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Nitrate Tolerance: Effect of Thiol Supplementation during Prolonged Nitroglycerin Infusion in an *in Vivo* Rat Model[1]

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ABSTRACT

Depletion of intracellular sulfhydryl groups has been considered a main reason for the development of nitrate tolerance during sustained nitrate therapy. Although administration of *N*-acetylcysteine, a sulfhydryl donor, potentiates the acute hypotensive effect of nitroglycerin (NTG), its role in the reversal of nitrate tolerance is controversial. In the present study, we developed a conscious *in vivo* rat model to study nitrate tolerance and nitrate-thiol interactions. Tolerance to NTG, as assessed by the blood pressure reduction in response to i.v. NTG bolus doses, developed after 24 h of i.v. NTG infusion. After 3 and 5 days of 0.2 mg/h NTG i.v., the dose-response relations for NTG-induced reduction in blood pressure were shifted to 25-fold higher doses ($P < .01$). Infusion of *N*-acetylcysteine (0.245, 1.225 and 6.125 mmol/kg/h for 4 h) and, to a lesser extent, equimolar doses of reduced glutathione, but not *N*-acetylserine, significantly potentiated the hypotensive effect of NTG, in a dose-dependent manner ($P < .05$). However, complete reversal of tolerance was not achieved. This animal model of nitrate tolerance is suitable for further investigations of nitrate-thiol interactions and shares similarities with nitrate tolerance development in humans. The results suggest that sulfhydryl supplementation may enhance the hypotensive effect of NTG in a dose-dependent manner. This effect is more likely to be achieved with *N*-acetylcysteine than with glutathione and may be related to differences in membrane permeability.

The vasodilating properties of organic nitrates attenuate rapidly during continued administration. This development of tolerance is imperfectly understood but has been attributed to a loss of nitrate effect on the vessel's smooth muscle (Needleman and Johnson, 1973). During its biotransformation, NTG interacts with sulfhydryl-containing compounds (*e.g.*, cysteine and glutathione) in vascular smooth muscle cells to generate vasoactive *S*-nitrosothiols and NO. A depletion of the intracellular thiol or "sulfhydryl pool" has been suggested as a main reason for the development of nitrate tolerance (Needleman and Johnson, 1973; Ignarro and Gruetter, 1980; Ignarro *et al.*, 1981; Torresi *et al.*, 1985). In clinical studies, supplementation with the sulfhydryl NAC counteracts nitrate tolerance, in accordance with this theory (May *et al.*, 1987; Packer *et al.*, 1987). However, other studies have failed to demonstrate such an effect (Parker *et al.*, 1987; Gruetter and Lemke, 1986). At present, there are only limited data regarding the effect of thiols on nitrate responsiveness in intact animals (Fung *et al.*, 1988; Munzel *et al.*, 1988). Recently, Fung *et al.* (1988) found a thiol-induced potentiation of NTG effects in nontolerant rats, but a similar effect has not been observed in nitrate-tolerant animals.

In the present study, firstly, we developed a conscious *in vivo* rat model to study development of nitrate tolerance and nitrate-thiol interactions. Secondly, we investigated the hemodynamic response to NTG and graded thiol infusions in conscious nitrate-tolerant rats.

Materials and Methods

Animals. Specific pathogen-free female Wistar rats (200 to 280 g) were used and housed under constant temperature and humidity conditions. Light was controlled to 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.

Rats were anesthesized with 1 to 3% halothane and N_2O/O_2 (2:1). Chronic catheterization of the left carotid artery and left and right jugular veins was performed by a technique modified from the method described by Gellai and Valtin (1979). One catheter (medical-grade Tygon catheters) was implanted with the tip in the ascending aorta, through the left carotid artery, and three separate catheters were placed in the superior vena cava *via* the left (two) and right (one) jugular veins. Catheters were filled with a solution of 50% glucose and 500 IU/

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ABBREVIATIONS: NTG, nitroglycerin; NO, nitric oxide; NAC, *N*-acetylcysteine; GSH, glutathione; NAS, *N*-acetylserine; MAP, mean arterial blood pressure; HR, heart rate.

ml heparin and plugged with a nylon pin. Each catheter was externalized through the back in the neck region and secured by a polyester felt disk placed in the subcutis.

***In vivo* induction of nitrate tolerance.** After implantation of permanent catheters, the rats were housed individually until they had regained their preoperative weight and appeared healthy (6 to 9 days after catheter implantation). At this time point (day 0), an osmotic minipump (Alza Corp.) was connected to one of the three i.v. catheters and placed s.c. For long-term exposure (7 days) to NTG, eight rats received NTG at a dose of 1 mg/h and another eight rats received NTG at a dose of 0.2 mg/h. The NTG was dissolved in a constant volume of ethanol, and each animal received 10 μl of 99% ethanol per h. A control infusion (99% ethanol) was performed in another series of seven rats. Development of tolerance was examined with four escalating bolus doses of NTG [0 mg (5% glucose/ethanol), 0.1 mg, 0.5 mg and 2.5 mg] . This dosing schedule was initiated immediately before the start of the NTG or control infusion (day 0) and repeated every 24 h throughout the 7-day study period. The different NTG bolus doses were separated by a 30-min interval, which allowed blood pressure to recover completely before administration of the next NTG bolus. The total volume of each bolus dose was 0.4 ml/animal, and each NTG bolus "schedule" lasted for 2 h. In the group infused with 0.2 mg/h, NTG bolus testing was additionally done 3 and 12 h after infusion start. Different catheters were used for constant infusion of NTG and for NTG bolus injections.

Blood pressure during base-line infusion conditions, as measured before NTG bolus administration, and blood pressure alterations during NTG bolus challenges were recorded continuously by Trantec pressure tranducers (model 60–800) connected to the left carotid catheter. HR was derived from the arterial pressure signal. Tracings of the variables were displayed on a Watanabe linear recorder (Watanabe Instruments Corp., Japan).

Animals were conscious and unrestrained throughout the experiment. Between NTG bolus dose testing they were housed in their usual surroundings.

At the time points 6 h and 1, 3, 5 and 7 days, blood samples (1 ml) for determination of plasma NTG were drawn from the arterial catheter into ice-cooled Eppendorf micro-test tubes containing 20 μl of 200 mM iodoacetamide solution. After centrifugation for NTG analysis, blood cells were suspended in 0.5 ml of 0.9% NaCl and reinfused to the rat.

Thiols and NTG effect during nitrate tolerance. The effect of NAS and different doses of NAC and GSH on the blood pressure response to NTG was studied in concious unrestrained nitrate-tolerant rats. From day 0, an i.v. infusion of NTG at a dose of 0.2 mg/h was established and continued throughout the protocol. Eight rats received 4-h infusions of NAC, GSH or NAS on days 3, 4 and 5. Infusions were given in a separate catheter in randomized order and in equimolar amounts of 1.225 mmol/kg/h (medium dose), in a volume of 1.5 ml/h. Before each of these infusions, NTG bolus dose testing was performed as described above. During the last 2 h of thiol infusion, the NTG bolus challenge doses were repeated (each animal served as its own control). In a similar manner (in two other groups of animals), the effect of a 5-fold lower (low dose, 0.245 mmol/kg/h; $n = 8$) and a 5-fold higher (high dose, 6.125 mmol/kg/h; $n = 7$) dose of NAC and GSH was tested in the same paired design.

NAS is identical to NAC except that it contains an hydroxy group in place of a sulfhydryl group. Differences in response to NAC and NAS were consequently interpretated as being related to the sulfhydryl group.

Drugs. NTG solutions were prepared from a 10:1 stock solution. For prolonged i.v. infusion NTG was dissolved in 99% ethanol, and for bolus injections NTG was diluted with 5% glucose. NAC, GSH and NAS were purchased from Sigma Chemical Co.; solutions were prepared in 5% glucose and adjusted to pH 7.4.

Plasma NTG assay. NTG was analyzed by gas chromatography, with electron capture detection. Twenty-five microliters of ethanolic solution of 14 ng of butantriol-trinitrate were added to an Extrelute 1 column (E. Merck Industrial Inc.). Two hundred-fifty microliters of rat plasma, prediluted 1:10 with outdated blood bank plasma, were added, followed by 500 μl of *n*-pentane. After 10 min, the compounds were eluted with 6 ml of *n*-pentane, which was evaporated under airflow at ambient temperature. The residue was immediately dissolved in 40 μl of toluene, and 1 μl was injected on a DB-210 column (15 m × 0.53 mm) (J & W) connected to a HP-5 (15 m × 0.53 mm) (HP) column. Injection and detection temperature were set at 200°C and the oven at 150°C. Relative s.d. on duplicates was 4.6%, and the minimum detected amount was 6 pg/injection.

Calculations and statistics. MAP was estimated as diastolic pressure + (systolic pressure − diastolic pressure)/3 (in mm Hg). The presented reduction in MAP after NTG bolus administration represents the difference between the pre-NTG-bolus value and the nadir value on the blood pressure response curve. Within 10 min, blood pressure in all rats recovered to the level recorded before NTG challenge. Within the same group of animals, responses were analyzed using analysis of variance for multiple comparisons of treatments, animal and period effects, followed by paired or unpaired *t* tests with Bonferroni's correction when appropriate. Values are presented as mean ± S.E.M. Statistical significance was set at the level of $P < .05$.

Results

Induction of tolerance. During infusion of NTG for 7 days, tolerance was manifested by a shift in the MAP response curve and a reduced maximum efficacy of NTG bolus doses (fig. 1). This tolerance appeared after 24 h, *i.e.*, tolerance to NTG developed between 12 and 24 h, and persisted for 7 days (fig. 1).

Before the start of infusion (day 0), the effects of NTG bolus doses were similar in the three treatment groups (control, 0.2 mg/h NTG and 1 mg/h NTG) (fig. 1). In the control experiment, *i.e.*, nontolerant animals, the responses to NTG bolus doses were dose dependent and completely reproducible throughout the study period, demonstrating that the NTG challenges did not provoke tolerance (fig. 1).

From day 1, during infusion of NTG (0.2 mg/h) and the remaining part of the infusion period, the blood pressure response to NTG challenge doses was significantly reduced, compared with the pretreatment response ($P < .01$). Furthermore, the typical dose-effect relation seen before the start of infusion (day 0) was blurred (fig. 1). At day 3, the fall in MAP in response to a bolus dose of 0.1, 0.5 and 2.5 mg of NTG was reduced by 16 ± 2 mm Hg ($P < .01$), 19 ± 3 mm Hg ($P < .01$) and 20 ± 2 mm Hg ($P < .01$), respectively. The NTG bolus dose needed to elicit a 10-mm Hg fall in MAP was approximately 25-fold higher, compared with preinfusion doses.

A marked shift in the dose-response curve was also observed during infusion of NTG at a dose of 1 mg/h (fig. 1). However, this response was not significantly different from that observed in the group infused with 0.2 mg/h NTG ($P > .05$).

Base-line MAP and HR are shown in table 1. Compared with control infusion, no significant changes in the base-line values of MAP were observed during infusion of 0.2 mg/h NTG for 7 days (table 1). HR was not affected by NTG infusion. During infusion of the high dose of NTG (1 mg/h), MAP was significantly lowered after 24 h (day 1) but returned to control levels within 3 days (table 1).

An initial small decrease in hematocrit values was observed during the control infusion period and may be related to the implantation of the infusion pump (table 1). An additional significant reduction in hematocrit levels was seen during NTG infusions, compared with control (table 1). This reduction developed within 24 h during infusion of the highest dose of NTG and within 3 days during infusion of 0.2 mg/h NTG.

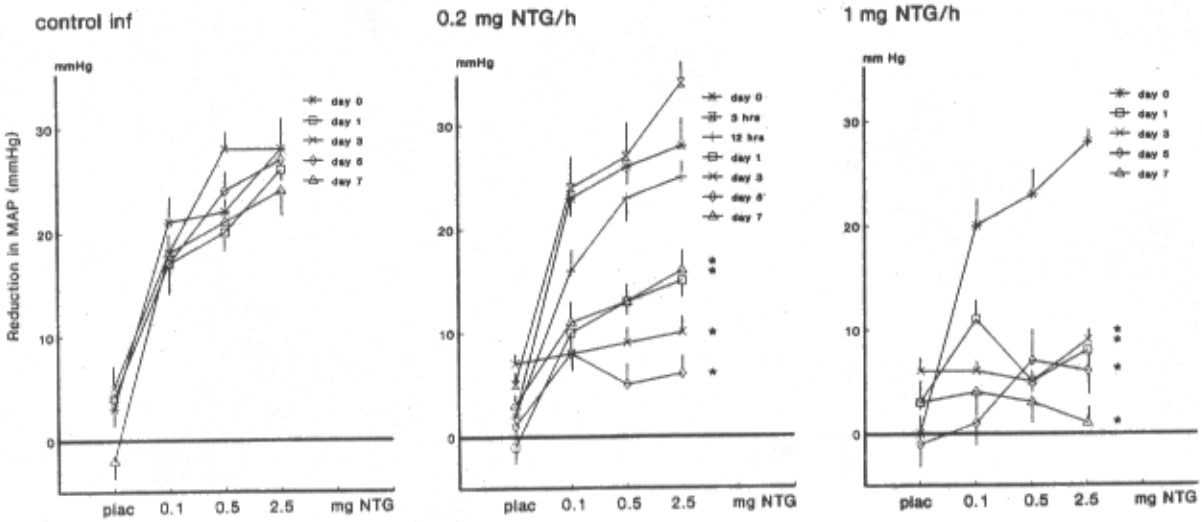

Fig. 1. Reduction in MAP in response to increasing bolus doses of NTG during infusion of 0.2 mg/h NTG ($n = 8$) or 1 mg/h NTG ($n = 8$) or control infusion ($n = 7$) for 7 days. Values are mean ± S.E.M. *, $P < .05$, compared with pretreatment levels (day 0).

TABLE 1

Effect (mean ± S.E.M.) of i.v. NTG/control infusions for 7 days on MAP, HR, plasma NTG and hematocrit (Hct) in unrestrained conscious rats

Day 0, values immediately before infusion start. $n = 8$ in both NTG groups and $n = 7$ in the control group.

	Day				
	0	1	3	5	7
MAP (mm Hg)					
Control	114 ± 2	112 ± 2	108 ± 2	113 ± 5	115 ± 5
NTG (0.2 mg/h)	117 ± 4	115 ± 4	114 ± 5	112 ± 4	120 ± 2
NTG (1 mg/h)	113 ± 5	90 ± 2*†	105 ± 3	109 ± 4	117 ± 2
HR (beats/min)					
Control	441 ± 8	454 ± 14	425 ± 16	460 ± 24	435 ± 10
NTG (0.2 mg/h)	438 ± 9	411 ± 18	428 ± 16	420 ± 22	
NTG (1 mg/h)	421 ± 14	468 ± 15	416 ± 21	446 ± 15	436 ± 16
Plasma NTG[a] (μg/liter)					
Control					
NTG (0.2 mg/h)	55 ± 18°	52 ± 9	42 ± 10	59 ± 7	42 ± 10
NTG (1 mg/h)		146 ± 33	128 ± 22	135 ± 41	100 ± 17
Hct (%)					
Control	45 ± 3	38 ± 3	36 ± 2	38 ± 1	42 ± 1
NTG (0.2 mg/h)	45 ± 2	36 ± 2	29 ± 1*†	30 ± 2*†	32 ± 1*†
NTG (1 mg/h)	42 ± 1	27 ± 3*†	29 ± 2*†	31 ± 1*†	32 ± 4*†

[a] $n = 6$.
° Blood samples collected 6 h after start of infusion ($n = 4$).
* Significantly ($P < .05$) different from pretreatment values.
† Significantly ($P < .05$) different from control.

Within each treatment group, plasma NTG levels were constant during the infusion period (table 1).

Sulfhydryl supplementation and reversal of tolerance. Tolerance could be achieved with a NTG dose of 0.2 mg/h, and this infusion rate was chosen in the protocols involving NAS, NAC and GSH. The presence of nitrate tolerance was substantiated by the attenuated blood pressure response to NTG bolus testing before the start of the 4-h infusions of NAC, GSH and NAS (table 2; fig. 2).

In nitrate-tolerant animals, NAC significantly potentiated the hypotensive effect of NTG, in a dose-dependent manner (table 2; fig. 2). After the highest and most effective dose of NAC, the fall in MAP was still less than that in nontolerant rats ($P < .05$), suggesting that full reversal of tolerance was not achieved. Whether this could be achieved by even higher doses of NAC was not investigated.

Infusion of GSH also potentiated the blood pressure-lowering effect of NTG (table 2; fig. 2), but on a molar basis the effect of GSH was less than that of NAC infusion. Only after infusion of GSH at the highest dose was an augmented effect on MAP seen for all bolus doses of NTG tested ($P < .05$). A trend towards an increased NTG responsiveness during infusion of the high dose of GSH, compared with the lower doses, did not reach statistical significance ($P > .05$).

NAS infusion (1.225 mmol/kg/h) did not influence the response to NTG bolus doses ($P > .05$) (table 2; fig. 2).

Compared with equimolar concentrations of GSH, the blood pressure-lowering effects of 0.5 and 2.5 mg of NTG were significantly ($P < .05$) increased during infusion of the medium and high NAC doses (table 2; fig. 2).

Base-line MAP and HR were not affected by infusion of thiols and NAS ($P > .05$).

TABLE 2

Reduction in MAP in response to NTG bolus doses before and after thiol infusions in nitrate-tolerant rats

Intravenous infusions of low (0.245 mmol/kg/h, $n = 8$), medium (1.225 mmol/kg/h, $n = 8$) and high (6.125 mmol/kg/h, $n = 8$) doses of NAC and GSH and a medium dose of NAS lasted for 4 h. The NTG bolus testing was performed before infusion and repeated during the last 2 h of the 4-h infusion period. Effects of NTG bolus doses (0.1, 0.5 and 2.5 mg) are given as mean ± S.E.M.

	MAP reduction					
	Preinfusion			Postinfusion		
	0.1*	0.5	2.5	0.1	0.5	2.5
	mm Hg					
NAC						
Low	4 ± 2	9 ± 1	11 ± 1	6 ± 2	11 ± 2	14 ± 2
Medium	4 ± 1	9 ± 2	10 ± 2	7 ± 2*	17 ± 2*,§	17 ± 2*,§
High	5 ± 1	9 ± 2	9 ± 2	11 ± 2*,†	17 ± 2*,§	22 ± 3*,†,§
GSH						
Low	4 ± 1	7 ± 1	9 ± 1	6 ± 3	10 ± 3	11 ± 1
Medium	5 ± 2	8 ± 1	9 ± 2	6 ± 2	11 ± 1*	13 ± 4
High	2 ± 2	5 ± 2	10 ± 2	6 ± 1*	13 ± 2*	17 ± 1*
NAS, Medium	7 ± 2	9 ± 2	10 ± 2	6 ± 3	8 ± 2	7 ± 2

* NTG (mg).
* $P < .05$, compared with preinfusion.
† $P < .05$, compared with a dose of 1.225 mmol/kg/h.
§ $P < .05$, compared with equimolar dose of GSH.

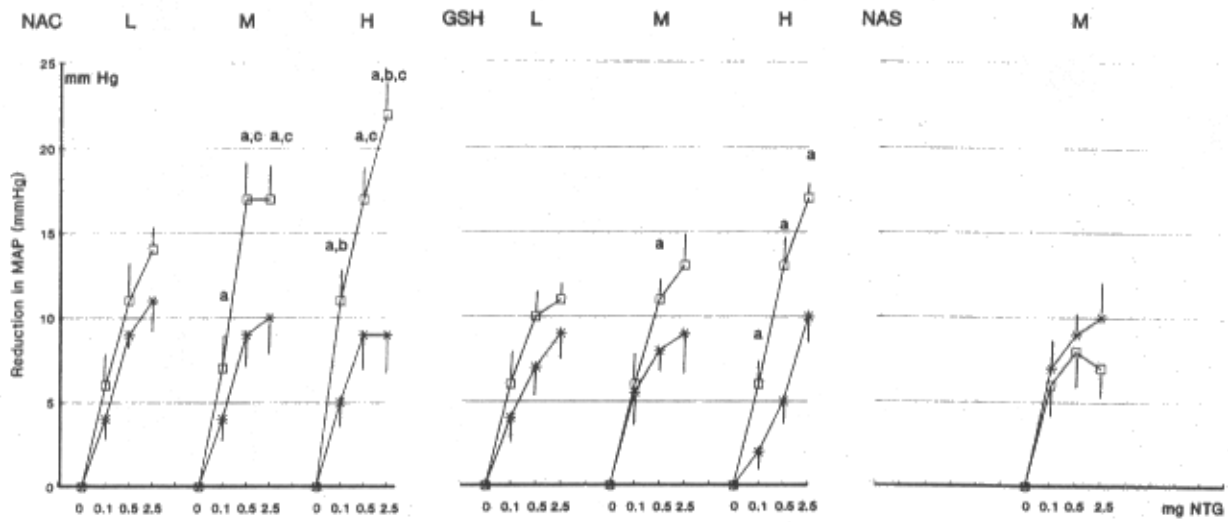

Fig. 2. Effect of NAC, GSH and NAS on the reduction in MAP in response to increasing bolus doses of NTG in nitrate-tolerant rats. The responses to NTG bolus doses were determined before (*) and repeated during the last 2 h of a 4-h infusion (□) of a low (L) (0.245 mmol/kg/h, $n = 8$), medium (M) (1.225 mmol/kg/h, $n = 8$) or high (H) (6.125 mmol/kg/h, $n = 7$) dose of NAC or GSH. NAS was infused at a medium dose. 0 mm Hg, the MAP response to a placebo bolus dose. Values are mean ± S.E.M. a, $P < .05$, compared with preinfusion; b, $P < .05$, compared with a dose of 1.225 mmol/kg/h; c, $P < .05$, compared with an equimolar dose of GSH.

Discussion

In the present study, using an *in vivo* rat model, tolerance to the blood pressure-lowering effects of NTG developed within 24 h during i.v. infusion of NTG, and the attenuated response to NTG was alleviated by NAC (and GSH) administration, in a dose-dependent manner. The animal model of NTG tolerance closely resembles human NTG tolerance; tolerance depends on constant NTG administration (regardless of dose), it does not develop after single doses of NTG and, although probably of multifactorial origin, it can be counteracted by sulfhydryls.

The magnitude and duration of the circulatory effects of organic nitrates are reduced during sustained therapy (Bogaert and De Schaepdryver, 1968; Thadani *et al.*, 1982; Needleman, 1970; Parker *et al.*, 1984). In rats, the time needed for development of nitrate tolerance during i.v. NTG infusions (determined as attenuated blood pressure response to NTG) has not been investigated previously. In the present *in vivo* study, the hypotensive effect of a NTG bolus challenge was unchanged during a period of 12 h, and tolerance appeared between 12 and 24 h after infusion start. The attenuated NTG response after 24 h developed in spite of high steady-state arterial plasma NTG levels and did not depend on the actual dose infused (0.2 or 1 mg/h). Interestingly, a similar time-span for the development of tolerance has been reported in clinical studies. After 24-h NTG infusion in patients with ischemic heart disease, systemic blood pressure returned to pretreatment values (May *et al.*, 1987) and the response to NTG bolus administration was significantly attenuated (May *et al.*, 1987; Zimrin *et al.*, 1988).

The organic nitrates cause vascular smooth muscle relaxation through a mechanism believed to involve the formation of NO (Ignarro, 1989). During their biotransformation, organic nitrates interact with free thiols in vascular smooth muscle cells

to generate NO and/or labile intermediate *S*-nitrosothiols that degrade spontaneously to NO (Ignarro *et al.*, 1981; Feelisch and Noack, 1987). NO, in turn, activates guanylate cyclase, with subsequent accumulation of cyclic guanosine monophosphate and vascular smooth muscle relaxation (Ignarro and Gruetter, 1980). Tolerance to NTG has been attributed to an intracellular depletion of thiols during prolonged exposure to nitrates (Needleman and Johnson, 1973; Ignarro *et al.*, 1981). According to this hypothesis, sustained exposure to nitrates may result in a reduced metabolic activation of NTG and diminished biotransformation. Alternatively, it has been proposed recently that NTG may directly inactivate guanylate cyclase by a process prevented by cysteine and facilitated by thiol depletion (Romanin and Kukovetz, 1989).

The present data are consistent with these findings, in that the attenuation of NTG effect during prolonged infusion of the drug was alleviated by NAC and GSH in a dose-dependent manner.

An effect of thiol supplementation on the hemodynamic responsiveness to NTG in nitrate-tolerant rats has not been reported previously. In the present study, as in a study with nontolerant rats (Fung *et al.*, 1988), the effect of NAC was observed 2 to 4 h after onset of NAC infusion. In humans, the maximum effect of NAC is seen after 30 to 60 min (Horowitz *et al.*, 1988). In contrast, no changes in the hypotensive effect of NTG were observed 10 min after NAC administration in a canine model (Munzel *et al.*, 1988). The reason for this discrepancy is not clear, but the findings suggest that a certain period is needed before a physiological response appears.

Recently, it was reported that NAC potentiates the acute effects of NTG in nontolerant rats (Fung *et al.*, 1988), and observations in humans have shown that NAC may enhance NTG responsiveness in both nitrate-tolerant and nontolerant patients (May *et al.*, 1987; Horowitz *et al.*, 1983; Winniford *et al.*, 1986). It is, therefore, possible that the nitrate-thiol interaction occurs by a mechanism not specifically related to tolerance and that intracellular sulfhydryl availability in general is a rate-limiting factor in the metabolism of nitrates. The present dose-effect relationship between NAC and NTG may support this assumption.

Alternatively, as suggested *in vitro*, NAC and GSH may be involved in an unspecific extracellular thiol-dependent formation of vasoactive *S*-nitroso-thiols (Fung *et al.*, 1988). However, the amino acid methionine has no extracellular sulfhydryl properties. Nevertheless, it is capable of augmenting sulfhydryl availability and reversing tolerance by means of its metabolic conversion to cysteine within the cell (Levy *et al.*, 1991). NAC and GSH may act extracellularly as sulfhydryl donors. In contrast to NAC (and methionine), GSH does not effectively cross cell membranes and, whereas NAC was effective in reversing nitrate tolerance, equimolar amounts of GSH were much less efficient. The results suggest that sulfhydryl compounds that pass intracellularly are more effective than those that remain extracellular. This finding, which is compatible with the intracellular effect of methionine, does not favor an important extracellular sulfhydryl-dependent contribution to nitrate metabolism. However, the present results do not allow a clear distinction between intra- and extracellular mechanisms. Such a distinction must be based on direct measurements of the relevant intracellular substances.

Even the highest dose of NAC infused (6.125 mmol/kg/h, equal to approximately 1 g/kg/h) did not completely normalize the blood pressure response to NTG, compared with pretreatment values. Furthermore, an almost maximal effect of NAC on the response to 0.1 and 0.5 mg of NTG was observed during infusion of a 5-fold lower dose. This observation is consistent with *in vitro* findings (Torresi *et al.*, 1985), suggesting that thiol supplementation may not be able to compensate for the total effect of nitrate tolerance and that other mechanisms also operate. One such mechanism may be nitrate-induced sodium and water retention due to activation of neurohormonal counter-regulatory mechanisms, especially the renin-angiotensin system (Packer *et al.*, 1987; Dupuis *et al.*, 1990). In the present study, hematocrit values were significantly reduced without signs of hemolytic anemia. In humans, a slight weight increase and expanded plasma volume have been described (Packer *et al.*, 1987; Dupuis *et al.*, 1990). The fall in hematocrit during nitrate therapy in the present study is compatible with these findings and supports the hypothesis that multiple mechanisms contribute to the development of nitrate tolerance.

From the present results, we conclude, firstly, that sulfhydryl supplementation may enhance the hypotensive effect of NTG in a dose-dependent manner in conscious nitrate-tolerant rats and that this effect is better achieved with NAC than with GSH and, secondly, that the development of nitrate tolerance in humans and rats shares similarities, which render a model with chronically catheterized conscious rats suitable for *in vivo* investigation of the mechanisms and determinants of nitrate-thiol interactions.

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