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ALDH-2 deficiency increases cardiovascular oxidative stress—Evidence for indirect antioxidative properties

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Abstract

Mitochondrial aldehyde dehydrogenase (ALDH-2) reduces reactive oxygen species (ROS) formation related to toxic aldehydes; additionally, it provides a bioactivating pathway for nitroglycerin. Since acetaldehyde, nitroglycerin, and doxorubicin treatment provoke mitochondrial oxidative stress, we used ALDH-2$^{-/-}$ mice and purified recombinant human ALDH-2 to test the hypothesis that ALDH-2 has an *indirect* antioxidant function in mitochondria. Antioxidant capacity of purified ALDH-2 was comparable to equimolar doses of glutathione, cysteine, and dithiothreitol; mitochondrial oxidative stress was comparable in C57Bl6 and ALDH-2$^{-/-}$ mice after *acute* challenges with nitroglycerin or doxorubicin, whereas *chronic* acetaldehyde, nitroglycerin, and doxorubicin treatment dose-dependently increased mitochondrial ROS formation and impaired endothelial function to a greater extent in ALDH-2$^{-/-}$ mice. Maximal nitroglycerin dose applied *in vivo* lead to a ''super-desensitized'' nitroglycerin response in isolated ALDH-2$^{-/-}$ aortas, inaccessible in C57Bl6 mice. Our results suggest that ALDH-2 has an indirect antioxidative property independent of its thiol-moiety in disease states of cardiovascular oxidative stress.

Keywords: Mitochondrial aldehyde dehydrogenase; Reactive oxygen species; Vascular function; Animal models of human disease

In the last years, the mitochondrial aldehyde dehydrogenase (ALDH-2) has gained growing attention as a nitroglycerin reductase [1]. This added a new functionality to the well described dehydrogenase and esterase functions of the enzyme and linked the bioactivation process of nitroglycerin with the oxidative stress concept of nitrate tolerance as proposed by our laboratory [2] and by others [3–5].

Nevertheless, the major role of ALDH-2 is still the elimination of toxic aldehydes like acetaldehyde, the major metabolite of ethanol. Accumulation of toxic aldehydes (that can be viewed as ''reactive carbonyls'') such as malondialdehyde (MDA) or 4-hydroxynonenal (4-HNE) results in MDA- and 4-HNE-protein adducts, leading to lipid peroxidation, protein/enzyme dysfunction, structural damage and apoptosis in alcohol related disorders [6] like alcoholic liver disease [7], heart disease [8] and gastrointestinal cancer [9]. Additionally, they can activate NAPDH oxidases, as described for the Kupffer cell NADPH oxidase in the pathogenesis of alcohol liver disease, for instance [10].

ALDH-2 carries three thiol groups in its catalytic center; two of these -SH groups work as electron donors in the

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nitrate reduction reaction of nitroglycerin, leaving a disulfide bond in the active center. We have recently demonstrated that the activity of ALDH-2 is redox regulated: its activity is inhibited by superoxide and peroxynitrite on the one hand and the activity is increased by dithiols like dihydrolipoic acid on the other [11]. It is therefore conceiving to assume that ALDH-2 may have *direct* antioxidant properties due to its potent reductase function with its highly activated sulfhydryl groups making it a bioactivating enzyme for all potent organic nitrates [12].

In a recent study it was speculated that ALDH-2 significantly contributes to overall antioxidative potential on a cellular level and plays an important role in protecting cells and rats from nitroglycerin-induced tolerance and endothelial dysfunction [13]. We here sought to determine whether ALDH-2 deficiency contributes to increased oxidative stress and cardiovascular complications in response to chronic pro-oxidant stimuli. Nitroglycerin treatment provokes mitochondrial oxidative stress [14], presumably by inhibition of the respiratory chain leading to an increase of the electron leakage [4,15]. Anthracyclines like doxorubicin are used as established chemotherapeutic agents in the systemic treatment of cancer and are known to induce cardiovascular complications and mitochondrial oxidative stress [16–18]. We therefore employed nitroglycerin and doxorubicin (acutely and chronically) treated ALDH-2$^{-/-}$ mice as well as purified human ALDH-2 as tools to further characterize the potential antioxidative role of mitochondrial aldehyde dehydrogenase and to distinguish between *direct* and *indirect* antioxidative properties of the enzyme.

Materials and methods

Animals and in vivo treatment. Young adult (10weeks) male ALDH-2$^{-/-}$ mice were age matched with C57Bl6 mice (wild type, WT). The generation of the ALDH-2 null mutant was described elsewhere [19]. Mice were equipped with micro osmotic pumps from Alzet (Cupertino, CA) containing 450 mM GTN solved in ethanol (undiluted, e.g. 100 μg/h/4d, diluted 1:1 with ethanol, e.g. 50 μg/h/4d, diluted 1:9, e.g. 10 μg/h/4d) or ethanol as a control. Mice were also treated *in vivo* with acetaldehyde (1.2 μl/d *s.c.* for 3 d) and doxorubicin according to two protocols: 48 μg/d for 3 d using micro osmotic pumps for *s.c.* infusion and 200 μg *i.p.* over night. In additional experiments, untreated male Wistar Rats were used (250 g of weight). Animals were killed by exsanguination under isoflurane anesthesia (5% inhalant in room air).

Isometric tension studies. Vasodilator responses to acetylcholine (ACh), diethylamine NONOate (DEA/NO) and nitroglycerin (glycerol trinitrate, GTN) were assessed with endothelium-intact isolated murine aortic rings mounted for isometric tension recordings in organ chambers and preconstricted with prostaglandin $F_{2\alpha}$, as described previously [12].

Mitochondrial ROS formation. Isolated mitochondria were prepared from mice and rat hearts according to previously published protocols and ROS formation was detected by L-012 (100 μM) ECL as recently described [14,20].

Expression, purification, activity, and antioxidant capacity of human ALDH-2. To generate pET16B-hALDH2, a bacterial expression plasmid coding for a His-tagged human ALDH2 protein (His-hALDH2), the plasmid pT7-7-hALDH2 (kindly provided by K.K. Ho and H. Weiner, Purdue University, West Lafayette, USA) was digested with NdeI and HindIII [21]. Antioxidant capacity of purified human ALDH-2 (10–330 nM) was assessed by scavenging of peroxynitrite-derived free radicals or the peroxynitrite anion which were generated by 3-morpholino sydnonimine (Sin-1, 20 μM). Sin-1 has a half-life of 45 min at pH 7.4 and 37 °C. Peroxynitrite and derived free radicals were detected by dihydrorhodamine 123 (10 μM) fluorescence (excitation at 500 nm and emission at 535 nm) in PBS using a plate reader (Twinkle, Berthold Techn., Bad Wildbad, Germany). ALDH-2 antioxidant capacity was compared to those of glutathione, cysteine and dithiothreitol (0.01–10 μM). Fluorescence was followed over 90 min—the results at 45 min are shown.

For description of statistical analysis and extended methods, please refer to the Supplementary material.

Results

Activity and antioxidant capacities of human ALDH-2

The isolated and purified human ALDH-2 enzyme was tested with regard to its potential antioxidant properties. Compared to equimolar amounts of glutathione (GSH), dithiothreitol (DTT), and cysteine, ALDH-2 did not differ in its peroxynitrite scavenging properties (see Fig. 1A). Additionally, the ROS formation of isolated rat cardiac mitochondria from untreated Wistar rats was not increased upon *in vitro* inhibition of ALDH-2 by the selective inhibitor, daidzin (200 μM, see Fig. 1B). Finally, acute challenges with GTN or antimycin A caused similar augmentation in mitochondrial ROS in WT and ALDH-2$^{-/-}$ samples, ruling out an important role of ALDH-2 as a direct ROS scavenger (see Fig. 1C).

Mitochondrial ROS formation upon in vivo treatment

In isolated mouse heart mitochondria, the L-012 derived chemiluminescence signal increased stepwise according to the GTN dose applied *in vivo* (see Fig. 2A). Importantly, the signal was about 20% higher in the ALDH-2$^{-/-}$ mice as compared to WT for each dosage. The signal could be blocked by coincubating the mitochondrial suspension with the respiratory chain complex I inhibitor, rotenone (100 μM).

Chronic doxorubicin treatment caused an increase in mitochondrial ROS levels that was 25–30% higher in the null mutant than in the WT (Fig. 2B) whereas short-time treatment with the drug resulted in no significantly different change of mitochondrial ROS formation between the two strains (not shown). Chronic acetaldehyde treatment increased mitochondrial ROS formation significantly higher in ALDH-2$^{-/-}$ than in WT (by 48.2 ± 6.5% higher), whereas acute *in vitro* challenges did not differently enhance the signal (not shown).

Isometric tension studies

The first two GTN doses (10 and 50 μg/h) did not result in significant alterations of the ACh concentration–relaxation curve (neither in the WT nor in the ALDH-2$^{-/-}$ group) (Fig. 3A and B). Only the highest applied dose (100 μg/h) caused a marked right-shift in the ACh concentration–relax-

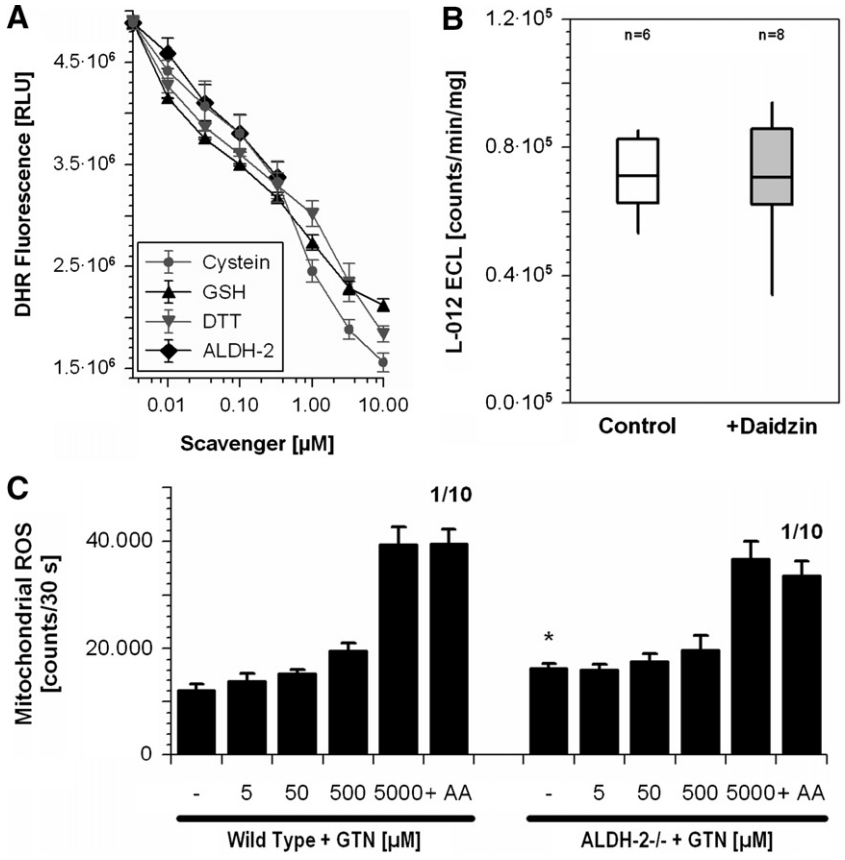

Fig. 1. Antioxidant capacity of ALDH-2. (A) Scavenger properties of thiol compounds and human recombinant ALDH-2 were assessed in a system of 20 μM Sin-1 as a peroxynitrite donor and 10 μM dihydrorhodamine 123 in PBS. (B) Isolated rat heart mitochondria were incubated with the specific inhibitor of ALDH-2, daidzin (200 μM) and ROS were measured by L-012 (100 μM)-enhanced chemiluminescence (ECL). (C) ROS were determined in cardiac mitochondria from WT and ALDH-2$^{-/-}$ mice by L-012 ECL upon *in vitro* challenges with GTN or antimycin A (AA, 0.02 mg/ml). The signal obtained with AA is shown by a 10-fold diminished scale. Data are means ± SEM of 3 (A), 6–8 (B) and 4 (C) independent experiments.

ation curve compatible with endothelial dysfunction in both animal groups (Fig. 3A and B). In contrast, the GTN response showed dose-dependency in WT and was stepwise shifted to the right with increasing GTN doses (Fig. 3C) thereby indicating the development of tolerance. As observed for ACh responses, the first two GTN doses, again, caused no rightward shift in the ALDH-2$^{-/-}$ animals (Fig. 3D). Only the highest applied dose resulted in an additional rightward shift of the GTN concentration–relaxation-curve in ALDH-2$^{-/-}$ mice (Fig. 3D).

Chronic doxorubicin treatment impaired endothelial function (ACh response, Fig. 3E) and smooth muscle function (DEA/NO response, Fig. 3F) to a greater extent in ALDH-2$^{-/-}$ mice as compared to WT mice. In contrast, short-time treatment of mice with doxorubicin over night did not reveal these differences between the two groups (data not shown) indicating that ALDH-2 deficiency promotes cardiovascular complications in the long run but not acutely.

Discussion

The main finding of our study is, that the mitochondrially located enzyme ALDH-2 seems to provide indirect antioxidant properties presumably by preventing the accumulation of toxic aldehydes rather than direct ROS scavenging activity based on its chemical structure. This explains best the observed more pronounced tolerance in response to *in vivo* nitroglycerin administration in ALDH-2$^{-/-}$ mice compared to WT but lack of any significant differences upon acute challenges with acetaldehyde, GTN or antimycin A. Further support for the indirect antioxidative properties of ALDH-2 came from more pronounced vascular dysfunction in ALDH-2$^{-/-}$ mice upon chronic treatment with the anthracycline doxorubicin whereas no significant changes were observed in response to short-time *in vivo* challenges.

In the recent literature as well as in seminal papers published 40 years ago, the mitochondria and in particular the respiratory chain have been suspected to be involved in tolerance development in response to chronic nitroglycerin exposure [4,15]. It has been demonstrated recently, that nitroglycerin tolerance could be prevented by supplying mitochondrial targeted antioxidants [4], low-molecular-weight dithiols like reduced lipoic acid [11] or by upregulating intrinsic antioxidant enzymes like heme oxygenase-1 [22]. All these measures kept the nitroglycerin bioactivation

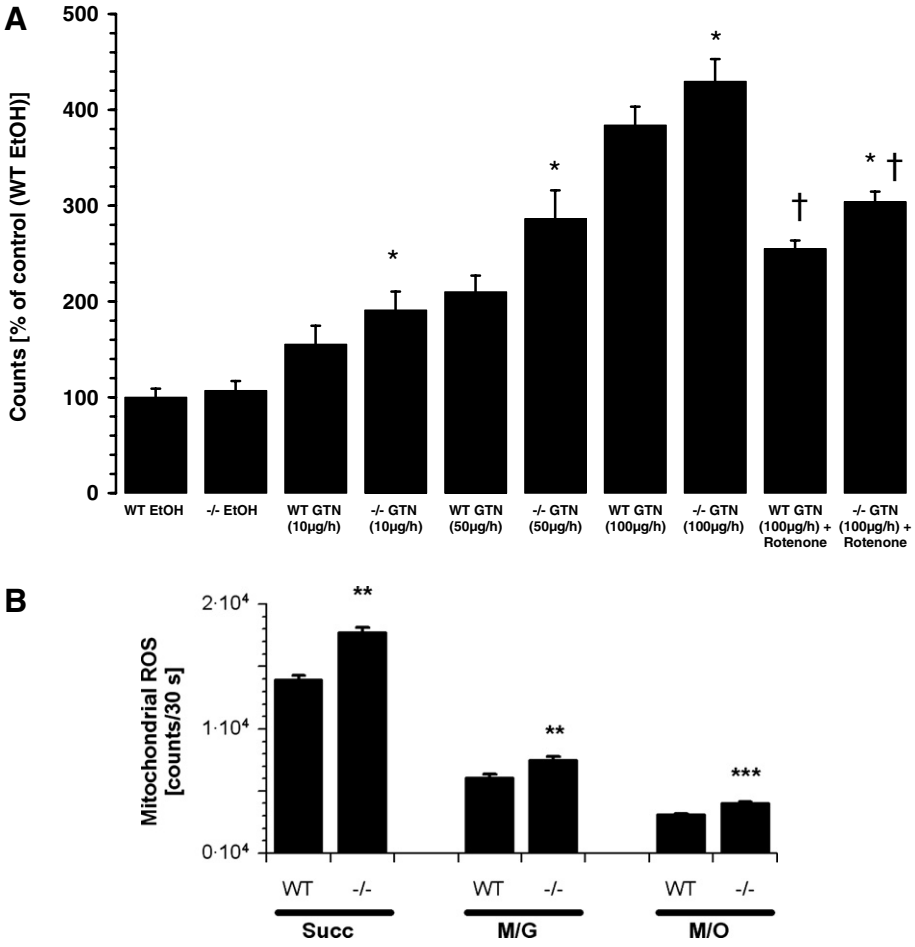

Fig. 2. Mitochondrial ROS formation for chronic nitroglycerin treatment. (A) Isolated cardiac mitochondria where stimulated with succinate (5 mM), the chemiluminescence signal was detected using a single photon counter in the presence of the dye L-012 (100 μM) and in the absence or presence of 100 μM rotenone, a respiratory chain inhibitor at complex I. *X*-axis depicts GTN *in vivo* treatment with the dose applied per hour. WT: C57Bl6, −/−: ALDH-2$^{-/-}$. Data are means ± SEM of 10–11 (C57Bl6) and 8–10 (ALDH-2$^{-/-}$) experiments. $^{*}p < 0.05$ vs WT on the same treatment; $^{\dagger}p < 0.05$ vs GTN (100 μg/h). (B) Mitochondrial ROS formation was measured by L-012 (100 μM) ECL in isolated cardiac mitochondria WT vs ALDH-2$^{-/-}$ treated with doxorubicin, (48 μg/d/3d) upon stimulation with succinate (Succ, 5 mM), malate/glutamate (M/G, 2.5 mM) or malate/oxaloacetate (M/O, 2.5 mM). Data are means ± SEM of 8–18 independent experiments with tissue from 3–6 animals/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. WT on the same treatment.

pathway alive by preserving ALDH-2-activity. Vice-versa it was shown that deficiency in manganese superoxide dismutase (the mitochondrial isoform) potentiated the phenomena of nitroglycerin-induced tolerance and endothelial dysfunction [14]. For doxorubicin-induced cardio-toxicity several mechanisms have been postulated [18] but an important constituent of the cardio-toxic cascade seems to be doxorubicin-induced oxidative stress [17,23], especially mitochondrial ROS formation [16].

The results of the present investigation indicate, that in WT, the stepwise increase in mitochondrial ROS formation induced by GTN treatment is paralleled by a dose dependent increase in nitrate tolerance, as depicted in the stepwise right-shift of the GTN-concentration–relaxation-curve in Fig. 3C. The 100 μg/h dose results in nitrate tolerance in WT, comparable to the impaired GTN-response in sham-treated ALDH-2$^{-/-}$ mice. This indicates that the ALDH-2 is fully inhibited and therefore cannot function as a GTN reductase anymore. Interestingly, ALDH-2$^{-/-}$ mice treated with lower doses of GTN such as 10 or 50 μg/h for 4 days, did not develop a state of nitrate tolerance that is different from the sham-treated ALDH-2$^{-/-}$ mice. Only the 100 μg/h dose resulted in a further right shift of the GTN-concentration–relaxation-curve, indicating that there seems to be a threshold, below which the increasing amount of mitochondrial ROS in ALDH-2$^{-/-}$ mice (see Fig. 2A), does not lead to a further aggravation of the degree of nitrate tolerance, whereas above that threshold (in our model: 100 μg/h GTN for 4d) the mitochondrial ROS formation increases sharply about 80 percent and leads to a further right-shift of the GTN-response (see Fig. 3D), pointing to the involvement of oxidation of sensitive sulfhydryl-groups in enzymes of the proposed "low-affinity-pathway" or depletion of thiol pools for unspecific activation of nitroglycerin [5,24]; most probably, this oxidative depletion of thiols or inhibitory aldehyde–protein interaction is caused by accumulation of toxic aldehydes occurring in the "chronic-treatment situation". This concept is supported by the finding, that chronic (in contrast to

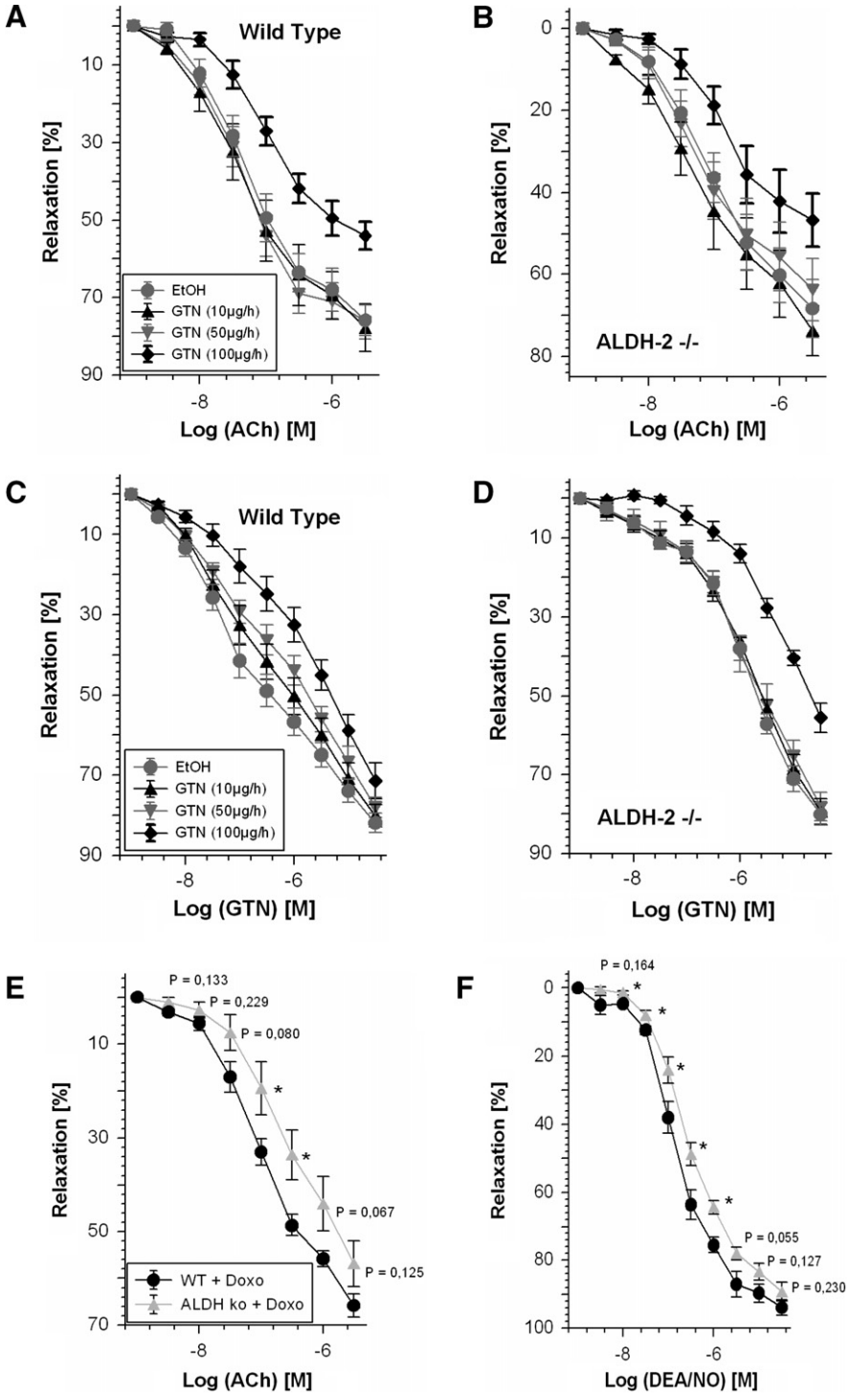

Fig. 3. Vascular function upon chronic nitroglycerin and doxorubicin treatment. Concentration–relaxation curves for ACh (10^{-9} to $10^{-5.5}$ M) and GTN (10^{-9} to $10^{-4.5}$ M) were obtained by isometric tension recordings in aortic segments from wild type (WT, C57B6) treated with GTN 10, 50 or 100 µg/h or the solvent ethanol (1.0 µl/h/4d) (A,C). Aorta from ALDH-2$^{-/-}$ mice (ALDH-2 ko) treated with the same protocol were also investigated (B,D). Data are means ± SEM of 10–11 (C57B6) and 8–10 (ALDH-2$^{-/-}$) independent experiments. For statistical analysis, see Supplementary Table 1. (E,F) Concentration–relaxation curves for ACh (10^{-9} to $10^{-5.5}$ M) and DEA/NO (10^{-9} to $10^{-4.5}$ M) were obtained by isometric tension recordings in aortic segments from wild type (WT, C57B6) or ALDH-2$^{-/-}$ mice treated with doxorubicin (48 µg/d/3d). Data are means ± SEM of 9 (ACh) and 6 (DEA/NO) independent experiments with tissue from 3–6 animals/group. $^{*}p < 0.05$ vs. WT on the same treatment.

short term/acute) treatment with doxorubicin lead to a more pronounced increase in mitochondrial ROS formation (Fig. 2B) and to a greater extent of endothelial (Fig. 3E) as well as smooth muscle (Fig. 3F) dysfunction in ALDH-2$^{-/-}$ mice as compared to C57Bl6 control mice.

A recent study suggested that ALDH-2 might function as a direct antioxidant, since inhibition of ALDH-2 by benomyl resulted in an increase in superoxide formation in cultured cells [13]. However, it should be realized that benomyl is a sulfhydryl-depleting compound and at concentrations above 10 μM will lead to disturbances in cellular redox potential and lead to increased oxidative stress.

In the present study, we could not establish a direct antioxidant effect of ALDH-2: the capability of purified ALDH-2 to scavenge peroxynitrite being the presumably most important reactive species occurring in nitrate tolerance, was not different from that of other thiol-containing compounds like GSH, DTT or cysteine used at the same concentration (see Fig. 1A). *In vivo*, these low molecular weight sulfhydryl compounds occur in mitochondria in concentrations several magnitudes higher than ALDH-2, arguing against an important role of ALDH-2 as a direct ROS scavenger. Additionally, selectively inhibiting ALDH-2 by daidzin in isolated rat cardiac mitochondria did not alter the chemiluminescence signal acutely (see Fig. 1B), and acute challenges of isolated mitochondria with either acetaldehyde (data not shown), antimycin A or GTN (see Fig. 1C) yielded essentially the same levels of ROS-formation in ALDH-$2^{-/-}$ and C57Bl6 control mice.

With our new findings, we would like to put forward another hypothesis, how ALDH-2 could work as an *indirect* antioxidant. It is well known, that increase of cardiac oxygen free radicals can lead to the formation of toxic aldehydes like malondialdehyde (MDA), 4-hydroxynonenal (HNE), and other aldehyde lipid peroxidation products. These aldehydes are substrates for ALDH-2 and other isoforms of aldehyde dehydrogenases, with varying k_m of different isoforms for different species of aldehydes [25–27]. Interestingly, ALDH-2 activity can also be impaired by aldehydes like MDA or HNE [26,27]. Accumulation of HNE can increase oxidative damage to the organism, as shown for instance for the liver [28], brain [29], erythrocytes [30], and cardiac myocytes [31]. In the latter, HNE is suspected to activate ATP-dependent K^+-channels [31]; since these channels are important for the stability of mitochondrial membrane potential, it is tempting to speculate, that HNE can stimulate mitochondrial ROS formation through a depolarization of mitochondrial membrane potential. Additionally, HNE formed within mitochondria by respiratory chain derived superoxide [32], is able to cause uncoupling of the mitochondrial respiratory chain itself and can be mechanistically involved in the production of mitochondrial superoxide [33].

In conclusion, the mitochondrial aldehyde dehydrogenase is critical for nitroglycerin bioactivation. Inactivation of this enzyme — as it occurs in the setting of nitrate tolerance as well as by diminished enzyme activity in ALDH-$2^{-/-}$ mice or humans carrying the loss of function Asian variant of ALDH-2 [3] — can lead to an increase in mitochondrial oxidative stress. Besides GTN-triggered nitrate tolerance and endothelial dysfunction, this postulate was also verified for doxorubicin-induced toxicity. This phenomenon can be attributed to the aldehyde-detoxifying and therefore *indirect* antioxidant effects of this enzyme rather than *direct* ROS scavenging activity.

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There are no financial disclosures.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.12.089.

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