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Medium-chain fatty acids and glutathione derivatives as inhibitors of S-nitrosoglutathione reduction mediated by alcohol dehydrogenase 3

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ABSTRACT

Alcohol dehydrogenase 3 (ADH3) has emerged as an important regulator of protein S-nitrosation in its function as S-nitrosoglutathione (GSNO) reductase. GSNO depletion is associated with various disease conditions, emphasizing the potential value of a specific ADH3 inhibitor. The present study investigated inhibition of ADH3-mediated GSNO reduction by various substrate analogues, including medium-chain fatty acids and glutathione derivatives. The observed inhibition type was non-competitive. Similar to the Michaelis constants for the corresponding ω-hydroxy fatty acids, the inhibition constants for fatty acids were in the micromolar range and showed a clear dependency on chain length with optimal inhibitory capacity for eleven and twelve carbons. The most efficient inhibitors found were undecanoic acid, dodecanoic acid and dodecanedioic acid, with no significant difference in inhibition constant. All glutathione-derived inhibitors displayed inhibition constants in the millimolar range, at least three orders of magnitudes higher than the Michaelis constants of the high-affinity substrates GSNO and S-hydroxymethylglutathione. The experimental results as well as docking simulations with GSNO and S-methylglutathione suggest that for ADH3 ligands with a glutathione scaffold, in contrast to fatty acids, a zinc-binding moiety is imperative for correct orientation and stabilization of the hydrophilic glutathione scaffold within a predominantly hydrophobic active site.

1. Introduction

Alcohol dehydrogenase 3 (ADH3) exhibits dual functions as glutathione-dependent formaldehyde dehydrogenase and S-nitrosoglutathione (GSNO) reductase [1,2]. By oxidizing S-hydroxymethylglutathione (HMGSH), the spontaneous glutathione adduct of formaldehyde, ADH3 is a central player in the detoxification of formaldehyde [3–5]. Through the irreversible GSNO reductase activity, ADH3 has been shown to affect the transnitrosation equilibrium between GSNO and S-nitrosated proteins, arguing for an important role in nitric oxide homeostasis [1,6–9]. Importantly, GSNO depletion associates with medical conditions as diverse as cerebral ischemia, Alzheimer's disease, sclerosis, and asthma [9–13]. Genetic and functional evidence suggests a deleterious effect of ADH3 activity under asthmatic conditions where ADH3 mediates the reductive inactivation of GSNO, a protective bronchodilator, in airway lining fluid [9,14]. Thus, a specific ADH3 inhibitor might be of direct clinical use for the prevention of asthma-exacerbating effects, and possibly for various other disease conditions.

Previous studies have identified dodecanoic acid, 4-pentylpyrazole, and 1,10-phenanthroline as inhibitors of ADH3-mediated alcohol oxidation activity [15–19]. Of those, 4-pentylpyrazole and 1,10-phenanthroline have been shown to equally inhibit other ADH classes, whereas dodecanoic acid, interacting with the ADH3-specific Arg114, has the potential to be a more specific inhibitor [20,21]. Notably, except for two reports on non-competitive inhibition by glutathione (GSH) at millimolar concentrations, inhibition of GSNO reduction activity has not been assessed previously [1,7]. Therefore, the present study investigated inhibition of ADH3-mediated GSNO reduction activity by fatty acids and fatty acid-derived compounds of different chain length as well as by GSH and GSH-derived compounds.

2. Materials and methods

2.1. Enzyme purification and chemicals

Recombinant human ADH3 was expressed in *Escherichia coli* and purified to homogeneity in a three-step procedure, essentially

Abbreviations: ADH3, alcohol dehydrogenase 3; GSH, glutathione; GSNO, S-nitrosoglutathione; HMGSH, S-hydroxymethylglutathione; MGSH, S-methylglutathione.

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as described previously [1,22]. Protein concentration was determined using the molar extinction coefficient of ADH3 at 280 nm (37 900 M^{-1} cm^{-1}) [1]. All chemicals were purchased from Sigma–Aldrich or Merck, unless stated otherwise. During experiments, GSNO, NADH and GSH solutions were protected from light and kept on ice.

2.2. Enzyme kinetics

Steady-state kinetics was performed in 0.1 M potassium phosphate buffer (pH 7.5) or 0.1 M glycine buffer (pH 10.0) containing 0.1 mM NADH or 2.4 mM NAD^+ for studies of reductive and oxidative reactions, respectively, unless stated otherwise in the corresponding table legends. Enzyme concentrations in the assays were 0.63–1.3 μg/ml (16–33 nM, per monomer) for inhibition of GSNO reduction and 14 μg/ml (360 nM) for ω-hydroxy fatty acid oxidation. GSNO concentrations were varied from 2.5 μM to 80 μM. Concentrations of ω-hydroxy fatty acids were varied from 100 μM to 1600 μM for 8-hydroxyoctanoic acid, from 20 μM to 300 μM for 10-hydroxydecanoic acid, and from 10 μM to 200 μM for 12-hydroxydodecanoic acid. At least three different inhibitor concentrations were used in each of the inhibition assays. Fatty acid concentrations were varied from 25 μM to 200 μM (except for octanoic acid, 100–400 μM; nonanoic acid, 50–300 μM; decanedioic acid, 50–250 μM), from 3 mM to 12.5 mM for GSH, from 1.3 mM to 6.3 mM for S-methylglutathione (MGSH), and from 1 mM to 2 mM for S-acetamidoglutathione. Enzymatic activity was monitored with a Hitachi U-3000 spectrophotometer by following NADH formation or consumption at 340 nm at room temperature (22 °C). Initial reaction velocities were calculated using the molar extinction coefficient for NADH (ε = 6220 M^{-1} cm^{-1}) or the additive one for NADH and GSNO (ε = 7060 M^{-1} cm^{-1}). Regression analysis was performed with the enzyme kinetics module of SigmaPlot 8.0 (SPSS). Kinetic parameters are derived from at least three experiments using the model for non-competitive inhibition.

2.3. Synthesis of S-acetamidoglutathione

S-acetamidoglutathione was synthesized by mixing 10 mM glutathione with 20 mM iodoacetamide in 10 mM ammonium bicarbonate and incubating overnight at room temperature (22 °C). Reactants and products were resolved on a strong anion-exchange column (Resource Q, GE Healthcare) and product peaks were identified by electrospray ionization mass spectrometry, as described previously [1,6]. Fractions including S-acetamidoglutathione were lyophilized overnight and dissolved in 500 μl water for inhibition experiments. Concentration of S-acetamidoglutathione was determined by amino acid analysis using a Biochrom 20 Plus ninhydrin-based analyzer (GE Healthcare).

2.4. Protein–ligand docking simulations

Protein–ligand docking simulations were performed with ICM 3.5 (Molsoft LLC). ICM applies the ECEPP/3 molecular mechanics force field for conformational energy computation and performs ligand docking by a global optimization of the energy function based on a Monte Carlo minimization algorithm [23–25]. The structure of human ADH3 (pdb: 1MC5) was obtained from the RCSB protein data bank and hydrogen atoms were added, followed by local energy minimization. Ligands were initially placed within the active site, using restraints derived from the structure with HMGSH (Zn, Pro56, Glu57, and Arg114). For each ligand, three docking simulations were performed omitting all ligand restraints and allowing for flexibility of the amino acid side chains constituting and surrounding the active site (amino acid residues 44–47, 54–58, 66, 91–95, 114, 173, 293 and 317) [26,27]. All docking simulations included 100 000 iterative Monte Carlo cycles, each followed by 100 steps of local energy minimization. The state with the lowest energy from each docking was further evaluated. For calculation of binding free energy, Coulomb and solvation energy contributions were calculated using the REBEL method, the side chain entropy term was calculated based on the exposed surface area of the flexible side chains and the hydrophobic energy change was calculated using a constant surface tension of 20 cal/$Å^2$ [28].

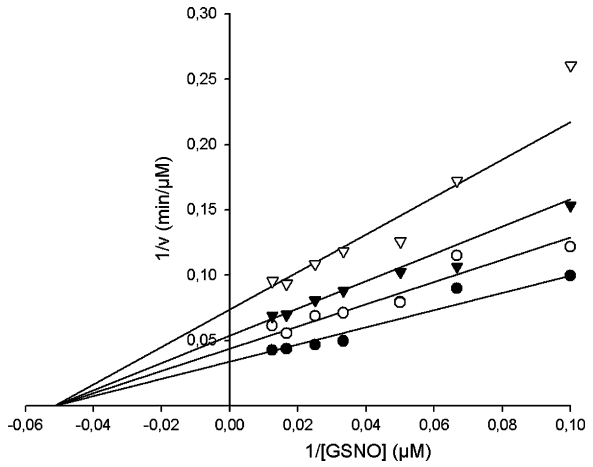

Fig. 1. Lineweaver–Burk plot demonstrating the non-competitive nature of inhibition of ADH3-mediated GSNO reduction by dodecanoic acid. Inhibitor concentrations were 0 μM (●), 50 μM (○), 100 μM (▼) and 200 μM (▽).

3. Results and discussion

In light of recent evidence suggesting a deleterious effect of ADH3 by its GSNO reductase activity, the present study assessed inhibition of ADH3-mediated GSNO reduction by analogues of two different substrate groups, ω-hydroxy fatty acids and glutathione-derived compounds.

3.1. Inhibition type

Dodecanoic acid had been previously investigated for inhibitory properties and shown to competitively inhibit ADH3-mediated alcohol oxidation with respect to HMGSH and 12-hydroxydodecanoic acid [16,29]. Here, with GSNO as variable substrate and a constant concentration of NADH, in most cases the data fit best to the model for non-competitive inhibition (Fig. 1). For alcohol dehydrogenase-catalyzed reactions, it is not uncommon that substrate analogues show competitive inhibition in one reaction direction but uncompetitive or non-competitive inhibition in the reverse direction. This observation is frequently explained by the inhibitor's preference for binding one of the two possible enzyme–cofactor complexes (the enzyme–NAD^+ or the enzyme–NADH complex) [29,30]. For the substrate analogues tested here, this suggests the formation of enzyme–inhibitor and enzyme–NAD^+–inhibitor complexes, in line with a random bi-bi mechanism, as proposed previously [29]. Also the model for uncompetitive inhibition provided a good, and for a few cases a slightly better, data fit. Irrespective of chosen inhibition model the results were qualitatively the same, but evaluation with the uncompetitive inhibition model resulted in lower K_i values (e.g. the K_i dropped from 177 μM to 101 μM for dodecanoic acid). The obtained K_i for dodecanoic acid is in both cases in reasonable agreement with the one from a previous study on 12-hydroxydodecanoic acid oxidation where the inhibition was of competitive nature (148 μM) [16]. Notably, the inhibition type is of importance for inhibitor-based drug development, as non-competitive and uncompetitive