SUPPLEMENTAL DATA

Monitoring white blood cell mitochondrial aldehyde dehydrogenase activity – implications for nitrate therapy in humans

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Supplemental Table 1. Constitution of the human blood cell fractions used in this study.

<table>
<thead>
<tr>
<th>Blood cell fraction *</th>
<th>Cell type</th>
<th>Whole blood</th>
<th>Buffy coat</th>
<th>Granulocytes</th>
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</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>6.3±0.7</td>
<td>7.0±1.5</td>
<td>33.5±9.2</td>
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<tr>
<td>Erythrocytes</td>
<td>5.0±0.1</td>
<td>0.9±0.6</td>
<td>0.8±0.6</td>
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<tr>
<td>Thrombocytes</td>
<td>186±16</td>
<td>202±51</td>
<td>40±9</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>64.9±3.4</td>
<td>5.5±1.1</td>
<td>72.9±4.7</td>
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</tr>
<tr>
<td>Lymphocytes</td>
<td>24.9±3.1</td>
<td>76.3±2.7</td>
<td>2.5±1.1</td>
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<tr>
<td>Monocytes</td>
<td>6.0±0.4</td>
<td>15.4±2.7</td>
<td>23.4±4.4</td>
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<tr>
<td>Eosinophils</td>
<td>1.8±0.4</td>
<td>2.2±1.2</td>
<td>1.2±0.4</td>
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<tr>
<td>Basophils</td>
<td>0.7±0.2</td>
<td>0.2±0.1</td>
<td>0.1±0.0</td>
<td></td>
</tr>
</tbody>
</table>

* Data is mean from 7-8 samples from volunteers. † [×1000/μl].
Supplemental Table 2. Effect of acute oral (15µg/kg) and chronic subcutaneous (0.73-6.6µg/kg/min) GTN treatment on vascular reactivity of Ach and nitroglycerin in rat aorta.

<table>
<thead>
<tr>
<th>In vivo treatment</th>
<th>Acetylcholine (Ach)</th>
<th>Nitroglycerin (GTN)</th>
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<tbody>
<tr>
<td></td>
<td>Potency (pD2)</td>
<td>Efficacy (%)</td>
</tr>
<tr>
<td>Control (oral)</td>
<td>7.25±0.08 (n=26)</td>
<td>88±2 (n=26)</td>
</tr>
<tr>
<td>GTN (oral)</td>
<td>6.94±1.00 (n=16)*</td>
<td>72±4 (n=16)***</td>
</tr>
<tr>
<td>Control (ethanol, s.c.)</td>
<td>7.07±0.09 (n=26)</td>
<td>72±3 (n=26)</td>
</tr>
<tr>
<td>GTN (diluted 1:8, s.c.)</td>
<td>6.83±0.07 (n=20)*</td>
<td>63±3 (n=20)*</td>
</tr>
<tr>
<td>GTN (diluted 1:2, s.c.)</td>
<td>6.65±0.08 (n=20)**</td>
<td>58±5 (n=20)*</td>
</tr>
<tr>
<td>GTN (undiluted, s.c.)</td>
<td>6.66±0.06 (n=13)**</td>
<td>70±3 (n=13)</td>
</tr>
</tbody>
</table>

* P<0.05, ** P<0.01, *** P<0.001 vs. Control.

Potency is –log EC50 (where EC50 is the concentration that causes half-maximal relaxation) and efficacy is defined as maximal relaxation obtained with the highest employed concentration of the vasodilator.
Supplemental Figure 1. Organic nitrate bioactivating capacity of white blood cells (WBCs). Representative chromatograms for the conversion of pentaerythritol trinitrate-dansyl adduct (PETriN-fluorophore, KL-61) to its dinitrate metabolite by WBCs prior (black) and post (blue) GTN treatment of a volunteer. The red trace shows the chromatogram of 1 µM KL-61 standard.
**Supplemental Figure 2.** Effect of in vivo administration of GTN or PETN on serum S-nitroso-BSA, nitrite and nitrate in human volunteers. Effect of GTN or PETN treatment on concentrations of nitric oxide end products nitrite, nitrate and S-nitroso-BSA in serum or plasma. The data are mean ± SEM of 3 volunteers/group. S-nitroso-BSA, nitrite and nitrate concentrations were assessed by GC-MS. The data are mean ± SEM of 6 independent measurements for in vivo treatment of 3 volunteers per group. *, p<0.05 vs. PETN in vivo-treated group.
Supplemental Figure 3. Effect of in vitro and in vivo treatment with GTN on WBC ALDH-2 activity in human volunteers. ALDH-2 activity in isolated white blood cells (WBCs) was assessed by an HPLC method that measures the conversion of 2-hydroxy-3-nitro-benzaldehyde (2H3N-BA) to its benzoic acid product. WBCs were treated in vitro with daidzin (200 µM), GTN (4.5 µM) ± DTT (1mM), benomyl (100 µM) or PETN (5 µM). In vivo, GTN (0.8mg) and PETN (80 mg) were orally administrated. The data are mean ± SEM of 15-30 independent measurements for in vivo treatment of 6 volunteers/group and were normalized on the number of WBCs. The in vitro experiments represent 6-18 independent experiments. *, p<0.05 vs. control group; #, p<0.05 vs. GTN in vitro-treated group; §, p<0.05 vs. GTN in vivo-treated group; $, p<0.05 vs. PETN in vivo-treated group.
**Supplemental Figure 4.** Effect of in vitro incubations with GTN and co-incubation with dithiol compounds on WBC ALDH-2 activity in human volunteers. Restoration of GTN (4.5 µM)-impaired WBC ALDH-2 activity by in vitro incubation with reduced lipoic acid (DHLA) or DTT. The data are mean ± SEM of 3-5 independent measurements and were normalized on the number of WBCs. *, p<0.05 vs. control group, #, p<0.05 vs. GTN in vitro-treated group.
**Supplemental Figure 5.** Effect of in vitro challenges with GTN, DTT and inhibitors on ALDH-2 activity in whole blood from human volunteers. Impairment of ALDH-2 activity by daidzin (200 µM), benomyl (100 µM), in vitro bolus of GTN (4.5 or 45 µM) and restoration of GTN (4.5 µM)-impaired activity by DTT (1 mM) co-incubation. The last bar represents the effect of in vivo GTN treatment (30 min after a single sublingual dose). The data are mean ± SEM of 4 independent measurements. *, p<0.05 vs. control group.
Supplemental Figure 6. Effects of chronic subcutaneous treatment of rats with GTN (0, 0.73, 2.2 and 6.6 µg/kg/min). WBC ALDH-2 activity was assessed by an HPLC method and based on the conversion of 2-hydroxy-3-nitro-benzaldehyde to its 2-hydroxy-3-nitro-benzoic acid product. The data are mean ± SEM of 15 independent measurements from 5-7 animals/group and was normalized on the number of WBCs. *, p<0.05 vs. control group; #, p<0.05 vs. 1:9 GTN in vivo-treated group; §, p<0.05 vs. 1:2 GTN in vivo-treated group.
**Supplemental Figure 7.** Effects of chronic subcutaneous treatment of rats with GTN (0, 0.73, 2.2 and 6.6 µg/kg/min). Mitochondrial (upper panel) and vascular (lower panel) ROS formation was measured using L-012 (100µM) ECL. Mitochondria were stimulated by malate/glutamate (each 2.5mM). The data are mean ± SEM of 18 (mitochondria) and 8-10 (aorta) independent measurements from 5-7 animals/group. *, p<0.05 vs. control group.
Extended Methods

Materials. PETN (Pentalong, 80mg/tablet) was purchased from Actavis Deutschland GmbH (Langenfeld, Germany). GTN (Nitrolingual, 0.8mg/capsule) for use in volunteers was obtained from G. Pohl-Boskamp (Hohenlockstedt, Germany). LA (Alpha-Lipon AL 600, 600mg/tablet) was obtained from ALIUD PHARMA GmbH (Laichingen, Germany). GTN stock solutions in water (Nitrolingual infusion solution (1mg/ml) for vascular isometric tension studies) or in ethanol (100g/l) for in vivo treatment of rats were purchased from G. Pohl-Boskamp and Unikem (Copenhagen, Denmark), respectively. L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt) was obtained from Wako Pure Chemical Industries (Osaka, Japan), all other chemicals of analytical grade from Sigma-Aldrich, Fluka or Merck.

Isolation of white blood cells (WBCs). The procedure is described in reference (Daiber et al., 2004a). Erythrocytes in 15ml heparin-supplemented human blood were separated by sedimentation on addition of an equal volume of dextran solution (MW 485,000, 40mg/ml PBS). The leukocyte-containing supernatant was centrifuged on Histopaque-1077 from Sigma for 30min at 500g at 20°C resulting in a neutrophil (PMN)-containing pellet and the monocyte/lymphocyte-enriched (WBCs) “buffy coat” between the aqueous and Ficoll phases. The WBC-fraction was collected and purified by further centrifugation for 10min at 500g followed by resuspension in PBS. For some experiments, the PMN pellet was freed from residual erythrocytes by hypotonic lysis and centrifugation at 500g, else it was discarded.

Nitrate bioactivation. The dansyl-tagged PETN metabolite (KL-61) used for this assay is a pentaerithrityl trinitrate (PETriN)-dansyl-adduct whose breakdown to the denitrated pentaerithrityl dinitrate (PEDiN)-dansyl-adduct can be assessed in biological fluids by a HPLC method with fluorescence detection. Briefly, WBCs were incubated with 1µM KL-61 for 20min at room temperature, diluted 1:1 (v/v) in acetonitrile to extract the lipophilic nitrate-fluorophores, centrifuged for 5min at 10,000g and the supernatant subjected to HPLC analysis. The system consisted of a 125x4mm C18-Nucleosil (100-3) column from Macherey & Nagel (Düren, Germany), an isocratic elution method (40% 50mM citric acid buffer pH 2, 60% acetonitrile) and fluorescence detection (Ex. 350, Em. 555nm), the pumps and detectors were from Jasco (Gross-Umstadt, Germany). The flow rate was 0.8ml/min, typical retention times were 5.7min for the PEDiN-derivative and 10.2 min for the PETriN-derivative.

ALDH-2 activity in isolated WBCs, rat aortic tissue and isolated rat cardiac mitochondria. WBCs in PBS were incubated with 2-hydroxy-3-nitrobenzaldehyde (100µM)
for 60min at 37°C. The samples were stored over night at -20°C and centrifuged for 10min at 2,000g. The supernatant was purified by size-exclusion centrifugation through a Microcon YM-10 filter device from Millipore Corporation (Billerica, MA) as previously described (Daiber et al., 2004b). The HPLC system was similar to the one described in (Wenzel et al., 2007c). The dehydrogenase activity of ALDH-2 in isolated rat heart mitochondria was determined by measuring the conversion of 2-hydroxy-3-nitrobenzaldehyde (200µM, 20min at 37°C) to the product 2-hydroxy-3-nitrobenzoic acid by a previously published HPLC protocol (Daiber et al., 2004b; Wenzel et al., 2007c). For determination of vascular ALDH dehydrogenase activity, isolated aortic rings were incubated with 2-hydroxy-3-nitrobenzaldehyde (100µM, 60min at 37°C) and the benzoic acid product was measured in the incubation solution as described (Sydow et al., 2004; Wenzel et al., 2007c). WBCs were isolated from human blood and WBC ALDH-2 activity was measured as described for human samples. WBCs in PBS were incubated with 2-hydroxy-3-nitrobenzaldehyde (100µM) for 60min at 37°C. The samples were stored over night at -20°C and after thawing centrifuged for 10min at 2,000g and the supernatant was purified by size-exclusion centrifugation through a Microcon YM-10 filter device from Millipore Corporation (Billerica, MA) as previously described in detail (Daiber et al., 2004b). The HPLC system was similar to the one described in (Wenzel et al., 2007c).

Detection of ROS production. Mitochondrial suspensions were diluted to a final protein concentration of 0.1mg/ml in 0.5ml of PBS buffer containing L-012 (100µM). ROS production was detected after stimulation with succinate or malate/glutamate (5mM or 2.5mM final concentration). The CL was registered at intervals of 30s over 5min with a Lumat chemiluminometer (Berthold Techn., Bad Wildbad, Germany) and the signal was expressed as counts/min at 5min. Vascular superoxide production in intact aortic rings (5mm) was determined using lucigenin (5µM)-ECL without any co-factor stimulation as described (Oelze et al., 2006). The CL was registered at intervals of 60s over 20min with a Lumat chemiluminometer and was normalized for the dry weight of aortic tissue.

Extended Results

Maximal plasma levels of organic nitrates and their metabolites. For a single sublingual administration of GTN (0.8mg) the plasma levels peaked at 5-10min and reached the following concentrations: 13nM GTN, 71.4nM 1,2-GDN and 7.4nM 1,3-GDN. Therefore, the here reported changes in serum S-nitrosoprotein levels of 40-70nM may be easily reached
but require an almost complete conversion of GTN (13+71.4+7.4=91.8nM) to GDN metabolites and S-nitrosoproteins. In addition, conversion of GDN metabolites to GMN (mononitrates) may also contribute to serum S-nitrosoprotein levels. Finally, it seems to be feasible that the here reported S-nitrosoprotein levels are reached at 30min after GTN administration since conversion of GDN to GMN metabolites may be a slower process. The pharmacokinetics of GTN are quite complex and subject to ongoing discussion and reviewed by T. Taylor et al. (*Progress in Drug Metabolism* 10, 207-336 (1987)). The here used data were published by A.J. Woodward et al. (Woodward et al., 1984).

For a single oral administration of PETN (80mg) the plasma levels of dinitrate (PEDN) and mononitrate (PEMN) peaked at 1-3h and 6-9h, respectively. The metabolites reached the following concentrations: 60nM PEDN and 331nM PEMN. Therefore, the here reported changes in serum S-nitrosoprotein levels of 40-60nM may be easily reached and require a 50% conversion of PETN and PETriN to S-nitrosoproteins during denitration to PEDN. Finally, it seems to be feasible that the here reported S-nitrosoprotein levels are reached at 120min after PETN administration since already the fast conversion of PETN and PETriN to PEDN yields sufficiently high NOx equivalents, ignoring the slower conversion to PEMN yielding 5-fold higher PEMN plasma concentrations over S-nitrosoprotein serum concentrations. The pharmacokinetics of PETN are quite complex and subject to ongoing discussion and reviewed by D. Stalleicken et al. (*PETN Schriftenreihe – Steinkopff Verlag* (2007)). The here used data were published by Weber et al. (Weber et al., 1995).

**Blood cells within the “buffy coat” fractions.** At the beginning of the study we tested whole blood, the “buffy coat” and the granulocyte fraction with respect to their ALDH-2 activity. Among all blood cells, WBCs in the buffy coat fraction had the highest ALDH-2 activity. The “buffy coat” fraction typically contained 75% lymphocytes, 15% monocytes but only 5% neutrophils (see Table I). Despite having a high number of thrombocytes, these cells did not contribute significantly to the overall ALDH-2 activity, since platelets only contain a very limited amount of mitochondria and hence display only marginal ALDH-2 activity (not shown). Granulocytes have an appreciable amount of ALDH-2 protein but tend to start the oxidative burst in response the isolation process which therefore may impair the activity of the redox-sensitive ALDH-2. In whole blood other ALDH-isoforms limit substantially the accurate measurement of ALDH-2 activity.

**Extended Discussion**
Role of ALDH-2 for organic nitrate bioactivation and nitrate tolerance. Using animal studies and the isolated enzyme we have previously demonstrated that ALDH-2 activity is a very sensitive marker of nitrate tolerance (Daiber et al., 2004b; Sydow et al., 2004; Daiber et al., 2005) and that this enzyme is sensitive to oxidative inhibition and redox-regulation (Wenzel et al., 2007a). Recently, ALDH-2 inactivation was also confirmed to occur in bypass vessels in response to chronic GTN treatment in humans (Hink et al., 2007). In addition, ALDH-2 deficiency was associated with attenuated hemodynamic effects of GTN in two independent studies (Mackenzie et al., 2005; Li et al., 2006). Since it was previously reported that aldehyde oxidizing activity in lymphocytes is considerably attributable to the ALDH-2 isoform (Dyck, 1990), we chose to use this easily accessible source of ADH-2 as a possible marker of the effects of GTN ex vivo. Because peripheral blood mononuclear cells (PBMCs) contain large amounts of ALDH-2 protein that is highly active, endothelial progenitor cells as a subfraction of PBMCs can be analyzed by FACS using the expression of ALDH-2 as a marker (Povsic et al., 2007). In preliminary experiments, we detected an appreciable and consistent aldehyde converting activity in WBCs that was suppressed by the specific ALDH-2 inhibitor daidzin. Importantly, neutrophil ALDH-2 activity was less pronounced and decreased rapidly upon isolation of the cells, probably due to neutrophil activation and subsequent oxidative inactivation of the enzyme. We have previously observed that isolated neutrophils tend to start oxidative burst without exogenous stimulation and form aggregates (Daiber et al., 2004a). Since platelets contain only a very small number of mitochondria and erythrocytes predominantly contain other ALDH isoforms, these cells do not seem suitable to monitor GTN effects on ALDH-2 activity (Daiber, unpublished).

In vitro versus in vivo nitrate effects and tolerance. Of note, important differences exist in the time course and mechanisms of nitrate tolerance induced by in vitro versus in vivo GTN. Incubation of tissues with high concentrations of GTN causes the overload of nitrate bioactivating mechanisms, which are exposed to these high concentrations in a time frame that is too short to allow regeneration of these mechanisms (particularly, of a reduced thiol in the catalytic site of ALDH-2). In contrast, in vivo tolerance is a more complex phenomenon that is mediated by the activation of counter-regulatory mechanisms at the humoral, genomic and proteomic level. Given these differences, the term “tachyphylaxis” should be used to identify the rapid desensitization of the vasodilator effects of nitrates. Although the precise mechanisms remain obscure, tachyphylaxis has been proposed to be based on the depletion of reductive co-factors such as thiols and dihydrolipoic acid, which results in redox-based inhibition of the bioactivating ALDH-2 (Wenzel et al., 2007b) and/or other essential vascular
enzymes. The mechanistic basis of in vivo nitrate tachyphylaxis may resemble that of in vitro tolerance with all the limitations due to the exclusion the activation of neurohormonal systems in response to the blood pressure lowering effects of nitrates. Therefore, the model employed here (acute in vivo administration of nitrates) is an interesting composite of clinical (in vivo) and in vitro tolerance. Of note, while there are substantial differences in the induction of ROS-mediated side effects between GTN and PETN when the latter is administered orally, these differences disappear in the setting of parenteral PETN administration. Although in vivo short-term infusion of PETN results in lesser ROS formation as compared to GTN (Schwemmer and Bassenge, 2003), in a human study, Parker et al. showed that tachyphylaxis developed in equal measure after repeated injections of GTN or pentaerythrityl trinitrate (PETriN) (Parker et al., 1975). This is in accordance with the lack of protective properties of PETN and its metabolites in case of acute parenteral administration, which results in systemic overload of the drug and potential harmful effects that resemble those of in vitro nitrate tolerance.

Extended References


