0026-895X/05/6803-579–588$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2005 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 68:579–588, 2005

Vol. 68, No. 3
11585/3046571
Printed in U.S.A.

Heterozygous Deficiency of Manganese Superoxide Dismutase in Mice (Mn-SOD$^{+/-}$): A Novel Approach to Assess the Role of Oxidative Stress for the Development of Nitrate Tolerance

Andreas Daiber, Matthias Oelze, Silke Sulyok, Meike Coldewey, Eberhard Schulz, Nicolai Treiber, Ulrich Hink, Alexander Mülsch, Karin Scharffetter-Kochanek, and Thomas Münzel

Klinikum der Johannes Gutenberg-Universität Mainz, Medizinische Klinik II, Kardiologie, Mainz, Germany (A.D., M.O., E.S., U.H., A.M., T.M.); University of Ulm, Department of Dermatology and Allergology, Ulm, Germany (S.S., N.T., K.S.-K.); and Universitätsklinikum Hamburg-Eppendorf, Medizinische Klinik III, Angiologie und Kardiologie, Hamburg, Germany (M.C.)

Received February 1, 2005; accepted June 1, 2005

ABSTRACT

Nitroglycerin (GTN)-induced tolerance was reported to be associated with increased levels of reactive oxygen species (ROS) in mitochondria. In the present study, we further investigated the role of ROS for the development of nitrate tolerance by using heterozygous manganese superoxide dismutase knockout mice (Mn-SOD$^{+/-}$). Mn-SOD is acknowledged as a major sink for mitochondrial superoxide. Vasodilator potency of mouse aorta in response to acetylcholine and GTN was assessed by isometric tension studies. Mitochondrial ROS formation was detected by 8-amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)dione sodium salt (L-012)-enhanced chemiluminescence and mitochondrial aldehyde dehydrogenase (ALDH-2) activity was determined by a high-performance liquid chromatography-based assay. Aortic rings from Mn-SOD$^{+/-}$ mice showed normal endothelial function and vasodilator responses to GTN. In contrast, preincubation of aorta with GTN or long-term GTN infusion caused a marked higher degree of tolerance as well as endothelial dysfunction in Mn-SOD$^{+/-}$ compared with wild type. Basal as well as GTN-stimulated ROS formation was significantly increased in isolated heart mitochondria from Mn-SOD$^{+/-}$ mice, correlating well with a marked decrease in ALDH-2 activity in response to in vitro and in vivo GTN treatment. The data presented indicate that deficiency in Mn-SOD leads to a higher degree of tolerance and endothelial dysfunction associated with increased mitochondrial ROS production in response to in vitro and in vivo GTN challenges. These data further point to a crucial role of ALDH-2 in mediating GTN bioactivation as well as development of GTN tolerance and underline the important contribution of ROS to these processes.

Although organic nitrates such as nitroglycerin (glyceryl trinitrate, GTN) have been used for over a century in the therapy of cardiovascular diseases such as stable and unstable angina (Abrams, 1995) the underlying mechanisms of nitrate bioactivation and development of nitrate tolerance remain elusive. The anti-ischemic effects of organic nitrates are due largely to venous and coronary artery dilation as well as improvement of collateral blood flow, which all decrease myocardial oxygen consumption and are mediated by nitric oxide or a related species. However, the use of organic nitrates is limited because of the rapid development of tolerance and cross-tolerance to endothelium-dependent and -independent vasodilators. Impairment of the NO-signaling pathway by increased formation of reactive oxygen species (ROS) (Munzel et al., 1995b) as well as an impaired biotransformation of organic nitrates may contribute to the development of tolerance and cross-tolerance. The mitochondrial aldehyde dehydrogenase (ALDH-2), which is subjected to an

This work was supported by grants from the German Research Foundation (Deutsche Forschungsgemeinschaft) (Mu1079/6-1, now SFB 553/C17, to T.M.), by grants from the VW-Stiftung (AZ: I/76333 and I/79003 to K.S.-K.), and by the European Union through the CELLAGE project (QLK6-CT-2001-00616 to K.S.-K.).
A.D. and M.O. contributed equally to this work
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.011585.

ABBREVIATIONS: GTN, glyceryl trinitrate (nitroglycerin); ROS, reactive oxygen species; ALDH-2, aldehyde dehydrogenase; Mn-SOD, manganese superoxide dismutase (mitochondrial isoform); Cu,Zn-SOD, copper/zinc superoxide dismutase (cytosolic and extracellular isoforms); Mn-SOD$^{+/-}$, heterozygous Mn-SOD deficiency; wt, wild-type; L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)dione sodium salt; DHE, dihydroethidium; ACh, acetylcholine; P-VASP, phosphorylated vasodilator stimulated phosphoprotein; CL, chemiluminescence; PETN, pentaerythritol tetranitrate; Cre, a site-specific bacteriophage P1-derived recombinase; lox P, recognition site of Cre.

oxidative mechanism-based inactivation, has recently been identified as a GTN-metabolizing enzyme and a possible important component in the processes leading to tolerance (Chen et al., 2002). Our laboratory further substantiated this concept in an animal model of in vivo tolerance and extended previous observations by demonstrating that mitochondria are a major source of ROS formation in response to short- and long-term GTN challenges (Daiber et al., 2004b; Sydow et al., 2004). These results provided indeed the missing link between tolerance and cross-tolerance, the oxidative stress concept, and the concept that tolerance is secondary to decreased GTN biotransformation. Because mitochondrial ROS formation seems to play a major role for development of tolerance and cross-tolerance, one could hypothesize that a deficiency in mitochondrial superoxide dismutase (Mn-SOD) would render vascular tissue more susceptible for the development of tolerance.

There are two different types of Mn-SOD–deficient mice. Removal of exon 1 and 2 shows lethality at 21 days as a result of neuronal abnormalities (Lebovitz et al., 1996), whereas removal of exon 3 shows lethality at 10 days with dilated ventricular cardiomyopathy (Li et al., 1995). The lack of Mn-SOD causes an increase in mitochondrial superoxide levels, which in turn leads to destruction of iron-sulfur-cluster [4Fe-4S] proteins (Flint et al., 1993). In Mn-SOD$^{-/-}$ mice, aconitase activity in the heart is decreased by 42.6%, which, combined with a decrease in succinic acid dehydrogenase activity, impairs the citric acid cycle and lead to lipid accumulation in the liver and muscle (Li et al., 1995). The estimation that 1 to 2% of all electrons transported by the respiratory chain will ultimately result in the formation of superoxide justifies the importance of Mn-SOD for survival in all mammals (Robinson, 1998). The importance of Mn-SOD was also underlined by the finding that Cu,Zn-SOD overexpression could not compensate for the lack of Mn-SOD: the lethality of these animals was unchanged (Copin et al., 2000). The expression of Mn-SOD, in contrast to Cu,Zn-SOD isoforms (cytosolic and extracellular), can be induced by cytokines (Hennet et al., 1993) and oxidative stress (Shull et al., 1991), but Mn-SOD is also subject to oxidative inactivation, namely nitration and dimerization of essential tyrosine residues. Overexpression of Mn-SOD in mice protected from myocardial ischemia/reperfusion injury (Jones et al., 2003), in cells, however, this condition was associated with a hydrogen peroxide-induced up-regulation of matrix-degrading metalloproteinase-1 (Wenk et al., 1999).

This is the first study to use heterozygous Mn-SOD deficiency (Mn-SOD$^{+/-}$) in mice as a tool to assess the role of oxidative stress for the development of in vitro nitrate tolerance and cross-tolerance upon short-term GTN treatment of isolated murine aortic rings. The expression of Mn-SOD in Mn-SOD$^{+/-}$ mice is decreased by approximately 50% compared with wild-type (wt) animals, leading to distinct ultrastructural damage of the myocard, with swelling and disruption of mitochondria and accumulation of lipid droplets, increased nitrotyrosine formation and lipid peroxidation as well as activation of apoptosis signaling pathways in the heart in vivo (Strassburger et al., 2005). Cu,Zn-SOD deficiency is well characterized with respect to the vascular system and endothelial dysfunction (Lynch et al., 1997; Didion et al., 2002), but little is known about vascular consequences of Mn-SOD deficiency. One report presented data seeming to indicate that endothelial function (response to acetylcholine) in Mn-SOD$^{+/-}$ mice was not altered compared with wt animals (Andresen et al., 2004). With the present studies, we sought to focus on 1) the short-term GTN responsiveness as well as on GTN tolerance development in wt mice and Mn-SOD$^{+/-}$ mice in response to in vitro and in vivo GTN challenges, and 2) whether ALDH-2 activity and the GTN bioactivation are affected by Mn-SOD deficiency.

Materials and Methods

Materials. For induction of in vitro tolerance and isometric tension studies, GTN was used from a Nitrolingual infusion solution (1 mg/ml) from G. Pohl-Boskamp (Hohenlockstedt, Germany). For induction of in vivo tolerance, GTN was used from a solution in ethanol (102 g/liter), which was obtained from UNIKEM (Copenhagen, Denmark). L-012 was purchased from Wako Pure Chemical Industries (Osaka, Japan). Dihydroethidium (hydroethidine, DHE) was obtained from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade and were obtained from Sigma Chemie (Deisenhofen, Germany), Fluka (Buchs, Switzerland), or Merck (Darmstadt, Germany).

Animal Model, In Vitro and In Vivo Nitrate Tolerance. In the present study, we used female mice aged 4 to 8 months on a mixed genetic background (C57BL/6 × 129/Ola). Experiments were performed with 16 wt and 16 Mn-SOD$^{+/-}$ mice. Mn-SOD$^{+/-}$ mice were generated according to a published procedure (Strassburger et al., 2005) in the laboratory of author K.S.-K. In brief, male mice carrying two SOD2flox alleles were crossed to K14Cre females that, because of Keratin 14 expression in oocytes, also express Cre recombinase in maturing oocytes (Hafner et al., 2004). Because Cre recombinase remains active in oocytes until the paternally inherited SOD2flox allele becomes accessible after fertilization, all animals derived from such breedings carry a stably deleted SOD2 allele (SOD2−). SOD2$^{+/-}$ mice were further bred with wt animals of the outbred strain more than 10 times. The absence of the K14Cre allele in the heterozygous offspring was proven by Southern blot analysis. The deletion of exon 3 of the Mn-SOD gene was determined by Southern blot analysis. Because exon 3 of the Mn-SOD gene codes for the domain important for tetramer formation of the Mn-SOD, deletion of this domain results in a complete loss of the activity of the enzyme. The deficiency of the Mn-SOD activity was determined using a specific activity assay, as described recently (Strassburger et al., 2005).

In vitro tolerance development as a result of GTN treatment was assessed by ex vivo incubation of murine vessels with 200 μM GTN for 30 min at 37°C in Krebs-HEPES buffer (5.78 g/liter NaCl, 0.35 g/liter KCl, 0.37 g/liter $CaCl_2$, 0.30 g/liter $MgSO_4$, 2.1 g/liter $NaHCO_3$, 0.14 g/liter K_2HPO_4, 5.21 g/liter HEPES, and 2.0 g/liter D-glucose) followed by a 1-h wash-out phase and subsequent recording of concentration-response curves with GTN. In vivo tolerance was induced by long-term infusion of mice with GTN by implanted micro-osmotic pumps (0.5 μl/h for 7 days; model 1007D; ALZET Osmotic Pumps, Cupertino, CA). Infusion of the solvent ethanol served as a control. To determine the infusion rate of GTN that causes tolerance, female wt mice (C57BL, 5–6 months old) were infused with either ethanol or GTN at high (100 μg/h, 220 nmol/min/kg) and low (16 μg/h, 35 nmol/min/kg) dosages for 3 d. Based on these results, four female wt (C57BL/6 × 129/Ola) and four female Mn-SOD$^{+/-}$ mice (all 6–8 months old) were infused with ethanol, and the same number of animals was infused with GTN (16 μg/h, 35 nmol/min/kg) for 4 d. After this period, the animals were sacrificed, and aortas as well as hearts were subjected to further analysis. 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 and of the University Hospital Mainz.

Isometric Tension Studies. Vasodilator responses to GTN and acetylcholine (ACh) were assessed with endothelium-intact isolated murine aortic rings mounted for isometric tension recordings in organ chambers, as described previously (Munzel et al., 1995a). In brief, mice were anesthetized using CO_2. Thoracic aortas were rapidly removed and cut into ring segments of approximately 3-mm length and mounted in organ chambers for isometric tension recording. Preliminary studies revealed that the optimum resting tension for tone development in response to 80 mM KCl was 1.00 g, which was achieved by gradual stretching over 1 h. After preconstriction with prostaglandin $F_{2\alpha}$ to reach 50 to 80% of maximal tone induced by KCl, a concentration-response to increasing concentrations of the endothelium-dependent vasodilator ACh (1 nM to 3 μM) and the endothelium independent vasodilator GTN (1 nM to 30 μM) was established as described.

Western Blot Analysis. Aortic segments (1 cm) from wt and Mn-SOD$^{+/-}$ mice were incubated for 5 min with GTN (0.1 μM), frozen, and homogenized in liquid nitrogen. The expression of the phosphorylated vasodilator stimulated phosphoprotein (P-VASP) was determined as described previously (Oelze et al., 2000). Immunoblotting was performed with a mouse monoclonal P-VASP phosphoserine 239 antibody (clone 16C2, 1.5 μg/ml; Calbiochem, Schwalbach, Germany). Detection was performed by enhanced chemiluminescence with peroxidase conjugated anti–rabbit/mouse secondary antibodies (1:10,000; Vector Laboratories, Burlingame, CA).

ALDH-2 Dehydrogenase and Esterase Activity in Isolated Mouse Heart Mitochondria and Dehydrogenase Activity in Isolated Aortic Segments. The activity of ALDH in isolated mitochondria was determined by measuring the conversion of benzaldehyde to benzoic acid. Mouse heart mitochondria were prepared according to a previously published method (Raha et al., 2000) that was slightly modified (Daiber et al., 2004b). 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 30 min at room temperature. In some experiments, mitochondria were incubated with GTN (5 or 50 μM) for 30 min before ALDH substrate addition. For measurement of ALDH-2 dehydrogenase activity, benzaldehyde (400 μM) was added to the mitochondrial suspension, and the samples were incubated for another 30 min at 37°C. For determination of vascular dehydrogenase activity, aortic rings of 3 to 4 mm in length were incubated with benzaldehyde (400 μM) for 30 min at 37°C. For measurement of ALDH-2 esterase activity, methylbenzoate (1 mM) was added, and the samples were incubated for another 30 min at 37°C. Mitochondrial samples were sonicated, centrifuged at 20,000*g* (4°C) for 20 min, and the supernatant was purified by size-exclusion centrifugation through a Microcon YM-10 filter device from Millipore (Bedford, MA). Two hundred microliters of each sample was subjected to high-performance liquid chromatography analysis. The details were published recently (Daiber et al., 2004b).

Measurement of Reactive Oxygen Species Production from Isolated Heart Mitochondria and from Isolated Aortic Segments. Mitochondrial stock solutions were diluted to final total protein concentrations of approximately 0.1 mg/ml in 0.5 ml of PBS. The dye L-012 (100 μM) was used as described previously (Daiber et al., 2004a) to quantify ROS after addition of the complex II substrate succinate (final concentration, 4 mM). Chemiluminescence was monitored over 5 min using a Lumat LB9507 from Berthold Technologies (Bad Wildbad, Germany), and the signal at 5 min was expressed in counts per minute. ROS production was quantified in mitochondria from wt and Mn-SOD$^{+/-}$ mice in the presence or absence of GTN (50 μM) or antimycin A (20 μg/ml). Vascular ROS production was qualitatively detected by DHE (0.1 μM)-derived fluorescence in aortic tissue sections as described previously (Hink et al., 2001) and by L-012 (100 μM)-derived chemiluminescence from isolated aortic rings (length, 3–4 mm) in Krebs-HEPES buffer (composition as described above). Chemiluminescence was monitored over 20 min using a Lumat LB9507 and the signal at 20 min expressed in counts per minute.

Statistical Analysis. Results are expressed as mean ± S.E.M. One-way analysis of variance (with Bonferroni's or Dunn's correction for comparison of multiple means) was used for comparisons of vasodilator potency and efficacy, L-012–derived chemiluminescence, ALDH-2 dehydrogenase and esterase activity, and protein expression. The EC_{50} value for each experiment was obtained by log-transformation.

Results

Vasodilator Responses. The basal response to ACh was almost identical in both wt and Mn-SOD$^{+/-}$ mice (Fig. 1A; Table 1). Upon pretreatment of vessels with 200 μM GTN, those from Mn-SOD$^{+/-}$ mice showed a highly significant degree of endothelial dysfunction (cross-tolerance to ACh) that was absent in those from wt mice. The efficacy of ACh in Mn-SOD$^{+/-}$ aorta was dramatically changed upon GTN pretreatment (maximal relaxation, 52 ± 4% versus 69 ± 4% in untreated wt tissue). Similar results were obtained for the response to GTN (Fig. 1B; Table 1). The Mn-SOD$^{+/-}$ aorta showed a decrease in maximal relaxation compared with wt aorta. Both groups showed a significant degree of tolerance upon pretreatment with 200 μM GTN that was significantly more pronounced in aortas from Mn-SOD$^{+/-}$ mice (Fig. 1B; Table 1).

To assess the role of Mn-SOD deficiency on development of in vivo tolerance, mice were subjected to long-term infusion with GTN. In a preceding experiment, wt mice were treated with high and low doses of GTN or with solvent alone to determine the infusion rate of GTN required to induce tolerance in mice. As determined by isometric tension studies with aortic rings in organ baths, the low dose of GTN (16 μg/h) induced neither nitrate tolerance nor cross-tolerance and had a tendency to shift the ACh and GTN dose-response curve slightly to the right (Fig. 1, C and D; Table 2). In contrast, the high dose of GTN (100 μg/h) induced a marked degree of nitrate tolerance and cross-tolerance, as predicted by the right-shifted dose-response curves to ACh and GTN, and significantly decreased efficacy of both vasodilators as well as a significantly reduced potency of GTN (Fig. 1, C and D; Table 2).

To test the GTN-induced NO-signaling, we performed Western blots to determine the phosphorylation state of VASP, a ubiquitous substrate of the cGMP-dependent protein kinase. No significant difference in P-VASP levels between ethanol and GTN (16 μg/h) in vivo infusion of wt mice was observed, whereas P-VASP expression was significantly decreased in GTN-infused Mn-SOD$^{+/-}$ mice compared with the ethanol-infused control mice, respectively (Fig. 1E). P-VASP levels in both Mn-SOD-deficient groups were significantly decreased compared with the wt groups, indicating an impaired GTN bioactivation and/or NO-signaling in the deficient animals.

Mitochondrial and Vascular Reactive Oxygen Species Formation. The formation of mitochondrial ROS was detected by a chemiluminescence (CL)-based assay using the luminol analog L-012. Isolated murine heart mitochondria were assessed for basal ROS production and for ROS production in the presence of GTN and antimycin A. ROS formation

was significantly increased by 80% in Mn-SOD$^{+/-}$ mitochondria compared with wt mitochondria (56,802 ± 3919 versus 31,368 ± 2871 cpm; see Fig. 2A). In the presence of 50 μM GTN, the CL signal in Mn-SOD$^{+/-}$ mitochondria increased by 100% and that in wt mitochondria by 150% (113,611 ± 9736 versus 78,616 ± 5071 cpm). The presence of antimycin A, which preferentially induces generation of mitochondrial superoxide, significantly increased the CL signal in Mn-SOD$^{+/-}$ mitochondria by 58%, whereas the CL signal in wt mitochondria increased by 88% (89,863 ± 9550 versus 58,972 ± 5192 cpm) (Fig. 2A). Mitochondrial ROS were also detected in isolated heart mitochondria from in vivo ethanol or GTN (16 μg/h)-treated wt and Mn-SOD$^{+/-}$ mice. GTN infusion had no effect on mitochondrial ROS production of wt mice, whereas it significantly increased that in Mn-SOD$^{+/-}$ mice (Fig. 2B). In vitro challenges of isolated mitochondria from in vivo-treated animals with GTN (25 μM) elevated ROS formation in both groups, but the absolute increase was higher in Mn-SOD–deficient mice (Fig. 2B). The ROS-induced signals in ethanol, GTN (16 μg/h) in vivo, and GTN in

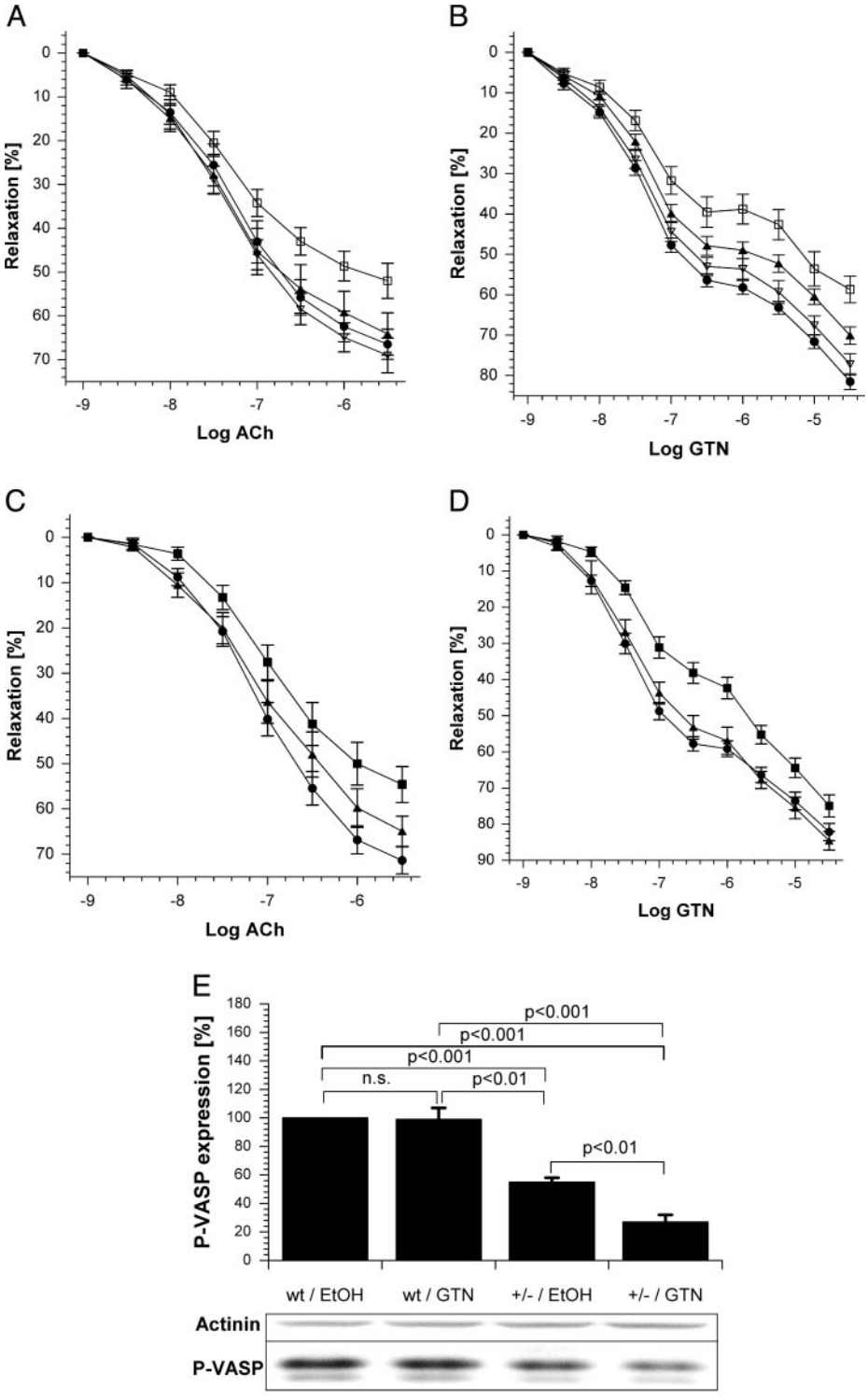

Fig. 1. Effect of heterozygous Mn-SOD deficiency on vasodilator activity of ACh and GTN in murine aorta and on development of in vitro and in vivo tolerance upon short-and long-term GTN challenges. Concentration-response curves to ACh (A) or GTN (B) in wt aorta (●), GTN (200 μM) in vitro-treated wt aorta (▲), Mn-SOD$^{+/-}$ aorta (▽), and GTN (200 μM) in vitro-treated Mn-SOD$^{+/-}$ aorta (□) were recorded upon preconstriction with prostaglandin $F_{2\alpha}$. Data represent the mean ± S.E.M. of 10 to 12 (GTN-treated) or 17 to 18 (untreated) independent experiments. Concentration-response curves to ACh (C) or GTN (D) in aorta from in vivo ethanol-treated (circles), in vivo GTN (16 μg/h)-treated (triangles), or in vivo GTN (100 μg/h)-treated wt mice (squares) were recorded upon preconstriction with prostaglandin $F_{2\alpha}$. Data represent the mean ± S.E.M. of 12 to 14 (ethanol-treated), 5 to 7 [GTN (16 μg/h)-treated], or 9 to 11 [GTN (100 μg/h)-treated] independent experiments. For statistical analysis, see Tables 1 and 2. E, P-VASP expression was determined as a measure of GTN (0.1 μM)-elicited cGMP-dependent protein kinase (cGK-I) activation (NO-signaling) in ethanol or GTN (16 μg/h) in vivo infused wt and Mn-SOD$^{+/-}$ mice. Data represent the mean ± S.E.M. of four independent experiments and a representative blot.

vivo plus in vitro treated Mn-SOD$^{+/-}$ mice were significantly higher than those in similarly treated wt mice, indicating the increased basal oxidative stress in deficient animals (Fig. 2B). Differences in the signal intensities between Fig. 2, A and B may be due to long-term ethanol infusion in the second set of experiments.

Vascular ROS formation was detected by DHE-derived fluorescence and L-012–derived chemiluminescence. Vascular ROS production from isolated aortic segments of wt mice was not significantly altered upon infusion with GTN (16 μg/h) in vivo compared with the ethanol-treated control mice (Fig. 2C). Likewise, vascular ROS formation in ethanol infused deficient mice was not significantly changed compared with the wt animals, but GTN in vivo infusion elevated the signal significantly compared with the wt treatment groups (Fig. 2C). Figure 2D shows representative DHE stainings of tissue sections from wt and Mn-SOD$^{+/-}$ aorta. The staining of Mn-SOD$^{+/-}$ material is more intense compared with wt material, indicating an increased basal production of vascular superoxide in Mn-SOD$^{+/-}$ mice.

Effects of Mn-SOD Deficiency on Mitochondrial ALDH-2 Dehydrogenase, Esterase Activity, and Vascular ALDH Dehydrogenase Activity. Basal enzyme activities were not significantly altered in Mn-SOD$^{+/-}$ compared with wt mitochondria (Fig. 3, A and B). The addition of 5 μM GTN attenuated the dehydrogenase activity in both groups. The decrease amounted to 31% in Mn-SOD$^{+/-}$ mitochondria and to only 21% in those from wt mice (decreases from 13.3 $\pm$ 0.8 to 9.2 $\pm$ 0.8 μM and 14.8 $\pm$ 0.8 to 11.7 $\pm$ 0.9 μM, respectively) (Fig. 3A). In contrast, the esterase activity in wt mitochondria was almost unaffected by pretreatment with 50 μM GTN (43.6 $\pm$ 4.9 versus 38.5 $\pm$ 6.9 μM), whereas esterase activity in Mn-SOD$^{+/-}$ mitochondria was significantly decreased by 32% in the presence of GTN (45.3 $\pm$ 4.9 versus 30.8 $\pm$ 3.4 μM) (Fig. 3B).

Vascular dehydrogenase activity in isolated aortic segments from ethanol-infused deficient mice was not significant different compared with similarly treated wt mice (Fig. 3C). GTN (16 μg/h) in vivo infusion caused no significant decrease of ALDH activity compared with ethanol controls of the same animal group, but GTN-infused Mn-SOD$^{+/-}$ mice showed a significantly lower vascular ALDH activity compared with ethanol-treated wt mice (Fig. 3C). Long-term infusion with either ethanol or an ethanolic solution of GTN increased mitochondrial ALDH-2 activity significantly ($p < 0.001$ for wt versus wt/EtOH) in comparison with noninfused animals, and ALDH-2 activity increased in deficient mice (Fig. 3, compare D and A). In vivo GTN treatment decreased ALDH-2 dehydrogenase activity in both animal groups, and the decrease was significantly stronger in deficient mice (Fig. 3D).

Discussion

We (Sydow et al., 2004) and others (Chen et al., 2002; de la Lande et al., 2004b; Kollau et al., 2004; Zhang et al., 2004) have shown previously that ALDH-2 biotransforms GTN in vitro and in vivo and that inhibition of this enzyme markedly decreases the vasodilator potency of GTN. So far, only one study has questioned the contribution of ALDH-2 to GTN bio-activation (DiFabio et al., 2003). The loss of ALDH-2 activity was associated with or was secondary to mitochondrial ROS formation upon long- or short-term challenges to GTN in vitro and in vivo (Daiber et al., 2004b; Sydow et al., 2004). With the present studies, we can demonstrate for the first time that increased oxidative stress within mitochondria from mice with heterozygous Mn-SOD deficiency (Mn-SOD$^{+/-}$) predisposes vascular tissue to develop tolerance as well as cross-tolerance (endothelial dysfunction) in response to in vitro and in vivo GTN challenges. These results point to

TABLE 1

EC_{50} values and maximal relaxation of aorta with or without acute *in vitro* challenges from wt and Mn-SOD$^{+/-}$ mice in response to ACh and GTN

In vitro GTN indicates that vessels in these groups were incubated for 30 min in the presence of 200 μM GTN, followed by a 1-h washout period before isometric tension measurement. EC_{50} values were normalized to maximal relaxation.

Group	Potency, EC_{50}		Efficacy	
	ACh	GTN	ACh	GTN
	−log M		*% maximal relaxation*	
wt	7.24 $\pm$ 0.13 (n = 18)	7.18 $\pm$ 0.05 (n = 18)	66.48 $\pm$ 3.45 (n = 18)	81.48 $\pm$ 1.95 (n = 18)
wt, in vitro GTN	7.17 $\pm$ 0.31 (n = 10)	7.10 $\pm$ 0.07 (n = 12)	64.12 $\pm$ 4.82 (n = 10)	70.05 $\pm$ 2.15‡ (n = 12)
Mn-SOD$^{+/-}$	7.23 $\pm$ 0.16 (n = 17)	7.13 $\pm$ 0.07 (n = 16)	69.02 $\pm$ 3.98 (n = 17)	77.15 $\pm$ 2.64 (n = 16)
Mn-SOD$^{+/-}$, in vitro GTN	7.25 $\pm$ 0.11 (n = 10)	6.82 $\pm$ 0.13* (n = 12)	51.99 $\pm$ 4.02† (n = 10)	58.74 $\pm$ 3.32§¶ (n = 12)

* Significance (P = 0.017) versus Mn-SOD$^{+/-}$ group.
† Significance (P = 0.036) versus Mn-SOD$^{+/-}$ group.
‡ Significance (P = 0.00187) versus wt group.
§ Significance (P = 0.00052) versus Mn-SOD$^{+/-}$ group.
¶ Significance (P = 0.0413) versus wt/in vitro GTN group.

TABLE 2

EC_{50} values and maximal relaxation in response to ACh and GTN of aorta from wt mice treated in vivo with GTN or sham (ethanol)

Presented are a separate set of experiments in which relaxation of aorta from wt mice treated in vivo with ethanol or GTN (low and high dose) was assessed. EC_{50} values were normalized on maximal relaxation.

Group	Potency, EC_{50}		Efficacy	
	ACh	GTN	ACh	GTN
	−log M		*% maximal relaxation*	
wt, in vivo ethanol	7.10 $\pm$ 0.07 (n= 14)	7,22 $\pm$ 0.04 (n = 12)	71.38 $\pm$ 2.97 (n = 14)	82.17 $\pm$ 2.38 (n = 12)
wt, in vivo GTN (16 μg/h)	7.09 $\pm$ 0.13 (n = 7)	7.03 $\pm$ 0.09 (n = 5)	64.92 $\pm$ 3.32 (n = 7)	84.61 $\pm$ 2.56 (n = 5)
wt, in vivo GTN (100 μg/h)	6.95 $\pm$ 0.11 (n = 11)	6.43 $\pm$ 0.15**† (n = 9)	54.59 $\pm$ 3.97** (n = 11)	74.96 $\pm$ 3.07*†† (n = 9)

* Significance ($P < 0.01$) versus wt, in vivo ethanol group (** means $P < 0.005$).
† Significance ($P < 0.05$) versus wt, in vivo GTN (16 μg/h) group (†† means $P < 0.01$).

a crucial role of ROS within mitochondria in determining vascular GTN biotransformation and vascular responsiveness to endothelium dependent and independent nitrovasodilators, respectively.

It is noteworthy that the degree of tolerance and cross-tolerance was markedly higher in vessels from Mn-SOD$^{+/-}$ versus wt mice (Fig. 1, A and B; Tables 1 and 2). Moreover, basal mitochondrial and vascular ROS formation and ROS production, in response to in vitro and in vivo challenges of GTN and the complex III inhibitor antimycin A, was substantially increased in Mn-SOD$^{+/-}$ animals (Fig. 2). Therefore, the redox-sensitive ALDH-2 was found to be inhibited upon in vitro and in vivo challenges with GTN, and this effect was more obvious in Mn-SOD$^{+/-}$ mice compared with wt animals (Fig. 3). In particular, the ALDH-2 esterase activity, which has been proposed to be crucial for GTN bio-activation (Chen et al., 2002) was strikingly more susceptible to GTN-mediated inactivation in Mn-SOD–deficient mice (Fig. 3).

Heterozygous Mn-SOD Deficiency Does Not Affect NO-Signaling/Endothelial Function but Makes Vessels More Susceptible to Nitrate Tolerance and Cross-Tolerance. The vasodilator potency and efficacy in response to the endothelium-dependent vasodilator ACh was not significantly different in Mn-SOD$^{+/-}$ compared with wild-type mice. This observation was in agreement with recent reports from Andresen et al. (2004) that neither the basal response to ACh was changed in Mn-SOD–deficient mice nor the ACh response upon treatment with the complex III inhibitor antimycin A, which probably would yield mitochondrial ROS. This is even more surprising because DHE staining clearly demonstrated increased ROS production throughout the vascular wall. It is interesting, however, that upon preincubation of the vessels

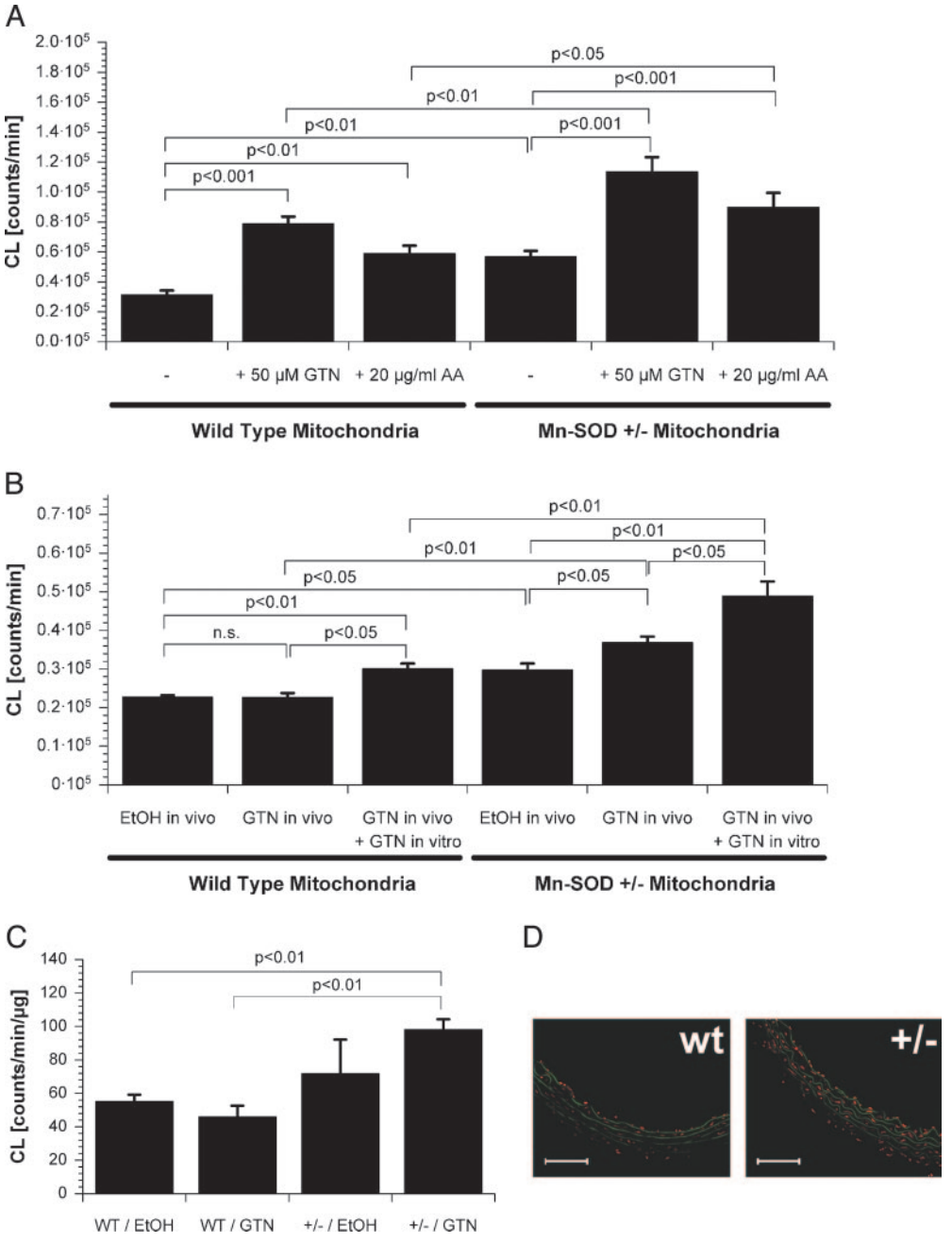

Fig. 2. Effect of heterozygous Mn-SOD deficiency on basal, antimycin A-, and GTN (in vitro and in vivo)-stimulated mitochondrial ROS formation. A, the formation of mitochondrial ROS was detected using L-012 (100 μM)-derived CL in mitochondria isolated from murine hearts. Mitochondrial suspensions (0.2 mg/ml final protein) were treated with either GTN or antimycin A (AA). The measurements were initiated by adding succinate (4 mM). Data represent the mean ± S.E.M. of 32 to 33 (basal), 18 to 19 (GTN), or 6 (AA) independent experiments. Mitochondrial (B) and vascular (C) ROS were also detected in isolated heart mitochondria from in vivo ethanol or GTN (16 μg/h)-treated wt and MnSOD$^{+/-}$ mice. The effects of short-term GTN (25 μM) challenges on mitochondrial ROS were also tested. The chemiluminescence signal (counts per minute) of vascular ROS formation was normalized to dry weight (milligrams) of aortic segments. Data represent the mean ± S.E.M. of three independent experiments. D, the basal formation of ROS was detected by microscopy using DHE (0.1 μM)-derived fluorescence in aortic tissue sections from wt or Mn-SOD$^{+/-}$ mice. The auto-fluorescence of the lamina is stained in green, and scale bars represent 100 μm. Images shown were recorded at 20× magnification and are representative of three (wt) or four (Mn-SOD$^{+/-}$) animals.

with GTN (200 μM), there was a quite marked loss of ACh efficacy in the Mn-SOD$^{+/-}$ mice that was absent in the wt animals, indicating that GTN treatment—presumably by inducing oxidative stress in mitochondria (Daiber et al., 2004b; Sydow et al., 2004)—influenced the ACh response and therefore caused cross-tolerance (endothelial dysfunction) in Mn-SOD–deficient mice (Fig. 1A; Table 1).

It is interesting that we could not detect significantly decreased responsiveness to the endothelium-independent nitrovasodilator GTN in vessels from Mn-SOD–deficient mice compared with wt control mice. Upon preincubation of the isolated vessels from wt and Mn-SOD$^{+/-}$ with GTN (200 μM), however, there was a significant loss of GTN potency in both animal groups, but it was more pronounced in the Mn-SOD$^{+/-}$ mice (Fig. 1B; Table 1). There was no significant difference in the efficacy of GTN pretreated Mn-SOD$^{+/-}$ and wt vessels (Table 1). These observations were further supported by the effects of in vivo GTN infusion (16 μg/h, 35 nmol/min/kg) in deficient mice on GTN-dependent NO-signaling (as measured by P-VASP levels; Fig. 1E). This indicates that Mn-SOD deficiency makes the vessels more susceptible to in vitro and in vivo nitrate tolerance. To address whether this phenomenon might be related to increased oxidative stress in Mn-SOD$^{+/-}$ mice, we measured superoxide production in mitochondria from wt and Mn-SOD$^{+/-}$ in the presence and absence of GTN.

Heterozygous Mn-SOD Deficiency Increases Basal, GTN-, and Antimycin A-Triggered Mitochondrial and Vascular ROS Formation, Which May Be a Key Event for the Development of Nitrate Tolerance and Cross-Tolerance. Oxidative stress plays an important role in the development of nitrate tolerance and cross-tolerance (Munzel et al., 1995b; Hink et al., 2003; Schwemmer and Bassenge, 2003). We have previously identified superoxide and/or peroxynitrite as the reactive species formed in tolerant vessels. In addition, scavengers of peroxynitrite and derived free radicals, such as ebselen and uric acid, normalized luminol-enhanced chemiluminescence in vessels from GTN-treated animals, restored the activity of the cGMP-dependent kinase I, and subsequently improved GTN tolerance in isolated rings (Hink et al., 2003). Increased vascular peroxynitrite formation also led to increased protein tyrosine nitration of the prostacyclin synthase and was associated with an almost complete inhibition of vascular prostaglandin I_2 formation in the setting of tolerance (Warnholtz et al., 2002; Hink et al., 2003). Nitration of prostacyclin synthase is a specific footprint of peroxynitrite in vivo formation (Zou et al., 1999).

Herein, we provide further evidence that a considerable part of the organic nitrate-induced oxidative stress may originate from mitochondrial nitrate metabolism. Using L-012–dependent chemiluminescence (Daiber et al., 2004a), we detected peroxynitrite and superoxide in isolated rat heart mitochondria under basal conditions as well as upon in vitro and in vivo administration of GTN or antimycin A. As expected, basal mitochondrial ROS formation was significantly increased in mitochondria from Mn-SOD$^{+/-}$ mice compared with the wt group (Fig. 2, A and B). As expected, the absolute increase upon stimulation with bolus or long-term GTN as well as antimycin A was significantly more pronounced in mitochondria from Mn-SOD$^{+/-}$–deficient animals (Fig. 2, A and B). This indeed indicates that the antioxidant defense system in Mn-SOD–deficient mitochondria is impaired and that ROS formation is increased under basal conditions. Vas-

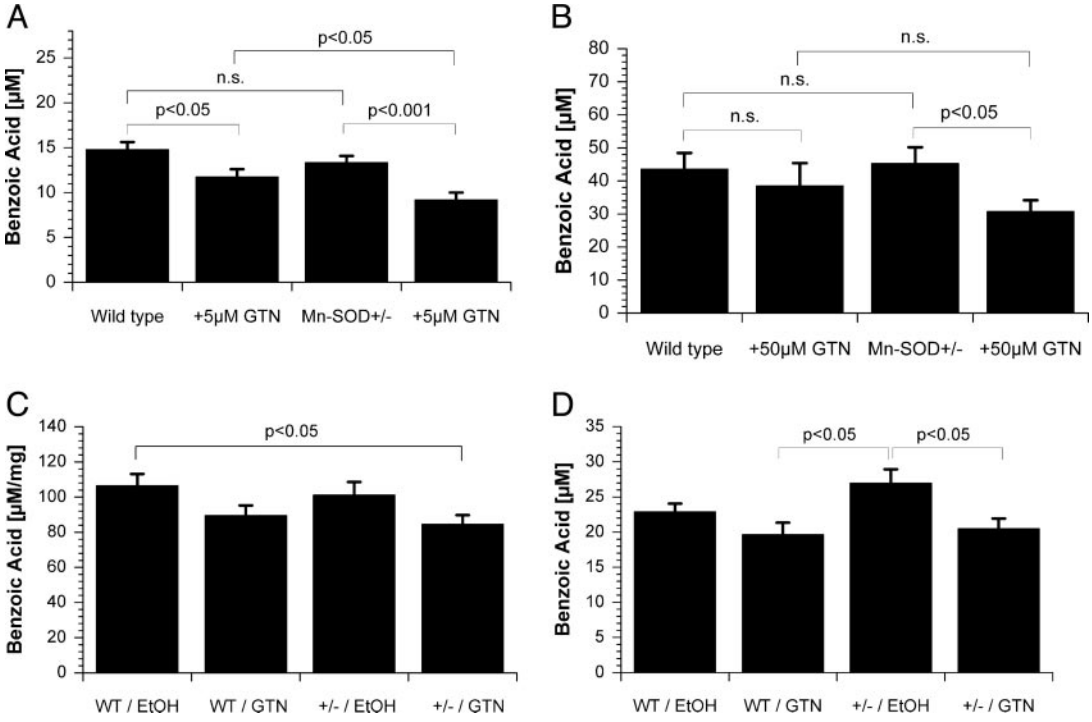

Fig. 3. Effect of heterozygous Mn-SOD deficiency on basal ALDH-2 activity and GTN-dependent inhibition of ALDH-2. The activities of ALDH-2 were determined by high-performance liquid chromatography-based measurements of the conversion of benzaldehyde (A, dehydrogenase activity) or methylbenzoate (B, esterase activity) to benzoic acid in suspensions of mouse heart mitochondria (from wt or Mn-SOD$^{+/-}$ mice) that were treated or not treated with GTN (5 or 50 μM). Data represent the mean ± S.E.M. of 15 to 20 (dehydrogenase) or 13 to 16 (esterase) independent experiments. Vascular (C) and mitochondrial (D) dehydrogenase activity was also measured in aortic segments from in vivo ethanol or GTN (16 μg/h)-treated wt or Mn-SOD$^{+/-}$ mice. Vascular dehydrogenase activity was normalized on dry weight (milligrams) of aortic segments. Data represent the mean ± S.E.M. of four (aorta) and seven (mitochondria) independent experiments.

cular ROS formation was detected by two methods (DHE-dependent oxidative fluorescent microtopography and L-012–derived chemiluminescence) and also showed that basal as well as GTN-triggered vascular ROS formation was increased in tissue from Mn-SOD$^{+/-}$ mice (Fig. 2, C and D).

Heterozygous Mn-SOD Deficiency Decreases ALDH-2 Dehydrogenase and Esterase Activity in Response to GTN and Thereby Impairs the Mitochondrial GTN Bioactivation Leading to Tolerance and Endothelial Dysfunction. The mitochondrial isoform of ALDH (ALDH-2) was previously identified as a GTN bioactivating enzyme (Chen et al., 2002). We recently demonstrated that ALDH-2 is sensitive to oxidative inactivation by different organic nitrates as well as by oxidants such as hydrogen peroxide or peroxynitrite (Daiber et al., 2004b). ALDH-2 contains three cysteine residues in the catalytic center, rendering the dehydrogenase activity highly sensitive toward oxidative inactivation (Senior and Tsai, 1990, 1991). In addition to its dehydrogenase activity, ALDH-2 also exhibits esterase activity, which has been proposed to be essential for the bioactivation of GTN (Chen et al., 2002). This activity also involves oxidation-sensitive cysteine residues (Tsai and Senior, 1991). Because of increased oxidative stress in the Mn-SOD$^{+/-}$ mitochondria, we found that ALDH-2 dehydrogenase activity and esterase activity was significantly decreased in mitochondria but also in isolated aortic rings upon in vitro or in vivo treatment with GTN (Fig. 3).

Mechanistic Implications. Mn-SOD together with glutathione peroxidase make up the most important antioxidant defense enzymes in mitochondria. Complete deficiency is disastrous for the organism and causes death within 2 to 3 weeks (Li et al., 1995; Lebovitz et al., 1996). We report herein that heterozygous Mn-SOD deficiency increases the basal but also the GTN- and antimycin A-induced formation of mitochondrial ROS. Because of GTN-induced ROS production, the GTN bioactivating enzyme ALDH-2 (Daiber et al., 2004b) or its repair system, which may involve mitochondrial lipoic acid stores and/or the glutathione/glutathione reductase system (A. Daiber, unpublished observation), will be impaired. Inactivation of ALDH-2 will subsequently slow down the mitochondrial bioactivation of GTN, which will be manifested by the phenomenon of nitrate tolerance and further point to a crucial role of this enzyme in the bioactivation process of GTN. A hypothetical unifying scheme is shown in Fig. 4. With respect to the importance of oxidative stress for the development of nitrate tolerance, it is worth mentioning that not all organic nitrates induce oxidative stress. Less potent nitrates, such as isosorbide dinitrate and isosorbide-5-mononitrate, will probably generate less mitochondrial ROS than GTN (Daiber et al., 2004b). In addition, for the highly potent pentaerythritol tetranitrate (PETN), studies have shown that this nitrate induces neither oxidative stress nor nitrate tolerance (Jurt et al., 2001). This is because of intrinsic antioxidative responses triggered by PETN, such as increased expression of the protective proteins heme oxygenase-1 and ferritin (Oberle et al., 2003).

Although GTN-triggered ROS formation within mitochondria explains the tolerance phenomenon caused by impaired GTN biotransformation, it is difficult to understand why this process should also cause the phenomenon of endothelial dysfunction, because ROS formed within mitochondria will not easily cross the mitochondrial membrane. It is possible,

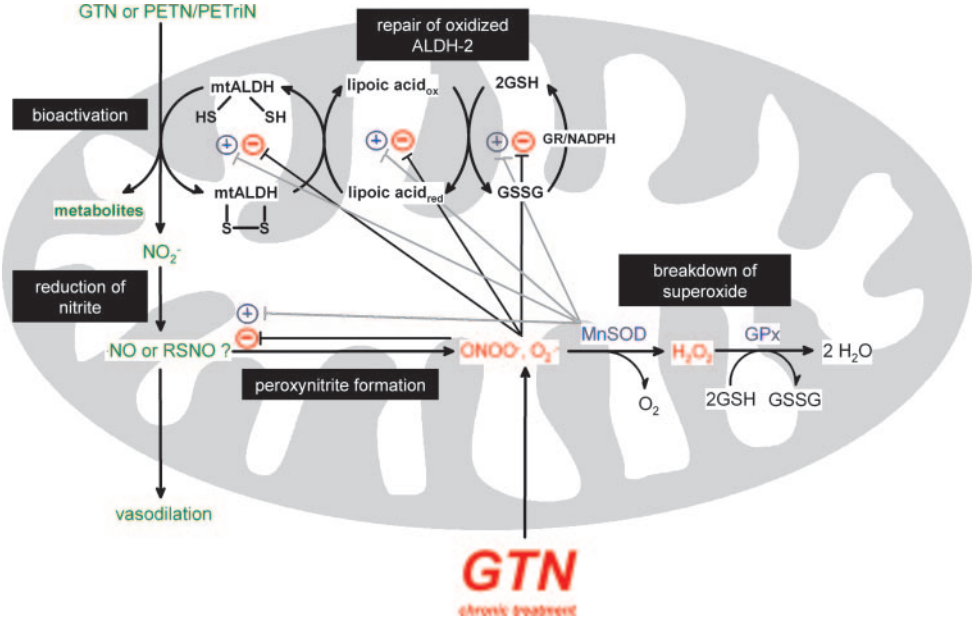

Fig. 4. Hypothetical scheme illustrating the mechanisms underlying oxidative stress-dependent development of nitrate tolerance in response to GTN treatment. Under normal conditions, GTN (as well as PETN and its trinitrate metabolite PETriN) is bioactivated by mitochondrial ALDH, yielding 1,2-glyceryl dinitrate and nitrite, which undergoes further reduction (by the mitochondrial respiratory chain or acidic disproportionation), and finally yields a vasodilator (either NO, *S*-nitrosothiol, or a related species). ALDH-2 is inactivated during bioactivation of GTN by oxidation of essential thiol groups to the disulfide. ALDH-2 reductase activity is restored by reduced lipoic acid; the oxidized form is reduced by the glutathione (GSH)-glutathione reductase system (GR/NADPH). Long-term GTN treatment induces mitochondrial ROS and reactive nitrogen species formation (ROS/RNS). The diffusion-limited reaction of nitric oxide and superoxide yields peroxynitrite ($ONOO^-$). This oxidative stress may inhibit GTN bioactivation by inactivation of ALDH-2 or inhibition of the ALDH-2 repair system, including lipoic acid as well as the glutathione/glutathione reductase system. Moreover, superoxide may decrease the bioavailability of the vasodilator released from GTN bioactivation. Mn-SOD and glutathione peroxidase (GPx) will protect the bioactivation system from oxidative damage by breakdown of superoxide and hydrogen peroxide. Mn-SOD may prevent the formation of peroxynitrite, which is a highly toxic compound to the respiratory chain at different sites of mitochondrial respiration.

however, that GTN-derived ROS (superoxide and peroxynitrite) might react with iron-sulfur cluster proteins, disrupting the respiratory chain (Flint et al., 1993). Therefore, it seems conceivable to conclude that GTN initiates a vicious cycle of mitochondrial ROS formation that could further be exaggerated by oxidative inactivation of Mn-SOD (MacMillan-Crow et al., 1996). Some of these ROS might escape the mitochondrial space and impair NO-signaling by direct reaction with NO or by an oxidative inactivation of soluble guanylyl cyclase (Brune et al., 1990; Mulsch et al., 1997). On the other hand, GTN-triggered mitochondrial ROS might lead to opening of mitochondrial ATP-dependent potassium channels (Zhang et al., 2001) and thereby trigger further ROS production (Lebuffe et al., 2003). The exact components of this molecular cascade are still not well determined, and this hypothesis remains rather speculative. However, it is interesting to note that mitochondrial ROS production and subsequent K-ATP channel opening might determine both GTN-induced protective (preconditioning-mimetic) effect (Dawn and Bolli, 2002) and, upon long-term GTN treatment, increased oxidative damage (Munzel et al., 1995b), leading to tolerance and endothelial dysfunction. Finally, we would like to emphasize that nitrate tolerance and cross-tolerance are probably multifactorial phenomena, and other processes contribute to the degree of tolerance as demonstrated by the marked effects of endothelium denudation of tolerant vessels on GTN responsiveness (de la Lande et al., 2004a; Munzel et al., 1995b). With respect to the "oxidative stress concept," there are also other sources of ROS that may trigger the development of tolerance, such as GTN-activated NADPH oxidases (Munzel et al., 1995a; Schwemmer and Bassenge, 2003), and probably an uncoupled NO-synthase.

Acknowledgments

We thank Dr. Tommaso Gori for helpful discussions. The expert technical assistance of Claudia Kuper and Yasamin Nazirizadeh is gratefully acknowledged.

References

Abrams J (1995) The role of nitrates in coronary heart disease. *Arch Intern Med* **155:**357–364.

Andresen JJ, Faraci FM, and Heistad DD (2004) Vasomotor responses in MnSOD-deficient mice. *Am J Physiol* **287:**H1141–H1148.

Brune B, Schmidt KU, and Ullrich V (1990) Activation of soluble guanylate cyclase by carbon monoxide and inhibition by superoxide anion. *Eur J Biochem* **192:**683–688.

Chen Z, Zhang J, and Stamler JS (2002) Identification of the enzymatic mechanism of nitroglycerin bioactivation. *Proc Natl Acad Sci USA* **99:**8306–8311.

Copin JC, Gasche Y, and Chan PH (2000) Overexpression of copper/zinc superoxide dismutase does not prevent neonatal lethality in mutant mice that lack manganese superoxide dismutase. *Free Radic Biol Med* **28:**1571–1576.

Daiber A, Oelze M, August M, Wendt M, Sydow K, Wieboldt H, Kleschyov AL, and Munzel T (2004a) Detection of superoxide and peroxynitrite in model systems and mitochondria by the luminol analogue L-012. *Free Radic Res* **38:**259–269.

Daiber A, Oelze M, Coldewey M, Bachschmid M, Wenzel P, Sydow K, Wendt M, Kleschyov AL, Stalleicken D, Ullrich V, et al. (2004b) Oxidative stress and mitochondrial aldehyde dehydrogenase activity: a comparison of pentaerythritol tetranitrate with other organic nitrates. *Mol Pharmacol* **66:**1372–1382.

Dawn B and Bolli R (2002) Role of nitric oxide in myocardial preconditioning. *Ann NY Acad Sci* **962:**18–41.

de la Lande IS, Siebert TE, Bennett CL, Stafford I, and Horowitz JD (2004a) Influence of the endothelium on ex vivo tolerance and metabolism of glyceryl trinitrate in rat aorta. *Eur J Pharmacol* **486:**201–207.

de la Lande IS, Stepien JM, Philpott AC, Hughes PA, Stafford I, and Horowitz JD (2004b) Aldehyde dehydrogenase, nitric oxide synthase and superoxide in ex vivo nitrate tolerance in rat aorta. *Eur J Pharmacol* **496:**141–149.

Didion SP, Ryan MJ, Didion LA, Fegan PE, Sigmund CD, and Faraci FM (2002) Increased superoxide and vascular dysfunction in CuZnSOD-deficient mice. *Circ Res* **91:**938–944.

DiFabio J, Ji Y, Vasiliou V, Thatcher GR, and Bennett BM (2003) Role of mitochondrial aldehyde dehydrogenase in nitrate tolerance. *Mol Pharmacol* **64:**1109–1116.

Flint DH, Tuminello JF, and Emptage MH (1993) The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *J Biol Chem* **268:**22369–76.

Hafner M, Wenk J, Nenci A, Pasparakis M, Scharffetter-Kochanek K, Smyth N, Peters T, Kess D, Holtkotter O, Shephard P, et al. (2004) Keratin 14 Cre transgenic mice authenticate keratin 14 as an oocyte-expressed protein. *Genesis* **38:**176–181.

Hennet T, Richter C, and Peterhans E (1993) Tumour necrosis factor-alpha induces superoxide anion generation in mitochondria of L929 cells. *Biochem J* **289 (Pt 2):**587–592.

Hink U, Li H, Mollnau H, Oelze M, Matheis E, Hartmann M, Skatchkov M, Thaiss F, Stahl RA, Warnholtz A, et al. (2001) Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ Res* **88:**E14–E22.

Hink U, Oelze M, Kolb P, Bachschmid M, Zou MH, Daiber A, Mollnau H, August M, Baldus S, Tsilimingas N, et al. (2003) Role for peroxynitrite in the inhibition of prostacyclin synthase in nitrate tolerance. *J Am Coll Cardiol* **42:**1826–1834.

Jones SP, Hoffmeyer MR, Sharp BR, Ho YS, and Lefer DJ (2003) Role of intracellular antioxidant enzymes after in vivo myocardial ischemia and reperfusion. *Am J Physiol* **284:**H277–H282.

Jurt U, Gori T, Ravandi A, Babaei S, Zeman P, and Parker JD (2001) Differential effects of pentaerythritol tetranitrate and nitroglycerin on the development of tolerance and evidence of lipid peroxidation: a human in vivo study. *J Am Coll Cardiol* **38:**854–859.

Kollau A, Hofer A, Russwurm M, Koesling D, Keung WM, Schmidt K, Brunner F, and Mayer B (2004) Contribution of aldehyde dehydrogenase to mitochondrial bioactivation of nitroglycerin. Evidence for activation of purified soluble guanylyl cyclase via direct formation of nitric oxide. *Biochem J* **385:**769–777.

Lebovitz RM, Zhang H, Vogel H, Cartwright J Jr, Dionne L, Lu N, Huang S, and Matzuk MM (1996) Neurodegeneration, myocardial injury and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc Natl Acad Sci USA* **93:**9782–9787.

Lebuffe G, Schumacker PT, Shao ZH, Anderson T, Iwase H, and Vanden Hoek TL (2003) ROS and NO trigger early preconditioning: relationship to mitochondrial KATP channel. *Am J Physiol* **284:**H299–H308.

Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH and et al. (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* **11:**376–381.

Lynch SM, Frei B, Morrow JD, Roberts LJ 2nd, Xu A, Jackson T, Reyna R, Klevay LM, Vita JA, and Keaney JF Jr (1997) Vascular superoxide dismutase deficiency impairs endothelial vasodilator function through direct inactivation of nitric oxide and increased lipid peroxidation. *Arterioscler Thromb Vasc Biol* **17:**2975–2981.

MacMillan-Crow LA, Crow JP, Kerby JD, Beckman JS, and Thompson JA (1996) Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc Natl Acad Sci USA* **93:**11853–8.

Mulsch A, Bauersachs J, Schafer A, Stasch JP, Kast R, and Busse R (1997) Effect of YC-1, an NO-independent, superoxide-sensitive stimulator of soluble guanylyl cyclase, on smooth muscle responsiveness to nitrovasodilators. *Br J Pharmacol* **120:**681–689.

Munzel T, Giaid A, Kurz S, Stewart DJ, and Harrison DG (1995a) Evidence for a role of endothelin 1 and protein kinase C in nitroglycerin tolerance. *Proc Natl Acad Sci USA* **92:**5244–5248.

Munzel T, Sayegh H, Freeman BA, Tarpey MM, and Harrison DG (1995b) Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance. *J Clin Investig* **95:**187–194.

Oberle S, Abate A, Grosser N, Hemmerle A, Vreman HJ, Dennery PA, Schneider HT, Stalleicken D, and Schroder H (2003) Endothelial protection by pentaerithrityl trinitrate: bilirubin and carbon monoxide as possible mediators. *Exp Biol Med (Maywood)* **228:**529–534.

Oelze M, Mollnau H, Hoffmann N, Warnholtz A, Bodenschatz M, Smolenski A, Walter U, Skatchkov M, Meinertz T, and Munzel T (2000) Vasodilator-stimulated phosphoprotein serine 239 phosphorylation as a sensitive monitor of defective nitric oxide/cGMP signaling and endothelial dysfunction. *Circ Res* **87:**999–1005.

Raha S, McEachern GE, Myint AT, and Robinson BH (2000) Superoxides from mitochondrial complex III: the role of manganese superoxide dismutase. *Free Radic Biol Med* **29:**170–180.

Robinson BH (1998) The role of manganese superoxide dismutase in health and disease. *J Inherit Metab Dis* **21:**598–603.

Schwemmer M and Bassenge E (2003) New approaches to overcome tolerance to nitrates. *Cardiovasc Drugs Ther* **17:**159–173.

Senior DJ and Tsai CS (1990) Esterase activity of high-Km aldehyde dehydrogenase from rat liver mitochondria. *Biochem Cell Biol* **68:**758–763.

Shull S, Heintz NH, Periasamy M, Manohar M, Janssen YM, Marsh JP, and Mossman BT (1991) Differential regulation of antioxidant enzymes in response to oxidants. *J Biol Chem* **266:**24398–403.

Strassburger M, Bloch W, Sulyok S, Schuller J, Keist AF, Schmidt A, Wenk J, Peters T, Wlaschek M, Krieg T, et al. (2005) Heterozygous deficiency of manganese superoxide dismutase results in severe lipid peroxidation and spontaneous apoptosis in murine myocardium in vivo. *Free Radical Biol Med* **38:**1458–1470.

Sydow K, Daiber A, Oelze M, Chen Z, August M, Wendt M, Ullrich V, Mulsch A, Schulz E, Keaney JF Jr, et al. (2004) Central role of mitochondrial aldehyde dehydrogenase and reactive oxygen species in nitroglycerin tolerance and cross-tolerance. *J Clin Investig* **113:**482–489.

Tsai CS and Senior DJ (1991) *Chem* studies of high-Km aldehyde dehydrogenase from rat liver mitochondria. *Biochem Cell Biol* **69:**193–197.

Warnholtz A, Mollnau H, Heitzer T, Kontush A, Moller-Bertram T, Lavall D, Giaid A, Beisiegel U, Marklund SL, et al. (2002) Adverse effects of nitroglycerin treatment on endothelial function, vascular nitrotyrosine levels and cGMP-dependent protein kinase activity in hyperlipidemic Watanabe rabbits. *J Am Coll Cardiol* **40:**1356–1363.

Wenk J, Brenneisen P, Wlaschek M, Poswig A, Briviba K, Oberley TD, and Scharffetter-Kochanek K (1999) Stable overexpression of manganese superoxide dismutase in mitochondria identifies hydrogen peroxide as a major oxidant in the AP-1-mediated induction of matrix-degrading metalloprotease-1. *J Biol Chem* **274:**25869–76.

Zhang DX, Chen YF, Campbell WB, Zou AP, Gross GJ, and Li PL (2001) Characteristics and superoxide-induced activation of reconstituted myocardial mitochondrial ATP-sensitive potassium channels. *Circ Res* **89:**1177–1183.

Zhang J, Chen Z, Cobb FR, and Stamler JS (2004) Role of mitochondrial aldehyde dehydrogenase in nitroglycerin-induced vasodilation of coronary and systemic vessels: an intact canine model. *Circulation* **110:**750–755.

Zou MH, Leist M, and Ullrich V (1999) Selective nitration of prostacyclin synthase and defective vasorelaxation in atherosclerotic bovine coronary arteries. *Am J Pathol* **154:**1359–1365.

Address correspondence to: Dr. Andreas Daiber, Klinikum der Johannes Gutenberg-Universität Mainz, II. Medizinische Klinik, Labor für Molekulare Kadiologie, Verfügungsgebäude für Forschung und Entwicklung, Raum 00349, Obere Zahlbacher Str. 63, 55101 Mainz, Germany. E-mail: andreas.daiber@bioredox.com