Mechanisms of Interaction between the Sulfhydryl Precursor L-Methionine and Glyceryl Trinitrate

T. Münzel, MD; A. Mülsch, PhD; J. Holtz, MD; H. Just, MD; D.G. Harrison, MD; and E. Bassenge, MD

Background. L-Methionine potentiates systemic hemodynamic effects of intravenous glyceryl trinitrate (GTN) in tolerant and nontolerant patients to a similar extent as N-acetylcysteine (NAC). This potentiation of GTN action by L-methionine has been attributed to enhanced intracellular formation of nitrosothiols, known to be potent stimulators of soluble guanylyl cyclase. This study was performed to analyze directly the effects of L-methionine on GTN-induced dilation of large epicardial arteries and the venous capacitance system of the dog in the tolerant and nontolerant states. Cultured rat aortic vascular smooth muscle cells and purified guanylyl cyclase were used to study potential intracellular and extracellular mechanisms responsible for this interaction.

Methods and Results. In awake nontolerant dogs, L-methionine (100 mg/kg) potentiated the tachycardic response to GTN (5.0 and 15 µg/kg/min) and enhanced the hypotensive action of GTN (1.5 and 5.0 µg/kg/min) in anesthetized, nonreflexic dogs. In nontolerant and tolerant dogs, however, L-methionine did not alter the dose-response of large epicardial artery dilation to intravenous GTN challenges and did not modify nitrate tolerance of the low pressure system of the dog. The infusion of L-methionine (100 mg/kg) significantly increased plasma methionine levels (from 52±12 to 1,141±239 µM), cystine levels (from 12±4 to 26±7 µM), but not homocysteine levels. In vitro, the L-methionine conversion product L-cysteine (0.1–1.0 mM) but not homocysteine significantly enhanced the augmentation of purified guanylyl cyclase activity by GTN (100 µM). Incubation of cultured rat aortic smooth muscle cells with L-methionine (10 µM or 1 mM) did not result in a significant increase of free intracellular sulfhydryl group content.

Conclusions. The L-methionine conversion product L-cysteine mediates tolerance independent the potentiation of GTN action. This may result from an L-cysteine-induced formation of a vasoactive metabolite of GTN (nitric oxide) or nitrosothiol. This effect occurs primarily in the resistance vessel circulation, not in large epicardial arteries and veins. The lack of effect of L-methionine on sulfhydryl group content in large conductance vessels indicates that hepatic L-methionine metabolism constitutes the significant source of L-cysteine. These findings strongly suggest that administration of sulfhydryl-group precursor L-methionine does not represent a therapeutic alternative to a nitrate-free interval to restore nitrate sensitivity in tolerant large epicardial arteries and veins. (Circulation 1992;86:995–1003)

KEY WORDS • glyceryl trinitrate • L-methionine

Several mechanisms have been proposed to explain the tolerance phenomenon after chronic administration of organic nitrates. Neurogenic counterregulatory mechanisms,1,2 intravascular volume expansion,3 impaired biotransformation4–6 (denitration of glyceryl trinitrate [GTN]), and desensitization of the target enzyme guanylyl cyclase7,8 have all been identified as possible mechanisms. Clinical strategies to reverse nitrate tolerance have mainly focused on the administration of thiol compounds9–12 since experimental studies have suggested that depletion of intracellular sulfhydryl pools might be responsible for the induction of nitrate tolerance.13 However, in vitro and in vivo data on sulfhydryl groups and GTN interactions are becoming increasingly contradictory.14–17 In a recent study,18 we found a tolerance-independent potentiation of effects of GTN by the sulfhydryl donor N-acetylcysteine (NAC) on canine epicardial arteries and peripheral resistance vessels. No reversal of a GTN-specific tolerance by NAC in large epicardial arteries and the venous capacitance system of the dog was observed; however, the augmentation of GTN effects in the tolerant and nontolerant states by NAC was attributed to the existence of an extracellular pathway since the stimulatory effect of GTN on the target enzyme guanylyl cyclase was potentiated in the presence of canine plasma.18 Furthermore, Fung et al19 demonstrated that tolerance in

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vascular strips was partially reversed in the presence of human plasma, indicating an extracellular interaction. Thus, clinical and experimental observations of NAC effects on GTN actions cannot be used to evaluate the significance of a potential intracellular sulfhydryl depletion and its replenishment for GTN tolerance in vivo. In contrast to NAC, L-methionine does not contain a free sulfhydryl group. If methionine is to act as a sulfhydryl donor, intracellular conversion of methionine to cysteine via the transulfuration pathway is necessary. Thus, methionine-induced potentiation of GTN action would point more specifically to an intracellular interaction between the sulfhydryl donor and the organic nitrate. The aim of the present study was, therefore, to analyze directly in vivo the effectiveness of sulfhydryl group supplementation by L-methionine on tolerant large coronary arteries and veins of the dog. Purified guananyl cyclase and cultured vascular smooth muscle cells of rat aorta were used to study potential intracellular or extracellular mechanisms responsible for this interaction.

Methods

Animals and Experimental Preparation

Seventeen dogs weighing 23–31 kg were used in different experimental protocols (Table 1). In all dogs (groups A–C) a common carotid artery was translocated into a cutaneous loop at the ventral surface of the neck. For chronic instrumentation (group C), the dogs were thoracotomized while under pentobarbital anesthesia with sterile conditions. The dogs were equipped for continuous measurement of coronary artery flow and external epicardial artery diameter of the circumflex branch of the left coronary artery diameter, as described previously. Dogs with <100-μm epicardial artery dilation in response to a GTN test injection were excluded. Coronary flow was measured with a Gould SP 2202 flowmeter, coronary artery external diameter was measured with ultrasonic time crystals, and arterial pressure was measured with a Statham P-23 pressure transducer connected to the carotid artery cannula; heart rate was derived from an arterial pressure signal. Anesthetized (27 mg/kg plus 0.5 mg/kg/min pentobarbital), areflexic dogs without GTN pretreatment (group B, n=5) were used to test the effect of GTN on mean arterial and central venous pressures before and after methionine administration. At the end of the experimental protocol, a polyethylene catheter was inserted into the external jugular vein transcutaneously for long-term GTN infusion (5 days) via a small mobile precision infusion pump (model AS30C, Baxter, Travenol). Intervals of at least 2 weeks were allowed before starting long-term GTN infusions and the subsequent assessment of venous tone in the tolerant state (group B, n=4; GTN-pretreated dogs). Venous tone was assessed using a modification of the technique suggested by Gauer’s group (Echt et al.).

In anesthetized dogs (27 mg/kg plus 0.05 mg/kg/min pentobarbital i.v.) with autonomic blockade (10 mg/kg plus 10 mg/kg/hr hexamethonium, 0.5 mg/kg methylatropine, 2 mg/kg nadolol) and vascular preconstriction with norepinephrine (0.15 μg/kg/min), 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 simultaneous changes in blood volume during a cycle of blood infusion, withdrawal, and reinfusion for 11 minutes, as described in detail previously. At the end of the experiments, the dogs were killed with an overdose of pentobarbital.

Experimental Protocols

Systemic hemodynamic measurements were performed in nontolerant dogs of group A (n=4) in response to three high-dose GTN test infusions (1.5, 5.0, and 15.0 μg/kg/min) before and after L-methionine (30, 100, and 300 mg/kg). L-Methionine was infused over 10 minutes at a rate of 10 ml/min starting 20 minutes after the last GTN test infusion. Five minutes later, the three short-term GTN challenges were applied again with the same sequence, duration, and intervals.

In the dogs of group B (n=5, protocol 1, Table 1), the systemic hemodynamic response to GTN (0.5, 1.5, and 5.0 μg/kg/min) before and after L-methionine administration (100 mg/kg) was measured with dogs under ganglionic blockade and pentobarbital anesthesia. After completion of the protocol, a polyethylene catheter was inserted into the external jugular vein percutaneously for long-term GTN administration. Then, 2 weeks were allowed for recovery before starting long-term GTN administration. After 5 days of GTN infusion, the dogs were anesthetized with pentobarbital, and venous tone was assessed in the tolerant state under autonomic

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

<table>
<thead>
<tr>
<th>Group (No. of dogs)</th>
<th>Instrumentation</th>
<th>Experimental analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (n=4)</td>
<td>Arterial line</td>
<td>Heart rate and blood pressure response to 1.5, 5.0, and 15.0 GTN* i.v. before and after Meth (30, 100, and 300 mg/kg)</td>
</tr>
<tr>
<td>Group B (n=5)</td>
<td>Central venous catheter</td>
<td>Anesthesia, AB, and NE Hemodynamic response to 0.5, 1.5, and 5.0 GTN* i.v. before and after Meth</td>
</tr>
<tr>
<td></td>
<td>Arterial line</td>
<td>Anesthesia, AB and NE venous response to 3.0, 6.5, and 16.5 GTN* i.v. before and after Meth (n=4)</td>
</tr>
<tr>
<td>Group C (n=8)</td>
<td>Coronary flowmeter</td>
<td>Awake, epicardial artery response to 0.15, 0.5, and 1.5 GTN* i.v. before and after Meth</td>
</tr>
<tr>
<td></td>
<td>Coronary artery crystals</td>
<td>Awake, epicardial artery response to 3.0, 6.5, and 16.5 GTN* before and after Meth (n=5)</td>
</tr>
</tbody>
</table>

GTN, glyceryl trinitrate; AB, autonomic blockade (10 mg/kg+10 mg/kg/hr hexamethonium, 0.5 mg/kg methylatropine, 2 mg/kg nadolol), NE, norepinephrine (i.v. infusion 0.15 μg/kg/min); Meth, 100 mg/kg L-methionine.

*GTN dosages were given in μg/kg/min.
blockade before and after L-methionine (group B, GTN-pretreated dogs, n=4).

For the assessment of large coronary artery diameter and hemodynamic effects before and after L-methionine in the nontolerant chronically instrumented dog (group C, n=8), two small doses of GTN (0.15 and 0.5 \( \mu \)g/kg/min) were infused intravenously for 5 minutes each until steady-state conditions were reached. When coronary artery diameter returned to baseline, an L-methionine infusion (100 \( \mu \)g/kg i.v.) was started and continued for 10 minutes. Then, the dogs were challenged again with the same GTN concentrations (Figure 2).

Thereafter, tolerance was induced by infusing 1.5 \( \mu \)g/kg/min GTN for 5 days (group C, GTN-pretreated dogs, n=5). Four days after starting the long-term GTN infusion, coronary artery diameter did not differ significantly from pretreatment diameters, indicating a near-complete loss of its initial vasodilator potency. The effects of methionine on nitrate-tolerant epicardial arteries were assessed on day 5. During the entire protocol (group C, tolerant dogs), the dogs received a continuous infusion of GTN (1.5 \( \mu \)g/kg/min). Additional GTN infusions were administered for 5 minutes each, yielding final concentrations of 3.0, 6.5, and 16.5 \( \mu \)g/kg/min. After a 15-minute pause, L-methionine (100 mg/kg) was infused for 10 minutes before rechallenging the dog with the same high GTN concentrations. During the protocols, changes in heart rate, mean arterial pressure, coronary flow, and coronary diameter were continuously recorded with a Watanabe linear recorder.

**Determination of Plasma Methionine, Cystine, and Homocysteine Levels**

The amino acid analysis was performed with an amino acid analyzer (LKB-Amino Acid Analysis System, Pharmacia, Freiburg, Germany) using a modification of the method originally described by Spackman et al.\(^{25}\) With this technique, separation and quantitative determination of amino acids are performed after reaction with ninhydrin, yielding a purple substance absorbing maximally at 570 nm. This absorbance is a linear function of the amount of \( \alpha \)-amino groups present, and the reaction provides a quantitative colorimetric assay for all organic compounds with \( \alpha \)-amino groups. The reaction product with amino acids is monitored at 440 nm. The color recovery for individual amino acids differ slightly. The coefficient of variation (CV) for standard mixtures is 1.5% at a loading of 10 nmol of amino acid (n=10). For samples, the CV value was 2% at the same loading level.

**Determination of Free Sulphydryl Group Content in Rat Aortic Vascular Smooth Muscle Cells After Incubation With L-Methionine (10 \( \mu \)M and 1 mM)**

Rat aortic vascular smooth muscle cells were grown in six-well plates to confluence. Subsequently, the cells
were incubated with either medium alone (DMEM) or DMEM with 10 μM or 1 mM L-methionine. The cells then were removed from the culture dish by trypsinization, washed four times with a physiological buffer containing no methionine, suspended in 3 ml of physiological buffer, and lysed with liquid nitrogen. The total content of free sulfhydryl groups was determined using the Elmans reaction.

Guanylyl Cyclase Assay

Soluble heme-containing guanylate cyclase was purified from bovine lung to apparent homogeneity according to the method of Gerzer et al.27 The activity of purified guanylate cyclase was determined in test tubes by measuring the [3H]cyclic GMP from (α-32P)-GTP as described in detail.27 Various concentrations of cysteine and homocysteine were tested on the enzyme stimulated with GTN (100 μM).

Drugs and Solutions

The drugs and intravenous solutions used were pentobarbital sodium (Ceva, Bad Segeberg, FRG), nitroglycerin (Pohl-Boskamp, Hohenlockstedt, FRG), methionine, cyclic GMP sodium salt (Sigma, München, FRG), hexamethonium bromide, methylatropine, (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, Pflülingen, FRG), dextran 60 (Makroderx, Schwa, Glandorf, FRG), L-cysteine, IBMX (Serva, Heidelberg FRG), [α-32P]GTP, and [125I]2-O-succinyl-(iodotyrosine methylester)-cyclic GMP (New England Nuclear, Dreieich, FRG).

Statistical Analysis

All values are presented as mean±SEM. For comparison within a protocol, an ANOVA for multiple comparisons within the same group followed by a t test with Bonferroni’s correction for the number of comparisons was applied. Single comparisons were made by paired t test. A value of p<0.05 was considered significant.

Results

L-Methionine and Nitrate Responsiveness in Awake, Nontolerant Dogs (Group A)

Blood pressure and heart rate in response to 1.5, 5.0, and 15.0 μg/kg/min were determined before and after the infusion of L-methionine at 30, 100, and 300 mg/kg in four dogs. All dogs tolerated L-methionine with respect to the two lower methionine concentrations, but three of four dogs became restless and vomited during infusion of methionine at the high concentration (300 mg/kg). The tachycardic response to GTN (5.0 and 15.0 μg/kg/min) was significantly enhanced after 100 mg/kg L-methionine but not after 30 mg/kg L-methionine (Figure 1).

L-Methionine and Nitrate Responsiveness in the Anesthetized, Areflexic, Nontolerant Dogs (Group B)

Figure 2 shows the effects of L-methionine on the GTN dose–response curves in dogs under anesthesia and autonomic blockade. Autonomic blockade was used to minimize neurogenic counterregulation during ad-
TABLE 2. Effects of Methionine on the Nitrate-Tolerant Capacitance System of the Dog Under Anesthesia and Autonomic Blockade

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>1.5 (µg/kg/min)</th>
<th>15.0 (µg/kg/min)</th>
<th>Control</th>
<th>Meth</th>
<th>1.5 (µg/kg/min)</th>
<th>15.0 (µg/kg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEVC (mL/mm Hg · kg)</td>
<td>1.81±0.1</td>
<td>2.13±0.08*</td>
<td>2.69±0.07*</td>
<td>1.79±0.08</td>
<td>1.71±0.07</td>
<td>2.02±0.07*</td>
<td>2.53±0.07*</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>133±6</td>
<td>127±8</td>
<td>115±11*</td>
<td>129±7</td>
<td>128±6</td>
<td>122±6</td>
<td>112±6*</td>
</tr>
<tr>
<td>CVP (mm Hg)</td>
<td>3.71±0.57</td>
<td>3.27±0.48*</td>
<td>2.05±0.52*</td>
<td>4.19±0.55</td>
<td>4.41±0.61</td>
<td>3.45±0.63*</td>
<td>2.53±0.51*</td>
</tr>
</tbody>
</table>

GTN, glyceryl trinitrate; TEVC, total effective vascular compliance; MAP, mean arterial pressure; CVP, central venous pressure. Values are mean±SEM.

Autonomic blockade: hexamethionium (10 mg/kg and 10 mg/kg/hr), nadolol (2 mg/kg), and methylatropine (0.5 mg/kg).

*p<0.05 compared with control values.

ministration of a vasodilator and to unmask even small potenations of GTN effects by L-methionine. The decrease in mean arterial and central venous pressures in response to GTN at 1.5 and 5.0 µg/kg/min was significantly increased after administration of 100 mg/kg L-methionine.

Effects of L-Methionine on GTN-Induced Coronary Vasodilation in Awake, Nontolerant, and Tolerant Dogs (Group C)

Figure 3 shows the dose-dependent epicardial artery dilation to 0.15 and 0.5 µg/kg/min GTN before and after L-methionine administration, respectively. Increasing GTN concentrations caused a dose-dependent epicardial artery dilation that was not modified after infusion of L-methionine (100 mg/kg). There also was no enhancement of the GTN effects on mean arterial pressure, heart rate, and coronary flow. This figure also illustrates that L-methionine infusion per se had no effect on systemic and coronary hemodynamics since coronary artery diameter and all hemodynamic variables did not change significantly after methionine infusion. Under continuous exposure to GTN (1.5 µg/kg/min for 5 days), the coronary artery diameter returned to control levels, indicating a complete development of tolerance in large epicardial arteries in agreement with previous observations. The GTN concentration for half-maximal dilation was shifted to 16-fold higher dosages. In the tolerant state (Figure 4), high-dose GTN challenges induced a dose-dependent increase in coronary artery diameter that was not significantly altered by infusion of L-methionine at 100 mg/kg.

L-Methionine and the Nitrate-Tolerant Capacitance System (Group B, Anesthetized and GTN-Pretreated Dogs)

The effects of L-methionine on the nitrate-tolerant canine capacitance system under anesthesia, ganglionic blockade, and continuous GTN infusion (1.5 µg/kg/min) are depicted in Table 2. The dose–response curve of GTN-induced venodilation was shifted to 13-fold higher dosages compared with that of nontolerant dogs in a previous study. Short-term GTN challenges (1.5 and 15.0 µg/kg/min) in nitrate-tolerant dogs caused a dose-dependent decrease in venous tone, that was not significantly modified by L-methionine infusion (100 mg/kg). There also was no potentiation of hemodynamic actions in response to GTN challenges in nitrate-tolerant dogs since changes in mean arterial central venous pressures were not significantly different before and after L-methionine infusion.

Effects of L-Methionine Infusion (100 mg/kg) on Plasma Methionine, Cystine, and Homocysteine Levels

Table 3 summarizes the effects of L-methionine infusion in a concentration of 100 mg/kg on plasma methionine, cystine, and homocysteine levels. L-Methionine caused a significant increase in plasma methionine levels (from 52±12 to 1,141±239 µM) and plasma cystine levels (from 12±4 to 26±7 µM) but not in homocysteine concentrations.

Extracellular Enhancement of Nitrate-Induced Guanylyl Cyclase by L-Cysteine

In a cell-free in vitro system, the activity of purified soluble guanylate cyclase was significantly enhanced above basal values by GTN in a concentration of 100 µM (Figure 5). The GTN-induced stimulation of guanylyl cyclase activity was significantly enhanced by L-cysteine (0.1+1 mM) but not by homocysteine. Compared with plasma cystine levels achieved with L-methionine infusion (20–30 µM; see Table 3), higher L-cysteine concentrations are required in this guanylyl cyclase assay. This may be due to suboptimal conditions for enzyme activation in this assay and therefore does not

TABLE 3. Plasma Concentrations of Methionine, Cystine, and Homocysteine After the Infusion of Methionine (100 mg/kg, n=4)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control 1</th>
<th>Control 2</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine (µmol/l)</td>
<td>52±12</td>
<td>62±2</td>
<td>984±195*</td>
<td>1,141±239*</td>
<td>932±53*</td>
<td>825±52*</td>
<td>722±53*</td>
</tr>
<tr>
<td>Cystine (µmol/l)</td>
<td>12±4</td>
<td>11±3</td>
<td>23±7*</td>
<td>26±7*</td>
<td>24±6*</td>
<td>18±4*</td>
<td>16±3</td>
</tr>
<tr>
<td>Homocysteine (µmol/l)</td>
<td>2.1±0.4</td>
<td>BD</td>
<td>BD</td>
<td>2.05±0.2</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
</tr>
</tbody>
</table>

BD, below detection limit.

*p<0.05 compared with control.

n=4.
argue against a role of such an interaction in vivo with lower L-cysteine concentrations.

Effects of L-Methionine on Sulfhydryl Group Content in Rat Aortic Smooth Muscle Cells in Culture

Figure 6 illustrates that the basal sulfhydryl content of cultured vascular smooth muscle cells was not significantly altered by incubation with L-methionine in a concentration of 10 μM or 1 mM. This finding might indicate that vascular smooth muscle cells of large conductance vessels cannot convert L-methionine to L-cysteine via the transsulfuration pathway. This observation, however, does not exclude that a minor but well-compartmentalized conversion could take place in critical intracellular sites, but this conversion cannot be monitored by looking at the whole vessel content.

Discussion

The objective of the present investigation was to determine whether the sulfhydryl precursor L-methionine can augment GTN actions in vivo and in vitro in the tolerant and nontolerant states. Although we could establish a small potentiation by L-methionine of GTN-induced changes in systemic hemodynamics in nontolerant dogs, this potentiation did not include a modification of the GTN-induced large epicardial arterial and venous dilation in tolerant and nontolerant dogs. The significant increase in plasma cystine levels in response to L-methionine infusion, together with the observed enhancement of GTN biotransformation in the guanylyl cyclase assay by L-cysteine, indicates that at least part of the L-methionine–induced potentiation of GTN action may be due to an interaction between the L-methionine conversion product L-cysteine and the organic nitrate. The inability of L-methionine to reverse nitrater tolerance in large epicardial arteries and veins in vivo, however, strongly argues against sulfhydryl group depletion as the predominant tolerance mechanism in these vessels.

Nitrate Tolerance and the Sulfhydryl Concept

The sulfhydryl group concept of nitrate tolerance is based on in vitro findings that chronic administration of organic nitrates leads to tolerance and is accompanied by intracellular sulfhydryl group depletion. Ignarro and coworkers demonstrated that in a cell-free system, activation of partially purified guanylyl cyclase requires the presence of sulfhydryl groups. Furthermore, they observed that S-nitroso-cysteine caused a guanylyl cyclase activation that resembled that induced by nitric oxide. Based on these observations, they hypothesized that S-nitrosothiols may be reactive intermediates required for vascular smooth muscle relaxation by organic nitrates.

More recently, however, Grütter and Lemke demonstrated that intracellular sulfhydryl group replenishment with cysteine (10-fold increase in sulfhydryl group content) had no influence on degree of tolerance of isolated bovine coronary arteries or on GTN-induced cyclic GMP accumulation. Subsequent studies in nontolerant and tolerant humans reported a potentiation of GTN effects by sulfhydryl donor NAC and sulfhydryl precursor L-methionine. However, the exact mechanism responsible for this potentiation remains controversial. Using chronically instrumented dogs as an in vivo model for nitrate tolerance, we found that the potentiation of GTN action by NAC was restricted to the nontolerant state, predominantly involved potentiation of GTN effects on resistance vessels, and did not include reversal of a high degree tolerance (shift to 17-fold higher dosages) in epicardial arteries and the venous capacitance system. The demonstration of an additional extracellular pathway for the interaction between NAC and GTN led us to propose a modified sulfhydryl concept. We and others suggested that exogenously administered sulfhydryl groups augment GTN action mainly by intravascular (extracellular) formation of either nitric oxide or nitrosothiol compounds at the resistance vessel level. This mechanism appears to be responsible for the potentiation of GTN action in nontolerant patients and for the partial reversal of a low-grade tolerance in humans.

Recent experimental data appear to support our hypothesis. Sellke and coworkers demonstrated that vessel size has great impact on the sensitivity to nitroglycerin. Biotransformation of GTN (i.e., the release of nitric oxide) in microvessels (diameter, <100 μM) was
Mechanism of Interaction Between L-Methionine and Organic Nitrates

The observed potentiation of GTN action by L-methionine in nontolerant and tolerant patients has also been linked with augmented intracellular nitrosothiol formation and subsequent enhanced guanylyl cyclase activation. In contrast to NAC, L-methionine does not contain sulfhydryl groups, and a metabolic, intracellular conversion to L-cysteine via cystathionine pathway (or transsulfuration pathway; see Figure 7) is required if L-methionine is to act as a sulfhydryl donor. This pathway, however, functions mainly in the liver and only to a lesser degree in other cells. After the intracellular conversion to L-cysteine, the amino acid is released from the liver into the plasma and undergoes rapid spontaneous oxidation at neutral pH to form cystine, which in turn is efficiently taken up by most cells. Cystine is then reduced to L-cysteine intracellularly by transhydrogenation with glutathione.

The present experiments provide four important results that may help to clarify possible mechanisms of interaction between L-methionine; its conversion product, L-cysteine; and the organic nitrate. First, infusion of L-methionine in a concentration of 100 mg/kg causes a significant increase in plasma cystine concentrations (Table 3), which indicates that this concentration of methionine is sufficient to increase intracellular sulfhydryl group content.

Second, we observed no change in intracellular sulfhydryl group content of rat aortic vascular smooth muscle cells after incubation with 1 mM L-methionine, a concentration comparable to plasma concentrations achieved with L-methionine infusions (1.1 mM; see Table 3). This observation strongly suggests that similar to skeletal muscle, vascular smooth muscle cannot degrade L-methionine to cysteine via the transsulfuration pathway, probably because of very low activity or absence of the required enzymes. Therefore, the observed changes in plasma cystine concentrations in response to L-methionine infusion are most likely due to enhanced intrahepatic methionine metabolism (see Figure 7). This concept is further strengthened by the observation that L-cysteine but not L-methionine potentiates the GTN-induced relaxation of canine coronary arterioles in vitro (unpublished observation).

Third, the augmentation of GTN-induced activation of purified soluble guanylyl cyclase activity is signifi-
cantly enhanced by L-cysteine. This suggests that an interaction between the L-methionine conversion product, L-cysteine, and GTN leads to a GTN metabolite formation (nitric oxide or nitrosothiol), which in turn enhances the vasodilator properties of the organic nitrate.

Fourth, the hemodynamic findings with respect to the interaction between L-methionine and GTN are striking similar to reported NAC-GTN interaction. L-Methionine enhanced GTN effects on systemic hemodynamics (Figures 1 and 2) similar to observations in nontolerant humans.12 However, it failed to modify GTN dose—response behavior of nontolerant and tolerant large epicardial arteries and tolerant veins. This indicates that L-methionine potentiates GTN actions predominantly in vessel regions such as peripheral resistance vessels that have been proposed to be nitrate resistant.30,39

Based on the literature and the present results, we propose the following mechanism for the interaction between L-methionine and GTN (see Figure 7). L-Methionine is intrahepatically converted to L-cysteine, released into the plasma and oxidized to cystine, taken up by microvessels, and reduced to L-cysteine, thereby replenishing sulfhydryl group stores necessary for improved biotransformation of GTN in very small vessels.33 This mechanism would explain the ability of L-methionine to potentiate the hypotensive action of GTN in the nontolerant state and the lack of effect on GTN-induced dilation of large coronary arteries in the tolerant and nontolerant states.

It appears implausible that other products in the conversion cascade from L-methionine to L-cysteine (e.g., adenosine, ADP, or α-keto-butyrate) contribute to the observed potentiation of GTN action since the infusion of methionine per se had no vasodilator effects. Furthermore, an extracellular interaction between L-homocysteine and the organic nitrate also appears unlikely since plasma homocysteine levels did not change significantly (Table 3) in response to L-methionine infusion. Furthermore, in contrast to L-cysteine, homocysteine failed to augment the nitroglycerin-induced activation of purified guanylyl cyclase in a cell-free buffer system (Figure 5).

Role of Desensitization of Guanylyl Cyclase and Pseudotolerance
It should be stressed that the development of nitrate tolerance is multifactorial.2,40 Desensitization of vascular smooth muscle to organic nitrates (true tolerance)18,22 and intravascular volume expansion,3 along with activation of neurogenic vasoconstrictor forces (both causing pseudotolerance,1,2), may contribute to blunting of GTN effects. Several aspects, however, strongly suggest that true tolerance rather than pseudotolerance develops in our model.

At first, the dose—response behavior for epicardial artery dilation in response to the spontaneously nitric oxide—releasing SIN-1, the vasoactive metabolite of sildoninimine molsidomine, is preserved in the nitrate— tolerant state.22 This makes it unlikely that mechanisms such as intravascular volume expansion and neurogenic counterregulation mechanisms contribute substantially to the tolerance of epicardial arteries in our model. Second, the administration of ganglionic blockade18 and chronic concomitant administration of ACE inhibitors (unpublished observation) did not reverse completely attenuated responsiveness of epicardial arteries to nitroglycerin in the tolerant state. This nitroglycerin—specific loss of responsiveness of epicardial arteries might involve a decreased liberation of nitric oxide from GTN,41 which in turn might be related to an impairment of the cytochrome P-450.42,43

Clinical Implications
The original impulse to use sulfhydryl donors was to reverse nitrate tolerance or to prevent tolerance by their concomitant administration. Although some improvement in hemodynamics in tolerant and nontolerant patients has been reported, the results are disappointing. High intravascular concentrations are apparently required to see this interaction, the degree of potentiation is small in nontolerant patients, and the reversal of tolerance in patients with heart failure is incomplete and short lived. Our results do not support a critical role of diminished intracellular sulfhydryl groups in the development of nitrate tolerance in large epicardial arteries and veins. We conclude that sulfhydryl group supplementation does not represent a therapeutic alternative to a nitrate—free interval as a means of restoring blood vessel sensitivity to nitrates.

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