Supplementary information

Wenzel et al - Organic nitrates bioactivation in ALDH-2$^{-/-}$ mice

Isometric tension studies in isolated mouse aortic segments. Concentration-relaxation-curve in respone PETN and PEDN with or without preincubation with benomyl (10µM), to isosorbide mononitrate (ISMN) and spermine NONOate was performed with $PGF_{2\alpha}$-preconstricted mouse aortic rings. ISMN stock was 5 M in DMSO and spermine NONOate stock was 100 mM in 0.1 M NaOH. The PETN and PEDN data without preincubation with benomyl are the same as depicted in figure 2.

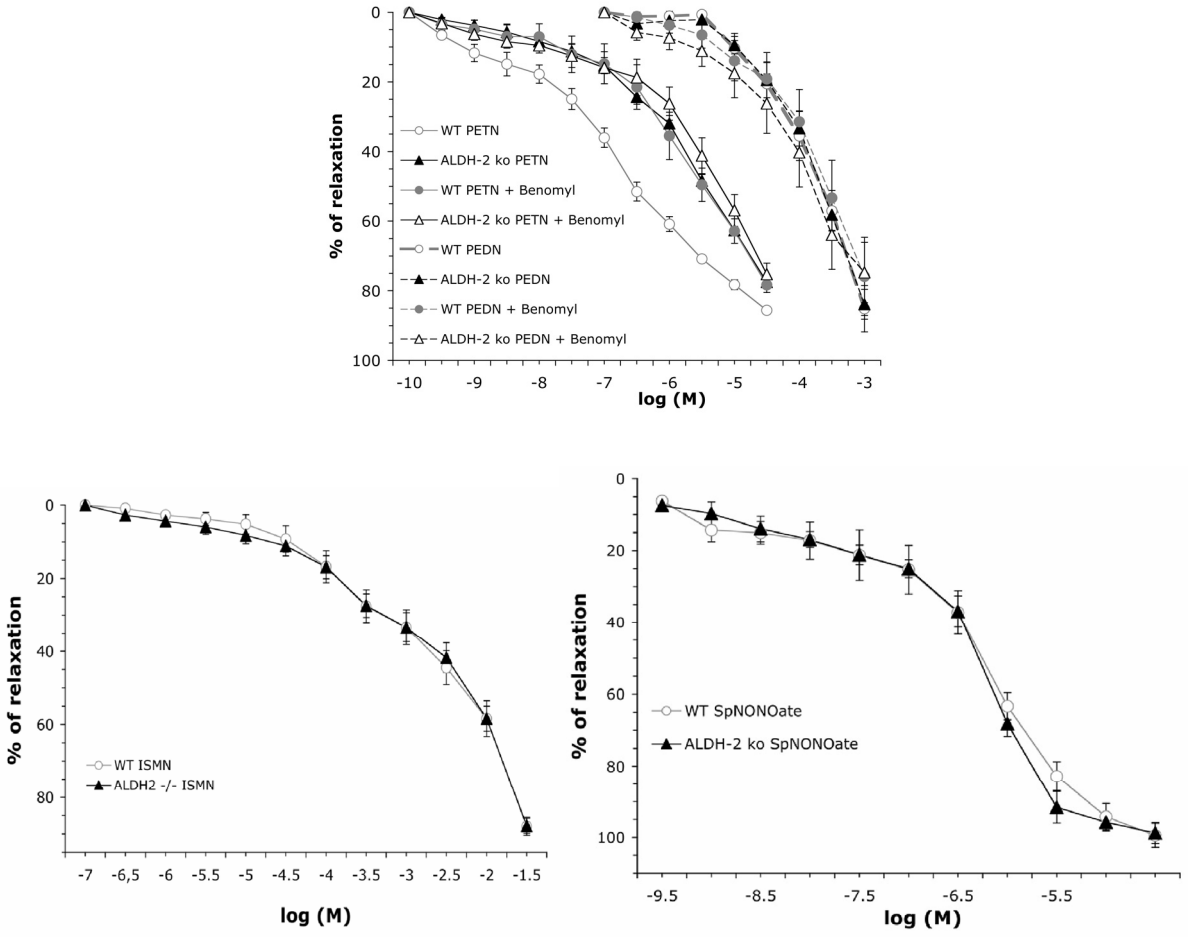

Figure 1S. Concentration-relaxation-curves in response various vasodilators. Top: PETN +/- benomyl vs. PEDN (broken line) +/- benomyl; Bottom: ISMN (left) and spermine NONOate (right) Symbols: Open grey circles, wt; filled grey circles, wt + benomyl; filled black triangles, ALDH-2$^{-/-}$; open black triangles, ALDH-2$^{-/-}$ + benomyl.

Table 1S. Vasodilator potency and efficacy of PETN and PEDN following benomyl preincubation, ISMN and spermine NONOate in isolated aortic rings from WT- vs. ALDH-2$^{-/-}$ mice.

	PETN + 10μM Benomyl	PEDN + 10μM Benomyl	ISMN	spermine NONOate		PETN + 10μM Benomyl	PEDN + 10μM Benomyl	ISMN	spermine NONOate
WT [i]	6.12±0.23[a,b] (n=8)	3.98±0.18[c] (n=7)	2.61±0.19 (n=8)	6.26±0.07 (n=4)	WT [j]	77.2±2.32[a,b] (n=8)	76.8±0.18[c] (n=7)	87.96±2.42 (n=8)	100.0±0.0 (n=4)
ALDH-2$^{-/-}$ [i]	5.77±0.27[a,b] (n=6)	4.25±0.17[c] (n=6)	2.54±0.15[z] (n=7)	6.30±0.05[z] (n=4)	ALDH-2$^{-/-}$ [j]	75.3±3.21[a,b] (n=6)	78.2±8.51[c] (n=6)	87.78±2.07[z] (n=7)	98.1±1.88[z] (n=4)

[a] $p<0.05$ vs WT PETN; [b] n.s. vs ALDH-2$^{-/-}$ PETN; [c] n.s. vs WT PEDN;
[z] n.s. vs WT ISMN or WT spermineNONOate, respectively. (n) indicates number of experiments.
[i] $-\log(EC_{50})$ [M] of concentration-relaxation curves ± SEM
[j] Maximal relaxation (% relaxation) of concentration-relaxation-curves ± SEM

Measurement of ALDH-2 activity in isolated mouse heart mitochondria using an optimized substrate with high specificity for the mitochondrial ALDH isoform. Hearts from wild type and ALDH-2$^{-/-}$ mice 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^{-1}) was kept on ice and diluted to approximately 1 mg ml^{-1} protein in 0.25 ml of PBS and preincubated for 10 min at room temperature in the presence or absence of the ALDH inhibitor daidzin (200 μM). For measurement of ALDH-2 dehydrogenase activity, 2-hydroxy-3-nitro-benzaldehyde (100 μM) 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 in a Microcon YM-10 filter device from Millipore (Bedford, USA). 100 µl of each sample were subjected to HPLC analysis. The system consisted of a Gynkotek pump and detector and a C_{18}-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^{-1}, detected at 360 nm, and quantified using internal and external standards (2-hydroxy-3-nitro-benzoic acid and 2-hydroxy-3-nitro-benzaldehyde). The typical retention times were 3.4 and 5.6 min, respectively.

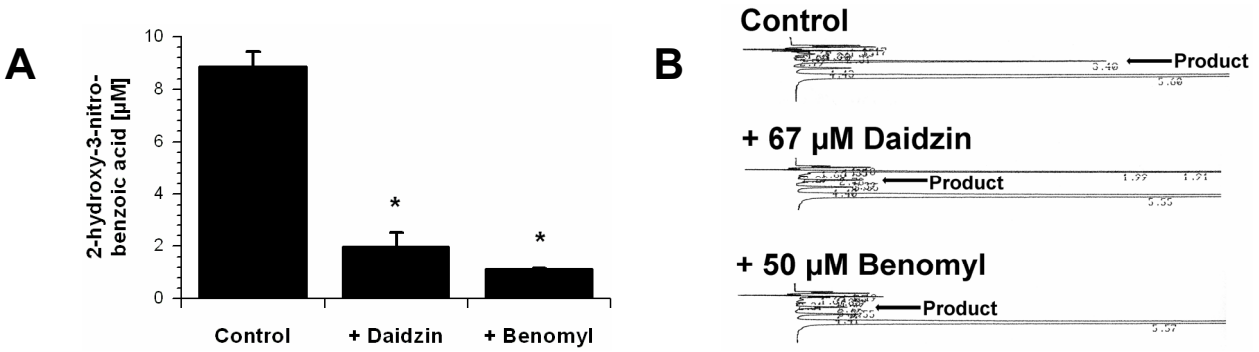

Figure 2S. (**A**) The high specificity of the modified substrate (2-hydroxy-3-nitro-benzaldehyde) for ALDH-2 activity could be demonstrated by specific inhibition of the conversion to the benzoic acid product by daidzin, a highly specific inhibitor of the mitochondrial isoform. The medium of the smooth muscle cells (10^6 cells per well using 4 well plates) was replaced by PBS (5 ml) and cells were incubated with the vehicle (0.1 % DMSO), 67 µM daidzin or 50 µM benomyl followed by 35 min incubation with 2-hydroxy-3-nitro-benzaldehyde at 37 °C. An aliquot of 100 µl of the supernatant was subjected to HPLC analysis. Since also cytosolic isoforms such as ALDH-1 are active in intact cells, the data clearly demonstrate that most of the conversion of the modified substrate is due to ALDH-2 activity which can be inhibited by daidzin. (**B**) Representative chromatograms for three independent experiments.

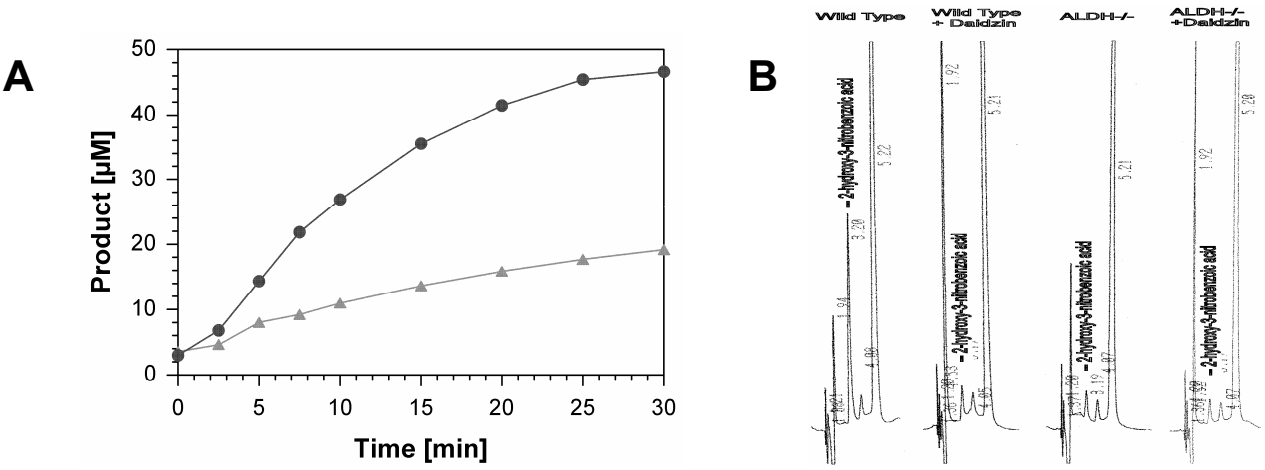

Figure 3S. (**A**) The high specificity of the modified substrate (2-hydroxy-3-nitro-benzaldehyde) for ALDH-2 activity could also be demonstrated by kinetic measurements of the conversion to the benzoic acid product in isolated mitochondria where the ALDH-2 is the dominant isoform. The isolated mitochondria (1 mg ml^{-1}) were incubated with 50 µM benzaldehyde (triangles) or 2-hydroxy-3-nitro-benzaldehyde (circles) and aliquots of 100 µl were subjected to HPLC analysis after the indicated time intervals. The modified substrate showed a much higher rate of conversion which was almost completely inhibited by daidzin. (**B**) Representative chromatograms for the data presented in figure 3B in the manuscript.

Quantification of PETN and PEDN in mouse heart mitochondria. The biotransformation of PETN and PEDN in isolated mouse heart mitochondria of wild type and ALDH-$2^{-/-}$ mice was measured by an HPLC-based method with chemiluminescent nitrogen detection (CLND) detection, modified from a previously published method (Seeling and Lehmann, 2006). After addition of 50 µl conc. H_3PO_4 each mitochondria sample was extracted three times with 3 ml of freshly distilled ethyl acetate. The organic layer was thoroughly shaken with anhydrous sodium sulphate, filtered, dried in a nitrogen stream and then resolved in 1ml of the HPLC-eluent (methanol/water). Analyses of the samples were performed isocratically on a Chromolith Performance RP-18e (100x4.6) column / Chromolith RP-18e Guard Cartridge (5x4.6) (Merck, Darmstadt) with methanol (50 %)/water (50 %). The flow was 0.5 ml min^{-1}; the injection volume was 100 µl and the split ratio of UV:CLND was set at 3.2:1.8 (UV detection at 215 nm). The system was calibrated with a 1 µM-PEDN solution and with 0.25

µM-, 1 µM- and 10 µM-PETN solutions. The HPLC system consisted of a LC-10 Series HPLC connected to an UV-detector SPD-10A (Shimadzu Europa GmbH, Duisburg, Germany) and to an Antek 8060 Nitrogen Specific HPLC Detector (ANTEK Instruments L.P., Houston, Texas, USA), respectively. The rate of retrieval was 87 % for PEDN and 94 % for PETN.

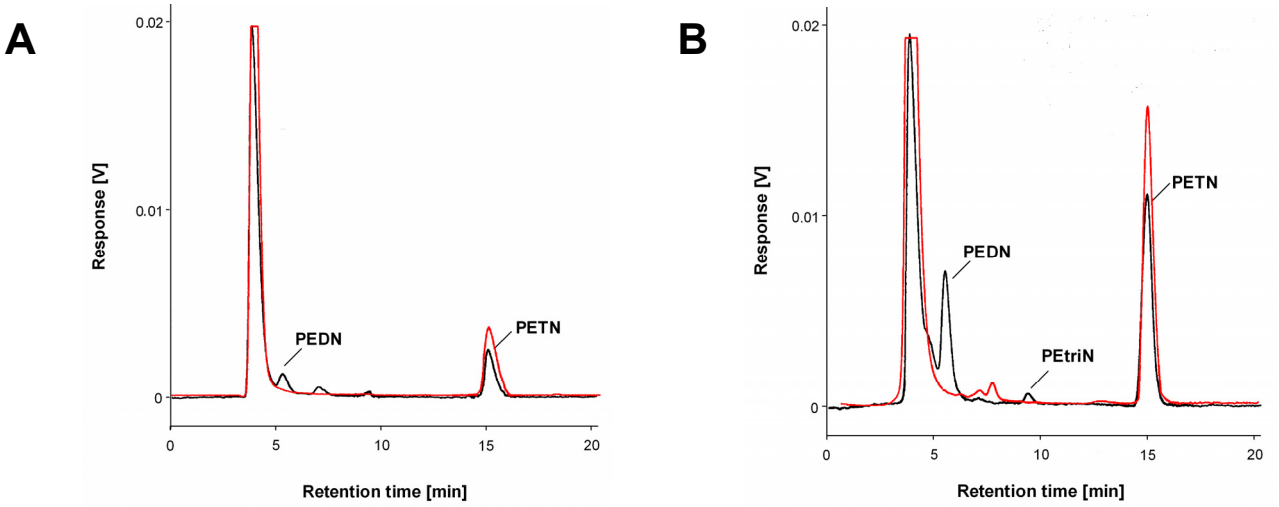

Figure 4S. Original CLND chromatograms of mitochondrial samples which were incubated with 0.25 µM PETN (**A**) or 1 µM PETN (**B**). The rate of conversion of PETN to PEDN is higher in the wild type samples (black line) as compared to the ALDH-2$^{-/-}$ samples (red line) indicating that ALDH-2 is essential for biotransformation of PETN and ist trinitrate PETriN. The chromatograms are representative for three independent measurements.

Table 2S. Biotransformation of PETN and PEDN in heart mitochondria of wild type and ALDH-2$^{-/-}$ mice measured by an HPLC-based method with CLND detection.

Sample (conc., subst.)	**PEDN [µM]**	**PETriN [µM]**	**PETN [µM]**
WT 1 (1 µM PETN)	0.22 ± 0.04	0.02	0.64 ± 0.08
WT 3 (0.25 µM PETN)	0.05 ± 0.01	n.d.	0.12 ± 0.04
WT 2 (1 µM PEDN)	0.78 ± 0.12	-	-
WT 4 (10 µM PEDN)	8.1 ± 0.3	-	-
-/- 5 (1 µM PETN)	n. d.	n. d.	0.87 ± 0.04

-/- 7	(0.25 μM PETN)	n. d.	n. d.	0.19 ± 0.02
-/- 6	(1 μM PEDN)	0.82 ± 0.13	-	-
-/- 8	(10 μM PEDN)	8.4 ± 0.0	-	-

Development of an HPLC-based assay with optical detection for determination of PETriN bioactivation by ALDH-2. The purpose of this new PETriN derivative (KL-61) was to develop an easy HPLC-based assay to assess organic nitrate bioactivation by ALDH-2 by means of optical UV/Vis detection. KL-61 was synthesized in the laboratory of J. Lehmann (Jena, Germany) (for structure see figure 4S) which is PETriN linked to a chromophore allowing optical UV/Vis detection at 370 nm (and even fluorescence detection). We observed increased hydrolysis of this compound in mitochondrial preparations which required careful and fast handling of the samples which could not be injected by an autosampler but had to be subjected to analysis immediately after thawing. Prior to the assay, liver tissue was removed from freshly killed wild type and ALDH-$2^{-/-}$ mice and a mitochondria preparation was carried out following the protocol described above. The mitochondrial preparation (5 or 15 mg ml^{-1}) was incubated with 2.5 or 20 μM of KL-61 (MW: 504.43 Da) for 30 sec or 2.5 minutes and then snap frozen. After thawing, suspensions were centrifuged for 1 min at 20.000g. The supernatant was rapidly mixed with acetonitril (1:1 dilution). Bioactivation of KL-61 by ALDH-2 could be optically detected (see figure 5S) and was confirmed by LC/MSMS measurements (see figure 6S).

Chromatography was carried out on a Thermo Electron Surveyor HPLC System consisting of a solvent delivery system from Thermo Electron (Dreieich, Germany). Separation was carried out on a Thermo Aquasil (Thermo Electron, Dreieich, Germany) C18 column (2 mm x 5 cm, 5 μm) using a mobile phase consisting of 0.1% formic acid, 2mg l^{-1} Ammoniumchloride in water (solvent "A") and 0.1% formic acid, 2mg l^{-1} Ammoniumchloride in acetonitrile (solvent

"B") at a flow rate of 0.4 mL min^{-1}. The gradient used was as follows: Isocratic conditions of 30% B were maintained for 2 min followed by a linear increase to 80% B in 8 min. The column then was washed with 80% B for 1 min and equilibrated at 30% B for 3 min prior to each injection. 10µl of the supernatant were injected onto the HPLC-System. The HPLC System was interfaced to a TSQ Quantum Discovery triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Electron, Dreieich, Germany). The mass spectrometer was operated in negative ion (3800V, Cap. Temp: 270°C) and multiple-reaction-monitoring (MRM) mode. Nitrogen served as auxiliary gas and argon as collision gas (1.5 torr, 12 eV). The monitored transitions were m/z 539 → 250, m/z 494 → 250 and m/z: 449 → 250 (chloride adducts of organic nitrates).

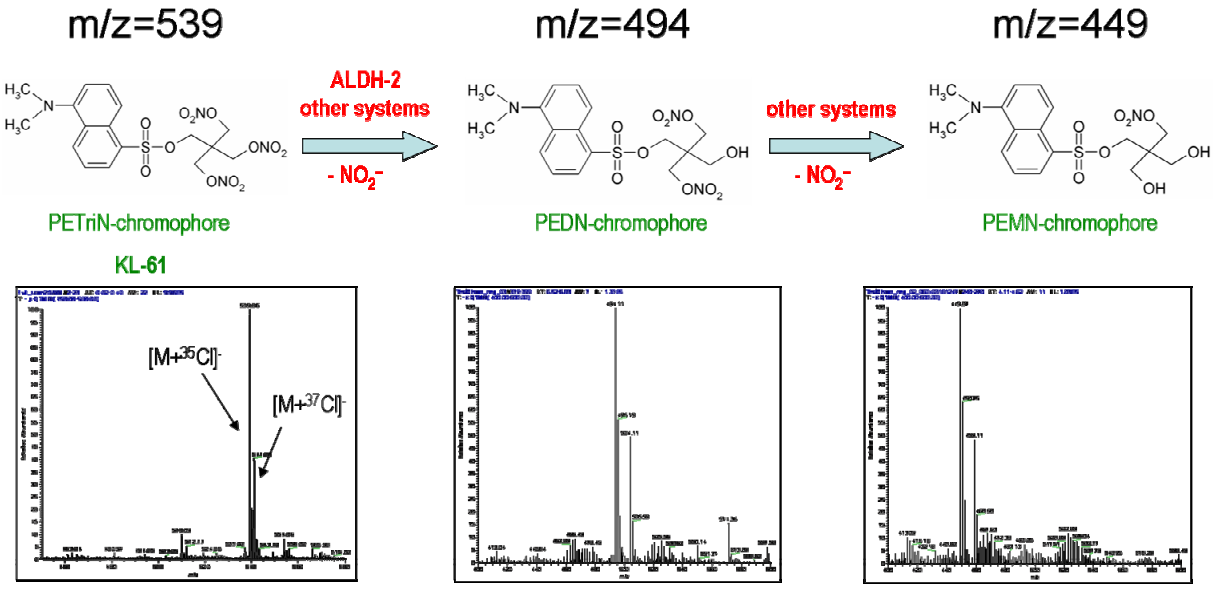

Figure 5S. Structure of the PETriN-chromophore KL-61 and its bioactivation to the di- and mononitrate metabolite. MS spectra are shown for each compound.

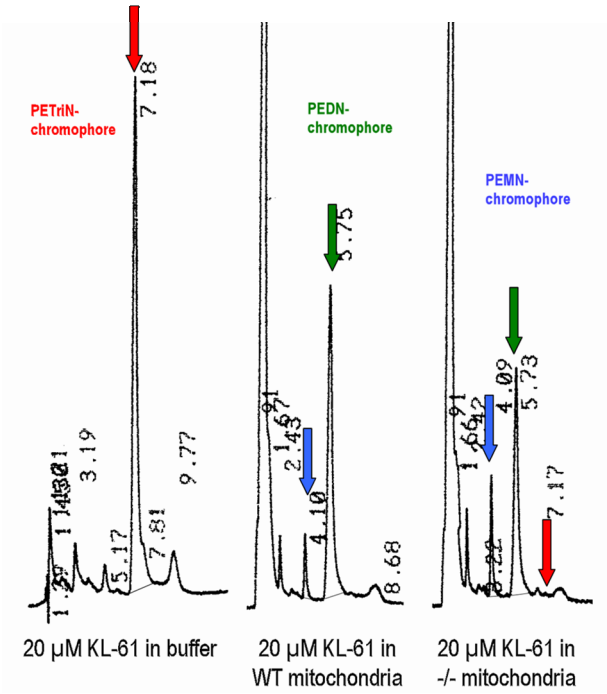

Figure 6S. Chromatograms of KL-61 (20 µM) incubations in isolated rat liver mitochondria (5 mg ml^{-1}). Mobile phase consisted of 57 % acetonitrile/43 % 50 mM citrate pH 2, the column was a Nucleosil C_{18} (125x4) 100-3 from Macherey & Nagel (Düren, Germany) and detection was set at 370 nm. Chromatograms are representative for 2-3 independent measurements.

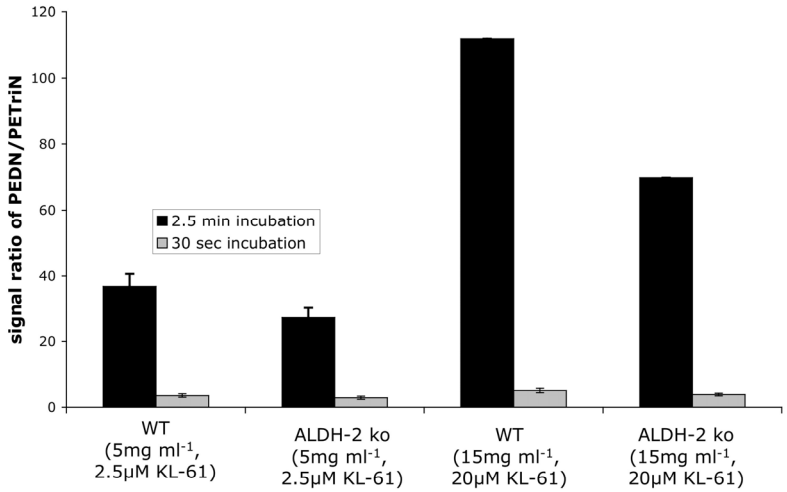

Figure 7S. Incubation of isolated liver mitochondria for 2.5 min with the PETriN Chromophore KL-61 (LC MSMS/manual injection). Values are the ratio of mass intensities of PEDN-Chromophore/PETriN-Chromophore. Black, 2.5 min incubation; grey: 30 s incubation. Data are representative for 1-3 independent measurements.

NO spin trapping - Methods. Vascular NO production was measured by electron paramagnetic resonance (EPR) spin trapping technique as described previously (Kleschyov *et al.*, 2000a; Kleschyov *et al.*, 2000b). Briefly, aortas from the wild type or ALDH-2 knock-out mice were collected in ice-cold Krebs solution, cleaned of adhering tissue, cut into segments (2 mm long) and then transferred in 24 well plate. The vascular segments were pre-incubated at 37°C for 90 min period during which the medium was changed 3 times. After that, aortic segments were incubated at 37°C for 60min with 100 μM colloid iron(II)-dithiocarbamate complex ($Fe(DETC)_2$) and with 0.3 mM L-NAME in the absence or in the presence of 20 μM PETN. EPR spectra were recorded at 77 K using an X-band spectrometer Miniscope 200 (Magnettech, Berlin). The instrument settings were 10mW microwave power, 1mT amplitude modulation, 100kHz modulation frequency, 60 seconds sweep time and 3 number of scans. The vascular NO production was expressed in EPR arbitrary units and normalized per mg of the sample dry weight. **Results.** The basal aortic NO production was bellow detection limit in both types of mice (not shown). In the presence of 20 μM PETN, aortas developed a characteristic triplet EPR signal (g=2.035; A_N= 1.3 mT), which reflects NO production from PETN. The NO donor properties of PETN were similar in aortas from both ALDH-$2^{+/+}$ and ALDH-$2^{-/-}$ mice (Figure 8S). **Discussion.** Our EPR spin trapping experiments indicate that ALDH-2 does not contribute to vascular biotransformation of PETN to NO when used in high pharmacological concentrations (low affinity pathway). These data are consistent with our previous work showing that in rat aorta, the ALDH-2 inhibitor benomyl has no effect on metabolism of GTN to NO (Kleschyov *et al.*, 2003; Nunez *et al.*, 2005) and once more challenge the hypothesis that NO is the active principle of highly potent organic nitrates like PETN or GTN.

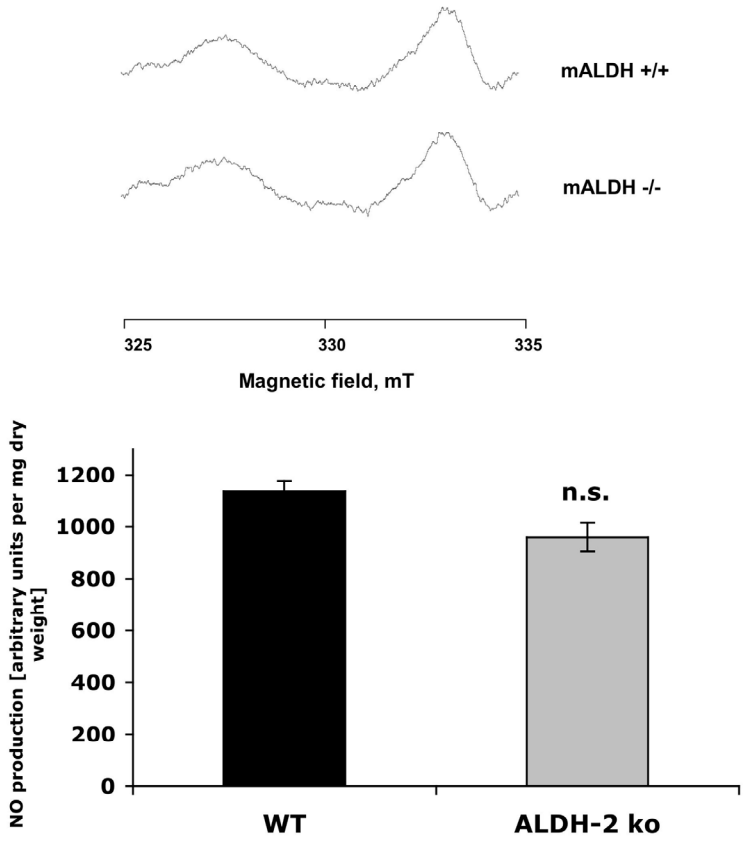

Figure 8S. Electron paramagnetic resonance (EPR) spin trapping of the PETN (20 μM)-derived NO in aortas from the WT and ALDH-2$^{-/-}$. EPR settings were as described in the Methods section. Top panel: representative EPR spectra, bottom panel: quantification of the vascular NO production. Mean ± SEM of 3 independent experiments.

Synthesis of 1,3-GDN.

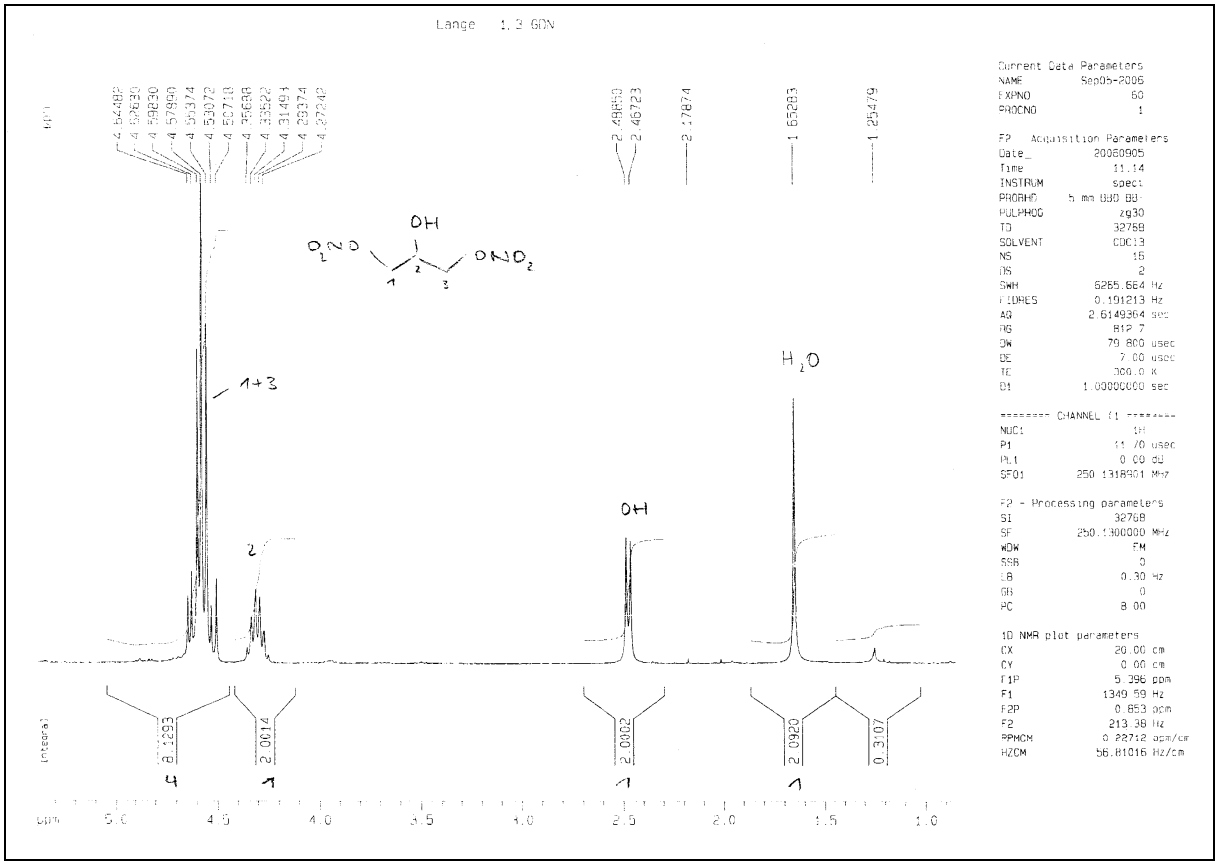

Figure 9S. [1]H-NMR spectrum of 1,3-GDN. The compound was synthesized according to a published method from the dibromo-compound (Shan *et al.*, 2004). Purification was achieved by semipreparative HPLC chromatography.

Cited Literature

Kleschyov AL, Mollnau H, Oelze M, Meinertz T, Huang Y, Harrison DG, Munzel T (2000a). Spin trapping of vascular nitric oxide using colloid Fe(II)-diethyldithiocarbamate. *Biochem Biophys Res Commun* **275:** 672-7.

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