Supplementary information to “Explaining the phenomenon of nitrate tolerance” by Munzel et al

GTN downstream signaling.

Pre-eminent cGMP effectors are cGMP-dependent protein kinases (cGK-I and cGK-II) and cyclic nucleotide-gated (CNG) ion channels. The mechanisms whereby cGK-I, the isoform expressed in vascular smooth muscle, lowers agonist-induced contractile tone have been reviewed in detail recently and are only shortly summarized here. Principal vasodilating mechanisms include a cGMP/cGK-I-induced decrease of agonist-induced intracellular free Ca\textsuperscript{2+} levels in smooth muscle cells and a desensitization of contractile elements to Ca\textsuperscript{2+}. The former is accomplished by several independent processes: A cGK-I-induced phosphorylation of the IP\textsubscript{3}-receptor-associated cGK-substrate (IRAG) prevents IP\textsubscript{3}-dependent release of Ca\textsuperscript{2+} from the sarcoplasmatic reticulum. Secondly, cGK-I reduces extracellular Ca\textsuperscript{2+} entry through potential-operated channels by phosphorylation of the Ca\textsuperscript{2+}-activated (Maxi)K\textsuperscript{+}-channel (BK\textsubscript{Ca}), thereby increasing the open probability of this channel and hyperpolarizing the smooth muscle cell membrane. Furthermore, cGK-I may accelerate Ca\textsuperscript{2+} uptake into the SR by phospholamban-dependent and/or -independent activation of the SR Ca\textsuperscript{2+}-ATPase (SERCA) thereby also inhibiting capacitative Ca\textsuperscript{2+} entry via Ca\textsuperscript{2+}-store-operated Ca\textsuperscript{2+} channels in smooth muscle and endothelial cells. In addition, contractile elements are desensitized to Ca\textsuperscript{2+} by cGK-I-dependent activation of myosin light chain (MLC) phosphatase, and by inhibition of RhoA/Rho-kinase-dependent intracellular contractile signaling, both resulting in dephosphorylation of MLC.

The endothelium might influence the mechanism of GTN-induced
vasorelaxation\textsuperscript{8}. In endothelium intact coronary arteries GTN relaxation was partially inhibited by iberiotoxin (specific inhibitor of BK\textsubscript{Ca}-channels), though not in endothelium denuded vessels. Thus, GTN relaxes endothelium-intact vessels also by activating endothelial and/or smooth muscle cell BK\textsubscript{Ca}-channels. This effect appeared to be mediated by sGC/cGMP, since relaxations to GTN were sensitive to inhibition by the sGC inhibitor NS2028\textsuperscript{9}, and NS2028-insensitive relaxation was not blocked by iberiotoxin.

GTN can also affect vascular eicosanoid production\textsuperscript{10}, which lead to the speculation that GTN might affect vascular tone by this mechanism, too. Indeed, GTN was shown to activate cyclooxygenase 1 (COX-1)\textsuperscript{11}, leading to enhanced PGI\textsubscript{2}-formation in cultured endothelial cells and vascular tissue\textsuperscript{12,13}.

**The low potency pathway for bioactivation of GTN**

The low potency pathway leads to formation of measurable amounts of NO in vascular tissues in vivo\textsuperscript{14} and in vitro\textsuperscript{15} (print version figure 1). Therefore, NO is a vasoactive principle of of GTN applied in higher concentrations. Previous studies, which focused on the identification of enzymes and/or low molecular mass factors which could generate NO from GTN, identified cysteine, N-acetyl-cysteine and thiosalicylic acid\textsuperscript{16,17}, as well as deoxyhemoglobin, deoxymyoglobin\textsuperscript{18}, cytochrome P\textsubscript{450} (CYP)\textsuperscript{19} and xanthine oxidase\textsuperscript{20}. Since the non-enzymatic reaction of GTN with thiols requires high concentrations (mM) of these thiols as well as GTN (µM range) this reaction may lack physiological significance. While NO formation by desoxy-hemoproteins and xanthine oxidase will be confined to tissues of low oxygen tension (hypoxia), the CYP pathway is likely to account for NO formation from GTN accumulating in high concentrations in liver, lung and kidney\textsuperscript{14}. Induction of hepatic
CYP isoforms by glucocorticoids and other agents increases NO and cGMP formation from GTN in these tissues\textsuperscript{21}, whereas 3d infusion of GTN decreases hepatic CYP expression\textsuperscript{22,23}. A similar effect was not observed with sodium nitroprusside infusion, indicating that the decrease of CYP expression may specifically depend on GTN metabolism by CYP.

In addition to its function in hepatic metabolism of GTN CYP is also a favorable candidate for catalyzing NO formation from GTN in vascular tissues exposed to high concentrations of GTN (figure 1 print version). Different isoforms of CYP have been shown to account for NO release from 10 µM GTN in isolated blood vessels of human\textsuperscript{24} and animal origin, the most active CYP isoform in rats being CYP1A2\textsuperscript{25}. Induction of vascular CYP1A2 by 5d treatment of rats with i.p. acetone strongly increased NO release from GTN by isolated veins, abdominal arteries and thoracic aorta, whereas down-regulation of CYP1A2 by 48 h GTN infusion decreased vascular NO formation\textsuperscript{25}.

\textit{The high potency pathway for bioactivation of GTN}

More recently Chen et al. suggested that the mitochondrial isoform of aldehyde dehydrogenase (ALDH-2) is responsible for bioactivation of GTN (figure 1 print version)\textsuperscript{26}. The isolated enzyme generates nitrite (NO\textsubscript{2}\textsuperscript{-}) and glycerol-1,2-dinitrate from GTN, and the reaction is accelerated by NAD\textsuperscript{+} probably by an allosteric action. Unspecific inhibitors of this enzyme (disulfiram, cyanamide, chloral hydrate) and high substrate concentrations (acetaldehyde) attenuated the vasorelaxing, cGMP-eliciting and blood pressure lowering activity of GTN in rats, and inhibited the organic nitrate reductase activity of ALDH-2. Since the enzyme is enriched in the mitochondria isolated mitochondria can mediate activation of isolated sGC by GTN in
a NAD⁺-dependent fashion, and this activation was acutely prevented by daidzin, a highly specific inhibitor of ALDH-2. Using isolated rat aortic rings we recently demonstrated a marked attenuation of the GTN vasodilator potency following incubation with acetaldehyde and choral hydrate, as previously observed in rabbit aortic rings, as well as with benomyl and daidzin. In addition, activation of cGK-I (as assessed by P-VASP) and vasodilation by GTN were markedly inhibited by the ALDH-2 inhibitor benomyl (10µM), whereas benomyl did not modify SNP- or ACh-induced phosphorylation of VASP and vasorelaxation.

We also showed that treatment of RAW 264.7 macrophages with GTN or exposure to benomyl or daidzin reduced GTN bioactivation (1,2-GDN formation). These results confirmed the observations by Chen et al. and pointed to a specific role of ALDH-2 in the cGMP-mediated GTN induced vasorelaxation. By depleting endothelial cells of functional mitochondria (so-called \( \rho^0 \) cells) by means of 5 d exposure to ethidiumbromide we could show that GTN-stimulated increases in cGMP were markedly attenuated by this treatment. Interestingly, inhibition of ALDH-2 by benomyl did not completely abolish the vasodilator and cGK-I stimulating activity of GTN. The concentration-response curve was shifted to the right and with higher concentrations of GTN a maximal relaxant response was still achieved. This finding supports our hypothesis of two independent pathways accounting for bioactivation of GTN, were only the high potency pathway is dependent on ALDH-2 activity (figure 1 print version). This mechanism has been proposed to explain the nitrate reductase activity of ALDH-2. The enzyme also exhibits nitrate-esterase activity which forms a hypothetic thionitrate-intermediate from GTN in its active center, with concomitant release of glycerol-1,2-dinitrate. The intermediate then spontaneously stabilizes by nucleophilic attack of an adjacent second cystein thiol-group under formation of a
disulfide bridge and with nitrite as the leaving group. The inactive thiol-oxidized enzyme can be reduced and re-activated by thiol donors like dithiothreitol and 2-mercaptoethanol. Our recent observations suggest that mitochondrial lipoic acid may function as the natural reducing agent (A. Daiber, unpublished observations). Chen et al. suggested that NO could form from NO$_2^-$ either via intermediacy of nitrous acid (HNO$_2$), or by catalysis of components of the mitochondrial respiratory chain, like cytochrome c oxidase (figure 1 print version). NO formation from therapeutic concentrations of GTN (< 1 µM) by this enzyme or isolated mitochondria has, however, not been demonstrated yet. The important role of ALDH-2 for NTG bioactivation was further substantiated by recent studies$^{30,31}$. Zhang et al. showed that GTN-induced increases in coronary blood flow in chronically instrumented dogs were completely abolished using the ALDH-2-inhibitor cyanamide. In principle this concept is a revival of the Needleman “thiol theory” which already suggested an interaction of organic nitrates with the mitochondria (swelling and increased oxygen uptake) as well as a depletion of mitochondrial thiol pools$^{32}$.

**Effect of GTN-induced superoxide/peroxynitrite production on prostacyclin synthase.**

GTN induced production of peroxynitrite may also adversely affect the activity of the PGI$_2$-synthase (PGI-S) (figure 2 print version). Recent studies have shown that PGI-S is a preferential nitration target of peroxynitrite$^{33}$. More importantly, tyrosine nitration of PGI-S presumably at tyrosine 430$^{34}$ resulted in an almost complete inhibition of the enzyme activity leading to decreased PGI$_2$ formation$^{33}$. Since the non-metabolized PGH$_2$ can activate the TxA$_2$/PGH$_2$ receptor of vascular smooth muscle cells and thereby counteract GTN-mediated vasodilation, peroxynitrite can be
considered as a mediator of endothelial dysfunction as well as of "nitrate tolerance" at the level of the smooth muscle. Our recent data obtained in nitrate tolerant rats and rabbits go along with this concept. GTN treatment increased the luminol-derived chemiluminescence signal in rat and rabbit aorta, which was effectively inhibited by peroxynitrite quenchers such as uric acid as well as by ebselen, compatible with increased vascular peroxynitrite formation. Western blots of 3-nitrotyrosine immunoprecipitates exposed to a polyclonal antibody directed against PGI-S detected a single 52 kDa protein band and revealed a marked increase in the PGI-S signal from tolerant aortas compared to controls, while total PGI-S expression was not modified by GTN tolerance. The immunoprecipitation results were in accordance with immunohistochemical stainings of control and tolerant aorta with PGI-S and 3-nitrotyrosine antibodies showing increased nitration of PGI-S within the endothelium of tolerant vessel sections. As a functional consequence of tyrosine nitration of PGI-S, the conversion of [14C]-PGH₂ into [14C]-PGI₂ (measured by its stable metabolite 6-keto-PGF₁α) was markedly inhibited. We also observed a shift to increased PGE₂ formation in tolerant tissue. These findings suggest that tyrosine nitration accounts for the observed inhibition of the activity of the enzyme in the setting of nitrate tolerance. The functional inhibition of PGI-S activity in tolerant tissue was indirectly confirmed by experiments using U51605, the dual blocker of PGI-S and TXA₂-S, which has previously been shown to mimic the effects of authentic peroxynitrite on in vitro vasorelaxation. U51605 shifted the concentration-response relationship for GTN significantly to the right. This finding was interpreted as indication for accumulation of non-metabolized PGH₂, which is able to elicit vasoconstriction via activation of the TXA₂/PGH₂-receptor of the vascular smooth muscle. In contrast, incubation of tolerant tissue with U51605 failed to modify the
remaining vasodilator responses to GTN (which were already right-shifted), because
tolerance and U51605 use the same mediator to counteract vasorelaxation, i.e.,
PGH₂.

**Do all nitrates induce tolerance?**

A number of studies have shown that clinical signs of tolerance also develop
with chronic administration of ISDN and ISMN (studies before 1990 reviewed in³⁷),
indicating that tolerance development is uniform to all organic nitrates if applied inclinically effective dosage for longer periods of time. A remarkable exception is
pentaerythritol tetranitrate (PETN)³⁸. In contrast to other long-acting nitrates, PETN
induces tolerance-free vasodilation in humans³⁹,⁴⁰ and prevents endothelial
dysfunction as well as atherogenesis in cholesterol-fed rabbits⁴¹. It was shown that
PETN possesses antioxidant properties, which could explain its specific
pharmacological profile⁴⁰. In contrast to all other organic nitrates, PETN and its
metabolite pentaerythritol trinitrate (PETriN) induce the antioxidant defense protein
heme oxygenase-1 (HO-1), also known as a chaperone, heat shock protein 32
(hsp32), and increase the formation of the antioxidant molecule bilirubin and the
(weak) vasodilator carbon monoxide (CO)⁴². In addition, PETN and PETriN increase
the expression of another antioxidant protein, ferritin, via the HO-1-dependent
release of free iron from endogenous heme sources⁴³. All together, these defense
mechanisms protect endothelial cells from hydrogen peroxide-induced toxicity, and
might explain the previously observed anti-atherogenic activity of PETN ⁴¹,⁴⁴. In
addition, we could show that PETN and PETriN in contrast to GTN did not affect the
nitrate esterase activity of ALDH-2 , and did not elicit ROS formation in isolated
arteries and mitochondria, adding a further mechanism to explain the lack of
tolerance development to PETN\textsuperscript{28}.

However, with regard to the clinical use of PETN there is an ongoing debate
that an anti-anginally effective dose was not applied in those clinical studies where
administration of PETN in patients with angina did not generate nitrate tolerance.
Indeed, there are studies arguing in both directions, e.g., lack of tolerance
development vs. lack of anti-anginal efficacy because of insufficient dosing\textsuperscript{45,46}.
However, this debate tends to neglect the influence of very special pharmacokinetics
and pharmacodynamics of PETN, the mode of application, the parameters chosen for
identifying anti-anginal efficacy and tolerance, and the patient group selected. Today
PETN is used only as a peroral long-acting nitrate, with a slow onset of action.
Therefore, it is not suitable for acutely relieving anginal pain. However, with
continuous therapy by 80 mg PETN applied 8 hourly, the incidence of anginal attacks
in patients with confirmed angina pectoris and the consumption of nitroglycerin spray
is significantly reduced, even after 4 weeks of continuous therapy\textsuperscript{47}. In a more recent
randomized, double-blinded, cross-over and placebo-controlled clinical study with 15
men with stable angina\textsuperscript{48}, 100 mg PETN p.o. after 2 h exhibited similar anti-anginal
efficacy as 80 mg ISDN and 15 mg NTG (slow release), but loss of efficacy after 6 h
was more rapid with PETN than with the other nitrates. To conclude, these and other
studies suggest that PETN clearly exhibits and maintains anti-anginal activity after
acute and chronic therapy. However, compared to other long-acting nitrates, the
duration of action after single oral application is shorter. Therefore, the current dosing
regimen requires 3 x 80 mg daily in order to achieve continuous anti-anginal
protection. Finally, a recent multi-center clinical study demonstrated the non-
inferiority of PETN vs. ISDN with even partial superiority of the tetranitrate\textsuperscript{49}. 
Old and new strategies to prevent the development of tolerance and cross-tolerance.

The most widely accepted approach to prevent the tolerance phenomenon has been a nitrate free interval. This procedure, however, bears several risks. Since oxidative stress is important for tolerance and cross-tolerance, antioxidants or drugs, which are able to reduce oxidative stress within vascular tissue are able to positively influence both phenomena. Recent studies in patients with CAD and heart failure indeed demonstrated that the development of tolerance is beneficially influenced by vitamin C, vitamin E as well as by ACE-inhibitors and statins. Most likely sources of ROS in the setting of tolerance are the mitochondria (see printed part), an uncoupled NOS III (see printed part) and NADPH oxidases which are activated by the RAAS (AT II, DAG, PKC).

Nitrate free interval:

Several groups demonstrated that tolerance to the hemodynamic effects of GTN can be avoided using a nitrate free interval or eccentric dosing approaches. A potential problem related to a nitrate free interval may be the development of rebound ischemia. Cessation of chronic GTN-treatment in experimental animals was not able to normalize endothelial dysfunction and hypersensitivity to vasoconstrictors. During the nitrate free interval the frequency of angina symptoms as well as of silent angina was significantly increased. By treating patients intermittently with GTN patches for a 5 d period Azevedo et al. have shown that this kind of regimen may prevent the development of tolerance. On the other hand, acute nitrate withdrawal increased the coronary vasoconstrictor responses to ACh, suggesting that the
rebound phenomenon may be secondary to the development of endothelial dysfunction. Similar data have been obtained with animal experiments where a nitrate free interval improved tolerance, but failed to correct completely endothelial dysfunction and even enhanced supersensitivity to vasoconstrictors such as serotonin and phenylephrine. These data clearly indicate that GTN-induced endothelial dysfunction cannot be prevented by a nitrate free interval. These earlier findings can now be rationalized on the basis of nitrate-induced mitochondrial superoxide formation and inhibition of ALDH-2, which occur in endothelial cells as well as in smooth muscle cells. Endothelial cells may be even more susceptible to GTN-induced damage than smooth muscle cells, since due to their orientation to the vessel lumen they are exposed to higher concentrations of GTN than the smooth muscle cells. A further detrimental effect experienced primarily by the endothelium is the loss of extracellular SOD activity in nitrate tolerant state. As a consequence of reduced ecSOD activity, plasma levels of important antioxidants like α- and β-carotene are reduced, exacerbating the susceptibility of endothelial cells to oxidative damage. Moreover, this leads to formation of toxic carotenoid cleavage products. These products inhibit mitochondrial respiration and increase mitochondrial oxidative stress, thus contributing to nitrate tolerance.

**Nitrate free interval and the rebound phenomenon:**

As pointed out before, one of the most attractive approaches to prevent tolerance development is a 8-12 h nitrate free interval with the disadvantage of lacking protection during this period. Another potential problem can be the development of rebound ischemia. In patients with stable angina Freedman et al. observed an increase in the duration of silent ischemia compared to patients treated
with placebo\textsuperscript{58}. These data go along with reports demonstrating decreased angina threshold after patch removal in smaller\textsuperscript{59} and multicenter trials (so called TIDES II trial)\textsuperscript{60}. Experimental studies provided some insight into the underlying mechanism\textsuperscript{61}. Treatment of rabbits for 3 d with GTN increased vasoconstrictor sensitivity of the tolerant vasculature, induced tolerance, endothelial dysfunction and increased production of reactive oxygen species. Importantly, increased superoxide production and endothelial dysfunction were almost completely prevented by a nitrate free interval, while the supersensitivity to vasoconstrictors persisted\textsuperscript{61}. Thus, the observed increased ACh-induced constriction in the coronary circulation in patients treated intermittently with GTN most likely represents increased vasoconstrictor sensitivity of the smooth muscle to ACh, rather than an attenuation of endothelium-dependent NO-release in response to acute ACh challenges\textsuperscript{54}. More recent clinical trials failed to demonstrate rebound phenomena in patients with CAD treated with nitrates\textsuperscript{62,63}, which may be explained at least in part by the concomitant treatment with an ACE-Inhibitors\textsuperscript{64,65} or beta receptor blockers\textsuperscript{66}.

**Sulfhydryl group donors:**

Studies in non-tolerant and tolerant animals and humans reported a potentiation of GTN bioactivity by sulfhydryl donors N-acetylcysteine\textsuperscript{51,67,68} and L-methionine\textsuperscript{69-71}, but this finding was interpreted by direct non-enzymatic interaction of certain thiols with GTN. The recent findings that oxidation of critical cysteinyi residues of ALDH-2 may account for nitrate tolerance may now better explain the beneficial effects of SH-group donors on tolerance, since thiol-reducing agents such as dithiothreitol or mercaptoethanol are able to fully restore impaired nitrate reductase activity of the ALDH-2 in vitro. It remains to be shown whether thiol donors act by the
same mechanism in GTN tolerant patients. With respect to our recent observations (involvement of lipoic acid in the reduction of inactivated ALDH-2) a co-therapy with lipoic acid could be of clinical interest.

**Angiotensin converting enzyme inhibitors and AT-1 receptor blockers:**

As discussed previously, vasodilator therapy with GTN is associated with a transient activation of neuro-hormonal vasoconstrictor forces. These counter-regulatory mechanisms may offset the direct vascular effects of GTN within 1 day and together with sodium and water retention may counterbalance the venodilator effects of this drug. Since activation of the RAAS plays an important role in tolerance development, co-treatment with an angiotensin converting enzyme inhibitor or an angiotensin II receptor antagonist should prevent tolerance. However the clinical data concerning the efficacy of ACE-inhibitors in preventing tolerance are contradictory. While Berkenboom et al. 72 and Mehra et al.73 showed that long-term treatment with ramipril prevented nitrate tolerance to GTN and ISDN, respectively, Daka et al. found no improvement of GTN tolerance in patients treated with GTN plus captopril compared to GTN alone74. Studying normal subjects, Katz et al found that tolerance to the venous forearm circulation was prevented by concomitant administration of captopril or enalapril 75, suggesting that the SH-group moiety in ACE-inhibitors is not responsible for the positive effects with respect to tolerance. Similarly, Muiesan et al. showed that the combination therapy of benazepril with transdermal GTN induced a significant increase in exercise duration 22 h post-dosing, while GTN given alone was no longer effective at this time point 76.

Uri Elkayam et al. studied the effects of high dose transdermal GTN (50 and 100 mg respectively) in patients pretreated with an ACE-inhibitor during a three
month period and observed that the combination therapy markedly improved exercise
time in response to GTN administration, but not in response to placebo treatment\textsuperscript{77}. In addition, GTN significantly decreased end-diastolic and end-systolic dimensions of the left ventricle and augmented fractional shortening\textsuperscript{77} indicating that nitrates may represent an important adjunctive therapy in patients pretreated with an ACE-inhibitor.

Although it is difficult to explain the different efficacy of various ACE-inhibitors in these particular studies in preventing or reversing tolerance it is quite possible that the use of higher concentrations of ACE-inhibitors may have influenced the different outcome of these studies\textsuperscript{65}. By using a well characterized animal model of nitrate tolerance, we demonstrated that high doses of enalapril (1 mg/kg) prevented the development of tolerance in large coronary arteries and the rebound constriction following cessation of GTN long-term therapy\textsuperscript{65}. Low concentrations of enalapril (0.2 mg/kg/day) failed to prevent tolerance and rebound and also failed to increase plasma renin activity for a 24 h period, although this concentration had already a marked effect on angiotensin I pressor responses. This may indicate that a persistent inhibition of angiotensin II formation might be a prerequisite for the observed beneficial effects of high dose enalapril on tolerance development and rebound responses\textsuperscript{65}.

Several experimental studies have demonstrated that nitrate tolerance in animal models at the resistance and conductance vessel level was markedly attenuated using AT1-receptor blocker such as losartan\textsuperscript{78,79}. Importantly, these studies also showed that the improvement of tolerance was also accomplished by giving SOD mimetics such as manganese-containing porphyrins, and was also associated with a reduction of oxidative stress in vascular tissue\textsuperscript{78,79}. A recent study
from patients with coronary spastic angina seems to confirm these preclinical observations\textsuperscript{80}. A total of 64 patients were treated with trans-dermal GTN (10mg/d) for a total of 3d. Of these 21 patients were concomitantly treated with an AT1 receptor blocker candesartan (8mg/d). By measuring the brachial artery diameter with ultrasound, the authors found that tolerance to GTN therapy was completely abolished by co-treatment with the AT1 receptor blocker\textsuperscript{80}. In addition, GTN-induced increases in plasma thioredoxin levels (a marker of oxidative stress), were completely inhibited by candesartan, indicating that neuro-hormonal activation during GTN therapy and the subsequent increases in circulating angiotensin II levels may contribute at least in part to the phenomenon of nitrate tolerance via increases in oxidative stress. However, these beneficial effects of sartan therapy on nitrate tolerance in human were questioned by two negative studies. Treatment of healthy volunteers with 75 mg losartan daily for one week could not prevent vascular and hemodynamic tolerance to continuous NTG therapy\textsuperscript{81}. In patients with stable angina pectoris 100 mg losartan daily together with 20 mg transdermal NTG could not prevent tolerance development to the anti-ischemic effect of NTG after 4 weeks of treatment\textsuperscript{82}. A weakness of the latter study was that the exercise test was stopped by most patients due to physical exhaustion, not because of angina, therefore it did not assess the anti-anginal effect of NTG therapy. The discrepant findings between these studies may be explained by the different study groups: patients with spastic angina\textsuperscript{80}, unstable angina\textsuperscript{83}, stable angina\textsuperscript{82}, and healthy volunteers\textsuperscript{81}, and the different functional tests for verifying tolerance development. Clearly, further studies are required to clarify this issue.

\textbf{Hydralazine:}
Favorable interactions between hydralazine and nitrates have been demonstrated in the Veterans Heart Failure Trials (V-HeFT) and in the African-American Heart Failure Trial (A-HeFT). This particular combination has been shown to have beneficial effects on left ventricular function, exercise capacity and most notably in survival in a large patient population with severe heart failure. Hydralazine has also been shown to prevent the development of GTN tolerance in both experimental animals and in humans with congestive heart failure. Hydralazine is a strong arteriolar dilator and stimulates reflex increases in vasoconstrictor stimuli including circulating catecholamines and plasma renin activity (reflecting increased circulating angiotensin II levels). This would seem, upon first inspection, to worsen rather than improve tolerance by enhancing the neuro-hormonal counter-regulatory adjustments to the nitrate. An explanation for this apparent paradox may be provided by our finding that a combination therapy of rats with hydralazine and GTN completely prevented the GTN induced increase in vascular superoxide production and tolerance. Further, acute addition of hydralazine in clinically relevant concentrations to segments of aorta from control and GTN-treated animals markedly reduced vascular superoxide steady state levels. Hydralazine was only effective when administered in vivo or incubated with intact rings, but had no effect when added to the vascular homogenates. One explanation for this finding is that the effect of hydralazine requires the intact cell to exert its effect, possibility via its hyperpolarizing properties. This possibility is strengthened by the fact that another hyperpolarizing agent, the $K_{ATP}$ channel activator pinacidil also markedly inhibited vascular superoxide production. In performing relaxation studies we found that tolerance to GTN and cross-tolerance to SIN-1 were completely normalized by hydralazine treatment.
Considering the importance of ALDH-2, is there a relationship to mitochondrial membrane potential and superoxide formation? It appears so. The mitochondrial membrane potential controls mitochondrial matrix volume and intermembrane space, and this in turn controls mitochondrial metabolism. Shrinking of the matrix and increased intermembrane space leads to uncoupling of electron flow and increased mitochondrial superoxide formation.

Several recent studies suggest that hydralazine may be also a potent direct free radical scavenger, and these antioxidant properties of hydralazine were linked to alterations in vascular gene expression. Leiro and colleagues demonstrated an inhibitory effect of hydralazine on inducible NOS/COX-2 gene and protein expression in rat peritoneal macrophages. Hydralazine at 0.1-10 mM inhibited both extracellular and intracellular ROS production by inflammatory macrophages, by a ROS-scavenging mechanism probably affecting superoxide generation by xanthine oxidase and NADPH oxidase. Knowles and coworkers tested whether hypoxia-inducible factor (HIF)-regulating proline hydroxylase might be a target of hydralazine. They found that hydralazine inhibited prolyl hydroxylase domain (PHD) activity and induced nonhydroxylated HIF-1α, taken as evidence for HIF stabilization specifically by inhibition of PHD enzyme activity. Consequently, hydralazine induced a rapid and transient expression of HIF-1α and downstream targets of HIF (endothelin-1, adrenomedullin, heme oxygenase 1, and vascular endothelial growth factor (VEGF)) in endothelial and smooth muscle cells and induced endothelial cell-specific proliferation. In experimental animals hydralazine induced HIF-1alpha and VEGF protein in tissue extracts and elevated plasma VEGF levels. Thus, hydralazine initiates a pro-angiogenic phenotype and might be beneficial in ischemic disease.
According to preliminary studies in our laboratory, hydralazine is a scavenger of superoxide and an excellent quencher of peroxynitrite-derived free radicals.

**Antioxidants:**

The demonstration of increased superoxide formation in endothelial and smooth muscle cells in GTN tolerance suggests that treatment with antioxidants may prevent this phenomenon. Indeed, studies published by Eberhard Bassenge's group and others demonstrated that concomitant treatment with vitamin C and SOD mimetics such as manganese-porphyrine preserved the sensitivity of the vasculature to organic nitrates. In chronically instrumented dogs, vitamin C completely prevented the development of nitrate tolerance. Recent data also indicate that the antioxidant vitamin E prevents GTN tolerance in forearm veins in humans and restores the depressed cGMP-response of tolerant platelets. In addition, concomitant treatment of patients with congestive heart failure with vitamin C and GTN completely prevented the development of hemodynamic tolerance.

**Carvedilol**

A promising therapeutic approach to prevent nitrate tolerance may be provided by the 3rd generation β-blockers with antioxidant properties, like carvedilol. Watanabe et al. analyzed the effect of carvedilol (10 mg twice a day) and arotinolol (a β-blocker without antioxidant properties) on nitrate tolerance in 24 patients with untreated hypertension. Carvedilol maintained the increase in forearm blood flow (FBF) after sublingual administration of 0.3 mg NTG, whereas this response was lost in the placebo and arotinolol group after 3 days application of a 20 mg/24 h GTN tape. In a similar study the authors observed that carvedilol (2.5 mg per day)
prevented development of nitrate tolerance in patients with chronic heart failure treated for 6 days with 10 mg/24 h GTN tape, whereas metoprolol (30 mg once a day) and doxazosin (0.5 mg once a day) were ineffective. These results indicate that carvedilol may prevent nitrate tolerance in patients with chronic heart failure during continuous therapy with GTN.

In a recent study the effects of 5 d co-infusion of GTN, either with a carvedilol metabolite (BM920228) with antioxidant properties, or with vitamin C (Vit-C) on various hemodynamic parameters were analyzed in chronically instrumented dogs. Co-infusion of either antioxidant prevented development of vascular nitrate tolerance (loss of coronary dilation). In addition, in vitro BM920228 exhibited $O_2^-$ radical scavenging activity similar to Vit-C, and superoxide dismutase (SOD). This finding suggests that the anti-oxidative activity of carvedilol may be based on the direct scavenging of superoxide radicals and presumably other ROS.

**Protein kinase C antagonists:**

As described in the printed part), an activation of protein kinase C and increased sensitivity to vasoconstrictors contributes to nitrate tolerance via. Consistent with this mechanism, in vivo treatment of rats with the protein kinase C antagonists N-benzoyl-staurosporine not only prevented increased constrictions of isolated vessels to catecholamines and thromboxane, but also the development of nitrate tolerance. This observation may suggest that activation of one or several isoforms of protein kinase C within the vasculature may specifically impair the GTN biotransformation process, via promotion of oxidative stress. There are, however, no patient studies with PKC antagonists available.
Statins

In a recent study in normocholesterolemic rats long-term (5 w) treatment with HMG-coenzyme A-inhibitors pravastatin and atorvastatin prevented nitrate tolerance induced by GTN s.c. injections (50 mg/kg/d) for the last three days\textsuperscript{50}. Also, GTN-induced increase in superoxide formation in thoracic aorta was prevented by statin treatment. The protective effect of the statins on GTN-induced relaxant responses and superoxide formation was abolished, when the rats received L-NAME (100 mg/kg/d) concomitantly with GTN. Statin treatment alone increased basal cGMP levels, in accordance with earlier findings that statins improve NOS III activation, presumably via stimulation of Akt phosphorylation\textsuperscript{103}, but do not alter the expression of the enzyme\textsuperscript{50}. The authors concluded that one mechanism whereby statins counteract nitrate tolerance is accomplished by preventing uncoupling of NOS. According to these authors a second mechanism is related to statin-induced downregulation of NADPH oxidases, as shown by Wassmann et al.\textsuperscript{104}, since they observed an acute loss of the protective effect of statins after addition of NADPH to the organ baths. Interestingly, simvastatin pretreatment (1 h) of isolated rat neonatal cardiac myocytes was shown to prevent the hydrogen peroxide-induced depolarization of the mitochondrial membrane potential\textsuperscript{105}. This effect was abolished by L-NAME and 5-hydroxydecanoate, a blocker of ATP-sensitive K\textsuperscript{+} channels. The authors concluded that the statin protected the cardiomyocytes from oxidative injury by acute activation of NOS III via phosphorylation of Akt\textsuperscript{103} thereby increasing bioavailability of NO. NO in turn could activate the (hypothetical) mitochondrial K\textsubscript{ATP} channel, thus counteracting membrane depolarization, uncoupling, superoxide generation and apoptosis. Further studies will have to show by which mechanism statins prevent nitrate tolerance. However, it is evident that the mitochondria are a
common target of GTN and statins.
Cited literature:


89. Leiro JM, Alvarez E, Arranz JA, Cano E, Orallo F. Antioxidant activity and inhibitory effects of hydralazine on inducible NOS/COX-2 gene and protein


