Published in final edited form as:

Nitric Oxide. 2013 November 30; 35: 137–143. doi:10.1016/j.niox.2013.09.005.

IN VITRO ORGANIC NITRATE BIOACTIVATION TO NITRIC OXIDE BY RECOMBINANT ALDEHYDE DEHYDROGENASE 3A1

Shunxin Lin^a, Nathaniel A. Page^a, Sun Mi Fung^a, and Ho-Leung Fung^a

^aDepartment of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York, Buffalo, New York 14214, United States

Abstract

Organic nitrates (ORNs) are commonly used anti-ischemic and anti-anginal agents, which serve as an exogenous source of the potent vasodilator nitric oxide (NO). Recently, both mitochondrial aldehyde dehydrogenase-2 (ALDH2) and cytosolic aldehyde dehydrogenase-1a1 (ALDH1A1) have been shown to exhibit the ability to selectively bioactivate various ORNs *in vitro*. The objective of the present research was to examine the potential role of ALDH3A1, another major cytosolic isoform of ALDH, in the *in vitro* bioactivation of various ORNs, and to estimate the enzyme kinetic parameters toward ORNs through mechanistic modeling. The extent of bioactivation was assayed by exposing recombinant ALDH3A1 to various concentrations of ORNs, and measuring the concentration-time profiles of released NO via a NO-specific electrode. Metabolite formation kinetics was monitored for nitroglycerin (NTG) using LC/MS/MS. Our results showed that ALDH3A1 mRNA and protein were highly expressed in C57BL/6 mouse aortic, cardiac, and hepatic tissues, and it was able to release NO from several ORNs, including NTG, isosorbide dinitrate (ISDN), isosorbide-2-mononitrate (IS-2-MN), and nicorandil with similar $V_{\max}$ (0.175 – 0.503 nmol/min/mg of ALDH3A1), and K_m values of 4.01, 46.5, 818 and $5.75 \times 10^3 \mu\text{M}$ respectively. However, activation of isosorbide-5-mononitrate (IS-5-MN) by ALDH3A1 was undetectable *in vitro*. ALDH3A1 was also shown to denitrate NTG, producing primarily glyceryl 1, 2-dinitrate (1, 2-GDN) in preference to glyceryl 1, 3-dinitrate (1, 3-GDN). Therefore, ALDH3A1 may contribute to the bioactivation of ORNs *in vivo*.

Keywords

Organic nitrates; Aldehyde dehydrogenase 3A1; Nitric oxide; Kinetics

© 2013 Elsevier Inc. All rights reserved.

Address for correspondence: Ho-Leung Fung, Ph.D., Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York, 372 Kapoor Hall, Buffalo, NY 14214-8033, Telephone: +1 7166454843, Fax: +1 7168296569, hlfung@buffalo.edu.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Shunxin Lin, Nathaniel Page, Ho-Leung Fung

Conducted experiments: Shunxin Lin, Nathaniel Page, Sun Mi Fung

Contributed new reagents or analytic tools: Shunxin Lin, Nathaniel Page

Performed data analysis: Shunxin Lin, Nathaniel Page, Sun Mi Fung

Wrote or contributed to the writing of the manuscript: Shunxin Lin, Nathaniel Page, Ho-Leung Fung

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. INTRODUCTION

Organic nitrates (ORNs), including nitroglycerin (NTG), isosorbide dinitrate (ISDN), isosorbide-5-mononitrate (IS-5-MN), nicorandil and pentaerythritol tetranitrate have been widely used as anti-ischemic and anti-anginal agents. They are chemically and enzymatically activated to produce nitric oxide (NO), which then activates soluble guanylyl cyclase (sGC) in the vascular smooth muscle cells. The activated sGC catalyzes GTP to cGMP, leading to vasorelaxation. Although NTG has been used clinically for over 130 years, the mechanisms of ORN bioactivation and tolerance are still incompletely understood.

In 2002, Chen and Stamler first demonstrated that mitochondrial aldehyde dehydrogenase (ALDH2) was able to bioactivate NTG to release NO [1; 2]. These results were later confirmed by an *ex vivo* aortic relaxation assay [3; 4]. Recently, Berretta et al. [5] showed that in mouse aorta, NTG bioactivation was mainly contributed by cytosolic rather than mitochondrial ALDH2. This enzyme however was not responsible for the bioactivation of IS-5-MN or ISDN [3].

A purified cytosolic ALDH isoform, ALDH1A1, has also been shown to activate NTG *in vitro* [6]. However, it was shown that the relaxation potencies of NTG and IS-5-MN were identical in ALDH1A1 wildtype and knockout mice [7], suggesting that this isoform may not be relevant to mediate ORN bioactivation *in vivo*.

A third ALDH isoform, ALDH3A1 is a cytosolic homodimer containing 453 amino acids, sharing about 25% sequence identity with ALDH2 and ALDH1A1 [8]. The catalytic active site of ALDH3A1 resides at the cysteine residue at the 243 position [9]. ALDH3A1 protects cells against lipid peroxidation products via oxidation of reactive aldehydes to their corresponding carboxylic acids, and it is present in the cornea of most mammalian species where it protects against oxidative damage mediated by UV radiation [10]. In addition, ALDH3A1 is of interest in the field of oncology due to its overexpression in many tumors where it mediates chemoresistance to cyclophosphamide derivatives via metabolic inactivation of the active metabolites of these drugs [11].

The role of ALDH3A1 in the bioactivation of ORNs has not been previously examined. Therefore, the present research is aimed at determining the potential role of ALDH3A1 in the *in vitro* bioactivation of several ORNs to NO and the estimation of the corresponding enzyme kinetic parameters (K_m and V_{max}). Due to the rapid degradation of NO and the ability of ORNs to inactivate their metabolizing enzymes, traditional methods of estimating Michaelis–Menten enzyme kinetics, such as utilizing the Lineweaver–Burk plot are not feasible. Mathematical modeling was therefore carried out to develop a suitable biochemical kinetic model for the generation of meaningful comparative kinetic parameters.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

NTG (10% in lactose) was purchased from Copperhead Chemical Company Inc. (Tamaqua, PA). Glycerol 1, 2-dinitrate (1, 2-GDN) and glycerol 1, 3-dinitrate (1, 3-GDN) were purchased from Cerilliant (Austin, TX). 1, 2, 4-Butanetriol-1, 4-dinitrate (BTDN) was obtained from Absolute Standards, Inc. (Hamden, CT). Isosorbide-2-mononitrate (IS-2-MN), IS-5-MN and ISDN were obtained from Schwarz Pharma (Monheim, Germany). Nicorandil was purchased from Tocris Bioscience (Ellisville, Missouri). HPLC grade water and methanol were obtained from Burdick & Jackson (Muskegon, MI). Ammonium chloride was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). The following items were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO): dithiothreitol (DTT),

superoxide dismutase (SOD), nicotinamide adenine dinucleotide phosphate (NADP⁺), nicotinamide adenine dinucleotide (NAD⁺), glutathione, isopropyl β -D-1-thiogalactopyranoside, methanol, magnesium chloride, disodium hydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), beta-mercaptoethanol, benzaldehyde, glycine, tween-20 and tris base.

2.2 Animals

Adult male C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) were anaesthetized with ketamine (90 mg/kg) and xylazine (9 mg/kg) by IP injection, and aorta, heart, and liver tissues were removed immediately after sacrifice. The isolated tissues were rapidly frozen at -80°C . All experiments were conducted with the approval of the University at Buffalo Institutional Animal Care and Use Committee.

2.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from the collected mouse tissues using the SV Total RNA Isolation System (Promega, part # TM048, Madison, WI). RNA purity and concentration were measured using a NanoDrop UV/Vis spectrophotometer (Thermo Scientific, Wilmington, DE). First strand cDNA was synthesized via reverse transcription using StrataScriptTM reverse transcriptase. PCR analysis was performed using murine ALDH1A1, ALDH2, and ALDH3A1 PCR primers and RT² SYBR Green qPCR Master Mix, as described by the manufacturer (Qiagen, Frederick, MD). Finally, the results were normalized with Alien RNA technology (Agilent Technologies, Santa Clara, CA).

2.4 Western Blot Analysis

Mouse aortic tissue was homogenized with a Kontes glass homogenizer in ice-cold 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (50 mM HEPES, 70 mM sucrose, 220 mM mannitol, and 1 mM ethylene glycol tetraacetic acid, pH 7.4) containing 10% (v/v) of a commercial protease inhibitor cocktail (Sigma #P2714) and 2% triton X-100. The crude homogenate was centrifuged at $3000 \times g$ for 10 minutes to precipitate cellular debris. The supernate was retained and protein content was quantified using the Lowry assay [12] with bovine serum albumin as the standard. Western blot analysis was performed using 10 μg total protein supernate via 4–15% Tris-HCl sodium dodecyl sulfate-polyacrylamide gel electrophoresis – SDS-PAGE (Bio-Rad Laboratories, Hercules, CA) and with electroblotting onto nitrocellulose membrane. The membrane was blocked with 1% bovine serum albumin and probed with rabbit anti-murine ALDH3A1 antibody (diluted 1:1000, Abcam, Cambridge, MA). Antibody binding was detected using an anti-rabbit antibody horseradish peroxidase conjugate (diluted 1:12,500, GE Healthcare UK Limited, UK). Protein visualization was performed using enhanced chemiluminescence substrates as described by the manufacturer (Pierce ECL Western Blotting Substrate, Thermo Scientific) and recorded with a Kodak 2000 mm Image Station.

2.5 Cloning, Expression and Purification of Recombinant Murine ALDH3A1

A pET30a expression vector containing murine ALDH3A1 cDNA was kindly provided by Dr. R.A. Prough of the University of Louisville (Louisville, KY). The vector was transfected into BL21-Gold (DE3) pLysS competent *Escherichia coli* cells (Stratagene, La Jolla, CA) by heat shock at 42°C for 30 seconds. The *E. coli* cells were initially grown in lysogeny broth (LB), and then selected using agar plates with kanamycin (30 $\mu\text{g}/\text{ml}$) and chloramphenicol (10 $\mu\text{g}/\text{ml}$). A single colony was selected and cultured in 5 ml of LB containing kanamycin (30 $\mu\text{g}/\text{ml}$) and chloramphenicol (10 $\mu\text{g}/\text{ml}$) at 37°C with agitation overnight. For production of recombinant ALDH3A1, the plasmid containing bacteria were cultured in one liter of LB containing kanamycin (30 $\mu\text{g}/\text{ml}$) and chloramphenicol (10 $\mu\text{g}/\text{ml}$)

ml) until the $OD_{600} \approx 0.6$. The recombinant ALDH3A1 expression was induced by addition of 0.4 mM isopropyl β -D-1-thiogalactopyranoside overnight at room temperature. Cells were lysed by Bugbuster protein extraction reagent (Novagen, Darmstadt, Germany) containing 2 mM dithiothreitol and 10% glycerol, and 125 units of benzonase nuclease per gram of wet cell pellet. The produced rALDH3A1 with its his-tag N-terminal was purified using a nickel-charged nitrilotriacetic acid (Ni-NTA) column (Thermo Scientific, Rockford, IL) and concentrated by ultrafiltration with a molecular cutoff of 30 kDa. The removal of imidazole was performed using a Zeba Spin desalting column (10 ml column, Thermo Scientific, Rockford, IL). Protein concentration was measured using the Lowry assay with bovine serum albumin as the standard. Protein purity was monitored using SDS-PAGE stained with coomassie blue.

2.6 Determination of rALDH3A1 Dehydrogenase Activity

The dehydrogenase activity of rALDH3A1 was measured spectrophotometrically by monitoring the reduction of $NADP^+$ at 340 nm (molar absorptivity₃₄₀ = 6220 M⁻¹ cm⁻¹) in the presence of various concentrations of the substrate benzaldehyde, at room temperature as previously described [13]. The incubation buffer was 50 mM phosphate buffered saline (PBS, pH 7.4), containing 1 mM $NADP^+$, 1 mM EDTA and 0.01% beta-mercaptoethanol. The Michaelis-Menten enzyme kinetic constants for dehydrogenase activity were estimated using SigmaPlot 12 (Systat Software, San Jose, CA) by fitting the data into a one-site saturation ligand binding equation.

2.7 Measurement of Denitration of NTG by rALDH3A1 Using LC/MS/MS

Denitration of NTG was measured using liquid chromatography tandem mass spectrometry with electrospray ionization as previously described by Miyayama et al. [14]. Standard solutions containing NTG, 1, 2-GDN, 1, 3-GDN and the internal standard BTDN were dissolved in 1:1 v/v PBS (50 mM, pH 7.4): 100% methanol, with final concentrations of the three nitrates ranging from 0.375 to 15 ng/ml and BTDN at 2.5 ng/ml. A gradient mobile phase containing a mixture of solvent A (0.025 mM NH_4Cl in water) and solvent B (0.025 mM NH_4Cl in methanol) was used. For the determination of initial rates of dinitrate formation, NTG at 1, 2.5, 5, 10, 25, 100, 250, and 500 μ M was incubated in 50 mM PBS, pH 7.4 (final volume of 300 μ l) at 37°C with 4 μ g of purified rALDH3A1, 0.2 mM $NADP^+$, 0.4 mM GSH, and 0.4 mM DTT. Samples of 30 μ l were removed at 0 and 2 minutes and immediately mixed with 30 μ l of 5 μ g/ml BTDN in 100% methanol. The samples were then centrifuged at 4°C, 16,000 $\times$ g for 10 minutes to remove protein precipitates. An aliquot of 20 μ l of the supernate was injected into the LC/MS/MS for the measurement of nitrate concentrations. Mass/charge ratios of precursor ions for NTG, 1, 2-GDN, 1, 3-GDN and BTDN were 262, 217, 217 and 231 with retention times of 11.82, 8.68, 7.77 and 9.22 minutes, respectively. The time course of NTG denitration and dinitrate formation were studied via incubation of 0.1 μ M of NTG over 120 minutes in the same reaction mixture described above.

2.8 Measurement of *in vitro* Activation of ORNs by rALDH3A1 Using an NO Electrode

NO formation was measured using an amiNO-700 Nitric Oxide Sensor (Innovative Instruments, Inc. FL) at 37°C, using the method described recently by Tsou et al. [15]. Various concentrations of ORNs were added to a solution containing 500 mM triethanolamine (pH 7.4), 1000 units/ml of SOD, 2 mM DTT, 2 mM of $NADP^+$, 5 mM $MgCl_2$, maintained at 37°C and with a total volume of 2 ml. The reaction was initiated by the addition of 0.15 mg/ml of pre-warmed (at 37°C) rALDH3A1. The time-dependent NO concentrations generated from various ORNs were then monitored. The system was calibrated daily with acidified nitrite as described by the manufacturer.

2.9 Estimation of rALDH3A1 Kinetic Parameters via Mechanistic Modeling of NO Kinetics

When examining the enzyme kinetics of ORN metabolism in relation to NO-generation several additional reactions must be accounted for (Fig. 1). First, NO degrades rapidly in solution with a half-life of approximately 5.6 seconds [16], so its kinetics of degradation must be considered. Second, ORNs have been known to inactivate cysteine-containing enzymes, both reversibly and irreversibly [1; 17; 18; 19], and it is not known whether the rate constant for enzyme inactivation and that for enzyme regeneration are identical for each ORN. These rate constants, together with those of the classical Michaelis-Menten parameters of reversible formation of the reaction complex (RC), and the catalytic rate of product formation must be used together to generate meaningful comparative estimates of K_m and V_{max} from the various ORNs.

The differential equations used to describe the time-dependent concentration change of each molecular species shown in Fig. 1 are as follows:

$$\frac{d[ORN]}{dt} = (k_{-1} * [RC]) - (k_1 * [ORN] * [ALDH]) \quad (1)$$

$$\frac{d[ALDH]}{dt} = ((k_{-1} + k_{cat}) * [RC]) + k_r * [ALDH_{inact}] - (k_1 * [ORN] * [ALDH]) \quad (2)$$

$$\frac{d[RC]}{dt} = (k_1 * [ORN] * [ALDH]) - ((k_{-1} + k_{cat} + k_{inact}) * [RC]) \quad (3)$$

$$\frac{d[NO]}{dt} = (k_{cat} * [RC]) - (k_{deg} * [NO]) \quad (4)$$

$$\frac{d[ALDH_{inact}]}{dt} = k_{inact} * [RC] - k_r * [ALDH_{inact}] \quad (5)$$

In these equations, [ALDH], [RC], and [ALDH_{inact}] are the concentrations of effective enzyme in the reaction, ORN/enzyme reaction complex, and inactivated enzyme, respectively. The rate constants k_1 and k_{-1} are second order association and first order dissociation rate constants of ORN to rALDH3A1. k_{inact} is the first order enzyme inactivation constant, k_r is the first order enzyme reactivation constant, and k_{cat} is the first order catalytic constant of conversion of ORN into NO. NO elimination is described by first order degradation rate constant k_{deg} .

The initial condition for Eq.1 was set to the added concentrations of ORNs, while initial RC, NO, and ALDH_{inact} concentrations were set to zero. The initial concentration of effective ALDH was estimated by fitting because it was not known whether all the added rALDH3A1 were metabolically active or if all the active sites were accessible for reaction. Since k_{deg} is independent of the ORNs added, it was fixed at 1.16 min^{-1} based on results from preliminary experiments. ORN and ALDH binding affinity K_m and maximum rate of NO generation V_{max} were determined as secondary parameters using the following standard equations [20]:

$$K_m = \frac{k_{-1} + k_{cat}}{k_1} \quad (6)$$

$$V_{max} = [ALDH]_0 * k_{cat} \quad (7)$$

2.10 Data Analysis

Data were presented as mean $\pm$ standard deviations. NO concentration-time profiles from NTG, ISDN, IS-2-MN and nicorandil were used for parameter estimation. Points were selected from the averaged NO concentrations observed during the reaction between ORNs and rALDH3A1 at a given initial ORN concentration. NO concentration-time profiles were also normalized with the initial ORN concentration before kinetic analysis. The added rALDH3A1 concentration was 1.5 mg/ml, or approximately 29 μ M.

ADAPT 5 Pharmacokinetic/Pharmacodynamic Systems Analysis Software (Biomedical Simulations Resource, Los Angeles, 2009) was used for model development. All parameters were estimated using maximum likelihood. The variance model was defined as: $VAR_i = (\sigma_{inter} + \sigma_{slope} \times Y(i))^2$, where VAR_i was the variance of the i th data point, σ_{inter} and σ_{slope} were the variance model parameters, and $Y(i)$ is the i th predicted value from the pharmacokinetic model. The goodness-of-fit was assessed by Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), coefficient of variation (CV), and visual inspection.

The $\Delta\Delta C_t$ method (as described by the manufacturer, Qiagen, Frederick, MD) and one-way ANOVA followed by Bonferroni correction were performed for RT-PCR data using SPSS 19 (SPSS Inc. Chicago IL). Peak and cumulative NO concentrations were obtained using Non-Compartmental Analysis in Phoenix WinNonlin 6.1 (Pharsight, Mountain View, CA).

3. RESULTS

3.1 ALDH Isoform mRNA and Protein Expression in C57BL/6 Mice

mRNA expression of three isoforms of ALDH, viz., 1A1, 2 and 3A1 were assessed in various mouse tissues (Fig. 2), and the results were normalized against mRNA expression of ALDH2 in the aorta. We did not normalize the expression results against a house-keeping gene (e.g., GAPDH) because its expression was not identical in different tissues, and we believe that it would be more informative to compare the expression levels vs. the most prevalently accepted nitrate metabolizing ALDH isoform, viz., ALDH2. ALDH1A1 expression was high in the liver, but not detectable in aortic tissues. In contrast, mRNA expressions of ALDH2 and ALDH3A1 were quite similar in aorta tissue. Preliminary Western blot analysis showed detectable ALDH3A1 protein expression in aorta (Fig. 2 insert). Mouse heart expressed all three isoforms of ALDH examined.

3.2 Dehydrogenase and Denitration Activities of Recombinant ALDH3A1

The identity of the rALDH3A1 prepared according to the described method has been previously verified [13], and the present preparation exhibited a single protein band of 54 kDa in SDS-PAGE analysis (data not shown). The dehydrogenase activity of the prepared recombinant ALDH3A1 was found as follows: $V_{max} = 30.3 \pm 3.3 \mu\text{mol/min/mg}$, and $K_m = 138 \pm 28 \mu\text{M}$ ($n=3$ experiments). The kinetics of rALDH3A1-mediated denitration of NTG and production of 1, 2-GDN and 1, 3-GDN were determined using LC/MS/MS. The 1, 2-dinitrate metabolite was shown to be preferentially formed vs. the 1, 3-GDN isomer (73 nM vs. 14 nM produced in 120 min, respectively, at the initial NTG concentration of 100 nM,

Fig. 3A). At this substrate concentration, the half-life of rALDH3A1-mediated NTG disappearance was estimated at 10.1 ± 1.5 min. We have shown in previous studies, using identical experimental conditions, that enzyme-free preparations in the same medium did not cause any significant NTG degradation [15].

The initial rates of dinitrate formation by rALDH3A1 at various added concentrations of NTG were measured over the first two minutes of the reaction, during which linear metabolite formation was observed. Fig. 3B shows that the initial rate of 1, 2-GDN formation was saturable, with $K_m = 6.13 \pm 0.90 \mu\text{M}$ and $V_{\max} = 147 \pm 5 \text{ pmol/min/mg}$. The goodness of fit was satisfactory, as judged by a correlation coefficient $R^2 = 0.980$ and coefficients of variation of 14.6% for K_m and 3.23% for $V_{\max}$, respectively. In contrast, the kinetics for 1, 3-GDN production appeared to be more complex (Fig. 3B) and could not be adequately described by the Michaelis-Menten equation. No further attempts were made to describe the 1, 3-GDN kinetic data mathematically. The ratio of 1, 3-GDN/1, 2-GDN production was approximately 0.1 up to $100 \mu\text{M}$ of added NTG. However, at 250 and $500 \mu\text{M}$ NTG, the formation of 1, 3-GDN increased significantly relative to 1, 2-GDN.

3.3 *In vitro* NO-generating Activity of Recombinant ALDH3A1 towards Various ORNs

Under the conditions studied, rALDH3A1 was shown to generate NO from NTG, ISDN, IS-2-MN and nicorandil (Fig. 4A–4D), but negligible NO release was observed from IS-5-MN (data not shown). The NO concentration data at each time point were reproducible, with average CV% of 19.7% (range of 8.41% to 39.5%) for the triplicate experiments. Our results showed that, in spite of the different sensitivity of ORNs toward the enzyme, a maximum NO concentration of slightly higher than 150 nM was produced at the highest ORN concentrations. Visual analysis of the curves also suggested that the disappearance of NO from the system appeared to be slower when higher initial concentrations of ORNs were used, particularly for NTG and ISDN. Intriguingly, the cumulative NO produced, when plotted against the initial ORN concentration added (Fig. 5), showed a bell-shaped curve for NTG, ISDN and nicorandil, exhibiting decreased cumulative NO production at higher ORN concentrations after a maximum had been observed. For IS-2-MN, cumulative NO production appeared to be relatively constant over the ORN concentration range studied.

3.4 Estimation of rALDH3A1 Kinetic Parameters toward ORNs

The reaction scheme shown in Fig. 1 was able to capture the NO generation time profiles of NTG, ISDN, IS-2-MN, and nicorandil satisfactorily (Fig. 4A–4D). All the parameters, except for k_r for ISDN, were well estimated with CV% of less than 20% (Table 1). Therefore, based on both objective criteria and visual inspection, it was concluded that the reaction scheme proposed could adequately describe the NO concentration vs. time profiles observed for all ORNs studied. Our kinetic model estimated that NTG reacted with rALDH3A1 with the highest affinity ($K_m = 4.01 \mu\text{M}$), and ISDN, IS-2-MN and nicorandil exhibited lower enzyme affinity toward rALDH3A1, with K_m values of 46.5, 818, and $5.75 \times 10^3 \mu\text{M}$ respectively. All these four ORNs were estimated to have similar maximal rate of NO generation when reacting with rALDH3A1, yielding $V_{\max}$ values of $0.175 - 0.503 \text{ nmol/min/mg}$ of protein.

4. DISCUSSION

Because ALDH3A1 is present in the smooth muscle cells of various rat vascular tissues including carotid and basilar arteries, vena cava, and jugular vein[18] and because of its sequence similarity to ALDH1A1 and ALDH2, it is possible that ALDH3A1 may be an important metabolic enzyme for the *in vivo* activation of ORN. The present *in vitro* studies demonstrate, for the first time, that ALDH3A1 mRNA is expressed in C57BL/6 mouse

aorta, heart and liver tissues, and its protein is present in the aorta. In addition, similar to ALDH2 and ALDH1A1, rALDH3A1 can denitrate NTG to produce 1, 2-GDN and 1, 3-GDN. Under the experimental conditions utilized, we found that rALDH3A1 was able to activate NTG, ISDN, IS-2-MN and nicorandil and release NO *in vitro*, but it exhibited poor activity toward IS-5-MN.

The dehydrogenase activity of the prepared recombinant ALDH3A1 exhibited $V_{\max}$ value ($30.3 \pm 3.3 \mu\text{mol/min/mg}$) compared to that reported in the literature ($28.6 \pm 1.6 \mu\text{mol/min/mg}$) [13]. However, our K_m value was approximately 3.5 times higher ($138 \pm 28 \mu\text{M}$ vs. $37 \pm 6 \mu\text{M}$). This difference could possibly be due to a number of factors, including different experimental conditions and reagents, and possible partial protein aggregation. Denitration of NTG produces glyceryl dinitrates and mononitrates, inorganic NO_x^- ions, and NO [21]. Production of 1, 2-GDN is associated with vasorelaxation and tolerance induction [22; 23; 24], thus it has been used as an index of the “mechanism-based metabolic pathway”. Production of 1, 3-GDN is thought to be unrelated to relaxation and tolerance, the formation of which presumably reflects the “clearance-based metabolism” [25; 26].

In this study, we measured both dinitrate concentrations from NTG denitration using LC/MS/MS and NO generations from different ORNs. The results revealed that rALDH3A1-catalyzed NTG metabolism producing 1, 2-GDN as the predominant metabolite. The ratio of the cumulative amounts of 1, 2-GDN to 1, 3-GDN formed, measured at 120 minutes, after the reaction between 100 nM of NTG and rALDH3A1, was 5.21 (Fig. 3A) compared to the observed ratios of 9.0 and 4.98 for rALDH1A1 [7] and ALDH2 purified from mouse macrophage cells [1], respectively, at the same added NTG concentration. Thus, 1, 2-GDN is the primary metabolite of NTG metabolism by all three isoforms of ALDH. However, this metabolite specificity diminishes at increasing NTG concentrations. Using initial rate measurements over 2 minutes, we found that, for rALDH3A1, the amount of 1, 2-GDN produced was 10 times more than 1, 3-GDN up to 100 μM of NTG, but formation rate of 1, 3-GDN increased significantly when the NTG concentration was higher than 250 μM . The same phenomenon was also observed in the denitration of NTG by rALDH1 and rALDH2 [6]. However, formation of 1, 3-GDN did not exceed 1, 2-GDN formation even at 1 mM and 0.5 mM NTG for rALDH2 and rALDH3A1, respectively, while 1, 3-GDN formation predominated in the rALDH1A1 catalyzed reaction at NTG 1 mM.

The rate of 1, 2-GDN formation from rALDH3A1 obeyed classic Michaelis-Menten kinetics with $K_m = 6.13 \pm 0.90 \mu\text{M}$ and $V_{\max} = 147 \pm 5 \text{ pmol/min/mg}$, supporting the hypothesis of enzymatic generation rather than chemical denitration [1; 27]. On the other hand, the rate of 1, 3-GDN formation (Fig. 3B) indicates a more complex mechanism, indicating the likelihood of multiple pathways. Similar patterns of 1, 3-GDN formation by rALDH1 and rALDH2 were previously observed [6], who also demonstrated that its formation was not affected by substrate-competitive inhibitors. Under similar conditions, the reaction of NTG with rALDH3A1 was three times faster than that with rALDH1A1 ($T_{1/2} = 10.1 \pm 1.5 \text{ min}$ vs. $T_{1/2} = 27.5 \pm 2.3 \text{ min}$), suggesting that NTG may be a better substrate for ALDH3A1 when compared to ALDH1A1.

The biochemical mechanism of ORN bioactivation by ALDH remains incompletely understood, but it is hypothesized that a thionitrate intermediate is formed upon interaction of nitro moiety of ORNs with thiol group(s) of metabolizing enzymes [28]. This thionitrate intermediate then undergoes further reactions to produce NO and oxidized thiol end-products. Free radical intermediates that are formed in the protein can either interact with each other to inactivate the enzyme, or proceed to generate oxygen species and superoxides that lead to enhanced degradation of NO [29]. Thus, the net amount of NO that was measured was derived from a number of reactions, including its formation, its degradation

and the partial inactivation of the enzyme. Determination and comparison of the enzyme kinetic parameters, viz., K_m and V_{max} must take these processes into consideration. The final estimated k_{-1} , k_{inact} , k_r , and k_{cat} values were similar among all ORNs studied (Table 1), supporting the view that once a common intermediate was formed when different ORNs interacted with rALDH3A1, the same down-stream reactions would occur. On the other hand, the estimated k_1 values are several orders of magnitude smaller than the other parameters, which indicates that formation of this intermediate is the rate-limiting step of the reaction. For the ORNs studied, the magnitudes of k_1 are in the following descending order, NTG > ISDN > IS-2-MN > nicorandil ($1.56 \times 10^{-4} > 6.84 \times 10^{-5} > 1.11 \times 10^{-6} > 2.43 \times 10^{-7} \text{ min}^{-1} \text{ nM}^{-1}$). When the Michaelis-Menten kinetic parameters were compared, it was observed that NTG possessed the smallest estimated K_m value, reflecting its highest affinity to the enzyme among the ORNs studied. In addition, the estimated K_m and V_{max} values of NTG derived from the model were similar to those calculated from 1, 2-GDN formation from NTG denitration by rALDH3A1 (Fig. 3B). The model estimated K_m and V_{max} values to be $4.01 \mu\text{M}$ and $0.191 \text{ nmol/min/mg}$, while the corresponding values from denitration reaction are $6.13 \mu\text{M}$ and $0.147 \text{ nmol/min/mg}$, respectively. The small discrepancies between the estimations may be due to the different experimental methods and conditions utilized between the two studies. It is also uncertain whether 1, 2-GDN and NO are produced at an equal molar ratio. The estimated effective rALDH3A1 concentration was $16.3 \mu\text{M}$, which indicated that our enzyme preparation might not be completely pure or that not all active sites of the enzyme were accessible to ORNs. In conclusion, we have demonstrated that rALDH3A1 can denitrate NTG, and produced NO selectively from various ORNs. Comparative enzyme parameters were estimated for several ORNs, based on known mechanisms of NO degradation and reversible and irreversible enzyme inactivation. Our kinetic model may be applicable for the quantitative comparison of enzyme kinetics for the bioactivation of different ORNs by different bioactivating enzymes.

Acknowledgments

The authors would like to thank Donna M Ruszaj for her help on LC/MS/MS experiment, Diane Letina for her assistance on RNA work, and Dr. Joseph P Balthasar and Dr. Yanguang Cao for their advice on the mathematical modeling of kinetic data. This work was supported in part by NIH grant HL081580.

References

1. Chen Z, Zhang J, Stamler JS. Identification of the enzymatic mechanism of nitroglycerin bioactivation. *Proc Natl Acad Sci U S A*. 2002; 99:8306–11. [PubMed: 12048254]
2. Chen Z, Foster MW, Zhang J, Mao L, Rockman HA, Kawamoto T, Kitagawa K, Nakayama KI, Hess DT, Stamler JS. An essential role for mitochondrial aldehyde dehydrogenase in nitroglycerin bioactivation. *Proc Natl Acad Sci U S A*. 2005; 102:12159–64. [PubMed: 16103363]
3. Daiber A, Oelze M, Coldewey M, Bachschmid M, Wenzel P, Sydow K, Wendt M, Kleschyov AL, Stalleicken D, Ullrich V, Mulsch A, Munzel T. Oxidative stress and mitochondrial aldehyde dehydrogenase activity: a comparison of pentaerythritol tetranitrate with other organic nitrates. *Mol Pharmacol*. 2004; 66:1372–82. [PubMed: 15331769]
4. Daiber A, Wenzel P, Oelze M, Schuhmacher S, Jansen T, Munzel T. Mitochondrial aldehyde dehydrogenase (ALDH-2)—maker of and marker for nitrate tolerance in response to nitroglycerin treatment. *Chem Biol Interact*. 2009; 178:40–7. [PubMed: 18834868]
5. Beretta M, Wolkart G, Scherthaner M, Griesberger M, Neubauer R, Schmidt K, Sacherer M, Heinzel FR, Kohlwein SD, Mayer B. Vascular bioactivation of nitroglycerin is catalyzed by cytosolic aldehyde dehydrogenase-2. *Circ Res*. 2012; 110:385–93. [PubMed: 22207712]
6. Beretta M, Gruber K, Kollau A, Russwurm M, Koesling D, Goessler W, Keung WM, Schmidt K, Mayer B. Bioactivation of nitroglycerin by purified mitochondrial and cytosolic aldehyde dehydrogenases. *J Biol Chem*. 2008; 283:17873–80. [PubMed: 18450747]

7. Page, NA. Role of aldehyde dehydrogenase in the metabolism and action of organic nitrate vasodilators. Department of Pharmaceutical Sciences, University at Buffalo; Buffalo: 2011. p. 246
8. Perozich J, Nicholas H, Lindahl R, Hempel J. The big book of aldehyde dehydrogenase sequences. An overview of the extended family. *Adv Exp Med Biol.* 1999; 463:1–7. [PubMed: 10352663]
9. Wymore T, Nicholas HB, Hempel J. Molecular dynamics simulation of class 3 aldehyde dehydrogenase. *Chem Biol Interact.* 2001; 130–132:201–7.
10. Estey T, Chen Y, Carpenter JF, Vasiliou V. Structural and functional modifications of corneal crystallin ALDH3A1 by UVB light. *PLoS One.* 2010; 5:e15218. [PubMed: 21203538]
11. Lindahl R. Aldehyde dehydrogenases and their role in carcinogenesis. *Critical reviews in biochemistry and molecular biology.* 1992; 27:283–335. [PubMed: 1521460]
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951; 193:265–75. [PubMed: 14907713]
13. Makia NL, Bojang P, Falkner KC, Conklin DJ, Prough RA. Murine hepatic aldehyde dehydrogenase 1a1 is a major contributor to oxidation of aldehydes formed by lipid peroxidation. *Chem Biol Interact.* 2011; 191:278–87. [PubMed: 21256123]
14. Miyayama T, Tsou PS, Fung SM, Fung HL. Simultaneous determination of nitroglycerin and dinitrate metabolites in metabolism studies using liquid chromatography-mass spectrometry with electrospray ionization. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2006; 835:21–6.
15. Tsou PS, Page NA, Lee SG, Fung SM, Keung WM, Fung HL. Differential metabolism of organic nitrates by aldehyde dehydrogenase 1a1 and 2: substrate selectivity, enzyme inactivation, and active cysteine sites. *The AAPS journal.* 2011; 13:548–55. [PubMed: 21818694]
16. Kelm M, Schrader J. Control of coronary vascular tone by nitric oxide. *Circ Res.* 1990; 66:1561–75. [PubMed: 2160870]
17. Krishnatreya AS, Kamei T, Wang H, Qu J, Fung HL. Identification of nitroglycerin-induced cysteine modifications of pro-matrix metalloproteinase-9. *Rapid communications in mass spectrometry: RCM.* 2011; 25:2291–8. [PubMed: 21766372]
18. Murphy TC, Arntzen R, Picklo MJ Sr. Nitrate-based vasodilators inhibit multiple vascular aldehyde dehydrogenases. *Cardiovasc Toxicol.* 2005; 5:321–32. [PubMed: 16244377]
19. Shen ML, Lipsky JJ, Naylor S. Role of disulfiram in the in vitro inhibition of rat liver mitochondrial aldehyde dehydrogenase. *Biochem Pharmacol.* 2000; 60:947–53. [PubMed: 10974203]
20. Silverman RB. Mechanism-based enzyme inactivators. *Methods Enzymol.* 1995; 249:240–83. [PubMed: 7791614]
21. Slack CJ, McLaughlin BE, Brien JF, Marks GS, Nakatsu K. Biotransformation of glyceryl trinitrate and isosorbide dinitrate in vascular smooth muscle made tolerant to organic nitrates. *Can J Physiol Pharmacol.* 1989; 67:1381–5. [PubMed: 2516767]
22. Fung HL, Poliszczuk R. Nitrosothiol and nitrate tolerance. *Zeitschrift fur Kardiologie.* 1986; 75(Suppl 3):25–7. [PubMed: 3099484]
23. Brien JF, McLaughlin BE, Breedon TH, Bennett BM, Nakatsu K, Marks GS. Biotransformation of glyceryl trinitrate occurs concurrently with relaxation of rabbit aorta. *The Journal of pharmacology and experimental therapeutics.* 1986; 237:608–14. [PubMed: 3084761]
24. Bennett BM, McDonald BJ, Nigam R, Simon WC. Biotransformation of organic nitrates and vascular smooth muscle cell function. *Trends in pharmacological sciences.* 1994; 15:245–9. [PubMed: 7940987]
25. Ji Y, Anderson DJ, Bennett BM. Role of microsomal glutathione transferase 1 in the mechanism-based biotransformation of glyceryl trinitrate in LLC-PK1 cells. *Biochemical pharmacology.* 2009; 77:1702–8. [PubMed: 19428324]
26. Gori T, Parker JD. Nitrate tolerance: a unifying hypothesis. *Circulation.* 2002; 106:2510–3. [PubMed: 12417551]
27. Fung HL, Poliszczuk R. Nitrosothiol and nitrate tolerance. *Z Kardiol.* 1986; 75(Suppl 3):25–7. [PubMed: 3099484]
28. Fung HL. Biochemical mechanism of nitroglycerin action and tolerance: is this old mystery solved? *Annu Rev Pharmacol Toxicol.* 2004; 44:67–85. [PubMed: 14744239]

29. Munzel T, Sayegh H, Freeman BA, Tarpey MM, Harrison DG. Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance. *J Clin Invest.* 1995; 95:187–94. [PubMed: 7814613]

Highlights

1. Aldehyde dehydrogenase (ALDH3A1) can selectively activate various organic nitrates to NO in vitro.
2. ALDH3A1 mRNA and protein are expressed in mouse aorta and other tissues
3. A mathematical model was developed to estimate kinetic parameters meaningfully
4. This model may be used for the quantification of enzyme kinetics when NO is generated.

Fig. 1.
Mechanistic model of NO generation from organic nitrates

Fig. 2. Relative mRNA expression of three ALDH isoforms in C57BL/6 mice in aorta, heart and liver tissues. Data were normalized against ALDH2 expression in the aorta. The insert shows the detection of ALDH3A1 protein in mouse aorta.

Fig. 3.

(Panel A). Concentration-time profiles of NTG, 1, 2-GDN and 1, 3-GDN after 0.1 μ M NTG was incubated with rALDH3A1 in the presence of cofactors as described in METHODS 2.7. Each point represents mean $\pm$ S.D. of triplicates. (Panel B): Rate of 1, 2-GDN formation over the first 2 minutes at different initial concentrations of NTG. The line was drawn from fitting the data with the Michaelis-Menten equation ($R^2 = 0.980$), with $V_{\max} = 147 \pm 5$ pmol/min/mg (mean $\pm$ S.E., CV = 3.23%), $K_m = 6.13 \pm 0.90$ μ M (mean $\pm$ S.E., CV = 14.6%). Each point represents mean $\pm$ S.D. of four independent experiments.

Fig. 4. Concentration-time profiles of NO generated from various ORNs when incubated with rALDH3A1 *in vitro*; (A) NTG, (B) ISDN, (C) IS-2-MN, and (D) nicorandil. Symbols are experimentally observed concentrations (each point represents the averaged NO concentration from triplicates), and lines are model-predicted concentration.

Fig. 5. Cumulative NO concentrations generated from different initial ORN concentrations in their reactions with rALDH3A1 (each point represents mean $\pm$ S.D. of triplicates). Panel A contains data for NTG and ISDN while panel B contains data for IS-2-MN and nicorandil.

Table 1

Enzyme kinetic parameters generated by the Mechanistic Model.

Parameters	NTG Estimated (CV%)	ISDN Estimated (CV%)	IS-2-MN Estimated (CV%)	Nicorandil Estimated (CV%)
ALDH ₀ (nM)	1.65 × 10 ⁴ (fixed)			
k ₁ (min ⁻¹ nM ⁻¹)	1.56 × 10 ⁻⁴ (7.76)	6.84 × 10 ⁻⁵ (10.4)	1.11 × 10 ⁻⁶ (5.05)	2.43 × 10 ⁻⁷ (10.7)
k ₋₁ (min ⁻¹)	0.610 (14.3)	3.17 (12.1)	0.890 (8.26)	1.35 (9.63)
k _{inact} (min ⁻¹)	0.295 (2.33)	0.327 (4.25)	0.251 (2.47)	0.506 (9.06)
k _r (min ⁻¹)	1.28 × 10 ⁻³ (17.1)	1.50 × 10 ⁻³ (31.3)	2.12 × 10 ⁻³ (8.41)	3.47 × 10 ⁻³ (9.77)
k _{cat} (min ⁻¹)	1.74 × 10 ⁻² (2.80)	1.59 × 10 ⁻² (4.65)	2.07 × 10 ⁻² (2.68)	4.58 × 10 ⁻² (9.60)
k _{deg} (min ⁻¹)	1.16 (fixed)			
σ _{inter}	0.141 (17.8)	0.0898 (19.4)	0.213 (15.2)	0.200 (15.2)
σ _{slope}	0.183 (6.02)	0.251 (5.96)	0.128 (6.02)	0.189 (5.77)
K _m (nM)	4.01 × 10 ³ (11.2)	4.65 × 10 ⁴ (8.52)	8.18 × 10 ⁵ (6.96)	5.75 × 10 ⁶ (11.5)
V _{max} (nmol/min/ mg of protein)	0.191 (2.80)	0.175 (4.65)	0.228 (2.68)	0.503 (9.60)

ALDH₀ = initial concentration of active rALDH3A1; k₁ = second order association rate constant of ORN and rALDH3A1; k₋₁ = first order dissociation rate constant of ORN-rALDH3A1 complex; k_{inact} = first order rate constant of rALDH3A1 inactivation; k_r = first order rate constant of inactivated rALDH3A1 reactivation; k_{cat} = first order rate constant of NO liberation; k_{deg} = first order rate constant of NO degradation; σ_{inter} and σ_{slope} = the variance model parameters; K_m = ORN concentration at ½ maximal rate of NO generation; V_{max} = Maximal rate of NO generation.