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RESEARCH PAPER

Different effects of ascorbate deprivation and classical vascular nitrate tolerance on aldehyde dehydrogenase-catalysed bioactivation of nitroglycerin

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Background and purpose: Vascular tolerance to nitroglycerin (GTN) may be caused by impaired GTN bioactivation due to inactivation of mitochondrial aldehyde dehydrogenase (ALDH2). As relaxation to GTN is reduced but still sensitive to ALDH2 inhibitors in ascorbate deficiency, we compared the contribution of ALDH2 inactivation to GTN hyposensitivity in ascorbate deficiency and classical *in vivo* nitrate tolerance.
Experimental approach: Guinea pigs were fed standard or ascorbate-free diet for 2 weeks. Reversibility was tested by feeding ascorbate-deficient animals standard diet for 1 week. Nitrate tolerance was induced by subcutaneous injection of 50 $mg \cdot kg^{-1}$ GTN 4 times daily for 3 days. Ascorbate levels were determined in plasma, blood vessels, heart and liver. GTN-induced relaxation was measured as isometric tension of aortic rings; vascular GTN biotransformation was assayed as formation of 1,2- and 1,3-glyceryl dinitrate (GDN).
Key results: Two weeks of ascorbate deprivation had no effect on relaxation to nitric oxide but reduced the potency of GTN ~10-fold in a fully reversible manner. GTN-induced relaxation was similarly reduced in nitrate tolerance but not further attenuated by ALDH inhibitors. Nitrate tolerance reduced ascorbate plasma levels without affecting ascorbate in blood vessels, liver and heart. GTN denitration was significantly diminished in nitrate-tolerant and ascorbate-deficient rings. However, while the ~10-fold preferential 1,2-GDN formation, indicative for active ALDH2, had been retained in ascorbate deficiency, selectivity was largely lost in nitrate tolerance.
Conclusions and implications: These results indicate that nitrate tolerance is associated with ALDH2 inactivation, whereas ascorbate deficiency possibly results in down-regulation of ALDH2 expression.
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Keywords: ascorbate level; bioactivation; biotransformation; guinea pig; nitrate tolerance; nitric oxide; nitroglycerin; vascular relaxation; mitochondrial aldehyde dehydrogenase; vitamin C

Abbreviations: ALDH, aldehyde dehydrogenase; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine (diethylamine NO/NOate); DTT, dithiothreitol; GDN, glyceryl dinitrate; GTN, glyceryl trinitrate (nitroglycerin)

Introduction

Subsequent to the discovery that mitochondrial aldehyde dehydrogenase (ALDH2) catalyses bioactivation of the anti-anginal drug nitroglycerin (glyceryl trinitrate; GTN) (Chen *et al.*, 2002), numerous studies have confirmed that this enzyme contributes critically to GTN-induced vasodilation in rodents and humans (see Mayer and Beretta, 2008). The mechanism of ALDH2-catalysed GTN bioactivation is not completely understood. It has been proposed that the enzyme denitrates GTN to 1,2-glyceryl dinitrate (GDN) and inorganic nitrite that is reduced to nitric oxide (NO) by components of the mitochondrial respiratory chain (Chen and Stamler, 2006). Alternatively, ALDH2 may catalyse direct 3-electron reduction of GTN to yield NO which activates soluble guanylate cyclase, resulting in cGMP-mediated vasorelaxation (Kollau *et al.*, 2005; Beretta *et al.*, 2008). However, several groups failed to detect NO formation in blood vessels exposed to pharmacologically relevant low GTN concentrations, raising the possibility that GTN-induced vasodilation may

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be independent of NO (Kleschyov *et al.*, 2003; Núñez *et al.*, 2005; Miller *et al.*, 2008).

It has been proposed that oxidative inactivation of ALDH2 by GTN, which is reversed by reducing agents such as dithiothreitol (DTT) (Chen *et al.*, 2002; Kollau *et al.*, 2005) or dihydrolipoic acid (Wenzel *et al.*, 2007a), accounts for reduced vascular sensitivity to GTN in nitrate tolerance (Sydow *et al.*, 2004). However, this view has been challenged by the observation that ALDH inhibitors caused identical rightward shifts of GTN concentration-response curves in control and nitrate-tolerant blood vessels (DiFabio *et al.*, 2003; De La Lande *et al.*, 2004). Besides mechanism-based oxidation of ALDH2, GTN may trigger formation of superoxide and peroxynitrite in endothelial cells and thereby further aggravate vascular dysfunction through oxidative stress-mediated reduction of NO bioavailability (Münzel *et al.*, 2005).

Recently we reported on the slow development of vascular tolerance to GTN in ascorbate-deprived guinea pigs (Wölkart *et al.*, 2008). The effect of ascorbate deficiency was not accompanied by cross-tolerance to NO, pointing to selective interference with GTN bioactivation. The processes underlying the striking modulation of GTN vasodilation by ascorbate are unknown and it is still unclear whether a common mechanism accounts for the reduced vasodilatory potency of GTN in classical nitrate tolerance and ascorbate deficiency. The present study was designed to compare these two experimental models of GTN hyposensitivity with respect to vascular ascorbate levels and ALDH2 activity. Our data suggest that reversible vascular hyposensitivity to GTN in ascorbate deficiency and classical nitrate tolerance are caused by two different mechanisms, which both result in reduced GTN bioactivation by ALDH2.

Methods

Animals and experimental groups

All animal care and experimental protocols were in accordance with the Austrian law on experimentation with laboratory animals (last amendment, 2004), which is based on the US National Institutes of Health guidelines. Normotensive Dunkin Hartley guinea pigs of either sex (initial body weight 300–400 g) were randomized into the following four groups: control, ascorbate-deficient, ascorbate-replenished and GTN-tolerant. Control animals were fed pelleted commercial guinea pig food (Altromin 3023 containing 1 $g \cdot kg^{-1}$ ascorbate; Altromin, Lage, Germany). Ascorbate deficiency was induced by feeding animals ascorbate-free diet (Altromin C3015, Altromin, Lage, Germany) for 2 weeks, as described by Wölkart *et al.* (2008). The ascorbate-replenished group received ascorbate-free diet for 2 weeks, followed by feeding with standard diet for 1 week. Nitrate tolerance was induced in animals on the standard diet, by subcutaneous injection of 50 $mg \cdot kg^{-1}$ GTN 4 times a day over the last 3 days of the 2 weeks feeding protocol. At the end of the treatment period, thoracic aortas were removed, placed in chilled buffer, and immediately used for functional studies and determination of GTN denitration, measured as formation of 1,2- and 1,3-GDN. Common carotid artery, posterior vena cava, heart and liver were removed and immediately frozen in liquid nitrogen for determination of tissue ascorbate levels.

Aortic ring bioassays

Aortic ring bioassays were performed by measuring isometric tension as previously described (Wölkart *et al.*, 2008). Briefly, aortic rings were suspended in 5 mL organ baths, containing oxygenated Krebs-Henseleit buffer (composition in $mmol \cdot L^{-1}$: NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.2, D-glucose 10.1; pH 7.4), and equilibrated for 90 min at 2 g tension. Maximal contractile activity was determined with a depolarizing solution of 100 $mmol \cdot L^{-1}$ KCl. Rings that did not readily contract to high K^+ were considered to be damaged and discarded. Following removal of the high K^+ solution, rings were precontracted with the thromboxane A_2 receptor agonist 9,11-dideoxy-11a, 9a-epoxymethanoprostaglandin F2α (U-46619) to an equivalent level of ~5 g (i.e. ~90% of maximal contractile activity). Finally, cumulative concentration-response curves were established in separate tissues, using GTN (1 $nmol \cdot L^{-1}$–100 $\mu mol \cdot L^{-1}$) or 2,2-diethyl-1-nitroso-oxyhydrazine (diethylamine NO/NOate; DEA/NO; 1 $nmol \cdot L^{-1}$–10 $\mu mol \cdot L^{-1}$). To test for the effects of ALDH inhibitors, rings were preincubated with chloral hydrate (0.5 $mmol \cdot L^{-1}$) or daidzin (0.3 $mmol \cdot L^{-1}$) for 45 min before addition of GTN or DEA/NO. The contractile force corresponding to each agonist concentration was recorded and is expressed as per cent of precontraction values (=baseline).

Determination of plasma and tissue ascorbate levels

At the time points indicated in Figure 1, blood was drawn from the femoral vein, plasma separated by centrifugation, acidified with an equal volume of meta-phosphoric acid (10%) and centrifuged again. The clear supernatant was stored at –70°C for analysis by HPLC. Blood vessels, heart and liver were homogenized with a Potter-Elvehjem glass homogenizer

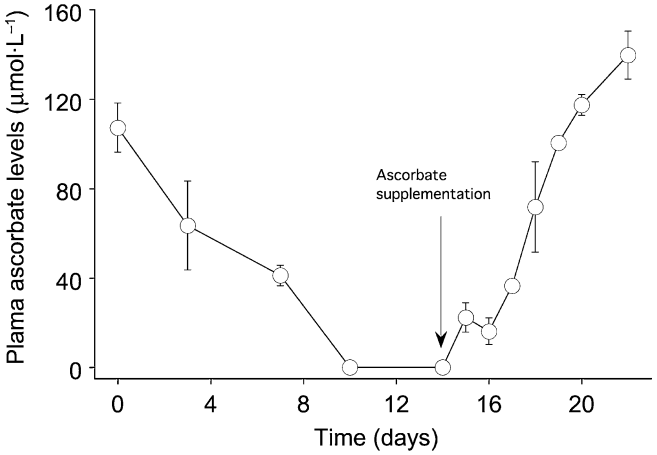

Figure 1 Reversible reduction of plasma ascorbate levels in guinea pigs upon ascorbate deprivation. Guinea pigs were fed ascorbate-free diet for 2 weeks and then switched to a standard diet containing 1 g ascorbate kg^{-1} for 1 week. Ascorbate plasma levels were measured by HPLC and UV detection as described in *Methods*. Data are mean values ± SEM (n = 3 in each group of animals).

in 5% (vol/vol) meta-phosphoric acid (10 µL per mg wet weight), containing 1 mmol·L^{-1} EDTA and 0.1 mmol·L^{-1} diethylene triamine pentaacetic acid (DTPA), and centrifuged at 20 000× *g* for 10 min. Supernatants were stored at −70°C. Ascorbate was determined by HPLC and UV detection at 264 nm as described (Karlsen *et al.*, 2007; Wölkart *et al.*, 2008). Briefly, aliquots (10 µL) of the samples were loaded on a LiChrospher 100 RP-18 HPLC column with 5 µm particle size (Merck, Darmstadt, Germany) and eluted at a flow rate of 1 mL min^{-1} with water containing acetonitrile (2%; vol/vol), NaH_2PO_4 (2.5 mmol·L^{-1}), dodecyltrimethyl ammonium chloride (2.5 mmol·L^{-1}) and DTPA (1.25 mmol·L^{-1}). The method was calibrated daily with ascorbate standard solutions (5–300 µmol·L^{-1}).

GTN denitration

The rates of GTN denitration catalysed by guinea pig aortic rings were determined as conversion of GTN into 1,2- and 1,3-GDN in the absence and presence of the ALDH inhibitors daidzin (0.1 mmol·L^{-1}) or chloral hydrate (10 mmol·L^{-1}). Freshly removed guinea pig aortas were cleaned and cut into three pieces (20–40 mg each) that were equilibrated at 37°C for 15 min in organ baths containing 5 mL of oxygenated phosphate buffer (50 mmol·L^{-1} K_2HPO_4, 80 mmol·L^{-1} NaCl, 3 mmol·L^{-1} $MgCl_2$, 1 mmol·L^{-1} EDTA, 1 mmol·L^{-1} EGTA, 1 mmol·L^{-1} NAD^+, pH 7.4) with or without ALDH inhibitors. Each piece was then weighed, placed into 0.2 mL of the same buffer, diced with scissors and incubated at 37°C for 10 min in the presence of 2 µmol·L^{-1} ^{14}C-labelled GTN, 2 mmol·L^{-1} GSH and 2 mmol·L^{-1} DTT. Reactions were terminated by flash-freezing and thawing the samples twice in liquid nitrogen. Products were extracted with 2 mL diethyl ether and quantified by radio-TLC as described (Kollau *et al.*, 2005). Blanks determined in the absence of tissue were subtracted for calculation of denitration rates.

Statistical analysis

Data are presented as mean values ± SEM of *n* experiments. Dose-response curves of different ring segments from a single animal were averaged and treated as individual experiment (*n* = 1). The averaged curves were fitted to a Hill-type model giving estimates of agonist potency (EC_{50}) and efficacy (E_{max}). One-way unpaired analysis of variance (ANOVA) with *post hoc* Bonferroni-Dunn test was used for comparison between groups using StatView® (Version 5.0). Values of $P < 0.05$ were considered statistically significant.

Materials

GTN, as Nitropohl® ampoules (G. Pohl-Boskamp GmbH. & Co, Hohenlockstedt Germany), containing 4.4 mmol·L^{-1} GTN in 250 mmol·L^{-1} glucose, was obtained from a local pharmacy. [2-^{14}C]GTN (50 mCi·mmol^{-1}, American Radiolabeled Chemicals) was supplied locally by Humos Diagnostica (Maria Enzersdorf, Austria). DEA/NO (ALEXIS Corp., Lausen, Switzerland) was purchased via Eubio (Vienna, Austria). Daidzin was a kind gift from Professor Wing-Ming Keung (Harvard Medical School, USA). All other chemicals were from Sigma-Aldrich GmbH (Vienna, Austria).

Results

Ascorbate levels in plasma and selected tissues

As shown in Figure 1, ascorbate levels in guinea pig plasma progressively decreased upon feeding the animals an ascorbate-free diet and were below the detection limit of the method (≤5 µmol·L^{-1}) after 10 days of ascorbate deprivation. This effect was fully reversed within 1 week of feeding the ascorbate-deficient animals on a standard diet containing ascorbate. The ascorbate content of carotid artery, vena cava, heart and liver of guinea pigs on the standard diet is shown in Figure 2. Two weeks of ascorbate deprivation reduced these levels close to or below the detection limit of the method (0.02 pmol·g^{-1}; $n = 3$; data not shown). Although plasma ascorbate levels were reduced by about 50% in nitrate-tolerant animals ($n = 14$ and $n = 6$ for control and nitrate tolerance respectively), the levels in arterial and venous blood vessels, heart and liver were not significantly affected by long-term application of GTN ($n = 6$ each; Figure 2).

Reversibility of vascular nitrate tolerance induced by ascorbate deprivation

As reported earlier (Wölkart *et al.*, 2008), 2 weeks of ascorbate deprivation caused an about 10-fold reduction in the potency of GTN to relax guinea pig aortic rings that was apparent as an increase in the EC_{50} from 0.048 ± 0.013 µmol·L^{-1} to 0.41 ± 0.09 µmol·L^{-1} (Figure 3A; $P = 0.03$). The effect of ascorbate deficiency was fully reversed by restoring ascorbate to the diet for 1 week (EC_{50} = 0.052 ± 0.007 µmol·L^{-1}). As shown in Figure 3B, neither of the treatments significantly affected

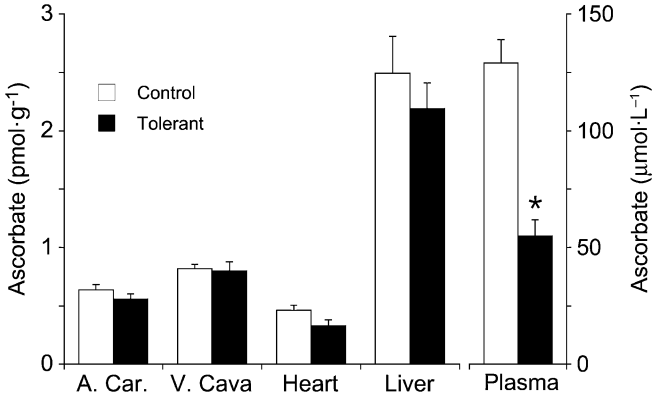

Figure 2 Effect of nitrate tolerance on ascorbate levels in plasma and selected tissues. Ascorbate was determined in plasma and homogenized tissues obtained from control and nitrate-tolerant guinea pigs as described in *Methods*. Data are mean values ± SEM measured in tissues obtained from 14 (plasma, control), 9 (carotid artery and heart, control), 8 (vena cava, control) or 6 (all tissues, nitrate-tolerant) animals. Nitrate tolerance caused a significant reduction of plasma ascorbate (*$P < 0.05$) but had no significant effect on the ascorbate content of blood vessels, heart or liver.

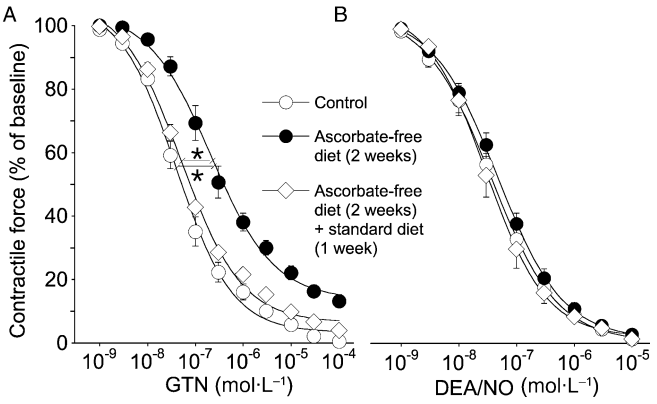

Figure 3 Reversibility of nitrate tolerance induced by 2 weeks of ascorbate deprivation. Relaxation to GTN (A) and DEA/NO (B) of aortic rings obtained from control, ascorbate-deprived and ascorbate-replenished guinea pigs was measured as described in *Methods*. Data are mean values ± SEM of six animals. Four (GTN) or two (DEA/NO) rings from a single animal were averaged and counted as individual experiment ($n = 1$). *$P < 0.05$ versus ascorbate-deprived.

relaxation of aortic rings to DEA/NO, indicating that ascorbate deprivation did not result in tolerance to NO.

Role of ALDH2 in GTN-induced vasodilation and classical nitrate tolerance

To clarify the contribution of ALDH2 inactivation in classical nitrate tolerance, we tested the effects of ALDH inhibitors on relaxation of aortic rings obtained from guinea pigs treated for 3 days with GTN. As shown in Figure 4A, daidzin (0.3 mmol·L^{-1}) and chloral hydrate (5 mmol·L^{-1}) markedly decreased the potency of GTN to dilate aortic rings from non-tolerant animals, reflected by an increase in the EC_{50} from 0.057 ± 0.013 μmol·L^{-1} to 0.20 ± 0.03 μmol·L^{-1} (P = 0.002) or 0.69 ± 0.18 μmol·L^{-1} (P = 0.006) respectively. Figure 4B shows that repeated *in vivo* application of GTN (to induce nitrate tolerance) caused a pronounced decrease in the vasorelaxant potency of GTN (EC_{50} = 0.64 ± 0.12 μmol·L^{-1}; P = 0.0005). Neither daidzin nor chloral hydrate caused a further rightward shift of the concentration-response curve in nitrate-tolerant blood vessels, suggesting that, in contrast to ascorbate deprivation (Wölkart *et al.*, 2008), long-term application of GTN causes inactivation of ALDH2. The ALDH inhibitors had no effect on relaxation to DEA/NO in control (Figure 4C) or nitrate-tolerant (Figure 4D) rings.

ALDH activity in ascorbate-deficient and nitrate-tolerant aortic rings

The effects of ascorbate deprivation and nitrate tolerance on ALDH-catalysed GTN denitration measured as formation of 1,2-GDN by aortic rings are shown in Table 1. Control vessels catalysed 1,2-GDN formation with a rate of about 0.8 nmol·min^{-1}·g^{-1} that was reduced to ~0.1 nmol·min^{-1}·g^{-1} by daidzin and chloral hydrate, suggesting a major contribution of ALDH2 to vascular GTN denitration. Long-term administration of GTN resulted in about 50% reduction of 1,2-GDN formation. Interestingly, ascorbate deprivation had a similar effect, even though aortic ring bioassays suggested that vascular ALDH2 was not inactivated in ascorbate deficiency (Wölkart *et al.*, 2008). To resolve this discrepancy we had a closer look on the formation of 1,3-GDN, which reflects non-specific GTN metabolism that is increased rather than decreased by ALDH inhibitors (Kollau *et al.*, 2005). As shown in Table 1, the rates of 1,3-GDN formation were increased two- to threefold by daidzin and chloral hydrate in control and ascorbate-deficient blood vessels. In contrast, ALDH inhibitors had no effect on 1,3-GDN formation by GTN-tolerant rings. Calculation of 1,2-/1,3-GDN ratios (Table 1) revealed about 10-fold preference for formation of the 1,2-isomer in control and ascorbate-deficient rings. In the presence of daidzin or chloral hydrate, the ratios were ≤0.65 under all experimental conditions, showing that inhibition and/or inactivation of ALDH2 results in loss of selective GTN denitration.

Discussion

The present study confirms and extends our previous findings on significantly decreased sensitivity of ascorbate-deficient blood vessels to GTN-induced relaxation (Wölkart *et al.*, 2008). Lack of cross-tolerance to the direct NO donor DEA/NO appears to exclude reduced vascular NO bioavailability due to oxidative stress as underlying mechanism [for further details on this issue see the correspondence following publication of our paper (Daiber and Gori, 2008; Mayer, 2008)]. In addition, the unchanged response to DEA/NO also indicates that overall vascular function was not compromised by ascorbate deficiency, as might have been expected from the ascorbate requirement of collagen synthesis (Mahmoodian and Peterkofsky, 1999). It is conceivable that the ascorbate-independent pathway of collagen synthesis described for mice (Parsons *et al.*, 2006) is present in guinea pigs as well and ensures proper vessel morphology in ascorbate deficiency. Rapid reversibility of impaired relaxation by feeding back ascorbate to the deficient animals suggests that ascorbate deprivation had no profound effects on vascular function but attenuated GTN-induced relaxation through dynamic regulation of an enzymatic pathway that contributes to GTN bioactivation (see below).

Together with the reports on protection by ascorbate against the development of nitrate tolerance (Bassenge *et al.*, 1998; Hinz and Schröder, 1998; Watanabe *et al.*, 1998a,b), our intriguing observation that long-term application of GTN reduced plasma ascorbate levels by more than 50% led us to speculate that nitrate tolerance is caused by GTN-triggered depletion of vascular ascorbate. To address this issue, we additionally measured ascorbate levels in guinea pig arterial and venous vascular beds (carotid artery and vena cava respectively), as well as heart and liver. The levels measured in plasma, heart and liver agree reasonably well with previous reports (Berger *et al.*, 1989; Tokumaru *et al.*, 1996; Dalloz *et al.*, 1999); reference data on ascorbate content of blood vessels were not found in the literature. Long-term treatment of guinea pigs with GTN did not cause a significant reduction of ascorbate levels in any of the tissues investigated, indicating that the lower plasma concentration of nitrate-tolerant

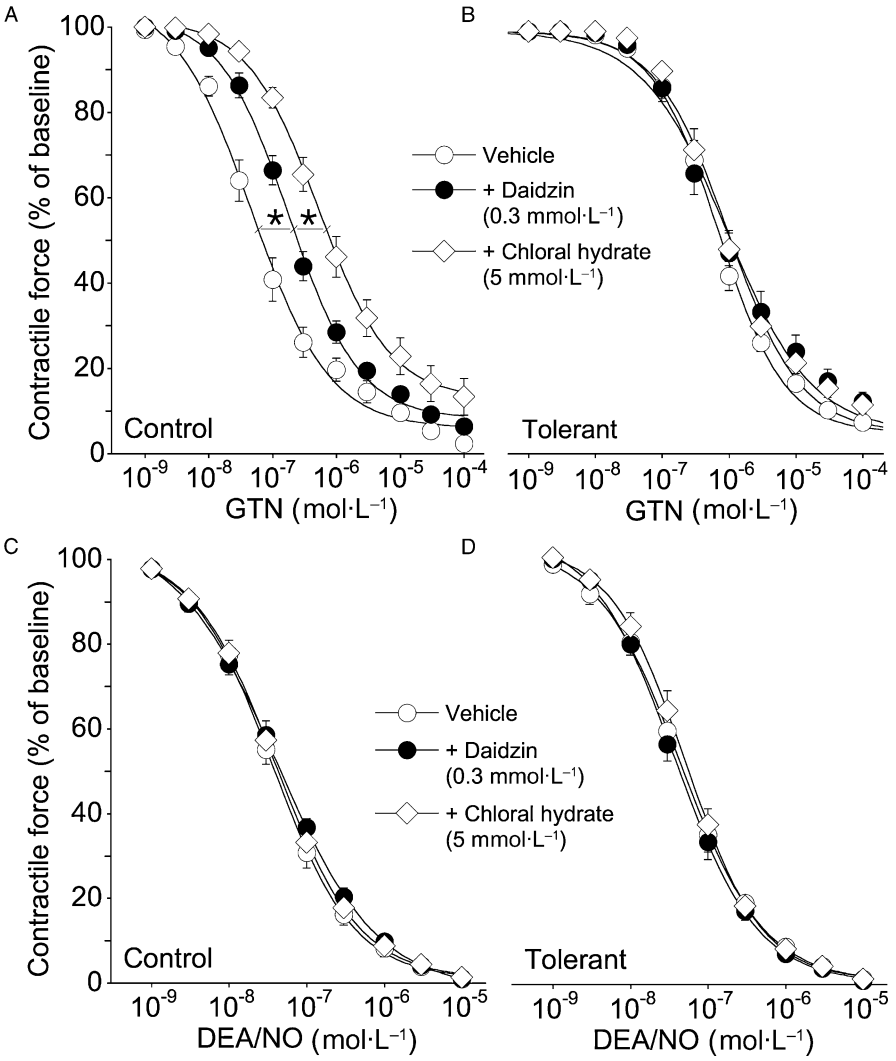

Figure 4 Effects of ALDH inhibitors on GTN-induced relaxation of control and nitrate-tolerant aortic rings. Relaxation to GTN (A and B) and DEA/NO (C and D) of aortic rings obtained from control (A and C) and nitrate-tolerant (B and D) guinea pigs in the absence and presence of the ALDH inhibitors daidzin (0.3 mmol·L^{-1}) and chloral hydrate (5 mmol·L^{-1}). Data are mean values ± SEM of six animals. Two rings from a single animal were averaged and counted as an individual experiment. *$P < 0.05$.

Table 1 Vascular biotransformation of GTN to 1,2- and 1,3-GDN

Treatment	*1,2-GDN(pmol·min^{-1}·g^{-1})*	*1,3-GDN (pmol·min^{-1}·g^{-1})*	*Ratio (1,2-/1,3-GDN)*
Control	0.77 ± 0.097	0.08 ± 0.018	11.92 ± 1.69
daidzin	0.11 ± 0.013	0.20 ± 0.040	0.63 ± 0.07
chloral hydrate	0.11 ± 0.011	0.19 ± 0.016	0.60 ± 0.02
Nitrate-tolerant	0.32 ± 0.062[a]	0.12 ± 0.045	3.03 ± 0.58[a]
daidzin	0.07 ± 0.029	0.16 ± 0.063	0.47 ± 0.05
chloral hydrate	0.13 ± 0.026	0.23 ± 0.042	0.54 ± 0.04
Ascorbate-deficient	0.36 ± 0.036[a]	0.05 ± 0.011	7.94 ± 1.14
daidzin	0.09 ± 0.021	0.18 ± 0.026	0.53 ± 0.10
chloral hydrate	0.11 ± 0.021	0.21 ± 0.040	0.53 ± 0.03
Ascorbate-replenished	0.63 ± 0.066[b]	≤0.04[c]	≥14[c]
Daidzin	0.06 ± 0.024	0.17 ± 0.035	0.39 ± 0.05
chloral hydrate	0.12 ± 0.029	0.25 ± 0.053	0.52 ± 0.08

Aortic rings from guinea pigs subjected to various treatments were incubated at 37°C for 10 min with 2 μmol·L^{-1} ^{14}C-labelled GTN in the absence and presence of the ALDH inhibitors daidzin (0.1 mmol·L^{-1}) or chloral hydrate (10 mmol·L^{-1}). The rates of 1,2- and 1,3-GDN formation are expressed as mean values ± SEM of three independent experiments performed in duplicates. The ratios of 1,2- to 1,3-GDN formation were calculated from individual data sets ($n = 6$).

[a]$p < 0.05$ versus control (standard diet, non-tolerant).

[b]$p < 0.05$ versus ascorbate-deficient.

[c]The rates of 1,3-GDN formation were close to or below the detection limit of the method.

animals is still sufficient to maintain normal ascorbate tissue levels, presumably through high-affinity uptake (Tsukaguchi *et al.*, 1999; Wilson, 2005; Aguirre and May, 2008). Obviously, we cannot exclude that nitrate tolerance is associated with depletion of a distinct ascorbate pool within vascular smooth muscle cells, but this appears rather unlikely and would be hard to test experimentally. Moreover, the considerably delayed development of nitrate tolerance upon ascorbate deprivation (Wölkart *et al.*, 2008) also suggests that classical nitrate tolerance is not due to depletion of vascular ascorbate.

There is a large body of evidence suggesting that ALDH2 contributes critically to GTN-induced vasodilation in rodents (Chen *et al.*, 2002; 2005; DiFabio *et al.*, 2003; De La Lande *et al.*, 2004; Sydow *et al.*, 2004; Daiber *et al.*, 2005; Kollau *et al.*, 2005; Esplugues *et al.*, 2006; Szöcs *et al.*, 2007) and humans (Mackenzie *et al.*, 2005; Hink *et al.*, 2007; Huellner *et al.*, 2008). Probably the most convincing evidence was provided by Chen *et al.* (2005), who showed that vasodilation induced by low concentrations of GTN (1–100 $nmol \cdot L^{-1}$) was completely abolished in ALDH2 knockout mice, while the response to isosorbide mononitrate, which is not metabolized by ALDH2 (Wenzel *et al.*, 2007b), and to the direct NO donor sodium nitroprusside was not affected by ALDH2 gene deletion. These findings are hard to reconcile with a recent report claiming that activation of endothelial NO synthase is essentially involved in vascular bioactivation of low GTN concentrations (Bonini *et al.*, 2008). Since relaxation of guinea pig aorta to GTN was not affected by removal of the endothelium (see supplementary figure S3 in Wölkart *et al.*, 2008), endothelial NO synthase-derived NO does not appear to contribute to GTN-induced vasodilation in our experimental model.

It is less clear to what extent inactivation of vascular ALDH2 is involved in the development of nitrate tolerance induced by long-term application of GTN. While Sydow *et al.* (2004) found that inhibition of ALDH2 does not affect relaxation of nitrate-tolerant blood vessels to GTN, DiFabio *et al.* (2003) and De la Lande *et al.* (2004) reported on virtually identical effects of ALDH inhibitors on GTN-induced relaxation of control and nitrate-tolerant vessels. Our present findings showing that neither daidzin nor chloral hydrate caused a further decrease in the potency of GTN to relax aortic rings from nitrate-tolerant guinea pigs (see Figure 4) supports the view that inactivation of vascular ALDH2 represents a major cause of nitrate tolerance. Interestingly, ALDH inhibitors attenuated residual vascular GTN denitration in nitrate-tolerant aortas even though GTN-induced relaxation was not affected, indicating that ALDH2-catalysed 1,2-GDN formation does not necessarily reflect GTN bioactivation. According to recent data from our laboratory, approximately 10% of total GTN turnover catalysed by wild-type ALDH2 results in direct 3-electron reduction of GTN to NO (Beretta *et al.*, 2008). As site-directed mutagenesis indicates that NO formation may occur independently of GTN denitration yielding inorganic nitrite (Beretta *et al*, unpublished experiments), it is conceivable that ALDH2-catalysed reduction of GTN to NO is more severely affected by long-term application of the nitrate than clearance-based denitration.

The present results indicate that classical nitrate tolerance is essentially different from tolerance induced by ascorbate deprivation, in which the sensitivity of GTN-induced relaxation to ALDH inhibitors was increased rather than decreased (Wölkart *et al.*, 2008) [see also the correspondence following publication of this article (Daiber and Gori, 2008; Mayer, 2008)]. To further investigate this issue, we determined the rates of GTN denitration by aortic rings in the absence and presence of daidzin and chloral hydrate as a measure of vascular ALDH activity. At first glance, reduced rates of 1,2-GDN formation by ascorbate-deficient and nitrate-tolerant rings might indicate ALDH2 inactivation as a common cause of reduced vascular GTN sensitivity. However, a closer look on the denitration data revealed striking differences with respect to the ratios of 1,2- and 1,3-GDN formation. ALDH2-catalysed denitration of GTN results in highly selective formation of 1,2-GDN (Chen *et al.*, 2002; DiFabio *et al.*, 2003; Kollau *et al.*, 2005), which has been recognized as relevant co-product of GTN bioactivation two decades ago (Brien *et al.*, 1986; Bennett *et al.*, 1989), and this selectivity is largely abolished upon inactivation of ALDH2 with enzyme inhibitors (Kollau *et al.*, 2005; Beretta *et al.*, 2008). Although most studies on ALDH2 inactivation in nitrate tolerance report only the rates of vascular aldehyde oxidation and/or 1,2-GDN formation, there are two papers showing preferential reduction of 1,2-GDN formation by nitrate-tolerant human saphenous vein (Sage *et al.*, 2000) and rat aorta (DiFabio *et al.*, 2003). In line with these earlier reports, we found that the 1,2-/1,3-GDN ratio was reduced from 11.9 to 3.0 in nitrate-tolerant guinea pig aorta. For comparison, complete enzyme inhibition with daidzin or chloral hydrate yielded 1,2-/1,3-GDN ratios of ≤ 0.65. In contrast to classical nitrate tolerance, ascorbate deprivation did not significantly alter the 1,2-/1,3-GDN ratio of vascular GTN biotransformation, suggesting that the reduced 1,2-GDN formation by ascorbate-deficient aortic rings was not a consequence of ALDH2 inactivation but resulted from reduced expression levels of active ALDH2. Down-regulation of vascular ALDH2 in ascorbate deficiency would also explain the considerably delayed development of nitrate tolerance in this experimental model. We are currently investigating this issue in a genetic mouse model of ascorbate deficiency (Maeda *et al.*, 2000; Parsons *et al.*, 2006) that allows quantification of ALDH2 mRNA and protein expression levels in blood vessels.

In conclusion, this study suggests that GTN hyposensitivity in both classical nitrate tolerance and ascorbate deficiency is at least partially due to reduced vascular ALDH2 activity. However, different mechanisms appear to underlie GTN hyposensitivity in these two models: while long-term exposure to GTN most likely results in ALDH2 inactivation, evident as loss of ALDH inhibitor sensitivity of GTN-induced relaxation and markedly decreased 1,2-/1,3-GDN ratios, ascorbate deficiency does not appear to cause ALDH2 inactivation. Reversible down-regulation of active ALDH2 expression in ascorbate deficiency has to be considered as possible explanation for our observations, but definitive conclusions await determination of ALDH2 expression levels. Taken together, our results emphasize the importance of ALDH2 in GTN bioactivation and add a new aspect to the complex mechanism underlying the development of nitrate tolerance *in vivo*.

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Conflicts of interest

None.

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