J Mol Med (1997) 75:891–900

REVIEW

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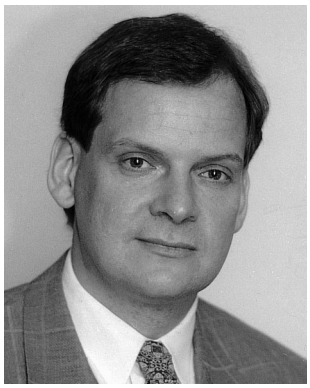
Evidence for a role of oxygen-derived free radicals and protein kinase C in nitrate tolerance

Received: 9 December 1996 / Accepted: 23 June 1997

Abstract The anti-ischemic effects of organic nitrates are rapidly attenuated due to the development of nitrate tolerance. The mechanisms underlying this phenomenon likely involve several independent factors. As a vasodilator, nitroglycerin activates compensatory neurohumoral mechanisms such as the renin-angiotensin system and increases catecholamine and vasopressin levels, all of which may attenuate its vasodilator potency. Tolerance may be also due to the inability of the vessel to dilate after prolonged treatment with the nitrate. More recent experimental studies have challenged traditional tolerance concepts by demonstrating that tolerance is not associated with sulfhydryl group depletion, reduced nitroglycerin biotransformation, or desensitization of the target enzyme guanylyl-cyclase. Experimental and clinical observations suggest that tolerance may be the consequence of intrinsic abnormalities of the vasculature, including enhanced endothelial production of oxygen-derived free radicals secondary to an activation of NAD(P)H-dependent oxidases and an activation of PKC. Superoxide degrades nitric oxide derived from nitroglycerin (NTG) while C activation causes enhanced sensitivity of the vasculature to circulating neurohormones such as catecholamines, angiotensin II, and serotonin, all of which may compromise the vasodilator potency of NTG. Interestingly, these vascular consequences of in vivo NTG treatment such as superoxide production and PKC activation can be mimicked in vitro by incubating cultured endothelial and smooth muscle cells with angiotensin II. Furthermore, nitrate tolerance and rebound following sudden cessation of prolonged NTG therapy can be prevented by concomitant treatment with high-dose angiotensin-converting enzyme inhibition, angiotensin type 1 receptor blockade, or antioxidants such as hydralazine. Thus one can conclude that neurohumoral counterregulatory mechanisms such as increased circulating levels of angiotensin II may be at least in part responsible for tolerance mechanisms at the cellular level.

Key words Nitrate tolerance · PKC · Superoxide · Endothelin-1 · NAD(P)H oxidase

Abbreviations *PKC* Protein kinase C · *NTG* Nitroglycerin



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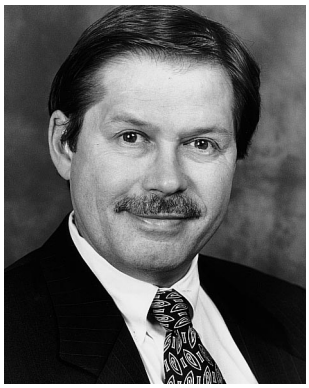

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Communicated by: Christian Holubarsch and Hanjörg Just

Introduction

NTG has been one of the most widely used anti-ischemic drugs for more than a century. Given in acute situations,

organic nitrates are excellent agents for the treatment of stable effort angina, mixed angina, unstable angina, and postmyocardial infarction. The efficacy of NTG in chronic situations is limited by the rapid development of nitrate tolerance in patients with coronary artery disease and heart failure [1–3]. The mechanisms underlying the tolerance remain poorly defined but likely involve several independent mechanisms. This review focuses on recent experimental and clinical observations regarding nitrate tolerance and summarized its potential implications with respect to concomitant drug treatment for preventing or reversing tolerance.

The challenge of traditional tolerance concepts

Several mechanisms have been suggested to account for the phenomenon of nitrate tolerance. Mechanisms extraneous (so-called pseudo-tolerance) to the vessel wall include neurohumoral counterregulatory mechanisms and intravascular volume expansion [1, 4, 5]. Tolerance has also been shown to be due to an inability of the vascular smooth muscle to convert NTG to nitric oxide (so-called "true vascular tolerance") [6]. There has been substantial debate as to the extent to which these two mechanisms contribute to the loss of antianginal efficacy during chronic NTG therapy, and the notion that neurohumoral mechanisms are a predominant cause has achieved substantial popularity [4, 7]. The decrease in blood pressure caused by NTG causes baroreflex stimulation leading to a variety of neurohumoral adjustments. These include increases in catecholamine levels and release rates [8] increases in plasma vasopressin [4, 9], plasma renin activity [4, 9], and aldosterone levels [4, 9]. These changes are not NTG specific but are also observed during therapy with other vasodilators.

NTG therapy is also associated with a marked increase in intravascular volume which may attenuate the preload effects. During continuous NTG infusion for 72 h there is a consistent drop in hematocrit in patients with coronary artery disease [9]. A decrease in hematocrit during long-term NTG has been demonstrated by several groups and very likely reflects intravascular volume expansion secondary to a transvascular shift of fluid due to an alteration in Starling forces and/or a phenomenon related to an aldosterone-mediated salt and water retention [1, 4, 5]

As mentioned above, vascular tolerance is thought to be secondary to an inability of the vascular tissue to respond to NTG. Vessels from animals pretreated with NTG demonstrate blunted vasodilatation to the environment. Four intracellular tolerance mechanisms have traditionally been hypothesized to be responsible for attenuation of NTG action following chronic exposure. These include: a desensitization of the target enzyme guanylyl-cyclase [10], an increase in phosphodiesterase activity (leading to an enhanced cGMP breakdown) [11, 12], intracellular sulfhydryl group depletion [13–15], and impaired NTG biotransformation [16]. There is experimental evidence supporting many of these hypotheses. In particular the loss of NTG biotransformation has been demonstrated in vitro in both intact organs and cultured cells [17–19]. The enzymes involved have been demonstrated to be membrane bound and not to be identical with glutathione-*S*-transferases. Following in vitro incubation with NTG, nitric oxide production in endothelial and smooth muscle cells has been shown to be decreased, as is relaxation to the organic nitrate. These observations have led several groups to suggest that at least in vitro tolerance is in part due to decreased NTG biotransformation. Almost 25 years ago Needleman and Jonhnson [13–15] postulated the well-known "sulfhydryl-group depletion concept." Their concept postulates that nitrates react with sulfhydryl-groups (so-called nitrate receptor) leading to the formation of a disulfide linkage, and that this change in the configuration of the nitrate receptor explains lower affinity to NTG in response to chronic treatment. The concept that the target enzyme guanylyl-cyclase becomes desensitized during NTG treatment is based on observations from Murad's group [10] demonstrating that in response to chronic NTG treatment there is cross-tolerance to both endothelium-dependent and endothelium-independent nitrovasodilators, and that in tolerant tissue the cGMP formation in response to acute NTG challenges is severely blunted.

All three traditional concepts of cellular tolerance, sulfhydryl-group depletion, impaired biotransformation, and desensitization of the target enzyme guanylyl-cyclase, have recently been challenged by experimental findings. Boesgard et al. [20] showed that in vivo treatment with high-dose NTG does not result in significant changes in the intracellular sulfhydryl-group concentrations in arterial or venous tissue. Thiol supplementation increases NTG responsiveness in both tolerant and nontolerant states mainly via an extravascular rather than intracellular interaction between sulfhydryl-groups and the organic nitrates [21, 22]. Using spin-trapping technique, Laursen et al. showed that in vivo conversion of NTG to nitric oxide is changed in neither veins nor arteries. In contrast, higher concentrations of NTG seem to induce rather than to desensitize the enzyme [23]. More recent data also show that tolerance is almost completely reversed by removing the endothelium, making it very unlikely that the soluble guanlyly-cyclase of smooth muscle cells is responsible for the attenuation of NTG vasodilator effects in the setting of nitrate tolerance [23, 24]

Dissociation of neurohormonal adjustments and tolerance development in large epicardial arteries

Some insight into the role of neurohormones in nitrate tolerance can be gained from examining the time course of tolerance in certain vessel regions and its relationship to neurohumoral activation. Further, the time course of tolerance may not be the same in the systemic and coronary vasculatures. For example, as mentioned above, it has been shown that a large portion of the intravascular

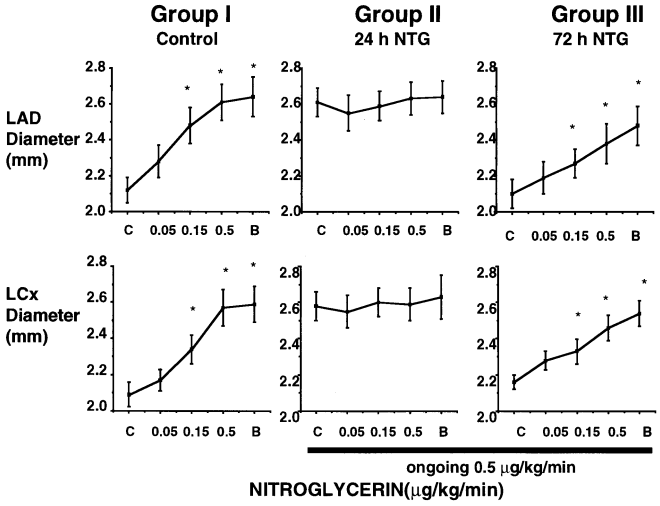

Fig. 1 Effects of increasing intravenous and intracoronary NTG on large coronary artery diameter [left anterior descending (*LAD*) and left circumflex (*LCx*)] in patients without () and with 24 (*group II*) and 72 h NTG pretreatment (*group III*). Under ongoing NTG infusions patients pretreated with NTG for 24 h did not respond with a further increase in diameter, indicating a maximal dilated coronary artery. In contrast, those pretreated with NTG for 3 days responded with a diameter increase that was not statistically different from that of group I, which is strongly suggestive of tolerance development in large epicardial arteries. Increasing concentrations of NTG were given intravenously for 7 min each. *B* 0.2 mg NTG bolus intracoronary. Data are given as mean±SEM (*open squares*). *Signifcantly different vs. baseline values (after Bonferonni correction for the numbers of comparisons, *n*=4). (Adapted from [9])

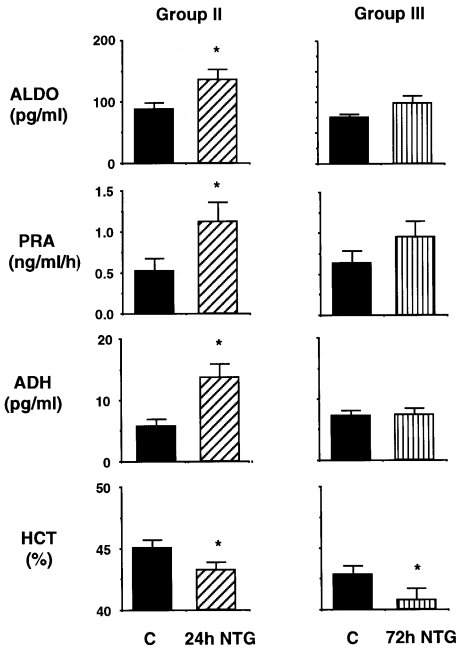

Fig. 2 Effects of 24 and 72 h NTG infusion on plasma renin activity (*PRA*), plasma aldosterone levels (*ALDO*), plasma vasopressin levels (*ADH*), and hematocrit (*HCT*). Long-term NTG infusion caused a transient increase in plasma vasopressin and aldosterone levels and a transient increase in plasma renin activity but a persistent drop in hematocrit, indicating intravascular volume expansion. Data are presented as mean±SEM. (Adapted from [9])

fluid shift occurs within the first hour of treatment [5]. During this period, however, the effects of NTG on the pulmonary capillary wedge pressure in patients with coronary artery disease and heart failure are usually preserved [25]. Over a longer period of infusion, 24–48 h, the pulmonary capillary wedge pressure rises to the pretreatment value [1, 26] with little or no additional volume retention, suggesting that mechanisms independent of volume retention are involved. Therefore a significant decrease in hematocrit may be used more as a marker for NTG treatment rather than a marker for tolerance development. In addition, the persistent drop in hematocrit actually lends support to the conclusion that, for example, tolerance to epicardial artery effects does not follow the same time line as tolerance in other vascular beds.

One possibility is that neurohumoral adjustments such as increases in vasopressin, activation of the renin-angiotensin system, and increases in circulating catecholamine levels produce increases in vasoconstrictor tone, overcoming the NTG vasodilatation. Recent observations by Parker et al. [4] demonstrate that therapy with NTG patches is associated with transient activation of the renin-angiotensin system, increases in vasopressin and catecholamine levels, and signs of intravascular volume expansion. Similarly, by using slightly higher levels of NTG intravenously we have found that tolerance in epicardial coronary arteries does not coincide with the activation time course of these neurohumoral parameters [9] (Figs. 1, 2). In these studies in patients with stable coronary artery disease the increase in these parameters during NTG therapy was also transient, and not observed after 72 h of therapy. During the period in which these parameters were highest (24–48 h) the epicardial coronary artery response to NTG was preserved. Well after these neurohumoral parameters had returned to normal, however, the vasodilatation of the epicardial coronary arteries to NTG was virtually lost. These findings indicate that increased levels of circulating vasoconstricting neurohormones are unlikely to be responsible for the loss of effect of NTG on the epicardial coronary arteries and strongly suggests tolerance at the cellular level.

New tolerance concepts: evidence for a role of oxygen-derived free radicals and PKC

Role for superoxide in nitrate tolerance

Recently we have defined a new mechanism partially responsible for NTG tolerance and cross-tolerance to other endothelium-dependent and edothelium-independent vasodilators [40]. In an animal model of nitrate tolerance we found that aortic segments from rabbits demonstrate a great degree of tolerance to NTG and cross-tolerance to acetylcholine and the sydnonimine of SIN-1. Using a different animal model we established a similar pattern of tolerance to that of Molina et al. [10]who demonstrated in addition to an attenuation of NTG responsiveness cross-tolerance to endothelium-dependent and endotheli-

um-independent vasodilators. The removal of the endothelium, however, markedly attenuated tolerance and cross-tolerance to NTG and the nitrovasodilator SIN-1, making an involvement of the guanylyl-cyclase unlikely (Fig. 3). This observation led us to hypothesize that the endothelium either releases chronically a vasoconstrictor or that nitric oxide released from NTG becomes chemically inactivated before stimulating the vascular smooth muscle guanylyl-cyclase.

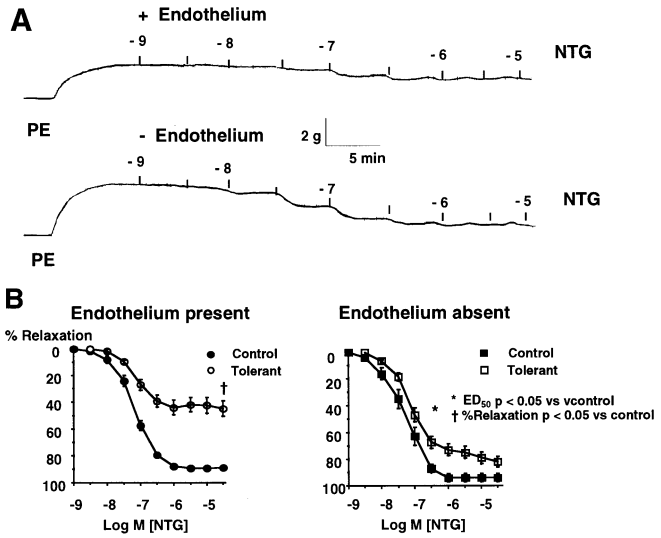

Fig. 3 **A** Experimental record demonstrating the effect of endothelial removal on the relaxations to NTG (1 nM–30 μM) in tolerant rabbit aortic ring segments. Both segments were preconstricted with phenylephrine, and relaxations to cumulative concentrations of NTG were examined. In the presence of the endothelium#, the vessel relaxed maximally 37% and in its absence 78%. **B** Mean data demonstrating NTG-induced relaxations in both control and NTG-tolerant vessels with and without endothelium. Data are mean±SEM. (Adapted from [24])

In support of the latter hypothesis we found that the superoxide $O_2^{-}\cdot$ levels in tolerant vessels were about twice those of control vessels (Fig. 4A). Interestingly, removal of the endothelium increased $O_2^{-}\cdot$ production in control vessels but paradoxically decreased it in tolerant vessels. This observation indicates that the endothelium indeed represents the major source of $O_2^{-}\cdot$ production in nitrate tolerance. $O_2^{-}\cdot$ binds very rapid with NO to form the highly reactive peroxinitrite ($ONOO^{-}$) with a cytotoxic potential which is about 1000 times higher than that of H_2O_2, and which has a substantially shorter half-life, and which is much less potent in stimulating guanylyl-cyclase [55, 56] (Fig. 4B). $ONOO^{-}$ also protonates to peroxynitrous acid (ONOOH) to yield an oxidant with the reactivity of hydroxyl radical (·OH) via metal-independent mechanisms. Peroxynitrite in pure form causes oxidative damage to protein, lipid, carbohydrate, DNA, subcellular organelles, and cell systems. The hypothesis that superoxide and/or peroxynitrite plays in important role in tolerance is strengthened by our observations that attenuated NTG responses in the setting of nitrate tolerance can be almost completely corrected by preincubation of vessels with a liposomal superoxide dismutase preparation (Fig. 5).

These studies also provided some insight into the potential sources of $O_2^{-}\cdot$ production in nitrate tolerance. $O_2^{-}\cdot$ production was completely normalized by adding diphenylene iodonium, a potent inhibitor of flavoprotein-containing oxidases. These include mitochondrial oxidases, nitric oxide synthase, xanthine oxidase, and plasmalemmal NADH-dependent and NADPH-dependent oxidases. Using specific inhibitors we excluded mitochondrial enzymes, nitric oxide synthase, or the xanthine oxidase as major $O_2^{-}\cdot$ sources, indicating a potential role for NAD(P)H-driven oxidases [57]. Recently it has become more apparent that vascular tissues possess these membrane-bound oxidase-specific activity-utilizing NADH and NADPH as cofactors for superoxide production [58, 59].

To address an involvement of this oxidase in tolerance we examined superoxide production by homogenates of

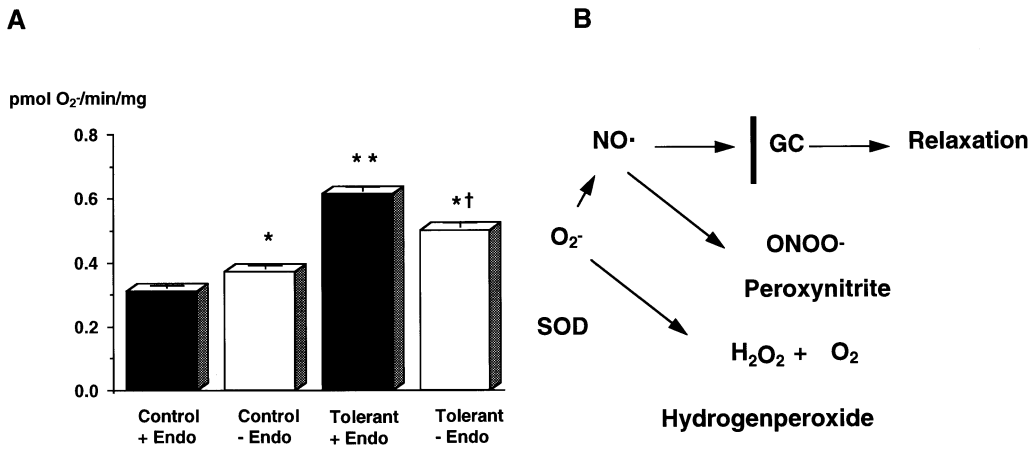

Fig. 4 **A** Superoxide levels in aortic segments from control and NTG-treated rabbits. $O_2^{-}\cdot$ levels were estimated by lucigenin chemiluminescence in the presence and absence of the endothelium. Data are expressed as mean±SEM. **P*<0.05 vs. control with and without endothelium; ***P*<0.001 tolerant vs. control vessels with endothelium; †*P*<0.05 tolerant vessel without vs. with endothelium. (Adapted from [24]) **B** Superoxide ($O_2^{-}\cdot$) rapidly reacts with nitric oxide to form the highly reactive intermediate peroxynitrite ($ONOO^{-}$). The bimolecular reaction between NO and $O_2^{-}\cdot$ is three times faster than the enzymatic dismutation of $O_2^{-}\cdot$ catalayzed by superoxide dismutase

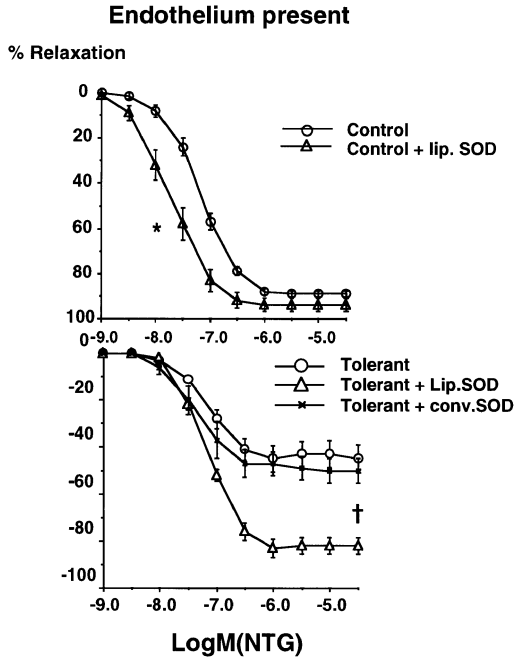

Fig. 5 Effect of liposomal entrapped superoxide dismutase (*SOD*) on NTG dose response in control NTG-tolerant rabbit aorta. Control and tolerant aortic segments were incubated at 37°C in a HEPES/Krebs buffer for one hour containing 600 U/ml SOD in this liposomal preparation. Segments were preconstricted with phenylephrine, and relaxations to cumulative concentrations of NTG were examined. In addition, in tolerant rabbit aorta the effects of conventional SOD on NTG dose response were tested. Liposomal entrapped SOD nicely reversed nitrate tolerance while conventional SOD was virtually ineffective. Data are expressed as mean±SEM. (Adapted from [24])

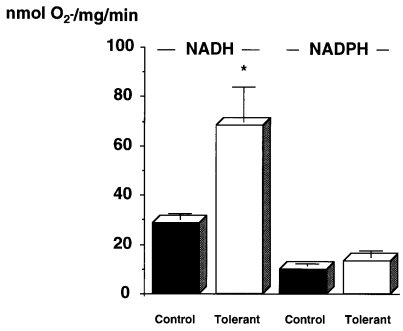

Fig. 6 Effects of in vivo NTG treatment on NADH and NADPH oxidase activity in aortas from rabbits. In vivo treatment with NTG increased superoxide ($O_2^{-}\cdot$) production in response to NADH almost 2.5-fold. (Adapted from [27])

aortas from normal and nitrate-tolerant animals. The use of homogenates allowed us to add various substrates to characterize the oxidases involved. As previously reported, the superoxide production evoked by addition of NADH is substantially (approximately threefold) greater than that observed upon addition of NADPH [58, 59]. Also consistent with previous reports, the oxidase activity is predominantly in the particulate fraction [59]. NTG treatment for ▮3d causes an almost threefold increase in activity of the NADH-oxidase in the nitrate-tolerant vessel homogenates (Fig. 6). Likewise, the activity of the membrane fractions of tolerant vessels is substantially higher than that in control membranes. The mechanism by which NTG treatment increases the activity of these oxidases remains unclear; however, it may involve activation by neurohumoral stimuli such as angiotensin II.

The concept that increased superoxide production is responsible for nitrate tolerance is further substantiated by recent experimental data showing that hydralazine prevents the NTG-induced increase in vascular superoxide production and in parallel the development of nitrate tolerance [27]. In this study we found that a commonly used vasodilator, hydralazine, potently inhibits the development of nitrate tolerance and in parallel prevents the NTG-induced activation of the vascular NAD(P)H oxidase (Fig. 7). Interestingly, hydralazine was effective as an antioxidant only when administered in vivo or in intact rings, and had no effect when administered to vascular homogenates.

One possibleexplanation is that hydralazine prevents assembly of the oxidase rather than directly inhibiting the enzyme. Another possibility is that the effect of hydralazine requires the intact cell to exert its effect, possibly via its known hyperpolarizing effects. This concept is strengthened by the observation that hyperpolarizing agents such as pinacaidil also inhibit vascular $O_2^{-}\cdot$ production, and that the antioxidant effect of hydralazine is inhibited by pretreating rings with depolarizing potassium chloride concentrations [27]. This would imply that the activity of these oxidases, which are membrane associated are regulated by the membrane potential. Further support of a potential role of $O_2^{-}\cdot$ in nitrate tolerance was provided by studies showing that angiotensin II infusion increases vascular superoxide production via activation of the membrane associated NADH oxidase and that under these circumstances the vasodilator potency of NTG is impaired [28].

Role for PKC in nitrate tolerance

Much less attention has been devoted to a potential role for enhanced vasoconstriction in nitrate tolerance. Previous studies with high-dose NTG have shown increased sensitivity to α-adrenergic receptor mediated contractions to epinephrine and norepinephrine [10, 29]. Since the hypersensitivity to catecholamines canould be blocked by a specific α_1-receptor antagonist, an α-adrenergic receptor mediated phenomenon has been suggested to be responsible for this phenomenon [29]. More recent experimental data, however, indicate that after prolonged NTG treatment enhanced vasoconstrictor sensitivity is not restricted to sympathomimetic agents and is also demonstrated for vasoconstrictors such as serotonin, angiotensin II, and potassium chloride [30] (Fig. 8). This observation suggests that enhanced sensitivity to vasoconstrictors is not specific to any one agonist but may involve a common intracellular signaling process.

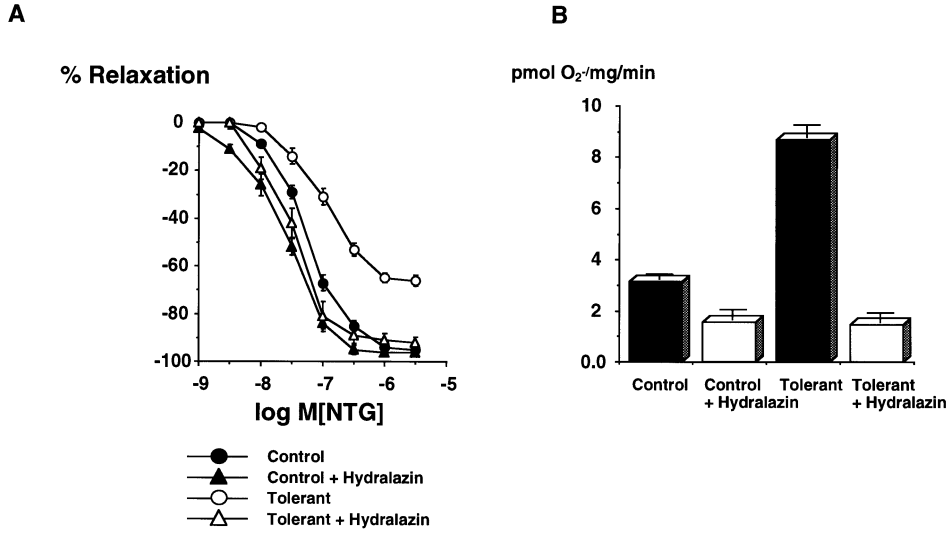

Fig. 7A, B Effect of 3d treatment with NTG alone or in combination with hydralazine on vascular reactivity to NTG and vascular superoxide production. NTG treatment alone caused a marked degree of tolerance. Combination therapy with hydralazine completely preserved the vascular sensitivity to NTG (**A**) and in addition completely prevented the NTG induced increase in superoxide production (**B**). (Adapted from [27])

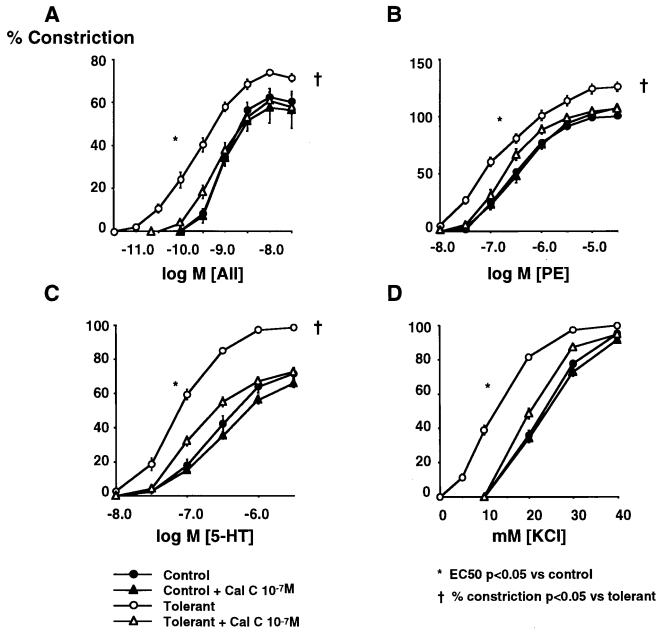

Fig. 8 of three days of NTG treatment on sensitivity to angiotensin II (**A**), phenylephrine (**B**), serotonin (**C**), and potassium chloride (**D**). Nitrate tolerance was associated with an increase in sensitivity to all constrictors, which was largely corrected by calphostin C (100 nM). Data are expressed as mean±SEM. (Adapted from [30])

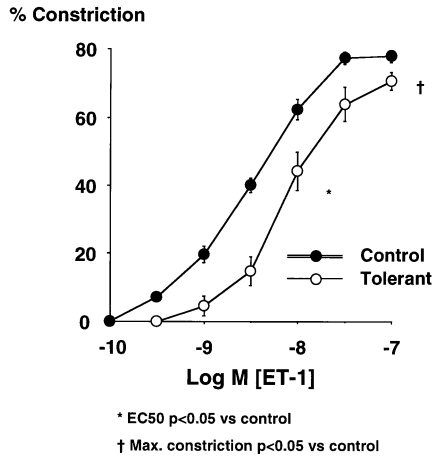

Fig. 9 Effects of 3 days' NTG treatment on constrictions induced by endothelin-1. Data are expressed as mean±SEM. (Adapted from [30])

An interesting observation is that constrictions in nitrate tolerant vessels in response to phenylephrine or angiotensin II are rather sustained despite repeated washing these drugs from the organ chambers. Since sustained vascular contractions are mediated by PKC [31], we hypothesized that this increased sensitivity to vasoconstrictors is due to activation of PKC. This concept is strengthened by the observation that the hypersensitivity of the tolerant vasculature is shared with a direct stimulator of PKC, the phorbolester phorbolester 12,13 dibutyrate, and that this hypersensitivity is corrected by the administration of the PKC inhibitors calphostin C and staurosporin (Fig. 9). Moreover, more recent experimental data demonstrated that in vivo treatment with a PKC antagonist prevented the development of a hypersensitivity to vasoconstrictors such as phenylephrine and thromboxane and simultaneously prevented the development of nitrate tolerance suggesting that the attenuation of the NTG vasodilator effects may be mediated at least in part by a PKC activation within endothelial/and or smooth muscle cells [32].

Surprisingly, contractions induced by endothelin-1, a classical activator of PKC, are paradoxically attenuated in nitrate tolerance (Fig. 10). A possible explanation of this paradox is that it is related to the binding of existing receptors by locally (autocrine) produced endothelin [33]. Indeed, immunocytochemical analysis reveals intense endothelin-1 and large-endothelin staining in NTG-

treated animals, but no staining in aortas from control animals is observed (Fig. 11).

How does locally (autocrine-) produced endothelin-1 mediate the increase in sensitivity to these various agonists? In separate experiments we found that threshold concentrations of endothelin-1 added to control vessels markedly enhance constrictions in response to angiotensin II, serotonin, KCl, and phenylephrine, thereby exactly mimicking the vascular hypersensitivity in response to a 3-day in vivo treatment with NTG (Fig. 12). Furthermore, adding the PKC inhibitor calphostin C completely inhibited this hypersensitivity to vasoconstrictors. We therefore postulate that autocrine-produced endothelin serves as a priming stimulus for PKC which in turn mediates hypersensitivity to a variety of vasoconstrictors. Interestingly, at least two other conditions have been shown to be associated with altered reactivity, possibly due to autocrine-produced endothelin, such as atherosclerosis and pulmonary hypertension [34, 35]. Thus nitrate tolerance may share with these diseases a common mechanism underlying sensitivity to vasoconstrictor stimuli.

The mechanism whereby nitrate treatment increases vascular superoxide production or induces endothelin expression in vascular smooth muscle cells remains unclear. In cultured vascular smooth muscle cells, angiotensin II in nanomolar concentrations activates NAD(P)H-driven, membrane-associated oxidases which in turn have been recently proposed to represent the major source of $O_2^{-}\cdot$

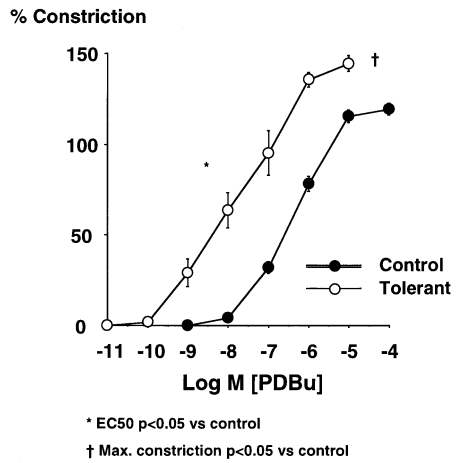

Fig. 10 Effects of 3 days' NTG treatment on constrictions induced by a diract activator of PKC, phorbolester 12,13 dibutyrate (*PDBu*). Data are expressed as mean±SEM. (Adapted from [30])

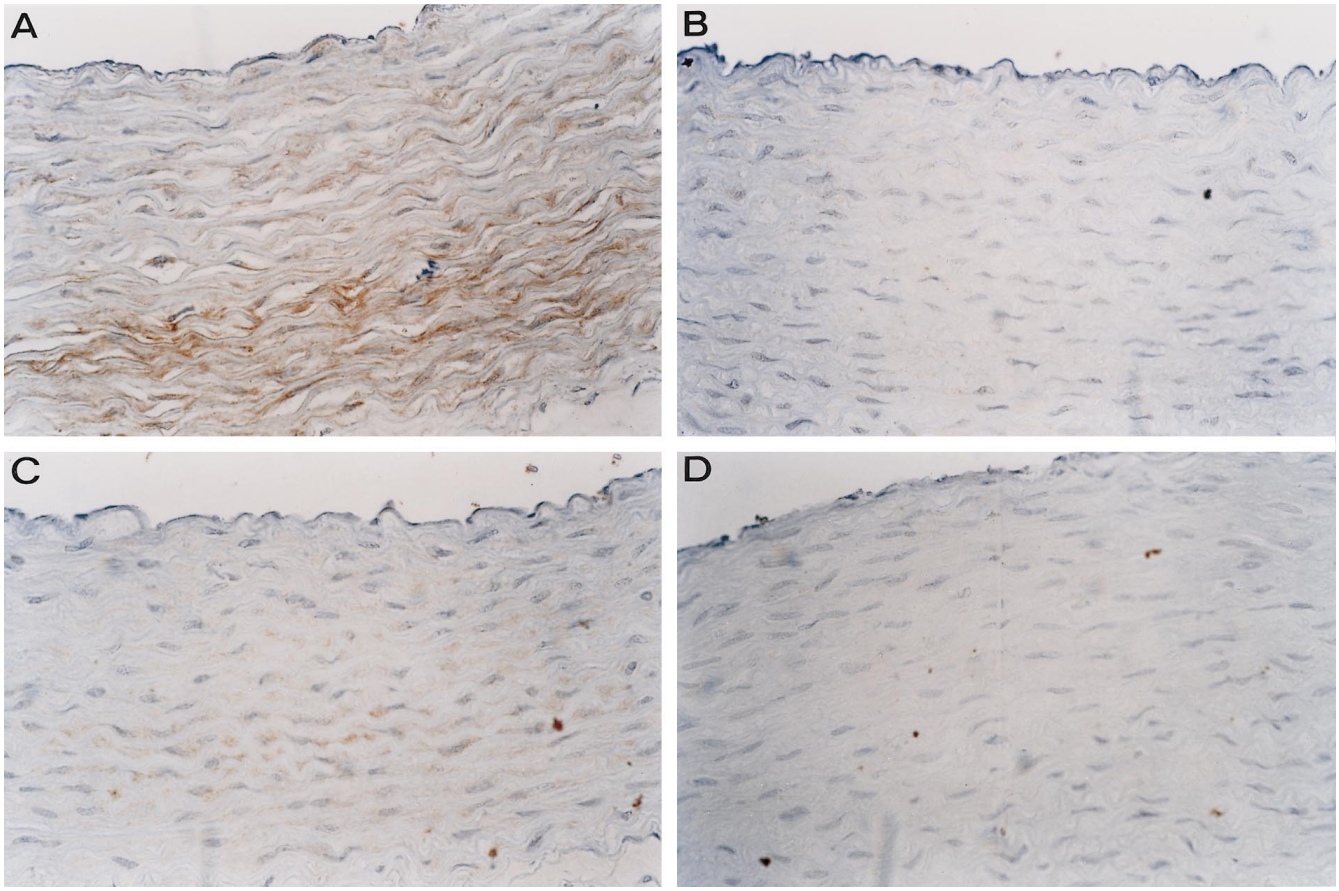

Fig. 11 Endothelin-1 (**A**, **B**) and large endothelin-1 (**C**, **D**) immunoreactivity in rabbit aortic segments. In NTG-tolerant (**A**, **C**) segments both endothelin-1 and large endothelin-1 immunoreactivity (*brown stain*) were present in the media. Normal (**B**, **D**) rabbit aortas did not exhibit positive staining for either endothelin-1 or large endothelin-1. (Adapted from [30])

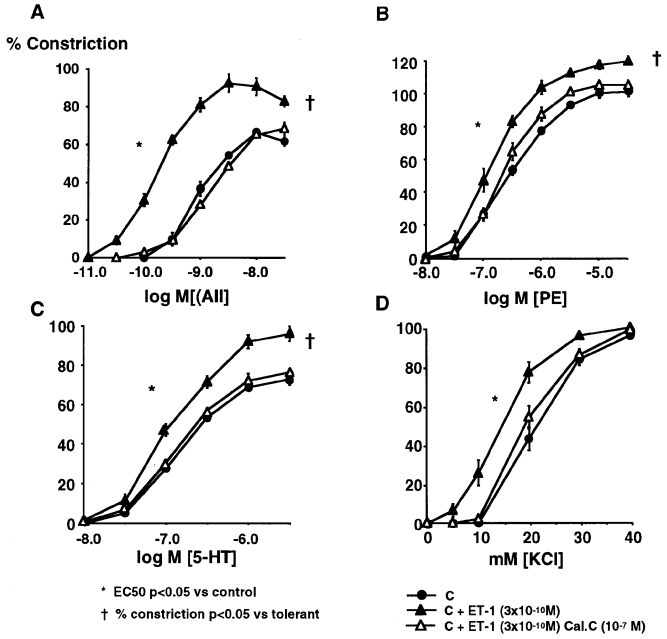

Fig. 12 Effects of preincubation of normal aortic segments with threshold concentrations of endothelin-1 (10 min) on contractions caused by angiotensin II (**A**), phenylephrine (**B**), serotonin (**C**), and potassium chloride (**D**) in the presence and absence of calphostin C. Data are expressed as mean±SEM. (Adapted from [30])

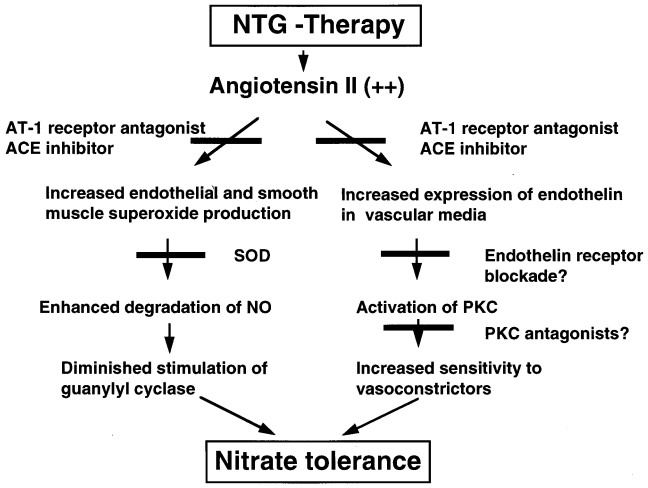

Fig. 13 Proposed mechanism for nitrate tolerance. NTG therapy increases via baroreflex mechanisms circulating levels of angiotensin II. Angiotensin II in turn activates vascular superoxide producing oxidases and induces autocrine endothelin production within vascular smooth muscle cells. Superoxide inactivates nitric oxide released from NTG and leads to diminished stimulation of the smooth muscle guanylyl-cyclase, and enhanced endothelin production serves as a priming stimulus for PKC mediating the hypersensitivity to vasoconstrictors. Either phenomenon may attenuate the vasodilator potency of NTG, thereby causing nitrate tolerance. Tolerance may be prevented by blocking the angiotensin II induced superoxide or endothelin production or antioxidants, endothelin receptor blockers, or PKC antagonists

production in nitrate tolerance [36, 37]. Moreover, in cultured smooth muscle cells, angiotensin II induces the expression of pre-proendothelin mRNA via stimulation of the angiotensin type 1 receptor subtype in a PKC-dependent mechanism [38, 39]. Thus it is conceivable to conclude that enhanced circulating angiotensin II levels, which have been encountered during the in vivo treatment with NTG [30], play a key role in initiating cellular events which ultimately lead to the attenuation of the NTG vasodilator effects during prolonged treatment periods.

Preliminary data indicate that this observation may have important clinical implications. Heitzer et al. [40] demonstrated that a 48-h treatment of patients suffering from stable coronary artery disease with intravenous NTG was associated with a marked hypersensitivity of forearm resistance vessels to angiotensin II and phenylephrine. This increase in sensitivity to vasoconstrictors was completely corrected by treating patients concomitantly with the angiotensin-converting enzyme inhibitor enalapril, suggesting an involvement of the renin-angiotensin system in mediating this phenomenon.

More recent data confirm our concept for a role of PKC in nitrate tolerance by demonstrating that in vivo treatment with a PKC inhibitor inhibits the development of a NTG-induced hypersensitivity to phenylephrine and also to a thromboxane agonist. Excitingly, PKC inhibition has also been found to prevent the development of nitrate tolerance [32]. What is the link between PKC and increased vascular (endothelial) superoxide production? The demonstration that a PKC-inhibitor prevents the development of nitrate tolerance [32] may suggest that activation of one or more PKC subtypes in the endothelium are involved in the reversible inactivation of NTG-metabolizing enzymes. PKC has been shown to activate NAD(P)H-dependent superoxide-producing oxidases in phagocytes [37]. As stated above, these oxidases represent the most important source of superoxide in endothelial and smooth muscle cells [37, 41] and also have been shown to be activated in the setting of nitrate tolerance [27, 36]. It is therefore tempting to speculate that activation of PKC during NTG therapy in turn activates oxidases in the vascular endothelium, resulting in increased superoxide production and consequently enhanced nitric oxide degradation and/or decreased NTG biotransformation.

Perspectives: do nitrates inhibit or accelerate the atherosclerotic process?

By comparing results obtained from vessels from hyperlipidemic animals and animals treated for several days with NTG, it is interesting to note that these two conditions are sharing many characteristics. Chronic NTG treatment and hypercholesterolemia are associated with increased endothelial superoxide production [24, 42], with endothelial dysfunction [24, 43, 44], with increased sensitivity to vasoconstrictors secondary to an activation

of PKC [30, 45] and increased autocrine production of endothelin-1 within the vascular media [30, 34]. These observations would explain at least in part why munition workers with high industrial NTG exposure show compared to age matched population signs for accelerated atherosclerosis in coronary arteries [46]. It is therefore tempting to speculate that NTG treatment initiates or even accelerates the atherosclerotic process rather than preventing it. The demonstration of angiotensin II mediated enhanced vascular superoxide production and activation of PKC secondary to autocrine endothelin production within the tolerant vasculature suggests that in addition to angiotensin-converting enzyme inhibitors or angiotensin type 1 receptor blockers the administration of antioxidants such as hydralazine, vitamin E, and vitamin C and, for example, endothelin receptor blockers prevent the development of nitrate tolerance (see Fig. 13). More recent data indeed indicate that concomitant treatment with antioxidants such as vitamin C and vitamin E preserve the sensitivity of the vasculature to the organic nitrates [47–49] and even prevent the development of venous tolerance in healthy volunteers [50].

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