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Original article

Human carbonyl reductase 1 as efficient catalyst for the reduction of glutathionylated aldehydes derived from lipid peroxidation

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ABSTRACT

Human recombinant carbonyl reductase 1 (E.C. 1.1.1.184, hCBR1) is shown to efficiently act as aldehyde reductase on glutathionylated alkanals, namely 3-glutathionyl-4-hydroxynonanal (GSHNE), 3-glutathionyl-nonanal, 3-glutathionyl-hexanal and 3-glutathionyl-propanal. The presence of the glutathionyl moiety appears as a necessary requirement for the susceptibility of these compounds to the NADPH-dependent reduction by hCBR1. In fact the corresponding alkanals and alkenals, and the cysteinyl and γ-glutamyl-cysteinyl alkanals adducts were either ineffective or very poorly active as CBR1 substrates. Mass spectrometry analysis reveals the ability of hCBR1 to reduce GSHNE to the corresponding GS-dihydroxynonane (GSDHN) and at the same time to catalyze the oxidation of the hemiacetal form of GSHNE, generating the 3-glutathionylnonanoic–δ-lactone. These data are indicative of the ability of the enzyme to catalyze a disproportion reaction of the substrate through the redox recycle of the pyridine cofactor. A rationale for the observed preferential activity of hCBR1 on different GSHNE diastereoisomers is given by molecular modelling. These results evidence the potential of hCBR1 acting on GSHNE to accomplish a dual role, both in terms of HNE detoxification and, through the production of GSDHN, in terms of involvement into the signalling cascade of the cellular inflammatory response.

1. Introduction

Lipid peroxidation is considered as a toxic consequence of oxidative stress, due to the generation of highly reactive saturated and unsaturated aldehydes, such as acrolein, malondialdehyde, 4-hydroxy-*trans*-2-nonenal (HNE), 4-hydroxy-*trans*-2-hexenal (HHE) and *trans*-2-nonenal [1,2]. These compounds are involved in the development of several pathological conditions [1–3], associated with the formation of corresponding adducts with proteins and nucleic acids. Studying their cellular detoxification mechanism is thus important. The main enzymes involved in the detoxification pathway are glutathione S-transferases (GSTs) [4], which are responsible for the conjugation of unsaturated aldehydes with glutathione (GSH), aldehyde dehydrogenases [5–8], aldo-keto reductases [5,9–11], alcohol dehydrogenases [5,12] and cytochromes P450 [5,13–15]. In particular, members of aldo-keto reductases family, such as AKR1B1 [9,10] and AKR1B10 [11], and of the short chain dehydrogenase/reductases family, such as carbonyl reductase 1 (CBR1) [16,17] have been reported to efficiently transform the above mentioned aldehydes and/or their glutathionylated adducts.

Carbonyl reductase 1 [E.C. 1.1.1.184] is a monomeric NADPH-dependent enzyme of 277 amino acids widely diffused in mammals [18]. In humans, it has been found in the brain, liver, kidney, stomach, small intestine, and epidermis [19,20]. In terms of its reductase activity, the enzyme has a broad substrate specificity on various carbonyl substrates, showing a marked activity towards *orto* or *para* xenobiotic quinone derivatives (*e.g.*, 9,10-phenanthrenequinone or menadione) but also towards endogenous substrates, such as eicosanoids, steroids and lipid derived carbonyl

Abbreviations: GSDHN, 3-glutathionyl-1,4-dihydroxynonane; GSHNE, 3-glutathionyl-4-hydroxynonal; GSHNA-lactone, 3-glutathionyl-4-hydroxynonanoic-δ-lactone; HHE, 4-hydroxy-2-hexenal; HNE, 4-hydroxy-2-nonenal; ONE, 4-oxo-*trans*-2-nonenal; 9,10-PQ, 9,10-phenanthrenquinone; BSA, bovine serum albumin; CBR1, carbonyl reductase 1; EIC, extracted ion chromatograms; DMSO, dimethyl sulfoxide; DTT, D,L-dithiotreitol; hCBR1, human recombinant carbonyl reductase 1; ΔRT, interval of retention time; IPTG, isopropyl-β-D-thiogalactopyranoside; MRFA, Met-Arg-Phe-Ala peptide; MD, molecular dynamic; PG, prostaglandin; SDS, sodium dodecyl sulphate; TIC, total ion chromatogram

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compounds [21–23].

Even though the potential of CBR1 to reduce aldehydes has been reported, aldehydes, among carbonyl compounds, are poor substrates for human CBR1 [23]. The case of 4-oxo-*trans*-2-nonenal (ONE) is indicative, as it carries both an oxo and an aldehyde group; ONE has been reported to be efficiently reduced at the oxo group thus generating HNE, with only a marginal reduction (approximately 10%) of the aldehydic moiety [17]. In fact, Doorn et al. associated the possibility for the aldehydic group to be reduced by CBR1 with the simultaneous presence in the molecule of both the aldehydic carbonyl and the oxo groups.

The recognition of carbonyl compounds as a substrate by CBR1 seems to be dependent on the presence of C227, which is a reactive cysteine located in the proximity of the substrate-binding site, and suggested as being involved in the correct substrate orientation [24]. The residues W229, A235 and M241 also appear to be important in the appropriate allocation of the hydrophobic moieties of substrates into the active site [23]. Near the active site, a glutathione-binding site has also been identified [25,26], which is involved in the recognition of glutathionylated substrates.

The relevance of the glutathione moiety in the recognition of potential substrates by CBR1 was reported for the first time in a study on prostaglandin (PG) metabolism. The reduction of the 9 keto group of PGA_1 occurred only for the glutathione adduct of PGA_1 ($GSPGA_1$), and was almost negligible for the free PGA_1 [25,27]. Conversely, both menadione and glutathionylated-menadione (GS-menadione) were comparable as reductable CBR1 substrates [27]. In addition, $GSPGA_1$ and GS-menadione were shown to be efficient inhibitors of the 15-hydroxyprostaglandin dehydrogenase activity of CBR1 on PGB_1 [27].

The C227 residue does not appear to be very critical in the binding of glutathionylated molecules. Thus, the C227S CBR1 mutant, while inactive in terms of menadione reduction, is still active, in fact more active than the wild type enzyme, on GS-menadione [24]. Similar results have been obtained when PGA_1 and $GSPGA_1$ were compared as substrates for the C227S CBR1 mutant [24]. These findings highlight the strong addressing effect exerted by the glutathionyl moiety of the substrates for their correct allocation within the active site. One exception was observed for S-nitrosoglutathione, which is efficiently reduced by CBR1 [26], and for which a prominent role of C227, but not of the glutathione-binding site, in its susceptibility to reduction has been established [28].

A new glutathionylated substrate of CBR1, 3-glutathionyl-4-hydroxynonal (GSHNE), has recently been reported [29]. The enzyme was demonstrated to act as an efficient $NADP^+$ -dependent dehydrogenase able to oxidize GSHNE to the corresponding 3-glutathionyl-4-hydroxynonanoic-δ-lactone (GSHNA-lactone). The present work shows the ability of CBR1 to catalyze an efficient NADPH-dependent reduction of glutathionylated aldehydes. In the presence of CBR1, GSHNE can therefore undergo a divergent redox transformation, ending with the generation of the oxidation as well as the reduction product through a disproportion reaction. This evidence reinforces the prominent role of CBR1 in the metabolism of lipid peroxidation products.

2. Materials and methods

2.1. Materials

9,10-phenanthrenquinone (9,10-PQ), bovine serum albumin (BSA), cysteine, CysGly, dimethyl sulfoxide (DMSO), D,L-dithiotreitol (DTT), γGluCys, 5,5′-dithiobis-(2-nitrobenzoicacid), GSH, hexanal, isopropyl-β-D-thiogalactopyranoside (IPTG), Met-Arg-Phe-Ala peptide (MRFA), $NADP^+$, N-acetyl-cysteine, nonanal, propanal, protease inhibitors cocktail, sodium dodecyl sulphate (SDS), *trans*-2-hexenal, *trans*-2-nonenal, *trans*-2-propenal (acrolein) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HHE was purchased from Cayman Chemicals (AnnArbor, MI, USA). Whatman DEAE-cellulose (DE-52) and Sephacryl S200 were purchased from GE Healthcare (Little Chalfont, UK). Blue Sepharose and Bradford reagent were purchased from Bio-Rad (Hercules, CA, USA). YM10 membranes (10 kDa cut-off) were purchased from Amicon Millipore (Darmstadt, Germany). Dialysis tubing (10 kDa cut-off) was purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). All inorganic chemicals were of reagent grade from BDH (VWR International, Poole, Dorset, UK). All solvents were HPLC grade from J.T. Baker Chemicals (Center Valley, PA, USA). NADPH was from Carbosynth (Compton, England).

2.1.1. RNA extraction and cDNA synthesis

Total RNA was extracted from a human astrocytoma cell line (ADF) with TRI® Reagent (Sigma), following the manufacturer's instructions. cDNA was prepared from total RNA by reverse transcription, using 200 units of Super Script™ III Reverse Transcriptase (Invitrogen) and 0.5 μg of an oligo-dT primer in a final volume of 50 μl. The mixture also contained 0.5 mM of each dNTP (GE-Healthcare), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 0.1 mg/ml BSA in 50 mM Tris-HCl, pH 8.3. This reaction mixture was incubated at 42 °C for 60 min. The product was directly used for PCR amplification or stored at −20 °C. As reported [29], the corresponding cDNA sequence completely matched with the human wild type counterpart.

2.1.2. Polymerase chain reaction

Aliquots of 1 μl of crude cDNA were amplified in a Bio-Rad Gene Cycler™ thermocycler, using 2.5 units of *Thermus aquaticus* DNA polymerase (GE-Healthcare), 1 mM of each dNTP (GE-Healthcare), 1 μM of each PCR primer, 50 mM KCl, 2.5 mM $MgCl_2$ and 0.1 mg/ml BSA in 10 mM Tris-HCl, pH 8.3, containing 0.1% v/v Triton X-100. At the 5′-end, we used the specific primer 5′-CAT ATG TCG TCC GGC ATC CAT GT-3′, which corresponds to the sequence encoding the first six amino acids of the mature protein and containing an Nde I restriction site for ligation into the expression vector, and at the same time provided the ATG codon for an additional methionine at position 1. At the 3'-end, the specific primer: 5′-GAA TTC CTA CCA CTG TTC AAC TCT CTT-3′ encoded the last six amino acids, followed by a stop codon and an Eco RI restriction site. After an initial denaturation step at 95 °C for 5 min, we performed 35 amplification cycles (1 min at 95 °C, 30 s at 50 °C, 1 min at 72 °C), which were followed by a final step of 7 min at 72 °C. An amplification product of about 800 bp was obtained, in agreement with the expected size (831 bp).

2.1.3. Cloning and sequencing

The crude PCR product was ligated into a pGEM (Promega) vector without further purification, using a 1:5 plasmid to insert molar ratio and incubating the mixture overnight, at room temperature. After transformation of *E. coli* XL-1 Blue competent cells with the ligation product, positive colonies were selected by PCR using the plasmid primers SP6 and T7, and were grown in LB/ampicillin medium. DNA was extracted using the Plasmid Mini-Prep Kit (Euroclone) and custom sequenced at Eurofins MWG, Ebersberg, Germany.

2.1.4. Cloning in expression vector

pGEM plasmid containing the appropriate sequence was digested with Nde I and Eco RI restriction enzymes for 2 h at 37 °C and the digestion product was separated on agarose gel. The fragment obtained was purified from gel using the QIAEX II Extraction kit (Qiagen) and ligated into the expression vector pET30