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Site-Directed Mutagenesis of Aldehyde Dehydrogenase-2 Suggests Three Distinct Pathways of Nitroglycerin Biotransformation[S]

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ABSTRACT

To elucidate the mechanism underlying reduction of nitroglycerin (GTN) to nitric oxide (NO) by mitochondrial aldehyde dehydrogenase (ALDH2), we generated mutants of the enzyme lacking the cysteines adjacent to reactive Cys302 (C301S and C303S), the glutamate that participates as a general base in aldehyde oxidation (E268Q) or combinations of these residues. The mutants were characterized regarding acetaldehyde dehydrogenation, GTN-triggered enzyme inactivation, GTN denitration, NO formation, and soluble guanylate cyclase activation. Lack of the cysteines did not affect dehydrogenase activity but impeded GTN denitration, aggravated GTN-induced enzyme inactivation, and increased NO formation. A triple mutant lacking the cysteines and Glu268 catalyzed sustained formation of superstoichiometric amounts of NO and exhibited slower rates of inactivation. These results suggest three alternative pathways for the reaction of ALDH2 with GTN, all involving formation of a thionitrate/sulfenyl nitrite intermediate at Cys302 as the initial step. In the first pathway, which predominates in the wild-type enzyme and reflects clearance-based GTN denitration, the thionitrate apparently reacts with one of the adjacent cysteine residues to yield nitrite and a protein disulfide. The predominant reaction catalyzed by the single and double cysteine mutants requires Glu268 and results in irreversible enzyme inactivation. Finally, combined lack of the cysteines and Glu268 shifts the reaction toward formation of the free NO radical, presumably through homolytic cleavage of the sulfenyl nitrite intermediate. Although the latter reaction accounts for less than 10% of total turnover of GTN metabolism catalyzed by wild-type ALDH2, it is most likely essential for vascular GTN bioactivation.

Introduction

Nitroglycerin [glyceryl trinitrate (GTN)] has been used for the treatment of angina pectoris since the late 19th century. Bioactivation of GTN in vascular smooth muscle is thought to yield NO that activates soluble guanylate cyclase (sGC), resulting in cGMP-mediated vasorelaxation (Fung, 2004). Although GTN activates sGC nonenzymatically in the presence of cysteine (Gorren et al., 2005) or high concentrations of ascorbate (Kollau et al., 2007) via NO release, there is a large body of evidence (Mayer and Beretta, 2008) indicating that vascular bioactivation of GTN involves an enzymatic reaction catalyzed by mitochondrial aldehyde dehydrogenase (ALDH2) (EC 1.2.1.3). In 2002, Stamler and coworkers (Chen et al., 2002) showed that ALDH2 denitrates GTN to 1,2-glyceryl dinitrate (1,2-GDN) and nitrite and proposed that reduction of nitrite by components of the mitochondrial electron transfer chain could provide the link between ALDH2-catalyzed GTN metabolism and vasorelaxation. However, we found that 1) GTN activates sGC in the presence of purified ALDH2 (Kollau et al., 2005), 2) ALDH2 catalyzes the reduction of GTN to NO, accounting for 5 to 10% of total GTN turnover (Beretta et al., 2008a; Wenzl et al., 2009), and 3) mitochondrial biotransformation of nitroglycerin is not affected by respiratory substrates or inhibitors (Kollau et al., 2009), suggesting that ALDH2 catalysis is sufficient for GTN bioactivation in blood vessels.

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ABBREVIATIONS: GTN, glyceryl trinitrate (nitroglycerin); NO, nitric oxide; sGC, soluble guanylate cyclase; ALDH2, mitochondrial aldehyde dehydrogenase; GDN, glyceryl dinitrate; TEA, triethanolamine; DTT, dithiothreitol; SOD, superoxide dismutase; DTPA, diethylene triamine pentaacetic acid; WT, wild-type; oxyHb, oxyhemoglobin; metHb, methemoglobin.

The mechanism underlying ALDH2-catalyzed GTN reduction is not known, but the reaction seems to occur within the active site pocket with Cys302 serving as the common essential nucleophile (Larson et al., 2007; Wenzl et al., 2009). It was proposed by Stamler and coworkers (Chen et al., 2002) that GTN denitration resembles ester hydrolysis by ALDH2 (Mann and Weiner, 1999) (i.e., nucleophilic attack of a nitro group of GTN by Cys302, resulting in formation of a thionitrate intermediate and release of the corresponding alcohol, preferentially 1,2-GDN). The thionitrate would then release nitrite either through nucleophilic attack of one of the adjacent cysteine residues (Cys301 or Cys303), resulting in formation of a disulfide in the active site or through Glu268-aided hydrolysis, yielding a sulfenic acid derivate of Cys302 that could undergo S-thiolation (Biswas et al., 2006) and would also lead to the formation of a disulfide with one of the adjacent cysteine residues. Both possibilities would agree well with the fact that NAD^+ is not essential but increases reaction rates and with the observation that GTN-triggered oxidative inactivation of ALDH2 is partially prevented by thiols such as dithiothreitol (DTT) or dihydrolipoic acid (Wenzel et al., 2007; Beretta et al., 2008b). Of interest, we found that mutation of Glu268 resulted in unaltered GTN denitration by ALDH2 in the absence of NAD^+, whereas the nucleotide cofactor led to pronounced inhibition of 1,2-GDN formation by E268Q-ALDH2, probably as a result of involvement of Glu268 in the structural organization of the NAD^+ binding pocket. Another interesting feature of E268Q-ALDH2 was that fractional NO formation from GTN was increased to up to 50% of total GTN turnover (Wenzl et al., 2009), pointing to an independent three-electron reduction pathway of GTN denitration, in addition to the two-electron reduction pathway yielding nitrite, that is predominant in the wild-type enzyme.

In the present study we investigated the role of the cysteine residues adjacent to the catalytically active Cys302 in GTN bioactivation as well as mechanism-based oxidative enzyme inactivation, which represents an attractive explanation for the phenomenon of nitrate tolerance because long-term exposure of blood vessels to GTN causes inactivation of ALDH2 (Towell et al., 1985; Daiber et al., 2004; Chen et al., 2007). Besides single and double mutants of Cys301 and Cys303 (C301S-ALDH2, C303S-ALDH2, and C301S/C303S-ALDH2, hereafter referred to as the "double mutant"), we also tested E268Q/C301S/C303S-ALDH2 (hereafter referred to as the "triple mutant") to exclude the possibility that Glu268 substituted for either of the cysteines in the reaction.

Materials and Methods

Materials. Bovine lung sGC was purified as described previously (Russwurm and Koesling, 2005). Human ALDH2 was expressed in *Escherichia coli* BL21(DE3) and purified as described previously (Zheng et al., 1993; Beretta et al., 2008a). Concentrations are expressed per monomer, assuming a molecular mass of 54 kDa. Sephacryl S-300 HR was obtained from GE Healthcare Europe GmbH (Vienna, Austria). [α-^{32}P]GTP (400 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Vienna, Austria). [2-^{14}C]GTN (50–60 mCi/mmol) was from American Radiolabeled Compounds, purchased through Humos Diagnostica GmbH (Maria Enzersdorf, Austria). Nitropohl ampoules (G. Pohl-Boskamp GmbH and Co., Hohenlockstedt Germany), containing 4.4 mM GTN in 250 mM glucose, were obtained from a local pharmacy; dilutions were made in 50 mM triethanolamine (TEA)/HCl buffer. 2,2-Diethyl-1-nitroso-oxyhydrazine (Enzo Life Sciences Corporation, Lausen, Switzerland) was dissolved and diluted in 10 mM NaOH. All other chemicals were from Sigma-Aldrich GmbH (Vienna, Austria).

Site-Directed Mutagenesis. E268Q, C301S, C303S, C301S/C303S, and E268Q/C301S/C303S mutations were inserted using the QuikChange II site-directed mutagenesis kit (Stratagene, Heidelberg, Germany). The following mutagenic sense primers were used to introduce the mutation (bold type) and to add the BamHI restriction site (underlined) as a silent mutation for screening of mutants to the cDNA encoding for WT-ALDH2: 5′-GTT CTT CAA CCA GGG CCA G**TC C**TG CTG TGC CGG aTC CCG GAC CTT CGT G-3′ for C301S, 5′-GTT CTT CAA CCA GGG CCA GTG CTG C**TC T**GC CGG aTC CCG GAC CTT CGT G-3′ for C303S, and 5′-GTT CTT CAA CCA GGG CCA G**TC C**TG C**TC T**GC CGG aTC CCG GAC CTT CGT G-3′ for C301S/C303S. The E268Q mutation was introduced to the cDNA encoding for WT-ALDH2 as described previously (Wenzl et al., 2009). For the E268Q/C301S/C303S mutation, the mutagenic sense primer 5′-GCA GCA ACC TtA AGA GAG TGA CCT TG**C AG**C TGG GGG GGA AG-3′ was used to introduce the E268Q mutation (bold type) and to add the AflII restriction site (underlined) as a silent mutation for screening of mutants to the cDNA encoding for C301S/C303S-ALDH2. To confirm the desired mutations, sequencing of cDNAs was performed with an ABI 373A automated DNA sequencer (Applied Biosystems, Carlsbad, CA). All mutants were expressed in *E. coli* BL21(DE3) and purified as described previously (Zheng et al., 1993; Beretta et al., 2008a).

Determination of GTN Denitration. The rates of GTN metabolism yielding 1,2- and 1,3-GDN were determined according to a protocol described previously (Kollau et al., 2005). WT and mutated ALDH2 proteins (4 μg each) were incubated with ^{14}C-labeled GTN ($\geq$2 μM as indicated) at 37°C for 10 min in a final volume of 0.2 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 3 mM $MgCl_2$ and 1 mM NAD^+ in the absence and presence of 2 mM dithiothreitol (DTT). GTN concentrations $\geq$10 μM were adjusted by addition of unlabeled GTN. Reaction products were extracted twice with 1 ml of diethyl ether, separated by thin-layer chromatography, and quantified by liquid scintillation counting. Blank values were determined in the absence of protein under identical conditions and subtracted. The results are mean values $\pm$ S.E. determined in three to five independent experiments. Because 1,3-GDN yields in the concentration range investigated in the present study are all but negligible (Beretta et al., 2010), only the 1,2-GDN yields are presented.

Determination of GTN-Derived NO. NO formation was measured with a Clark-type electrode (WPI Inc., Berlin, Germany) that was calibrated daily with acidified nitrite as described previously (Mayer et al., 1995). The electrode was equilibrated in 50 mM TEA/HCl, pH 7.4, containing 1000 U/ml superoxide dismutase (SOD), 0.1 mM diethylene triamine pentaacetic acid (DTPA), 1 mM NAD^+, 3 mM $MgCl_2$, and purified WT or mutated ALDH2 (250 μg/ml) in the absence or presence of 2 mM DTT at 37°C, followed by addition of GTN (final concentration of 0.1 mM). The incubation volume was 0.5 ml. Output current was recorded in three to six separate experiments.

Oxyhemoglobin Assay. Conversion of oxyhemoglobin (oxyHb) to methemoglobin (metHb) by NO was measured by monitoring the absorbance difference between 420 and 401 nm, which represent the peak and trough in the oxyHb minus metHb Soret absorption difference spectrum. OxyHb was prepared from bovine hemoglobin by reduction with a molar excess of sodium dithionite and purification by gel chromatography (Sephadex G25). ALDH2 (250 μg/ml) was incubated in 0.25 ml of potassium phosphate (50 mM, pH 7.4) containing 1000 U/ml SOD, 10 U/ml catalase, 0.1 mM DTPA, 1 mM NAD^+, 3 mM $MgCl_2$, and 10 μM oxyHb in the absence or presence of 2 mM DTT at 37°C, followed by addition of GTN (0.1 mM). NO concentrations were calculated with an extinction coefficient ($\Delta\Delta\varepsilon_{420-401}$ = 91.9 mM $\cdot$ cm^{-1}) that was determined with $K_3Fe(CN)_6$ in three to five separate experiments. Control experiments in the

absence of ALDH2 and DTT showed direct oxidation of oxyHb by GTN. This artifactual reaction, which did not occur in the presence of DTT, was subtracted from all affected data (essentially the slow phase in the absence of DTT).

Determination of sGC Activity. The sGC preparation used for the experiments in the present study exhibited a maximal specific activity of ~20 μmol cGMP · min^{-1} · mg^{-1} (tested with 10 μM 2,2-diethyl-1-nitroso-oxyhydrazine) and contained 2 mM DTT to protect the enzyme from oxidation, yielding a final DTT concentration of 10 μM in the assays. Purified sGC (50 ng) was incubated at 37°C for 2 min in 100 μl with GTN in the presence of WT or mutant ALDH2 (2 μg each) as indicated in the text and figure legends. Assay mixtures contained 50 mM TEA/HCl, pH 7.4, 0.5 mM [α-^{32}P]GTP (~250,000 cpm), 3 mM $MgCl_2$, and 1 mM cGMP. NAD^+ (1 mM) and SOD (1000 U/ml) were present as indicated. Reactions were terminated by addition of 0.45 ml of zinc acetate (120 mM) and 0.45 ml of sodium bicarbonate (120 mM), followed by isolation and quantification of [^{32}P]cGMP (Schultz and Böhme, 1984). Blank values were determined in the absence of sGC and subtracted. The results are mean values ± S.E. determined in three to five independent experiments.

ALDH2 Inactivation by GTN. GTN-triggered inactivation of ALDH2 was determined as described previously (Beretta et al., 2008b). Dehydrogenase activity of WT or mutant ALDH2 (33 μg/ml each) was measured by monitoring the formation of NADH spectrophotometrically at 340 nm (ε_{340} = 6.22 mM · cm^{-1}) at 25°C. The initial reaction mixture consisted of 0.4 mM acetaldehyde, 0.4 mM NAD^+, and 10 mM $MgCl_2$ in 50 mM potassium phosphate, pH 7.4. After 2 min, reactions were started by addition of ALDH2. Approximately 4 min later, 50 μM GTN was added to inactivate the enzyme. After complete inactivation, 0.4 mM DTT was added to reactivate the enzyme. The linear absorbance increases after addition of ALDH2 and DTT yielded initial (v_0) and restored ($v_{restored}$) activities, respectively. The gradual inactivation after GTN addition was fitted to a single-exponential curve yielding the apparent inactivation rate constant (k_{inact}). The results are mean values ± S.E. determined in four to seven independent experiments.

Results

GTN Denitration. As shown in Fig. 1A, GTN was denitrated within 10 min to ~1 mol of 1,2-GDN/mol of ALDH2 monomer in the absence of DTT by WT-ALDH2 as well as by C301S-ALDH2, C303S-ALDH2, and the double mutant, indicating that these enzyme species are inactivated by GTN after one turnover, as reported earlier for WT-ALDH2 (Chen and Stamler, 2006; Beretta et al., 2008b). We previously found that E268Q-ALDH2 exhibits strongly reduced activities, particularly in the presence of NAD^+ (Wenzl et al., 2009). In line with those observations, E268Q-ALDH2 was virtually inactive, whereas the triple mutant formed 4-fold less 1,2-GDN than the double mutant (Fig. 1A).

Figure 1B shows that WT-ALDH2 catalyzed maximally ~14 turnovers in the presence of DTT, whereas mutation of Cys301 or Cys303, alone or in combination, decreased the yield to ~3 turnovers. Because NAD^+ was present in the assay, GTN denitration by E268Q-ALDH2 was not detectable at any GTN concentration tested (Wenzl et al., 2009). GTN denitration by the triple mutant surprisingly was more pronounced, with a maximum of ~2 turnovers at ≥30 μM GTN. The concentration-response curves suggest that the triple mutant exhibits low affinity for GTN (EC_{50} = ~20 μM) compared with WT-ALDH (EC_{50} = ~3 μM). The lack of effect of increasing GTN concentrations on denitration catalyzed by the three cysteine mutants most likely reflects complete inactivation of the enzyme within the 10-min incubation period (see below).

NO Formation. There was a striking discrepancy between GTN denitration and formation of NO catalyzed by the different ALDH2 variants. In the absence of DTT, all cysteine mutants (C301S and C303S as well as double and triple mutants) yielded NO peak concentrations that were between

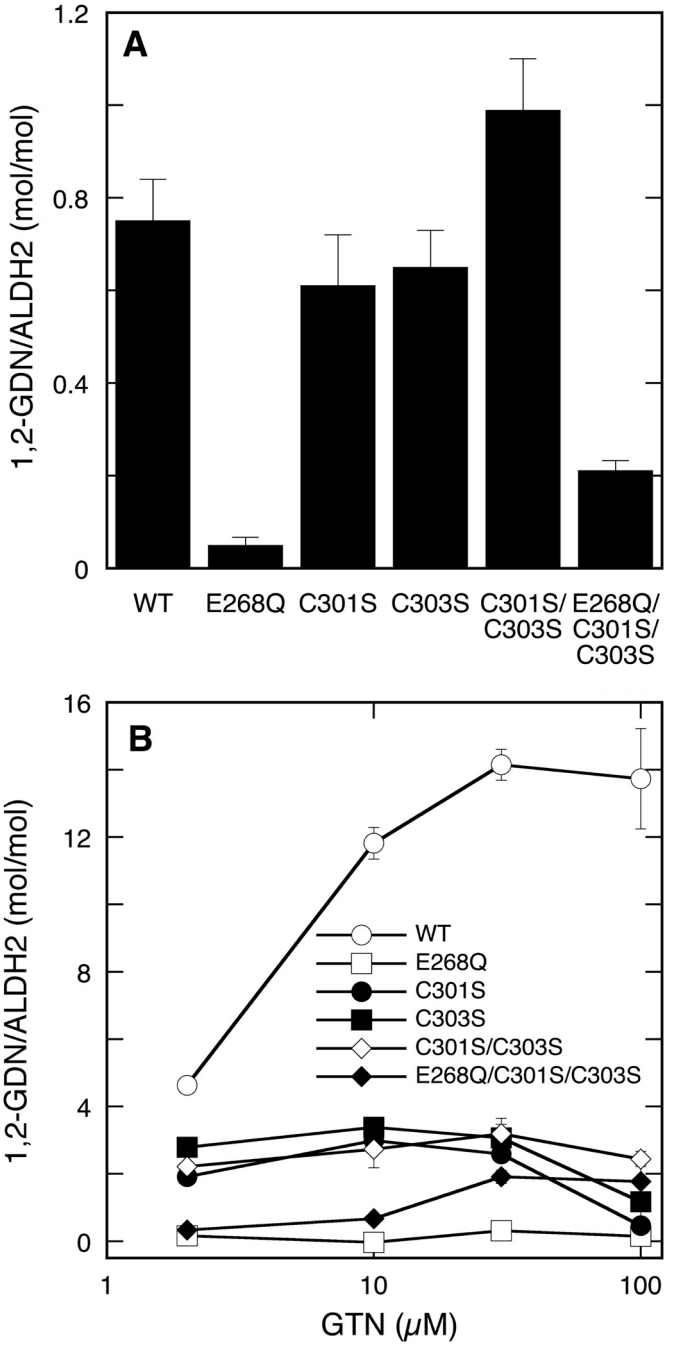


Fig. 1. GTN denitration by wild-type and mutant ALDH2. WT or mutant ALDH2 (C301S, C303S, C301S/C303S, E268Q, or E268Q/C301S/C303S, 0.36 μM final) was incubated with 2 μM or the indicated concentration of ^{14}C-labeled GTN in the absence (A) or presence (B) of 2 mM DTT at 37°C for 10 min in a final volume of 200 μl of potassium phosphate (50 mM, pH 7.4), containing 3 mM $MgCl_2$ and 1 mM NAD^+. Reaction products were extracted and quantified by radio thin-layer chromatography as described under *Materials and Methods*. Data are the mean values ± S.E. of three to five independent experiments and are expressed as moles of 1,2-GDN catalyzed by 1 mol of ALDH2 monomer.

2.5- and 4.4-fold higher than those observed with the wild-type enzyme (Fig. 2; Table 1). Initial rates of NO formation were between 2.8- and 7.2-fold higher than those with the wild type. Thus, mutation of these residues apparently caused a shift of GTN turnover toward formation of NO. A similar, although less pronounced, shift was observed in the presence of DTT, which increased the peak concentration of NO formed by WT-ALDH2 8-fold but caused only between 2- and 4-fold increases of NO peak heights for the mutants. As a result, in the presence of DTT, incubation of GTN with C301S-ALDH2 and wild-type enzyme yielded the same NO peak heights, whereas the other cysteine mutants showed between 1.3- and 1.5-fold higher NO peaks. All four mutants catalyzed NO formation with between 1.4- and 2.9-fold higher initial rates than WT-ALDH2 (Table 1; Supplemental Fig. S1). If we consider that the wild-type enzyme denitrated approximately 7 times more GTN than the mutants (Fig. 1B), these data indicate that the fraction of GTN reduced to NO was increased upon mutation of Cys301, Cys303, and/or Glu268.

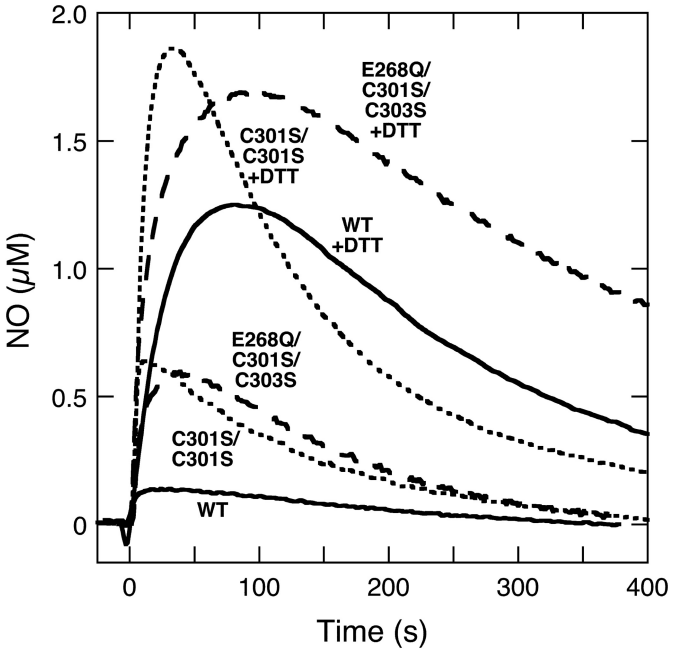


Fig. 2. Formation of GTN-derived NO in the presence of WT and mutant ALDH2. ALDH2 (WT, C301S/C303S, or E268Q/C301S/C303S, 4.5 μM final) was incubated in 0.5 ml of 50 mM TEA/HCl, pH 7.4, containing 1000 U/ml SOD, 3 mM $MgCl_2$, 0.1 mM DTPA, 1 mM NAD^+, and 0.1 mM GTN at 37°C in the absence and presence of 2 mM DTT. NO formation was monitored with a Clark-type electrode. The traces shown are representative of three to six similar experiments. a.u., arbitrary units.

The mutant enzymes also differed from WT-ALDH2 with respect to the decay rates of the NO signal, particularly in the presence of DTT (Fig. 2; Table 1). C303S-ALDH2 and the double mutant exhibited approximately 2-fold higher decay rates than the wild-type enzyme, whereas the decay of the signal observed in the presence of the triple mutant was slower. Because the observed rate of NO decay correlates with the rate of ALDH2 inactivation (Beretta et al., 2010), these data suggest that ALDH2 inactivation is accelerated upon mutation of one or both cysteines, whereas mutating Glu268 protects against inactivation. The latter observation agrees with previous results obtained with the single E268Q mutant (Wenzl et al., 2009).

All experiments described above were performed in the presence of SOD. We have previously shown that the presence of SOD is essential for the electrochemical detection of NO with WT-ALDH2 but not with E268Q-ALDH2 (Wenzl et al., 2009). Likewise, when we omitted SOD from the reaction mixture in the present study, NO was not detectable with WT-ALDH2 and the three Cys mutants, whereas NO formation was clearly observed in the absence of SOD with the triple mutant (Supplemental Fig. S2). We previously proposed that this phenomenon might be due to significant superoxide production by the WT enzyme but not by E268Q-ALDH. For an alternative interpretation, see Scheme 2 under *Discussion*.

We repeated some of the electrode experiments with 2 μM instead of 0.1 mM GTN (Supplemental Fig. S3). It was shown previously that NO formation is close to the electrochemical detection limit with 1 μM GTN for the WT enzyme (Beretta et al., 2010). In the present study we attained a peak concentration of ~0.13 μM with 2 μM GTN. Considerably higher peaks were obtained with the double and triple mutants (0.67 and 0.60 μM, respectively), whereas a lower peak was observed for E268Q-ALDH2 (~0.07 μM).

Oxyhemoglobin Oxidation. The NO electrode experiments provided unequivocal evidence for the formation of NO, but because the curves are complex functions of simultaneous NO formation and disappearance, this method does not allow reliable quantification of the amount of NO released, precluding use of the data for calculation of turnover numbers. Therefore, we also measured NO formation with the oxyhemoglobin assay. Although more susceptible to possible interferences by redox-active agents than the electrochemical method, this assay has the advantage of measuring product accumulation in addition to reaction rates. As illus-

TABLE 1

GTN-derived NO formation in the presence of WT and mutant ALDH2

WT or mutant ALDH2 (4.5 μM) was incubated in 0.5 ml of TEA/HCl (50 mM, pH 7.4) containing 3 mM $MgCl_2$, 1 mM NAD^+, 0.1 mM DTPA, 1000 U/ml SOD, and 0.1 mM GTN at 37°C in the absence and presence of 2 mM DTT. NO formation was monitored with a Clark-type electrode. Data are mean values ± S.E. of three to six independent experiments.

ALDH2 Variant	$[NO]_{peak}$		NO Formation Rate		NO Half-Life	
	−DTT	+DTT	−DTT	+DTT	−DTT	+DTT
	μM		$nmol \cdot min^{-1} \cdot mg^{-1}$		s	
WT	0.15 ± 0.02	1.21 ± 0.03	3.2 ± 0.5	11.5 ± 0.8	194 ± 30	295 ± 31
E268Q	0.14 ± 0.01	0.17 ± 0.02	3.9 ± 0.2	2.9 ± 0.3	232 ± 20	228 ± 37
C301S	0.57 ± 0.07	1.20 ± 0.07	12.2 ± 1.5	16.6 ± 1.3	174 ± 7	239 ± 12
C303S	0.37 ± 0.04	1.52 ± 0.04	8.9 ± 1.6	29.4 ± 1.1	152 ± 7	153 ± 7
C301S/C303S	0.66 ± 0.02	1.83 ± 0.02	23.4 ± 2.0	32.9 ± 2.1	123 ± 10	139 ± 7
E268Q/C301S/C303S	0.50 ± 0.05	1.73 ± 0.05	11.6 ± 0.4	33.1 ± 3.1	155 ± 16	416 ± 6

trated by Fig. 3 and Supplemental Fig. S4, metHb formation by ALDH/GTN-derived NO was biphasic, with a rapid phase occurring within the 1st min of incubation and a slow phase that continued beyond 20 min. The relative amounts of NO produced in the fast phase by the various enzymes (Table 2) correlated well with the corresponding peak concentrations observed with the NO electrode (Table 1), suggesting that the results of the oxyhemoglobin assay do indeed represent NO formation.

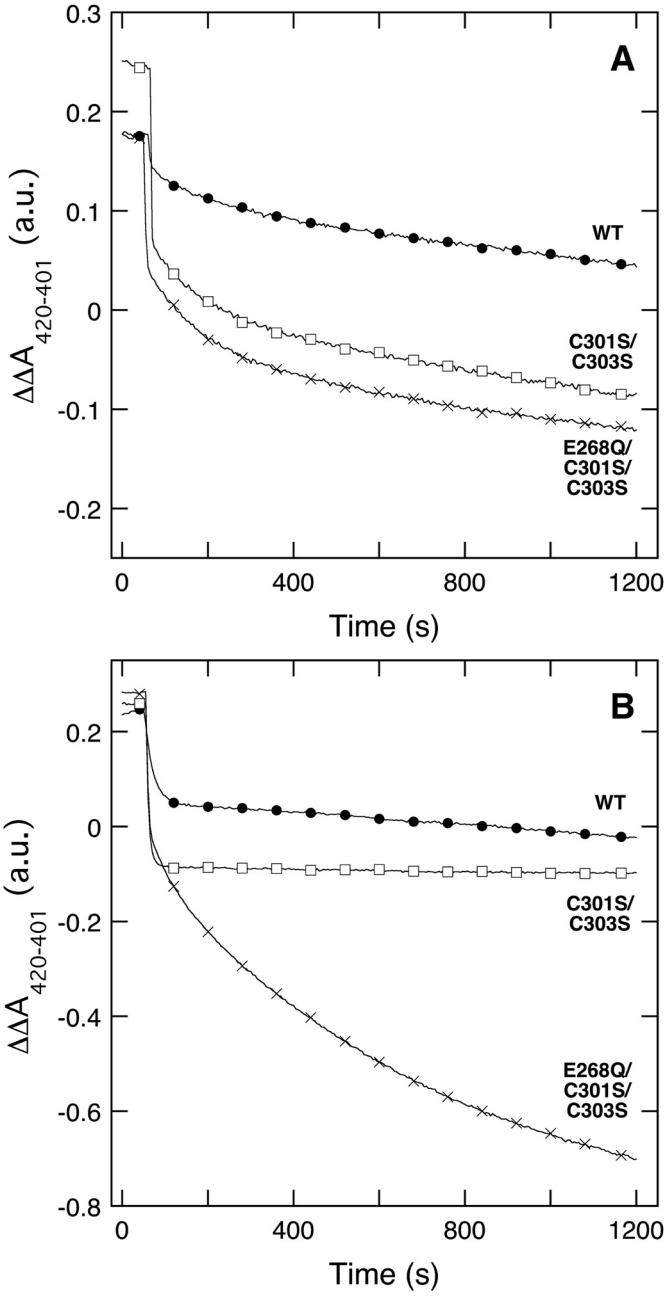


Fig. 3. Formation of metHb from oxyHb by GTN-derived NO in the presence of WT and mutant ALDH2. WT or mutant ALDH2 (WT, C301S/C303S, or E268Q/C301S/C303S, 4.5 μM final) was incubated in a final volume of 250 μl of potassium phosphate (50 mM, pH 7.4) containing 1000 U/ml SOD, 10 U/ml catalase, 0.1 mM DTPA, 1 mM NAD^+, 3 mM $MgCl_2$ and 10 μM oxyHb in the absence (A) or presence (B) of 2 mM DTT at 37°C, followed by addition of GTN (0.1 mM). The traces plot the changes in the absorption difference between 420 and 401 nm (peak minus trough in the oxyHb/metHb difference spectrum) over time ($\Delta\Delta A_{420-401}$) and are representative of three to five similar experiments.

GTN alone caused some oxyHb autoxidation ($\Delta\Delta A_{420-401}$ = 0.12 ± 0.01) that, unlike enzyme-mediated NO formation, did not show a fast initial phase and was virtually abolished by DTT (Supplemental Fig. S5). After correction for that artifact, $[NO]_{slow}$ was negligible in the absence of DTT, with the possible exception of the mutants lacking Glu268 (Table 2). In the absence of DTT, none of the enzymes catalyzed stoichiometric amounts of NO from GTN within 20 min (~0.3–2 μM NO catalyzed by ~5 μM ALDH2), although elimination of Cys301 and Cys303, as well as additional mutation of Glu268, led to significant increases in NO formation. In the presence of DTT, all enzyme species produced ~2–3 μM NO in 20 min, with the notable exception of the triple mutant, which generated ~10 μM NO, corresponding to 2.2 ± 0.3 turnovers (Table 2).

Soluble Guanylate Cyclase Activation. Activation of purified sGC was measured in the presence of wild-type and mutated ALDH2 to test for GTN bioactivation at therapeutically relevant submicromolar concentrations of the nitrate (Fig. 4A). In the absence of both NAD^+ and SOD, none of the ALDH2 variants stimulated sGC beyond basal activity determined with 0.1 μM GTN in the absence of ALDH2 (~0.2 μmol cGMP · min^{-1} · mg^{-1}). NAD^+ did not affect basal cGMP formation (not shown) but increased sGC activity in the presence of WT-ALDH2 approximately 10-fold (1.8 ± 0.5 μmol cGMP · min^{-1} · mg^{-1}). A similar effect of NAD^+ was observed with C301S-ALDH2, C303S-ALDH2, and the double mutant. In line with a previous report (Wenzl et al., 2009), NAD^+ did not affect sGC activation mediated by E268Q-ALDH2. It is noteworthy, however, that the nucleotide cofactor caused an ~7-fold increase in sGC activity in the presence of the triple mutant.

SOD caused an increase in basal sGC activity (~1.4 μmol cGMP · min^{-1} · mg^{-1}, not shown), probably as a result of stabilization of airborne NO (Friebe et al., 1998). Addition of wild-type or mutant ALDH2 (2 μg each) caused only slightly higher rates of cGMP formation in the presence of SOD (between 2.2 ± 0.3 and 2.7 ± 0.4 μmol · min^{-1} · mg^{-1}), but pronounced effects were observed in the combined presence of SOD and NAD^+. Under these conditions, WT-ALDH2 stimulated sGC activity to 8.9 ± 0.6 μmol cGMP · min^{-1} · mg^{-1}. In the presence of the cysteine mutants (C301S, C303S, and the double mutant) the rates of cGMP formation were even higher, ranging from 12.4 ± 0.5 to 14.7 ± 0.7 · min^{-1} · mg^{-1}. As reported previously (Wenzl et al., 2009), hardly any activation was observed in the presence of E268Q-ALDH2 under these conditions. The effect of the triple mutant was modest, most likely reflecting low apparent GTN affinity (see below).

Figure 4B illustrates the effect of GTN concentration on GTN-triggered sGC activation mediated by the various ALDH2 species in the presence of SOD and NAD^+. In the presence of WT-ALDH2, GTN exhibited an EC_{50} of 52 ± 15 nM, and mutation of Glu268 led to an approximately 50-fold rightward shift of the curve. The double mutant was the most effective ALDH2 variant, yielding an EC_{50} as low as 17 ± 8 nM and significantly higher maximal activity than the wild type (18.7 ± 1.0 versus 11.7 ± 0.4 cGMP · min^{-1} · mg^{-1}). Again, mutation of Glu268 to Gln caused a large (~28-fold) rightward shift of the curve.

Acetaldehyde Dehydrogenation. Because Cys301 and Cys303 may be involved in oxidative enzyme inactivation

through formation of a disulfide bridge with Cys302 after one turnover of GTN denitration, we also investigated inhibition by GTN and restoration by DTT of the aldehyde dehydrogenase activity of the three cysteine mutants in comparison to WT-ALDH2 (Fig. 5). These experiments were not performed with E268Q-ALDH2 and the triple mutant, because in the absence of Glu268 dehydrogenase activity is hardly detectable under the applied conditions. Figure 5 shows that oxidation of acetaldehyde (v_0) was not significantly affected by mutation of Cys301 and/or Cys303. Although single mutation of these residues did not affect the rate of ALDH2 inactivation, the double mutant was inactivated ~2.5 times faster than WT-ALDH [k_{inact} $(15.2 \pm 1.3) \cdot 10^{-3} \cdot s^{-1}$ versus $(6.3 \pm 0.7) \cdot 10^{-3} \cdot s^{-1}$]. The activity of GTN-exposed WT-ALDH2 was restored to approximately 45% of v_0 by DTT ($v_{restored}$ in Fig. 5). The cysteine mutants exhibited significantly lower restored activities (~20–30% of v_0), indicating that mutation of these residues shifts the effect of GTN toward DTT-irreversible enzyme inactivation.

Discussion

In their initial report on the role of ALDH2 in GTN bioactivation, Stamler and coworkers (Chen et al., 2002) showed that the enzyme reduces GTN to 1,2-GDN and nitrite and proposed a mechanism involving formation of a disulfide bridge between the catalytic nucleophile Cys302 and one of the adjacent cysteines. The inactivation of ALDH2 after each turnover was shown to be DTT-reversible, suggesting that it contributes to the development of nitrate tolerance. Although this mechanism may account for the main route of ALDH2-catalyzed GTN denitration, our recent work demonstrating an additional pathway yielding NO instead of nitrite (Beretta et al., 2008a) and partially DTT-irreversible inactivation of the enzyme by GTN (Beretta et al., 2008b) indicates that the reaction between ALDH2 and GTN may be more complex than originally assumed. In the present study, we mutated the cysteine residues adjacent to the catalytic nucleophile Cys302 and the putative general base Glu268 in human ALDH2 to clarify their function in GTN metabolism and GTN-induced enzyme inactivation. The results not only confirmed that GTN denitration can be dissociated from NO formation and ALDH2 inactivation but also demonstrated that NO formation and ALDH2 inactivation represent different processes. For the sake of brevity and clarity, we limit the discussion to those aspects of the results that suggest three distinct pathways for the reaction of ALDH2 with GTN. A detailed discussion of other aspects is provided as supplemental data.

NO Formation and ALDH2 Inactivation Can Be Distinguished from Clearance-Based GTN Denitration. WT-ALDH2 produced ≤1 Eq of 1,2-GDN/ALDH2 from GTN in the absence of DTT, whereas DTT increased the yield approximately 20-fold. In line with the hypothesis that GTN denitration results in DTT-reversible disulfide formation between Cys302 and one of the adjacent Cys residues, we found that mutating one or both of these residues impeded multiple turnover, without affecting single turnover.

Mutating the adjacent Cys residues did not affect NO formation to the same extent. Consequently, the mutants showed a considerable increase in the relative yield of NO compared with GTN denitration (from 5% for WT-ALDH2 to 22% for the double mutant in the presence of DTT). These results suggest two distinct pathways for GTN denitration with the minor one yielding NO. They also demonstrate that, at least in vitro, Cys301 or Cys303 does not participate in NO formation and suggest competition between disulfide formation and NO generation.

Mutation of Cys301 and Cys303 also affected inactivation of the enzyme. The double mutant was inactivated 2- to 3-fold faster than WT-ALDH2, and all three Cys mutants showed more pronounced irreversible inactivation, indicating that this process as well can be separated from GTN denitration.

NO Formation Can Be Distinguished from ALDH2 Inactivation. The results with the Cys mutants (C301S, C303S, and the double mutant) demonstrate that ALDH2-catalyzed NO formation can be distinguished from the main GTN denitration pathway. The results obtained with the Glu268 mutants (E268Q-ALDH2 and the triple mutant) show that NO formation can be dissociated from ALDH2 inactivation as well. As reported and discussed by us previously (Wenzl et al., 2009), E268Q-ALDH2 exhibits generally low activity accompanied by a higher relative contribution of NO formation. The triple mutant combined some of the characteristics of E268Q and the Cys mutants. Like these mutants, it exhibited a large contribution of NO production. Like E268Q-ALDH2, it displayed rather low activity, although not to the same extent. It shared with the three Cys mutants a higher production of NO than WT-ALDH2 in single turnover

TABLE 2

Formation of metHb from oxyHb by GTN-derived NO

WT or mutant ALDH2 (4.5 μM) was incubated for 20 min in 0.25 ml of potassium phosphate (50 mM, pH 7.4) containing 1000 U/ml SOD, 10 U/ml catalase, 0.1 mM DTPA, 1 mM NAD^+, 3 mM $MgCl_2$, and 10 μM oxyHb in the absence or presence of 2 mM DTT, followed by addition of 0.1 mM GTN at 37°C. Blank values were determined in the absence of protein under otherwise identical conditions and subtracted. Curves were fitted as explained under *Materials and Methods* to estimate fast and slow contributions to NO formation. Please note that in many instances, especially in the presence of DTT, NO was still being formed at the end of the incubation, so that $[NO]_{slow}$ and the NO/ALDH stoichiometry merely represent the values observed after 20 min. Results are mean values ± S.E. determined in three to five independent experiments.

ALDH2 Species	$[NO]_{fast}$		$[NO]_{slow}$		$[NO]_{20\ min}$/[ALDH]	
	−DTT	+DTT	−DTT[a]	+DTT	−DTT[b]	+DTT
			μM			
WT	0.36 ± 0.01	1.97 ± 0.07	−0.1 ± 0.2	0.94 ± 0.13	0.08 ± 0.01	0.65 ± 0.03
E268Q	0.35 ± 0.03	0.50 ± 0.03	0.4 ± 0.2	1.95 ± 0.01	0.17 ± 0.04	0.54 ± 0.01
C301S	1.06 ± 0.02	1.5 ± 0.2	−0.2 ± 0.2	0.87 ± 0.15	0.24 ± 0.01	0.53 ± 0.06
C303S	0.52 ± 0.02	2.15 ± 0.09	−0.2 ± 0.2	0.1 ± 0.6	0.12 ± 0.01	0.50 ± 0.13
C301S/C303S	1.2 ± 0.3	2.9 ± 0.4	0.2 ± 0.3	0.20 ± 0.04	0.27 ± 0.09	0.69 ± 0.09
E268Q/C301S/C303S	1.18 ± 0.06	3.1 ± 0.3	0.9 ± 0.4	6.9 ± 1.4	0.46 ± 0.09	2.22 ± 0.33

[a] The slow phase in the absence of DTT did not significantly differ from zero except for the mutants lacking Glu268.
[b] The slow phase was ignored for the estimation of the NO/ALDH stoichiometry in the absence of DTT for WT- C301S-, and C303S-ALDH2.

(i.e., in the absence of DTT). However, unlike the other three Cys mutants, the triple mutant did not experience rapid inactivation. Therefore, it sustained catalysis over at least 20 min, resulting in severalfold higher NO yields despite a low GTN denitration rate. Indeed, the triple mutant converts GTN almost exclusively to 1,2-GDN and NO.

Most importantly, the triple mutant produced superstoichiometric NO (in 20 min >2 NO/ALDH active site), which demonstrates that NO formation is a catalytic process that is distinguishable from enzyme inactivation. Three main conclusions can be drawn from the present data: clearance-based denitration of GTN, NO formation, and irreversible ALDH2 inactivation are distinct processes, Cys301 and Cys303 are critically involved in the main denitration pathway, and Glu268 plays a central role in irreversible enzyme inactivation.

Proposed Reaction Mechanism. As illustrated in Scheme 1, the results suggest that ALDH2-catalyzed GTN biotransformation involves three distinct pathways. In a first step common to all three, GTN reacts with Cys302 of the enzyme (E_{red}) to form an intermediate (E_{int}). The main reaction catalyzed by the wild-type enzyme results in formation of nitrite and DTT-reversible inactivation (path *a*). This pathway requires Cys301 and Cys303. A smaller fraction of the enzyme (path *b*) is inactivated irreversibly in a reaction that is strongly promoted by Glu268. The third pathway yields NO in a reaction that results in formation of a DTT-reducible oxidized enzyme species (path *c*). This reaction cycle predominates for the triple mutant.

A more concrete, but inevitably more speculative version of the proposed model is presented in Scheme 2. GTN denitration (A) requires Cys302 and seems to yield 1,2-GDN almost exclusively. Accordingly, and in line with previous proposals (Chen et al., 2002), the intermediate (E_{int} in Scheme 1) may be a thionitrate. As previously suggested (Beretta et al., 2008a), the thionitrate may reversibly rearrange to a sulfenyl

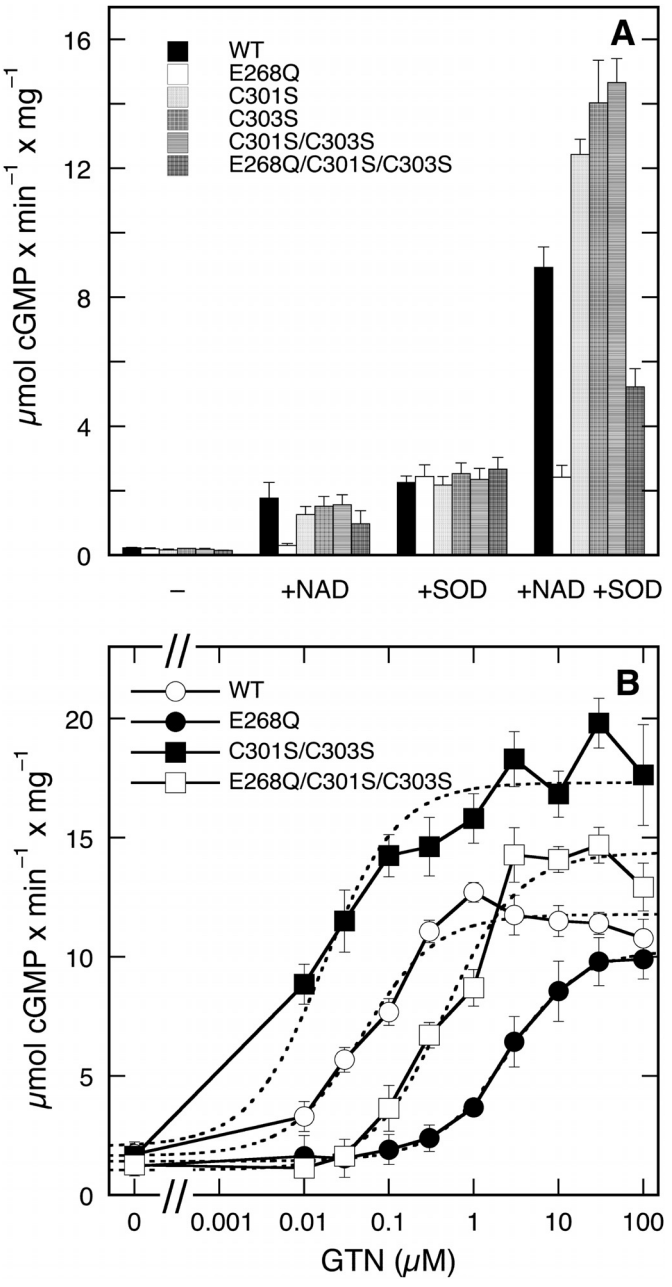


Fig. 4. Effects of NAD^+ and SOD on sGC activation by GTN in the presence of WT and mutant ALDH2. A, purified bovine lung sGC (50 ng) was incubated at 37°C for 2 min in a final volume of 100 μl with 0.1 μM GTN and 0.36 μM WT or mutant ALDH2 (C301S, C303S, C301S/C303S, E268Q, or E268Q/C301S/C303S). NAD^+ (1 mM) and SOD (1000 U/ml) were present as indicated. Assay mixtures contained 50 mM TEA/HCl, pH 7.4, 0.5 mM [α-^{32}P]GTP (~250,000 cpm), 3 mM $MgCl_2$, and 1 mM cGMP. [^{32}P]cGMP was isolated and quantified as described under *Materials and Methods*. Data are mean values ± S.E. of three to five independent experiments. B, GTN concentration dependence. Purified bovine lung sGC (50 ng) was incubated at 37°C for 2 min as described above with 1 mM NAD^+ and 1000 U/ml SOD and the indicated concentrations of GTN in the presence of WT or mutant (E268Q, C301S/C303S, or E268Q/C301S/C303S) ALDH2 (0.36 μM each). Data are mean values ± S.E. of three to five independent experiments.

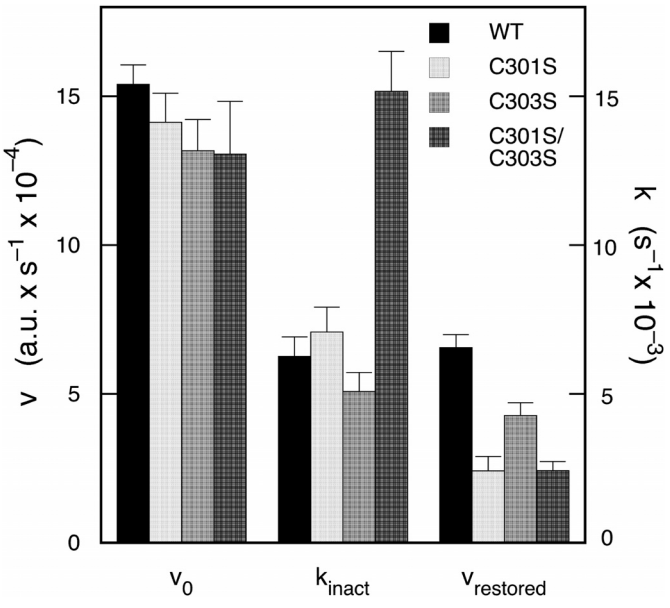
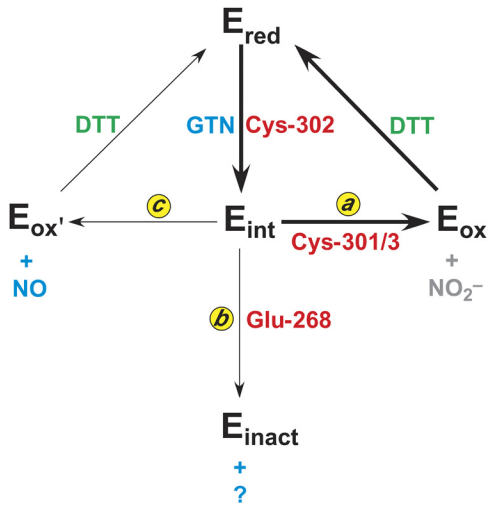


Fig. 5. Inactivation of WT and mutant ALDH2 by GTN. GTN-induced inactivation of WT and mutant ALDH2 (C301S, C303S, and C301S/C303S) was assayed in the presence of 0.4 mM acetaldehyde, 0.4 mM NAD^+, and 10 mM $MgCl_2$ in 250 μl of potassium phosphate (50 mM, pH 7.4). After 2 min, the reaction was started by addition of ALDH2 (0.59 μM) and monitored to obtain initial rates of acetaldehyde oxidation (v_0). Approximately 4 min later, 50 μM GTN was added for determination of inactivation rate constants (k_{inact}) by fitting to a single exponential curve. After complete inactivation, 0.4 mM DTT was added to determine restored activities ($v_{restored}$). Data are mean values ± S.E. of four to seven independent experiments.

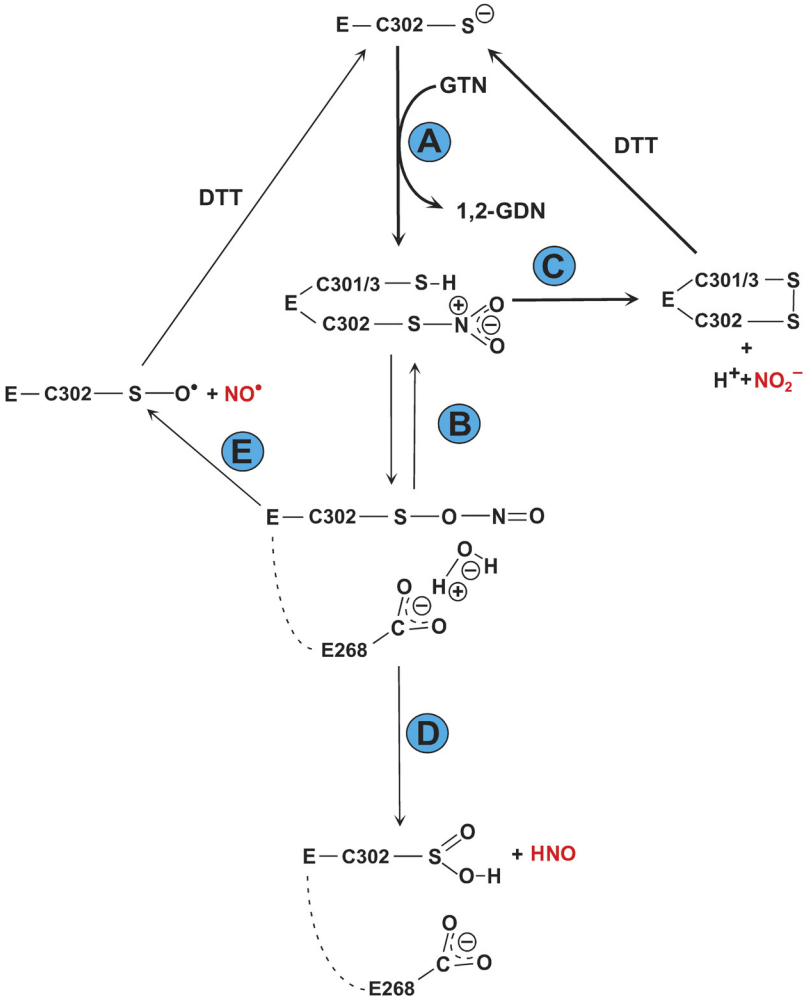


Scheme 1. Three pathways for ALDH2-catalyzed GTN denitration are inferred. Because all reactions strictly depend on Cys302, we propose an initial step, common to all three pathways, in which the enzyme (E_{red}) reacts with GTN to form an intermediate (E_{int}). From there, the main pathway results in DTT-reversible inactivation and formation of nitrite (path *a*). This pathway requires the presence of Cys301 and Cys303. A smaller fraction of the enzyme (path *b*) is inactivated irreversibly in a reaction that is strongly promoted by Glu268. The present results identify a third pathway that yields NO and results in DTT-reversible inhibition (path *c*). This reaction cycle, which will produce NO in superstoichiometric amounts, is the dominant pathway for the triple mutant.

nitrite as an alternative intermediate (B). The main pathway catalyzed by the wild-type enzyme most likely involves formation of nitrite and a disulfide between Cys302 and one of the vicinal cysteines (C). DTT can regenerate the reduced cysteine residues, thereby enabling catalytic conversion of GTN to 1,2-GDN and nitrite (clearance-based GTN metabolism).

Accepting the putative role of Glu268 as a general base catalyst, this residue is expected to affect the reaction by promoting electrophilic attack of the intermediate on a nascent hydroxide ion. How this might affect the outcome of the reaction is unclear. One attractive possibility, illustrated in Scheme 2, is that the intervention of Glu268 results in heterolysis of the sulfenyl nitrite, which might yield a protein-sulfinate and nitroxyl (D). This would explain both irreversible inactivation (by formation of a sulfinate that is not reducible by DTT) and SOD-dependent NO formation (because SOD oxidizes nitroxyl to NO) (Zeller et al., 2009).

For the third pathway, we suggest homolytic cleavage of the sulfenyl nitrite (E). DTT may react with the sulfinyl radical or a subsequently formed species to regenerate Cys302, thereby enabling multiple turnovers of NO formation. Notwithstanding the lack of information on the structure of reaction intermediates and the oxidized species formed upon exposure of the protein to GTN, the present study demonstrates that ALDH2 can produce NO directly from GTN in a catalytic process and, thus, supports our proposal that ALDH2-catalyzed reduction of GTN to NO



Scheme 2. Hypothetical reaction mechanism for ALDH2-catalyzed GTN denitration. A, GTN denitration requires Cys302 and yields 1,2-GDN almost exclusively. Thus, the intermediate formed in the reaction between GTN and ALDH2 is most likely a Cys302-centered thionitrate. B, reversible rearrangement of the thionitrate may yield a sulfenyl nitrite as an alternative intermediate. C, the main pathway for the wild-type enzyme probably involves formation of nitrite and a disulfide bridge between Cys302 and one of the vicinal cysteines. Certain thiols such as DTT enable multiple turnovers of this pathway by reduction of the disulfide. D, Glu268 may stimulate hydrolysis of the sulfenyl nitrite, yielding nitroxyl and an irreversibly inactivated sulfinate at Cys302. E, homolysis of the sulfenyl nitrite may yield NO and a sulfinyl radical. Superstoichiometric NO formation with the triple mutant suggests that DTT can regenerate the reduced Cys302 residue from this or a subsequently formed enzyme species.

essentially contributes to vascular bioactivation of GTN in vivo.

Physiological Relevance. The surprising discovery that bioactivation of GTN involves catalysis by ALDH2 (Chen et al., 2002) raised the question of how nitrite, the main product of GTN bioconversion by ALDH2, is linked to vascular relaxation. Originally it was suggested that reduction of nitrite by components of the mitochondrial respiratory chain might provide that link. However, known affinities of nitrite for such components appear too low, and interfering with respiration did not affect mitochondrial biotransformation of GTN (Kollau et al., 2009), suggesting that ALDH2 catalysis alone is sufficient.

As a possible solution, we found that ALDH2 catalyzed direct NO formation from GTN in a minor pathway, accounting for 5 to 10% of the total yield (Beretta et al., 2008a). We also demonstrated that ALDH2 is irreversibly inactivated by GTN during catalysis, which might offer a potential mechanism for the development of nitrate tolerance (Beretta et al., 2008b). However, these issues remain controversial, partly because of the lack of a detailed mechanism for the proposed reactions. In addition, the apparent linkage between NO formation and ALDH2 inactivation seemed to limit the production of NO to one turnover, which greatly diminished the potential physiological significance of the observed reactions. The present study goes some way in answering the remaining questions. In summary,

1. ALDH2 catalyzes three distinct pathways of GTN biotransformation that involve different active site residues.
2. The assignment of specific functions of active site residues to the different catalytic pathways allows the first data-based attempt to describe the complex mechanism of ALDH2-catalyzed GTN bioconversion.
3. Clearance-based metabolism producing nitrite and bioactivation, which yields NO involve two separate pathways, implying that 1,2-glyceryl dinitrate formation measured in blood vessels may not be a valid measure for vascular ALDH2-catalyzed GTN bioactivation. (Although GTN denitration is a necessary first step in the formation of NO, the thionitrate is metabolized along three different pathways, only one of which yields NO. Accordingly, denitration is not inseparably linked to NO formation, and the NO/1,2-GDN ratio may vary widely.)
4. The demonstration that NO formation is catalytic and occurs through a pathway distinct from that resulting in irreversible enzyme inactivation greatly increases the potential in vivo relevance of direct NO formation.
5. Finally, the observation that direct NO formation can be dissociated from irreversible inactivation and clearance-based denitration may pave the way for the design of ALDH2 substrates that are more efficient vasodilators and less prone to develop vascular tolerance than GTN.

Authorship Contributions

Participated in research design: Wenzl, Beretta, Russwurm, Koesling, Schmidt, Mayer, and Gorren.

Conducted experiments: Wenzl, Beretta, and Griesberger.

Contributed new reagents or analytic tools: Russwurm and Koesling.

Performed data analysis: Wenzl, Beretta, Griesberger, Schmidt, Mayer, and Gorren.

Wrote or contributed to the writing of the manuscript: Wenzl, Mayer, and Gorren.

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