Role of Reduced Lipoic Acid in the Redox Regulation of Mitochondrial Aldehyde Dehydrogenase (ALDH-2) Activity

IMPLICATIONS FOR MITOCNDRIAL OXIDATIVE STRESS AND NITRATE TOLERANCE*

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Chronic therapy with nitroglycerin results in a rapid development of nitrate tolerance, which is associated with an increased production of reactive oxygen species. We have recently shown that mitochondria are an important source of nitroglycerin-induced oxidants and that the nitroglycerin-bioactivating mitochondrial aldehyde dehydrogenase is oxidatively inactivated in the setting of tolerance. Here we investigated the effect of various oxidants on aldehyde dehydrogenase activity and its restoration by dihydrolipoic acid. In vivo tolerance in Wistar rats was induced by infusion of nitroglycerin (6.6 μg/kg/min, 4 days). Vascular reactivity was measured by isometric tension studies of isolated aortic rings in response to nitroglycerin. Chronic nitroglycerin infusion lead to impaired vascular responses to nitroglycerin and decreased dehydrogenase activity, which was corrected by dihydrolipoic acid co-incubation. Superoxide, peroxynitrite, and nitroglycerin itself were highly efficient in inhibiting mitochondrial and yeast aldehyde dehydrogenase activity, which was restored by dithiol compounds such as dihydrolipoic acid and diithiothreitol. Hydrogen peroxide and nitric oxide were rather insensitive inhibitors. Our observations indicate that mitochondrial oxidative stress (especially superoxide and peroxynitrite) in response to organic nitrate treatment may inactivate aldehyde dehydrogenase thereby leading to nitrate tolerance. Glutathionylation obviously amplifies oxidative inactivation of the enzyme providing another regulatory pathway. Furthermore, the present data demonstrate that the mitochondrial dithiol compound dihydrolipoic acid restores mitochondrial aldehyde dehydrogenase activity via reduction of a disulfide at the active site and thereby improves nitrate tolerance.

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Organic nitrates such as nitroglycerin (glyceryl trinitrate, GTN) have been used for over a century in the therapy of cardiovascular diseases like myocardial infarction, unstable angina, and arterial hypertension (1). However, the usefulness of organic nitrates is limited by tolerance, which develops shortly after onset of treatment. The mechanisms underlying nitrate tolerance remain only in part defined and are most likely multifactorial (2). Previously, we found that 3 days of nitrate treatment doubled vascular superoxide (O2−) production (3), which was also found in human bypass material from GTN-treated patients (4).

Chen et al. (5) identified the mitochondrial aldehyde dehydrogenase (ALDH-2) as a GTN-metabolizing enzyme and a possible important component in the processes leading to tolerance. This concept was supported by recent studies in ALDH-2-deficient mice (ALDH-2−/−) (6). Our laboratory further substantiated this concept in an animal model of in vivo tolerance and extended previous observations by demonstrating that mitochondria are a major source of reactive oxygen species formation in response to acute and chronic GTN challenges (7, 8). The importance of the ALDH-2 concept for clinical nitrate tolerance was proven by two independent clinical studies in Asian subjects with a point-mutated, dysfunctional ALDH-2 (9, 10). Because mitochondrial reactive oxygen species formation seems to play a major role for development of tolerance and cross-tolerance, we verified this hypothesis in mice with heterozygous deficiency in mitochondrial superoxide dismutase (Mn-SOD−/−) that were more susceptible for the development of in vitro nitrate and cross-tolerance (tachyphylaxis) (11).

Previous studies implicated that ALDH-2 is oxidatively inhibited by organic nitrates (5, 8, 11–13), and it has been known that ALDH-2 has redox-sensitive thiol groups, which are subject to oxidative inactivation (14–16), which may also have important implications for alcohol-induced cell damage and cardiotoxicity (17, 18). Despite this knowledge, very little is known about the nature of ALDH-2-inhibiting oxidants, espe-
cially, about restoration of enzyme activity. Because chronic GNTN treatment increases vascular superoxide and peroxynitrite formation (3, 19–21) and because reduced α-lipoic acid/lipoamide represent potent dithiol reductants with their own reductase system in mitochondria (22–24) the present study was focused on these compounds. We here sought to determine the role of reduced α-lipoic acid/lipoamide for maintenance and restoration of ALDH-2 activity and to gain new insights in the oxidative inactivation process of ALDH-2 by various oxidants.

**EXPERIMENTAL PROCEDURES**

**Reagents**—For isometric tension studies, GNTN was used from a Nitrolingual infusion solution (1 mg/ml) from G. Pohl-Boskamp (Hohenlockstedt, Germany). For induction of in vivo tolerance, GNTN was used from a solution in ethanol (102 g/liter), which was obtained from UNIKEM (Copenhagen, Denmark). Sin-1 and SPE/NO were from Cayman Chemicals (Ann Arbor, MI). Reduced (±)-α-lipoic acid (dihydrolipoic acid), (±)-α-lipoamide (lipoic acid-ε-lysine), and XO (buttermilk, grade IV) were purchased from Sigma-Aldrich, and yeast aldehyde dehydrogenase (lot 93300620, EC 1.2.1.5) was from Roche Diagnostics (Mannheim, Germany). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich, Fluka, or Merck.

**Animals and in Vivo Treatment**—All animal treatment was in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and was granted by the Ethics Committee of the University Hospital Mainz. We used 30 male Wistar rats (250 g) from Charles River (Sulzfeld, Germany). In vivo tolerance was induced by chronic infusion of rats with GNTN in ethanol (rate: 100 μg/kg/dose: 6.6 μg/kg/min for 4 days) by implanted osmotic pumps (Alzet, model 1007D, 1 μl/h for 7 days) from Durect Corp. (Cupertino, CA). Infusion of the solvent ethanol served as a control. The detailed protocol was recently published (7, 8). One should note that the ethanol dose was 300 μg/kg/h, which is far below doses reached in alcohol abuse or animal studies on alcoholism, thus toxic effects of ethanol metabolism can be excluded.

**Isometric Tension Studies**—Vasodilator responses to GNTN were assessed with endothelium-intact isolated rat aortic rings mounted for isometric tension recordings in organ chambers, as described previously (3, 25). In vitro tolerance (tachyphylaxis) was induced by performing two consecutive concentration-relaxation curves with GNTN (EC100 = 31.6 μM) in the same samples. Dihydrolipoic acid and lipoamide (each 0.1 mM) were added to the samples together with the ALDH-2 cofactor NAD+ (1 mM) was added to the samples as described above.

**Detection of Glutathionylated Proteins in Mitochondrial Suspension in the Presence of GNTN/Peroxynitrite and GSH—SDS-PAGE and Western blotting with the same samples as used for HPLC-based measurement of ALDH-2 activity was performed as recently described (28, 29). Briefly, the samples were treated with Triton X-100, boiled with Laemmli buffer without mercaptoethanol, and subjected to gel electrophoresis and Western blotting. For detection of glutathionylated proteins we followed the protocol provided by the manufacturer and a previously published procedure (30). The first antibody against GSH-α-dia-duct proteins was a monoclonal mouse antibody from ViroGen (Watertown, MA) at a dilution of 1:1000. Detection was performed by enhanced chemiluminescence with peroxidase-conjugated anti-mouse secondary antibody (1:10,000, Vector Laboratories, Burlingame, CA).

For immunoprecipitation of ALDH-2 250 μg of protein of each sample was incubated with 2.5 μg of rabbit-polycyonal anti-ALDH-2 antibody (31) coupled on protein A-Sepharose
Restoration of ALDH-2 Activity by Dihydrolipoic Acid

**FIGURE 1. Effect of oxidants on ALDH-2 dehydrogenase activity in isolated rat heart mitochondria.** The ALDH-2 activity was determined by an HPLC-based assay measuring the conversion of the substrate benzoic acid to the product benzoic acid. The effects of authentic peroxynitrite (5 min, room temperature), the peroxynitrite generator Sin-1 or GTN (90 min, 37 °C) on ALDH-2 dehydrogenase activity were tested in sonicated mitochondrial suspensions (0.5 mg/ml total protein). Data are mean ± S.E. of three independent experiments. *p < 0.05 versus control.

**TABLE 1**

Concentrations of oxidants that cause half-maximal inhibition of yeast ALDH

| Reactive species | IC_{50} μM or milliunits/ml |
|------------------|-----------------------------|
| GTN              | 4.44 ± 0.94                 |
| H₂O₂             | >1000                       |
| ONOO⁻             | 7.92 ± 4.12                |
| Sin-1            | 16.30 ± 4.41               |
| SPE/NO           | 58.98 ± 13.81              |
| SPE/NO plus HX/XO| 8.48 ± 2.14                |
| HX/XO            | ~1 milliunit/ml = 17 μM O₂/90 min* |

* 1 unit/ml yeast ALDH (Roche Applied Science) in phosphate-buffered saline (1 ml) plus oxidant (ONOO⁻, 5 min at room temperature; Sin-1, SPE/NO, HX (1 mM)/XO, HX (1 mM)/XO (0.625 milliunit/ml) plus SPE/NO, H₂O₂, GTN 90 min 37 °C) plus 1 mM NAD⁺ plus 1 mM propionaldehyde; measurement at 340 versus 450 nm for 120 s.

* Based on previous published release rates (36).

(—Yeast ALDH—To study the inhibitory effects of various reactive oxygen and nitrogen species in more detail we used highly purified yeast ALDH along with an optical activity assay. Although the sequence similarity between yeast ALDH and the mitochondrial isoform ALDH-2 in rat is <50% (using ExPASy similarity alignment), the yeast enzyme may serve as a crude model to study oxidative damage, because its thiol active site is also highly sensitive to oxidative inactivation. Indeed, we found that yeast ALDH was easily oxidized and inactivated by peroxynitrite, Sin-1, GTN, and superoxide-generating system HX/XO, which caused half-maximal inhibition (IC_{50}) in the lower micromolar range (Table 1). The NO-donor SPE/NO alone required rather high concentrations (~100–120 μM based on NO concentration, because each SPE/NO releases 1.7–2 NO molecules), which were dramatically decreased in the presence of the superoxide-generating system HX/XO (~17 μM based on NO concentration) (Table 1). This increased inhibitory potency in the presence of NO and superoxide source is probably due to peroxynitrite formation and could be blocked by ~50% in the presence of SOD (see supplemental information, Fig. VI). H₂O₂ was not a potent inhibitor of yeast ALDH and required a concentration of more than 1 mM for half-maximal inhibition (Table 1). The detailed concentration-inhibition curves for each oxidant along with effects of antioxidants are presented in the supplemental information.

We also tested the effect of low molecular weight thiol compounds on ALDH activity upon treatment with peroxynitrite, Sin-1, and GTN (see supplemental information). Simultaneous administration of peroxynitrite and GSH decreased rather than increased the activity pointing to an inhibitory effect of GSH. Simultaneous administration of GTN and GSH also lacked any protective effect. Only an addition of Sin-1 and GSH resulted in minor protection of ALDH activity. In contrast, the diithiol compounds DTT and dihydrolipoic acid caused a more pronounced protection of ALDH activity. To ensure that this observation was due to restoration of dehydrogenase activity in oxidized ALDH rather than direct scavenging of oxidants (peroxynitrite and GTN), we added the diithiols after completion of

**RESULTS**

**Oxidative Inactivation of ALDH-2 in Isolated Mitochondria—** ALDH-2 dehydrogenase activity in isolated rat heart mitochondria was inhibited in a dose-dependent manner by authentic peroxynitrite as well as by *in situ* generated peroxynitrite from Sin-1 (Fig. 1). The inhibitory potency on a molar basis was almost identical for both of these oxidants. GTN was even more powerful in inhibiting the enzyme activity (Fig. 1).

**Detection of Yeast ALDH Activity in Response to Treatment with Different Reactive Oxygen and Nitrogen Species—** Yeast ALDH (1 unit/ml, 680 nM in phosphate-buffered saline) was incubated with increasing concentrations of peroxynitrite (5 min at 37 °C), Sin-1, SPE/NO, HX (1 mM)/XO, or SPE/NO plus HX (1 mM)/XO (0.625 milliunit/ml) (90 min at 37 °C). In some experiments antioxidant enzymes such as Cu,Zn-SOD and/or catalase (300–1000 units/ml) or low molecular weight thiol compounds such as GSH, DTT, and dihydrolipoic acid (up to 1 mM) were co-incubated together with the oxidants. Upon addition of 1 mM propionaldehyde and 1 mM NAD⁺ the ALDH dehydrogenase activity was determined spectrophotometrically by following the formation of NADH by the absorption at 340 versus 450 nm for 120 s at room temperature.

**Statistical Analysis—** Results are expressed as mean ± S.E. One-way analysis of variance (with Bonferroni or Dunn correction for comparison of multiple means) was used for comparisons of vasodilator potency and efficacy, ALDH-2, and yeast ALDH activity. The EC_{50} value for each experiment was obtained by log-transformation. *p values < 0.05 were considered significant. Extended experimental procedures are provided in the supplemental information.
Restoration of ALDH-2 Activity by Dihydrolipoic Acid

Although not significant, the addition of 100 μM dihydrolipoic acid tended to increase this activity, 250 μM dihydrolipoic acid further normalized it, whereas 500 μM dihydrolipoic acid had no additional effect (Fig. 3). Similar observations were made with another complex III inhibitor myxothiazol (25 μM) where dihydrolipoic acid (100 μM) showed the tendency to restore impaired ALDH-2 activity (supplemental information). In contrast, when ALDH-2 activity was inhibited by the thiol-targeted ALDH inhibitor benomyl (25 μM) or the specific ALDH-2 inhibitor daidzin (100 μM), dihydrolipoic acid (100 μM) failed to show any protective effect on ALDH-2 activity (supplemental information). This excludes nonspecific interaction of dihydrolipoic acid with the dehydrogenase activity assay.

Detection of Glutathionylated Proteins in Mitochondrial Suspension in the Presence of GTN/Peroxynitrite and GSH—The ALDH-2 activity was significantly attenuated by GTN bolus incubation of isolated rat heart mitochondria. Interestingly, co-incubation with high GSH concentrations resulted in an additional decrease in activity that was associated with heavy antibody staining against glutathionylated proteins (Fig. 3A and supplemental information, Figs. XIII and SXIV).

**FIGURE 2.** Restoration of ALDH-2 dehydrogenase activity by dihydrolipoic acid and DTT in heart mitochondria from tolerant (in vivo GTN treated) rats and upon treatment with antimycin A. The ALDH-2 activity was determined by an HPLC-based assay measuring the conversion of the highly specific substrate 2-hydroxy-3-nitrobenzaldehyde to its benzoic acid product. A, the effects of dihydrolipoic acid (LA) and DTT (0.1 or 1 mM) on ALDH-2 dehydrogenase activity were tested in mitochondrial suspensions (1 mg/ml total protein) from in vivo ethanol (sham)- or GTN (tolerant)-treated rats. Data are mean ± S.E. of 4 (ethanol in vivo group) and 8–19 (GTN in vivo group) independent experiments. *, p < 0.05 versus ethanol in vivo (without treatment) and #, p < 0.05 versus GTN in vivo (without treatment). B, the effects of dihydrolipoic acid (0.1, 0.25, or 0.5 mM) on ALDH-2 dehydrogenase activity were tested in mitochondrial suspensions from untreated rats upon preincubation with antimycin A. This complex III inhibitor blocks mitochondrial respiratory chain and thereby generates superoxide, which causes oxidative ALDH-2 inactivation. Data are mean ± S.E. of 3–4 independent experiments. *, p < 0.05 versus control.

Also peroxynitrite induced glutathionylation when GSH was added after decay (completed oxidations) of peroxynitrite (Fig. 3B and supplemental information, Figs. XIII and SXIV). However, when co-incubated with peroxynitrite, GSH prevented ALDH-2 inactivation probably by direct scavenging of peroxynitrite and derived free radicals (Fig. 3C). To further elucidate whether ALDH-2 is among the glutathionylated proteins, we performed immunoprecipitation of ALDH-2 followed by Western blotting against GSH-adducts. As shown in Fig. 3D by immunoprecipitation, ALDH-2 was glutathionylated upon incubation with peroxynitrite followed by addition of GSH. However, we were not able to detect similar staining in the presence of GTN/GSH indicating that glutathionylation under these conditions is of minor importance or reversible (see Reactions 1 and 2 under “Discussion”). For immunoprecipitation we used a specific antibody against ALDH-2 (see supplemental Fig. XV).
determined by isometric tension studies of isolated vessels in organ baths. In vivo treatment of rats with GTN led to the development of nitrate tolerance as indicated by a severe right shift of the concentration-relaxation curve to the vasodilator GTN as compared with aortic rings from sham-treated rats (Fig. 4A). This right shift was caused by a 10-fold decreased sensitivity of the tolerant vessels to GTN or a 10-fold decreased potency (ED$_{50}$) of this nitrovasodilator in vessels from GTN-treated rats (supplemental Table I). In the presence of 100 $\mu$M dihydrolipoic acid in the organ bath the impaired potency of GTN in tolerant vessels was almost normalized, and the ED$_{50}$ was 8-fold improved (Fig. 4A and supplemental Table I).

A similar observation was made when aortic rings of untreated controls were subjected to two consecutive GTN concentration-relaxation curves. The highest GTN concentration during the first treatment (ED$_{100}$ = 31.6 $\mu$M) induced in vitro tolerance (tachyphylaxis) as indicated by a right shift of the subsequent GTN-concentration-relaxation curve (Fig. 4B). This caused a 10-fold decrease in the vasodilator potency of GTN in the tolerant vessels (Table I, supplemental information), which was normalized in the presence of lipoamide in the organ bath to the level of the untreated control (Fig. 4B and supplemental Table I). The cellular uptake of lipoamide is facilitated by membrane transporters, and mitochondrial lipoamide dehydrogenase has a higher selectivity for this compound as compared with the free lipoic acid. Reduction may also occur by other (cytosolic) enzymes such as thioredoxin reductases and glutathione reductases (22–24).

**DISCUSSION**

ALDH-2 is the mitochondrial isoform of aldehyde dehydrogenases and is so far known to exert three enzymatic activities. The traditional activities comprise the dehydrogenase activity, which requires the cofactor NAD$^+$ and the esterase activity (for details see Fig. 5). Both activities seem to involve cysteine thiol groups (15, 32) and are inhibited by oxidants and thiol-targeted compounds such as Ellman’s reagent (8). In 2002, an additional function was attributed to this enzyme when Chen et al. (5) identified ALDH-2 as a GTN-bioactivating enzyme. Bioactivation of GTN and other nitrates is a reduction reaction and requires electrons from active site-located cysteine thiol groups, which are oxidized to a disulfide bridge during the catalytic cycle (Fig. 5). The activity of oxidized ALDH-2 is restored by DTT and reduced lipoic acid as demonstrated by the present study. For the interested reader we provide an extended discussion in the supplemental information.

According to the “oxidative stress concept in nitrate tolerance” vascular and mitochondrial oxidants seem to play a major role for the development of nitrate and cross-tolerance in response to chronic organic nitrate treatment (2–4, 11). More-
FIGURE 4. Effect of dihydrolipoic acid or reduced lipoamide on GTN potency in isolated rat aortic vessel segments upon chronic (in vivo) or acute (in vitro) treatment with GTN. A, concentration-relaxation curves for GTN (10^{-9} to 10^{-4.5} M) were established by isometric tension studies in vessels from rats upon treatment with ethanol (inverse triangles) or GTN in ethanol (circles). The effect of dihydrolipoic acid (100 μM) on GTN potency was tested in aorta from in vivo GTN treated (tolerant) rats (triangles). Data are mean ± S.E. of 7–24 independent experiments. B, concentration-relaxation curves for GTN (10^{-9} to 10^{-4.5} M) in vessels from untreated (circles) and GTN (31.6 μM)-treated (triangles) rats. The effect of lipoamide (100 μM) (upon reduction by the cellular/mitochondrial reductases) on GTN potency was tested in aorta upon in vitro GTN (31.6 μM) treatment (inverse triangles). Data are mean ± S.E. of 12–32 independent experiments.

FIGURE 5. Enzyme activities of the mitochondrial aldehyde dehydrogenase (ALDH-2). The ALDH-2 has two long known enzyme activities. The dehydrogenase activity requires the cofactor NAD^{+} (blue) and converts aldehydes to the respective carboxylic acids (e.g. acetaldehyde to acetic acid). The esterase activity does not require cofactors and converts carboxylic acid esters (probably also esters from other acids) to the free acid and the respective alcohol. These enzyme activities involve thiol-dependent catalysis (red and purple), which are inhibited by thiol-oxidizing compounds. The third activity is the “reductase activity,” which was recently identified by Chen et al. and is responsible for bioactivation of organic nitrates such as GTN yielding nitrite and the dinitrate (1,2-GDN). This activity differs from the others, because it reduces the nitrogen of the dissociated nitrite. The electrons for this reduction are not provided by NADH (because NAD^{+} accelerates this reduction) but by cysteine-thiols at the active site of the enzyme (red) that are thereby converted to a disulfide. Reductase activity of this oxidized ALDH-2 can be restored by dithiol compounds such as DTT and dihydrolipoic acid. 1,2-GDN, 1,2-glyceryl dinitrate.

over, oxidative inhibition of prostacyclin synthase (20) as well as mitochondrial ALDH activity (8) may present other key events in the development of nitrate tolerance. Peroxynitrite production was indirectly evidenced by protein tyrosine nitration (21), especially of prostacyclin synthase (20), which is a valid biomarker for peroxynitrite formation in vivo (33). Additional support for this concept is based on the recent identification of the mitochondria as a major source of oxidative stress in tolerant animals (7, 8, 12) and increased tolerance susceptibility of Mn-SOD^{+/-} mice (11). Mn-SOD^{+/-} mice have partial (50%) deficiency in mitochondrial SOD (Mn-SOD) (34) and show increased basal mitochondrial oxidative stress as well as increased oxidant levels and decreased ALDH-2 activity in response to GTN infusion as well as increased susceptibility to GTN-induced tolerance (11). The involvement of mitochondria in the development of nitrate tolerance was previously postulated by the “thiol concept” of Needleman and coworkers (35).

Bolus authentic peroxynitrite, in situ Sin-1 generated peroxynitrite and GTN showed similar efficacy in inhibiting the enzyme in mitochondrial suspensions (Fig. 1). We further substantiated this finding by using purified yeast ALDH as a model of oxidative ALDH-2 inactivation (Table 1 and supplemental information). The results point to an intermediary formation of peroxynitrite from the reaction of NO with superoxide, which has previously been shown for the inactivation of yeast alcohol dehydrogenase by simultaneous formation of superoxide and NO (36). In accordance with the above described hypothesis, we observed a severe decrease in ALDH-2 activity in mitochondria from GTN in vivo-treated rats but also in response to acute supplementation with the complex III inhibitor antimycin A (Figs. 2 and 3). Antimycin A blocks the mitochondrial respiratory chain and thereby uncouples electron flow to molecular oxygen resulting in increased superoxide formation, which inhibits ALDH-2 activity.

It has been previously shown that DTT but not GSH may restore the activity of oxidized ALDH-2 (5). The fact that DTT is a synthetic compound and GSH could not reactivate ALDH-2 implicated the existence of another physiologically relevant low molecular weight dithiol reductant. Lipoic acid and its predominant metabolite lipoamide occur at considerable concentrations within mitochondria and are converted to the reduced species by specific reductases such as the mitochondrial NADH-dependent dihydrolipoamide dehydrogenase as well as cytosolic NADPH-dependent glutathione reductase (22) but also by mitochondrial and cytosolic thioredoxin reductases (24). Reduced lipoic acid was able to at least partially restore ALDH-2 activity in isolated mitochondria from in vivo GTN-treated rats but also to improve ALDH-2 activity in sham-treated controls (Fig. 24). DTT even completely normalized ALDH-2 activity in mitochondria from tolerant rats. Dihydrolipoic acid also dose-dependently improved ALDH-2 activity upon induction of mitochondrial superoxide formation by anti-
A last possible explanation for only partial restoration of dehydrogenase activity by dithiols in the presence of bolus GTN in vitro could be that the organic nitrate was not degraded during incubation and at excess concentrations blocked protective effects of dithiols. The almost complete restoration of vasodilator responses by reduced lipoic acid (in the setting of in vivo tolerance) and lipoamide (in the setting of in vitro tolerance, tachyphylaxis) may involve also direct antioxidative properties of the reduced and oxidized forms of these compounds besides ALDH activity restoration (39).

To address this issue in more detail, the effects of thiol compounds were studied using yeast ALDH. GSH had only minor effect, whereas DTT and lipoic acid could partially restore ALDH activity (see supplemental information). The lack of protective effects of GSH is best explained on a molecular basis: oxidized ALDH-2 or yeast ALDH contains a disulfide bridge (-SS-) or a sulfenic acid (-SOH) that require two electrons for reduction. GSH nucleophilically attacks the sulfur atoms and provides the two electrons resulting in a mixed disulfide. Because the active site of aldehyde dehydrogenases is rather narrow, it is conceivable that only one GSH molecule can enter the active site. Because complete reduction of oxidized ALDH always requires two GSH molecules, this reaction will end up with the inactive mixed disulfide (G-SS-ALDH). DTT and reduced lipoic acid in a first step also form a mixed disulfide with oxidized ALDH but, in contrast to GSH, can complete the reduction via intramolecular thiol exchange. Moon et al. (14) found that S-nitrosogluthathione is highly effective in inhibiting ALDH-2 activity, probably by transnitrosylation of the active site thiols of ALDH-2. For the interested reader we provide an Extended Discussion in the supplemental information.

**CONCLUSION**

Taken together, these lines of evidence imply that reduced lipoic acid/lipoamide restores activity of oxidized ALDH-2 and yeast ALDH and that this dithiol is the physiological reductant for this enzyme, which is highly susceptible for oxidative inactivation by superoxide, peroxynitrite, and GTN. Our data support a role of dihydrolipoic acid and lipoamide in the restoration of impaired ALDH-2 reductase activity, which is essential for organic nitrate bioactivation and thereby may prevent/improve nitrate tolerance in response to chronic GTN treatment. However, sulfonic acid formation and/or glutathionylation could provide pathways for irreversible ALDH-2 inhibition requiring de novo synthesis of the enzyme. The sequence of catalytic steps is shown in the hypothetical scheme in Fig. 6. These findings may have important implications for the clinical use of organic nitrates that are bioactivated by ALDH-2 but also for alcoholism. ALDH-2 is one of the major enzymes that are responsible for metabolism of toxic aldehydes, especially of acetaldehyde, which is formed at high concentrations during alcohol abuse. The oxidative inhibition of ALDH-2 by alcohol-induced reactive oxygen and nitrogen species would inevitably lead to accumulation of these toxic aldehydes, which in turn have been reported to activate NADPH oxidases thereby initiating a vicious circle that will finally result in cell damage or death of the whole organism.
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