Complex I Dysfunction and Tolerance to Nitroglycerin
An Approach Based on Mitochondrial-Targeted Antioxidants

Juan V. Esplugues, Milagros Rocha, Cristina Nuñez, Irene Bosca, Sales Ibiza, Jose R. Herance, Angel Ortega, Juan M. Serrador, Pilar D’Ocon, Victor M. Victor

Abstract—Nitroglycerin (GTN) tolerance was induced in vivo (rats) and in vitro (rat and human vessels). Electrochemical detection revealed that the incubation dose of GTN (5×10⁻⁶ mol/L) did not release NO or modify O₂ consumption when administered acutely. However, development of tolerance produced a decrease in both mitochondrial O₂ consumption and the K_m for O₂ in animal and human vessels and endothelial cells in noncompetitive action. GTN tolerance has been associated with impairment of GTN biotransformation through inhibition of aldehyde dehydrogenase (ALDH)-2, and with uncoupling of mitochondrial respiration. Feeding rats with mitochondrial-targeted antioxidants (mitoquine [MQ]) and in vitro coinoculation with MQ (10⁻⁶ mol/L) or glutathione (GSH) ester (10⁻⁴ mol/L) prevented tolerance and the effects of GTN on mitochondrial respiration and ALDH-2 activity. Biotransformation of GTN requires functionally active mitochondria and induces reactive oxygen species production and oxidative stress within this organelle, as it is inhibited by mitochondrial-targeted antioxidants and is absent in HUVEC_p⁰ cells. Experiments analyzing complex I–dependent respiration demonstrate that its inhibition by GTN is prevented by mitochondrial-targeted antioxidants. Furthermore, in presence of succinate (10×10⁻³ mol/L), a complex II electron donor added to bypass complex I–dependent respiration, GTN-treated cells exhibited O₂ consumption rates similar to those of controls, thus suggesting that complex I was affected by GTN. We propose that, following prolonged treatment with GTN in addition to ALDH-2, complex I is a target for mitochondrially generated reactive oxygen species. Our data also suggest a role for mitochondrial-targeted antioxidants as therapeutic tools in the control of the tolerance that accompanies chronic nitrate use. (Circ Res. 2006;99:1067-1075.)

Key Words: nitroglycerin ＊ endothelium ＊ oxidative stress ＊ mitochondria ＊ antioxidant

The vasodilatory actions of nitroglycerin (glyceryl trinitrate [GTN]) have generally been attributed to its bioconversion into the relaxant agent nitric oxide (NO), which acts on the enzyme soluble guanylate cyclase (sGC). However, most studies that support the existence of such a pathway have demonstrated increases of NO only when GTN concentrations considerably exceeded the plasma levels reached during clinical dosing. Moreover, the involvement of other NO-related species in the actions of GTN when used at clinically relevant concentrations is also under debate. Different enzymes have been implicated in the bioconversion of GTN, in particular, glutathione S-transferases, the cytochrome p450 system, and xanthine oxidoreductase, although the most recent evidence suggests a central role for mitochondrial aldehyde dehydrogenase (ALDH)-2. The medical use of GTN is limited by the development of tolerance, which occurs following prolonged administration or the application of high doses. This phenomenon has been related to various mechanisms, in particular, desensitization of sGC, and, mainly, impairment of GTN biotransformation by inhibition of ALDH-2. These actions, like others associated with GTN, have been linked to an increase in the production of reactive oxygen species (ROS), as well as mitochondrial dysfunction. The present study confirms the theory that tolerance to GTN (both in vivo and in vitro) is related to the oxidative stress that leads to an inhibition of ALDH-2. Furthermore, through the use of mitochondrial antioxidants such as mitoquine (MQ), or glutathione (GSH) ester, we have confirmed the mitochondria to be the main location for the development of tolerance. Finally, we have identified mitochondrial complex I as a target at which the initial oxidative stress responsible for GTN tolerance takes place.

Materials and Methods

Umbilical cords obtained from the Department of Gynecology (Faculty of Medicine of Valencia) were cut into rings (5 mm) and placed in Krebs solution (in 118 NaCl, KCl 4.75, 6mol/L) or glutathione (GSH) ester, we have confirmed the mitochondria to be the main location for the development of tolerance. Finally, we have identified mitochondrial complex I as a target at which the initial oxidative stress responsible for GTN tolerance takes place.

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CaCl₂ 1.9, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, and glucose 10.1).

Human umbilical vein cells (HUVECs) and human umbilical arterial endothelial cells (HUAECs) were cultured in medium 199 (Cambrex, Walkersville, Md) as previously described. Male Sprague–Dawley rats (200 to 250 g; Harlan, Barcelona, Spain) were decapitated and 5 mm rings obtained from their thoracic aortas.

To generate HUVEC₆₀ cells, 50 ng/mL ethidium bromide was added to the medium for 7 days to inhibit mitochondrial gene transcription and activity. Pyruvate (110 mg/mL) and uridine (50 µg/mL) were used as alternative energy and nucleotide sources. Lack of mitochondrial gene expression was evaluated by Western blot analysis for cytochrome c oxidase subunit I (Molecular Probes, Leiden, The Netherlands), and the absence of respiratory function was measured by analyzing mitochondrial oxygen (O₂) consumption.

All protocols complied with European Community guidelines for the use of experimental animals and were approved by the Ethics Committee of the University of Valencia.

**Vascular Contractility Studies**

Rings were suspended in an organ bath containing Krebs solution (37°C), as described previously. GTN tolerance was induced in vitro by incubating rings for 3 hours with GTN (10⁻⁶ mol/L). Rings from the in vivo nitrate-tolerance experiments were obtained from rats that had been allowed free access for 14 days to tap water with or without MQ or triphenylphosphonium (TPP) (10⁻⁴ mol/L) and that had been treated with a GTN patch for the last 3 days. All of these parameters were selected from preliminary experiments and were similar to those used by other authors.

Relaxation-response curves were obtained by adding cumulative concentrations of GTN (10⁻¹⁰ to 10⁻⁴ mol/L) or DETA-NO (10⁻⁶ to 10⁻⁴ mol/L). Some experiments were performed in aortic denudated rings, and, when necessary, either MQ (10⁻⁶ mol/L) or GSH ester (10⁻⁴ mol/L) was added 1 hour before the 3-hour GTN incubation period and maintained thereafter. In preliminary experiments the lipophilic cation linker TPP (10⁻⁶ mol/L), responsible for targeting MQ to mitochondria, showed no effect on vascular responses. When necessary, experiments were performed in the presence of non–mitochondrial-targeted antioxidants such as uric acid (UA) (10⁻⁴ mol/L; scavenger of ONOO⁻), ebselen (Eb) (10⁻⁴ mol/L; scavenger of ONOO⁻ and H₂O₂), and tempol (TP) (10⁻³ mol/L; scavenger of O₂⁻). The adequate concentration for producing 50% relaxation (pEC₅₀) was obtained from a nonlinear regression analysis with GraphPad software.

**ALDH Activity**

The activity of ALDH-2 in homogenates of aortic rings was determined by measuring the conversion of propionaldehyde to propionic acid and was expressed as the mean rate of absorbance (0.0125 A₅₆₂ was equivalent to 1 nmol/mg per minute).

**Measurements of O₂ Consumption and NO Production**

The aorta from 2 animals (100±1.04 mg wet weight) or human umbilical arteries or veins (196±2.3 or 199±3.6 mg wet weight, respectively) were cut into rings and resuspended in Krebs solution.
HUVECs, HUVEC<sub>p</sub> cells, and HUAECs were resuspended (5×10<sup>6</sup> cells/mL) in Krebs supplemented with l-arginine (3×10<sup>-4</sup> mol/L) and HEPES (25×10<sup>-3</sup> mol/L). Induction of GTN tolerance (in vivo or in vitro) and the use of mitochondrial and non-mitochondrial-targeted antioxidants were similar to those described in the vascular reactivity studies. When administered acutely, the dose of GTN used (5×10<sup>-5</sup> mol/L) had no effect on mitochondrial O<sub>2</sub> consumption, despite producing maximal relaxations of vascular rings. The aforementioned tissues were then placed in a gas-tight chamber, and O<sub>2</sub> consumption was measured with a Clark-type O<sub>2</sub> electrode (Rank Brothers, Bottisham, UK).<sup>6</sup> Sodium cyanide (10<sup>-6</sup> mol/L) confirmed that O<sub>2</sub> consumption was mainly mitochondrial. Measurements were collected using the data-acquisition device Duo.18 (WPI, Stevenage, UK). A hyperbolic function was used to describe the relationship between O<sub>2</sub> concentration and the rate of O<sub>2</sub> consumption (V<sub>O<sub>2</sub></sub>). The maximal rate of O<sub>2</sub> consumption (V<sub>O<sub>2</sub></sub>max)) and the apparent O<sub>2</sub> affinity Michaelis–Menten constant (K<sub>M</sub>) (10<sup>-6</sup> mol/L) were calculated according to their analogy with the Michaelis–Menten constant.

NO concentration in the cell medium was monitored throughout the 3-hour incubation period with GTN by means of an NO electrode (ISO-NOP; WPI, Stevenage, UK) as described previously.<sup>22</sup> The NO donor DETA-NO (10<sup>-3</sup> mol/L) was added at the end of each experiment as an internal control. Changes in intracellular NO were also evaluated by incubating cells with the fluorescent probe diamodifluororescin diacetate (DAF-FM DA) (1×10<sup>-6</sup> mol/L). Thereafter, the medium was changed to HBSS, supplemented with glucose (20×10<sup>-3</sup> mol/L), l-arginine (3×10<sup>-4</sup> mol/L), and DAF-FM, incubated for 30 minutes and measured using a Fluoroscan plate reader (TL, Franklin, Mass.). GSH levels were measured with the Biomol assay kit (Plymouth Meeting, Pa).

### Measurement of ROS Production, GSH Content, and Complex I Activity

Two methods were used to evaluate ROS. Total ROS production was assessed following incubation (30 minutes) with the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (5×10<sup>-6</sup> mol/L) as described elsewhere.<sup>24</sup> Quantitative assessment of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was performed with the Amplex Red H<sub>O</sub>2/Peroxidase Assay kit (Molecular Probes, Eugene, Ore.).<sup>25,26</sup> GSH content was assessed following incubation (30 minutes) with the fluorescent probe monochlorobimane (MCB) (40×10<sup>-6</sup> mol/L). GSH levels were also measured by confocal microscopy (Leica, Heidelberg, Germany), following incubation (30 minutes) with 5-chloromethyfluorescein diacetate (CMFDA) (1×10<sup>-6</sup> mol/L). The ratio oxidized glutathione (GSSG)/GSH was calculated as previously described.<sup>27</sup> Proteins were determined using the BCA protein assay kit (Pierce, Rockford, Ill.).

The activity of mitochondrial complex I was assessed by calculating the NADH oxidation rate.<sup>28</sup> Briefly, a cellular homogenate (20 μL, 0.3 mg) was added to 1 mL of potassium phosphate buffer (10<sup>-3</sup> mol/L) containing NADH (10<sup>-4</sup> mol/L) at 37°C. Basal absorbance (340 nm) was recorded for 1 minute, and 5 μL of ubiquinone (10<sup>-3</sup> mol/L) was then added, and the rate of NADH oxidation (taken as complex I activity) over 2 minutes was measured. The NADH oxidation rate was calculated from the time-dependent decrease in the slope of absorbance using an extinction coefficient for NADH of 6.81×10<sup>-3</sup> mol·cm<sup>-1</sup> at 340 nm. Isolated complex I substrates malate (0.4×10<sup>-3</sup> mol/L) and glutamate (3×10<sup>-3</sup> mol/L), the complex II substrate succinate (10<sup>-3</sup> mol/L), or the complex I inhibitor rotenone (6×10<sup>-5</sup> mol/L).<sup>29</sup>

### Drugs and Solutions

Phenylephrine (Phe), acetylcholine (ACh), 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), sodium cyanide, KCl, ubiquinone, succinate, rotenone, glutamate, malate, diguiniton, arginine, HEPES, uridine, TPP, glucose, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), trypan blue, GSH reductase (GR), NADH, TP, UA, Eb, methylpyrazole, H<sub>O</sub>2, and hemoglobin were obtained from Sigma-Aldrich (St Louis, Mo). Propionaldehyde was from Fluka (Milano, Italy). The GTN used is a clinically used preparation (Solinitrina, Allmirall, Barcelona, Spain). Patches of GTN were from Schering-Plough (Madrid, Spain). Na-pyruvate was from GIBCO-BRL (Gaithersburg, Md). Ethidium bromide was from SERVA (Heidelberg, Germany). HBSS and medium 199 were from Cambrex (Verviers, Belgium). DETA-NO was from Alexis (San Diego, Calif). DAF-FM, and DCFH-DA were from Calbiochem (San Diego, Calif). MCB and CMFDA were from Molecular Probes (Eugene, Ore). MQ was synthesized according to the published method.<sup>30</sup>

### Data Analysis

Unless stated otherwise all values are mean±SEM of at least 5 experiments. Statistical analysis was performed with 1-way ANOVA with post hoc corrections, followed by the Student t test for unpaired samples (GraphPad Software). Significance was defined as P<0.05.

### Results

#### Tolerance to GTN Vasorelaxation

Figure 1 shows dose-dependent relaxation curves by GTN (10<sup>-10</sup> to 10<sup>-4</sup> mol/L) in precontracted (Phe 10<sup>-6</sup> mol/L) rings. Figure 1A represents results from rat aorta in which tolerance was induced in vitro and in vivo. Data are the mean±SEM of 5–7 independent experiments. *P<0.001 vs control, †P<0.05 and ‡P<0.001 vs GTN.

**Table 1. pEC<sub>50</sub> of the Dose-Dependent Relaxation Curves by GTN in Rat Aortic Rings and Artery and Vein Rings from Human Umbilical Cord**

<table>
<thead>
<tr>
<th></th>
<th>In Vitro</th>
<th>In Vivo</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Rat Aorta</td>
<td>Human Umbilical Artery</td>
</tr>
<tr>
<td>Control</td>
<td>8.30±0.10</td>
<td>8.30±0.14</td>
</tr>
<tr>
<td>MQ</td>
<td>8.06±0.11</td>
<td>8.19±0.18</td>
</tr>
<tr>
<td>GSH</td>
<td>8.05±0.09</td>
<td>8.12±0.06</td>
</tr>
<tr>
<td>Eb</td>
<td>8.28±0.06</td>
<td></td>
</tr>
<tr>
<td>UA</td>
<td>8.25±0.07</td>
<td></td>
</tr>
<tr>
<td>TP</td>
<td>8.11±0.05</td>
<td></td>
</tr>
<tr>
<td>GTN</td>
<td>7.11±0.06*</td>
<td>6.81±0.07*</td>
</tr>
<tr>
<td>MQ</td>
<td>7.82±0.07*</td>
<td>7.83±0.16†</td>
</tr>
<tr>
<td>GSH</td>
<td>8.03±0.12†</td>
<td>8.12±0.12‡</td>
</tr>
<tr>
<td>Eb</td>
<td>7.63±0.08†</td>
<td></td>
</tr>
<tr>
<td>UA</td>
<td>7.67±0.07†</td>
<td></td>
</tr>
<tr>
<td>TP</td>
<td>7.32±0.09†</td>
<td></td>
</tr>
</tbody>
</table>

Concentration range of GTN was 10<sup>-10</sup> to 10<sup>-4</sup> mol/L. Rat aortic rings and artery and vein rings from human umbilical cord were precontracted with Phe (10<sup>-6</sup> mol/L), in the presence of MQ (10<sup>-5</sup> mol/L), GSH ester (10<sup>-4</sup> mol/L), Eb (10<sup>-4</sup> mol/L), UA (10<sup>-4</sup> mol/L), or TP (10<sup>-3</sup> mol/L). GTN tolerance was induced in vitro or in vivo. Data are the mean±SEM of 5–7 independent experiments. *P<0.001 vs control, †P<0.05 and ‡P<0.001 vs GTN.
GTN, although they continued to differ from those of controls (91.6±2% of controls, P<0.05 versus both).

Treatment with MQ (in vivo or in vitro) or GSH ester (in vitro) during the induction of tolerance restored the acute vasorelaxant effects of GTN, suggesting that mitochondrial ROS were involved in the development of GTN tolerance. Addition of non–mitochondrial-targeted antioxidants (UA, Eb, and TP) following the in vitro induction of tolerance only partially restored the acute effects of GTN (Figure 1C). pEC50 values with DETA-NO (10^-4 mol/L) were similar in controls (6.06±0.07) and GTN-tolerant (5.96±0.05) aortas. Incubation with DETA-NO (5×10^-6 mol/L; 3 hours) did not induce tolerance to the effects of the GTN (7.98±0.07) administered later.

**GTN Biotransformation**

ALDH activity was significantly (P<0.001) diminished in GTN-tolerant aortas when compared with that of controls (1.1±0.1 vs 2.8±0.2 nmol/mg per minute, respectively). Presence of MQ or GSH ester during the in vitro induction of GTN tolerance prevented such a decrease (2.7±0.2 and 2.5±0.2 nmol/mg per minute, respectively).

### Mitochondrial O2 Consumption

Figure 2 and Table 2 show that the rate of O2 consumption, the apparent Km for O2, and VO2max decreased in vessels in which GTN tolerance was induced in vivo (Figure 2A) or in vitro (Figure 2B). Treatment with MQ (in vivo or in vitro) or GSH ester (in vitro) during the induction of tolerance prevented these inhibitory effects of GTN. Following the in vitro induction of tolerance, addition of non–mitochondrial-targeted antioxidants partially (P<0.05) restored the inhibitory effects of GTN (Table 2). Presence of the NO scavenger hemoglobin (10^-5 mol/L) or the sGC inhibitor ODQ (5×10^-6 mol/L) did not modify the effects of GTN on O2 consumption (data not shown).

Figure 2C shows representative traces of the electrochemical detection of NO in a medium containing HUVECs.
Addition of GTN (5 × 10^{-6} mol/L) did not induce any NO signal, whereas a robust increase was noted following DETA-NO (10^{-5} mol/L). Similar results were obtained with the NO-sensitive fluorescent probe DAF-FM (data not shown).

Role of Mitochondria in GTN-Induced cGMP Production

To confirm the role of functioning mitochondria in GTN bioactivity, we created HUVEC<sup>0</sup> cells and considered the absence of cytochrome c oxidase subunit I as an index (Figure 3A). These cells were associated with a 99% reduction in O_2 consumption in a closed respiration chamber. A, Representative traces showing the rate of O_2 consumption in rat aorta of rats pretreated with MQ (14 days) and in which GTN tolerance was induced in vivo. B, Representative traces showing the rate of O_2 consumption in HUVECs and HUVEC<sup>0</sup> cells (in which the expression was absent). Tubulin was used as a control charge. B, Representative traces showing the rate of O_2 consumption in HUVECs and HUVECs following in vitro preincubation with GTN (5 × 10^{-6} mol/L) or GTN+MQ (10^{-6} mol/L). C, cGMP levels in HUVECs and HUVECs<sup>0</sup> cells. In some cases, cells were preincubated with GTN (5 × 10^{-6} mol/L). Coincubation with MQ (10^{-6} mol/L) or GSH ester (10^{-4} mol/L) prevented the effect on cGMP levels of GTN tolerance in HUVECs. Addition of DETA-NO (10^{-4} mol/L) increased cGMP levels in both HUVECs and HUVECs<sup>0</sup> cells. Data are mean±SEM of 3 to 5 separate experiments. *P<0.001 vs control, †P<0.001 vs cells with mitochondria.

Addition of GTN (5 × 10^{-6} mol/L) did not induce any NO signal, whereas a robust increase was noted following DETA-NO (10^{-5} mol/L). Similar results were obtained with the NO-sensitive fluorescent probe DAF-FM (data not shown).
consumption (Figure 3B). In HUVECs (Figure 3B), preincubation with MQ during the induction of tolerance prevented the inhibitory effects of GTN on mitochondrial O2 consumption. The addition of GTN increased cGMP levels in HUVECs, but such a response was absent when cells have been preincubated with GTN and in HUVECp0 cells (Figure 3C). Coincubation with either MQ or GSH ester prevented the effects induced by the incubation with GTN in HUVECs. Both HUVECs and HUVECp0 cells exhibited increased cGMP production following addition of DETA-NO (10^-6 mol/L). Preincubation of HUVECs with DETA-NO (5×10^-6 mol/L; 3 hours) did not modify the increase in cGMP that follows acute addition of GTN (data not shown).

**ROS Production and GSH Levels**

Preincubation of HUVECs with GTN significantly increased the fluorescence of DCFH-DA, indicating an augmented production of ROS (Figure 4A). A similar increase was observed when the effects of GTN preincubation were evaluated by confocal microscopy (Figure 4B). Incubation with GTN also increased H2O2 (Figure 4C). In all cases, coincubation with MQ and GSH ester reversed the effects of GTN. Similar results were obtained in HUAECs (data not shown). Neither incubation of HUVECp0 cells with GTN nor acute administration of GTN in both HUVECs and HUVECp0 cells produced any increase in ROS-related fluorescence. Pretreatment with GTN produced increases in H2O2 in aortic rings with and without endothelium (Figure 4D), and coincubation with MQ or GSH ester prevented such an increase (data not shown). Although the removal of the endothelium reduced the level of fluorescence, the production of ROS continued to be substantial, thus suggesting that both muscle and endothelial cells are capable of generating ROS following incubation with GTN.

Oxidative stress is related to both an increase in ROS production and a decrease in antioxidant content. As shown in Figure 5A, preincubation of HUVECs with GTN significantly decreased the fluorescence of MCB, indicating a reduction in GSH levels. A similar diminution in CMFDA fluorescence was observed when the effects of GTN were evaluated by confocal microscopy (Figure 5B). Treatment with MQ reversed these effects, and, as expected, GSH ester boosted the levels of the fluorescence signal. Figure 5C shows how incubation with GTN increased the GSSG:GSH ratio, an index of oxidative stress, whereas MQ and GSH ester prevented this effect.

**GTN Impairs Mitochondrial O2 Consumption by Inhibiting Complex I: Reversal by Mitochondrial-Targeted Antioxidants**

Figure 6A shows the inhibitory effects of preincubation with GTN on mitochondrial complex I activity, as calculated from the rate of NADH oxidation in HUVECs. Coincubation with either MQ or GSH ester reversed this effect. The specificity of the action of GTN was further characterized through an alternative method involving cells permeabilized with digitonin and measurement of isolated complex I–dependent respiration. Figure 6B shows that HUVECs respiring on the complex I substrates malate (0.4×10^-3 mol/L) and glutamate (30×10^-3 mol/L) were inhibited by nearly 90% with the complex I inhibitor rotenone (6×10^-6 mol/L). Cells incubated with GTN respired very poorly with malate and glutamate, whereas rotenone-sensitive respiration did not differ to that observed in the absence of the inhibitor. When succinate (10×10^-3 mol/L), a complex II electron donor, was added to bypass complex I–dependent respiration, GTN-treated cells exhibited O2 consumption rates similar to those of controls, suggesting that complex I was the main target of GTN preincubation. Coincubation with MQ or GSH ester prevented the effects of GTN preincubation on the levels of complex I–dependent respiration.
Discussion

The present study demonstrated how prolonged in vivo administration or in vitro preincubation with GTN result in a reduced vascular relaxation when the drug is administered acutely, thus resembling the clinical induction of GTN tolerance.\textsuperscript{11,21,22} The dose of GTN used for incubation does not release NO and following the induction of tolerance, it reduced mitochondrial O$_2$ consumption in a noncompetitive manner in vascular tissue and endothelial cells, which is contrary to that observed when administered acutely.\textsuperscript{6} It has been suggested that biotransformation of GTN requires a functionally active mitochondria and leads to an increased production of ROS and oxidative stress that coincides with an impairment of mitochondrial ALDH-2 activity.\textsuperscript{10,11} We have delved further into these processes by using HUVEC$^\text{p}$ cells and mitochondrial-targeted antioxidants to show that MQ and GSH ester prevent the induction of GTN tolerance and block any increase in the production of ROS and any reduction in ALDH-2 activity within the mitochondria. Finally, we propose that complex I of the electron transport chain is a target of the ROS that is generated during the induction of GTN tolerance and can cause a decrease in mitochondrial O$_2$ consumption. Collectively, these findings extend the concept that mitochondrial dysfunction plays a key role in the appearance of nitrate tolerance.

MQ is the result of covalently linking ubiquinone to a TPP cation, causing a several-hundred-fold accumulation within the mitochondria.\textsuperscript{30} The active antioxidant form of MQ is the reduced ubiquinol form, which is regenerated by the electron transport chain, and selectively blocks mitochondrial oxidative damage by detoxifying ROS.\textsuperscript{16,17} This and other mitochondrial-targeted antioxidants have been shown to protect this organelle from oxidative stress in isolated cells\textsuperscript{32} and living tissues\textsuperscript{20} in hypoxia,\textsuperscript{26} apoptosis,\textsuperscript{29} and cancer.\textsuperscript{18} In our experiments, continuous pretreatment with GTN produced a substantial reduction in its vasorelaxant effects of GTN when administered acutely, but not in those of acute DETA-NO. These experiments argue against desensitization of sGC as a possible mechanism for GTN tolerance. Administration in vivo of MQ prevented the reduction in the relaxant effects of GTN. This effect was equally reproduced in vitro by MQ and GSH ester, another antioxidant that increases GSH levels in mitochondria.\textsuperscript{18} Furthermore, as previously shown,\textsuperscript{11,33} non–mitochondrial-targeted antioxidants such as UA, Eb, and TP partially reproduced the effects of MQ and GSH ester, probably as a result of their access to the mitochondria.

In further experiments, vascular tissues and cells in which GTN tolerance had been induced in vivo or in vitro exhibited a reduction of O$_2$ consumption and the $K_m$ for O$_2$ in a way that indicated of a noncompetitive action. Administration in vivo of MQ or in vitro of either MQ or GSH ester prevented these effects. Once more, UA, Eb, and TP partially reproduced the effects of MQ and GSH ester. In this way, taking into account the specificity of these non–mitochondrial-targeted antioxidants,\textsuperscript{33} ONOO$^-$, H$_2$O$_2$, and O$_2^-$ would be involved in the impairment of mitochondrial respiration.

The dose of GTN used in vitro did not result in an electrochemical detection of NO release, thus confirming our previous hypothesis that biotransformation of these clinically relevant doses of GTN does not release free NO.\textsuperscript{6} In HUVECs, the acute addition of GTN increased cGMP levels and ALDH-2 activity, whereas this response was absent following preincubation with GTN. Coincubation with either MQ or GSH ester prevented the effects induced by incubation with GTN. In HUVEC$^\text{p}$ cells, GTN-stimulated increases in cGMP were abolished, thus confirming the necessity for GTN to be metabolized by functional mitochondria. This corroborates previous results obtained in mouse macrophages and porcine endothelial cells.\textsuperscript{10,11} Collectively, this evidence gives weight to the idea that ALDH-2 contributes to GTN bioconversion, and the subsequent increase in cGMP levels, whereas inhibition of this enzyme is implicated in GTN tolerance.

It has been widely postulated that continuous exposure to GTN would lead to an increase in the production of ROS,\textsuperscript{15,34–39} which is related to the development of tolerance and cross-tolerance.\textsuperscript{11} Our results with varying fluorescence
methods confirm this hypothesis, because incubation with GTN increased the release of ROS in vascular cells and tissues. The mitochondria seems the origin of said ROS production, as they were absent in HUVEC cells, whereas treatment with MQ and GSH ester reversed the increase in both ROS and oxidative stress that followed incubation with GTN. It is important to highlight the important contribution of vascular muscle cells to this genesis of ROS, as demonstrated by the maintenance of a substantial production in endothelium-denuded vessels.

The subsequent oxidative stress and complex I impairment following long-term treatment with GTN could inactivate ALDH-2 or inhibit the ALDH-2 repair system, both of which result in the impairment of GTN bioactivation. In addition, a potential interaction with superoxide may decrease the bioavailability of the vasodilator released following GTN bioactivation. ROS are highly toxic to various sites of mitochondrial respiratory chain, and inhibition of complex I would seem to be the most likely consequence. We demonstrate that GTN tolerance with MQ and GSH ester prevented the effects of GTN, highlighting ROS-mediated damage in complex I as the likely cause of the respiratory deficiency.

In conclusion, the present study confirms that prolonged exposure to GTN induces oxidative stress and highlights the mitochondria as both the source and target of ROS. In addition, we propose that the activity of mitochondrial complex I is diminished following continuous incubation with GTN. Our data provide fresh insight into the mechanisms responsible for nitrate tolerance, suggesting a potential role for mitochondrial-targeted antioxidants as a therapeutic tool in the prevention or control of the tolerance that accompanies the chronic use of GTN in patients.

Acknowledgments
We thank B. Norman for editorial assistance and F. Rodriguez and C. Amezcu for providing help.

Sources of Funding
This study was financed by Generalitat Valenciana grant 2006-341 and Contrato-Investigador Fondo de Investigacion Sanitaria (FIS) (CP03/00024) (both to V.M.V.) and Ministerio de Educacion y Ciencia grant SAF2005-01366 (to J.V.E.). V.M.V. is the recipient of a Contrato (FIS).

Disclosures
None.

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Circ Res. 2006;99:1067-1075; originally published online October 19, 2006;
doi: 10.1161/01.RES.0000250430.62775.99
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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