more than 3,000 members, from minimally 71 members in the human, and becomes one of the largest protein families to date [9, 42, 104]. Initially, the SDRs have been divided into two large families, “classical” with 250-odd residues and “extended” with 350-odd residues, which differ, among others, in glycine residue pattern in the coenzyme-binding regions. Later, based on multiple sequence alignment with known SDRs as seed sequences, the SDR superfamily were clustered into five families, namely “intermediate”, “divergent” and “complex” families, in addition to the already established “classical” and “extended” families [105]. Carbonyl reductases belong to the group of “classical” SDRs.

Members of the SDR family are identified through the occurrence of typical sequence motifs that are arranged in a specific manner, called the Rossmann fold [42]. Rossmann fold is characteristic of many NADH- and NADPH-binding domains [22, 42]. The overall structure of SDRs is built up of sequence of alternating α-helices and β-strand. These β-strands form a central

four- or five-stranded β-sheet with flanking α-helices on each side [42]. This $\beta\alpha\beta$ motif is a characteristic of the Rossmann-fold necessary to bind the dinucleotide cofactor [42, 100]. The Rossmann fold in the SDR superfamily is very stable, tolerating, despite little sequence conservations [42]. It is less readily understood how a protein conformation as consistent as the Rossmann fold has no structurally conserved residues [22].

The three-dimensional structures and the comparison of sequences of several SDR enzymes revealed the importance of common characteristics and provide the basis for understanding the molecular organization of short-chain dehydrogenases in general and elucidating possible catalytic roles of amino acids conserved in this family of enzymes. The most conserved sequence motif of the SDRs is the Tyr-x-x-x-Lys segment (x for any amino acid) found in the active site [55]. Nearly all "classical" SDR enzymes used Ser, Tyr, Lys motif as catalytic residues [54]. These amino acids constituting the catalytic triad are critically important for enzyme function [42]. They appear to maintain a fixed postition relative to the scaffolding of the $\beta\alpha\beta$ folding and the cofactor position [42].

All SDRs probably share a common reaction mechanism. After cofactor binding, a hydroxyl or carbonyl substrate binds. Herewith, the so-called substrate binding loop becomes well ordered and covers the substrate as well as the catalytic centre from the aqueous enviroment [42]. According to three-dimensional structure of $3\alpha/20\beta$-hydroxysteroid dehydrogenase, the model for catalytic activity has been suggested. Tyr has been proposed to be the proton donor in a carbonyl reduction and Lys facilitates the proton transfer [35]. The Ser also participates in catalysis by stabilizing the reaction intermediates or as part of a proton-relay network [42]. The Tyr/Lys mechanism would constitute an explanation of the strict conservation of this residues [54].

Most of the partially conserved residues are at the core of the Rossmann fold and are primarily hydrophobic [22]. The only conserved residues in the substrate-binding cleft are those of the catalytic triad: Ser, Tyr, and Lys. No conserved residues can be found in the substrate-binding site [42]. This is consistent with the wide variety of structures used as substrates by different members of the family [22]. SDR enzymes usually have a substrate binding loop of more than 20 residues, which covers the active site and becomes well ordered after the hydroxyl or carbonyl substrate binding [42]. When the α-carbon backbone of the cofactor binding domains of known SDR's structures is superimposed, the conserved residues appear at the core of the structure and in the cofactor-binding domain, but not in the substrate-binding pocket [22].

In the N-terminal region of the SDR proteins, pattern of three glycine residues important for cofactor binding has been identified [105]. The glycine residues are located at comparable points in their sequences and form a turn between a β-strand and α-helix that border on the

cofactor-binding site [22]. Structural analysis has shown that the glycine residues interact with the pyrophosphate moiety of the cofactor [42]. Glycine residues are spaced differently in individual subfamilies of the SDR superfamily [105]. For example, the motif characteristic for the subfamily of the "classical" SDRs is Gly-x-x-x-Gly-x-Gly [53]. Both NAD(H) and NADP(H) bind to the classical $\beta\alpha\beta$ motif of the Rossmann-fold. The specificity for NADP(H), rather than NAD(H), is conferred by two highly conserved basic residues in the N-terminal end of the peptid chain [42].

Most enzymes of the SDR superfamily appear as either homodimers or as homotetramers. Only few members occur as monomers such as human CBR1 and CBR3. Two types of oligomerization can be found, these are called the P-axis and Q-axis interface [42]. The P-axis interface comprises interparalel association of two α-helices G and two β-strands G of each subunit [35]. This type of oligomerization is usually found in homotetrameric SDRs [42]. The Q-axis interface consists of α-helices E and F of each of the two subunits forming a four-helix bundle [35]. The monomeric enzyme CBR1 has additional extraloop domain between the Q-interfacial β-strand E and α-helical F, which is believed to prevent oligomerization along the Q-axis interface [42].

2.4 Carbonyl reductases of the SDR superfamily

Carbonyl reducing enzymes from the SDR superfamily differ, among others, in their oligomerization behavior and subcelullar localization. In human, the monomeric enzymes include cytosolic CBR1 and CBR3, tetrameric enzymes are mitochondrial dicarbonyl/L-xylulose reductase (DCXR) and peroxisomal dehydrogenase/reductase 4 (DHRS4). Pluripotent HSD belonging to SDRs is dimeric microsomal 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) [42, 101]. Hep27 (gene symbol DHRS2) is also SDR reducing carbonyl compounds [114]. Further, sequencing of the human genome revealed the existence of a third human isoform of carbonyl reductase named carbonyl reductase 4 (CBR4).

2.4.1 CBR1

CBR1 was first described in 1973 [108]. This member of the SDR superfamily is a cytosolic and monomeric NADPH-dependent carbonyl reductase. The gene is mapped to chromosome 21q22.12, contains three exons and encodes for a 277 amino acid protein with a molecular weight of 30.375 [133].

CBR1 can be detected in all organs by immunohistochemical staining, but the amount of staining varied widely [30]. The highest concentrations were found in liver, stomach, small intestine, epidermis, kidney, neuronal, and glial cells of the central nervous system [30, 43]. In a recent study based on PCR method, CBR1 mRNA was detected at highest levels in liver and kidney [82].

CBR1 catalyzes the NADPH-dependent reduction of various carbonyl compounds. The best substrates are quinones (e.g. menadione, phenanthrenequinone), followed by ketoaldehydes, aromatic aldehydes, and the biogenic aldehydes [42]. Since CBR1 catalyze reduction of wide spectrum of xenobiotic carbonyls, this enzyme fulfills an important role in the phase I metabolism of xenobiotics [100]. Moreover, this enzyme may protect against reactive oxygen species (ROS), because it reduces a variety of reactive carbonyl compounds.

CBR1 is an important determinant in the metabolism of p- and o-quinones derived from polycyclic aromatic hydrocarbons (PAH) [75]. In humam liver and placenta, CBR1 acts as a major quinone reductase [49]. Enzymatic reduction of several o-quinones has been observed to result in redox cycling of the quinones, leading to the generation of semiquinones and the superoxide anion [68]. Thus, a possible protective role against quinone toxicity exerted by CBR1 depends on expression and activity of superoxide dismutase and further metabolism, reactivity, and excretion of the hydroquinone formed [100].

Aldehydes and ketones derived from lipid peroxidation were found to be substrates for CBR1 [21]. Lipid peroxidation products comprise highly reactive lipid aldehydes, such as 4-oxonon-2-enal (4ONE), 4-hydroxynon-2-enal, and acrolein, which are able to modify proteins and DNA. Detoxification of these electrophilic aldehydes can occur through reaction with glutathione or, as recently demonstrated, through reductive metabolism of the carbonyl moieties or reduction of the double bond, leading to lipid products that are less reactive and can be further metabolized or excreted [24]. CBR1 play a significant role in 4ONE metabolism and detoxification, since CBR1 catalyzes hydride transfer to the ketone but also to the aldehyde and C=C of 4ONE [21].

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is other xenobiotic substrate of CBR1 [3]. NNK is a potent pulmonary carcinogen presented in tobacco [3]. CBR1 was identified as the major contributor to NNK carbonyl reduction in human liver and lung cytosol [3].

CBR1 has been recognized as a metabolizing enzyme for several therapeutic agents such as haloperidol, metyrapone, loxoprofen, and anthracyclines, namely doxorubicin (DOXO) and daunorubicin (DRC) [75]. CBR1 reduced DOXO and DRC to doxorubicinol (DOXol), and daunorubicinol (DRCol), respectively [30]. Since resulting metabolites are cardiotoxic and has diminished tumor cell killing activity, CBR1 is though to contribute to the unpredictable pharma-

cology of anthracycline drugs [80]. Moreover, CBR1 expression and activity may be modulated by environmental factors acting via recently identified xenobiotic response elements in the CBR1 promoter. Therefore, the variability in the CBR1 expression may affect outcomes of therapies with CBR1 substrates [56].

CBR1 was shown to accomplish the reduction of the 9-keto group of prostaglandin E_2 (PGE_2) to form prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and thus to inactivate PGE_2. The enzyme was later recognized to be identical to prostaglandin-9-reductase. In addition to 9-keto reduction, CBR1 was shown to catalyze the NADP-dependent oxidation of the hydroxyl group at position 15 of prostaglandins. Therefore, CBR1 has also been named NADP-linked 15-hydroxy-prostaglandin dehydrogenase. It should be noted that oxo-reduction at position 9 and hydroxy-dehydogenation at position 15 are the inactivation steps of prostaglandines [42]. Since the Km values for prostaglandins are rather high and the turnover numbers are low, doubts about a physiological role of CBR1 in prostaglandin metabolism appeared [133]. However, several studies have shown that CBR1 may play important role in prostaglandins metabolism. In detail, CBR1 was revealed to have a function in modulating in the metastasis of cancer cells by decreasing PGE_2 level [82]. Prostaglandins, especially PGE_2, are known to play an important role in modulating tumor growth and metastasis in a variety of human tumors [44]. Moreover, study on patients with non-small-cell lung carcinoma reported that reduced CBR1 mRNA expression in non-small-cell lung cancer was significantly correlated with active angiogenesis and tumor progression while enhanced CBR1 mRNA expression was associated with a favorable prognosis [120]. Additionally, interference with prostaglandin metabolism may also affect other physiological roles. The ovarian carbonyl reductase in rats has been found to be regulated by gonadotropins and was suggested to be important for the ovulation process [27]. Study in pigs has shown that porcine CBR1 may be involved in the regulation of the $PGE_2/PGF_{2\ \alpha}$ ratio and thereby in the implantation and early placentation in the pig. Porcine CBR1 is considered to be identical with PG 9-ketoreductase in the pig [130].

Significant interindividual variability in carbonyl reductase activity has been documented in liver, erythrocytes, and in breast and lung tumors. The molecular basis of such disparities and its potential impact on CBR1-mediated drug metabolism remain to be elucidated. It has been suggested that the interindividual differences in CBR activity may in part reflect variable rates of CBR1 gene transcription [59]. Observed induction of CBR1 by xenobiotics such as 2,(3)-t-butyl-4-hydroxyanisole and β-naphtoflavone, has indicated the transcriptional regulation via aryl hydrocarbon receptor (AhR) pathway [29]. Recently, the functional characterization of the promoter of human CBR1 has confirmed this hypothesis by several lines of evidence. [59] The powerful AhR ligand is the polycyclic aromatic hydrocarbon benzo[a]pyrene (B[a]P), one

of the best-characterized carcinogens in cigarette smoke. B[*a*]P induces CBR1 expression significantly in AhR-proficient mice but fails to induce CBR1 in AhR-deficient animals. Consequently, B[*a*]P may modulate CBR1 expression in the lungs of smokers via the AhR pathway, which in turn has an impact on the CBR1-mediated detoxification of other smoke carcinogens relevant to the pathogenesis of lung cancer such as NNK [59].

An unusual property of CBR1 is that it exists in vivo in three forms with different charges and slightly different molecular masses. These multiple isoforms of carbonyl reductase result from covalent modification of a lysine residue at position 239 by an autocatalytic process involving the formation of a Schiff base between the ε-amino group of lysine and 2-oxocarboxylic acids, such as pyruvate and 2-oxoglutarate. This chemical modification is unique to human CBR1 and rat carbonyl reductase and has not been found in any other SDR, nor in another oxidoreductase [112].

Human CBR1 orthologues with sequence identities of more than 80 % to CBR1 have been identified in several other species, such as pig, rabbit, hamster, rat and mouse. The enzyme from pig testes was the first monomeric member within the SDR superfamily for which the three-dimensional structure has been solved [42].

2.4.2 CBR3

CBR3 was first identified 62 kilobase apart from the CBR1 gene on chromosome 21 in year 1998 [131]. This enzyme consists of 277 amino acids and has molecular weight of 30.850 [127]. CBR3 as well as CBR1 is a cytosolic and monomeric NADPH-dependent carbonyl reductase belonging to the SDR superfamily [42]. CBR1 and CBR3 have 72 % identity and 85 % similarity at the amino acid level, which is remarkably high compared to the usually observed low identity levels among other members of the SDR superfamily [23].

The Rossmann-fold consensus sequence (Gly-x-x-x-Gly-x-Gly) of CBR1s is completely conserved with that of CBR3's in the N-terminal part of the enzymes. A Ser/Tyr-x-x-x-Lys motif of CBR1s, which is a catalytic triad conserved among classical SDR enzymes, is also identical with that of CBR3's. On the other hand, Lys-239 in CBR1, which is autocatalytically modified with 2-oxocarboxylic acids, was substituted with Asp in CBR3 [82].

Recent study has detected the expression of CBR1 and CBR3 on mRNA level in all tested tissues but the expression patterns of the genes for CBR1 and CBR3 were clearly different. The highest expression of CBR3 mRNA was detected in ovary, pancreas, and intestine, lower in lung,

liver, spleen, and colon, and other tissues. Notably, sex-specific CBR3 mRNA expression was observed, that is, lower expression in prostate and testis than that in ovary. The level of CBR3 mRNA was shown be lower compared with that of CBR1 [82].

Several studies have described a wide range of interindividual variability in the metabolism of drugs that are CBR substrates. The genetic polymorphisms in CBR1 and CBR3 was proposed as the key for this variability. Initially, a single nucleotide polymorphism in CBR3 was characterized that encodes for a V244 to M244 change in the protein. The CBR3 V244M genotype distributions differed significantly among ethnic groups. CBR3 V244 and CBR3 M244 isoforms have distinctive catalytic properties. The comparative three-dimensional analysis revealed that the V244M substitution is positioned in a region critical for interactions with the NADP(H) cofactor [60]. Up to now, six natural variants of CBR3 have been identified [127].

Furthermore, polymorphisms in CBR1 and CBR3 were assessed in 101 Southwest Asian breast cancer patients receiving DOX containing therapy. Of the five CBR3 coding region variants identified, three were nonsynonymous: C4Y, E124K, and V244M. The CBR3 C4Y variant was associated with altered doxorubicin pharmacokinetics, in addition to significantly improved tumor reduction. This suggests lower carbonyl reductase activity in patients with substitution of tyrosine for cysteine at the fourth amino acid at the amino terminal [28]. No associations with CBR1 polymorphisms were observed. This study takes into account that both carbonyl reductases, CBR1 and CBR3, are responsible for phase I metabolism of DOX [28]. In a later study, CBR1 has been suggested as a major hepatic DOX reductase while CBR3 showed no or very little DOX reduction. Moreover, the very low expression of CBR3 transcripts argues against any major role of this isozyme in drug disposition [56].

CBR3 was shown to be induced in cell line oral squamosus cell carcinoma cells by 9-cis retinoic acid (RA). Retinoids act as cytostatic agents in the prevention of epithelial carcinogenesis. The results suggested that CBR3 is a mediator of cytostatic effects by RA. Moreover, the expression of CBR3 among oral squamosus cell carcinoma cells was downregulated in highly invasive tumors compared with less invasive ones. A potential retinoid X receptor responsive element, which could be target for the 9-cis-RA, was observed in the promoter of CBR3. Since the maximal up-regulation of CBR3 mRNA by RA required longer than 6 days, there may be indirect mechanism for this gene's activation rather than direct transactivation via responsive element [99].

Despite the high sequence similarity between CBR1 and CBR3, unexpected differences in substrate specifity have appeared. CBR3 shows only low or no activity towards substrate that are metabolized by CBR1 in humans [81]. CBR3 also exhibited considerably lower catalytic effi-

ciency of carbonyl reductase activity than chinese hamster orthologues of CBR1 (Chinese hamster CBR1, Chinese hamster CBR2) and CBR3 (Chinese hamster CBR3) [81]. 9,10-phenanthrenequinone and isatin are to date the only known substrates for CBR3. Although, they are model substrates rather than physiological. Menadione was initially reported as a substrate for CBR3 [60]. However, kinetic data from recently studies ruled out menadione as a substrate for CBR3 [23, 81, 82].

In order to elucidate the enzymatic mechanism of CBR3, differences in sequence alignment and catalytic activity of CBR3 and other orthologues was compared. Thryptophan 230, a highly conserved amino acid in most CBR proteins, is located in the hinge region at the substrate-binding loop. Human CBR3 possesses rigid amino acid, proline, at that position instead. Based on this knowledge, the role of amino acid residue at position 230 has been investigated. Results shown that the substitution of the amino acid at position 230 residue alone is not sufficient to affect enzymatic properties [23, 81]. However, residue 230 have importance in ligand binding [23]. Looking at the crystal structures, the catalytic cleft of CBR3 has to be regarded as being more hydrophilic in comparison to CBR1. Therefore, the spectrum of possible substrates for CBR3 might comprise polar substances like sugars or polyols [23].

The substrate spectrum and the physiological importance of this enzyme are poorly characterized and partly controversial. Further studies on the catalytic activity and gene regulation of CBR3 are necessary to elucidate its roles in the metabolism of endogenous and exogenous compounds, and in the pathogenesis of diseases [75].

2.4.3 CBR4

The gene Cbr4 is found on chromosome 4 (4q32.3) and its encoded 237-amino acid protein is currently annotated as CBR4. CBR4 shows low sequence identity ($<30\,\%$) with the six known CBRs and enzymes with CBR activity. A very recent study has characterized CBR4 as a novel mitochondrial quinone reductase, since the recombinant tetrameric protein exhibits NADPH-dependent reductase activity for o- and p-quinones, but not for other aldehydes and ketones [25]. The highest catalytic efficiency (kcat/Km) has been showed for 9,10- phenanthrenequinone, followed by 1,4-benzoquinone, and the lowest catalytic efficiency for menadione. Although the endogenous substrate of CBR4 is unknown at present, this enzyme may play a role in protection against cytotoxicity of exogenous quinones, because of its low Km values for the quinones and, in particular, its localization in the mitochondria. Mitochondria participate in key steps of the apoptotic signal cascade induced by oxidative stress [25].

2.4.4 Tetrameric carbonyl reductase

Tetrameric forms of carbonyl reductase are expressed in mitochondria, as it is known for carbonyl reductases in the lungs of mouse, guinea pig, and pig, or in peroxisomes in pig. These homotetrameric forms have occasionally been termed carbonyl reductase 2 (CBR2) [42]. However, a homologous enzyme is not found in the human genome. CBR2 enzymes reduce various aliphatic, alicyclic, and aromatic carbonyl compounds, and it is suggested that CBR2 might function in the detoxification of xenobiotic carbonyls and carbonyls derived from lipid peroxidation [75].

2.4.5 Dimeric carbonyl reductase: 11β-HSD1

11β-HSD is a microsomal enzyme from SDR superfamily that catalyzes the reversible interconversion of receptor-active 11-hydroxy glucocorticoids (cortisol) to their receptor-inactive 11-oxo metabolites (cortisone) [113]. Two different isoforms of 11β-hydroxysteroid dehydrogenase have been described so far, 11β-HSD1 and 11β-HSD2, which differ in their biological properties and tissue distribution [69]. Although displaying dehydrogenase and reductase activities in vitro, the dominant in vivo function of the type 1 enzyme is obviously glucocorticoid 11-oxoreduction [42]. 11β-HSD2 seems to work exclusively as dehydrogenase by inactivating 11-hydroxyglucocorticoids and thereby effectively protects mineralocorticoid receptor from occupancy and transactivation by corticosterone, but not aldosterone. Thus, 11β-HSD2 is responsible for ensuring mineralocorticoid specificity of aldosterone [113]. The biological functions of 11β-HSD1 are less well understood. 11β-HSD1 has been referred to as a enzyme with a micromolar affinity for glucocorticoids which is in contrast to low glucocorticoid plasma levels and high binding affinities of glucocorticoids to the glucocorticoid receptor (both in low nanomolar concentrations) [71]. This enzyme is active as dimer and oligomeric enzymes often display kinetics other than that described by the Michaelis-Menten equation. When applying sigmoidal dose response calculation, cooperative kinetics of 11β-HSD1 action with cortisone and dehydrocortisone were observed. Accordingly, this enzyme dynamically adapts to low (nanomolar) as well as to high (micromolar) substrate concentrations, thereby providing the fine-tuning required as a consequence of great variations in circadian plasma glucocorticoid levels [71].

Increases in the activity and expression of 11β-HSD1 have been implicated in the pathogenesis of many common conditions including obesity, insulin resistance, type 2 diabetes, and the metabolic syndrome [126]. 11β-HSD1 knockout mice opposes the pathogenic lipid and lipoprotein profile and exhibited the improved hepatic insulin sensitivity and glucose tolerance [85]. In

contrast, transgenic mice overexpressing 11β-HSD1 selectively in adipose tissue had developed visceral obesity that was exaggerated by a high-fat diet and also exhibited pronounced insulin-resistant diabetes and hyperlipidemia [73]. Hence, a selective 11β-HSD1 inhibition has been proposed as a novel therapeutic strategy in the metabolic syndrome [1].

Interestingly, evidence is emerging that 11β-HSD1 fulfills an additional role in the metabolism of xenobiotic carbonyl compounds [72]. 11β-HSD1 was demonstrated to be capable of acting as carbonyl reductase in the detoxification of aldehydes, ketones, and quinones [42]. As observed with glucocorticoid reduction, xenobiotic carbonyl reduction showed cooperative kinetics indicating that the enzyme may dynamically adapt to low as well as to high substrate concentrations [72]. Most carbonyl compounds are lipid soluble and are expected to distribute themselves in membranes rich in lipids. Therefore, microsomal 11β-HSD1 rather than cytoplasmic reductases may play a significant role in the reductive metabolism of these substances [70]. In addition, quinone toxicity may result in lipid peroxidation and deleterious effects on the phospholipid bilayer. 11β-HSD1 may therefore be more important as a protective device against lipid peroxidation and subsequent membrane damage than cytosolic NAD(P)H:quinone oxidoreductase and CBR1. In addition, UDP-glucuronosyltransferase, a glucuronic-acid-conjugating enzyme important in quinone detoxification, is located in the endoplasmic reticulum. It is conceivable that 11β-HSD1 and glucuronosyltransferase cooperate in the detoxification of quinones [70]. Next, 11β-HSD1 has important role in the detoxification of NNK, a tobacco-derived carcinogen, since it is able to reduce NNK even in the low nanomolar scale [69].

2.4.6 Other SDR-type carbonyl reducing enzymes

The SDR enzymes DCXR, (dehydrogenase/reductase 2) DHRS2, and DHRS4 constitute a cluster of sequence-related SDR enzymes whose physiological roles are insufficiently understood at present [100].

DCXR is a mitochondrial enzyme with a large range of activities towards dicarbonyl and sugar compounds. These properties suggest that DCXR is involved in the uronate cycle of glucose metabolism [89]. DHRS4 is a peroxisomal enzyme which reduces all-trans retinal with low catalytic efficiency. It has been reported that DHRS4 reduces 3-keto-C_{19}/C_{21}-steroids into 3β-hydroxysteroids. Therefore, this enzyme may be involved in the metabolism of 3-ketosteroids [74]. DHRS2 was originally isolated as a cell-cycle regulated protein and found to be member of the SDR family [114]. All these carbonyl reducing enzymes show activity toward various α -dicarbonyl compounds and are proposed as detoxification enzymes [74, 89, 114].

2.5 Biological functions of carbonyl reductases

Carbonyl reductases are ubiquitous in nature and catalyze the NADPH reduction of a large number of biologically and pharmacologically active substrates [132]. The biological function of carbonyl reducing enzymes from the SDR superfamily is not fully understood. The role of several HSDs in steroid metabolism is known, however, some exhibit activity toward nonsteroid substrates. The best substrates for carbonyl reductases are quinones, e.g. quinones derived from polycyclic aromatic hydrocarbons (PAH), and several xenobiotic aldehyds and ketones. Many of these compounds exert toxic effects in biological systems and hence, carbonyl reductases may serve as a general catalyst in the detoxification of these compounds [43]. On the other hand, physiological roles of carbonyl reductases in the endogenous metabolism of prostaglandins, steroids, or tetrahydrobiopterin synthesis were proposed [42].

2.5.1 Roles in steroid and prostaglandin metabolism

HSD from the SDRs, namely 11β-HSD1, and several HSDs from the AKRs catalyze position-specific and stereospecific reactions of steroids. Thus, they play pivotal roles in the biosynthesis and inactivation of steroid hormones. Carbonyl reductases may also have role in steroid metabolism, because their ability to metabolize steroids [46]. They are known to interconvert PGE_2 and $PGF_{2\alpha}$. This suggests a further regulatory role of this enzymes in prostaglandin function [27]. Moreover, the activity of carbonyl reductases has been found to be regulated by estrogens and gonadotropins, and was proposed to be important for the ovulation process [42].

2.5.2 Roles in oxidative stress

Carbonyl reduction plays important role in oxidative stress. Oxidative stress is defined by increase in the levels of ROS. Major sources for ROS production include respiration, inflammatory mechanisms, endogenous metabolism (e.g., through peroxisomal or flavin-containing enzymes), metabolic activation of xenobiotics, or through direct prooxidative effects of environmental agents (e.g., metals, radiation, chlorinated compounds) [100]. Oxidative stress results in the oxidation of cellular components, and in many cases, reactive carbonyls are produced as a consequence. Examples are the lipid peroxidation end products such as malondialdehyde, acrolein, 4-hydroxy-2-nonenal, and 4-oxo-2-nonenal. Since carbonyl compounds are highly reactive, they are capable of forming adducts with proteins and nucleotides. Some carbonyl compounds cause the mutation of genes through modification of DNA and, hence, are mutagenic [45]. Consider-

able evidence is now emerging that it is the presence of these carbonyls rather than the initial oxidative insult that leads to the cellular damage observed. Therefore, reactive carbonyl products could be involved in the progression of diseases, including neurodegenerative disorders, diabetes, atherosclerosis, diabetic complications, reperfusion after ischemic injury, hypertension, and inflammation [24].

Mammals have evolved several mechanisms to suppress oxidative stress and minimize damage by ROS. The one mechanism is an antioxidative system comprised of enzymes and low molecular weight compounds such as vitamins (A, C, and E) and glutathione. This system scavenges harmful ROS before they have an opportunity to react with other important molecules and terminates the subsequent chain reaction. The other is a reduction-oxidation system that not only functions to detoxify harmful oxidants but reductively repairs oxidized molecules as well [31]. Carbonyl compounds produced by oxidative stress can undergo spontaneous and enzyme catalyzed GSH conjugation and either oxidation by aldehyde dehydrogenases or carbonyl reduction catalyzed by AKR or SDR enzymes [42].

2.5.3 Quinone detoxification

Reactions of quinone compounds are of special toxicological interest [102]. Quinones may be toxic to cells by a number of mechanisms including redox cycling, arylation, intercalation, induction of DNA strand breaks, generation of site-specific free radicals, and interference with mitochondrial respiration. The role of oxidative stress and redox cycling is regarded to be of a big significance in the quinone toxicity [84]. Human exposure to quinones can occur via drug therapy, the diet, or airborne pollutants. In addition, mammals themselves synthesize quinones [42].

Quinones may undergo one-electron reduction by microsomal NADPH-cytochrome P450 reductase, microsomal NADH-cytochrome b5 reductase, and mitochondrial NADH-ubiquinone oxidoreductase. Resulting semiquinones may be toxic per se or react with molecular oxygen forming superoxide anion radical and regenerating the parent quinone, which is then available for rereduction and hence undergoes a redox cycling. The net result of this redox cycling is an oxidative stress [84].

Two-electron reduction of quinones has generally been considered to be a detoxication pathway since the resulting hydroquinone may be conjugated and excreted [84]. In principle, hydroquinones may undergo autooxidation and generate active oxygen species. However, in the presence of conjugating enzymes, they are rapidly converted to the glucuronyl or sulfate conjugates, which prevent their reoxidation to quinones. Consequently, enzymes catalyzing the two-

electron reduction of quinones to hydroquinones may protect the cell against oxidative stress from quinone compounds by competition with single-electron reduction pathways [70]. Two-electron reduction of quinones is catalyzed by cytosolic NAD(P)H: quinone reductase and two enzymes from the SDR superfamily, these are cytosolic CBR1 and microsomal 11-βHSD1 [42].

2.5.4 Protection against tobacco smoke-derived lung cancer

The tobacco specific NNK is the most potent carcinogen presented in tobacco and suggested to be an important factor in the etiology of lung cancer in smokers. [40] NNK requires metabolic activation by cytochrome P-450 resulting to the formation of reactive electrophilic species that alkylate DNA. Detoxification occurs mainly by carbonyl reduction to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which can be further subjected to glucuronidation and excretion in urine [117]. It has been characterized five different enzymes catalyzing NNK carbonyl reduction in humans, CBR1 and 11β-HSD1 from the SDR superfamily and three members of the AKR superfamily (AKR1C1, AKR1C2 and AKR1C4) [42]. CBR1 was identified as the major contributor to NNK carbonyl reduction in human liver and lung cytosol [3].

Any impact on NNK carbonyl reduction could have consequences with regard to NNK-induced carcinogenesis [42]. For example, a variety of factors may influence the NNK detoxification capacity of 11β-HSD1. These factors include differences in expression levels which may result from environmental or genetic factors, mutational aberrations in enzyme activity, or interactions with endogenous or exogenous 11β-HSD1 substrates or inhibitors. Consequently, this conditions may cause an impaired detoxification capacity of 11β-HSD1 that may shift the NNK/NNAL equilibrium towards cytochrome P450 mediated NNK activation and lead to the accumulation of deleterious NNK metabolites [69].

2.5.5 Carbonyl reduction in drug metabolism

Carbonyl reduction has a significant role in drug metabolism. Carbonyl reductases may reduce carbonyl-containing drugs to less lipophilic alcohol metabolites. These reduced alcohol metabolites can be than excreted, as well as rapidly conjugated by phase II metabolizing enzymes, which facilitate their elimination [42]. Carbonyl reduction has been shown to be important in inactivation of drugs, such as warfarin, haloperidol, DRC and DOXO. On the other hand, the respective carbinols formed may retain their therapeutic potency, thus prolonging the pharmacodynamic effect, or, in some instances, a compounds gain activity through carbonyl reduction, as is the

case for pentoxifylline, and propranolol [42].

2.5.6 Role in anthracycline therapy

Anthracyclines such as DRC and DOXO are the most valuable cytostatic agents in chemotherapy, but their usefulness is limited by intrinsic or acquired resistance towards these drugs. Anthracycline resistance is not merely the result of alterations in drug uptake and retention, but is also mediated by enzymatic anthracycline detoxification [4]. DOXO and DRC undergo reduction mediated by NADPH-dependent cytoplasmic aldo/keto- or carbonyl-reductases resulting in formation of C-13-alcohol metabolites, DOXol, and DRCol, respectively [80]. Human tissues express at least two DOX-reducing enzymes, these are CBR1 and AKR1C3. CBR1 seems to be a major DOX reductase [34, 56]. Three enzymes capable of catalyzing DRC carbonyl reduction have been identified in human liver: AKR1A1 and AKR1C2 from the AKR superfamily and CBR1 from the SDR superfamily.

DOXol and DRCol are the major metabolites in patients [5]. Interestingly, they have significantly lower antineoplastic potency compared to the parent drugs, in terms of inhibiting tumor cell growth in vitro. Hence, elevated levels of carbonyl-reducing enzymes constitute a mechanism in the development of resistance [118]. Moreover, DOXol and DRCol are regarded to be responsible for cardiotoxicity, which is a serious side effect observed upon anthracycline chemotherapy [80]. Therefore, prevention of carbonyl reduction may represent a potential approach to enhancing the safety and efficiency of cytostatics in clinical chemotherapy [42].

2.6 Theories on CBR3 regulation

The expression of genes could be regulated in gene-specific way at any one of several stages. The major control point for most genes is initiation of transcription. Regulatory transcription factors provide common control of a large number of target genes. The transcription factor binds to response element located in promoter or in enhancer regions of response genes, thereby stimulates, or sometimes inhibits, transcription from those genes [63]. The activity of transcription factor is itself regulated in response to intrinsic or extrinsic signals [63]. A diversity of compounds can bind to transcriptional factors and act as agonists or antagonists. In the absence of ligand, the ligand-binding domain of many transcriptional factors is bound to transcriptional co-repressor complexes. Transcriptional factors exist either in the cytoplasm or nucleus. The latter are termed as nuclear receptors and their ligands include steroid hormones. Transcrip-

tional factors can form monomers, homodimers (e.g., estrogen receptor and mineralocorticoid receptor), or heterodimers. Many nuclear receptors form obligate heterodimers with the retinoid X receptor [18].

While the regulation of CBR3 gene expression is completely unknown, the regulation of CBR1 seems to be mediated by AhR, this mean by xenobiotic response element (XRE) [59]. Since CBR1 and CBR3 are close related, the transcription regulation of both enzymes may be identical. Furthermore, the states in which is expression of CBR3 increased could indicate other possible mechanisms involved in its regulation. This data may be obtained from the Gene Expression Omnibus (GEO). GEO is a public repository that archives and freely distributes microarray and other forms of high-throughput data submitted by the scientific community. Results published in GEO report about the increased CBR3 expression, among others, in human microvascular endothelial cells (HMEC) and macrovascular human umbilical vein endothelial cells (HUVEC) stimulated with tumor necrosis factor alpha (TNF-α), and in synovial tissues from patients with rheumatoid arthritis [90–92, 129]. Since nuclear factor κB (NF-κB) is activated with TNF-α and is overexpressed in inflamed synovium in rheumatoid arthritis, CBR3 could be regulated through NF-κB pathway [26]. Next, elevated CBR3 level was reported in small and large airway epithelial cells from cigarette smokers [12, 39, 93, 94]. Cigarette smoke is a complex mixture containing thousands of toxicants, many of which can be potent activators of Nrf2, either directly as electrophiles or indirectly by causing redox imbalance and oxidative stress [57]. Cigarette smoke also contains benzo(*a*)pyrene, a powerful AhR ligand [124]. Therefore, CBR3 could be target gene of Nrf2 or AhR. In addition, oxidatively modified LDL, other source of oxidative stress, induced expression of CBR3 in retinal pigment epithelial ARPE-19 cells [95, 136], indicating the possible involvement of Nrf2-pathway in CBR3 regulation.

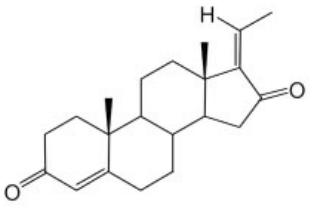
2.6.1 NF-κB

Nuclear factor κB (NF-κB) plays a pivotal role in regulating genes involved in the control of the immune system as well as in the response to injury and infection [111]. NF-κB is maintained in an inactive form in the cytoplasm by association with its specific inhibitor, IκB [26]. The major pathway leading to NF-κB activation is based on inducible IκB degradation. Upon stimulation with many NF-κB inducers, IκB kinase (IKK) is rapidly phosphorylated, which targets the inhibitor for ubiquitination and subsequent degradation [111]. The liberated NF-κBs enter the nucleus, where they form homo- or heterodimer and thereby regulate the transcription of diverse genes encoding cytokines, growth factors, cell adhesion molecules, and pro- and anti-apoptotic proteins [26]. Activated NF-κB can then be down-regulated through multiple mechanisms in-

cluding the well characterized feedback pathway whereby newly synthesized IκB protein binds to nuclear NF -κB and exports it out to the cytosol [2].

NF-κB is activated by a range of stimuli, including various pro-inflammatory cytokines (e.g., TNF-α and IL-1), growth factors, DNA-damaging agents and viral proteins [14]. By reason of a large diversity of agents that might activate NF-κB, it has been proposed that agents activating NF-κB do so by increasing oxidative stress within the cell. However, this model was not found to be universal, since the dependence between NF-κB activation and intracellular ROS generation was only detected in certain cell lines [111].

2.6.2 Guggulsterone



Z-Guggulsterone

Guggulipid, an extract from the tree called Commiphora mukul, is used to treat a variety of disorders in humans, including dyslipidemia, obesity, and inflammation [10, 11]. The active ingredient in guggulipid are the cis- and trans-stereoisomers of 4,17(20)-pregnadiene-3,16-dione, referred to as Z-guggulsterone (Z-GS) and E-guggulsterone (E-GS), respectively.

GS has been shown to attenuate intestinal inflammation. The anti-inflammatory action appeared to be mediated by inhibiting NF-κB signaling by way of IKK blockade [15]. The hypolipidemic activity of GS has been suggested to be mediated by antagonism of the receptor for bile acids, the farnesoid X receptor (FXR) [11, 134]. Later studies have demonstrated that GS is a promiscuous steroid receptor ligand [10, 11]. Both stereoisomers of GS act as antagonists of mineralocorticoid receptor (MR), glucocorticoid receptor (GR), and androgen receptor (AR), and agonists of progesterone receptor (PR), pregnane X receptor (PXR) and estrogen receptor α (ERα) at concentrations well below those required to block FXR [11].

2.6.3 AhR

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor known to mediate most of the toxic and carcinogenic effects of a wide variety of environmental contaminants such as dioxins and related aromatic hydrocarbons [6]. The AhR is present in the cytosol in complex with the chaperone Hsp90 and co-chaperones. Ligand binding leads to nuclear translocation, release of chaperones and heterodimerization with aryl hydrocarbon nuclear translocator (ARNT) [8]. This heterodimer binds to a partially characterized set of co-activators and/or co-repressors and the resulting complex interacts with XREs in the regulatory region of target genes, including genes coding for Phase I and II biotransformation enzymes and genes involved in regulation of cell development, proliferation and differentiation [8].

The aryl hydrocarbon receptor repressor (AhRR) is a negative regulator of AhR, competing with it for formation of a heterodimer with the ARNT [79]. Since the AhRR itself is induced by AhR ligands, the AhR and AhRR form a regulatory feedback loop in the AhR signal transduction pathway [79]. Direct interaction of the AhR with the retinoblastoma protein and NF-κB has been identified. AhR signaling also includes cross-talk with a number of protein kinases [8, 106].

Typical ligands are PAHs, halogenated aryl hydrocarbons (HAHs), and numerous dietary plant constituents such as indole 3-carbinol and curcumin [50]. In addition, a variety of endogenous chemicals have been identified that can bind to the AhR and/or activate AhR-dependent gene expression. Although the majority of these chemicals are relatively weak when compared to 2,3,7,8-tetrachlorodibenzo-[p]-dioxin, the most potent HAH [8, 50].

2.6.4 Nrf2

Nuclear factor erythroid 2-related factor 2 (Nrf2) is the central protein that interacts with the antioxidant response element (ARE) to activate gene transcription in response to an oxidative stress signal [96]. Kelch ECH associating protein 1 (Keap1), a cytosolic repressor protein that binds to Nrf2, retains it in the cytoplasm, and targets it for ubiquitination and proteasome degradation. This mechanism promotes low basal gene expression of the cytoprotective enzymes and proteins in cells under normal physiological conditions [57]. The modification of key cysteine residues on Keap-1 during electrophilic or oxidative stress lead to a disruption of the low-affinity interaction between Nrf2 and Keap-1 [20]. Furthermore, phosphorylation by protein kinases is required for Nrf2 release. Three major signal transduction pathways have been proposed as being involved in transducing oxidative stress signals to Nrf2: the protein kinase C, the mitogen-activated protein

kinase cascades, and the phosphatidylinositol 3-kinase [37]. Released Nrf2 quickly translocates to the nucleus and before the binding to ARE, Nrf2 heterodimerizes with members of the small Maf family of transcription factors [57]. Proteins that are encoded by the ARE gene battery include enzymes associated with glutathione biosynthesis, redox proteins with active sulfhydryl moieties, and xenobiotic detoxification enzymes [96]. Nrf2 transcription itself is transactivated by ARE [86].

Two classes of anticarcinogenic enzyme inducers were described, these are monofunctional and bifunctional [121]. The inducers of ARE target genes are also distinguished as monofunctional and bifunctional. The monofunctional inducers can only act through the ARE-mediated pathway [96]. Monofunctional inducers induce phase II enzymes and antioxidant enzymes. They are electrophilic compounds capable of reacting with sulfhydryl groups. The bifunctional inducer can induce both phase I and phase II enzymes. The bifunctional inducers bind to and activate the AhR, which then lead to activation of transcription through the XRE [37]. The bifunctional inducers can also activate transcription through the ARE via a separate pathway, but at first, they have to be metabolized by the monooxygenase system to compounds that are chemically similar to the monofunctional inducers. This is facilitated by their ability to induce CYP 1A1, thus increasing their own metabolism [96].

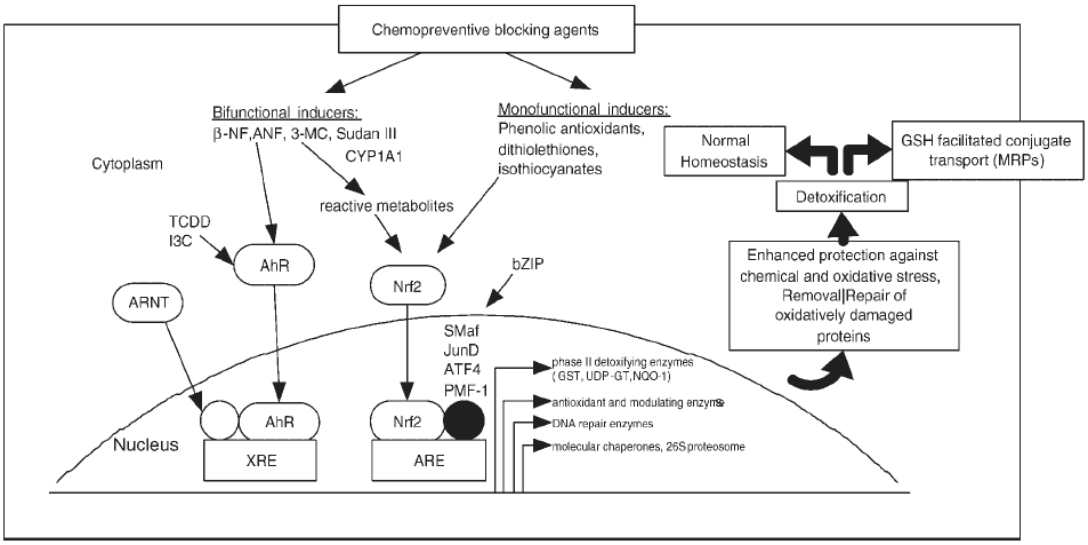


Figure 2.1: Mono and bifunctional inducers, according to Giudice et al., 2006. ANF, α-naphthoflavone; ATF4, activating transcription factor; 4β-NF, β-naphthoflavone; bZip, basic region leucine zipper; GST, glutathione S-transferase; I3C, indole-3-carbinol; NQO1, NAD(P)H:quinone oxidoreductase1; PMF-1, polyamine-modulated factor-1 protein; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; UDP-GT, UDP-glucuronosyltransferase

Results from several studies implies that AhR and Nrf2 have direct links. It was demonstrated that the Nrf2 gene expression is at least partly regulated by AhR inducers by activating multiple XRE elements in its promoter [78]. In contrast to XRE, ARE is capable to mediate a response to monofunctional inducer tert-butylhydroquinone (TBHQ) in mutant cell lines lacking either a functional AhR or CYP 1A1. Thus, the data strongly confirm the proposal that induction of Phase II enzymes may also be mediated by an AhR-independent mechanism [96].

3 Aims of the diploma thesis

The aims of this thesis were:

1. to reveal suitable cell line for investigation of CBR3 regulation
2. to study effect of TNF-α on CBR3 expression
3. to study effect of guggulsterone on CBR3 expression
4. to study effect of AhR-ligands on CBR3 expression
5. to study effect of Nrf2-ligands on CBR3 expression

4 Material

4.1 Chemicals

Substance	Producer
Acetic Acid	Sigma, Steinheim (Germany)
Acrylamide/bis-Acrylamide 30 %	Sigma, Steinheim (Germany)
Agarose NEEO Ultra Qualität	Carl Roth, Karlsruhe (Germany)
Amonium Peroxide Sulfate	Carl Roth, Karlsruhe (Germany)
Benzo[k]fluoranthene	Sigma, Steinheim (Germany)
Bovine Serum Albumine	Behringwerke, Marburg (Germany)
Bromophenolblue	Fluka, Buchs (Switzerland)
Chloroform	Carl Roth, Karlsruhe (Germany)
Curcumin	Sigma, Steinheim (Germany)
Dexamethasone	Sigma, Steinheim (Germany)
Dibenzoyl methane	Sigma, Steinheim (Germany)
Diethyl maleate	Sigma, Steinheim (Germany)
Disodium Phosohate Dihydrate	Merck, Darmstadt (Germany)
D,L-Sulforaphane	Sigma, Steinheim (Germany)
EDTA	Sigma, Steinheim (Germany)
Estradiol	Sigma, Steinheim (Germany)
Ethanol	Carl Roth, Karlsruhe (Germany)
Glycerol	Carl Roth, Karlsruhe (Germany)
Glycine	Sigma, Steinheim (Germany)
Hydrogen Chloride	Merck, Darmstadt (Germany)
Isopropanol	Merck, Darmstadt (Germany)
Kalium Chloride	Merck, Darmstadt (Germany)
Kalium Dihydrogenphosphate	Sigma, Steinheim (Germany)
Kodak x-ray Developer LX 24	Kodak
Kodak x-ray Fixer AL 4	Kodak
Methanol	Carl Roth, Karlsruhe (Germany)

MG-132	Calbiochem, Darmstadt (Germany)
N,N,N´,N´-Tetramethylethylendiamin	Carl Roth, Karlsruhe (Germany)
Pefablock sc	Fluka, Buchs (Switzerland)
Powdered Milk Blotting grade	Carl Roth, Karlsruhe (Germany)
Rifampicine	Sigma, Steinheim (Germany)
Mifepristone	Sigma, Steinheim (Germany)
Sodium Chloride	Baker, Griesheim (Germany)
Sodium Dodecyl Sulfate (SDS)	Sigma, Steinheim (Germany)
Sodium Hydroxide	Merck, Darmstadt (Germany)
tert-butylhydroquinone	Sigma, Steinheim (Germany)
TNF-α	Cell System Biotechnologie, St. Katharinen(Germany)
Tris Base	Sigma, Steinheim (Germany)
Tween 20	Sigma, Steinheim (Germany)
Z-Guggulsterone	Sigma, Steinheim (Germany)

4.2 Enzymes and related products

dNTP mix (PCR grade)	Promega, Mannheim (Germany)
DTT (0,1 M)	Promega, Mannheim (Germany)
Hot Star Taq Plus DNA Polymerase (5U/ µl)	Qiagen, Hilden (Germany)
Phire Hot Start DNA Polymerase	Biozym, Oldendorf (Germany)
RevertAid H minus M-MuLV Reverse Transcriptase (200U/ µl)	Fermentas, St. Leon-Rot (Germany)
Ribolock RNAse inhibitor	Fermentas, St. Leon-Rot (Germany)
RQ1 DNase Stop Solution	Promega, Mannheim (Germany)
RQ1 RNAse-free DNAse (1U/ µl)	Promega, Mannheim (Germany)
SuperScript II Reverse Transcriptase (200U/ µl)	Invitrogen, Karlsruhe (Germany)
Taq DNA Polymerase (5U/ µl)	PEQLAB, Erlangen (Germany)

4.3 Antibodies

CBR3 antibody (goat)	Abcam, Cambridge (UK)
Goat-secondary antibody HRP conjugated	Abcam, Cambridge (UK)

4.4 Kits

BCA Protein Assay Kit	Novagen, Darmstadt (Germany)
ECL advance Western Blotting detection kit	Amersham Biosciences, Freiburg (Germany)
ECL Western Blotting detection kit	Amersham Biosciences, Freiburg (Germany)

4.5 Markers and stains

6× Loading Dye Solution	Fermentas, St. Leon-Rot (Germany)
GelGreen Nucleic Acid Stain	Biotrend, Köln (Germany)
GelRed Nucleic Acid Stain	Biotrend, Köln (Germany)
Gene Ruler 100 bp DNA Ladder Plus	Fermentas, St. Leon-Rot (Germany)
Ponceau S (concentrate)	Sigma, Steinheim (Germany)
Protein Molecular Weight Marker	Fermentas, St. Leon-Rot (Germany)
Rainbow Marker	Amersham Biosciences, Freiburg (Germany)
SYBR Safe DNA Gel Stain	Invitrogen GmbH, Karlsruhe (Germany)

4.6 Apparatus

Blotter	Pegasus Semi-Dry-Blotter	Phase, Lübeck (Germany)
Gel documentation system	Image Master VDS + FUJIFILM	
	Imaging System FTI-500	Pharmacia Biotech, Freiburg (Germany)
	Gel IX Imager	Intas,Göttingen (Germany)
PCR-Thermocycler	T Gradient	Biometra, Göttingen (Germany)
Power Supply	Power Pack 35/60	Phase, Lübeck (Germany)
	Power Pack 300	BioRad, München (Germany)
Sonicator	Labsonic 1510	B. Braun, Melsungen (Germany)
Spectrophotometer	GeneQuant II	Pharmacia Biotech, Freiburg (Germany)
Ultracentrifuge	TGA 65(Rotor: TFT 70.38)	Kontron Analytik GmbH, (Switzerland)
Vacuum concentrator	SPD121P Speed Vac	Thermo, Engelsbach (Germany)

4.7 Expendable supplies

96-well microtest plates	Sarstedt, Nürnbrecht (Germany)
Biosphere MicroTube 1,5 ml, RNAse-free	Sarstedt, Nürnbrecht (Germany)
Eppendorfs	Sarstedt, Nürnbrecht (Germany)
Hyperfilm ECL (18x24cm)	Amersham Biosciences, Freiburg (Germany)
Multiply PCR cups 0,2 ml	Sarstedt, Nürnbrecht (Germany)
Multiply-µStrip of 8 tubes 0,2 ml	Sarstedt, Nürnbrecht (Germany)
Nitrocellulose Membran Hybond ECL	Amersham Biosciences, Freiburg (Germany)

4.8 Cell lines

Cell line	Cell type	Origin	Supplier	Received at passage number
A-549	human lung carcinoma	established from an explanted lung tumor which was removed from a 58-year-old Caucasian man in 1972	DSMZ	unknown
Caco-2	human colon adenocarcinoma	established from the primary colon tumor(adenocarcinoma) of a 72-year-old Caucasian man in 1974	DSMZ	unknown
HCT-116	human colon carcinoma	established from the primary colon carcinoma of an adult man	kindly provided by Prof. Dr. J. Abel, IUF, Düsseldorf	17
HT-29	human colon adenocarcinoma	established from the primary tumor of a 44-year-old Caucasian woman with colon adenocarcinoma in 1964	DSMZ	unknown
SW-480	human colon adenocarcinoma	established from the tumor of a 50-year-old Caucasian man with colon adenocarcinoma (grade 4, Duke class B)	DSMZ	unknown
TC-7	human colon adenocarcinoma	a subclone of the caco-2 cell line	kindly provided by Prof. P. Münzel, University of Tübingen	26

5 Methods

5.1 Cell culture

Cell culture experiments were kindly carried out by Dr. Bettina Ebert. Caco-2, HCT-116, SW-480, HT-29 and A-549 cells were cultured in Dulbecco's modified Eagle medium (DMEM, high glucose (4.5g/l), containing sodium pyruvate), with 2 mM L-glutamine, 1 % (v/v) non-essential amino acids and 10 %(v/v) heat-inactivated FCS, without antibiotics. Heat-inactivation of FCS was performed at 56 °C for 30 min. TC-7 cells were grown in medium supplemented 20 % (v/v) heat-inactivated FCS. The cells were incubated at 37 °C in an atmosphere of 5 % CO_2. The medium was changed every 2-3 days. After reaching confluence, the cells were subcultured via standard method using Trypsin-EDTA-solution (0.2 % Trypsin).

For experiments, cells were seeded on petri dishes (9.6 cm^2 or 78.5 cm^2 growth area) or six-well plates (9.3 cm^2 growth area). After reaching 80-95 % confluence, or 4-15 days after differentiation as was the case of Caco-2 cells, they were treated with test compounds freshly dissolved in culture medium. Control treated with culture medium containing 0.1 % dimethylsulfoxid (v/v) were included in each experiment. After incubation, the medium was removed, the cell monolayer was washed twice with icecold phosphate-buffered saline (PBS) and cells were harvested for RNA or protein isolation.

Since FCS contains hormones and growth factors, experiments with estradiol (E_2), TNFα, or GS requiredincubation of cells in serum free DMEM (overnight) and the experiments with these substances were performed under serum-free conditions as well. In addition, experiments with E_2 required not only serum free conditions, but also medium without phenol red, because phenol red possess estrogenic activity.

Composition of PBS buffer:	137.0 nM	NaCl
	2.7 nM	KCl
	8.8 nM	$Na_2HPO_4 \cdot 2\ H_2O$

5.2 Gene expression experiments based on semi-quantitative RT-PCR

The gene expression level of CBR3 was investigated by polymerase chain reaction (PCR) using complementary DNA (cDNA) as a template. Total RNA was isolated from cells after treatment and then, cDNA was synthesized by reverse transcription with oligo (dT)primer. This cDNA represents poly(A)$^+$ mRNA and after PCR amplification, the intensity of a band reflects the mRNA amount of the respective gene.

5.2.1 RNA isolation

The total RNA was isolated from cells using TRIzol Reagent. This is a phenol-based reagent which maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform separates the solution into an aqueous phase and an organic phase. The RNA is recovered from aqueous phase by precipitation with isopropyl alcohol.

Total RNA was isolated using TRIzol reagent according to the manufacturer's protocol. In detail, after removing the culture medium, the cell monolayers were washed twice with PBS and subsequently harvested by adding 1 ml of TRIzol reagent to each well. To reduce the viscosity and and to shear genomic DNA, the cell lysate was passed several times through a blue tip and subsequently stored at -80 °C until further analysis. RNA isolation was continued by thawing the samples and adding 0.2 ml of chloroform per 1 ml of TRIzol reagent. Tubes were shaken by hand for 15 seconds. The samples were centrifuged at 12,000 g for 15 minutes at 4 °C to separate phases. Then the aqueous phase was transferred to a fresh tube and RNA was precipitated from the aqueous phase by adding 0.5 ml of isopropyl alcohol per 1 ml of TRIzol reagent. The total RNA was collected by centrifugation at 12,000 g for 15 minutes at 4 °C. The pellets were washed by dislocating the pellet by vortexing with 0.7 ml of 75 % ethanol per 1 ml of TRIzol reagent used for the initial homogenization and the solution was centrifuged at 12,000 g for 10 minutes at 4 °C. All leftover ethanol was removed and washing procedure was repeated once. The RNA pellets were dried in vacuum dry for 4-8 min at room temperature and subsequently dissolved in 50-170 µl DEPC-treated water by gentle pipetting and incubated for 10 minutes at 55 to 60 °C. The concentrations of the RNA samples were determined by the use of UV spectrophotometry. The absorbance at 260 nm (OD 260) of diluted RNA (1:30 dilution) was measured. The concentration of the RNA in sample was calculated as follows:
RNA concentration (ng/µl) = (OD 260) × (dilution factor) × 40

Moreover, the ratio of the absorbance at 260 nm and 280 nm was measured to estimate the purity of a RNA sample. The A_{260}/A_{280} ratio should be ≥ 1.8 [115].

5.2.2 First strand cDNA synthesis

Isolated RNA was converted to cDNA with a reverse transcriptase, because DNA is much more stable. At first, the DNA contamination was removed from sample using DNAse I. The DNase I digestion reaction consisted of the appropriate amount of RNA sample containing 2 µg RNA, 2 µl DNase I, 2 µl 10 × Reaction Buffer and DEPC-water to a final volume of 20 µl (see table 5.1). After incubation at 37 °C for 30 minutes, 2 µl of Stop Solution were added to terminate the reaction. Subsequently incubation at 65 °C for 10 minutes inactivated the DNase I.

Table 5.1: DNase digestion reaction-mix

Component	Amount
RNA	2.0 - 4.0 µg
RNase-Free DNase I	2.0 µl
10× Reaction Buffer	2.0 µl
DEPC-water	ad 20.0 µl
Incubation 30 min at 37 °C	
DNase Stop Solution	2.0 µl
Total volume	22.0 µl

Reverse transcription of RNA was performed with Superscript II reverse transcriptase or the RevertAid H Minus M-MuLV Reverse Transcriptase. In both manners, the solution with 2 µg RNA after DNAse treatment was used (see table 5.1). To this solution were added other components (see table 5.2, 5.3). The mixture was incubated at 42 °C for 60 min. Then, the reaction was inactivated by heating at 70 °C for 15 min by the use of Superscript II reverse transcriptase or for 10 min. when RevertAid H Minus M-MuLV Reverse Transcriptase was used.

Table 5.2: SuperscriptII reverse transcriptase reaction-mix

Component	Volume µl
DNase digestion reaction-mix	22.0
oligo $(dT)_{15}$ (500 µg/ml)	2.0
dNTP mix (10 mM each)	2.0
0,1M DTT	2.0
5× First strand Buffer	7.0
Incubation 1 min. at 42 °C	
Superscript II RT (200U / µl)	1.0
Total volume	36.0

Table 5.3: RevertAid H Minus M-MuLV reverse transcriptase reaction mix

Component	Volume [µl]
DNase digestion reaction-mix	22.0
oligo $(dT)_{15}$ (500 µg/ml)	1.0
dNTP mix (10 mM each)	3.5
Ribolock RNAse inhibitor	0.5
5× Reaction Buffer	7.0
Incubation 1 min. at 42 °C	
RevertAid H Minus M-MLV RT (200U/ µl)	1.0
Total volume	35.0

5.2.3 PCR

PCR is a method used to amplify specific regions of a DNA strand. Hot-start PCR is a technique that provides high PCR specificity and often increases the yield of the specific PCR product. Other advantage is that all reaction components can be combined at room temperature [115]. Three different DNA Polymerases were used: Taq Polymerase (PEQLAB), HotStarTaq Plus DNA Polymerase (Qiagen) and Phire Hot Start DNA Polymerase (Finnzymes). QIAGEN Taq Polymerase is a recombinant 94 kDa DNA polymerase originally isolated from Thermus aquaticus, and expressed in E. coli. HotStarTaq Plus DNA Polymerase is in an inactive state with no polymerase activity at ambient temperatures. Phire Hot Start DNA Polymerase is a PCR enzyme with improved yields in shorter time. Cycling times for HotStarTaq Plus DNA Polymerase were optimalized for thermal cycler used. In detail, the lengths of the denaturation, annealing, and final extension were extended.

Master-Mix was prepared from appropriate amounts of double distilled water, Buffer (purchased together with polymerase), dNTPs (10 mM of each deoxyribonucleotide triphosphate), Primer-mix (forward and reverse primer, each 25 µM) and polymerase (see Table 5.10). When PEQLAB Taq Polymerase or HotStar Taq Plus was used, 3 µl of cDNA was added to 22 µl of Master-Mix (see Table 5.4, 5.6), and by using Phire Hotstart Polymerase 2 µl of cDNA to 18 µl of Master Mix (see Table 5.8). A negative control without template DNA was included in every experiment. Thermo cycler conditions for each DNA Polymerase are indicated in Table 5.5, 5.7, and 5.9. The number of cycles varied according to primer and cell line used (see Table 5.11). PCR products were examined on 1 % agarose gels.

Table 5.4: Reaction mixture for 1 PCR assay with Taq DNA Polymerase reaction-mix

Component	Volume[µl]
ddH_2O	17.6
10× Taq Reaction Buffer	2.5
dNTPs (10mM each)	0.5
Primer mix (12.5 µM each)	1.25
Taq DNA Polymerase (5U/ µl)	0.125
Master-mix	*22.0*
cDNA	3.0
Total volume	25.0

Table 5.5: Thermal cycler conditions for Taq DNA Polymerase

Step	Duration	Temperature
Initial denaturation	3 min	94 °C
Denaturation	45 sec	94 °C
Annealing	45 sec	56 °C
Extension	45 sec	72 °C
Final extension	10 min	72 °C
Pause		4 °C

Table 5.6: Reaction mixture for 1 PCR assay with Hot Star Taq Plus DNA Polymerase

Component	Volume[µl]
ddH_2O	17.9
10× CoralLoad PCR Buffer	2.5
dNTPs (10mM each)	0.5
Primer mix (12.5 µM each)	1.0
HotStarTaq Plus DNA Polymerase (5U/ µl)	0.125
Master-mix	*22.0*
cDNA	3.0
Total volume	25.0

Table 5.7: Thermal cycler conditions for Hot Star Taq Plus DNA Polymerase

Step	Duration	Temperature
Activation	5 min	95 °C
Denaturation	45 sec	94 °C
Annealing	45 sec	56 °C
Extension	45 sec	72 °C
Final extension	10 min	72 °C
Pause		4 °C

Table 5.8: Reaction mixture for 1 PCR assay with Phire Hot Start DNA Polymerase

Component	Volume[µl]
ddH_2O	12.4
5× Phire Reaction Buffer	4.0
dNTPs (10mM each)	0.4
Primer mix (12.5 µM each)	0.8
Phire Hot Start DNA Polymerase	0.4
Master-mix	*18.0*
cDNA	2.0
Total volume	20.0

Table 5.9: Thermal cycler conditions for Phire Hot Start DNA Polymerase

Step	Duration	Temperature
Activation	30 sec	98 °C
Denaturation	15 sec	98 °C
Annealing	15 sec	53-59 °C
Extension	20 sec	72 °C
Final extension	2 min	72 °C
Pause		4 °C

Table 5.10: Primers-sequence

Gene		Sequence (5'-3')	T_m [°C]
β actin	forward	ACTCTTCCAGCCTTCCTTCCT	59.8
	reverse	AGGTTTTGTCAAGAAAGGGTGT	56.5
CBR1	forward	GGACGTGCTGGTCAACAAC	58.8
	reverse	TCCTCTGCTCACTCAGTTTCCT	60.3
CBR3	fowrard	GCTCAACGTACTGGTCAACAAC	57.9
	reverse	ATCCTCGATAAGACCGTGACC	60.3
COX-2	forward	GAATGGGGTGATGAGCAGTT	57.3
	reverse	GGTCAATGGAAGCCTGTCAT	57.3
cyclophilin	forward	TTCCAGTCCCAGGAAGTGTC	59.4
	reverse	CTCACTGCAGACTGACCCAA	59.4
CYP1A1	forward	TGATTGAGCACTGTCAGGAGA	57.9
	reverse	GGTTGATCTGCCACTGGTTTA	57.9
CYP3A4	forward	CTAGCACATCATTTGGACTG	55.3
	reverse	ACAGAGCTTTGTGGGACT	53.7
GAPDH	forward	TGGAGGACTCATGACCACA	57.3
	reverse	TTCTAGACGGCAGGTCAGGT	59.4

Table 5.11: Primers-conditions for PCR

Gene	Product length	Polymerase	Annealing T [°C]	Cycles
β actin	394	Taq Polymerase	56	21
		HotStarTaq Plus Polymerase	56	18-19
		Phire Hot Start Polymerase	59	18-19
CBR1	398	Taq Polymerase	56	34
		Phire Hot Start Polymerase	59	30
CBR3	372	Taq Polymerase	56	34
		HotStarTaq Plus Polymerase	56	29-37
		Phire Hot Start Polymerase	59	28-34
COX-2	397	Taq Polymerase	56	34
		Phire Hot Start Polymerase	59	32-33
cyclophilin	368	Phire Hot Start Polymerase	59	25-26
CYP1A1	393	Taq Polymerase	56	34
		Phire Hot Start Polymerase	59	35
CYP3A4	325	Phire Hot Start Polymerase	53	39
GAPDH	238	Phire Hot Start Polymerase	59	23-26

5.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis is a method used to separate DNA, or RNA molecules by size. 1 % agarose gels were used. Agarose gels were prepared from Agarose NEEO Ultra Qualität, TAE-buffer and a stain for visualisation double-stranded DNA. Different nucleic acid stains were used, namely SYBR Safe DNA Gel Stain, GelRed Nucleic Acid Stain, or GelGreen according to manufactor's instructions. GeneRuler 100 bp Plus was used to check the size of the PCR products. Each sample was mixed before loading with 6 × Loading Dye Solution with the exception of the products of PCR with Hot Star Taq Plus DNA Polymerase. In this case, 10x CoralLoad PCR Buffer contains a red dye that eliminates the need for dye addition before loading DNA onto an agarose gel.

Composition of TAE buffer, pH 8.0:	40.0 mM	Tris
	40.0 mM	Acetic acid
	1.0 mM	0,5 M Na_2EDTA

5.3 Western blot analyses

Western blot is an analytical method allowed to detect specific proteins. It involves gel electrophoresis to separate proteins, and the immobilization of proteins on membranes, where they are detected using monoclonal or polyclonal antibodies.

5.3.1 Harvesting of cells and samples preparation

Samples were prepared from cell culture. For harvesting the cells, the cell monolayer was washed twice with ice-cold PBS, and cells were scraped into 500 µl ice-cold PBS supplemented with Pefabloc (500 µM). The whole cell lysate was stored at - 80 °C. The cells were homogenized by passing through a 20- and 26-gauge hypodermic needle and occasionally with ultrasonication. In some case, cell debris was removed by centrifugation (5 min/12000 g at 4 °C).

5.3.2 Determination of protein concentration

In order to load a known quantity of protein onto SDS gel, the protein concentration in samples were determined by the the bicinchoninic acid (BCA) method based on a biuret reaction.

The BCA method was performed in 96-well plate. Two replicate wells were loaded with 100 µl Elution-Buffer (PBS) (blank standard), standard bovine serum albumin solution or protein sample. To each well, 100 µl BCA working reagent was added and incubated for 15 min by 60 °C. The plate was cooled to room temperature and the absorbance was measured at 595 nm on a plate reader. The absorbance of the blank standard was subtracted from all others standard and protein samples. Using the standard curve, the amount of protein present in the original sample was calculated.

5.3.3 SDS polyacrylamide gel electrophoresis

Proteins were separated according to size with SDS polyacrylamide gel electrophoresis (SDS-PAGE). Samples to be run on SDS-PAGE were first boiled in sample buffer containing Sodium Dodecyl Sulfate (SDS). SDS is an anionic detergent and binds strongly to, and denatures, the proteins [116]. The samples were applied onto a stacking gel which is located on top of a separating gel.

Compounds for the separating gel (10 %) as described in Table 5.12 were brought together, transferred to the gel cassette, overlayed with ddH_20 and left to polymerize for 30-45 min. The overlaying water was poured off and the stacking gel (4 %) was prepared according to Table 5.13. After loading the stacking gel onto the separating gel by using a pasteur pipette, the well-forming comb was placed into this solution. It was left to polymerize for 30-45 min.

Composition of Separating gel buffer, pH 8.8:	1.5 mM	Tris
	0.4 % (w/v)	SDS
Composition of Stacking gel buffer, pH 6.8:	0.5 mM	Tris
	0.4 % (w/v)	SDS

Table 5.12: Composition of separating gel 10 %

Component	Volume
30 % Acrylamide solution	2.0 ml
Separating gel buffer	1.5 ml
ddH_2O	2.5 ml
TEMED	7.0 µl
APS	30.0 µl

Table 5.13: Composition of stacking gel 4 %

Component	Volume
30 % Acrylamide solution	0.5 ml
Stacking gel buffer	938.0 µl
ddH_2O	2.3 ml
TEMED	5.0 µl
APS	35.0 µl

Samples were diluted with Sample buffer to a final concentration 8 or 16 µg/µl and heated at 95 °C for 5 min. 80-320 µg of proteins were loaded into the wells. Recombinant CBR3 protein prepared with Novagen's pET-15b vector and provided by Y. El-Hawari was used as a positive control. Recombinant protein was loaded in amount of 5-10 ng. The molecular weigth of recombinant CBR3 (rCBR3) was higher than that of CBR3 from cells, because rCBR3 possesses a polyhystidine-tag. Gel was run on constant voltage, 60-80V in stacking gel and 100-120V in separating gel.

Composition of Sample buffer:	100 mM	TrisHCl
	4 % (w/v)	SDS
	20 % (v/v)	Glycerol
	0.2 % (w/v)	Bromophenolblue
	2 mM	DTT
Composition of SDS-Electrophoresis buffer:	25 mM	Tris
	192 mM	Glycine
	0.1 % (v/v)	SDS

5.3.4 Blotting

The proteins were transferred from the polyacrylamide gel onto a membrane with semi-dry transfer method. Nine sheets of Whatman filter paper, polyacrylamide gel and nitrocellulose membrane (Hybond ECL) were soaked in transfer buffer for several minutes. The sandwich of 3 sheets of wet whatman filter paper, polyacrylamide gel, membrane, and 6 sheets of whatman filter paper was placed on the cathode of the semi-dry blotting apparatus (see Figure 5.1). The transfer was run approximately at 0.8 mA/cm^2 for 45 min. The membrane was stained with 0.1% Ponceau red to assure equal loading and transfer. Then, the membrane was destained by washing in PBS-T.

Composition of Western-Blot (Transfer) buffer:	48 mM	Tris
	39 mM	Glycine
	20 % (v/v)	Methanol
	0.0375 % (w/v)	SDS
Composition of PBS-T buffer:	0.1 % (v/v)	Tween 20
	99.9 % (v/v)	PBS

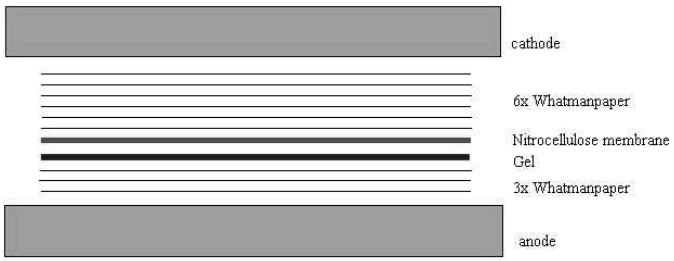


Figure 5.1: Blotting: semi-dry transfer method

5.3.5 Detection of protein

The proteins immobilized on membrane were detected by immunological procedure. Non-specific binding of antibodies was reduced by blocking the unoccupied membrane sites with 3 or 5 %(w/v) non-fat dry milk in PBS-T for 1 hour at room temperature on an orbital shaker. After blocking, the blot was incubated with antibodies. The primary antibody is specific for the protein of interest, and, at appropriate concentrations, should not bind any of the other proteins on the membrane. The secondary antibody binds to the primary antibody. The western blotting was performed with secondary antibody conjugated to horseradish peroxidase (HRP).

The primary antibody was anti-CBR3 anti-goat antibody diluted in a ratio 1:2,000 in 1-5 % (w/v) non-fat dry milk in PBS-T. The incubation was performed overnight at 4 °C. After the incubation, the membrane was washed three times briefly and four times for 15 min with PBS-T. Next, the membrane was incubated in the diluted anti-goat-HRP-conjugated secondary antibody for 1 hour at room temperature on an orbital shaker. The dilution was 1:4,000 in 1-5 % (w/v) non-fat dry milk in PBS-T. After the incubation, the membrane was washed three times briefly and four times for 15 min with PBS-T. The immune complexes were visualised using the ECL™ detection kit (ECL™ plus and ECL™ advance) following the manufacturer's instructions. The membrane was incubated for several minutes with mixed detection solutions and exposed to Hyperfilm ECL for 15 sec - 10 min.

6 Results

6.1 Constitutive expression of CBR3

6.1.1 Constitutive level of CBR3 mRNA in five colon carcinoma cell lines and one lung carcinoma cell line

In a recent study by Miura et al., CBR3 mRNA was detected in all tested tissues, such as lung, intestine, and colon. The level of CBR3 mRNA was clearly different in particular tissues and CBR3 was expressed at a lower level than that of CBR1 [82]. In order to select suitable cell lines for future experiments, the constitutive level of CBR3 mRNA was investigated in five colon carcinoma cell lines, namely Caco-2, HCT-116, HT-29, SW-480, and TC-7, and one lung carcinoma cell line A-549. TC-7 cell line is subclone of Caco-2 cells isolated from late passage [110]. Caco-2 cells is an established model for the human small intestine, since the cells differentiate spontaneously into polarized epithelial cell monolayers and show all functional (e.g., expression of brush border membrane enzymes) and morphological characteristics of human small intestinal enterocytes (e.g., tight junctions, microvilli) [76, 110].

The cell lines were cultured under standard conditions. Cell lines A-549, Caco-2, HCT-116, and HT-29 were harvested after reaching 90-100 % confluence. TC-7 cells were harvested 14 days after reaching confluent state. CBR3, CBR1, and β-actin (as a housekeeping gene) mRNA levels were determined by RT-PCR. Because the level of β-actin differed between the cell lines, a second commonly used housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used to confirm equal loading and RNA integrity. The highest CBR3 mRNA levels were detected in A-549 cells and SW-480 cells, whereas HCT-116 and TC-7 cells showed lower levels of CBR3 mRNA. The lowest amount of CBR3 mRNA was detected in Caco-2 and HT-29 cells (see Figure 6.1).

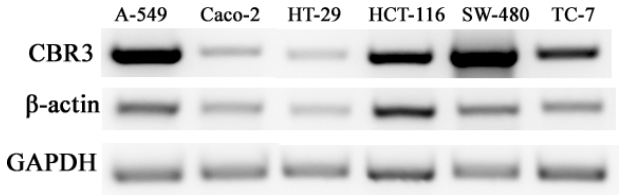


Figure 6.1: Constitutive level of CBR3 in different cell lines

By contrast, the amount of CBR1 mRNA was saturated in all cell lines, except HCT-116 with no detected CBR1 mRNA, whereas CBR3 gene appeared to be saturated just in samples from SW-480 and A-549 cells. The PCR was performed simultaneously with the same cycle number for both CBR3 and CBR1 assay. Based on these identical conditions, CBR3 was expressed at a lower level than that of CBR1 in Caco-2, HT-29 and TC-7 cells. The level of CBR3 and CBR1 mRNA was almost the same in A-549 cells and SW-480 cells (see Figure 6.2).

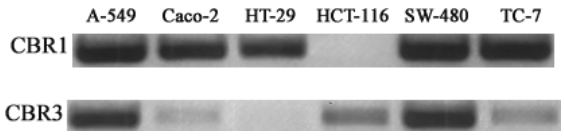


Figure 6.2: Constitutive level of CBR1 and CBR3 in different cell lines

6.1.2 CBR3 expression in Caco-2 cells cultured for various times

Caco-2 cell undergo in culture a process of spontaneous differentiation which starts as soon as the cells achieve confluence [76]. Some properties of the Caco-2 cells have been reported to change as the differentiation proceeds,e.g. up-regulation of genes involved in xenobiotic and drug metabolism [110]. To determine if changes in mRNA expression of CBR3 may occur in Caco-2 cells with progression in differentiation, the cells were cultured for different times and CBR3 mRNA level were determined.

The CBR3 mRNA level showed to be lower in subconfluent and confluent cells than in cells cultured for 4, 8, 12, or 15 days after reaching confluence. The level of CBR3 mRNA was detected lower in cells grown for 8 days after confluence but level of β-actin mRNA was also lower. After normalization to level of housekeeping gene, the expression of CBR3 mRNA seems to be stable in cells cultured for 4-15 days after reaching confluence (see Figure 6.3).

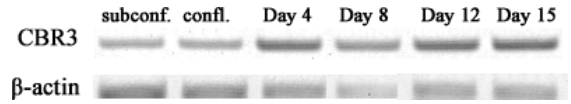


Figure 6.3: Level of CBR3 in Caco-2 cells, various age

6.2 Effect of TNF-α on expression of CBR3 mRNA in human colon carcinoma cell lines

Data from microarray analysis provided to the public on Gene Expression Omnibus (GEO) clearly indicate that CBR3 mRNA expression may be regulated by inflammatory processes in vitro (HUVEC and HMEC) and in vivo (synovial tissues from patients with rheumatoid arthritis) [90–92, 129]. The treatment of HUVEC and HMEC with TNF-α (2 ng/ml, 5 h) led to substantial increase in CBR3 mRNA expression [129]. TNF-α, an important pro-inflammatory cytokine, is a rapid and potent activator of NF-κB [16]. In order to investigate effect of TNF-α on CBR3 in colon carcinoma cell lines, the level of CBR3 mRNA was determined in cells after treatment with TNF-α.

6.2.1 Effect of TNF-α on CBR3 mRNA expression in Caco-2 cells

Caco-2 cells are known to be responsive to TNF-α mediated activation of NF-κB, and therefore, they are a suitable model for this experiment [52, 61, 128]. COX-2 mRNA level was used as a control gene to confirm the activation of NF-κB by TNF-α.

Caco-2 cells were incubated at age 3 or 7 days after reaching confluence with TNF-α (50 ng/ml) for 8 hours. TNF-α did not obviously affect level of CBR3 mRNA, although the level of COX-2 mRNA was slightly increased, indicating the activation of NF-κB-signaling by TNF-α under the applied experimental conditions (see Figure 6.4).

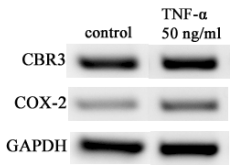


Figure 6.4: Caco-2 cells, incubation for 8h

6.2.2 Effect of TNF-α on CBR3 mRNA expression in HT-29 cells

HT-29 cells are also known to be responsive to TNF-α-mediated activation of NF-κB [52, 61, 128].

After reaching 90-100 % confluence, HT-29 cells were incubated with TNF-α (50 ng/ml) for 8 hours. In contrast to Caco-2 cells, the incubation of HT-29 cells with TNF-α led to a substantial increase in CBR3 mRNA level (see Figure 6.5).

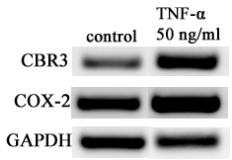


Figure 6.5: HT-29 cells, incubation for 8h

6.3 Effect of Z-guggulsterone on expression of CBR3 mRNA in Caco-2 cells

As mentioned above, the pro-inflammatory cytokine TNF-α induced CBR3 expression in HMEC and HUVEC [129]. While TNF-α-treatment was able to elevate the CBR3 mRNA level in HT-29 cells, no effect on CBR3 transcription could be observed in Caco-2 cells. In order to elucidate the relationship of CBR3 level and inflammatory state, the effect of Z-guggulsterone (Z-GS) on CBR3 mRNA level was tested. GS is a plant sterol used to treat a variety of disorders in human, including inflammatory diseases [10, 11]. The anti-inflammatory action of GS in intestinal epithelial cells was shown to be mediated by inhibiting NF-κB signaling [15]. In addition, GS acts as antagonist of GR, AR and MR and activates PR, PXR and ERα [10, 11].

It has been described that GS inhibits NF-κB by blocking IKK activity in Caco-2 cells [15]. Therefore, Caco-2 cells are a suitable model to study the effect of GS mediated via NF-κB on transcription CBR3.

Caco-2 cells at age 7 days after reaching confluence were treated for 8 hours with Z-GS (25 µM) and/or TNF-α (50 ng/ml). The induction of CBR3 transcription was detected in cells treated with Z-GS. CBR3 mRNA level was also induced in cells treated simultaneously with Z-GS and TNF-α. TNF-α alone did not affect CBR3 mRNA level (see Figure 6.6).

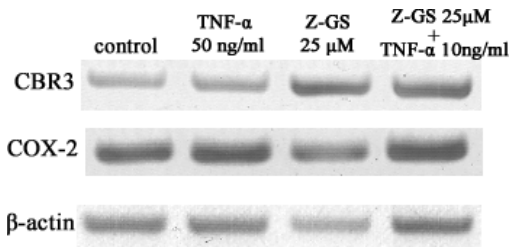


Figure 6.6: Caco-2 cells, incubation for 8h

6.4 Effect of substances affecting the same transcriptional pathways as GS

In the present study, increase in CBR3 mRNA level was observed in Caco-2 cells treated with Z-GS. From experiments using reporter gene assays it is known that GS is able to interact with several nuclear receptors, acting as agonist in some cases (PR, PXR and ERα) and as antagonist in others (GR, AR and MR) [10, 11]. In order to elucidate which nuclear factor may be involved in the detected induction of CBR3 mRNA, the effect of various substances on CBR3 transcription was tested. Tested substances were prototypical ligands of several nuclear factors, which GS could affect.

6.4.1 Effect of PR- and GR-antagonist mifepristone on CBR3 mRNA expression in Caco-2 cells

Mifepristone (RU 486) is known to be able to block PR-activity of guggulsterone [11]. In Caco-2 cells, expression of PR was detected [64].

Caco-2 cells were incubated for 8 hours with RU 486 (10 µM) or/and Z-GS (25 µM). No change in CBR3 transcription was observed in cells treated with RU 486. Co-treatment with RU 486 did not reverse the increased CBR3 mRNA level mediated by Z-GS (see Figure 6.7).

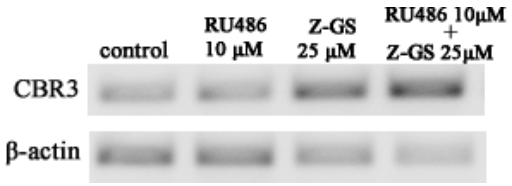


Figure 6.7: Caco-2 cells, incubation for 8h

6.4.2 Effect of GR- and PXR-agonist dexamethasone on CBR3 mRNA expression in Caco-2 cells

Caco-2 cells are known to express GR and to be sensitive to dexamethasone (DEX) mediated activation of GR-target gene [119]. Based on this, Caco-2 cells were used to investigate the effect of GR-activation on CBR3 mRNA level.

The incubation of Caco-2 cells on day 4 after reaching confluence with DEX (20 µM) was performed for 8 hours. DEX did not alter CBR3 transcription (see Figure 6.8).

6.4.3 Effect of PXR-agonist rifampicine on CBR3 mRNA expression in Caco-2 cells

PXR expression and response to rifampicine (RIF) in Caco-2 cells is well established [32]. Moreover, CYP3A4 level was used in present study as a positive control, since CYP3A4 is a PXR-target gene [38].

Caco-2 cells were incubated 4 days after reaching confluence with RIF (20 µM) for 8 hours. No change in CBR3 transcription was detected (see Figure 6.8).

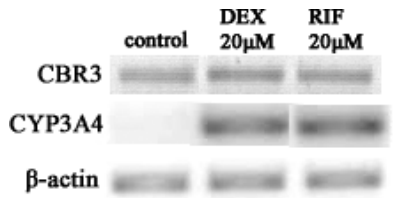


Figure 6.8: Caco-2 cells, incubation for 8h

6.4.4 Effect of ER-agonist estradiol on CBR3 mRNA expression in Caco-2 cells

Estradiol (E_2) binds to the estrogen receptor (ER) to activate gene expression or repression of target genes [109]. Caco-2 cells are known to express ERα and ERβ [36, 64].

Cells were cultured with 10 nM E_2 for 8 hours three days after reaching confluence. A moderately lower level of CBR3 mRNA was detected than in untreated cells (see Figure 6.9). GAPDH, or cyclophilin mRNA level was used as a reference gene, because E_2 may affect expression of β-actin.

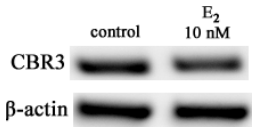


Figure 6.9: Caco-2 cells, incubation for 8h

6.4.5 Effect of ER-agonist estradiol on CBR3 mRNA expression in HT-29 cells

Colon cell line HT-29 express ERα and ERβ [36]. In contrast to Caco-2 cells, HT-29 cells represent cell line established from colon adenocarcinoma of a woman.

HT-29 cells were treated with E_2 (10 nM) for 8 hours. A slight increase of CBR3 mRNA could be observed (see Figure 6.10). GAPDH or cyclophilin mRNA level was used as a reference gene.

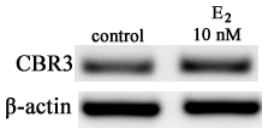


Figure 6.10: HT-29 cells, incubation for 8h

6.5 Regulation of CBR3 via AhR

A recent study by Lakhman et al. has shown that AhR pathway contributes to the transcriptional regulation of CBR1 [59]. Since CBR1 and CBR3 share 72 % identity in amino acid level, the regulation of CBR3 via AhR was investigated.

6.5.1 Effect of benzo[*k*]fluoranthene on CBR3 mRNA expression in Caco-2 cells

AhR agonists have been previously reported to induce the expression of AhR target genes in Caco-2 cells [83]. CYP1A1 was used as a positive control, since it is an established AhR target gene [124].

Caco-2 cells after reaching 70% confluence were incubated for 48 hours with AhR agonist benzo[*k*]fluoranthene (B[*k*]F) (5 µM). The results showed a distinct elevation of CBR3 mRNA expression. As expected, CYP1A1 mRNA levels were increased as well (see Figure 6.11).

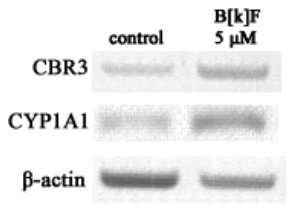


Figure 6.11: Caco-2 cells, incubation for 48h

6.5.2 Effect of B[*k*]F on CBR3 mRNA expression in HT-29 cells

HT-29 cells are known to be responsive to both AhR-activators [58].

Cells were incubated for 48 hours with B[*k*]F (5 µM). A pronounced increase in CBR3 mRNA level was detected. CYP1A1, control gene for AhR activation, was also induced in treated cells (see Figure 6.12).

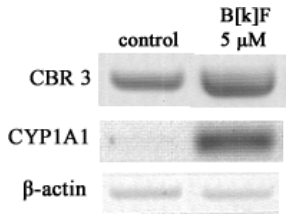


Figure 6.12: HT-29 cells, incubation for 48h

6.5.3 Effect of B[*k*]F and *tert*-butylhydroquinone (TBHQ) on CBR3 mRNA expression in HCT-116 cells

B[*k*]F can affect not only AhR- but also Nrf2-pathway, especially after longer exposure [37]. Hence, the incubation was performed for shorter time. Moreover, the effect of the monofunctional inducer of Nrf2-pathway, namely TBHQ, was tested in this experiment. HCT-116 cells were detected to be sensitive to both AhR- and Nrf2-mediated signal transduction [7, 65].

Cells were treated with B[*k*]F (5 µM or 10 µM) for 8 and 24 hours, or TBHQ (50 µM) for 8 hours. The level of CYP1A1 mRNA was determined as a positive control for AhR-driven transcription. Whereas B[*k*]F induced CBR3 mRNA level only after 24-hour treatment, the CYP1A1 level was considerably increased after both 8 and 24 hours. B[*k*]F induced CBR3 as well as CYP1A1 after 24 hours in a concentration-dependent manner. TBHQ induced CBR3 transcription after 8 hour but did not affect the level of CYP1A1 mRNA (see Figure 6.13).

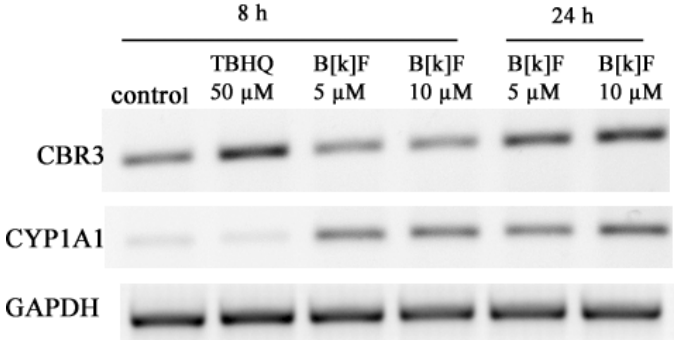


Figure 6.13: HCT-116 cells, incubation for 8 or 24h

6.6 Regulation of CBR3 via Nrf2

Experiments with B[*k*]F and TBHQ in colon carcinoma cell lines suggest that induction of CBR3 mRNA was mediated via Nrf2 pathway rather than AhR. To confirm this hypothesis, the effect of specific Nrf2-ligands, namely TBHQ, D,L-sulforaphane (SUL) and diethyl maleate (DEM), or AhR ligand, namely dibenzoyl methane (DBM), on CBR3 transcription was investigated (see Figure 6.14).

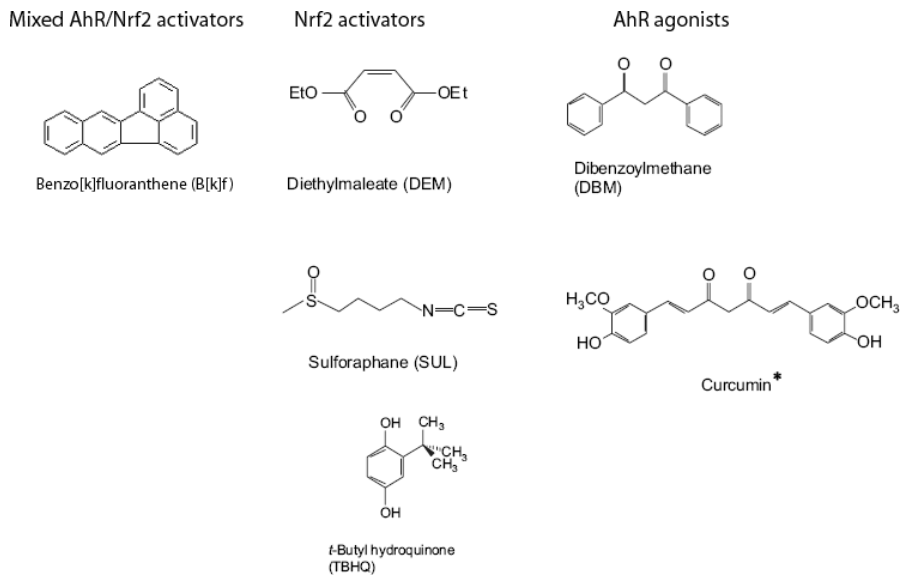


Figure 6.14: AhR- and Nrf2 activators as described in the literature [17, 37],*=curcumin has been described as AhR ligand with both agonistic and antagonistic activity [97, 107] and also Nrf2-activator [33]

6.6.1 Effect of AhR- and/or Nrf2-activators on CBR3 mRNA expression in HT-29 cells

HT-29 cells are known to be responsive to both AhR- and Nrf2-activators [58, 66].

HT-29 cells were incubated for 4 and 8 hours with either TBHQ (50 µM), SUL (20 µM), DEM (100 µM), or DBM (50 µM). The level of β-actin was inconsistent. Other reference genes, namely cyclophilin and GAPDH, were used but the signal of bands was also obviously distinct. However, after normalization to any of reference gene tested, the considerable induction in CBR3 mRNA was detected. mRNA was induced dramatically in both times upon exposure

to Nrf2 activators; these were DEM, SUL, and TBHQ. In contrast, specific AhR activator DBM did not affect its expression (see Figure 6.15).

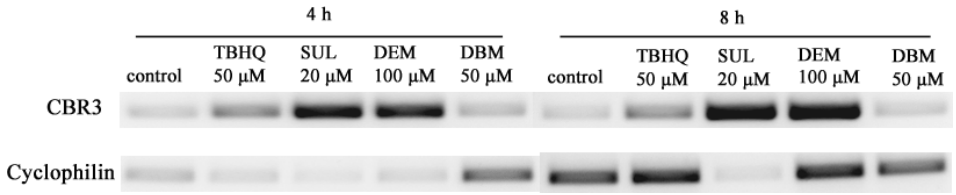


Figure 6.15: HT-29 cells, incubation for 4 or 8h

6.6.2 Effect of AhR- and/or Nrf2-activators on CBR3 mRNA expression in HCT-116 cells

HCT-116 cells were treated for 8 hours with TBHQ (50 or 100 µM), SUL (10 or 20 µM), DEM (100 or 200 µM), DBM (50 µM) and curcumin (50 µM). Curcumin has been described as AhR ligand with both agonistic and antagonistic activity [97, 107] and Nrf2-activator [33]. While TBHQ, DEM and SUL induced substantially CBR3 mRNA level, DBM and curcumin did not affect CBR3 transcription (see Figure 6.16).

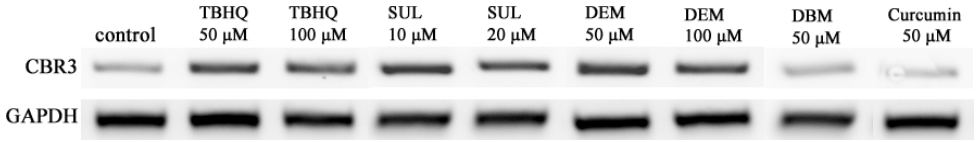


Figure 6.16: HCT-116 cells, incubation for 8h

6.6.3 Effect of Nrf2-activator on CBR3 mRNA expression in SW-480 cells

SW-480 cells are known to be responsive to induction of Nrf2-target genes after exposure to Nrf2-activators [137].

In this experiment, the proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-leucinal (MG-132) was used with purpose to block degradation of Nrf2, thus to cause higher induction via Nrf2

pathway [62]. Cells were incubated with DEM (50, 100, or 200 µM), or simultaneously with DEM (100 µM) and MG-132 (20 µM). The incubation was performed for 6 hours. The house-keeping gene β-actin did not show consistent transcription. When the values were normalized relative to the expression of β-actin, the CBR3 mRNA level was slightly induced upon exposure to DEM at concentration 100 µM(see Figure 6.17).

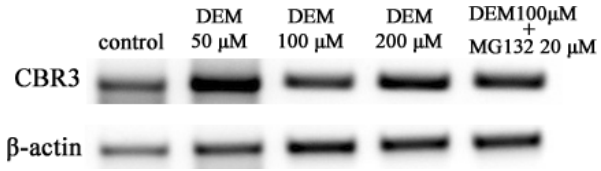


Figure 6.17: SW-480 cells, incubation for 6h

6.6.4 Effect of AhR- and/or Nrf2-activators on CBR3 mRNA expression in A-549 cells

Lung adenocarcinoma cell line A-549 is known to be sensitive to induction of both AhR- and Nrf2-target genes [19, 125].

The incubation of A-549 cells was performed with TBHQ (50 µM) alone or in combination with MG-132 (20 µM), SUL (10 µM) alone or with MG-132 (20 µM), and DEM (100 µM) for 6 hours. A slight induction of CBR3 mRNA was detected in cells treated with combination TBHQ and MG-132. In cells treated with a combination of SUL and MG-132, the level of CBR3 mRNA was slightly decreased compared to untreated cells. The CBR3 mRNA level in cells incubated with DEM was lower than in control cells without treatment. However, the level of reference gene, namely β-actin was also lower in this sample (see Figure 6.18).

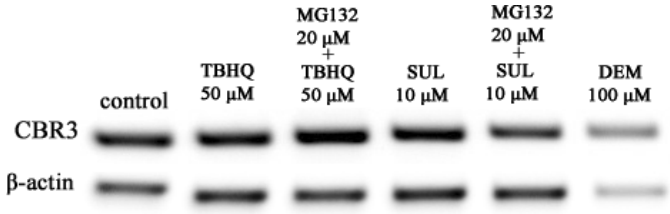


Figure 6.18: A-549 cells, incubation for 6h

6.7 Detection of CBR3 protein in colon cell lines and lung cell line

In order to investigate the induction of CBR3 on protein level, the western blot analysis was carried out. Samples from Caco-2, HCT-116, HT-29, TC-7, and A-549 cells, either untreated or treated with compounds previously observed to induce CBR3 mRNA level, were used. Two bands that may correspond to CBR3 were detected in untreated A-549 cells (see Figure 6.19). In samples from other cells, numerous, or in contrast no bands was observed on exposed film (see Figure 6.20). Different conditions for blotting, blocking, and incubation was tested. Either whole cell lysate or cytosol was used and 80 to 320 µg of proteins were loaded. Recombinant CBR3 protein used as a positive could be detect in an amountas low as 5 ng, confirming that both antibodies were working properly. Moreover, a detection of other protein performed simultaneously was successful.

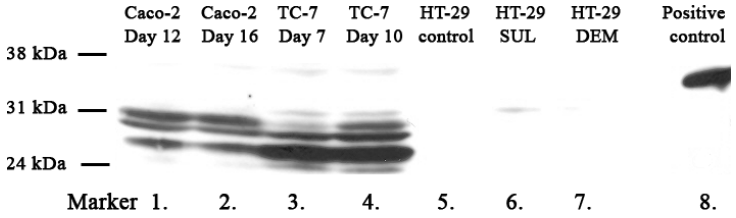


Figure 6.19: Western blotting:1. Caco-2 day 12 after confluence, 2. Caco-2 day 16 after confluence, 3. TC-7 day 7 after confluence, 4. TC-7 day 10 after confluence, 5. HT-29 control, 6. HT-29 SUL 20 µMfor 4 hours, 7. HT-29 DEM 100 µM for 4 hours, 8. recombinant CBR3. It was loaded 80 µg protein into well (samples 1.-4.), 160 µg(samples 5.-7.), or 6 ng (sample 8).

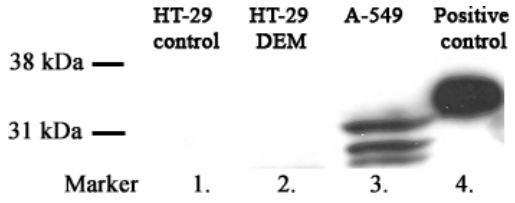


Figure 6.20: Western blotting:1. HT-29 control, 2. HT-29 DEM 100 µM for 24 hours, 3. A-549 confluent, 4. recombinant CBR3. It was loaded 200 µg protein into well (sample 1.-3.), or 5 ng (sample 4.)

7 Discussion

Carbonyl reductases play an important role in xenobiotic metabolism. Human monomeric carbonyl reductase CBR3 is poorly characterized since its discovery in 1998. Substrates and biological function of CBR3 are unknown as well as regulation of its expression. Despite the high similarity of CBR3 with CBR1 in amino acid level, both isoforms seem to play distinct roles. CBR3 has no or very low catalytic activity towards substrates of CBR1. Moreover, the expression patterns of the genes for CBR1 and CBR3 are different and CBR3 mRNA levels in tissues were found to be lower than CBR1 mRNA [82].

In the present study, the constitutive level of CBR3 mRNA was determined in five colon carcinoma cell lines and one lung carcinoma cell line. The level of the reference gene β-actin was very inconsistent although the same amount of cDNA from each cell line was used for PCR and the experiment has been repeated. The reason for this inconsistent level could be unequal transcription of β-actin in particular cell lines. Hence, β-actin was not suitable as housekeeping gene for comparison of CBR3 mRNA level between various cell lines. GAPDH was used as a normalizer instead. HCT-116 and TC-7 cells seem to express slightly higher level of GAPDH. This is in line with higher cell metabolism reflected by higher glucose consumption and the need for more frequent medium changes in comparison to all other cell lines. However, the level of CBR3 mRNA was clearly distinct in all cell lines tested. A particularly high level was detected in the lung cell line A-549. This is in agreement with the reported higher expression of CBR3 in lung than colon tissue [82]. Interestingly, the approximately same high level was also detected in colon carcinoma cell line SW-480. HCT-116 and TC-7 cells express lower levels of CBR3 mRNA, whereas Caco-2 and HT-29 cells have the lowest constitutive level of CBR3 from all cell lines tested. For the reason, Caco-2 and HT-29 cells were chosen as suitable model cell lines for the investigation of the regulation of CBR3. Especially HT-29 cells turned out to be the best model for this purpose because a low constitutive expression of CBR3 implicates a higher inducible expression in this cell line.

Caco-2 cells undergo in culture a process of spontaneous differentiation which starts as soon as the cells achieve confluence [76]. Differentiation of cells is a dynamic process that is accompanied by a series of changes in gene expression [88]. To investigate, whether a change in CBR3

mRNA may occur throughout differentiation, the level of CBR3 in Caco-2 cells grown for various times was determined. Transcription of CBR3 was higher in cells after reaching confluence than in cells in subconfluent and 100 % confluent state. The mRNA level of CBR3 was stable in Caco-2 cells in age between 4 and 15 day after confluence. This finding indicates that CBR3 may be of some importance in mature Caco-2 cells.

The induction of CBR3 expression in HMEC and HUVEC stimulated with TNF-α and in synovial tissues from patients with rheumatoid arthritis has been previously reported [90–92, 129]. Hence, the effect of TNF-α was investigated in colon cell lines. The induction of CBR3 mRNA was detected in HT-29 cells upon exposure to TNF-α but not in Caco-2 cells. Obviously, HT-29 cells are more responsive to effects mediated by TNF-α, or NFκB respectively. This effect could be based on differences in the expression of TNF-α receptors and/or components of the NFκB signaling cascade. In addition, a prototypical NFκB-target gene, COX-2 was induced in HT-29 cells, whereas not as high in Caco-2 cells.

GS, a plant sterol with anti-inflammatory properties, was tested in order to investigate the effect of substance with contradictory activity on NFκB than TNF-α. Z-GS induced CBR3 mRNA level in Caco-2 cells and this induction was not affected upon co-treatment with TNF-α. These findings suggest that effect of Z-GS was not mediated via NFκB pathway. Since GS was described as antagonist of MR, GR, and AR, and agonist of PR, PXR and ER-α [11], the prototypical ligands of some of these transcriptional factors were tested.

PR-antagonist RU 486 alone did not have an effect on CBR3 transcription. Moreover, RU 486 could not reverse the inducing effect of Z-GS. Considering that GS also act as GR-antagonist, the GR-antagonistic activity of RU 486 could counteract its possible PR-mediated effect on CBR3. PXR agonists RIF and DEX did not affect CBR3 mRNA level. DEX also acts as activator of GR-target genes. The findings suggest that neither PR- nor PXR- nor GR-ligands affect CBR3 transcription. Next, E_2 also failed to affect CBR3 transcription. However, not all possible transcriptional pathways were examined. Moreover, the CBR3 induction in Caco-2 cells upon exposure to Z-GS may be due to alteration activity of various transcription factors simultaneously. In summary, the mechanism by which Z-GS induces CBR3 mRNA transcription could not be elucidated with the methods used in the present study.

The regulation of CBR1 is mediated by AhR-pathway [59]. Since the high similarity between CBR1 and CBR3, the effect of AhR activator B[*k*]F on CBR3 transcription was tested. The CBR3 mRNA level was increased after treatment with 5 µM B[*k*]F for 48 hours. B[*k*]F was described as bifunctional inducer [37]. Thus, B[*k*]F can be metabolized by AhR-target gene CYP1A1 and its metabolites activate Nrf2-pathway. In addition, its metabolization leads to the

generation of ROS, which also activates Nrf2-target genes. Since the incubation with B[*k*]F for 48 hours caused induction of CYP1A1 in tested cell lines, the possible involvement of the Nrf2-pathway was supposed. Next, HCT-116 cells were incubated with B[*k*]F (5 μM and 10 μM) for 8 and 24 h. The CBR3 mRNA level was induced only after 24 hour-treatment with B[*k*]F. This indicates that the effect of B[*k*]F on CBR3 expression is mediated by its metabolites and/or ROS generated by the action of CYP1A1 and obviously not by the unaltered compound itself. Moreover, Nrf2-activator TBHQ induced CBR3 transcription, and, in agreement with its monofunctional activity, did not affect mRNA level of AhR-target gene CYP1A1. Other tested Nrf2-activators, namely DEM and SUL, induced also CBR3 transcription, whereas AhR-agonist DBM did not change the level of CBR3 mRNA in HCT-116 cells. No effect could be observed with AhR-ligand and Nrf2-activator curcumin, probably due to its ability to affect various signaling pathways [47].

The induction of CBR3 by Nrf2-activators was not observed on the same level in all tested cell lines. HT-29 cells was the most responsive to induction by Nrf2-activators, such as TBHQ, DEM and SUL. A clear but lower induction of CBR3 mRNA by these compounds was also observed in HCT-116 cells. In the other cell lines tested, SW-480, the level of CBR3 was induced after incubation with DEM with the lower concentration (50 μM) turned out to be the most effective. Observed induction of CBR3 level in lungs of cigarette smokers [12, 39, 93, 94] indicate that CBR3 can be induced in lungs due to oxidative stress. Therefore, Nrf2-ARE pathway could be involved in the mechanism of induction. However, the Nrf2-activator tested did not alter level of CBR3 mRNA in lung cell line A-549. Together, cell lines A-549, HCT-116 and SW-480 seem not to be able to increase the expression of CBR3 as HT-29 cells do. This difference can be due to their high constitutive level of CBR3 mRNA in comparison with low detected level in HT-29 cells. Further, the CBR3 induction could depend upon the cellular context. Therefore, HT-29 cells could be used as suitable model for future investigations of CBR3 regulation.

To determine whether the observed induction of CBR3 mRNA expression were translated into increased levels of protein, the western blotting was performed. However, no results were obtained, because CBR3 protein was not specifically detected and optimisation of the method was not successful. Only in A-549 cells, two strong bands were detected, that may correspond to CBR3 protein. Based on the position of rCBR3, the band of CBR3 is probably the higher one. The appearance of two bands could be due to the degradation of protein, but a protease inhibitor always was used and samples were kept on ice. Since the positive control had a signal also in small amount loaded, CBR3 seems to be expressed on very low level in cell lines tested. In the short time available for experiments, the optimisation of conditions was not successful. It can be suggested that loading of a higher amount of proteins could led to better result. Another

possibility would be to increase the concentration of CBR3 protein in the samples by using immunoprecipitation.

In conclusion, the present study demonstrates that the mRNA level of CBR3 is obviously increased in HT-29 and HCT-116 cells after exposure to Nrf2 activators, whereas AhR-ligands have no effect after short incubation times (8h). Moreover, TNF-α induced CBR3 mRNA level in HT-29 cells. TNF-α is known to stimulate ROS production [67, 87] and in addition, the up-regulation of NF-κB-target genes led to increase in ROS level [41]. Since ROS are able to activate Nrf2 [57], the induction of CBR3 mRNA observed in cells after exposure to TNF-α could be mediated via Nrf2 pathway. Further, GS was shown to induce CBR3 mRNA level in Caco-2 cells, but the underlying mechanism could not be elucidated. The induction of CBR3 via Nrf2-pathway observed in this study is in agreement with the detected increased level of CBR3 mRNA in lungs from cigarette smokers [12, 39], in synovial tissues from patients with rheumatoid arthritis [92], and in retinal pigment epithelial cells exposed to oxidatively modified LDL [136]. In all these states, the Nrf2-target genes can be induced [57, 87]. Together, several lines of evidence suggest the regulation of CBR3 expression via Nrf2-ARE pathway. To confirm this hypothesis, future studies are necessary. They may be performed with HT-29 cells, since in the present study, it was identified to be the most responsive cell line from all cell lines tested. In order to confirm that Nrf2 is involved in the transcriptional regulation of CBR3, the promotor analysis and reporter gene assay could be used. In addition, the induction of CBR3 on protein level needs to be investigated.

Abstract

The enzymatic reactions carried out by enzymes from the short-chain dehydrogenase/reductase (SDR) superfamily can be grouped mainly as NAD(P)(H)-dependent oxidoreductions acting on a highly diverse set of substrates. The SDR superfamily has several members capable mediating carbonyl reduction and therefore affecting endogenous processes of both xenobiotic and endogenous ligands. Two monomeric carbonyl reductases, namely carbonyl reductase 1 (CBR1) and carbonyl reductase 3 (CBR3), have been found in humans. Whereas substrates and gene regulation of CBR1 have been described, CBR3 is poorly characterized. Despite the high similarity of CBR3 with CBR1 in amino acid level, both isoforms seem to play distinct roles. In the present study, the five colon carcinoma cell lines (Caco-2, HCT-116, SW-480, HT-29 and TC-7) and one lung carcinoma cell line (A-549) were used to investigated the regulation of CBR3. The constitutive level of CBR3 mRNA was clearly distinct in all cell lines tested. HT-29 cells turned out to be the best model for investigations of CBR3 regulation from all cell lines used. Several lines of evidence suggest the regulation of CBR3 expression via Nrf2-antioxidant response element (ARE) pathway. Next, TNF-α induced the CBR3 mRNA level in HT-29 cells. Further, natural product guggulsterone was shown to induce CBR3 mRNA level in Caco-2 cells, but the underlying mechanism of effct of this promiscuous steroid receptor ligand could not be elucidated.

Abstrakt

Reakce katalyzované enzymy z nadrodiny short-chain dehydrogenasy/reduktasy (SDR) zahrnují převážně NAD(P)(H) dependentní oxidoredukční reakce probíhající se strukturně rozmanitými substráty. SDR nadrodina obsahuje několik členů, kteří zprostředkovávají redukci karbonylových sloučenin a tím ovlivňují endogenní procesy ligandů ze skupiny jak xenobiotik, tak endogenních látek. U člověka byly nalezeny dvě monomerní karbonyl reduktasy, konkrétně karbonyl reduktasa 1 (CBR1) a karbonyl reduktasa 3 (CBR3). Zatímco substráty i genetická regulace enzymu CBR1 byly popsány, CBR3 je jen velmi málo charakterizována. Navzdory velké podobnosti enzymů CBR1 a CBR3 na úrovni aminokyselin se zdá, že obě isoformy mají rozdílné funkce. V této práci byla zkoumána regulace CBR3 s použitím pěti buněčných linií odvozených z karcinomu kolonu (Caco-2, HCT-116, SW-480, HT-29 a TC-7) a jedna buněčná linie z karcinomu plic (A-549). Konstitutivní hladina CBR3 mRNA se v jednotlivých testovaných buněčných liniích výrazně lišila. Ze všech testovaných buněčných linií se jako nejvhodnější pro studium regulace CBR3 ukázaly buňky HT-29. Výsledky naznačují, že exprese CBR3 je regulována prostřednictvím dráhy Nrf2-antioxidant response element (ARE). Dále bylo zjištěno, že TNF-α indukuje CBR3 mRNA v buňkách HT-29. Navíc, guggulsteron zvyšoval v Caco-2 buňkách hladinu CBR3 mRNA, ale mechanismus působení teto přírodní látky ovlivňující vícero steroidních transkripčních receptorů se nepodařilo odhalit.

Bibliography

[1] P. Alberts, L. Engblom, N. Edling, M. Forsgren, G. Klingström, C. Larsson, Y. Rönquist-Nii, B. Ohman, and L. Abrahmsén. Selective inhibition of 11beta-hydroxysteroid dehydrogenase type 1 decreases blood glucose concentrations in hyperglycaemic mice. *Diabetologia*, 45(11):1528–1532, Nov 2002.

[2] W. Y. Almawi and O. K. Melemedjian. Negative regulation of nuclear factor-kappab activation and function by glucocorticoids. *J Mol Endocrinol*, 28(2):69–78, Apr 2002.

[3] A. Atalla and E. Maser. Carbonyl reduction of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (nnk) in cytosol of mouse liver and lung. *Toxicology*, 139(1-2):155–166, Nov 1999.

[4] W. Ax, M. Soldan, L. Koch, and E. Maser. Development of daunorubicin resistance in tumour cells by induction of carbonyl reduction. *Biochem Pharmacol*, 59(3):293–300, Feb 2000.

[5] O. S. Bains, R. H. Takahashi, T. A. Pfeifer, T. A. Grigliatti, R. E. Reid, and K. W. Riggs. Two allelic variants of aldo-keto reductase 1a1 exhibit reduced in vitro metabolism of daunorubicin. *Drug Metab Dispos*, 36(5):904–910, May 2008.

[6] R. Barouki, X. Coumoul, and P. M. Fernandez-Salguero. The aryl hydrocarbon receptor, more than a xenobiotic-interacting protein. *FEBS Lett*, 581(19):3608–3615, Jul 2007.

[7] A. Begleiter, M. K. Leith, J. A. Thliveris, and T. Digby. Dietary induction of nqo1 increases the antitumour activity of mitomycin c in human colon tumours in vivo. *Br J Cancer*, 91(8):1624–1631, Oct 2004.

[8] K. W. Bock and C. Köhle. Ah receptor: dioxin-mediated toxic responses as hints to deregulated physiologic functions. *Biochem Pharmacol*, 72(4):393–404, Aug 2006.

[9] J. E. Bray, B. D. Marsden, and U. Oppermann. The human short-chain dehydrogenase/reductase (sdr) superfamily: a bioinformatics summary. *Chem Biol Interact*, 178(1-3):99–109, Mar 2009.

[10] D. E. Brobst, X. Ding, K. L. Creech, B. Goodwin, B. Kelley, and J. L. Staudinger. Guggulsterone activates multiple nuclear receptors and induces cyp3a gene expression through the pregnane x receptor. *J Pharmacol Exp Ther*, 310(2):528–535, Aug 2004.

[11] T. P. Burris, C. Montrose, K. A. Houck, H. E. Osborne, W. P. Bocchinfuso, B. C. Yaden, C. C. Cheng, R. W. Zink, R. J. Barr, C. D. Hepler, V. Krishnan, H. A. Bullock, L. L. Burris, R. J. Galvin, K. Bramlett, and K. R. Stayrook. The hypolipidemic natural product guggulsterone is a promiscuous steroid receptor ligand. *Mol Pharmacol*, 67(3):948–954, Mar 2005.

[12] B. J. Carolan, A. Heguy, B.-G. Harvey, P. L. Leopold, B. Ferris, and R. G. Crystal. Up-regulation of expression of the ubiquitin carboxyl-terminal hydrolase l1 gene in human airway epithelium of cigarette smokers. *Cancer Res*, 66(22):10729–10740, Nov 2006.

[13] I. Chantret, A. Barbat, E. Dussaulx, M. G. Brattain, and A. Zweibaum. Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: a survey of twenty cell lines. *Cancer Res*, 48(7):1936–1942, Apr 1988.

[14] L.-F. Chen and W. C. Greene. Shaping the nuclear action of nf-kappab. *Nat Rev Mol Cell Biol*, 5(5):392–401, May 2004.

[15] J. H. Cheon, J. S. Kim, J. M. Kim, N. Kim, H. C. Jung, and I. S. Song. Plant sterol guggulsterone inhibits nuclear factor-kappab signaling in intestinal epithelial cells by blocking ikappab kinase and ameliorates acute murine colitis. *Inflamm Bowel Dis*, 12(12):1152–1161, Dec 2006.

[16] B. H. Dekelbab, S. F. Witchel, and D. B. DeFranco. Tnf-alpha and glucocorticoid receptor interaction in l6 muscle cells: a cooperative downregulation of myosin heavy chain. *Steroids*, 72(9-10):705–712, Sep 2007.

[17] M. S. Denison and S. R. Nagy. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol*, 43:309–334, 2003.

[18] I. D'Errico and A. Moschetta. Nuclear receptors, intestinal architecture and colon cancer: an intriguing link. *Cell Mol Life Sci*, 65(10):1523–1543, May 2008.

[19] O. Döhr and J. Abel. Transforming growth factor-beta1 coregulates mrna expression of aryl hydrocarbon receptor and cell-cycle-regulating genes in human cancer cell lines. *Biochem Biophys Res Commun*, 241(1):86–91, Dec 1997.

[20] A. T. Dinkova-Kostova, W. D. Holtzclaw, R. N. Cole, K. Itoh, N. Wakabayashi, Y. Katoh, M. Yamamoto, and P. Talalay. Direct evidence that sulfhydryl groups of keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci U S A*, 99(18):11908–11913, Sep 2002.

[21] J. A. Doorn, E. Maser, A. Blum, D. J. Claffey, and D. R. Petersen. Human carbonyl reductase catalyzes reduction of 4-oxonon-2-enal. *Biochemistry*, 43(41):13106–13114, Oct 2004.

[22] W. L. Duax, D. Ghosh, and V. Pletnev. Steroid dehydrogenase structures, mechanism of action, and disease. *Vitam Horm*, 58:121–148, 2000.

[23] Y. El-Hawari, A. D. Favia, E. S. Pilka, M. Kisiela, U. Oppermann, H.-J. Martin, and E. Maser. Analysis of the substrate-binding site of human carbonyl reductases cbr1 and cbr3 by site-directed mutagenesis. *Chem Biol Interact*, 178(1-3):234–241, Mar 2009.

[24] E. M. Ellis. Reactive carbonyls and oxidative stress: potential for therapeutic intervention. *Pharmacol Ther*, 115(1):13–24, Jul 2007.

[25] S. Endo, T. Matsunaga, Y. Kitade, S. Ohno, K. Tajima, O. El-Kabbani, and A. Hara. Human carbonyl reductase 4 is a mitochondrial nadph-dependent quinone reductase. *Biochem Biophys Res Commun*, 377(4):1326–1330, Dec 2008.

[26] R. O. Escárcega, S. Fuentes-Alexandro, M. García-Carrasco, A. Gatica, and A. Zamora. The transcription factor nuclear factor-kappa b and cancer. *Clin Oncol (R Coll Radiol)*, 19(2):154–161, Mar 2007.

[27] L. L. Espey, S. Yoshioka, D. Russell, T. Ujioka, B. Vladu, M. Skelsey, S. Fujii, H. Okamura, and J. S. Richards. Characterization of ovarian carbonyl reductase gene expression during ovulation in the gonadotropin-primed immature rat. *Biol Reprod*, 62(2):390–397, Feb 2000.

[28] L. Fan, B.-C. Goh, C.-I. Wong, N. Sukri, S.-E. Lim, S.-H. Tan, J.-Y. Guo, R. Lim, H.-L. Yap, Y.-M. Khoo, P. Iau, H.-S. Lee, and S.-C. Lee. Genotype of human carbonyl reductase cbr3 correlates with doxorubicin disposition and toxicity. *Pharmacogenet Genomics*, 18(7):621–631, Jul 2008.

[29] G. L. Forrest, S. Akman, S. Krutzik, R. J. Paxton, R. S. Sparkes, J. Doroshow, R. L. Felsted, C. J. Glover, T. Mohandas, and N. R. Bachur. Induction of a human carbonyl reductase gene located on chromosome 21. *Biochim Biophys Acta*, 1048(2-3):149–155, Apr 1990.

[30] G. L. Forrest and B. Gonzalez. Carbonyl reductase. *Chem Biol Interact*, 129(1-2):21–40, Dec 2000.

[31] J. Fujii, Y. Iuchi, S. Matsuki, and T. Ishii. Cooperative function of antioxidant and redox systems against oxidative stress in male reproductive tissues. *Asian J Androl*, 5(3):231–242, Sep 2003.

[32] D. Gardner-Stephen, J.-M. Heydel, A. Goyal, Y. Lu, W. Xie, T. Lindblom, P. Mackenzie, and A. Radominska-Pandya. Human pxr variants and their differential effects on the regulation of human udp-glucuronosyltransferase gene expression. *Drug Metab Dispos*, 32(3):340–347, Mar 2004.

[33] R. Garg, S. Gupta, and G. B. Maru. Dietary curcumin modulates transcriptional regulators of phase i and phase ii enzymes in benzo[a]pyrene-treated mice: mechanism of its anti-initiating action. *Carcinogenesis*, 29(5):1022–1032, May 2008.

[34] M. Gavelová, J. Hladíková, L. Vildová, R. Novotná, J. Vondrácek, P. Krcmár, M. Machala, and L. Skálová. Reduction of doxorubicin and oracin and induction of carbonyl reductase in human breast carcinoma mcf-7 cells. *Chem Biol Interact*, 176(1):9–18, Oct 2008.

[35] D. Ghosh, Z. Wawrzak, C. M. Weeks, W. L. Duax, and M. Erman. The refined three-dimensional structure of 3 alpha,20 beta-hydroxysteroid dehydrogenase and possible roles of the residues conserved in short-chain dehydrogenases. *Structure*, 2(7):629–640, Jul 1994.

[36] L. A. Gilad, T. Bresler, J. Gnainsky, P. Smirnoff, and B. Schwartz. Regulation of vitamin d receptor expression via estrogen-induced activation of the erk 1/2 signaling pathway in colon and breast cancer cells. *J Endocrinol*, 185(3):577–592, Jun 2005.

[37] A. Giudice and M. Montella. Activation of the nrf2-are signaling pathway: a promising strategy in cancer prevention. *Bioessays*, 28(2):169–181, Feb 2006.

[38] B. Goodwin, M. R. Redinbo, and S. A. Kliewer. Regulation of cyp3a gene transcription by the pregnane x receptor. *Annu Rev Pharmacol Toxicol*, 42:1–23, 2002.

[39] B.-G. Harvey, A. Heguy, P. L. Leopold, B. J. Carolan, B. Ferris, and R. G. Crystal. Modification of gene expression of the small airway epithelium in response to cigarette smoking. *J Mol Med*, 85(1):39–53, Jan 2007.

[40] S. S. Hecht. Biochemistry, biology, and carcinogenicity of tobacco-specific n-nitrosamines. *Chem Res Toxicol*, 11(6):559–603, Jun 1998.

[41] E. Ho and T. M. Bray. Antioxidants, nfkappab activation, and diabetogenesis. *Proc Soc Exp Biol Med*, 222(3):205–213, Dec 1999.

[42] F. Hoffmann and E. Maser. Carbonyl reductases and pluripotent hydroxysteroid dehydrogenases of the short-chain dehydrogenase/reductase superfamily. *Drug Metab Rev*, 39(1):87–144, 2007.

[43] N. Inazu, N. Inaba, and T. Satoh. Immunohistochemical localization and physiological regulation of carbonyl reductase in immature rat ovary. *Jpn J Pharmacol*, 54(1):13–21, Sep 1990.

[44] E. Ismail, F. Al-Mulla, S. Tsuchida, K. Suto, P. Motley, P. R. Harrison, and G. D. Birnie. Carbonyl reductase: a novel metastasis-modulating function. *Cancer Res*, 60(5):1173–1176, Mar 2000.

[45] Y. Iuchi, T. Kaneko, S. Matsuki, T. Ishii, Y. Ikeda, K. Uchida, and J. Fujii. Carbonyl stress and detoxification ability in the male genital tract and testis of rats. *Histochem Cell Biol*, 121(2):123–130, Feb 2004.

[46] N. Iwata, N. Inazu, S. Takeo, and T. Satoh. Carbonyl reductases from rat testis and vas deferens. purification, properties and localization. *Eur J Biochem*, 193(1):75–81, Oct 1990.

[47] A. Jacob, R. Wu, M. Zhou, and P. Wang. Mechanism of the anti-inflammatory effect of curcumin: Ppar-gamma activation. *PPAR Res*, 2007:89369, 2007.

[48] J. Jakubíková, J. Sedlák, J. Bod'o, and Y. Bao. Effect of isothiocyanates on nuclear accumulation of nf-kappab, nrf2, and thioredoxin in caco-2 cells. *J Agric Food Chem*, 54(5):1656–1662, Mar 2006.

[49] J. Jarabak and R. G. Harvey. Studies on three reductases which have polycyclic aromatic hydrocarbon quinones as substrates. *Arch Biochem Biophys*, 303(2):394–401, Jun 1993.

[50] A. Jeuken, B. J. G. Keser, E. Khan, A. Brouwer, J. Koeman, and M. S. Denison. Activation of the ah receptor by extracts of dietary herbal supplements, vegetables, and fruits. *J Agric Food Chem*, 51(18):5478–5487, Aug 2003.

[51] Y. Jin and T. M. Penning. Aldo-keto reductases and bioactivation/detoxication. *Annu Rev Pharmacol Toxicol*, 47:263–292, 2007.

[52] C. Jobin, S. Haskill, L. Mayer, A. Panja, and R. B. Sartor. Evidence for altered regulation of i kappa b alpha degradation in human colonic epithelial cells. *J Immunol*, 158(1):226–234, Jan 1997.

[53] H. Jörnvall, J. O. Höög, and B. Persson. Sdr and mdr: completed genome sequences show these protein families to be large, of old origin, and of complex nature. *FEBS Lett*, 445(2-3):261–264, Feb 1999.

[54] H. Jörnvall, B. Persson, M. Krook, S. Atrian, R. Gonzalez-Duarte, J. Jeffery, and D. Ghosh. Short-chain dehydrogenases/reductases (sdr). *Biochemistry*, 34(18):6003–6013, May 1995.

[55] H. Jörnvall, M. Persson, and J. Jeffery. Alcohol and polyol dehydrogenases are both divided into two protein types, and structural properties cross-relate the different enzyme activities within each type. *Proc Natl Acad Sci U S A*, 78(7):4226–4230, Jul 1981.

[56] N. Kassner, K. Huse, H.-J. Martin, U. Gödtel-Armbrust, A. Metzger, I. Meineke, J. Brockmöller, K. Klein, U. M. Zanger, E. Maser, and L. Wojnowski. Carbonyl reductase 1 is a predominant doxorubicin reductase in the human liver. *Drug Metab Dispos*, 36(10):2113–2120, Oct 2008.

[57] T. W. Kensler, N. Wakabayashi, and S. Biswal. Cell survival responses to environmental stresses via the keap1-nrf2-are pathway. *Annu Rev Pharmacol Toxicol*, 47:89–116, 2007.

[58] C. Köhle, O. A. Badary, K. Nill, B. S. Bock-Hennig, and K. W. Bock. Serotonin glucuronidation by ah receptor- and oxidative stress-inducible human udp-glucuronosyltransferase (ugt) 1a6 in caco-2 cells. *Biochem Pharmacol*, 69(9):1397–1402, May 2005.

[59] S. S. Lakhman, X. Chen, V. Gonzalez-Covarrubias, E. G. Schuetz, and J. G. Blanco. Functional characterization of the promoter of human carbonyl reductase 1 (cbr1). role of xre elements in mediating the induction of cbr1 by ligands of the aryl hydrocarbon receptor. *Mol Pharmacol*, 72(3):734–743, Sep 2007.

[60] S. S. Lakhman, D. Ghosh, and J. G. Blanco. Functional significance of a natural allelic variant of human carbonyl reductase 3 (cbr3). *Drug Metab Dispos*, 33(2):254–257, Feb 2005.

[61] A. Lang, M. Lahav, E. Sakhnini, I. Barshack, H. H. Fidder, B. Avidan, E. Bardan, R. Hershkoviz, S. Bar-Meir, and Y. Chowers. Allicin inhibits spontaneous and tnf-alpha induced

secretion of proinflammatory cytokines and chemokines from intestinal epithelial cells. *Clin Nutr*, 23(5):1199–1208, Oct 2004.

[62] D. H. Lee and A. L. Goldberg. Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol*, 8(10):397–403, Oct 1998.

[63] B. Lewin. *Genes VI*. Oxford Univ Pr, March 1997.

[64] T. Lin. Vitamin d receptor regulation via estrogen administration: A possible link to colon cancer. *Journal of Undergraduate Research*, 2, Nov 2000.

[65] A. I. Loaiza-Pérez, S. Kenney, J. Boswell, M. Hollingshead, M. C. Alley, C. Hose, H. P. Ciolino, G. C. Yeh, J. B. Trepel, D. T. Vistica, and E. A. Sausville. Aryl hydrocarbon receptor activation of an antitumor aminoflavone: basis of selective toxicity for mcf-7 breast tumor cells. *Mol Cancer Ther*, 3(6):715–725, Jun 2004.

[66] H. Lou, S. Du, Q. Ji, and A. Stolz. Induction of akr1c2 by phase ii inducers: identification of a distal consensus antioxidant response element regulated by nrf2. *Mol Pharmacol*, 69(5):1662–1672, May 2006.

[67] N. Mariappan, R. N. Soorappan, M. Haque, S. Sriramula, and J. Francis. Tnf-alpha-induced mitochondrial oxidative stress and cardiac dysfunction: restoration by superoxide dismutase mimetic tempol. *Am J Physiol Heart Circ Physiol*, 293(5):H2726–H2737, Nov 2007.

[68] E. Maser. Xenobiotic carbonyl reduction and physiological steroid oxidoreduction. the pluripotency of several hydroxysteroid dehydrogenases. *Biochem Pharmacol*, 49(4):421–440, Feb 1995.

[69] E. Maser, J. Friebertshäuser, and B. Völker. Purification, characterization and nnk carbonyl reductase activities of 11beta-hydroxysteroid dehydrogenase type 1 from human liver: enzyme cooperativity and significance in the detoxification of a tobacco-derived carcinogen. *Chem Biol Interact*, 143-144:435–448, Feb 2003.

[70] E. Maser and U. C. Oppermann. Role of type-1 11beta-hydroxysteroid dehydrogenase in detoxification processes. *Eur J Biochem*, 249(2):365–369, Oct 1997.

[71] E. Maser, B. Völker, and J. Friebertshäuser. 11 beta-hydroxysteroid dehydrogenase type 1 from human liver: dimerization and enzyme cooperativity support its postulated role as glucocorticoid reductase. *Biochemistry*, 41(7):2459–2465, Feb 2002.

[72] E. Maser, V. Wsol, and H.-J. Martin. 11beta-hydroxysteroid dehydrogenase type 1: purification from human liver and characterization as carbonyl reductase of xenobiotics. *Mol Cell Endocrinol*, 248(1-2):34–37, Mar 2006.

[73] H. Masuzaki, J. Paterson, H. Shinyama, N. M. Morton, J. J. Mullins, J. R. Seckl, and J. S. Flier. A transgenic model of visceral obesity and the metabolic syndrome. *Science*, 294(5549):2166–2170, Dec 2001.

[74] T. Matsunaga, S. Endo, S. Maeda, S. Ishikura, K. Tajima, N. Tanaka, K. T. Nakamura, Y. Imamura, and A. Hara. Characterization of human dhrs4: an inducible short-chain dehydrogenase/reductase enzyme with 3beta-hydroxysteroid dehydrogenase activity. *Arch Biochem Biophys*, 477(2):339–347, Sep 2008.

[75] T. Matsunaga, S. Shintani, and A. Hara. Multiplicity of mammalian reductases for xenobiotic carbonyl compounds. *Drug Metab Pharmacokinet*, 21(1):1–18, Feb 2006.

[76] V. Meunier, M. Bourrié, Y. Berger, and G. Fabre. The human intestinal epithelial cell line caco-2; pharmacological and pharmacokinetic applications. *Cell Biol Toxicol*, 11(3-4):187–194, Aug 1995.

[77] U. A. Meyer. Overview of enzymes of drug metabolism. *J Pharmacokinet Biopharm*, 24(5):449–459, Oct 1996.

[78] W. Miao, L. Hu, P. J. Scrivens, and G. Batist. Transcriptional regulation of nf-e2 p45-related factor (nrf2) expression by the aryl hydrocarbon receptor-xenobiotic response element signaling pathway: direct cross-talk between phase i and ii drug-metabolizing enzymes. *J Biol Chem*, 280(21):20340–20348, May 2005.

[79] J. Mimura, M. Ema, K. Sogawa, and Y. Fujii-Kuriyama. Identification of a novel mechanism of regulation of ah (dioxin) receptor function. *Genes Dev*, 13(1):20–25, Jan 1999.

[80] G. Minotti, P. Menna, E. Salvatorelli, G. Cairo, and L. Gianni. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev*, 56(2):185–229, Jun 2004.

[81] T. Miura, Y. Itoh, M. Takada, H. Tsutsui, T. Yukimura, T. Nishinaka, and T. Terada. Investigation of the role of the amino acid residue at position 230 for catalysis in monomeric carbonyl reductase 3. *Chem Biol Interact*, 178(1-3):211–214, Mar 2009.

[82] T. Miura, T. Nishinaka, and T. Terada. Different functions between human monomeric carbonyl reductase 3 and carbonyl reductase 1. *Mol Cell Biochem*, 315(1-2):113–121, Aug 2008.

[83] P. A. Münzel, S. Schmohl, F. Buckler, J. Jaehrling, F. T. Raschko, C. Köhle, and K. W. Bock. Contribution of the ah receptor to the phenolic antioxidant-mediated expression of human and rat udp-glucuronosyltransferase ugt1a6 in caco-2 and rat hepatoma 5l cells. *Biochem Pharmacol*, 66(5):841–847, Sep 2003.

[84] T. J. Monks, R. P. Hanzlik, G. M. Cohen, D. Ross, and D. G. Graham. Quinone chemistry and toxicity. *Toxicol Appl Pharmacol*, 112(1):2–16, Jan 1992.

[85] N. M. Morton, M. C. Holmes, C. Fiévet, B. Staels, A. Tailleux, J. J. Mullins, and J. R. Seckl. Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11beta-hydroxysteroid dehydrogenase type 1 null mice. *J Biol Chem*, 276(44):41293–41300, Nov 2001.

[86] H. Motohashi, T. O'Connor, F. Katsuoka, J. D. Engel, and M. Yamamoto. Integration and diversity of the regulatory network composed of maf and cnc families of transcription factors. *Gene*, 294(1-2):1–12, Jul 2002.

[87] H. Motohashi and M. Yamamoto. Nrf2-keap1 defines a physiologically important stress response mechanism. *Trends Mol Med*, 10(11):549–557, Nov 2004.

[88] H. Y. Naim. Secretion of human intestinal angiotensin-converting enzyme and its association with the differentiation state of intestinal cells. *Biochem J*, 316 (Pt 1):259–264, May 1996.

[89] J. Nakagawa, S. Ishikura, J. Asami, T. Isaji, N. Usami, A. Hara, T. Sakurai, K. Tsuritani, K. Oda, M. Takahashi, M. Yoshimoto, N. Otsuka, and K. Kitamura. Molecular characterization of mammalian dicarbonyl/l-xylulose reductase and its localization in kidney. *J Biol Chem*, 277(20):17883–17891, May 2002.

[90] NCBI. Geo profiles. http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS1542, February 16 2009.

[91] NCBI. Geo profiles. http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS1543, February 16 2009.

[92] NCBI. Geo profiles. http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS2126, February 16 2009.

[93] NCBI. Geo profiles. http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS1304, February 17 2009.

[94] NCBI. Geo profiles. http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS2491, February 17 2009.

[95] NCBI. Geo profiles. http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS2307, February 17 2009.

[96] T. Nguyen, P. J. Sherratt, and C. B. Pickett. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annu Rev Pharmacol Toxicol*, 43:233–260, 2003.

[97] S. Nishiumi, K.-I. Yoshida, and H. Ashida. Curcumin suppresses the transformation of an aryl hydrocarbon receptor through its phosphorylation. *Arch Biochem Biophys*, 466(2):267–273, Oct 2007.

[98] S. Nobel, L. Abrahmsen, and U. Oppermann. Metabolic conversion as a pre-receptor control mechanism for lipophilic hormones. *Eur J Biochem*, 268(15):4113–4125, Aug 2001.

[99] S. Ohkura-Hada, N. Kondoh, A. Hada, M. Arai, Y. Yamazaki, M. Shindoh, Y. Kitagawa, M. Takahashi, T. Ando, Y. Sato, and M. Yamamoto. Carbonyl reductase 3 (cbr3) mediates 9-cis-retinoic acid-induced cytostatis and is a potential prognostic marker for oral malignancy. *Open Dent J*, 2:78–88, 2008.

[100] U. Oppermann. Carbonyl reductases: the complex relationships of mammalian carbonyl- and quinone-reducing enzymes and their role in physiology. *Annu Rev Pharmacol Toxicol*, 47:293–322, 2007.

[101] U. C. Oppermann, C. Filling, and H. Jörnvall. Forms and functions of human sdr enzymes. *Chem Biol Interact*, 130-132(1-3):699–705, Jan 2001.

[102] U. C. Oppermann and E. Maser. Molecular and structural aspects of xenobiotic carbonyl metabolizing enzymes. role of reductases and dehydrogenases in xenobiotic phase i reactions. *Toxicology*, 144(1-3):71–81, Apr 2000.

[103] T. M. Penning and J. E. Drury. Human aldo-keto reductases: Function, gene regulation, and single nucleotide polymorphisms. *Arch Biochem Biophys*, 464(2):241–250, Aug 2007.

[104] B. Persson, Y. Kallberg, J. E. Bray, E. Bruford, S. L. Dellaporta, A. D. Favia, R. G. Duarte, H. Jörnvall, K. L. Kavanagh, N. Kedishvili, M. Kisiela, E. Maser, R. Mindnich, S. Orchard, T. M. Penning, J. M. Thornton, J. Adamski, and U. Oppermann. The sdr

(short-chain dehydrogenase/reductase and related enzymes) nomenclature initiative. *Aldose Reductase Mediates Mitogenic Signaling in Vascular Smooth Muscle Cells*, 178(1-3):94–98, Mar 2009.

[105] B. Persson, Y. Kallberg, U. Oppermann, and H. Jörnvall. Coenzyme-based functional assignments of short-chain dehydrogenases/reductases (sdrs). *Chem Biol Interact*, 143-144:271–278, Feb 2003.

[106] A. Puga, C. Ma, and J. L. Marlowe. The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. *Biochem Pharmacol*, 77(4):713–722, Feb 2009.

[107] A. L. Rinaldi, M. A. Morse, H. W. Fields, D. A. Rothas, P. Pei, K. A. Rodrigo, R. J. Renner, and S. R. Mallery. Curcumin activates the aryl hydrocarbon receptor yet significantly inhibits (-)-benzo(a)pyrene-7r-trans-7,8-dihydrodiol bioactivation in oral squamous cell carcinoma cells and oral mucosa. *Cancer Res*, 62(19):5451–5456, Oct 2002.

[108] M. M. Ris and J. P. von Wartburg. Heterogeneity of nadph-dependent aldehyde reductase from human and rat brain. *Eur J Biochem*, 37(1):69–77, Aug 1973.

[109] S. Safe and K. Kim. Non-classical genomic estrogen receptor (er)/specificity protein and er/activating protein-1 signaling pathways. *J Mol Endocrinol*, 41(5):263–275, Nov 2008.

[110] Y. Sambuy, I. D. Angelis, G. Ranaldi, M. L. Scarino, A. Stammati, and F. Zucco. The caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on caco-2 cell functional characteristics. *Cell Biol Toxicol*, 21(1):1–26, Jan 2005.

[111] S. Schoonbroodt and J. Piette. Oxidative stress interference with the nuclear factor-kappa b activation pathways. *Biochem Pharmacol*, 60(8):1075–1083, Oct 2000.

[112] M. A. Sciotti, S. Nakajin, B. Wermuth, and M. E. Baker. Mutation of threonine-241 to proline eliminates autocatalytic modification of human carbonyl reductase. *Biochem J*, 350 Pt 1:89–92, Aug 2000.

[113] J. R. Seckl. 11beta-hydroxysteroid dehydrogenase in the brain: a novel regulator of glucocorticoid action? *Front Neuroendocrinol*, 18(1):49–99, Jan 1997.

[114] N. Shafqat, J. Shafqat, G. Eissner, H.-U. Marschall, K. Tryggvason, U. Eriksson, F. Gabrielli, H. Lardy, H. Jörnvall, and U. Oppermann. Hep27, a member of the short-chain dehydrogenase/reductase family, is an nadph-dependent dicarbonyl reductase expressed in vascular endothelial tissue. *Cell Mol Life Sci*, 63(10):1205–1213, May 2006.

[115] G. Shrimpf. *Gentechnische Methoden: eine Sammlung von Arbeitsanleitungen*. Spektrum Akademischer Verlag GmbH, Heidelberg, 3 edition, 2002.

[116] R. J. Slater. *Experiments in Molecular Biology*. Humana Press, 1986.

[117] M. Soldan, G. Nagel, M. Losekam, M. Ernst, and E. Maser. Interindividual variability in the expression and nnk carbonyl reductase activity of 11beta-hydroxysteroid dehydrogenase 1 in human lung. *Cancer Lett*, 145(1-2):49–56, Oct 1999.

[118] M. Soldan, K. J. Netter, and E. Maser. Induction of daunorubicin carbonyl reducing enzymes by daunorubicin in sensitive and resistant pancreas carcinoma cells. *Biochem Pharmacol*, 51(2):117–123, Jan 1996.

[119] S. Takabe, K. Mochizuki, and T. Goda. De-phosphorylation of gr at ser203 in nuclei associates with gr nuclear translocation and glut5 gene expression in caco-2 cells. *Arch Biochem Biophys*, 475(1):1–6, Jul 2008.

[120] K. Takenaka, E. Ogawa, H. Oyanagi, H. Wada, and F. Tanaka. Carbonyl reductase expression and its clinical significance in non-small-cell lung cancer. *Cancer Epidemiol Biomarkers Prev*, 14(8):1972–1975, Aug 2005.

[121] P. Talalay. Mechanisms of induction of enzymes that protect against chemical carcinogenesis. *Adv Enzyme Regul*, 28:237–250, 1989.

[122] B. Testa and S. D. Krämer. The biochemistry of drug metabolism–an introduction: part 1. principles and overview. *Chem Biodivers*, 3(10):1053–1101, Oct 2006.

[123] B. Testa and S. D. Krämer. The biochemistry of drug metabolism–an introduction: Part 2. redox reactions and their enzymes. *Chem Biodivers*, 4(3):257–405, Mar 2007.

[124] M. Till, D. Riebniger, H. J. Schmitz, and D. Schrenk. Potency of various polycyclic aromatic hydrocarbons as inducers of cyp1a1 in rat hepatocyte cultures. *Chem Biol Interact*, 117(2):135–150, Jan 1999.

[125] R. Tirumalai, T. R. Kumar, K. H. Mai, and S. Biswal. Acrolein causes transcriptional induction of phase ii genes by activation of nrf2 in human lung type ii epithelial (a549) cells. *Toxicol Lett*, 132(1):27–36, Jun 2002.

[126] J. W. Tomlinson. 11beta-hydroxysteroid dehydrogenase type 1 in human disease: a novel therapeutic target. *Minerva Endocrinol*, 30(1):37–46, Mar 2005.

[127] UniProt. http://www.uniprot.org/uniprot/O75828, February 19 2009.

[128] S. Vallee, S. Laforest, F. Fouchier, M. P. Montero, C. Penel, and S. Champion. Cytokine-induced upregulation of nf-kappab, il-8, and icam-1 is dependent on colonic cell polarity: implication for pkcdelta. *Exp Cell Res*, 297(1):165–185, Jul 2004.

[129] D. Viemann, M. Goebeler, S. Schmid, U. Nordhues, K. Klimmek, C. Sorg, and J. Roth. Tnf induces distinct gene expression programs in microvascular and macrovascular human endothelial cells. *J Leukoc Biol*, 80(1):174–185, Jul 2006.

[130] A. Waclawik and A. J. Ziecik. Differential expression of prostaglandin (pg) synthesis enzymes in conceptus during peri-implantation period and endometrial expression of carbonyl reductase/pg 9-ketoreductase in the pig. *J Endocrinol*, 194(3):499–510, Sep 2007.

[131] K. Watanabe, C. Sugawara, A. Ono, Y. Fukuzumi, S. Itakura, M. Yamazaki, H. Tashiro, K. Osoegawa, E. Soeda, and T. Nomura. Mapping of a novel human carbonyl reductase, cbr3, and ribosomal pseudogenes to human chromosome 21q22.2. *Genomics*, 52(1):95–100, Aug 1998.

[132] B. Wermuth. Purification and properties of an nadph-dependent carbonyl reductase from human brain. relationship to prostaglandin 9-ketoreductase and xenobiotic ketone reductase. *J Biol Chem*, 256(3):1206–1213, Feb 1981.

[133] B. Wermuth, K. M. Bohren, G. Heinemann, J. P. von Wartburg, and K. H. Gabbay. Human carbonyl reductase. nucleotide sequence analysis of a cdna and amino acid sequence of the encoded protein. *J Biol Chem*, 263(31):16185–16188, Nov 1988.

[134] J. Wu, C. Xia, J. Meier, S. Li, X. Hu, and D. S. Lala. The hypolipidemic natural product guggulsterone acts as an antagonist of the bile acid receptor. *Mol Endocrinol*, 16(7):1590–1597, Jul 2002.

[135] C. Xu, C. Y.-T. Li, and A.-N. T. Kong. Induction of phase i, ii and iii drug metabolism/transport by xenobiotics. *Arch Pharm Res*, 28(3):249–268, Mar 2005.

[136] Y. Yamada, J. Tian, Y. Yang, R. G. Cutler, T. Wu, R. S. Telljohann, M. P. Mattson, and J. T. Handa. Oxidized low density lipoproteins induce a pathologic response by retinal pigmented epithelial cells. *J Neurochem*, 105(4):1187–1197, May 2008.

[137] J. Zhang, T. Ohta, A. Maruyama, T. Hosoya, K. Nishikawa, J. M. Maher, S. Shibahara, K. Itoh, and M. Yamamoto. Brg1 interacts with nrf2 to selectively mediate ho-1 induction in response to oxidative stress. *Mol Cell Biol*, 26(21):7942–7952, Nov 2006.