

Nitrate tolerance impairs nitric oxide-mediated vasodilation in vivo

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Abstract

Objectives: Nitroglycerin (NTG) is metabolized to nitric oxide (NO) in vascular smooth muscle cells. It is currently not clear whether prolonged exposure to NTG and tolerance development directly affects endogenous NO-mediated vasodilation in vivo. This study investigates NO-mediated vasodilation in conscious chronically catheterized rats before and after development of nitrate tolerance. The effect of the thiol compound N-acetylcysteine (NAC), which may affect NTG responsiveness, was also studied. **Methods:** Nitrate tolerance was induced by a 72-h intravenous infusion of NTG and confirmed by a 65–68% reduction in the hypotensive response to NTG ($P < 0.05$). The hypotensive effects of acetylcholine (ACh) and sodium nitroprusside, (SNP) and possible NAC-mediated changes in the responses to these compounds were examined in nontolerant and nitrate-tolerant rats. Furthermore, the hypertensive response to the NO synthase inhibitor, NG-nitro-L-arginine methyl ester (L-NAME) was measured. **Results:** Nitrate tolerance was associated with a significantly attenuated hypotensive response to ACh (before 24 ± 1 mmHg; after 17 ± 2 mmHg, $n = 7$, $P < 0.05$). Similarly, the response to SNP was reduced from 32 ± 1 mmHg to 26 ± 3 mmHg ($n = 7$, $P < 0.05$). NTG-vehicle (placebo) did not affect the response to ACh and SNP ($P > 0.05$). NAC augmented the effect of NTG, ACh and SNP in both nontolerant and nitrate-tolerant animals ($P < 0.05$). The hypertensive response to L-NAME ($n = 8$), was reduced by 67% (from 34 ± 6 mmHg to 11 ± 1 mmHg, $P < 0.05$) after induction of nitrate tolerance. **Conclusions:** The results suggest (1) that nitrate tolerance in vivo is associated with cross tolerance to NO-mediated vasodilation produced by both exogenous and endogenous nitrovasodilators and (2) that also responses to nitrovasodilator agents other than NTG are improved by the addition of NAC.

Keywords: Nitrates; Nitrate tolerance; Nitric oxide; Acetylcholine; N-acetylcysteine; L-NAME; Rat, anesthetized

1. Introduction

Nitric oxide (NO) is an important endogenous regulator of vascular tone and accounts for the clinical effects of organic nitrates [1,2]. Continuous administration of organic nitrates (e.g. nitroglycerin (NTG) and isosorbide-dinitrate (ISDN)) leads, however, to tolerance of their hemodynamic and clinical effects [3–5]. The phenomenon of nitrate tolerance is complex and still not understood. Some of the mechanisms underlying tolerance development may include reduced bioconversion of the vasoinactive parent compound to NO [2,6,7] and neurohormonal activation, counteracting nitrate-induced NO-mediated vasodilation [8–10]. However, other mechanisms may also play a role [11,12].

Recent in vitro data suggest that nitrate tolerance may be associated with an impaired function of NO probably caused by nitrate-mediated changes in the oxidative milieu [13]. In addition, exogenously added NO causes a reversible inhibition of the synthesis of the endothelium-derived NO in vitro [14]. Thus, it is possible that prolonged nitrate therapy directly affects endogenous NO-mediated vasodilation. However, despite important clinical implications, the in vivo effect of nitrates on the endogenous vascular NO pathway is currently not clear.

The purpose of this study was to determine whether nitrate tolerance affects the endogenous NO pathway in vivo. Endothelium-dependent and endothelium-independent NO-mediated vasodilation was measured before and after development of nitrate tolerance. Thiol compounds have previously been shown to modify nitrate responsive-

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ness [15–21], thus, the influence of the thiol compound N-acetylcysteine (NAC) on NO-mediated vasodilation was also investigated.

2. Materials and methods

2.1. Animals

Specific-pathogen-free female Wistar rats (200 to 250 g) were housed under constant temperature and humidity conditions. Light was controlled in a 12/12 h light–dark cycle. Before and during the experimental period, all rats had free access to a standard rat chow and tap water. The rats were anesthetized with 1 to 3% halothane and N₂O/O₂ (2:1). One catheter (medical-grade Tygon catheters) was implanted with the tip in the ascending aorta through the left carotid artery, and 3 separate catheters were placed in the superior vena cava via the left (2) and right (1) jugular vein. Catheters were filled with a solution of 50% glucose and 500 IU heparin/ml and plugged with a nylon pin. Each catheter was externalized through the back in the neck region and secured by a polyester felt disc placed in the subcutis. After implantation of catheters, the rats were housed individually until they had regained their preoperative weight and appeared healthy (6 to 8 days after catheter implantation). All experiments were performed in conscious unrestrained rats.

2.2. Induction of nitrate tolerance

At the end of the recovery period (day 0), the hemodynamic response to NTG was determined from the blood pressure lowering effect of intravenous bolus infusions (0.4 ml over 60 s) of NTG (0 mg (placebo) and 5 mg NTG) separated by a 20 min interval. An osmotic minipump (Alza Corp.) was then placed subcutaneously and connected to one of the 3 i.v. catheters. NTG or placebo (98% ethanol (NTG-vehicle)) was delivered from the minipump at a constant rate of 0.2 mg/h i.v. (10 μ l/h) throughout the study period. To confirm development of nitrate tolerance, the blood pressure lowering effect of NTG/placebo bolus infusions was reexamined at day 3 in all treatment groups. Baseline blood pressure (before NTG/placebo bolus infusion) and blood pressure changes during NTG/placebo bolus infusions were recorded continuously by pressure transducers (Baxter Corp, Uden, Netherlands) connected to the arterial catheter. Tracings were displayed on a Graphtec linear recorder (Watanabe Instruments Corp., Japan). The investigation conforms with the Guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1985).

2.3. Experimental protocol

Six different groups of conscious chronically catheterized rats were studied. In the 3 groups receiving prolonged NTG infusion, nitrate tolerance was confirmed by the response to NTG bolus infusions as described above.

2.3.1. NO-mediated vasodilation in nontolerant and nitrate-tolerant rats

Two groups of rats were investigated. Seven rats received longterm infusion of NTG (0.2 mg/h) for 3 days (NTG-tolerant group). Another seven rats received NTG-vehicle infusion for a similar period of time (NTG-placebo). In each group a NO-mediated reduction in mean arterial blood pressure (MAP) was induced before and after 3 days (72 h) of NTG/placebo infusion. Endothelium-dependent NO-mediated vasodilation was examined using acetylcholine (ACh) and endothelium-independent NO-mediated vasodilation using sodium nitroprusside (SNP). Both ACh (10 μ g/kg) and SNP (20 μ g/kg) were administered as a bolus i.v. infusion (0.4 ml over 60 s) separated by a 20 min interval allowing MAP to recover to the baseline value. Based on previous dose-response experiments, submaximal (in the range of ED₅₀–ED₇₅) doses of ACh and SNP were chosen and expected to elicit a fall in MAP of approximately 30 mmHg in normal untreated rats. Isotonic saline was used as control.

2.3.2. Thiol supplementation and NO-mediated vasodilation in nontolerant and nitrate-tolerant rats

On day 3, after completion of the experiments described above, the same two groups of rats received NAC (5 mmol/kg per h intravenously (1.5 ml/h) for 3 h) in addition to the ongoing NTG/placebo infusion. The hemodynamic responses to ACh, SNP and NTG were repeated during the last infusion hour.

2.3.3. Nitrate tolerance and NO synthase inhibition

The hemodynamic effect of endothelial NO synthase inhibition by N^G-nitro-L-arginine-methyl ester (L-NAME) was studied in 2 other groups of rats ($n = 8$ in each group). One group of normal nontolerant rats (control) and one group of nitrate-tolerant rats (NTG-tolerant + L-NAME) received intravenous L-NAME (1.5 mg/kg) for 60 s. From dose-response experiments 1.5 mg L-NAME per kg was expected to increase MAP by approximately 30 mmHg in normal nontolerant rats.

2.3.4. NO-independent vasodilation in nontolerant and nitrate-tolerant rats

In order to investigate the effect of nitrate tolerance on NO-independent vasodilation, the effect of the calcium channel antagonist nimodipine (200 μ g/kg, 0.4 ml over 1 min) was investigated before and after development of nitrate tolerance in one additional group of rats receiving

NTG for 72 h ($n = 6$) and a comparable control group receiving NTG vehicle ($n = 4$).

2.4. Drugs

NTG solutions were prepared from a stock solution (100 mg NTG in 1 ml of 98% ethanol). For prolonged infusion, NTG was further diluted in 98% ethanol (20 mg/ml). All other solutions were prepared in 0.9% saline and adjusted to pH 7.4. Ach, SNP, L-NAME were purchased from Sigma Chemical Co, St. Louis, MO. NAC (Mucomyst[®]) was kindly delivered by ASTRA A/S, Copenhagen, Denmark.

2.5. Calculations and statistics

Mean arterial blood pressure (MAP) was estimated as diastolic pressure plus (systolic minus diastolic pressure)/3 in mmHg. The reported changes in MAP to ACh and SNP represent the difference between the baseline MAP (immediately before ACh and SNP) and the nadir on the blood pressure curve after ACh and SNP. The hypertensive effect of L-NAME was calculated as the maximum increase from baseline MAP after i.v. L-NAME administration. All data are presented as mean \pm s.e.m. In the 2 groups receiving NAC, differences within each group were determined by a repeated measures analysis of variance. In the other experiments differences within groups (pre- and posttreatment means) were determined by Student's paired *t*-test. Within each set of experiments comparisons between the study group and the control group were done with Student's *t*-test for unpaired samples. Statistical significance was assumed when $P < 0.05$.

3. Results

3.1. Induction of nitrate tolerance

In the 3 groups of rats infused with NTG for 3 days, development of nitrate tolerance was confirmed by a 68%, 67% and 65% reduction (compared with preinfusion) in the hypotensive effect of bolus NTG (NTG-tolerant group: from 25 ± 3 mmHg to 8 ± 1 mmHg (Fig. 1); NTG-tolerant/L-NAME group: from 34 ± 6 to 11 ± 1 mmHg; NTG-tolerant/nimodipine group: from 26 ± 2 to 9 ± 1 mmHg, $P < 0.05$). The response to NTG in the groups infused with NTG-vehicle did not change during the 3-day infusion period ($P > 0.05$) (Fig. 1). Baseline MAP values (before NTG bolus challenges) were similar in all treatment groups and did not change ($P > 0.05$) during the experimental period (0 h vs 72 h, NTG-tolerant group: 110 ± 2 vs 107 ± 5 mmHg; NTG-tolerant/L-NAME group: 106 ± 4 vs 109 ± 6 ; NTG-tolerant/nimodipine group: 111 ± 5 vs 108 ± 3 mmHg; NTG-placebo: 106 ± 4 vs 108 ± 5 mmHg). Bolus doses of saline and NTG-placebo

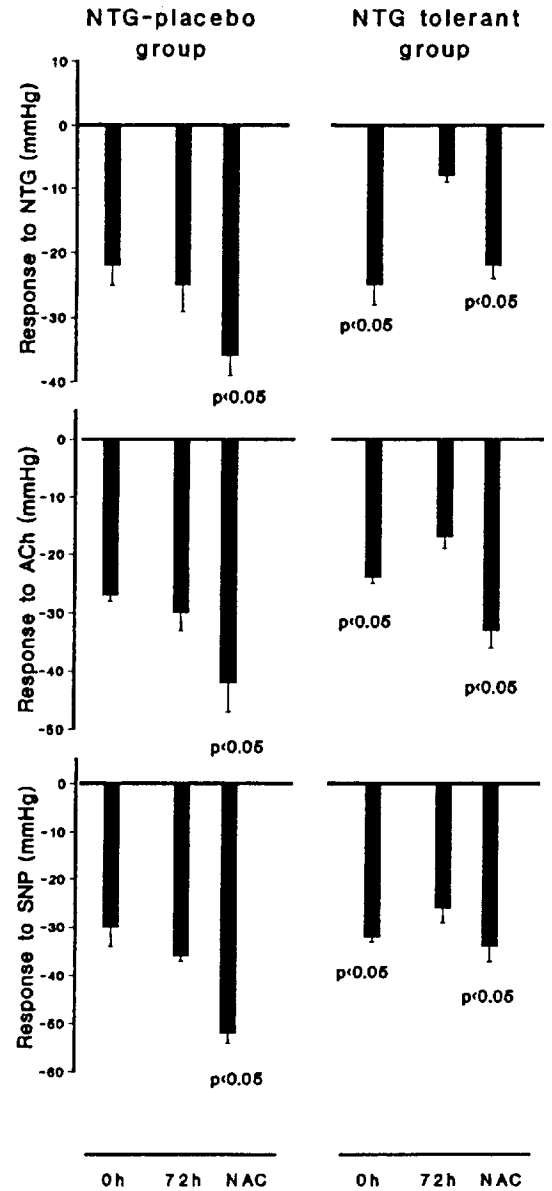


Fig. 1. Bar graphs showing the hypotensive effects of NTG, ACh and SNP in two groups of rats ($n = 8$ in each group) before (0 h) and after a 72 h (72 h) intravenous infusion of either NTG vehicle (NTG-placebo, left panel) or NTG 0.2 mg/h (NTG-tolerant, right panel). In each group the effect of additional thiol supplementation with NAC (5 mmol/kg per h for 3 h) is also shown (NAC). $P < 0.05$: significantly different from 72 h.

caused no significant changes in MAP in any of the experiments (data not shown).

3.2. NO-mediated vasodilation in nontolerant and nitrate-tolerant rats

The hypotensive effect of ACh was reduced by 29% after development of nitrate tolerance (NTG group); (from 24 ± 1 mmHg at day 0 (before NTG infusion) to 17 ± 2 mmHg at day 3 after 72 h of NTG infusion) (Fig. 1). Similarly, the fall in MAP induced by SNP was reduced by 19% from 32 ± 1 mmHg to 26 ± 3 mmHg ($P < 0.05$)

after development of nitrate tolerance (Fig. 1). The data suggest that development of nitrate tolerance *in vivo* is associated with a significant attenuation of both endothelium-dependent (ACh) and endothelium-independent (SNP) NO-mediated vasodilation. The effect of nitrate tolerance on the responses to ACh and SNP is, however, less pronounced than the effect on NTG responsiveness ($P < 0.05$) (NTG group). Infusion of NTG-vehicle (placebo group) did not affect responses to ACh and SNP (Fig. 1).

3.3. Thiol supplementation and NO-mediated vasodilation in nontolerant and nitrate-tolerant rats

NAC administration significantly potentiated the effect of all the 3 drugs used to induce NO-mediated vasodilation (i.e. NTG, ACh and SNP) in both nitrate-nontolerant and nitrate-tolerant rats (Fig. 1). Intriguingly, the responses to NTG, ACh and SNP in nitrate-tolerant rats treated with NAC were not different from either the initial NTG responses before longterm infusion of NTG or the responses seen after 72 h in the NTG-placebo group (Fig. 1).

3.4. Nitrate tolerance and NO synthase inhibition

An effect of nitrate tolerance development on endothelial NO regulation is further substantiated by the observation of a marked reduction (62%) in the blood pressure response to NO synthase inhibition with L-NAME (Fig. 2). In the group of normal nontolerant rats (L-NAME group) MAP increased by 29 ± 6 mmHg whereas MAP increased only 11 ± 1 mmHg in nitrate-tolerant animals (NTG-tolerant/L-NAME group) ($P < 0.05$).

3.5. NO-independent vasodilation in nontolerant and nitrate-tolerant rats

In contrast to the drugs inducing NO-mediated vasodilation, NO-independent (and endothelium-independent) va-

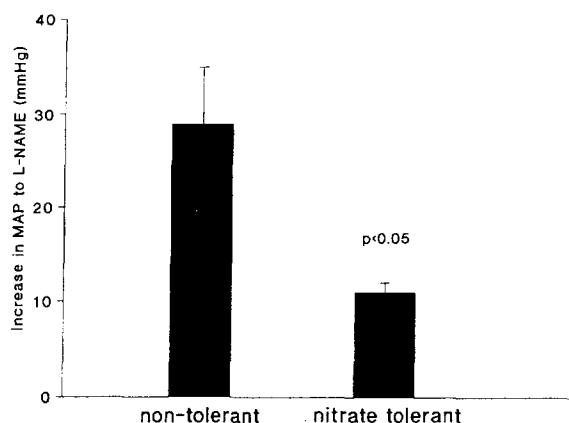


Fig. 2. Bar graph showing the increase in mean arterial pressure (MAP) after infusion of the NO synthase inhibitor L-NAME (1.5 mg/kg) in normal nontolerant rats ($n = 8$) and nitrate-tolerant rats ($n = 8$). $P < 0.05$: significantly different from control.

sodilation by nimodipine was not affected by the development of nitrate tolerance. Nimodipine reduced MAP by 26 ± 4 mmHg before the NTG infusion and by 25 ± 3 mmHg after the development of nitrate tolerance ($P < 0.05$). In the corresponding control animals the responses were 25 ± 2 and 24 ± 2 mmHg respectively.

4. Discussion

A major finding of this *in vivo* study is that development of nitrate tolerance is associated with significant impairments of the vascular effects of endothelium-dependent NO agonist (ACh) and antagonist (L-NAME) treatment. Thus, prolonged administration of an exogenous nitrovasodilator and development of *in vivo* nitrate tolerance significantly interferes with the endogenous nitrovasodilator pathway for vascular relaxation.

Vascular (endothelial) NO is produced from L-arginine [22]. The process is controlled by the enzyme, NO-synthase, which can be stimulated by both receptor-dependent agonists (e.g. ACh) and physical stimuli or inhibited by L-arginine analogs (e.g. L-NAME) [23]. NO activates soluble guanylate cyclase and the subsequent increase in cyclic GMP leads to smooth muscle relaxation [24]. Organic nitrates and SNP are converted directly to NO and “bypass” the endogenous NO synthesis. However, they parallel the biological actions of endogenous NO due to the common final pathway (i.e. activation of guanylate cyclase).

In the present study marked tolerance to the hemodynamic effects of NTG developed after intravenous infusion of NTG in conscious rats. However, not only the response to NTG, but also the responses to ACh, L-NAME and SNP were significantly attenuated, suggesting the existence of a certain degree of cross tolerance between NTG and other compounds modulating NO-mediated vasorelaxation. Interestingly, NO-independent vasodilation, as measured by the response to nimodipine, was not affected by tolerance development, suggesting that the observed cross tolerance is specific for nitrovasodilator substances.

Data from previous *in vitro* studies have been conflicting [25–35]. In general, experiments performed strictly *in vitro*, incubating vessels with high concentrations of NTG [25–29], have not been able to show the existence of cross tolerance to other nitrovasodilators whereas more physiological approaches using *in vivo* NTG exposure and subsequent *in vitro* testing [11,13,32] are more compatible with the present strictly *in vivo* setup. Thus, the present finding is compatible with recent reports [13,34] stressing the differences between results obtained by *in vitro* methods of tolerance development as compared with methods of *in vivo* nitrate tolerance development.

Mechanisms responsible for a nitrate-induced development of cross tolerance between NTG and other NO releasing substances may include either a downregulation

of endogenous endothelial NO synthesis and/or an impaired effect of NO itself (e.g. desensitization of guanylate cyclase). In vitro, administration of different NO-donor agents including NTG may inhibit endothelial NO synthase [14]. It is tempting to speculate that a NO-induced (NTG) negative feed-back regulation on endothelial NO synthase and thus decreased endogenous vasodilator forces may contribute partly to the hemodynamic signs of nitrate tolerance. However, while our findings of decreased responses to ACh and L-NAME are compatible with a nitrate-induced inhibition of endothelial NO synthase, such a mechanism does not explain the reduced effect of NTG and SNP in this and other studies [11,13,30–33,35]. It is therefore of note that Münzel and coworkers [13] recently reported that NTG treatment is associated with an increased endothelial production of reactive oxygen species in nitrate-tolerant aorta segments. These changes may affect the efficacy of NO per se [36]. The fact that the antioxidant, superoxide dismutase (scavenging O_2^-) markedly enhanced relaxations to NTG, SNP and ACh (all releasing NO by different mechanisms) [13] further suggests that increased oxidative stress and increased degradation of NO by reactive oxygen species may contribute to the development of both nitrate tolerance and cross tolerance. This hypothesis may account for (1) the observed reduction in endothelium-dependent NO-mediated vasoreactivity (ACh and L-NAME), (2) the reduced hypotensive effect of SNP and (3) the potentiated hypotensive effect of NTG, SNP and ACh after administration of NAC which has both extracellular and intracellular antioxidant properties.

The reduced response to L-NAME in tolerant rats suggests that nitrate tolerance inhibits endogenous NO-synthase activity and/or that basally produced NO is rapidly inactivated. In as much as the response to L-NAME may be considered as a marker of the contribution of basally produced NO to the regulation of vascular tone, the present results are compatible with the assumption that nitrate tolerance not only has a detrimental effect on agonist-induced NO-mediated vasodilation but also affects the basal regulation of vascular tone.

Tolerance to NTG was significantly more pronounced than tolerance to ACh and SNP. This observation stresses that other (nitrate specific) mechanisms (e.g. impaired bioconversion of NTG) also may contribute to in vivo tolerance development to organic nitrates.

Coadministration of thiols and NTG have previously been shown to augment the in vivo circulatory response to NTG both in nontolerant [34,37,38] and nitrate-tolerant conditions [5,19,21]. Data on in vitro NTG/thiol interactions are, however, more divergent [15–17,39–41]. The mechanisms underlying such an interaction are not clear, but in addition to the specific cofactor requirement of thiol compounds for the conversion of organic nitrates to NO [1,34,42], thiols like NAC may induce the formation of vasoactive nitrosothiols and improve the action of NTG on

the microcirculation [41,43,44]. As suggested by the lack of tolerance specificity and the present finding of a thiol-induced change in the in vivo effect of other NO donors as well, the effect of thiols may be rather nonspecific. Since antioxidants like superoxide dismutase [13] and ascorbic acid [45] may also increase the vascular effects of SNP and ACh, it is possible that an effect of thiol supplementation may at least partly be related to its vascular reducing capacity. In this context it is interesting that NAC has been shown to prolong the half live of endothelium-derived relaxing factor in vitro [46,47].

In summary, this study shows that hemodynamic tolerance to NTG is accompanied by an in vivo attenuation of both endothelium-dependent (ACh, L-NAME) and endothelium-independent NO-mediated effects (SNP). It is concluded that (1) nitrate tolerance in vivo is associated with cross tolerance to NO-mediated vasodilation produced by both exogenous and endogenous nitrovasodilators and (2) reduced effects of NO-dependent vasodilators may contribute to the development of nitrate tolerance in vivo.

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