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Oxidative Stress and Mitochondrial Aldehyde Dehydrogenase Activity: A Comparison of Pentaerythrityl Tetranitrate (PETN) with Other Organic Nitrates

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Running title: Role of Oxidative Stress and ALDH-2 in Nitrate Tolerance

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Abbreviations:

GTN, glyceryl trinitrate (nitroglycerin); GDN, glyceryl dinitrate; ISDN, isosorbide dinitrate; ISMN, isosorbide-5-mononitrate; PETN, pentaerythrityl tetranitrate; PETriN, pentaerythrityl trinitrate; PEDN, pentaerythrityl dinitrate; PEMN, pentaerythrityl mononitrate; ACh, acetylcholine; DTT, dithiothreitol; GSH, glutathione; SNP, sodium nitroprusside; ALDH-2, mitochondrial aldehyde dehydrogenase; L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt; sGC, soluble guanylyl cyclase; cGK-I, cGMP-dependent protein kinase; VASP, VAsodilator Stimulated Phosphoprotein; PEG-SOD, polyethylene-glycolated superoxide dismutase; Sin-1, 3-morpholino sydnonimine.

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Abstract

Mitochondrial aldehyde dehydrogenase (ALDH-2) was recently identified to be essential for bio-activation of glyceryl trinitrate (GTN). We now assessed whether other organic nitrates are bio-activated by a similar mechanism. The ALDH-2 inhibitor benomyl reduced the vasodilator potency, but not the efficacy, of GTN, pentaerythrityl tetranitrate (PETN) and pentaerythrityl trinitrate in phenylephrine-constricted rat aorta, whereas vasodilator responses to isosorbide dinitrate, isosorbide-5-mononitrate, pentaerythrityl dinitrate, pentaerythrityl mononitrate and the endothelium-dependent vasodilator acetylcholine were not affected. Similarly, benomyl decreased GTN and PETN-elicited phosphorylation of the cGMP-activated protein kinase substrate vasodilator-activated phosphoprotein (VASP), but not that elicited by other nitrates. The vasodilator potency of organic nitrates correlated with their potency to inhibit ALDH-2 dehydrogenase activity in mitochondria from rat heart, and to increase mitochondrial superoxide formation, as detected by chemiluminescence. In contrast, mitochondrial ALDH-2 esterase activity was not affected by PETN and its metabolites, whereas it was inhibited by benomyl, GTN applied in *vitro* and *in vivo*, and some sulfhydryl-oxidants. The bio-activation related metabolism of GTN to glyceryl-1,2-dinitrate by isolated RAW-macrophages was reduced by the ALDH-2 inhibitors benomyl and daidzin, as well as by GTN concentrations > 1 μM. We conclude that mitochondrial ALDH-2, specifically its esterase activity, is required for bio-activation of the organic nitrates with high vasodilator potency, such as GTN and PETN, but not for the less potent nitrates. Interestingly, ALDH-2 esterase activity was inhibited by GTN only, not by the other nitrates tested. This difference might explain why GTN elicits mitochondrial superoxide formation and nitrate tolerance with the highest potency.

Organic nitrates such as nitroglycerin (glyceryl trinitrate, GTN) are widely used in the therapy of cardiovascular diseases such as stable and unstable angina (Abrams, 1995). The anti-ischemic effects of organic nitrates are largely due to venous and coronary artery dilation as well as improvement of collateral blood flow, which all decrease myocardial oxygen consumption. Their use, however, is limited due to the rapid development of tolerance and cross-tolerance characterized by decreased sensitivity of the vasculature to the organic nitrates and to endothelium-dependent vasodilators, respectively (Mangione and Glasser, 1994; Warnholtz et al., 2002). It is thought that organic nitrates induce vasorelaxation by generating nitric oxide (NO) or a related S-nitrosothiol (SNO). Both enzymatic and non-enzymatic mechanisms of organic nitrate metabolism/bio-activation have been proposed. The list of candidate enzymes includes glutathione S-transferases (Lau et al., 1992), the cytochrome P450 system (Schroder, 1992), xanthine oxidoreductase (O'Byrne et al., 2000) and mitochondrial aldehyde dehydrogenase (ALDH-2) (Chen et al., 2002). The active metabolite(s) of GTN activate the target enzyme soluble guanylyl cyclase (sGC), increasing tissue levels of the second messenger cGMP. cGMP in turn activates a cGMP-dependent protein kinase (cGK-I) which mediates vasorelaxation by phosphorylating proteins that regulate intracellular Ca^{2+} levels (Ignarro, 2002; McDonald and Murad, 1995). An impairment of the NO-signaling pathway by increased formation of reactive oxygen species (ROS) (Munzel et al., 1995) as well as an impaired biotransformation of organic nitrates may contribute to the development of tolerance and cross-tolerance. Recently, the mitochondrial aldehyde dehydrogenase (ALDH-2) has been identified as a GTN-metabolizing enzyme, which is subjected to an oxidative mechanism-based inactivation (Chen et al., 2002; Sydow et al., 2004). These results provide the missing link between tolerance and cross-tolerance, the oxidative stress concept and a decreased GTN biotransformation. However, in a recent study the ALDH-2 concept was criticized (DiFabio et al., 2003), because an appreciable number of Asiatic people possess a mutation of mtALDH-2 which causes a loss of function, but yet

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show no signs of inherited tolerance to GTN. This apparent discrepancy may be explained, if one recalls that this mutation affects the co-factor binding (Sladek, 2003), thereby probably leaving the ALDH-2 esterase and GTN reductase activity intact.

Several organic nitrates have been reported to induce tolerance. Examples are isosorbide-5-mononitrate (ISMN), isosorbide dinitrate (ISDN) and GTN (Abrams, 1987). In contrast, pentaerythrityl tetranitrate (PETN) treatment has been described to cause less tolerance (Fink and Bassenge, 1997; Jurt et al., 2001) and to induce less side effects, such as headache (Pfaffenrath et al., 1998). In animal studies using dogs or rabbits it was shown that chronic therapy with PETN causes no tolerance and less oxidative stress and has beneficial effects on the progression of atherosclerosis in hyperlipidemic rabbits (Mullenheim et al., 2001; Schwemmer and Bassenge, 2003). These effects may be explained by the property of PETN to induce ferritin and heme oxygenase-1, as shown in cultured endothelial cells (Oberle et al., 2003; Oberle et al., 1999). Increased expression of ferritin could decrease the tissue levels of free iron and thereby reduce the formation of free radicals induced by a Fenton-type reaction. The protective effects of increased heme oxygenase-1 expression has been shown to be secondary to increased degradation of porphyrins to form bilirubin, a potent antioxidant (Yamaguchi et al., 1996), as well as carbon monoxide, which is a weak stimulator of soluble guanylyl cyclase and inhibits platelet aggregation (Brune and Ullrich, 1987).

With the present study, we sought to determine whether or not biotransformation of GTN by ALDH-2 represents a common pathway of organic nitrate bio-activation, and whether or not all organic nitrates induce oxidative stress during bio-activation, irrespective of their molecular structure. Therefore, ISMN, ISDN, GTN, PETN and its metabolites PETriN, PEDN, PEMN were tested for their effects on mitochondrial ROS formation and ALDH-2 activity. Moreover, we assessed the effect of the ALDH-2 inhibitor, benomyl, on the vasodilator potency of these different organic nitrates in isolated, endothelium-intact rat aortic rings, and observed a marked difference between PETN and GTN on one hand, and the other

nitrates on the other hand. Our present findings help to establish a unifying concept for the molecular mechanism underlying nitrate tolerance and cross-tolerance. Thus, *in vivo* GTN tolerance primarily impairs the high potency pathway and is due to oxidative inactivation of ALDH-2.

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Materials and Methods

Materials. GTN was purchased as an ethanolic stock solution (102 g/l) from UNIKEM (Copenhagen, Denmark). For organ chamber tension studies, GTN was used from a Nitrolingual infusion solution (1 mg/ml) from G.Pohl-Boskamp (Hohenlockstedt, Germany). PETN (with 80 % (w/w) lactose), PETriN, PEDN and PEMN were kindly donated by Alpharma-Isis GmbH (Langenfeld, Germany). ISDN (with 60 % (w/w) lactose) was obtained from Alexis Biochemicals (Ca, USA), ISMN from Acros Organics (NJ, USA) and Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid), DTNB) and PEG-SOD from Sigma-Aldrich (Steinheim, Germany). PETN and PETriN are barely soluble in aqueous solutions and were used from stock solutions in DMSO. For better comparison, all organic nitrates were used from stock solutions in DMSO. 1,2- and 1,3-GDN were purchased from Cerilliant (TX, USA). Peroxynitrite (oxoperoxonitrate (1-)) was synthesized according to the quenched-flow method as previously described (Saha et al., 1998). Benomyl (methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate) was purchased from Chem Service (PA, USA), daidzin (7-glucoside of 4',7-dihydroxy-isoflavone) from Indofine Chemical Company Inc. (NJ, USA), Sin-1 from Calbiochem (CA, USA) and L-012 from Wako Pure Chemical Industries (Japan). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich, Fluka or Merck.

Animal Model, *In Vivo* Nitrate Tolerance. Wistar rats treated with ethanol (sham) or nitroglycerin (3 d, 0.48 µmol/h) were studied, as described recently (Munzel et al., 2000). All animals were treated in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and was granted by the Ethics Committee of the University Hospital Eppendorf.

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Isometric tension studies. Vasodilator responses to organic nitrates and other vasodilators were assessed with isolated aortic rings mounted for isometric tension recordings in organ chambers, as described previously (Munzel et al., 1995). In selected experiments, vessels from rats were incubated with benomyl, an irreversible inhibitor of ALDH (10 µM). *In vitro* tolerance development as a result of PETN treatment was assessed by *ex vivo* incubation of rat vessels with 100 µM PETN for 30 min at 37 °C in Krebs-HEPES buffer (composition in g/l: 5.78 NaCl, 0.35 KCl, 0.37 $CaCl_2$, 0.30 $MgSO_4$, 2.1 $NaHCO_3$, 0.14 K_2HPO_4, 5.21 HEPES and 2.0 D-glucose) followed by a 1 h wash-out phase, and subsequent recording of concentration-response curves with PETN. Cross-tolerance of PETN to GTN was assessed by measurement of vasodilator responses to PETN with aortic rings pre-exposed to GTN *in vitro* (100 µM for 30 min at 37 °C) or *in vivo* (3 d chronic infusion).

Activity Assay for cGMP-dependent Protein Kinase (cGK-I) and Soluble Guanylyl Cyclase (sGC). Aortic segments (1 cm) from GTN and sham-treated rats were frozen and homogenized in liquid nitrogen. Immunoblotting was performed with a mouse monoclonal antibody (16C2) specific for P-VASP at serine239, as described (Mulsch et al., 2001; Schulz et al., 2002). The effect of benomyl (10 µM) on VASP phosphorylation was tested in the absence or presence of either acetylcholine (0.1 µM), sodium nitroprusside (0.1 µM), PETN (0.05 µM), GTN (0.1 µM), ISDN (10 µM) or ISMN (1 mM). For PETN (0.05 µM), GTN (0.1 µM) and ISDN (100 µM) the activation of sGC was determined by formation of cGMP which was measured by a non-radioactive assay kit from Amersham (Freiburg, Germany). The effect of ALDH-2 inhibition on the organic nitrate-dependent stimulation of sGC was determined by addition or exclusion of benomyl (10 µM).

ALDH-2 Dehydrogenase and Esterase Activity in Isolated Rat Heart Mitochondria. The activity of ALDH in isolated mitochondria was determined by measuring the conversion of

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benzaldehyde to benzoic acid. Rat heart mitochondria were prepared according to a previously published method (Raha et al., 2000). Hearts from sham-treated rats were homogenized in HEPES buffer (composition in mM: 50 HEPES, 70 sucrose, 220 mannitol, 1 EGTA and 0.033 BSA) and centrifuged at 1500g (10 min at 4 °C) and 2000g for 5 min (the pellets were discarded). The supernatant was then centrifuged at 20000g for 20 min, and the pellet was resuspended in 1 ml of HEPES buffer. The latter step was repeated and the pellet resuspended in 1 ml of Tris buffer (composition in mM: 10 Tris, 340 sucrose, 100 KCl and 1 EDTA). The mitochondrial fraction (total protein approximately 5-10 mg/ml) was kept on ice and diluted to approximately 1 mg/ml protein in 0.25 ml of PBS and preincubated for 10 min at room temperature in the presence or absence of the ALDH-2 inhibitors benomyl (1 µM) or chloralhydrate (10 µM) as well as peroxynitrite (500 µM), hydrogen peroxide (10 mM), Ellman's reagent (5 mM) or organic nitrates (50-5000 µM, except PETN due to its limited solubility). For measurement of ALDH-2 dehydrogenase activity, benzaldehyde (200 µM) was added and the samples were incubated for another 30 min at 37 °C. ALDH activity in cytosolic fractions was measured in the 20,000g supernatant of heart homogenates (2 mg/ml total protein) upon incubation with 200 µM NAD^+ and 200 µM benzaldehyde for 30 min at 37 °C. To determine the oxidative inactivation of ALDH-2 by Sin-1-derived peroxynitrite, isolated mitochondria (1.6 mg/ml in PBS) were lysed by sonication (to provide direct access for peroxynitrite and PEG-SOD), incubated for 90 min at 37 °C in the presence or absence of Sin-1 (0.1 or 1 mM) and PEG-SOD (500 U/ml) or DTT (1 mM). Then 1 mM NAD^+ and 0.5 mM benzaldehyde were added to the samples and further incubated for 30 min at room temperature. For measurement of ALDH-2 esterase activity, methylbenzoate (1 mM) was added and the samples were incubated for another 30 min at 37 °C. Samples were sonicated, centrifuged at 20000g (4 °C) for 20 min, and the supernatant was purified by size exclusion centrifugation through a Microcon YM-10 filter device from Millipore (Bedford, USA). 200 µl of each sample were subjected to HPLC analysis. The system consisted of a LKB pump, a

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Knaur UV/Vis detector and a C18-Nucleosil 125x4 100-3 reversed phase column from Macherey & Nagel (Düren, Germany). The mobile phase contained acetonitrile (35 v/v %) in 50 mM citric acid buffer (65 % (v/v)) pH 2.2. The substrate and its products were isocratically eluted at a flow rate of 0.8 ml/min, detected at 275 nm and quantified using internal and external standards (benzoic acid, benzaldehyde and methylbenzoate). The typical retention times were 4.2, 6.2 and 12.6 min, respectively.

Measurement of Reactive Oxygen Species Production from Isolated Heart Mitochondria. Mitochondrial stock solutions were diluted to final total protein concentrations of approximately 0.1 mg/ml in 0.5 ml PBS. The dye L-012 (100 µM) was used as described (Daiber et al., 2004) to quantify ROS following addition of the complex II substrate succinate (4 mM final concentration). Chemiluminescence was monitored over 5 min using a Lumat LB9507 from Berthold Techn. (Germany) and the signal at 5 min was expressed as counts/min. ROS production was quantified in mitochondria from control rats exposed to increasing concentrations of organic nitrates (50-5000 µM, except PETN due to its limited solubility) in vitro.

Measurement of GTN Biotransformation in RAW 264.7 Macrophages by GC-MS Analysis. RAW 264.7 macrophages were cultured to confluency in 75 cm^2 flasks in DMEM from Biochrom (Berlin, Germany) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 4 mM glutamine, 1 mM pyruvate and 1.5 mg/ml carbonate. Cells ($50x10^6$) were incubated in 10 ml of Hanks' buffered salt solution containing 10 % fetal calf serum for 30 min with 1-10 µM GTN. To determine the effect of ALDH-2 inhibition on GTN biotransformation the cells were incubated with 1 µM GTN in the presence of either benomyl (50 µM) or daidzin (50 µM). The culture medium was then discarded, the cells were scraped from the culture bottle in 1 ml $AgNO_3$ (20 mM) solution (Chen et al., 2002) and were frozen

at -20 °C. After thawing, the cells were sonicated and GTN as well as its metabolites 1,2- and 1,3-GDN were extracted with 2 ml ethylacetate (25 % (v/v))/pentane (75 % (v/v)) as previously described (Gerardin et al., 1982). The organic phase was centrifuged for 10 min at 1600g and evaporated using a gentle nitrogen stream. The remaining sample was dissolved in acetonitrile. An aliquot of each sample (20 µl) was injected into a GC-MS unit GC Star 3400 CX, MS Saturn 3 from Varian (Darmstadt, Germany). GTN and its metabolites were separated on a Optima delta 6 (0.25 µm film, 30 m length, 0.25 mm diameter) GC column from Macherey and Nagel (Düren, Germany). The nitrates were detected at the mass of the nitro group (m/z = 46) using EI mode. The temperatures were 150 °C for the injector, 280 °C for the transferline and 170 °C for the iontrap. The temperature program for the column was 80 °C for 4 min, 150 °C at 5.75 min, 166 °C at 13.75 min, 300 °C at 16.43 - 18.43 min. The helium flow was 2 ml/min and typical retention times were 11.8 min for 1,2-GDN, 12.2 min for 1,3-GDN and 13.2 min for GTN.

Statistical Analysis. Results are expressed as mean±SEM. One-way ANOVA (Bonferroni's or Dunn's method) was used for comparisons of vascular responses, L-012 chemiluminescence, cGK-I activity, cGMP responses and ALDH-2 dehydrogenase and esterase activity. The EC_{50} value for each experiment was obtained by log-transformation. Significance is indicated by * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ and respective other symbols.

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Results

Vasodilator responses. In endothelium-intact rat aorta the vasodilator potency of the different organic nitrates roughly correlated with the number of $–ONO_2$ groups present in the molecule. PETN and GTN exhibited the highest potency (EC_{50} 8.5 and 22 nM, respectively; Fig. 1A and Tabel 1), followed by PETriN (EC_{50} 0.2 µM), ISDN (EC_{50} 5 µM) and ISMN (EC_{50} 170 µM). The vasodilator potency of PEDN (EC_{50} 25 µM) was lower than that of PETriN, and PEMN exhibited the lowest potency (EC_{50} 1 mM) (Fig. 1B and Tabel 1). PETN and GTN showed similar potency as the biological inducer of relaxation ACh (EC_{50} 35 nM).

The role of ALDH-2 in the bio-activation of the organic nitrates was assessed by the addition of benomyl (10 µM) to the organ bath solution. Benomyl induced a marked right-shift of the concentration-response curves of PETN, PETriN and GTN (Fig. 1A and B), indicating that ALDH-2 is involved in the bioactivation of these nitrates. In contrast, benomyl did not significantly affect the vasodilator potency of ISDN and ISMN (Fig. 1A, Tabel 1), thereby excluding involvement of ALDH-2 in the bioactivation process of these two nitrates. Similarly, benomyl did not affect the vasodilator potency of the higher-denitrated PETN metabolites PEDN and PEMN (Fig. 1B). Therefore, bio-activation of PEDN and PEMN, similar to ISDN and ISMN presumably involves another, ALDH-2-independent pathway(s). The vasodilator response to the endothelium-dependent agonist ACh was not affected by benomyl (Fig. 1B and Tabel 1).

In additional experiments with higher concentrations of benomyl (100 µM), we ascertained that the concentration of benomyl used (10 µM) was maximally efficacious with regard to ALDH-2 inhibition (data not shown). Thereby we excluded the possibility that higher concentrations of organic nitrates surmounted inhibition by benomyl (as observed by the right-shift of the concentration-response curve) because of incomplete inhibition of ALDH-2. We can not entirely exclude that the carbamoylating compound benomyl inhibits other targets besides ALDH-2, but Chen et al. showed that acetaldehyde, a natural substrate of

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ALDH-2, also inhibits GTN-dependent relaxation, probably by acting as a competitive inhibitor for GTN biotransformation (Chen et al., 2002). In a recent study, we made a similar observation with the specific ALDH-2 inhibitor daidzin, further supporting an important role for ALDH-2 in the bioactivation of GTN (Sydow et al., 2004).

In order to assess the influence of so-called *in vitro* tolerance on the vasodilator responsiveness of PETN, isolated rat aortic rings were exposed for 30 min to high concentrations of PETN (100 µM) or GTN (100 µM), followed by a 1 h washout-period, before assessment of vasodilator responses. Pre-treatment with PETN caused a small, but significant right-shift of the PETN concentration-response curve (EC_{50} increased from 8.5 to 27 nM; Fig. 1C), indicating a weak degree of tolerance/tachyphylaxis. In contrast, *in vitro* treatment with high concentrations of GTN induced a larger right-shift of the PETN vasodilator response curve (EC_{50} increased from 8.5 to 85 nM). This effect was even more pronounced when using aortic rings from *in vivo* GTN treated rats (EC_{50} increased to 182 nM; Fig. 1C and Tabel 1). These results indicate that PETN induces a weak *in vitro* auto-tolerance (tachyphylaxis) and GTN a pronounced *in vitro* and *in vivo* cross-tolerance to PETN.

Effect of ALDH-2 Inhibition on Activation of cGK-I and sGC by Organic Nitrates, SNP and ACh. cGK-I activity was assessed by the phosphorylation state of VASP at serine239 (P-VASP) detected by means of Western blotting. The 48 kD protein VASP (on blots appearing as a 45 and 50 kD double band) is a prominent and ubiquitous cGK-I substrate (Eigenthaler et al., 1993) and can be taken as a monitor for cGK-activity in intact cells and tissues (Mulsch et al., 2001). ACh, SNP, PETN, GTN, ISDN and ISMN caused a concentration-dependent increase in P-VASP levels (Fig. 2A). The bar graph shows summarized densitometric data of P-VASP levels from at least 3 independent experiments with aortae from different animals, demonstrating a significant stimulation of cGK-I activity by all vasodilators used. The stimulatory effect of the organic nitrates on cGK-I activity increased with increasing number

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of $–ONO_2$ groups (PETN > GTN > ISDN > ISMN) and therefore these results are in accordance with those obtained by isometric tension recordings. Co-incubation with the ALDH-2 inhibitor benomyl (10 μM) significantly decreased the P-VASP levels in PETN- and GTN-stimulated vessels almost to control levels, whereas with all other organic nitrates, SNP and ACh VASP phosphorylation was not significantly altered (Fig. 2A). These results corroborate our vasodilator experiments showing that ALDH-2 specifically bioactivates PETN and GTN, but not the lower nitrated organic nitrates. The failure of benomyl to affect ACh- and SNP-dependent VASP phosphorylation proves that this ALDH-2 inhibitor does not interfere with the endothelium-dependent and -independent NO signalling pathway, i.e. NO synthase activity and sGC activation.

The activation of sGC by different organic nitrates was detected by measuring cGMP levels in rat aortic tissue with a cGMP-specific EIA. Treatment of isolated blood vessels with GTN (0.1 μM) or PETN (0.05 μM) caused a marked 2.5- to 3.5-fold increase in cGMP levels, which was almost reduced to control levels in the presence of benomyl (10 μM; Fig. 2B). In contrast, the 5-fold increase in cGMP induced by ISDN (100 μM) was slightly, though not significantly reduced by addition of benomyl (Fig. 2B). These results support our conclusion that GTN and PETN requires ALDH-2-dependent bioactivation to elicit a cGMP response, whereas ISDN-induced cGMP is largely independent of this bioactivation pathway. It should be noted that the high and low potency organic nitrates were employed at different concentrations in order to achieve similar pronounced effects with all organic nitrates tested.

Mitochondrial Reactive Oxygen Species Formation. The formation of mitochondrial ROS was detected by a chemiluminescence (CL)-based assay using the probe L-012. Isolated rat heart mitochondria were incubated *in vitro* with high concentrations of organic nitrates (50 μM – 5 mM) in the presence of L-012 (100 μM) and CL was recorded for 5 min (see Methods). ISMN had no marked effect on ROS formation even in the highest concentration

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applied (5 mM), whereas ISDN at 5 mM showed a significant 2-fold increase in the CL signal (Fig. 3A). With GTN, a marked concentration-dependent increase in ROS formation was achieved, resulting in a near 7-fold increase above control at 5 mM GTN (Fig. 3A). PETN (500 µM) induced only a 3-fold increase in CL and thus was less efficacious than 500 µM GTN (5-fold increase). With PETN and its metabolites the capability to elicit mitochondrial ROS formation decreased with the loss of $–ONO_2$ groups (PETN > PETriN > PEDN ≥ PEMN) (Fig. 4A).

Inhibition of ALDH-2 Dehydrogenase Activity by Organic Nitrates. The activity of ALDH-2 in isolated rat heart mitochondria was measured by the conversion of benzaldehyde to benzoic acid, as described previously (Sydow et al., 2004). ISMN, even at high concentration (5 mM) did not alter the activity of ALDH-2 (Fig. 3B). ISDN induced a clear concentration-dependent decrease in ALDH-2 activity, maximally 50 % at 5 mM, whereas GTN already at intermediate concentrations (500 µM) completely inhibited the enzyme (the remaining signal corresponds to background activity) (Fig. 3B). In contrast, PETN was a less potent inhibitor of ALDH-2 activity (Fig. 3B). ALDH-2 activity was not significantly affected by PEDN and PEMN treatment (Fig. 4B). Surprisingly, PETriN and PETN appeared to be equally effective in inhibiting ALDH-2 dehydrogenase activity. When comparing Figs. 3A and 3B, and 4A and 4B it becomes evident that the potency of the different organic nitrates to inhibit ALDH-2 dehydrogenase activity is mirrored by their potency to elicit mitochondrial ROS formation. These findings therefore demonstrate a direct correlation between organic nitrate-dependent ROS formation and ALDH-2 dehydrogenase inhibition. However, one should note that also cytosolic ALDH activity was inhibited upon *in vivo* treatment with GTN (60.8 ± 4.5 µM in control cytosol versus 23.1 ± 0.5 µM in cytosol from tolerant animals, n = 3; MV ± SEM).

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Inhibition of ALDH-2 dehydrogenase activity by Sin-1-derived peroxynitrite. To demonstrate the sensitivity of ALDH-2 dehydrogenase activity towards oxidative inactivation, we assessed the effect of the peroxynitrite generator 3-morpholino-sydnonimine (Sin-1) on ALDH-2 activity. Sin-1 releases peroxynitrite constantly over a time period of 90 min (at pH 7.4 and 37 °C) to generate low steady state concentrations of peroxynitrite (Daiber et al., 2002). In mitochondria from control animals, the presence of Sin-1 (1 mM) caused a 60 % loss of ALDH-2 activity (15.7 ± 2.1 µM versus 6.1 ± 1.1 µM benzoic acid/30min, n=3; MV±SEM). Sin-1 at a concentration of 100 µM still caused 30 % inhibition of ALDH-2 (15.7±2.1 µM versus 9.8±1.7 µM benzoic acid/30min, n=3; MV±SEM). The Sin-1 (1mM)-dependent decrease in ALDH-2 activity was partially prevented in the presence of PEG-SOD (500 U/ml, 6.1±1.1 µM versus 10.4±0.8 µM benzoic acid/30min, n=3; MV±SEM) and was completely prevented by addition of DTT (1 mM, 6.1±1.1 µM versus 14.8±1.1 µM benzoic acid/30min, n=3; MV±SEM). DTT (1 mM) was also able to restore ALDH-2 activity in isolated mitochondria from *in vivo* GTN-treated, tolerant animals (10.1±1.2 µM versus 17.1±1.1 µM benzoic acid/30min, n=3; MV±SEM) to the level of control.

Inhibition of ALDH-2 Esterase Activity by Organic Nitrates. Esterase activity in isolated rat heart mitochondria was measured by the conversion of methylbenzoate to benzoic acid. Since several esterases are present in mitochondria, special precautions had to be taken to specifically determine the contribution of ALDH-2 to total esterase activity. The horizontal line 1 in Figure 5A (right axis) defines the level of total esterase activity in control mitochondria, whereas line 2 defines the oxidation-resistant esterase activity. The esterase activity of ALDH-2 involves redox-sensitive sulfhydryl groups and should be inhibited by oxidants (Senior and Tsai, 1990; Tsai and Senior, 1991). Therefore, this activity is represented by the difference between lines 1 and 2. ALDH-2 esterase activity was almost completely inhibited by thiol-oxidants like peroxynitrite (500 µM) and hydrogen peroxide (10 mM; Fig.

5A). Interestingly, also GTN applied *in vitro* and *in vivo* inhibited ALDH-2 esterase activity to a comparable extent (Fig. 5A). In contrast, PETN, ISDN and ISMN did not significantly affect esterase activity (Fig. 5A). The mixed disulfide-forming reagent DTNB caused complete inhibition of total esterase activity, whereas the ALDH inhibitors benomyl (1 μM) and chloral hydrate (10 μM) inhibited only the oxidant-sensitive esterase activity (Fig. 5B), supporting the conclusion that ALDH-2 esterase activity accounts for the oxidant-sensitive esterase. PETN and its metabolites did not alter esterase activity (Fig. 5B).

Effect of ALDH-2 Inhibition on GTN Biotransformation in RAW 264.7 Macrophages. According to a previous study (Chen et al., 2002), RAW 264.7 macrophages contain appreciable amounts of ALDH-2. In order to confirm that ALDH-2 accounts for GTN biotransformation, we measured the formation of 1,2- and 1,3-GDN from GTN by these macrophages by means of GC-MS analysis. As shown in Fig. 6, the ratio of 1,2-GDN/1,3-GDN increased 4-fold with decreasing GTN concentrations (from 10 to 1 μM). This finding indicates, that the pattern of biotransformation is highly sensitive to the GTN concentration used, in accordance with a substrate-based inactivation of ALDH-2 by excess GTN. Experiments with the ALDH-2 inhibitors benomyl and daidzin (50 μM each) were performed in the presence of 1 μM GTN. Both inhibitors significantly decreased the 1,2-GDN/1,3-GDN ratio to approximately 1 (Fig. 6).

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Discussion

Previously, we (Sydow et al., 2004) and others (Chen et al., 2002) showed that ALDH-2 significantly biotransforms GTN *in vitro* and *in vivo* and that inhibition of this enzyme markedly decreased the vasodilator potency of GTN. In the present study we sought to determine whether ALDH-2 is also required for the bio-activation of other commonly used organic nitrates.

We now show that ALDH-2 inhibition by benomyl decreases the vasodilator potency of PETN and PETriN in rat aorta, but not that of ISDN, PEDN, ISMN, and PEMN (Fig. 1, Tabel 1). These findings were corroborated by our observation that only GTN and PETN-induced activation of both cGMP-stimulated protein kinase (cGK-I) (assessed by P-VASP formation) and sGC (assessed by cGMP formation) and were decreased by ALDH-2 inhibitors, whereas activation of these enzymes by the other organic nitrates were not affected by ALDH-2 inhibition (Figs. 2A, B). Moreover, we show that GTN, PETN, and PETriN exhibit the highest potency to induce mitochondrial ROS formation (Figs. 3A,4A) and to inhibit ALDH-2 dehydrogenase activity (Figs. 3B,4B). In general, the vasodilator potency, the dependence on ALDH-2-catalyzed bioactivation, the stimulation of mitochondrial ROS and the inhibition of ALDH-2 dehydrogenase activity increase with the number of $–ONO_2$ groups present in the organic nitrate molecule. A remarkable deviation from this general behaviour was the finding that PETN and PETriN did not inhibit ALDH-2 esterase activity, whereas GTN did (Fig. 5).

The Requirement of ALDH-2 for cGMP-dependent Vasodilator Activity of Organic Nitrates is Confined to Nitrates with High Vasodilator Potency. The vasodilator potency of the different organic nitrates tested here roughly correlated with the number of nitrate groups present in the molecule. It decreased in the order PETN > GTN > PETriN > ISDN > PEDN > ISMN > PEMN (Tabel 1). This order is in accordance with previous studies in the

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same and in other vessel types (Parker et al., 1975). ALDH-2 inhibition by benomyl specifically affected the vasodilator responses to GTN, PETN and PETriN, but not to mono and dinitrates (Tabel 1), indicating a specific interference with bio-activation of higher-nitrated organic nitrates. Benomyl did not influence ACh-induced relaxations, i.e. it did not interfere with the endothelium-derived NO signalling pathway. In nitrate-tolerant vessels benomyl was unable to further decrease vasodilator responses to GTN (Tabel 1)(Sydow et al., 2004), suggesting that both benomyl and nitrate tolerance interfere with the same bio-activation step specifically required for GTN/PETN/PETriN. The results obtained by vasorelaxation experiments were corroborated by the measurement of cGK-I and sGC activation. GTN- and PETN-dependent activation of cGK-I and sGC, which resulted in increased levels of P-VASP and cGMP, respectively, could be suppressed to control levels by benomyl (Fig. 2). In contrast, VASP phosphorylation and cGMP formation induced by all other organic nitrates showed no significant alteration upon addition of benomyl. Moreover, neither basal, nor ACh- or SNP-stimulated P-VASP levels were affected by benomyl, thereby excluding an unspecific impairment of NO/cGMP-signalling by this compound.

Two independent pathways accounting for vasodilator activity of organic nitrates. In many vascular beds, the vasodilator-response curve to GTN consists of a high- and a low-potency component (Ahlner et al., 1986), suggesting that bio-activation may not be uniform for a given nitrate and between different nitrates. Superoxide generating sGC inhibitors like LY83583 and methylene blue (Malta, 1989), as well as the G_i-inhibitory pertussis toxin (Ahlner et al., 1988) primarily abolish the high potency component, which can be overcome by higher concentrations of GTN. In contrast, the much less potent ISDN and ISMN exhibit a monophasic dose-response curve. Our previous (Sydow et al., 2004) and present findings showing that the maximal relaxation to GTN, PETN and PETriN is not affected by benomyl,

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supports the hypothesis of at least two pathways of bioactivation for these organic nitrates. The high potency pathway may now be identified as the ALDH-2-dependent bioactivation step.

Organic Nitrate-induced Oxidative Stress and Inhibition of ALDH-2 Dehydrogenase and Esterase Activity. Oxidative stress plays an important role in the development of nitrate tolerance and cross-tolerance (Hink et al., 2003). Superoxide and peroxynitrite were identified as the reactive species formed in tolerant vessels, and peroxynitrite scavengers such as uric acid and ebselen normalized endothelial dysfunction in tolerant vessels. We here present evidence that at least a part of the organic nitrate induced oxidative stress may originate from mitochondrial nitrate metabolism. Using L-012-dependent chemiluminescence (Daiber, 2004) we detected peroxynitrite and superoxide in isolated rat heart mitochondria upon *in vitro* administration of organic nitrates. Interestingly, the potency of organic nitrates to generate mitochondrial superoxide and peroxynitrite, as well as to inhibit ALDH-2 dehydrogenase activity, increased with their vasodilator potency. This is not contradictory, since ALDH-2 contains three cysteine residues in the catalytic center (Fig. 7), rendering the dehydrogenase activity highly sensitive towards oxidative inactivation (Senior and Tsai, 1990; Tsai and Senior, 1991). In addition to its dehydrogenase activity ALDH-2 also exhibits esterase activity (Scheme 1), which has been proposed to be essential for the bioactivation of GTN (Chen et al., 2002). This activity also involves oxidation-sensitive cysteine residues, different from the dehydrogenase activity (Tsai and Senior, 1991). As depicted in Scheme 2, the bioactivation of GTN by ALDH-2 could lead to oxidative inactivation of the enzyme, presumably by formation of disulfide, and/or sulfenic and sulfinic acid. Indeed, 30-40 % of total (DTNB-sensitive) mitochondrial esterase activity was sensitive to inhibition by oxidants such as peroxynitrite and hydrogen peroxide, and was inhibited to a similar extent by benomyl and chloralhydrate. Therefore, this part of ALDH-2 esterase activity is expected to be

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essential for bioactivation of GTN and PETN, and to be impaired by nitrate tolerance. Indeed, we could demonstrate that GTN *in vivo* and *in vitro* treatment inhibited the mitochondrial esterase activity to a similar extent. In contrast, all other nitrates did not affect esterase activity. In accordance with the oxidative stress concept, previous reports have shown that antioxidants such as ascorbic acid positively influence the development of nitrate tolerance (Daniel and Nawarskas, 2000; Schwemmer and Bassenge, 2003) and that oxidatively inactivated ALDH-2 activity can be restored by addition of DTT (Chen et al., 2002; Sydow et al., 2004).

Another characteristic feature of the high potency bioactivation pathway is the rapid desensitization (tachyphylaxis) by GTN concentrations > 1µM (Chen et al., 2002). At the molecular level, tachyphylaxis can now be related to auto-inhibition of GTN bioactivation by mitochondrial ALDH-2, since in RAW 264.7 macrophages GTN concentrations > 1 µM decreased the formation of 1,2-GDN, which is associated with vasorelaxation and sGC activation (Brien et al., 1988). Furthermore, the formation of the 1,2-GDN metabolite was inhibited by the ALDH inhibitors benomyl and daidzin (Fig. 6).

Comparison of GTN *vs*. PETN and PETriN. Though the highly potent nitrates share a common bioactivation pathway, we noted some remarkable differences between GTN and PETN/PETriN. Firstly, PETN and PETriN at similar concentrations elicited significantly less mitochondrial superoxide formation as compared to GTN. Secondly, PETN and PETriN caused less inactivation of ALDH-2 dehydrogenase activity as compared to GTN. Moreover, both PETN and PETriN, in contrast to GTN, did not inhibit mitochondrial esterase activity. These results may explain the previous findings that PETN lacks not only *in vivo* tolerance (Mullenheim et al., 2001; Schwemmer and Bassenge, 2003), but also as shown here, *in vitro* tolerance. In contrast, PETN bioactivity was reduced by cross-tolerance after treatment with GTN *in vivo* and *in vitro*, indicating that both nitrates share a common bioactivation pathway.

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In addition, preliminary results of an *in vivo* PETN study demonstrate that high dose PETN *in vivo* treatment neither causes significant tolerance versus PETN-dependent relaxation nor significant cross-tolerance versus GTN-dependent relaxation (work in progress, data not shown).

Mechanism of sGC activation by organic nitrates. The present ALDH-2 concept does not provide the molecular link between mitochondrial nitrite formation and sGC activation leading to vasorelaxation (see Scheme 2). Nitrite may activate sGC via intermediate formation of HNO_2 and disproportionation into NO_2 and NO. However, due to the low pK of HNO_2 this requires at least 1000-fold higher concentrations of nitrite at physiological intracellular pH than provided by vasorelaxing concentrations of GTN, or a pH around 4. Indeed, we could not detect release of NO from isolated blood vessels during GTN (< 1 μM)-induced relaxation (Kleschyov et al., 2003). Therefore, free diffusible NO may not account for sGC activation by low concentrations of GTN. Nevertheless, we may not completely rule out the possibility that our NO EPR detection approach fails to detect minute amounts of NO generated from GTN and PETN within discrete intracellular compartments (e.g. mitochondria). However, even with this possibility admitted, our hypothesis of the existence of two different bio-activation mechanisms accounting for organic nitrates with high and low vasodilator potency is not disproved. Moreover, our observations result in another attractive hypothesis: The vasodilator potency of organic nitrates correlated with their ability to induce mitochondrial reactive oxygen and nitrogen species formation (such as peroxynitrite). Peroxynitrite, at low steady state concentrations, has been reported to cause vasodilation (Villa et al., 1994). This implies that GTN- and PETN-dependent relaxation at concentrations < 1 μM may be induced by mitochondrial peroxynitrite formation.

In contrast, sGC activation by higher concentrations of nitrates appears to be mediated by free NO as we could detect the GTN- and ISDN- derived NO at concentrations exceeding

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10 µM. This ALDH-2-independent pathway is apparently shared by the other low potency organic nitrates. Consistently, benomyl no longer inhibits vasorelaxation at these high concentrations of GTN.

In summary, we provide further evidence for the hypothesis that at least two different pathways exist for bioactivation of organic nitrates (Ahlner et al., 1986; Kleschyov et al., 2003): The high affinity pathway metabolizes organic nitrates with high vasorelaxant potency (GTN, PETN, PETriN), depends on ALDH-2 esterase activity, is only operative in the sub-micromolar concentration range; it does not generate measurable quantities of free nitric oxide, and is subject to tachyphylaxis and tolerance by either long-lasting *in vivo* exposure to low concentrations of these organic nitrates, or short term *in vitro* exposure to concentrations exceeding 1 µM GTN. The low affinity pathway is operative at higher concentrations of the highly potent organic nitrates and for the organic nitrates with lower vasorelaxant potency (ISDN, ISMN, PEDN, PEMN). This pathway is independent of ALDH-2, yields measurable quantities of free NO and is less prone to tolerance development.

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Footnotes

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Legends to Figures

Fig. 1. Effect of ALDH-2 inhibition by benomyl on vasodilator activity of various organic nitrates in the rat aorta. (**A**) Dose-response curves of PETN (circles), GTN (triangles), ISDN (squares) and ISMN (diamonds) were recorded in the presence (open symbols) or absence (filled symbols) of benomyl (10 µM). The data is the mean ± SEM of 6-12 independent experiments. (**B**) Dose-response of PETN (circles), ACh (inverted triangles), PETriN (squares), PEDN (triangles) and PEMN (diamonds) in the presence (open symbols) or absence (filled symbols) of benomyl (10 µM). The data is the mean ± SEM of 4-10 independent experiments. (**C**) Dose-response of PETN (circles), PETN after *in vitro* incubation with PETN (100 µM) for 30 min at 37 °C (triangles), PETN after *in vitro* incubation with GTN (100 µM) for 30 min at 37 °C (squares) and PETN after *in vivo* treatment with GTN for 3 days (inverted triangles). The data is the mean ± SEM of 4-10 independent experiments. For statistical analysis see Table 1.

Fig. 2. Effects of various organic nitrates on NO/sGC-downstream signalling in the presence and absence of the ALDH-2 inhibitor benomyl. (**A**) The activity of cGMP-dependent kinase (cGK-I) in isolated rat aortic rings was measured *ex vivo* by determining the P-VASP levels upon stimulation with different organic nitrates (PETN, GTN, ISDN and ISMN) in the absence (black) or presence (grey) of the ALDH inhibitor benomyl (10 µM). For comparison, this ratio was determined under non-stimulated (basal) conditions or in response to the endothelium-dependent vasodilator acetylcholine (ACh) and the endothelium independent nitrovasodilator sodium nitroprusside (SNP) in the presence or absence of benomyl. The data is the mean ± SEM of 3-9 independent experiments. The upper part of the figure shows a typical blot for the determination of VASP phosphorylation. (**B**) Accumulation of cGMP in isolated rat aortic rings was measured ex vivo in response to GTN (0.1 µM), ISDN (100 µM)

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or PETN (0.05 μM) in the absence (black) or presence (grey) of benomyl (10 μM). Each well contained 0.1 μg protein from aortic homogenates. The data is the mean ± SEM of 3-8 independent experiments. * indicates significance versus control and # significance versus sample without benomyl treatment.

Fig. 3. Formation of mitochondrial reactive oxygen species (ROS) and inhibition of ALDH-2 dehydrogenase activity in response to different organic nitrates. (**A**) The formation of mitochondrial ROS was detected using L-012 (100 μM)-derived chemiluminescence in mitochondria isolated from rat hearts. Mitochondrial suspensions (0.2 mg/ml final protein) were treated with different organic nitrates (50-5000 μM). The measurements were initiated by adding succinate (4 mM). The data is the mean ± SEM of 3-5 independent experiments. (**B**) The inhibition of ALDH-2 was determined by HPLC-based measurements of the conversion of benzaldehyde to benzoic acid. Suspensions of rat heart mitochondria (1 mg/ml final protein) were treated with different nitrates (50-5000 μM). The data is the mean ± SEM of 3-6 independent experiments. * indicates significance versus control.

Fig. 4. Formation of mitochondrial reactive oxygen species (ROS) and inhibition of ALDH-2 dehydrogenase activity in response to PETN and its metabolites. (**A**) The formation of mitochondrial ROS was detected using L-012 (100 μM)-derived chemiluminescence in mitochondria isolated from rat hearts. Mitochondrial suspensions (0.2 mg/ml final protein) were treated with PETN or its metabolites PETriN, PEDN and PEMN (500 μM each). The measurements were initiated by adding succinate (4 mM). The data is the mean ± SEM of 7-10 independent experiments. (**B**) The inhibition of ALDH-2 was determined by HPLC-based measurements of the conversion of benzaldehyde to benzoic acid. Suspensions of rat heart mitochondria (1 mg/ml final protein) were treated with PETN or its metabolites (each 500

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μM). The data is the mean ± SEM of 5-11 independent experiments. * indicates significance versus control.

Fig. 5. Effects of organic nitrates, ALDH inhibitors and reactive oxygen species (ROS) on ALDH-2 esterase activity. (**A**) Activity of ALDH-2 was determined by HPLC-based measurements of the conversion of methylbenzoate to benzoic acid and methanol. Suspensions of rat heart mitochondria (1 mg/ml final protein) were pretreated with authentic peroxynitrite (PN), GTN, PETN, ISDN, ISMN (500 μM each) or hydrogen peroxide (H_2O_2, 10 mM). For comparison, mitochondria from *in vivo* GTN-treated, nitrate tolerant rats were used. Line number 1 indicates the level of total mitochondrial esterase activity in a control sample. The space between line number 1 and 2 indicates the part of total mitochondrial esterase activity that can be inhibited by ROS. The data is the mean ± SEM of 3-4 independent experiments. (**B**) Suspensions of rat heart mitochondria (0.5 mg/ml final protein) treated with Ellman's reagent (DTNB, 5 mM), PETN, PETriN, PEDN, PEMN (500 μM each) or the ALDH inhibitors benomyl (Beno, 1 μM) as well as chloralhydrate (Chloral, 10 μM). Line number 1 indicates the level of total mitochondrial esterase activity in a control sample. The space between line number 1 and 2 indicates the part of total mitochondrial esterase activity which is sensitive to ALDH inhibitors. The data is the mean ± SEM of 3-5 independent experiments. * indicates significance versus control.

Fig. 6. Effect of ALDH-2 inhibition on GTN biotransformation (1,2-GDN/1,3-GDN ratio) in RAW 264.7 macrophages. Cells ($50x10^6$) were incubated with GTN (1-10 μM). The GTN metabolites were measured by GC-MS (see insert for a typical chromatogram of injected standards (10 μM each)). The biotransformation of 1 μM GTN was also determined in the presence of the ALDH-2 inhibitors benomyl (50 μM) or daidzin (50 μM). The data is the

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mean ± SEM of 3-6 independent experiments. * indicates significance versus GTN (10 µM)-treated sample and # indicates significance versus GTN (1 µM)-treated sample.

Fig. 7. Crystal structure of bovine mitochondrial ALDH. The structure was rendered from the protein database file 1A4Z.pdb using the freeware program PyMol Molecular Graphics System (version 0.93) from DeLano Scientific LLC. The structure shows one monomer of the active tetramer complex. The active site contains 3 cysteine thiol groups (Cys301-303). One of these thiol groups (most recently from Cys301) is in close proximity to the nicotinamide ring of the co-factor NAD^+ and probably participates in the catalytic hydride transfer from an aldehyde to the co-factor. Moreover, these three cysteines provide optimal conditions for an oxidation-based inactivation of the enzyme (e.g. by formation of disulfide).

Scheme 1. ALDH-2 possesses two different enzymatical activities. The dehydrogenase activity catalyzes the conversion of aldehydes to carbonic acids using NAD^+ as a co-factor. In the present study this activity was measured by HPLC-based analysis of the conversion of benzaldehyde to benzoic acid. The esterase activity catalyzes the hydrolysis of esters to the free acid and an alcohol without requirement of a co-factor. In the present study this activity was measured by HPLC-based analysis of the hydrolysis of methylbenzoate to benzoic acid and methanol.

Scheme 2. GTN biotransformation by ALDH-2. GTN bioactivation has been postulated to essentially involve the ALDH-2 esterase activity yielding 1,2-GDN and nitrite, which is further bioactivated to vasodilating species. This reaction is not a simple ester hydrolysis but also involves reduction of the $–ONO_2$ group to nitrite and alcohol. Although the reaction has reductive character, NAD^+ accelerates the catalytic cycle indicating that sterical aspects may

play a role. During the catalytic cycle the enzyme is inactivated as envisaged by the formation of a disulfide bridge. The activity can partially be restored by dithiothreitol (DTT) but not via administration of glutathione (GSH) or cysteine (Cys).

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Table 1. EC_{50} values for different organic nitrates and the endothelium-dependent vasodilator ACh in the presence and absence of the ALDH-2 inhibitor benomyl.

Condition	EC_{50} w/o Benomyl[a]	EC_{50} with Benomyl[a]
GTN	-7.66 ± 0.08 (n=19)	-6.56 ± 0.13*** (n=10)
GTN upon GTN *in vivo*[b]	-6.54 ± 0.25††† (n=10)	-6.27 ± 0.08 (n=4)
PETN	-8.07 ± 0.06 (n=10)	-7.16 ± 0.06*** (n=6)
PETN upon 100 µM PETN[c]	-7.57 ± 0.13$^{\#}$ (n=6)	n.d.
PETN upon 100 µM GTN[c]	-7.07 ± 0.09$^{\#\#\#}$ (n=5)	n.d.
PETN upon GTN *in vivo*[b]	-6.74 ± 0.05$^{\#\#\#}$ (n=4)	n.d.
PETriN	-6.63 ± 0.11 (n=6)	-5.76 ± 0.16* (n=6)
ISDN	-5.29 ± 0.04 (n=12)	-5.28 ± 0.23 (n=7)
PEDN	-4.61 ± 0.13 (n=6)	-4.66 ± 0.06 (n=6)
ISMN	-3.77 ± 0.04 (n=7)	-3.51 ± 0.14 (n=6)
PEMN	-2.97 ± 0.18 (n=4)	-3.10 ± 0.11 (n=4)
ACh	-7.46 ± 0.07 (n=55)	-7.47 ± 0.08 (n=4)

n.d. means not determined, * indicates significance versus sample without benomyl treatment, † indicates significance versus relaxation of control in response to GTN and $^{\#}$ indicates significance versus relaxation of control in response to PETN.

[a] The vessels were preincubated in the presence or absence of 10 µM benomyl for 30 min.

[b] Dose response curves were recorded with tolerant vessels from rats which were treated for 3 d with GTN (0.48 µmol/h/kg).

[c] Dose response curves were recorded with vessels which were treated *in vitro* with either 100 µM PETN or GTN for 30 min at 37 °C.

Figure 1

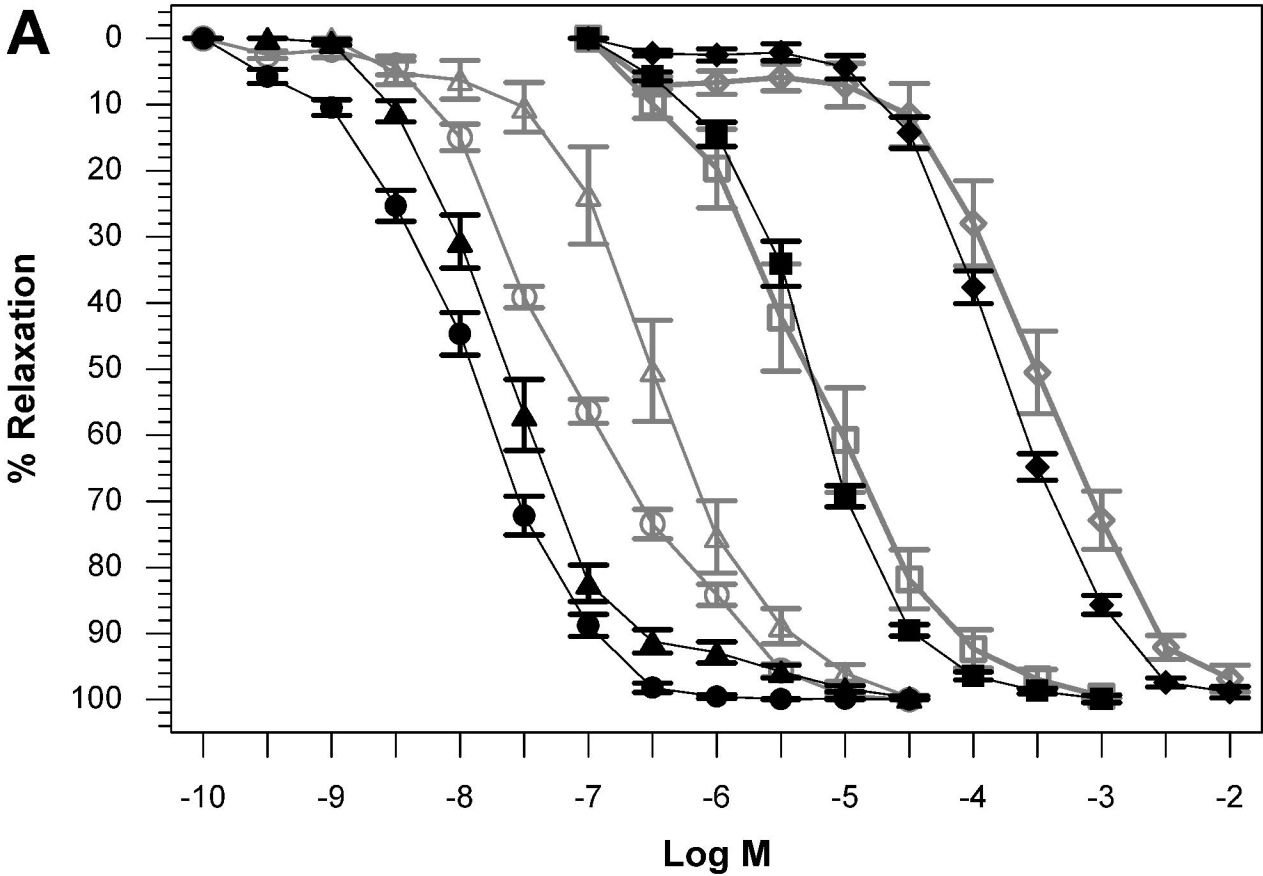


Figure 1

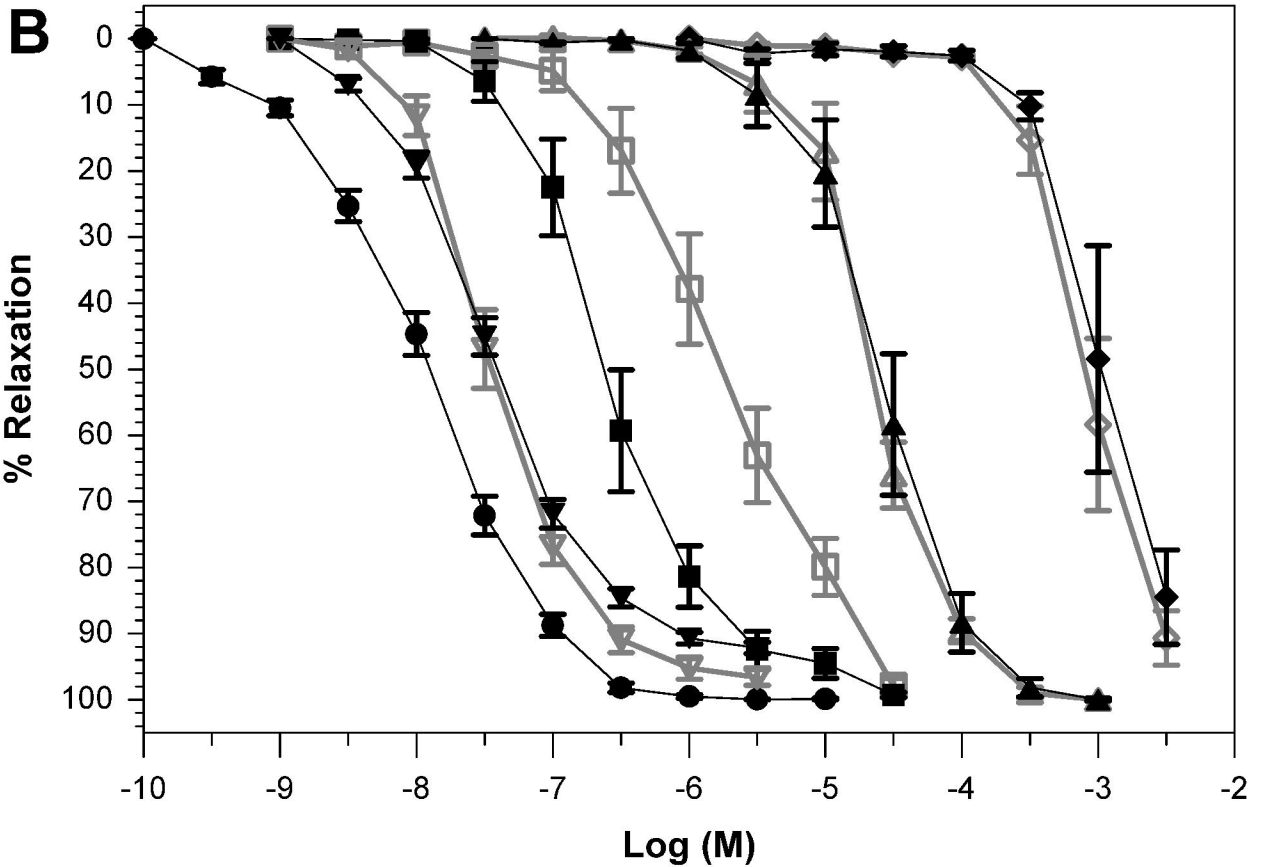


Figure 1

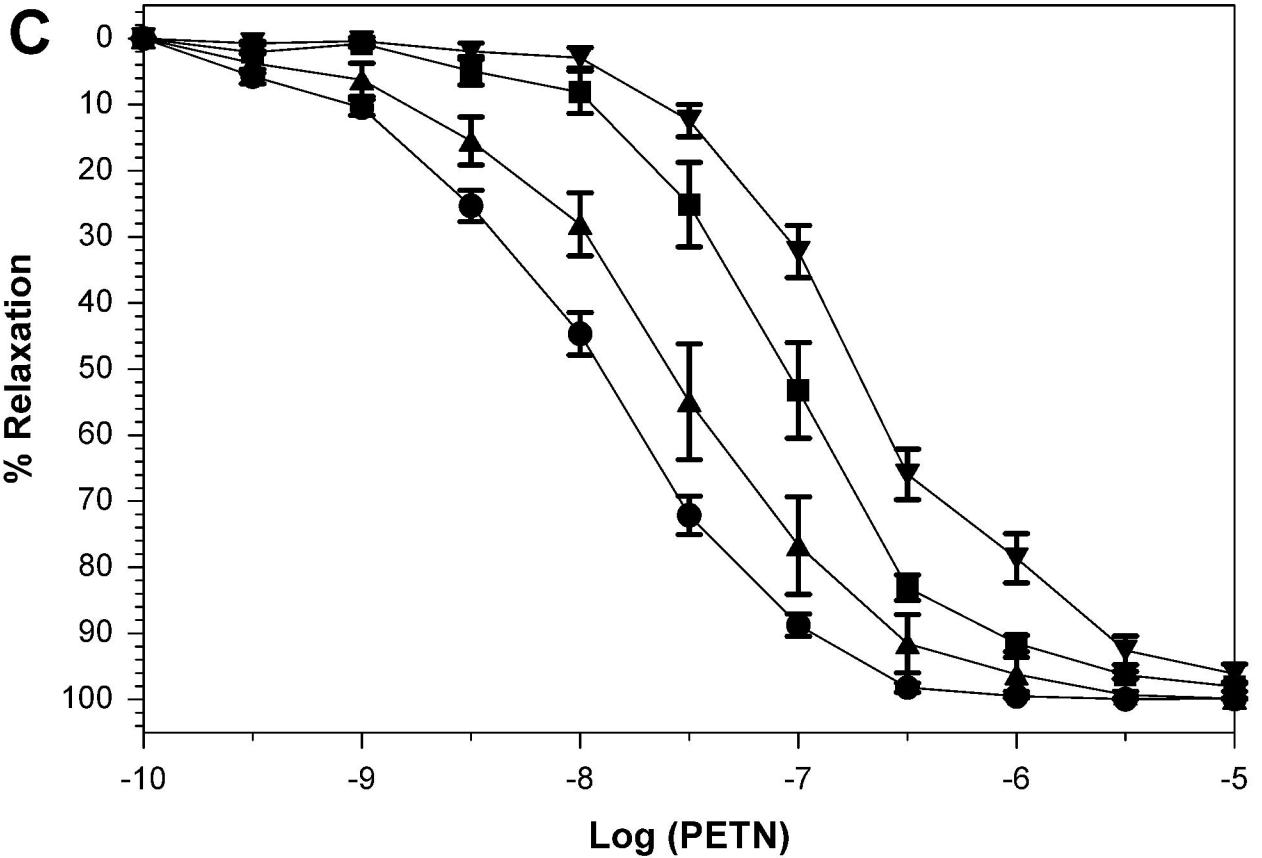


Figure 2

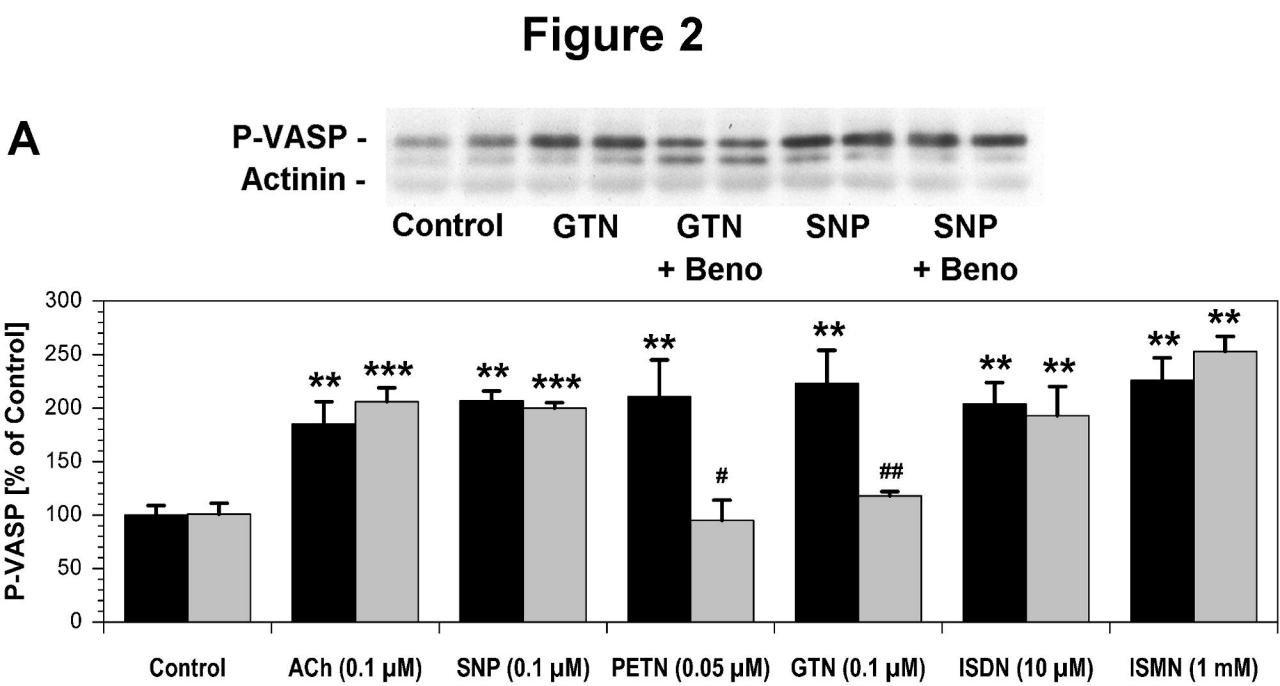

A
P-VASP -
Actinin -
Control
GTN
GTN + Beno
SNP
SNP + Beno
P-VASP [% of Control]
300
250
200
150
100
50
0
**
***
#
##
Control
ACh (0.1 µM)
SNP (0.1 µM)
PETN (0.05 µM)
GTN (0.1 µM)
ISDN (10 µM)
ISMN (1 mM)

Figure 2

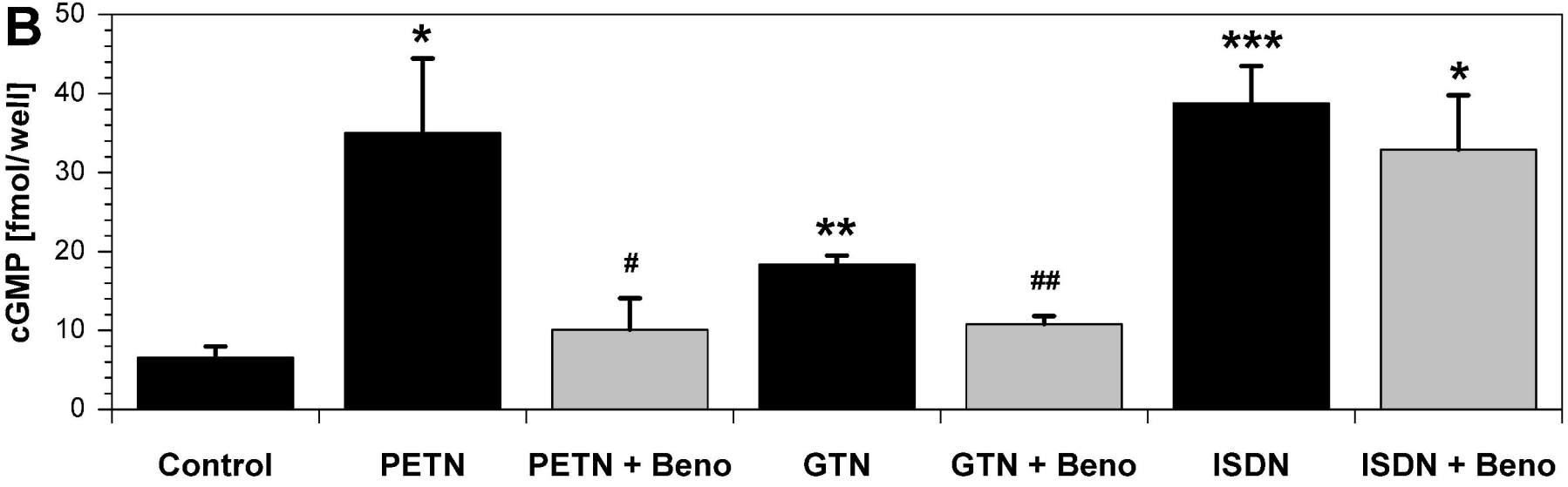

B
cGMP [fmol/well]
50
40
30
20
10
0
*
#
**
##
***
*
Control
PETN
PETN + Beno
GTN
GTN + Beno
ISDN
ISDN + Beno

Figure 3

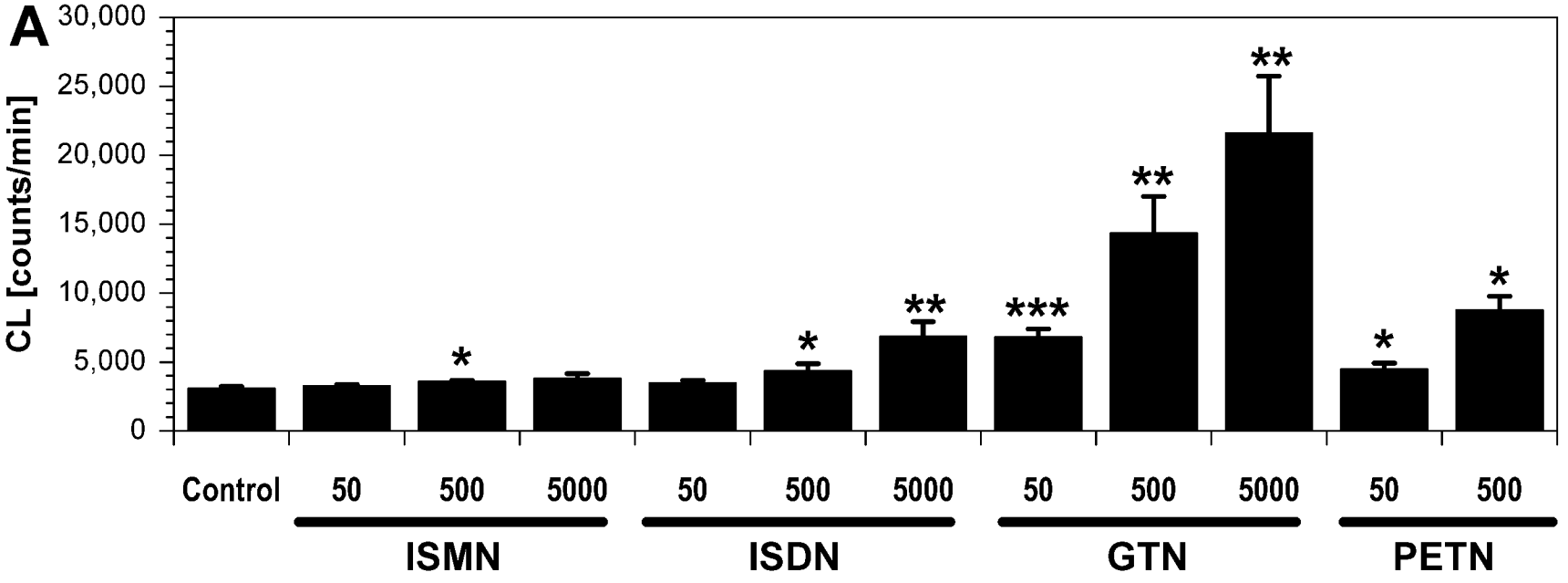

A
CL [counts/min]
30,000
25,000
20,000
15,000
10,000
5,000
0
Control
50
500
5000
50
500
5000
50
500
5000
50
500
ISMN
ISDN
GTN
PETN

Figure 3

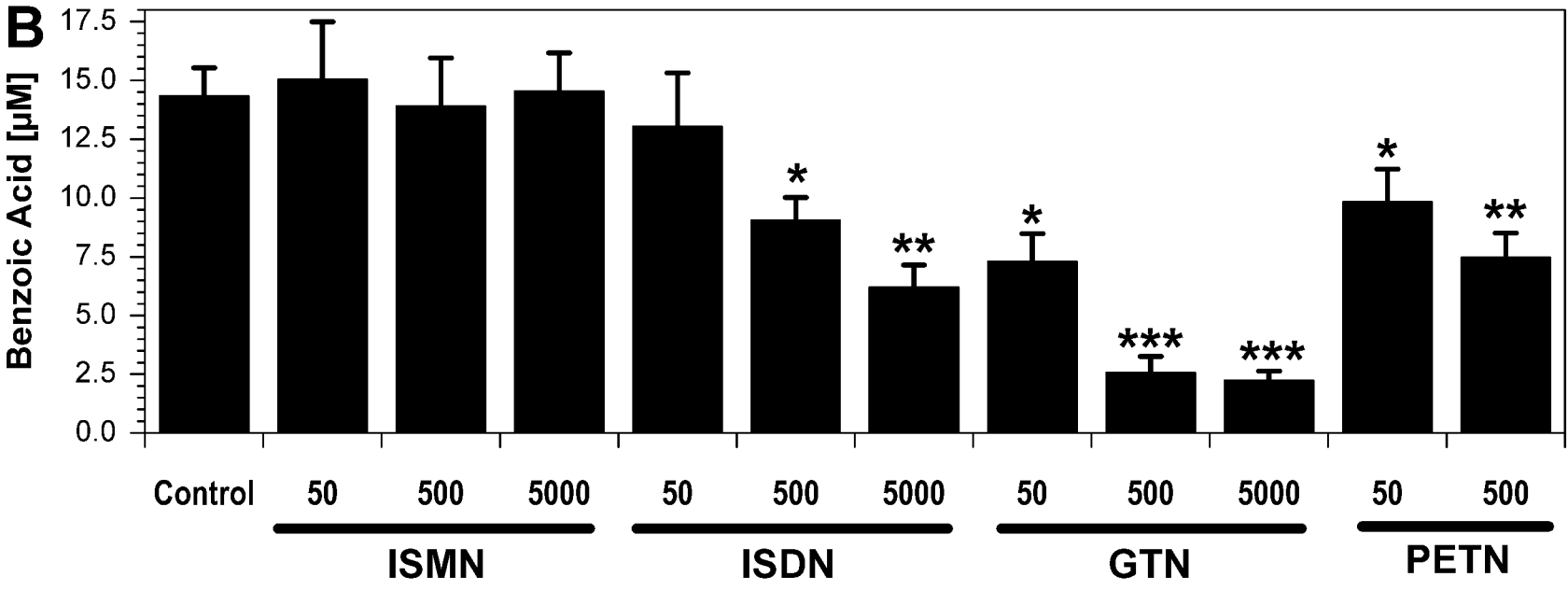

B
Benzoic Acid [μM]
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
*
**
*
***
***
*
**
Control
50
500
5000
50
500
5000
50
500
5000
50
500
ISMN
ISDN
GTN
PETN

Figure 4

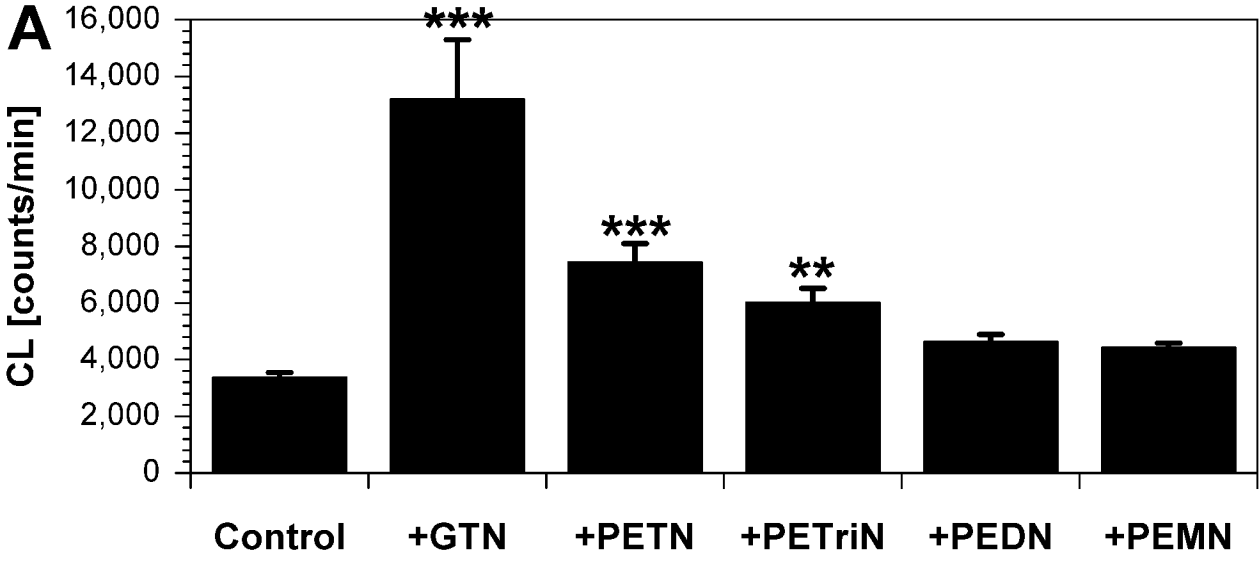

A
CL [counts/min]
16,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
***
***
**
Control
+GTN
+PETN
+PETriN
+PEDN
+PEMN

Figure 4

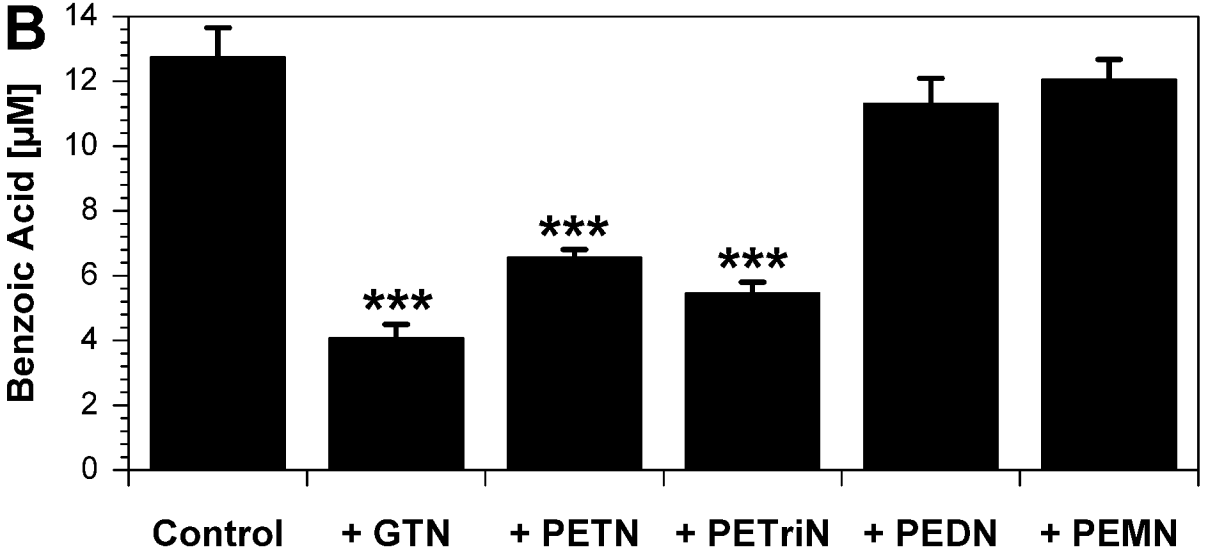

B
Benzoic Acid [µM]
14
12
10
8
6
4
2
0
***
***
***
Control
+ GTN
+ PETN
+ PETriN
+ PEDN
+ PEMN

Figure 5

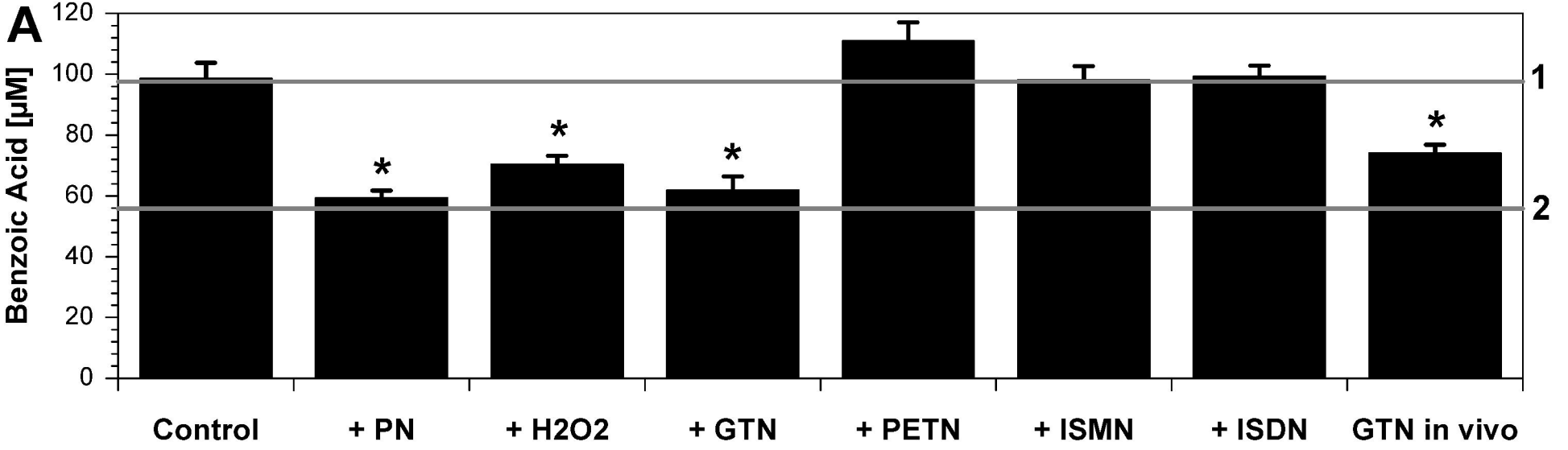

A
Benzoic Acid [µM]
120
100
80
60
40
20
0
1
2
*
*
*
*
Control
+ PN
+ H2O2
+ GTN
+ PETN
+ ISMN
+ ISDN
GTN in vivo

Figure 5

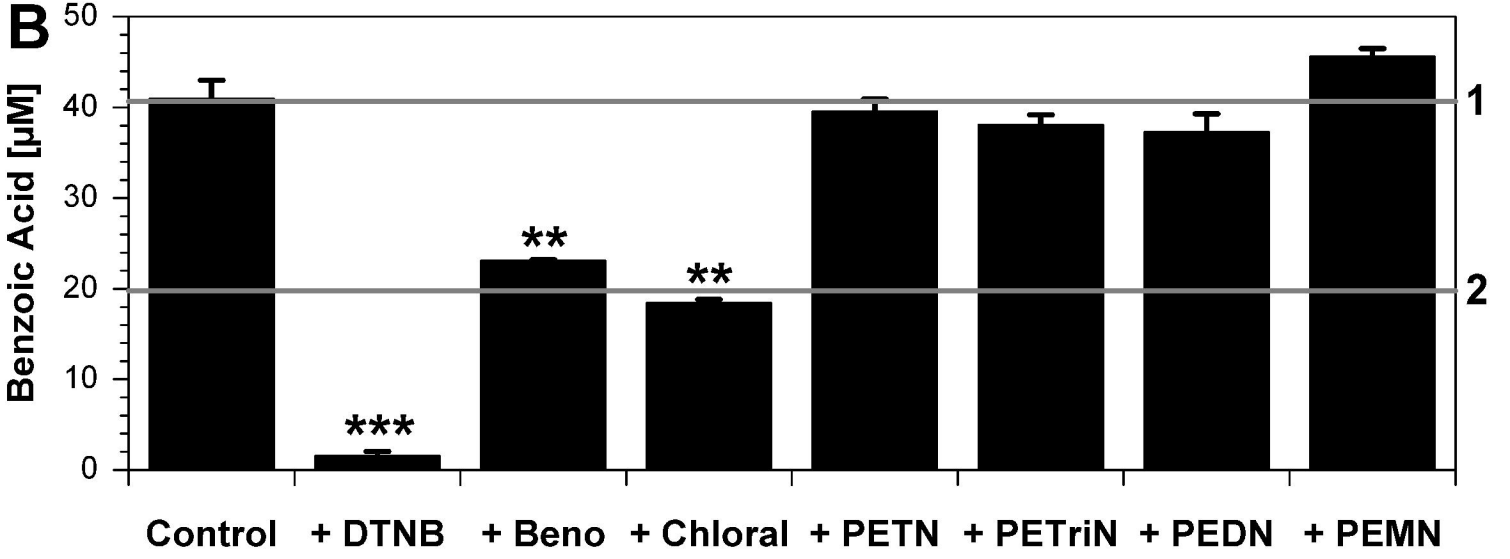

B
Benzoic Acid [µM]
50
40
30
20
10
0
***
**
**
1
2
Control
+ DTNB
+ Beno
+ Chloral
+ PETN
+ PETriN
+ PEDN
+ PEMN

Figure 6

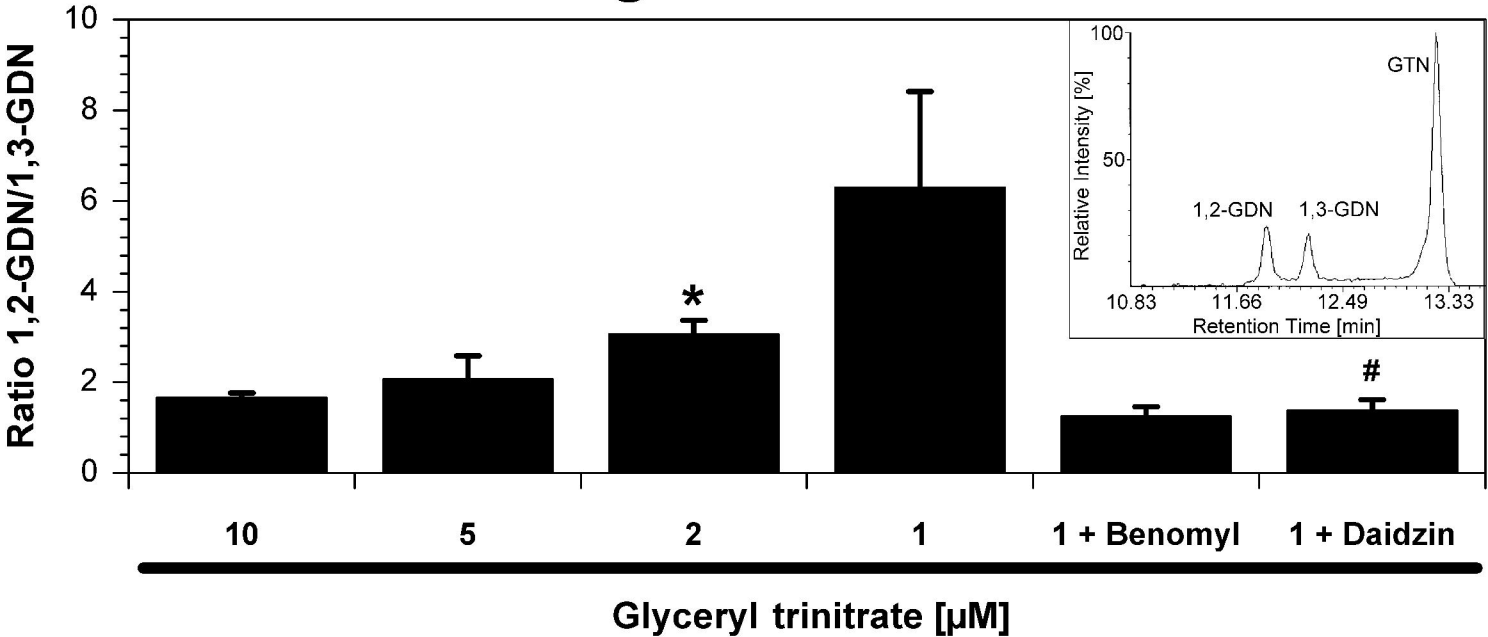
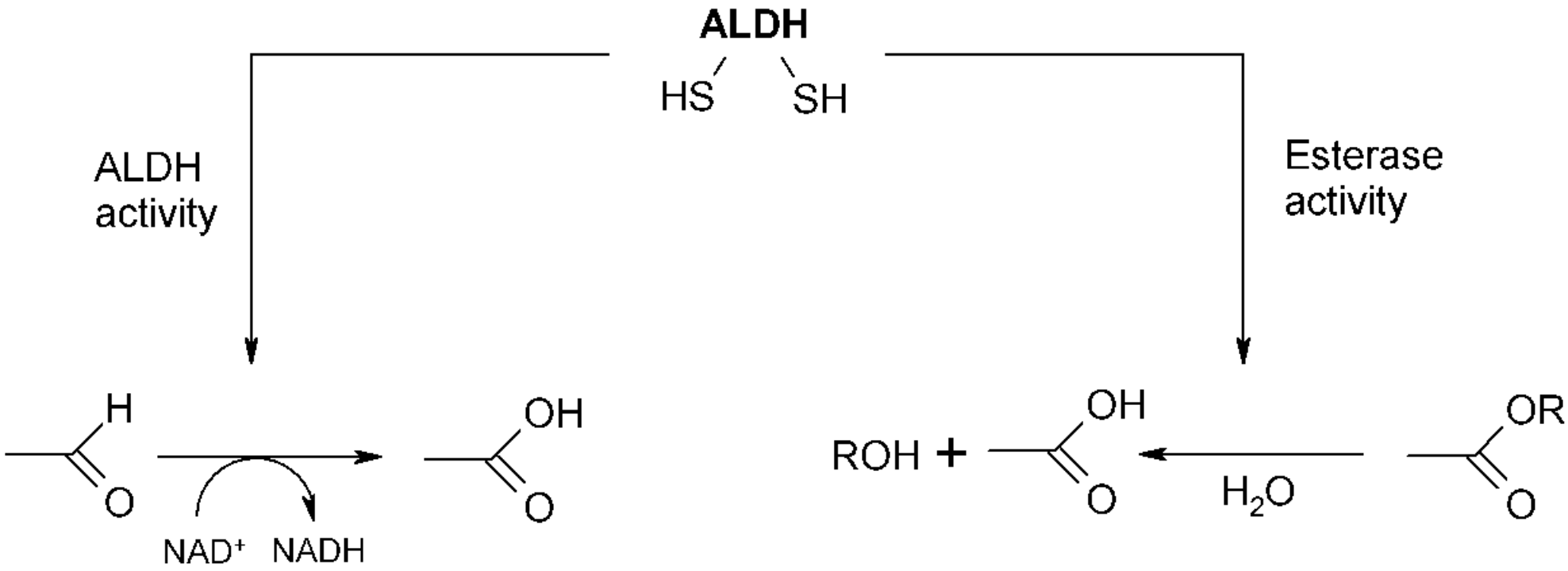
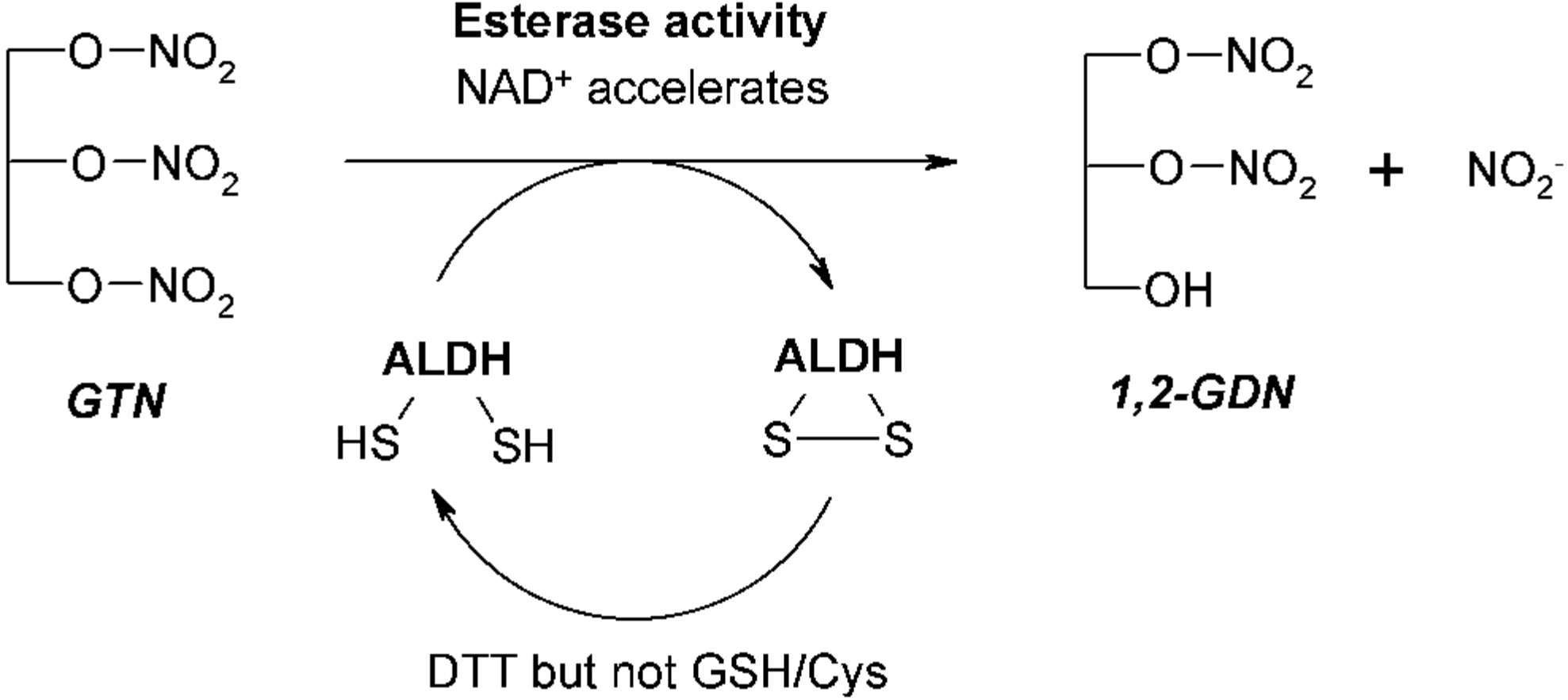

Ratio 1,2-GDN/1,3-GDN
10
8
6
4
2
0
*
#
10
5
2
1
1 + Benomyl
1 + Daidzin
Glyceryl trinitrate [µM]
Relative Intensity [%]
100
50
GTN
1,2-GDN
1,3-GDN
10.83
11.66
12.49
13.33
Retention Time [min]

Figure 7

Scheme 1


ALDH
HS
SH
ALDH
activity
Esterase
activity
H
O
NAD+
NADH
OH
O
ROH +
OH
O
H2O
OR
O

Scheme 2


Esterase activity
NAD+ accelerates
O—NO2
O—NO2
O—NO2
GTN
ALDH
HS
SH
ALDH
S—S
O—NO2
O—NO2
OH
+
NO2-
1,2-GDN
DTT but not GSH/Cys