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Original Contribution

# NADP$^+$-dependent dehydrogenase activity of carbonyl reductase on glutathionylhydroxynonanal as a new pathway for hydroxynonenal detoxification

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ABSTRACT

An NADP$^+$-dependent dehydrogenase activity on 3-glutathionyl-4-hydroxynonanal (GSHNE) was purified to electrophoretic homogeneity from a line of human astrocytoma cells (ADF). Proteomic analysis identified this enzymatic activity as associated with carbonyl reductase 1 (EC 1.1.1.184). The enzyme is highly efficient at catalyzing the oxidation of GSHNE ($K_M$ 33 μM, $k_{cat}$ 405 min$^{-1}$), as it is practically inactive toward *trans*-4-hydroxy-2-nonenal (HNE) and other HNE-adducted thiol-containing amino acid derivatives. Combined mass spectrometry and nuclear magnetic resonance spectroscopy analysis of the reaction products revealed that carbonyl reductase oxidizes the hydroxyl group of GSHNE in its hemiacetal form, with the formation of the corresponding 3-glutathionylnonanoic–δ-lactone. The relevance of this new reaction catalyzed by carbonyl reductase 1 is discussed in terms of HNE detoxification and the recovery of reducing power.



The pathophysiological effects of oxidative stress on cells and tissues are assumed to be related, at least in part, to endogenous lipid peroxidation and to the highly reactive molecules consequently produced, of which the most abundant is *trans*-4-hydroxy-2-nonenal (HNE) [1–3]. HNE is generated as a racemic mixture of 4*R* and 4*S* enantiomers primarily from the peroxidation of ω-6 polyunsaturated fatty acids [4]. It may account for more than 95% of the enals produced [5,6]. Basal HNE concentrations in tissue and plasma range from 0.8 to 2.8 μM [7], and there is evidence of increased levels up to 4.5 mM in peroxidizing membranes under oxidative stress conditions [8].

HNE plays an important role in the pathogenesis of several diseases, including atherosclerosis and Alzheimer disease [6,9,10]. The toxicity of HNE depends on its high chemical reactivity, which is associated with the presence of an aldehydic group, a double bond, and a secondary alcohol at the chiral center $C_4$ [11]. HNE can react with nucleophiles via 1,2 and 1,4 addition [7,12,13]. The main protein modification associated with HNE occurs via 1,4-Michael addition with Cys, Lys, and His residues [7,12,14]. A number of proteins, including albumin, amyloid β peptide, α-synuclein, aldose reductase, and several glutathione *S*-transferase (GST) isoforms, have been reported to be covalently modified in vitro by HNE [15–25]. On the other hand, the 1,2-addition of the carbonyl group to the primary amine of Lys has also been reported, which generates the corresponding Schiff base [13,24].

As a signaling molecule, HNE is involved in modulating in a concentration-dependent manner several cellular processes, such as proliferation, differentiation, and apoptosis. High concentrations of HNE have thus been reported to cause cell cycle arrest [26], differentiation [27], and apoptosis [28,29]. Lower concentrations, at least in some cell types, appear to induce proliferation [30]. Intracellular control of HNE levels is thus required to modulate the cell cycle.

HNE metabolism involves reduction of aldehyde to the corresponding 1,4-dihydroxynonene by aldose reductase [31,32] and alcohol dehydrogenase [33,34] or oxidation of aldehyde to the 4-hydroxynonenoic acid by aldehyde dehydrogenase [35–37]. However, the formation of 3-glutathionyl-4-hydroxynonanal (GSHNE) is considered the main pathway of HNE metabolism [38–40]. GSHNE,

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which is present in solution essentially (95%) in its cyclic hemiacetal form [7,41], can be produced both in a spontaneous reaction between HNE and glutathione (GSH) and in a reaction efficiently catalyzed by GST [7,42]. Of the isoforms of GST in mammalian tissues, a subgroup of the α-class isozymes shows the highest catalytic efficiency for HNE [43–46].

As GSHNE is the most significant HNE derivative, its metabolism represents the main pathway for the control of HNE levels. Depletion of intracellular GSH by buthionine sulfoximine or by oxidative insult reduces GSHNE levels with a consequent increase in free HNE amounts and in cellular toxicity [47,48]. However, because GSHNE is an inhibitor of GST activity, the adduct needs to be removed from the cell by extrusion and/or intracellular metabolism [49,50].

As with HNE, GSHNE is susceptible to both oxidative and reductive transformations. Thus, aldose reductase reduces GSHNE to 1,4-dihydroxynonane–glutathione (GSDHN) [32], which has been shown to be involved in the proliferation and inflammation cell signaling cascades [51–53]. On the other hand, GSHNE can be oxidized by NAD-dependent aldehyde dehydrogenase to 4-hydroxynonanoic acid–glutathione (GSHNA) [54] or its lactone form [55]. These oxidized derivatives can be further metabolized by cytochrome P450 4A, with the subsequent production of ω-hydroxylated and carboxylated metabolites [38,55–57]. Finally, the glutathione moiety of these adducts can be transformed into mercapturic acid in the kidney and the resulting metabolites are excreted into urine [58–60].

The present study on human astrocytoma ADF cells highlights a highly efficient and specific dehydrogenase activity of carbonyl reductase 1 (CBR1) on GSHNE but not on HNE, which was never recognized and reported before. This novel activity of CBR1 is here discussed in terms of detoxification and recovery of cell reducing power.

## Materials and methods

### *Materials*

GSH, Cys, CysGly, γGluCys, bovine serum albumin, sodium dodecyl sulfate (SDS), 5,5′-dithiobis-(2-nitrobenzoic acid), *trans*-2-nonenal, $NADP^+$, NADPH, 9,10-phenanthrenquinone (9,10-PQ), and sequencing-grade trypsin were purchased from Sigma–Aldrich (St. Louis, MO, USA). 4-Oxononenal (ONE), prostaglandin $B_1$ ($PGB_1$), and 4-hydroxy-2-nonenal mercapturic acid were purchased from Cayman Chemicals (Ann Arbor, 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). RPMI 1640, penicillin, streptomycin, glutamine, and fetal bovine serum were obtained from Lonza (Basel, Switzerland). 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).

### *Cell cultures*

ADF cells were kindly provided by Dr. W. Malorni, Istituto Superiore di Sanità, Rome, Italy. ADF cells were cultured on 10-cm-diameter dishes in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 50 mU/ml penicillin/streptomycin, and 2 mM glutamine at 37 °C, in a humidified 5% $CO_2$ atmosphere. Cell plates were washed with a phosphate-buffered saline (PBS) (0.08% w/v NaCl, 0.002% w/v KCl, 0.002% w/v $KH_2PO_4$, 0.006% $Na_2HPO_4$), and cells were harvested with a scraper and stored as suspensions in PBS ($8 \times 10^7$ cells/ml) at −80 °C until use.

### *Assay of carbonyl reductase*

The activity of CBR1 was determined at 37 °C using GSHNE as the substrate to be oxidized. The reaction was monitored following the increase in absorbance at 340 nm due to the reduction of $NADP^+$ ($\varepsilon_{340}=6.22\ mM^{-1}\ cm^{-1}$). The reaction mixture contained 0.1 mM GSHNE, 0.18 mM $NADP^+$ in 50 mM sodium phosphate buffer, pH 8.4. One unit of enzyme activity is the amount that catalyzes the conversion of 1 μmol of substrate/min under the above assay conditions.

The dehydrogenase activity of CBR1 was evaluated as described above on various substrates at the reported concentrations (see Results). In the case of $PGB_1$, 2% ethanol was present in the assay mixture. The NADPH-dependent reductase activity of CBR1 was determined under the conditions described above on various substrates at the reported concentrations, following the decrease in absorbance at 340 nm due to the oxidation of 0.18 mM NADPH. In the case of 9,10-PQ and menadione, the assay mixture contained dimethyl sulfoxide (DMSO) at a fixed final concentration of 2 and 1.25% (v/v), respectively. DMSO and ethanol (2% final concentration) had no effect on the activity of CBR1, as measured under standard conditions. $K_M$ and $k_{cat}$ values were determined by linear regression analysis of kinetic data of double reciprocal plots using GraphPad software. The $k_{cat}$ values were calculated on the basis of a molecular mass of 31 kDa.

### *Purification of the GSHNE $NADP^+$-dependent dehydrogenase activity*

A GSHNE $NADP^+$-dependent dehydrogenase (GSHNE-DH) activity was purified from ADF cells. All procedures were carried out at 4 °C. Cell lysates were obtained through a freezing and thawing protocol, followed by centrifugation at 10,000 *g* for 30 min. The supernatant was diluted 1:1 with 4 mM dithiothreitol (DTT) in 10 mM sodium phosphate, pH 7, and referred to as the "crude extract." This was applied onto a DE-52 column (2.5 × 9 cm), which was eluted with 2 mM DTT in 10 mM sodium phosphate buffer pH 7 (SB), at a flow rate of 17 ml/h, collecting 3-ml fractions. SB containing 0.2 M NaCl was then applied. Fractions displaying activity were pooled and concentrated using an Amicon YM10 membrane. Thus, the concentrated protein solution was applied on a Sephacryl S200 column (1.6 × 80 cm), which was eluted with SB containing 0.1 M NaCl, at a flow rate of 20 ml/h, collecting 1.7-ml fractions. The active fractions were pooled and applied on a Blue-Sepharose column (1.2 × 8 cm) equilibrated with SB at 10 ml/h; column elution was performed with SB supplemented with 0.1 mM $NADP^+$ and NaCl at various concentrations (see details in supplementary materials), collecting 1.5-ml fractions. Fractions displaying GSHNE-DH activity were pooled, concentrated to a protein concentration of 0.01 mg/ml using an Amicon YM10 ultrafiltration membrane, and stored at 4 °C until used.

### *Synthesis of aldehydes*

Diethyl acetals of HNE and 4-hydroxy-2-noninal were prepared according to a previous procedure [61]. 4-Hydroxy-2-nonanal diethyl acetal was prepared by catalytic hydrogenation (PtO in ethyl acetate) of 4-hydroxy-2-noninal diethyl acetal [62]. Free aldehydes were prepared by acid hydrolysis (pH 3.0) of the diethylacetals for 1 h, at 4 °C. The concentration of HNE and *trans*-2-nonenal was determined by measuring the absorbance at 224 nm using an extinction coefficient of $13.7\ mM^{-1}\ cm^{-1}$ [61].