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# Discrepancies Between Nitroglycerin and NO-Releasing Drugs on Mitochondrial Oxygen Consumption, Vasoactivity, and the Release of NO

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*Abstract*—It has been generally acknowledged that the actions of glyceryl trinitrate (GTN) are a result of its bioconversion into NO. However, recent observations have thrown this idea into doubt, with many studies demonstrating that NO is present only when there are high concentrations of GTN. We have explored this discrepancy by developing a new approach that uses confocal microscopy to directly detect NO. Intracellular levels of NO in the rat aortic vascular wall have been compared with those present after incubation with 3 different NO donors (DETA-NO, 3-morpholinosydnonimine, and *S*-nitroso-*N*-acetylpenicillamine), endothelial activation with acetylcholine, or administration of GTN. We have also evaluated the relaxant effects of these treatments on isolated rings of aorta following activation of the enzyme soluble guanylyl cyclase and their inhibitory action on mitochondrial respiration, which is an index of the interaction of NO with the enzyme of the electron transport chain cytochrome C oxidase. In the case of the various NO donors and acetylcholine, we detected a concentration-dependent relationship in the intensity of vascular relaxation and degree of NO fluorescence and an increase in the Michaelis constant ($K_m$) for $O_2$. GTN did not produce similar effects, and although clinically relevant concentrations of this compound caused clear, concentration-related relaxations, there was neither any increase in NO-related fluorescence nor an augmented $K_m$ for $O_2$. The nature of these differences suggests that these concentrations of GTN do not release free NO but probably a different species that, although it interacts with soluble guanylyl cyclase in vascular smooth muscle, does not inhibit $O_2$ consumption by vascular mitochondria. (*Circ Res*. 2005;97:1063-1069.)

**Key Words:** glyceryl trinitrate ■ nitric oxide ■ mitochondria ■ vascular relaxation ■ NO donors

It is generally acknowledged that the action of glyceryl trinitrate (GTN) is a result of its bioconversion into the vasorelaxant agent NO.[1,2] This idea is based on reports, both in vitro and in vivo,[3–7] that suggest that exposure to GTN leads to the formation of NO. However, in most of these studies, NO was observed only when GTN concentrations considerably exceeded the plasma levels reached during clinical dosing, whereas a number of further reports question the direct formation of NO.[8–10] Furthermore, the precise mechanism by which GTN is biotransformed is also currently a subject of controversy.[11]

Using confocal microscopy to observe the cells of vessels, we detected endothelium-synthesized NO and compared it with that released by different NO donors and with GTN. Furthermore, the vasodilatatory effects of NO and its consequences for mitochondrial respiration have also been evaluated. By studying these 2 targets of NO,[12,13] we identified inconsistencies between the effects of NO, produced both endogenously and pharmacologically, and those obtained with GTN used at clinically relevant concentrations. The nature of these discrepancies suggests that the actions of GTN on the vasculature are indeed unrelated to its bioconversion to NO.

## Materials and Methods

### Animals

Male Sprague–Dawley rats (200 to 250 g; Harlan Laboratories, Barcelona) were decapitated and their thoracic aortas removed, cleaned of adhering tissues in Krebs solution, and cut into rings of 1 mm (for image analysis) or 5 mm (all other experiments). Unless otherwise stated, all results are mean±SEM of at least 5 experiments, each in a different animal. All protocols complied with the European Community guidelines for the use of experimental animals and were approved by the Ethics Committee of the University of Valencia.

## Contractility Studies

Aortic rings were suspended in a 5-mL organ bath containing Krebs solution at 37°C. The solution was constantly gassed with 12% $O_2$, 5% $CO_2$, and 83% $N_2$, producing an $O_2$ concentration of 12 to $13 \times 10^{-5}$ mol/L in the bath, which was monitored with a dissolved $O_2$ meter (ISO2; World Precision Instruments, Stevenage, Herts, UK). This level of $O_2$ is similar to that present in the aortic blood.[13] Following an equilibration period of 75 to 90 minutes, rings were submaximally (75% to 85% of maximal response) contracted with $10^{-6}$ mol/L phenylephrine (Phe), and the presence of a functional endothelium was confirmed by eliciting a relaxant response (>90%) to $10^{-5}$ mol/L acetylcholine (ACh). Only those vessels exhibiting this response were included in what remained of the experiment.

Following a further equilibration period of 30 to 45 minutes, rings were contracted with a depolarizing solution ($6 \times 10^{-2}$ mol/L KCl). After being washed, the vessels returned to the baseline and a further Phe concentration of $10^{-6}$ mol/L was added, giving a sustained contraction equivalent to the response to depolarization. Once such a stable tone was achieved, relaxation-response curves were obtained by adding cumulative concentrations of the NO donors (Z)-1-([2-aminoethyl]-*N*-[2-ammonioethyl]amino)diazen-1-IM1,2-diolate (DETA-NO) ($10^{-8}$ to $10^{-5}$ mol/L), *S*-nitroso-*N*-acetylpenicillamine (SNAP) ($10^{-10}$ to $10^{-5}$ mol/L), or 3-morpholinosydnonimine (SIN-1) ($10^{-11}$ to $10^{-6}$ mol/L); and ACh ($10^{-9}$ to $10^{-5}$ mol/L) or GTN ($10^{-10}$ to $10^{-6}$ mol/L). In some experiments, the soluble guanylyl cyclase (sGC) inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) ($5 \times 10^{-6}$ mol/L), the scavenger of NO and related species oxyhemoglobin (oxyHb) ($10^{-5}$ mol/L), or the NO synthase inhibitor *N*-$\omega$-nitro-L-arginine (L-NNA) ($10^{-4}$ mol/L) was added before the NO-producing drugs. The adequate concentration ($-\log[M]$) for producing 50% relaxation (pEC$_{50}$) was obtained from a nonlinear regression analysis with Graph Pad Software. The percentage of relaxation obtained with the highest concentration of each agent in the absence (control) or presence of ODQ ($5 \times 10^{-6}$ mol/L) or oxyHb ($10^{-5}$ mol/L) is represented by $E_{max}$.

## Confocal Microscopy Imaging of NO Release

NO was visualized with diaminorhodamine-4M (DAR-4M AM), a cell permeable, fluorescent precursor with a convenient profile for NO bioimaging. It exhibits low background fluorescence with longer wavelength excitation, allows detection throughout a wider pH range, and is more photostable than standard fluorochromes that have been used previously for NO imaging.[14] Inside the cell, the relatively nonfluorescent DAR-4M AM is converted into its triazole form, DAR-4M T, the fluorescence intensity of which is directly proportional to the concentration of NO, with a detection limit of $7 \times 10^{-9}$ mol/L.[14]

Aortic rings were incubated in the dark for 60 minutes in a Krebs solution that was constantly gassed (12% $O_2$, 5% $CO_2$, and 83% $N_2$) and that contained DAR-4M AM ($2.5 \times 10^{-6}$ mol/L). The vital cell nuclei staining fluorochrome Hoechst 33342 ($10^{-6}$ mol/L) was added to this solution after 30 minutes. Thereafter, the rings were mounted in a vertical position on $24 \times 50$-mm coverslips, fixed with a grid in Krebs solution, and placed in a heated chamber (37°C, 2 mL; Warner Instrument Corp, Hamden, Conn). Following a 10-minute stabilization period, which was considered basal, cumulative concentrations of DETA-NO ($10^{-7}$ to $10^{-4}$ mol/L), SNAP ($10^{-9}$ to $10^{-5}$ mol/L), SIN-1 ($10^{-9}$ to $10^{-6}$ mol/L), ACh ($10^{-6}$ to $10^{-4}$ mol/L), or GTN ($10^{-9}$ to $10^{-6}$ mol/L) were added every 10 minutes. In some experiments, before addition of the agents, tissues were preincubated (10 minutes) with L-NNA ($10^{-4}$ mol/L). Images were obtained every 2 minutes throughout the entire experiment, which lasted a maximum of 60 minutes. A concentration of DETA-NO ($10^{-4}$ mol/L) was added at the end of each experiment to monitor the sensitivity of our system, and, to avoid variability, the response obtained was considered a reference for normalizing the signal elicited in each experimental series. To ensure that the fluorochromes had no influence on the viability of the tissues, parallel experiments were performed in which rings were incubated with these compounds under the same experimental circumstances that confocal analysis and contractility studies were performed. No significant differences were found either in the

response to a single concentration of Phe ($10^{-6}$ mol/L) or in the concentration-response curves of relaxation to DETA-NO (pEC$_{50}$: $5.98 \pm 0.09$ in control versus $5.90 \pm 0.08$, n=4 and 5, respectively), ACh (pEC$_{50}$: $7.33 \pm 0.10$ in control versus $7.23 \pm 0.21$, n=5), and GTN ($8.08 \pm 0.06$ in control versus $8.40 \pm 0.13$, n=5 and 6, respectively).

Images were observed in water immersion ($\times 60$ magnification) and studied with a Radiance 2100 confocal microscope (Bio-Rad, Hempel Hempstead, UK) using a 405-nm diode laser to excite Hoechst 33342 and a 546-nm He-Ne laser to excite DAR-4M AM. Fluorescence was detected through 440/20 band pass (BP) and 570 long pass (LP) filters for Hoechst 33342 and DAR-4M AM, respectively. DAR-4M AM fluorescence was analyzed (Laserpix software; Bio-Rad) in portions of aortic ring comprising the entire width of the vessel, from the adventitia to the endothelium. Confocal microscope settings were adjusted to produce the optimum signal/noise ratio. The vessel wall exhibited basal red fluorescence, representing the autofluorescence of the elastin fibers, which was deducted from the total quantification of fluorescence in each specific measurement.

Further experiments were performed to exclude the interference on DAR-4M AM fluorescence by GTN or some of its main intermediate metabolites. Different concentrations ($10^{-9}$ to $10^{-4}$ mol/L) of GTN, its metabolites 1,2 glyceryl dinitrate (1,2-GDN) and 1,3 glyceryl dinitrate (1,3-GDN), and sodium nitrate (NaNO$_3$) or sodium nitrite (NaNO$_2$) were incubated in the presence of DAR-4M AM ($2.5 \times 10^{-6}$ mol/L) in a cell-free Krebs solution. Fluorescence was measured with a Fluoroskan plate reader (Thermo Labsystems) and $10^{-4}$ mol/L DETA-NO was added at the end of each experiment. No modification of the fluorochrome basal fluorescence was obtained after incubation with GTN or any of the compounds tested for 60 minutes. Furthermore, the increase in fluorescence observed after addition of DETA-NO was similar in the presence or absence of these agents (n=4 to 5 experiments; results not shown).

## Electrochemical Measurement of $O_2$ Consumption

The aorta of 2 animals ($97.65 \pm 1.04$ mg total wet weight) were cut in rings and placed in gas-tight chambers containing 1 mL of Krebs solution and gently agitated at 37°C. The $O_2$ consumption by the tissue was measured with a Clark-type $O_2$ electrode (Rank Brothers, Bottisham, UK) calibrated with an air-saturated Krebs solution, assuming an $O_2$ concentration of $2 \times 10^{-4}$ mol/L. Experiments were performed in the presence of DETA-NO ($10^{-8}$ to $10^{-4}$ mol/L), SNAP ($10^{-9}$ to $10^{-5}$ mol/L), SIN-1 ($10^{-9}$ to $10^{-6}$ mol/L), ACh ($10^{-7}$ to $10^{-4}$ mol/L), or GTN ($10^{-9}$ to $10^{-6}$ mol/L). Sodium cyanide ($10^{-3}$ mol/L) was used to confirm that $O_2$ consumption was mainly mitochondrial ($\approx 95\%$ to 99%). In some experiments, the reversibility of NO-induced inhibition of respiration was assessed by adding oxyHb ($10^{-5}$ mol/L) when the concentration of $O_2$ in the chamber was $10^{-4}$ mol/L. Measurements were collected using a data-acquisition device, Duo.18 (World Precision Instruments, Stevenage, UK). A hyperbolic function was used to describe the relationship between $O_2$ concentration and the rate of $O_2$ consumption (V$O_2$).[15] The maximal rate of $O_2$ consumption (V$O_{2max}$) and the apparent $O_2$ affinity Michaelis constant ($K_m$) ($10^{-6}$ mol/L) were calculated according to their analogy with the Michaelis–Menten constant. The rate of $O_2$ consumption at the interval between 3 to $2 \times 10^{-5}$ mol/L of $O_2$ (similar to conditions during hypoxia[16,17]) was calculated from the same experiments. To avoid variability, the rate of $O_2$ consumption was expressed as $10^{-9}$ mol $O_2$/min per $10^{-6}$ g protein. Proteins were determined by the BCA protein assay kit (Pierce, Rockford, Ill) using BSA as the standard. In some cases, the viability of the rings was later assessed by confirming that their contractile response to Phe ($10^{-6}$ mol/L) and ACh ($10^{-5}$ mol/L) did not differ from that of nonmanipulated vessels.

## Data Analysis

Values are expressed as mean$\pm$SEM. Statistical analysis was performed by 1-way ANOVA followed by the Student *t* test for unpaired samples (Graph Pad Software). Significance was defined as $P < 0.05$.

**TABLE 1.** pEC$_{50}$ and $E_{max}$ of the Concentration Response Curves of Relaxation Induced by the Different Drugs in Rat Aortic Rings Precontracted by Phe

| Drug | pEC$_{50}$ | $E_{max}$ | n |
|---|---|---|---|
| Ach | | | |
|   Control | 7.46±0.12 | 96.67±0.60 | 9 |
|   +ODQ | | 10.21±4.78‡ | 4 |
|   +OxyHb | | 16.63±6.92‡ | 4 |
| GTN | | | |
|   Control | 7.94±0.04 | 94.89±1.33 | 9 |
|   +ODQ | | 12.85±5.61‡ | 4 |
|   +OxyHb | | 47.12±16.22‡ | 4 |
| DETA-NO | | | |
|   Control | 6.08±0.01 | 99.74±0.36 | 5 |
|   +ODQ | | 0.53±0.49‡ | 6 |
|   +OxyHb | | 16.21±9.10‡ | 4 |
| SNAP | | | |
|   Control | 7.66±0.09 | 99.57±0.36 | 14 |
|   +ODQ | | 0 | 4 |
| SIN-1 | | | |
|   Control | 8.26±0.10 | 93.70±3.61 | 9 |
|   +ODQ | | 4.20±4.20‡ | 5 |

Phe concentration was $10^{-6}$ mol/L. Data are the mean±SEM of n experiments. ‡$P<0.001$ vs control.

## Drugs and Solutions

The composition of the Krebs solution was as follows ($10^{-3}$ mol/L): 118 NaCl, 4.75 KCl, 1.9 CaCl$_2$, 1.2 MgSO$_4$, 1.2 KH$_2$PO$_4$, 25 NaHCO$_3$, and 10.1 glucose. NaNO$_3$ and NaNO$_2$ were obtained from Panreac (Barcelona, Spain). 1,2-GDN and 1,3-GDN were purchased from LGC Promochem (Barcelona, Spain). Phe, ACh, L-NNA, sodium cyanide, ODQ, meth(a)emoglobin, and Hoechst 33342 were acquired from Sigma Aldrich (St Louis, Mo). DETA-NO was obtained from Alexis (Lausen, Switzerland). A clinically available preparation of GTN was used (Solinitrina from Allmirall Prodesfarma, Barcelona, Spain), and DAR-4M AM was obtained from Calbiochem (San Diego). OxyHb was prepared by reduction of human meth(a)emoglobin with a 10-fold molar excess of sodium dithionite, followed by dialysis against PBS. All drugs were dissolved in ultrapure water, except GTN, DAR-4M AM, and Hoechst 33342, which were dissolved in Krebs solution, and ODQ, which was dissolved in dimethyl sulfoxide (1:5000 [vol/vol]).

## Results

### Contractility Studies

Table 1 shows pEC$_{50}$ and $E_{max}$ of the relaxation curves induced in vessels precontracted in vitro with Phe ($10^{-6}$ mol/L) by cumulative concentrations of DETA-NO ($10^{-8}$ to $10^{-5}$ mol/L), SNAP ($10^{-10}$ to $10^{-5}$ mol/L), SIN-1 ($10^{-11}$ to $10^{-6}$ mol/L), ACh ($10^{-9}$ to $10^{-5}$ mol/L), and GTN ($10^{-10}$ to $10^{-6}$ mol/L), respectively. The presence in the organ bath of the sGC inhibitor ODQ ($5\times10^{-6}$ mol/L) prevented the vasorelaxant activity of all 5 substances. Likewise, incubation with the NO scavenger oxyHb inhibited the effects of DETA-NO, ACh, and GTN. The effects of ACh were inhibited by prior incubation with the NO synthesis inhibitor L-NNA ($10^{-4}$ mol/L) (data not shown).
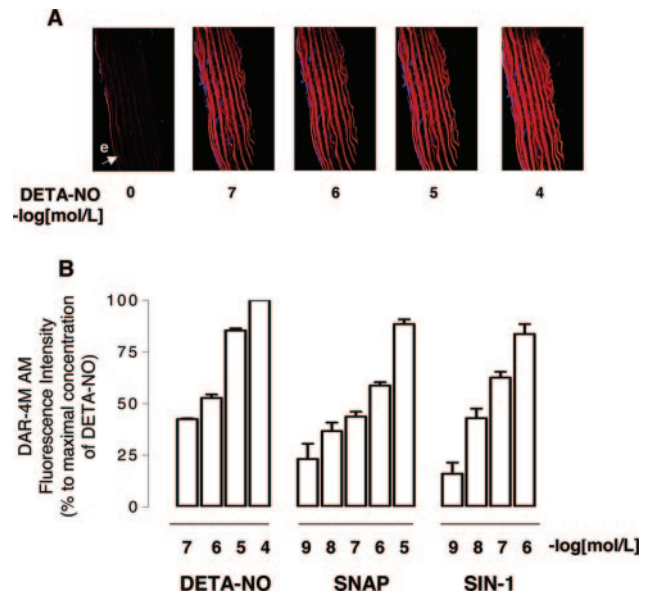


**Figure 1.** Detection by confocal microscopy of intracellular NO levels in isolated rat aortic rings. A, Images were obtained after addition of increasing concentrations of DETA-NO. Rings were loaded with DAR-4M AM ($2.5\times10^{-6}$ mol/L, 60 minutes) as an NO probe (red fluorescence) and Hoechst 33342 ($10^{-6}$ mol/L, 30 minutes) as a nuclei marker (blue fluorescence). e indicates endothelial layer. B, Bar diagrams show DAR-4M AM fluorescence intensity obtained with DETA-NO, SNAP, and SIN-1, expressed as percentage of basal fluorescence and normalized according to the signal elicited by $10^{-4}$ mol/L DETA-NO. Data are mean±SEM from 6 independent experiments.

### Confocal Microscopy Imaging of NO Release

Figure 1A shows representative confocal images of the rat aorta following incubation (60 minutes) with DAR-4M AM and concentration-dependent increases in the red fluorescent signal induced by addition of cumulative concentrations of DETA-NO ($10^{-7}$ to $10^{-4}$ mol/L) every 10 minutes. An analysis of the total changes in red fluorescence is presented in Figure 1B. Figure 1 also shows significant and concentration-dependent increases in NO-related DAR-4M AM fluorescence obtained with the other 2 NO donors used, SNAP ($10^{-9}$ to $10^{-5}$ mol/L) and SIN-1 ($10^{-9}$ to $10^{-6}$ mol/L). Addition of ACh at concentrations that produced maximal vasodilatation ($10^{-6}$ to $10^{-4}$ mol/L) increased DAR-4M AM fluorescence in a concentration-dependent manner (Figure 2A). This red fluorescence, although particularly abundant in the endothelial layer, permeated through the entire thickness of the vessel and was prevented by preincubation with L-NNA ($10^{-4}$ mol/L) (Figure 2B). Figure 2C summarizes the quantitative analysis of the total changes in red fluorescence. Incubation with L-NNA induced a slight but significant decrease in the signal, which could be interpreted as an inhibition of the basal release of NO. Incubation with GTN ($10^{-9}$ to $10^{-6}$ mol/L) did not increase DAR-4M AM fluorescence. Indeed, each increase in the concentration of GTN was accompanied by a significant reduction in red fluorescence, as represented in Figure 3A and quantified in Figure 3B.

To discard the possibility that such a reduction in the fluorescence was attributable to a direct interference on DAR-4M AM by GTN or any of its main intermediary
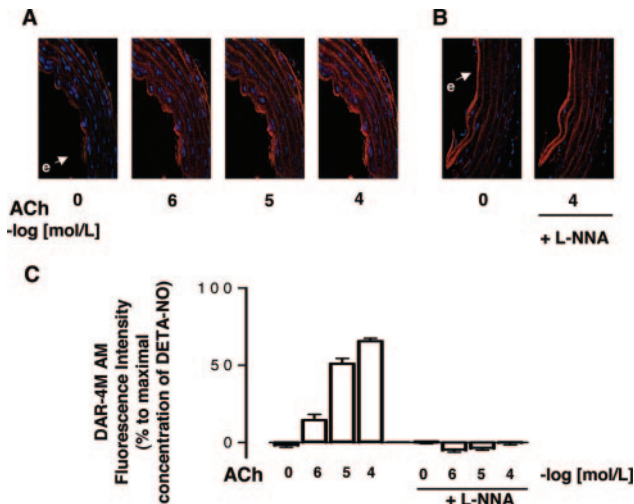
**Figure 2.** Detection by confocal microscopy of intracellular NO levels in isolated rat aortic rings. Images were obtained after stimulation with ACh in the absence (A) or presence (B) of L-NNA $10^{-4}$ mol/L. Rings were loaded with DAR-4M AM ($2.5 \times 10^{-6}$ mol/L, 60 minutes) as an NO probe (red fluorescence) and Hoechst 33342 ($10^{-6}$ mol/L, 30 minutes) as a nuclei marker (blue fluorescence). e indicates endothelial layer. C, Bar diagrams show DAR-4M AM fluorescence intensity, expressed as percentage of basal fluorescence and normalized according to the signal elicited by $10^{-4}$ mol/L DETA-NO. Data are mean±SEM from 3 independent experiments.

metabolites, $10^{-4}$ mol/L DETA was added at the end of each experiment and, in all cases, produced a significant increase in the fluorescence observed (Figure 3A). The magnitude of this fluorescence increase in the presence of $10^{-4}$ mol/L GTN was $71.27 \pm 17.95\%$ (n=4), and it was similar to that observed when $10^{-4}$ mol/L DETA was added in the absence of
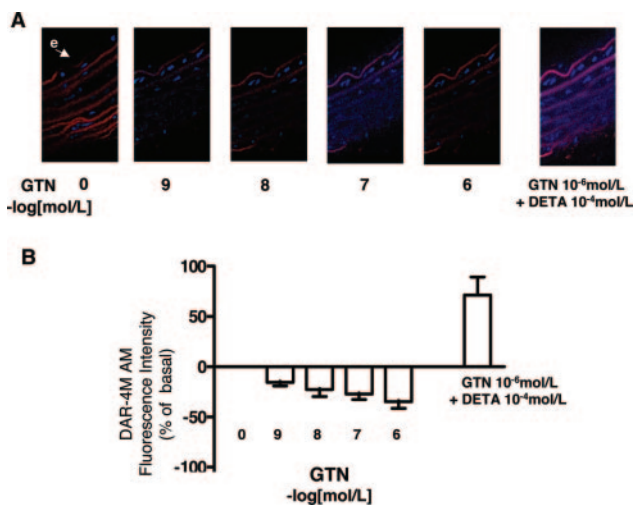


**Figure 3.** Detection by confocal microscopy of intracellular NO levels in isolated rat aortic rings. A, Images were obtained after addition of increasing concentrations of GTN and $10^{-4}$ mol/L DETA-NO in presence of GTN. Rings were loaded with DAR-4M AM ($2.5 \times 10^{-6}$ mol/L, 60 minutes) as an NO probe (red fluorescence) and Hoechst 33342 ($10^{-6}$ mol/L, 30 minutes) as a nuclei marker (blue fluorescence). e indicates endothelial layer. B, Bar diagrams show DAR-4M AM fluorescence intensity obtained with GTN, expressed as percentage of basal fluorescence. Data are mean±SEM from 6 independent experiments.

**TABLE 2.   Modulation of the Apparent $K_m$ for $O_2$ by Different Drugs in Rat Aortic Rings**

| Drug | $K_m$ ($10^{-6}$ mol/L) | n |
|---|---|---|
| Control | 30.4±2.5 | 8 |
| DETA (mol/L) | | |
| $10^{-8}$ | 38.2±14.7 | 4 |
| $10^{-7}$ | 46.5±9.1* | 5 |
| $10^{-6}$ | 96.9±13.1‡ | 4 |
| $10^{-5}$ | 116.1±12.1‡ | 5 |
| $10^{-4}$ | 142.3±16.5‡ | 4 |
| SNAP (mol/L) | | |
| $10^{-9}$ | 33.6±1.0 | 3 |
| $10^{-7}$ | 47.0±6.3* | 3 |
| $10^{-5}$ | 92.1±20.8† | 4 |
| SIN-1 (mol/L) | | |
| $10^{-9}$ | 30.7±3.2 | 3 |
| $10^{-7}$ | 47.4±12.6* | 3 |
| $10^{-6}$ | 80.7±20.6† | 3 |
| Ach (mol/L) | | |
| $10^{-7}$ | 39.7±5.2 | 5 |
| $10^{-6}$ | 61.9±13.9† | 5 |
| $10^{-5}$ | 75.2±6.7‡ | 6 |
| $10^{-4}$ | 90.3±16.4‡ | 3 |
| GTN (mol/L) | | |
| $10^{-9}$ | 28.6±2.6 | 4 |
| $10^{-8}$ | 31.8±1.1 | 5 |
| $10^{-7}$ | 28.0±9.5 | 5 |
| $10^{-6}$ | 26.2±3.5 | 5 |

Data are the mean±SEM from n independent experiments. *$P<0.05$, †$P<0.01$, ‡$P<0.001$ vs control.

GTN or in the presence of $10^{-5}$ mol/L SNAP or $10^{-6}$ mol/L SIN-1 (n=5 to 7 in each case).

## Effect on the Rate of $O_2$ Consumption

Table 2 represents the effects of DETA-NO ($10^{-8}$ to $10^{-5}$ mol/L), SNAP ($10^{-9}$ to $10^{-5}$ mol/L), SIN-1 ($10^{-9}$ to $10^{-6}$ mol/L), ACh ($10^{-7}$ to $10^{-4}$ mol/L), and GTN ($10^{-9}$ to $10^{-6}$ mol/L). DETA-NO, SNAP, SIN-1, and ACh did not modify (data not shown) the $Vo_{2max}$ of controls ($36.50 \pm 1.1 \times 10^{-9}$ mol $O_2$/min/ $10^{-6}$ g protein) but significantly and concentration-dependently increased the apparent $K_m$ for $O_2$ of isolated rings of rat thoracic aorta. Concentrations of GTN ($10^{-9}$ to $10^{-6}$ mol/L), which produced maximal relaxation in the aortic rings, neither modified the $Vo_{2max}$ nor increased the apparent $K_m$ for $O_2$. However, higher concentrations of GTN ($10^{-5}$ and $10^{-4}$ mol/L) produced a significant decrease in the $Vo_{2max}$ ($22.67 \pm 1.01$ and $12.85 \pm 1.12 \times 10^{-9}$ mol $O_2$/min per $10^{-6}$ g protein, respectively) and the apparent $K_m$ for $O_2$ ($20.5 \pm 3.93$ and $14.71 \pm 6.47$, respectively).

The implication of NO in the effects of DETA-NO, ACh, and GTN on mitochondrial $O_2$ consumption is shown by the representative traces of respiring rings in Figure 4. As suggested by the change in the curve slopes, the rate of $O_2$
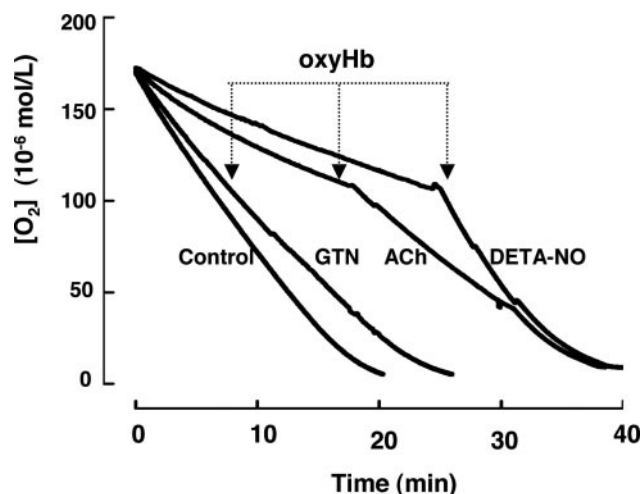
**Figure 4.** Representative traces showing the rate of $O_2$ consumption by rat aortic rings placed in a closed respiration chamber and allowed to consume $O_2$ in the absence (control) or presence of GTN ($10^{-6}$ mol/L), ACh ($10^{-5}$ mol/L), and DETA-NO ($10^{-5}$ mol/L). When the $O_2$ concentration reached $10^{-4}$ mol/L, addition of oxyHb ($10^{-5}$ mol/L) reverted the inhibition of $O_2$ consumption induced by ACh and DETA-NO but not by GTN.

consumption was decreased by incubation with concentrations of DETA-NO ($10^{-5}$ mol/L) and ACh ($10^{-5}$ mol/L), which produced maximal relaxation in the aortic rings. Scavenging of NO by addition of oxyHb reversed the inhibitory effects of both drugs on respiration. This effect was not shared by GTN ($10^{-6}$ mol/L). Presence of the sGC inhibitor ODQ ($5 \times 10^{-6}$ mol/L) did not modify the inhibitory effect on mitochondrial $O_2$ consumption observed with the 3 agents (data not shown). Figure 5 shows the effects of the various treatments on the $O_2$ consumption rate ($Vo_2$) of rat aortic rings in the interval during which $O_2$ concentrations in the bath were similar to those present in hypoxic conditions, between 2 to $3 \times 10^{-5}$ mol/L. Within the same range of concentrations that completely relax precontracted vessels, DETA-NO, SNAP, SIN-1, and ACh reduced $O_2$ consumption concentration dependently, whereas GTN did not modify $Vo_2$.

## Discussion

GTN has been used since 1879 in the treatment of ischemic syndromes related to coronary heart disease. Its principal
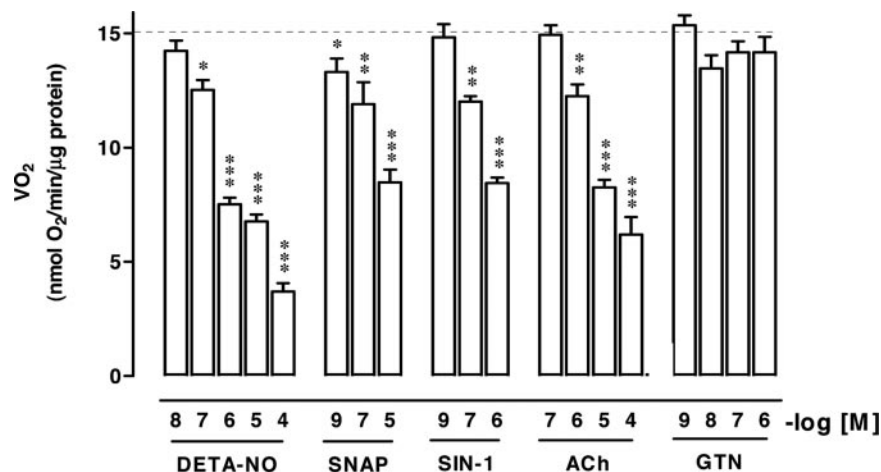
action has been accredited to the release of NO and subsequent activation of guanylyl cyclase, which should induce the relaxation of vessels, both coronary and peripheral. However, most studies have demonstrated that NO is released from GTN only at elevated pharmacological concentrations, beyond those achieved with therapeutic concentrations of the drug.[3–7] In our vascular relaxation experiments, the effects of GTN were similar to those of the 3 structurally different NO donors evaluated or the induction of endogenous production of NO with ACh. They all produced a potent and concentration-dependent relaxation of vascular rings, which was blocked by the presence of the NO scavenger oxyHb or the sGC inhibitor ODQ. However, this similitude of profiles disappeared when the NO was determined.

Using confocal microscopy, we performed a new technique for real-time detection of intracellular NO in isolated intact vessels. Incubation of the rat aorta with vasoactive concentrations of DETA-NO, SNAP, and SIN-1 produced concentration-dependent augmentations in DAR-4M AM fluorescence. Similarly, stimulation of the endothelium with maximal vasoactive concentrations of ACh induced an immediate increase in NO-related fluorescence, which was prevented by inhibition of NO synthase. This increase in a NO-related signal was not observed following incubation with GTN. Indeed, there was a small decrease in the intensity of DAR-4M AM fluorescence for which we presently have no explanation.

A similar absence of an NO signal produced by GTN has recently been reported with electron paramagnetic resonance spin trapping in a variety of rodent vessel.[11] This technique is also highly sensitive to NO but requires the use of frozen tissue and, therefore, yields single, static measurements. By comparison, our approach allows repetitive real-time assessments of the effects of drugs in a biologically active vessel at various concentrations and throughout a prolonged period of time, thus facilitating a clearer picture of the physiological implications.

The relaxant effects of GTN on the isolated vessels are compatible with an effect mediated by NO. However, our results, considered together with the measurement of NO levels by confocal microscopy, raise the possibility that these effects result from the release of another species that shares with NO the characteristics of being scavenged by oxyHb and



**Figure 5.** Effects of incubation with DETA-NO, SNAP, SIN-1, ACh, and GTN on the $O_2$ consumption rate ($Vo_2$) in rat aortic rings when the $O_2$ concentration in the medium reached 2 to $3 \times 10^{-5}$ mol/L. Data are mean±SEM from 3 to 8 independent experiments and *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control.

of stimulating sGC. This is not a new proposal, and results with other nitrates have shown that they could oxidize oxyHb at a rate that correlates with their vasodilatory potency but not with the release of NO,[18,19] thus suggesting the release of a species different from NO.

The idea that the effects of GTN are not a consequence of the release of NO is reinforced further by the analysis of mitochondrial oxygen consumption. The interaction of this mediator with the terminal enzyme of the electron transport chain, cytochrome C oxidase, is emerging as a major pathway through which NO exerts important physiological/pathological functions.[13,20] NO reduces the affinity of the enzyme for $O_2$, increasing its $K_m$ and, consequently, inhibiting mitochondrial $O_2$ consumption with an intensity that is inversely proportional to the concentration of $O_2$.[21–24] Our results with DETA-NO, SNAP, SIN-1, and ACh confirm this mechanism of action. They reduced the rate of $O_2$ consumption by the rat aorta in a concentration-dependent manner within the same concentration range that produced NO-mediated vasorelaxations and increased the fluorescent signals in confocal microscopy. Furthermore, they followed a pattern of competitiveness and reversibility previously described for the interaction between NO and cytochrome C oxidase: (1) competitive, because the affinity for $O_2$ is decreased, as shown by the concentration-dependent increase in the $K_m$ that occurs without a significant change in the $Vo_{2max}$[25,26]; and (2) reversible by oxyHb.[16,27]

The effects of GTN differ and are not compatible with the concept that this compound releases NO, which inhibits mitochondrial $O_2$ consumption in a competitive manner. Clinically used concentrations of GTN, which significantly relax isolated vessels by activation of sGC, do not modify $Vo_{2max}$ or the apparent affinity for $O_2$ represented by the $K_m$. Higher, supratherapeutic concentrations of GTN reduce $Vo_{2max}$ and decrease the $K_m$ in a way that is indicative of a noncompetitive action and probably related to the known GTN capacity to produce reactive oxygen species, such as superoxide and peroxynitrite,[28–30] that might compromise the function of different mitochondrial enzymes.[31,32] The differences between NO donors, ACh, and GTN are particularly evident if analyzed in a hypoxic context, which highlights the inhibition by NO of mitochondrial $O_2$ consumption. With a concentration of $O_2$ between 2 and $3 \times 10^{-5}$ mol/L, equivalent to hypoxic conditions,[17] all 3 NO donors and ACh, but not GTN, produce a significant and concentration-dependent inhibition of the mitochondrial $O_2$ consumption. It has recently been suggested that the bioactivation of GTN is mediated by the mitochondrial enzyme aldehyde dehydrogenase.[33] Our results do not support the idea that NO is 1 of the species formed as a product of GTN biotransformation, as it would be difficult to explain how such a NO diffuses from the mitochondria to activate the cytosolic sGC without inhibiting mitochondrial complex IV or being detected by confocal microscopy.

We conclude that the biotransformation of GTN does not release free NO. It remains to be investigated whether this leads to the release of another species that directly interacts with sGC in vascular smooth muscle without inhibiting $O_2$ consumption by vascular mitochondria. This variation in the bioactivity of GTN and NO donors is interesting insofar as its therapeutic implications. NO-producing agents produce vasodilatation in concentrations that also significantly inhibit $O_2$ consumption, but GTN does not. GTN is used to improve the blood flow in ischemic tissues, and the fact that this is achieved without any further reduction in the bioenergetic activity of the mitochondria could lie behind its long clinical success.

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