



Review

Nitrate tolerance as a model of vascular dysfunction: Roles for mitochondrial aldehyde dehydrogenase and mitochondrial oxidative stress

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Abstract:

Organic nitrates are a group of very effective anti-ischemic drugs. They are used for the treatment of patients with stable angina, acute myocardial infarction and chronic congestive heart failure. A major therapeutic limitation inherent to organic nitrates is the development of tolerance, which occurs during chronic treatment with these agents. The mechanisms underlying nitrate tolerance remain incompletely defined and are likely multifactorial. One mechanism seems to be a diminished bioconversion of nitroglycerin, another seems to be the induction of vascular oxidative stress, and a third may include neurohumoral adaptations. Recent studies have revealed that mitochondrial reactive oxygen species (ROS) formation and a subsequent oxidative inactivation of nitrate reductase, the mitochondrial aldehyde dehydrogenase (ALDH-2), play an important role in the development of nitrate and cross-tolerance. The present review focus first on the role of oxidative stress and second on the role of ALDH-2 in organic nitrate bioactivation leading to the development of tolerance and cross-tolerance (endothelial dysfunction) in response to nitroglycerin treatment. Recently, the role of mitochondrial oxidative stress in the development of nitrate tolerance was demonstrated in a mouse model with a heterozygous deletion of manganese superoxide dismutase (MnSOD^{+/-}), which is the mitochondrial isoform of this enzyme. Studies from our own laboratory have provided evidence for cross-talk between mitochondrial and cytosolic (Nox-dependent) sources of ROS. We close this review by focusing on the protective properties of the organic nitrate pentaerythrityl tetranitrate, which up-regulates enzymes that have strong antioxidative activity, such as heme oxygenase-1 and ferritin, thereby preventing the development of tolerance and endothelial dysfunction.

Key words:

organic nitrate, superoxide, peroxynitrite, mitochondrial aldehyde dehydrogenase, mitochondrial oxidative stress, vascular dysfunction

Clinical background

A number of cardiovascular diseases are associated with increased oxidative stress due to the formation of

reactive oxygen and nitrogen species, abbreviated as ROS and RNS [37, 38, 40]. It is important to note that these reactive species are not only byproducts of cardiovascular diseases but that they also contribute to

the development and progression of these diseases. For example, it has been shown for hypertension, hyperlipidemia, atherosclerosis and diabetes mellitus that higher levels of reactive oxygen and nitrogen species occur within the vasculature and that these species induce oxidative damage in vascular tissue [56]. This observation has been associated with endothelial dysfunction [9], which in turn has proven to be a valid early marker for cardiovascular events [34, 41]. Therefore, non-invasive flow-mediated dilation (FMD) in the forearm, invasive plethysmography (acetylcholine-dependent dilation in the forearm), and coronary vasoreactivity (acetylcholine infusion into the coronaries by a catheter) [76] are increasingly being used to determine endothelial function as a prognostic parameter. Endothelial dysfunction has been identified as a hallmark of most cardiovascular diseases [9], and it is always associated with vascular oxidative stress, decreased NO bioavailability, and/or impaired activity (uncoupling) of endothelial NO synthase [30, 61]. Conversely, patients with an impaired endothelial function have been shown to be at higher risk of cardiovascular events [41, 76]. Endothelial dysfunction of coronary arteries is associated with an increased risk of myocardial infarction [76] since dysfunctional vessels are prone to atherosclerosis and thrombus formation [87]. Coronary stenosis is another hallmark of endothelial dysfunction of the heart vasculature; depending on whether vulnerable plaques are involved or not, this condition is termed unstable or stable angina pectoris [2]. In the present review we wish to explain the basic mechanisms underlying oxidative stress-induced endothelial dysfunction and why nitroglycerin-triggered nitrate tolerance is a valid model for studying vascular oxidative stress and dysfunction.

Vascular (endothelial) dysfunction and oxidative stress

Vascular function is as essential as the heart beat, since it regulates blood pressure and thereby protects from hypertension and atherosclerosis in the long term [9, 40]. In addition, it maintains the function of the vessel walls as a blood barrier and thereby prevents leukocyte infiltration as well as inflammatory processes into the vascular wall [13, 50]. Finally, vascular function involves the release of anti-aggregation

mediators that suppress thrombus formation and vascular stenosis [94]. There is a complex network of regulatory systems of vascular tone, which consists of catecholamines, vasoactive peptides such as angiotensin-II or vasopressin, a set of vasoactive prostaglandins, and the water retention/excretion system. A central constituent of the regulatory instruments of the vascular system (especially for large vessels) is the synthesis of the endothelium-derived relaxing factor (EDRF), nitric oxide (NO), by endothelial NO synthase. NO is a potent vasodilator but also an anti-aggregation messenger [3, 72, 73]. The synthesis of the vasodilator NO, along with that of other compounds such as prostacyclin and natriuretic peptides, makes the endothelium one of the most important “organs” in the organism. At the same time this central role of the endothelium for regulating vascular tone also makes it a good target for oxidative stress, which can interfere at many points along the NO/cGMP signaling cascade [15].

Figure 1 summarizes the most important mechanisms by which reactive oxygen and nitrogen species impair vascular NO/cGMP signaling [85]. Typical risk factors for endothelial dysfunction are chronic smoking, hypertension, diabetes and hypercholesterolemia, which lead to an induction and/or (PKC-dependent) activation of vascular NADPH oxidases. NADPH oxidases produce superoxide, which reacts with NO produced by endothelial NO synthase to form peroxynitrite (ONOO^-) within the endothelium. This reaction leads to a decrease in NO bioavailability (a depletion of NO in favor of ONOO^-). Peroxynitrite is a potent oxidant that is much more potent than superoxide, and it can react directly with sulfhydryl moieties or decay to hydroxyl and nitrogen dioxide radicals that react with nearly all known biomolecules [5, 19]. Peroxynitrite is known to nitrate and inactivate prostacyclin synthase, thereby removing the potent vasodilator and anti-aggregation compound prostacyclin [46, 96]. In addition, peroxynitrite oxidizes tetrahydrobiopterin (BH_4), an essential cofactor of eNOS, thereby causing uncoupling of eNOS [49]. A semi-uncoupled NOS was reported to function as a peroxynitrite synthase, indicating that uncoupled NOS produces superoxide instead of NO, thereby turning a highly protective enzyme into a harmful one. Because of this possible switch of NOS enzymes from good to evil, they are also termed “Janus-faced” enzymes. In smooth muscle, the above-described risk factors also lead to an activation of NADPH oxidases,

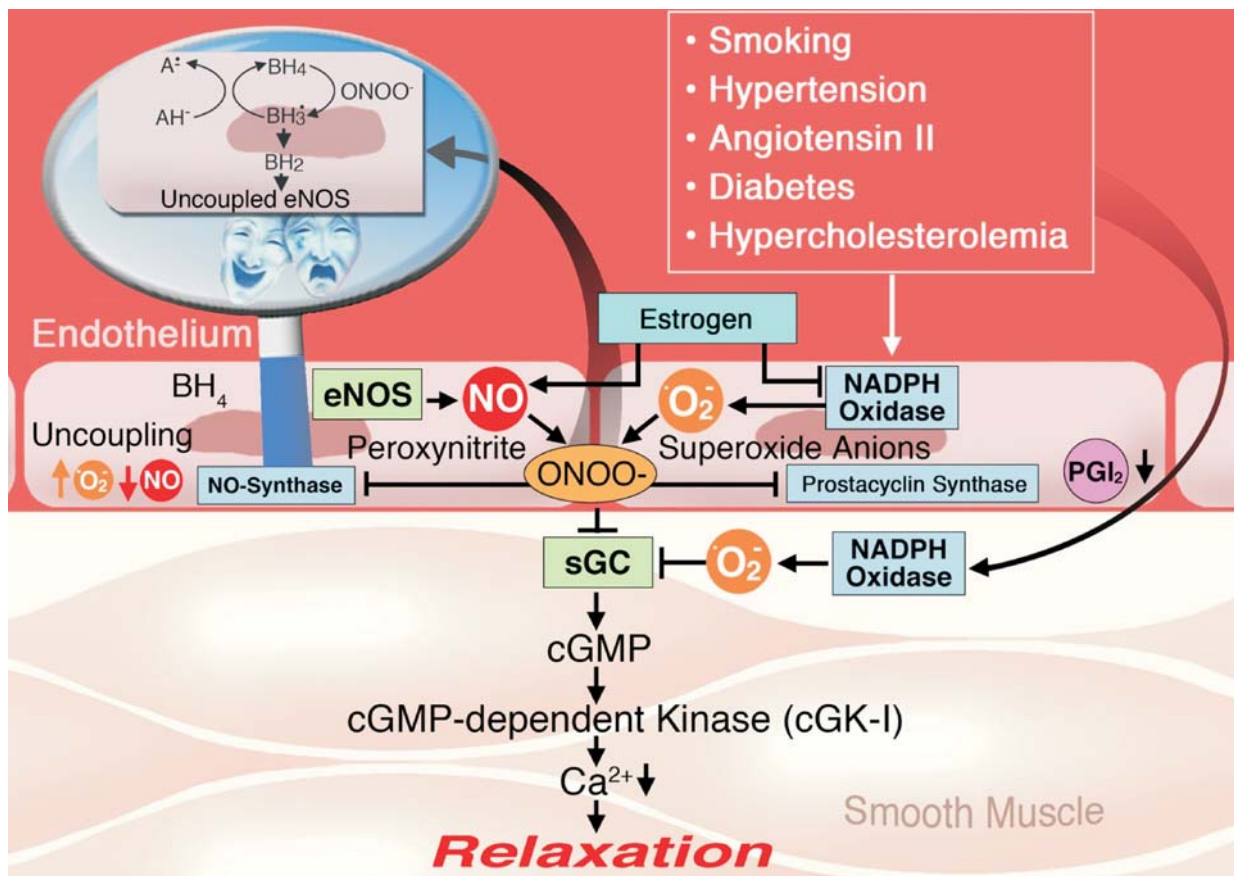


Fig. 1. Scheme illustrating the mechanisms underlying vascular (endothelial) dysfunction caused by oxidative stress. Known cardiovascular risk factors (e.g. smoking, hypertension, hyperlipidemia) increase superoxide ($O_2^{\cdot-}$) formation in endothelium and smooth muscle, both in the mitochondria and through the activation of NADPH oxidase by protein kinase C (PKC). Superoxide reacts with *NO , thereby decreasing *NO bioavailability in favor of peroxynitrite ($ONOO^-$) formation. Peroxynitrite causes uncoupling of endothelial NOS due to oxidation of tetrahydrobiopterin (BH_4) and nitration/inactivation of prostacyclin synthase (PGI_2S). Uncoupled NOS produces superoxide instead of NO, and nitrated PGI_2S produces no prostacyclin (PGI_2). Inhibition of smooth muscle soluble guanylyl cyclase (sGC) by superoxide and peroxynitrite contributes to vascular dysfunction as well as increased inactivation of cyclic GMP (cGMP) by phosphodiesterases (PDE). Adopted from Warnholtz et al. [85]. (Reproduced with the permission of Georg Thieme Verlag KG, Stuttgart, Germany.)

which may lead to a desensitization of soluble guanylyl cyclase (sGC), as well as to increased activity of phosphodiesterases (PDEs) that catalyze the breakdown of cGMP. Several additional factors contribute to vascular dysfunction: uncoupling of mitochondrial respiration, opening of the mitochondrial permeability transition pore (mPTP), formation of mitochondrial ROS and RNS, increased xanthine oxidase activity, and an increase in neutrophil and macrophage infiltration to the subendothelial tissue [4].

A number of studies using transgenic or knockout mouse models have provided molecular evidence that oxidative stress leads to vascular (endothelial) dysfunction and associated cardiovascular diseases. It has been shown that NO production in mice with deoxycorticosterone acetate (DOCA)-salt-induced hyper-

tension approaches the normal level following genetic deletion of the NADPH oxidase subunit $p47^{phox}$ and simultaneous administration with the NOS cofactor tetrahydrobiopterin [49]. These results demonstrate that NADPH oxidase-derived ROS contribute to the pathogenesis of hypertension and the associated endothelial dysfunction. Moreover, these results show that uncoupled NOS is a major source of ROS and that restoring eNOS function can cure hypertension in experimental systems. Another research group showed that in mice with myocardial infarction (MI), which significantly reduced the level of NO, genetic deletion of the NADPH oxidase subunit $p47^{phox}$ partially restored the normal bioavailability of NO [22]. At the same time, MI-induced ROS formation was lower in $p47^{phox}$ knockout mice as compared to wild type con-

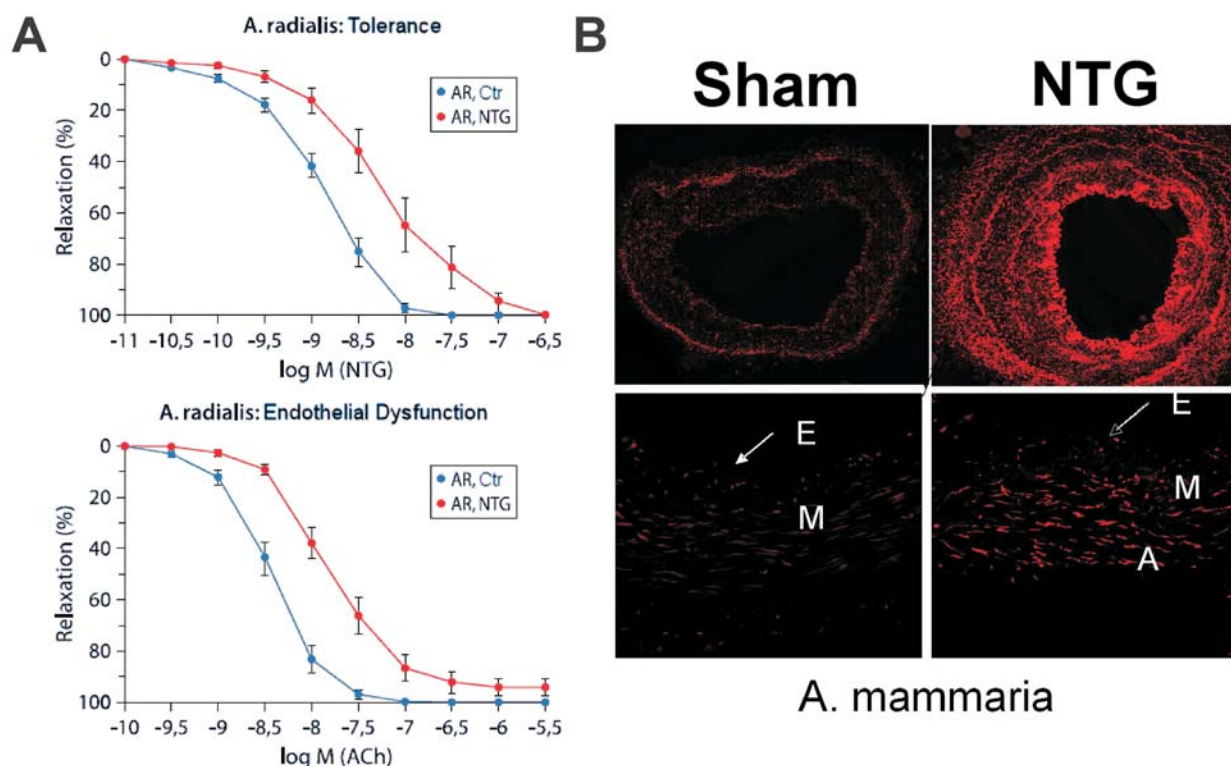


Fig. 2. Effects of treating patients undergoing bypass surgery for 48 h with nitroglycerin (NTG, 0.5 $\mu\text{g}/\text{kg}/\text{min}$ *via* infusion) on vasoreactivity of the radial artery (**left panels**) and vascular superoxide formation in aortic cryo-sections after incubation with dihydroethidine (red fluorescent staining, **right panel**). This nitroglycerin therapy leads to the development of nitrate tolerance, as demonstrated by a rightward shift in the nitroglycerin (NTG)-relaxation curve (**upper left panel**). In addition, the nitroglycerin therapy leads to the development of endothelial dysfunction, as demonstrated by a rightward shift in the acetylcholine (ACh)-relaxation curve (**lower left panel**). This loss of vascular function is accompanied by a dramatic increase in superoxide formation in the vascular wall of the mammary artery (NTG versus Sham). Adopted from Schulz et al. [77]. (Reproduced with permission of the American Heart Association.)

trols, heart function was better, and survival rate after MI was higher by 20%. Moreover, deletion of the NADPH oxidase subunits p47^{phox} and Nox1 has a protective effect on blood pressure and endothelial function in angiotensin-II-induced hypertension in mice [48, 53]. In contrast, overexpression of Nox1 in these transgenic mice causes a further increase in blood pressure [21]. There are many more examples that cannot be presented here.

Nitrate tolerance, vascular dysfunction and oxidative stress

It is well established that most organic nitrates cause nitrate tolerance and/or cross-tolerance to endothelium-dependent vasodilators such as acetylcholine

[35, 77, 78, 81]. Below we discuss pentaerythrityl tetranitrate as an example of an organic nitrate that fails to stimulate any tolerance development *in vivo*. In contrast, continuous nitroglycerin therapy lasting at least 48 h is known to significantly decrease the drug's potency in humans [75, 77] and experimental animals [58]. In the types of vessels studied, nitroglycerin caused concentration-dependent relaxation. The vessel most sensitive to nitroglycerin-induced relaxation was the A. radialis = V. saphena > A. mammaria (data for radial artery are shown in Fig. 2A). Nitroglycerin treatment caused a rightward shift of the nitroglycerin concentration-response relationship, consistent with nitrate tolerance. The degree of tolerance was most striking in A. radialis. Acetylcholine treatment in the vessel types studied caused a concentration-dependent relaxation, and this effect was greatest in A. radialis > A. mammaria > V. saphena (data for radial artery are shown in Fig. 2A). Treatment with nitroglycerin caused

a significant degree of endothelial dysfunction, as indicated by the rightward shift of the acetylcholine concentration-response relationship in *A. radialis*, whereas the changes in endothelial function observed in *A. mammaria* and *V. saphena* were less pronounced. These findings on decreased vascular reactivity went hand in hand with a dramatic increase in ROS formation throughout the vessel wall, as visualized by dihydroethidine-dependent fluorescence (Fig. 2B). In these previous studies, the integrity of NO/cGMP signal transduction was assessed by measuring phosphorylation of the vasodilator-stimulated phosphoprotein (VASP), which is a downstream target of cGMP-dependent kinase. In contrast to its lack of effect on VASP protein expression, *in vivo* treatment of patients with nitroglycerin caused a significant drop in the level of P-VASP and therefore in the P-VASP/VASP ratio in *A. mammaria* (not shown). These results demonstrate that *in vivo* treatment of patients with nitroglycerin for 48 h results in a significant degree of nitrate tolerance and endothelial dysfunction, which leads in turn to increased vascular oxidative stress and impaired NO/cGMP signaling. Therefore, nitrate tolerance obviously meets all the criteria of vascular (endothelial) dysfunction.

In addition to oxidative stress, other factors contribute to clinical tolerance [60]: one mechanism seems to be a diminished conversion of nitroglycerin to its active vasodilator metabolite. Other mechanisms likely include neurohumoral adaptations, such as increases in plasma volume, activation of the renin-angiotensin system, and increases in plasma levels of vasopressin and catecholamines. The extravascular effects serve to counteract the vasodilator and cardiac unloading actions of these agents. Cross-tolerance to other nitrovasodilators may be due to changes in the activity of the enzyme guanylate cyclase, which is the target of the nitric oxide released from these drugs, or perhaps to increases in the activity of the phosphodiesterases that degrade cGMP.

The first report on a role for oxidative stress in the development of nitrate tolerance was published in 1995 by Münzel and coworkers [66]. These authors found that superoxide levels were two-fold higher in aortic segments of nitrate-tolerant vessels with intact endothelium. Based on these findings, they suspected that the enhanced levels of superoxide in nitroglycerin-tolerant vessels might contribute not only to nitroglycerin tolerance, but also to cross-tolerance to 3-morpholino sydnonimine (Sin-1) and endogenous

NO production stimulated by acetylcholine. To test this hypothesis, they examined the effects of bovine Cu,Zn-SOD encapsulated in pH-sensitive liposomes. In nitroglycerin-tolerant aortic segments with intact endothelium, liposomal SOD markedly enhanced the relaxations evoked by nitroglycerin, Sin-1, and acetylcholine. The source of ROS formation in the case of nitrate tolerance was first thought to be the NADH oxidase since tolerant tissue homogenates showed a NADH-dependent increase in ROS formation (meanwhile it is accepted that classical Nox-dependent oxidases rather use NADPH instead of NADH as a co-factor). This finding was based mainly on the observation that the superoxide signal was largest in the presence of NADH, that it was reduced by NADPH, and that it was located in the fraction that contained mitochondria and membrane constituents (60,000 g pellet) and not in the cytosolic fraction [65]. More compelling data come from the observation that protein kinase C inhibition effectively suppressed nitroglycerin-induced vascular ROS formation and vasoconstrictor supersensitivity in tolerant vessels [62, 63]. Indirect evidence that chronic nitroglycerin treatment activates the renin-angiotensin-aldosterone system (RAAS) and therefore the angiotensin-II-dependent PKC/NADH oxidase is based on the highly protective effect of AT₁-receptor blockers [43, 47] and ACE inhibitors [6, 59], which are known to have pleiotropic effects including antioxidative properties. However, the NADH-based assay used to measure oxidase activity by lucigenin-enhanced chemiluminescence was called into question because of redox cycling of the chemiluminescent probe lucigenin. In addition, suspensions of 60,000 g pellets contain many cell organelles that complicate the identification of the ROS source in these preparations. We still believe that these former observations are correct but there is now good evidence that a large part of the signal is due to mitochondrial ROS formation and is probably not exclusively due to membrane-bound NADH oxidase. As presented in Figure 3, the 60,000 g pellet suspensions contain mitochondria and the inhibitory profile on the NADH-driven ROS signal resembles that of sonicated preparations of isolated mitochondria. Therefore, the NADH-triggered ROS signals previously observed in tolerant tissue or aortic homogenates may be due largely to mitochondrial ROS formation. As shown in Figure 3B and C, the inhibitory profile did not depend on the chemiluminescent probe used, since L-012 and lucigenin gave similar results.

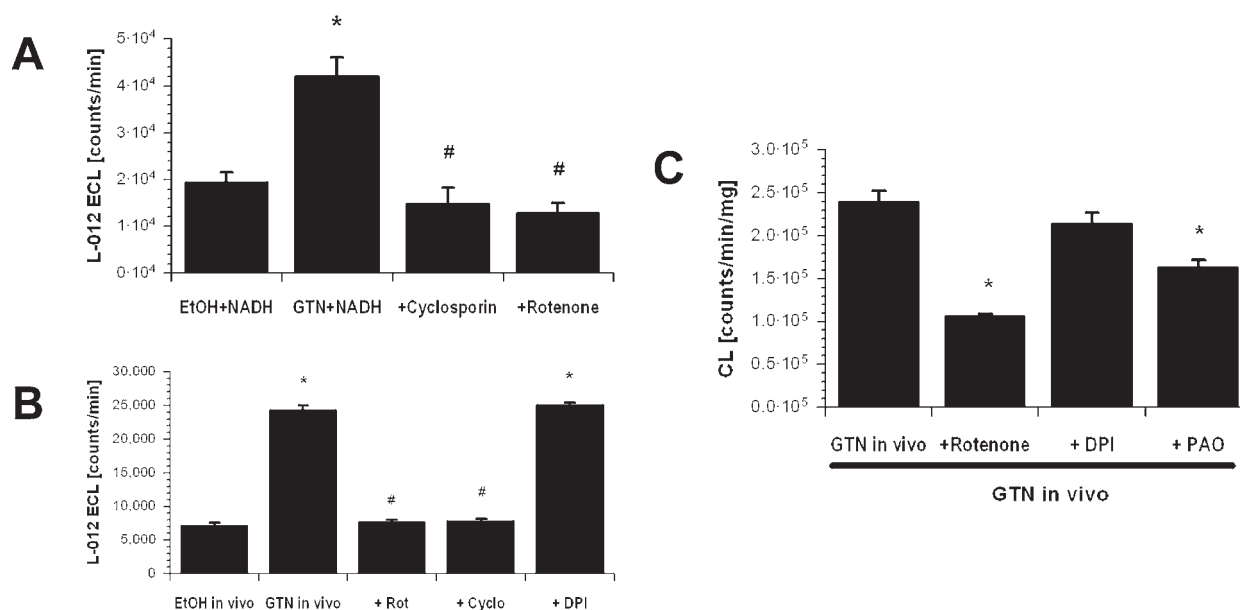


Fig. 3. Effects of nitroglycerin treatment *in vivo* (6.6 µg/kg/min for 4 d) on ROS formation from cardiac mitochondria and aortic homogenates. **(A)** ROS formation in sonicated cardiac mitochondria (0.1 mg/ml) was measured using L-012 (100 µM) ECL in the presence of NADH (200 µM) in PBS. Cyclosporine A (0.2 µM) was used as an inhibitor of mPTP and rotenone (5 µM) as an inhibitor of mitochondrial complex I. Data are the mean ± SEM of 14–16 independent experiments. * $p < 0.05$ vs. EtOH *in vivo*; # $p < 0.05$ vs. GTN *in vivo*. **(B)** ROS formation in aortic suspensions of 60,000 g pellets (0.1 mg/ml, from 750 g supernatants) was measured by L-012 (100 µM) ECL in the presence of NADH (200 µM). Inhibitors were rotenone (Rot), cyclosporine A (Cyclo) and diphenylene iodonium (DPI, 10 µM). Results were similar when 750 g fractions from aortic homogenates were used. Data are the mean ± SEM of 4–6 independent experiments. * $p < 0.05$ vs. EtOH *in vivo*; # $p < 0.05$ vs. GTN *in vivo*. **(C)** ROS formation in aortic 60,000 g fractions (from 750 g supernatants) was measured using lucigenin (250 µM) ECL in the presence of NADH (200 µM). Inhibitors were rotenone (Rot), diphenylene iodonium (DPI) and the NADPH oxidase inhibitory compound phenylarsenoxide (PAO, 10 µM). Results were similar when 750 g fractions from aortic homogenates were used. Data are the mean ± SEM of 6–10 independent experiments. * $p < 0.05$ vs. GTN *in vivo*

This excludes false-positive detection of ROS by lucigenin in this system. Similar inhibitory profiles were obtained when aortic or cardiac homogenates were stimulated by succinate, malate/glutamate or α -ketoglutarate (not shown). In addition to these reports, numerous studies exist supporting the oxidative stress concept in nitrate tolerance due to the protective effects of various antioxidants such as vitamin C, folic acid and hydralazine [14, 60].

Several studies investigated the nature of the reactive species being formed in the setting of nitrate tolerance. For example, there is good evidence that superoxide forms, since superoxide dismutases have significantly improved vascular reactivity in tolerant vessels. However, since nitroglycerin is thought to release NO as well, the formation of peroxynitrite from NO and superoxide can be expected. Indeed, some studies have reported increased levels of tyrosine-nitrated proteins in tolerant tissue, which is a marker of increased levels of peroxynitrite formation *in vivo* [86]. We have even observed higher concentrations of nitrated prostacyclin synthase and decreased prosta-

cyclin levels in tolerant tissue (Fig. 4A and B) [42]. Nitrated protein was detected by immunohistochemical methods as well as immunoprecipitation, and several controls were performed in order to exclude any false-positive staining by the 3-nitrotyrosine antibody. Nitration was prevented by the peroxynitrite scavenger ebselen and by uric acid, a scavenger of peroxynitrite-derived free radicals. Recently, we also detected 3-nitrotyrosine formation in isolated mitochondria treated with increasing levels of nitroglycerin (Fig. 4C). Indirect proof for a role of peroxynitrite in nitrate tolerance came from the observation that hydralazine, which effectively improves nitrate tolerance, is a powerful peroxynitrite scavenger and inhibitor of nitration of tyrosines in proteins [17]. Moreover, authentic or *in situ*-generated (Sin-1) peroxynitrite was most efficient at inhibiting the enzyme that activates nitroglycerin [88]. Three more independent reports have provided data that peroxynitrite plays a central role in the development and pathogenesis of nitrate tolerance [1, 28, 54].

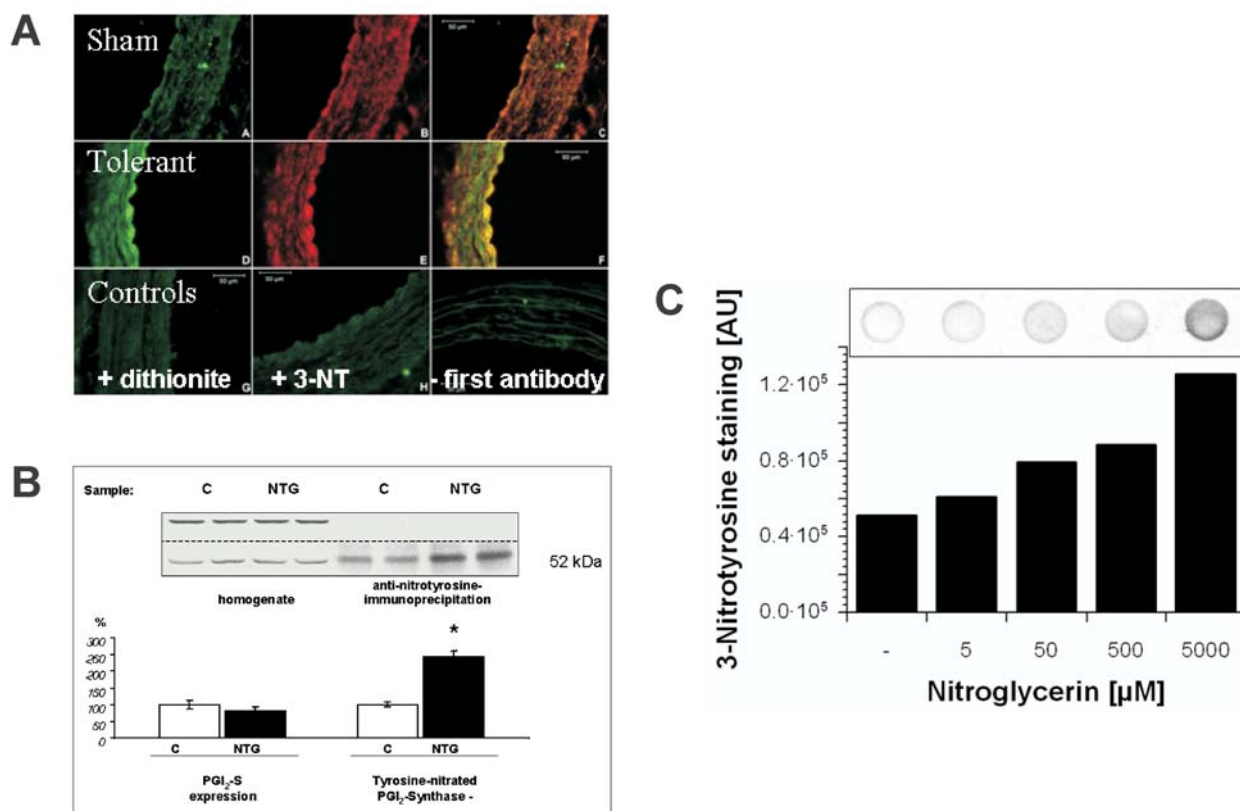


Fig. 4. (A) Immunohistochemical detection of nitrated prostacyclin-synthase (PGI₂-S) in slices of aortic rings from rats with and without nitroglycerin treatment (0.4 mg/h for 3 d). In tissue from sham-treated rats, (a) nitration was virtually absent, and (b) PGI₂-S expression was not significantly modified, and hence, (c) the overlay of both yielded only marginal background staining. In tolerant tissue, (d) nitrated protein gave a clear positive signal, (e) the amount of PGI₂-S was comparable to that in control tissue, and (f) the overlay resulted in deep yellow staining indicating co-localization of PGI₂-S and nitration. Specificity of the 3-nitrotyrosine antibody in tolerant tissue was confirmed in three control experiments: (1) the antibody was blocked by co-incubation with authentic 3-nitrotyrosine (h); (2) protein-bound 3-nitrotyrosine was reduced by sodium dithionite before antibody incubation (g); and (3) no specific staining was observed when only secondary antibody was used (i). Green fluorescence (Alexa 488-labeled secondary antibody) corresponds to nitrated protein; red fluorescence (Alexa 568-labeled secondary antibody), to PGI₂-S; and yellow, to the computer-generated overlay of both signals and, thus to nitrated PGI₂-S. (B) Western blot analysis of aortas from control and nitroglycerin (NTG)-treated rabbits. The left side shows the effects of nitroglycerin treatment on the expression of prostacyclin-synthase (PGI₂-S). The right side shows the amount of tyrosine-nitrated PGI₂-S present in 3-nitrotyrosine immunoprecipitates. $p < 0.05$ vs. control. Adopted from Hink et al. [42]. (Reproduction was granted by Rightslink®, Copyright Clearance Center.) (C) Nitrotyrosine-positive staining in mitochondria (0.1 mg/ml) treated with increasing concentrations of nitroglycerin (5, 50, 500 and 5000 μM) for 1 h. Dot blot analysis was used as described [93], and 12.5 μg of protein were loaded in each well

Mitochondrial oxidative stress and mitochondrial aldehyde dehydrogenase

The concept of NAD(P)H oxidase-driven ROS formation in nitrate tolerance was the most accepted one for nearly 10 years. It was in 2002 when Chen et al. showed that mitochondrial aldehyde dehydrogenase (ALDH-2) significantly metabolizes nitroglycerin *in vitro* and *in vivo* and that inhibition of this enzyme markedly decreases the vasodilator potency of nitroglycerin [12]. In fact, Towell et al. had already reported in 1985 that anti-anginals have antabus-like ef-

fects in red blood cells [84], suggesting a role of ALDH-2 in nitroglycerin bioactivation. In 2004 we demonstrated that ALDH-2 also functions in generating tolerance *in vivo*, and we provided the first insights into mitochondrial ROS formation in the setting of tolerance [80]. Formation of ROS in heart mitochondria from rats treated with nitroglycerin for three days was approximately 50% higher than in controls (Fig. 5A). Furthermore, incubation of isolated mitochondria from control animals with nitroglycerin (5 and 50 μM) caused a dose-dependent increase in ROS production (Fig. 5A). Uric acid (20 μM) nearly completely inhibited L-012-enhanced chemilu-

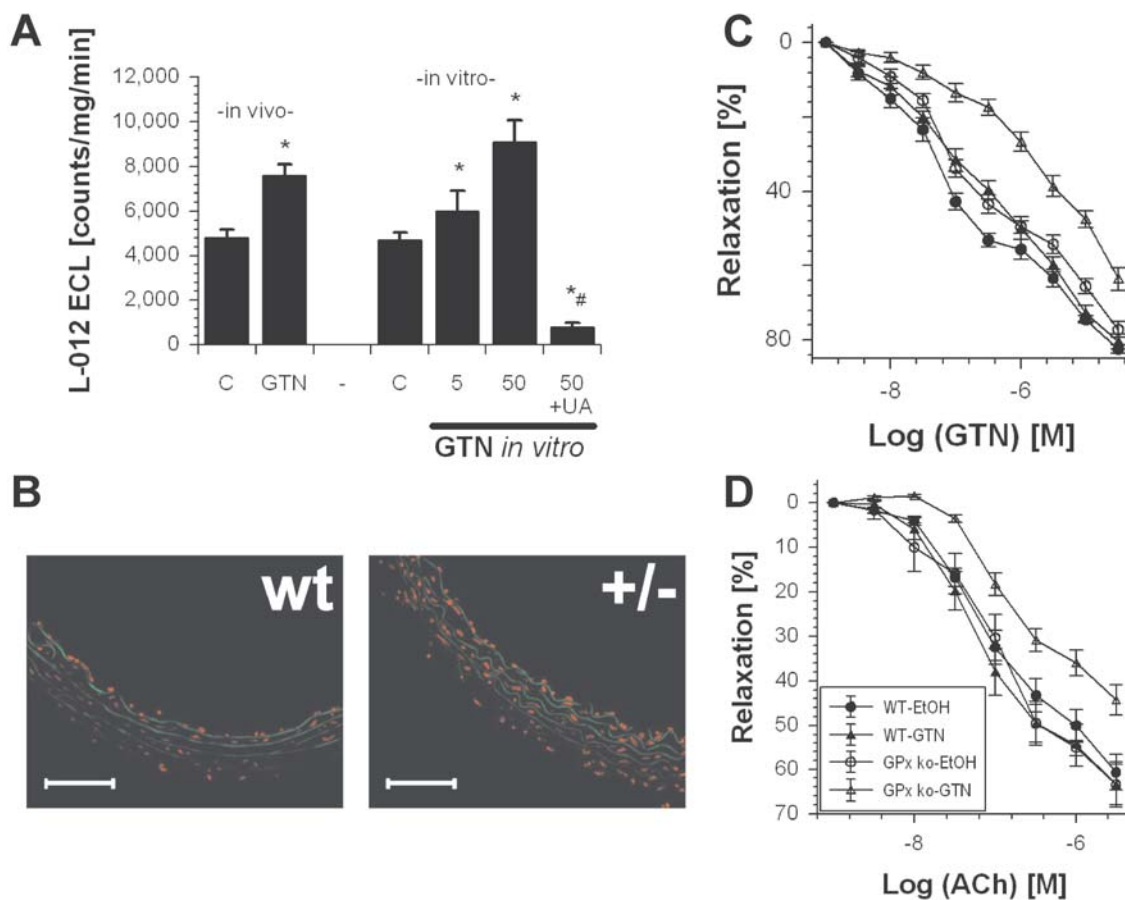


Fig. 5. (A) Effects of *in vivo* and *in vitro* nitroglycerin treatment (6.6 $\mu\text{g}/\text{kg}/\text{min}$ for 3 d) on mitochondrial production of ROS, as assessed by L-012 enhanced chemiluminescence. UA, uric acid. * $p < 0.05$ vs. control; # $p < 0.05$ vs. 50 μM GTN. Redrawn from Sydow et al. [80]. **(B)** The basal formation of ROS was detected by microscopy using fluorescence due to DHE (0.1 μM) in aortic tissue sections from wild-type 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 \times magnification. Adopted from Daiber et al. [18]. (Reproduced with the permission of the American Society for Pharmacology and Experimental Therapeutics.) **(C)** Vasodilator responses of isolated aortic vessel segments in response to chronic treatment of wild-type and Mn-SOD^{+/-} mice with low-dose nitroglycerin (12.5 $\mu\text{g}/\text{min}/\text{kg}$ for 4 d). Concentration-relaxation curves for nitroglycerin (10^{-9} to $10^{-4.5}$ M) in vessels from wild-type and Mn-SOD^{+/-} mice in response to treatment with ethanol or nitroglycerin in ethanol. The symbols are closed circles (ethanol-treated wild type), closed triangles (GTN-infused wild type), open circles (ethanol-infused Mn-SOD^{+/-}) and open triangles (GTN-infused Mn-SOD^{+/-}). Adopted from Mollnau et al. [57]. **(D)** Vasodilator responses of isolated aortic vessel segments in response to chronic treatment of wild-type and GPx-1^{-/-} mice with low-dose nitroglycerin (12.5 $\mu\text{g}/\text{min}/\text{kg}$ for 4 d). Isometric tension studies were performed as described above. The symbols are closed circles (ethanol-treated wild type), closed triangles (GTN-infused wild type), open circles (ethanol-infused GPx-1^{-/-}) and open triangles (GTN-infused GPx-1^{-/-}). Data are the mean \pm SEM of 6–11 independent experiments

minescence derived from nitroglycerin (50 μM). DTT and ebselen (each 100 μM) also completely prevented the increase in ROS caused by *in vitro* incubation with 5 mM nitroglycerin. In this study we also found that total ALDH activity was more than 50% lower in vessels from rats treated with nitroglycerin *in vivo* than in vessels from sham-treated controls. In addition, ALDH activity in isolated rat heart mitochondria was markedly inhibited by *in vitro* treatment with nitroglycerin (500 μM) and by *in vivo* treatment with nitroglycerin. Incubation of mitochondria from toler-

ant animals with DTT (2 mM) normalized ALDH activity.

A decrease of more than 50% in ALDH-2 activity was also observed when ROS formation was stimulated in isolated mitochondria using the complex III inhibitor antimycin A. Although Needleman and co-workers had already described the harmful effects of organic nitrates on mitochondria in the 1960s [44, 68], which included mitochondrial swelling, thiol depletion and impaired respiration, it took more than 40 years to reveal the pivotal role of mitochondria in

nitroglycerin toxicity [12, 16, 80]. The important role of mitochondria for nitroglycerin bioactivation is also consistent with the observation that this compound mimics ischemic preconditioning (IP) [24, 36], a process known to involve mitochondrial pathways and ROS formation [39, 51]. Interestingly, nitroglycerin-mediated ischemic preconditioning was lost in the presence of vitamin C, suggesting that ROS have an important role in this process. It should be also noted that ALDH-2 not only converts nitroglycerin to a vasodilator but also PETN and its trinitrate PETriN, but not classical di- or mononitrates (e.g. ISMN, ISDN) [16, 89].

In recent years, mitochondrial ROS have become established as central players in the nitrate tolerance that develops in response to nitroglycerin treatment *in vivo*. Therefore we hypothesized that a deficiency in mitochondrial superoxide dismutase (Mn-SOD) would render vascular tissue more susceptible to the development of tolerance. To test this hypothesis we used heterozygous deletion of manganese superoxide dismutase (Mn-SOD^{+/-}) in mice; this is the mitochondrial isoform of superoxide dismutases. The expression of Mn-SOD in Mn-SOD^{+/-} mice is approximately 50% lower than in wild-type animals, leading to distinct ultrastructural damage of the myocardium, with swelling and disruption of the 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* [79]. Basal ROS formation in vessels from Mn-SOD^{+/-} mice was significantly higher than in wild-type animals (Fig. 5B) [18]. In addition, nitroglycerin-driven vascular and mitochondrial ROS formation was higher in Mn-SOD^{+/-} mice, and also the ALDH activity in these samples was decreased by nitroglycerin in a more pronounced manner as compared to wild-type mice. Moreover, nitroglycerin potency was significantly impaired in response to low-dose nitroglycerin treatment *in vivo*, indicating that this low dose develops nitrate tolerance in Mn-SOD^{+/-} mice but not in wild-type controls (Fig. 5C) [57]. We have recently shown that Mn-SOD^{+/-} mice are also a good model for studying aging-induced mitochondrial ROS formation, mitochondrial DNA strand breaks and endothelial dysfunction [93]. A role of mitochondrial ROS for nitroglycerin-induced tolerance was shown by Esplugues et al. using mitochondria-targeted antioxidants (Mito-Q and a GSH-ester) and cells depleted of mitochondrial proteins (so-called rho⁰ cells) [27]. These authors ob-

served nitroglycerin-triggered mitochondrial complex I dysfunction and impaired respiration.

In glutathione peroxidase-1 knockout mice (GPx-1^{-/-}), treatment with low-dose nitroglycerin resulted in greater cross-tolerance to acetylcholine which is a measure for endothelial dysfunction as compared to wild-type mice, but the GPx-1 deletion had no significant effect on nitrate tolerance (nitroglycerin potency) (Fig. 5D). This came as a surprise, since several studies had previously shown that GPx-1 provides mitochondrial [33, 95] and vascular protection [83]. Therefore, it is not generally true that proteins with mitochondrial antioxidative properties prevent nitroglycerin-induced tolerance. This raises the question of which species are scavenged by these proteins: MnSOD removes superoxide and thereby prevents peroxynitrite formation; HO-1 produces bilirubin, which is a highly effective peroxynitrite scavenger; and GPx-1 catalyzes the breakdown of hydrogen peroxide. Perhaps the breakdown of mitochondrial hydrogen peroxide does not affect nitrate tolerance, but removal of cytosolic hydrogen peroxide prevents PKC activation and thereby NADPH oxidase-triggered oxidative stress and endothelial dysfunction.

This central role of mitochondrial ROS in nitrate tolerance may be explained by the structure of ALDH-2, the nitroglycerin-bioactivating enzyme. This enzyme has three adjacent cysteine thiol-groups in the active site (Fig. 6) [16]. One of these thiol groups participates directly in the enzymatic catalysis (aldehyde breakdown) of the protein. Therefore, oxidation of these thiol groups leads to inactivation of the enzyme and formation of a disulfide or at least a sulfenic acid group. This oxidative inhibition of ALDH-2 provides the missing link between the oxidative stress concept in nitrate tolerance [66] and diminished organic nitrate bioactivation [32, 69]. During the catalytic cycle of nitroglycerin bioactivation, the drug is denitrated, leading to the release of 1,2-glyceryldinitrate and the formation of a thionitrate (-SNO₂) intermediate (Fig. 7) [20]. Upon nucleophilic attack of a second adjacent thiol-group, nitrite is released and a disulfide is formed. This disulfide is either restored by dihydrolipoic acid, a natural, mitochondria-located reducing compound that has its own recycling systems (lipoamide reductase, thioredoxin reductase or glutathione reductase), or the disulfide state is "frozen" by glutathionylation, which seems to be a long-lasting inhibiting mechanism. Since an appreciable amount

Fig. 6. Crystal structure of bovine mitochondrial ALDH. The structure was rendered from the protein database file 1A4Z.pdb using the free-ware program PyMol Molecular Graphics System (version 0.93) from DeLano Scientific L.L.C. (San Carlos, CA). The structure shows one monomer of the active tetramer complex. The active site contains three cysteine thiol groups (Cys301–303). The Cys301 thiol group is near the nicotinamide ring of the cofactor NAD⁺ and probably participates in the catalytic hydride transfer from an aldehyde to the cofactor. These three cysteines provide optimal conditions for oxidation-based inactivation of the enzyme through formation of a disulfide. Adopted from Daiber et al. [16]. (Reproduced with the permission of the American Society for Pharmacology and Experimental Therapeutics.)

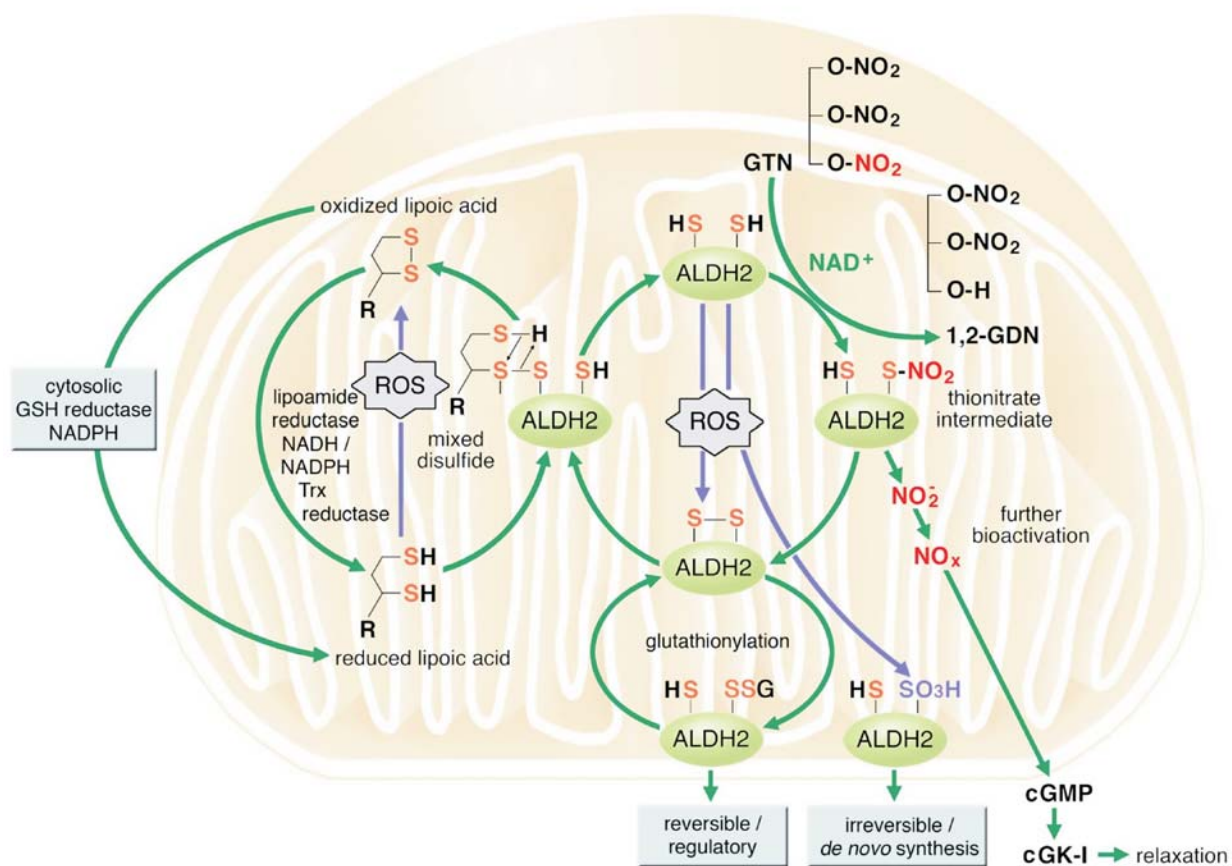


Fig. 7. Based on data from our and other laboratories, we postulate the following sequence of events for the metabolism of organic nitrates by ALDH-2. Two adjacent, reduced cysteine thiols are essential for organic nitrate bioactivation. In a first step, one of these thiols acts as a nucleophile to attack the nitrogen of the most sterically accessible nitric acid ester, yielding a thionitrate intermediate and the denitrated metabolite (1,2-GDN). Upon nucleophilic attack of the second thiol at this thionitrate, the disulfide forms and nitrite acts as the leaving group. The disulfide can also be formed when reactive oxygen and nitrogen species such as superoxide and peroxynitrite directly oxidize the thiols. In fact, more complete oxidation to sulfonic acid ($-SO_3H$) can occur, causing irreversible inhibition of the enzyme. Restoration of enzyme activity involves the dithiol compound dihydrolipoic acid, which is present in mitochondria and initially forms a mixed disulfide. Upon intramolecular nucleophilic attack this dissociates to oxidized lipoic acid and the reactivated ALDH-2. Oxidized lipoic acid is reduced by special lipoamide reductases in mitochondria or by glutathione reductases in the cytosol. In addition, this reduction of oxidized lipoic acid can be inhibited by oxidation in the setting of tolerance. The inorganic nitrite that was formed during the catalytic cycle requires further bioactivation to exert vasodilation. Adopted from Daiber et al. [20]. (Reproduced with the permission of Springer Verlag.)

of the enzyme cannot be reactivated by DTT or dihydrolipoic acid upon challenge with nitroglycerin *in vivo* or *in vitro*, we suggest that nitroglycerin-triggered ROS production causes irreversible inhibition of the enzyme *via* formation of the sulfonic acid (-SO₃H) group. We have demonstrated that dihydrolipoic acid and DTT at least partially restore the enzymatic activity of ALDH-2 in response to nitroglycerin treatment and that co-therapy with lipoic acid significantly improves nitrate tolerance in response to chronic nitroglycerin treatment [88]. Lipoic acid is already in use for the treatment of diabetic complications [7] and may gain further attention in the context of nitrate tolerance. In this previous report peroxy-nitrite was also shown to be the most efficient of all the reactive species tested at inactivating the enzyme. Superoxide was less efficient, and NO as well as hydrogen peroxide were quite ineffective. In a recent study, Dudek et al. supported our concept of ALDH-2 reactivation by lipoic acid and showed that lipoic acid co-therapy improves nitroglycerin-induced vasodilation (a decrease in blood pressure) in rabbits treated chronically with nitroglycerin [25]. According to preliminary data by Stamler and coworkers, DTT was most efficient at restoring the activity of inactivated ALDH-2, followed by dihydrolipoic acid, 2-mercaptoethanol, cysteine and glutathione [11].

Cross-talk between mitochondrial and NADPH oxidase-generated ROS

We have recently reported on the cross-talk between mtROS and cytosolic ROS/RNS in a model of increased mitochondrial oxidative stress (nitroglycerin-induced tolerance). In this system, endothelial dysfunction (sensitive to NADPH oxidases) and vascular dysfunction (sensitive to mitochondria) depended on the activation of different sources of ROS [90]. This cross-talk was blocked by *in vivo* and *ex vivo* administration of the mitochondrial permeability pore inhibitor cyclosporine A, which improved endothelial dysfunction without affecting nitrite tolerance. In contrast, the respiratory complex I inhibitor rotenone improved endothelial dysfunction and tolerance. Conversely, *in vivo* or *ex vivo* treatment with the K_{ATP} opener diazoxide caused a nitrate tolerance-like phenomenon in control animals, whereas the K_{ATP} inhibi-

tor glibenclamide improved tolerance in nitroglycerin-treated animals. Very similar effects of rotenone (Rot), cyclosporine A (CsA), diazoxide (Diaz) and glibenclamide (Glib) were recently demonstrated by another group in an experimental model of angiotensin-II induced hypertension [23]. A role for K_{ATP} channels in NADPH oxidase-driven activation of mitochondrial ROS formation *via* changes in the membrane potential has been proposed [8]. Mice homozygous for deletions of the gp91^{phox} and p47^{phox} genes developed tolerance but no endothelial dysfunction in response to nitroglycerin treatment. The findings of this study are summarized in Figure 8. The mechanism underlying this concept is based on mtROS-driven PKC activation, which in turn activates NADPH oxidases. The NADPH oxidase-dependent cytosolic ROS and RNS formation then uncouples eNOS, nitrates prostacyclin synthase and desensitizes sGC. Previous experimental studies have shown that increased oxidative stress in cellular tissue activates the oxidase through a positive feedback mechanism

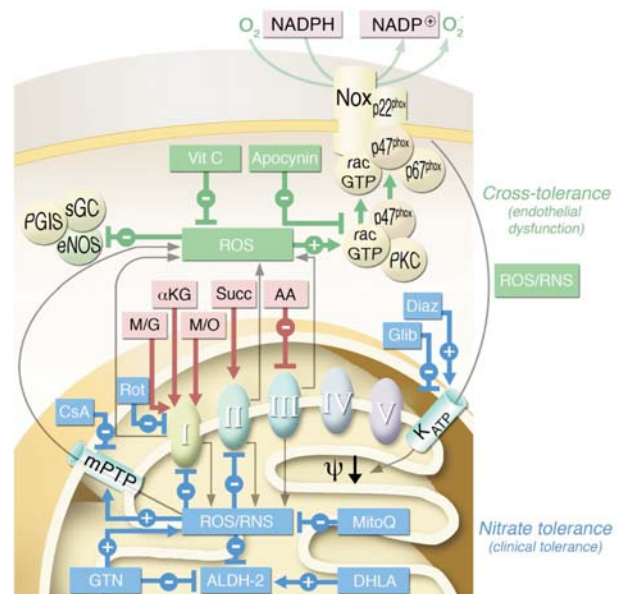


Fig. 8. Proposed hypothetical scheme of the cross-talk between mitochondrial and cytosolic (NADPH oxidase-derived) reactive oxygen and nitrogen species. CsA, cyclosporin A; Rot, rotenone; mPTP, mitochondrial permeability transition pore; Glib, glibenclamide; Diaz, diazoxide; DHLA, dihydrolipoic acid; M/G, malate/glutamate; αKG, α-ketoglutarate; M/O, malate/oxaloacetate, which decays to pyruvate; Succ, succinate; AA, antimycin A; PGIS, prostacyclin synthase; sGC, soluble guanylyl cyclase; eNOS, endothelial NO synthase; Vit C, vitamin C; PKC, protein kinase C; MitoQ, mitochondria-targeted quinone; K_{ATP}, ATP-dependent potassium channel. Adopted from Wenzel et al. [90]. (Reproduced with permission of Rightslink®, Copyright Clearance Center.)

[31]. Thus, nitroglycerin-induced mitochondrial superoxide production may cause a secondary activation of Nox. It has also been suggested that the hypotensive action of nitroglycerin activates the renin–angiotensin–aldosterone system [64], leading to increased circulating levels of angiotensin-II and aldosterone, and therefore to activation of NADPH oxidase. This concept is corroborated by the demonstration that *in vivo* treatment with an AT₁ receptor blocker prevented the development of nitroglycerin-induced endothelial dysfunction in an animal model of nitrate tolerance [47]. In addition, our findings may explain why treatment with an AT₁ receptor blocker did not prevent the development of nitroglycerin-induced nitrate tolerance in human subjects [52, 55].

We propose that a similar cross-talk exists in the aging vasculature, and that aging-induced mtROS can activate cytosolic sources of ROS and RNS, leading to age-related vascular dysfunction [93]. This proposal is based on the finding that mtROS formation increases with age and is higher in MnSOD^{+/-} mice as compared to wild-type controls, and that endothelial function is impaired with age and to a greater extent in MnSOD^{+/-} mice than in wild-type mice.

Antioxidative properties of pentaerythryl tetranitrate (PETN) and heme oxygenase-1

Previous studies have shown that long-term treatment with nitroglycerin results in clinical tolerance and endothelial dysfunction [77] and recent observations suggest that ISDN [78] and ISMN [81] induce severe endothelial dysfunction in patients. In the latter studies, endothelial dysfunction was measured by forearm blood flow in patients chronically treated with ISDN or ISMN. In contrast to other long-acting nitrates, PETN induces persistent vasodilation in humans [35, 45] and has been reported not to induce endothelial dysfunction. PETN also improved function of endothelial progenitor cells (EPC) in an experimental model of myocardial infarction [82]. In a study in humans, Jurt et al. demonstrated that PETN-treatment, in contrast to treatment with nitroglycerin, does not cause oxidative stress, based on measurements of MDA or isoprostane levels, which are indicative for increased levels of circulating lipid peroxides [45].

Recently, PETN was also shown to mimic ischemic preconditioning (IP), but in contrast to nitroglycerin, PETN-mediated IP was not inhibited by vitamin C. This observation favors an ROS-independent mechanism [24]. Previous *in vitro* studies suggest that PETN and its metabolite pentaerythryl trinitrate (PETriN) induce the antioxidant defense protein heme oxygenase-1 (HO-1), which by breaking down porphyrins produces the antioxidant molecule precursor biliverdin which is converted to bilirubin and the vasodilator carbon monoxide (CO) [70, 92]. Bilirubin is formed from biliverdin by biliverdin reductase [29]. HO-1 in turn stimulates the expression of a second antioxidant protein, ferritin, by triggering the release of free iron from endogenous heme sources [71]. HO-1 is a highly protective enzymatic system [26]. The combined effect of these defense mechanisms is to protect endothelial cells from hydrogen peroxide-induced toxicity, and they may explain the previously observed antiatherogenic actions of PETN *in vivo*. In addition, we showed recently that PETN and PETriN, in contrast to nitroglycerin, do not affect the nitrate esterase activity of ALDH-2, nor do they elicit ROS formation in isolated arteries or mitochondria. These findings provide evidence for yet another mechanism to explain the fact that PETN does not stimulate the development of tolerance [16]. Nevertheless, even more important is the observation that treatment with PETN *in vivo* induces heme oxygenase-1 and ferritin since this was never shown before [92]. Further key observations are the normalization of nitroglycerin-induced nitrate tolerance by cotreatment with hemin, which is a potent HO-1 inducer, and the induction of a tolerance-like phenomenon in PETN-treated rats by cotreatment with apigenin, an HO-1 suppressor. These results point to a crucial role for this enzyme in modulating the degree of tolerance in response to the use of organic nitrates. According to preliminary data from our group, PETN treatment induces nitrate tolerance in HO-1^{+/-} mice, and low-dose nitroglycerin treatment induces severe loss of nitroglycerin potency in these mice.

Outlook

We would like to close this overview with a personal perspective on recent developments in the fields of organic nitrates and nitrate tolerance. We presented

some preliminary data that may help to add some other pieces to the puzzle of nitrate tolerance. Recently, Chen et al. reported that activation of aldehyde dehydrogenase-2 reduces ischemic damage to the heart and, conversely, that inhibition of ALDH-2 by nitroglycerin or cyanamide treatment increased infarct area in experimental MI [10]. The cardioprotective role of ALDH-2 is well known from studies in knockout mice [74], and the antioxidative effects of ALDH-2 have been described for nitroglycerin-induced tolerance, doxorubicin-triggered vascular toxicity and aging-dependent endothelial dysfunction [91, 93]. Therefore, in addition to the oxidative damage that nitroglycerin causes directly, nitrate tolerance combined with ALDH-2 inactivation may contribute to cardiovascular risk. Based on a retrospective analysis using databases from two large-scale postinfarction studies, Nakamura et al. presented evidence that long-term nitrate therapy increases cardiovascular mortality [67]. In light of the data presented in the present review, this increased mortality may be secondary to nitrate-mediated inactivation of ALDH-2. Therefore, it may be that organic nitrates that do not inhibit ALDH-2 activity, such as PETN, may have better effects on prognosis in patients. However, future clinical studies must verify these experimental findings in the clinic.

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References:

1. Abou-Mohamed G, Johnson JA, Jin L, El-Remessy AB, Do K, Kaesemeyer WH, Caldwell RB, Caldwell RW: Roles of superoxide, peroxynitrite, and protein kinase c in the development of tolerance to nitroglycerin. *J Pharmacol Exp Ther*, 2004, 308, 289–299.
2. Abrams J: Mechanisms of action of the organic nitrates in the treatment of myocardial ischemia. *Am J Cardiol*, 1992, 70, 30B–42B.
3. Arnold WP, Mittal CK, Katsuki S, Murad F: Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. *Proc Natl Acad Sci USA*, 1977, 74, 3203–3207.
4. Bachschmid M, Schildknecht S, Ullrich V: Redox regulation of vascular prostanoid synthesis by the nitric oxide-superoxide system. *Biochem Biophys Res Commun*, 2005, 338, 536–542.
5. Beckman JS, Koppenol WH: Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. *Am J Physiol Cell Physiol*, 1996, 271, C1424–1437.
6. Berkenboom G, Fontaine D, Unger P, Baldassarre S, Preumont N, Fontaine J: Absence of nitrate tolerance after long-term treatment with ramipril: An endothelium-dependent mechanism. *J Cardiovasc Pharmacol*, 1999, 34, 547–553.
7. Bilska A, Wlodek L: Lipoic acid – the drug of the future? *Pharmacol Rep*, 2005, 57, 570–577.
8. Brandes RP: Triggering mitochondrial radical release: A new function for NADPH oxidases. *Hypertension*, 2005, 45, 847–848.
9. Cai H, Harrison DG: Endothelial dysfunction in cardiovascular diseases: The role of oxidant stress. *Circ Res*, 2000, 87, 840–844.
10. Chen CH, Budas GR, Churchill EN, Disatnik MH, Hurlley TD, Mochly-Rosen D: Activation of aldehyde dehydrogenase-2 reduces ischemic damage to the heart. *Science*, 2008, 321, 1493–1495.
11. Chen Z, Stamler JS: Bioactivation of nitroglycerin by the mitochondrial aldehyde dehydrogenase. *Trends Cardiovasc Med*, 2006, 16, 259–265.
12. Chen Z, Zhang J, Stamler JS: Identification of the enzymatic mechanism of nitroglycerin bioactivation. *Proc Natl Acad Sci USA*, 2002, 99, 8306–8311.
13. Cheng ZJ, Vapaatalo H, Mervaala E: Angiotensin II and vascular inflammation. *Med Sci Monit*, 2005, 11, RA194–205.
14. Daiber A, Gori T: Vascular tolerance to nitroglycerin in ascorbate deficiency – results are in favor of an important role of oxidative stress in nitrate tolerance. *Cardiovasc Res*, 2008, 79, 722–723.
15. Daiber A, Münzel T: Oxidative stress, redoxregulation and NO-bioavailability – experimental and clinical aspects (German). Steinkopff Verlag, Darmstadt, 2006.
16. Daiber A, Oelze M, Coldewey M, Bachschmid M, Wenzel P, Sydow K, Wendt M et al.: Oxidative stress and mitochondrial aldehyde dehydrogenase activity: A comparison of pentaerythritol tetranitrate with other organic nitrates. *Mol Pharmacol*, 2004, 66, 1372–1382.
17. Daiber A, Oelze M, Coldewey M, Kaiser K, Huth C, Schildknecht S, Bachschmid M et al.: Hydralazine is a powerful inhibitor of peroxynitrite formation as a possible explanation for its beneficial effects on prognosis in patients with congestive heart failure. *Biochem Biophys Res Commun*, 2005, 338, 1865–1874.
18. Daiber A, Oelze M, Sulyok S, Coldewey M, Schulz E, Treiber N, Hink U et al.: 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. *Mol Pharmacol*, 2005, 68, 579–588.
19. Daiber A, Ullrich V: Radical chemistry in the organism: nitrogen monoxide, superoxide and peroxynitrite (German). *Chemie in unserer Zeit*, 2002, 36, 366–375.

20. Daiber A, Wenzel P, Oelze M, Munzel T: New insights into bioactivation of organic nitrates, nitrate tolerance and cross-tolerance. *Clin Res Cardiol*, 2008, 97, 12–20.
21. Dikalova A, Clempus R, Lassegue B, Cheng G, McCoy J, Dikalov S, San Martin A et al.: Nox1 overexpression potentiates angiotensin II-induced hypertension and vascular smooth muscle hypertrophy in transgenic mice. *Circulation*, 2005, 112, 2668–2676.
22. Doerries C, Grote K, Hilfiker-Kleiner D, Luchtefeld M, Schaefer A, Holland SM, Sorrentino S et al.: Critical role of the NAD(P)H oxidase subunit p47phox for left ventricular remodeling/dysfunction and survival after myocardial infarction. *Circ Res*, 2007, 100, 894–903.
23. Doughan AK, Harrison DG, Dikalov SI: Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: Linking mitochondrial oxidative damage and vascular endothelial dysfunction. *Circ Res*, 2008, 102, 488–496.
24. Dragoni S, Gori T, Lisi M, Di Stolfo G, Pautz A, Kleinert H, Parker JD: Pentaerythryl tetranitrate and nitroglycerin, but not isosorbide mononitrate, prevent endothelial dysfunction induced by ischemia and reperfusion. *Arterioscler Thromb Vasc Biol*, 2007, 27, 1955–1959.
25. Dudek M, Bednarski M, Bilaska A, Iciek M, Sokołowska-Jeżewicz M, Filipek B, Włodek L: The role of lipoic acid in prevention of nitroglycerin tolerance. *Eur J Pharmacol*, 2008, 591, 203–210.
26. Dulak J, Deshane J, Jozkowicz A, Agarwal A: Heme oxygenase-1 and carbon monoxide in vascular pathobiology: Focus on angiogenesis. *Circulation*, 2008, 117, 231–241.
27. Esplugues JV, Rocha M, Nunez C, Bosca I, Ibiza S, Herance JR, Ortega A et al.: Complex I dysfunction and tolerance to nitroglycerin: An approach based on mitochondrial-targeted antioxidants. *Circ Res*, 2006, 99, 1067–1075.
28. Fan Q, Gao F, Zhang L, Christopher TA, Lopez BL, Ma XL: Nitrate tolerance aggravates postischemic myocardial apoptosis and impairs cardiac functional recovery after ischemia. *Apoptosis*, 2005, 10, 1235–1242.
29. Floreczyk UM, Jozkowicz A, Dulak J: Biliverdin reductase: New features of an old enzyme and its potential therapeutic significance. *Pharmacol Rep*, 2008, 60, 38–48.
30. Forstermann U, Munzel T: Endothelial nitric oxide synthase in vascular disease: From marvel to menace. *Circulation*, 2006, 113, 1708–1714.
31. Fukui T, Ishizaka N, Rajagopalan S, Laursen JB, Capers Q, Taylor WR, Harrison DG et al.: p22phox mRNA expression and NADPH oxidase activity are increased in aortas from hypertensive rats. *Circ Res*, 1997, 80, 45–51.
32. Fung HL: Biochemical mechanism of nitroglycerin action and tolerance: Is this old mystery solved? *Annu Rev Pharmacol Toxicol*, 2004, 44, 67–85.
33. Gao J, Xiong Y, Ho YS, Liu X, Chua CC, Xu X, Wang H et al.: Glutathione peroxidase 1-deficient mice are more susceptible to doxorubicin-induced cardiotoxicity. *Biochim Biophys Acta*, 2008, 1783, 2020–2029.
34. Gokce N, Keaney JF, Jr., Hunter LM, Watkins MT, Nedeljkovic ZS, Menzoian JO, Vita JA: Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease. *J Am Coll Cardiol*, 2003, 41, 1769–1775.
35. Gori T, Al-Hesayen A, Jolliffe C, Parker JD: Comparison of the effects of pentaerythritol tetranitrate and nitroglycerin on endothelium-dependent vasorelaxation in male volunteers. *Am J Cardiol*, 2003, 91, 1392–1394.
36. Gori T, Di Stolfo G, Sicuro S, Dragoni S, Lisi M, Forconi S, Parker JD: Nitroglycerin protects the endothelium from ischaemia and reperfusion: Human mechanistic insight. *Br J Clin Pharmacol*, 2007, 64, 145–150.
37. Griendling KK, FitzGerald GA: Oxidative stress and cardiovascular injury: Part I: Basic mechanisms and in vivo monitoring of ROS. *Circulation*, 2003, 108, 1912–1916.
38. Griendling KK, FitzGerald GA: Oxidative stress and cardiovascular injury: Part II: Animal and human studies. *Circulation*, 2003, 108, 2034–2040.
39. Hausenloy D, Wynne A, Duchon M, Yellon D: Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. *Circulation*, 2004, 109, 1714–1717.
40. Heistad DD: Oxidative stress and vascular disease: 2005 Duff lecture. *Arterioscler Thromb Vasc Biol*, 2006, 26, 689–695.
41. Heitzer T, Schlinzig T, Krohn K, Meinertz T, Munzel T: Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation*, 2001, 104, 2673–2678.
42. Hink U, Oelze M, Kolb P, Bachschmid M, Zou MH, Daiber A, Mollnau H et al.: Role for peroxynitrite in the inhibition of prostacyclin synthase in nitrate tolerance. *J Am Coll Cardiol*, 2003, 42, 1826–1834.
43. Hirai N, Kawano H, Yasue H, Shimomura H, Miyamoto S, Soejima H, Kajiwara I et al.: Attenuation of nitrate tolerance and oxidative stress by an angiotensin II receptor blocker in patients with coronary spastic angina. *Circulation*, 2003, 108, 1446–1450.
44. Jakschik B, Needleman P: Sulfhydryl reactivity of organic nitrates: Biochemical basis for inhibition of glyceraldehyde-P dehydrogenase and monoamine oxidase. *Biochem Biophys Res Commun*, 1973, 53, 539–544.
45. Jurt U, Gori T, Ravandi A, Babaei S, Zeman P, Parker JD: 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*, 2001, 38, 854–859.
46. Klumpp G, Schildknecht S, Nastainczyk W, Ullrich V, Bachschmid M: Prostacyclin in the cardiovascular system: New aspects and open questions. *Pharmacol Rep*, 2005, 57, Suppl, 120–126.
47. Kurz S, Hink U, Nickenig G, Borthayre AB, Harrison DG, Munzel T: Evidence for a causal role of the renin-angiotensin system in nitrate tolerance. *Circulation*, 1999, 99, 3181–3187.
48. Landmesser U, Cai H, Dikalov S, McCann L, Hwang J, Jo H, Holland SM, Harrison DG: Role of p47^{phox} in vascular oxidative stress and hypertension caused by angiotensin II. *Hypertension*, 2002, 40, 511–515.
49. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG: Oxidation of tet-

- rahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest*, 2003, 111, 1201–1209.
50. Lau D, Baldus S: Myeloperoxidase and its contributory role in inflammatory vascular disease. *Pharmacol Ther*, 2006, 111, 16–26.
51. Lim SY, Davidson SM, Hausenloy DJ, Yellon DM: Preconditioning and postconditioning: The essential role of the mitochondrial permeability transition pore. *Cardiovasc Res*, 2007, 75, 530–535.
52. Longobardi G, Ferrara N, Leosco D, Abete P, Furgi G, Cacciatore F, Corbi G et al.: Angiotensin II-receptor antagonist losartan does not prevent nitroglycerin tolerance in patients with coronary artery disease. *Cardiovasc Drugs Ther*, 2004, 18, 363–370.
53. Matsuno K, Yamada H, Iwata K, Jin D, Katsuyama M, Matsuki M, Takai S et al.: Nox1 is involved in angiotensin II-mediated hypertension: A study in Nox1-deficient mice. *Circulation*, 2005, 112, 2677–2685.
54. Mihm MJ, Coyle CM, Jing L, Bauer JA: Vascular peroxynitrite formation during organic nitrate tolerance. *J Pharmacol Exp Ther*, 1999, 291, 194–198.
55. Milone SD, Azevedo ER, Forster C, Parker JD: The angiotensin II-receptor antagonist losartan does not prevent hemodynamic or vascular tolerance to nitroglycerin. *J Cardiovasc Pharmacol*, 1999, 34, 645–650.
56. Minuz P, Fava C, Lechi A: Lipid peroxidation, isoprostanes and vascular damage. *Pharmacol Rep*, 2006, 58, Suppl, 57–68.
57. Mollnau H, Wenzel P, Oelze M, Treiber N, Pautz A, Schulz E, Schuhmacher S et al.: Mitochondrial oxidative stress and nitrate tolerance – comparison of nitroglycerin and pentaerithrityl tetranitrate in Mn-SOD^{+/-} mice. *BMC Cardiovasc Disord*, 2006, 6, 44.
58. Mülsch A, Oelze M, Kloss S, Mollnau H, Topfer A, Smolenski A, Walter U et al.: Effects of in vivo nitroglycerin treatment on activity and expression of the guanylyl cyclase and cGMP-dependent protein kinase and their downstream target vasodilator-stimulated phosphoprotein in aorta. *Circulation*, 2001, 103, 2188–2194.
59. Münzel T, Bassenge E: Long-term angiotensin-converting enzyme inhibition with high-dose enalapril retards nitrate tolerance in large epicardial arteries and prevents rebound coronary vasoconstriction in vivo. *Circulation*, 1996, 93, 2052–2058.
60. Münzel T, Daiber A, Mülsch A: Explaining the phenomenon of nitrate tolerance. *Circ Res*, 2005, 97, 618–628.
61. Münzel T, Daiber A, Ullrich V, Mülsch A: Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase. *Arterioscler Thromb Vasc Biol*, 2005, 25, 1551–1557.
62. Münzel T, Giaid A, Kurz S, Stewart DJ, Harrison DG: Evidence for a role of endothelin 1 and protein kinase C in nitroglycerin tolerance. *Proc Natl Acad Sci USA*, 1995, 92, 5244–5248.
63. Münzel T, Harrison DG: Evidence for a role of oxygen-derived free radicals and protein kinase c in nitrate tolerance. *J Mol Med*, 1997, 75, 891–900.
64. Münzel T, Heitzer T, Kurz S, Harrison DG, Luhman C, Pape L, Olschewski M, Just H: Dissociation of coronary vascular tolerance and neurohormonal adjustments during long-term nitroglycerin therapy in patients with stable coronary artery disease. *J Am Coll Cardiol*, 1996, 27, 297–303.
65. Münzel T, Kurz S, Rajagopalan S, Thoenes M, Berrington WR, Thompson JA, Freeman BA, Harrison DG: Hydralazine prevents nitroglycerin tolerance by inhibiting activation of a membrane-bound NADH oxidase. A new action for an old drug. *J Clin Invest*, 1996, 98, 1465–1470.
66. Münzel T, Sayegh H, Freeman BA, Tarpey MM, Harrison DG: Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance. *J Clin Invest*, 1995, 95, 187–194.
67. Nakamura Y, Moss AJ, Brown MW, Kinoshita M, Kawai C: Long-term nitrate use may be deleterious in ischemic heart disease: A study using the databases from two large-scale postinfarction studies. Multicenter myocardial ischemia research group. *Am Heart J*, 1999, 138, 577–585.
68. Needleman P, Hunter FE, Jr.: Effects of organic nitrates on mitochondrial respiration and swelling: Possible correlations with the mechanism of pharmacologic action. *Mol Pharmacol*, 1966, 2, 134–143.
69. Needleman P, Johnson EM, Jr.: Mechanism of tolerance development to organic nitrates. *J Pharmacol Exp Ther*, 1973, 184, 709–715.
70. Oberle S, Abate A, Grosser N, Hemmerle A, Vreman HJ, Dennery PA, Schneider HT et al.: Endothelial protection by pentaerithrityl trinitrate: Bilirubin and carbon monoxide as possible mediators. *Exp Biol Med (Maywood)*, 2003, 228, 529–534.
71. Oberle S, Schwartz P, Abate A, Schroder H: The antioxidant defense protein ferritin is a novel and specific target for pentaerithrityl tetranitrate in endothelial cells. *Biochem Biophys Res Commun*, 1999, 261, 28–34.
72. Palmer RM, Ferrige AG, Moncada S: Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, 1987, 327, 524–526.
73. Radomski MW, Palmer RM, Moncada S: The anti-aggregating properties of vascular endothelium: Interactions between prostacyclin and nitric oxide. *Br J Pharmacol*, 1987, 92, 639–646.
74. Ren J: Acetaldehyde and alcoholic cardiomyopathy: Lessons from the ADH and ALDH2 transgenic models. *Novartis Found Symp*, 2007, 285, 69–76; discussion 76–69, 198–199.
75. Sage PR, de la Lande IS, Stafford I, Bennett CL, Philipov G, Stubberfield J, Horowitz JD: Nitroglycerin tolerance in human vessels: Evidence for impaired nitroglycerin bioconversion. *Circulation*, 2000, 102, 2810–2815.
76. Schachinger V, Britten MB, Zeiher AM: Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation*, 2000, 101, 1899–1906.

77. Schulz E, Tsilimingas N, Rinze R, Reiter B, Wendt M, Oelze M, Woelken-Weckmuller S et al.: Functional and biochemical analysis of endothelial (dys)function and NO/cGMP signaling in human blood vessels with and without nitroglycerin pretreatment. *Circulation*, 2002, 105, 1170–1175.
78. Sekiya M, Sato M, Funada J, Ohtani T, Akutsu H, Watanabe K: Effects of the long-term administration of nicorandil on vascular endothelial function and the progression of arteriosclerosis. *J Cardiovasc Pharmacol*, 2005, 46, 63–67.
79. Strassburger M, Bloch W, Sulyok S, Schuller J, Keist AF, Schmidt A, Wenk J et al.: Heterozygous deficiency of manganese superoxide dismutase results in severe lipid peroxidation and spontaneous apoptosis in murine myocardium in vivo. *Free Radic Biol Med*, 2005, 38, 1458–1470.
80. Sydow K, Daiber A, Oelze M, Chen Z, August M, Wendt M, Ullrich V et al.: Central role of mitochondrial aldehyde dehydrogenase and reactive oxygen species in nitroglycerin tolerance and cross-tolerance. *J Clin Invest*, 2004, 113, 482–489.
81. Thomas GR, DiFabio JM, Gori T, Parker JD: Once daily therapy with isosorbide-5-mononitrate causes endothelial dysfunction in humans: Evidence of a free-radical-mediated mechanism. *J Am Coll Cardiol*, 2007, 49, 1289–1295.
82. Thum T, Fraccarollo D, Thum S, Schultheiss M, Daiber A, Wenzel P, Münzel T et al.: Differential effects of organic nitrates on endothelial progenitor cells are determined by oxidative stress. *Arterioscler Thromb Vasc Biol*, 2007, 27, 748–754.
83. Torzewski M, Ochsenhirt V, Kleschyov AL, Oelze M, Daiber A, Li H, Rossmann H et al.: Deficiency of glutathione peroxidase-1 accelerates the progression of atherosclerosis in apolipoprotein e-deficient mice. *Arterioscler Thromb Vasc Biol*, 2007, 27, 850–857.
84. Towell J, Garthwaite T, Wang R: Erythrocyte aldehyde dehydrogenase and disulfiram-like side effects of hypoglycemics and antianginals. *Alcohol Clin Exp Res*, 1985, 9, 438–442.
85. Warnholtz A, Buse J, Wild P, Münzel T: Prognostic value of endothelial dysfunction (German). *Kardiologie up2date*, 2006, 2, 218–225.
86. Warnholtz A, Mollnau H, Heitzer T, Kontush A, Moller-Bertram T, Lavall D, Giaid A et al.: 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*, 2002, 40, 1356–1363.
87. Wennmalm A: Endothelial nitric oxide and cardiovascular disease. *J Intern Med*, 1994, 235, 317–327.
88. Wenzel P, Hink U, Oelze M, Schuppan S, Schaeuble K, Schildknecht S, Ho KK et al.: Role of reduced lipoic acid in the redox regulation of mitochondrial aldehyde dehydrogenase (ALDH-2) activity. Implications for mitochondrial oxidative stress and nitrate tolerance. *J Biol Chem*, 2007, 282, 792–799.
89. Wenzel P, Hink U, Oelze M, Seeling A, Isse T, Bruns K, Steinhoff L et al.: Number of nitrate groups determines reactivity and potency of organic nitrates: A proof of concept study in ALDH-2^{-/-} mice. *Br J Pharmacol*, 2007, 150, 526–533.
90. Wenzel P, Mollnau H, Oelze M, Schulz E, Wickramanayake JM, Muller J, Schuhmacher S et al.: First evidence for a crosstalk between mitochondrial and NADPH oxidase-derived reactive oxygen species in nitroglycerin-triggered vascular dysfunction. *Antioxid Redox Signal*, 2008, 10, 1435–1447.
91. Wenzel P, Muller J, Zurmeyer S, Schuhmacher S, Schulz E, Oelze M, Pautz A et al.: ALDH-2 deficiency increases cardiovascular oxidative stress – evidence for indirect antioxidative properties. *Biochem Biophys Res Commun*, 2008, 367, 137–143.
92. Wenzel P, Oelze M, Coldewey M, Hortmann M, Seeling A, Hink U, Mollnau H et al.: Heme oxygenase-1. A novel key player in the development of tolerance in response to organic nitrates. *Arterioscler Thromb Vasc Biol*, 2007, 27, 1729–1735.
93. Wenzel P, Schuhmacher S, Kienhofer J, Muller J, Hortmann M, Oelze M, Schulz E et al.: MnSOD and ALDH-2 deficiency increase mitochondrial oxidative stress and aggravate age-dependent vascular dysfunction. *Cardiovasc Res*, 2008, 80, 280–289.
94. Willerson JT, Golino P, Eidt J, Campbell WB, Buja LM: Specific platelet mediators and unstable coronary artery lesions. Experimental evidence and potential clinical implications. *Circulation*, 1989, 80, 198–205.
95. Xiong Y, Shie FS, Zhang J, Lee CP, Ho YS: The protective role of cellular glutathione peroxidase against trauma-induced mitochondrial dysfunction in the mouse brain. *J Stroke Cerebrovasc Dis*, 2004, 13, 129–137.
96. Zou MH, Ullrich V: Peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibits bovine aortic prostacyclin synthase. *FEBS Lett*, 1996, 382, 101–104.

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