Nitrate Tolerance in Epicardial Arteries or in the Venous System is Not Reversed by N-Acetylcysteine In Vivo, but Tolerance-Independent Interactions Exist

Thomas Münzel, MD, Jürgen Holtz, MD, Alexander Mülsch, PhD, Duncan J. Stewart, MD, and Eberhard Bassenge, MD

N-acetylcysteine is assumed to reverse nitrate tolerance by replenishing depleted intracellular sulfhydryl groups, but data on interactions of N-acetylcysteine and nitrates in patients with stable angina are controversial and disappointing. Therefore, we studied the effect of N-acetylcysteine on nitrate responsiveness of epicardial arteries and of the venous system (assessed as changes in effective vascular compliance) in dogs (n=12) during long-term nitroglycerin treatment (1.5 μg/kg/min i.v. for 5–6 days). In dogs with nitroglycerin-specific tolerance (shift of venous or epicardial artery dilation to 15–17-fold higher dosages), N-acetylcysteine (100 mg/kg i.v.) had no dilator effect and did not alter the dose-response relations of nitroglycerin. Yet, in nontolerant dogs (n=17), N-acetylcysteine augmented (1.5–2.0-fold) the dilation of epicardial arteries and the reduction of peripheral vascular resistance induced by 0.5–1.5 μg/kg/min nitroglycerin. In vitro, the augmentation of purified guanylate cyclase activity by nitroglycerin (10–100 μM) was potentiated by N-acetylcysteine (0.01–1.0 mM) in saline or in canine plasma, but N-acetylcysteine alone was ineffective. We conclude that 1) N-acetylcysteine does not restore nitroglycerin responsiveness in tolerant epicardial arteries or veins in vivo, 2) a small, tolerance-independent augmentation of nitroglycerin-induced dilation may result from N-acetylcysteine–induced extracellular formation of a stimulant of guanylate cyclase from nitroglycerin. (Circulation 1989;79:188–197)

During continued administration of organic nitrates, their anti-ischemic effects are severely blunted.1–5 Proposed mechanisms of this phenomenon of tolerance include alterations in intracellular nitrate metabolism6,7 or the activation of neurohumoral systems counteracting the vasodilator potency of nitrates.8–11 In vitro findings stressed the critical role of intracellular sulfhydryl groups in activation of guanylate cyclase6,12,13 and nitrate tolerance development. These observations led to the study of the interactions between nitroglycerin and the sulfhydryl donor N-acetylcysteine (NAC) in nontolerant and tolerant patients.14–18 However, clinical studies have yielded conflicting results concerning the reversibility of nitrate tolerance by NAC.16,18 These studies did not analyze directly the nitrate and NAC interactions in vessels that are most important for the antianginal actions of nitrates, namely veins and large epicardial arteries. Therefore, we used a recently described canine model of nitrate tolerance17,19 to analyze directly the effectiveness of NAC in restoring nitrate sensitivity, specifically in large epicardial arteries and the venous capacitance system. To exclude a potential contribution of neurogenic mechanisms in the interaction between nitrate and NAC, we also used protocols with autonomic blockade.

Materials and Methods

Animals

Twenty-six mongrel dogs of either sex, weighing 18–31 kg, were used in different experimental protocols (Table 1) without repeating the same experiment in any dog. After thorough inspection by a veterinarian, the dogs were kept under quarantine.
for 3 weeks. Thereafter, they were familiarized with the laboratory and the staff, trained to lie quietly on the experimental table, assigned to one of the experimental groups (Table 1), chronically instrumented in anesthesia (see below), and allowed to recover from surgery for at least 10 days. Throughout these periods and during the experiments, the dogs were kept on a standard chow containing 2–4 meq/kg Na⁺ each day, with free access to tap water. They remained in a vigorous condition, and their body weight, rectal temperature, and hematocrit level were repeatedly checked. The care of the animals and the performance of the experimental protocols were under the supervision of an independent veterinarian in strict accordance with Federal Republic of Germany legislation and with the animal welfare regulations of the University of Freiburg, which correspond to the guidelines for animal welfare of the American Physiological Society.

For chronic instrumentation, the dogs were thoracotomized under pentobarbital anesthesia and sterile conditions. Dogs of groups 1 and 3 were equipped for the measurement of coronary flow and external epicardial coronary artery diameter of the circumflex branch of the left coronary artery as described previously.¹⁰ Criteria for inclusion of these dogs in the study were the same as in a previous study on nitrate tolerance.¹⁹ Three instrumented dogs were excluded because of a small epicardial artery dilation (<100 μm) in response to a nitroglycerin test injection. In the dogs of group 4, an electromagnetic flowmeter was placed around the aorta ascendens. During thoracotomy, a polyethylene catheter was implanted in the pulmonary artery of each dog for long-term drug application. Furthermore, a common carotid artery was translocated into a cutaneous loop at the ventral surface of the neck (for transcutaneous puncture), or alternatively, a catheter was implanted in the aorta descendens. Dogs of group 2 were not thoracotomized, they received only a polyethylene catheter for long-term drug application, which was inserted transcutaneously into the external jugular vein. All cables and catheters were tunneled subcutaneously to the dogs’ back. Postoperatively, the dogs received antibiotics for 1 week, and the catheters were flushed daily. At the end of the experiments, the dogs were killed by an overdose of pentobarbital.

### Experimental Protocols

For long-term nitroglycerin application in dogs of groups 1 and 2, a dosage of 1.5 μg/kg/min was infused continuously during 6 (group 1) or 5 days (group 2) with 0.5 ml/hr ethanol (99%) as solvent by portable battery-operated electrolytic pumps (Model 216, Sage Instruments, Cambridge, Massachusetts) as described earlier (Table 1).¹¹¹² In the dogs of group 1, the epicardial artery diameter of the resting conscious dogs was registered on 3 separate days before long-term nitroglycerin application and on each day during nitroglycerin infusion. The epicardial artery diameter was significantly greater than control diameters on the first 2 days of nitroglycerin treatment, but it was within the control range on days 4–6. The effect of NAC on the epicardial artery responsiveness to nitroglycerin under nitrate tolerance was studied in the conscious dogs of group 1 on day 5 of nitroglycerin infusion (Table 1).

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**Table 1. Groups of Dogs and Experimental Protocols**

<table>
<thead>
<tr>
<th>Group (chronic instrumentation)*</th>
<th>Pretreatment</th>
<th>Experimental analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong>&lt;br&gt;Coronary flowmeter coronary artery diameter gauge (n = 7)†</td>
<td>Long-term nitroglycerin i.v. (1.5) until end of day 6</td>
<td>Day 5: &lt;br&gt;Awake &lt;br&gt;Epicardial artery response to nitroglycerin 3.0–16.5 i.v. before and after NAC</td>
</tr>
<tr>
<td><strong>Group 2</strong> (none) (n = 5)</td>
<td>Long-term nitroglycerin i.v. (1.5) until end of day 5</td>
<td>Day 5: &lt;br&gt;Awake &lt;br&gt;Epicardial artery response to nitroglycerin 3.0 and 16.5 i.v. before and after NAC</td>
</tr>
<tr>
<td><strong>Group 3</strong>&lt;br&gt;Coronary flowmeter coronary artery diameter gauge (n = 10)</td>
<td>None</td>
<td>Day 5: &lt;br&gt;Awake &lt;br&gt;Epicardial artery response to nitroglycerin 0.15–1.5 i.v. before and after NAC</td>
</tr>
<tr>
<td><strong>Group 4</strong>&lt;br&gt;Aortic flowmeter (n = 7)</td>
<td>None</td>
<td>Day 5: &lt;br&gt;Awake &lt;br&gt;Peripheral resistance response to nitroglycerin 0.5–5.0 i.v. before and after NAC</td>
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* n, number of dogs. AB, autonomic blockade (hexamethonium 10 mg/kg + 10 mg/kg/hr; methyleatropine 0.5 mg/kg; nadolol 2 mg/kg); NE, norepinephrine (i.v. infusion 0.15 μg/kg/min); NAC, N-acetylcyisteine (100 mg/kg).

† Three dogs from group 3 included in group 1.

‡ Nitroglycerin dosages were given in micrograms per kilogram per minute.
protocol, the unsedated dogs rested quietly on their sides on the table for 2 hours with the implanted instruments connected. They received an ongoing basal infusion of 1.5 μg/kg/min nitroglycerin throughout the protocol. Additional intravenous test infusions of nitroglycerin were applied for 5 minutes each, yielding total dosages of 3.0, 6.4, and 16.5 μg/kg/min, respectively, with intervals of 10–15 minutes during basal infusions of nitroglycerin (1.5 μg/kg/min) between consecutive test infusions. NAC (100 mg/kg i.v.) was infused during 10 minutes at a rate of 10.0 ml/min, starting 15–20 minutes after the last nitroglycerin test infusion; 5 minutes later, the three test infusions of nitroglycerin were applied again with the same sequence, duration, and intervals. The same schedule for NAC infusion and for test infusions was applied in the conscious dogs of groups 3 and 4 (Table 1). However, in these groups, the dogs did not receive a continuous nitroglycerin infusion, and the test infusions amounted to 0.15, 0.5, and 1.5 μg/kg/min nitroglycerin in group 3 dogs and to 0.5, 1.5, and 5.0 μg/kg/min nitroglycerin in group 4 dogs, respectively (Table 1).

Although alternating between different infusions was performed in such a way that the dogs did not notice the manipulations, small movements and licking indicated that the dogs became aware of NAC, perhaps by taste. Two dogs of group 1, one of group 3, and one of group 4 vomited briefly during NAC infusions, but the protocol was continued as usual in these four dogs.

In dogs of group 1, the protocol of nitroglycerin test infusions before and after NAC was repeated under anesthesia (27 mg/kg+0.05 mg/kg/min pentobarbital i.v.), autonomic blockade (10 mg/kg+10 mg/kg/hr hexamethonium, 0.5 mg/kg methylatropine, and 2 mg/kg nadolol), vascular preconstriction by norepinephrine (0.15 μg/kg/min), and volume infusion (2.5 ml/kg/hr saline and 2.5 ml/kg/hr dextran) on day 6 of the ongoing nitroglycerin infusion (1.5 μg/kg/min). The dogs of group 2 were anesthetized with pentobarbital (27 mg/kg+0.05 mg/kg/min) on day 5 of ongoing nitroglycerin infusion. Catheters were positioned in the femoral artery and the right atrium for recording of mean arterial pressure and central venous pressure, respectively, and for changing the blood volume. The protocol with ongoing basal nitroglycerin infusion (1.5 μg/kg/min) and with two nitroglycerin test infusions (3.0 and 16.5 μg/kg/min) before and after NAC was performed during heparin administration (500 units/kg+250 units/kg/hr), volume infusion, autonomic blockade, and preconstriction by norepinephrine as in group 1. The effective vascular compliance (see below) was determined before and during each of the two nitroglycerin test infusions (lasting 15 minutes) and before the NAC infusion. Throughout the protocol, the anesthetized dogs breathed spontaneously through an endotracheal tube, and arterial blood gases and body temperature were kept within the normal range as described previously.11,21,22

**Measurements**

In the conscious resting dogs, the implanted electromagnetic flow probes were connected to a Gould Statham SP 2202 flowmeter (Cleveland, Ohio) for continuous measurement of coronary or aortic flow. The external diameter of the epicardial artery was monitored by ultrasonic transit time analysis between the implanted perivascular crystals, and the arterial pressure was obtained by a Statham P23 pressure transducer from either the punctured carotid artery in the cutaneous loop or the aortic catheter. Heart rate was derived from the arterial pressure signal. Mean and phasic tracings of all variables were recorded on a Watanabe linear recorder (Herrsching, FRG). In the dogs of group 2 under anesthesia, mean and phasic central venous and arterial pressures were recorded continuously, and the effective vascular compliance of the total vascular system was obtained by relating changes in central venous pressure to the changes in blood volume during a cycle of blood infusion, withdrawal, and reinfusion during 11 minutes as described previously.11,21,22

**Guanylate Cyclase Assay**

Soluble heme-containing guanylate cyclase was purified from bovine lung to apparent homogeneity by slightly modifying the method of Gerzer et al.24 The activity of purified guanylate cyclase was determined in test tubes by measuring the formation of [32P]cyclic guanosine monophosphate (GMP) from [α-32P]guanosine triphosphate (GTP). The incubation mixture contained [α-32P]GTP (0.1 mM, 0.2 μCi), triethanolamine-HCl buffer (30 mM, pH 7.4), MgCl2 (4 mM), glutathione (3 mM), ethyleneglycol-bis-(β-aminoethylether)-N,N',N'-tetraacetic acid (EGTA) (1 mM), bovine γ-globulin (0.1 mg/ml), isobutylmethyl-xanthine (0.2 mM), benzamidine (0.2 mM), creatine phosphate (5 mM), creatine phosphokinase (2.6 units), cyclic GMP (0.1 mM), and purified soluble guanylate cyclase (0.02 μM). All concentrations are final concentrations in a total volume of 100 μl. Incubation was for 10 minutes at 37° C. The enzyme reaction was stopped by the addition of 450 μl zinc acetate (120 mM) and 500 μl sodium carbonate (120 mM). The isolation of [32P]cyclic GMP and calculation of specific guanylate cyclase activity were performed as described previously.26 Various concentrations of NAC were tested on the basal enzyme activity and on the enzyme stimulated with nitroglycerin (1–100 μM). NAC was added to the incubation mixture as either a solution in canine plasma or in isotonic sodium chloride, resulting in a 1:1 final dilution of plasma or sodium chloride.

**Statistical Analysis**

Values are mean ± SEM. For comparison within a protocol, an analysis of variance for multiple comparisons within the same group, followed by a t test
Drugs Used

The following drugs and intravenous solutions were used: pentobarbital sodium (Ceva, Bad Segeberg, FRG), nitroglycerin (Pohl-Boskamp, Hohenlockstedt, FRG), N-acetylcysteine (NAC, Inphaz-arm, Gräfelfing, FRG), hexamethonium bromide, methylatropine, triethylamine-HCl buffer, MgCl₂, zinc acetate, and sodium carbonate (Merck, Darmstadt, FRG), nadolol (Von Heyden, Regensburg, FRG), heparin sodium and protamine HCl (Hoffman La Roche, Grenzach-Wyhlen, FRG), sodium bicarbonate 8.4% (Delta Pharma, Pfulling, FRG), dextran 60 (Makrodex, Schiwa, Glandorf, FRG), l-norepinephrine HCl (Hochst, Frankfurt, FRG) [α32P]GTP (NEN, Dreieich, FRG), glutathione, EGTA, bovine γ-globulin and benzamidine · HCl (Sigma, München, FRG), isobutylmethyl-xanthine (Serva, Heidelberg, FRG), creatine phosphate, creatine phosphokinase, GTP-disodium salt, and cyclic GMP (Böhringer, Mannheim, FRG).

Results

N-Acetylcysteine and Nitrate Responsiveness in Tolerance

The effects of nitroglycerin test infusions (3.0–16.5 µg/kg/min) on epicardial coronary artery diameter and on hemodynamics in conscious dogs (group 1) treated with long-term basal nitroglycerin infusions (1.5 µg/kg/min) are shown in Figure 1. The dose-dependent epicardial artery dilations in response to test infusions and the basal diameters between test infusions were not modified by NAC infusions. The tendency toward elevated basal values of heart rate and mean arterial pressure after NAC infusion were not significant.

In these dogs, tolerance to nitroglycerin had developed in epicardial arteries because the basal epicardial artery diameter (2.89±0.23 mm) of these dogs on day 5 of nitroglycerin infusion was not significantly different from artery diameter (2.90±0.21 mm) obtained on 3 separate days before long-term nitroglycerin application and because the epicardial artery responsiveness to nitroglycerin test infusions was identical to that in another group of dogs treated with this long-term dose in a previous study, in which arterial responsiveness before and during nitroglycerin application had been compared directly.19 Furthermore, heart rate and mean arterial pressure during nitroglycerin test infusions were similar to the respective values in the previous study, but heart rate in the intervals between test infusions was higher in the present study (88±2–93±3 beats/min in the present study vs. 70±2–74±3 beats/min in the previous study19). This may be the result of differences in the level of training for quiet rest and of differences in the protocols (shorter durations of a greater number of test infusions and intervals in the present series).

Similarly, NAC infusion did not modify epicardial artery dilation or hypotension in response to nitroglycerin test infusions under anesthesia, autonomic blockade, and preconstriction by norepinephrine in these tolerant dogs (group 1) on day 6 of ongoing basal nitroglycerin infusion (Figure 2). Thus, changes in autonomic activity in the conscious tolerant dogs are excluded from masking an NAC-induced augmentation of dilations in response to nitroglycerin test infusions. During this protocol under anesthesia, autonomic blockade, norepinephrine preconstriction, and ongoing basal nitroglycerin infusion, there was a continuous decline in basal mean arterial pressure (from 146±9 initially to 121±5 mm Hg before the last test infusion) and in coronary flow (from 50±10 to 45±9 ml/min), whereas the epicardial
artery diameter and heart rate remained essentially constant. NAC infusion did not modify any variable or the time course of the ongoing spontaneous decline in pressure and coronary flow.

The effects of NAC on nitroglycerin-induced venodilation under tolerance and ongoing basal nitroglycerin infusion in dogs of group 2 are summarized in Figure 3. The dose-response curve of nitroglycerin-induced venodilation in these dogs was shifted to 15-fold higher dosages compared with nontolerant dogs in a previous study. The nitroglycerin-induced augmentation in total effective vascular compliance, the variable of overall venous tone, and the basal value of this variable were not altered by NAC; also basal mean arterial pressure and nitroglycerin-induced hypotension (Figure 3) were not altered. The nitroglycerin-induced decline in central venous pressure (-1.8 ± 0.3 mm Hg with 16.5 μg/kg/min nitroglycerin) appeared attenuated after NAC (-1.1 ± 0.5 mm Hg), though the difference was not significant.

Potentiation of Nitrate Vasodilation by N-Acetylcysteine Without Tolerance

Nitroglycerin test infusions in conscious, resting dogs (group 3) without any nitroglycerin pretreatment induced dose-dependent epicardial artery dilation in the range of 0.15–1.5 μg/kg/min nitroglycerin (Figure 4). The calculated nitroglycerin dosage causing half-maximal epicardial artery dilation (i.e., 80 μm increase in diameter) was 0.3 μg/kg/min in these dogs of group 3, which is 17-fold lower than the respective dosage (5.1 μg/kg/min) in the tolerant dogs of group 1. After NAC in the nontolerant dogs of group 3, basal epicardial artery diameter and diameters during nitroglycerin test infusions were not significantly different from the respective values before NAC infusion (Figure 4). However, the relative increase under the second test infusion (0.5 μg/kg/min) was significantly (p < 0.05) higher after NAC (3.2 ± 0.4%) than before NAC (2.7 ± 0.2%). Furthermore, a significant elevation in heart rate occurred at a lower dosage of nitroglycerin test infusions after NAC than before it (Figure 4), and the hypotension with the highest nitroglycerin dosage tended to be more pronounced (Figure 4), indirectly suggesting an enhanced nitroglycerin effect on peripheral resistance after NAC.

This enhanced nitroglycerin effect was tested directly in the nontolerant dogs of group 4. A significantly greater decline in peripheral resistance was observed with 1.5 μg/kg/min nitroglycerin after NAC, whereas the tachycardia in response to 1.5 and 5.0 μg/kg/min was significantly greater after NAC (Figure 5). This indicates more activation of neurogenic counterregulation by nitroglycerin under NAC than by nitroglycerin alone.

Extracellular Enhancement of Nitrate-Induced Guanylate Cyclase Activation by N-Acetylcysteine

In a cell-free in vitro test system, the activity of purified soluble guanylate cyclase was significantly enhanced above basal values (absence of nitroglycerin and NAC) by all concentrations of nitroglycerin tested (1–100 μM; p < 0.05, n = 9 each), both in the assay in the presence of 50% isotonic NaCl and

![Figure 2](http://circ.ahajournals.org/)

Figure 2. Bar graph of changes of left circumflex coronary artery diameter (CDLF) and of mean arterial pressure (MAP) during nitroglycerin (GTN) test infusions under anesthesia, autonomic blockade, and norepinephrine infusion in dogs (n = 7) on continuous basal nitroglycerin infusion (1.5 μg/kg/min) for 6 days. Changes induced by test infusions did not differ significantly before and after N-acetylcysteine (NAC, 100 mg/kg i.v.).

![Figure 3](http://circ.ahajournals.org/)

Figure 3. Plot of effects of N-acetylcysteine (NAC) on venodilation induced by cumulative test infusions of nitroglycerin (GTN) in anesthetized dogs (n = 5) under autonomic blockade and norepinephrine preconstriction. Each test infusion lasted for 15 minutes; intervals before and after NAC infusion were 20–50 minutes. TEVC, total effective vascular compliance; CVP, central venous pressure; MAP, mean arterial pressure. Asterisks indicate significance of difference between value under test infusion and preceding basal value; (*)p = 0.10–0.05, *p < 0.05, **p < 0.01.
in the assay in the presence of 50% canine plasma. The nitroglycerin-induced stimulation of guanylate cyclase activity was significantly enhanced by NAC (0.01–1.0 mM) in both assays with 10 and 100 μM nitroglycerin. This enhancement was nitroglycerin concentration–dependent with 100 μM nitroglycerin (Table 2). Though the activity of the purified guanylate cyclase was strongly inhibited by unknown factors in the plasma, as compared with the activity in saline, the relative changes of guanylate cyclase activity by addition of nitroglycerin and NAC were comparable under both conditions (Table 2). NAC (0.01–1.0 mM) without nitroglycerin did not augment guanylate cyclase activity in either saline or canine plasma.

Discussion

This study confirms previous findings on the development of tolerance in epicardial arteries, as well as in the venous capacitance system, by long-term administration of nitroglycerin.11,19 There are three features of this tolerance model, which make it particularly useful for in vivo testing of the action of NAC, which should reverse tolerance by restoring nitroglycerin biotransformation, according to current hypotheses.27,28

First, there is true tolerance of vascular smooth muscle in the vessels most important to the antianginal action of nitroglycerin (large coronary arteries and veins) and not just pseudotolerance in these vessels because of regulatory influences. The shift of the dilatory response of the large epicardial conduit arteries to 17-fold higher nitroglycerin doses, induced by long-term nitroglycerin application (Figure 4 vs. Figure 1), did not result from a compensatory increase in constrictive neuroendocrine activity. The dose–response curve of the epicardial artery in the tolerant dogs was not shifted toward lower nitroglycerin dosages when reflex regulation was attenuated by autonomic blockade and anesthesia (compare Figures 2 and 1). The same is true for venous nitrate responsiveness, which has been analyzed under autonomic blockade both in tolerance11 (Figure 3 of the present study) and without tolerance.11,19

Second, the observed tolerance is specific for nitroglycerin. The responsiveness to SIN-1, the vasoactive metabolite of the antianginal prodrug molsidomine, is well preserved in this tolerance model in the venous11 and in the epicardial coronary arteries.19 This excludes potential subtle alterations in volume balance as the cause for the attenuated venous responsiveness to nitroglycerin, and it excludes the progressive pericoronary cica-

Figure 4. Plot of effects of test infusions of nitroglycerin (GTN) before and after N-acetylcysteine (NAC, 100 mg/kg i.v.) in conscious dogs (group 3, n=10) without nitroglycerin pretreatment. Steady-state values during test infusions (5 minutes) and at the end of 10–15 minute intervals are given. CDLC, left circumflex coronary artery diameter; MAP, mean arterial pressure; HR, heart rate; CFCL, left circumflex coronary flow. Asterisks indicate significant difference between value during test infusion and preceding basal value; *p<0.05, **p<0.01. Basal values of all variables did not differ significantly before and after NAC nor did absolute values of CDLC during nitroglycerin infusions. However, the percent increase of CDLC with 0.5 μg/kg/min nitroglycerin was higher after than before NAC infusion (p<0.05).

trization (which inevitably occurs in our hands at the site of implanted crystals19,20) as the reason for the reduced dilatory nitroglycerin responsiveness of epicardial arteries.

Third, the degree of tolerance in the model is relevant for the clinical situation, for instance, during sustained nitroglycerin infusion in the coronary care unit. Tolerance is obtained in vivo by the long-term intravenous administration of a “clinical” dosage of nitroglycerin, and all observations in the model are consistent with the interpretation that the tolerance development is restricted to the level of nitroglycerin biotransformation. Studies in vitro induced substantial tolerance by incubating vasculature at high concentrations of nitroglycerin and obtained a “downregulation” of the intracellular target of nitroglycerin, which is the soluble enzyme guanylate cyclase.29,30 In our model, however, the responsiveness to endothelium-derived relaxing factor, an endogenous activator of vascular soluble guanylate cyclase,31–33 and to SIN-1 (see above), another exogenous prodrug activator of this enzyme not requiring the biotransformation pathway of nitroglycerin,34 is well established.19 This argues strongly against a direct involvement of the enzyme in our model of moderate tolerance (approx-
imately 20-fold shift in nitroglycerin responsiveness) as well as in clinically occurring tolerance (where larger shifts have never been reported).

In our model, NAC application during ongoing nitroglycerin infusion did not dilate epicardial arteries or capacitance vessels, and it did not shift the respective dose-response curves toward lower dosages of nitroglycerin (Figures 1–3). Regarding the characteristics of the model, these results clearly indicate that a substantial and specific tolerance to the vascular antianginal actions of nitroglycerin cannot be reversed by NAC. Thus, the direct analysis of relevant vascular responses in our experimental study is in agreement with a more indirect clinical study, demonstrating the ineffectiveness of NAC in augmenting exercise tolerance under nitrate tolerance. In that study, tolerance was induced by long-term oral application of isosorbide dinitrate, but the cross tolerance between nitroglycerin and isosorbide dinitrate indicates a common cellular mechanism. By analyzing the antianginal efficacy of NAC and isosorbide dinitrate in patients, the investigators derived the same conclusion as is obtained from our direct analysis of venous and epicardial artery vasomotion.

At first glance, this conclusion seems to contradict several recent reports on an augmentation of nitrate responsiveness by NAC. However, a more detailed inspection reveals that there is not necessarily a contradiction. Four studies and a preliminary report on enhanced nitroglycerin responsiveness during NAC application have been published and are summarized in Table 3. The NAC-induced increase in the nitroglycerin responsiveness in these studies amounts to a twofold to threefold shift toward lower nitroglycerin dosages and is not specific for tolerant patients: the shift is comparable in patients with and without nitroglycerin tolerance and is similar to the borderline shift in arterial and arteriolar nitroglycerin responsiveness observed in our nontolerant dogs of groups 3 and 4 (Figures 4 and 5).

In our tolerant dogs, the nitroglycerin application lasted longer, and the degree of tolerance induced thereby was more pronounced than in the clinical studies with an NAC-induced enhancement of nitrate responsiveness (Table 3). This could be one factor that partially explains the discrepancies. Furthermore, a part of the apparent discrepancy between documented positive NAC effects on nitroglycerin hemodynamics and a lack of NAC action on the antianginal efficacy of nitrates could result from the fact that the enhancement of nitroglycerin responsiveness by NAC cannot be ascribed for sure to those vascular segments that determine the antianginal efficacy of nitroglycerin. In two of the studies, the increase in coronary sinus outflow in response to intracoronary nitroglycerin was doubled by NAC.

**FIGURE 5.** Plot of the lowering of total peripheral resistance (TPR) by nitroglycerin (GTN) test infusions. The decline in TPR is accentuated after N-acetylcysteine (NAC, 100 mg/kg i.v.) in conscious dogs (group 4 with implanted aortic flowmeter, n = 7) without nitroglycerin pretreatment. Steady-state values during test infusions (5 minutes) and at the end of 10–15-minute intervals are given. MAP, mean arterial pressure; HR, heart rate. Asterisks indicate significant difference between value during test infusion and preceding basal value; *p < 0.05, **p < 0.01. Basal values of all variables did not differ significantly before and after NAC.
Yet, in both studies, it was not clear whether and to what extent this increase resulted from an effect on stenosed epicardial arteries or from a dilatation of coronary arterioles in normally perfused myocardial areas in response to the intracoronary nitroglycerin (an action certainly not important for antianginal efficacy). In two studies, the shift in nitrate responsiveness was assessed as the dosage necessary for a certain preload reduction. However, in one of the studies, this was done in patients suffering from heart failure with high filling pressures. Under this condition, with ventricles demonstrating marked afterload dependency, the nitrate effect on preload may be mediated largely by arteriolar dilatation, whereas the venous nitroglycerin responsiveness during heart failure probably is less than normal. In one study in heart failure, a continuous nitroglycerin infusion during 48 hours resulted in clear hemodynamic deterioration with signs of augmented neuroendocrine activation (see Table 3; these effects were not observed in a parallel group during intermittent administration of nitroglycerin). In these patients treated with ongoing nitroglycerin, NAC induced a very shortlasting improvement, which probably resulted from an additive action of the ongoing nitroglycerin and the NAC on peripheral resistance vessels. Again, such an action may not be of primary importance for antianginal effectiveness in patients without failure.

Recently, it was speculated that NAC might have augmented nitrate responsiveness in these studies by an extracellular interaction with nitroglycerin, forming an active compound. This has been proposed previously by others and has been documented in vitro and in human plasma. The augmentation of nitroglycerin-induced activation of purified soluble guanylate cyclase by NAC in cell-free buffer or in canine plasma (Table 2) clearly demonstrates the existence of such an interaction. Compared with clinically occurring nitroglycerin concentrations in plasma, very high nitroglycerin levels are required in this guanylate cyclase assay. This may result from the fact that the chemical conditions for enzyme activation in the assay are not optimal, differing from the not yet fully identified intracellular conditions. Thus, the very high nitroglycerin concentrations in our assay do not argue against the role of such extracellular interaction of NAC and nitroglycerin in vivo with lower nitroglycerin concentrations.

Such an extracellular interaction in vivo, resulting in the chemical formation of a stimulant of the target enzyme guanylate cyclase, probably the nitric oxide radical or a nitrosothiol, would elegantly

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**Table 3. Actions of N-Acetylcysteine* on Nitrate Responsiveness In Vivo**

<table>
<thead>
<tr>
<th>Diagnosis (reference)</th>
<th>Induction of tolerance</th>
<th>Under nitrate tolerance</th>
<th>Effect of N-acetylcysteine</th>
<th>Without nitrate tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD no CHF14</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>0.36 nitroglycerin† i.c. (bolus): increase in CF: from 30% to 50%</td>
</tr>
<tr>
<td>CAD no CHF15</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>Nitroglycerin dosage (i.v. infusion): -10% MAP: from 0.37 to 0.13</td>
</tr>
<tr>
<td>CAD no CHF16</td>
<td>24 hr i.v. infusion 0.64 nitroglycerin</td>
<td>CF response to 0.14-1.43 nitroglycerin i.c. (bolus) reduced to ½ of control</td>
<td>CF response to nitroglycerin i.c. restored to control</td>
<td>Nitroglycerin dose (bolus): 10 mm Hg PCWP: from 0.19 to 0.06</td>
</tr>
<tr>
<td>CHF17</td>
<td>48 hr i.v. infusion 6.4 nitroglycerin</td>
<td>Initial effect of 6.4 nitroglycerin on SVR (-25%) attenuated 48 hr later to -11% with elevations in HR, PRA, body weight</td>
<td>Transient (&lt;90 min) reduction in SVR (-21%) and in HR under ongoing nitroglycerin infusion</td>
<td>...</td>
</tr>
<tr>
<td>CHF18</td>
<td>24 hr i.v. infusion nitroglycerin</td>
<td>Nitroglycerin dose (bolus): -10 mm Hg PCWP: from 1.6 to 8.4</td>
<td>Nitroglycerin dose (bolus): -10 mm Hg PCWP: from 8.4 to 3.6</td>
<td>Nitroglycerin dose (bolus): 10 mm Hg PCWP: from 7.8 to 3.2</td>
</tr>
<tr>
<td>No enhancement</td>
<td>7-10 days 30 mg ISDN q.i.d.</td>
<td>Attenuated hypotension to 30 mg ISDN at rest; exercise tolerance 3 hr after ISDN not improved</td>
<td>No improvement of ISDN action on exercise tolerance</td>
<td>...</td>
</tr>
</tbody>
</table>

CAD, coronary artery disease; CHF, congestive heart failure; MAP, mean arterial pressure; PCWP, pulmonary capillary wedge pressure; CF, coronary flow (measured as coronary sinus outflow); SVR, systemic vascular resistance; HR, heart rate; PRA, plasma renin activity; ISDN, isosorbide dinitrate.

*N-Acetylcysteine was given as an intravenous infusion at a dose of 100 mg/kg or 140 mg/kg, or 200 mg/kg was given orally.

†Nitroglycerin dosages were given as micrograms per kilogram per minute for infusions and as microgram per kilogram for bolus injections, assuming 70 kg body weight (if not specified).
explain most of the existing observations. In the only clinical study\textsuperscript{18} without any NAC-induced enhancement of nitrate interaction (see Table 3), isosorbide dinitrate was used instead of nitroglycerin. In human plasma in vitro, Fung et al\textsuperscript{40} demonstrated the formation of a nitrosothiol from nitroglycerin in presence of NAC, and they reported that NAC only potentiated the degradation of nitroglycerin, not of isosorbide dinitrate. The enhancement of the action of ongoing nitroglycerin infusion by NAC is moderate in extent (no more than twofold to threefold enhancement, Table 3) and very shortlasting.\textsuperscript{17} This is in agreement with the data on plasma kinetics of NAC in humans,\textsuperscript{43} demonstrating with intravenous application a clearance with a half-life of 2 hours and, with oral application, an early peak within 40 minutes, followed by a similar clearance. Furthermore, the reported NAC-induced enhancements of nitroglycerin effects do not demonstrate any specificity for tolerance (Table 3). On the contrary, they occur in humans and in dogs without nitroglycerin pretreatment (Table 3 and Figures 4 and 5) and in humans with a very low degree of tolerance (approximately twofold shift of nitroglycerin responsiveness, Table 3) but not in those vascular segments of dogs, which have a documented 15- to 17-fold shift in nitroglycerin responsiveness (Figures 1–3). This could mean that the transiently formed putative extracellular intermediate (the nitric oxide radical?) has an effect too weak to be detectable in markedly nitroglycerin-hyporesponsive vasculature. At present, the proposed extracellular interaction of NAC and nitroglycerin is documented only in vitro, but its role in vivo must remain an attractive hypothesis.

The postulated reversal of nitrate tolerance by NAC has been considered in three contexts: as a support for models of intracellular nitroglycerin biotransformation, as an approach to enhance vasodilator potency of continuous nitroglycerin in heart failure, and as a possibility to obtain long-term antiangiogenic protection from nitroglycerin by preventing or reversing nitrate tolerance. In its original form, the NAC hypothesis proposed the most pronounced augmentations of nitroglycerin actions in cases with the lowest initial responsiveness to nitroglycerin.\textsuperscript{15} Together with the proposal of an NAC-induced replenishment of intracellular sulphhydryl group deficiency as the underlying mechanism, this implicates a tolerance-specific augmentation of nitroglycerin efficacy by NAC, with one specific mechanism (intracellular sulphhydryl group deficiency) involved in both tolerance induction and tolerance reversal. What became apparent, however, from clinical studies (Table 3) and from our data on NAC in nontolerant animals, is a tolerance-independent interaction, which may occur extracellularly. This means that the induction of nitrate tolerance and the NAC-mediated augmentation of nitrate response are due to different mechanisms and may only accidentally balance each other at a rather low degree of tolerance. Our study shows that a sizeable, nitroglycerin specific tolerance in defined vascular sections cannot be reversed by NAC in vivo.

The implications of this finding in the above three contexts are not identical. It is not clear whether or not NAC application in vivo really can modify intracellular availability of sulphhydryl groups, which were claimed as critical for nitroglycerin biotransformation.\textsuperscript{12} Thus, our result cannot yield arguments for or against theoretical models of intracellular nitroglycerin metabolism.\textsuperscript{6,44} In congestive heart failure, enhancing transiently the nitroglycerin-induced arteriolar dilation by NAC should be possible as has been demonstrated convincingly in patients with modest tolerance.\textsuperscript{17} However, one should take into account the very short duration of this effect compared with other possibilities of vasodilator therapy in this syndrome. Finally, there is the intention to obtain a permanent anti-ischemic protection of the jeopardized myocardium in coronary heart disease by long-term nitroglycerin without tolerance development. Certainly, this is a desirable goal, but our study and the available data in patients indicate that nitroglycerin specific tolerance at the level of intracellular nitroglycerin biotransformation in vessels cannot be reversed by NAC application in vivo. However, other potential interactions of NAC and nitroglycerin, such as the proposed extracellular formation of the nitric oxide radical and the potentiation of nitroglycerin-induced inhibition of platelet aggregation by NAC,\textsuperscript{45} are not excluded by our study and may become therapeutically relevant in the future.\textsuperscript{46}

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**KEY WORDS** • soluble guanylate cyclase • antiangiinal action • effective vascular compliance • autonomic blockade
Nitrate tolerance in epicardial arteries or in the venous system is not reversed by N-acetylcysteine in vivo, but tolerance-independent interactions exist.

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