UltraRapid Communication

Does Nitric Oxide Mediate the Vasodilator Activity of Nitroglycerin?

Andrei L. Kleschyov, Matthias Oelze, Andreas Daiber, Yale Huang, Hanke Mollnau, Eberhard Schulz, Karsten Sydow, Birgit Fichtlscherer, Alexander Mülsch, Thomas Münzel

Abstract—Nitroglycerin (glyceryl trinitrate, GTN) relaxes blood vessels primarily via activation of the soluble guanylyl cyclase (sGC)/cGMP/cGMP-dependent protein kinase (cGK-I) pathway. Although the precise mechanism of sGC activation by GTN in the vascular wall is unknown, the mediating role of nitric oxide (NO) has been postulated. We tested the GTN/NO hypothesis in different types of isolated rat and rabbit blood vessels using two novel approaches: (1) EPR spin trapping using colloid Fe(DETC)₂ and (2) analysis of cGK-I–dependent phosphorylation of the vasodilator-stimulated phosphoprotein at Ser239 (P-VASP). For comparison, another organic nitrate, isosorbide dinitrate (ISDN), and endothelium-dependent vasodilator, calcium ionophore A23187, were tested. We found a marked discrepancy between GTN’s strong vasoactivity (vasodilation and augmentation of P-VASP) and its poor NO donor properties. In aortas precontracted with phenylephrine, GTN, ISDN, and A23187 induced nearly full relaxations (>80%) and doubling of vascular P-VASP content at concentrations of 100 nmol/L, 100 μmol/L, and 1 μmol/L, respectively. GTN applied in vasorelaxant concentrations (10 to 1000 nmol/L) did not significantly increase the basal vascular NO production, in contrast to ISDN and A23187. The absence of GTN-derived NO was confirmed in rabbit vena cava and renal artery. A significant increase in vascular NO formation was observed only at suprapharmacological GTN concentrations (>10 μmol/L). The concentration dependency of NO formation from GTN was comparable to that of ISDN, although the latter exhibits 100-folds lower vasorelaxant potency. We conclude that GTN activates the sGC/cGMP/cGK-I pathway and induces vasorelaxation without intermediacy of the free radical NO. The full text of this article is available online at http://www.circresaha.org. (Circ Res. 2003;93:e104-e112.)

Key Words: nitroglycerin | nitric oxide | cGMP | vasodilation | spin trapping

Since 1879, nitroglycerin (glyceryl trinitrate, GTN) is widely used for treatment of angina pectoris. It is believed that the beneficial therapeutic effect of GTN is due to selective vasodilation of coronary arteries and venous capacitance vessels with minimal effect on arteriolar tone.

Several evidences indicate that the principal mechanism of GTN-induced smooth muscle relaxation is the activation of the intracellular enzyme, soluble guanylyl cyclase (sGC), and subsequent elevation of the cyclic guanosine-3′,5′-monophosphate (cGMP) levels. Among other effects, the elevated cGMP level leads to activation of cGMP-dependent protein kinase (cGK-I), which in turn mediates vasorelaxation via phosphorylation of several proteins regulating intracellular Ca²⁺ mobilization.

However, the precise mechanism by which GTN activates vascular sGC is still controversially discussed. According to the hypothesis presently favored, GTN undergoes intracellular bioconversion into the putative metabolite nitric oxide (NO), which is known as a direct activator of sGC and as a smooth muscle relaxant. After the discovery of endothelium-derived NO, the idea of NO being the active principle of GTN has been widely accepted by the scientific community, and it has been often speculated that GTN can replace the compromised endothelial NO production in patients with coronary heart disease. The results from several studies seemed to support the GTN/NO hypothesis by demonstrating the formation of NO in cells and tissues exposed to GTN either in vitro or in vivo. However, in all of these studies GTN was applied in concentrations in vivo and in vitro far exceeding the therapeutic range. Strikingly, none of the studies answered the critical question whether and how therapeutically effective low GTN concentrations affect vascular NO levels.

To address this issue, we used two novel experimental approaches used for direct assessment of NO/cGMP/cGK-I signaling in intact vascular segments: (1) EPR spin trapping utilizing colloid Fe-diethylthiophcarmate, Fe(DETC)₂, which is a direct and sensitive method for quantification of vascular NO production and (2) analysis of the phosphorylation state of vasodilator-stimulated phosphoprotein.

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From the Division of Cardiology (A.L.K., M.O., A.D., Y.H., H.M., E.S., K.S., T.M.), University Hospital Hamburg-Eppendorf, Hamburg, Germany; and the Institute of Cardiovascular Physiology (B.F., A.M.), Johann Wolfgang Goethe University Hospital, Frankfurt, Germany.
Correspondence to Andrei L. Kleschyov, Division of Cardiology, University Hospital Hamburg-Eppendorf, Martinistr 52, Hamburg 20246, Germany. E-mail kleschyov@uke.uni-hamburg.de
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(VASP) at Ser239 (P-VASP), which is a reliable biochemical marker of vascular cGK-I activity.\textsuperscript{39–43} We compared the potency of GTN, isosorbide dinitrate (ISDN), and calcium ionophore (A23187) to affect vascular tone, vascular P-VASP content, and NO production. Surprisingly, we found that, in contrast to other NO-related vasodilators, GTN exhibited a striking dissociation between its vascular activity (increase in P-VASP and relaxation) and NO donor properties. These results challenge the widely accepted GTN/NO hypothesis and suggest that at therapeutically effective concentrations, GTN activates the vascular sGC/cGK-I pathway and produces a vasodilation independently of its biotransformation into the free radical NO.

**Materials and Methods**

**Animals and Vessel Preparations**

Studies were performed using different types of blood vessels obtained from male Wistar rats (thoracic aorta) and male New Zealand White rabbits, NZWR (thoracic aorta, renal artery, and vena cava). Rats and rabbits were supplied by Charles River (Sulzfeld, Germany); they were kept and used in accordance with the guidelines for animal experimentation at the University Hospital Eppendorf, Hamburg, Germany. Some of the rats were made tolerant to GTN according to the recently described protocol.\textsuperscript{44} The rats were killed by a cervical dislocation under ether narcosis and rabbits by an overdose of sodium pentobarbital; blood vessels were rapidly dissected, cleaned of adhering fat, and kept in ice-cold Krebs-HEPES solution of the following composition (in mmol/L): NaCl 99.01; KCl 4.69; CaCl\(_2\) 2.50; MgSO\(_4\) 1.20; K\(_2\)HPO\(_4\) 1.03; NaHCO\(_3\) 25.0; glucose 11.1; Na–HEPES 20.00; pH 7.4.

**Isometric Tension Studies**

Aortic rings (3-to-4 mm long) were mounted under a passive tension (3 g for rat aorta and 5 g for rabbit aorta) in organ baths filled with Krebs-bicarbonate solution (37 °C) with indomethacin (10 μmol/L) as described previously.\textsuperscript{45} The cumulative concentrations of the vasodilators were added to rings preconstricted with phenylephrine (70% of maximal tone induced by KCl; 0.6 mmol/L for rat aorta and 0.2 μmol/L for rabbit aorta).

**NO Spin Trapping and EPR Spectroscopy**

The rate of NO production in intact vascular segments was measured by EPR spin trapping utilizing colloid Fe(DETC)\(_2\), as described previously.\textsuperscript{34,35} Briefly, the 0.4 mmol/L colloid Fe(DETC)\(_2\) was prepared in oxygen-free conditions by mixing two parent solutions: Na-DETC and FeSO\(_4\)\(_2\)H\(_2\)O. The vascular segments (about 50 to 70 mm\(^2\) of intima surface) were thoroughly cleaned and placed in a 24-well plate filled with 750 μL Krebs-HEPES solution. The vasodilators under study were added in a volume of 10 μL, just before the addition of 250 μL of the Fe(DETC)\(_2\) solution. The vascular segments were incubated for 3 minutes at 37°C, removed, and frozen in liquid nitrogen. In some experiments, the vascular segments were preincubated for 20 minutes with the inhibitor of NO synthase, \textit{N}-nitro-L-arginine methyl ester (L-NAME; 1 mmol/L) or G-nitro-L-arginine methyl ester (L-NAME; 1 mmol/L). In some rings, endothelium was removed by rubbing of the intimal surface with a cotton ball.

EPR spectra were recorded at 77K using an X-band spectrometer MS 100 (Magnetech, Germany). Instrument settings were 10-mW microwave power, 1.0-mT amplitude modulation, 100-kHz modulation frequency, 60-second sweep time, and 10 scans.

Nonstimulated blood vessels incubated for 30 minutes with colloid Fe(DETC)\(_2\) exhibited the triplet EPR signal (g = 2.035; A\(_\text{iso}\) = 1.26 mT) characteristic of NO-Fe(DETC)\(_2\) (Figure 1). The signal reflects the absolute amount of NO-Fe(DETC)\(_2\) (and thus NO) generated in a vascular segment during the incubation with Fe(DETC)\(_2\). The NO-Fe(DETC)\(_2\) signal usually largely dominated over the associated Cu-DETC signal and thus the total amplitude of the signal was taken as a measure. After correction of baseline, the amplitude was estimated in arbitrary units, which then were converted into absolute amount of NO-Fe(DETC)\(_2\) (pmol/sample).

The coefficient of variation of EPR estimates of a vascular sample recorded 10 times was 5.2% (n = 10), demonstrating a good reproducibility of the EPR technique.

The characteristic signal increased 3-fold when vessels were stimulated with acetylcholine (ACh; 1 μmol/L); removal of the endothelium or inhibition of NO synthase with L-NAME (1 mmol/L) prevented the appearance of the signal (Figure 1). The rate of bioconversion of GTN into NO at high GTN concentrations was estimated by comparison of the absolute amount of NO-Fe(DETC)\(_2\) (pmol) generated in tissue after inhibition of vascular NO production by L-NAME with the absolute amount of GTN added to the
incubation medium (100 nmol/mL) and was expressed as a percent. The experiments involving mechanical separation of adventitia and endothelium removal have shown that in spite of NO originated from endothelium, the lipophilic Fe(DETC)_2/NO-Fe(DETC)_2 complexes were proportionally distributed between all three tunicae, thus demonstrating the excellent vascular permeability of the NO-trapping agent. Additionally, in agreement with previous studies, colloid Fe(DETC)_2 rapidly and quantitatively reacted with S-nitrosoglutathione (GS-NO) (10 to 1000 nmol/L), giving rise to the NO-Fe(DETC)_2 EPR signal. Thus, using this protocol both, free NO and S-nitrosothiols potentially contribute to the NO-Fe(DETC)_2 formed in blood vessels during the incubation period. In contrast, colloid Fe(DETC)_2 did not react with sodium nitrite (up to 100 μmol/L).

The efficiency of NO spin trapping was not significantly affected by the additives present in the GTN formulation, "Trinitrosan" (Merck), which was used in this study as a stock solution of GTN. Also, no effect of the organic polar solvent dimethylsulfoxide (DMSO, final concentration 0.1%) was observed. Finally, our NO spin trapping protocol did not modify the vascular ALDH activity as measured by conversion of benzaldehyde into benzoic acid followed by HPLC analysis (26.4±2.2 and 28±2.1 μmol/L per mg, before and after incubation with colloid Fe(DETC)_2, respectively). Thus, the potential interference of colloid Fe(DETC)_2 with this important GTN metabolizing enzyme was excluded.

**Determination of P-VASP**

The vascular P-VASP content was measured by immunoblotting technique as described previously. Briefly, aortic rings were incubated at 37°C for 10 minutes with or without the vasodilator under study, then frozen and homogenized in liquid nitrogen. The pulverized tissue was vortexed with ice-cold homogenization buffer, centrifuged at 3000g to remove insoluble material, and subjected to SDS-PAGE electrophoresis/electroblotting. The mouse monoclonal antibody 16C2 was used for the recognition of P Ser239 -VASP. Densitometric values of the P-VASP bands were normalized against the actin bands. As a positive control of P-VASP, a protein extract prepared from the sodium nitroprusside-stimulated human platelets (1 μg protein) was used.

cGMP Assay

The vascular cGMP content was measured by the cGMP enzyme immunoassay system from Amersham Pharmacia Biotech. The IBMX-pretreated rat aortic segments were incubated at 37°C for 10 minutes with GTN (100 nmol/L) in the presence or absence of the inhibitor of mALDH, benomyl (1 μmol/L). The segments were frozen and homogenized in liquid nitrogen and then processed according to the manufacturer's instructions.

**Statistical Analysis**

Results were expressed as mean±SEM of n experiments. To compare EPR spin trapping data, the Student's t test was used. To compare P-VASP formation, one-way ANOVA was used. A value of P<0.05 was considered as significant.

**Chemicals**

All reagents were from Sigma unless otherwise indicated. GTN was from Merck and ISDN from Alexis. MGD, sodium salt was synthesized according to the published method. GS-NO was prepared as described previously.

**Results**

**Isometric Tension Studies**

In endothelium-intact aortas precontracted with phenylephrine, the vasodilators under study induced nearly complete relaxation at the following concentrations: GTN at 100 to 1000 nmol/L, ISDN at 100 μmol/L, and A23187 at 1 μmol/L. The E_{50}, −logM values were as follows: GTN in rat aorta, 7.5±0.1; GTN in rabbit aorta, 7.6±0.1; ISDN in rat aorta, 5.6±0.4; A23187 in rabbit aorta, 7.0±0.1 as calculated from 5 to 30 experiments (Figures 2 and 3).

**Effect of Vasodilators on Vascular NO Production**

The rate of NO production in nonstimulated endothelium-intact rat aortas was 1.5±0.1 pmol/cm^2××× min. Exposure of
aortas to the vasorelaxing concentrations of GTN (10 to 1000 nmol/L) did not significantly affect the vascular NO production (Figure 2A). Vascular NO production was increased in a concentration-dependent manner only when aortas were exposed to the suprapharmacological concentrations of GTN (10 to 1000 μmol/L) (Figure 2A). Preincubation of aortas with L-NAME (1 mmol/L) totally suppressed the basal vascular NO production, but did not affect the GTN (100 μmol/L)-dependent surplus of NO formation (data not shown). The bioconversion of GTN (100 nmol/mL) into NO by an entire rat thoracic aorta during 30 minutes was less than 0.1%. Incubation of aortas in the presence of vasorelaxing concentrations of ISDN (100 to 1000 μmol/L) was associated with a significant increase in vascular NO production (Figure 2B).

In endothelium-intact rabbit aortas, the vascular NO production was significantly higher than in rat aortas (3.2±0.2 pmol/cm²×min; P<0.05). Again, no increase in NO production was observed when rabbit aortas were exposed to pharmacologically relevant (10 to 1000 nmol/L) GTN concentrations, but suprapharmacological (10 to 1000 μmol/L) GTN concentrations induced significant NO formation (Figure 3A). The stimulation of rabbit aorta with A23187 at concentrations providing more than 50% relaxation (100 and 1000 nmol/L) was associated with a significant increase in vascular NO production (Figure 3B).

Effect of Vasodilators on P-VASP
Exposure of the rat aortas to increasing GTN concentrations (1 nmol/L, 100 nmol/L, and 10 μmol/L) led to a concentration-dependent increase in vascular P-VASP content; the difference started to be significant at 100 nmol/L (Figure 4A). ISDN applied in vasorelaxing concentrations (5 to 500 μmol/L) also significantly increased the P-VASP content (Figure 4B). In rabbit aorta, both GTN and A23187, when applied in vasorelaxing concentrations, significantly increased the aortic P-VASP content (Figures 5A and 5B).

Effect of GTN on NO Production in Different Types of Blood Vessels
There were no significant differences between the rates of basal NO production in endothelium-intact rabbit renal artery and vena cava. Exposure of both types of blood vessels to a pharmacologically effective GTN concentration (100 nmol/L) did not modify NO levels in renal artery and venous tissue (Figure 6).

Effect of GTN Tolerance on GTN-Derived NO
Aortas isolated from GTN-treated rats exhibited a significant shift to the right in the GTN concentration-relaxation curve (EC₅₀, −logM values 6.7±0.2 versus 7.5±0.1; P<0.05). The capacity of GTN-tolerant aortas to generate NO from suprapharmacological concentrations of GTN (100 μmol/L) was unaltered (control, 5.0±0.3 versus tolerant, 5.2±0.6 pmol/cm²×min, respectively; n=4).

Effect of Benomyl on GTN-Induced cGMP and GTN-Derived NO
Rat aortas preincubated with the inhibitor of mtALDH, benomyl (1 μmol/L) demonstrated a significant shift to the right in the GTN concentration-relaxation curve, without affecting the maximal relaxation (EC₅₀, −logM values 6.6±0.2 versus 7.5±0.1; P<0.05). Incubation of aortas with
vasorelaxing concentration of GTN (100 nmol/L) significantly increased the vascular cGMP levels from 7.4±2.3 to 18.4±1.1 fmol/µg protein (P<0.05; n=3). Benomyl (1 µmol/L) significantly inhibited the GTN-induced cGMP formation (10.8±1.0 fmol/µg protein; P<0.05; n=3). The capacity of benomyl-treated rat aortas to generate NO at suprapharmacological GTN concentrations (100 µmol/L) was not significantly different from that of control aortas (control, 4.1±0.5 versus benomyl, 3.8±0.4 pmol/cm²×min; n=3). Similar results were obtained on benomyl-treated rabbit aortas exposed to 100 µmol/L GTN (control, 9.9±1.2 versus benomyl, 9.2±1.1 pmol/cm²×min; n=3).

**Discussion**

With the present studies, we demonstrate that GTN in the nanomolar concentration range increases vascular VASP phosphorylation and induces vasodilatation without eliciting a significant increase in vascular NO formation. Thus, the role of free NO as the vasoactive principle of GTN is questioned.

**GTN and sGC/cGMP Downstream Signaling**

There is abundant evidence that the mechanism of GTN-induced smooth muscle relaxation is related to activation of the sGC/cGMP/cGK-I signaling pathway. In the present study, we observed that incubation of vascular tissue with GTN, concentration-dependently increased the phosphorylation of VASP at Ser239, a process known to be predominantly induced by cGMP. A nearly complete relaxation was associated with an increase in vascular P-VASP content by about 200%, independently of whether GTN, ISDN, or A23187 were applied. These data highlight the requirement of sGC/cGMP/cGK-I activation for the vasodilator activity of GTN, as well as ISDN and A23187.

**Figure 4.** Effect of different concentrations of glyceryl trinitrate (GTN) (A) and isosorbide-dinitrate (ISDN) (B) on phosphorylation of vasodilator-stimulated phosphoprotein at Ser239 (P-VASP) in rat aortas. Mean±SEM of 3 to 5 independent experiments.

**Figure 5.** Effect of different concentrations of glyceryl trinitrate (GTN) (A) and Ca²⁺ ionophore (A23187) (B) on phosphorylation of vasodilator-stimulated phosphoprotein at Ser239 (P-VASP) in rabbit aortas. Mean±SEM of 3 to 5 independent experiments.

**Figure 6.** Effects of pharmacological concentration of glyceryl trinitrate (GTN; 100 nM) on NO production in different types of rabbit blood vessels. Mean±SEM of 3 to 5 independent experiments.
The GTN/NO Paradigm
The idea that NO mediates the vasoactivity of GTN originated from the observation that GTN activated isolated sGC only in the presence of low molecular weight thiols, such as cysteine, suggesting the possible nonenzymatic release of NO from GTN. Because NO was found to be a direct and potent activator of sGC,\(^\text{20,21}\) it was proposed that this free radical might mediate the bioactivity of GTN, as well as other nitrovasodilators.\(^\text{5,21,22}\) Later, this concept has been supported by detection of NO in the GTN/cysteine system using methemogoblin formation from oxyhemoglobin as a measure of NO.\(^\text{33}\) Subsequent studies, however, disproved the specificity of the oxyhemoglobin assay.\(^\text{54,55}\) Furthermore, it has been demonstrated, that the production rate of NO in GTN/cysteine system is too low to account for the sGC activation.\(^\text{17,56}\) Moreover, it was found that the mechanism of sGC activation by GTN/cysteine is apparently different from that elicited by NO, as it does not involve the formation of heme-N0 species but rather the oxidation of sGC heme group.\(^\text{18}\) These very recent results not only argue against the idea of cysteine-dependent, nonenzymatic conversion of GTN into NO in blood vessels as it was originally suggested, but also rise the possibility that GTN may exert his activity via an NO-independent activation of vascular sGC.

Some evidences suggest that in native environment, the mechanism of GTN/sGC interaction might be different from that studied on isolated sGC. Thus, in intact cells and vascular tissue, GTN activates sGC and induces vasorelaxation at picomolar/nanomolar concentrations depending on the vessel and cell phenotype.\(^\text{13,57}\) In contrast, micromolar/millimolar concentrations of GTN necessary to stimulate sGC activity in broken cell/tissue preparations\(^\text{7,13,17,58,59}\) to our knowledge, such striking difference has been observed for neither putative NO donors nor other organic nitrates. The reason of why GTN is somewhat 1000 to 100 000 times more potent activator of sGC in intact cells than in cell-free preparations is puzzling. Whatever the reason, experiments on GTN/sGC using tissue homogenates or subcellular fractions\(^\text{48,60–62}\) should be interpreted with caution.

GTN/NO in Blood Vessels
Previous studies, which demonstrated NO formation from GTN in cultured cells or isolated blood vessels used indirect methods such as the oxyhemoglobin assay,\(^\text{27,29}\) ozone-induced chemiluminescence,\(^\text{30}\) or a Clark-type electrode.\(^\text{31}\) Interestingly, in these experiments, suprapharmacological (usually high micromolar) GTN concentrations were required for a reliable detection of GTN-derived NO, despite the fact that these methods are sensitive enough to detect NO production from the nonstimulated endothelial cells. Therefore, negative results obtained at lower GTN concentrations may suggest the absence of the GTN-derived NO.

To study the potential vascular bioconversion of GTN into NO our EPR approach is more suitable than previous methods\(^\text{27,29–31}\) that detect the extracellular NO concentration and thus heavily determined by the balance between NO generation and NO consumption reactions. In contrast, our approach allows measuring the absolute amount of NO formed within a blood vessel during a selected period of time. Because of the high lipophilicity and the high reaction rate constant with NO (1.1×10^7 mol/L s\(^{-1}\))\(^\text{63}\) the Fe(DETC)\(_2\) complexes can very efficiently compete with NO consumption reactions within the vascular wall. These advantages allow us to specifically assess NO production even in nonstimulated intact vascular segments.\(^\text{34,35}\)

In the present study, we found that GTN, in nanomolar concentrations, increased cGMP levels, P-VASP formation, and induced vasorelaxation without increasing vascular NO production. This rather surprising observation was made for GTN only, whereas other NO-related vasodilators, such as ISDN and A23187 activated the vascular cGMP/cGK-I pathway and induced full vasorelaxation in association with the elevation of vascular NO levels. Moreover, essentially the same results were obtained both in rat and rabbit aortas, indicating that the ability GTN to induce vasodilation without affecting vascular NO levels is a general phenomenon.

It is well known that the vascular responsiveness to GTN varies greatly depending on the blood vessel type.\(^\text{13}\) With regard to the GTN/NO hypothesis, it is generally assumed that GTN-hypersensitive blood vessels possess a higher capacity to metabolize GTN to NO.\(^\text{13,64}\) Our present results do not favor this view as not only the aorta but also vena cava and renal artery did not exhibit any elevation of NO levels on exposure to pharmacological GTN concentrations.

A concentration-dependent increase in the vascular wall NO production was detected only in the presence of suprapharmacological GTN concentrations (>10 μmol/L); this was associated with further augmentation of the P-VASP content. These data were in general agreement with the previous studies using different NO detection methods\(^\text{27,29–31}\) and confirm that GTN is indeed an NO donor at these high concentrations. In the present study, we have found that GTN was comparably effective to ISDN as an NO donor, despite being two to three orders more potent as a stimulator of P-VASP formation and vasodilatation. Thus, ISDN served as a valuable control, indicating the ability of our method to detect vasoactive amounts of NO formed within vascular smooth muscle.

A characteristic feature of prolonged GTN action in the vasculature is the development of GTN-tolerance. A possible explanation in accordance with the GTN/NO hypothesis is that either a decrease in GTN bioconversion into NO\(^\text{48,65}\) or a decrease in bioavailability of GTN-derived NO due to enhanced superoxide formation\(^\text{45}\) accounts for GTN tolerance. We have demonstrated in this study that capacity of GTN-tolerant aortas to produce NO from 100 μmol/L GTN was not different from control aortas. These results are consistent with a previous study\(^\text{66}\) and argue against the idea of decreased bioconversion of GTN into NO in GTN-tolerant vessels. These results also do not support a role for NO in GTN-induced vasodilatation.

GTN/NO Paradigm Revised
Our present findings may explain early studies from the late 1980th showing that the GTN concentration-effect curve for vascular smooth muscle relaxation has two distinct phases.\(^\text{13,67–69}\) Although both phases involved cGMP,\(^\text{11,68}\) only the
low-concentration phase was abolished by induction of GTN tolerance, was apparently vessel type specific,13,67,68 and was associated with predominant biotransformation of GTN into 1,2 GDN,52,57,70 Interestingly, the inhibitor of sGC, methylene blue inhibited the first phase noncompetitively, but the second phase competitively, implying potentially different mechanisms of GTN action.13,68 Based on these results, it was postulated that two different enzymatic systems might be responsible for GTN-mediated vascular relaxation: (1) a high-affinity, low-capacity system that operates at picomolar/nanomolar (clinically relevant) concentrations, and (2) the low-affinity, high-capacity system, which requires GTN in the micromolar/millimolar range.13 Unfortunately, further characterization these two apparently distinct GTN pathways has not been accomplished. Intriguingly, the biphasic concentration-effect curve has been described for GTN only; ISDN exhibits a monophasic curve13,71 (and this study), which roughly corresponds to the second phase of the GTN relaxation curve. Because only nanomolar concentrations fall into therapeutic range of the drug, only the first enzymatic system may have a pharmacological significance. The full appreciation of the biphasic GTN effect may also imply that experiments using the high GTN concentrations only27,29 were misinterpreted as they were related to the second GTN pathway.

Our current data are generally consistent with the GTN/NO hypothesis in that GTN produces the vasoactive amounts of NO at high, micromolar concentrations. This NO production is probably related to the “second” GTN pathway, which is not affected by in vivo GTN tolerance state. The enzyme responsible for this NO producing “second pathway” is unknown; however, the possible implication of mALDH48 is unlikely, because the inhibitor of mALDH, benomyl, had no effect on the NO production at 100 µmol/L GTN. The GTN-derived NO may be important under special condition requiring high GTN concentrations for relaxation (eg, high concentration of a pressor agent13). In clinical setting, the implication of the GTN/NO pathway is probably limited to cases where GTN is infused at high doses to induce the local vasodilatation.72

Despite the apparent link between GTN biotransformation into 1,2 GDN/nitrite and GTN vasoactivity,73 the mechanistic explanation for this phenomenon is lacking. Several purified enzymes are known to metabolize GTN; among them glutathione S-transferase,74,75 cytochrome P450,76,77 xanthine oxidoreductase,78 and mALDH.48 The selective transformation of GTN into 1,2 GDN has been described for the deoxy-heme-containing proteins79 and mALDH.48 However, none of the enzymes released appreciable amounts of free NO, but nitrite.48,62 In this regard, some of investigators proposed that GTN-derived nitrite may account for GTN bioactivity after conversion into S-nitrosothiols8 or into unidentified “NO bioactivity.”44 However, with regard to intracellular concentrations of nitrite, which are in the micromolar range,80 it is difficult to imagine how nanomolar concentrations of GTN-derived nitrite could appreciably increase NO levels.

Recently, mALDH has been described as the principal GTN bioactivating enzyme.48 In the present study, inhibition of mALDH by benomyl suppressed the GTN-induced relaxation and cGMP accumulation at low GTN concentrations, although it did not modify the relaxation and NO production in response to high, suprapharmacological GTN concentrations. These data are in accordance with the concept of a biphasic concentration-dependent metabolism and activity of GTN and may support a role of the mALDH in the high-affinity, pharmacologically relevant, GTN pathway. Because the vasoactive mALDH activity was not modified by colloid Fe(DETC)2, it is unlikely that potential inhibition of this enzyme by the spin trap may cause the lack of NO detection at low GTN concentrations.

Our present experiments on intact vascular segments clearly indicate that the first, high-affinity GTN/GK-I pathway may not rely on NO donor properties of GTN. Other possibilities may be considered apart from the mALDH/GTN bioactivation concept. One may hypothesize that yet unidentified mechanism of GC/GK-I activation by GTN may take place in intact vessels. This might be due to some labile cofactors or due to existence of the GTN-sensitive pool of GC. A striking corollary of this hypothesis is that GTN may mimic some endogenous signaling molecules beyond NO. Another possibility is that in native milieu, GTN/sGC interaction results in the formation of NO–heme moieties directly on sGC, without liberation of free NO. Such possibility seems to be not excluded from chemical standpoint.16

Conclusion
The critical analysis of the literature reveals that unequivocal evidences supporting the GTN/NO hypothesis are lacking. Our NO spin trapping data suggest that GTN may apparently exert its activity independently of its NO-releasing properties. These results urge a reconsideration of our current view on GTN as an efficient intravascular NO donor. These results may encourage the efforts searching for new NO-independent activation of the sGC/PK-I signaling pathways, which might affect or mimic this widely used drug.

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