

# Bioactivation of Pentaerythrityl Tetranitrate by Mitochondrial Aldehyde Dehydrogenase

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## ABSTRACT

Mitochondrial aldehyde dehydrogenase (ALDH2) contributes to vascular bioactivation of the antianginal drugs nitroglycerin (GTN) and pentaerythrityl tetranitrate (PETN), resulting in cGMP-mediated vasodilation. Although continuous treatment with GTN results in the loss of efficacy that is presumably caused by inactivation of ALDH2, PETN does not induce vascular tolerance. To clarify the mechanisms underlying the distinct pharmacological profiles of GTN and PETN, bioactivation of the nitrates was studied with aortas isolated from ALDH2-deficient and nitrate-tolerant mice, isolated mitochondria, and purified ALDH2. Pharmacological inhibition or gene deletion of ALDH2 attenuated vasodilation to both GTN and PETN to virtually the same degree as long-term treatment with GTN, whereas treatment with PETN did not cause tolerance. Purified ALDH2 catalyzed bioactivation of PETN,

assayed as activation of soluble guanylate cyclase (sGC) and formation of nitric oxide (NO). The  $EC_{50}$  value of PETN for sGC activation was  $2.2 \pm 0.5 \mu\text{M}$ . Denitration of PETN to pentaerythrityl trinitrate was catalyzed by ALDH2 with a specific activity of  $9.6 \pm 0.8 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  and a very low apparent affinity of  $94.7 \pm 7.4 \mu\text{M}$ . In contrast to GTN, PETN did not cause significant inactivation of ALDH2. Our data suggest that ALDH2 catalyzes bioconversion of PETN in two distinct reactions. Besides the major denitration pathway, which occurs only at high PETN concentrations, a minor high-affinity pathway may reflect vascular bioactivation of the nitrate yielding NO. The very low rate of ALDH2 inactivation, presumably as a result of low affinity of the denitration pathway, may at least partially explain why PETN does not induce vascular tolerance.

## Introduction

Organic nitrates are used clinically for the treatment of angina pectoris and other cardiovascular diseases (Abrams, 1995; Daiber et al., 2008, 2009). The most widely used organic nitrates are nitroglycerin (GTN), isosorbide mononitrate, and isosorbide dinitrate. PETN, a drug used in former East Germany, is currently being reintroduced into Western markets (Daiber and Münzel, 2010). It is generally accepted that the drugs are bioactivated in blood vessels to release NO or a related activator of sGC, resulting in cGMP-mediated vasodilation (Fung, 2004).

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**ABBREVIATIONS:** GTN, glycerol trinitrate (nitroglycerin); ALDH2, aldehyde dehydrogenase-2; Anti-A, antimycin-A; CLM, calf liver mitochondria; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine; DMSO, dimethyl sulfoxide; DTPA, diethylene triamine pentaacetic acid; DTT, dithiothreitol; GDN, glycerol dinitrate; PETN, pentaerythrityl tetranitrate; PETriN, pentaerythrityl trinitrate; sGC, soluble guanylate cyclase; SOD, superoxide dismutase; TEA, triethanolamine; TLC, thin-layer chromatography; TTFA, thenoyltrifluoroacetone; WT, wild type; U-46,619, 9,11-dideoxy-11 $\alpha$ ,9 $\alpha$ -epoxy-methanoprostaglandin  $F_{2\alpha}$ .

The mechanisms underlying vascular bioconversion of organic nitrates to NO are not well understood. Recent evidence suggests that at least two pathways are involved: a low-affinity pathway that is common to all nitrates, and a high-affinity pathway responsible for bioactivation of GTN and PETN, which exert therapeutic effects at much lower doses than the isosorbide nitrates. Although the low-affinity pathway may reflect a combination of various enzymatic and possibly nonenzymatic reactions (Fung, 2004), the high-affinity pathway seems to be catalyzed exclusively by ALDH2 (Chen et al., 2002, 2005). Meanwhile, there is general agreement that ALDH2 is essentially involved in vasorelaxation induced by GTN (DiFabio et al., 2003; Kollau et al., 2005; Huellner et al., 2008) and PETN (Daiber et al., 2004; Wenzel et al., 2007a) but does not contribute to the vascular effects of the isosorbide nitrates (Chen et al., 2005).

Continuous administration causes rapid loss of the hemodynamic effects of GTN (Ahlner et al., 1991). Among the various mechanisms that have been proposed to explain this nitrate tolerance (Fung, 2004; Mayer and Beretta, 2008), inactivation of ALDH2 (Chen et al., 2002) and superoxide-mediated oxidation of GTN-derived NO (Münzel et al., 2005) are the most promising candidates at present. Denitration of GTN results in mechanism-based inactivation of ALDH2 (Beretta et al., 2008b), GTN-tolerant blood vessels exhibit markedly reduced ALDH2 activity (Sage et al., 2000; DiFabio et al., 2003; Sydow et al., 2004; Hink et al., 2007; Wenzl et al., 2009b), and ALDH2(−/−) mice do not become tolerant (Chen et al., 2005). Taken together, these results suggest that vascular tolerance to GTN is explained by inactivation of ALDH2. In addition, oxidative stress leading to superoxide-mediated inactivation of NO in GTN-exposed blood vessels may essentially contribute to the development of nitrate tolerance (Münzel et al., 2005). Animal studies have shown that continuous administration of PETN does not cause any of the deleterious effects observed with GTN (i.e., nitrate tolerance) (Fink and Bassenge, 1997; Jurt et al., 2001; Müllenheim et al., 2001; Gori et al., 2003), endothelial dysfunction (Jurt et al., 2001), enhanced oxidative stress (Jurt et al., 2001; Daiber et al., 2004), or inactivation of ALDH2 (Daiber et al., 2004). Whereas the lack of superoxide formation may reflect an antioxidative effect (Oberle et al., 2002), the lack of ALDH2 inactivation by PETN is unexplained.

ALDH2 seems to catalyze at least two distinct reactions with 1,2-GDN formed as coproduct. Whereas a two-electron reduction pathway, which accounts for ~90% of turnover and yields nitrite, may reflect clearance-based denitration of GTN, a minor three-electron reduction pathway yields free NO radical and may reflect GTN bioactivation (Beretta et al., 2008a, 2010; Wenzl et al., 2009a). Direct NO formation may explain how ALDH2-catalyzed GTN bioconversion is linked to sGC activation and resolve puzzles posed by the initial proposal on the involvement of nitrite reduction by the mitochondrial respiratory chain (Chen et al., 2002) (e.g., localization of ALDH2 in cytosolic rather than mitochondrial fractions of rat aorta) (DiFabio et al., 2003), lack of inhibition of cytochrome c oxidase by GTN (Núñez et al., 2005), and identical rates of GTN-induced cGMP formation at low and high rates of mitochondrial respiration (Kollau et al., 2005, 2009).

Despite circumstantial evidence indicating that PETN is a substrate of ALDH2, enzymatic bioactivation of this drug has not been investigated so far. The present study provides evidence for ALDH2-catalyzed bioactivation of PETN that results in the formation of NO and activation of sGC. The very low rate of ALDH2 inactivation, most likely as a result of the low affinity of PETN for the clearance-based denitration pathway, may explain why PETN does not cause vascular tolerance.

## 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) using pT7.7 as the host vector for cDNA and purified by *p*-hydroxyacetophenone affinity chromatography and size-exclusion chromatography as described previously (Beretta et al., 2008a). [ $\alpha$ - $^{32}$ P]GTP (400 Ci/mmol) was obtained from PerkinElmer Vertrieb GmbH (Vienna, Austria). [ $^{14}$ C]GTN (50–60 mCi/mmol) and [ $^3$ H]formaldehyde (10 Ci/

mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO) via Humos Diagnostika GmbH (Salzburg, Austria). 1,2-GDN, 1,3-GDN, and GTN were obtained from LGC Standards (Wesel, Germany). Daidzin was a kind gift from Dr. Wing-Ming Keung (Harvard Medical School, Boston, MA). For aortic ring experiments, daidzin was diluted in DMSO and for sGC assays, it was dissolved in 50 mM TEA at 160  $\mu$ M maximally. PETN [with 90% (w/w) lactose] was kindly donated by Schwarz-Pharma (Zwickau, Germany). Because PETN is barely soluble in aqueous solutions, stock solutions in DMSO were used. 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 TEA, pH 7.4. DEA/NO was purchased from Alexis Corporation (Lausen, Switzerland) via Eubio (Vienna, Austria) and diluted in 10 mM NaOH. Cu,Zn-SOD was from Sigma-Aldrich GmbH (Vienna, Austria). All other chemicals were either from Sigma-Aldrich GmbH or Merck KGaA (Darmstadt, Germany).

**Synthesis of [ $^3$ H]PETN.** One milliliter of [ $^3$ H]formaldehyde (1 mCi, 10 Ci/mmol) was mixed with 16.2  $\mu$ l of formaldehyde solution [36.5% (w/w)] and 62 mg of calcium oxide. After the addition of 2.5  $\mu$ l of acetaldehyde, the vessel was tightly closed, and temperature was raised slowly to 50°C in a waterbath under constant stirring. After incubation for 2 h, the reaction mixture was transferred to a 1-ml tube, and the precipitate was removed by centrifugation at 4°C at 20,000g for 1 min. The supernatant was added drop-wise to a mixture of 770  $\mu$ l of sulfuric acid [85% (w/w)] and 1230  $\mu$ l of nitric acid [63% (w/w)] under constant stirring on ice. After incubating for 30 min on ice, any precipitate was removed by filtration. PETN was extracted twice with dichloromethane. The extracts were washed and dried on water-free MgSO<sub>4</sub>, which was subsequently removed by centrifugation. The supernatant was collected, and the solvent was removed under a gentle stream of nitrogen. The residue was redissolved in 250  $\mu$ l of acetone and separated by thin-layer chromatography. Standard solutions of PETN, pentaerythrityl trinitrate (PETriN), pentaerythrityl dinitrate, and pentaerythrityl mononitrate were loaded on the same plate. Water-saturated toluene/ethyl acetate/1-butanol [6:3:1 (v/v)] was used as solvent. The standard lane was sprayed with a solution of diphenylamine [1 g in 100 ml of H<sub>2</sub>SO<sub>4</sub> 75% (w/w)]. The corresponding areas in the sample lane were scraped off, and PETN was extracted twice with 500  $\mu$ l of acetone. After measuring total radioactivity by liquid scintillation counting, acetone was removed under nitrogen. The residue of purified PETN was redissolved in DMSO giving a final concentration of 6000 dpm/ $\mu$ l.

**Animals.** All animals received care in accordance with the Austrian law on experimentation with laboratory animals (last amendment, 2005), which is based on the U.S. National Institutes of Health guidelines. Studies were performed on male Sprague-Dawley rats (initial body weight, 400–500 g), wild-type C57BL/6 mice of either gender (25–30 g), or ALDH2(−/−) mice of either gender (25–30 g). ALDH2(−/−) animals were obtained by breeding heterozygous mice purchased from the Texas Institute for Genomic Medicine (Houston, TX). These parental mice had a 129/Sv  $\times$  C57BL/6 background and were obtained by knocking out one copy of the ALDH2 gene via gene-trap insertion in the OST7285 Omnibank ES cell line. The mouse line was amplified by breeding ALDH2(−/−) animals with C57BL/6 wild-type mice (F<sub>1</sub> progeny). F<sub>1</sub> heterozygous mice were intercrossed to generate F<sub>2</sub> ALDH2(−/−) or ALDH2 WT animals. For genotyping, mice tail tips (~2 mm length) were lysed using the DirectPCR lysis reagent (Peqlab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol. Mice genotype was determined with two polymerase chain reactions to amplify the wild-type allele (using the primers 5'-AGCCTCCAAGTGTAGGGTAATCC-3' and 5'-TAAGAGCACTGAATG CTCTTCTGG-3') and/or the knockout allele (with the primers 5'-AGCCTCCAAGTGTAGGGTAATCC-3' and 5'-ATAAACCTCTTGGCAG TTGCATC-3').

**Aortic Ring Experiments.** Animals were anesthetized with urethane (1 g/kg), and the aortas were removed, cleaned, and cut into

small rings of approximately 3 mm length. Rings were suspended in 5-ml organ baths for isometric tension measurement containing oxygenated Krebs-Henseleit buffer (composition: 118.4 mM NaCl, 25 mM NaHCO<sub>3</sub>, 4.7 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, and 10.1 mM D-glucose, pH 7.4). After equilibration for 90 min at 0.5 (mice) or 2 g (rats), rings were contracted with the thromboxane A<sub>2</sub> receptor agonist 9,11-dideoxy-11 $\alpha$ ,9 $\alpha$ -epoxymethanoprostaglandin F<sub>2 $\alpha$</sub>  (U-46,619; 50 nM). When contractions had reached a stable plateau, cumulative dose-response curves were established using either PETN (10<sup>-10</sup>–10<sup>-4</sup> M) or GTN (10<sup>-9</sup>–10<sup>-4</sup> M). Where indicated, rings were preincubated for 45 min with chloral hydrate (5 mM) or daidzin (0.3 mM) before the addition of PETN or GTN. Contractile force corresponding to each agonist concentration was recorded and expressed as a percentage of precontraction (baseline) (Wölkart et al., 2008). To test for the development of nitrate tolerance, PETN in DMSO (60 nmol/min/kg) or solvent (0.5  $\mu$ l/h) was infused to WT mice through implanted miniosmotic pumps (Alzet; Durect Corporation, Cupertino, CA). GTN was injected subcutaneously twice a day (20 mg/kg/d) for 4 days. For both drugs, the daily dose was 85  $\mu$ mol/kg.

**Isolation of CLM.** Calf livers were obtained from a local slaughterhouse. Isolation was performed at 4°C in a sucrose solution (250 mM) containing Tris (10 mM), pH 7.4, EGTA (5 mM), pH 7.4, and MgCl<sub>2</sub> (2 mM). Fat and connective tissue were removed, and 300 g of tissue was cut into thin slices. Blood was removed by washing the slices with 500 ml of ice-cold isolation buffer under intensive squeezing by hand and decanting the solution. Washing was repeated with fresh buffer until the decanted solution was colorless. Tissue slices were transferred into a fresh glass beaker and filled up with isolation buffer, giving a final volume of 1000 ml. A crude mince was obtained by mixing the tissue with an Ultra-Turrax tissue blender (IKA Works Inc., Wilmington, NC) for 30 s on ice, and the pH was immediately readjusted if necessary. The minced tissue was further homogenized using a Potter-Elvehjem glass homogenizer with motor driven Teflon pestle for approximately 30 s in 50-ml aliquots. The homogenate was centrifuged at 600g for 10 min for removal of tissue fragments and nuclei. Supernatants were collected and a mitochondria-enriched fraction was obtained by centrifuging for 15 min at 1500g. The mitochondrial pellets were resuspended in isolation buffer. Mitochondria were sedimented by centrifuging twice for 5 min at 15,000g. The final mitochondrial pellets were resuspended in a total volume of 15 ml of isolation buffer. Protein concentration was determined photometrically using the Bradford assay and adjusted to 20 to 30 mg/ml. Mitochondrial suspensions were stored frozen at -70°C until use (Kollau et al., 2005).

**Determination of sGC Activity.** Purified bovine lung sGC (50 ng) was incubated at 37°C for 10 min in a final volume of 0.1 or 0.2 ml of with the indicated concentrations of donor compounds (PETN, GTN, or DEA/NO) in the presence of purified ALDH2 or CLM as indicated in the figure legends. Assay mixtures contained 50 mM TEA, 0.5 mM [ $\alpha$ -<sup>32</sup>P]GTP (~250,000 cpm), 1 mM cGMP, 3 mM MgCl<sub>2</sub>, 2 or 0.1 mM DTT, and 0.1 mM DTPA. ALDH2, SOD, NAD, phosphocreatine, creatine phosphokinase, succinate, antimycin-A (Anti-A), TTFA, daidzin, and chloral hydrate were present as specified in the figure legends. Reactions were terminated by the addition of 0.45 ml of zinc acetate (120 mM) and 0.45 ml of sodium bicarbonate (120 mM). After centrifugation, supernatants were applied to Al<sub>2</sub>O<sub>3</sub> columns, which had been pretreated with perchloric acid (100 mM). Columns were washed with H<sub>2</sub>O and [<sup>32</sup>P]cGMP eluted with sodium acetate (50 mM) and quantified by liquid scintillation counting. Blank values determined in the absence of sGC were subtracted.

**Determination of NO Formation.** NO formation was measured electrochemically with a Clark-type electrode (World Precision Instruments, Berlin, Germany). The electrode was connected to an Apple Macintosh computer equipped with LabChart software via a data acquisition system (PowerLab 2/26; ADInstruments Pty Ltd, Bella Vista, NSW, Australia). NO measurements were performed under constant stirring at 37°C. The electrode was calibrated daily

with acidified nitrite after equilibration in 1 ml of potassium iodide (100 mM) in H<sub>2</sub>SO<sub>4</sub> (100 mM). When the signal became stable, baseline was adjusted to 0, and 5  $\mu$ l of NaNO<sub>2</sub> (100  $\mu$ M) was injected four to five times. The average of the obtained NO peaks was set to 0.5, and units were converted to micromoles. For the determination of enzymatic NO formation, the electrode was equilibrated in 500  $\mu$ l of 50 mM TEA, pH 7.4, containing 3 mM MgCl<sub>2</sub>, 2 mM DTT, 0.1 mM DTPA, and 125  $\mu$ g of purified ALDH2 in the absence or presence of SOD and NAD as indicated in the figure legend, followed by injection of PETN to give a final concentration of 10  $\mu$ M.

**Determination of GTN Denitration.** Radiolabeled GTN was incubated with 50  $\mu$ g of ALDH2 at 37°C for 10 min in a total volume of 200  $\mu$ l of 50 mM TEA, pH 7.4, containing 1 mM NAD, 0.1 mM DTT, 3 mM MgCl<sub>2</sub>, and 2 mM EDTA. Reaction products were extracted twice with 1 ml of diethyl ether, followed by the evaporation of the solvent under a gentle stream of nitrogen. The residues were dissolved in 50  $\mu$ l of ethanol from which 40  $\mu$ l was transferred to a silica-gel TLC plate. Radioactivity of the residual 10- $\mu$ l aliquots was determined by liquid scintillation counting. 1,2-GDN, 1,3-GDN, and GTN standards were applied on the same plate. Water-saturated toluene/ethyl acetate [9:1 (v/v)] was used as mobile phase. Separated products were localized by spraying markers with a solution of diphenylamine (1 g in 100 ml of methanol) and exposure to UV light. The corresponding radioactive spots were scraped off, and radioactivity was determined by liquid scintillation counting. Blank levels were determined in the absence of ALDH2. The sum of radioactivity (corrected for blanks) was taken as 100% and was used to calculate the relative amounts of each product, which were transformed into absolute values through the known amount of applied [<sup>14</sup>C]GTN (Kollau et al., 2005).

**Determination of PETN Denitration.** Radiolabeled PETN was incubated with 50  $\mu$ g of ALDH2 under the same conditions as described above for GTN denitration. Reaction products were extracted twice with 1 ml of dichloromethane followed by evaporation of the solvent under a gentle stream of nitrogen. The residues were dissolved in 50  $\mu$ l of acetone, of which 40  $\mu$ l was transferred to a silica-gel TLC plate. PETN, PETriN, pentaerythrityl dinitrate, and pentaerythrityl mononitrate standards were applied on the same plate. Water-saturated toluene/ethyl acetate/1-butanol [6:3:1 (v/v)] was used as mobile phase. Separated products were localized by spraying markers with a solution of diphenylamine [1 g in 100 ml of H<sub>2</sub>SO<sub>4</sub> 75% (w/w)]. The corresponding radioactive spots were scraped off and radioactivity was determined by liquid scintillation counting. Blank levels were determined in the absence of ALDH2.

**ALDH2 Inactivation by PETN and GTN.** To determine the rate of inactivation of dehydrogenase activity of ALDH2 by PETN and GTN, the formation of NADH from NAD was measured by monitoring the optical absorbance in a 10-mm quartz cuvette (Hellma, Mühlheim, Germany) at 340 nm in a Hewlett-Packard 8452A Diode Array spectrophotometer. The initial reaction mixture consisted of 0.4 mM acetaldehyde and 0.4 mM NAD in phosphate buffer, pH 7.4. After 2 min, ALDH2 was added, and the reaction was allowed to proceed for 2 min longer. Thereafter, PETN or GTN (50  $\mu$ M each) was added. Approximately 13 min after the start of the experiment, 0.4 mM DTT was added to test for enzyme reactivation. The total volume of the samples was 250  $\mu$ l and the total time over which the reaction was monitored was 20 min. Temperature was kept at 25°C by a refrigerating circulation water bath (Lauda RM6; Bartelt GmbH, Graz, Austria). The various segments of the time traces were fitted linearly, except for the inactivation, which was fitted to a single-exponential curve as described previously (Beretta et al., 2008b).

**Statistical Analysis.** Data are presented as mean values  $\pm$  S.E.M. of *n* experiments. Individual dose-response curves were fitted to a Hill-type model giving estimates of agonist potency (EC<sub>50</sub>). Statistical significance was determined using Student's unpaired *t* test or two-way analysis of variance. *P* values <0.05 were considered statistically significant.



## Results

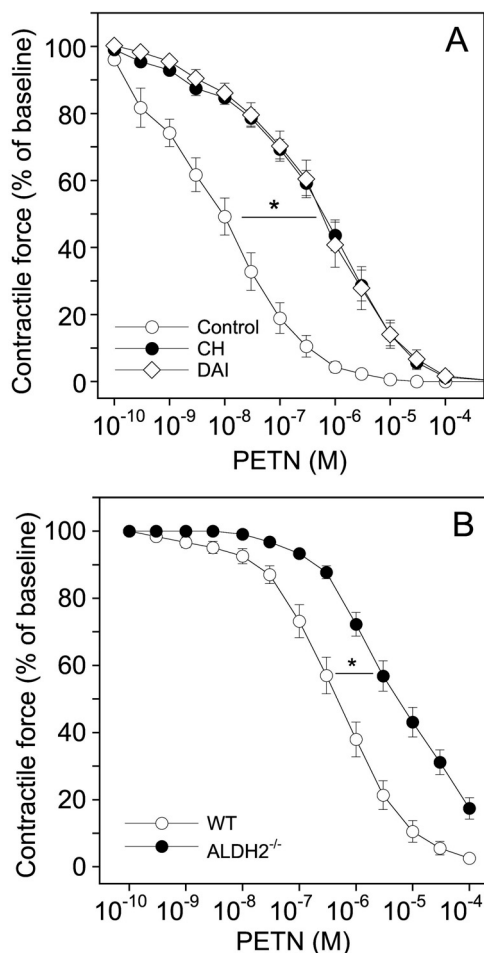
As shown in Fig. 1A, PETN caused relaxation of rat aortic rings with an  $EC_{50}$  value of  $0.03 \pm 0.01 \mu\text{M}$ . The potency of GTN was identical ( $0.03 \pm 0.01 \mu\text{M}$ ; data not shown). The ALDH2 inhibitors daidzin and chloral hydrate caused almost 100-fold rightward shifts of the PETN concentration-response curves, resulting in  $EC_{50}$  values of  $2.21 \pm 1.11$  and  $1.87 \pm 0.65 \mu\text{M}$ , respectively. Figure 1B shows that ALDH2 gene deletion increased the  $EC_{50}$  value of PETN to induce relaxation of mouse aorta from  $0.8 \pm 0.1$  to  $8.8 \pm 2.3 \mu\text{M}$ .

To test for the development of tolerance and/or cross-tolerance, we measured the relaxation of aortic rings isolated from mice that had been treated with GTN (20 mg/kg/day s.c., twice per day), PETN (60 nmol/min/kg), or vehicle DMSO (0.5  $\mu\text{l/h}$ ) for 4 days. The daily dose for both drugs was 85  $\mu\text{mol/kg}$ . Figure 2 shows that treatment with GTN led to pronounced tolerance of the blood vessels to GTN (Fig. 2A) and cross-tolerance to PETN (Fig. 2B), whereas in vivo application of PETN did not affect sensitivity of the blood vessels to either of the nitrates. The ALDH2 inhibitor chloral hydrate atten-

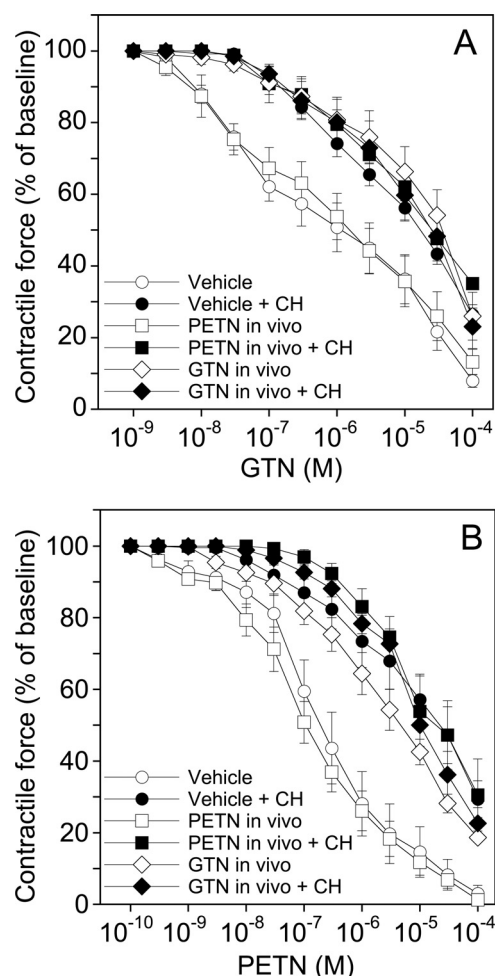
uated relaxation of nontolerant aortas to virtually the same extent as in vivo treatment with GTN.

The potential role of mitochondrial respiration in the bioactivation of PETN was studied using an established bioassay that is based on the activation of purified sGC. Figure 3 shows that DEA/NO-stimulated sGC activity was reduced by approximately 30% by CLM but was not further affected by succinate, mitochondrial poisons, or chloral hydrate. Basal sGC activity was not significantly affected by CLM (data not shown). In the presence of CLM, PETN (10  $\mu\text{M}$ ) increased the rates of cGMP formation from  $0.83 \pm 0.53$  to  $3.32 \pm 0.36 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ . The effect of the nitrate was completely blocked by the ALDH2 inhibitors daidzin and chloral hydrate but was not affected by succinate, Anti-A, or TTFA, indicating that mitochondrial bioactivation of PETN by ALDH2 does not require electron transfer from the respiratory chain.

Previous results showed that purified ALDH2 provokes NO-mediated activation of purified sGC by GTN (Kollau et al., 2005; Beretta et al., 2008a); the data in Fig. 4 indicate that a similar reaction occurs with PETN. As shown in Fig. 4A, up to 100  $\mu\text{M}$  PETN caused only very minor increases in cGMP formation in the absence of SOD and the ALDH2 cofactor NAD. In the presence of NAD, PETN activated sGC



**Fig. 1.** Effects of ALDH2 inhibition in rat aorta (A) or gene deletion in mice (B) on PETN-induced vasorelaxation. A, PETN-induced relaxation of aortic rings isolated from Sprague-Dawley rats in the absence and presence of the ALDH2 inhibitors chloral hydrate (CH; 5 mM) or daidzin (DAI; 0.3 mM). Data are mean values  $\pm$  S.E.M. from 11 to 15 rings obtained from 5 to 7 animals. \*,  $P < 0.05$ . B, PETN-induced relaxation of aortic rings isolated from C57BL/6 (WT) and ALDH2<sup>-/-</sup> mice. Data are mean values  $\pm$  S.E.M. of 18 rings obtained from 5 animals. \*,  $P < 0.05$ .



**Fig. 2.** Relaxation of aortic rings isolated from mice treated with GTN or PETN. WT mice were treated for 4 days with either GTN (GTN in vivo) or PETN (PETN in vivo) or vehicle followed by determination of aortic relaxation in response to GTN (A) or PETN (B) in the absence and presence of chloral hydrate (CH; 5 mM). Data are mean values  $\pm$  S.E.M. of six rings obtained from three animals.

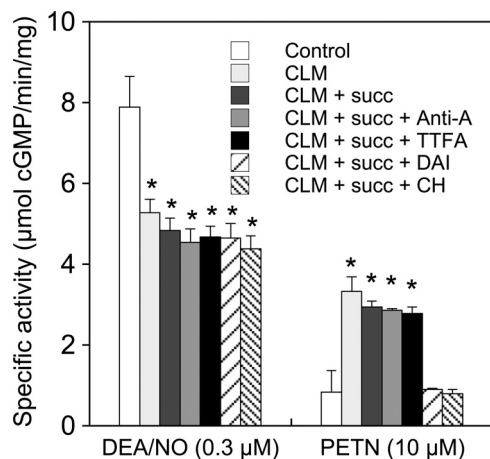
with an apparent  $EC_{50}$  of  $5.5 \pm 0.8 \mu\text{M}$  and maximal effects at 30 to 100  $\mu\text{M}$  ( $2.20 \pm 0.20 \mu\text{mol cGMP} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ). SOD (1000 U/ml) increased the rates of PETN-induced cGMP formation to  $8.50 \pm 1.24$  and  $19.31 \pm 0.82 \mu\text{mol cGMP} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  in the absence and presence of NAD, respectively. The corresponding  $EC_{50}$  values were  $6.8 \pm 2.6$  and  $2.2 \pm 0.5 \mu\text{M}$ , respectively. Thus, both efficacy and potency of PETN were markedly increased by the combined presence of NAD and SOD.

The reaction of PETN with purified ALDH2 led to the formation of free NO radical that was detectable with an NO-selective electrode. As shown in Fig. 4B, PETN (10  $\mu\text{M}$ ) caused a pronounced NO signal in the presence of NAD and SOD, with an initial rate of  $1.67 \pm 0.17 \text{ nmol NO} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  and a peak concentration of  $0.51 \pm 0.06 \mu\text{M}$ . In the absence of NAD or SOD, NO was hardly detectable. Note that the electrochemical method is approximately 3 orders of magnitude less sensitive than the sGC bioassay, which detects NO in the low picomolar range. Thus, NO formation may have been close to the detection limit of the electrode in the absence of NAD or SOD. The experiments shown were obtained with Cu,Zn-SOD (for financial reasons), but a few key experiments conducted with manganese-SOD gave identical results (data not shown). The superoxide scavenger Tempol (10–100 mM; Tocris Bioscience, Ellisville, MO) also led to detectable NO formation from PETN with initial rates comparable with those measured in the presence of SOD. However, the NO peak concentration measured in the presence of 100 mM Tempol was approximately 40% lower than with SOD, apparently because of an additional component of NO decay, presumably reflecting secondary reactions that were not further studied (data not shown).

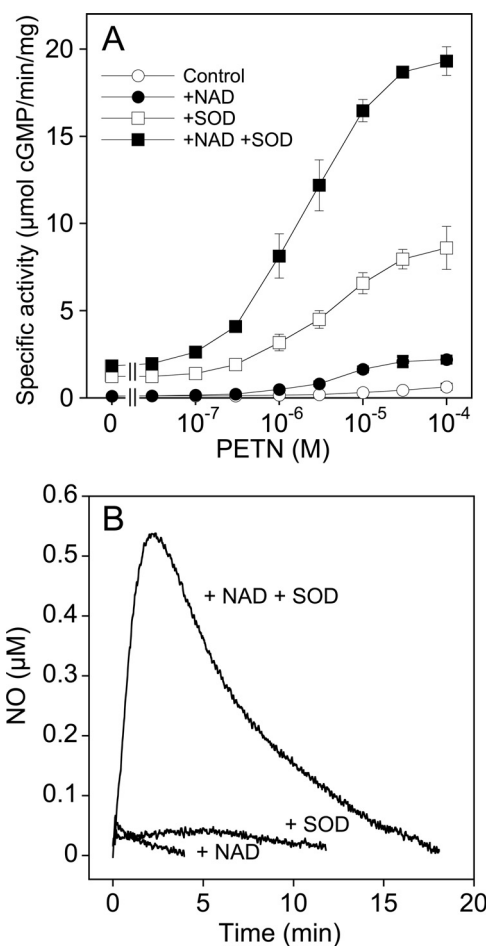
Denitration of PETN to yield PETriN was measured by radio TLC using [ $^3\text{H}$ ]PETN as substrate according to a modified protocol that had been established for quantification of  $^{14}\text{C}$ -labeled GTN metabolites (Kollau et al., 2005). Although

the nonenzymatic reaction of GTN with DTT, which is routinely included at a concentration of 2 mM for ALDH2 reactivation (Beretta et al., 2008b), is negligible within the time frame of the assay (Kollau et al., 2005), incubation of PETN with 2 mM DTT gave rise to unacceptably high blank levels, suggesting considerable nonenzymatic formation of PETriN (data not shown). Note that the nonenzymatic reaction is apparently not associated with PETN bioactivation, because incubation of the nitrate with DTT did not result in stimulation of sGC in the absence of added mitochondria or ALDH2 (see Fig. 3). Reducing the DTT concentration to 0.1 mM and increasing the amount of added ALDH2 to 50  $\mu\text{g}$ /assay led to reasonably low blank levels, allowing reliable determination of denitration rates.

As shown in Fig. 5, PETriN formation occurred with a  $V_{\text{max}}$  of  $9.6 \pm 0.8 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  (at 1 mM PETN) and an  $EC_{50}$  of  $94.7 \pm 7.4 \mu\text{M}$ . The corresponding values for GTN were



**Fig. 3.** Activation of sGC by DEA/NO and PETN in the presence of CLM. Purified sGC (50 ng) was incubated at  $37^\circ\text{C}$  for 10 min in 50 mM TEA buffer, pH 7.4, containing [ $\alpha$ - $^{32}\text{P}$ ]GTP ( $\sim 250,000$  cpm), 1 mM cGMP, 3 mM  $\text{MgCl}_2$ , 2 mM DTT, 1 mM NAD, 0.1 mM DTPA, 5 mM phosphocreatine, 152 U/ml creatine phosphokinase, and 1000 U/ml SOD with either 0.3  $\mu\text{M}$  DEA/NO or 10  $\mu\text{M}$  PETN in the absence (control) or presence of 1.5 mg/ml CLM and 10 mM succinate (succ), 10  $\mu\text{M}$  Anti-A, 10  $\mu\text{M}$  TTFA, 100  $\mu\text{M}$  daidzin (DAI) or chloral hydrate (CH; 1 mM) as indicated. Samples were analyzed for [ $^{32}\text{P}$ ]cGMP formation as described in the Supplemental Materials. Data are the mean values  $\pm$  S.E.M. of three independent experiments. \*,  $P < 0.05$  versus control.



**Fig. 4.** Activation of sGC by PETN (A) and formation of PETN-derived NO (B) in the presence of ALDH2. A, purified sGC (50 ng) was incubated at  $37^\circ\text{C}$  for 10 min in 0.1 ml of 50 mM TEA buffer, pH 7.4, with 4  $\mu\text{g}$  of ALDH2, and the indicated concentrations of PETN in the absence or presence of 1000 U/ml SOD and 1 mM NAD. Assay mixtures further contained [ $\alpha$ - $^{32}\text{P}$ ]GTP ( $\sim 250,000$  cpm), 1 mM cGMP, 3 mM  $\text{MgCl}_2$ , 2 mM DTT, and 0.1 mM DTPA. Samples were analyzed for  $^{32}\text{P}$ -cGMP as described in the Supplemental Materials. The data are the mean values  $\pm$  S.E.M. of three to seven independent experiments. B, ALDH2 (125  $\mu\text{g}$ ) was incubated at  $37^\circ\text{C}$  in 0.5 ml of 50 mM TEA buffer, pH 7.4, containing 3 mM  $\text{MgCl}_2$ , 2 mM DTT, and 0.1 mM DTPA in the presence of 1000 U/ml SOD and 1 mM NAD (unless indicated otherwise). After equilibration, PETN was added at  $t = 0$  to give a final concentration of 10  $\mu\text{M}$ . NO formation was monitored with a Clark-type electrode. The traces shown are representative for three to four similar experiments.

$17.4 \pm 1.2 \text{ nmol } 1,2\text{-GDN} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  ( $V_{\text{max}}$  at  $0.1 \text{ mM}$ ) and  $24.7 \pm 1.9 \text{ } \mu\text{M}$  ( $\text{EC}_{50}$ ). Denitration of PETN ( $0.1 \text{ mM}$ ) was inhibited to approximately 10% of control by chloral hydrate and daidzin (data not shown). As also shown in Fig. 5, PETN and GTN activated sGC with  $\text{EC}_{50}$  values of approximately  $0.1 \text{ } \mu\text{M}$  under identical assay conditions.

PETN- and GTN-triggered inactivation of the aldehyde dehydrogenase activity of purified ALDH2 was monitored spectrophotometrically at  $340 \text{ nm}$  (reduction of NAD to NADH). As illustrated by the representative traces shown in Fig. 6, initial ALDH2 dehydrogenase activity decreased rapidly upon addition of GTN ( $50 \text{ } \mu\text{M}$  final;  $\circ$ ) and was partially restored by DTT ( $0.4 \text{ mM}$  final), whereas PETN ( $50 \text{ } \mu\text{M}$ ;  $\bullet$ ) did not cause significant inactivation of the enzyme. The apparent inactivation rate constants for GTN and PETN were  $5.90 \pm 0.22 \times 10^{-3} \cdot \text{s}^{-1}$  and  $0.48 \pm 0.22 \times 10^{-3} \cdot \text{s}^{-1}$ , respectively ( $n = 7$ ).

## Discussion

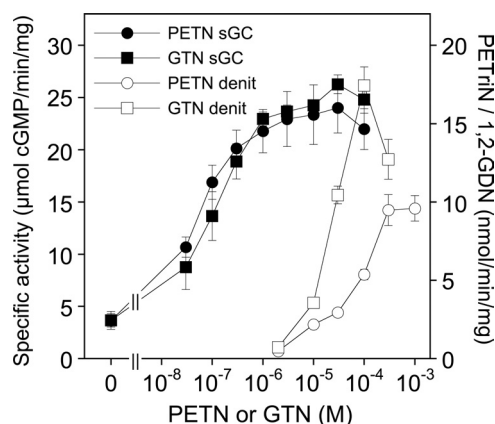
Previous studies have shown that vasorelaxation to PETN is attenuated by pharmacological inhibition or gene deletion of ALDH2 (Daiber et al., 2004; Mollnau et al., 2006; Wenzel et al., 2007a). Although these studies provided circumstantial evidence for a role of ALDH2 in vascular bioactivation of PETN, enzymatic metabolism of the tetranitrate has not been demonstrated so far. In the present study, we investigated the bioactivation of PETN at the level of isolated blood vessels, isolated mitochondria, and purified ALDH2.

Inhibition of PETN-induced relaxation of rat aorta by the nonspecific ALDH inhibitor chloral hydrate and the ALDH2-specific drug daidzin (Keung and Vallee, 1993), apparent as 100-fold rightward shifts of the concentration-response curves, as well as significantly attenuated relaxation upon gene deletion of ALDH2 strongly suggest that vascular

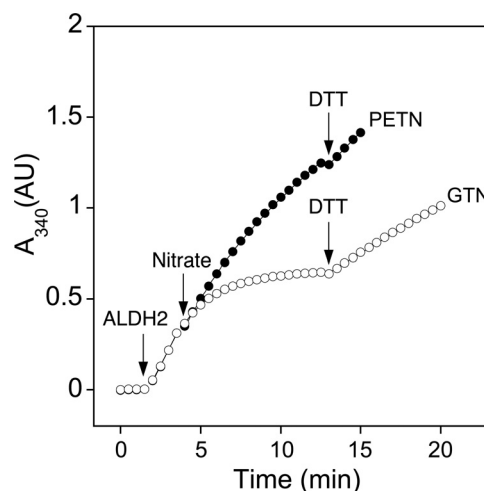
ALDH2 is essential for vasodilation in response to therapeutically relevant low concentrations of PETN. PETN was approximately 25-fold less potent on mouse ( $\text{EC}_{50} = 0.8 \text{ } \mu\text{M}$ ) than on rat ( $\text{EC}_{50} = 0.03 \text{ } \mu\text{M}$ ) aortas. A similar difference in potency of PETN has been reported previously [ $\text{EC}_{50}$  values of  $8.5 \text{ nM}$  in rat (Daiber et al., 2004) and  $170 \text{ nM}$  in mouse (Wenzel et al., 2007a) aorta].

Although several factors may synergistically contribute to the development of nitrate tolerance (Fung, 2004; Daiber et al., 2008; Mayer and Beretta, 2008), there is little doubt that mechanism-based inactivation of ALDH2 is the main culprit. The present data, showing that treatment of mice with GTN attenuated aortic relaxation in response to GTN and PETN to virtually the same degree as pharmacological inhibition or gene deletion of ALDH2, support this view. As observed previously with laboratory animals and humans (Fink and Bassege, 1997; Jurt et al., 2001; Müllenheim et al., 2001; Gori et al., 2003), in vivo administration of PETN did not affect the vasodilatory potency of PETN or GTN. Considering that purified ALDH2 was rapidly inactivated by GTN but not by PETN, the lack of tolerance development to PETN ties in well with the frame of the ALDH2 inactivation hypothesis of nitrate tolerance. This does not exclude, however, the contribution of other factors, in particular antioxidative effects of PETN (Mollnau et al., 2006; Wenzel et al., 2007b).

A main focus of our research in the past couple of years has been the link between ALDH2-catalyzed GTN bioconversion and activation of sGC. Taking into account the well established mitochondrial localization of ALDH2 in liver and the nitrite reductase activity of mammalian mitochondria, Stamler and coworkers proposed that GTN-derived nitrite is reduced by the mitochondrial respiratory chain to NO (Chen et al., 2002). However, ALDH2 is mainly cytosolic in blood vessels (DiFabio et al., 2003) and respiratory substrates or inhibitors of mitochondrial respiration did not affect GTN bioactivation by isolated mitochondria (Kollau et al., 2005,



**Fig. 5.** Effects of GTN and PETN concentration on denitration (unfilled symbols) and sGC activation (filled symbols) in the presence of ALDH2. Denitration (denit) of GTN and PETN was assayed as formation of 1,2-GDN and PETriN, respectively. ALDH2 ( $50 \text{ } \mu\text{g}$ ) was incubated at  $37^\circ\text{C}$  for 10 min in a total volume of  $0.2 \text{ ml}$  of  $50 \text{ mM}$  TEA buffer, pH 7.4, containing increasing concentrations of either [ $^3\text{H}$ ]PETN or [ $^{14}\text{C}$ ]GTN,  $1 \text{ mM}$  NAD,  $0.1 \text{ mM}$  DTT,  $3 \text{ mM}$   $\text{MgCl}_2$ , and  $2 \text{ mM}$  EDTA. Reaction products were extracted and quantified by radio TLC as described. Activation of sGC by PETN and GTN was determined by incubation of purified sGC ( $50 \text{ ng}$ ) with ALDH2 ( $25 \text{ } \mu\text{g}$ ) at  $37^\circ\text{C}$  for 10 min in a total volume of  $0.1 \text{ ml}$  of  $50 \text{ mM}$  TEA buffer, pH 7.4, containing  $0.5 \text{ mM}$  [ $\alpha\text{-}^{32}\text{P}$ ]GTP ( $\sim 250\,000 \text{ cpm}$ ),  $1 \text{ mM}$  cGMP,  $1 \text{ mM}$  NAD,  $0.1 \text{ mM}$  DTT,  $3 \text{ mM}$   $\text{MgCl}_2$ ,  $0.1 \text{ mM}$  DTPA, and  $1000 \text{ U/ml}$  SOD. The samples were analyzed for [ $^{32}\text{P}$ ]cGMP as described in the Supplemental Methods. Data are mean values  $\pm$  S.E.M. of three to four independent experiments.



**Fig. 6.** ALDH2 inactivation by PETN and GTN. Shown are representative time traces for the formation of NADH from NAD monitored spectrophotometrically at  $340 \text{ nm}$ . At  $t = 0$  the cuvette contained  $0.4 \text{ mM}$  acetaldehyde and  $0.4 \text{ mM}$  NAD in  $50 \text{ mM}$  phosphate buffer, pH 7.4. At  $t = 2 \text{ min}$ , reaction was initiated by the addition of ALDH2 ( $33 \text{ } \mu\text{g/ml}$ ). At  $t = 4 \text{ min}$ , either PETN or GTN ( $50 \text{ } \mu\text{M}$  each) was added. At  $t = 13 \text{ min}$ , DTT ( $0.4 \text{ mM}$ ) was added to test for reversibility of enzyme inactivation. The dots represent the data points, whereas the continuous lines are best fits to the data. The traces are representative of seven independent experiments.



2009), raising doubt about the involvement of the mitochondrial electron transport chain. In the present study, we found that sGC was stimulated by PETN in the presence of mitochondria in a chloral hydrate- and daidzin-sensitive manner (see Fig. 3), indicating that the organic nitrate is bioactivated by mitochondrial ALDH2. Because neither the respiratory chain substrate succinate nor the complex II and III inhibitors TTFA and Anti-A affected PETN-induced activation of sGC, ALDH2-catalyzed bioactivation of PETN does not seem to be linked to mitochondrial respiration.

The observation that incubation of PETN with purified ALDH2 led to pronounced stimulation of sGC and formation of free NO radical demonstrates that ALDH2 alone is sufficient for PETN bioactivation. In the presence of NAD and SOD, PETN increased cGMP formation with an  $EC_{50}$  of approximately 2  $\mu$ M. This value is significantly higher than the potency of the nitrate in blood vessels, but activation of 3 to 4% of the vascular sGC pool seems to be sufficient for maximal relaxation (Kollau et al., 2005; Mergia et al., 2006). Thus, there is good reason to assume that vascular bioactivation of PETN is explained by NO formation and sGC activation catalyzed by purified ALDH2. Bioactivation of PETN was essentially dependent on the presence of NAD and SOD. The effect of NAD is not surprising because the nucleotide is a cofactor of ALDH2 and has profound effects on active site geometry (Wenzl et al., 2009a). The effect of SOD, however, warrants some comments. Although the ALDH2/GTN reaction generates relatively low amounts of superoxide and there is no obvious reaction mechanism that would account for superoxide formation (Wenzl et al., 2009a), it cannot be excluded that SOD acts through scavenging of superoxide. Because there is substantial evidence for increased superoxide generation in GTN-tolerant blood vessels (Münzel et al., 2005), we considered the ALDH2/GTN reaction as a potential source of superoxide in nitrate tolerance. This possibility is challenged by the present results demonstrating that bioactivation of PETN, which does not cause vascular oxidative stress (Jurt et al., 2001; Daiber et al., 2004), is also largely dependent on the presence of SOD. However, our data do not exclude that ALDH2-catalyzed metabolism of GTN triggers the generation of superoxide from other sources (e.g., mitochondria or NADPH oxidase). Further work is necessary to clarify the role of SOD in the bioactivation of organic nitrates.

The results shown in Fig. 5 indicate that ALDH2 is able to catalyze denitration of PETN to PETriN, although compared with GTN, apparent affinity and  $V_{max}$  were approximately 4- and 2-fold lower, respectively. This difference was not observed when bioactivation of the organic nitrates was assayed as stimulation of sGC under identical conditions. PETN and GTN increased the rates of cGMP formation with apparent  $EC_{50}$  values of approximately 0.1  $\mu$ M. The  $EC_{50}$  values were determined under rather unusual conditions to meet the requirements of the PETN denitration assay and do not reflect true affinity constants. Nevertheless, it is stunning to notice that denitration of both PETN and GTN was not even detectable at concentrations of the drugs that gave rise to maximal sGC activation ( $\sim 1$   $\mu$ M).

Our previous work strongly suggests that ALDH2-catalyzed biotransformation of GTN involves at least two distinct reactions yielding nitrite and NO, respectively (Beretta et al., 2008a, 2010; Wenzl et al., 2009a), and the present data suggest

a similar scenario for PETN. We have not yet resolved the 3D-structure of GTN-bound ALDH2, but molecular modeling revealed a large number of different possible binding modes for GTN, depending on the selected docking parameters (K. Gruber, personal communication). Thus, distinct binding conformations of the nitrates may give rise to either clearance-based nitrite formation or bioactivation yielding NO. According to our data, PETN exhibits significantly lower affinity for the clearance-based pathway than GTN, and it is likely that this pathway is associated with inactivation of ALDH2. This would explain the low inactivation rate constant obtained with PETN and, possibly, why PETN does not induce nitrate tolerance.

#### Authorship Contributions

*Participated in research design:* Griesberger, Wölkart, Russwurm, Koesling, Schmidt, Gorren, and Mayer.

*Conducted experiments:* Griesberger, Kollau, Wölkart, Wenzl, and Beretta.

*Contributed new reagents or analytic tools:* Russwurm and Koesling.

*Performed data analysis:* Griesberger, Kollau, Wölkart, Wenzl, Beretta, Schmidt, and Gorren.

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

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